WHOLE GENOME SEQUENCE CHARACTERIZATION OF PASTEURELLA MULTOCIDA ISOLATED FROM DIFFERENT ANIMAL SPECIES

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A
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BY
VIRAL B. AHIR
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DEPARTMENT OF ANIMAL BIOTECHNOLOGY
COLLLGE OF VETERINARY SCIENCE AND A.H.
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Dedicated to
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ABSTRACT

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ABSTRACT

"WHOLE GENOME SEQUENCE CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* ISOLATED FROM DIFFERENT ANIMAL SPECIES"

Name of Student | **Name of Major Advisor**

Viral B. Ahir | **Dr. M. K. Jhala**

Department of Animal Biotechnology

College of Veterinary Science and Animal Husbandry

Anand Agricultural University, Anand-388 001

Gujarat, India

Pasteurella multocida is a commensal microorganism of the upper respiratory track of many animal and avian species and is responsible for wide range of diseases in domestic animals and poultry. Despite vaccination of the dairy animals particularly against Haemorrhagic Septicaemia (HS), several outbreaks occur regularly in Gujarat as well as in other parts of India. Whole genome sequencing is a recent advanced approach for understanding of genetic makeup of an organism as well for identification of virulence genes/factors responsible for the disease process in host.

In order to sequence whole genome of *P. multocida* and to elucidate virulence associated genes, five isolates of *P. multocida* were sequenced using pyrosequencing based approach of 454 GS FLX Titanium. All the five isolates *viz.* P52 vaccine strain (P52VAC), poultry (Anand1_poultry), goat (Anand1_goat), buffalo (Anand1_buffalo) and cattle (Anand1_cattle) were identified and characterized based on biochemical and cultural characters and subsequently confirmed by PM-PCR. For sequencing of the whole genome of organisms, dsDNA libraries were prepared for all the five isolates and quantity as well as quality checks were done using Agilent Bioanalyzer as well as TBS flurometer. dsDNA library of each of the five isolates was amplified using emPCR and positive clonal amplified DNA beads were used for sequencing

after annealing of sequencing primers. After completion of the sequencing run, data generated in form of images were converted into reads using GS Run Browser.

After signal processing, total 118843, 113997, 105729, 134886 and 31346 reads were generated which yielded 42,598,100 (42.59Mb), 29,000,497 (29.00Mb), 21,890,353 (21.89Mb), 39,756,349 (39.75Mb) and 7,429,658 (7.42Mb) of sequence bases for P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle, respectively. Coverage obtained for P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle was 18.87, 12.85, 9.70, 17.61 and 3.29 respectively.

All the reads after signal processing were mapped with the reference genome available for a poultry isolate Pm70 at NCBI using GS Reference mapper. Mapping of the isolates P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle resulted in 38,079,806 (89.52%), 20,085,356 (87.38%), 19,867,143 (90.81%), 25,095,466 (63.22%) and 6,145,156 (82.87%) mapped bases with 105327, 97674, 95092, 86765 and 24967 mapped reads. Remaining reads which were not mapped by GS Reference Mapper, were used for *de novo* assembly using GS *De Novo* Assembler for finding sequences which code for plasmid of *P. multocida*. None of the *de novo* assembled sequences matched to plasmid.

For sequence analysis and finding of virulence associated genes in *P. multocida*, two different annotation pipelines were used *viz.* Rapid Annotation using Subsystem Technology (RAST) and Prokaryotic Genome Automatic Annotation Pipeline (PGAAP). For RAST analysis, all the contigs generated after reference mapping with Pm70 uploaded at RAST server. RAST is a subsystem based annotation pipeline which generated 2,273,366bp (2.27Mb), 2,227,943bp (2.22Mb), 2,285,382bp

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(2.28Mb), 2,045,610bp (2.04Mb) and 1,438,517bp (1.43Mb) of genome with 209, 489, 349, 2188, and 3152 contigs for P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle, respectively and 68, 54, 54, 40 and 0 RNA. Based on RAST analysis, highest abundance of subsystem were assigned to 'amino acids and derivatives', 'carbohydrates', 'protein metabolism' and 'cofactor and vitamins, prosthetic groups and pigments'. As expected, no subsystem was assigned to 'photosynthesis' and 'motility and chemotaxis' group as *Pasteurella* is a non-motile organism and is not photosynthetic.

Due to less coverage (3.29X) obtained for the Anandl_cattle isolate, it was omitted from the RAST based comparative analysis. Subsystem based genes/proteins assigned to the other four isolates under 'virulence, disease and defence' category ranged from 47 to 54 in number. There were presence of *DedA, DedD* and *toxin* under 'colicin and bacteriocin production' in P52 vaccine strain, poultry and goat isolates. Genes *gyrA, gyrB, Parc* and *ParD* under 'resistance to fluroquinolones' were present in all the four isolates. There was also presence of negative regulator of betalactamase expression, *BLR* gene leading to resistance expressed by this organism as well as multidrug resistance efflux pump cluster genes, *MATE* (Multidrug and toxin extrusion), *MacA* and *MacB* (Macrolide specific efflux protein) in P52 vaccine strain, poultry and goat isolates.

For PGAAP analysis, all the reads generated after sequencing run were submitted to the PGAAP pipeline of NCBI after removing sequences less than 200bp. PGAAP analysis revealed genome size of 2,273,366bp (2.27Mb), 2,227,943bp (2.22Mb), 2,285,382bp (2.28Mb), 2,045,610bp (2.04Mb) and 1,438,517bp (1.43Mb) with 40.40%, 40.20%, 40.50%, 40.90% and 41.00% of G+C contents for P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle, respectively.

Total number of coding sequences (CDS) were 2066, 2337, 2319, 3258 and 3623; total number of protein encoding genes (PEG) were 2194, 2284, 2266, 3218 and 3590, and total number of RNA assigned were 64, 53, 53, 41 and 33 for P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle, respectively.

Deciphering virulence mechanism is one of the most useful application of bacterial genomics to understand the molecular intricacies involved in disease mechanism as well as for understanding host-pathogen interactions. For this purpose, genes associated with virulence were downloaded from annotation files available at (http://www.ncbi.nlm.nih.gov/genome/genomes/912?) in 'Protien' column/section to find out gene locus/id. After manually searching for the virulence associated genes, 55 important genes were selected based on the available literature. These 55 genes grouped under seven broad categories *viz.* capsule, fimbriae and adhesion, iron metabolism, outer membrane protein, superoxide dismutase, sialic acid metabolism and transcription regulation. Out of these seven categories, all the five genes falling under three categories i.e. *SodA* and *SodC* under superoxide dismutase, *NanH* and *NanB* under sialic acid metabolism and *Fis* under transcription regulation category were present in all the five isolates.

Nine genes involved in capsule production were found, out of which, *PglA* and *Kmt1* were present in all the five isolates, while *HyaE* was present only in the goat isolate. *HexA* and *HexC* genes were absent in buffalo and cattle isolates, while *HexB* and *HexD* were absent in goat and cattle isolates. *KpsF* gene was absent in poultry and cattle isolates. Gene *LctP* was present only in goat and cattle isolates.

Sixteen genes were found under the category of fimbriae and adhesion, of which, *Hsf, PfhB2, PfhR, PflB, PlpB,* and *Plp4* genes were found in all the five

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isolates of *P. multocida* studied. *HofC* gene was absent only in P52 vaccine strain, whereas *PlpE* gene was absent in cattle as well as buffalo isolates. *ComE* gene was absent in P52 vaccine strain, while *TadE* was absent in buffalo isolate. *PfhB1* and *PlpP* genes were absent in cattle isolate, while *RcpA* and *RcpB* were absent in buffalo isolate. *ClpB* gene was absent in P52 vaccine strain and cattle isolates, whereas *TadF* was absent in buffalo and cattle isolates.

For iron metabolism, 16 genes were found, of which, *ExbB, FbpB, HbpA, HgbA, HemU, OmpW, Rfb* and *RffG* genes were present in all the five isolates studied. Genes *FbpA* and *TonB* were absent in cattle isolate, *TbpA* and TonB dependent lactoferrin and transferrin receptor were absent in goat isolate. Gene *FbpC* was absent in buffalo isolate. TonB-dependent receptor was present in poultry and goat isolates only. P52 vaccine strain was having a presence of translocation protein TolB, whereas *HemR* gene was absent.

For outer membrane proteins, out of nine genes found, *HasR, LppB, LspB, OmpH* and *PtfA* genes were present in all the five isolates of *P. multocida*. Outer membrane protein LolB was found in P52 vaccine strain as well as in cattle isolate. *VacJ* gene was absent in goat and cattle isolates. *Oma87* gene was absent only in poultry isolate. Gene *PfhA* was found present only in P52 vaccine strain.

This study is apparently the first attempt in India involving local *P. multocida* isolates from four different species and a vaccine strain for the purpose of identifying virulence genes/virulence associated genes using modern biotechnological tools like pyrosequencing based whole genome sequencing. The study aids in data of whole genome sequencing of bacterial pathogens particularly for *P. multocida* and also provides new insight into their genomic characters and possible molecular

mechanisms involved in disease process. The present findings would provide a much needed base for further screening of virulence associated genes and identification of certain markers for early diagnosis as well as characterization of *P. multocida*, which continues to pose challenges as a menace against the health management of animals. Genes which have been found in all the *P. multocida* isolates under the study can be explored as specific probes for the early diagnosis of the disease. Further, future scientific endeavors targeting the vaccine design for *P. multocida* may get a scientific support from this data, so as to formulate modern and more effective vaccines, for better animal health.

Dr. M. K. Jhala, PhD
Associate Director of Research
Anand Agricultural University
Anand - 388001
Gujarat State (India)

CERTIFICATE

This is to certify that the thesis entitled **"WHOLE GENOME SEQUENCE CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* ISOLATED FROM DIFFERENT ANIMAL SPECIES"** submitted by **VIRAL B. AHIR (Reg. No. 04-1070-2009/14)** in partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** in the subject of **ANIMAL BIOTECHNOLOGY** of the Anand Agricultural University is a record of *bona fide* research work carried out by him under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

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MAJOR ADVISOR

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ACKNOWLEDGEMENT

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Place: *Anand*
Date: *31. 03. 2015*

(Viral B. Ahir)

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ACRONYMS

%	Per cent
µL	Micro liter
°C	Degree Celsius
µg	Micro gram
µg/ml	Microgram Per Millilitre
µL	Micro liter
5S, 16S and 23S	5 Svedberg, 16 Svedberg, 23 Svedberg
Ab intio	From the Beginning
ATP	Adenosine triphosphate
BA	Blood Agar
BLAST	Basic Local Alignment Search Tool
bp	Base-Pair
CDS	Coding Domain Sequence
CPSs	Capsupar Polysachharide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
DBIM	DNA Bead Incubation Mix
ddNTPs	Dideoxy Nucleotide Triphisphate
DNA	Deoxyribonucleic acid
dsDNA	Double Stranded DNA
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetraacetic Acid
emPCR	Emulsion Polymerase Chain Reaction
et al.	Et alii
etc.	Et cet·er·a
FAO	Food and Agriculture Organization
FIGfams	Fellowship of Interpretation of Genome Families
G	Gram(S)
GOLD	Genome Online Database
Hz	Hertz
i.e.	id est (that is)
INSDSC	International Nucleotide Sequence Database Collaboration
IROMPs	Iron Regulated Outer Membrane Proteins
Lf	Lactoferrin
Lib	Library
LPS	Lipopolysachharide
M	Molar
MALDI-TOF	Matrix Assisted Laser Desorption Ionization - Time Of Flight

MCA	MacConkey Agar
Mg	Mili gram
MIDs	Multiplex Identifires
Min	Minutes
mL	Mili liter
mM	Mili molar
Mm	Mili meter
MPC	Magnetic Particle Concentrator
mRNA	Messenger Ribonucleic Acid
NCBI	National Center For Biotechnology Information
NGS	Next-generation Sequencing
nm	Nanometer
nr	Non Redundant
nt	Nucleotide
OIE	Office International des Epizootics
OMP	Outer Membrane Protein
ORF	Open Reading Frame
P. multocida	*Pasteurella multocida*
PEG	Protein Encoding Gene
PCR	Polymerase Chain Reaction
pg	Pico gram
PGAAP	Prokaryotic Genomes Automatic Annotation Pipeline
pH	Hydrogen ion concentration
PM-PCR	*Pasteurella multocida* Polymerase Chain Reaction
RAST	Rapid Annotation Subsystem Technology
RL	Rapid Library
rpm	Rotation Per Minute
sec.	Seconds
SMRT	Single Molecule Real Time
SNP	Single Nucleotide Polymorphism
spp.	Species
TBE	Tris borate EDTA
TE	Tris-EDTA
Tf	Transferrin
tRNA	Transfer Ribonucleic Acid
VF	Virulence Factors
viz.	Videlicet
WGS	Whole Genome Sequencing

DEFINITIONS

Annotation	The process of identifying the locations of genes and all of the coding regions in a genome and determining what those genes do
Assembly	It refers to the process of taking a large number of short DNA Sequences and putting them back together to create a representation of the original chromosome from which the DNA originated
Contig	It is a set of overlapping DNA segments that together represent a consensus region of DNA
Coverage	It is the number of times a nucleotide is read during the sequencing process
L50	It is the number of scaffolds that accounts for more than 50% of the genome assembly
Mapping	A method used for determining the location and relative distances between genes on a chromosome
N50	It is the length for which the collection of all contigs of that length or longer contains at least half of the sum of the lengths of all contigs
Read	Reads are obtained from the data files produced by DNA sequencing instruments. In shotgun sequencing, DNA sequences obtained from each cloned fragment of DNA and each nucleotide sequences is called a read. The reads are used later to reconstruct the original sequence
Read Length	It is the number of DNA bases sequenced
Scaffold	It consists of overlapping contigs separated by gaps of known length
Subsystem	Collection of functional roles jointly involved in a biological process or complex

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INTRODUCTION

CHAPTER I

INTRODUCTION

Animal diseases are gaining incremental significance because of their potential impact on human health and economy, their importance for conservation of rare species, and their potential for insight into the co-evolution of hosts and pathogens. Due to relatively small genomes of infectious pathogens, NGSMs (Next Generation Sequencing Methods) have been applied extensively for sequencing genomes of a range of pathogens. These include viruses such as avian influenza (Hoper *et al.,* 2009) or avian borna virus (Gancz *et al.,* 2009), bacteria such as MRSA *Staphylococcus* (Highlander *et al.,* 2007) and avian mycobacteria (Paustian *et al.,* 2008), and protozoa such as *Toxoplasma gondii* (Bontell *et al.,* 2009). These methods have also been used in genome-sequencing or genetic-association studies to identify mutations responsible for genetic disorders or diseases (ten Bosch and Grody, 2008; Vasta *et al.,* 2009). In addition, researchers are beginning to apply NGSMs for diagnosis or identification of pathogenic organisms by random screening (Adams *et al.,* 2009; Nakamura *et al.,* 2008, Nakamura *et al.,* 2009; Jones *et al.,* 2009) and by targeted methods using PCR amplicons (Jordan *et al.,* 2009). Targeted tagged pyrosequencing methods have also been used to assess variation in host immune-system genes, such as those of the Major histocompatibility complex (MHC) (Babik *et al.,* 2009; Bentley *et al.,* 2009; Wiseman *et al.,* 2009) and immunoglobulins (Glanville *et al.,* 2009).

Bacteria must retain their genetic information from one generation to the next. On the other hand, bacteria need to evolve strategies allowing the generation of new genetic diversity in order to survive and adapt to the continually changing environmental conditions and niches in which they live. Therefore, genetic plasticity

mirrors different bacterial lifestyles and physiological versatilities (Dobrindt and Hacker, 2001). There are three main forces which are shaping bacterial genomes, gene gain, gene loss and gene change.

Determination of entire genome sequences, however, is only the first step in understanding the inner working of an organism. The next critical step is to elucidate the functions of these sequences and give biochemical, physiological, and ecological meaning to the information. Sequence analysis indicates that the biological functions of substantial portions of complete genomes are unknown. Defining the role of each gene in the complex cellular machine and network is a formidable task (DeRisi *et al.*, 1997; Hieter and Boguski, 1997; Strauss and Falkow, 1997). In addition, genomes contain hundreds to thousands of genes, many of which encode multiple proteins that interact and function together as multicomponent systems or apparatuses for accomplishing specific cellular processes. The products of many genes are often coregulated in complex signal transduction networks, and understanding how the genome functions as a whole to give life to complete organisms presents an even greater challenge (DeRisi *et al.*, 1997).

The use of small subunit ribosomal RNA sequence data for understanding the evolutionary relationships of organisms was introduced by Woese in 1987. Molecular ecological studies based upon 16S rDNA sequencing, and its application to microbial ecology have revolutionised microbial ecology. 16S rDNA is too conserved to define organisms at a taxonomic level which corresponds to any appropriate concept of the species, whether a taxonomic (Goodfellow *et al.*, 1997) or a natural (Ward, 1998) species concept. 16S rDNA sequencing has provided a phylogenetic framework to understand the bacterial diversity at the level of domains, phyla down to genera (Woese, 1987). It is the initial tool in identifying species and the only tool which

enables the identification of prokaryotic "phylotypes" and their enumeration in metagenomic studies until recently when increasing amounts of data allow identification using other techniques (Venter *et al.,* 2004). But, 16S sequences are too conserved to effectively discriminate prokaryotes at the species level (Stackebrandt and Goebel, 1994).

The first sequence of an entire bacterial genome (*Haemophilus influenzae*) was published in 1995 (Fleischmann *et al.,* 1995). Since then, the total number of publicly available genome sequences has grown rapidly (http://igweb.integrated genomics.com/GOLD/) and there has been an increasing interest in the use of these genome sequence data to assess evolutionary relationships among bacterial species (Eisen, 2000; Wolf *et al.,* 2002). The total number of prokaryotic genome projects running worldwide are 32,437, out of which 3,658 are completed, 767 are chromosomes with gaps, 11,034 are in scaffold and 16,978 are in contigs (http://www.ncbi.nlm.nih.gov/genome/browse/) till date. The comparison of single genes (especially the 16S rRNA gene) to infer phylogenetic relationships among bacteria has been widely used for several decades (Wheelis *et al.,* 1992; Eisen, 1995; *Garrity* and Holt, 2001), but there has been considerable debate whether a tree based on any single gene can accurately represent the evolution of a species, considering the possibility of horizontal gene transfer (Lan and Reeves, 1996; Ochman *et al.,* 2000) and the possibility of degradation of the phylogenetic signal because of saturation for amino acid substitutions (Forterre and Philippe, 1999) as complicating factors.

The study of these genomes by both computational and experimental approaches has significantly advanced understanding of the physiology and pathogenicity of many microbes and provided insights into the mechanisms and history of genome evolution (Raskin *et al.,* 2006). The genomic DNA sequence

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encodes all of the heritable information responsible for microbial replication, virulence, host specificity, and ability to evade the immune system. A comprehensive knowledge of a pathogen's genome provides all of the necessary information required for cost-effective and targeted research into disease prevention and treatment (May *et al.,* 2001).

Whole genome amplification methods are used to generate large amounts of genomic DNA from small or precious samples. In the field of species identification, the ability to rapidly amplify genomes from limited DNA samples can significantly increase the classification accuracy by enabling the large-scale screening of several loci using high-throughput technologies. Several whole genome analysis strategies have been developed either using PCR or isothermal amplifications, each one with their own specific strengths and limitations (Pereira *et al.,* 2008).

Pasteurella multocida is a small, gram-negative, non-motile, non-spore forming coccobacillus, facultative anaerobe belonging to the family *Pasteurellaceae* with bipolar staining features having 0.2-0.4 X 0.6-2.5 μm size. It is a member of the normal flora of the upper respiratory and gastrointestinal tract of many domestic and wild animals (Zurlo, 2000). *Pasteurellaceae* family includes 15 genera with four main genera namely *Pasteurella*, *Haemophilus*, *Actinobacillus* and *Lonepinella* (Hunt *et al.,* 2000; Kuhnert and Christensen, 2008), which are obligate parasites or commensals of vertebrate animals, colonizing mainly the mucosal surface of the upper respiratory tract, oropharynx and other parts of their hosts.

Pasteurella multocida is a causative agent of several economically significant animal diseases occuring in numerous species viz: cattle, buffaloes, sheep, goats, poultry, turkeys, yak, deer, hippopotamus, mink, monkey, cat, elk, tortoise, snow

leopard, lion, rabbits, horses and camels (Shivachandra *et al.,* 2011). Serious infectious diseases which include fowl cholera, bovine hemorrhagic septicemia (HS), porcine atrophic rhinitis and lapine snuffles are caused by *P. multocida*, whereas *M. haemolytica* is the causitive agent of shipping fever or pneumonic pasteurellosis. *P. multocida* strains can be differentiated into 5 serogroups (A, B, D, E, and F), based on capsule antigens, and into 16 serotypes based on lipopolysaccharide (LPS) structures. Each serogroup produces a distinct capsular polysaccharide, with serogroups A, D and F producing capsules composed of hyaluronic acid (HA) (Cifonelli *et al.,* 1970), heparin and chondroitin (DeAngelis *et al.,* 2002), respectively. *P. mutocida* plays a secondary role in pathogenesis of the disease or acts in combination with other bacterial, viral or parasitic infections. HS is Office International des Epizooties (OIE) listed disease and case fatality approaches 100% if treatment is not followed at the initial stage of infection in infected animals in endemic areas of Africa and Asia (De Alwis, 1999; FAO, 2005; OIE, 2009). The disease has a high global index as an animal health constraint to poor farmers (Perry *et al.,* 2002).

The structures of the serogroup B and E capsules are not known but preliminary compositional analysis suggests that these capsules have a more complex structure than those produced by serogroups A, D and F (Muniandy *et al.,* 1992). Fowl cholera is generally caused by the A:1, A:3 or A:4 strains (Harper *et al.,* 2007); whereas serogroups B:2 (Asian serotype) and E:2 (African serotype) are implicated in hemorrhagic septicemia and serogroup D responsible for atrophic rhinitis (Campoy *et al.,* 2006). Hemorrhagic septicemia is endemic in most parts of tropical Asia, Africa, and India and causes high mortality in livestock (Bain *et al.,* 1982). Various other serotypes, namely A:1, A:1,3, A:3, A:4, B:1, B:2,5, B:3,4, E:2,5, F:3, F:3,4 have also

been isolated from HS outbreaks (De Alwis, 1999; Biswas *et al.,* 2004; Kumar *et al.,* 2004; Kumar *et al.,* 2009).

Haemorrhagic septicemia is considered to be the most economically important disease of livestock in South East Asia and causes significant economic losses in India and Africa (Verma and Jaiswal, 1998). Most of the oceanic countries such as Australia, New Zealand, Japan and Western Europe have not reported the disease (De Alwis, 1999). Cattle and buffalo are the most common hosts, but pigs, sheep, goats, deer, and camels are also susceptible to infection and disease (Dawkins *et al.,* 1991; Blackall *et al.,* 2000). It is presumed that HS may occur in developed countries with high water buffalo population and where conditions are similar to those of tropical Asia and Africa (Benkirane and De Alwis, 2002). In India, during the past four decades, it has been found that HS accounted for 46-55% of all bovine deaths. During the 12 years period between 1974 to 1986, it accounted for 58.7% of the aggregate of deaths due to five endemic diseases, viz. foot-and-mouth disease (FMD), rinderpest, blackquarter, anthrax and HS (Dutta *et al.,* 1990). In an active surveillance study in Sri Lanka, it was shown that in the 1970's, around 15% buffaloes and 8% cattle in the HS endemic areas died of HS annually. During the same period, the passive reporting systems recorded only 1200 to 1500 deaths a year in cattle and buffalo populations of approximately 2.5 million (De Alwis and Vipulasiri, 1981; Benkirane and De Alwis, 2002). Despite the application of advanced investigation and diagnostic techniques on the organism and the affected animal species, *Pasteurella* infections still continue to contribute to heavy losses in animal production.

Because of the economic relevance of *P. multocida* infections, research work in several fields concerning this bacterial species, such as molecular characterization of pathogenic factors and construction of mutants to be used as vaccines, is

increasing. Nevertheless, few genetic tools for *P. multocida* genetic manipulation are available (Campoy *et al.,* 2006). Little is known about the molecular basis for pathogenesis, but the genome does contain some interesting virulence functions, for example two hemagglutinins that are similar to filamentous hemagglutinins from pathogens such as *Bordetella pertusis* and *Haemophilus influenzae*. Therefore, whole genome sequencing and its analysis by the modern biotechnological and bioinformatics tools, may not only help to establish and confirm the presence and behaviour of few reported genes, but can also lead to some new insight in genome and its functional behaviour. Also, as per the literature, whole genome sequencing of *P. multocida* has apparently invited less scientific attention before, and such an scientific endeavours on local isolates of *P. multocida* from different animal species, seems to have potential for revealing novel understanding of this important pathogen causing various important diseases in animals and birds.

Considering the above perspectives, present work was undertaken with following objectives:

OBJECTIVES:

1.1 Whole genome sequence characterization and annotation of *Pasteurella multocida* from different species using pyrosequencing based 454 sequencing technology.

1.2 Identification of virulence associated genes from *Pasteurella multocida* of different species.

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REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Bacteria are the most abundant group of organisms and a major cause of animal diseases and mortality. Bacterial cells account for most of the earth's biomass (Whitman *et al.,* 1998). Successful colonization of a host is essential to the life cycle of the pathogen, and the dynamics of the host-pathogen interaction determine the outcome of the interaction, including the severity of disease (Wilson, 2012).

Bacterial genomes display variation in size, even among strains of the same species. As the microorganisms have very little non-coding or repetitive DNA, the variation in genome size usually reflects differences in gene repertoire. Some species, particularly bacterial parasites and symbionts, have undergone massive genome reduction and simply contain a subset of the genes present in their ancestors (Moran, 1996). However, in free-living bacteria, such gene loss can not explain the observed disparities in genome size because ancestral genomes would have had to contain improbably large numbers of genes. A substantial fraction of the difference in gene contents in free-living bacteria is due to the presence of ORFans, that is, open reading frames (ORFs) that have no known homologs and are consequently of no known function (Fischer and Eisenberg, 1999).

Pasteurella multocida is capable of causing disease in a wide range of animals and birds and subdivided into four subspecies; *multocida*, which includes the type species and three other viz. *gallicida*, *septica* and *tigris* (Boyce *et al.,* 2010). There is a clear correlation between capsular type and the disease, with serotypes A and F typically associated with fowl cholera, serotypes B and E with haemorrhagic septicaemia in cattle and serotype D strains expressing PMT toxin with atrophic

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rhinitis in swine. Unclearity is whether the expression of a particular type of lipopolysaccharide or the type and amount of proteins presented on the bacterial surface also contribute to the disease specificity (Harper *et al.*, 2006).

Table 2.1: Diseases caused by *Pasteurella multocida*

Disease	Capsular serogroup	Species affected	Virulence factors
Animal bite associated sepsis	Usually not determined, but where given usually A, occasionally D	Any, but most commonly humans, cats, dogs	Not established
Haemorrhagic septicaemia	B and E	Cattle, buffalo, pigs,goats, deer, camels, rarely horses	Capsule, fimbriae, filamentous haemagglutinin
Avian cholera	A, F and very rarely D	Chickens, turkeys, ducks, wild birds	Capsule, lipopolysaccharide (LPS), iron acquisition proteins, fimbriae, filamentous haemagglutinin, sialic acid uptake
Atrophic rhinitis	D and rarely A	Pigs and rabbits	*Pasteurella multocida* toxin (PMT)
Snuffles	A and D	Rabbits	Not established
Enzootic pneumonia, shipping fever	A, D and rarely F	Cattle, sheep and pigs	Not established. Co-infection with other bacterial or viral species important

(Wilkie *et al.*, 2012)

The cell surface protects Gram-negative bacteria against a range of harsh environments and is critical for interaction of the bacterium with the host. Virulent *P. multocida* strains produce a polysaccharide capsule. The serogroups A and B capsule contains hyaluronic acid, while serogroups D and F capsules contain heparin and chondroitin, respectively. The outer membrane (OM) functions as a selective barrier that prevents the entry of many toxic molecules into the cell, a property that is crucial for bacterial survival in many environments (Hatfaludi *et al.*, 2010).

P. multocida is a commensal organism in many mammalian hosts. However, it can potentially cause disease in almost all species if it gains access to the blood stream. Pathogenesis is a result of complex interactions between specific host factors (e.g. species, age, immune status) and specific bacterial virulence factors (e.g. LPS, capsule, adhesins, OM proteins). Therefore, disease pathogenesis is versatile and depends on the bacterial strain, the animal model and the changing response of both the host and bacteria to the interaction (Boyce *et al.,* 2010).

Little progress is made in understanding exactly how *P. multocida* invades mucosal surfaces to gain access to the blood and how the host responds to the infection. However, significant advances have been made in identifying bacterial factors critical to *P. multocida* pathogenesis. The capsule and lipopolysaccharide are essential for normal disease progression in fowl cholera and there is some progress in understanding the bacterial response to the *in vivo* environment (Boyce *et al.,* 2004) and identifying bacterial factors required for disease progression (Fuller *et al.,* 2000; Harper *et al.,* 2003).

2.1 VIRULENCE FACTORS OF *P. multocida*

Discovering virulence factors of pathogenic bacteria is a key in understanding pathogenesis and for identification of targets for novel drugs and design of new vaccines. Comparative genomics, transcriptomics and proteomics have become the popular tools in discovering the virulence factors in bacterial pathogens. Once the putative virulence factors are identified by genomics and/or proteomics, their functions and mechanisms can be further investigated by phenotypic analyses including mutagenesis, biochemical methods and/or structural biology. Combination

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of these techniques will accelerate the developments of therapeutic drugs and vaccines in combating bacterial diseases (Wu *et al.,* 2008).

Comparative genomics is a popular tool to identify virulence factors and genes involved in environmental persistence of pathogens. The goal is to correlate those differences to biological function and to gain insight into selective evolutionary pressures and patterns of gene transfer or loss, particularly within the context of virulence in pathogenic species. Comparisons can be performed either with genome sequence or by using microarray-based methods (Raskin *et al.,* 2006).

In essence, the ability of pathogenic bacteria to cause disease in a susceptible host is determined by multiple virulence factors acting individually or together at different stages of infection. Virulence factors are often involved in direct interactions with the host tissues or in concealing the bacterial surface from the host's defense mechanisms. Chen *et al.* (2005) created a database called virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/). Bacterial genome sequences rapidly add candidate virulence genes to such databases.

Bacterial virulence factors can be divided into several groups on the basis of the mechanism of virulence and function. These are (1) membrane proteins, which play roles in adhesion, colonization and invasions, promote adherence to host cell surfaces, are responsible for resistance to antibiotics, and promote intercellular communication; (2) Polysaccharide capsules that surround many bacterial species and have anti-phagocytic property; (3) Secretory proteins, such as toxin, which can modify the host cell environment and are responsible for some host cell-bacteria interactions (Wu *et al.,* 2008).

There are many virulence factors which are responsible for the diseases due to *Pasteurella multocida* in animals and birds, which are described below.

2.1.1 Capsule

Capsules are a common bacterial virulence determinant and are associated with pathogenesis and immunity in many genera of bacteria (Finlay and Falkow, 1989; Moxon and Kroll, 1990). *Pasteurella multocida* can be classified by serological methods into five capsule groups designated A, B, D, E and F. The composition and structure of the capsular material found in *P. multocida* serotypes A, D and F are very similar to mammalian glycosaminoglycans and consist mainly of hyaluronan, heparosan and unsulphated chondroitin, respectively (Pandit and Smith, 1993; Rimler, 1994; DeAngelis, 1996; DeAngelis and Padgett-McCue, 2000). The genes required for the synthesis and transport of these capsular types are encoded within a single region on the genome (Townsend *et al.,* 2001). In type A, D and F strains, an additional gene encoding a heparosan synthase was identified outside of the known capsule biosynthesis region. The synthase encoded by this gene, renamed *hssB* (formerly *pglA*), is transcribed 10-fold less than the synthase within the capsule operon, uses a different acceptor and gives rise to smaller molecular weight polymer products and the expression of this gene may give rise to capsular variation (DeAngelis and White, 2004).

Generally strains that possess a capsule are more virulent than their acapsular variants (Heddleston *et al.,* 1964; Snipes *et al.,* 1987; Tsuji and Matsumoto, 1989). The important role of capsule in the pathogenesis of *P. multocida* has been clearly demonstrated as genetically defined, acapsular mutants constructed from both serogroup A and B strains were strongly attenuated in mice (Boyce and Adler, 2000;

Chung *et al.,* 2001). The serogroup A acapsular mutant was also shown to be avirulent in chickens and was unable to establish growth in chicken muscle (Chung *et al.,* 2001). Despite its apparent lack of persistence, vaccination of chickens with high doses of this acapsular mutant stimulated protective immunity (Chung *et al.,* 2005). Immunity is generally serotype specific. Empirically derived, live, avirulent strains have been used as vaccines in both poultry and cattle (Derieux, 1984; Myint *et al.,* 1987), but the basis for attenuation is not known and therefore reversion to virulence occurs (Hofacre and Glisson, 1986). However, a major advantage of live, attenuated vaccines is the fact that they are able to stimulate cross-protection against different *P. multocida* serotypes.

Capsule plays a significant role in resistance to phagocytosis, and this has been demonstrated *in vitro* by Harmon *et al.* (1991) and others, who have correlated sensitivity to phagocytosis with the presence and thickness of the bacterial capsule (Truscott and Hirsh, 1988; Harmon *et al.,* 1991; Pruimboom *et al.,* 1996). Studies using murine macrophages clearly demonstrated that a genetically defined acapsular serotype B mutant was more susceptible to uptake than its wild-type parent (Boyce and Adler, 2000). Resistance to complement-mediated lysis is clearly important for virulence and experiments on *P. multocida* type A strains have shown that serum resistance correlates with the possession of a capsule (Snipes and Hirsh, 1986; Hansen and Hirsh, 1989). A genetically defined acapsular mutant was no longer serum resistant in normal avian serum compared with the serotype A wild-type parent and complemented mutant (Chung *et al.,* 2001). Jaques *et al.* (1993) reported non-capsulated variants of atrophic rhinitis strains of *P. multocida*, which were shown to have reduced virulence for mice and piglets.

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Arumugam *et al.* (2011) isolated 114 strains of *Pasteurella multocida* from different domestic animal species (cattle, buffalo, sheep, goat, pig, rabbit, dog, cat), avian species (chicken, duck, turkey) and wild animals (deer, tiger, orang utan, marmoset). The serogroups of *P. multocida* were determined by both conventional capsular serotyping and a multiplex PCR assay targeting specific capsular genes. Based on the conventional serotyping method, 114 strains of *P. multocida* were subtyped into 55 species-specific (untypeable strains) *P. multocida*, 15 serogroup A, 23 serogroup B and 21 serogroup D. Based on the multiplex PCR assay on the specific capsular genes associated with each serogroup, 114 strains were further divided to 22 species-specific *P. multocida* (KMT1 - 460 bp), 53 serogroup A (A - 1,044 bp), 33 serogroup B (B - 760 bp) and 6 serogroup D (D - 657 bp). No serogroup E (511 bp) or F (851 bp) was detected among the Malaysian *P. multocida* isolates.

2.1.2 Lipopolysaccharide

Lipopolysaccharide stimulates humoral immunity and is considered to be a protective antigen. Wijewardana *et al.* (1990) raised monoclonal antibodies (MAb) against the lipopolysaccharide from a serotype A strain, which were bactericidal and protected mice against homologous challenge. An opsonic monoclonal antibody against lipopolysaccharide from a serotype B strain of *P. multocida* partially protected mice against *P. multocida* infection (Ramdani and Adler, 1991). Intravenous inoculation of lipopolysaccharide from serotype B:2 strains could reproduce clinical signs of haemorrhagic septicaemia in buffalo (Horadagoda *et al.*, 2002), but the endotoxic properties of lipopolysaccharide from serotype A strains was less clear. Various studies have indicated that chicken embryos and mice are highly susceptible, but that turkey poults are relatively resistant (Ganfield *et al.*, 1976; Rhoades and Rimler, 1987; Mendes *et al.*, 1994).

P. multocida requires a complete lipopolysaccharide structure in order to replicate *in vivo* and cause disease (Harper *et al.,* 2004). A *galE* mutant of *P. multocida* had significantly reduced viability in mice. The role of *galE* in bacteria is the epimerization of UDP-glucose to UDP-galactose before lipopolysaccharide assembly, and this mutant probably expressed an altered lipopolysaccharide (Fernandez de Henestrosa *et al.,* 1997).

The complete lipopolysaccharide structure of strains VP161 and X73 belonging to Heddleston serotype 1 and Pm70, a Heddleston serotype 3 strain, was determined and all possessed a structure similar to the lipopolysaccharide or lipo-oligosaccharide (LOS) of *Neisseria* spp. and *Haemophilus* spp. The *P. multocida* lipopolysaccharide isolated from the two Heddleston type I strains contains terminal phosphocholine residues, which in other bacteria plays a key role in bacterial adhesion to, and invasion of, host cells by binding directly to the platelet-activating factor (PAF) receptor (Cundell *et al.,* 1995; Schenkein *et al.,* 2000; Swords *et al.,* 2000; Serino and Virji, 2002).

In addition to phosphocholine residues, all the *P. multocida* strains have phosphoethaolamine residues attached to various sites on their lipopolysaccharide (St Michael *et al.,* 2005). In other Gram-negative bacteria, phosphoethaolamine is added to a number of sites within the inner core of lipopolysaccharide by specific transferases, and in *Neisseria meningitidis*, the expression and position of these residues affects the ability of the bacteria to resist the innate immune response (Ram *et al.,* 2003).

2.1.3 Fimbriae and adhesins

Attachment to host cells and/or extracellular matrix proteins is a primary prerequisite for bacterial infections; adhesins that mediate such adherence are potential virulence factors. There are many genes, including *ptfA*, *fimA*, *flp1*, *flp2*, *hsf-1* and *hsf-2* on *P. multocida* genome that encode proteins similar to fimbriae or fibrils in other bacteria. Fimbriae play a role in surface adhesion, as fimbriae have been observed on some *P. multocida* serotype A strains that were able to adhere to mucosal epithelium, but not on the surface of those strains unable to adhere (Glorioso *et al.,* 1982; Rebers *et al.,* 1988; Isaacson and Trigo, 1995; Ruffolo *et al.,* 1997). Type IV fimbriae (pili) have been isolated and characterized from *P. multocida* serotypes A, B and D (Ruffolo *et al.,* 1997) and are often associated with virulence in other bacteria because of their role in attachment to host cell surfaces. Type IV fimbriae have been used as vaccines against ovine footrot and bovine keratoconjunctivitis caused by *Dichelobacter nodosus* and *Moraxella bovis*, respectively (Hatfaludi *et al.,* 2010).

The subunit gene, *ptfA*, has been isolated and sequenced from a number of strains and the predicted protein sequences showed significant variation between strains (Doughty *et al.,* 2000). Siju *et al.* (2007) cloned, sequenced and expressed in *E. coli* the *ptfA* gene from *P. multocida* serogroup B:2. Sequence alignment showed that the *P. multocida* B:2 and A:1 genes were 78.4% identical at the nucleotide level, with the first 200 bp being 100% identical. Rabbit antiserum raised against the recombinant PtfA (serotype B:2) protein detected an 18 kDa protein in *P. multocida* B:2, A:1 and *Pseudomonas aeruginosa* whole cell lysates. However, the role of fimbrial structures in *P. multocida* virulence is still unproven.

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P. multocida Pm70 genome contains a region encoding proteins with significant similarity to the *Flp* pilin locus in *Actinobacillus actinomycetemcomitans*, encoding a type IV fimbrial subfamily (Kachlany *et al.,* 2001[b]; May *et al.,* 2001). This locus encodes proteins predicted to be the Flp pilin subunits and the proteins required for the pilin assembly. *Flp* pili gene products in *P. multocida* are required for virulence in mice. Two genes, *flp1*, encoding a Flp pilin subunit and *tadD*, predicted to encode a component of the secretion apparatus required for Flp pilin assembly, have been inactivated in two independent signature tagged mutagenesis (STM) studies and both mutants were significantly attenuated in mice (Kachlany *et al.,* 2001[a]; Kachlany *et al.,* 2001[b]; Harper *et al.,* 2003; Wilkie *et al.,* 2012).

Two *P. multocida* genes, *pfhaB1* and *pfhaB2*, share significant similarity with a class of genes that encode filamentous haemagglutinins, which in *Bordetella pertussis* play a major role in the colonization of the upper respiratory tract (Kimura *et al.,* 1990; Mooi *et al.,* 1992). Mutation of these genes in *P. multocida* resulted in significantly reduced virulence in mice (Fuller *et al.,* 2000). A *pfhaB2* mutant was constructed in a fowl cholera strain, P1059, and shown to be highly attenuated in turkeys when administered intranasally, but only moderately attenuated when given intravenously, suggested a significant role of *pfhaB2* in initial colonization or invasion (Tatum *et al.,* 2005).

2.1.4 Toxins

Most *P. multocida* strains that cause fowl cholera, haemorrhagic septicaemia or pneumonia are not known to express any toxins. The dermonecrotic toxin, PMT, expressed mainly by serogroup D strains, is the only toxin identified to date and is responsible for the clinical and pathological signs of atrophic rhinitis (Foged *et al.,*

1987; Rimler and Rhoades, 1989). Both purified native and recombinant PMT toxin can be used to experimentally induce clinical signs of the disease (Foged *et al.,* 1987; Lax and Chanter, 1990).

In pigs, this toxin leads to atrophy of nasal turbinates, where bone resorption occurs due to uncontrolled proliferation of osteoclasts, and regeneration of bone is prevented by the inhibition of osteoblasts (Sterner-Kock *et al.,* 1995; Mullan and Lax, 1998). PMT is also a potent mitogen, inducing many cellular effects including rearrangements in the actin cytoskeleton (Zywietz *et al.,* 2001). Like cholera and pertussis toxins, PMT activates dendritic cells to mature cells but, unlike the other toxins, it is a poor adjuvant and appears to suppress the antibody response (Bagley *et al.,* 2005). PMT blocks chemotaxis-induced migration of dendritic cells to regional lymph nodes and might, therefore, in a natural infection, limit the development of an adaptive immune response (Blocker *et al.,* 2006).

The PMT toxin gene resides on a lysogenic bacteriophage belonging to the *Siphoviridae* family. As the PMT toxin has no signal sequence and no known mechanism of export, the lytic phase of the bacteriophage may mediate release of the toxin (Pullinger *et al.,* 2004). The toxin is an effective immunogen, a toxoid developed by deletion mutagenesis of the cloned PMT toxin gene was found to protect mice and their offspring against challenge with purified PMT (Petersen *et al.,* 1991). A genetically modified PMT toxin, where there were two key amino acid substitutions, led to a non-toxigenic protein that protected pigs against experimental challenge with the wild-type strain (To *et al.,* 2005).

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2.1.5 Iron regulated and iron acquisition proteins

Iron is an essential element which must be acquired by bacteria in order to survive. Because of its inherent toxicity, the level of free iron available *in vivo* is very limited and *P. multocida,* like other bacterial species, has developed multiple mechanisms for iron uptake. Sequence analysis of *P. multocida* Pm70 revealed that a relatively large proportion of the genome (over 2.5%) encodes 53 proteins with similarity to proteins involved in iron uptake or acquisition (May *et al.,* 2001). Whole genome microarray studies have identified a large number of genes (up to 12%) with altered expression under iron-limiting conditions or in response to defined sole iron sources (Paustian *et al.,* 2001; Paustian *et al.,* 2002). Analysis of the outer membrane sub-proteome under iron-limiting conditions identified only two proteins, OmpW and Pm803, with increased expression (Boyce and Adler, 2006).

Iron, essential for bacterial growth, is largely unavailable *in vivo* as a result of binding by host proteins such as transferrin and lactoferrin. Pathogenic bacteria, such as *P. multocida,* overcome these severe iron limitations by expressing a number of iron regulated proteins and low molecular weight siderophores which are able to sequester iron from the host proteins. Proteins which are expressed under iron-limiting conditions may include outer membrane proteins (OMPs) involved in iron acquisition or other virulence factors such as toxins and haemolysins (Chawak *et al.,* 2001).

Comparisons of *P. multocida* grown in iron-rich, iron-depleted media or *in vivo* has demonstrated that many high molecular weight outer membrane proteins are regulated by iron levels and have therefore been called iron-regulated outer membrane proteins (IROMPs) (Snipes *et al.,* 1988; Choi-Kim *et al.,* 1991). *P. multocida* grown

under iron-limited conditions induces a stronger protective response in mice compared with the same strain grown under iron-replete conditions (Kennett *et al.*, 1993). IROMPs therefore play a significant role in cross-protective immunity (Glisson *et al.*, 1993; Ruffolo *et al.*, 1998).

Transferrin receptors utilized by bacterial species in *Pasteurellaceae* and *Neisseriaceae* families usually consist of two iron binding receptors TbpA and TbpB (Gray-Owen and Schryvers, 1996). The transferrin receptor in bovine strains of *P. multocida* is composed of only a single protein TbpA (Ogunnariwo and Schryvers, 2001). However, this receptor may not be present in all *P. multocida* strains. PCR and DNA hybridization study found that the *tbpA* gene is present only in some bovine and ovine clinical isolates (Ewers *et al.*, 2006).

Iron acquisition proteins are believed to play a role in the disease process; serogroup A mutants with inactivated ExbB, ExbD, TonB or HgbA proteins are attenuated in mice (Fuller *et al.*, 2000; Bosch *et al.*, 2002[a]). ExbB, ExbD and TonB are part of the TonB transport complex, required to transport and provide energy for iron sequestration and HgbA is predicted to be a haemoglobin-binding protein required for the acquisition of iron from host proteins (Bosch *et al.*, 2002[b]). Expression of *P. multocida hgbA* gene increased six fold under iron limitation (Paustian *et al.*, 2001) and also during growth in blood of infected chickens (Boyce *et al.*, 2002). Several of these iron-acquisition proteins have been tested as potential vaccine antigens; none has elicited protection (Boyce *et al.*, 2010).

Haemoglobin-binding protein of 113.5 kDa, designated HgbB from *P. multocida* was identified by Cox *et al.* (2003). Bioinformatics analysis of HgbB revealed the presence of putative Fur and TonB binding sites. However, inactivation

of the *hgbB* did not result in reduced bacterial virulence in mice, nor did it decrease the ability of *P. multocida* to bind haemoglobin. Furthermore, when used as a vaccine antigen, recombinant insoluble HgbB did not confer any protection to mice against wild-type infection.

The iron component of haemoglobin, haem, can be utilised as a source of essential iron by many microorganisms. Pathogenic bacteria commonly secrete exotoxins such as haemolysin that can lyse cells and release haem. *P. multocida* has been shown to lyse erythrocytes under anaerobic conditions (Krewulak and Vogel, 2008).

Adler *et al.* (1999) demonstrated that OMPs produced under iron-limiting conditions, mimic the concentration of iron *in vivo*, could induce cross-protective immunity in mice whereas, OMPs produced under iron replete conditions stimulated only homologous protection (Ruffolo *et al.*, 1998).

2.1.6 Sialic acid metabolism

Many pathogenic bacteria have evolved mechanisms to evade recognition by the immune system based on the surface exposure of sialic acid. A common theme in this host-mimicry is the incorporation of sialic acid as a component of either the capsular polysaccharide or LPS. It has been hypothesised that sialidases contribute to the virulence of some pathogenic organisms, especially those that inhabit and invade mucosal surfaces (Schauer, 2009). Sialidases act to remove sialic acid from host glycosylated proteins and lipids for use as a carbon source. These enzymes also enhance bacterial virulence by unmasking key host receptors and/or reducing the effectiveness of host defences such as mucin. Most *P. multocida* strains produce

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sialidase both as cell bound and as extracellular sialidases (Scharmann *et al.,* 1970; Drzeniek *et al.,* 1972; White *et al.,* 1995).

Two sialidases *NanH* and *NanB* have been cloned and characterized from a fowl cholera isolate of *P. multocida* (Mizan *et al.,* 2000). These sialidases differed in their specificity, with both able to utilize 2,3′ sialyl lactose, but only *NanB* was able to fully utilize 2,6′ sialyl lactose. The presence of two sialidases with slightly different specificities would enhance the metabolic capacity of *P. multocida* in the host (Mizan *et al.,* 2000).

P. multocida is capable of scavenging sialic acid from the environment for both the sialylation of cell components and for nutrients via a catabolic breakdown pathway. The uptake, but not catabolism, of sialic acid was shown to be essential for virulence in mice (Steenbergen *et al.,* 2005). Two genes, *pm0188* and *pm0508*, encoding sialic acid transferases, have been identified in the *P. multocida* genome (Yu *et al.,* 2005).

2.1.7 Hyaluronidase

Although, the role of hyaluronidase in pathogenesis of *P. multocida* infections has not been determined, it is present in many of the serotype B strains of *P. multocida* that cause bovine haemorrhagic septicaemia.

A study of 74 *P. multocida* strains representing all capsular serotypes found that only the type B strains, isolated from haemorrhagic septicaemia infections, produced hyaluronidase (Carter and Chengappa, 1980). Another study of 176 strains of *P. multocida* representing different serotypes also found hyaluronidase activity confined to serotype B, but more specifically B:2, and it was suggested that a test for

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hyaluronidase activity could be used to presumptively identify B:2 strains (Rimler and Rhoades, 1994).

2.1.8 Outer membrane proteins

Outer membrane proteins (OMPs) have been separated into OM integral and lipoproteins, and then further separation of these two groups into functional categories. Bacterial OM lipoproteins have been grouped according to functional type either as structural proteins, enzymes, receptors or transporters performing essential functions at the membrane-aqueous interface (Babu *et al.,* 2006). For integral membrane proteins, the functional categories include: small b-barrel membrane anchors, nonspecific porins, specific channels, energy-dependent transport-efflux, energy-dependent transporter-influx, protein secretion pore, OM usher proteins, adhesins, membrane integral enzymes and protein pore-forming toxins (Nikaido, 2003; Wimley, 2003).

A protein of 39 kDa was identified in *P. multocida* A:3 strain P1059 and its expression was shown to correlate with the presence and amount of capsule present on the cell (Borrathybay *et al.,* 2003[a]; Ali *et al.,* 2004[a]). *P. multocida* adhere and invade chicken embryo fibroblasts, and this adherence was inhibited by both monoclonal and polyclonal antibodies raised against the 39 kDa protein (Borrathybay *et al.,* 2003[b]; Ali *et al.,* 2004[a]; Ali *et al.,* 2004[b]). Passive immunization of mice with a monoclonal antibody against the 39 kDa protein or active immunization with affinity purified 39 kDa protein, demonstrated that antibodies raised against this protein were cross-protective against serovars A:1 and A:3 (Ali *et al.,* 2004[a]; Ali *et al.,* 2004[b]). This protein was identified as PlpB (*Pasteurella* lipoprotein B), using peptide mass fingerprinting (Tabatabai and Zehr, 2004) and is predicted to be an ABC (ATP-

binding cassette) transport protein required for the uptake of methionine into the cell (Merlin *et al.,* 2002).

One of the major outer membrane proteins of *P. multocida* is OmpH. Antibodies raised against this protein provide some protection against the disease. Monoclonal antibodies specific for OmpH passively protected mice against *P. multocida* challenge (Marandi and Mittal, 1997) and vaccination with the native, but not recombinant, OmpH protein elicited protective immunity in birds against homologous challenge (Luo *et al.,* 1997). In addition, antibodies raised to an OmpH synthetic peptide, Cyclic-L2, provided partial protection in chickens against homologous challenge (Luo *et al.,* 1999). OmpH expression is regulated by the Fur (ferric uptake regulator) protein and responds to glucose and iron concentrations (Bosch *et al.,* 2001). Garrido *et al.* (2008) identified two copies of OmpH (OmpH1 and OmpH2) in serogroup A strain PM108.

Examining the ability of *P. multocida* to bind host extracellular matrix proteins have shown that the bacteria can adhere to fibronectin and collagen type IX. Proteins identified as possible adhesins include OmpA, Oma87, Pm1069 and the iron related proteins, Tbp (transferrin binding protein) and the putative TonB receptor HgbA (Dabo *et al.,* 2005). Bovine *P. multocida* strains are able to utilise iron from bovine transferrin, but not from transferrin of other species, whereas, avian strains are unable to capture iron from any transferrin molecules including those from avian source (Hatfaludi *et al.,* 2010). *P. multocida* has a single, novel TbpA receptor capable of efficiently mediating iron acquisition from bovine transferrin without the involvement of a second receptor protein TbpB.

Veken *et al.* (1994) examined *P. multocida* B:2,5 haemorrhagic septicaemia strains for TbpA homologues. An 82 kDa protein was present in B:2,5 strains and was found to bind bovine transferrin. However, no proteins that bound transferrin could be isolated from a B:3,4 serotype strains. Shivachandra *et al.* (2005) amplified the *tbpA* gene from chicken (A:1), buffalo (B:2), pig (D:1) and duck (F:3) *P. multocida* strains. Sequence analysis of the *tbpA* gene from serotype B:2 showed 98% identity to the A:1 strain. However, Ewers *et al.* (2006) analysed 289 strains for distribution of virulence genes and no *tbpA*-specific PCR fragment was amplified from avian or pig strains. The *tbpA* gene was present in only 31.5% of the strains. In this study, *tbpA* was found exclusively in isolates from bovine (70.2% of all tested bovine strains), sheep (80%) or buffalo (57.1%) strains.

Another 30.1 kDa PlpB protein involved in methionine binding, represents the periplasmic binding component of a methionine uptake ABC transporter. PlpB was initially identified as a cross-protective antigen, recombinant PlpB failed to protect either mice or chickens against *P. multocida* challenge, despite stimulating an antibody response (Wu *et al.*, 2007), but later found that the cross-protective antigen *is* PlpE. Immunisation with recombinant *P. multocida* PlpE conferred protective immunity, with 80-100% of mice and 63-100% of chickens protected against heterologous challenge (Wu *et al.*, 2007). PlpE is the first *P. multocida* recombinant protein to stimulate high level cross-serotype protective immunity.

2.1.9. Transcriptional regulators

Fis is a growth phase-dependent, nucleoid-associated protein which plays a role in the transcriptional regulation of a number of genes in diverse bacterial species (Grainger and Busby, 2008). In *Escherischia coli*, Fis is expressed at high levels in

actively growing cells (50,000 molecules per cell in early exponential growth phase) and expression drops to very low levels during stationary phase (Ball *et al.,* 1992; Bradley *et al.,* 2007). In addition to growth phase regulation, levels of Fis are negatively regulated by the stringent response during nutrient starvation (Ninnemann *et al.,* 1992). Fis can act as both, a positive or negative regulator of transcription and it has both direct and indirect effects on gene transcription. In *E. coli* and *Salmonella*, Fis binds to a degenerate 15-bp consensus sequence GNtYAaWWWtTRaNC, inducing DNA bending, but only a few of the sequences fitting this consensus are high affinity binding sites (Pan *et al.,* 1996; Shao *et al.,* 2008). Fis is involved in the regulation of genes encoding a wide range of functions, including quorum sensing in *Vibrio cholerae* (Lenz and Bassler, 2007), and certain virulence factors in pathogenic *E. coli* (Goldberg *et al.,* 2001; Kelly *et al.,* 2004) and *Salmonella* (Kelly *et al.,* 2004) and *Erwinia chrysanthemi* (Saldana *et al.,* 2009). The absence of functional Fis protein led to the loss of capsule expression in *P. multocida.* The presence of functional Fis protein is also required for the expressions of virulence gene *pfhB2* and its predicted secretion partner *lspB2* in *P. multocida* (Steen *et al.,* 2010).

Other virulence factors such as neuraminidases, iron sequestering proteins and metabolic enzymes play key roles in acquiring and utilizing substrates for growth within the host, often a relatively nutrient poor and hostile environment (Harper *et al.,* 2006).

Adler *et al.* (1999) identified potential protective antigens or virulence genes to be used as candidates for attenuating mutations or as the basis for vaccine antigen delivery systems. The gene encoding an outer membrane protein, Oma87, Type 4 fimbriae, fimbrial subunit protein PtfA, four *bex* genes involved in capsule transport, transferrin binding protein Tbp1, *mesA* gene responsible for haemolytic phenotype

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under anaerobic conditions were studied using different approaches for their use as candidate vaccine antigens.

Ewers *et al.* (2006) isolated 289 strains from *Pasteurella multocida* wild type and reference strains viz. cattle (104), small ruminants (15), buffaloes (7), swine (52), rabbits (20), poultry (20), cats (54), dogs (8), human (2), and of unknown host origin (7) from various clinically healthy and diseased hosts for detecting capsule biosynthesis genes (*capA, B, D, E* and *F*) and 14 virulence associated genes. Capsule type A strains were highly adapted to bovines (92.3%) and poultry (85.7%), while *capA* (34.9%) and *capD* (58.1%) positive strains in swine. *capD* positive strains also from small ruminants (34.9%) and *capF* was detected in wild type strains from diseased cattle (2.2%) and cats (7.4%). None of the isolates harboured *capE*, while *capB* was exclusively found in all strains from buffaloes. Nearly all isolates showed a combination of genes encoding outer membrane proteins, colonization factors, iron aquisition factors and superoxide-dismutases without any clue for host specificity. In contrast, the transferrin binding protein encoding gene *tbpA* (31.5%) was limited to ruminant strains and only 37.0% of all *P. multocida* strains harboured *pfhA*, coding for a filamentous hemagglutinin, supposed to be a putative adhesion and serum resistance factor. *PfhA* revealed a strong positive association to the outcome of disease in bovine hosts and in combination with *toxA* to that in swine. The dermonecrotoxin encoding *toxA*, present in 12.5% of all strains, was detected in isolates from swine, small ruminants, cattle and poultry. A significant association to the disease status, however, was only existent in swine, although with 66.7% found a notably high prevalence of the toxin gene among strains from small ruminants. The genes *toxA, tbpA* and *pfhA* as well as capsule biosynthesis genes were supposed to be important epidemiological marker genes for characterizing *P. multocida* field strains.

Atashpaz *et al.* (2009) developed a reliable multiplex PCR method for rapid detection of four virulence genes, i.e., *tbpA, pfhA, toxA* and *hgbB* to determine the epidemiological correlation between *P. multocida* infection and existence of virulence genes in *P. multocida.* Eighty seven strains of *P. multocida* isolated from various clinically healthy and infected hosts were examined by uniplex PCR method for each virulence associated genes. For rapid detection of four virulence genes, they developed a multiplex PCR method to benefit the epidemiological investigations.

Tang *et al.* (2009) isolated a total of 233 of *P. multocida* isolates obtained from 2,912 cases of clinical respiratory disease in pigs in China. Serogroup A, *P. multocida* isolates were obtained from 92 cases (39.5%), and serogroup D isolates from 128 cases (54.9%); 12 isolates (5.2%) were untypeable. All the isolates were characterized for their susceptibilities to 20 antibiotics and the presence of 19 genes for virulence factors (VFs). Use of PCR showed that colonization factors (*ptfA, fimA,* and *hsf-2*), iron acquisition factors, sialidases (*nanH*) and outer membrane proteins occurred in most porcine strains. The VFs *pfhA, tadD, toxA,* and *pmHAS* were each present in <50% of strains. Various VFs exhibited distinctive associations with serogroups: concentrated in serogroup A, concentrated in serogroup D, or occurring jointly in serogroups A and D.

Garcia *et al.* (2011) obtained 205 *P. multocida* isolates from pigs, which were phenotypically and genetically characterised by determining their biovar, capsular type, virulence-associated genes and pulsed-field gel electrophoresis (PFGE) profiles. All the isolates were identified as *P. multocida* subspecies *multocida* and most were assigned to biovar 3 (58%) and biovar 2 (39.5%). Biovar 1 represented 24% of the isolates. According to the capsular type, a great majority of the isolates (79.0%) belonged to capsular type A, 18.5% belonged to capsular type D and 24% were of

capsular type F. All the isolates harboured *ompH, psl, oma87, ptfA, nanB, nanH, tonB, hgbA, sodA* and *sodC* genes, while none of them possessed the transferrin-binding protein gene *tbpA*. The prevalence of *toxA, pfhaA* and *hgbB* genes was variable (7.8, 40.5 and 60.5% of the isolates, respectively). After PFGE typing, isolates of biovar 2 and 3 were grouped in two different clusters (A and B) at a level of 45 per cent similarity. In addition, isolates of biovar 2 and 3 exhibited statistically significant differences ($P<0.05$) in the virulence-associated *hgbB* and *pfhA* genes (biovar 3 was *hgbB+ pfhA-*, while biovar 2 was *hgbB- pfhA+*).

Furian *et al.* (2013) investigated 12 genes related to virulence in 25 samples of *P. multocida* isolated from fowl cholera cases in southern region of Brazil through the development of multiplex PCR protocols. The *ompH, oma87, sodC, hgbA, hgbB, exBD-tonB* and *nanB* genes were present in 100% of the samples (25/25), the *sodA* and *nanH* genes were present in 96% (24/25), *ptfA* was present in 92% (23/25), and *pfhA* was present in 60% (15/25). Gene *toxA* was not identified in any of the samples. Five different genetic profiles were obtained, of which P1 (negative to *toxA*) was the most common.

Verma *et al.* (2013) performed isolation of 23 isolates of *P. multocida,* obtained from 335 cases of various clinically healthy and diseased cattle. These isolates were examined for capsule synthesis genes (*capA, B, D, E* and *F*) and 11 virulence associated genes (*tbpA, pfhA, toxA, hgbB, hgbA, nanH, nanB, sodA, sodC, oma87* and *ptfA*) by PCR. A total of 19 *P. multocida* isolates belonging to capsular type B and 4 of capsular type A were isolated. All the isolates of capsular type B harboured the virulence associated genes: *tbpA, pfhA, hgbA, sodC* and *nanH*, coding for transferrin binding protein, filamentous hemagglutinin, haemoglobin binding protein, superoxide dismutase and neuraminidases, respectively; while isolates

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belonging to capsular type A also carried *tbpA, pfhA, hgbA* and *nanH* genes. Only 50% of capsular type A isolates contained *sodC* gene while 100% of capsular type B isolates had *sodC* gene. Genes *nanB* and *toxA* were absent in all the 23 isolates. In capsular type A isolates, either *sodA* or *sodC* gene was present and these genes did not occur concurrently. The presence of virulence associated gene *ptfA* revealed a positive association with the disease outcome in cattle and could therefore be an important epidemiological marker gene for characterizing *P. multocida* isolates.

Jamali *et al.* (2014) isolated *Pasteurella multocida* from calves with respiratory infection in Iran. *P. multocida* was detected in 141/169 bovine respiratory infection cases on Iranian dairy and beef farms. *P. multocida* were grouped into serogroups A (126/141), D (12/141), and B (3/141). Of the *P. multocida* isolates, all harboured *psl, ompH, oma87, fimA, ptfA, nanB* and *nanH* genes, 139/141 had *hsf-2*, and 115/141 had *pfhA,* and *tadD.*

Khamesipour *et al.* (2014) isolated 30 *Pasteurella multocida* strains from 333 pneumonic and apparently health slaughter cattle, examined for capsule biosynthesis genes and 23 virulence-associated genes by polymerase chain reaction (PCR). Of the isolates, 23 belonged to capsular type A, 5 to capsular type D and 2 isolates were untypeable. The distribution of the capsular types in pneumonic lungs and in apparently healthy lungs was statistically similar. All the virulence genes tested were detected among the isolates derived from pneumonic lungs; whereas isolates derived from apparently healthy lungs carried 16 of the 23 genes. The frequently detected genes among the isolates from pneumonic lungs were *exbD, hgbA, hgbB, ompA, ompH, oma87*, and *sodC*; whereas *tadD, toxA, and pmHAS* genes occurred less frequently. Most of the adhesins and superoxide dismutases and all of the iron acquisition and protectin proteins occurred at significantly ($p \leq 0.05$) higher

frequencies in isolates from pneumonic lungs. Isolates from apparently healthy lungs did not carry the following genes; *hsf-1, hsf-2, tadD, toxA, nanB, nanH,* and *pmHAS.* One adhesion (*hsf-1*) and two iron acquisition (*exbD* and *tonB*) genes occurred at significantly ($p \leq 0.05$) higher frequencies among *capA* isolates.

Sarangi *et al.* (2014) studied 108 *P. multocida* isolates recovered from various host animals such as cattle, buffalo, swine, poultry (chicken, duck, and emu) and rabbits, which were screened for 8 virulence associated genes. The results revealed some unique information on the prevalence of virulence associated genes among Indian isolates. With the exception of *toxA* gene, all other virulence associated genes were found to be regularly distributed among host species. Association study between capsule type and virulence genes suggested that *pfhA, nanB,* and *nanH* genes were regularly distributed among all serotypes with the exception of *capD*, whereas *toxA* gene was found to be positively associated with *capD* and *capA*. The frequency of *hgbA* and *nanH* genes among swine isolates of Indian origin was found to be less in comparison to its equivalents around the globe. Very high prevalence of *tbpA* gene was observed among poultry, swine and rabbit isolates. Likewise, very high prevalence of *pfhA* gene (95.3%) was observed among Indian isolates, irrespective of host species origin.

Sarangi *et al.* (2015) studied a total of 88 *P. multocida* isolates of small ruminant origin, which were subjected to virulence gene profiling for 19 genes by PCR and antibiogram study employing 17 different antibiotics. Virulence genes like *exbB, exbD, tonB, oma87, sodA, sodC, nanB* and *plpB* (100% prevalence) and *ptfA* and *hsf-2* (>90% prevalence) were found to be uniformly distributed among the isolates. A very high prevalence (95.45%) of *pfhA* gene was observed in the present study. Dermonecrotoxin gene (*toxA*) was observed in 48.9% of the isolates with

highest occurrence among serotype A isolates and interestingly, one of each isolate of serotype B and F were found to carry this gene.

2.2 WHOLE GENOME SHOTGUN SEQUENCING

The basic methodology used for sequencing a large genome is double-barreled shotgun sequencing introduced by Sanger and colleagues (Sanger *et al.,* 1977[b]). Shotgun sequencing was initially applied by Sanger to the genome of bacteriophage λ (Sanger *et al.,* 1980; Sanger *et al.,* 1982). Over the years, shotgun sequencing has been applied to genomic segments of increasing length. In the 1980s, it was applied to plasmids, chloroplasts (Ohyama *et al.,* 1986), viruses (Goebel *et al.,* 1990), and cosmid clones of length roughly 40 kbp, which were considered to be the limit of this approach. In the 1990s, mitochondria (Oda *et al.,* 1992), bacterial artificial chromosomes of length 200 Kbp carrying genomic DNA from human or other organisms, as well as the entire 1,800 Kbp genome of the bacterium *H. influenzae* (Fleischmann *et al.,* 1995), were sequenced.

Compared to clone-based shotgun sequencing, Whole Genome Sequencing (WGS) poses a much harder computational challenge, especially in large, repeat-rich genomes. WGS assembly became possible through advances in algorithms and software systems, rather than in either computer processor speed or sequencing accuracy.

The most common technique employed for modern genome projects is whole genome shotgun sequencing (Pittendrigh *et al.,* 2005). Sequencing DNA, sometimes called "reading", involves using chemical reactions to find the exact sequence of nucleotides that make up that molecule. Current laboratory technologies only permit reading sequences of less than 1,000 nucleotides at a time. Genomes of interest are

typically millions or even billions of nucleotides long. In order to determine the genome sequence, it must first be segmented into smaller pieces called fragments. First, many copies of the entire genome are collected and randomly cut. Next, the fragments are sorted by length and groups of fragments with desired sizes are selected. A group of fragments whose approximate sizes are known is called a library. The fragments are placed into a cloning vector such as the bacteria *E. coli*, which makes many copies of the sequence. Finally, the first 500-1000 nucleotides of each fragment are read using chemical reactions. The result consists of thousands or millions of segments, each 500-1000 nucleotides long, that are known to come from somewhere in the genome.

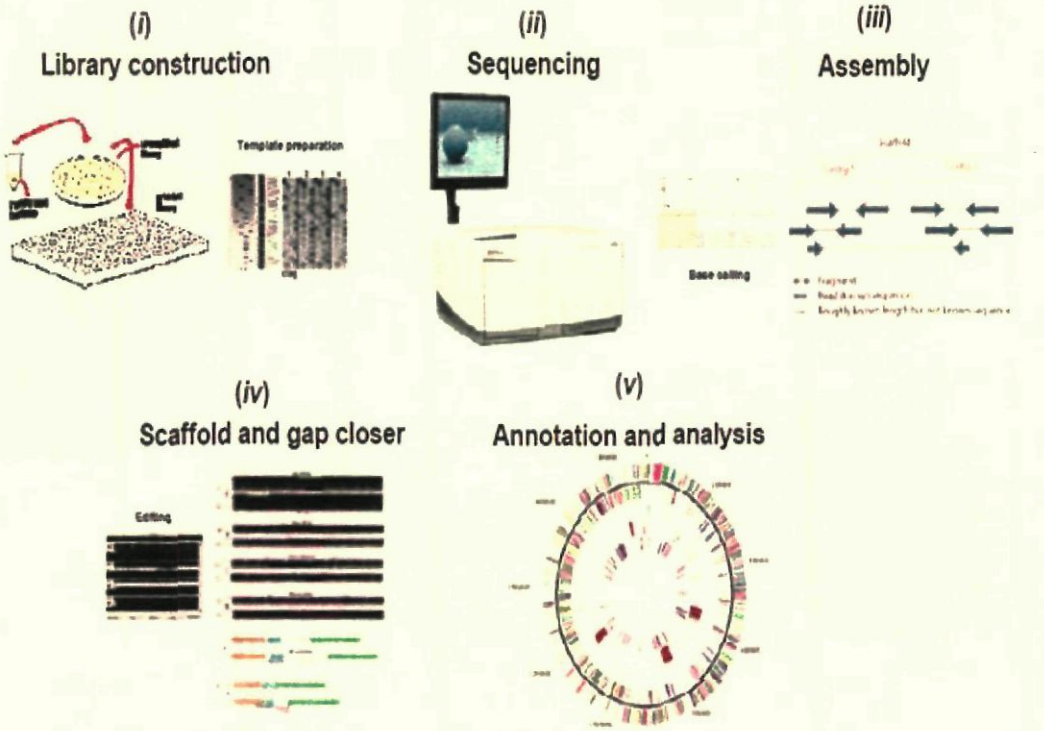

Figure 2.1: Main steps required for the whole-genome shotgun sequencing of bacterium and generation of genomic data (Fraser and Fleischmann, 1997).

2.2.1 DNA sequencing technologies

The first DNA genome, that of the 5,386-nucleotide, single-stranded bacteriophage ϕX174 was determined in 1977 using one of the technologies of DNA sequencing invented at the time (Sanger *et al.*, 1977[a]). Since then, sequencing of whole genomes as well as of individual genomic regions and genes has become a major focus of modern biology and transformed the field of modern genetics. However, in the 1970s and for almost another decade, DNA sequencing was barely automated and therefore very tedious process which allowed determining only a few hundred nucleotides in an experiment.

In the late 1980s, semi-automated sequencers with higher throughput became available (Swerdlow and Gesteland, 1990), still only able to determine a few sequences at a time. One breakthrough in the early 1990s was the development of capillary array electrophoresis and appropriate detection systems (Zagursky and McCormick, 1990; Kambara and Takahashi, 1993; Ueno and Yeung, 1994; Kim *et al.*, 1996).

In 1998, the MegaBACE 1000 and the ABI Prism 3700 DNA Analyzer became the first commercial 96 capillary sequencers, a development which then was termed high-throughput sequencing. Only within the last few years, alternative sequencing strategies like pyrosequencing (Ronaghi *et al.*, 1996; Margulies *et al.*, 2005), reversible terminator chemistry (Bentley *et al.*, 2008; Turcatti *et al.*, 2008), sequencing-by-ligation (Shendure *et al.*, 2005), virtual terminator chemistry (Harris *et al.*, 2008) and real-time sequencing (Korlach *et al.*, 2008) were developed or converged into new instruments. These new instruments require us to completely redefine the term "high-throughput sequencing", as they outperform the older Sanger-sequencing technologies by a factor of 100 to 1,000 in daily throughput and reduce

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the cost of sequencing one million nucleotides (1Mb) to 4% - 0.1% of that associated with Sanger sequencing. This large difference in throughput led scientists and companies to introduce new terms like "next generation sequencing" (Ansorge, 2009; Metzker, 2009) or "ultra-high-throughput sequencing" (Fox *et al.,* 2009) for this group of new technologies.

2.2.2 Next generation sequencing technologies

Next generation sequencing (NGS) technologies include a number of steps that can be broadly grouped as template preparation, clonal amplification, sequencing, data acquisition and analysis. The unique combination of specific protocols distinguishes one technology from another and determines the type of data produced from each platform. These differences in data output present challenges when comparing platforms based on data quality and cost. Although quality scores and accuracy estimates are provided by each manufacturer, there is no consensus that a 'quality base' from one platform is equivalent to that from another platform (Metzker, 2010). NGS platforms available to dates are listed below and discussed further with particular emphasis on Roche/454's GS-FLX Titanium.

- Roche/454's GS-FLX Titanium
- Illumina/ Solexa's Genome Analyzer
- Life/ APG's SOLiD (Support Oligonucleotide Ligation Detection)
- Polonator G.007
- Helicose Biosciences - Heliscope
- Single Molecule Real Time Sequencing of Pacific Biosciences - SMRT
- Life Technologie's Ion Torrent PGM/ Protone
- Other upcoming sequencing technologies

2.2.2.1 Roche/454's GS-FLX Titanium

The first large-scale adaption of the pyrosequencing technique, invented by 454 Life Sciences (Patrick, 2007) and later commercialized by Roche (Morozova and Marra, 2008), is a high-throughput system. The 454 Genome Sequencer (GS) is based on the pyrosequencing approach developed by Paul Nyren and Mostafa Ronaghi at the Royal Institute of Technology, Stockholm in 1996 (Ronaghi *et al.,* 1996). In contrast to the Sanger technology, pyrosequencing is based on iteratively complementing single strands and simultaneously reading out the signal emitted from the nucleotide being incorporated (also called "sequencing by synthesis" or "sequencing during extension"). Electrophoresis is therefore no longer required to generate an ordered read out of the nucleotides, as the read out is done simultaneously with the sequence extension.

In the pyrosequencing process, one nucleotide at a time is washed over several copies of the sequence to be determined, causing polymerases to incorporate the nucleotide if it is complementary to the template strand. The incorporation stops if the longest possible stretch of complementary nucleotides has been synthesized by the polymerase. In the process of incorporation, one pyrophosphate per nucleotide is released and converted to adenosine triphosphate (ATP) by an ATP sulfurylase. The ATP drives the light reaction of luciferases present and the emitted light signal is measured. To prevent the deoxyadenosine triphosphate (dATP) provided in a typical sequencing reaction from being used directly in the light reaction, deoxy-adenosine-5'-alpha-thio-triphosphate (dATPαS), which is not a substrate of the luciferase, is used for the base incorporation reaction of adenine. Standard deoxyribose nucleotides are used for all other nucleotides. After capturing the light intensity, the remaining

unincorporated nucleotides are washed away and the next nucleotide is provided (Kircher *et al.*, 2009).

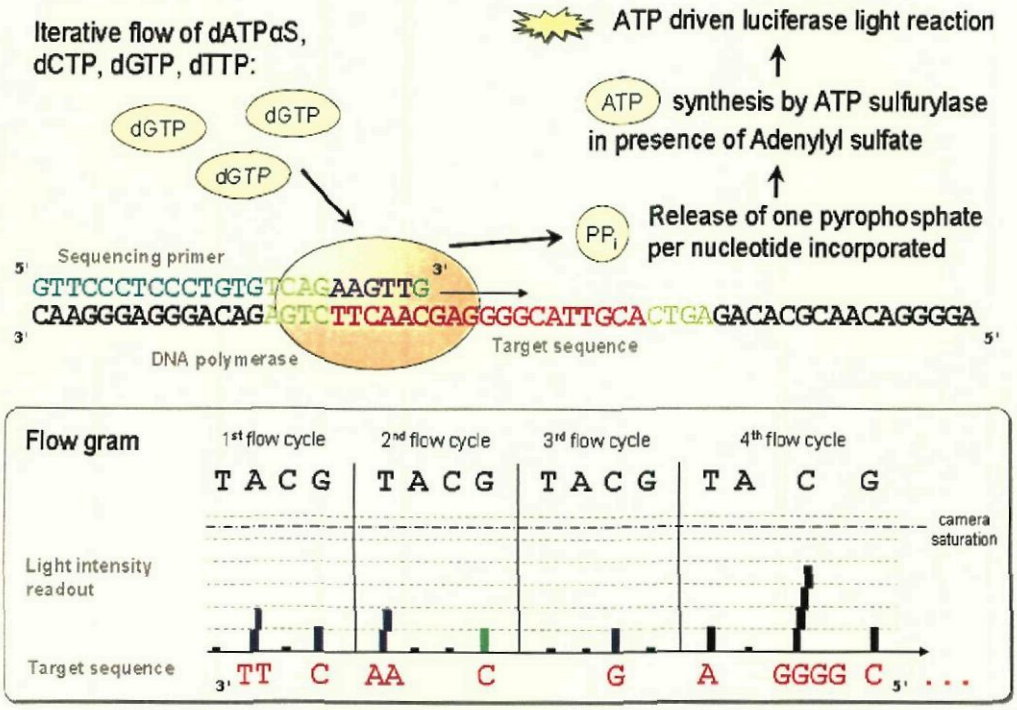

Figure 2.2: The pyrosequencing process. One of four nucleotides is washed sequentially over copies of the sequence to be determined, causing polymerases to incorporate complementary nucleotides. The incorporation stops if the longest possible stretch of the available nucleotide has been synthesized. In the process of incorporation, one pyrophosphate per nucleotide is released and converted to ATP by an ATP sulfurylase. The ATP drives the light reaction of luciferases present and a light signal proportional (within limits) to the number of nucleotide incorporations can be measured.

Later pyrosequencing technology was parallelized on a picotiter plate allowing high-throughput sequencing (Margulies *et al.*, 2005). The sequencing plate has about two million wells - each of them able to accommodate exactly one 28µm diameter bead covered with single stranded copies of the sequence to be determined. The beads

are incubated with a polymerase and single-strand binding proteins and together with smaller beads carrying the ATP sulfurylases and luciferases, gravitationally deposited in the wells. Free nucleotides are then washed over the sequencing plate and the light emitted during the incorporation is captured for all wells in parallel using a high resolution CCD (Charged-couple device) camera, exploiting the light-transporting features of the plate used.

One of the main prerequisites for applying this array-based pyrosequencing approach is covering individual beads with multiple copies of the same molecule. This is done by first creating sequencing libraries in which every individual molecule gets two different adapter sequences, one at the 5' end and one at the 3' end of the molecule. In the case of the 454/Roche sequencing library preparation (Margulies *et al.*, 2005), this is done by sequential ligation of two pre-synthesized oligos. One of the adapters added is complementary to oligonucleotides on the sequencing beads and thus allows molecules to be bound to the beads by hybridization.

Low molecule to bead ratios and amplification from the hybridized double stranded sequence on the beads (kept separate using polymerase chain reaction in an water-in-oil emulsion, (i.e emulsion PCR) makes it possible to grow beads with thousands of bound copies of a single starting molecule. Using the second adapter, beads covered with molecules can be separated from empty beads (using capture beads with oligonucleotides complementary to the second adapter) and are then used in the sequencing reaction.

The average substitution (excluding insertion/deletions) error rate is in the range of 10^{-3} to 10^{-4} (Margulies *et al.*, 2005; Quinlan *et al.*, 2008), which is higher than the rates observed for Sanger sequencing but is the lowest average substitution

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error rate of the new sequencing technologies. However in Sanger sequencing, *in vitro* amplifications performed for the sequencing preparation causes a higher background error rate, that is, the error introduced into the sample before it enters the sequencing process. In addition, in bead preparation (i.e. emulsion PCR step), a fraction of the beads end up carrying copies of multiple different sequences. These "mixed beads" will participate in a high number of incorporations per flow cycle, resulting in sequencing reads that do not reflect real molecules. Most of these reads are automatically filtered during the software post-processing of the data. The filtering of mixed beads may however cause a depletion of real sequences with a high fraction of incorporations per flow cycle.

A large fraction of the errors observed for GS FLX are small insertions or deletions (InDels), mostly arising from inaccurate calling of homopolymer length, and single basepair deletions or insertions caused by signal-to-noise thresholding issues (Quinlan *et al.,* 2008). Most of these problems can be resolved by higher coverage (Wicker *et al.,* 2006; Green *et al.,* 2008; Quinlan *et al.,* 2008).

As for Sanger sequencing, the error rate increases with the position in the sequence. In the case of 454 sequencing, this is caused by 1) a reduction in enzyme efficiency or loss of enzymes which results in a reduction of the signal intensities, 2) some molecules on the beads no longer being elongated and 3) by an increasing, so-called, phasing effect. Phasing is observed when a population of DNA molecules amplified from the same starting molecule (ensemble) is sequenced, and describes the process whereby not all molecules in the ensemble are extended in every cycle (Kircher and Kelso, 2010).

Current 454/Roche GS FLX Titanium makes it possible to sequence about 1.5 million such beads in a single experiment and to determine sequences of length between 300-500nt. The length of the reads is determined by the number of flow cycles, i.e. the number of times all four nucleotides have been washed over the plate, as well as by the base composition and the order of the bases in the sequence to be determined. Currently, 454/Roche limits this number to 200 flow cycles, resulting in an expected average read length of about 400nt. This is largely due to limitations imposed by the efficiency of polymerases and luciferases which drops over the sequencing run resulting in decreased base qualities.

2.2.2.2 Illumina/ Solexa's Genome Analyzer

The reversible terminator technology used by the Illumina Genome Analyzer employs the sequencing by synthesis concept that is most similar to that used in Sanger sequencing i.e. the incorporation reaction is stopped after each base, the label of the base incorporated is read out with fluorescent dyes and the sequencing reaction is then continued with the incorporation of the next base (Bentley *et al.,* 2008; Turcatti *et al.,* 2008).

2.2.2.3 Life/ APG's SOLiD (Support Oligonucleotide Ligation Detection)

The principle behind SOLiD sequencing technology is sequencing-by-ligation, which is very different from the other approaches of pyrosequenicng. The sequence extension reaction is not carried out by polymerases but rather by ligases (Shendure *et al.,* 2005). In the sequencing-by-ligation process, a sequencing primer is hybridized to single-stranded copies of the library molecules to be sequenced. A mixture of 8mer probes carrying four distinct fluorescent labels compete for ligation to the sequencing primer. The fluorophore encoding, which is based on the two 3' most nucleotides of

the probe, is read. Three bases including the dye are cleaved from the 5' end of the probe, leaving a free 5' phosphate on the extended (by five nucleotides) primer, which is then available for further ligation. After multiple ligations (typically up to 10 cycles), the synthesized strands are melted and the ligation product is washed away before a new sequencing primer (shifted by one-nucleotide) is annealed. Starting from the new sequencing primer, the ligation reaction is repeated.

2.2.2.4 Polonator G.007

The Polonator G.007 is ligation detection sequencing, which decodes the base by the single-base probe in nonanucleotides (nonamers), not by dual-base coding (Timp *et al.,* 2010). The fluorophore-tagged nonamers will be degenerated by selectively ligating onto a series of anchor primers, whose four components are labeled with one of four fluorophores with the help of T4 DNA ligase, which correspond to the base type at the query position. In the ligation progress, T4 DNA ligase is particularly sensitive to mismatches on 3'-side of the gap which is a benefit to improve the accuracy of sequencing. After imaging, the Polonator chemically strips the array of annealed primer-fluorescent probe complex; the anchor primer is replaced and the new mixture of fluorescently tagged nonamers is introduced to sequence the adjacent base (Deamer and Akeson, 2000).

2.2.2.5 Helicose Biosciences - Heliscope

Helicos is able to sequence individual molecules instead of molecule ensembles created by an amplification process. Single-molecule sequencing has the advantage that it is not affected by biases or errors introduced in a library preparation or amplification step, and may facilitate sequencing of minimal amounts of input DNA. Using methods able to detect non-standard nucleotides, it could also allow for

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the identification of DNA modifications, common to most in the *in vitro* amplification process (Kircher and Kelso, 2010).

2.2.2.6 Single Molecule Real Time Sequencing of Pacific Biosciences - SMRT

Another technology for sequencing individual molecules is Pacific Biosciences's SMRT (Single Molecule Real Time) sequencing technology (Korlach *et al.,* 2008). This technology performs the sequencing reaction on silicon dioxide chips with a 100nm metal film containing thousands of tens of nanometers diameter holes, so called zero-mode waveguides (ZMWs) (Eid *et al.,* 2009). Each ZMW is used as a nano visualization chamber, providing a detection volume of about 20 zeptoliters (10^{-21} liters). At this volume, a single molecule can be illuminated while excluding other labelled nucleotides in the background - saving time and sequencing chemistry by omitting wash steps. In Recent new sequencing technologies like Helicos and Pacific Biosciences systems, the sequencing of single molecules without prior library preparation or amplification is achieved.

2.2.2.7 Life Technologie's Ion Torrent PGM/ Protone

A completely different approach to next-generation sequencing is embodied in an instrument system that detects the release of hydrogen ions, a by-product of nucleotide incorporation, as quantitated changes in pH through a novel coupled silicon detector. This instrument was commercialized in 2010 by Ion Torrent (Rothberg *et al.,* 2011). Enriched beads are primed for sequencing by annealing a sequencing primer and are deposited into the wells of an ion chip, a specialized silicon chip designed to detect pH changes within individual wells of the sequencer as the reaction progresses stepwise. Ion chip has an upper surface that serves as a

microfluidic conduit to deliver the reactants needed for the sequencing reaction. The lower surface of the ion chip interfaces directly with a hydrogen ion detector that translates released hydrogen ions from each well into a quantitative readout of nucleotide bases that were incorporated in each reaction step.

2.2.2.8 Other upcoming sequencing technologies

Three other systems viz. Oxford Nanopore's BASE technology (Clarke *et al.*, 2009), IBM's silicon based nanopores (IBM Research, 2009) and Life Technologies single molecule sequencing technology based on quantum dots (Thompson and Milos, 2011) have also been reported.

Oxford Nanopore's BASE technology offers the potential to identify individual nucleotide modifications (e.g. 5-methyl-cytosine versus cytosine) during the sequencing process (Clarke *et al.*, 2009). In Nanopore sequencing, a multilayer metal/dielectric nanopore device is developed which utilizes the interaction of the DNA backbone charges with a modulated electric field to trap and slowly release an individual DNA molecule. For Life Technologies quantum dots, light-emitting semiconductor nanocrystals of 2-10 nm diameter are attached to DNA polymerases. These dots can be laser excited with a specific wavelength and then via fluorescence resonance energy transfer (FRET) enable light emission from the fluorescently labelled nucleotides at a different wave length while the polymerase incorporates them during complementary strand synthesis. These quantum dots do not only provide/transfer the energy for the fluorescence signal, they also enhance the signal strength.

2.2.3 Whole genome sequencing of *P. multocida*

The first complete genome sequence of a common avian clone of *Pasteurella multocida*, Pm70, was reported by May *et al.* (2001). The genome of Pm70 is a single circular chromosome 2,257,487 base pairs in length and contains 2,014 predicted coding regions, 6 ribosomal RNA operons, and 57 tRNAs. Genome-scale evolutionary analyses based on pairwise comparisons of 1,197 orthologous sequences between *P. multocida, Haemophilus influenzae* and *Escherichia coli* suggested that *P. multocida* and H. influenzae diverged ≈270 million years ago and the ϒ subdivision of the proteobacteria radiated about 680 million years ago. Two undescribed open reading frames, accounting for ≈1% of the genome, encode large proteins with homology to the virulence-associated filamentous hemagglutinin of *Bordetella pertussis*. Consistent with the critical role of iron in the survival of many microbial pathogens *in silico* and whole genome microarray analyses identified more than 50 Pm70 genes with a potential role in iron acquisition and metabolism.

Liu *et al.* (2012) completed whole genome sequencing of *Pasteurella multocida* HN06, a Toxigenic Strain of Serogroup D by using the Illumina Solexa platform. A total of 4,792,617 paired-end reads were generated from a genomic DNA library with 300- to 500-bp fragments, giving an ~360-fold coverage of the genome. All reads were *de novo* assembled using the Velvet package, and 61 contigs of more than 500 bp were generated. Gaps between contigs were closed by primer walking. Protein-coding sequences (CDSs) were predicted by use of the programs Glimmer and GeneMarks. tRNAs and rRNAs were identified using tRNAscan-SE and RNAmmer, respectively. *P. multocida* HN06 consists of a circular chromosome with 2,402,218 bp and a plasmid, pHN06, with 5,360 bp. The average GC contents of the chromosome and plasmid are 40.2% and 47.5%, respectively. The HN06 genome

encodes 2,279 CDSs with an average size of 928 bp, accounting for about 88% of the genome. Additionally, 56 tRNA genes and 6 rRNA operons were identified. Approximately 48.4% of CDSs were assigned to functional COGs (cluster of orthologous groups), 3.9% had general function predictions only, and the remaining proteins had unknown functions.

Abrahante *et al.* (2013) sequenced two virulent strains of *Pasteurella multocida,* P1059 and X73, using 454 pyrosequencing. The assembly generated 2.30 Mbp (40.21% GC, 24 contigs, 18 contigs >500 bp) and 2.26 Mbp (40.30% GC, 39 contigs, 28 contigs >500 bp) genomes for P1059 and X73, respectively. Genome annotation using the RAST server revealed that P1059 contained 2,144 predicted coding regions, a gene density of 88.8%, an average coding size of 961 bp, 50 tRNAs, and 4 rRNA operons. X73 contained 2,085 predicted coding regions, a gene density of 88.30%, an average coding size of 964 bp, 51 tRNAs, and 4 rRNA operons. Multigenome comparative analysis identified the presence of a filamentous hemagglutinin *pfhB1* gene, which was highly conserved in all three strains including Pm70. The *phfB2* gene was identical in X73 and P1059 strains but shared only 90% sequence identity with the Pm70 strain. A novel gene, *pfhB3*, was present in both X73 and P1059, but absent from Pm70. Another novel gene, *pfhB4*, was unique to the P1059 strain. The *plpE* gene was present in all three strains and highly conserved. There was presence of a chondroitin synthase gene (*fcbD*) in Pm70 and the hyaluronan synthase gene (*hyaD*) in both P1059 and X73. The *pcgDABC* gene cluster involved in the decoration of phosphocholine to the outer core of the lipopolysaccharide (LPS) was present only in X73, the pm1138 gene encoding a glycosyltransferase was present in all the three strains.

Eidam *et al.* (2013) performed whole genome sequencing of *Mannheimia haemolytica* Strain 42548 from a case of bovine respiratory disease using the 454 GS-FLX titanium XL system (Titanium GS70 chemistry) and the genome analyzer (GA) IIx (Illumina). 454 shotgun sequencing produced 135,530 single-end reads with an average length of 612 bases (28.6X coverage), while Illumina sequencing resulted in 3,696,221 of 112-bp paired-end reads (116X coverage). The initial hybrid *de novo* assembly performed using the MIRA software resulted in 316 contigs. Gaps between contigs were closed using PCR amplification, subsequent to Sanger sequencing. Gap closure was done using the Gap4 (v.4.11) software. The genome of *M. haemolytica* 42548 consists of a single chromosome of 2.73 Mb with a GC content of 41.05%. The initial gene prediction was done using YACOP and Glimmer, while rRNA and tRNA were identified with RNAmmer and tRNAscan. This revealed a total number of 2,888 genes, including 6 rRNA gene clusters and 61 tRNA genes. Protein genes annotated using Swiss-Prot and TrEMBL resulted in 2,807 protein-encoding genes with assigned functions.

Hauglund *et al.* (2013) carried whole genome sequencing of *Mannheimia haemolytica* strain D153 isolated from pneumonic calf lung using three platforms: the Roche (454) GS FLX titanium, resulting in 26-fold coverage; the Illumina GA IIx, resulting in 1,700-fold coverage; and the pacific bioscience Research (PacBio RS), resulting in 30 fold coverage. The completed D153 genome sequence consists of 2.68 Mb, with a G+C content of 41.04%. The draft genome sequence of *M. haemolytica* D193 was determined using the Roche platform alone, which yielded 39-fold coverage. Assembly against the closed D153 reference genome using the CLC software yielded 50 contigs consisting of 2.68 Mb, with a G+C content of 41.08%, an N50 of 139,865 bp, and 100% of contigs with lengths of >500 bp. Annotation of both

genome sequences was accomplished with the NCBI Prokaryotic Genome Annotation Pipeline. Strain D153 contained a total of 2,766 genes, including 2,641 predicted protein-encoding genes, 43 frame shifted pseudogenes, 20 rRNA genes, and 62 tRNA genes. Strain D193 contained a total of 2,801genes, including 2,689 predicted protein encoding genes, 39 frameshifted pseudogenes, 16 rRNA genes, and 57 tRNA genes. One CRISPR array was detected in each isolate. In contrast to the multi-resistant *M. haemolytica* isolate 42548, both strains D153 and D193 lack the genes *aphA1*, *strA*, *strB*, and *sul2*. *tetR* and *tetH* were detected in strain D193 but not D153.

Lainson *et al.* (2013[a]) performed whole genome sequencing for three bovine isolates (2000, RIIF, 1500E) from the United Kingdom and one bovine isolate from United States (P1933) using an Illumina Solexa Genome Analyzer. *De novo* assembly was carried out using Velvet version 0.7, and the resulting sequence data were submitted to NCBI analysis using the Prokaryotic Genomes Annotation Pipeline (PGAP). The cumulative sequence lengths and the numbers of predicted coding sequences are consistent with those of related *P. multocida* strains sequenced previously (accession numbers NC_017764, NC_016808, NC_17027, and NC_002663). Assembled contig sequences were mapped to the reference genome Pm70 using Nucmer. This showed extensive sequence similarity, with a high proportion of each genome mapping to Pm70 with an identity of 98% or greater.

Lainson *et al.* (2103[b]) isolated *P. multocida* strain 671/90 which is an A:3 serotype isolated from infected calf lung tissue and sequenced using Illumina PE and 454 platforms and produced 26 contigs ranging in size from 124 to 578,571 bp, with a cumulative size of 2,246,063 bp. The G+C content was found to be 40.25% and predicted 2,115 genes, of which 2,064 were protein coding, 5 encoded rRNAs, and 46

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encoded tRNA sequences. Strain 671/90 was having extensive homology 95.98%, and mapped with an identity of ≥98% to Pm70 strain.

Yap *et al.* (2013) sequenced strain PMTB isolated from buffalo carcass, performed using an Illumina genome analyzer. A total of 7,760,284 single-end reads were generated from a genomic library with 300- to 500-bp fragments with approximately 100-fold genome coverage. PMTB has a genome size of 2,203,419 bp, a coding percentage of 87.3%, a G+C content of 40.44%, and 9 unclosed gaps. From the analysis of PGAAP results, strain PMTB contains 2,021 coding sequences (CDS) with an average size of 951bp. Among the 2,021 CDS, 1,796 were assigned to functional clusters of orthologous groups (COGs), 51 had general function prediction only, and the remaining proteins had unknown functions. The *toxA* gene, which is frequently detected in genomes of serotype D, was confirmed by PCR assay to be absent in the genome of strain PMTB. The integrative conjugative elements of *P. multocida* (*ICEPmu1*) were not found in the draft genome sequence of strain PMTB. A comparative analysis of the protein sequences encoded by the genome of *P. multocida* serotype F:3 strain Pm70 and that of PMTB revealed that at 50% identity, 217 protein sequences encoded by strain PMTB represented unique proteins. Of these unique proteins, 22 were phage or transposon related.

Abrahante *et al.* (2014) performed whole-genome sequencing of two strains, 2213 and 3213 of *P. multocida* isolated from the blood of carcasses of two buffaloes (*Bubalus bubalis*) using the Illumina MiSeq platform. A total of 6,253,700 and 5,550,478 paired-end 150-bp reads of each genome were assembled into 34 and 32 contigs, respectively, for strains 2213 and 3213, using Velvet assembly software available on the Galaxy Suite (Minnesota Supercomputing Institute). Major gaps (>50 bp) were resolved on the GenBank assembly algorithm in an iterative manner in order

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to obtain a single assembled whole genome sequence. An annotation file was generated by Rapid Annotations using Subsystems Technology (http://rast.nmpdr.org /rast.cgi), and cross-verified using a re-annotation performed by the Genbank annotation resource. RAST annotation of strain 2213 using strain 36950 as a reference produced a full genome of 2,309,333 bp carrying 2,163 coding sequences classified into 425 metabolic or virulence subsystems and 59 predicted RNAs. Strain 3213 produced a genome or 2,307,438 bp carrying 2,160 coding sequences that were classified into 424 subsystems and 57 predicted RNAs.

Davenport *et al.* (2014) performed sequencing of *P. multocida* subspp. *multocida* ATCC 43137, a type-A LPS strain commonly used as a reference strain in pathogenicity studies using Illumina technology. Genome assembly was performed by the Los Alamos National Laboratory (LANL) Genome Science Group, and 300-fold 100-bp paired-end (270 ± 30 bp insert) Illumina data assembled in Newbler (version 2.6), Velvet (version 1.2.08), and AllPaths (version 42298). Consensus sequences from all assemblers were computationally shredded and assembled with a subset of read pairs from the long-insert library using Phrap (version SPS-4.24). The resulting assembly was brought to closed and finished status through both manual and computational finishing efforts using Consed and in-house scripts. The assembled genome sequence was corrected by mapping Illumina reads back to the final consensus sequences using Burrows-Wheeler Alignment (BWA), SAMtools and inhouse scripts. Annotations were completed at LANL using an automated system utilizing the ergatis workflow manager and in-house scripts. The 2.27 Mbp (40.4% G + C content) complete assembly of *P. multocida* subspp. *multocida* ATCC 43137 assembly includes 2,076 coding sequences, 19 rRNA, and 58 tRNA sequences.

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Preliminary review of the annotated genome indicates resistance genes for multiple toxic metals, iron acquisition, hemagglutinin, and genes for LPS production.

Vaid *et al.* (2014) sequenced *P. multocida* isolate strain VTCCBAA264, a B:2 serotype, from buffalo intestine using 454 pyrosequencing. A total of 123,415 reads were with ~23X coverage of the genome with 78 contigs ranging in size from 710 to 319,560 bp, with a total size of 2,280,332 bp. Annotation using RAST server showed G+C content of 40.4% with 2,176 predicted genes, out of which, there were 2,127 protein-encoding genes, 4 coded rRNAs, and 45 encoded tRNAs. Among the 2,127 coding sequences (CDSs), 2,074 (97.5%) were assigned to functional clusters of orthologous groups (COGs), 1,863 (87.6%) were assigned to FIGfams (fellowship for interpretation of genome families) and 1,527 (71.8%) to KEGG (Kyoto Encyclopedia of Genes and Genomes). One clustered regularly interspaced short palindromic repeats (CRISPRs) array was also detected. The genes for antimicrobial resistance including translation elongation factor, DNA gyrase subunit A, topoisomerse IV subunit B, and DNA directed RNA polymerase subunit, were found among others. Genes for resistance to flouroquinolones and negative regulators of beta-lactamase expression were also reported.

2.3 SEQUENCE ANALYSIS STRATEGIES

Genome sequencing presents challenges to computer analysis at all levels, from sequence assembly to large-scale genome comparisons. Genome sequence analysis is a multistep process that starts with establishing the maximally accurate assembled sequence, and proceeds through functional predictions to higher-level genome comparisons (Koonin *et al.,* 1996).

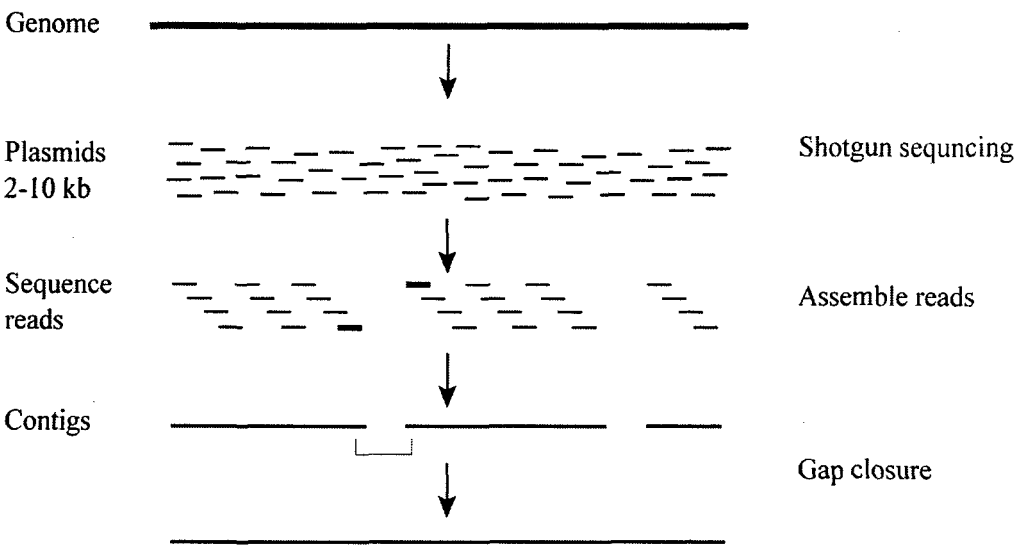

Figure 2.3: Whole genome shotgun sequencing. The obtained contigs are ordered into scaffolds using information from read pairs spanning the gaps.

2.3.1 Bacterial genome assembly

The process of deciphering the sequence of a genome from the small DNA fragments and any other additional genome information available is called "assembling" the genome. At present, the sequencing process is often a 10x-fold genome coverage followed by two phases: assembly and finishing. Currently, sequencing reads generated by different sequencing technologies range from 35-1000 bp. In order to obtain a complete bacterial genome, the fragments need to be aligned and joined together using a computer program called an assembler. Assemblers can join sequences together based on overlapping regions between the sequences, assuming that the two sequence reads have originated from the same place in the genome. After assembly, a collection of contiguous pieces (contigs) instead of an entire chromosome are usually obtained. This is often due to non-random shearing of DNA, intrinsic cloning bias and repeated regions in the genome. Increasing the sequencing coverage of the genome will help to reduce the number of contigs. Lander and Waterman (1988) showed that sequencing a 1 Mbp genome using Sanger

chemistry resulted in a small number of contigs (~5) if 8 to 10 times genome coverage was attained.

In addition to the assembler, another computer program called a scaffolder is used if paired-end or mate-pair reads are available. Scaffolder can link distant sequences together based on the distance between the two ends of the original template, i.e. 3 kb or 8 kb. Therefore, the scaffolder is able to define the size of gaps between contigs and orient the contigs into a draft genome (Pop *et al.,* 2004). To assemble next-generation sequencing reads, *de novo* assembly becomes more of a challenge because only short overlaps can be considered. Therefore, higher coverage of the genome is required for assembly of shorter reads, resulting in large volumes of data (Miller *et al.,* 2010). However, one study has shown that it is possible to assemble a large portion of the *E. coli* K12 MG1655 genome using read lengths of 20-50 nucleotides, given that the reads were error-free (Whiteford *et al.,* 2005). Since then, several bacterial genomes have been *de novo* assembled using short read sequence data, including *Helicobacter acinonychis* (Dohm *et al.,* 2007; Hernandez *et al.,* 2008), *Staphylococcus aureus* (Hernandez *et al.,* 2008), *Bacillus subtilis* (Srivatsan *et al.,* 2008; Nishito *et al.,* 2010), *Pseudomonas aeruginosa* (Salzberg *et al.,* 2008), *Pseudomonas syringae* (Reinhardt *et al.,* 2009; Studholme *et al.,* 2009) and *Erwinia pyrifoliae* (Smits *et al.,* 2010). Furthermore, combining the read data from more than one type of sequencing platform can improve *de novo* assembly of a bacterial genome significantly (Aury *et al.,* 2008; Reinhardt *et al.,* 2009, Smits *et al.,* 2010).

2.3.1.1 Construction of contigs and scaffolds

The placement of the reads along a reference genome implicitly defines a set of contigs or contiguous regions of the assembly, as well as the relative order and

orientation of these contigs in a structure which is commonly known as a scaffold (Pop *et al.,* 2004). This phase consists of three major steps. Firstly, each overlap is evaluated based on the depth of coverage of two regions in the overlap. Secondly, poorly differentiated ends of every sequence read are identified and trimmed. Thirdly, reads are assembled into contigs based on unique overlaps.

Contigs are corrected and linked into scaffolds based on pairs, and scaffolds are also corrected based on read pairs. The joining of the fragments is modeled as a mathematical weighted graph, where nodes are fragments and the weights of edges are the number of overlapping nucleotides. The fragments are joined based upon maximum overlap using a greedy algorithm (Qin *et al.,* 2003). In a greedy algorithm, most nodes having maximum (or minimum) scores are collapsed first.

To join contigs, the fragments with larger nucleotide sequence overlaps are joined first. Contigs are constructed by processing overlaps with an adjusted score greater than a cutoff. Initially, each read is a contig by itself. The overlaps are ranked in a decreasing order of their adjusted scores, and they are considered one by one, in order, for the construction of contigs. The overlap being considered is called the current overlap. For a current overlap between two reads, if the reads are in different contigs and two contigs have an overlap consistent with the current overlap, the two contigs are merged into a larger contig.

The computation is performed on one processor with enough memory to hold the overlaps and contigs. Read pairs are used to order and orient contigs into a scaffold, as follows. Initially, each contig is a scaffold by itself. Unsatisfied read pairs are partitioned into groups such that all reads in a group link a pair of scaffolds. The groups of unsatisfied read pairs are considered in a decreasing order of their sizes. If

the number of read pairs in the group is sufficiently large, the read pairs link two scaffolds, and the two scaffolds can be combined by using the read pairs in the group, then the two scaffolds are combined into an even larger scaffold.

2.3.1.2 Generation of consensus sequences

For each group of overlapping reads in the refined layout, a multiple alignment is computed to generate a consensus sequence for the genomic region covered by those reads. The multiple alignment is computed in a series of rounds. In each round, a pairwise alignment of each read to the current consensus sequence is computed and the resulting multiple alignment is used to generate a new consensus sequence. The process terminates when the new consensus sequence is the same as the one in the previous round. This essentially utilises the algorithm described by Anson and Myers (1997). For each scaffold, a set of repetitive reads that are linked by read pairs to unique reads in the scaffold are identified. For each gap in the scaffold, a subset of repetitive reads that may fall into the gap are selected from the set on the scaffold. An attempt is made to close the gap with the subset of repetitive reads. After all gaps in the group of scaffolds are considered for closure, a consensus sequence is generated for each contig and a list order and oriented contig consensus sequence is recorded for each scaffold.

Generation of a consensus sequence for each contig is based on multiple alignments of reads in the contig, which is constructed as follows. The reads in the contig are sorted in an increasing order of their position in the contig. A multiple alignment is constructed by repeatedly aligning the current read with the current alignment, and the resulting alignment is the current alignment for the iteration. The reads in the contig are considered one by one, in order. For each column of the final

multiple alignment, a weighted sum of quality values is calculated for base type. The base type with the largest sum of quality value is taken as the consensus base for the column.

2.3.1.3 Scaffold and gap closure

The availability of complete edited genome sequences of bacterial pathogens free of gaps is of great utility as it gives access to complete gene sets and provides for studies of genome organisation, genome comparisons and functional genomics, including the development of microarrays (Fraser *et al.,* 2000). Once one or two genomes of a given organism are sequenced to completion, the diversity of the species can be assessed through comparative genome hybridisation (CGH) using microarrays (Tettelin *et al.,* 2001), or by generating draft sequences of other strains of interest and comparing them to the reference genome(s). These two approaches are rapid and cost effective whilst generating genome-scale information on species diversity.

2.3.2 Bacterial genome annotation

In general, genome annotation can be performed at two main levels, static and dynamic (Medigue and Moszer, 2007). At the static level, the main features of the genome can be obtained, such as protein coding genes, functional RNA products, GC content, codon usage, genomic islands, motifs, chemical and structural properties of proteins and their sub-cellular localisation. In contrast, the dynamic view can exhibit gene context, gene order, regulatory networks, protein interaction networks, metabolic networks as well as phyloprofile and gene fusion/fission information obtained by comparative genomics (Medigue and Moszer, 2007). Nowadays, bacterial genomes are often annotated using automated pipelines, which can be either web-based or run locally (Stothard and Wishart, 2006). Web-based annotation pipelines are more

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convenient for small research groups or smaller sequencing facilities that lack computing resources and expertise that is necessary to maintain or implement the software (Stothard and Wishart, 2006). Bacterial Annotation System (BASys) is a automated bacterial annotation web server (http://wishart.biology.ualberta.ca/basys), which uses more than 30 programs to determine nearly 60 annotation subfields for each gene (Van Domselaar *et al.,* 2005). With this system, results can be generated in about 24 h for a 5 megabase genome and can be browsed and evaluated using a navigable graphical map. However, BASys is not able to analyze partially assembled genomes (Van Domselaar *et al.,* 2005).

In 2008, the RAST (Rapid Annotation using Subsystem Technology) Server (http://rast.nmpdr.org/), a fully automated annotation service for complete or draft archaeal and bacterial genome was built (Aziz *et al.,* 2008). Annotations provided by the service include protein-encoding, rRNA and tRNA genes, gene function and metabolic network. On the completion of annotation, the annotated genome can be downloaded in a variety of formats or browsed in SEED Viewer for up to 120 days (Aziz *et al.,* 2008). The SEED is an annotation/analysis tool provided by the Fellowship for Interpretation of Genomes (FIG) (Overbeek *et al.,* 2005). WeGAS (http://ns.smallsoft.co.kr:8051) is another web-based microbial genome annotation system (Lee *et al.,* 2009). Like the RAST Server, it is capable of handling both ongoing and completed microbial genome projects. The user can start genome annotation with contigs and the process can be monitored during each analysis. The annotation pipeline includes seven major modules, which are gene prediction, homology search, promoter search, pathway mapping, motif search, COG (Clusters of Orthologous Groups of proteins) assignment and GO (Gene Ontology) assignment. A genome browser is also available to view the detailed results (Lee *et al.,* 2009).

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Recently, another prokaryotic genome annotation web server called Integrative Services for Genomics Analysis (ISGA) became available to the community (http://isga.cgb.indiana.edu/) (Hemmerich *et al.,* 2010).

2.3.2.1 Genome annotation pipeline

Genome annotation falls into two distinct stages (Kuroda and Hiramatsu, 2004). The first stage is referred to as 'structural annotation' and involves the correct identification and localisation of distinct sequence elements such as genes, regulatory elements, transposons, repetitive elements and more. The second stage, termed 'functional annotation' attempts to predict the biological function for each of those elements and the biological process in which it takes part.

2.3.2.1.1 Structural annotation

Computational identification of protein-coding genes is an approach used in newly sequenced genomes. It is divided into three methods: (i) *ab initio* or *de novo* methods, which predict genes solely on the basis of local sequence characteristics (ii) similarity-based methods, which utilise sequence similarity to known genes and (iii) *comparative* methods, which employ sequence comparison between multiple, related genomes to identify conserved genes. Gene prediction is the most visible part of this phase and involves identification of the genes encoded in the sequence, which include the boundaries of the regions that act as templates for transcription, the initiator and terminator of translation, splice sites, promoter and regulatory regions and perhaps several other biologically important elements. In bacterial genomes, gene prediction is largely a matter of identifying long ORFs (open reading frame). For a known sequence, ORFs are predicted using a variety of programs such as GeneMark (Besemer *et al.,* 2001), GeneMark.hmm (Lukashin and Borodovsky, 1998),

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GENESCAN (McEvoy *et al.,* 1998), Glimmer (Aggarwal and Ramaswamy, 2002) and Glimmer3 (Delcher *et al.,* 2007). Each program applies to the sequence for searching the potential ORFs or gene encoding regions. A genome can be divided into two parts, one comprises the protein and RNA encoding genes and the other is the non-coding DNA (Pearson and Lipman, 1988).

2.3.2.1.2 Functional annotation

Once the genes and other structural sequences in a genome have been identified, the next stage in annotation is to predict the molecular function and biological role of these elements. Functional annotation is the process of assigning function to the genes expressed in the genome, discovering the mechanisms of regulation associated with different regulatory sites and more generally, assigning function to structural elements annotated in the genome. Functional annotation is divided into two parts, functional annotation based on homology or based on protein signature.

Homology-based function prediction involves the use of database searches to identify genes that are similar to a query sequence and which preferably have a known experimentally determined function. Similar to structural annotation, the focus in functional annotation is on the function of genes and their products. A widely used tool for homology searches in databases is the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.,* 1997). BLAST is based on a search and alignment algorithm and is used to compare a nucleotide or protein sequence against nucleotide or protein databases. Similarity scores between the query sequence and database homologous reflect their local pairwise alignments. Homology searches are carried out on the predicted ORFs in an attempt to determine function as well as other

information about the potential gene and its protein product (Rubin, 2001). Other sequence similarity search tools such as FASTA (Pearson and Lipman, 1988) and variations of BLAST are commonly used algorithms for sequence comparisons (Altschul *et al.,* 1997). FASTA generates tables of short query sequences to compare to the database and BLAST expands upon the short matches to find the best scoring matches for the query sequence. The possible ORFs are classified by comparison to the entire sequence. Many different databases are available for searching including the NCBI non-redundant database (NCBI nr) (Altschul *et al.,* 1997), the KEGG database for examining metabolic pathways (the Kyoto Encyclopedia of Genes and Genomes, Japan) (Kanehisa *et al.,* 2008), the Clusters of Orthologous Groups (COG) (Tatusov *et al.,* 2003) and the STRING-extended COG database (179 microbial genomes, version 7.0) (von Mering *et al.,* 2007).

The limitation of homology-based function prediction, when tools such as BLAST are used, is that the presence of structural domains and motifs that make up a protein are not properly analysed. BLAST may report the most significant database matches for a query sequence that are based solely on the presence of one common, conserved protein domain. Other domains in the sequence may be different and be indicative of an alternative function.

2.3.3 Data analysis

The process of sorting quality reads and aligning and analyzing hundreds of thousands or millions of base pairs is both time intensive and computationally intensive. Technology for data acquisition is proceeding faster than information technology in many cases and data-processing time may well exceed sample-handling time until new methods of analysis are developed.

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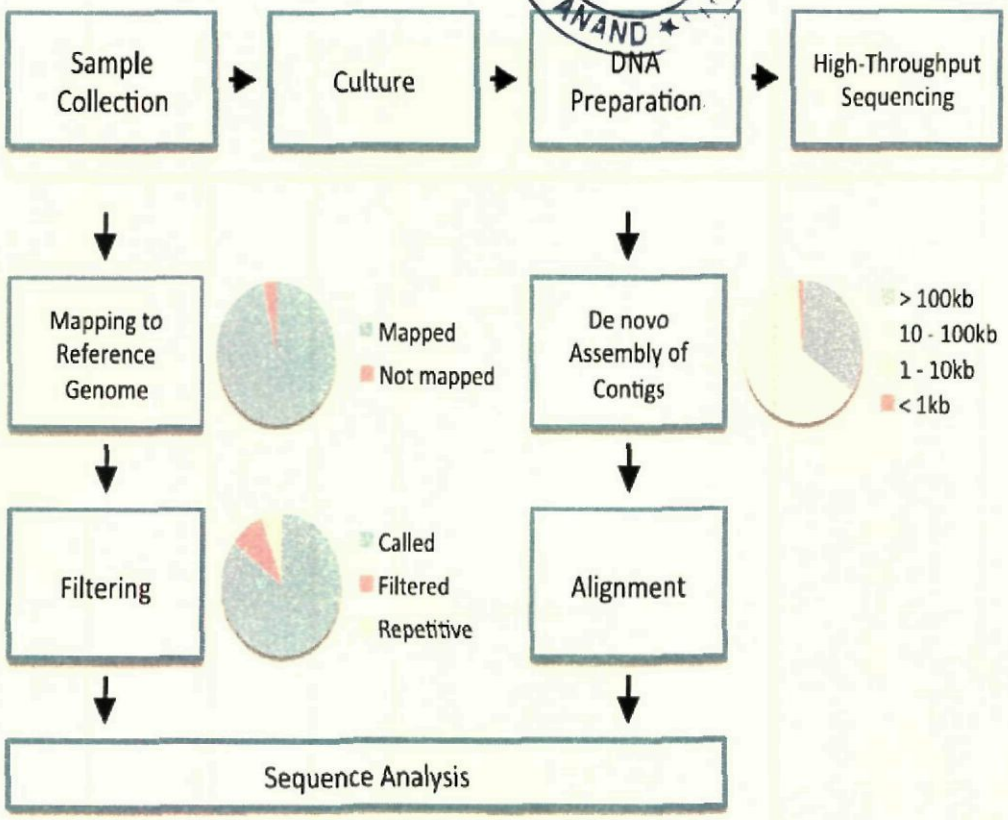
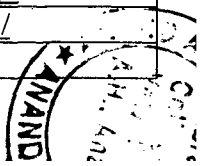


Figure 2.4: Workflow for high-throughput whole genome sequencing in bacteria

Explanation of Figure 2.4: Sample collection: A biological sample (e.g., blood) is collected. **Culture:** Bacterial colonies are isolated from the sample by culturing on appropriate media. **DNA Preparation:** DNA is extracted from the colonies and a DNA library is prepared ready for sequencing. **High-Throughput Sequencing:** Millions of short sequence reads are yielded, typically several hundred nucleotides long or less. To reconstruct the genome, one of two approaches is generally adopted. **Mapping to Reference Genome:** In reference-based mapping, the short sequences are mapped (i.e., aligned) to a reference genome using an algorithm. Preferably, the reference genome is of high quality, complete, and closely related. The pie chart illustrates that not all reads necessarily map to the reference genome (e.g., because of novel regions not present in the reference). **Filtering:** Short reads cannot

be mapped reliably to repetitive regions of the reference genome, so these are identified and filtered out. Sites that are problematic for other reasons (e.g., because too few reads have mapped or because the consensus nucleotide is ambiguous) are also filtered out. The pie chart illustrates that some portion of the reference genome does not get called due to filtering. In the mapped genome, these positions will receive an ambiguity code (i.e., N rather than A, C, G, or T). ***De novo* Assembly of Contigs:** An alternative to mapping is *de novo* assembly, in which no reference genome is used. An algorithm is used to assemble short reads into longer sequences known as contigs. The number and length of contigs will depend on general factors such as the length of sequence reads and the total amount of DNA sequence produced, as well as local factors such as the presence of repetitive regions. The pie chart shows an example of the proportion of all reads that assemble into contigs of a given length. **Alignment:** For further analysis, it is necessary to align local regions (e.g., genes) or whole genomes using appropriate algorithms. There is a trade-off in computational terms between the length of region and the number of sequences that can be aligned. **Sequence Analysis:** The two approaches produce sequence alignments that represent pairwise alignments against a reference (mapping) or multiple alignments one to another (*De novo* assembly). These alignments can be analyzed directly, or processed further to detect variants such as single nucleotide polymorphisms, insertions, and deletions (Wilson, 2012). Various bioinformatics tools/software and the useful databases at present, are shown in Table 2.2

Tool	Description/ Features	Web address/URL
Assembly tools		
CAP3	Alignment/assembly/Roche	http://pbil.univ-lyon1.fr/cap3.php
Abyss	Alignment/assembly/Illumina	www.bcgsc.ca/platform/bioinfo/software/abyss
Phrap	Alignment/assembly/Illumina/Roche	http://www.phrap.org/consed/consed.html
Velvet	Alignment/assembly/Roche/ABI/Illumina	http://www.ebi.ac.uk/%7Ezerbino/velvet
Gene prediction tools		
Glimmer	Microbial gene-finding system	www.cbcb.umd.edu/software/glimmer/
GeneMark	Gene prediction in bacteria, archaea and metagenomes	http://opal.biology.gatech.edu/GeneMark/
EasyGene	Gene predictor in prokaryotic DNA	www.cbs.dtu.dk/services/EasyGene/
FgenesB	Bacterial operon and gene prediction	http://linux1.softberry.com/
REGANOR	Gene prediction sever and database	www.cebitec.uni-bielefeld.de/
Prodigal	Prokaryotic dynamic programming gene finding algorithm	http://prodigal.ornl.gov/
Automatic and mannual annotation pipelines/Tools		
GenColors	Comparative genomics and annotation platform	http://www.genoscope.cns.fr/
MicroScope	Comparative genomics and annotation platform	http://www.genoscope.cns.fr/
KAAS	KEGG automatic annotation server	www.genome.jp/tools/kaas/
AutoFACT	Automated annotation tool	http://megasun.bch.umontreal.ca/
BASys	Bacterial annotation system	http://basys.ca/basys/cgi/submit.pl
IGS	IGS prokaryotic annotation pipeline	http://ae.igs.umaryland.edu/cgi/
CMR	Comprehensive microbial resource and annotation	http://cmr.jcvi.org/
PGAAP	NCBI prokaryotic automatic annotation pipeline	www.ncbi.nlm.nih.gov/genomes/
GenDB	Prokaryotic genomes annotation system	www.cebitec.uni-bielefeld.de/
MANATEE	Manual functional annotation tool	http://manatee.sourceforge.net/
HAMAP	Automated and manual annotation of microbial proteomes	http://us.expasy.org/sprot/hamap/
RAST	Rapid annotation using subsystem technology	www.nmpdr.org/FIG/wiki/view.cgi/
xBASE	Bacterial genome annotation service	http://www.xbase.ac.uk/annotation/
Blast2GO	Annotation and sequence analysis tool	http://www.blast2go.com/



Databases and resources		
NCBI	Genbank, RefSeq, TPA and PDB, databanks for storage and downloadable genomic information	http://www.ncbi.nlm.nih.gov/
EMBL	Nucleotide sequence database	http://www.ebi.ac.uk/embl
GOLD	Data resource for genomic and matagenomic projects	http://www.genomesonline.org/
KEGG	An integrated database resource, provides genomic, chemical and systemic information	http://www.kegg.jp/
IMG	Resource for comparative analysis and annotation	http://img.jgi.doe.gov/
JCVI	Comprehensive microbial resource (CMR)	http://www.jcvi.org/
MBGD	Database analysis of orthologous, paralogous, motifs, gene order and annotation	http://mbgd.genome.ad.jp/
RDP	Ribosomal database, bacterial RNA sequences, alignments and tools for RNA analysis	http://rdp.cme.msu.edu/
Rfam	RNA database	http://rfam.sanger.ac.uk/
GtRNAdb	RNA database, tRNA gene predictions	http://lowelab.ucsc.edu/GtRNAdb/
UniProt	Protein resource and functional information	http://www.uniprot.org
UniProtKB	Curated protein database (UniProtKB/Swiss-Prot and UniProtKB/TrEMBL)	http://www.uniprot.org/help/uniprotkb
Gene Ontology (GO)	GO database, annotation of genes, protein and sequences	http://www.geneontology.org/
METACYC	Database for metabolic pathways	http://metacyc.org

(Ali *et al.*, 2013)

Table 2.2: Gene Prediction tools, an automatic and manual annotation pipelines, databases and resources. Tools for comparative genomics/proteomics analysis.

2.3.3.1 Gene prediction

Gene identification is the first task after completion of any genome sequencing. If a putative protein encoded by an uncharacterized ORF shows statistically significant similarity to another protein of known function, the ORF in question is a new gene and predicts its likely function (Borodovsky *et al.,* 1994). Even if the homolog of the new protein has not been characterized, useful information is produced in the form of conserved motifs that may be important for protein function. The methods of choice for the initial database screening are those, that translate the query nucleotide sequence in all six reading frames and compare the resulting putative protein sequences to the protein sequence database (Gish and States, 1993).

Sequence analysis methods that distinguish between coding and non-coding regions in DNA on the basis of their different statistical properties are therefore indispensable for gene identification (Fickett and Tung, 1992; Gelfand, 1995). The non-homogenous Markov models using in-phase hexamer statistics (Borodovsky *et al.,* 1994; Krogh *et al.,* 1994), and hidden Markov models (Krogh *et al.,* 1994), have proved particularly effective in bacterial gene prediction. Many of the genes originally predicted by these statistical methods have subsequently proved to be homologous to newly described genes or have been confirmed experimentally, thus supporting the robustness of the prediction methods (Borodovsky *et al.,* 1995).

2.3.3.2 Functional prediction

Most of the sequence similarities are detectable with standard database-searching methods, such as BLASTP (Altschul *et al.,* 1990; Altschul *et al.,* 1994). Additional approaches to similarity analysis, including methods for identifying motifs, produce a significant increase in sensitivity. Proteins with closely related homologs, database screening

with conserved motifs frequently provides additional connections to functionally well characterized proteins.

2.3.3.3 Comparative genomics

Comparative genomics is the study of the differences and similarities in genome structure and organisation in different organisms. Comparative genome sequencing is an important tool in the ongoing effort to exploit conservation in order to annotate and analyse genes and architectural features of genomes. Comparative genomic analysis can be used to define the basic concepts to describe and understand genome evolution (Wolfe and Li, 2003), which is closely linked to gene evolution. The relationship between evolutionary and bioinformatics analyses is evidently reciprocal and synergistic. The intimate relationship between evolution and bioinformatics analysis is nicely illustrated by the fact that one of the first computational analyses of sequences was a phylogenetic analysis, i.e. a study of molecular evolution (Fitch and Margoliash, 1967; Prager and Wilson, 1978). Based on these bioinformatic studies of sequences, many important and intrinsically relevant results for the study of evolution have been obtained. Comparative genomics has revolutionised taxonomy and the understanding of the interplay between phenotype and genotype (Forst and Schulten, *2001).* Understanding relationships between the genomes of different species can yield insight into many aspects of evolution, and is especially valuable for the identification of genes and regulatory regions.

2.3.3.4 Finishing a sequencing project (Genome finishing)

Genome finishing involves determining the order and orientation of the consensus sequences of contigs obtained from Phrap assemblies of random draft genomic sequences (Lee and Vega, 2004; de la Bastide and McCombie, 2007). This process consists of linking contig ends using information embedded in each sequence file that relates the sequence to the

original cloned insert. Since inserts are sequenced from both ends, a link can be established between these paired ends in different contigs, and thus the contigs can be ordered and orientated. Since genomes may carry numerous copies of insertion sequences, these repeated elements confuse the Phrap assembly program.

It is thus necessary to break these contigs apart at the repeated sequences and individually join the proper flanking regions using paired-end information, or using results of comparisons against a similar genome. Larger repeated elements such as the small subunit ribosomal RNA operon require verification using polymerase chain reaction (PCR) amplification and sequencing. Tandem repeats require manual intervention and typically rely on single nucleotide polymorphisms to be resolved. Filling remaining gaps requires PCR amplification and sequencing. Once the genomes have been closed, low quality regions are addressed by re-sequencing reactions.

For smaller, less complex genomes, the computational scaffolding process may be able to produce complete chromosomes. However, for more complex organisms, the scaffolding process will still result in hundreds or thousands of scaffolds that must be sorted into chromosomes and ordered and oriented. If a closely related genome has already been *finished*, this may be done computationally. Additionally, depending on the quality of sequence desired, any gaps between scaffolds may need to be filled in using very low-throughput techniques. Most non-model organisms never make it to a high degree of finishing and such genome sequences are known as draft assemblies.

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MATERIALS AND METHODS

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CHAPTER III

MATERIALS AND METHODS

The present study was undertaken to characterize and annotate whole genome sequence of *Pasteurella multocida* isolated from different species of animals and identification of virulence associated genes.

3.1 ISOLATION AND CONFIRMATION OF *P. multocida* FROM DIFFERENT SPECIES

3.1.1 Collection of samples and isolation of *P. multocida*

All the samples suspected of *Pasteurella multocida* were collected from four different animal species *viz.* buffalo, cattle, goat and poultry, whereas P52 is a vaccine strain used for vaccination against Haemorrhagic septicaemia. Details of the isolates are given in Table 3.1.

Table 3.1: Details of *Pasteurella multocida* isolates from different animal species

Sr. No.	Species	Isolate designation	Place	Type of tissue
1	Buffalo	Anand1_buffalo	Kapadvanj, Kheda district	Lung
2	Cattle	Anand1_cattle	Gutal, Nadiad, Kheda district	Liver
3	Goat	Anand1_goat	RBRU farm, Veterinary College, Anand district	Nasal swab
4	Poultry	Anand1_poultry	Adas, Anand district	Liver
5	Vaccine Strain P52	P52VAC	Animal Vaccine Institute, Gandhinagar district	Blood

Blood agar (BA) was used as a primary culture medium for preliminary isolation of the *P. multocida* isolates from the suspected samples. A loopful of

triturated tissue sample was first inoculated on BA and incubated at 37°C for 24 hours. Next day, non-haemolytic single colony from BA was transferred to MacConkey agar (MCA) and incubated at 37°C for 24 hours. The isolates which failed to grow on MCA was preliminary presumed to be *P. multocida.* Single non haemolytic colony of these isolates from the primary culture was re-streaked on fresh BA plate and incubated at 37°C for 24 hours to obtain single colony of pure culture of the isolates. The isolates were obtained and stored on blood agar slant in duplicate for further identification by biochemical tests and other studies. All the isolates were stained by Gram's method and observed under microscope to check the purity of growth.

3.1.2 Biochemical characterization of *P. multocida* isolates

Presumptive *P. multocida* isolates were primarily identified by biochemical tests *viz.*, oxidase, catalase, indole, citrate utilization and nitrate reduction as per the methods described by Barrow and Feltham (1993) with some minor modifications.

3.1.2.1 Oxidase test

Standard oxidase discs (HiMedia, Mumbai) were used to perform the test. A single colony was just touched on the disc with platinum loop/ match stick. Immediate development of blue colour was considered as positive.

3.1.2.2 Catalase test

This test was performed by taking 2-3 drops of three per cent H_2O_2 on clean grease free glass slide and single colony was mixed with the help of wire loop. Immediate formation of gas bubbles was considered as positive test.

3.1.2.3 Indole test

Few drops of xylene were added to a two day old broth of the isolate in two mL of tryptone water and mixed thoroughly to dissolve indole and about 0.2 mL of

Kovac's reagent (HiMedia, Mumbai) was added. Pink ring of xylene was considered as positive reaction.

3.1.2.4 Citrate utilization

Slant of Simmon's citrate agar (HiMedia, Mumbai) was inoculated with the culture and incubated at 37°C for four days. Growth with a development of intense blue color of the medium was considered as a positive reaction.

3.1.2.5 Nitrate reduction

Added few drops of two days old broth culture in 2 mL peptone water containing 0.1 per cent potassium nitrate and then incubated at 37°C for two days. Presence of nitrate was detected by adding approximately 4-5 drops of sulfanilic acid and 4-5 drops of alpha naphthylamine reagent to nitrate broth culture. Development of a distinct red colour (which may turn to brown rapidly) was considered as positive test.

3.1.3 PCR based detection of *P. multocida*

3.1.3.1 DNA Extraction

The genomic DNA of *P. multocida* isolates were extracted according to Wilson (1987) with minor modification. The culture was prepared by inoculating the isolates in BHI (Brain heart infusion) broth and incubated at 37°C for 24 hours in a shaker water bath.

3.1.3.1.1 Reagents used for DNA extraction

- Tris-EDTA (pH 8.0)

  10mM Tris-HCL

  1mM EDTA

- Sodium dodecyl sulphate (SDS) (10% w/v)
- Proteinase K solution (20mg/mL, w/v)

- Lysozyme (10mg/mL)
- 5M Sodium Chloride
- 7.5M Ammonium acetate
- 10% cetylmethyl ammonium bromide (CTAB)
- Saturated phenol (pH 8.0)
- Chloroform
- Isoamyl alcohol
- Absolute ethanol
- 70% ethanol
- RNAse A
- Nuclease free water

3.1.3.2 Procedure of genomic DNA isolation by Proteinase K-SDS method

- ✓ Overnight grown culture cells (10 mL) in BHI broth were centrifuged at 10,000 rpm for 10 minutes.
- ✓ Supernatant was discarded and pellet was resuspended in 2 mL Tris-EDTA (pH 8.0).
- ✓ To resuspended pellet, 250 µL SDS (10% w/v), 10 µL of proteinase K solution (20 mg/mL; Qiagen, Germany) and 50 µL of lysozyme (20mg/mL; Fermentas, USA) was added, and incubated for 1 hour at 37°C.
- ✓ After adding 500 µL of 5M NaCl and 100 µL CTAB, the tube was incubated in water bath for 10 minutes at 65°C and then centrifuged at 10,000 rpm for 10 minutes at 4°C.
- ✓ Supernatant was transferred to fresh microfuge tube and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and

centrifuged at 10,000 rpm for 10 minutes at 4°C and upper phase was transferred to clean microfuge tube.

- ✓ Equal volume of Chloroform: Isoamyl alcohol (24:1) was added, mixed well by inverting, spun for 10 min. at 10,000 rpm and upper aqueous phase was transferred to clean microfuge tube.
- ✓ Supernatant was transferred in fresh microfuge tube in which 1/10th volume of 7.5M ammonium acetate and double volume of chilled absolute ethanol was added followed by centrifuging at 12,000 rpm for 15 minutes at 4°C and ethanol was discarded.
- ✓ The pellet was washed in 70% ethanol, followed by centrifugation at 11,000 rpm for 5 minutes and finally dried at 65°C for ~10 minutes.
- ✓ Pellet was resuspended in 250 µL of nuclease free molecular biology grade water, 2 µL of RNase A was added and incubated at 37°C for 1 hour and then at 75°C for another 15 minutes.

3.1.3.3 Quality checking and quantitation of DNA

Quality and quantity of DNA was calculated by using Nanodrop 1000 spectrophotometry (Thermo Scientific, USA) as well as agaose gel electrophoresis (0.8%). Optical density (OD) at 260 and 280 were taken in spectrophotometer with distilled water as reference.

3.1.3.4 PM-PCR for identification of *P. multocida*

For PM-PCR based identification of *P. multocida* genomic DNA, following primer sequence as described by Townsend *et al.* (1998) and yielding the expected product of 460bp for the subsequent use in GS FLX Titanium (Roche, Switzerland) sequencing experiment.

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Primer KMT1SP6 5'- GCT GTA AAC GAA CTC GCC AC - 3'

Primer KMT1T7 5'- ATC CGC TAT TTA CCC AGT GG - 3'

3.2 LIBRARY PREPARATION OF *P. multocida* DNA

3.2.1 DNA fragmentation (Nebulization)

- ✓ Before starting DNA library preparation, quality and quantity of DNA was checked with help of Nanodrop spectrophotometer (Thermo Scientific, USA) and agarose gel electrophoresis.
- ✓ Nebulizer unit was assembled and 500ng of DNA was loaded at bottom of nebulizer cup and TE buffer (pH 8.0) was added to make final volume to 100 µL, followed by 500 µL of nebulizing buffer (Roche, Switzerland).
- ✓ Nitrogen gas was vented through nebulizing unit at 30 psi for 1 minute, and the sample was collected carefully so to get recovery greater than 300 µL. To this, 2.5 mL of buffer PBI (Qiagen, Germany) was added and swirled properly.
- ✓ DNA fragments were purified by using mini elute columns.
- ✓ To the column, 750 µL of the sample was added and centrifuged at 10,000 rpm for 15 seconds.
- ✓ The flow through was discarded and the above step was repeated thrice in the same column. Finally centrifuged for a minute and the remaining solution was discarded.
- ✓ Added 750 µL of PE buffer (Qiagen, Germany), centrifuged at 13,000 rpm for one minute after which, the flow through was discarded.
- ✓ The tubes were centrifuged for 15 sec. and then rotated 180° and centrifuged for another 15 seconds.

- ✓ The column was transferred to a 1.5 mL centrifuge tube and 16 µL of elution buffer (TE buffer) was added.
- ✓ Centrifuged for 1 minute at 13,000 rpm. The eluant was again passed through the same column and centrifuged.
- ✓ The eluted sample (approximately 16 µL), was transferred to 200 µL PCR tubes.

3.2.2 Fragment end polishing

Θ End repair mix preparation

Table 3.2: Preparation of end polishing reaction mixture

Reagents	**Quantity**
RL 10X PNK Buffer	2.5 µL
RL ATP	2.5 µL
RL dNTP	1.0 µL
RL T4 polymerase	1.0 µL
RL PNK	1.0 µL
RL Taq polymerase	1.0 µL
Total volume	**9.0 µL**

- ✓ To the PCR tubes containing the samples, 9 µL of end polishing reaction mixture was added.
- ✓ The tubes were vortexed followed by centrifugation at 10,000 rpm for few seconds.
- ✓ The samples were run in the thermocycler (Applied Biosystems) with the below set parameters.
  - 25° C for 20 minutes
  - 72° C for 20 minutes
  - 4° C on hold.

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3.2.3 Bead preparation

- ✓ The AMPure beads (Backman Coulter, USA) were vortexed for 20 seconds.
- ✓ Fractionated 125 µL of the beads to a 2 mL centrifuge tube.
- ✓ Placed the tubes in a magnetic particle concentrator (MPC; Invitrogen, USA) until all the beads pelleted to one side.
- ✓ The supernatant was then pipetted out carefully.
- ✓ To the tubes, 73 µL of TE buffer was added and the tubes were vortexed for 5 seconds.
- ✓ To the beads, 500 µL of sizing solution was added, vortexed for 5 seconds and centrifuged for 2 seconds.
- ✓ The tubes were then placed on ice until further use.
- ✓ In a tube, 5 mL of 70% ethanol was prepared by mixing 3.5 mL of 100% ethanol and 1.5 mL of molecular grade water (Milli-Q, Merck Millipore, USA).

3.2.4 Adaptor ligation

- ✓ To each of the tubes, 1 µL of RL (Rapid library) MIDs adaptors (Roche, Switzerland) was added, after the completion of the end repair programmed cycles.
- ✓ To the mixture, 1 µL of Ligase was then added, the tubes were vortexed and centrifuged in a micro centrifuge for 2 seconds at 10,000 rpm.
- ✓ The tubes were incubated at 25 °C for 10 minutes.

3.2.5 Small fragments removal

- ✓ To the bead solution, 73 µL of the sample was added. Vortexed and spun in the microcentrifuge for 5 seconds at 10,000 rpm.

- ✓ After incubation, the tubes were placed on the MPC until all the beads were pelleted and carefully discarded the supernatant.
- ✓ To the tubes, 100 µL of TE buffer was added and vortexed for 5 seconds.
- ✓ To the tubes, 500 µL of sizing solution was added and vortexed for 5 seconds.
- ✓ The tubes were then incubated at room temperature for 5 minutes.
- ✓ They were then placed on the MPC till all the beads pelleted on the wall and discarded the remaining supernatant.
- ✓ The above steps were repeated once. The tubes were placed on the MPC and the beads were washed twice with 70% ethanol followed by their complete removal.
- ✓ The tubes were then air dried for 2 minutes at room temperature.
- ✓ To the tubes, 53 µL of TE buffer was then added, vortexed for 5 seconds and centrifuged for 2 sec. in a microcentrifuge.
- ✓ The tubes were placed on the MPC, the beads were pelleted and 50 µL of supernatant which contained the library was transferred to a new centrifuge tube.

3.2.6 Library quantitation

3.2.6.1 Preparation of standard for TBS 380 fluorometer

- ✓ For generation of standard curve, 8 tubes were labeled (1-8).
- ✓ Tube 1: 2.5 X 10^9 molecules/µL solution of RL standard (Roche, Switzerland) was prepared by mixing 90 µL of RL standard with 90 µL of TE Buffer.
- ✓ The remaining 7 tubes (2-8) were filled with 60 µL of TE buffer.
- ✓ From tube 1 to tube 2, 120 µL of solution was transferred.

- ✓ The tube was vortexed for 5 seconds and spun in a minicentrifuge for 2 seconds.
- ✓ From tube 2 to tube 3, 120 µL of solution was transferred.
- ✓ The tube was vortexed for 5 seconds and spun in a minicentrifuge for 2 seconds.
- ✓ The serial dilution was proceeded as above for the remaining 5 tubes.

3.2.6.2 Sample library quantitation using TBS 380 fluorometer

- ✓ To 8 cuvettes, 50 µL of 8 dilutions of the RL standard were transferred.
- ✓ As the blank, 50 µL of TE buffer was taken.
- ✓ The TBS 380 fluorometer (Promega, USA) was set on the blue channel with the blue cuvette holder insert.
- ✓ The standard value was set to 250.
- ✓ The fluorometer was calibrated with the blank and 2.5 X 10^9 molecules/µL solution RL standard.
- ✓ The relative fluorescence units (RFU) was read and recorded for each dilution.
- ✓ To a cuvette, 50 µL of the sample library was transferred. The fluorescence was read and recorded.
- ✓ The sample library was transferred back to its tube with 20 µL pipette.

3.2.6.3 Generation of rapid library (RL) standard curve and calculation of sample concentration

- ✓ To generate the standard curve of the fluorescence readings and to calculate the library sample concentration, the rapid library quantitation calculator was used (http://454.com/my454/tools-downloads/rapid-library calc.asp).

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3.2.6.4 Library quality assessment

- ✓ To assess the quality of library, 1 µL of aliquot of the DNA library was run on a Bioanalyzer High Sensitivity DNA chip (Agilent Technologies, USA).
- ✓ The quality of the DNA library was assessed for the below characteristics.

Table 3.3: Quality assessment of 454 library

Library characteristics	Expected results
Average fragment length	Between 600 bp to 900 bp
Lower size cut off	<10% below 350 bp

3.3 EMULSION PCR

3.3.1 Emulsion based clonal amplification

3.3.1.1 Preparation of the live and mock amplification mixture

- ✓ All the reagents used for emPCR other than enzymes were thawed, vortexed for 5 seconds and centrifuged (including enzymes) for 10 seconds on bench top minicentrifuge.
- ✓ The Live amplification mix for small volume (SV) as well as for large volume (LV) were prepared as per Table 3.4 and vortexed for 5 seconds and stored at +2 to +8°C till used.

Table 3.4: Preparation of live amplification mix

Reagents	2 Cups (LV)	4 tubes (SV)
Mol. Bio. Grade water	2100 µL	280 µL
emPCR Additive	3000 µL	360 µL
5X amplification mix	1720 µL	190 µL
Amplification primer	600 µL	55 µL
emPCR enzyme mix	400 µL	50 µL
PPiase	10 µL	2 µL
Total	**7830 µL**	**937 µL**
(LV:- Large Volume; SV:- Small Volume)		

- ✓ The mock amplification mix was prepared by diluting 2 mL of 5X mock amplification mix with 8 mL of molecular biology grade water in a 15 mL Falcon tube, vortexed for 10 seconds and then stored at +2 to +8°C till used.

3.3.2 DNA library capture

- ✓ Small volume emPCR was first done for getting best ratio of molecule/beads to set large volume emPCR.
- ✓ Capture bead wash buffer TW (Roche, Switzerland) was diluted 10 times to get 1X capture bead wash buffer TW (10 mL) with molecular biology grade water.
- ✓ Single tube of DNA capture beads was vortexed, and centrifuged for 10 seconds, rotating tube 180° and again centrifuged for 10 seconds, followed by discarding supernatant.

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- ✓ The beads were washed twice with 1 mL of 1X capture bead buffer TW and supernatant was discarded by spinning tubes as done in above step.
- ✓ Washed DNA capture bead pellet was resuspended in 640 µL of 1X capture bead wash buffer and then evenly distributed in 1.7 mL tubes.
- ✓ The beads were pelleted and correct amount of DNA library (after heat denature at 95^{0}C for 2 minutes) was added to each tube and vortexed for 5 seconds. The amount of DNA library to be added was calculated on basis of required DNA molecules per bead using formula;

$$\text{µl of library per tube} = \frac{\text{Molecules per bead desired X Number of beads per tube}}{\text{Library concentration (molecules/µl)}}$$

Number of beads per tube = 2.4 X 10^6

- ✓ For small volume emPCR, sample dsDNA library after fluorometer analysis, was diluted in TE buffer to obtain 1 X 10^7 molecules/µL. From 1 X 10^7 molecules/µL of DNA library, 20 µL of dsDNA library was further diluted into 30 µL of TE buffer, to prepare a 4 X 10^6 molecules/ µL. Different amount of dsDNA library and beads were added as per Table 3.5 and vortexed for 5 seconds.

Table 3.5: Amount of library to be used for various molecules/bead ratio

Tube no.	Amount of lib. (µL)	Molecules/bead
Tube 1	1.2	2.0
Tube 2	2.4	4.0
Tube 3	4.8	8.0
Tube 4	9.6	16.0

- ✓ Finally, large volume emPCR was done by taking appropriate ratio of DNA library and capture bead from recovery of small volume emPCR.
- ✓ Capture bead wash buffer TW was diluted 10 times to get 1X capture bead wash buffer TW (10 mL) with molecular biology grade water.
- ✓ The two tubes of DNA capture beads were vortexed and pelleted by centrifuging twice for 10 seconds by rotating microfuge tube to 180°.
- ✓ The supernatant was discarded and beads were washed twice with 1 mL 1X bead wash buffer, vortexed for 5 seconds and again pelleted.
- ✓ DNA library was thawed and correct aliquot was added to each tube of DNA capture bead.
- ✓ The amount of library used was calculated by following equation:

$$\mu l \text{ of library per tube} = \frac{\text{Molecules per bead desired X Number of beads per tube}}{\text{Library concentration (molecules/}\mu l)}$$

Number of beads per tube = 35×10^6

3.3.3 Emulsification and amplification for small volume

- ✓ Emulsion oil was pre-mixed on the tissue lyser (Qiagen, Germany) at 25 Hz for 2 minutes.
- ✓ In each tube of oil, 290 µL of 1x mock amplification was added and inverted 2-3 times.
- ✓ Content in the tube was mixed at 25 Hz for 5 minutes.
- ✓ The prepared DNA library (~255 µL) was added to each tube and mixed in tissue lyser at 15 Hz for 5 minutes.
- ✓ After emulsification, 50 µL of the content was dispensed in 96 well PCR plates (Thermo Fisher Scientific, USA).
- ✓ Plates were then placed in a thermocycler (Applied Biosystem, USA) with amplification program as follow:

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Table 3.6: Amplification cycles for small volume emPCR

	Stage 1	Stage 2	Stage 3
Number of cycles	1	50	-
Cycle parameters	4 min at 94°C	30 sec. at 94°C, 4.5 min. at 58°C, 30 sec. at 68°C	10°C until used

3.3.4 Emulsification and amplification for large volume

- ✓ Tubes containing emulsion oil were mounted in tissue lyser and mixed at 28 Hz for 2 minutes.
- ✓ To these, 5 mL of 1X Mock amplification mix was added in each cup of oil and again mixed at 28 Hz for 5 minutes in tissue lyser.
- ✓ Captured library whole content (~3.75 mL) was added and mixed at 12 Hz for 5 minutes.
- ✓ Emulsified emPCR amplification mix was dispensed in 4 plates each with 96 wells (50 µL per well) and sealed.
- ✓ Plates were then placed in a thermocycler with amplification program as follows:

Table 3.7: Amplification cycles for large volume emPCR

	Stage 1	Stage 2	Stage 3
Number of cycles	1	50	-
Cycle parameters	4 min at 94°C	30 sec. at 94°C, 4.5 min at 58°C, 30 sec. at 68°C	10°C until used

3.3.5 Bead recovery (Vacuum assisted emulsion breaking)

- ✓ Added 187.5 mL of molecular biology grade water (Milli-Q; Millipore, USA) to 62.5 mL of 4X enhancing fluid TW (Roche, Switzerland) stock to make 1X concentrate and placed on ice.
- ✓ Added 72 mL of molecular biology grade water to 8 mL of 10X annealing buffer TW (Roche, Switzerland) stock to make 1X concentrate and kept on ice.
- ✓ 50 mL conical tube was fixed to each of the two lids and the connector located at one end of the tubing was inserted into the top opening of the 8 pronged transpette.
- ✓ The other end of the tubing was connected to a vacuum source.
- ✓ Vacuum was turned on and emulsions were aspirated from eight wells at a time, using a slow circular motion of the transpette tips at the bottom of the wells.
- ✓ The amplified DNA beads were collected in two 50 mL tubes. The plate was rinsed twice with 100 µL of isopropanol (Merck, USA) per well.

3.3.6 Bead washes and recovery for large volume emulsions (LVE)

- ✓ The contents of two (or more) 50 mL collection tubes were mixed by transferring their contents (in pairs) back and forth four times or until the bead suspension were of about the same density.
- ✓ Isopropanol was added to final volume of 40 mL in each tube and vortexed.
- ✓ The beads were pelleted in a centrifuge at 930g for 5 min (2,000 rpm).
- ✓ The supernatant was carefully poured into a waste container and the bead pellets were rinsed twice with 35 mL of isopropanol by centrifugation as mentioned above.

- ✓ The bead pellets were rinsed once again with 35 mL of 1X enhancing fluid TW.
- ✓ The DNA bead suspension was then transferred to two 1.7 mL tubes for each emulsion cup processed.
- ✓ Each of the 50 mL collection tubes were then rinsed with 600 µL of 1X enhancing fluid TW and added this rinse to the 1.7 mL tubes.
- ✓ Again 1.7 mL tubes were centrifuged in a bench top minifuge.
- ✓ The supernatant was removed and rinsed twice with 1 mL of 1X enhancing fluid TW.
- ✓ The bead pellets were then resuspended in a final 1 mL of 1X enhancing fluid TW.

3.3.7 DNA library bead enrichment

Θ Preparation for Indirect Enrichment

- ✓ At this point, the beads carry double-stranded clonally amplified DNA products, and were suspended in 1X enhancing fluid TW.
- ✓ A stock was prepared of melt solution by mixing 125 µL of NaOH (10N) in 9.875 mL of molecular biology grade water.
- ✓ The collected DNA beads were pelleted using bench top minifuge.
- ✓ Supernatant was discarded and the ssDNA beads were prepared by melting the dsDNA amplification products (only one strand is attached to the beads) using one mL of melt solution per tube, vortexed and incubated for 2 minutes.
- ✓ The supernatant was removed and discarded.
- ✓ The beads were washed twice with 1 mL of 1X annealing buffer TW per tube each time.

- ✓ 45 µL of 1X annealing buffer TW and 25 µL of enrichment primer was added per tube and vortexed to mix completely.
- ✓ The enrichment primers were annealed to the bead-bound ssDNA by placing the tubes in a heat block at 65°C for 5 min and then promptly cooling on ice for 2 min.
- ✓ Added 800 µL of 1X enhancing fluid TW and vortexed.
- ✓ Centrifuged the beads, pelleted as above, and the supernatant was removed.
- ✓ Beads were then washed twice more by centrifugation, with 1 mL of 1X enhancing fluid TW per tube.
- ✓ Supernatant was removed, each bead pellet was resuspended in 800 µL of 1X enhancing fluid TW and vortexed.

3.3.8 Preparation of enrichment beads

- ✓ The tube of enrichment beads was placed in a MPC, pelleting the paramagnetic enrichment beads against the side of the tube.
- ✓ The supernatant was then removed and discarded.
- ✓ The pelleted beads were washed twice with 1 mL of 1X enhancing fluid TW, using the MPC.
- ✓ At the end after removing the supernatant, the tube was removed from the MPC and 320 µL of 1X enhancing fluid TW was added.

3.3.9 Enrichment of the DNA carrying beads

- ✓ To each tube of amplified DNA beads, 80 µL of washed enrichment beads were added and vortexed to mix completely.
- ✓ Then rotated on a LabQuake tube roller (Thermolyne) at ambient temperature (+15°C to +25°C) for 5 minutes.

- ✓ The tubes were placed in the MPC and allowed to stand for 3-5 minutes to pellet the paramagnetic enrichment beads against the side of the tubes.
- ✓ The supernatant was carefully removed and discarded from each tube using 1000 µL pipette.
- ✓ The beads were then washed with 1 mL of 1X enhancing fluid TW per tube until there were no visible beads remaining in the supernatant (usually 6-10 washes for LVE)
- ✓ Removed the tubes from the MPC and bead pellets were re-suspended in 700 µL of melt solution.
- ✓ Then vortexed for 5 seconds and placed back into the MPC to pellet the enriched beads.
- ✓ Transferred the supernatants containing enriched DNA beads to a separate 1.7 mL microfuge tube.
- ✓ Pelleted enriched DNA beads by centrifugation and the bead pellets were washed three times with 1 mL of 1X annealing buffer TW (by centrifugation) to completely neutralize the melt solution.
- ✓ Each of the final bead pellets were re-suspended in 200 µL of 1X annealing buffer TW.

3.3.10 Sequencing primer annealing

- ✓ Fifty microliters of sequencing primer was added and vortexed.
- ✓ Tubes were then placed in a heat block at 65°C for 5 min, and then kept promptly on ice for 2 minutes.
- ✓ Added 800 µL of 1X annealing buffer TW to the tube, pelleted and the supernatant was removed.
- ✓ Bead pellets were then washed twice with 1 mL of 1X annealing buffer TW.

- ✓ Resuspended each of the final bead pellets by adding 1 mL of annealing buffer TW.
- ✓ Beads were then stored (immobilized, clonally amplified DNA library) at +2°C to +8°C.

3.4 SEQUENCING EXPERIMENT AND DATA PROCESSING

3.4.1 Sample preparation procedure

3.4.1.1 Preparation of bead buffer 2 (BB2)

- ✓ 1.2 mL of titanium supplement CB (Roche, Switzerland) was added to the 200 mL bottle of pre-chilled titanium bead buffer (Roche, Switzerland) to prepare BB2.
- ✓ Thirty four microliters of apyrase solution was added to the bottle of titanium bead buffer plus titanium supplement CB, mixed by inversion.
- ✓ BB2 was poured into the tray to completely submerge the PTP (Pico titre plate; Roche, Switzerland) device.
- ✓ The PTP device was left submerged in BB2 at room temperature on the lab bench for 10 minutes.

3.4.1.2 Preparation of packing beads

- ✓ Packing beads were washed three times with BB2 using one microliter of BB2 to the tube of packing beads, vortexed and centrifuged at 10,000 rpm for 5 minutes.
- ✓ Supernatant was discarded and beads were re-suspended by adding 550 µL of BB2.

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3.4.1.3 Preparation of DNA beads (Sample and control)

- ✓ Sample DNA beads were spiked with control DNA beads included in the GS sequencing kit (Roche, Switzerland) which serves as internal control for sequencing reaction.
- ✓ DNA library was vortexed and transferred 30 (x4) µL to a 1.7 mL tube. Appropriate volume of control DNA bead 10 (x4) µL was added to each DNA library bead tube.
- ✓ The beads were centrifuged for 1 minute at 10,000 rpm twice. DNA bead incubation mix (DBIM; Roche, Switzerland) was prepared side by side in 15 mL tube and vortexed.

Table 3.8: Preparation of DNA bead incubation mixture

Loading region size	**All size**
PTP size	70X75 mm
BB2 (µL)	1570
Polymerase cofactor (µL)	150
DNA polymerase (µL)	300
Total volume (µL)	**2020**

- ✓ The appropriate volume of DBIM (320(x4) µL) was transferred to DNA beads, vortexed and placed on LabQuak tube roller for 15 minutes at room temperature.
- ✓ The DNA beads were combined with the packing beads to make bead layer 2.

3.4.1.4 Preparation of enzyme and PPiase beads (Bead layers 1, 3 and 4)

- ✓ Vortexed and then pelleted the enzyme beads and the PPiase beads using a magnetic particle concentrator (MPC) as follows.

- ✓ The two tubes of enzyme beads and one tube of PPiase beads were placed on the MPC till the pellet was formed. Supernatant was discarded carefully with pipettor and removed the tubes from MPC.
- ✓ Washed the enzyme and PPiase beads three times using 1 mL of BB2 to each tube of enzyme beads and 500 µL to each tube of PPiase beads and vortexed till the beads were resuspended completely.
- ✓ The tubes were then placed on MPC till the pellet was formed.
- ✓ Supernatant was discarded with pipettor and the tubes were removed from MPC and original volume of BB2 was added to each tube.

Table 3.9: Types of beads

Reagent label	No. of tubes	Volume/tube
Enzyme beads	2	1000 µL
PPiase beads	1	500 µL

- ✓ The beads for layers 1, 3 and 4 were prepared in three separately labelled tubes of an appropriate size. This was done by diluting the enzyme beads (layer 1: Enzyme beads pre-layer; and layer 3: Enzyme beads post-layer) and the PPiase beads (layer 4) in BB2 as follow.

Table 3.10: Preparation of beads for layers 1, 3 and 4

Bead layer	BB2 (µL)	Enzyme beads (µL)	PPiase beads (µL)	Total volume (µL)
Layer 1	3250	550	---	3800
Layer 3	2500	1300	---	3800
Layer 4	3340	---	460	3800

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3.4.1.5 Combining DNA and Packing Beads

✓ On completion of incubation of DNA beads with DBIM (DNA bead incubation mix), packing beads and BB2 were added to DNA beads as per Table 3.11.

Table 3.11: Packing beads and BB2 addition to DNA beads

Loading region size	Medium
PTP size	70X75 mm
DNA Beads µL	350 (x4)
Packing beads µL	100
BB2 µL	210
Total beads µL	660

✓ The DNA and packing bead mixture was then incubated on LabQuake tube roller for 5 minutes, and unused packing beads were discarded.

✓ PTP device was removed, wiped and placed on BDD (Bead deposition device).

✓ Bead loading gasket was placed on BDD base by laying it on PTP and assembled.

3.4.2 Picotitreplate (PTP) device preparation

3.4.2.1 Bead layer 1: Enzyme beads pre-layer

✓ The bead suspension for layer 1 was vortexed for 5 seconds and 660 µL was carefully drawn for uniform bead distribution throughout region.

✓ The assembled BDD along with counterweight were centrifuged for 5 minutes at 1620g, seals were removed and BDD was discarded using pipettor.

3.4.2.2 Bead layer 2: DNA and packing beads

- ✓ Tube of beads of layer 2 was centrifuged and mixed.
- ✓ Six hundred sixty µL was dispensed over the first layer through loading port and sealed.
- ✓ The assembled BDD along with counterweight were centrifuged for 10 minutes at 1620g, seals were removed and BDD was discarded.

3.4.2.3 Bead layer 3: Enzyme beads post-layer

- ✓ Tube of enzyme beads of layer 3 was centrifuged and mixed.
- ✓ Six hundred sixty µL was dispensed over the first layer through loading port and sealed.
- ✓ The assembled BDD along with counterweight were centrifuged for 5 minutes at 1620g, seals were removed and BDD was discarded.

3.4.2.4 Bead layer 4: PPiase beads

- ✓ Tube of PPiase beads was centrifuged briefly and mixed.
- ✓ Six hundred sixty µL was dispensed to over first layer through loading port and sealed.
- ✓ The assembled BDD along with counterweight were centrifuged for 5 minutes at 1620g, seals were removed and BDD was discarded.

3.4.3 Sequencing run

3.4.3.1 Preparation and loading the sequencing reagents cassette

- ✓ The titanium supplement CB (Roche, Switzerland) was added to each bottle of titanium buffer CB as per Table 3.12.

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Table 3.12: Addition of titanium supplement CB

Reagent label	No. of bottles	Volume of titanium supplement CB added per bottle	Volume of DTT added per bottle
Titanium buffer CB	4	6.6 mL	1000 µL

- ✓ Forty five µL of inhibitor TW was transferred from the sequencing reagent tray to 1.7 mL tube.
- ✓ Five µL of the concentrated PPiase reagent was added to 45 µL aliquot of inhibitor TW and vortexed.
- ✓ 13.2 µL of the diluted PPiase reagent was added to inhibitor TW tube.
- ✓ 260 µL of apyrase reagent to the tube of the buffer for apyrase reagent.
- ✓ 3000 µL of dATP reagent to the tube of buffer for dATP.
- ✓ The entire sequencing reagent tray was inverted several times to uniformly mix the contents of all tubes and the sequencing reagent tray loaded into the instrument.

3.4.3.2 PTP cartridge loading and sequencing run

- ✓ PTP was carefully lifted and inserted into cartilage from BDD after deposition of forth layer, discarding supernatant and uncasing top of BDD.
- ✓ Started the sequencing run using various parameters for sequencing run PTP id, Name of sequencing run, PTP type (4 Region),. Number of cycles (200) for nucleotide flow and sequencing run type was full processing,

3.4.4 Data transfer and signal processing

- ✓ The data generated was transferred to GS FLX cluster with help of file transfer protocol (FTP) and signals present in form of images were

processed, giving reads using "GS Run Browser" software (Roche, Switzerland).

✓ The sequencing depth was calculated using following formula:

$$\text{Sequencing depth (coverage)} = \frac{\text{Total number of bases in reads}}{\text{Total number of bases in reference}}$$

3.5 BIOINFORMATICS ANALYSIS

The reads generated using 454 sequencing run were further analysed using various bioinformatics tools. Preliminary analysis was done using two different softwares viz., GS Reference Mapper (Roche, Switzerland) for mapping the genome of *Pasteurella multocida* isolates and another was GS *De Novo* Assembler (Roche, Switzerland), used for assembling reads which were not assigned to the reference genome. Remaining reads which were not mapped and assembled were singleton reads and used for the BLASTX analysis. The detailed procedure for above software is given below.

3.5.1 GS Reference Mapper

The GS Reference Mapper (Roche, Switzerland) aligns sequencing reads against a reference sequence consisting of one or more sequences, with or without associated annotations. The GS Reference Mapper software is an interactive application used to create mapping projects, add or remove reads from the project, specify reference sequences, annotations and other project parameters, run the mapping algorithms on the project data, and view the output produced by the mapping computations. The application can be accessed via a Graphical User Interface (GUI) or from a command line interface (CLI).

Mapping generates consensus sequences of the reads that align against the reference and also computes statistics for variations found in the reads, relative to the

reference. Data were output to a variety of file formats, including FASTA, ACE, BAM or consed files.

When the mapping algorithms run, the software performs the following operations:

- For each read, search for its best alignment to the reference sequence(s) (a read may align to multiple positions in the reference); this is done in 'nucleotide' space.
- Perform multiple alignments for the reads that align contiguously to the reference in order to form "contigs." From the contigs' multiple alignments, consensus base call sequences were produced using the signals of the reads in the multiple alignments (performed in 'flowspace').
- Identify subsets of the reads that vary relative to the reference to form lists of putative variations (nucleotide differences and structural variants). For each putative variation, the reads supporting the variation will be in the subset.
- Evaluate these lists of putative variations to identify High-Confidence nucleotide differences (HCDiffs), structural variations (HCStructVars) and larger-scale structural rearrangements (HCStructRearrangements).
- Output the following information:
  - ✓ Contig consensus sequence(s) and associated quality values
  - ✓ Alignments of the reads to the reference
  - ✓ Position-by-position metrics of the depth and consensus accuracy (quality values) for each position in the aligned reference
  - ✓ The positions and alignments of identified differences.

For performing mapping of our five isolates of *Pasteurella multocida*, we used Pm70, isolated from poultry as a reference sequence available for the *P. multocida*.

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The mapping project was created using reads that were available with 454 pyrosequencing approach. Multiplex Identifiers (MIDs) tab was selected to scan for the presence of MIDs, and the reads containing the selected MIDs were made eligible for mapping in the project. The input parameters kept for mapping were kept with (Seed step- 12, Seed length- 16, Seed count- 1, Hit-per-seed limit- 70, Minimum overlap length- 40, Minimum overlap identity- 90, Alignmnet identity score- 2, Allignmnet difference score- -3, Repeat score threshold- 12) for all *Pasteurella* isolates.

3.5.2 GS *De Novo* Assembler

The GS *De Novo* Assembler (Roche, Switzerland) application constructs *De Novo* assemblies of the reads from one or more sequencing runs, using the "read flowgrams" (SFF files) as input. The GS *De Novo* Assembler software, an interactive application used to create assembly projects, add or remove reads from the project, specify project parameters, run the assembly algorithms on the project data, and view the output produced by the assembly computations. The application can be accessed via a graphical user interface (GUI) or from a command line interface (CLI).

Input data was combined from one or several regions of one or several runs of interest. Assembly generates a consensus sequence of the sample DNA library, output as one or more contiguous sequences (contigs) in FASTA, ACE or consed files.

During the assembly process, the software:

- ✓ Identifies pairwise overlaps between reads,
- ✓ Constructs multiple alignments of overlapping reads and divides or introduces breaks into the multiple alignments in regions where consistent differences were found between different sets of reads. This step results in a preliminary set of "contigs" that represents the assembled reads.

- ✓ Attempts to resolve branching structures between contigs,
- ✓ Generates consensus base calls of the contigs by using quality and flow signal information for each nucleotide flow included in the contigs multiple alignments,
- ✓ Outputs the contig consensus sequences and corresponding quality scores, along with an ACE file of the multiple alignments and assembly metrics files.

Read overlaps and multiple alignments were made in "nucleotide" space while the consensus base calling and quality value determination for contigs were performed in "flowspace". Work in flowspace allows the quality-weighted averaging of processed flow signals (a continuous variable) at each nucleotide flow of the sequencing run(s) and allows the use of information from the "negative flows", i.e. flows where no nucleotide incorporation is detected. The use of flowspace in determining the properties of the consensus sequence results in an improved accuracy for the final base calls.

The assembler produces contigs from the multiple alignments of overlapping read sequences. The GUI provides tools that allow the contig consensus sequences, the multiple alignments of the reads that form the contig, and the flowgrams of these reads to be viewed interactively.

The new assembly project was created in *GS De Novo* Assembler and when a project opens, the overview tab was displayed. The read data file (.sff) was added before an assembly computation was performed. Use Multiplex Filtering" checkbox was selected which allows to screen multiple libraries of *Pasteurella multocida* prepared using distinct MID tags and sequenced together, on the same PTP device. The input parameter for assembly were kept (Seed step-12, Seed length- 16, Seed

count- 1, Minimum overlap length- 40, Minimum overlap identity- 90, Alignmnet identity score- 2, Allignmnet difference score- -3) for all *P. multocida* isolates.

3.5.3 RAST annotation server

RAST server was developed by Aziz *et al.* (2008), designed to rapidly call and annotate the genes of a complete or essentially complete prokaryotic genome. RAST, Rapid Annotations based on Subsystem Technology, uses a "Highest Confidence First" assignment propagation strategy based on manually curated subsystems and subsystem-based protein families that automatically guarantees a high degree of assignment consistency. For analysis of all our five *Pasteurella* isolates, contigs generated after reference mapping with Pm70 were uploaded to RAST server (http://rast.nmpdr.org/).

RAST used existing tools built by other research teams to first identify both the tRNA and rRNA encoding genes. For the tRNA genes, it uses tRNAscan-SE (Lowe and Eddy, 1997) and to identify the rRNA encoding genes, uses a tool "search_for_rnas" (McNeil *et al.*, 2007). Then, the server will not consider retaining any protein-encoding gene that significantly overlaps any of these regions.

Once the tRNA and rRNA gene-encoding regions were removed from consideration, an initial call using GLIMMER2 was made. Using this small set of representatives, it searches the protein-encoding genes from the new genome for occurrences of these Fellowship of Interpretation of Genome families (FIGfams). This was a very rapid step, since only the new genome was being searched, and it was being searched using a small set of representative protein sequences. The outcome of this initial scan was a small set (normally, 8-15 genes) that can be used to estimate the closest phylogenetic neighbours of the newly-sequenced genome. The putative genes

that remain can be used to search against the entire collection of FIGfams. This was done by blasting against a representative set of sequences from the FIGfams to determine potential families that need to be checked, and then checking against each family.

The final assignments of functions were made to the remaining putative genes. If similarities were computed in the preceding step, these similarities can be accessed and functions can be asserted. Once assignments of function was made, an initial metabolic reconstruction was formed.

3.5.4 NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP)

NCBI Prokaryotic Genome Automatic Annotation Pipeline was designed to annotate bacterial and archaeal genomes (chromosomes and plasmids). Genome annotation, a multi-level process that includes prediction of protein-coding genes, as well as other functional genome units such as structural RNAs (5S, 16S and 23S), tRNAs, small RNAs, pseudogenes, control regions, direct and inverted repeats, insertion sequences, transposons and other mobile elements (http://www.ncbi.nlm.nih.gov/genome/ annotation_prok/).

PGAAP developed by NCBI is an automatic annotation pipeline that combines *ab initio* gene prediction algorithms with homology based methods. The pipeline is capable of annotating both complete genomes and draft WGS genomes consisting of multiple contigs. Since our genome sequenced were not in a single scaffold, the genome assembly is in multiple pieces and were submitted using WGS submission portal (https://submit.ncbi.nlm.nih.gov/subs/wgs1/) after removing the sequences which were less than 200bp (using in house script).

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3.5.4.1 Gene finding using GeneMarkS+

A gene prediction program GeneMarkS+, integrates information about protein alignments, frameshifted genes, non-coding RNA, and DNA statistical patterns typical for protein-coding and non coding regions into gene predictions. GeneMarkS+ is an extension of the GeneMarkS *ab initio* gene finding program (Besemer *et al.*, 2001).

Detecting frameshifts is a critical component of resolving ambiguities in automated annotation, and provides important feedback in assessing the quality of an assembly. Frameshift detection in the pipeline was implemented as a two-step process. Proteins from the target set were aligned to the genome with ProSplign (a global alignment algorithm) that detects alignments with frameshifts. These regions were passed to GeneMarkS+ and reported in the final output as pseudogenes. In a second step, newly predicted GeneMarkS+ genes were evaluated for potential frameshifts. All the proteins were aligned to the search set used for protein identification and naming. Several categories of potential frameshift were handled currently, including a set of tandem models collocated within a threshold of 200 nucleotides and aligned to the same search protein as well as singleton partial alignments of sufficient quality, were identified as a potential frameshifted region. All candidate search proteins were then aligned to the region with ProSplign and evaluated for frameshifts. If at least four candidate search proteins align in a given region with a frameshift, the original models were replaced with a new gene feature with a pseudo qualifier covering the maximal extent of aligned frameshifted protein.

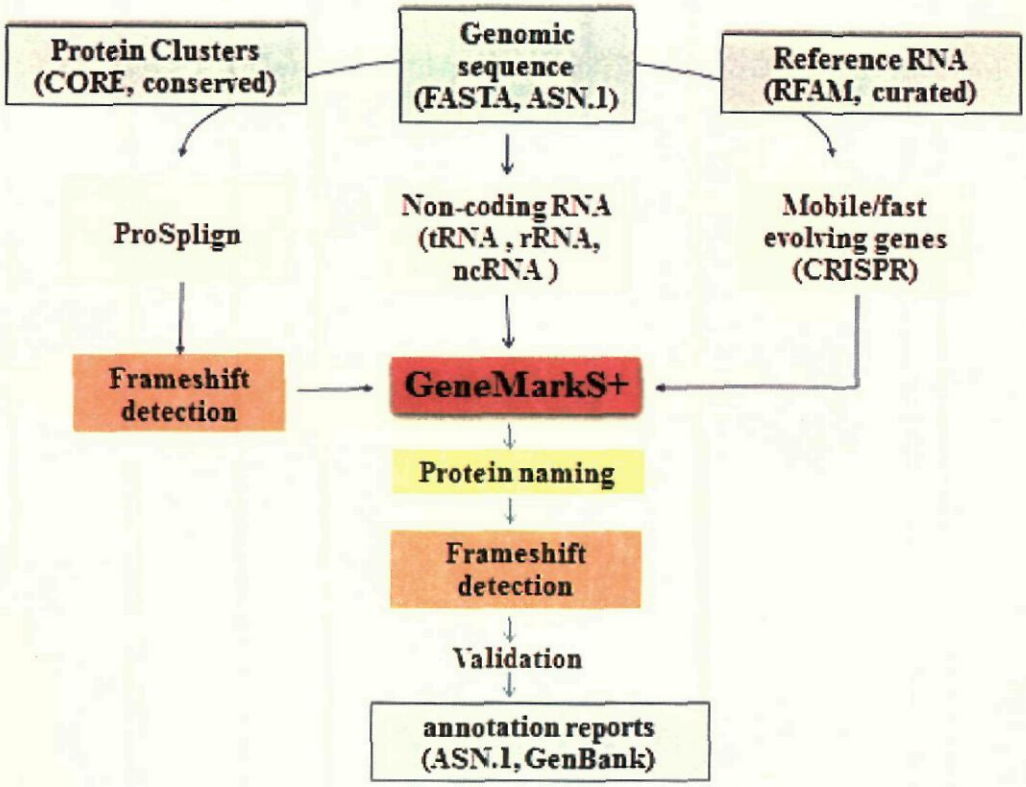

Figure 3.1: Workflow for the GeneMarkS+ used by NCBI database

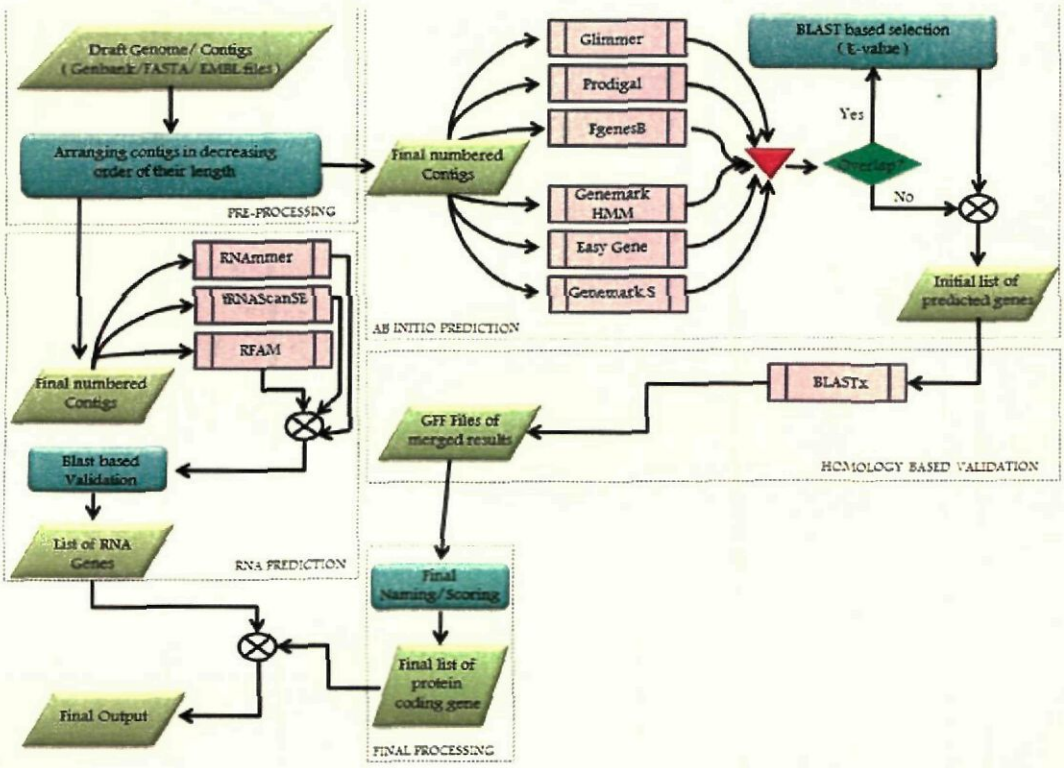

Figure 3.2: Overview of gene prediction pipeline

3.5.4.2 Annotation results of PGAAP

The annotation pipeline produces ASN.1 files (*.sqn) ready for GenBank submission. It can also convert ASN.1 to traditional GenBank flat file format for manual review. The summary report is generated as a part of the output file and includes the total number of predictions by each feature type.

3.5.5 tRNA prediction

For tRNA prediction tRNAscan-SE, available at http://lowelab.ucsc.edu/tRNAscan-SE/ was used (Schattner *et al.*, 2005). Raw sequences generated after sequencing were uploaded to tRNAscan-SE 1.21 server for the tRNA prediction.

3.5.6 rRNA prediction

For rRNA prediction, RNAmmer tool was used, available at http://www.cbs.dtu.dk/services/RNAmmer (Lagesen *et al.*, 2007). All the contigs were uploaded to RNAmmer 1.2 server in FASTA format.

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RESULTS AND DISCUSSIONS

CHAPTER IV

RESULTS AND DISCUSSION

Bacteria are the most predominant form of free-living life on earth and are ubiquitous. Many of them are causing diseases and hence, understanding their evolution, adaptation, and virulence functions helps in developing intervention procedures involving modern biotechnological tools for our benefit. This also leads to improve our understanding of how the host and bacterial interaction occurs and the mechanisms of overcoming the host defence mechanisms to establish an infection. Bacteia are also excellent systems for studying adaptation, both at the level of genome content and at the level of controlling gene expression and protein activity in response to changing environmental and host conditions.

Genome sequence analysis has enabled the development of various clinico-microbiological tools for pathogen detection, identification or genotyping by identification of sequence fragments specific at distinct taxonomic levels (genus, species, strain, clone), for the detection of genes associated with antibiotic resistance or virulence and for the identification of deficient metabolisms to aid development of optimized culture media. However, whole-genome sequencing (WGS), by giving access to the full genetic repertoire of an isolate, has demonstrated an undisputed discriminatory power for deciphering the whole disease process. In addition to identifying bacteria at various taxonomic levels, WGS also offers an opportunity to detect various genetic markers, such as virulence factors or antibiotic resistance-associated genes. Identifying and characterizing the virulence factors of pathogens are crucial for understanding the pathogenesis of the diseases that they cause and for developing dedicated molecular tools to detect specific virulence markers. Genomics has played an important role in the identification of virulence factors in bacteria.

Three main strategies are used to identify virulence factor encoding genes in genomes: first, comparison of genomes from strains or species exhibiting diverse degrees of virulence; second, identification of laterally transferred genomic islands, assuming that virulence genes are often acquired by this mechanism and third, comparing the genome against databases of known virulence markers.

Diseases caused by many members of *Pasteurellacae* family are very harmful, acute and economic loss to the farmers is very high. Despite vaccination to the animals particularly against Haemorrhagic Septiceamin (HS), several outbreaks occur in Gujarat as well as in other parts of India on regular basis. Whole genome sequencing is a recent advanced approach for understanding of genetic makeup of an organism as well for identification of virulence factors responsible for the disease process in host. The first whole genome sequence of *P. multocida* isolated from poultry was done in 2001 by May *et al.* (2001). After that, no other attempt for complete genome sequencing of *P. multocida* was apparently carried out during the subsequent decade. Recently, in last 2-3 years, whole genome sequencing of pig, bovine and poultry (virulent) isolates were carried out (Liu *et al.,* 2012; Abrahante *et al.,* 2013; Lainson *et al.,* 2013[a]; Lainson *et al.,* 2013[b]). None of these whole genome sequencing projects involved Indian isolates of *P. multocida* till date, except for Vaid *et al.* (2014) and Abrahante *et al.* (2014) at Minnesota, USA (for the buffalo isolates). This might be the first study for the whole genome sequencing of *P. multocida* from different speices of animals to desipher more insight into the virulence factors responsible for various important diseases in different animal species and poultry.

The study was conducted to sequence whole genome of *Pasteurella multocida* of different species, to annotate their genome and finding the virulence associated genes which were either reported earlier or previously not identified and/or

characterized. These newly identified genes or the protein products may directly or indirectly be involved in virulence of the bacteria and thereby the disease process.

Whole genome sequencing of four *Pasteurella multocida* field isolates viz. poultry, goat, buffalo and cattle as well as P52 (vaccine strain) was carried out at Department of Animal Biotechnology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand. All the isolates of *P. multocida* were identified by cultural and biochemical characters and were subsequently confirmed by PM-PCR. All the isolates were subsequently processed for the whole genome sequencing using pyrosequencing based 454 GS-FLX Titanium (Roche, Switzerland).

4.1 ISOLATION AND CHARACTERIZATION OF *P. multocida* ISOLATES

For the isolation of *P. multocida*, blood agar (BA) was used as a primary culture medium for preliminary isolation. The samples were initially inoculated on BA plates and incubated at 37°C for 24 hours. The non-haemolytic moderate sized, round, greyish, smooth or mucoid colonies which failed to grow on MacConkey agar (MCA) were primarily presumed to be of *P. multocida*. On the basis of Gram's staining, the isolates were found to be Gram-negative coccobacillary organisms. These isolates were further confirmed to be *P. multocida* by various prescribed biochemical tests and were found to be positive for oxidase, catalase, indole production and nitrate reduction. The isolates were designated as P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle, for P52 (vaccine strain), poultry, goat, buffalo and cattle isolates of *P. multocida*, respectively. Such an approach for isolation and characterization of *P. multocida* has also been used by Carter and Chengappa (1980) and Wijewardana *et al.* (1986).

4.1.1 Nucleic acid isolation

From pure culture of the organism, DNA was isolated according to method described by Wilson (1987) from all the isolates of *P. multocida.* The cultures were grown in BHI (Brain heart infusion) broth overnight at 37°C. Bacterial cells were harvested by centrifugation of broth culture and the pellet thus obtained was subjected to enzymatic digestion by proteinase-K-SDS method. Nucleic acid was extracted by standard phenol-chloroform method and precipitated using ethanol.

The qualitative and quantitative evaluation of DNA was carried out using agrose gel electrophoresis and NanoDrop Spectrophotometer ND 1000 (Thermo Scientific, USA). The Spectrophotometric evaluation of the extracted DNA revealed 680, 878, 843, 784 and 528 ng/μL concentration for P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle respectively, as well as 260/280 ratio of 1.87, 1.93, 1.83, 1.89 and 1.92. Further, agarose gel electrophoresis was performed, which revealed fine quality genomic DNA (Plate 4.1).

4.1.2 PM-PCR based identification

For the PCR based identification and thereby confirmation of *P. multocida,* the protocol described by Townsend *et al.* (1998), specific for PM-PCR using KMT1SP6 and KMT1T7 primers were used with some minor modification. All the five isolates including P52 vaccine strain showed the expected amplified product of approximately 460bp, thus confirming them to be the isolates of *P. multocida* (Plate 4.2). Usefulness of PM-PCR using KMT1SP6 and KMT1T7 primers has also been reported earlier (Townsend *et al.,* 1998; Townsend *et al.,* 2001; Kro *et al.,* 2011; OIE, 2012).

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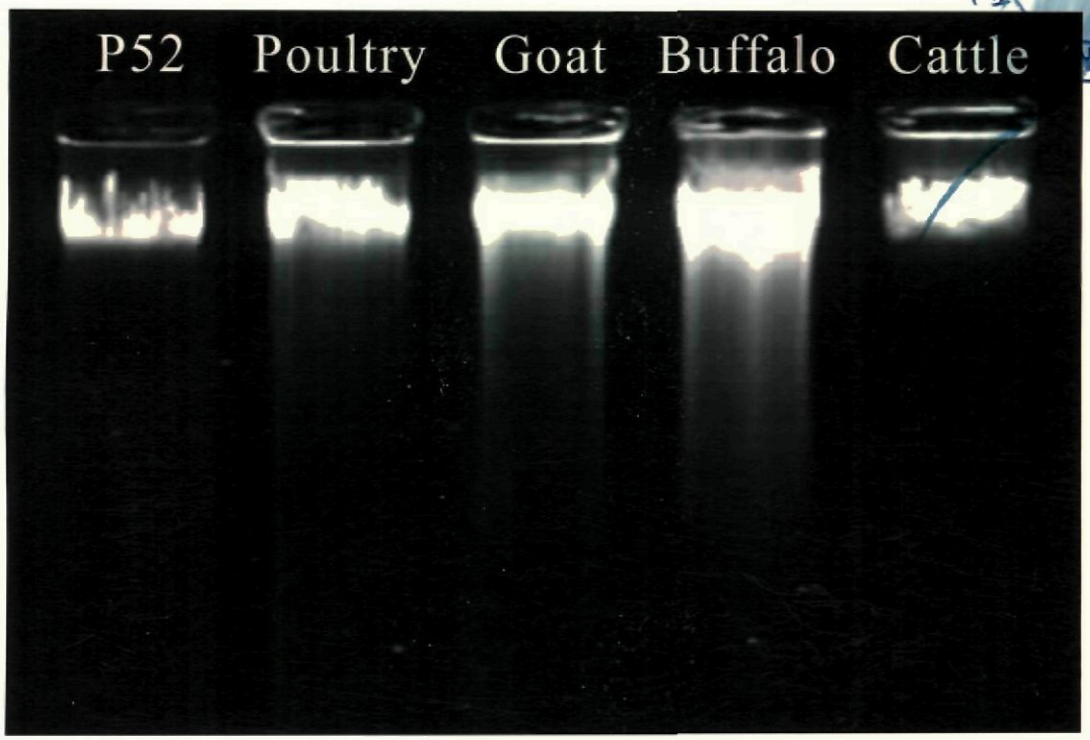

Plate 4.1: Agarose gel electrophoresis of extracted genomic DNA of *P. multocida* isolates

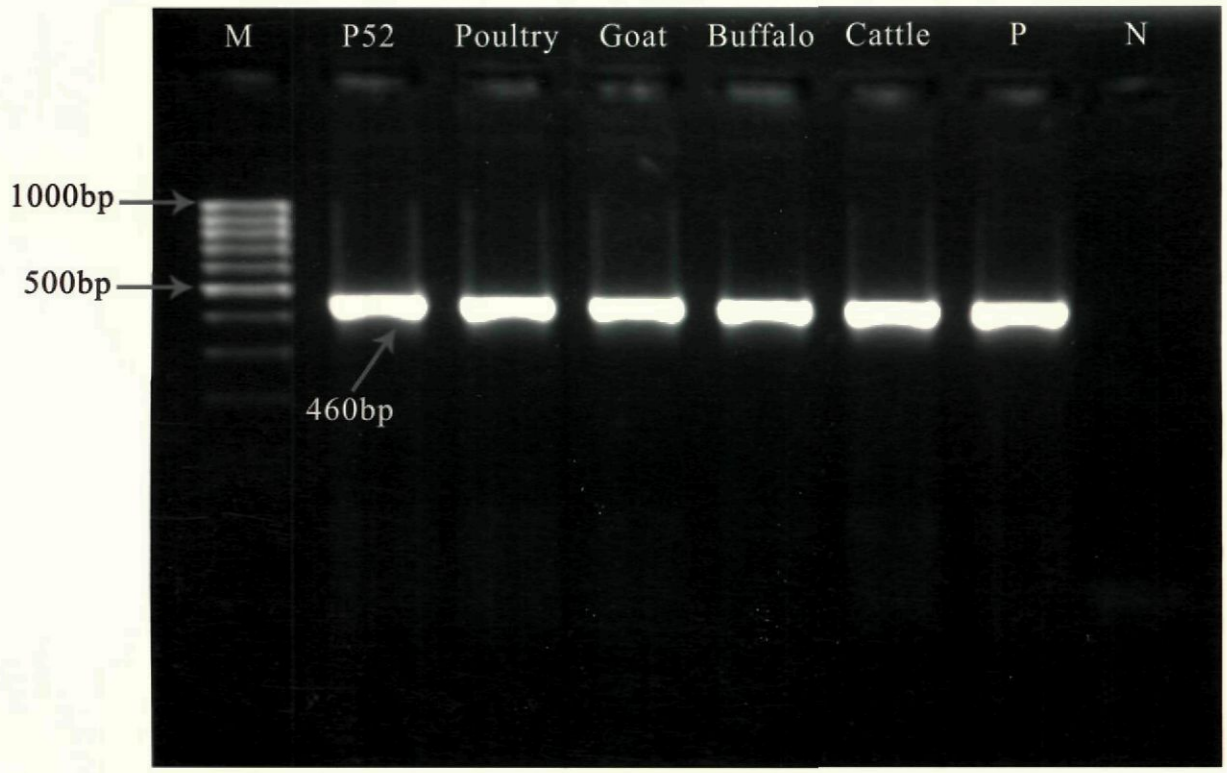

Plate 4.2: PM PCR based identification of *P. multocida* isolates showing 460bp product size for all the isolates. M: Molecular marker (100bp), P: Positive control, N: Negative control

4.2 WHOLE GENOME SEQUENCING OF *P. multocida* ISOLATES

4.2.1 DNA nebulization, library quantity and quality assessment

4.2.1.1 Library quantity assessment using rapid library calculator (Flurimeter assay)

DNA extracted from all the five isolates as in section 4.1.1 was used for the 454 GS FLX DNA library preparation. All the samples (DNA of the isolates) were nebulized and MIDs adaptor ligated as per the protocols in section 3.2.

Library quantitaion was done by comparing sample with known concentration of the standard by preparing a standard curve (Plate 4.3), available at (http://454.com/my454/tools-downloads/rapid-library-calc.asp). Taking standard curve as the reference, reading of unknown sample was calculated as 7.27 X 10^8 molecules/µL, 5.85 X 10^8 molecules/µL, 7.93 X 10^8 molecules/µL, 3.93 X 10^8 molecules/µL and 3.01 X 10^8 molecules/µL for P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle respectively.

Stock sample (dsDNA library) was diluted to get 1 X 10^7 molecules/µL by mixing stock library sample and TE Buffer (pH 8.0) as per Table 4.1.

Table 4.1: Stock library preparation

Sample name	Stock library sample	TE buffer
P52VAC	6.9 µL	493.1 µL
Anand1_poultry	8.5 µL	491.5 µL
Anand1_goat	6.3 µL	493.7 µL
Anand1_buffalo	14.8 µL	485.2 µL
Anand1_cattle	16.6 µL	483.4 µL

4.2.1.2 Bioanalyser assay

For library quantitation, size selected dsDNA fragments were run on High Sensitivity DNA Chip in Bioanalyzer 2100 (Agilent Technologies, USA). Bioanalyzer results showed uniform distribution of DNA fragments across the length with average fragment size of 997bp, 892bp, 867bp, 783bp and 763bp as well as concentration of 2,706.62, 605.61, 822.70, 1,395.41 and 694.12 pg/µL for P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle, respectively (Plate 4.4).

The DNA library, diluted to final concentration of 1 X 10^6 molecules/µL by taking 10µL of dsDNA and 90µL TE buffer (pH 8.0) for all the samples, denatured and finally used for emPCR. After small volume emPCR, beads were recovered, sequencing primers were attached and then percentage enrichment was calculated using following formula;

$$\% \text{ enriched beads} = \frac{\text{Number of final enriched beads}}{\text{Total number of beads}} \times 100$$

Tubes with obtained different molecules/beads ratio with % beads recovery details were as per Table 4.2.

Table 4.2: Percentage bead recovery of small volume emPCR

Tube no.	Molecules/beads	No. of beads recovered	% bead recovery
Tube 1	2.0	0.72 X 10^5	3
Tube 2	4.0	2.16 X 10^5	9
Tube 3	8.0	3.84 X 10^5	16
Tube 4	16.0	6.00 X 10^5	25

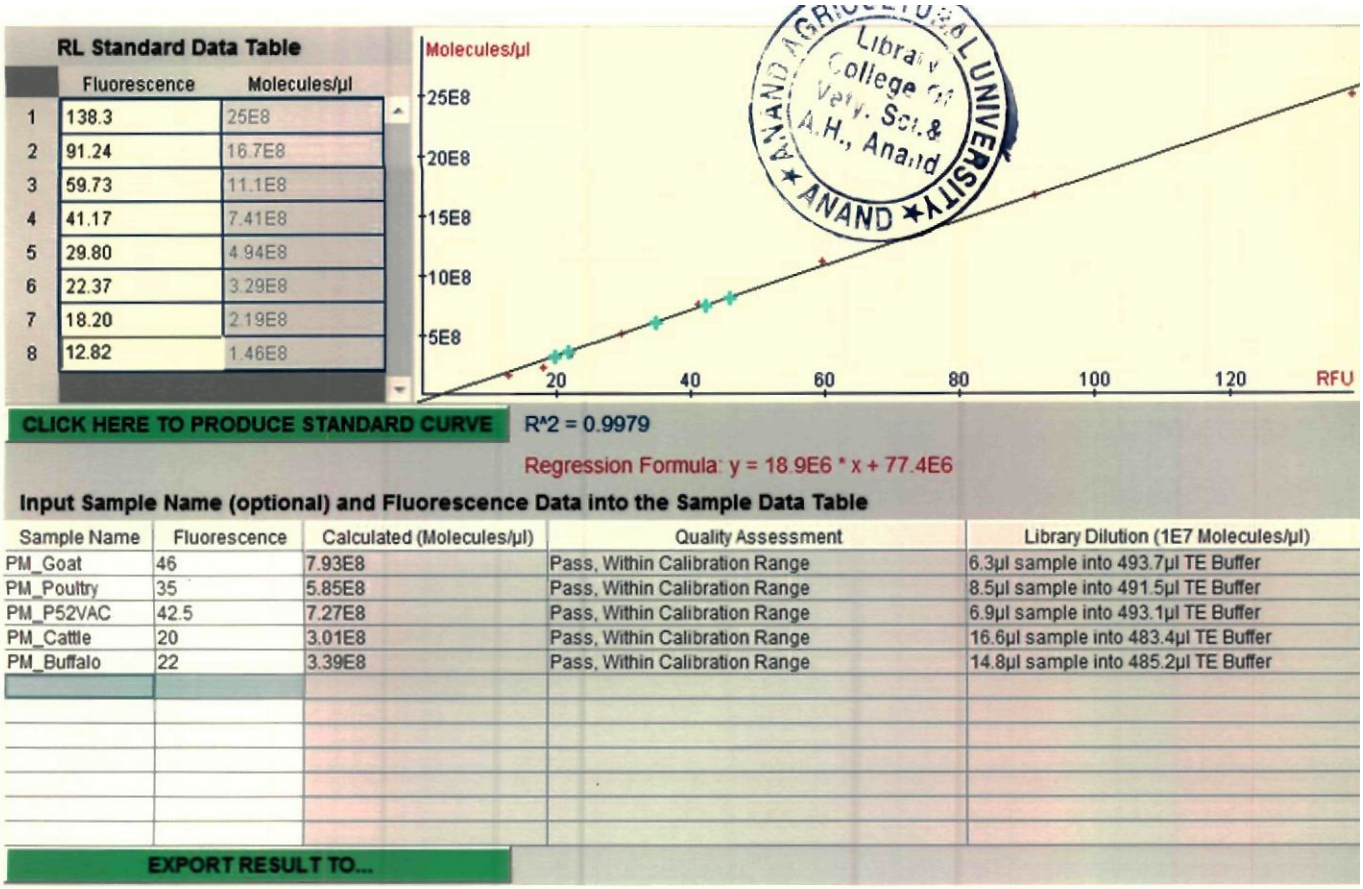

Plate 4.3: GS FLX library calculation for all the five isolates of *P. multocida*

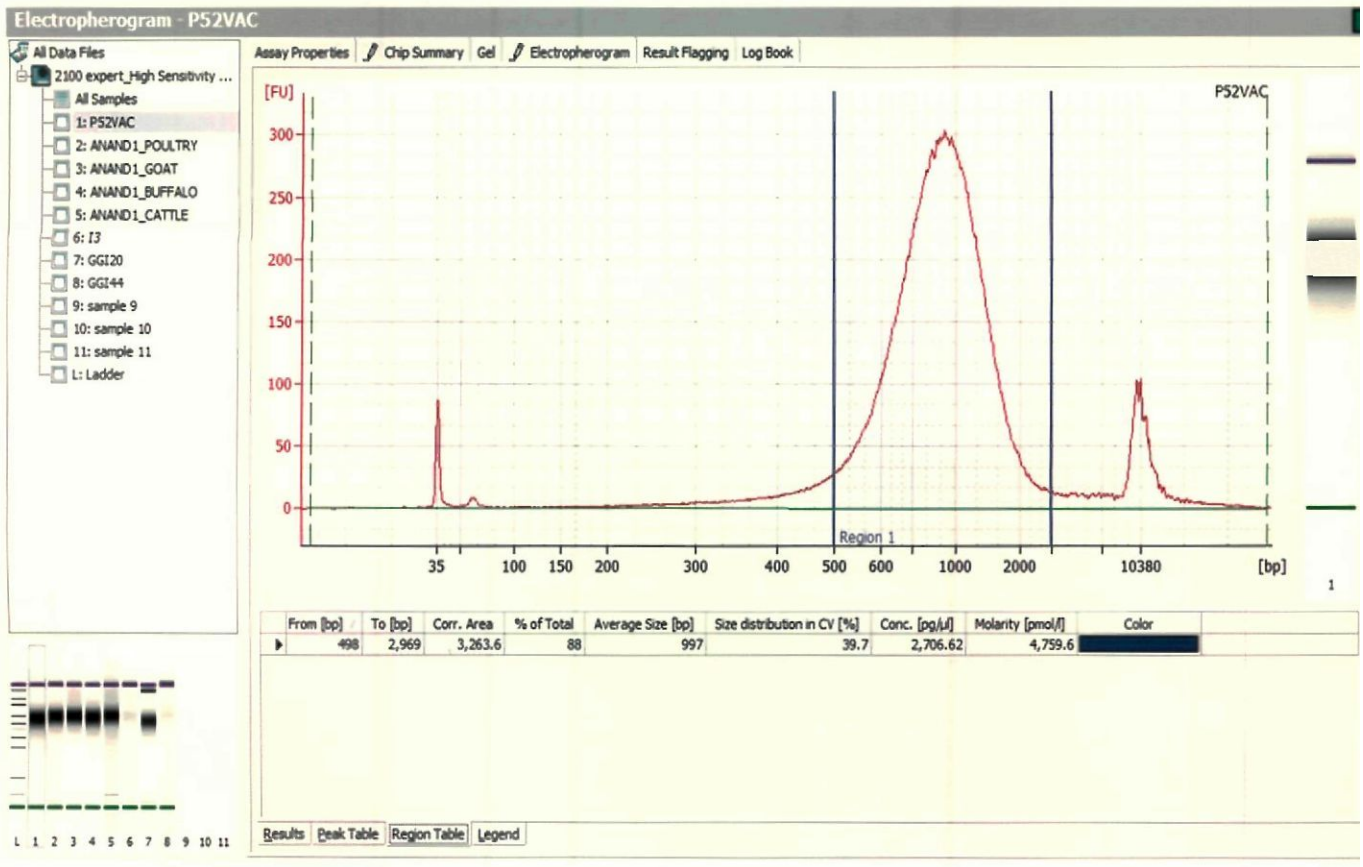

Plate 4.4: Flurogram of a nebulized DNA sample after dsDNA library preparation

From percentage bead recovery of small volume (SV) emPCR, ideal bead recovery (i.e. 9.0%) having 4.0 molecules/bead was considered for LV (large volume) emulsion PCR. The clonally amplified DNA beads were enriched, annealed sequencing primers and finally DNA beads were used for sequencing run. On completion of sequencing run, raw data in form of images was transferred to GS FLX cluster via file transfer protocol (ftp), where signal (data) processing was done.

4.3 SEQUENCE ANALYSIS

After signal processing, reads were generated for all five *P. multocida* isolates and sequencing depth was calculated using below mentioned formula.

$$\text{Sequencing depth (Coverage)} = \frac{\text{Total number of bases in reads}}{\text{Total number of bases in reference genome}}$$

Whole genome sequence available for *P. multocida* Pm70 strain was used for mapping of all the five isolates using GS Reference Mapper version 2.3 and remaining reads which were not mapped, were assembled using GS *De novo* Assembler (Newbler) version 2.3 to check whether any read contains sequences for plasmid as well as for unique sequences related to *P. multocida*. Detailed results of mapping and assembly are given in Tables 4.3 and 4.4 respectively.

To confirm the results of mapping and assembly, clustal multiple sequence alignment of the isolates was also done with Pm70 reference strain sequence using MUSCLE. A representative clustal multiple alignement (for *HemU* DNA sequence) is shown in Plate 4.5, which shows sequence match for all the five isolates of *P. multocida* with Pm70. Currently available GS FLX Titanium instrument is having a capacity to sequence around 400 - 500bp at a time in 200 cycles of nucleotides (A, T, G and C) flow. As shown in Plate 4.6, most of the reads were in the size range of 300 - 500bp.

```
CLUSTAL multiple sequence alignment by MUSCLE (3.8)

Anand1_goat         ATGAATGTTACAGCAAGACTGTGGCTGCCGCCGATTCTGCTGGTTCTGGTTGCAATTTCA
Pm70                ATGAATGTTACAGCAAGACTGTGGCTGCCGCCGATTCTGCTGGTTCTGGTTGCAATTTCA
Anand1_poultry      ATGAATGTTACAGCAAGACTGTGGCTGCCGCCGATTCTGCTGGTTCTGGTTGCAATTTCA
Anand1_cattle       ATGAATGTTACAGCAAGACTGTGGCTGCCGCCGATTCTGCTGGTTCTGGTTGCAATTTCA
Anand1_buffalo      ATGAATGTTACAGCACATCTGTGGCTGCCGCCGATTCTGCTGGTTCTGGTTGCAATTTCA
P52VAC              ATGAATGTTACAGCACATCTGTGGCTGCCGCCGATTCTGCTGGTTCTGGTTGCAATTTCA
                    ***************   ******************************************

Anand1_goat         GCATGCCTGATTGGCCAATATCATATTGCATTTCATGATGTTTGGCAAGCAGTTCTGCAT
Pm70                GCATGCCTGATTGGCCAATATCATATTGCACTGCAAGATGTTTGGCAAGCAGTTCTGCAT
Anand1_poultry      GCATGCCTGATTGGCCAATATCATATTGCACTGCAAGATGTTTGGCAAGCAGTTCTGCAT
Anand1_cattle       GCATGCCTGATTGGCCAATATCATATTGCACTGCAAGATGTTTGGCAAGCAGTTCTGCAT
Anand1_buffalo      GCATGCCTGATTGGCCAATATCATATTGCACTGCAAGATGTTTGGCAAGCAGTTCTGCAT
P52VAC              GCATGCCTGATTGGCCAATATCATATTGCACTGCAAGATGTTTGGCAAGCAGTTCTGCAT
                    ******************************* * ** ************************

Anand1_goat         ATTGCAGCAGATAATCCGAGAACAGATGCAGAAACAGTTCTGTGGAATATTAGACTGCCG
Pm70                ATTGCAGCAGATAATCCGAGAACAGATGCAGAAACAGTTCTGTGGAATATTAGACTGCCG
Anand1_poultry      ATTGCAGCAGATAATCCGAGAACAGATGCAGAAACAGTTCTGTGGAATATTAGACTGCCG
Anand1_cattle       ATTGCAGCAGATAATCCGAGAACAGATGCAGAAACAGTTCTGTGGAATATTAGACTGCCG
Anand1_buffalo      ATTGCAGCAGATAATCCGAGAACAGATGCAGAAACAGTTCTGTGGAATATTAGACTGCCG
P52VAC              ATTGCAGCAGATAATCCGAGAACAGATGCAGAAACAGTTCTGTGGAATATTAGACTGCCG
                    ************************************************************

Anand1_goat         AGAATTTTTACAGCAATTATTGTTGGCGCAGCACTGTCAGTTGCAGGCGCAACATATCAA
Pm70                AGAATTTTTACAGCAATTATTGTTGGCGCAGCACTGTCAGTTGCAGGCGCAACATATCAA
Anand1_poultry      AGAATTTTTACAGCAATTATTGTTGGCGCAGCACTGTCAGTTGCAGGCGCAACATATCAA
Anand1_cattle       AGAATTTTTACAGCAATTATTGTTGGCGCAGCACTGTCAGTTGCAGGCGCAACATATCAA
Anand1_buffalo      AGAATTTTTACAGCAATTATTGTTGGCGCAGCACTGTCAGTTGCAGGCGCAACATATCAA
P52VAC              AGAATTTTTACAGCAATTATTGTTGGCGCAGCACTGTCAGTTGCAGGCGCAACATATCAA
                    ************************************************************

Anand1_goat         GGCATGTTTAAAAATCCGCTGGTTTCACCGGATATTCTGGGCGTTACAGCAGGCGCAGGC
Pm70                GGCATGTTTAAAAATCCGCTGGTTTCACCGGATATTCTGGGCGTTACAGCAGGCGCAGGC
Anand1_poultry      GGCATGTTTAAAAATCCGCTGGTTTCACCGGATATTCTGGGCGTTACAGCAGGCGCAGGC
Anand1_cattle       GGCATGTTTAAAAATCCGCTGGTTTCACCGGATATTCTGGGCGTTACAGCAGGCGCAGGC
Anand1_buffalo      GGCATGTTTAAAAATCCGCTGGTTTCACCGGATATTCTGGGCGTTACAGCAGGCGCAGGC
P52VAC              GGCATGTTTAAAAATCCGCTGGTTTCACCGGATATTCTGGGCGTTACAGCAGGCGCAGGC
                    ************************************************************
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Plate 4.5: Clustal multiple alignment of all *P. multocida* isolates with Pm70 reference strain for *HemU* DNA sequence using MUSCLE

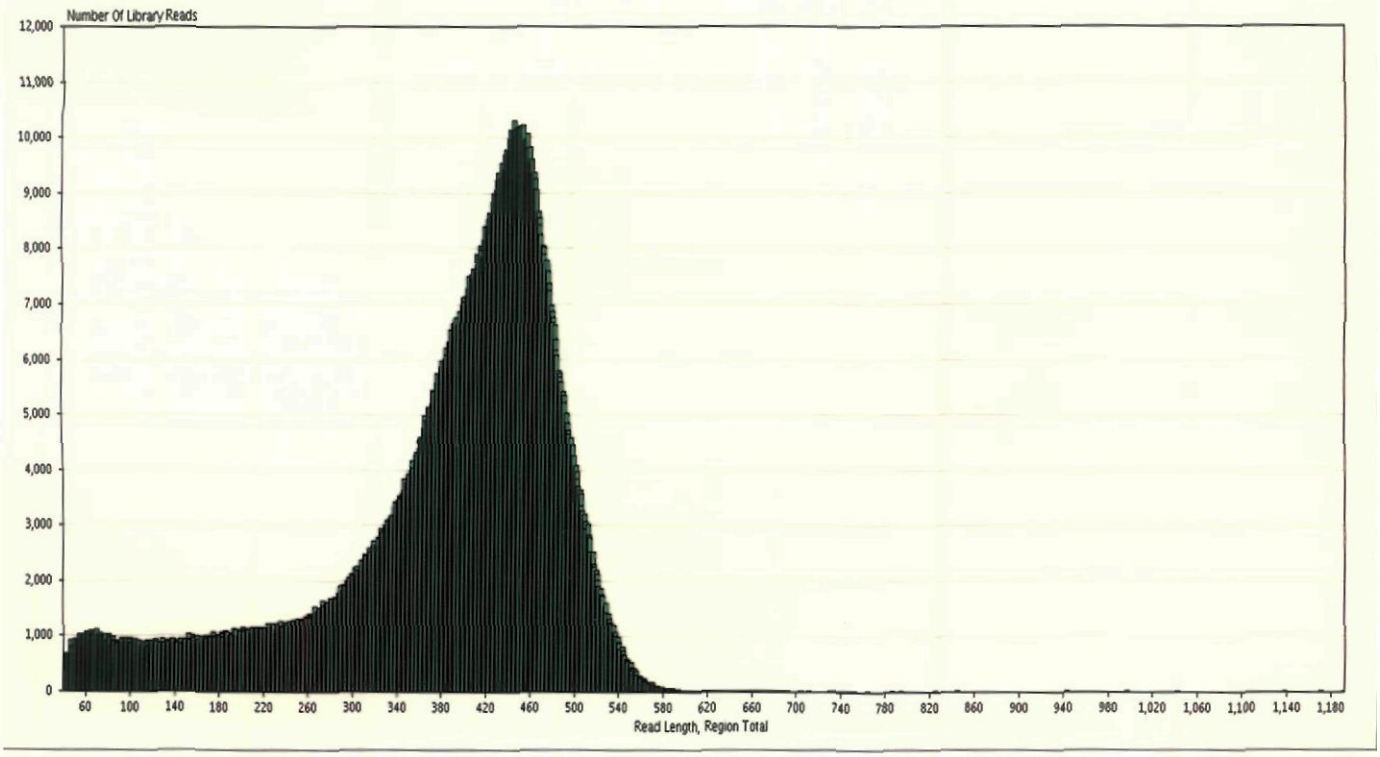


Plate 4.6: Read length distribution of a representative sample (P52VAC) of *P. multocida*

Table 4.3: Summary of data generated by GS FLX cluster after reference mapping with Pm70

Mapping	P52VAC	Anand1_poultry	Anand1_goat	Anand1_buffalo	Anand1_cattle
Total Sequence reads	118843	113997	105729	134886	31346
Total Sequence Bases	42,598,100	29,000,497	21,890,353	39,756,349	7,429,658
Average Read Length	358	202	207	294	237
Coverage	18.87	12.85	9.70	17.61	3.29
Total Mapped Reads	105327	97674	95092	86765	24967
Total Mapped Reads (%)	88.63%	85.70%	89.95%	64.34%	79.67%
Total Mapped Bases	38,079,806	20,085,356	19,867,143	25,095,466	6,1451,56
Total Mapped Bases (%)	89.52%	87.38%	90.81%	63.22%	82.87%
Unmapped Reads	11308	12439	8743	42316	4820
Unmapped Reads (%)	9.52%	10.91%	8.270%	31.38%	15.38%
Total Contigs	155	402	257	227	1998
Total Contig Bases	2,069,867	2,024,810	2,097,193	2,064,639	1,819,413
Average. Contig Size	18716	10096	24768	1057	1339
Largest Contig	169703	88697	55720	90166	10792
N50* Contigs	44873	20025	23885	25302	1550

N50* is the length for which the collection of all contigs of that length or longer contains at least half of the sum of the lengths of all contigs.

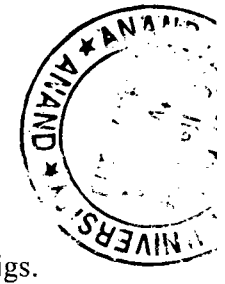
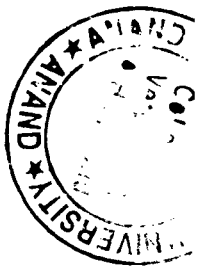


Table 4.4: Summary of data generated by GS FLX cluster after *De novo* assembly

De Novo Assembly	P52VAC	Anand1_poultry	Anand1_goat	Anand1_buffalo	Anand1_cattle
Total unmapped reads	11308	12439	8743	42316	4820
Total unmapped bases	3,911,529	2,467,578	1,752,951	13,962,856	1,104,849
Total aligned reads	10775	10797	7287	24709	3002
Total aligned reads (%)	95.29%	62.28%	89.95%	58.40%	79.67%
Total aligned bases	3,834,503	2,197,589	1,490,730	7,805,067	7,522,44
Total aligned bases (%)	98.03	68.10%	90.81%	55.90%	82.87%
Total Contigs	78	157	123	1359	192
Total contigs bases	207,091	213,470	192,527	1,235,377	143,000
Average contig size	2655	1360	1565	909	745
Largest contig size	34017	10692	17034	1057	3616
N50 contig	6306	2142	4255	969	1044



In our experiment, all sequencing runs were filtered for individual samples based on MIDs tags attached at the time of adaptor ligation step, which resulted into 118843, 113997, 105729, 134886 and 31346 reads with 42,598,100 (42.59Mb), 29,000,497 (29.00Mb), 21,890,353 (21.89Mb), 39,756,349 (39.75Mb) and 7,429,658 (7.42Mb) sequence bases for P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle, respectively. The coverage obtained were 18.87, 12.85, 9.70, 17.61 and 3.29 for P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle, respectively. All the isolates were mapped with Pm70 strain isolated from poultry as a reference strain (May *et al.*, 2001) and generated 38,079,806 (89.52%), 20,085,356 (87.38%), 19,867,143 (90.81%), 25,095,466 (63.22%) and 6,145,156 (82.87%) mapped bases with 105327, 97674, 95092, 86765 and 24967 mapped reads for P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle, respectively. Mapping results generated 155, 402, 257, 227 and 1998 contigs for P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle, respectively (Table 4.3).

Remaining reads which were not mapped to the reference strain Pm70, were assembled using GS *De novo* Assembler (Newbler) version 2.3 which generated 10775 (95.29%), 10797 (62.28%), 7287 (89.95%), 24709 (58.40%) and 3002 (79.67%) total aligned reads with 3,834,503 (98.03%), 2,197,589 (68.10%), 1,490,730 (90.81%), 7,805,067 (55.90%) and 752,244 (82.87%) total aligned bases for P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle, respectively (Table 4.4). *De novo* assembly of reads were carried out for searching for plasmid. None of the *De novo* assembled reads matched to plasmid using BLAST hits.

Whole genome sequencing experiments were also carried out by May *et al.* (2001), Liu *et al.* (2012), Abrahante *et al.* (2013), Hauglund *et al.* (2013), Lainson *et al.* (2013[a]), Lainson *et al.* (2013[b]), Yap *et al.* (2013), Abrahante *et al.* (2014); Davenport *et al.* (2014) and Vaid *et al.* (2014) for *P. multocida*. Since first whole genome sequence of *P. multocida* was carried out in 2001 when no whole genome sequencing platforms were available at that time, May *et al.* (2001) used Sanger sequencing approach. Afterward, as various whole genome sequencing platforms became available, the attempts for sequencing the whole bacterial genome increased. For Pm70, a poultry isolate, genome size is 2,257,487bp (2.25Mb) in length which is in a single scaffold as reported by May *et al.* (2001). After a decade of Pm70 sequencing, Liu *et al.* (2012) reported sequencing of HN06, a toxigenic *P. multocida* isolate of pig using Illumina Solexa platform and generated a total of 4,792,617 paired-end reads with ~360-fold coverage of the genome. *P. multocida* HN06 consists of a circular chromosome with 2,402,218bp (2.40Mb) and a plasmid, pHN06, with 5,360 bp.

Two virulent strains of *P. multocida,* P1059 and X73 of poultry were sequenced using 454 pyrosequencing by Abrahante *et al.* (2013). The assembly generated 2.30Mb and 2.26 Mbp genomes for P1059 and X73, respectively. *P. multocida* strain 671/90 belonging to A:3 serotype and isolated from infected calf lung tissue was sequenced by Lainson *et al.* (2103[b]) using Illumina and 454 platforms, which produced 26 contigs with a genome size of 2,246,063bp (2.24Mb).

Whole genome sequencing for three bovine isolates (2000, RIIF, 1500E) from the United Kingdom and one bovine isolate from United States (P1933) using an Illumina Solexa Genome Analyzer was reported by Lainson *et al.* (2013[a]). *De novo* assembly

generated 261, 243, 265 and 269 contigs with 2,210,534bp (2.21Mb), 2,195,634bp (2.19Mb), 2,210,576bp (2.21Mb) and 2,441,686bp (2.44Mb) genome for 2000, RIIF, 1500E and P1933 isolates respectively.

PMTB strain isolated from buffalo carcass was sequenced using an Illumina genome analyzer and a total of 7,760,284 single-end reads were generated with approximately 100-fold genome coverage. PMTB has a genome size of 2,203,419bp (2.20Mb) as reported by Yap *et al.* (2013).

P. multocida subspecies *multocida* ATCC 43137, a type-A LPS strain commonly used as a reference strain in pathogenicity was studied by Davenport *et al.* (2014) using Illumina technology, which recorded 2.27Mb of genome size.

Whole genome sequencing of two strains, 2213 and 3213 of *P. multocida* isolated from the blood of carcasses of two buffaloes (*Bubalus bubalis*) by Abrahante *et al.* (2014) were carried out using the Illumina MiSeq platform. A total of 6,253,700 and 5,550,478 paired-end reads resulted into 34 and 32 contigs, respectively, for strains 2213 and 3213 with genome size of 2,309,333bp (2.30Mb) and 2,307,438bp (2.30Mb).

Another strain VTCCBAA264, a B:2 serotype of *P. multocida*, isolated from buffalo intestine was sequenced using 454 pyrosequencing by Vaid *et al.* (2014). A total of 123415 reads with ~23X coverage and 78 contigs with a total genome size of 2,280,332bp (2.28Mb) was obtained.

4.3.1 RAST analysis

For RAST analysis, all the reads of *P. multocida* were submitted to the RAST server available at (http://rast.nmpdr.org/rast.cgi). RAST produces two classes of asserted gene functions: subsystem-based assertions are based on recognition of functional

variants of subsystems, while non subsystem-based assertions are filled in using more common approaches based on integration of evidence from a number of tools. RAST distinguishes these two classes of annotation and uses the relatively reliable subsystem-based assertions as the basis for a detailed metabolic reconstruction (Aziz *et al.,* 2008). Out of 27 RAST subsystems, as expected, subsystems for "Photosynthesis" and "Motility and Chemotaxis" were not assigned to any of the five isolates. The remaining 25 subsystems assigned with common genes/proteins for four isolates i.e. P52VAC, Anand1_poultry, Anand1_goat and Anand1_buffalo are shown in Table 4.5. Cattle isolate is omitted from this comparison as only 10 out of 27 subsystems were assigned, that too with lower counts because of the very less coverage obtained for this isolate during sequencing.

4.3.1.1 P52VAC

RAST analysis of P52VAC revealed genome size of 2,273,366bp with 209 contigs. Total number of subsystems defined was 419, while number of coding sequences were 2165 and number of RNAs were 68.

Based on subsystem analysis, out of 2165 coding sequences, 67% (1446 CDS) of genome was having defined subsystem based classification, while rest of the 33% (719 CDS) of genome was not having defined subsystem. Of the defined subsystem, highest abundance of subsystem were amino acids and derivatives (248 CDS), carbohydrates (244 CDS), protein metabolism (238 CDS), cofactor and vitamins, prosthetic groups, pigments (217 CDS) (Plate 4.7).

The most characteristic feature of P52VAC observed was that it contains colicin and bacteriocin production cluster which includes *DedA, DedD* and *toxin*. Bacteriocins

are antimicrobial peptides with different sizes, microbial targets and mechanisms of action produced by a large variety of bacteria. They are characterized by a bactericidal or bacteriostatic activity against strains of the same species or closely related species and differ from most therapeutic antibiotics due to their narrow activity spectrum and their proteinaceous nature (Zihler *et al.*, 2009).

There was also presence of a gene cluster with resistance to fluroquinolones which includes *gyrA* and *gyrB* genes coding for DNA gyrase as well as *ParC* and *ParD* genes coding for topoisomerase IV subunit. This implies to the antibiotic resistance shown by the bacteria against the most commonly employed antibiotics for the treatment of diseases caused by *P. multocida.* Other important feature observed in the P52VAC strain is the presence of negative regulator of betalactamase expression, which is coded by *BLR* gene cluster leading to resistance expressed by the organism. There was also presence of multidrug resistance efflux pump cluster of genes which were mainly *MATE* (multidrug and toxin extrusion), *MacA* and *MacB* (macrolide specific efflux protein). Beside all these, genome of *P. multocida* P52VAC contains all the proteins or genes required for the normal function for their survival. The common genes or proteins which have been shown to be of significance in *P. multocida* or other bacterial spp. as per the available literature and found in our study as per RAST are listed in Table 4.5 with their potential role.

Table 4.5 Coding sequences (CDS) in different functional groups in four isolates of *P. multocida*

Functional group	Isolate*	No. of CDS	Genes/ Proteins	Function
Cofactors, Vitamins, Prosthetic Groups, Pigments	P52VAC	217	***tRNAglu, GltR, GAST, PBS, PBD, UroS, UroM, UroD, CPOan, SAM, PPO, FC,*** *KpsS*(+), *KpsC*(+), *KpsE*(-), *KpsM*(-), *KpsT*(-)	Heme and siroheme biosynthesis, Capsular polysaccharides biosynthesis and assembly
	Anand1_poultry	235	***tRNAglu, GltR, GAST, PBS, PBD, UroS, UroM, UroD, CPOan, SAM, PPO, FC,*** *KpsS*(+), *KpsC*(+), *KpsE*(+), *KpsM*(+), *KpsT*(+)	
	Anand1_goat	242	***tRNAglu, GltR, GAST, PBS, PBD, UroS, UroM, UroD, CPOan, SAM, PPO, FC,*** *KpsS*(+), *KpsC*(+), *KpsE*(+), *KpsM*(+), *KpsT*(+)	
	Anand1_buffalo	294	***tRNAglu, GltR, GAST, PBS, PBD, UroS, UroM, UroD, CPOan, SAM, PPO, FC,*** *KpsS*(-), *KpsC*(-), *KpsE*(-), *KpsM*(-), *KpsT*(-)	
Cell Wall and Capsule	P52VAC	146	***NeuC, NeuA, NeuS, NanA,NanH, NanK, NanE, NanX, NanTRAP, NagE, NagA, NagB, GlmS, GlmM, GlmU, ManX***	Sialic acid metabolism
	Anand1_poultry	150	***NeuC, NeuA, NeuS, NanA,NanH, NanK, NanE, NanX, NanTRAP, NagE, NagA, NagB, GlmS, GlmM, GlmU, ManX***	
	Anand1_goat	152	***NeuC, NeuA, NeuS, NanA,NanH, NanK, NanE, NanX, NanTRAP, NagE, NagA, NagB, GlmS, GlmM, GlmU, ManX***	
	Anand1_buffalo	179	***NeuC, NeuA, NeuS, NanA,NanH, NanK, NanE, NanX, NanTRAP, NagE, NagA, NagB, GlmS, GlmM, GlmU, ManX***	

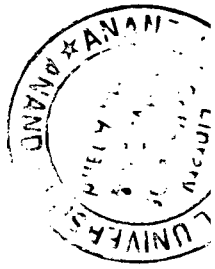
Virulence, Disease and Defense	P52VAC	47	*DedA*(+), *toxin*(+), *PurF*(+), ***gyrA, gyrB, parC, parE,*** *BLR*(+), *TolC*(+), *MATE*(+), *MacA*(+), *MacB*(+), *MtrF*(+), *AcrB*(+)	Colicin and bacteriocin production cluster, Resistance to fluroquinolones, Betalactamase negative regulator, Multidrug efflux pump
	Anand1_poultry	54	*DedA*(+), *toxin*(+), *PurF*(+), ***gyrA, gyrB, parC, parE,*** *BLR*(+), *TolC*(+), *MATE*(+), *MacA*(+), *MacB*(+), *MtrF*(+), *AcrB*(+)	
	Anand1_goat	50	*DedA*(+), *toxin*(+), *PurF*(+), ***gyrA, gyrB, parC, parE,*** *BLR*(+), *TolC*(-), *MATE*(-), *MacA*(-), *MacB*(-), *MtrF*(-), *AcrB*(-)	
	Anand1_buffalo	54	*DedA*(-), *toxin*(-), *PurF*(-), ***gyrA, gyrB, parC, parE,*** *BLR*(-), *TolC*(+), *MATE*(+), *MacA*(+), *MacB*(-), *MtrF*(-), *AcrB*(-)	
Potassium metabolism	P52VAC	12	*MSC*(+), ***TkA, FKB, AATP, TrkG, KtrA, KtrB***	Potassium homeostasis, Hyperosmotic potassium uptake
	Anand1_poultry	17	*MSC*(+), ***TkA, FKB, AATP, TrkG, KtrA, KtrB***	
	Anand1_goat	11	*MSC*(+), ***TkA, FKB, AATP, TrkG, KtrA, KtrB***	
	Anand1_buffalo	14	*MSC*(-), ***TkA, AATP, FKB, TrkG, KtrA, KtrB***	
Miscellaneous	P52VAC	35	***Met, YjeE, QUEA, Tgt,*** *HflC*(+), ***HflK, DedA***	Scaffold protein for [4Fe-4S] cluster assembly, DedA family inner membrane protein
	Anand1_poultry	35	***Met, YjeE, QUEA, Tgt,*** *HflC*(+), ***HflK, DedA***	
	Anand1_goat	33	***Met, YjeE, QUEA, Tgt,*** *HflC*(+), ***HflK, DedA***	
	Anand1_buffalo	37	***Met, YjeE, QUEA, Tgt,*** *HflC*(-), ***HflK, DedA***	
Phage, Prophage, Transposable elements, Plasmid	P52VAC	17	*SpellVar*(+), *Rep*(-), *dPol 3*(+), *Hel* (+), *TrmL*(+), *Port*(+)	Phage packaging machinery, Phage replication
	Anand1_poultry	29	*SpellVar*(-), *Rep*(-), *dPol 3*(-), *Hel* (-), *TrmL*(+), *Port*(+)	
	Anand1_goat	51	*SpellVar*(-), *Rep*(+), *dPol 3*(+), *Hel* (-), *TrmL*(-), *Port*(+)	
	Anand1_buffalo	07	*SpellVar*(-), *Rep*(-), *dPol 3*(+), *Hel* (+), *TrmL*(-), *Port*(-)	
Membrane Transport	P52VAC	86	*SapA*(+), *SapB*(+), *SapC*(+), *SapD*(+), *SapF*(+), ***oppA, oppB, oppC,*** *oppD*(-), **oppF,** *TonB*(+), ***TonR, ExbB, ExbD, TolA, TolB, HTAS, pal, HasA***	Peptide ABC transporter system Sap, ABC transported oligopeptide, Ton and Tol transport systems
	Anand1_poultry	86	*SapA*(+), *SapB*(+), *SapC*(+), *SapD*(+), *SapF*(+), ***oppA, oppB, oppC,*** *oppD(-),* ***oppF,*** *TonB*(+), ***TonR, ExbB,***	

			ExbD, TolA, TolB, HTAS, pal, HasA	
	Anand1_goat	96	*SapA*(+), *SapB*(+), *SapC*(+), *SapD*(+), *SapF*(+), ***oppA, oppB, oppC,*** *oppD*(-), ***oppF,*** *TonB*(+), ***TonR, ExbB, ExbD, TolA, TolB, HTAS, pal, HasA***	
	Anand1_buffalo	106	*SapA*(-), *SapB*(-), *SapC*(-), *SapD*(-), *SapF*(-), ***oppA, oppB, oppC,*** *oppD*(+), ***oppF,*** *TonB*(-), ***TonR, ExbB, ExbD, TolA, TolB, HTAS, pal, HasA***	
Iron acquisition and metabolism	P52VAC	65	***pitA, pitC, pitD, R1, R2, R3,*** *R5*(-), ***R6,*** *HutX*(+), *HutZ*(+), *HBP*(+), *fhuC*(+), *ExB*(+), *TonB*(+), *FCR*(+), *ParA*(+), *ParB*(+), *RP2*(+), *HBP*(+), *Rec*(+), *TonB*(+), *POHIU*(+)	Iron acquisition in Streptococcus, Iron transport system including ABC transporter, Ferric iron ABC tansporter, Heme hemin uptake and utilization system in Gram negatives, Hemin transport system
	Anand1_poultry	46	***pitA, pitC, pitD, R1, R2, R3,*** *R5*(-), ***R6,*** *HutX*(-), *HutZ*(-), *HBP*(-), *fhuC*(-), *ExB*(-), *TonB*(-), *FCR*(-), *ParA*(-), *ParB*(-), *RP2*(-), *HBP*(+), *Rec*(+), *TonB*(+), *POHIU*(-)	
	Anand1_goat	69	***pitA, pitC, pitD, R1, R2, R3,*** *R5*(+), ***R6,*** *HutX*(+), *HutZ*(+), *HBP*(+), *fhuC*(+), *ExB*(+), *TonB*(+), *FCR*(+), *ParA*(+), *ParB*(+), *RP2*(+), *HBP*(+), *Rec*(+), *TonB*(+), *POHIU*(+)	
	Anand1_buffalo	25	***pitA, pitC, pitD, R1, R2, R3,*** *R5*(+), ***R6,*** *HutX*(-), *HutZ*(-), *HBP*(-), *fhuC*(-), *ExB*(-), *TonB*(-), *FCR*(-), *ParA*(-), *ParB*(-), *RP2*(-), *HBP*(-), *Rec*(-), *TonB*(-), *POHIU*(-)	
RNA Metabolism	P52VAC	172	*RNase III*(+), ***ExoII, RNaseR, RNaseE, RraA, RraB, Orn, IscR, CMLDR***	RNA processing and degradation (Bacterial), Rrf2 family transcriptional regulators
	Anand1_poultry	138	*RNase III*(+), ***ExoII, RNaseR, RNaseE, RraA, RraB, Orn, IscR, CMLDR***	
	Anand1_goat	181	*RNase III*(+), ***ExoII, RNaseR, RNaseE, RraA, RraB, Orn, IscR, CMLDR***	
	Anand1_buffalo	139	*RNase III*(-), ***ExoII, RNaseR, RNaseE, RraA, RraB, Orn, IscR, CMLDR***	

Nucleosides and Nucleotides	P52VAC	85	***MazG, Dut, MutT, NudC,*** *NudE*(+), ***NudF, NudH, cytk, pyrR1,nt5, udk-C1, PURPH, cytk, ndk, ctpscytd,*** *thred*(+), ***cydethyk, thydyk, PrsA, PurF, PurD, PurN, PurT, PurL, PurQ, PurM, PurE, PurK, PurC, PurB, PuH1/P, pyrAA, pyrAB, pyrD, pyre, pyrF, pyrRI,*** *pyrRII*(+), ***uraA***	Nucloside triphosphate pyrophosphohydrolase, Nudix protein, Pyrimidin conversions, *de novo* purine biosynthesis, *de novo* pyrimidine synthesis
	Anand1_poultry	89	***MazG, Dut, MutT, NudC,*** *NudE*(+), ***NudF, NudH, cytk, pyrR1,nt5, udk-C1, PURPH, cytk, ndk, ctpscytd,*** *thred*(+), ***cydethyk, thydyk, PrsA, PurF, PurD, PurN, PurT, PurL, PurQ, PurM, PurE, PurK, PurC, PurB, PuH1/P, pyrAA, pyrAB, pyrD, pyre, pyrF, pyrRI,*** *pyrRII*(+), ***uraA***	
	Anand1_goat	84	***MazG, Dut, MutT, NudC,*** *NudE*(+), ***NudF, NudH, cytk, pyrR1,nt5, udk-C1, PURPH, cytk, ndk, ctpscytd,*** *thred*(+), ***cydethyk, thydyk, PrsA, PurF, PurD, PurN, PurT, PurL, PurQ, PurM, PurE, PurK, PurC, PurB, PuH1/P, pyrAA, pyrAB, pyrD, pyre, pyrF, pyrRI,*** *pyrRII*(+), ***uraA***	
	Anand1_buffalo	117	***MazG, Dut, MutT, NudC,*** *NudE*(-), ***NudF, NudH, cytk, pyrR1,nt5, udk-C1, PURPH, cytk, ndk, ctpscytd,*** *thred* (-), ***cydethyk, thydyk, PrsA, PurF, PurD, PurN, PurT, PurL, PurQ, PurM, PurE, PurK, PurC, PurB, PuH1/P, pyrAA, pyrAB, pyrD, pyre, pyrF, pyrRI,*** *pyrRII* (-), ***uraA***	
Protein Metabolism	P52VAC	238	*needs*(-), **SelA, *SelB, SelD, DsbA,*** *2-SeU*(-), *DsbB*(+), ***DsbC, DsbE, CcdA,*** *DnaJ*(-), ***DnaK, GrpE, HscA,*** *HscB*(+), ***HtpG, ClpB, ClpP, ClpX, ClpQ, ClpB, DegS, DegQ, hslU, hslV, ClpP, ClpX, lonI,*** *lonII*(+)	Selenocystein metabolism, Periplasmic disulfide interchange, Protein chaperons, Proteoslysis in bacteria, Proteosome bacterial
	Anand1_poultry	224	*needs*(+), ***SelA, SelB, SelD, DsbA,*** *2-SeU*(-), *DsbB*(+), ***DsbC, DsbE, CcdA,*** *DnaJ*(+), ***DnaK, GrpE, HscA,*** *HscB*(-), ***HtpG, ClpB, ClpX, ClpQ, ClpB, DegS, DegQ, hslU, hslV, ClpP, ClpX, lonI,*** *lonII*(+)	

	Anand1_goat	252	*needs*(-), ***SelA, SelB, SelD, DsbA,*** *2-SeU*(-), *DsbB*(+), ***DsbC, DsbE, CcdA,*** *DnaJ*(+), ***DnaK, GrpE, HscA,*** *HscB* (+), ***HtpG, ClpB, ClpX, ClpQ, ClpB, DegS, DegQ, hslU, hslV, ClpP, ClpX, lonI,*** *lonII*(+)	
	Anand1_buffalo	200	*needs*(+), ***SelA, SelB, SelD, DsbA,*** *2-SeU*(+), *DsbB*(-), ***DsbC, DsbE, CcdA,*** *DnaJ*(-), ***DnaK, GrpE, HscA,*** *HscB*(+), ***HtpG, ClpB, ClpX, ClpQ, ClpB, DegS, DegQ,, hslU, hslV, ClpP, ClpX, lonI,*** *lonII*(-)	
Cell Division and Cell Cycle	P52VAC	32	*MreB*(+), *MreC*(+), *MreD*(+), *RodA*(+), *Maf*(+), *FtsA*(+), *MraZ*(+), *FtsI*(+), *FtsQ*(+), *ZipA*(+), *ZapA*(+), *MinC*(+), ***IspA, YciA, SpoT, RelA***	Bacterial cytoskeleton, Intracellular septation in enterobacteria, Stringent response ppGpp
	Anand1_poultry	39	*MreB*(+), *MreC*(+), *MreD*(+), *RodA*(+), *Maf*(+), *FtsA*(+), *MraZ*(+), *FtsI*(+), *FtsQ*(+), *ZipA*(+), *ZapA*(+), *MinC*(+), ***IspA, YciA, SpoT, RelA***	
	Anand1_goat	33	*MreB*(+), *MreC*(+), *MreD*(+), *RodA*(+), *Maf*(+), *FtsA*(+), *MraZ*(+), *FtsI*(+), *FtsQ*(+), *ZipA*(+), *ZapA*(+), *MinC*(+), ***IspA, YciA, SpoT, RelA***	
	Anand1_buffalo	27	*MreB*(-), *MreC*(-), *MreD*(-), *RodA*(-), *Maf*(-), *FtsA*(-), *MraZ*(-), *FtsI*(-), *FtsQ*(-), *ZipA*(-), *ZapA*(-), *MinC*(-), ***IspA, YciA, SpoT, RelA***	
Regulation and Cell signaling	P52VAC	34	***FNR,*** *CGA*(-), ***AC, PDE, APRT, Clp,*** *LsrK*(+), ***LsrR, LsrA,*** *LsrC*(+), ***LsrB, LsrD, LuxS, Ycjx, UcjF***	cAMP signaling in bacteria, Autoinducer 2 transport and processing, Conserve operon linked to TyrR and possible involved in virulence
	Anand1_poultry	51	***FNR,*** *CGA*(+), ***AC, PDE, APRT, Clp,*** *LsrK(-),* ***LsrR, LsrA,*** *LsrC*(+), ***LsrB, LsrD, LuxS, Ycjx, UcjF***	
	Anand1_goat	43	***FNR,*** *CGA*(+), ***AC, PDE, APRT, Clp,*** *LsrK*(+), ***LsrR, LsrA,*** *LsrC*(+), ***LsrB, LsrD, LuxS, Ycjx, UcjF***	
	Anand1_buffalo	54	***FNR,*** *CGA*(+), ***AC, PDE, APRT, Clp,*** *LsrK*(+), ***LsrR,***	

			LsrA, *LsrC* (-), ***LsrB, LsrD, LuxS, Ycjx, UcjF***	
Secondary Metabolism	P52VAC	4	***APRT, PRAI, Tsa, Tsb***	Auxin biosynthesis
	Anand1_poultry	4	***APRT, PRAI, Tsa, Tsb***	
	Anand1_goat	5	***APRT, PRAI, Tsa, Tsb***	
	Anand1_buffalo	0		
DNA Metabolism	P52VAC	95	*Cas1*(+), *Cas2*(+), *Csy2*(+), *Csy4*(+), *Csa1*(+), *Csa2*(+), *Csa3*(+), *Csa4*(+), ***hyp1, dnaX,*** *hyp2*(+), ***recR, Topo-III***	CRISPRs, DNA processing cluster
	Anand1_poultry	107	*Cas1*(-), *Cas2*(-), *Csy2*(-), *Csy4*(-), *Csa1*(-), *Csa2*(-), *Csa3*(-), *Csa4*(-), ***hyp1, dnaX,*** *hyp2*(+), ***recR, Topo-III***	
	Anand1_goat	101	*Cas1*(+), *Cas2*(+), *Csy2*(-), *Csy4*(+), *Csa1*(+), *Csa2*(+), *Csa3*(+), *Csa4*(+), ***hyp1, dnaX,*** *hyp2*(+), ***recR, Topo-III***	
	Anand1_buffalo	134	*Cas1*(+), *Cas2*(+), *Csy2*(+), *Csy4*(+), *Csa1*(+), *Csa2*(+), *Csa3*(+), *Csa4*(+), ***hyp1, dnaX,*** *hyp2*(-), ***recR, Topo-III***	
Fatty Acids, Lipids and Isoprenoids	P52VAC	78	***3EE, 3EZ, DXS, ISPC, ISPD, ISPE, ISPF, GTT, UppS, DMAT, Cdl, BCCP, BC, AccA, AccD, ACP, ACPS,*** *FAB_FH*(+), ***FabD, FabG, FabZ, FabB, FabA***	Isoprenoide, Isoprenoid biosynthesis, Cardiopilin metabolism, Fatty acid biosynthesis FASII
	Anand1_poultry	77	***3EE, 3EZ, DXS, ISPC, ISPD, ISPE, ISPF, GTT, UppS, DMAT, Cdl, BCCP, BC, AccA, AccD, ACP, ACPS,*** *FAB_FH*(-), ***FabD, FabG, FabZ, FabB, FabA***	
	Anand1_goat	81	***3EE, 3EZ, DXS, ISPC, ISPD, ISPE, ISPF, GTT, UppS, DMAT, Cdl, BCCP, BC, AccA, AccD, ACP, ACPS,*** *FAB_FH*(+), ***FabD, FabG, FabZ, FabB, FabA***	
	Anand1_buffalo	101	***3EE, 3EZ, DXS, ISPC, ISPD, ISPE, ISPF, GTT, UppS, DMAT, Cdl, BCCP, BC, AccA, AccD, ACP, ACPS,*** *FAB_FH*(+), ***FabD, FabG, FabZ, FabB, FabA***	



Nitrogen Metabolism	P52VAC	23	***NiRes1, NiRes2, NaRper, Reg, GS, GlnE, GlnD, PII***	Nitrate and nitrite ammonification, Ammonia assimilation
	Anand1_poultry	26	***NiRes1, NiRes2, NaRper, Reg,GS, GlnE, GlnD, PII***	
	Anand1_goat	26	***NiRes1, NiRes2, NaRper, Reg,GS, GlnE, GlnD, PII***	
	Anand1_buffalo	14	***NiRes1, NiRes2, NaRper, Reg,GS, GlnE, GlnD, PII***	
Dormancy and Sporulation	P52VAC	2	***SulA, SpoVC***	Persister cell, Sporulation associated protein
	Anand1_poultry	2	***SulA, SpoVC***	
	Anand1_goat	2	***SulA, SpoVC***	
	Anand1_buffalo	3	***SulA, SpoVC***	
Respiration	P52VAC	74	***TtrA, TtrB, TtrC,*** *TtrS*(+), *TtrR*(+)	Tetrathionate respiration
	Anand1_poultry	82	***TtrA, TtrB, TtrC,*** *TtrS*(-), *TtrR*(-)	
	Anand1_goat	80	***TtrA, TtrB, TtrC,*** *TtrS*(+), *TtrR*(+)	
	Anand1_buffalo	91	***TtrA, TtrB, TtrC,*** *TtrS*(+), *TtrR*(+)	
Stress Response	P52VAC	77	***Fe-stress, Fr, Redox,*** H_2O_2(+), *Fnr*(+), ***AhpC, SOD,*** *CspA*(+), *CspD*(+), *DnaJ*(+), *DnaK*(+), *GrpE*(+), *SnpB*(+), *Hsp15*(+), *Rsml*(+), ***UrpA, UrpE***	Oxidative stress, Cold shock, Heat shock, Universal stress protein
	Anand1_poultry	68	***Fe-stress, Fr, Redox,*** H_2O_2(+), *Fnr*(+), ***AhpC, SOD,*** *CspA*(+), *CspD*(+), *DnaJ*(-), *DnaK*(-), *GrpE*(-), *SnpB*(-), *Hsp15*(-), *Rsml*(-), ***UrpA, UrpE***	
	Anand1_goat	86	***Fe-stress, Fr, Redox,*** H_2O_2(+), *Fnr*(+), ***AhpC, SOD,*** *CspA*(+), *CspD*(+), *DnaJ*(+), *DnaK*(+), *GrpE*(+), *SnpB*(+), *Hsp15*(+), *Rsml*(+), ***UrpA, UrpE***	
	Anand1_buffalo	70	***Fe-stress, Fr, Redox,*** H_2O_2(-), *Fnr*(-), ***AhpC, SOD,*** *CspA*(-), *CspD*(-), *DnaJ*(-), *DnaK*(-), *GrpE*(-), *SnpB*(-), *Hsp15*(-), *Rsml*(-), ***UrpA, UrpE***	

Metabolism of Aromatic Compounds	P52VAC	8	*HPAH*(+), ***HPADO, HPAHb***	Aromatic amin catabolism
	Anand1_poultry	8	*HPAH*(+), ***HPADO, HPAHb***	
	Anand1_goat	8	*HPAH*(+), ***HPADO, HPAHb***	
	Anand1_buffalo	22	*HPAH*(-), ***HPADO, HPAHb***	
Amino Acids and Derivatives	P52VAC	248	***CysE, CysK, CysZ, CysB, CysT, HSDH, HSAT, HS, CTGS, CTBL, MS, MTHFR, MT, SAMS, SAHCN, RHMCCysE, CysK***	Cystein biosynthesis, Methionine biosynthesis
	Anand1_poultry	267	***CysE, CysK, CysZ, CysB, CysT*(-), *HSDH, HSAT, HS, CTGS, CTBL, MS, MTHFR, MT, SAMS, SAHCN, RHMCCysE, CysK***	
	Anand1_goat	254	***CysE, CysK, CysZ, CysB, CysT, HSDH, HSAT, HS, CTGS, CTBL, MS, MTHFR, MT, SAMS, SAHCN, RHMCCysE, CysK***	
	Anand1_buffalo	337	***CysE, CysK, CysZ, CysB, CysT, HSDH, HSAT, HS, CTGS, CTBL, MS, MTHFR, MT, SAMS, SAHCN, RHMCCysE, CysK***	
Sulfur Metabolism	P52VAC	19	***DsrE***, *DsrH*(+), ***DsrC-4, TcyP, MetI, MetC***	Sulfite reduction-associated complex, L-cystein uptake and metabolism
	Anand1_poultry	18	***DsrE***, *DsrH*(+), ***DsrC-4, TcyP, MetI, MetC***	
	Anand1_goat	17	***DsrE***, *DsrH*(+), ***DsrC-4, TcyP, MetI, MetC***	
	Anand1_buffalo	17	***DsrE***, *DsrH*(-), ***DsrC-4, TcyP, MetI, MetC***	
Phosphorus Metabolism	P52VAC	23	***IP, THb, THa, PhoH, LAT, PhoU, EPP***, *PhoR*(+), ***PhoB, ptsS, ptsA, PtsC, PtsB***	Phosphate metabolism
	Anand1_poultry	23	***IP, THb, THa, PhoH, LAT, PhoU, EPP***, *PhoR*(+), ***PhoB, ptsS, ptsA, PtsC, PtsB***	

	Anand1_goat	23	***IP, THb, THa, PhoH, LAT, PhoU, EPP,*** *PhoR*(+), ***PhoB, ptsS, ptsA, PtsC, PtsB***	
	Anand1_buffalo	32	***IP, THb, THa, PhoH, LAT, PhoU, EPP,*** *PhoR*(-), ***PhoB, ptsS, ptsA, PtsC, PtsB***	
Carbohydr-ates	P52VAC	244	*XylA*(+), ***XylB,*** *Xyl_T*(+), *Xyn_T*(+), *mdh*(+), *gck*(+), *eno*(+), *MTFC*(+), *GlyA*(+), *sdhB*(+), *sdhA*(+), *scsA*(+), *gltA*(+), ***LldD, LldEFG, LldP***	Xylose utilization, Serine-glyoxanate cycle, Lactate utilization
	Anand1_poultry	237	*XylA*(-), ***XylB***, *Xyl_T* (-), *Xyn_T*(-), *mdh*(-), *gck*(-), *eno*(-), *MTFC* (-), *GlyA*(-), *sdhB*(-), *sdhA*(-), *scsA*(-), *gltA*(-), ***LldD, LldEFG, LldP***	
	Anand1_goat	243	*XylA*(-), ***XylB***, *Xyl_T*(-), *Xyn_T*(-), *mdh*(+), *gck*(+), *eno*(+), *MTFC*(+), *GlyA*(+), *sdhB*(+), *sdhA*(+), *scsA*(+), *gltA*(+), ***LldD, LldEFG, LldP***	
	Anand1_buffalo	239	*XylA*(-), ***XylB***, *Xyl_T*(+), *Xyn_T*(+), *mdh*(-), *gck*(-), *eno* (-), *MTFC*(-), *GlyA*(-), *sdhB*(-), *sdhA*(-), *scsA*(-), *gltA*(-), ***LldD, LldEFG, LldP***	

- Cattle isolate Anand1_cattle is omitted due to very less coverage obtained.
- Genes/proteins in bold letters indicate their common presence in all the four isolates.
- Genes/proteins with (+) sign indicate their presence in one or more isolates.
- Genes/proteins with (-) sign indicate their absence in one or more isolates.

4.3.1.2 Anand1_poultry

RAST analysis of Anand1_poultry revealed genome size of 2,227,943bp with 489 contigs. Total number of subsystem defined was 406, while number of coding sequences (CDS) were 2365 and number of RNAs were 54.

Based on subsystem analysis, 65% (1529 CDS) of genome was having defined subsystem based classification, while rest of the 35% (836 CDS) of genome was not having defined subsystem. Of the defined subsystem, highest abundance of subsystem were Amino acids and derivatives (267 CDS), carbohydrates (237 CDS), cofactor and vitamins, prosthetic groups, pigments (235 CDS), and protein metabolism (224 CDS) (Plate 4.8).

The genome of Anand1_poultry contains genes for resistance to antibiotics and toxic compounds, which includes lysozyme inhibitors, copper homeostasis, cobalt-zinc-cadmium resistance, arsenic resistance, copper homeostasis: copper tolerance, resistance to fluoroquinolones, beta-lactamase and multidrug resistance efflux pumps. The multidrug resistance efflux pumps confer resistance to natural substances produced by the host, including bile, hormones and host-defence molecules. In addition, some efflux pumps of the resistance nodulation division (RND) family have been shown to have a role in the colonization and the persistence of bacteria in the host. So, they have roles in bacterial pathogenicity. Genes for resistance to fluoroquinolones include *ParC*, *ParE* which code for topoisomerase IV subunit as well as *gyrA* and *gyrB,* which code for DNA gyrase subunit B and lead to the resistance of bacteria against commonly used fluroquinolone group of antibiotics against Gram negative bacteria. The list of genes required for the normal survival, replication, protein synthesis as well as for bacterial pathogenicity are listed in Table 4.5 as per RAST server.

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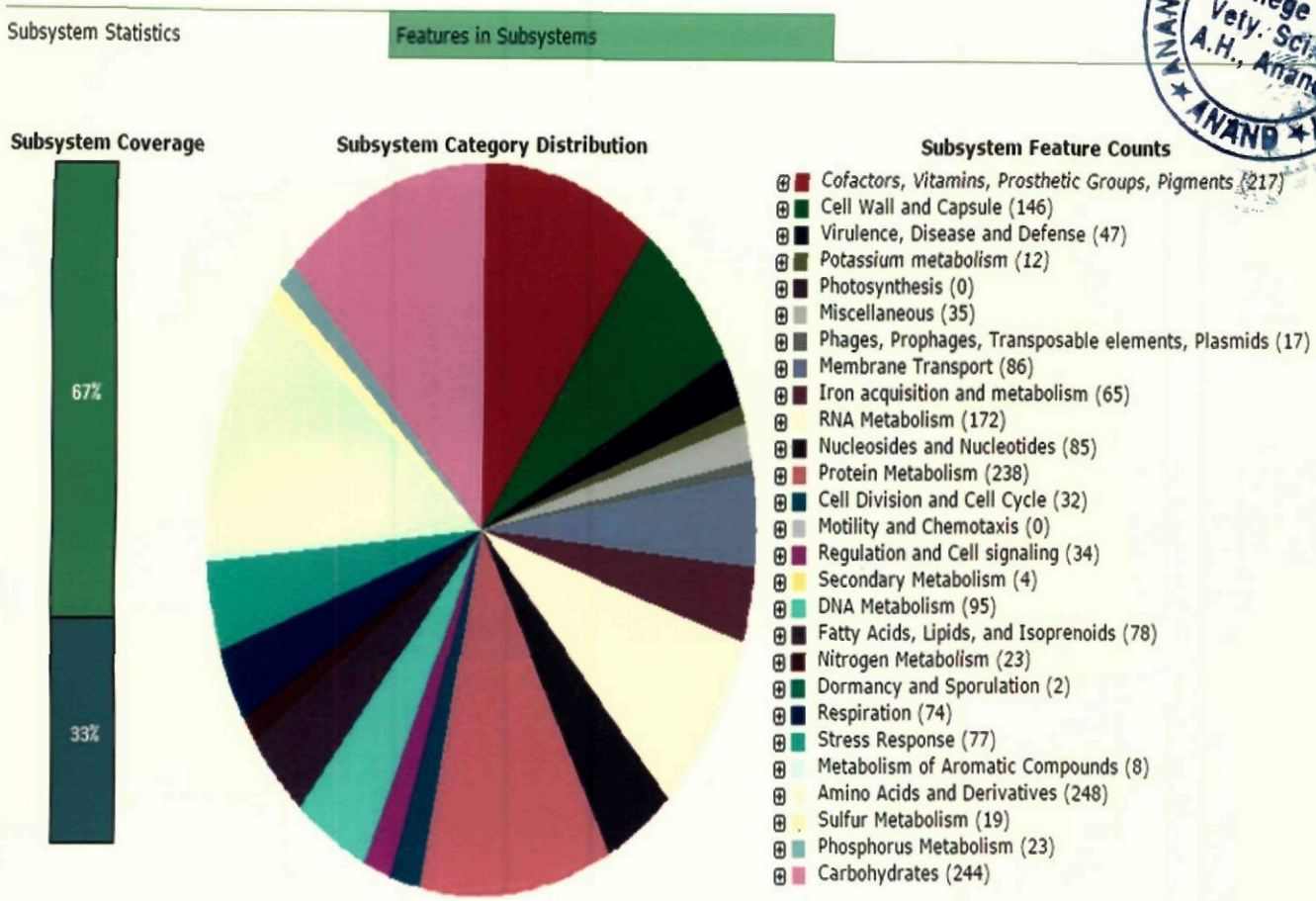

Plate 4.7: Subsystem distribution of P52VAC isolate of *P. multocida* (based on RAST annotation server)

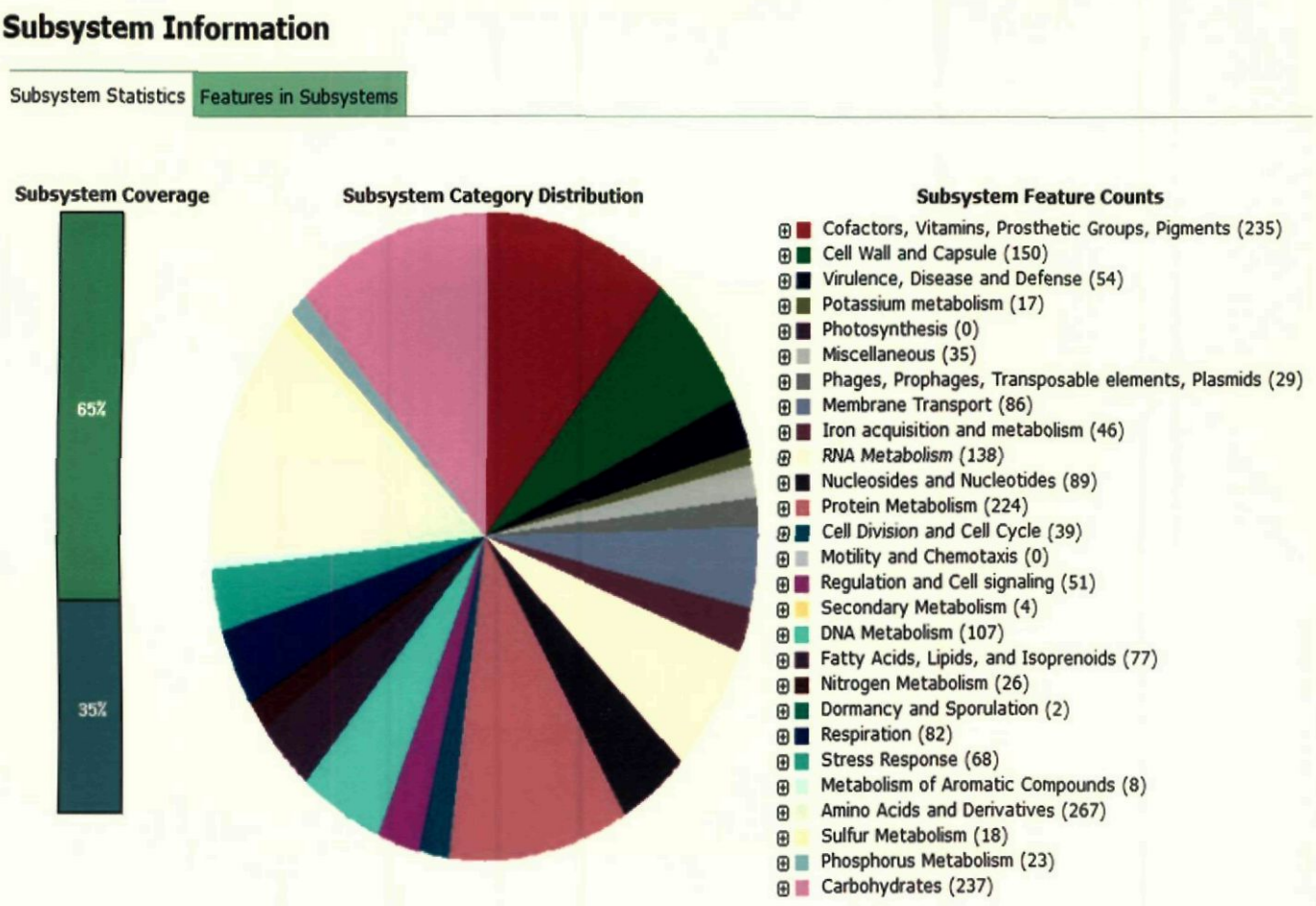

Plate 4.8: Subsystem distribution of Anand1_poultry isolate of *P. multocida* (based on RAST annotation server)

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4.3.1.3 Anand1_goat

RAST analysis of Anand1_goat revealed genome size of 2,285,382bp with 349 contigs. Total number of subsystems defined was 419, number of coding sequences (CDS) were 2340 and number of RNAs were 54.

Based on subsystem analysis, 66% (1543 CDS) of genome was having defined subsystem based classification, while rest of the 34% (797 CDS) of genome was not having defined subsystem. Of the defined subsystem, highest abundance of subsystem were *Amino acids and derivatives* (254 CDS), *protein metabolism* (252 CDS), carbohydrates (243 CDS), and cofactor and vitamins, prosthetic groups, pigments (242 CDS) (Plate 4.9).

For Anand1_goat isolate, important feature is the presence of periplasmic hemin binding protein (HBP and ExB, TonB). Hemin is the oxidized form of heme that is found in extracellular environments, and it is the form of heme transported by bacterial uptake systems. Hemin iron transport and utilization systems have been identified in numerous bacterial species and were initially described in gram-negative pathogens. It is an outer membrane receptor and a periplasmic binding protein-dependent ABC-type transporters required for hemin uptake. Certain Gram negative species also obtain hemin iron through the use of hemophores, which are low-molecular-weight secreted hemin binding proteins that are able to extract hemin from hemoglobin and then transfer the hemin to receptors in the bacterial outer membrane (Allen and Schmitt, 2009).

There was also presence of Hemin ABC transporter particularly permease protein, Arsenic efflux pump protein, Multidrug resistance transporter, Bcr/CflA family, Multi antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family of MDR efflux pumps, resistance-nodulation-cell division superfamily (RND) efflux

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system, outer membrane lipoprotein CmeC in the genome of Anand1_goat isolate. The genetic elements encoding efflux pumps may be encoded on chromosomes and/or plasmids, thus contributing to both intrinsic (natural) and acquired resistance respectively. As an intrinsic mechanism of resistance, efflux pump genes can survive a hostile environment (e.g. in the presence of antibiotics) which allows for the selection of mutants that over-express these genes. Being located on transportable genetic elements as plasmids or transposons is also advantageous for the microorganisms as it allows for the easy spread of efflux genes between distant species. Antibiotics can act as inducers and regulators of the expression of some efflux pumps (Morita *et al.,* 2006). Expression of several efflux pumps in a given bacterial species may lead to a broad spectrum of resistance when considering the shared substrates of some multi-drug efflux pumps, where one efflux pump may confer resistance to a wide range of antimicrobials. The major facilitator superfamily (MFS) mainly present in the Gram positive bacteria was not present in any of the isolates studied in present study. The list of genes found present in the genome of Anand1_goat are listed in Table 4.5 as per RAST server.

4.3.1.4 Anand1_buffalo

RAST analysis of Anand1_buffalo revealed genome size of 2,045,610bp with 2188 contigs. Total number of subsystems defined was 345, number of coding sequences (CDS) were 2680 and number of RNAs were 40.

Based on subsystem analysis, 62% (1656 CDS) of genome was having defined subsystem based classification, while rest of the 38% (1024 CDS) of genome was not having defined subsystem. Of the defined subsystem, highest abundance of subsystem were amino acids and derivatives (357 CDS), cofactor and vitamins, prosthetic

groups, pigments (294 CDS), carbohydrates (239 CDS), and protein metabolism (200 CDS) (Plate 4.10).

The main distinguishing feature of Anand1_buffalo isolate was the presence of *Mycobacterium* virulence operon involved in protein synthesis. Additionally, there was presence of "Heme, hemin uptake and utilization systems in Gram negatives" which includes *ExB* gene, involved in ferric siderophore transport system, *FCR* which codes for the TonB-dependent hemin, ferrichrome receptor RP_2, which is a outer membrane receptor protein mostly involved in Fe transport. There was also presence of *HBP*, which is periplasmic heme binding protein and *HemR* gene coding for hemin receptor protein. Apart from all these, genes involve in protein metabolism, synthesis as well as for normal functions of bacteria were found as listed in Table 4.5.

4.3.1.5 Anand1_cattle

RAST analysis of Anand1_cattle revealed genome size of 1,438,517bp with 3152 contigs. Total number of subsystems defined was 19, number of coding sequences (CDS) were 1862 and number of RNAs were 0.

Based on subsystem analysis, 2% (32 CDS) of genome was having defined subsystem based classification, while rest of the 98% (1830 CDS) of genome was not having defined subsystem. This was due to less coverage obtained during sequencing of the isolate Anand1_cattle i.e. 3.29X. Out of 27 subsystems, only 10 were assigned and that too with lower counts. Hence, the subsystems assigned are very less. Of the defined subsystem, highest abundance of subsystems were cofactor and vitamins, prosthetic groups, pigments (14 CDS), amino acids and derivatives (9 CDS) and fatty acid, lipids and isoprenoids (9 CDS) (Plate 4.11). Possible reason of less coverage particularly for Anand1_cattle might be increased number of "mixed beads" or loading of probably less number of beads at the time of sequencing. Second

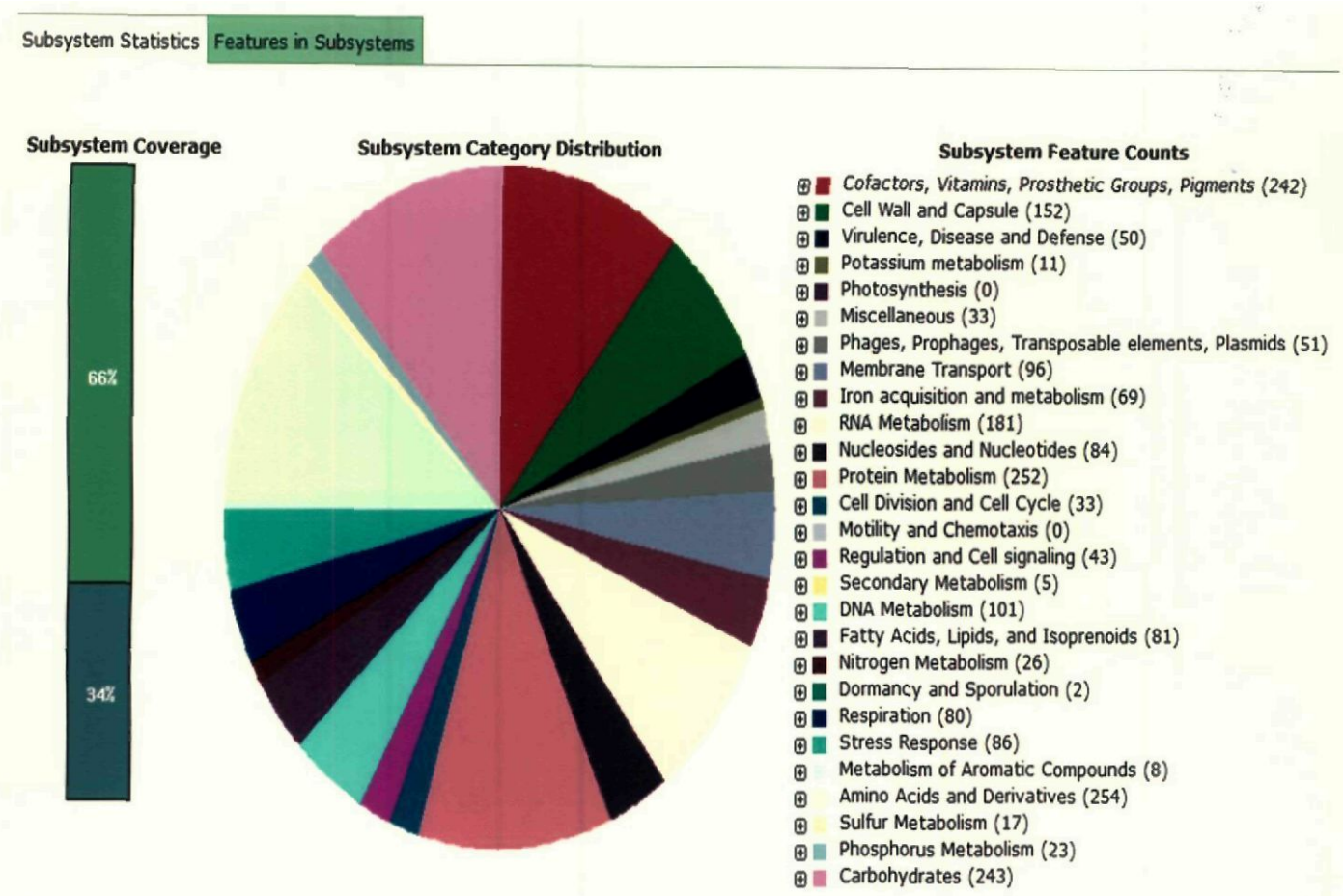

Plate 4.9: Subsystem distribution of Anand1_goat isolate of *P. multocida* (based on RAST annotation server)

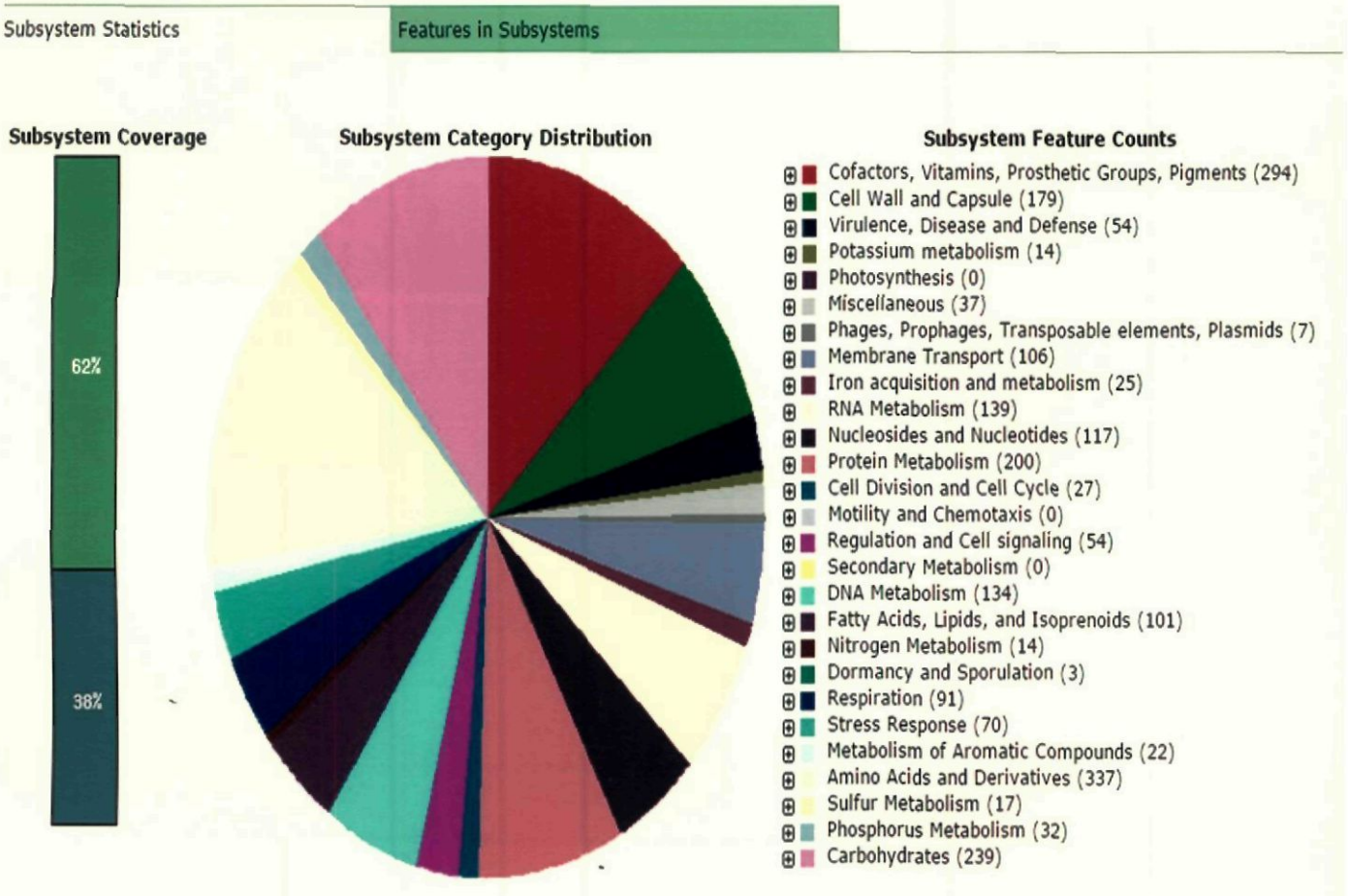

Plate 4.10: Subsystem distribution of Anand1_buffalo isolate of *P. multocida* (based on RAST annotation server)

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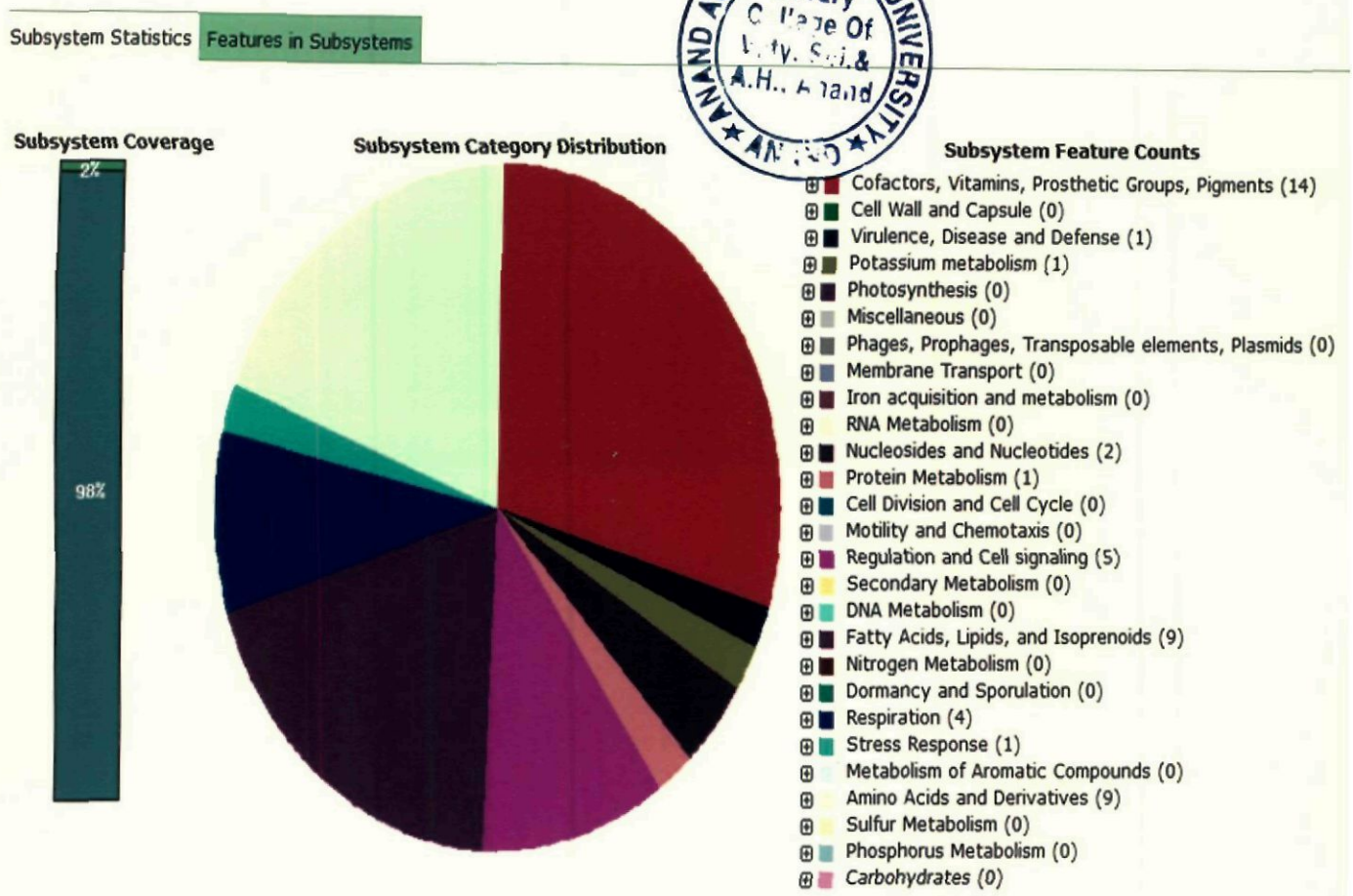

Plate 4.11: Subsystem distribution of Anand1_cattle isolate of *P. multocida* (based on RAST annotation server)

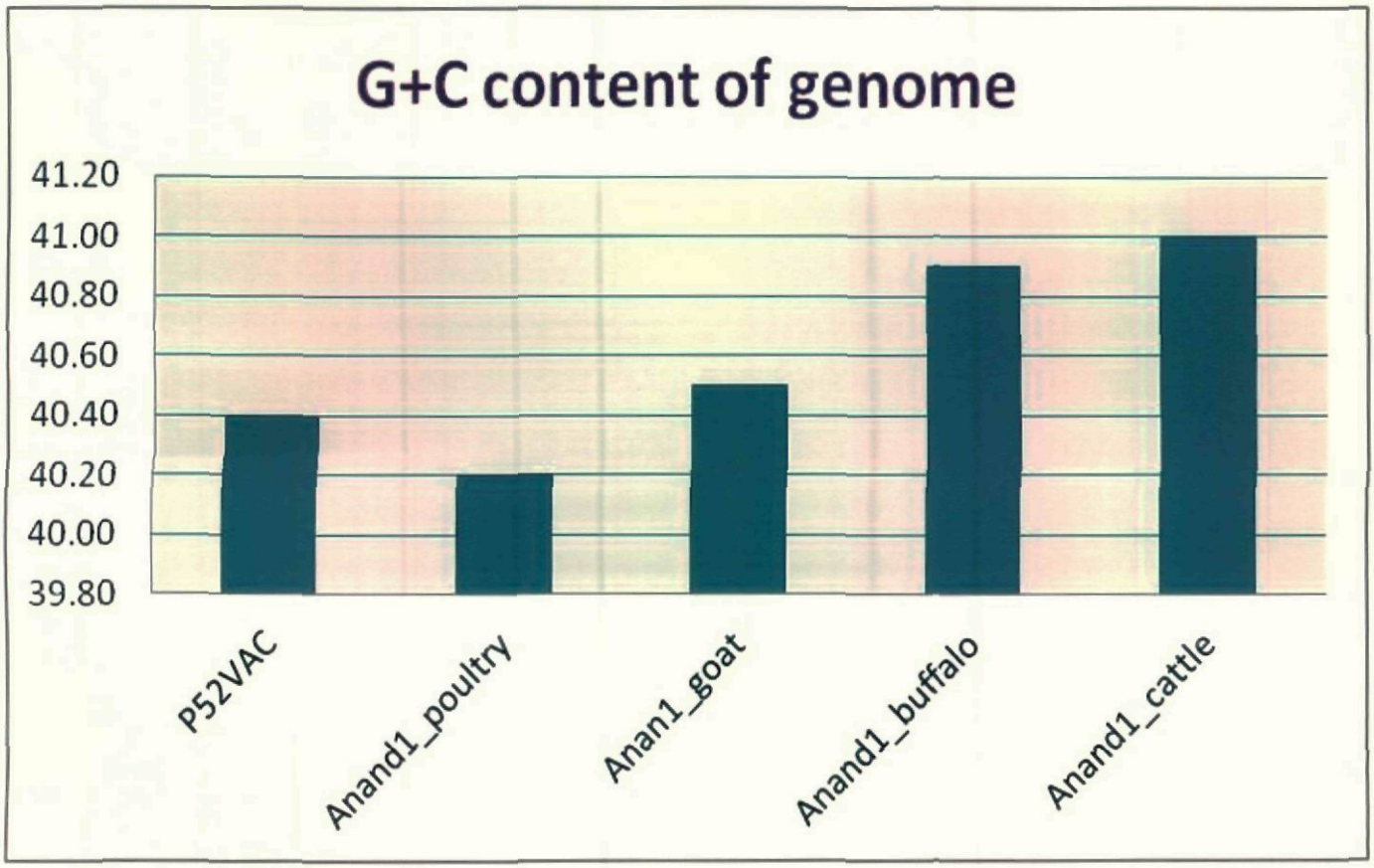

Plate 4.12: G+C content of the genome for all the five isolates of *P. multocida*

possibility may be related with improper adaptor ligation at the time of library preparation, which may ultimately leads to decreased amplification of DNA sequences during emPCR (454 GS Run Browser manual available at http://454.com/my454/tools-downloads/software-downloads.asp).

4.3.2 PGAAP analysis

For Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) analysis, all the reads were submitted to the PGAAP annotation pipeline of NCBI (National Center for Biotechnology Information) after removing reads which were less than 200bp (using in-house perlscript programme). Genomic statistics of all *P. multocida* isolates obtained by PGAAP analysis are given in Table 4.6. All the sequences were submitted to Genebank NCBI for open access under Genebank project id ALBZ00000000.1, AFRR00000000.1, AFRS00000000.1, ALBX00000000.1 and ALBY00000000.1 for P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle, respectively.

Comparative G+C content of the five isolates obtained by PGAAP analysis are shown in Table 4.6 and Plate 4.12. Number of CDS and PEG of all the isolates are depicted in Table 4.6 and Plate 4.13 based on PGAAP analysis. Representative graph (for Anand1_goat isolate) showing number of contigs number and contig length is shown after annotation using PGAAP pipeline in Plate 4.14.

Whole genome sequence comparison of the five isolates of *P. multocida* and Pm70 genome sequences using progressiveMauve alignment showing collinear blocks of genes is represented in Plate 4.15. Rectangles of similar colour shade show collinear blocks of genes. Areas of low identity within colinear blocks are shown by reduced hight of shading.

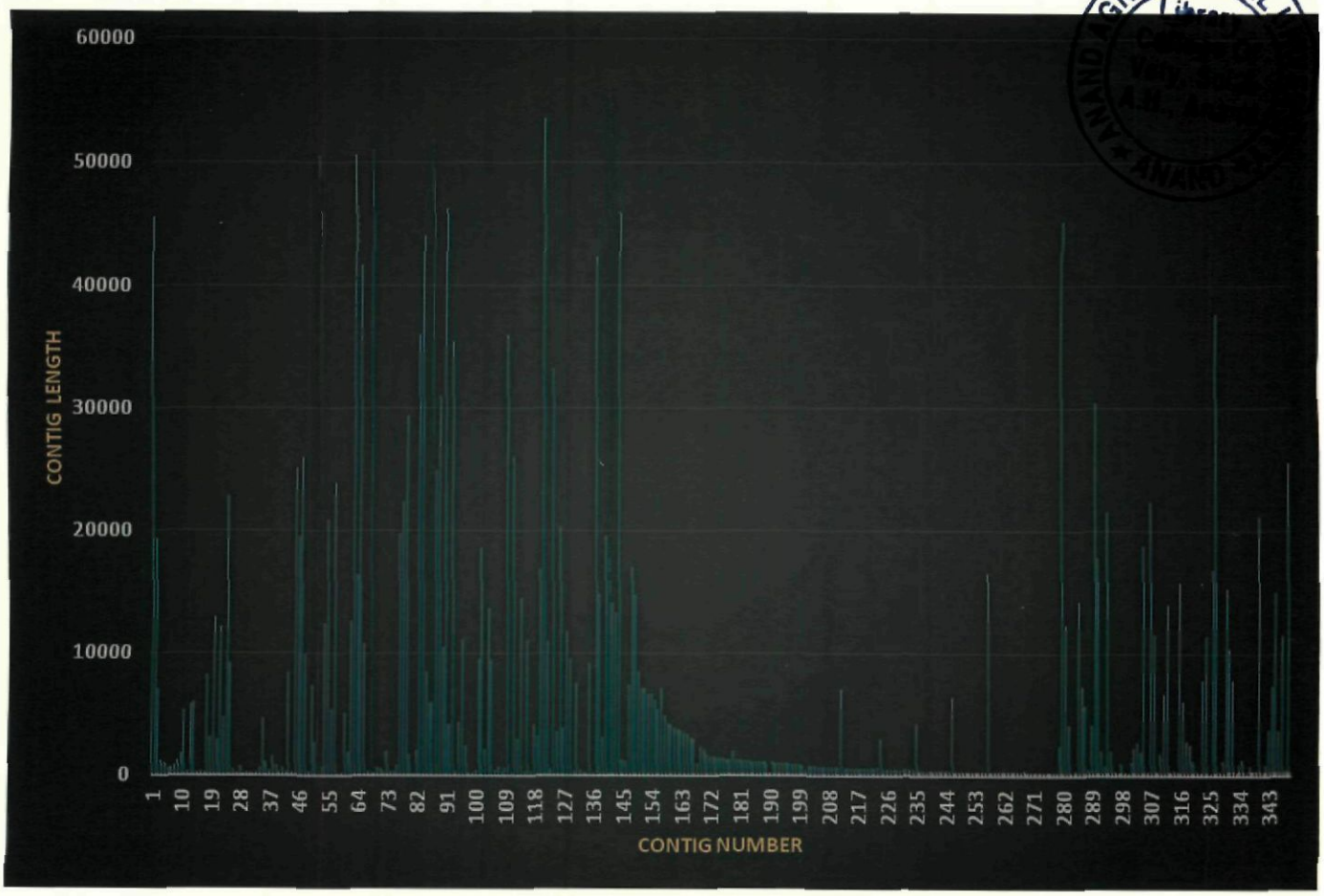

Plate 4.13: Graph showing number and length of contings for represantative sample (Anand1_goa isolate of *P. multocida*

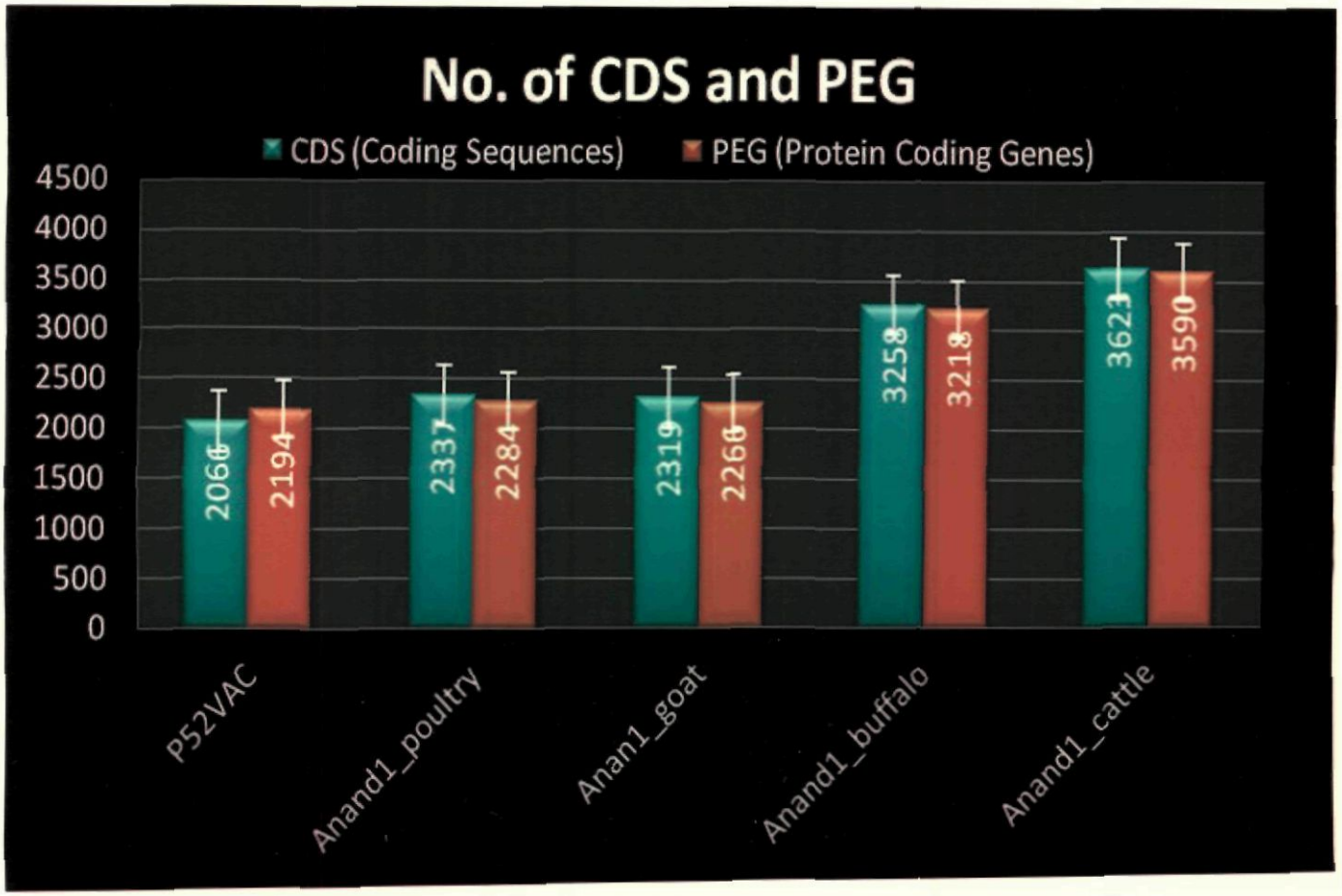

Plate 4.14: Number of CDS and PEG for all the isolates of *P. multocida*

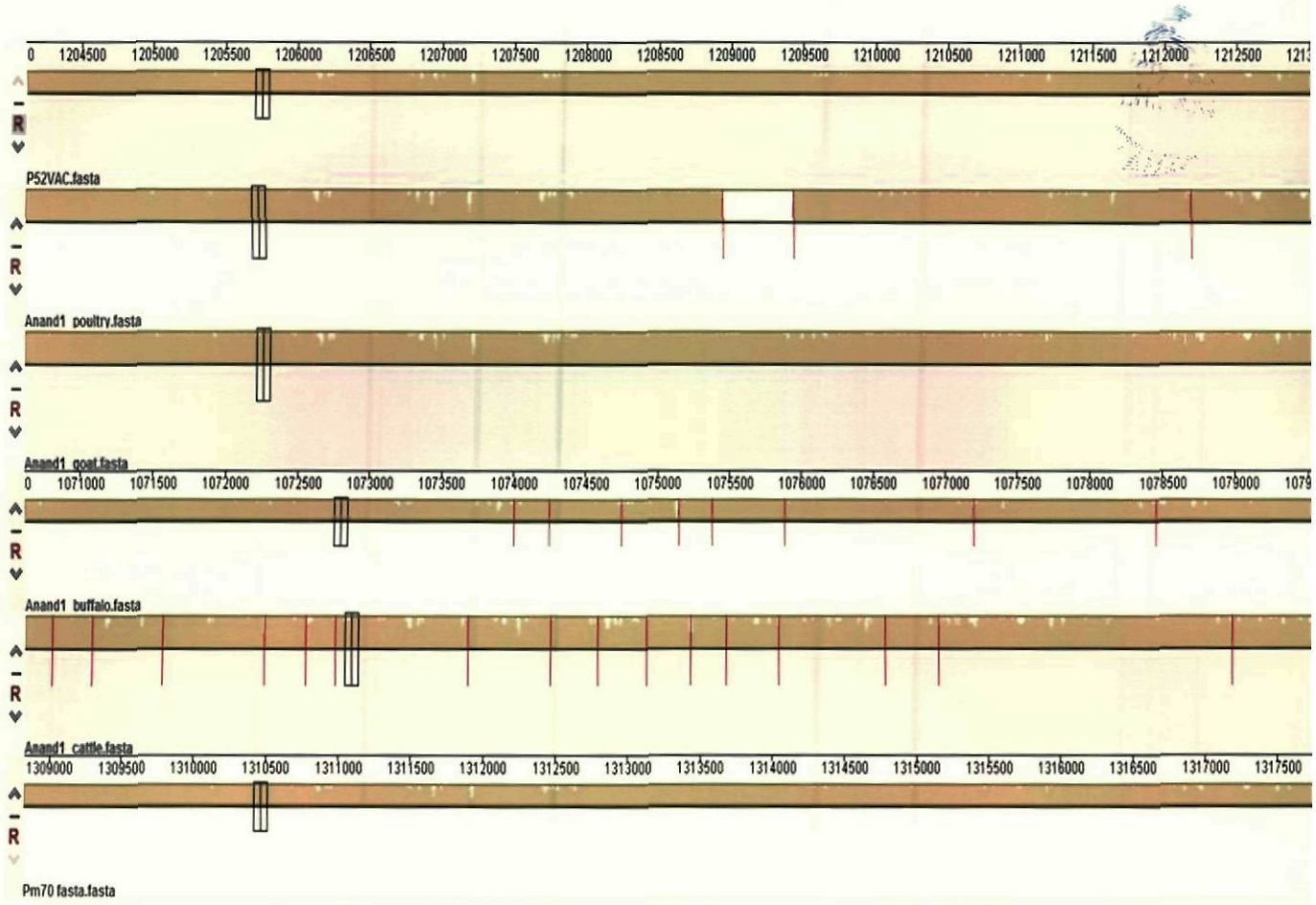

Plate 4.15: Comparison of the five isolates of *P. multocida* and Pm70 genome sequences using progressiveMauve alignment (Darling *et al.*, 2010)

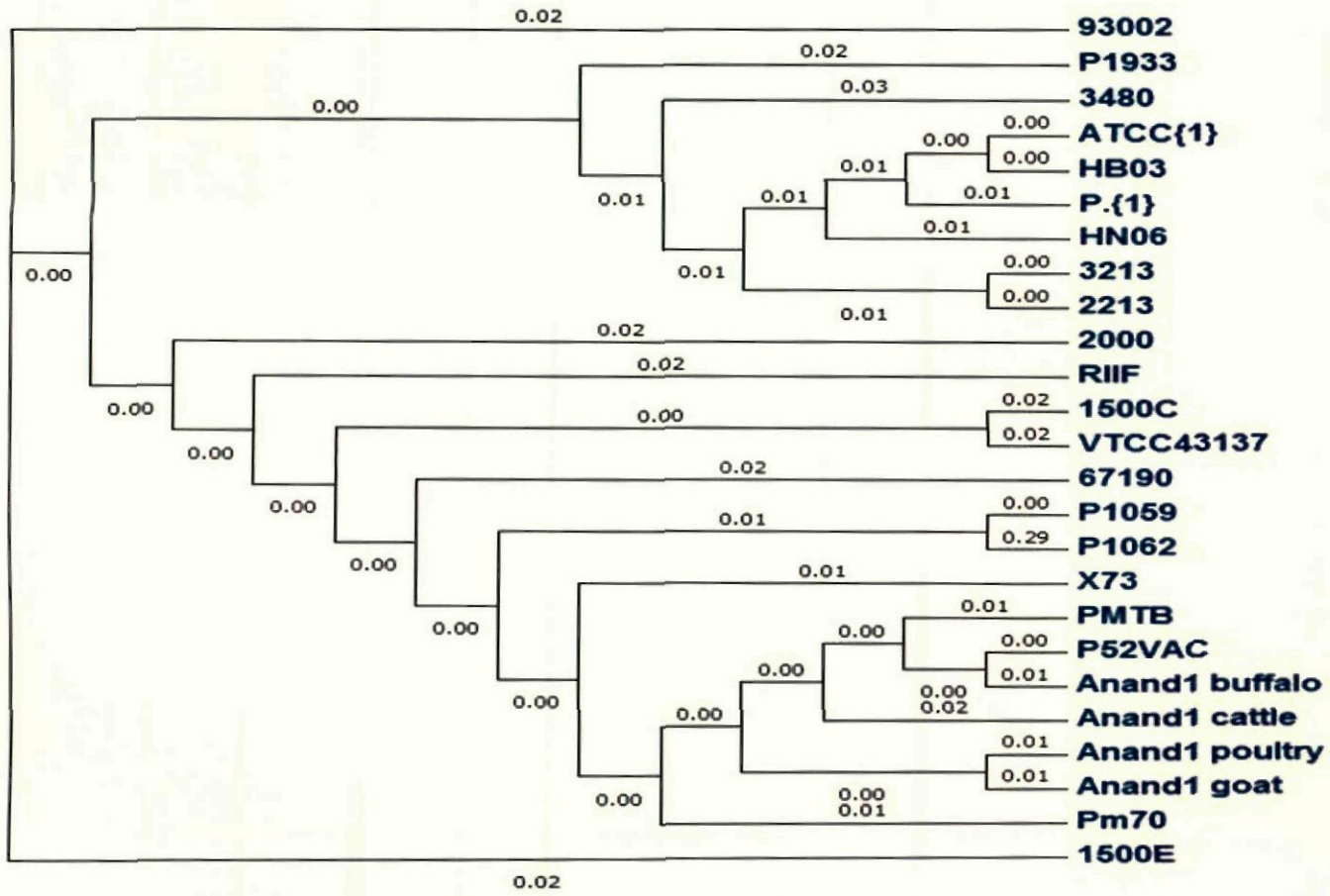

Plate 4.16: Phylogenetic comparision among *Pasteurella multocida* species whole genome (complete and incomplete) sequences using MEGA6

Table 4.6: Genomic statastics of *P. multocida* isolate of different species obtained by PGAAP analysis

	P52VAC	Anand1_poultry	Anand1_goat	Anand1_buffalo	Anand1_cattle
Size	2.27Mb	2.23Mb	2.29Mb	2.05Mb	1.44Mb
INSDC	ALBZ00000000.1	AFRR00000000.1	AFRS00000000.1	ALBX00000000.1	ALBY00000000.1
Coverage	18.87X	12.85X	9.70X	17.61X	3.29X
Contig/ Scaffold	209	489	349	2188	3152
Bioproject	PRJNA 180802	PRJNA 179832	PRJNA 179833	PRJNA 180800	PRJNA 180801
Taxonomic id	1077143	1032866	1032869	1032868	1032867
Capsule	B (B:2)	A	D	B (B:2)	B (B:2)
G+C content	40.40%	40.20%	40.50%	40.90%	41.00%
Coding region (CDS)	2,066	2,337	2,319	3,258	3,623
RNA genes	64	53	53	41	33
Protein coding genes (PEG)	2,194	2,284	2,266	3,218	3,590
N50	36,108	17,120	21,172	1,299	528
L50*	18	36	32	474	865

*L50 is the number of scaffolds that accounts for more than 50% of the genome assembly.

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Phylogenetic analysis is a useful measure for desiphering the genetic relationship among the different isolates of bacterial spp. For phylogenetic analysis, available whole genome sequences of *P. multocida* (complete and incomplete) in FASTA files were downloaded from (http://www.ncbi.nlm.nih.gov/genome/genomes/912?). A phylogenetic tree based on whole genome was constructed using CV tree (Zhao and Hao, 2009) and results were visualized in MEGA6 (Tamura *et al.,* 2013). Phylogenetic analysis revealed least genetic distance between isolates P52VAC and Anand1_buffalo, which grouped under the same cluster as compared to others. Anand1_cattle was found to be closely related to the isolate P52VAC and Anand1_buffalo. Anand1_poultry and Anand1_goat grouped in same cluster, however at a genetic distance from rest of the three isolates. Pm70 (poultry isolate) and PMTB (buffalo isolate) are closely related to our five isolates as compared to other isolates/strains from various parts of the world (Plate 4.16). Circular genome maps for all the five isolates of *P. multocida* were created using CGView server (Grant and Stothard, 2008) and are shown in Plates 4.17 to 4.21).

4.3.2.1 Virulence genes of *P. multocida*

Productive infection by bacterial pathogens relies on the expression of virulence factors that have wide ranging functions like competence, adherence, capsule synthesis and export, evading host immune responses etc. For all the isolates of *P. multocida* (i.e. P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle), gene locus or protein id were downloaded from http://www.ncbi.nlm.nih.gov/genome/genomes/912? under 'Protein' column/section. After downloading protein or gene locus individually, each were searched for the virulence associated genes manually falling under seven broad categories of virulence associated genes viz. capsule, fimbriae and adhesion, iron metabolism, outer

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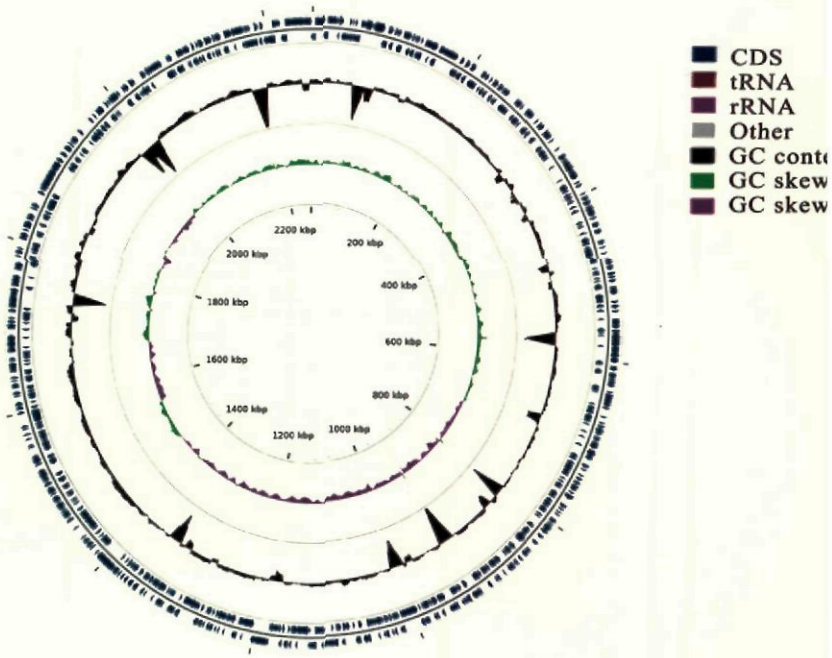

Plate 4.17: Circular genome map of P52VAC isolate of *P. multocida**

The map represents the circular view of the genome sequence of P52VAC *multocida* (Mapped Contigs). The circle was created using CGView server fo map creation. The outermost circular ring shows coding sequences (CDS followed by tRNA and rRNA respectively. Circle 4 shows the G+C conten The innermost ring towards the center represents the GC skew information i the (+) strand (green color) and (-) strand (dark pink color). *Reads wer mapped to reference genome using Genebank file and consensus sequence wa created. Finally annotated consensus sequence with reference sequence wa used to create circular map of genome.

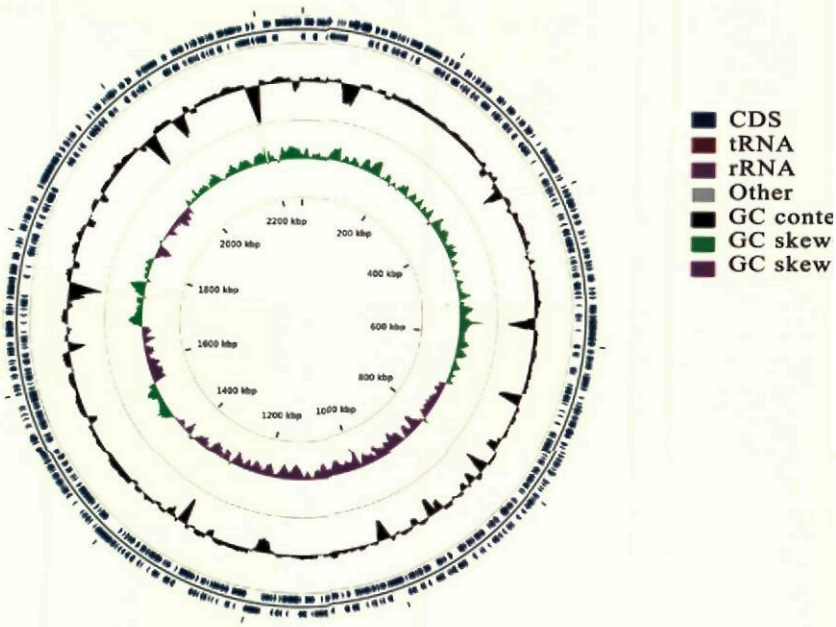

Plate 4.18: Circular genome map of Anand1_poultry isolate of *P. multocida**

The map represents the circular view of the genome sequence o Anand1_poultry *P. multocida* (Mapped Contigs). The circle was created using CGView server for map creation. The outermost circular ring shows coding sequences (CDS) followed by tRNA and rRNA respectively. Circle 4 shows the G+C content. The innermost ring towards the center represents the GC skew information in the (+) strand (green color) and (-) strand (dark pink color) *Reads were mapped to reference genome using Genebank file and consensus sequence was created. Finally annotated consensus sequence with reference sequence was used to create circular map of genome.

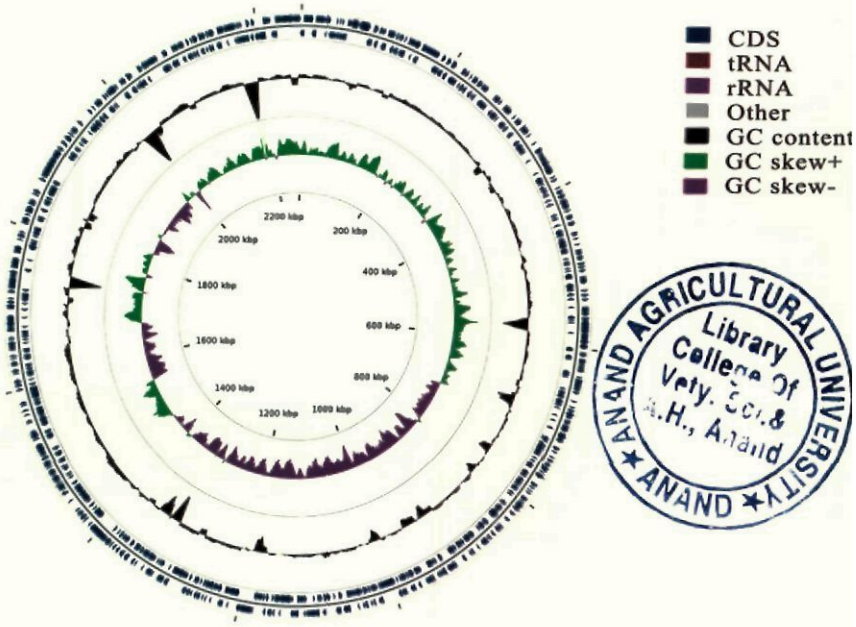

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Plate 4.19: Circular genome map of Anand1_goat isolate of *P. multocida**
The map represents the circular view of the genome sequence of Anand1_goat *P. multocida* (Mapped Contigs). The circle was created using CGView server for map creation. The outermost circular ring shows coding sequences (CDS) followed by tRNA and rRNA respectively. Circle 4 shows the G+C content. The innermost ring towards the center represents the GC skew information in the (+) strand (green color) and (-) strand (dark pink color). *Reads were mapped to reference genome using Genebank file and consensus sequence was created. Finally annotated consensus sequence with reference sequence was used to create circular map of genome.

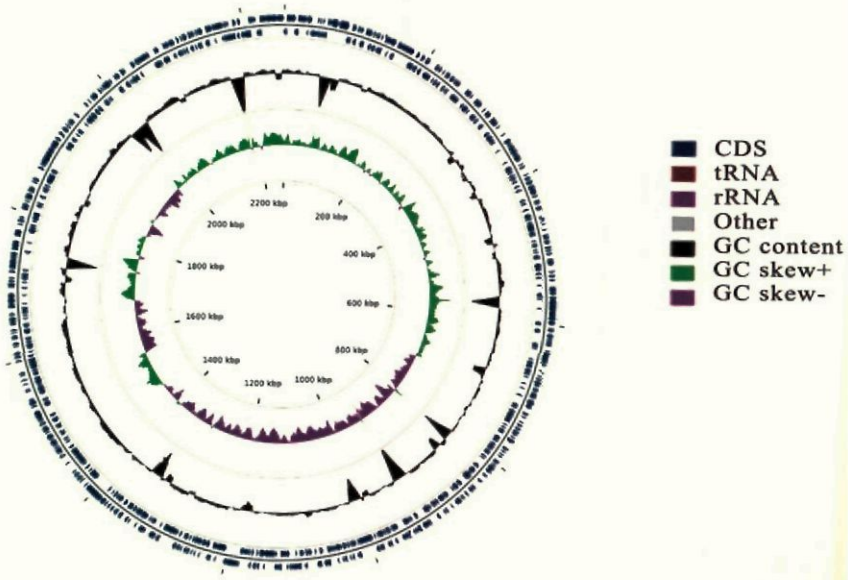

Plate 4.20: Circular genome map of Anand1_buffalo isolate of *P. multocida**
The map represents the circular view of the genome sequence of Anand1_buffalo *P. multocida* (Mapped Contigs). The circle was created using CGView server for map creation. The outermost circular ring shows coding sequences (CDS) followed by tRNA and rRNA respectively. Circle 4 shows the G+C content. The innermost ring towards the center represents the GC skew information in the (+) strand (green color) and (-) strand (dark pink color). *Reads were mapped to reference genome using Genebank file and consensus sequence was created. Finally annotated consensus sequence with reference sequence was used to create circular map of genome.

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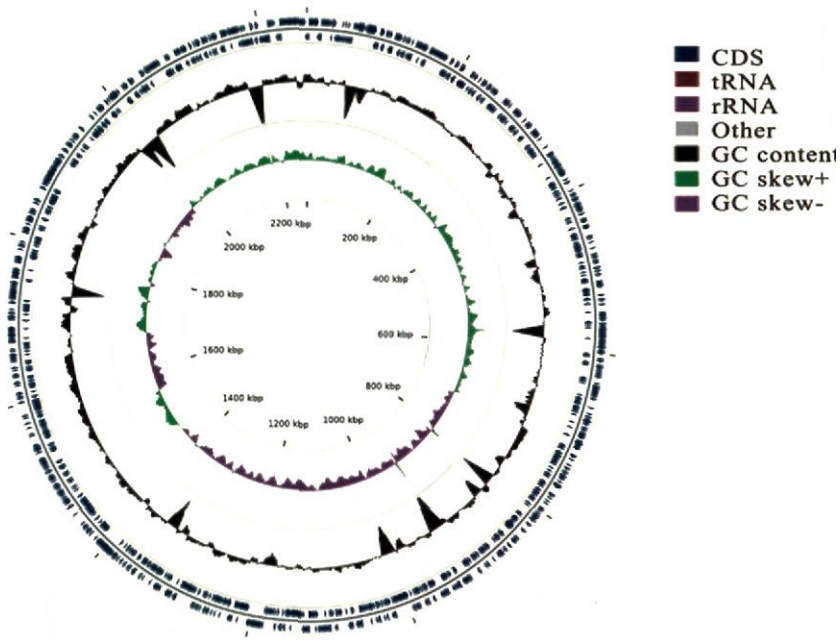

Plate 4.21: Circular genome map of Anand1_cattle isolate of *P. multocida**

The map represents the circular view of the genome sequence of Anand1_cattle *P. multocida* (Mapped Contigs). The circle was created using CGView server. The outermost circular ring shows coding sequences (CDS) followed by tRNA and rRNA respectively. Circle 4 shows the G+C content. The innermost ring towards the center represents the GC skew information in the (+) strand (green color) and (-) strand (dark purple color). *Reads were mapped to reference genome using Genebank file and consensus sequence was created. Finally annotated consensus sequence with reference sequence was used to create circular map of genome.

membrane protein, superoxide dismutase, sialic acid metabolism and transcription regulation. Out of these seven categories, all the five genes falling under three categories i.e. *SodA* and *SodC* under superoxide dismutase, *NanH* and *NanB* under sialic acid metabolism and *Fis* under transcription regulation category were present in all the five isolates (Tables 4.7 and 4.8).

Presence or absence of virulence associated genes under rest of the four categories viz. capsule, fimbriae and adhesion, iron metabolism and outer membrane protein was as follow.

A) Genes for capsule

Out of nine genes under this category, *PglA* and *Kmt1* were found in all the five isolates, while *HyaE* was found to be present only in goat isolate. *HexA* and *HexC* were absent in buffalo and cattle isolates, while *HexB* and *HexD* were absent in goat and cattle isolates. *KpsF* gene was found to be absent in poultry and cattle isolates. Gene *LctP* was present only in goat and cattle isolates. Out of nine genes falling under this category, P52VAC and goat isolates revealed maximum number of seven genes, while buffalo isolate revealed only three out of nine genes in this category.

B) Genes for fimbriae and adhesion

Out of 16 genes under this category, *Hsf, PfhB2, PfhR, PflB, PlpB,* and *Plp4* were found in all the five isolates of *P. multocida* studied, which indicated the importance of these genes in virulence. *HofC* gene was absent in P52VAC only, whereas *PlpE* was absent in cattle as well as buffalo isolate. *ComE* was absent in P52VAC isolate, while *TadE* was absent in buffalo isolate. *PfhB1* and *PlpP* genes were absent in cattle isolate, while *RcpA* and *RcpB* were absent in buffalo isolate.

Table 4.7: Virulence associated genes of *P. multocida* from different species of animals by PGAAP analysis

Sr. No.	Gene locus / Protein	P52VAC	Anand1_poultry	Anand1_goat	Anand1_buffalo	Anand1_cattle
			Capsule			
1	*HexA*	+	+	+	-	-
2	*HexB*	+	+	-	-	+
3	*HexC*	+	+	+	-	-
4	*HexD*	+	+	-	-	+
5	*PglA*	+	+	+	+	+
6	*Kmt1*	+	+	+	+	+
7	*KpsF*	+	-	+	+	-
8	*HyaE*	-	-	+	-	-
9	*LctP*	-	-	+	-	+
			Fimbriae and adhesion			
10	*ComE*	-	+	+	+	+
11	*ClpB*	-	+	+	+	-
12	*HofC*	+	+	+	+	-
13	*Hsf*	+	+	+	+	+
14	*PfhB1*	+	+	+	+	-
15	*PfhB2*	+	+	+	+	+
16	*PfhR*	+	+	+	+	+
17	*PflB*	+	+	+	+	+
18	*PlpB*	+	+	+	+	+
19	*PlpE*	+	+	+	-	-
20	*PlpP*	+	+	+	+	-
21	*RcpA*	+	+	+	-	+
22	*RcpB*	+	+	+	-	+

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23	*TadE*	+	+	+	-	+
24	*TadF*	+	+	+	-	-
25	*Plp4*	+	+	+	+	+
	Iron metabolism					
26	*ExbB*	+	+	+	+	+
27	*FbpA*	+	+	+	+	-
28	*FbpB*	+	+	+	+	+
29	*FbpC*	+	+	+	-	+
30	*HbpA*	+	+	+	+	+
31	*HgbA*	+	+	+	+	+
32	*HemR*	-	+	+	+	+
33	*HemU*	+	+	+	+	+
34	*OmpW*	+	+	+	+	+
35	*Rfb*	+	+	+	+	+
36	*RffG*	+	+	+	+	+
37	*TbpA*	+	+	-	+	+
38	*TonB*	+	+	+	+	-
39	TonB-dependent lactoferrin and transferrin receptor	+	+	-	+	+
40	TonB-dependent receptor	-	+	+	-	-
41	Translocation protein TolB	+	-	-	-	-
	Outer membrane protein (OMP)					
42	*HasR*	+	+	+	+	+
43	*LppB*	+	+	+	+	+
44	*LspB*	+	+	+	+	+
45	*OmpH*	+	+	+	+	+
46	Outer membrane lipoprotein LolB	+	-	-	-	+
47	Filamentous hemagglutinin outer membrane protein (*PfhA*)	+	-	-	-	-

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48	*PtfA*	+	+	+	+	+
49	*Oma87*	+	-	+	+	+
50	*VacJ*	+	+	-	+	-
Superoxide dismutase						
51	*SodA*	+	+	+	+	+
52	*SodC*	+	+	+	+	+
Sialic acid metabolism						
53	*NanB*	+	+	+	+	+
54	*NanH*	+	+	+	+	+
Transcription regulation						
55	*Fis*	+	+	+	+	+

(+) indicate presence of gene/protein in the particular isolate
(-) indicate absence of gene/protein in the particular isolate

Table 4.8: Unique virulence associated genes (Categorywise) in different isolates of *P. multocida*

Gene name	**Found only in isolate of**					**Absent only in isolate of**				
Isolate name	**P52**	**Poultry**	**Goat**	**Buffalo**	**Cattle**	**P52**	**Poultry**	**Goat**	**Buffalo**	**Cattle**
Capsule										
HexA									+	+
HexB								+	+	
HexC									+	+
HexD								+	+	

KpsF							+			+
HyaE			+							
LctP			+		+					
Fimbriae and adhesion										
ComE						+				
ClpB						+				+
HofC										+
PfhB1										+
PlpE									+	+
PlpP										+
RcpA									+	
RcpB									+	
TadE									+	
TadF									+	+
Iron metabolism										
FbpA										+
FbpC									+	

HemR						+				
TbpA								+		
TonB										+
TonB- dependent lactoferrin and Transferrin receptor								+		
TonB- dependent receptor		+	+							
TolB		+	+							
Translocation protein TolB	+									
Outer membrane protein										
Lipoprotein LolB	+				+					
PfhA	+									
Oma87		+								
VacJ								+		+

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ClpB was found to be absent in P52VAC and cattle isolates, whereas *TadF* was found to be absent in buffalo and cattle isolates. Out of 16 genes falling under ''fimbriae and adhesion'' category, P52 and goat isolates were having all the 16 genes present in their genome, while cattle isolate was having only 10 genes present out of 16 genes.

C) Genes for iron metabolism

Out of 16 genes falling under ''iron metabolism'' category, *ExbB, FbpB, HbpA, HgbA, HemU, OmpW, Rfb* and *RffG* were present in all the five isolates studied. Genes *FbpA* and *TonB* were absent in cattle isolate, *TbpA* and TonB-dependent lactoferrin and transferrin receptor were absent in goat isolate. Gene *FbpC* was absent in buffalo isolate. TonB-dependent receptor was found to be present in poultry and goat isolates only. Translocation protein TolB was found to be present and *HemR* was absent only in P52VAC strain, which is a unique finding of this isolate. Under this category, maximum genes were present in poultry isolate, having 15 out of 16 genes studied with absence of only translocation protein TolB. Least number of genes were 12 for cattle isolate, which might be due to less sequencing depth/ coverage.

D) Genes for outer membrane protein

The category of ''outer membrane proteins (OMPs)'' included nine genes, out of which, *HasR, LppB, LspB, OmpH* and *PtfA* were found in all the five isolates of *P. multocida*. Outer membrane protein LolB was found in P52VAC as well as in cattle isolate. But, the sequence of *LolB* gene was found to be truncated for the cattle isolate. Hence, genes *LolB* and *PfhA* were unique to the P52VAC strain. *VacJ* gene, which has been recently identified as a potential virulence factor, was absent in goat and cattle isolates. *Oma87* was absent only in poultry isolate. *PfhA* gene codes for filamentous haemagglutinin outer membrane protein, which was present only in

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P52VAC isolate. Out of nine genes falling in the category of "outer membrane protein", P52VAC revealed all the genes present in their genome, while poultry and goat isolates were having least number of six genes present out of nine genes.

Considering the literature available particularly for the virulence associated genes in *P. multocida*, following genes are described and discussed for their possible role in virulence or their association in the disease process and progression.

4.3.2.1.1 Capsule

Capsules are bacterial surface structures used by many Gram-negative pathogens to evade the host immune system. They are comprised of long carbohydrate chains, called capsular polysaccharides (CPSs), which possess a lipid at one end. The lipid is connected to the CPSs through an unusual linker consisting of five to nine residues of an acidic sugar 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) (Willis and Whitfield, 2013).

In the present study, we found capsule associated genes viz. *HexA, HexB, HexC, HexD, PglA, Kmt1, KpsF, HyaE and Lctp* in vaious isolates of *P. multocida* under the study. Chung *et al.* (1998) cloned the entire *cap* locus by chromosome walking using *P. multocida* based homologous probes and found that entire cap locus spans 16 kb and contains 11 ORFs in 3 regions. Region 1 contains four ORFs, hexA, hexB, hexC and hexD (hex for haluronic acid export). Region 2 contains five ORFs, hyaA, hyaB, hyaC, hyaD and hyaE (hya for hyaluronic acid), which encode proteins that are involved in the formation of precursor activated sugar monomers and the assembly of the capsular polysaccharide polymer. Region 3 consists of two genes, *phyA* and *phyB* (phy for phospholipid substitution of hyaluronic acid) that are transcribed in the opposite direction to that of Regions 1 and 2.

HexA, hexB, hexC and *hexD* encode proteins responsible for transport of the polysaccharide to the bacterial surface. Gene *hexA* was present in P52VAC, poultry and goat isolates only. Another capsular virulence gene *HexB* was observed in P52VAC, poultry and buffalo isolates. For HexB, Zhang *et al.* (2013) compared hexB deleted mutant ΔhexB and wild-type strain P-1059 as well as the complemented strain P-1059C, which demonstrated that ΔhexB was sensitive to the bactericidal action of chicken serum, whereas P-1059 and P-1059C were both resistant. The ΔhexB was highly attenuated in chickens by intravenously injection, and intramuscular administration of ΔhexB to chickens and stimulated significant protection against P-1059 and the homologous strain X-73(A:1).

Capsule biosynthetic locus (*HexC*) was found in P52VAC, poultry and goat isolates and absent in cattle and buffalo, which might be due to its non coverage during sequencing run of cattle and buffalo isolates. Chung *et al.* (2013) cloned and sequenced entire capsule locus of avian *P. multocida* X-73 (A:1) and the locus was divided into three regions, region 1 of which contains four genes, *hexD, hexC, hexB* and *hexA* predicted to encode proteins responsible for transport of the polysaccharide to the bacterial surface. The sequence analysis demonstrated that the *P. multocida* hexABCD were highly homologous at both nucleotide and amino acid levels to *Haemophilus influenza* bexABCD, *Actinobacillus pleuropneumoniae* cpxABCD and *Neisseria meningitidis* ctrABCD. In the serotype A:1 strain X-73, inactivation of the capsule transport gene *hexA* resulted in a mutant strain that was highly attenuated in both mice and chickens, and was more sensitive to the bactericidal activity of chicken serum (Chung *et al.*, 2001).

HexD, another capsular gene was found in P52VAC, poultry and buffalo isolates. Watt *et al.* (2003) demonstrated nucleotide sequence of the A:1 capsule locus

to be similar in organization to capsule loci in other bacteria, where it can be divided into three functional regions (hyaB, hexD, and phyB). Transcripts for hyaB, hexD, and phyB were detected in capsulated parental strains P-1039 and P-2100, as well as the P-2100 derivative strain (P-2100/sub), but were not detected in the non-capsulated variant strains.

PglA, a heparosan synthase outside of the known capsule biosynthesis region was found in all the isolates studied. *PglA* gene was previously reported in A, D and F strains of *P. multocida* only. In our experiment, it was also found in cattle, buffalo as well as in P52VAC strains which are of B type as well as in poultry isolate of A serotype and goat isolate of D serotype. Harper *et al.* (2006) reported that synthase encoded by *PglA* is transcribed 10-fold less than the synthase within the capsule operon, uses a different acceptor and gives rise to smaller molecular weight polymer products and the expression of this gene may give rise to capsular variation.

Kmt1, which is a *P. multocida* specific gene (for PM-PCR) was found to be present in all the isolates. This gene is routinely used for the identification of *Pasteurella* (OIE Terrestrial Mannual Chapter 2.4.12 on Haemorrhagic septicemia) and all the five isolates included in the present study were confirmed before conducting whole genome sequencing by PM-PCR using the primers specific for *Kmt1* (Plate 4.2). Sequencing analysis also confirmed the presence of this gene sequence in all the five isolates. Townsend *et al.* (1998) demonstrated that hybridization of the clone KMT1, revealed binding to all serotypes of *P. multocida*; however, type B and type E isolates could be distinguished from other strains on the basis of fragment size. In addition, the clone *KMT1* was able to distinguish HS-causing *P. multocida* B:2 from type B strains possessing other somatic serotypes. Arumugam *et al.* (2011) isolated 114 strains of *P. multocida* from different animal

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species and based on the multiplex PCR assay using specific capsular genes associated with each serogroup, 114 strains were further divided to 22 species-specific *P. multocida* (KMT1 - 460 bp), 53 serogroup A (A - 1,044 bp), 33 serogroup B (B - 760 bp) and 6 serogroup D (D - 657 bp). No serogroup E (511 bp) or F (851 bp) was detected among the Malaysian *P. multocida* isolates. PCR-based typing was more discriminative and could further subtype the previously untypeable strains.

KpsF, arabinose 5-phosphate isomerase, was found in P52VAC, goat and cattle isolates. The *kps* locus codes biosynthesis of the capsular polysialic acid virulence factor in *Escherichia coli* K1 and contains at least two convergently transcribed operons, designated region 1 and regions 2 plus 3. Transcription through *kpsF* was required for capsule production however, complementation analysis failed to indicate a clear requirement for the *kpsF* polypeptide. *KpsF* appeared to be the first gene of a region whose gene products are required for polysialic acid transport. The function of *kpsF* is unclear, however, primary structure analysis indicated two motifs commonly observed in regulatory proteins and homology with glucosamine synthase of *Rhizobium meliloti* (Cieslewicz and Vimr, 1996). *KpsF* mediates the interconversion of ribulose 5-phosphate and arabinose 5-phosphate. *KpsF* is required for 3-deoxy-D-manno-octulosonic acid (Kdo) biosynthesis in *Neisseria meningitidis*. Mutation of *kpsF* or the gene encoding the CMP-Kdo synthetase (kpsU/kdsB) in *N. meningitidis* resulted in expression of a lipooligosaccharide (LOS) structure that contained only lipid A and reduced capsule expression in the five invasive disease-associated meningococcal serogroups (A, B, C, Y, and W). The step linking meningococcal capsule and LOS biosynthesis was related to Kdo production, as the expression of capsule was wild type in a Kdo transferase (kdtA) mutant. Thus, in

addition to lipooligosaccharide assembly, Kdo is required for meningococcal capsular polysaccharide expression (Tzeng *et al.,* 2002).

HyaE encodes proteins required for polysaccharide biosynthesis and was found to be present only in the goat isolate. *HyaE* shows similarity to *kjiB*, a gene in the Region 2 of the *E. coli* K5 capsule locus. The function of this gene is not determined, but it is unique to the *E. coli* K5 Region 2 and there is no corresponding homologue in other *E. coli* K strains, consistent with the finding that Region 2 genes are usually capsule-type specific. The similarity of *HyaE* to *KfiB* is expected as the *E. coli* K5 capsule is also a polymer containing N-acetylglucosamine and glucuronic acid with the arrangement 4-GlçA-β(1,4)-GlcNAc-α(1). The same monosaccharide components is found in the *P. multocida* hyaluronic acid capsule. Both *hyaE* and *KfiB* therefore code for a function related to the common sugar monomers, in the synthesis of the N-acetylglucosamine component (Chung *et al.,* 1998).

LctP, lactate permease, involved in the uptake of lactate, is present in blood and is taken up by the bacterium as a carbon energy source and also converted to precursors of capsular and lipopolysaccharide sialic acid (Exley *et al.,* 2005). The up-regulation of *lctP* together with the phenotype of decreased survival that was observed for the deletion mutants in *ex vivo* model as well as in other relevant models (Exley *et al.,* 2005) confirmed its important role and indicated that membrane transporter plays a role in increasing complement resistance of *Neisseria meningitidis* strains. *lctP* deletion mutant is not completely killed in human blood even after 120 minutes of incubation, which might suggest that other carbon sources could be utilized by the bacterium to generate phospho-enol pyruvate, that in turn could be used to generate sialic acid (Leighton *et al.,* 2001).

Many virulent strains of *P. multocida* express capsule consisting of hyaluronic acid (HA) (types A and B), chondroitin (type F) or heparin (type D), which are similar to molecules found naturally in various hosts (Snipes *et al.,* 1987; Rimler and Rhoades, 1989; Tsuji and Matsumoto, 1989; DeAngelis, 1996). This 'molecular mimicry' prevents the mounting of a strong antibody response to capsular materials, impairs phagocytosis, reduces the action of complement killing and increases adherence to and survival in the host (Harmon *et al.,* 1991). Paradoxically, only *P. multocida* capsular B strains, and more specifically B:2 strains, produced hyaluronidase, an enzyme that specifically cleaves HA between its repeating disaccharides (N-acetylglucosamine and D-glucuronic acid). Decapsulation of *P. multocida* serotype A:3 with hyaluronidase increased phagocytosis by macrophages, whereas an unencapsulated variant of the bacteria was not internalized (Pruimboom *et al.,* 1996).

4.3.2.1.2 Fimbriae and adhesion

Microbial infections are typically initiated by the colonization of tissues by a specific mechanism that promotes adherence to host cells or tissues. A necessary step for the development of an infectious disease in the host organism is the formation of a firm link between the pathogen and the target cells of the host. The link is mediated by surface-exposed adhesive organelles and is required for internalization of bacteria or extracellular colonization of host tissues. The adhesive organelles mediate bacterial adhesion via a specific interaction with surface structures present on host cells. The adhesin binding to the target cells triggers subversive signals that allow pathogens to evade immune defense and facilitate bacterial colonization or invasion (Zav'yalov *et al.,* 2010). Fimbriae play a role in the surface adhesion, as fimbriae on some *P. multocida* serotype A strains that were able to adhere to mucosal epithelium, but not

on the surface of those strains unable to adhere (Glorioso *et al.,* 1982; Rebers *et al.,* 1988; Isaacson and Trigo, 1995; Ruffolo *et al.,* 1997). Type IV fimbriae (pili) from *P. multocida* serotypes A, B and D (Ruffolo *et al.,* 1997) are associated with virulence in other bacteria because of their role in attachment to host cell surfaces. However, the role of fimbrial structures in *P. multocida* virulence is still unproven (Harper *et al.,* 2006).

ComE is a fibronectin binding protein which is non fimbrial adhesion protein identified as a virulence factor in *P. multocida.* Mullen *et al.* (2008) identified ComE1 (12.7 kDa) using phage library of *P. multocida* and shown to interact with integrin-binding fibronectin type III (FnII) repeats and to be involved in bacterial competence and DNA uptake (Shivachandra *et al.,* 2011). This protein shares many structural and functional similarities with the fibronectin binding proteins of *S. aureus* and *S. pyogenes,* suggesting that this is the general mechanism of bacterial binding to fibronectin. In our study also, *ComE* was found to be present in all the five isolates except for P52VAC strain of *P. multocida.*

ClpB, caseinolytic peptidase B is a ATP-dependent chaperone (Heat shock protein) was present in poultry, goat and cattle isolates. ClpB belongs to a subclass of highly conserved proteins that include molecular chaperones, proteolysis regulators, and regulators of thermotolerance. It plays an important role in protection or recovery from a variety of stresses. In *E. coli,* clpB mutants exhibit a higher death rate at high temperatures, underscoring the importance of *ClpB* in thermotolerance. In *Saccharomyces cerevisiae,* the *ClpB* homolog, *Hsp104,* is also important for recovery from thermal stress as well as a variety of other stressful conditions (Fuge and Farr, 1993). Nanduri *et al.* (2009) studied the effects of three classes of antibiotics viz. amoxicillin, chlortetracycline, and enrofloxacin on the *P. multocida* transcriptome

using custom oligonucleotide microarrays and identified *ClpB* as a potential virulence factor which was down-regulated leading to decreased virulence.

HofC, an assembly protein in type IV pilin biogenesis, was found to be present in all the five isolates except for the cattle. Voss *et al.* (2014) reported that most Hop and Hom OMPs were susceptible to proteolysis, whereas Hor and Hof proteins were relatively resistant to extracellular protease digestion in *Helicobacter pylori*. Protease-susceptible OMPs contain a large protease-susceptible extracellular domain exported beyond the outer membrane and a protease-resistant domain at the C- terminus with a predicted β-barrel structure. Their results provided insights into the repertoire of surface-exposed *H. pylori proteins*, which may mediate bacterium-host interactions as well as the cell surface topology of these proteins.

Hsf is a fimbriae gene and was present in all the isolates of *P. multocida* studied. Fimbriae play a role in surface adhesion and attachment to host cells and extracellular matrix protein is a primary prerequisite for bacterial adhesion. This implies role of fimbriae as a potential virulence factor required for bacteria to induce disease process after entry in the host.

PfhB1 and PfhB2 are filamentous hemagglutinin proteins, and were present in all the five isolates except for the cattle, in which, pfhB1 was absent. *P. multocida* possesses two filamentous hemagglutinins, PfhB1 and PfhB2 (also named FhB1 and FhB2, respectively). *P. multocida* Pm70 genome sequence predicted two genes encoding for the filamentous hemagglutinin proteins (FhaB1 and FhaB2) that appear to increase bacterial dispersion and colonization (Fuller *et al.*, 2000; May *et al.*, 2001). These proteins are similar to the LspA1 and LspA2 filamentous hemagglutinins from *Haemophilus ducreyi*, mutations of which have been shown to affect virulence. A similar filamentous hemagglutinin in *Bordetella pertussis* and *B.*

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bronchiseptica is required for biofilm formation and colonization of the nose and trachea in mice. FhaB2 in *P. multocida* has been implicated in virulence, and expression of FhaB2 was found to be reduced by 4-fold in a non-mucoid *P. multocida* variant, AL1114. FhaB and its transporter FhaC form a two-partner secretion system that is similar to the FhaB-FhaC system in *Bordetella* species, LspA-LspB in *H. ducreyi*, and IbpA-IbpB in the pathogenic *Histophilus somni* strain 2336, which act to inhibit phagocytosis (Wilson and Ho, 2013). These genes share significant similarity with a class of genes that encode filamentous haemagglutinins, which in *Bordetella pertussis* plays a major role in the colonization of the upper respiratory tract (Kimura *et al.,* 1990; Mooi *et al.,* 1992). Mutation of these genes in *P. multocida* resulted in significantly reduced virulence in mice (Fuller *et al.,* 2000) and pfhaB2 mutant strain P1059 was shown to be highly attenuated in turkeys when administered intranasally, but only moderately attenuated when given intravenously (Tatum *et al.,* 2005). Ewers *et al.* (2006) showed that only 46% of the cattle isolates investigated harbored the *fha* gene, and a significant association was reported between disease status and bovine *P. multocida* strains harboring *pfhA* gene. Hypothetically, *P. multocida* fha may also function as a serum resistance factor based on sequence homology to *H. somni* immunoglobulin binding p76 protein (Cole *et al.,* 1993).

PfhR, hemoglobin receptor and iron transport, was present in all the isolates of *P. multocida*. Wheeler (2009) separated OMPs (Outer membrane proteins) by SDS-PAGE and identified by matrix-assisted light desorption/ionization time of flight mass (MALDI-TOF) in the high molecular weight (HMW) region. Iron-acquisition OMPs expressed by isolates were OMPs HasR, HemR, PfhR and hypothetical protein PM0741 from the invasive disease group as well as in porcine and ovine pneumonic group. HemR and PfhR were common to all invasive disease isolates. Ovine isolate

had a very similar OMP profile to porcine in iron-rich culture, and had reduced HMW OMP expression. Similar gene was present in the complete sequence of avian and porcine isolates of *P. multocida* (E-komon *et al.*, 2012).

PlpB, outer membrane lipoprotein 2, was found to be present in all the isolates studied. This gene encodes 39 kDa cross-protecting protein of *P. multocida* (Tabatabai and Zehr, 2004) and is predicted to be an ABC transport protein required for the uptake of methionine into the cell (Merlin *et al.*, 2002). Lipoprotein B (*PlpB*) from *P. multocida* serotype A isolate was shown to be an adherence factor which stimulated cross-protective immunity in mice and poultry (Dabo *et al.*, 2008). Expression of *PlpB* was related to the presence and amount of capsule present on the cell (Ali *et al.*, 2004[a]). This gene is essential for adherence to the host cell and was present in all the isolates of *P. multocida*. Wheeler (2009) also reported presence of *PlpB* in iron limited culture. Cooney and Lo (1993) reported the presence of *PlpB* in avian isolates only, whereas in our study, it was in addition to the avian isolate, also found in cattle, buffalo and goat isolates.

Pasteurella lipoprotein E (PlpE) is a protective surface antigen associated with *P. multocida* serotype A:1, A:3 and A:4 strains isolated from cattle with shipping fever (Wu *et al.*, 2007) and from birds with fowl cholera (Hatfaludi *et al.*, 2012). *PlpE* gene was found to be present in P52VAC, poultry and goat isolates in our study, which is consistent with the study of Wu *et al.* (2007) and Hatfaludi *et al.* (2012), as *PlpE* gene was found to be present in P52VAC which is a serotype B:2 strain. Using a bioinformatics approach, 98 genes in avian strain Pm70 and 107 genes in the non-toxinogenic porcine strain 3480 were identified as encoding putative OMPs (E-komon *et al.*, 2012). Of this combined list, 71 recombinant proteins were expressed and purified, albeit most as insoluble proteins, and tested as vaccine candidates. Only one

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protein, lipoprotein E (PlpE), was found to protect against *P. multocida* challenge in chicken and mice, which confirmed previously reported results using the *PlpE* cloned from the avian serotype A:1 strain X-73 (Wu *et al.,* 2007). However, a *PlpE* knockout mutant strain retained full virulence (Hatfaludi *et al.,* 2012). Conjugated vaccines comprised of multiple antigens, such as OmpH plus PlpE peptides, have also shown promise (Okay *et al.,* 2012[a]; Okay *et al.,* 2012[b]). Pandher *et al.* (1998) cloned and sequenced the gene encoding protein PlpE which is similar to an *Actinobacillus pleuropneumoniae* lipoprotein, OmLA. Affinity-purified, anti-PlpE antibodies recognized a protein in all serotypes of *P. haemolytica. P. haemolytica* and recombinant *E. coli* expressing PlpE are capable of absorbing anti-PlpE antibodies from bovine immune serum, which indicated that PlpE is surface exposed in *P. haemolytica.* In complement-mediated killing assays, a significant reduction in killing of *P. haemolytica* was observed by them when bovine immune serum was depleted of anti-PlpE antibodies, and used as the source of antibody. Results indicated PlpE is surface exposed and immunogenic in cattle and that antibodies against PlpE contribute to host defense against *P. haemolytica.* In our experiment, *PlpE* was found to be present in P52VAC, poultry and goat isolates. Similarly, Abrahante *et al.* (2013) also found *PlpE* gene coding for a cross-protection factor antigen present in all three strains (two virulent avian strains and Pm70) and highly conserved.

PlpP, involved in methionine binding, was found to be present in all the isolates of *P. multocida* studied except the cattle isolate. Initially, recombinant PlpB failed to protect either mice or chicken against *P. multocida* challenge but later it was found that PlpE is cross-protective (Wu *et al.,* 2007). Immunisation with recombinant *P. multocida* PlpE conferred protective immunity, with 80-100% of mice and 63-100% of chicken were protected against heterologous challenge. PlpE is the first *P.*

multocida recombinant protein to stimulate high level cross-serotype protective immunity.

RcpA (Rough colony protein A) and RcpB (Rough colony protein B), are outer membrane secretin family protein components of Tad (Tight adherence), which were found to be absent only in buffalo isolate. Tight adherence (*Tad*) locus, comprised of genes encoding for outer membrane proteins (OMPs) (*TadD, RcpA, RcpB* and *Flp1*), is involved in the Tad macromolecular transport system that is required for biofilm formation and colonization (May *et al.*, 2001). RcpA forms the multimeric outer membrane secretion channel (secretin) of the Flp pilus biogenesis apparatus. The 43-kDa protein (RcpA) was similar to precursor protein D of the general secretion pathway of Gram-negative bacilli, while the 20-kDa protein (RcpB) appeared to be unique. The genes encoding these proteins have been cloned from *Actinobacillus actinomycetemcomitans* 283 and sequenced. A BLASTX (gapped BLAST) search of the surrounding ORFs revealed homology with other fimbria-related proteins. Haase *et al.* (1999) reported that the genes encoding the 43-kDa (rcpA) and 20-kDa (rcpB) proteins may be functionally related to each other and to genes that may encode fimbria-associated proteins. The genes *TadB* and *RcpA* are part of a large operon which, in *A. actinomycetemcomitans*, is composed of 14 genes and mediates nonspecific adhesion to solid surfaces, whether they are biological surfaces or not (Auger *et al.*, 2009). *TadB* is a part of flp (fimbria like protein) operon, a widespread locus in bacteria known to be essential for virulence in *A. actinomycetemcomitans* and *Haemophilus ducreyi*. The expression of *P. multocida tad* genes was also up-regulated under starvation conditions (Paustian *et al.*, 2001).

The *flp-1* gene is found upstream of the tad operon in a low G+C region (similar G+C ratio to the tad operon) along with two other genes (rcpA and rcpB)

expressed specifically in rough adherent bacteria but not in smooth mutants (Haase *et al.,* 1999). The relationship between the tad operon and flp-1 is that the former is a secretion system for the later.

TadE and *TadF* gene loci code for tide adherence (Tad), which was found to be absent in buffalo isolate (for *TadE*) and in cattle as well as buffalo isolates (for *TadF*). The tad locus of *Actinobacillus actinomycetemcomitans* encodes a molecular transport system required for tenacious, non-specific adherence to surfaces and formation of extremely strong biofilms. This locus is dedicated to the biogenesis of Flp pili, which are required for colonization and virulence and 11 of the 14 *tad* locus genes are required for adherence and Flp pilus production (Perez *et al.,* 2006). TadE and TadF are pilin-like proteins, which are termed as pseudopilins (Tomich *et al.,* 2006). Using site-directed mutagenesis, Tomich *et al.* (2006) demonstrated that for processing of pre-Flp1, pre-TadE and pre-TadF are required for biofilm formation.

The LtxA toxin of *A. actinomycetemcomitans* had been thought to be entirely cell associated; either bound to cell surface-associated nucleic acids or within membranous vesicles which bud from the bacterium's surface. This means that the bacterium itself is toxic to target cells. Detailed analysis has revealed that adherent (or rough) colonies of *A. actinomycetemcomitans* do not secrete leukotoxin but non-adherent (smooth colonies) do excrete leukotoxin. Bacteria with mutations in the Tad operon were found to secrete the leukotoxin, suggesting that binding of this toxin to *A. actinomycetemcomitans* is in some manner dependent on proteins expressed by *Tad* genes.

Plp4, type IV fimbriae (pili), was found to be present in all the studied isolates of *P. multocida.* Pilli are associated with attachment of the bacteria to host cell surfaces in other bacteria also. Type IV fimbriae (pili) have been isolated and

characterized from *P. multocida* serotypes A, B and D (Ruffolo *et al.,* 1997) but not from cattle and buffalo. Cloning and sequence analysis of the ptfA gene from *P. multocida* B:2 (strain p52) indicated 78.4% similarity with that of the A:1 strain (Siju *et al.,* 2007). Treatment of the organism with pronase, heat, acidity or homogenization confirmed the involvement of the fimbriae in the attachment process. An anti-fimbrial antibodies specifically inhibited the adherence capabilities of different serotypes that are known to greatly vary for tracheal epithelial cells from different hosts (Harper *et al.,* 2006). This might be the first report of type IV fimbriae in *P. multocida* of cattle, buffalo and goat.

4.3.2.1.3 Iron metabolism

Virtually all bacterial pathogens require iron to infect vertebrates. The most abundant source of iron within vertebrates is in the form of heme as a cofactor of hemoproteins. Many bacterial pathogens have elegant systems dedicated to the acquisition of heme from host hemoproteins. Once internalized, heme is either degraded to release free iron or used intact as a cofactor in catalases, cytochromes, and other bacterial hemoproteins. Paradoxically, the high redox potential of heme makes it a liability, as heme is toxic at high concentrations. Although, a variety of mechanisms have been proposed to explain heme toxicity, the mechanisms by which heme kills bacteria are not well understood. Bacteria employ various strategies to protect against and eliminate heme toxicity. Factors involved in heme acquisition and detoxification have been found to contribute to virulence, underscoring the physiological relevance of heme stress during pathogenesis (Anzaldi and Skaar, 2010).

Iron homeostasis in the mammalian host limits the availability of iron to invading pathogens and is thought to restrict iron availability for microbes inhabiting

mucosal surfaces. The presence of surface receptors for the host iron-binding glycoproteins transferrin (Tf) and lactoferrin (Lf) in globally important Gram-negative bacterial pathogens of humans and food production animals suggested that Tf and Lf are important sources of iron in the upper respiratory or genitourinary tracts, where they exclusively reside (Morgenthau *et al.*, 2013).

ExbB, a biopolymer transport protein, functions as a part of the TonB-dependent energy transduction system for the import of iron-siderophore complexes and vitamin B12 across the outer membrane. The iron transport protein energy-transducing genes *exbB*, *exbD* and *tonB* were expressed at 2.1-fold elevated levels when transferrin was added to iron-free medium. Some of the other genes that were elevated in expression included those encoding the tryptophan transport protein Mtr, the transmembrane permease FbpB, and the branched-chain amino acid transporter AzlD. In the present study, *ExbB* gene was found to be present in all the isolates sequenced suggesting the importance of this gene in virulence of *P. multocida.*

The ferric iron-binding protein (Fbp) expressed by pathogenic *Neisseria* spp. plays a central role in the high-affinity acquisition of iron from human transferrin. Fbp participates in this process as a functional analogue of a Gram-negative periplasmic-binding protein component, which operates as a part of a general active transport process for the receptor-mediated, high-affinity transport of iron from human transferrin (Chen *et al.*, 1993). FbpA functions in complexing and transporting iron across the periplasmic space as the periplasmic component of the iron ABC transport system. The ABC family of transporters includes a broad and diverse group of import systems found in prokaryotes. ABC transporters generally consist of separate polypeptides that form an $A_1B_2C_2$ complex at the inner membrane of the cell during transport. Thus, in order for the ferric ion to reach the cytoplasm, it is donated to an

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inner membrane complex consisting of the inner transmembrane protein FbpB and the cytoplasmic ATPase FbpC. This high affinity bacterial iron uptake system results in iron from host transferrin being liberated at the cell surface, translocated through the periplasmic space, and deposited into the cytoplasm for use or storage without the incorporation of the transferrin protein into the bacterial cell (Shouldice *et al.,* 2003). Because the FbpABC pathway appears to be a common route for ferric iron uptake from several different sources, it is particularly an attractive therapeutic target. In our study also, we found the presence of *FbpA, FbpB* and *FbpC* in all the five isolates except for absence of *FbpA* and *FbpC* in cattle and buffalo isolates, respectively. This indicated importance of this *FbpA, FbpB* and *FbpC* gene cluster for the virulence or disease progression by *P. multocida.*

HbpA, a haemin-binding protein is found to be regulated by iron, manganese and haemin through a Fur-independent mechanism. One protein implicated in heme utilization is the heme-binding lipoprotein (HbpA). HbpA was initially identified as a potential constituent of a heme acquisition pathway following transformation of *H. influenzae* genomic DNA library into *Escherichia coli* and screening for recombinant clones with heme-binding activity. Expression of heme-binding activity by *E. coli* correlated with the expression of a protein of approximately 51-kDa, sized on SDS-PAGE gels, that was subsequently purified in a heme-agarose affinity purification protocol, from both recombinant *E. coli* and *H. influenzae*, and shown to be a lipoprotein. HbpA was localized to the periplasmic space and shown to be associated with both the inner membrane and the outer membrane in *H. influenzae*. HbpA serves to transport heme into the cytosol of *H. influenzae* subsequent to initial binding steps at the cell surface. However, no direct evidence has been presented for the role of HbpA in heme utilization (Morton *et al.,* 2005).

Garrido *et al.* (2003) inoculated purified HbpA protein into Swiss mice previous to a challenge treatment with wild-type *P. multocida* cells. No protective effect was obtained as compared to whole outer membrane proteins of this micro-organism used in the immunization procedure. This indicated that either other haemin-binding proteins are present in *P. multocida* cells or blocking HbpA by antibodies is not sufficient to prevent the infective process of the mice cells presumably because other iron receptors present in this bacterium are able to acquire the necessary amount of iron for survival.

HbpA, a haemin binding protein in immunization assays is not able to protect mice against a challenge with virulent *P. multocida* cells. These data are in agreement with the existence in *P. multocida* of several putative haem- or haemoglobin binding proteins (May *et al.,* 2001). HbpA was found to be present in all the isolates of *P. multocida* in our study. *HbpA* gene needs to be fully characterized for its role in disease due to *P. multocida*, as it may not be directly involved in virulence but it may work as virulence associated genes or protein.

HgbA is a necessary surface-located OM protein of bacterial species that interacts with Hb to import haem into the cell. HgbA may be sufficient as sole Hb receptor or HgbA may function in concert with another protein to mediate haem import. This later mechanism resembles transferrin mediated iron uptake in which two OM proteins, TbpA and TbpB, are required for efficient iron uptake (Srikumar *et al.,* 2004). Fuller *et al.* (2000) identified two loci containing potential virulence genes with homology to iron-acquisition-related genes *ExbB* and *HgbA* of *H. influenzae*. Bosch *et al.* (2002[b]) demonstrated that three ORFs (PM0298, PM0299 and PM300) of *P. multocida* constitute a single transcriptional operon. Mutants defective in PM0298 or PM0299, demonstrated that these genes were essential for the viability of the

pathogen (Bosch *et al.*, 2002[b]) The *HgbA* (PM300) gene product bound hemoglobin (Bosch *et al.*, 2002[b]) and hemin (Bosch *et al.*, 2004), but binding and virulence of the hgbA-mutant strain were not affected (Bosch *et al.*, 2002[b]). The *hgbA* gene is widespread in *P. multocida* strains regardless of the serotype or the source of the isolate. So, *hgbA* gene may be useful as a specific probe to detect *P. multocida* in field samples. In our study also, it was found to be present in all the five isolates studied. It has been demonstrated that some TonB-dependent iron receptors, such as transferrin and lactoferrin binding proteins, are able to induce protection when they are used as prophylactic immunogens. HgbA protein may also be useful as an antigen that provides protection against virulent *P. multocida* strains (Bosch *et al.*, 2002[a]).

The prevalence of *tonB, tbpA, hgbA* and *hgbB* genes in *P. multocida* bovine strains is reported to be 100, 70.2, 95.2 and 57.7%, respectively (Ewers *et al.*, 2006), and there was significant association between disease status and the presence of *hgb*B and *tbpA* as single virulence associated genes in *P. multocida* bovine isolates. The potential for *TbpA* to be a good vaccine candidate is questionable, because approximately 30% of bovine *P. multocida* strains lack the *TbpA* gene (Ewers *et al.*, 2006; Roehrig *et al.*, 2007).

HemR protein is involved in binding hemopexin bound heme from serum important for iron and porphyrin acquisition by a number of pathogens. HemR expressing pathogens enable the pathogen to obtain iron from a large number of heme containing proteins including hemoglobin, myoglobin, hemealbumin, and catalase, ensuring survival in the host (Tabatabai, 2008). HemR protein may be expressed only during hemin-limiting conditions (the environment of the human oral cavity). Since the HemR protein has significant homology with other iron-regulated outer membrane proteins, a potential role for this HemR protein in hemin transport was suggested

(Karunakaran *et al.,* 1997). HemR was found in all the isolates included in our study except for P52VAC.

HemU is a transmembrane protein required for transfer of hemin across the membrane, and was found to be present in all the isolates of our study. Heme, a major iron source, is transported through the outer membrane of Gram-negative bacteria by specific heme/hemoprotein receptors and through the inner membrane by heme-specific, periplasmic, binding protein-dependent, ATP-binding cassette permeases. *Yersinia enterocolitica* strains mutated in *hemU* or *hemV* genes were unable to use haemin as an iron source whereas those mutated in the *hemT* gene were able to use haemin as an iron source. *Escherichia coli* strains expressing only the haemin outer membrane receptor protein HemR from *Y. enterocolitica* were capable of using haemin as an iron source.

OmpW, an outer membrane protein is involved in protective immunity in *P. multocida* infections and the OMPs expressed exclusively *in vivo* may be involved in stimulating immunity to serotypes expressing different LPS types. It is also likely that many *P. multocida* virulence factors will be surface located, and these may include factors critical for colonisation and invasion, important steps in pathogenesis which are poorly understood (Boyce *et al.,* 2006). In our study, OmpW was found to be present in all the isolates imparting their role and importance in the pathogenesis of *P. multocida.* Further studies need to be carried out for the complete characterization of OmpW protein in the diseases caused by *Pasteurella.*

Rfb is a capsule transport protein and the O antigen is encoded by the rfb gene cluster. The Rfb gene was also present in all the isolates of *P. multocida* in our study. The Rfb region contains all the information necessary for synthesis of the components of the O-antigen, its assembly and export to the cell surface as demonstrated by

expression in a heterologous host (Morona *et al.,* 1991). Various rfb mutations have effects on the rhamnose contents of the cell wall polysaccharides produced, including a loss of O antigen production, a reduced level of LPS production, or production of LPS with a reduced amount or complete lack of rhamnose (Boels *et al.,* 2004). This gene also plays an important role by decreasing virulent component of the bacteria and thus imparts its role in pathogenesis of pasteurellosis.

RffG codes for the enzyme dTDP-D-glucose-4,6-dehydratase and participates in formation of O-specific polysaccharide or O antigen, which, joined together with lipid A via core oligosaccharide, forms lipopolysaccharide in the bacterial outer membrane. The enzyme also participates in formation of the polysaccharide part of the enterobacterial common antigen, a cell surface glycolipid. Gene *RffG* was present in all the five isolates of *P. multocida* in the present study and not fully characterized for their role in bacterial virulence and pathogenesis.

TbpA proteins are TonB-dependent, OM receptors that are thought to serve as channels through which ferric iron crosses the outer membrane after its release from bound transferrin (Ratledge and Dover, 2000). TbpA, transferin binding protein, was found to be present in all the isolates of *P. multocida* except for the goat isolate. TbpA binds to bovine transferrin and bovine *P. multocida* are able to utilize iron from bovine transferrin, but not from other species. In constrast, avian *P. multocida* are unable to utilize iron from any transferrin molecules including from avian sources (Hatfaludi *et al.,* 2010). Ewers *et al.* (2006) was not able to amplify *TbpA* gene from avian and pig strain which is in contrast with our result as *TbpA* was found to be present in the poultry and bovine isolates and absent in the goat isolate. Absence of *TbpB* gene in all our isolates is consistent with previous studies stating none of their isolates having *TbpB* gene in their genome (Wheeler, 2009). *TbpA* from *P. multocida*

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serotype B:2 strain was predicted to be a gated pore with several membrane-spanning regions (Shivachandra *et al.,* 2005). The specific role of TbpA in *P. multocida* virulence is still unknown.

TonB is a periplasmic protein and was found to be present in all the isolates studied except for the cattle. Transport of the iron into the bacterial cell by any of high-affinity systems requires the product of the *tonB* gene, which links both cytoplasmic and outer membranes thus enabling the transfer of energy necessary for this process involving tonB-dependent lactoferrin and transferrin receptor (Bosch *et al.,* 2002[a]). The function of TonB is apparently to transduce energy to the outer membrane receptors, by releasing tightly bound ligands into the periplasmic space. The ExbB and ExbD proteins, which are in the inner membrane stabilizing the TonB protein was present in all the isolates of poultry studied by them, which signifies its importance in iron utilization. A conserved region present in these receptors, known as the TonB box, is responsible for the interaction between them and the TonB protein. Binding of the iron-loaded molecules (siderophore, transferrin, etc.) to the outer membrane receptor induces a conformational change in it, which enables the iron to pass through the TonB pore (Ratledge and Dover, 2000). A TonB mutation reduces virulence in *Haemophilus influenzae*, *Bordetella pertussis* and *Pseudomonas aeruginosa*. Likewise, a wild-type *TonB* gene is required for intracellular growth and the intercellular spread of *Shigella dysenteriae*. ExbB, ExbD and TonB mutants of *P. multocida* showed poor growth in both BHI plates and liquid medium. All three components of ExbB, ExbD and TonB are equally required to support the infectious process of *P. multocida* cells (Bosch *et al.,* 2002[a]).

TonB-dependent lactoferrin and transferrin receptor was absent only in the goat isolate whereas, it was present in bovine, bubaline and avian isolates in our

study. Transferrin (Tf) is the predominant iron-containing extracellular protein within the body; therefore, Tf receptors play a critical role during infection. Lectoferrin (Lf) receptors may play an important role during colonization, but the relative importance for iron acquisition and protection from cationic antimicrobial peptides is uncertain. Assumptions that Lf receptors were primarily important on mucosal surfaces need to be revisited and the relative role in iron acquisition and protection from cationic antimicrobial peptides needs to be assessed (Morgenthau *et al.,* 2013).

TonB-dependent receptor was found to be present in poultry and goat isolates only, whereas it was absent in P52VAC, buffalo and cattle isolates or so as to say in bovine isolates. Ogunnariwo and Schryvers (2001) analyzed bovine respiratory isolates of *P. multocida* and demonstrated that six of nine strains tested were capable of growth dependent upon bovine transferrin and of specifically binding ruminant transferrins. In contrast to other species, binding of this protein to immobilized transferrin was specifically blocked by the N-lobe sub-fragment of bovine transferrin. *P. multocida* has a single, novel receptor protein (TbpA), that is capable of efficiently mediating iron acquisition from bovine transferrin without the involvement of a second receptor protein (TbpB).

Translocation protein TolB, is a periplasmic protein of the cell envelope Tol complex. It is partially membrane associated through an interaction with the outer membrane lipoprotein AL (peptidoglycan-associated lipoprotein), which also belongs to the Tol system. The interaction of TolB with outer membrane porins of *Escherichia coli* was investigated with a purified TolB derivative harboring a six-histidine tag. TolB interacted with the trimeric porins OmpF, OmpC, PhoE, and LamB but not with their denatured monomeric forms or OmpA. These interactions took place both in the presence and in the absence of lipopolysaccharide. Interactions of TolB with porins

might take place *in vivo* and participate in porin assembly. The Tol system as a whole may be involved in porin assembly in the outer membrane (Rigal *et al.,* 1997). TolB is a periplasmic protein that peripherally associates with the outer membrane and participates in peptidoglycan metabolism or OM lipoprotein assembly. TolB proteins are invloved in maintaining cell envelope integrity of *Escherichia coli.* TolB is found to be periplasmic, although it is partially membrane-associated and interact with membrane proteins (Bouveret *et al.,* 1995). TolB box is found in various TolB-dependent colicins and is required for the interaction of colicin with TolB. Colicins are proteins produced by some strains of *Escherichia coli* that are lethal for related strains of *E. coli.* In our study, TolB protein was found to be present in P52VAC strain only, which is generally used for the vaccination of animals against HS disease.

4.3.2.1.4 Outer membrane proteins

Outer membrane (OM) functions as a selective barrier that prevents the entry of many toxic molecules into the cell, a property that is crucial for bacterial survival in many environments. At the same time, the proteins embedded in the OM fulfill a number of roles that are critical for the bacterial cell, such as nutrient uptake, transport of molecules in and out of the cell, and interaction with the environment and host tissues. As cytoplasmic proteins are usually soluble and easier to characterise then membrane proteins, they have often been the subject of proteomic studies. Outer membrane protein (OMP) of Gram negative bacteria has a role in disease processes as it acts at an interface between the host and pathogen (Lin *et al.,* 2002). Thus, OMP variation among the isolates may help in epidemiological survey by assessing their inter-strain heterogeneity and can be used to assess intra-species diversity (Davies *et al.,* 2003). Despite progress in recent years, detailed knowledge of membrane proteins remains elusive (Hatfaludi *et al.,* 2010).

HasR, a heme acquisition system receptor in a bovine *P. multocida* isolate, is a protein, which is 98% identical to its homolog in the *P. multocida* Pm70 genome sequence, is surface exposed, is conserved among most *P. multocida* isolates and is an immunodominant iron-regulated outer membrane proteins (IROMPs) (Prado *et al.*, 2005). It is expressed under low-iron conditions *in vivo* and confers protection against challenge with ovine *P. multocida* serotype A:3 strain 232. This indicated that antibody responses in cattle are induced by *P. multocida* IROMPs, and that the 96 kDa HasR protein is an immunodominant IROMPs (Prado *et al.*, 2005). A whole-cell vaccine based on a serotype A:1 strain of *P. multocida* that has been inactivated by treatment with high iron concentrations has also been explored (Herath *et al.*, 2010). HasR protein has been described for *P. multocida* A:3 in cattle as an immunodominant, presumably a *fur* (ferric uptake regulator) gene dependent iron-regulated outer-membrane protein as is the expression of the HemR protein (Tabatabai, 2008). Dabo *et al.* (2008) reported that vaccination of calves with OMPs or IROMPs resulted in significant decrease in lesion scores following *P. multocida* challenge compared to vaccination with adjuvant alone. Vaccinated calves developed intense antibody responses to a 96 kDa protein band, and correlation between high anti-96 kDa antibodies and low lesion scores approached significance ($P<0.06$). The 96 kDa OMP was a homologue to the iron-regulated protein HasR, a heme acquisition receptor protein. In our study, HasR was found to be present in all the isolates of *P. multocida.*

LppB codes for lipoprotein B protein, and contains a prokaryotic membrane lipoprotein lipid attachment site profile from amino acids 1 to 17, and a LysM domain from amino acids 120 to 163 that is also found in a variety of enzymes involved in bacterial cell wall degradation and have a general peptidoglycan binding function. An

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experimental vaccine enriched with these recombinant lipoproteins generated high antibody titers in rabbits and sheep and exerted a protective effect in mice against septicemia induced by *Histophilus somni* bacterial challenge (Guzman-Brambila *et al.,* 2012). The outer-membrane lipoprotein-encoding gene, *lpp* was the most highly expressed gene during growth of *Y. pestis* in human plasma. A proteome analysis showed that Lpp is highly immunogenic upon *Y. pestis* EV76 injection into rabbits (Li *et al.,* 2005). Lpp represents a major constituent of the *Y. pestis* membrane, and may be important at various steps of the disease. Braun (murein) lipoprotein (Lpp), a major bacterial outer membrane component of Gram-negative bacteria in the family *Enterobacteriaceae*, contributed significantly to the development of septic shock. Virulence of *Salmonella enterica* is abolished by mutating one or the other of the two copies of the *Lpp* gene (Sha *et al.,* 2004) was also reported. In the present study, *LppB* gene was present in all the isolates of *P. multocida*.

LspB, a haemolysin secretion and activation protein, was found to be present in all the isolates of *P. multocida* studied. Haem-uptake systems are associated with haemolysins that liberate haem from host erythrocytes and other cell types. *P. multocida* is described as non-haemolytic under aerobic conditions. However, studies have reported production of an inner membrane-bound haemolysin, expressed under anaerobic conditions *in vitro* and is most active against avian red blood cells. The haemolysin production could enhance haem acquisition under certain *in vivo* conditions and could be associated with increased virulence of strains (Wheeler, 2009).

OmpH, Protein H, or porin H, is the major outer membrane protein in the envelope of *P. multocida*. This protein has been purified and characterized as a porin because it is structurally and functionally related to the superfamily of porins of

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Gram-negative bacteria. Bacterial porins are channel-forming transmembrane proteins which are found in the outer membranes of Gram-negative bacteria. They function as molecular sieves to allow the diffusion of small hydrophilic solutes through the outer membrane and also serve as receptors for bacteriophages and bacteriocins. Porins are highly immunogenic, exposing epitopes on the bacterial surface. They are generally conserved in a bacterial species or even in a bacterial family in that they have high homology in primary amino acid sequence and secondary structure and are antigenically related. These properties make porins attractive vaccine candidates for induction of homologous and heterologous immunity against Gram-negative bacterial infections. In native conformation, porin H is a homotrimer, stable in sodium dodecyl sulfate (SDS) at room temperature, and is dissociated into monomers upon boiling. The molecular masses of denatured monomers range between 34 and 42 kDa depending on the serotype and the electrophoretic system used for analysis (Luo *et al.,* 1997). In our study, we found the presence of OmpH in all the isolates studied.

Outer membrane lipoprotein LolB, is an essential lipoprotein that functions as an outer membrane receptor for lipoproteins. LolB is one of the essential outer membrane lipoproteins, being involved in the last step of lipoprotein sorting. It accepts lipoproteins from a periplasmic molecular chaperone, LolA, and mediates the outer membrane anchoring of lipoproteins through a largely unknown mechanism (Tsukahara *et al.,* 2009). The release and outer membrane localization of LolB per se are also dependent on LolA and LolB, respectively. All Lol proteins are essential for *E. coli* growth (Tokudaa and Matsuyama, 2004). Outer membrane lipoproteins of *Escherichia coli* are released from the inner membrane upon the formation of a complex with a periplasmic chaperone, LolA, followed by localization to the outer membrane. Localization of lipoproteins to the outer membrane generally requires an

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outer membrane lipoprotein, LolB, and occurs via transient formation of a LolB-lipoprotein complex. A mutant carrying the chromosomal *LolB* gene under the control of the lac promoter-operator grew normally in the absence of LolB induction, if the mutant did not possess the major outer membrane lipoprotein Lpp, suggesting that LolB is only important for the localization of Lpp. For *in vivo* function of LolB, Tanaka *et al.* (2001) constructed a chromosomal LolB null mutant harboring a temperature-sensitive helper plasmid carrying the LolB gene. At a nonpermissive temperature, depletion of the LolB protein due to loss of the LolB gene caused cessation of growth and a decrease in the number of viable cells (Tanaka *et al.*, 2001). In our study, LolB was found in P52VAC and cattle isolates only.

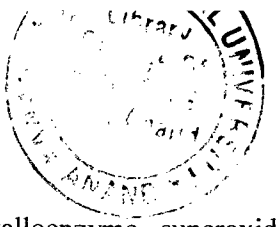
Filamentous haemagglutinin outer membrane protein, pfhA, showed a higher prevalence in capsular types A and F compared with capsular type D, and the *HghB* gene was more frequently detected in capsular D isolates than in capsular A isolates (Ewers *et al.*, 2006; Tang *et al.*, 2009). Gene *pfhA* was also not found in any of the four field isolates analyzed in our study. Ewers *et al.* (2006) also found this gene in 25% (2/8) of isolates from dogs, 18.5% from cats (10/54), and 21.2% (11/52) from swine. Tang *et al.* (2009) working with swine strains described that 15% of *P. multocida* tested positive to the *pfhA* gene (35/233). In our study also, we found the presence of filamentous hemagglutinin outer membrane protein in only P52VAC.

PtfA gene products assemble to form type IV fimbriae on the bacterial surface. This subunit is exported from the cell and polymerised to form the fimbrial strand by a complex multi-component pathway. Doughty *et al.* (2000) isolated type 4 fimbrial subunit protein (PtfA) from whole membrane fraction and identified as an 18-kDa protein. Sellyei *et al.* (2010) reported that among *fimA* (fimbriae), *hsf-1,2* (autotransporter adhesins), *pfhA* (filamentous hemagglutinin), *tad* (nonspecific tight

adherence protein) and *ptfA* (subunit of type 4 fimbriae), only *fimA*, *hsf-2* and *ptfA* are present in all or virtually all pathogenic isolates of *P. multocida*. These indicated a possible role of *ptfA* gene in the virulence of *P. multocida* in various hosts and disease conditions. Ewers *et al.* (2006) detected *ptfA* (99.0%), coding for a type IV fimbrial subunit as well as the outer membrane protein encoding gene *oma87* (99.7%) in most of the strains. The *PtfA* gene was demonstrated in most bovine *P. multocida* isolates and has been cloned (Adler *et al.*, 1999; Ewers *et al.*, 2006); however, immunogenicity was not reported. In our study also, we found *PtfA* to be present in all the five isolates signifying its role in pathogenesis of *P. multocida*.

Oma87 is a part of the outer membrane protein assembly complex, involved in assembly and insertion of beta-barrel proteins into the outer membrane. Omp87 (previously oma87) gene encoding Omp87 (87 kDa protein) was identified on the surface of all 16 serotypes of *P. multocida* isolates and considered as one of the immunodominant antigen (Kumar *et al.*, 2013). Oma87 was also found to be present in all the five isolates of present study.

Vacj is a virulence-associated chromosome locus *J* and a surface lipoprotein of *Shigella flexneri* and *Hemophilus influenzae*. VacJ is a highly conserved and widely distributed outer membrane lipoprotein of *P. multocida* strains, which are known to affect a wide range of domestic as well as wild animals and birds. Structurally, majority of triacylated bacterial lipoproteins are considered to be similar in each bacterium and known to play important diverse roles in bacterial physiology and virulence (Shivachandra *et al.*, 2014). *VacJ* gene was found to be present in our P52VAC, poultry and buffalo isolates.



4.3.2.1.5 Superoxide dismutase

SodA and SodC are the metalloenzyme superoxide dismutase (SOD), catalyses the conversion of superoxide radical anion to oxygen and hydrogen peroxide in the first of a series of protective reactions that remove cytotoxic free radicals generated during the reduction of molecular oxygen. Mechanisms to remove free superoxide are correspondingly widely found and three varieties of SOD being recognised. In the bacterial cytosol, iron- or manganese-cofactored SOD protects cellular proteins and DNA from free-radical attack. However, as the cytoplasmic membrane is impermeable to superoxide, these SODs cannot dismute superoxide produced outside the cell, leaving the cell surface and periplasmic contents vulnerable (Lainson *et al.,* 1996). Reactive oxygen and nitrogen species are the main actors of the innate defense responses of phagocytic cells. Accordingly, during macrophage infection, *Salmonella enterica* displayed an oxidative stress response illustrated by an increased expression of *sodB, sodC* and *hmpA*, the later being implicated in the *in vivo* detoxification of nitric oxide (La *et al.,* 2008). In our study, *SodA* and *SodC* genes were found in all the five isolates studied.

4.3.2.1.6 Sialic acid metabolism

Two genes, *NanB* and *NanH* were found in all the isolates studied using whole genome sequencing. Neuraminidase (sialidase) is prevalent among *P. multocida* strains and is expressed *in vivo* by *P. multocida* A:3 strains associated with bovine pneumonia (White *et al.,* 1995; Ewers *et al.,* 2006); both cell-bound and extracellular sialidases were reported (White *et al.,* 1995). Its role in bovine respiratory disease remains largely unknown. Evidence of neuraminidase function in *P. multocida* infection suggested that the protein is protective in mice (Ifeanyi and Bailie, 1992), is associated with virulence (Muller and Krasemann, 1974) and may contribute to

bacterial adherence, colonization and persistence (Mizan *et al.,* 2000). However, the relationship between the ability of *P. multocida* to produce the enzyme and to cause pneumonia in cattle has not been established. Two sialidase genes (*NanB* and *NanH*) were reported in 100 and 88.5% of *P. multocida* bovine strains investigated, respectively (Mizan *et al.,* 2000). Mutation in *NanH* resulted in a bacterium that was not deficient in sialidase production but had reduced enzyme activity (Mizan *et al.,* 2000).

Sialidases contribute to the virulence of pathogenic organisms which inhabit and invade mucosal surfaces (Schauer, 2009). The sialidases are enzymes that remove sialic acid conjugated to glycoproteins and glycolipids of eukaryotic cells (Hatfaludi *et al.,* 2010). Sialic acid is then used as a carbon source by the bacteria, or it is incorporated into the outer membrane, aiding in the infectious process and in the inhibition of the host immune system (Vimr and Lichtensteiger, 2002). These enzymes also enhance bacterial virulence by unmasking key host receptors and reducing the effectiveness of host defences. Sialidases *NanH* and *NanB*, have been cloned and characterized from a fowl cholera isolate of *P. multocida* (Mizan *et al.,* 2000). These sialidases differed in their specificity, with both able to utilize 2,3' sialyl lactose, but only NanB able to fully utilize 2,6' sialyl lactose. It was proposed that the presence of two sialidases with slightly different specificities would enhance the metabolic capacity of *P. multocida* in the host (Mizan *et al.,* 2000). The uptake of sialic acid was also shown to be essential for virulence in mice (Steenbergen *et al.,* 2005).

4.3.2.1.7 Transcription regulation

Fis is a transcriptional regulator, which has not previously been identified in virulence of *P. multocida*, was found to be present in all the isolates of *P. multocida*

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studied. Fis can act as both, a positive or negative regulator of transcription and it has both direct and indirect effects on gene transcription. The absence of functional Fis protein led to the loss of capsule expression in *P. multocida*. The presence of functional Fis protein is also required for the expression of virulence gene *pfhB2* and its predicted secretion partner *lspB2* in *P. multocida* (Steen *et al.*, 2010).

Apart from these virulence associated genes, there were some other genes present in one or more isolates of *P. multocida*, which are directly or indirectly involved in disease progression and yet to be fully characterized in *P. multocida*, which are listed in Table 4.9.

Table 4.9: List of probable virulence associated genes with their function

Genes	**Function**
GlpE	Thiosulfate sulfurtransferase
GlpT	Glycerol-3-phosphate transmembrane transporter
CcmB	Heme exporter protein
Clp like protease	Degradation of misfolded proteins
CRISPR associated helicase Cas3 family	Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a prokaryotic defense mechanism against foreign genetic elements
DsbA, DsbC, DsbD, DsbE	Disulfide bond froming protein
NfuA	Fe/S biogenesis protein
Ferric transporter ATP binding subunit	Help in iron transport
Ferric uptake regulation protein	Help in iron transport

hemH	Ferrochelatase (Heme biosynthesis)
HSP 90, HSP 33, GrpE, hslo	Heat shock protein
Heme exporter protein B and D	Iron metabolism
HemN family oxidoreductase	Iron metabolism
YfcA	Inner membrane protein
fecB, fecC, fecD, fecE	Iron dicitrate transporter
MsmB	Cold shock like protein CspC
SanA	Encodes a dehydrogenase which is essential for Nikkomycin biosynthesis
SapD, SapF	Heme-iron utilization

P. multocida LPS is highly antigenic and capable of producing widespread vascular alterations and death, but it is poorly immunogenic (Heddleston and Rebers, 1975; Tsuji and Matsumoto, 1988; Ryu and Kim, 2000). Generally, *P. multocida* LPS is immunogenic or protective only when complexed with proteins (Ganfield *et al.*, 1976; Tsuji and Matsumoto, 1988; Ryu and Kim, 2000) or ribosomes (Phillips and Rimler, 1984). Ryu and Kim (2000) demonstrated complete protection in mice vaccinated with *P. multocida* type A:3 LPS-protein complex; however, neither the LPS or protein alone nor the LPS and protein as a mixture provided protection. The LPS-protein complex induced both humoral and cell-mediated immunity (Ryu and Kim, 2000). However, studies by Lu *et al.* (1991) failed to show protection in mice against *P. multocida* infection following passive immunization with affinity-purified

rabbit anti-LPS serum. An opsonic but not bactericidal anti-LPS monoclonal antibody only partially protected against *P. multocida* infection in mice (Ramdani and Adler, 1991; Confer, 1993). LPS is also known as a stimulator of host defense against *P. multocida* infection (Iovane *et al.,* 1998; Galdiero *et al.,* 2000). LPS from a *P. multocida* serotype A strain enhanced humoral and cell-mediated immune responses in chickens (Maslog *et al.,* 1999).

The gene sequences which are found to be present in the present study are the sequences which were being sequenced at a time of sequencing experiment and thus were definitely present. For absence of particular gene sequence in any of the isolates, the sequences were either absent or might not be sequenced at the time of experiment. The whole genome seqreuencing is based on random shearing and sheared DNA (~ 400 -500 bp) were used for the sequencing experiments. Since our sequences were in a scaffold and not in a single sequence, further study needs to be carried out for filling up the gaps using paired end sequencing approach. For the cattle isolate, further sequencing should be carried out for increasing in coverage and ultimately reduces the contigs for better prediction of genes/proteins. Any predictions should be confirmed by experimental data to definitively assign a definite biological function.

P. multocida strains of bovine, avian, and porcine origin have recently been sequenced and comparatively reviewed by Boyce *et al.* (2012). The authors noted, based on the nine genomes sequenced to date, that there was "no clear correlation between phylogenetic relatedness and host predilection or disease". Information is sparse on the location and characterization of the genes responsible for differences in virulence of avian and other *P. multocida.* None of the isolates was having Pasteurella multocida toxin (PMT) in their genome, which is specific to pig isolate of serotype D as well as recently sequenced buffalo isolate by Yap *et al.* (2013).

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4.4 VACCINE DEVLOPMET FOR *P. multocida* - A way forward

Live-attenuated micro-organisms, inactivated bacteria, purified microbial components, polysaccharide carrier protein conjugates, recombinant proteins or DNA are used as vaccines (Freddy *et al.,* 2004). One of the most widely used vaccines in Asia is the whole cell formalin killed *P. multocida* P52 bacterin emulsified in aluminium hydroxide gel. This vaccine, however, does not provide long immunity (Qureshi and Saxena, 2014). It is therefore obvious that there are difficulties being encountered in producing vaccines for *P. multocida*, which may be due to the high antigenic variability and low reported work on whole genome sequence. In addition, the development of vaccines involves the detailed understanding of the mechanisms and mode of action of those virulent factors. Various studies have shown inconsistent efficacy of these vaccines (Moiser *et al.,* 1994). Other challenges being encountered in the determination of a strong candidate gene from the pool of potential genes discovered, is the need to test the protective properties on animal model. Furthermore, potential gene products or protein will have to be purified and characterized before its antigenic properties can be fully known.

Recombinant antigens can be obtained from different expression systems after the targeted antigen has been determined. Recombinant proteins used as subunit vaccines have been reported safe as subunit vaccine (Hussaini *et al.,* 2012). The cross-protective antigens still remain elusive (Shivachandra *et al.,* 2011). Several candidate virulence genes were identified by Ahmad (2014) which includes *plpB, plpE, plp-40, PMT, OmpA, OmpH, Momp* and *PtfA*. Other OMPs have been identified including *Omp16, Omp87* and iron-regulated OMPs such as *TbpA, HemR, HbpA, HasR* and *HgbA* (Wheeler, 2009). Analysis of *OmpH* from different serotypes showed a high degree of conservation (Meredith *et al.,* 2000). *OmpH* was shown to be of importance

in stimulating immunity to infection in animals (Davies and Lee, 2004). OmpA proteins are under evaluation as potential vaccine candidates (Shivachandra *et al.*, 2013). The *OmpA*, will be a favorable gene for the development of naturally and recombinant-derived vaccines (Shivachandra *et al.*, 2013).

The *ptfA* gene has also been proven to have a positive association with the disease outcome in cattle and hence could be used to develop broad cross-protective and disease specific subunit vaccine along with suitable adjuvants especially for HS in bovines and septicaemic pasteurellosis in sheep and goat (Shivachandra *et al.*, 2013). Controversies have surrounded the acceptance of the *plpB* as a candidate gene for the development of *P. multocida* vaccine. It was first purified and proved to confer protection against different serotypes of *P. multocida* (Rimler, 2001). The protein was confirmed to be *P. multocida* lipoprotein B by Tabatabai and Zehr (2004). Other reported candidate genes include the *AroA* gene, as deletion of *AroA* (A gene encoding the *P. multocida* toxin) of serotype B2 was used as an effective vaccine against HS (Ataei *et al.*, 2009).

Virulent genes *tbpA* and *pfhA* as well as capsule biosynthesis genes are supposed to be important epidemiological marker genes for characterizing *P. multocida* field strains (Ataei *et al.*, 2009). Study by Ewers *et al.* (2006) confirmed that *tbpa* and *pfha* are virulent genes. *TbpA* gene (82 kDa protein) first isolated in HS strains of capsule serotype B: 2, 5 in buffaloes and cattle (Veken *et al.*, 1994) have been shown to have strong homology with A1 and D1 (Shivachandra *et al.*, 2005). Verma *et al.* (2013) found high occurrence of *pfhA* and *tbpA* genes among *P. multocida* isolates from diseased as well as healthy cattle. Some virulence associated genes such as *sodA* and *sodC* were found in a higher percentage among isolates from diseased animals as compared to isolates obtained from apparently healthy animals.

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Shivachandra *et al.* (2013) suggested that a recombinant *OmpH* might be a useful vaccine candidate. Surface exposure and high number of copy makes the OmpA-like proteins favourite candidates for vaccine development (Shivachandra *et al.,* 2013). Synthetic porin (*OmpH*) peptides have been shown to induce limited protection against *P. multocida* (Luo *et al.,* 1999).

Several genes involved in virulence and immunity in *P. multocida* were localized and targeted as having potential in the development of subunit vaccines (Fuller *et al.,* 2000; May *et al.,* 2001; Hatfaludi *et al.,* 2010). Type 4 fimbriae (*PtfA*), capsule, OMP87 (*Omp87*), fragments of exotoxin (*PMT*), porin (*OmpH*), lipoprotein E (*plpE*), heme-binding proteins (*HasR, HemR* and *HgbA*), filamentous hemagglutinin (*fhaB2*), adhesin (*OmpA*) and transferrin binding protein (*TbpA*) have been considered as candidate antigens (Veken *et al.,* 1994; Ruffolo *et al.,* 1997; Paustian *et al.,* 2001; Bosch *et al.,* 2004; Prado *et al.,* 2005; Shivachandra *et al.,* 2005; Wu *et al.,* 2007; Hatfaludi *et al.,* 2010). Recombinant proteins used as subunit vaccines were safe for immunocompromised animals as they did not cause disease (Verma and Jaiswal, 1998; De Alwis, 1999).

The observations that bovine strains of *P. multocida* regularly harbored several virulence genes (Type 4 fimbrial gene *ptfA*, iron acquisition genes *tbpA*, *hgbA*, *hgbB*, HasR, and genes for multiple hemoglobin-binding proteins), independent of its capsule type and these genes are highly expressed in the host, which strongly suggest that their protein products are potential subunit vaccine candidates (Veken *et al.,* 1996; Ruffalo *et al.,* 1997; Bosch *et al.,* 2004). A surface exposed, highly immunogenic lipoprotein (plpE) of *P. multocida* conferred 80-100% protective immunity in mice and induced cross-serotype protective immunity (Wu *et al.,* 2007).

At present, there is no commercial recombinant HS subunit vaccine and cross-protective antigens still remain elusive.

This study is apparently the first attempt in India involving local *P. multocida* isolates from four different species and a vaccine strain for the purpose of identifying virulence/ virulence associated genes using modern biotechnological tools like pyrosequencing based whole genome sequencing. The study aids in data of whole genome sequencing of bacterial pathogens particularly for *P. multocida* and also provides new insight into their genomic characters and possible molecular mechanisms involved in disease process. The present findings would provide a much needed base for further screening of virulence associated genes and identification of certain markers for early diagnosis as well as characterization of *P. multocida*, which continues to pose challenges as a menace against the health management of animals. Genes which have been found in all the isolates under the study can be explored as specific probes for the early diagnosis of the *P. multocida* disease. Further, future scientific endeavors targeting the vaccine design for *P. multocida* may get a scientific support from this data, so as to formulate modern and more effective vaccines, for better animal health.

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SUMMARY AND CONCLUSIONS

CHAPTER V

SUMMARY AND CONCLUSIONS

5. 1 SUMMARY

Haemorrhagic septicemia is an important disease of cattle, buffaloes, poultry and other livestock species. For effective control of the disease, an efficacious and longer duration immunity vaccine is required. Identification and characterization of important immunogens of the bacteria help in designing an improved vaccine as well as identification of the OMPs and exploiting it would serve as a candidate for development of diagnostics.

Genome sequencing has revolutionized approach for understanding bacterial pathogenesis. As for obligate intracellular bacteria and other microbial species that cannot be cultured *in vitro*, genomics has provided virtually the only path for efficient discovery of virulence genes. Genomics study has greatly benefited research on organisms that can be cultured by increasing the pace of identification and characterization of virulence factors. Improved understanding of disease etiology helps to direct research into therapies. Genomics is a promising tool for investigating the differences between invasive and non-invasive bacteria at the population as well as at host levels.

Next generation sequencing technologies empower the use of highly parallel methodologies that allow study of all the genes or all the proteins of a pathogen in the context of its host or under various physiological or genetic states of interest. *P. multocida* whole genome sequences and advances in ability to genetically manipulate the organism will facilitate major advances in understanding of disease pathogenesis. Determining the *P. multocida* genes involved in bacterial adherence, as well as other

virulence associated genes, can assist in understanding of the molecular pathogenesis of *P. multocida*. Changes in host-pathogen interaction resulting from changes in environmental signals and the associated changing molecular mechanisms for virulence are most likely the causes of differences between commensal and pathogenic *P. multocida*.

In spite of its undesirably high cost at present, sequence-based identification of the virulent isolates is a convenient alternative and is recommended when unusual or atypical isolates are observed. It can also be used in molecular epidemiological study and for selecting/designing a suitable vaccine candidate.

For Whole genome sequencing of *P. multocida*, different isolates belonging to different species viz. P52 (vaccine strain), poultry (Anand1_poultry), goat (Anand1_goat), buffalo (Anand1_buffalo) and cattle (Anand1_cattle) were collected. All the isolates were confirmed based on cultural and biochemical characteristic as well as PM-PCR based identification method and found to be of *P. multocida*. For whole genome sequencing of all the five isolates, DNA was extracted using phenol-chloroform method. Extracted DNA were quality and quantity checked for confirming purity and nebulized using nitrogen gas. Sheared DNA fragments ends were polished using T4 DNA polymerase and Taq DNA polymerase. MIDs adaptors were ligated to each of the isolates and small fragments were removed using AMPure beads. dsDNA library prepared were quantity checked using TBS flurometer as well as with High Sensitivity DNA chip. Small volume Emulsion PCR was done to check which DNA molecules/beads ratio gives recovery of 6-12% after enrichment. Large volume PCR was done using 4.0 molecules/beads recovery (i.e. 9%). After recovering positively clonally amplified DNA beads, sequencing primers were attached to each of the fragments and sequined on 454 GS FLX Titanium whole genome sequencer.

Sequencing of DNA resulted into 118843, 113997, 105729, 134886 and 31346 reads with 42,598,100 (42.59Mb), 29,000,497 (29.00Mb), 21,890,353 (21.89Mb), 39,756,349 (39.75Mb) and 7,429,658 (7.42Mb) sequence bases for P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle, respectively. The coverage obtained were 18.87, 12.85, 9.70, 17.61 and 3.29 for P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle, respectively.

Reference mapping of reads with the first sequenced genome of *P. multocida* i.e. of Pm70, an avirulent strain isolated from poultry in Australia, was done using GS Reference Mapper and generated 38,079,806 (89.52%), 20,085,356 (87.38%), 19,867,143 (90.81%), 25,095,466 (63.22%) and 6,145,156 (82.87%) mapped bases with 105327, 97674, 95092, 86765 and 24967 mapped reads for P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle, respectively. Mapping results generated 155, 402, 257, 227 and 1998 contigs for P52VAC, Anand1_poultry, Anand1_goat, Anand1_buffalo and Anand1_cattle, respectively. Remaining reads which were not mapped to Pm70 were *De novo* assembled using *De novo* Assembly software to search for sequences associated with plasmid. None of the *De novo* assembled sequences matched with the plasmid using BLASTX.

For genome annotation, two different pipelines were used i.e. Rapid Annotation using Subsystem Technology (RAST) and Prokaryotic Genome Automatic Annotation Pipeline (PGAAP). RAST defined 27 major subsystems, out of which, two subsystems for 'photosynthesis' as well as 'motility and chemotaxis' were not assigned to any of the five isolates as expected. RAST analysis revealed genome size of 2,273,366bp (2.27Mb), 2,227,943bp (2.22Mb), 2,285,382bp (2.28Mb), 2,045,610bp (2.04Mb) and 1,438,517bp (1.43Mb) with 68, 54, 54, 40 and 0 RNA for

P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle, respectively.

RAST analysis revealed maximum subsystems assigned to 'amino acids and derivatives', 'carbohydrates', 'protein metabolism' and 'cofactor and vitamins, prosthetic groups and pigments'. Cattle isolate was omitted from the RAST comparison as very less subsystems were assigned with lower counts because of very less coverage (3.29X). Under 'virulence, disease and defence' subsystem, presence of *DedA, DedD* and *toxin* under colicin and bacteriocin production was noted in P52 vaccine strain, poultry and goat isolates. Genes *gyrA, gyrB, Parc* and *ParD* under 'resistance to fluroquinolones' subsystem were present in all the other four isolates. There was also presence of negative regulator of betalactamase expression, *BLR* gene leading to resistance expressed by this organism as well as multidrug resistance efflux pump cluster genes, *MATE* (Multidrug and toxin extrusion), *MacA* and *MacB* (Macrolide specific efflux protein) in P52 vaccine strain, poultry and goat isolates.

Second annotation was done using PGAAP pipeline developed by NCBI using reads more than 200bp in length. PGAAP analysis revealed genome size of 2,273,366bp (2.27Mb), 2,227,943bp (2.22Mb), 2,285,382bp (2.28Mb), 2,045,610bp (2.04Mb) and 1,438,517bp (1.43Mb) with 40.40%, 40.20%, 40.50%, 40.90% and 41.00% of G+C contents for P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle, respectively. Total number of coding sequences (CDS) were 2066, 2337, 2319, 3258 and 3623 and total number of protein encoding genes (PEG) were 2194, 2284, 2266, 3218 and 3590 respectively, for P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle. Total no. of RNA assigned were 64, 53, 53, 41 and 33 for P52VAC, Anandl_poultry, Anandl_goat, Anandl_buffalo and Anandl_cattle, respectively.

Based on PGAAP analysis, 55 genes were identified and grouped under seven categories viz. capsule, fimbriae and adhesion, iron metabolism, outer membrane protein, superoxide dismutase, sialic acid metabolism and transcription regulation. Genes *SodA* and *SodC* under superoxide dismutase, *NanH* and *NanB* under sialic acid metabolism and *Fis* under transcription regulation category were present in all the five isolates of *P. multocida*. Remaining 50 genes were present in varying frequency either in one or more isolates.

For capsule production, there were nine genes identified, out of which, genes *PglA* and *Kmt1* were present in all the five isolates. Gene *HyaE* was present only in goat isolate, whereas *LctP* gene was present in goat and cattle isolates only. Genes *HexA* and *HexC* were absent in cattle and buffalo isolates, while *HexB* and *HexD* were absent in goat as well as cattle isolates.

Under fimbriae and adhesion category, there were 16 genes, of which *Hsf, PfhB2, PfhR, PflB, PlpB,* and *Plp4* genes were present in all the five isolates. In P52 vaccine strain, *HofC* and *ComE* genes were absent; *TadE*, *RcpA* and *RcpB* were absent only in buffalo isolate. Gene *PlpE* and *TadF* were absent in buffalo as well as in cattle isolate. Genes *PlpP* and *PfhB1* were absent in cattle isolate whereas, *ClpB* was absent in P52 as well as in cattle isolate of *P. multocida*.

In iron metabolism category, 16 genes were present, out of which, *ExbB, FbpB, HbpA, HgbA, HemU, OmpW, Rfb* and *RffG* genes were present in all the five isolates of *P. multocida*. For cattle isolate, *FbpA* and *TonB* genes were absent, where as in goat isolate, *TbpA* and TonB dependent lactoferrin and transferrin receptor were absent. Gene *FbpC* was absent in buffalo isolate. TonB-dependent receptor was

present in poultry and goat isolates only. Translocation protein TolB was present and *HemR* absent only in P52 vaccine strain.

For the category of outer membrane proteins, *HasR, LppB, LspB, OmpH* and *PtfA* genes were present in all the five isolates of *P. multocida*, out of 9 genes falling in this category. *VacJ* gene was absent in goat and cattle isolates, whereas *Oma87* gene was absent only in poultry isolate. Gene *PfhA* was present only in P52 vaccine strain. Outer membrane protein LolB, was found present in P52 vaccine strain as well as in cattle isolate.

Based on the results obtained in this study, following conclusions could be drawn:

5.2 CONCLUSIONS

5.2.1 The genome size of *Pasteurella multocida* vaccine strain (P52), poultry, goat, buffalo and cattle isolates was 2.27Mb, 2.22Mb, 2.28Mb, 2.04Mb and 1.43Mb. The genomes of vaccine strain and the goat isolate were little larger than the reference Pm70 (2.25Mb) genome, while the genome of the poultry and buffalo isolates were smaller.

5.2.2 GC contents of the *P. multocida* vaccine strain (P52), poultry, goat, buffalo and cattle isolates were 40.40%, 40.20%, 40.50%, 40.90% and 41.00%, respectively and number of RNA genes were 64, 53, 53, 41 and 33.

5.2.3 The protein encoding genes (PEG) were 2194, 2284, 2266, 3218 and 3590 and the number of coding sequences (CDS) were 2066, 2337, 2319, 3258 and 3623 for *P. multocida* P52, poultry, goat, buffalo and cattle isolates, respectively.

5.2.4 Based on RAST ananlysis, maximum number of subsystems were assigned to 'amino acids and derivatives', 'carbohydrates', 'protein metabolism' and 'cofactor and vitamins, prosthetic groups and pigments'. No subsystems was assigned to 'photosynthesis' and 'motility and chemotaxis' group.

5.2.5 By PGAAP analysis, *SodA* and *SodC* genes under superoxide dismutase category, *NanH* and *NanB* genes under sialic acid metabolism category and *Fis* gene under transcription regulation category were present in all the five isolates of *P. multocida*.

5.2.6 Gene *HyaE*, which codes for hyaluronic acid and involved in capsular assembly was found only in the goat isolate. Possibility of targeting this gene for detecting *P. multocida* of goat origin should be further explored.

5.2.7 Gene *ComE* under fimbrie and adhesion category and *HemR* under iron metabolism category were absent only in P52 vaccine strain of *P. multocida*, whereas, genes *RcpA, RcpB* and *TadE* under the same category were absent only in buffalo isolate.

5.2.8 Type IV fimbriae encoding *Plp4* gene was uniquely present in all the five isolates and this might apparently be the first report of *Plp4* gene from *P. multocida* of ruminants using whole genome sequence.

5.2.9 Under iron metabolism category, gene *FbpC* was absent only in buffalo isolate. Genes *TbpA* and TonB-dependent lactoferrin and transferrin were absent only in goat isolate. Protein TolB was present only in P52 vaccine strain.

5.2.10 For outer membrane proteins, gene *PfhA* and outer membrane protein LolB were present only in P52 vaccine strain, whereas *Oma87* was present only in the poultry isolate.

5.2.11 Fis, a transcriptional regulator identified recently in the virulence of *P. multocida* was found to be present in all the five isolates (i.e. P52, poultry, goat, buffalo and cattle).

5.2.12 Gene *VacJ*, which codes for outer membrane lipoprotein of *P. multocida* and playing role in bacterial physiology and virulence was found in P52 vaccine strain, poultry and buffalo isolates.

5.2.13 Genes which are found commonly present in all the isolates regardless of the serotype as well as the genes found exclusively present or absent in the isolate of a particular species can be further explored as specific probe for the detection as well as vaccine designing for *P. multocida*.

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REFERENCES

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REFERENCES

Abrahante, J. E.; Johnson, T. J.; Hunter, S. S.; Maheswaran, S. K.; Hauglund, M. J.; Bayles, D. O.; Tatum, F. M. and Briggs, R. E. (2013). Draft Genome Sequences of Two Virulent Serotypes of Avian *Pasteurella multocida*. *Genome Announcements* **1(1):** e00058-12.

Abrahante, J. E.; Veeregowda, B. M.; Hogtapur, S. S.; Briggs, R. E.; Maheswaran, S. K. and Sreevatsan, S. (2014). Draft Genome Sequences of Two *Pasteurella multocida* Strains Isolated from Buffaloes in India with Hemorrhagic Septicemia Disease. *Genome Announc.* **2(4):** e00798-14.

Adams, I. P.; Glover, R. H.; Monger, W. A.; Mumford, R.; Jackeviciene, E.; Navalinskiene, M.; Samuitiene, M. and Boonham, N. (2009). Next-generation sequencing and metagenomics analysis: A universal diagnostic tool in plant virology. *Molecular Plant Pathology* **10(4):** 537-545.

Adler, B.; Bulach, D.; Chung, J.; Doughty, S.; Hunt, M.; Rajakumar, K.; Serrano, M.; van Zanden, A.; Zhang, Y. and Ruffolo, C. (1999). Candidate vaccine antigens and genes in *Pasteurella multocida*. *J. Biotechnol.* **73(2-3):** 83-90.

Aggarwal, G. and Ramaswamy, R. (2002). *Ab initio* gene identification: prokaryote genome annotation with GeneScan and GLIMMER. *J. Biosci.* **27(1):** 7-14.

Ahmad, A. M. (2014). Efforts towards the development of recombinant vaccines against *Pasteurella multocida*. *Science World Journal* **9(2):** 1-7.

Ali, H. A.; Sawada, T.; Hatakeyama, H.; Ohtsuki, N. and Itoh, O. (2004[a]). Characterization of a 39 kDa capsular protein of avian *Pasteurella multocida* using monoclonal antibodies. *Vet. Microbiol.* **100(1-2):** 43-53.

Ali, H. A.; Sawada, T. and Noda, K. (2004[b]). Protectivity of an immunoaffinity-purified 39 kDa capsular protein of avian *Pasteurella multocida* in mice. *J. Vet. Med. Sci.* **66(12):** 1603-1604.

Ali, A.; Soares, S. C.; Barbosa, E.; Santos, A. R.; Barh, D.; Bakhtiar, S. M.; Hassan, S. S.; Ussery, D. W.; Silva, A.; Miyoshi, A. and Azevedo, V. (2013). Microbial Comparative Genomics: An Overview of Tools and Insights Into The Genus *Corynebacterium. J. Bacteriol. Parasitol.* **4(2):** 1000167.

Allen, C. E. and Schmitt, M. P. (2009). HtaA Is an Iron-Regulated Hemin Binding Protein Involved in the utilization of Heme Iron in *Corynebacterium diphtheria. Journal of Bacteriology* **191(8):** 2638-2648.

Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215(3):** 403-410.

Altschul, S. F.; Boguski, M. S.; Gish, W.; Wootton, J. C. (1994): Issues in searching molecular sequence databases. *Nat. Genet.* **6(2):** 119-129.

Altschul, S. F.; Madden, T. L.; Schaffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25(17):** 3389-3402

Anson, E. L. and Myers, E. W. (1997). ReAligner: a program for refining DNA sequence multi-alignments. *J. Comput. Biol.* **4(3):** 369-383.

Ansorge, W. J. (2009). Next-generation DNA sequencing techniques. *Nature Biotechnology* **25(4):** 195-203.

Vety. Sci. & A.H., Anand ANAND UNIVERSITY

Anzaldi, L. L. and Skaar, E. P. (2010). Overcoming the heme paradox: heme toxicity and tolerance in bacterial pathogens. *Infection and Immunity* **78(12):** 4977-4989.

Arumugam, N. D.; Ajam, N.; Blackall, P. J.; Asiah, N. M.; Ramlan, M.; Maria, J.; Yuslan, S. and Thong, K. L. (2011). Capsular serotyping of *Pasteurella multocida* from various animal hosts - a comparison of phenotypic and genotypic methods. *Tropical Biomedicine* **28(1):** 55-63.

Ataei, S.; Burchmore, R.; Christopher, H. J.; Finucane, A.; Parton, R. and Coote, J. G. (2009). Identification of immunogenic proteins associated with protection against haemorrhagic septicaemia after vaccination of calves with a live-attenuated *aroA* derivative of *Pasteurella multocida* B:2. *Research in Veterinary Science* **87(2):** 207-210.

Atashpaz, S.; Shayegh, J. and Hejazi, M. S. (2009). Rapid virulence typing of *Pasteurella multocida* by multiplex PCR. *Res. Vet. Sci.* **87(3):** 355-357.

Auger, E.; Deslandes, V.; Ramjeet, M.; Contreras, I.; Nash, J. H.; Harel, J.; Gottschalk, M.; Olivier, M. and Jacques, M. (2009). Host-pathogen interactions of *Actinobacillus pleuropneumoniae* with porcine lung and tracheal epithelial cells. *Infection and Immunity* **77(4):** 1426-1441.

Aury, J. M.; Cruaud, C.; Barbe, V.; Rogier, O.; Mangenot, S.; Samson, G.; Poulain, J.; Anthouard, V.; Scarpelli, C.; Artiguenave, F. and Wincker, P. (2008). High quality draft sequences for prokaryotic genomes using a mix of new sequencing technologies. *BMC Genomics*. **9:** 603.

Aziz, R. K.; Bartels, D.; Best, A. A.; DeJongh, M.; Disz, T.; Edwards, R. A.; Formsma, K.; Gerdes, S.; Glass, E. M.; Kubal, M.; Meyer, F.; Olsen, G. J.; Olson, R.; Osterman, A. L.; Overbeek, R. A.; McNeil, L. K.; Paarmann, D.; Paczian, T.; Parrello, B.; Pusch, G. D.; Reich, C.; Stevens, R.; Vassieva, O.; Vonstein, V.; Wilke, A. and Zagnitko, O. (2008). The RAST server: rapid annotations using subsystems technology. *BMC Genomics* **9:**75.

Babik, W.; Taberlet, P.; Ejsmond, M. J. and Radwan, J. (2009). New generation sequencers as a tool for genotyping of highly polymorphic multilocus MHC system. *Molecular Ecology Resources* **9(3):** 713-719.

Babu, M. M.; Priya, M. L.; Selvan, A. T.; Madera, M.; Gough, J.; Aravind, L. and Sankaran, K. (2006). A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins. *J. Bacteriol.* **188(8):** 2761-2773.

Bagley, K. C.; Abdelwahab, S. F.; Tuskan, R. G. and Lewis, G. K. (2005). *Pasteurella multocida* toxin activates human monocyte-derived and murine bone marrow-derived dendritic cells *in vitro* but suppresses antibody production *in vivo*. *Infect. Immun.* **73:** 413-421.

Bain, R. V. S.; De Alwis, M. C. L.; Carter, G. R. and Gupta, B. K. (1982). Haemorrhagic septicaemia. FAO Animal Production and Health Paper **33:** 11-33.

Ball, C. A.; Osuna, R.; Ferguson, K. C. and Johnson, R. C. (1992). Dramatic changes in Fis levels upon nutrient upshift in *Escherichia coli*. *J. Bacteriol.* **174(24):** 8043-8056.

Barrow, G. H. and Feltham, R. K. A. (1993) Cowan and Steel's Manual for Identification of Medical Bacteria. Third edition. Cambridge University Press, Cambridge. pp. 331.

Benkirane, A. and De Alwis M. C. L. (2002). Haemorrhagic septicaemia, its significance, prevention and control in Asia. *Vet. Med. - Czech.* **47 (8):** 234-240.

Bentley, D. R.; Balasubramanian, S.; Swerdlow, H. P.; Smith, G. P.; Milton, J.; Brown, C. G.; Hall, K. P.; Evers, D. J.; Barnes, C. L.; Bignell, H. R.; Boutell, J. M.; Bryant, J.; Carter, R. J.; Keira Cheetham, R.; Cox, A. J.; Ellis, D. J.; Flatbush, M. R.; Gormley, N. A.; Humphray, S. J.; Irving, L. J.; Karbelashvili, M. S.; Kirk, S. M.; Li, H.; Liu, X.; Maisinger, K. S.; Murray, L. J.; Obradovic, B.; Ost, T.; Parkinson, M. L.; Pratt, M. R.; Rasolonjatovo, I. M.; Reed, M. T.; Rigatti, R.; Rodighiero, C.; Ross, M. T.; Sabot, A.; Sankar, S. V.; Scally, A.; Schroth, G. P.; Smith, M. E.; Smith, V. P.; Spiridou, A.; Torrance, P. E.; Tzonev, S. S.; Vermaas, E. H.; Walter, K.; Wu, X.; Zhang, L.; Alam, M. D.; Anastasi, C.; Aniebo, I. C.; Bailey, D. M.; Bancarz, I. R.; Banerjee, S.; Barbour, S. G.; Baybayan, P. A.; Benoit, V. A.; Benson, K. F.; Bevis, C.; Black, P. J.; Boodhun, A.; Brennan, J. S.; Bridgham, J. A.; Brown, R. C.; Brown, A. A.; Buermann, D. H.; Bundu, A. A.; Burrows, J. C.; Carter, N. P.; Castillo, N.; Chiara, E.; Catenazzi M.; Chang, S.; Neil Cooley, R.; Crake, N. R.; Dada, O. O.; Diakoumakos, K. D.; Dominguez-Fernandez, B.; Earnshaw, D. J.; Egbujor, U. C.; Elmore, D. W.; Etchin, S. S.; Ewan, M. R.; Fedurco, M.; Fraser, L. J.; Fuentes Fajardo, K. V.; Scott Furey, W.; George, D.; Gietzen, K. J.; Goddard, C. P.; Golda, G. S.; Granieri, P. A.; Green, D. E.; Gustafson, D. L.; Hansen, N. F.; Harnish, K.; Haudenschild, C. D.; Heyer, N. I.; Hims, M.

Sci.& A.H., Anand ANAND UNIVERSITY

M.; Ho, J. T.; Horgan, A. M.; Hoschler, K.; Hurwitz, S.; Ivanov, D. V.; Johnson, M. Q.; James, T.; Huw Jones, T. A.; Kang, G. D.; Kerelska, T. H.; Kersey, A. D.; Khrebtukova, I.; Kindwall, A. P.; Kingsbury, Z.; Kokko-Gonzales, P. I.; Kumar, A.; Laurent, M. A.; Lawley, C. T.; Lee, S. E.; Lee, X.; Liao, A. K.; Loch, J. A.; Lok, M.; Luo, S.; Mammen, R. M.; Martin, J. W.; McCauley, P. G.; McNitt, P.; Mehta, P.; Moon, K. W.; Mullens, J. W.; Newington, T.; Ning, Z.; Ling Ng, B.; Novo, S. M.; O'Neill, M. J.; Osborne, M. A.; Osnowski, A.; Ostadan, O.; Paraschos, L. L.; Pickering, L.; Pike, A. C.; Chris Pinkard, D.; Pliskin, D. P.; Podhasky, J.; Quijano, V. J.; Raczy, C.; Rae, V. H.; Rawlings, S. R.; Chiva Rodriguez, A.; Roe, P. M.; Rogers, J.; Rogert Bacigalupo, M. C.; Romanov, N.; Romieu, A.; Roth, R. K.; Rourke, N. J.; Ruediger, S. T.; Rusman, E.; Sanches-Kuiper, R. M.; Schenker, M. R.; Seoane, J. M.; Shaw, R. J.; Shiver, M. K.; Short, S. W.; Sizto, N. L.; Sluis, J. P.; Smith, M. A.; Ernest Sohna Sohna, J.; Spence, E. J.; Stevens, K.; Sutton, N.; Szajkowski, L.; Tregidgo, C. L.; Turcatti, G.; Vandevondele, S.; Verhovsky, Y.; Virk, S. M.; Wakelin, S.; Walcott, G. C.; Wang, J.; Worsley, G. J.; Yan, J.; Yau, L.; Zuerlein, M.; Rogers, J.; Mullikin, J. C.; Hurles, M. E.; McCooke, N. J.; West, J. S.; Oaks, F. L.; Lundberg, P. L.; Klenerman, D.; Durbin, R. and Smith, A. J. (2008). Accurate whole human genome sequencing using reversible terminator chemistry. *Nature* **456(7218):** 53-59.

Bentley, G.; Higuchi, R.; Hoglund, B.; Goodridge, D.; Sayer, D.; Trachtenberg, E. A. and Erlich, H. A. (2009). High-resolution, high-throughput HLA genotyping by next-generation sequencing. *Tissue Antigens* **74(5):** 393-403.

Besemer, J.; Lomsadze, A. and Borodovsky, M. (2001). GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for

A.H., Anand

finding sequence motifs in regulatory regions. *Nucleic Acids Res.* **29(12):** 2607-2618.

Biswas, A.; Shivachandra, S. B.; Saxena, M. K.; Kumar, A. A.; Singh, V. P. and Srivastava, S. K. (2004). Molecular variability among strains of *Pasteurella multocida* isolated from an outbreak of Haemorrhagic Septicaemia in India. *Veterinary Research Communications* **28(4):** 287-298.

Blackall, P. J.; Fegan, N.; Pahoff, J. L.; Storie, G. J.; McIntosh, G. B.; Cameron, R. D. A.; O'Boyle, D.; Frost, A. J.; Bara, M. R.; Marr, G. and Holder, J. (2000). The molecular epidemiology of four outbreaks of porcine pasteurellosis. *Vet. Microbiol.* **72(1-2):** 111-120.

Blocker, D.; Berod, L.; Fluhr, J. W.; Orth, J.; Idzko, M.; Aktories, K. and Norgauer, J. (2006). *Pasteurella multocida* toxin (PMT) activates Rho GTPases, induces actin polymerization and inhibits migration of human dendritic cells, but does not influence macropinocytosis. *Int. Immunol.* **18(3):** 459-464.

Boels, I. C.; Beerthuyzen, M. M.; Kosters, M. H.; Van Kaauwen, M. P.; Kleerebezem, M. and De Vos, W. M. (2004). Identification and functional characterization of the *Lactococcus lactis* rfb Operon, required for dTDP-Rhamnose Biosynthesis. *Journal of Bacteriology* **186(5):** 1239-1248.

Bontell, I. L.; Hall, N.; Ashelford, K. E.; Dubey, J. P.; Boyle, J. P.; Lindh, J. and Smith, J. E. (2009). Whole genome sequencing of a natural recombinant *Toxoplasma gondii* strain reveals chromosome sorting and local allelic variants. *Genome Biology* **10(5):** R53.

Borodovsky, M.; Koonin, E. V. and Rudd, K. E. (1994). New genes in old sequence: a strategy for finding genes in the bacterial genome. *Trends Biochem. Sci.* **19:** 309-313.

Borodovsky, M.; McIninch, J.; Koonin, E. V.; Rudd, K. E.; Medigue, C. and Danchin, A. (1995). Detection of new genes in a bacterial genome using Markov models for three gene classes. *Nucleic Acids Res.* **23(17):** 3554-3562.

Borrathybay, E.; Sawada, T.; Kataoka, Y.; Ohtsu, N.; Takagi, M.; Nakamura, S. and Kawamoto, E. (2003[a]). A 39 kDa protein mediates adhesion of avian *Pasteurella multocida* to chicken embryo fibroblast cells. *Vet. Microbiol.* **97(3-4):** 229-243.

Borrathybay, E.; Sawada, T.; Kataoka, Y.; Okiyama, E.; Kawamoto, E. and Amao, H. (2003[b]). Capsule thickness and amounts of a 39 kDa capsular protein of avian *Pasteurella multocida* type A strains correlate with their pathogenicity for chickens. *Vet. Microbiol.* **97(3-4):** 215-227.

Bosch, M.; Tarrago R.; Garrido, M. E.; Campoy, S.; Fernandez de Henestrosa, A. R.; Perez, A. M.; Badiola I. and Barbe J. (2001). Expression of the Pasteurella multocida *ompH* gene is negatively regulated by the Fur protein. *FEMS Microbiology Letters* **203(1):** 35-40.

Bosch, M.; Garrido, E.; Llagostera, M.; Perez De Rozas, A. M.; Badiola, I. and Barbe, J. (2002[a]). *Pasteurella multocida* exbB, exbD and tonB genes are physically linked but independently transcribed. *FEMS Microbiology Letters* **210(2):** 201-208.

Bosch, M.; Garrido, M. E.; Llagostera, M.; Perez De Rozas, A. M.; Badiola, I. and Barbe, J. (2002[b]). Characterization of the *Pasteurella multocida* hgbA Gene Encoding a Hemoglobin-Binding Protein Montserrat. *Infection and Immunity* **70(11):** 5955-5964.

Bosch, M.; Garrido, M. E.; Perez de Rozas, A. M.; Badiola, I.; Barbe, J. and Llagostera, M. (2004). *Pasteurella multocida* contains multiple immunogenic haemin- and haemoglobin-binding proteins. *Vet. Microbiol.* **99(2):** 103-112.

Bouveret, E.; Derouiche, R.; Rigal, A.; Lloubes, R.; Lazdunski, C. and Benedetti, H. (1995). Peptidoglycan-associated lipoprotein-TolB interaction. A possible key to explaining the formation of contact sites between the inner and outer membranes of Escherichia coli. *J. Biol. Chem.* **270(19):** 11071-11077.

Boyce, J. D. and Adler, B. (2000). The capsule is a virulence determinant in the pathogenesis of *Pasteurella multocida* M1404 (B:2). *Infect. Immun.* **68(6):** 3463-3468.

Boyce, J. D.; Wilkie, I.; Harper, M.; Paustian, M. L.; Kapur, V. and Adler, B. (2002). Genomic scale analysis of *Pasteurella multocida* gene expression during growth within the natural chicken host. *Infect. Immun.* **70:** 6871-6879.

Boyce, J. D.; Wilkie, I.; Harper, M.; Paustian, M. L.; Kapur, V. and Adler, B. (2004). Genomic-scale analysis of *Pasteurella multocida* gene expression during growth within liver tissue of chickens with fowl cholera. *Microbes Infect.* **6:** 290-298.

Boyce, J. D. and Adler, B. (2006). How does Pasteurella multocida respond to the host environment? *Current Opinion in Microbiology* **9(1):** 117-122.

Boyce, J. D.; Cullen, P. A.; Nguyen, V.; Wilkie, I. and Adler, B. (2006). Analysis of the *Pasteurella multocida* outer membrane sub-proteome and its response to the *in vivo* environment of the natural host. *Proteomics* **6(3):** 870-880

Boyce, J.; Harper, M.; Wilkie, I. and Adler, B. (2010). Pasteurella. In: Prescott, J. F. (Eds.), Pathogenesis of Bacterial Infections in Animals. Blackwell Publishing, IA, USA.

Boyce, J. D.; Seemann, T.; Adler, B. and Harper, M. (2012). Pathogenomics of *Pasteurella multocida*. *Current Topics in Microbiology and Immunology* **361:** 23-38.

Bradley, M. D.; Beach, M. B.; de Koning, A. P.; Pratt, T. S. and Osuna, R. (2007). Effects of Fis on *Escherichia coli* gene expression during different growth stages. *Microbiol.* **153(9):** 2922-2940.

Campoy, S.; Aranda, J.; Alvarez, G.; Barbe, J. and Llagostera, M (2006). Isolation and sequencing of a temperate transducing phage for *Pasteurella multocida*. *Appl. Environ. Microbiol.* **72(5):** 3154-3160.

Carter, G. R. and Chengappa, M. M. (1980). Hyaluronidase production by type B *Pasteurella multocida* from cases of hemorrhagic septicemia. *J. Clin. Microbiol.* **11(1):** 94-96.

Chawak, M. M.; Verma, K. C. and Kataria, J. M. (2001). Characterization and immunizing properties of outer membrane proteins of *Pasteurella multocida* grown in iron sufficient and iron restricted media. *Indian J. Comp. Microbial. Immunol. Infect. Dis.* **22:** 36-42.

Vety. Sci. & A.H., Anand
ANAND

Chen, C. Y.; Berish, S. A.; Morse, S. A. and Mietzner, T. A. (1993). The ferric iron-binding protein of pathogenic *Neisseria* spp. functions as a periplasmic transport protein in iron acquisition from human transferrin. *Mol. Microbiol.* **10(2):** 311-318.

Chen, L.; Yang, J.; Yu, J.; Yao, Z.; Sun, L.; Shen, Y. and Jin, Q. (2005). VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Res.* **33(Database issue):** D325-328.

Choi-Kim, K.; Maheswaran, S. K.; Felice, L. J. and Molitor, T. W. (1991). Relationship between the iron regulated outer membrane proteins and the outer membrane proteins of *in vivo* grown *Pasteurella multocida*. *Vet. Microbiol.* **28(1):** 75-92.

Chung, J. Y.; Zhang, Y. and Adler, B. (1998). The capsule biosynthetic locus of *Pasteurella multocida* A:1. *FEMS Microbiol. Lett.* **166(2):** 289-296.

Chung, J. Y.; Wilkie, I.; Boyce, J. D.; Townsend, K. M.; Frost, A. J.; Ghoddusi, M. and Adler, B. (2001). Role of capsule in the pathogenesis of fowl cholera caused by *Pasteurella multocida* serogroup A. *Infect. Immun.* **69(4):** 2487-2492.

Chung, J. Y.; Wilkie, I.; Boyce, J. D. and Adler, B. (2005). Vaccination against fowl cholera with acapsular *Pasteurella multocida* A:1. *Vaccine* **23(21):** 2751-2755.

Cieslewicz, M. and Vimr, E. (1996). Thermoregulation of kpsF, the first region 1 gene in the kps locus for polysialic acid biosynthesis in *Escherichia coli* K1. *Journal of Bacteriology* **178(11):** 3212-3220.

Cifonelli, J. A.; Rebers, P. A. and Heddleston, K. L. (1970). The isolation and characterisation of hyaluronic acid from *Pasteurella multocida. Carbohydr. Res.* **14:** 272-276.

Clarke, J.; Wu, H. C.; Jayasinghe, L.; Patel, A.; Reid, S. and Bayley, H. (2009). Continuous base identification for single-molecule nanopore DNA sequencing. *Nature Nanotechnolgy* **4(4):** 265-270.

Cole, S. P.; Guiney, D. G. and Corbeil, L. B. (1993). Molecular analysis of a gene encoding a serum-resistance-associated 76 kDa surface antigen of *Haemophilus somnus. J. Gen. Microbiol.* **139(9):** 2135-2143.

Confer, A. W. (1993). Immunogens of *Pasteurella. Vet. Microbiol.* **37(3-4):** 353-368.

Cooney, B. J. and Lo, R. Y. (1993). Three contiguous lipoprotein genes in *Pasteurella haemolytica* Al which are homologous to a lipoprotein gene in *Haemophilus influenzae* type b. *Infection and immunity* **61(11):** 4682-4688.

Cox, A. J.; Hunt, M. L.; Boyce, J. D. and Adler, B. (2003). Functional characterization of HgbB1 a new hemoglobin binding protein of *Pasteurella multocida. Microb. Pathog.* **34(6):** 287-296.

Cundell, D. R.; Gerard, N. P.; Gerard, C.; Idanpaan-Heikkila, I. and Tuomanen, E. I. (1995). *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. *Nature* **377(6548):** 435-438.

Dabo, S. M.; Confer, A. W. and Hartson, S. D. (2005). Adherence of *Pasteurella multocida* to fibronectin. *Vet. Microbiol.* **110:** 265-275.

Dabo, S. M.; Taylor, J. D. and Confer, A. W. (2008). *Pasteurella multocida* and bovine respiratory disease. *Animal Health Research Reviews* **8(2):** 129-150.

Davenport, K. W.; Daligault, H. E.; Minogue, T. D.; Bishop-Lilly, K. A.; Bruce, D. C.; Chain, P. S.; Coyne, S. R.; Frey, K. G.; Jaissle, J.; Koroleva, G. I.; Ladner, J. T.; Lo, C. C.; Palacios, G. F.; Redden, C. L.; Scholz, M. B.; Teshima, H. and Johnson, S. L. (2014). Complete Genome Sequence of Type Strain *Pasteurella multocida* subsp. *multocida* ATCC 43137. *Genome Announc.* **2(5):** e02070-14.

Davies, R. L.; MacCorquodale, R.; Baillie, S. and Caffery, B. (2003). Characterization and comparison of *Pasteurella multocida* strains associated with porcine pneumonia and atrophic rhinitis. *J. Med. Microbiol.* **52(1):** 59-67.

Davies, R. L. and Lee, I. (2004). Sequence diversity and molecular evolution of the heat-modifiable outer membrane protein gene (OmpA) of *Mannheimia* (Pasteurella) *haemolytica*, *Mannheimia glucosida*, and *Pasteurella trehalosi*. *Journal of Bacteriology* **186(17):** 5741-5752.

Dawkins, H. J. S.; Ramdani, R.; Johnson, B. and Spencer, T. L. (1991). Haemorrhagic septicaemia: correlation of vaccinal antibody responses in mice with protection against *Pasteurella multocida* strain M1404. *Vet. Microbiol.* **27:** 309-326.

De Alwis, M. C. L. (1999). Haemorrhagic septicaemia. Australian Centre for International Agricultural Research (ACIAR) Monograph **57:** 1-34.

De Alwis, M. C. L.; and Vipulasiri, A. A. (1981). An epizootiological study of haemorrhagic septicaemia in Sri Lanka. *Ceylon Vet. J.* **28:** 24-35.

de la Bastide, M. and McCombie. W. R. (2007). Assembling genomic DNA sequences with PHRAP. *Curr. Protoc. Bioinformatics* **17:** 11.4.1-11.4.15.

Deamer, D. W. and Akeson, M. (2000). Nanopores and nucleic acids: prospects for ultrarapid sequencing. *Trends in Biotechnology* **18(4):** 147-151.

DeAngelis, P. L. (1996). Enzymological characterization of the *Pasteurella multocida* hyaluronic acid synthase. *Biochemistry* **35(30):** 9768-9771.

DeAngelis, P. L. and Padgett-McCue, A. J. (2000). Identification and molecular cloning of a chondroitin synthase from *Pasteurella multocida* type F. *J. Biol. Chem.* **275(31):** 24124-24129.

DeAngelis, P. L.; Gunay, N. S.; Toida, T.; Mao, W. J. and Linhardt, R. J. (2002). Identification of the capsular polysaccharides of Type D and F *Pasteurella multocida* as unmodified heparin and chondroitin, respectively. *Carbohydr. Res.* **337(17):** 1547-1552.

DeAngelis, P. L. and White, C. L. (2004). Identification of a distinct, cryptic heparosan synthase from *Pasteurella multocida* types A, D, and F. *J. Bacteriol.* **186(24):** 8529-8532.

Delcher, A. L.; Bratke, K. A.; Powers, E. C. and Salzberg, S. L. (2007). Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics* **23(6):** 673-679.

Derieux, W. T. (1984). Response of broiler-type chickens to live *Pasteurella multocida*-duration of immunity and minimum dose. *Avian Dis.* **28(1):** 281-284.

Vety. Sci.& A.H., Anand ANAND UNIVERSITY

DeRisi, J. L.; Iyer, V. R. and Brown, P. O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278(5338):** 680-694.

Dobrindt, U. and Hacker, J. (2001). Whole genome plasticity in pathogenic bacteria. *Curr. Opin. Microbiol.* **4(5):** 550-557.

Dohm, J. C.; Lottaz, C.; Borodina, T. and Himmelbauer, H. (2007). SHARCGS, a fast and highly accurate short-read assembly algorithm for *de novo* genomic sequencing. *Genome Research* **17(11):** 1697-1706.

Doughty, S. W.; Ruffolo, C. G. and Adler, B. (2000). The type 4 fimbrial subunit gene of *Pasteurella multocida. Vet. Microbiol.* **72(1-2):** 79-90.

Drzeniek, R.; Scharmann, W. and Balke, E. (1972). Neuraminidase and N-acetylneuraminate pyruvate-lyase of *Pasteurella multocida. J. Gen. Microbiol.* **72(2):** 357-368.

Dutta, J.; Rathore, B. S.; Mullik, S. G.; Singh, R. and Sharma, G. C. (1990). Epidemiological studies and occurrence of haemorrhagic septicaemia in India. *Indian Vet. J.* **67:** 893-899.

Eid, J.; Fehr, A.; Gray, J.; Luong, K.; Lyle, J.; Otto, G.; Peluso, P.; Rank, D.; Baybayan, P.; Bettman, B.; Bibillo, A.; Bjornson, K.; Chaudhuri, B.; Christians, F.; Cicero, R.; Clark, S.; Dalal, R.; Dewinter, A.; Dixon, J.; Foquet, M.; Gaertner, A.; Hardenbol, P.; Heiner, C.; Hester, K.; Holden, D.; Kearns, G.; Kong, X.; Kuse, R.; Lacroix, Y.; Lin, S.; Lundquist, P.; Ma, C.; Marks, P.; Maxham, M.; Murphy, D.; Park, I.; Pham, T.; Phillips, M.; Roy, J.; Sebra, R.; Shen, G.; Sorenson, J.; Tomaney, A.; Travers, K.; Trulson, M.; Vieceli, J.;

Wegener, J.; Wu, D.; Yang, A.; Zaccarin, D.; Zhao, P.; Zhong, F.; Korlach, J. and Turner, S. (2009). Real-time DNA sequencing from single polymerase molecules. *Science* **323(5910):** 133-138.

Eidam, C.; Poehlein, A.; Brenner Michael, G.; Kadlec, K.; Liesegang, H.; Brzuszkiewicz, E.; Daniel, R.; Sweeney, M. T.; Murray, R. W.; Watts, J. L. and Schwarz, S. (2013). Complete Genome Sequence of *Mannheimia haemolytica* Strain 42548 from a Case of Bovine Respiratory Disease. *Genome Annoounc.* **1(3):** e00318-13.

Eisen, J. A. (1995). The RecA protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of RecAs and 16S rRNAs from the same species. *J. Mol. Evol.* **41(6):** 1105-1123.

Eisen, J. A. (2000). Assessing evolutionary relationships among microbes from whole-genome analysis. *Curr. Opin. Microbiol.* **3(5):** 475-480.

E-komon, T.; Burchmore, R.; Herzyk, P. and Davies, R. (2012). Predicting the outer membrane proteome of *Pasteurella multocida* based on consensus prediction enhanced by results integration and manual confirmation. *BMC Bioinformatics* **13:** 63.

Ewers, C.; Lubke-Becker, A.; Bethe, A.; Kiebling, S.; Filter, M. and Wieler, L. H. (2006). Virulence genotype of *Pasteurella multocida* strains isolated from different hosts with various disease status. *Vet. Microbiol.* **114(3-4):** 304-317.

Exley, R. M.; Goodwin, L.; Mowe, E.; Shaw, J.; Smith, H.; Read, R. C. and Christoph, M. (2005). *Neisseria meningitidis* lactate permease is required for nasopharyngeal colonization. *Infect. Immun.* **73:** 5762-5766.

FAO (1982) .Animal Production and Health Paper 33. Bain, R. V. S.; De Alwis, M. C. L.; Carter, G. R. and Gupta, B. K. Haemorrhagic septicaemia, p. 11-33.

FAO (2005). Production Year book, Statistics Division. Rome, Italy.

Fernandez de Henestrosa, A. R.; Badiola, I.; Saco, M.; Perez de Rozas, A. M.; Campoy, S. and Barbe, J. (1997). Importance of the *galE* gene on the virulence of *Pasteurella multocida*. *FEMS Microbiol. Lett.* **154(2):** 311-316.

Fickett, J. W. and Tung, C. S. (1992). Assessment of protein-coding measures. *Nucleic Acids Res.* **20(24):** 6441-6450.

Finlay, B. B. and Falkow, S. (1989). Common themes in microbial pathogenicity. *Microbiol. Rev.* **53(2):** 210-230.

Fischer, D. and Eisenberg, D. (1999). Finding families for genomic ORFans. *Bioinformatics* **15(9):** 759-762.

Fitch, W. M., and Margoliash, E. (1967). Construction of phylogenetic trees. *Science* **155(3760):** 279-284.

Fleischmann, R. D.; Adams, M. D.; White, O.; Clayton, R. A.; Kirkness, E. F.; Kerlavage, A. R.; Bult, C. J.; Tomb, J. F.; Dougherty, B. A.; Merrick, J. M.; McKenney, K.; Sutton, G.; FitzHugh, W.; Fields, C.; Gocyne, J. D.; Scott, J.; Shirley, R.; Liu, L.; Glodek, A.; Kelley, J. M.; Janice F.; Weidman, J. F.; Phillips, C. A.; Spriggs, T.; Hedblom, E. and Cotton, M. D. (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269(5223):** 496-512.

Sci.& Anand

Foged, N. T.; Pedersen, K. B. and Elling, F. (1987). Characterization and biological effects of the *Pasteurella multocida* toxin. *FEMS Microbiol. Lett.* **43:** 45-51.

Forterre, P. and Philippe, H. (1999). Where is the root of the universal tree of life? *Bioessays* **21(10):** 871-879.

Forst, C.V. and Schulten, K. (2001). Phylogenetic analysis of metabolic pathways. *J. Mol. Evol.* **52(6):** 471-489.

Fox, S.; Filichkin, S. and Mockler, T. C. (2009). Applications of ultra-high-throughput sequencing. *Methods in Molecular Biology* **553:** 79-108.

Fraser, C. and Fleischmann, R. (1997). Strategies for whole microbial genome sequencing and analysis. *Electrophoresis* **18:** 1207-1216.

Fraser, A. G.; Kamath, R. S.; Zipperlen, P.; Martinez-Campos, M.; Sohrmann, M. and Ahringer, J. (2000). Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408(6810):** 325-330.

Freddy, H.; Frank, P.; Koen, C.; Dominiek, M.; Richard, D. and Annemie, D. (2004). Efficacy of vaccines against bacterial diseases in swine: what can we expect? *Veterinary Microbiology* **100:** 255-268.

Fuge, E. K. and Farr, S. B. (1993). AppppA-binding protein E89 is the *Escherichia coli* heat shock protein ClpB. *Journal of Bacteriology* **175(8):** 2321-2326.

Fuller, T. E.; Kennedy, M. J. and Lowery, D. E. (2000). Identification of *Pasteurella multocida* virulence genes in a septicemic mouse model using signature-tagged mutagenesis. *Microb. Pathog.* **29(1):** 25-38.

Vety. Sci.& A.H., Anand
ANAND

Furian, T. Q.; Borges, K. A.; Rocha, S. L. S.; Rodrigues, E. E.; do Nascimento, V. P.; Salle, C. T. P. and Moraes, H. L. S. (2013). Detection of virulence-associated genes of *Pasteurella multocida* isolated from cases of fowl cholera by multiplex-PCR. *Pesq. Vet. Bras.* **33(2):** 177-182.

Galdiero, M.; Folgore, A.; Nuzzo, I. and Galdiero, E. (2000). Neutrophil adhesion and transmigration through bovine endothelial cells *in vitro* by protein H and LPS of *Pasteurella multocida. Immunobiology* **202(3):** 226-238.

Gancz, A. Y.; Kistler, A. L.; Greninger, A. L.; Farnoushi, Y.; Mechani, S.; Perl, S.; Berkowitz, A.; Perez, N.; Clubb, S.; DeRisi, J. L.; Ganem, D. and Lublin, A. (2009). Experimental induction of proventricular dilatation disease in cockatiels (*Nymphicus hollandicus*) inoculated with brain homogenates containing avian bornavirus 4. *Virology Journal* **6:** 100.

Ganfield, D. J.; Rebers, P. A. and Heddleston, K. L. (1976). Immunogenic and toxic properties of a purified lipopolysaccharide-protein complex from *Pasteurella multocida. Infect. Immun.* **14(4):** 990-999.

Garcia, N.; Fernandez-Garayzabal, J. F.; Goyache, J. Dominguez, L. and Vela, A. I. (2011). Associations between biovar and virulence factor genes in *Pasteurella multocida* isolates from pigs in Spain. *Vet. Rec.* **169(14):** 362.

Garrido, M. E.; Bosch, M.; Medina, R.; Bigas, A.; Llagostera, M.; Perez De Rozas, A. M.; Badiola, I. and Barbe, J. (2003). fur-independent regulation of the *Pasteurella multocida hbpA* gene encoding a haemin-binding protein. *Microbiology* **149(8):** 2273-2281.

Garrido, M. E.; Bosch, M.; Bigas, A.; Badiola, I.; Barbe, J. and Llagostera, M. (2008). Heterologous protective immunization elicited in mice by *Pasteurella multocida* fur ompH. *Int. Microbiol.* **11(1):** 17-24.

Garrity, G. M. and Holt, J. G. (2001). The road map to the manual. In Bergey's Manual of Systematic Bacteriolog, 1, 2nd edn. pp. 119-141.

Gelfand, M. S. (1995). Prediction of function in DNA sequence analysis. *J. Comput. Biol.* **2(1):** 87-115.

Gish, W. and States, D. J. (1993). Identification of protein-coding regions by sequence similarity searches. *Nat. Genet.* **3(3):** 266-272.

Glanville, J.; Zhai, W.; Berka, J.; Telman, D.; Huerta, G.; Mehta, G. R.; Ni, I.; Mei, L.; Sundar, P. D.; Day, G. M.; Cox, D.; Rajpal, A. and Pons, J. (2009). Precise determination of the diversity of a combinatorial antibody library gives insight into the human immunoglobulin repertoire. *Proceedings of the National Academy of Sciences* **106(48):** 20216-20221.

Glisson, J. R.; Contreras, M. D.; Cheng, I. H. N. and Wang, C. (1993). Cross protection studies with *Pasteurella multocida* bacterins prepared from bacteria propagated in iron-depleted medium. *Avian Dis.* **37(4):** 1074-1079.

Glorioso, J. C.; Jones, G. W.; Rush, H. G.; Pentler, L. J.; Darif, C. A. and Coward, J. E. (1982). Adhesion of type A *Pasteurella multocida* to rabbit pharyngeal cells and its possible role in rabbit respiratory tract infections. *Infect. Immun.* **35(3):** 1103-1109.

College Of Vet. Sci.& A.H., Anand UNIVERSITY ANAND

Goebel, S. J.; Johnson, G. P.; Perkus, M. E.; Davis, S. W.; Winslow, J. P. and Paoletti, E. (1990). The complete DNA sequence of Vaccinia virus. *Virology* **179(1):** 247-266.

Goldberg, M. D.; Johnson, M.; Hinton, J. C. and Williams, P. H. (2001). Role of the nucleoid-associated protein Fis in the regulation of virulence properties of enteropathogenic *Escherichia coli*. *Mol. Microbiol.* **41(3):** 549-559.

Goodfellow, M.; Manfio, G. P. and Chun, J. (1997). Towards a practical species concept for cultivable bacteria. In Species: the units of biodiversity, pp. 265-269. Edited by Claridge, M. F. Dawah, H. A. and Wilson, M. R. London: Chapman and Hall.

Grainger, D. C. and Busby, S. J. (2008). Global regulators of transcription in *Escherichia coli*: mechanisms of action and methods for study. *Adv. Appl. Microbiol.* **65:** 93-113.

Grant, J. R. and Stothard, P. (2008). The CGView Server: a comparative genomics tool for circular genomes *Nucleic Acids Research* **36(Web Server issue):** W181-W184.

Gray-Owen, S. D. and Schryvers, A. B. (1996). Bacterial transferrin and lactoferrin receptors. *Trends Microbiol.* **4(5):** 185-191.

Green, R. E.; Malaspinas, A. S.; Krause, J.; Briggs, A. W.; Johnson, P. L.; Uhler, C.; Meyer, M.; Good, J. M.; Maricic, T.; Stenzel, U.; Prufer, K.; Siebauer, M.; Burbano, H. A.; Ronan, M.; Rothberg, J. M.; Egholm, M.; Rudan, P.; Brajkovic, D.; Kucan, Z.; Gusic, I.; Wikstrom, M.; Laakkonen, L.; Kelso, J.; Slatkin, M. and Paabo, S. (2008). A complete Neandertal mitochondrial

genome sequence determined by high-throughput sequencing. *Cell* **134(3):** 416-426.

Guzman-Brambila, C.; Rojas-Mayorquin, A. E.; Flores-Samaniego, B. and Ortuno-Sahagun, D. (2012). Two outer membrane lipoproteins from *Histophilus somni* are immunogenic in rabbits and sheep and induce protection against bacterial challenge in mice. *Clinical and Vaccine Immunology* **19(11):** 1826-1832.

Haase, E. M.; Zmuda, J. L. and Scannapieco, F. A. (1999). Identification and molecular analysis of rough-colony-specific outer membrane proteins of *Actinobacillus actinomycetemcomitans. Infection and Immunity* **67(6):** 2901-2908.

Hansen, L. M. and Hirsh, D. C. (1989). Serum resistance is correlated with encapsulation of avian strains of *Pasteurella multocida. Vet. Microbiol.* **21(2):** 177-184.

Harmon, B. G.; Glisson, J. R.; Latimer, K. S.; Steffens, W. L. and Nunnally, J. C. (1991). Resistance of *Pasteurella multocida* A:3,4 to phagocytosis by turkey macrophages and heterophils. *Am. J. Vet. Res.* **52(9):** 1507-1511.

Harper, M.; Boyce, J. D.; Wilkie, I. W. and Adler, B. (2003). Signature tagged mutagenesis of *Pasteurella multocida* identifies mutants displaying differential virulence characteristics in mice and chickens. *Infect. Immun.* **71(9):** 5440-5446.

Harper, M.; Cox, A. D.; St Michael, F.; Wilkie, I. W.; Boyce, J. D. and Adler, B. (2004). A heptosyltransferase mutant of *Pasteurella multocida* produces a

College of Vety. Sci. & A.H., Anand

truncated lipopolysaccharide structure and is attenuated in virulence. *Infect. Immun.* **72(6):** 3436-3443.

Harper, M.; Boyce, J. D. and Adler, B. (2006). *Pasteurella multocida* pathogenesis: 125 years after Pasteur. *FEMS Microbiol. Lett.* **265(1):** 1-10.

Harper, M.; Cox, A.; St. Michael, F.; Parnas, H.; Wilkie, I.; Blackall, P. J.; Adler, B. and Boyce, J. D. (2007). Decoration of *Pasteurella multocida* lipopolysaccharide with phosphocholine is important for virulence. *J. Bacteriol.* **189(20):** 7384-7391.

Harris, T. D.; Buzby, P. R.; Babcock, H.; Beer, E.; Bowers, J.; Braslavsky, I.; Causey, M.; Colonell, J.; Dimeo, J.; Efcavitch, J. W.; Giladi, E.; Gill, J.; Healy, J.; Jarosz, M.; Lapen, D.; Moulton, K.; Quake, S. R.; Steinmann, K.; Thayer, E.; Tyurina, A.; Ward, R.; Weiss, H. and Xie, Z. (2008). Single-molecule DNA sequencing of a viral genome. *Science* **320(5872):** 106-109.

Hatfaludi, T.; Al-Hasani, K.; Boyce, J. D. and Adler, B. (2010). Outer membrane proteins of *Pasteurella multocida. Vet. Microbiol.* **144(1-2):** 1-17.

Hatfaludi, T.; Al-Hasani, K.; Gong, L.; Boyce, J. D.; Ford, M.; Wilkie, I. W.; Quinsey, N.; Dunstone, M. A.; Hoke, D. E. and Adler, B. (2012). Screening of 71 *P. multocida* proteins for protective efficacy in a fowl cholera infection model and characterization of the protective antigen PlpE. *PLoS One.* **7(7):** e39973.

Hauglund, M. J.; Tatum, F. M.; Bayles, D. O.; Maheswaran, S. K. and Briggs, R. E. (2013). Genome Sequences of *Mannheimia haemolytica* Serotype A1 Strains D153 and D193 from Bovine Pneumonia. *Genome Announc.* **1(5):** .00848-13.

ANAND

Heddleston, K. L.; Watko, L. P. and Rebers, P. A. (1964). Dissociation of a fowl cholera strain of *Pasteurella multocida*. *Avian Dis.* **8:** 649-657.

Heddleston, K. L. and Rebers, P. A. (1975). Properties of free endotoxin from *Pasteurella multocida*. *Am. J. Vet. Res.* **36(4):** 573-574.

Hemmerich, C.; Buechlein, A.; Podicheti, R.; Revanna, K. V. and Dong, Q. (2010). An Ergatis-based prokaryotic genome annotation web server. *Bioinformatics.* **26:** 1122-1124.

Herath, C.; Kumar, P.; Singh, M.; Kumar, D.; Ramakrishnan, S.; Goswami, T. K.; Singh, A. and Ram, G. C. (2010). Experimental iron-inactivated *Pasteurella multocida* A:1 vaccine adjuvanted with bacterial DNA is safe and protects chickens from fowl cholera. *Vaccine* **28(11):** 2284-2289.

Hernandez, D.; Francois, P.; Farinelli, L.; Osteras, M. and Schrenzel, J. (2008). *De novo* bacterial genome sequencing: millions of very short reads assembled on a desktop computer. *Genome Research* **18(5):** 802-809.

Hieter, P. and Boguski, M. (1997). Functional genomics: it's all how you read it. *Science* **278(5338):** 601-602.

Highlander, S. K.; Hulten, K. G.; Qin, X.; Jiang, H.; Yerrapragada, S.; Mason, E. O.; Jr. Shang, Y.; Williams, T. M.; Fortunov, R. M.; Liu, Y.; Igboeli, O.; Petrosino, J.; Tirumalai, M.; Uzman, A.; Fox, G. E.; Cardenas, A. M.; Muzny, D. M.; Hemphill, L.; Ding, Y.; Dugan, S.; Blyth, P. R.; Buhay, C. J.; Dinh, H. H.; Hawes, A. C.; Holder, M.; Kovar, C. L.; Lee, S. L.; Liu, W.; Nazareth, L. V.; Wang, Q.; Zhou, J.; Kaplan, S. L. and Weinstock, G. M. (2007). Subtle

Vety Sci.& A.H., Anand ANAND UNIVERSITY

genetic changes enhance virulence of methicillin resistant and sensitive *Staphylococcus aureus*. *BMC Microbiology* 7: 99

Hofacre, C. L. and Glisson, J. R. (1986). A serotypic survey of *Pasteurella multocida* isolated from poultry. *Avian Dis.* **30(3):** 632-633.

Hoper, D.; Hoffmann, B. and Beer, M. (2009). Simple, sensitive, and swift sequencing of complete H5N1 avian influenza virus genomes. *Journal of Clinical Microbiology* **47(3):** 674-679.

Horadagoda, N. U.; Hodgson, J. C.; Moon, G. M.; Wijewardana, T. G. and Eckersall, P. D. (2002). Development of a clinical syndrome resembling haemorrhagic septicaemia in the buffalo following intravenous inoculation of *Pasteurella multocida* serotype B:2 endotoxin and the role of tumour necrosis factor-alpha. *Res. Vet. Sci.* **72(3):** 194-200.

Hunt, M. L.; Adler, B and Townsend, M. (2000). The molecular biology of *Pasteurella multocida*. *Veterinary Microbiology* **72:** 3-25.

Hussaini, J.; Abdullahi, M. A. and Ismail, S. (2012). Expression and immunogenicity determination of recombinant clone of *Pasteurella multocida* serotype B against Haemorrhagic septicaemia: Towards a vaccine development. *Journal of Animal and veterinary advances* **11(3):** 351-356.

IBM Research (2009). IBM Research Aims to Build Nanoscale DNA Sequencer to Help Drive Down Cost of Personalized Genetic Analysis.

Ifeanyi, F. I. and Bailie, W. E. (1992). Passive protection of mice with antiserum to neuraminidase from *Pasteurella multocida* serotype A:3. *Vet. Res. Commun.* **16(2):** 97-105.

Iovane, G.; Galdiero, M.; Vitiello, M. and De Martino, L. (1998). Effect of *Pasteurella haemolytica* outer membrane proteins on bovine neutrophils. *FEMS Immunol. Med. Microbiol.* **20(1):** 29-36.

Isaacson, R. E. and Trigo, E. (1995). Pili of *Pasteurella multocida* of porcine origin. *FEMS Microbiol. Lett.* **132(3):** 247-251.

Jacques, M.; Kobisch, M.; Belanger, M. and Dugal, F. (1993). Virulence of capsulated and noncapsulated isolates of *Pasteurella multocida* and their adherence to porcine respiratory tract cells and mucus. *Infect. Immun.* **61(11):** 4785-4792.

Jamali, H.; Rezagholipour, M.; Fallah, S.; Dadrasnia, A.; Chelliah, S.; Velappan, R. D.; Wei, K. S. and Ismail, S. (2014). Prevalence, characterization and antibiotic resistance of *Pasteurella multocida* isolated from bovine respiratory infection. *Vet. J.* **202(2):** 381-383.

Jones, R. T.; Knight, R. and Martin, A. P. (2009). Bacterial communities of disease vectors sampled across time, space, and species. *ISME Journal* **4(2):** 223-231.

Jordan, J. A.; Jones-Laughner, J. and Durso, M. B. (2009). Utility of pyrosequencing in identifying bacteria directly from positive blood culture bottles. *Journal of Clinical Microbiology* **47(2):** 368-372.

Kachlany, S. C.; Planet, P. J.; DeSalle, R.; Fine, D. H. and Figurski, D. H. (2001[a]). Genes for tight adherence of *Actinobacillus actinomycetemcomitans*: from plaque to plague to pond scum. *Trends Microbiol.* **9(9):** 429-437.

Kachlany, S. C.; Planet, P. J.; DeSalle, R.; Fine, D. H.; Figurski, D. H. and Kaplan, J. B. (2001[b]). Flp-1, the first representative of a new pilin gene subfamily, is

College Of Vety. Sci.& A.H., Anand
ANAND VETERINARY UNIVERSITY

required for non-specific adherence of *Actinobacillus actinomycetemcomitans*. *Mol. Microbiol.* **40:** 542-554.

Kambara, H. and Takahashi, S. (1993). Multiple-sheath flow capillary array DNA analyser. *Nature* **361(6412):** 565-566.

Kanehisa, M.; Araki, M.; Goto, S.; Hattori, M.; Hirakawa, M.; Itoh, M.; Katayama, T.; Kawashima, S.; Okuda, S.; Tokimatsu, T. and Yamanishi, Y. (2008). KEGG for linking genomes to life and the environment *Nucleic Acids Res.* **36(Database issue):** D480-484.

Karunakaran, T.; Madden, T. and Kuramitsu, K. (1997). Isolation and characterization of a hemin-regulated gene, hemR, from *Porphyromonas gingivalis*. *J. Bacteriol.* **179(6):** 1898-1908.

Kelly, A.; Goldberg, M. D.; Carroll, R. K.; Danino, V.; Hinton, J. C. and Dorman, C. J. (2004) A global role for Fis in the transcriptional control of metabolism and type III secretion in *Salmonella enterica* serovar Typhimurium. *Microbiol.* **150(7):** 2037-2053.

Kennett, L.; Muniandy, N. and Mukkur, T. K. S. (1993). Comparative Protective Potential of Non-living Intact Cells and Purified Outer Membrane and Associated Proteins of *Pasteurella multocida* Type B:6 Grown Under Iron-Regulated Conditions. In: Pasteurellosis in Production Animals, ACIAR Proceedings No. 43 (Patten, B. E. ed.), pp. 144-149. ACIAR Press, Canberra.

Khamesipour, F.; Momtaz, H. and Azhdary Mamoreh, M. (2014). Occurrence of virulence factors and antimicrobial resistance in *Pasteurella multocida* strains isolated from slaughter cattle in Iran. *Front Microbiol* **5:** 536.

College of Vety Sci. & A.H., Anand ANAND AGRICULTURAL UNIVERSITY

Kim, S.; Yoo, H. J. and Hahn, J. H. (1996). Postelectrophoresis capillary scanning method for DNA sequencing. *Anal. Chem.* **68(5):** 936-6399.

Kimura, A.; Mountzouros, K. T.; Relman, D. A.; Falkow, S. and Cowell, J. L. (1990). *Bordetella pertussis* filamentous hemagglutinin: evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. *Infect. Immun.* **58(1):** 7-16.

Kircher, M.; Stenzel, U. and Kelso, J. (2009). Improved base calling for the Illumina Genome Analyzer using machine learning strategies. *Genome Biology* **10(8):** R83.

Kircher, M. and Kelso, J. (2010). High-throughput DNA sequencing - concepts and limitations. *Bioessays* **32(6):** 524-536.

Koonin, E. V.; Tatusov, R. L. and Rudd, K. E. (1996). Protein sequence comparison at a genome scale. *Meth. Enzymol.* **266:** 295-322.

Korlach, J.; Marks, P. J.; Cicero, R. L.; Gray, J. J.; Murphy, D. L.; Roitman, D. B.; Pham, T. T.; Otto, G. A.; Foquet, M. and Turner, S. W. (2008). Selective aluminum passivation for targeted immobilization of single DNA polymerase molecules in zero-mode waveguide nanostructures. *Proceedings of the National Academy of Sciences* **105(4):** 1176-1181.

Krewulak, K. D. and Vogel, H. J. (2008). Structural biology of bacterial iron uptake. *Biochim. Biophys. Acta.* **1778(9):**1781-1804.

Kro, J.; Bania, J.; Florek, M; Pliszczak-Krol, A. and Staroniewicz, Z. (2011). Polymerase chain reaction-based identification of clinically relevant

Pasteurellaceae isolated from cats and dogs in Poland. *Journal of Veterinary Diagnostic Investigation* **23(3):** 3532-537.

Krogh, A.; Mian, I. S. and Haussler, D. (1994). A hidden Markov model that finds genes in *E. coli* DNA. *Nucleic Acids Res* **22(22):** 4768-4778.

Kuhnert, P. and Christensen, H. (2008). Pasteurellaceae: Biology, Genomics and Molecular Aspects. Norfolk, UK: Caister Academic Press.

Kumar, A. A.; Shivachandra, S. B.; Biswas, A.; Singh, V. P.; Singh, V. P. and Srivastava, S. K. (2004). Prevalent serotypes of *Pasteurella multocida* isolated from different animal and avian species in India. *Veterinary Research Communications* **28(8):** 657-667.

Kumar, P.; Singh, V. P.; Agrawal, R. K. and Singh, S. (2009). Identification of *Pasteurella multocida* isolates of ruminant origin using polymerase chain reaction and their antibiogram study. *Tropical Animal Health Production* **41(4):** 573-578.

Kumar, A.; Yogisharadhya, R.; Ramakrishnan, M. A.; Viswas, K. N. and Shivachandra, S. B. (2013). Structural analysis and cross-protective efficacy of recombinant 87 kDa outer membrane protein (Omp87) of *Pasteurella multocida* serogroup B:2. *Microbial Pathogenesis* **65:** 48-56.

Kuroda, M. and Hiramatsu, K. (2004). Genome Sequencing and Annotation: An Overview. *Methods Mol. Bio.* **266:** 2-46.

La, M.; Raoult, D. and Renesto, P. (2008). Regulation of whole bacterial pathogen transcription within infected hosts. *FEMS Microbiol. Rev.* **32(3):** 440-460.

College of Vety. Sci. & A.H., Anand ANAND VERSITY

Lagesen, K.; Hallin, P.; Rodland, E. A.; Staerfeldt, H. H.; Rognes, T. and Ussery, D. W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* **35(9):** 3100-3108.

Lainson, F. A.; Thomson, N.; Rowe, H. A.; Langford, P. R.; Aitchison, K. D.; Donachie, W. and Kroll, J. S. (1996). Occurrence of [copper, zinc]-cofactored superoxide dismutase in *Pasteurella haemolytica* and its serotype distribution. *FEMS Microbiology Letters* **142(1):** 11-17.

Lainson, F. A.; Dagleish, M. P.; Fontaine, M.; Bayne, C. and Hodgson, J. C. (2013[a]). Draft Genome Sequences of Strains of *Pasteurella multocida* Isolated from the United Kingdom and the United States. *Genome Announc.* **1(5):** e00773-13.

Lainson, F. A.; Dagleish, M. P.; Fontaine, M. C.; Bayne, C. and Hodgson, J. C. (2013[b]). Draft Genome Sequence of *Pasteurella multocida* A:3 Strain 671/90. *Genome Announc.* **1(5):** e00803-13.

Lan, R. and Reeves, P. R. (1996). Gene transfer is a major factor in bacterial evolution. *Mol. Biol. Evol.* **13(1):** 47-55.

Lander, E. S. and Waterman, M. S. (1988). Genomic mapping by fingerprinting random clones: A mathematical analysis. *Genomics* **2(3):** 231-239.

Lax, A. J. and Chanter, N. (1990). Cloning of the toxin gene from *Pasteurella multocida* and its role in atrophic rhinitis. *J. Gen. Microbiol.* **136(1):** 81-87.

Lee, W. H. and Vega, V. B. (2004). Heterogeneity detector: finding heterogeneous positions in Phred/Phrap assemblies. *Bioinformatics* **20(16):** 2863-2864.

Lee, D.; Seo, H.; Park, C. and Park, K. (2009). WeGAS: a web-based microbial genome annotation system. *Bioscience, Biotechnology and Biochemistry* **73(1):** 213-216.

Leighton, M. P.; Kelly, D. J.; Williamson, M. P. and Shaw, J. G. (2001). An NMR and enzyme study of the carbon metabolism of *Neisseria meningitidis*. *Microbiology* **147:** 1473-1482.

Lenz, D. H. and Bassler, B. L. (2007). The small nucleoid protein Fis is involved in *Vibrio cholerae* quorum sensing. *Mol. Microbiol.* **63(3):** 859-871.

Li, B.; Jiang, L.; Song, Q.;Yang, J.; Chen, Z.; Guo, Z.; Zhou, D.; Du, Z.; Song, Y.; Wang, J.; Wang, H.;Yu, S. and Wang, J. (2005). Protein microarray for profiling antibody responses to *Yersinia pestis* live vaccine. *Infect. Immun.* **73(6):** 3734-3739.

Lin, J., Huang, S. and Zhang, Q. (2002). Outer membrane protein: key players for bacterial adaptation in host niches. *Microbes Infect.* **4:** 325-331.

Liu, W.; Yang, M.; Xu, Z.; Zheng, H.; Liang, W.; Zhou, R.; Wu, B. and Chen, H. (2012). Complete genome sequence of *Pasteurella multocida* HN06, a toxigenic strain of serogroup D. *J. Bacteriol.* **194(12):** 3292-3293.

Lowe, T. M. and Eddy, S. R. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res.* **25(5):** 955-964.

Lu, Y. S.; Lai, W. C.; Pakes, S. P. and Nie, L. C. (1991). A monoclonal antibody against a *Pasteurella multocida* outer membrane protein protects rabbits and mice against Pasteurellosis. *Infect. Immun.* **59(1):** 172-180.

Lukashin, A. V. and Borodovsky, M. (1998). GeneMark.hmm: new solutions for gene finding. *Nucleic Acids Res.* **26(4):** 1107-1115.

Luo, Y.; Glisson, J. R.; Jackwood, M. W.; Hancock, R. E.; Bains, M.; Cheng, I. H. and Wang, C. (1997). Cloning and characterization of the major outer membrane protein gene (ompH) of *Pasteurella multocida* X-73. *Journal of bacteriology* **179(24):** 7856-7864.

Luo, Y.; Zeng, Q.; Glisson, J. R.; Jackwood, M. W.; Cheng, I. H. and Wang, C. (1999). Sequence analysis of *Pasteurella multocida* major outer membrane protein (ompH) and application of synthetic peptides in vaccination of chickens against homologous strain challenge. *Vaccine* **17(7-8):** 821-831.

Marandi, M. V. and Mittal, K. R. (1997). Role of outer membrane protein H (ompH)- and ompA-specific monoclonal antibodies from hybridoma tumors in protection of mice against *Pasteurella multocida. Infect. Immun.* **65(11):** 4502-4508.

Margulies, M.; Egholm, M.; Altman, W. E.; Attiya, S.; Bader, J. S.; Bemben, L. A.; Berka, J.; Braverman, M. S.; Chen, Y. J.; Chen, Z.; Dewell, S. B.; Du, L.; Fierro, J. M.; Gomes, X. V.; Godwin, B. C.; He, W.; Helgesen, S.; Ho, C. H.; Irzyk, G. P.; Jando, S. C.; Alenquer, M. L.; Jarvie, T. P.; Jirage, K. B.; Kim, J. B.; Knight, J. R.; Lanza, J. R.; Leamon, J. H.; Lefkowitz, S. M.; Lei, M.; Li, J.; Lohman, K. L.; Lu, H.; Makhijani, V. B.; McDade, K. E.; McKenna, M. P.; Myers, E. W.; Nickerson, E.; Nobile, J. R.; Plant, R.; Puc, B. P.; Ronan, M. T.; Roth, G. T.; Sarkis, G. J.; Simons, J. F.; Simpson, J. W.; Srinivasan, M.; Tartaro, K. R.; Tomasz, A.; Vogt, K. A.; Volkmer, G. A.; Wang, S. H.; Wang, Y.; Weiner, M. P.; Yu, P.; Begley, R. F. and Rothberg, J. M. (2005). Genome

Vety. Sci.& A.H., Anand
ANAND ★ ANAND ★ UNIVERSITY

sequencing in microfabricated high-density picolitre reactors. *Nature* **437(7057):** 376-80.

Maslog, F. S.; Motobu, M.; Hayashida, N.; Yoshihara, K.; Morozumi, T.; Matsumura, M. and Hirota, Y. (1999). Effects of the lipopolysaccharide-protein complex and crude capsular antigens of Pasteurella multocida serotype A on antibody responses and delayed type hypersensitivity responses in the chicken. *J. Vet. Med. Sci.* **61(5):** 565-567.

May, B. J.; Zhang, Q.; Li, L. L.; Paustian, M. L.; Whittam, T. S. and Kapur, V. (2001). Complete genomic sequence of *Pasteurella multocida*, Pm70. **98(6):** 3460-3465.

McEvoy, C. R.; Seshadri, R. and Firgaira, F. A. (1998). Large DNA fragment sizing using native acrylamide gels on an automated DNA sequencer and GENESCAN software. *Biotechniques* **25(3):** 464-470.

McNeil, L. K.; Reich, C.; Aziz, R. K.; Bartels, D.; Cohoon, M.; Disz, T.; Edwards, R. A.; Gerdes, S.; Hwang, K.; Kubal, M.; Margaryan, G. R.; Meyer, F.; Mihalo, W.; Olsen, G. J.; Olson, R.; Osterman, A.; Paarmann, D.; Paczian, T.; Parrello, B.; Pusch, G. D.; Rodionov, D. A.; Shi, X.; Vassieva, O.; Vonstein, V.; Zagnitko, O.; Xia, F.; Zinner, J.; Overbeek, R. and Stevens, R. (2007). The National Microbial Pathogen Database Resource (NMPDR): a genomics platform based on subsystem annotation. *Nucleic Acids Res.* **35(Database issue):** D347-D353.

Medigue, C. and Moszer, I. (2007). Annotation, comparison and databases for hundreds of bacterial genomes. *Research in Micribiology* **158(10):** 724-736.

Mendes, S.; Carmichael, K. P.; Nunnally, J. C.; Glisson, J. R.; Cheng, I. H. and Harmon, B. G. (1994). Lesions resulting from attempted Shwartzman reaction in turkey poults inoculated with *Pasteurella multocida* lipopolysaccharide. *Avian Dis.* **38(4):** 790-796.

Meredith, L.; Hunta, B.; Adlera, K. and Townsend, M. (2000). The molecular biology of *Pasteurella multocida. Veterinary Microbiology* **72:** 3-25.

Merlin, C.; Gardiner, G.; Durand, S. and Masters, M. (2002). The *Escherichia coli* metD locus encodes an ABC transporter which includes Abc (MetN), YaeE (MetI), and YaeC (MetQ). *J. Bacteriol.* **184(19):** 5513-5517.

Metzker, M. L. (2009). Sequencing in real time. *Nat. Biotechnol.* **27(2):** 150-151.

Metzker, M. L. (2010). Sequencing technologies - the next generation. *Nature Review Genetics* **11(1):** 31-46.

Miller, J. R.; Koren, S. and Sutton, G. (2010). Assembly algorithms for next-generation sequencing data. *Genomics* **95(6):** 315-327.

Mizan, S.; Henk, A.; Stallings, A.; Maier, M. and Lee, M. D. (2000). Cloning and characterization of sialidases with 2-6' and 2-3' sialyl lactose specificity from *Pasteurella multocida. Journal of Bacteriology* **182:** 6874-6883.

Moiser, D. A.; Simons, K. R.; Chengapappa, M. M. and Confer, A. W. (1994). Antigenic composition of *Pasteurella haemolytica* serotype-1 supernatants from supplemented and non-supplemented media. *American Journal Vet. Res.* **55:** 348-352.

Mooi, F. R.; Jansen, W. H.; Brunings, H.; Gielen, H.; van der Heide, H. G.; Walvoort, H. C. and Guinee, P. A. (1992). Construction and analysis of *Bordetella pertussis* mutants defective in the production of fimbriae. *Microb. Pathog.* **12(2):** 127-135.

Moran, N. A. (1996). Accelerated evolution and Muller's rachet in endosymbiotic bacteria. *Proc. Natl. Acad. Sci.* **93(7):** 2873-2878.

Morgenthau, A.; Pogoutse, A.; Adamiak, P.; Moraes, T. F. and Schryvers, A. B (2013). Bacterial receptors for host transferrin and lactoferrin: molecular mechanisms and role in host-microbe interactions. *Future Microbiol.* **8(12):** 1575-1585.

Morita, Y.; Sobel, M. L. and Poole, K. (2006). Antibiotic inducibility of the MexXY multidrug efflux system of *Pseudomonas aeruginosa*: involvement of the antibiotic-inducible PA5471 gene product. *Antimicrob. Agents Chemother.* **188(5):** 1847-1855.

Morona, R.; Brown, M. H.; Yeadon, J.; Heuzenroeder, M. W. and Manning, P. A. (1991). Effect of lipopolysaccharide core biosynthesis mutation on the production of *Vibrio cholerae* O-antigen in *Escherichia coli* K-12. *FEMS Microbiol. Lett.* **66(3):** 279-285.

Morozova, O. and Marra, M. A. (2008). Applications of next-generation sequencing technologies in functional genomics. *Genomics* **92(5):** 255-264.

Morton, D. J.; Madore, L. L.; Smith, A.; Vanwagoner, T. M.; Seale, T. W.; Whitby, P. W. and Stull, T. L. (2005). The heme-binding lipoprotein (HbpA) of

College of Vety. Sc. & A.H., Anand
ANAND AGRICULTURAL UNIVERSITY

Haemophilus influenzae: role in heme utilization. *FEMS Microbiology Letters* **253(2):** 193-199.

Moxon, E. R. and Kroll, J. S. (1990). The role of bacterial polysaccharide capsules as virulence factors. *Curr. Topics Microbiol. Immun.* **150:** 65-86.

Mullan, P. B. and Lax, A. J. (1998). *Pasteurella multocida* toxin stimulates bone resorption by osteoclasts via interaction with osteoblasts. *Calcif. Tissue. Int.* **63(4):** 340-345.

Mullen, L. M.; Nair, S. P.; Ward, J. M.; Rycroft, A. N.; Williams, R. J.; Robertson, G.; Mordan, N. J. and Henderson, B. (2008). Novel adhesin from *Pasteurella multocida* that binds to the integrin-binding fibronectin FnIII9-10 repeats. *Infect. Immun.* **76(3):** 1093-1104.

Muller, H. E. and Krasemann, C. (1974). The virulence of *Pasteurella multocida* strains and their neuraminidase production (author's transl.). *Zentralbl. Bakteriol. Orig. A.* **229(3):** 391-400.

Muniandy, N.; Edgar, J.; Woolcock, J. B. and Mukkur, T. K. S. (1992). Virulence, purification, structure, and protective potential of the putative capsular polysaccharide of *Pasteurella multocida* type 6:B. In: Patten, B. E.; Spencer, T. L.; Johnson, R. B.; Lehane, L. eds. Pasteurellosis in production animals. Bali, Indonesia, 47-53.

Myint, A.; Carter, G. R. and Jones, T. O. (1987). Prevention of experimental haemorrhagic septicaemia with a live vaccine. *Vet. Rec.* **120(21):** 500-501.

Nakamura, S.; Maeda, N.; Miron, I. M.; Yoh, M.; Izutsu, K.; Kataoka, C.; Honda, T.; Yasunaga, T.; Nakaya, T.; Horii, T. and Iida, T. (2008). Metagenomic

diagnosis of bacterial infections. *Emerging Infectious Diseases* **14(11):** 1784-1786.

Nakamura, S.; Yang, C. S.; Sakon, N.; Ueda, M.; Tougan, T.; Yamashita, A.; Goto, N.; Takahashi, K.; Yasunaga, T.; Ikuta, K.; Mizutani, T.; Okamoto, Y.; Tagami, M.; Morita, R.; Maeda, N.; Kawai, J.; Hayashizaki, Y.; Nagai, Y.; Horii, T. and Iida, T. (2009). Direct metagenomic detection of viral pathogens in nasal and fecal specimens using an unbiased high-throughput sequencing approach. *PLoS One* **4(1):** e4219.

Nanduri, B.; Shack, L. A.; Burgess, S. C. and Lawrence, M. L. (2009). The transcriptional response of *Pasteurella multocida* to three classes of antibiotics. *BMC Genomics* **10(2):** S4.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* **67(4):** 593-656.

Ninnemann, O.; Koch, C. and Kahmann, R. (1992). The *E. coli* fis promoter is subject to stringent control and autoregulation. *EMBO J.* **11(3):** 1075-1083.

Nishito, Y.; Osana, Y.; Hachiya, T.; Popendorf, K.; Toyoda, A.; Fujiyama, A.; Itaya, M. and Sakakibara, Y. (2010). Whole genome assembly of a natto production strain *Bacillus subtilis* natto from very short read data. *BMC Genomics* **11:** 243.

Ochman, H.; Lawrence, J. G. and Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* **405(6784):** 299-304.

Oda, K.; Katsuyuki, Y.; Ohta, E.; Nakamura, Y.; Takemura, M.; Nozato, N.; Akashi, K.; Kanegae, T.; Ogura, Y.; Kohchi, T. and Ohyama K. (1992). Gene

Vety. Sci.& A.H., Anand
ANAND VETERINARY UNIVERSITY

organization deduced from the complete sequence of Liverwort *Marchantia polymorpha* mitochondrial DNA. *J. Mol. Biol.* **223(1):** 1-7.

Ogunnariwo, J. A. and Schryvers, A. B. (2001). Characterization of a novel transferrin receptor in bovine strains of *Pasteurella multocida. Journal of Bacteriology* **183(3):** 890-896.

Ohyama, K.; Fukuzawa, H.; Kohchi, T.; Shirai, H.; Sano, T.; Sano, S.; Umesono, K.; Shiki, Y.; Takeuchi, M.; Chang, Z.; Aota, S.; Inokuchi, H. and Ozeki, H. (1986). Chloroplast gene organization deduced from complete sequence of livewort *Marchantia polymorpha* chloroplast DNA. *Nature* **322:** 572-574.

OIE, World Organisation for Animal Health, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2009. OIE Terrestrial Manual 2008, World Organization for Animal Health, Paris, France, 2009.

OIE World Organisation for Animal Health, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012. OIE Terrestrial Manual 2012, World Organization for Animal Health, Paris, France, 2012.

Okay, S.; Ozcengiz, E.; Gursel, I. and Ozcengiz, G. (2012[a]). Immunogenicity and protective efficacy of the recombinant *Pasteurella* lipoprotein E and outer membrane protein H from *Pasteurella multocida* A:3 in mice. *Res. Vet. Sci.* **93(3):** 1261-1265.

Okay, S.; Ozcengiz, E. and Ozcengiz, G. (2012[b]). Immune responses against chimeric DNA and protein vaccines composed of PlpEN-OmpH and PlpEC-OmpH from *Pasteurella multocida* A:3 in mice. *Acta. Microbiol. Immunol. Hung.* **59(4):** 485-498.

Overbeek, R.; Begley, T.; Butler, R. M.; Choudhuri, J. V.; Chuang, H. Y.; Cohoon, M.; de Crecy-Lagard, V.; Diaz, N.; Disz, T.; Edwards, R.; Fonstein, M.; Frank, E. D.; Gerdes, S.; Glass, E. M.; Goesmann, A.; Hanson, A.; Iwata-Reuyl, D.; Jensen, R.; Jamshidi, N.; Krause, L.; Kubal, M.; Larsen, N.; Linke, B.; McHardy, A. C.; Meyer, F.; Neuweger, H.; Olsen, G.; Olson, R.; Osterman, A.; Portnoy, V.; Pusch, G. D.; Rodionov, D. A.; Rückert, C.; Steiner, J.; Stevens, R.; Thiele, I.; Vassieva, O.; Ye, Y.; Zagnitko, O. and Vonstein, V. (2005). The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Research* **33(17):** 5691-5702.

Pan, C. Q.; Finkel, S. E.; Cramton, S. E.; Feng, J. A.; Sigman, D.S. and Johnson, R. C. (1996). Variable structures of Fis-DNA complexes determined by flanking DNA-protein contacts. *J. Mol. Biol.* **264(4):** 675-695.

Pandher, K.; Confer, A. W. and Murphy, G. L. (1998). Genetic and immunologic analyses of PlpE, a lipoprotein important in complement-mediated killing of *Pasteurella haemolytica* serotype 1. *Infect. immune.* **66(12):** 5613-5619.

Pandit, K. K. and Smith, J. E. (1993). Capsular hyaluronic acid in *Pasteurella multocida* type A and its counterpart in type D. *Res. Vet. Sci.* **54(1):** 20-24.

Patrick, K. (2007). 454 life sciences: illuminating the future of genome sequencing and personalized medicine. *Yale J. Biol. Med.* **80(4):** 191-194.

Paustian, M. L., Zhu, X.; Sreevatsan, S.; Robbe-Austerman, S.; Kapur, V. and Bannantine, J. P. (2008). Comparative genomic analysis of *Mycobacterium avium* subspecies obtained from multiple host species. *BMC Genomics* **9:** 135.

Vety. S.A.&
A.H., Anand
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Paustian, M. L.; May, B. J. and Kapur, V. (2001). *Pasteurella multocida* gene expression in response to iron limitation. *Infection and Immunity* **69(6):** 4109-4115.

Paustian, M. L.; May, B. J.; Cao, D.; Boley, D. and Kapur, V. (2002). Transcriptional response of *Pasteurella multocida* to defined iron sources. *J. Bacteriol.* **184(23):** 6714-6720.

Pearson, W. R. and Lipman, D. J. (1988). Improved tools for biological sequence comparison *Proc. Natl. Acad. Sci.* **85(8):** 2444-2448.

Pereira, F.; Carneiro, J. and Amorim, A. (2008). Identification of species with DNA-based technology: Current progress and challenges. *Recent Patents on DNA and Gene Sequences* **2(3):** 187-200.

Perez, B. A.; Planet, P. J.; Kachlany, S. C.; Tomich, M.; Fine, D. H. and Figurski, D. H. (2006). Genetic analysis of the requirement for flp-2, tadV, and rcpB in *Actinobacillus actinomycetemcomitans* biofilm formation. *J. Bacteriol.* **188(17):** 6361-6375.

Perry, B. D.; Randolph, T. F.; McDermott, J. J.; Sones, K. R. and Thornton, P. K. (2002). Animal diseases and their impact on poor. In: Investing in Animal Health Research to Alleviate Poverty. Nairobi, Kenya: ILRI (International Livestock Research Institute) pp. 49-59.

Petersen, S. K.; Foged, N. T.; Bording, A.; Nielsen, J. P.; Riemann, H. K. and Frandsen, P. L. (1991). Recombinant derivatives of *Pasteurella multocida* toxin: candidates for a vaccine against progressive atrophic rhinitis. *Infect. Immun.* **59(4):** 1387-1393.

Phillips, M. and Rimler, R. B. (1984). Protection of chickens by ribosomal vaccines from *Pasteurella multocida*: dependence on homologous lipopolysaccharide. *Am. J. Vet. Res.* **45(9):** 1785-1789.

Pittendrigh, B. R.; Clark, J. M.; Johnston, J. S.; Lee, S. H.; Romero-Severson, J. and Dasch, G. A. (2005). Proposal for the sequencing of a new target genome: White paper for a human body louse project. *Journal of Medical Entomology* **46(6):** 1103-1111.

Pop, M.; Kosack, D. S. and Salzberg, S. L. (2004). Hierarchical scaffolding with Bambus. *Genome Res.* **14(1):** 149-159.

Prado, M. E.; Dabo, S. M. and Confer, A. W. (2005). Immunogenicity of iron regulated outer membrane proteins of *Pasteurella multocida* A:3 in cattle: molecular characterization of the immunodominant heme acquisition system receptor (HasR) protein. *Vet. Microbiol.* **105(3-4):** 269-280.

Prager, M. and Wilson, A. C. (1978). Construction of phylogenetic trees for proteins and nucleic acids: empirical evaluation of alternative matrix methods. *J. Mol. Evol.* **11(2):** 129-142.

Pruimboom, I. M.; Rimler, R. B.; Ackermann, M. R. and Brogden, K. A. (1996). Capsular hyaluronic acid-mediated adhesion of *Pasteurella multocida* to turkey air sac macrophages. *Avian Dis.* **40(4):** 887-893.

Pullinger, G. D.; Bevir, T. and Lax, A. J. (2004). The *Pasteurella multocida* toxin is encoded within a lysogenic bacteriophage. *Mol. Microbiol.* **51(1):** 255-269.

College of Vety. Sc. & A.H., Anand
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Qin, Z. S.; McCue, L. A.; Thompson, W.; Mayerhofer, L.; Lawrence, C. E. and Liu, J. S. (2003). Identification of co-regulated genes through Bayesian Clustering of predicted regulatory binding sites. *Nature Biotechnology* **21(4):** 435-439.

Quinlan, A. R.; Stewart, D. A.; Stromberg, M. P. and Marth, G. T. (2008). Pyrobayes: an improved base caller for SNP discovery in pyrosequences. *Nature Methods* **5(2):** 179- 81.

Qureshi, S. and Saxena, H. M. (2014). Estimation of titers of antibody against *Pasteurella multocida* in cattle vaccinated with haemorrhagic septicemia alum precipitated vaccine. *Veterinary World* 7 **(4):** 224-228.

Ram, S.; Cox, A. D.; Wright, J. C.; Vogel, U.; Getzlaff, S.; Boden, R.; Li, J.; Plested, J. S.; Meri, S.; Gulati, S.; Stein, D. C.; Richards, J. C.; Moxon, E. R. and Rice, P. A. (2003). Neisserial lipooligosaccharide is a target for complement component c4b. Inner core phosphoethanolamine residues define C4b linkage specificity. *J. Biol. Chem.* **278(51):** 50853-50862.

Ramdani, and Adler, B. (1991). Opsonic monoclonal antibodies against lipopolysaccharide (LPS) antigens of *Pasteurella multocida* and the role of LPS in immunity. *Vet. Microbiol.* **26(4):** 335-347.

Raskin, D. M.; Seshadri, R.; Pukatzki, S. U. and Mekalanos, J. J. (2006). Bacterial genomics and pathogen evolution. *Cell* **124(4):** 703-714.

Ratledge, C. and Dover, L. G. (2000). Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.* **54:** 881-941.

Rebers, P. A.; Jensen, A. E. and Laird, G. A. (1988). Expression of pili and capsule by the avian strain P-1059 of *Pasteurella multocida*. *Avian Dis.* **32:** 313-318.

Reinhardt, J. A.; Baltrus, D. A.; Nishimura, M. T.; Jeck, W. R.; Jones, C. D. and Dangl, J. L. (2009). *De novo* assembly using low-coverage short read sequence data from the rice pathogen *Pseudomonas syringae pv. oryzae*. *Genome Research* **19(2):** 294-305.

Rhoades, K. R. and Rimler, R. B. (1987). Effects of *Pasteurella multocida* endotoxins on turkey poults. *Avian Dis.* **31(3):** 523-526.

Rigal, A.; Bouveret, E.; Lloubes, R.; Lazdunski, C. and Benedetti, H. (1997). The TolB protein interacts with the porins of *Escherichia coli*. *Journal of Bacteriology* **179(23):** 7274-7279.

Rimler, R. B. (1994). Presumptive identification of *Pasteurella multocida* serogroups A, D and F by capsule depolymerisation with mucopolysaccharidases. *Vet. Rec.* **134(8):** 191-192.

Rimler, R. B. (2001). Purification of a cross-protective antigen from *Pasteurella multocida* grown *in vitro* and *in vivo*. *Avian Dis.* **45:** 572-580.

Rimler, R. B. and Rhoades, K. R. (1989). *Pasteurella multocida*. In: Pasteurella and Pasteurellosis (Adlam, C. and Rutter, J. M. eds.), pp. 37-73. Academic Press Limited, London.

Rimler, R. B. and Rhoades, K. R. (1994). Hyaluronidase and chondroitinase activity of *Pasteurella multocida* serotype B:2 involved in hemorrhagic septicaemia. *Vet. Rec.* **134(3):** 67-68.

Roehrig, S. C.; Tran, H. Q.; Spehr, V.; Gunkel, N.; Selzer, P. M. and Ullrich, H. J. (2007). The response of *Mannheimia haemolytica* to iron limitation:

College Of Vety. Sci.& A.H., Anand
ANAND ★ ANAND ★ UNIVERSITY

implications for the acquisition of iron in the bovine lung. *Vet Microbiol.* **121(3-4):** 316-329.

Ronaghi, M.; Karamohamed, S.; Pettersson, B.; Uhlen, M. and Nyren, P. (1996). Real-time DNA sequencing using detection of pyrophosphate release. *Analytical Biochemistry* **242(1):** 84-89.

Rothberg, J. M.; Hinz, W.; Rearick, T. M.; Schultz, J.; Mileski, W.; Davey, M.; Leamon, J. H.; Johnson, K.; Milgrew, M. J.; Edwards, M.; Hoon, J.; Simons, J. F.; Marran, D.; Myers, J. W.; Davidson, J. F.; Branting, A.; Nobile, J. R.; Puc, B. P.; Light, D.; Clark, T. A.; Huber, M.; Branciforte, J. T.; Stoner, I. B.; Cawley, S. E.; Lyons, M.; Fu, Y.; Homer, N.; Sedova, M.; Miao, X.; Reed, B.; Sabina, J.; Feierstein, E.; Schorn, M.; Alanjary, M.; Dimalanta, E.; Dressman, D.; Kasinskas, R.; Sokolsky, T.; Fidanza, J. A.; Namsaraev, E.; McKernan, K. J.; Williams, A. and Roth, G. T. (2011). An integrated semiconductor device enabling non-optical genome sequencing. *Nature* **475(7356):** 348-352.

Rubin, H. (2001). Etymology of epigenetics. *Science* **294(5551):** 2477-2478.

Ruffolo, C. G.; Tennent, J. M.; Michalski, W. P. and Adler, B. (1997). Identification, purification, and characterization of the type 4 fimbriae of *Pasteurella multocida*. *Infect. Immun.* **65(1):** 339-343.

Ruffolo, C. G.; Jost, B. H. and Adler, B. (1998). Iron-regulated outer membrane proteins of *Pasteurella multocida* and their role in immunity. *Vet. Microbiol.* **59(2-3):** 123-137.

Ryu, H. l. and Kim, C. J. (2000). Immunologic reactivity of a lipopolysaccharide-Protein complex of type A *P. multocida* in mice. *Vet. Sci.* **1(2):** 87-95.

Saldana, Z.; Xicohtencati-Cortes, J.; Avelino, F.; Phillips, A. D.; Kaper, J. B.; Puente, J. L. and Giron, J. A. (2009). Synergistic role of curli and cellullose in cell adherance and biofilm formation and attaching and effacing *Escherichia coli* and identification of Fis as a negative regulator of curli. *Environ. Micro.* **11(4):** 992-1006.

Salzberg, S. L.; Sommer, D. D.; Puiu, D. and Lee, V. T. (2008). Gene-boosted assembly of a novel bacterial genome from very short reads. *PLoS Computational Biology* **4(9):** e1000186.

Sanger, F.; Air, G. M.; Barrell, B. G.; Brown, N. L.; Coulson, A. R.; Fiddes, C. A.; Hutchison, C. A.; Slocombe, P. M. and Smith, M. (1977[a]). Nucleotide sequence of bacteriophage phi X174 DNA. *Nature* **265(5596):** 687-695.

Sanger, F.; Nicklen, S. and Coulson, A. R. (1977[b]). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences* **74(12):** 5463-5467.

Sanger, F.; Coulson, A. R.; Barrell, B. G.; Smith, A. J. H. and Roe, B. A. (1980). Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* **143(2):** 161-178.

Sanger, F.; Coulson, A. R.; Hong, G. F.; Hill, D. F. and Petersen, G. B. (1982). Nucleotide sequence of bacteriophage λ DNA. *J. Mol. Biol.* **162(4):** 729-773.

Sarangi, L. N.; Priyadarshini, A.; Kumar, S.; Thomas, P.; Gupta, S. K.; Nagaleekar, V. K. and Singh, V. P. (2014). Virulence genotyping of *Pasteurella multocida* isolated from multiple hosts from India. *Scientific World Journal* Article ID **814109:** 1-10.

College Of Vety. Sci. & A.H., Anand

Sarangi, L. N.; Thomas, P.; Gupta, S. K.; Priyadarshini, A.; Kumar, S.; Nagaleekar, V. K.; Kumar, A. and Singh, V. P. (2015). Virulence gene profiling and antibiotic resistance pattern of Indian isolates of *Pasteurella multocida* of small ruminant origin. *Comp. Immunol. Microbiol. Infect. Dis.* **38:** 33-39.

Scharmann, W. R.; Drzeniek, R. and Blobel, H. (1970). Neuraminidase of *Pasteurella multocida. Infect. Immun.* **1(3):** 319-320.

Schattner, P.; Brooks, A. N. and Lowe, T. M. (2005). The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs. *Nucleic Acids Res.* **33(2):** 686-689.

Schauer, R. (2009). Sialic acids as regulators of molecular and cellular interactions. *Curr. Opin. Struct. Biol.* **19(5):** 507-514.

Schenkein, H. A.; Barbour, S. E.; Berry, C. R.; Kipps, B. and Tew, J. G. (2000). Invasion of human vascular endothelial cells by *Actinobacillus actinomycetemcomitans* via the receptor for platelet-activating factor. *Infect. Immun.* **68(9):** 5416-5419.

Sellyei, B.; Banyai, K. and Magyar, T. (2010). Characterization of the *ptfA* gene of avian *Pasteurella multocida* strains by allele-specific polymerase chain reaction. *J. Vet. Diagn. Invest.* **22(4):** 607-610.

Serino, L. and Virji, M. (2002). Genetic and functional analysis of the phosphorylcholine moiety of commensal *Neisseria lipopolysaccharide. Mol. Microbiol.* **43(2):** 437-448.

Sha, J.; Fadl, A. A.; Klimpel, G. R.; Niesel, D. W.; Popov, V. L. and Chopra, A. K. (2004). The two murein lipoproteins of *Salmonella enterica* serovar

Typhimurium contribute to the virulence of the organism. *Infect. Immun.* **72(7):** 3987-4003.

Shao, Y.; Feldman-Cohen, L. S. and Osuna, R. (2008). Biochemical identification of base and phosphate contacts between Fis and a high-affinity DNA binding site. *J. Mol. Biol.* **380(2):** 327-339.

Shendure, J.; Porreca, G. J.; Reppas, N. B.; Lin, X.; McCutcheon, J. P.; Rosenbaum, A. M.; Wang, M. D.; Zhang, K.; Mitra, R. D. and Church, G. M. (2005). Accurate multiplex polony sequencing of an evolved bacterial genome. *Science* **309(5741):** 1728-1732.

Shivachandra, S. B.; Kumar, A. A.; Amaranath, J.; Joseph, S.; Srivastava, S. K. and Chaudhuri, P. (2005). Cloning and characterization of *tbpA* gene encoding transferrin binding protein (TbpA) from *Pasteurella multocida* B:2 (strain P52). *Veterinary Research Communications* **29(6):** 537-542.

Shivachandra, S. B.; Viswas, K. N. and Kumar, A. A. (2011). A review of hemorrhagic septicemia in cattle and buffalo. *Anim. Health. Res. Rev.* **12(1):** 67-82.

Shivachandra, S. B.; Kumar, A.; Yogisharadhya, R.; Ramakrishnan, M. A. and Viswas, K. N. (2013). Carboxyl terminus heterogeneity of type IV fimbrial subunit protein of *Pasteurella multocida* isolates. *Veterinary Research Communications* **37:** 269-275.

Shivachandraa, S. B.; Kumar, A.; Yogisharadhya, R. and Viswas, K. N. (2014). Immunogenicity of highly conserved recombinant VacJ outer membrane lipoprotein of *Pasteurella multocida. Vaccine* **32(2):** 290- 296.

Shouldice, S. R.; Dougan, D. R.; Williams, P. A.; Skene, R. J.; Snell, G.; Scheibe, D.; Kirby, S.; Hosfield, D. J.; McRee, D. E.; Schryvers, A. B. and Tari, L. W. (2003). Crystal structure of *Pasteurella haemolytica* ferric ion-binding protein A reveals a novel class of bacterial iron-binding proteins. *Journal of Biological Chemistry* **278(42):** 41093-41098.

Siju, J, Kumar, A. A.; Shivachandra, S. B.; Chaudhuri, P.; Srivastava, S. K. and Singh, V. P. (2007). Cloning and characterization of Type IV fimbrial gene (*ptfA*) of *Pasteurella multocida* Serogroup B:2 (Strain P52). *Veterinary Research Communications* **31:** 397-404.

Smits, T. H.; Jaenicke, S.; Rezzonico, F.; Kamber, T.; Goesmann, A.; Frey, J. E. and Duffy, B. (2010). Complete genome sequence of the fire blight pathogen *Erwinia pyrifoliae* DSM 12163T and comparative genomic insights into plant pathogenicity. *BMC Genomics* **11:** 2.

Snipes, K. P. and Hirsh, D. C. (1986). Association of complement sensitivity with virulence of *Pasteurella multocida* isolated from turkeys. *Avian Dis.* **30(3):** 500-504.

Snipes, K. P.; Ghazikhanian, G. Y. and Hirsh, D. C. (1987). Fate of *Pasteurella multocida* in the blood vascular system of turkeys following intravenous inoculation: comparison of an encapsulated, virulent strain with its avirulent, acapsular variant. *Avian Dis.* **31(2):** 254-259.

Snipes, K. P.; Hansen, L. M. and Hirsh, D. C. (1988). Plasma and iron regulated expression of high molecular weight outer membrane proteins by *Pasteurella multocida*. *Am. J. Vet. Res.* **49(8):** 1336-1338.

Srikumar, R.; Mikael, L. G.; Pawelek, P. D.; Khamessan, A.; Gibbs, B. F.; Jacques, M. and Coulton, J. W. (2004). Molecular cloning of haemoglobin-binding protein HgbA in the outer membrane of *Actinobacillus pleuropneumoniae*. *Microbiology* **150(6):** 1723-1734.

Srivatsan, J.; Han, Y.; Peng, J.; Tehranchi, A. K.; Gibbs, R.; Wang, J. D. and Chen, R. (2008). High-precision, whole-genome sequencing of laboratory strains facilitates genetic studies. *PLoS Genetics* **4(8):** e1000139.

St Michael, F.; Li, J. and Cox, A. D. (2005). Structural analysis of the core oligosaccharide from *Pasteurella multocida* strain X73. *Carbohydr. Res.* **340:** 1253-1257.

Stackebrandt, E. and Goebel, B. M. (1994). A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44:** 846-849.

Steen, J. A.; Harrison, P.; Seemann, T.; Wilkie, I.; Harper, M. Adler, B. and Boyce, J. D. (2010). Fis is essential for capsule production in *Pasteurella multocida* and regulates expression of other important virulence factors. *PLoS Pathog.* **6(2):** e1000750.

Steenbergen, S. M.; Lichtensteiger, C. A.; Caughlan, R.; Garfinkle, J.; Fuller, T. E. and Vimr, E. R. (2005). Sialic acid metabolism and systemic pasteurellosis. *Infect. Immun.* **73(3):** 1284-1294.

Sterner-Kock, A.; Lanske, B.; Uberschar, S. and Atkinson, M. J. (1995). Effects of the *Pasteurella multocida* toxin on osteoblastic cells *in vitro*. *Vet. Pathol.* **32:** 274-279.

Stothard, P. and Wishart, D. S. (2006). Automated bacterial genome analysis and annotation. *Current Opinion in Microbiology* **9(5):** 505-510.

Strauss, E. and Falkow, S. (1997). Microbial pathogenesis: genomics and beyond. *Science* **276(5313):** 707-712.

Studholme, D. J.; Ibanez, S. G.; MacLean, D.; Dangl, J. L.; Chang, J. H. and Rathjen, J. P. (2009). A draft genome sequence and functional screen reveals the repertoire of type III secreted proteins of *Pseudomonas syringae* pathovar tabaci 11528. *BMC Genomics* **10:** 395.

Swerdlow, H. and Gesteland, R. (1990). Capillary gel electrophoresis for rapid, high resolution DNA sequencing. *Nucleic Acids Research* **18(6):** 1415-1419.

Swords, W. E.; Buscher, B. A.; Ver Steeg Ii, K.; Preston, A.; Nichols, W. A.; Weiser, J. N.; Gibson, B. W. and Apicella, M. A. (2000). Non-typeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. *Mol. Microbiol.* **37(1):** 13-27.

Tabatabai, L. B. and Zehr, E. S. (2004). Identification of five outer membrane-associated proteins among cross-protective factor proteins of *Pasteurella multocida*. *Infect. Immun.* **72(2):** 1195-1198.

Tabatabai, L. B. (2008). Identification of *Pasteurella multocida* CHAPS-soluble outer membrane proteins. *Avian Diseases* **52(1):** 147-149.

Tamura, K; Stecher, G.; Peterson, D.; Filipski, A. and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* **30(12):** 2725-2729.

College Of Vety. Sci.& A.H., Anand
ANAND ★ ANAND ★ UNIVERSITY

Tanaka, K.; Matsuyama, S. I. and Tokuda, H. (2001). Deletion of lolB, encoding an outer membrane lipoprotein, is lethal for *Escherichia coli* and causes accumulation of lipoprotein localization intermediates in the periplasm. *Journal of Bacteriology* **183(22):** 6538-6542.

Tang, X.; Zhao, Z.; Hu, J.; Wu, B.; Cai, X.; He, Q. and Chen, H. (2009). Isolation, antimicrobial resistance, and virulence genes of *Pasteurella multocida* strains from swine in China. *Journal of Clinical Microbiology* **47(4):** 951-958.

Tatum, F. M.; Yersin, A. G. and Briggs, R. E. (2005). Construction and virulence of a *Pasteurella multocida* fhab2 mutant in turkeys. *Microb. Pathog.* **39:** 9-17.

Tatusov, R. L.; Fedorova, N. D.; Jackson, J. D.; Jacobs, A. R.; Kiryutin, B.; Koonin, E. V.; Krylov, D. M.; Mazumder, R.; Mekhedov, S. L.; Nikolskaya, A. N.; Rao, B. S.; Smirnov, S.; Sverdlov, A. V.; Vasudevan, S.; Wolf, Y. I. ; Yin, J. J. and Natale, D. A. (2003). The COG database: an updated version includes eukaryotes. *BMC Bioinformatics* **4:** 41.

ten Bosch, J. R., and Grody, W. W. (2008). Keeping up with the next generation: Massively parallel sequencing in clinical diagnostics. *Journal of Molecular Diagnostics* **10(6):** 484-492.

Tettelin, H.; Nelson, K. E.; Paulsen, I. T.; Eisen, J. A.; Read, T. D.; Peterson, S.; Heidelberg, J.; DeBoy, R. T.; Haft, D. H.; Dodson, R. J.; Durkin, A. S.; Gwinn, M.; Kolonay, J. F.; Nelson, W. C.; Peterson, J. D.; Umayam, L. A.; White, O.; Salzberg, S. L.; Lewis, M. R.; Radune, D.; Holtzapple, E.; Khouri, H.; Wolf, A. M.; Utterback, T. R.; Hansen, C. L.; McDonald, L. A.; Feldblyum, T. V.; Angiuoli, S.; Dickinson, T.; Hickey, E. K.; Holt, I. E.;

Loftus, B. J.; Yang, F.; Smith, H. O.; Venter, J. C.; Dougherty, B. A.; Morrison, D. A.; Hollingshead, S. K. and Fraser, C. M. (2001). Complete genome sequence of a virulent isolate of *Streptococcus pneumonia. Science* **293(5529):** 498-506.

Thompson, J. F. and Milos, P. M. (2011). The properties and applications of single-molecule DNA sequencing. *Genome Biology* **12(2):** 217.

Timp, W.; Mirsaidov, U. M.; Wang, D.; Comer, J.; Aksimentiev, A. and Timp, G. (2010). "Nanopore sequencing: electrical measurements of the code of life," *IEEE Transactions on Nanotechnology* **9(3):** 281-294

To, H.; Someno, S. and Nagai, S. (2005). Development of a genetically modified nontoxigenic *Pasteurella multocida* toxin as a candidate for use in vaccines against progressive atrophic rhinitis in pigs. *Am. J. Vet. Res.* **66:** 113-118.

Tokuda, H. and Matsuyama, S. (2004). Sorting of lipoproteins to the outer membrane in *E. coli. Biochim. Biophys. Acta.* **1693(1):** 5-13.

Tomich, M.; Fine, D. H. and Figurski, D. H. (2006). The TadV protein of *Actinobacillus actinomycetemcomitans* is a novel aspartic acid prepilin peptidase required for maturation of the Flp1 pilin and TadE and TadF pseudopilins. *J. Bacteriol.* **188(19):** 6899-6914.

Townsend, K. M.; Frost, A. J.; Lee, C. W.; Papadimitriou, J. M. and Dawkins, H. J. (1998). Development of PCR Assays for Species- and Type-Specific Identification of *Pasteurella multocida* Isolates. *Journal of Clinical Microbiology* **36(4):** 1096-1100.

Townsend, K. M.; Boyce, J. D.; Chung, J. Y.; Frost, A. J. and Adler, B. (2001). Genetic organization of *Pasteurella multocida* cap loci and development of a multiplex capsular PCR typing system. *J. Clin. Microbiol.* **39(3):** 924-929.

Truscott, W. M. and Hirsh, D. C. (1988). Demonstration of an outer membrane protein with antiphagocytic activity from *Pasteurella multocida* of avian origin. *Infect. Immun.* **56(6):** 1538-1544.

Tsuji, M. and Matsumoto, M. (1988). Evaluation of relationship among three purified antigens from *Pasteurella multocida* strain P-1059 and of their protective capacities in turkeys. *Am. J. Vet. Res.* **49(9):**1516-1521.

Tsuji, M. and Matsumoto, M. (1989). Pathogenesis of fowl cholera: influence of encapsulation on the fate of *Pasteurella multocida* after intravenous inoculation into turkeys. *Avian Dis.* **33(2):** 238-247.

Tsukahara, J.; Mukaiyama, K.; Okuda, S.; Narita, S. and Tokuda, H. (2009). Dissection of LolB function - lipoprotein binding, membrane targeting and incorporation of lipoproteins into lipid bilayers. *FEBS Journal* **276(16):** 4496-4504.

Turcatti, G.; Romieu, A.; Fedurco, M. and Tairi, A. P. (2008). A new class of cleavable fluorescent nucleotides: synthesis and optimization as reversible terminators for DNA sequencing by synthesis. *Nucleic Acids Research* **36(4):** e25.

Tzeng, Y. L.; Datta, A.; Strole, C.; Kolli, V. S.; Birck, M. R.; Taylor, W. P.; Carlson, R. W.; Woodard, R. W. and Stephens, D. S. (2002). KpsF is the arabinose-5-phosphate isomerase required for 3-deoxy-D-manno-octulosonic acid

biosynthesis and for both lipooligosaccharide assembly and capsular polysaccharide expression in *Neisseria meningitides. J. Biol. Chem.* **277(27):** 24103-24113.

Ueno, K. and Yeung, E. S. (1994). Simultaneous monitoring of DNA fragments separated by electrophoresis in a multiplexed array of 100 capillaries. *Analytical Chemistry* **66(9):** 1424-1431.

Vaid, R. K.; Shanmugasundaram, K.; Boora, A.; Bera, B. C.; Shukla, B. N.; Anand, T.; Singha, H.; Riyesh, T.; Virmani, N.; Barua, S.; Ahir, V. B.; Koringa, P. G.; Sajnani, M. R.; Bhatt, V. D.; Rana, N.; Singh, K. P.; Malik, P.; Singh, R. K. and Joshi, C. G. (2014). Draft Genome Sequence of Pasteurella multocida subsp. multocida B:2 Strain VTCCBAA264 Isolated from Bubalus bubalis in North India. *Genome Announc.* **2(4):** e00755-14.

Van Domselaar, G. H.; Stothard, P.; Shrivastava, S.; Cruz, J. A.; Guo, A.; Dong, X.; Lu, P.; Szafron, D.; Greiner, R. and Wishart, D. S. (2005). BASys: a web server for automated bacterial genome annotation. *Nucleic Acids Research* **33 (Web Server issue):** W455-459.

Vasta, V.; Ng, S. B.; Turner, E. H.; Shendure, J. and Hahn, S. H. (2009). Next generation sequence analysis for mitochondrial disorders. *Genome Medicine* **1(10):** 100.

Veken, J. W.; Oudega, B.; Luirink, J. and de Graaf, F. K. (1994). Binding of bovine transferrin by *Pasteurella multocida* serotype B:2,5, a strain which causes haemorrhagic septicaemia in buffalo and cattle. *FEMS Microbiol. Lett.* **115(2-3):** 253-257.

Vety. Sci.& A.H., Anand
ANAND

Veken, J. W.; Shah, N. H.; Klaasen, P.; Oudega, B. and de Graaf, F. K. (1996). Binding of host iron-binding proteins and expression of iron-regulated membrane proteins by different serotypes of *Pasteurella multocida* causing haemorrhagic septicaemia. *Microb. Pathog.* **21(1):** 59-64.

Venter, J. C.; Remington, K.; Heidelberg, J. F.; Halpern, A. L; Rusch, D.; Eisen, J. A.; Wu, D. Y.; Paulsen, I.; Nelson, K. E.; Nelson, W.; Fouts, D. E.; Levy, S.; Knap, A. H.; Lomas, M. W.; Nealson, K.; White, O.; Peterson, J.; Hoffman, J.; Parsons, R.; Baden-Tillson, H.; Pfannkoch. C.; Rogers, Y. H. and Smith, H. O. (2004). Environmental genome shotgun sequencing of the sargasso Sea. *Science* **304(5667)**: 66-74.

Verma, R. and Jaiswal, T. N. (1998). Haemorrhagic septicaemia vaccines. *Vaccine* **16(11-12):** 1184-1192.

Verma, S.; Sharma, M.; Katoch, S.; Verma, L.; Kumar, S.; Dogra, V.; Chahota, R.; Dhar, P. and Singh, G. (2013). Profiling of virulence associated genes of *Pasteurella multocida* isolated from cattle. *Veterinary Research Commun.* **37(1):** 83-89.

Vimr, E. and Lichtensteiger, C. (2002). To sialylate, or not to sialylate: that is the question. *Trends Microbiol.* **10(6):** 254-257.

von Mering, C.; Jensen, L. J.; Kuhn, M.; Chaffron, S.; Doerks, T.; Kruger, B. and Snel, B. and Bork, P. (2007). STRING 7- recent developments in the integration and prediction of protein interactions. *Nucleic Acids Res.* **35(Database issue):** D358-362.

Vety. Sci.& A.H., Anand
ANAND

Voss, B. J.; Gaddy, J. A.; McDonald, W. H. and Cover, T. L. (2014). Analysis of surface-exposed outer membrane proteins in *Helicobacter pylori*. *J. Bacterio.* **196(13):** 2455-2471.

Ward, D. M. (1998). A natural species concept for prokaryotes. *Curr. Opin. Microbiol.* **1(3):** 271-277.

Watt, J. M.; Swiatlo, E.; Wade, M. M. and Champlin, F. R. (2003). Regulation of capsule biosynthesis in serotype A strains of *Pasteurella multocida*. *FEMS Microbiology Letters* **225(1):** 9-14.

Wheeler, R. (2009). Outer membrane proteomics of *Pasteurella multocida* isolates to identify putative host-specificity determinants. *Bioscience horizons* **2(1):** 1-6.

Wheelis, M. L.; Kandler, O. and Woese, C. R. (1992). On the nature of global classification. *Proc. Natl. Acad. Sci.* **89:** 2930-2934.

White, D. J.; Jolley, W. L.; Purdy, C. W. and Straus, D. C. (1995). Extracellular neuraminidase production by a *Pasteurella multocida* A-3 strain associated with bovine pneumonia. *Infect. Immun.* **63(5):** 1703-1709.

Whiteford, N.; Haslam, N.; Weber, G.; Prugel-Bennett, A.; Essex, J. W.; Roach, P. L.; Bradley, M. and Neylon, C. (2005). An analysis of the feasibility of short read sequencing. *Nucleic Acids Research* **33(19):** e171.

Whitman, W. B.; Coleman, D. C. and Wiebe, W. J. (1998). Prokaryotes: the unseen majority. *Proceedings of the National Academy of Sciences* **95(12):** 6578-6583.

Wicker, T.; Schlagenhauf, E.; Graner, A.; Close, T. J.; Keller, B. and Stein, N. (2006). 454 sequencing put to the test using the complex genome of barley. *BMC Genomics* **7:** 275.

Wijewardana, T. G.; De Alwis, M. C. L. and Bastianz, H. L. G. (1986). Cultural, biochemical and pathogenicity studies on strains of *Pasteurella multocida* isolated from carrier animals and outbreaks of haemorrhagic septicaemia. *Sri Lanka Vet. J.* **34:** 43-57.

Wijewardana, T. G.; Wilson, C. F.; Gilmour, N. J. and Poxton, I. R. (1990). Production of mouse monoclonal antibodies to *Pasteurella multocida* type A and the immunological properties of a protective anti-lipopolysaccharide antibody. *J. Med. Microbiol.* **33(4):** 217-222.

Wilkie, I. W.; Harper, M.; Boyce, J. D. and Adler, B. (2012). *Pasteurella multocida*: diseases and pathogenesis. *Curr. Top. Microbiol. Immunol.* **361:** 1-22.

Willis L. M. and Whitfield, C. (2013). KpsC and KpsS are retaining 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) transferases involved in synthesis of bacterial capsules. *Proceedings of the National Academy of Sciences* **110(51):** 20753-20758.

Wilson, K. (1987). Preparation of genomic DNA from bacteria. In: Ausubel F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. G.; Smith, J. A. and Struhl, K. (eds.) *Current Protocols in Molecular Biology* **1:** pp. 2.4.1-2.4.5. John Wiley and Sons, New York.

Wilson, D. J. (2012). Insights from Genomics into Bacterial Pathogen Populations. *PLOS Pathogen* **8(9):** e1002874.

Wilson, B. A. and Ho, M. (2013). *Pasteurella multocida*: from zoonosis to cellular microbiology. *Clinical Microbiology Reviews* **26(3):** 631-655.

Wimley, W. C. (2003). The versatile beta-barrel membrane protein. *Curr. Opin. Struct. Biol.* **13(4):** 404-411.

Wiseman, R. W.; Karl, J. A.; Bimber, B. N.; O'Leary, C. E.; Lank, S. M.; Tuscher, J. J.; Detmer, A. M.; Bouffard, P.; Levenkova, N.; Turcotte, C. L.; Szekeres, E., Jr. Wright, C.; Harkins, T. and O'Connor, D. H. (2009). Major histocompatibility complex genotyping with massively parallel pyrosequencing. *Nature Medicine* **15(11):** 1322-1326.

Woese, C. R. (1987). Bacterial evolution. *Microbiol. Rev.* **51(2):** 221-271.

Wolf, Y. I.; Rogozin, I. B.; Grishin, N. V. and Koonin, E. V. (2002). Genome trees and the tree of life. *Trends Genet.* **18(9):** 472-479.

Wolfe K. H. and Li, W. H. (2003). Molecular evolution meets the genomics revolution. *Nature Genetics Supplement* **33:** 255-265.

Wu, H. J.; Wang, A. H. and Jennings, M. P. (2008). Discovery of virulence factors of pathogenic bacteria. *Current Opinion in Chemical Biology* **12(1):** 93-101.

Wu, J. R.; Shien, J. H.; Shieh, H. K.; Chen, C. F. and Chang, P. C. (2007). Protective immunity conferred by recombinant *Pasteurella multocida* lipoprotein E (PlpE). *Vaccine* **25(21):** 4140-4148.

Yap, H. Y.; Ghazali, K.; Wan Mohamad Nazarie, W. F.; Mat Isa, M. N.; Zakaria, Z. and Omar, A. R. (2013). Draft Genome Sequence of *Pasteurella multocida*

subsp. *multocida* Strain PMTB, Isolated from a Buffalo. *Genome Announc.* **1(5):** 00872-13.

Yu, H.; Chokhawala, H.; Karpel, R.; Wu, B.; Zhang, J.; Zhang, Y.; Jia, Q. and Chen, X. (2005). A multifunctional *Pasteurella multocida* sialyltransferase: a powerful tool for the synthesis of sialoside libraries. *J. Am. Chem. Soc.* **127(50):** 17618-17619.

Zagursky, R. J. and McCormick, R. M. (1990). DNA sequencing separations in capillary gels on a modified commercial DNA sequencing instrument. *Biotechniques* **9(1):**74-79.

Zav'yalov, V; Zavialov, A.; Zav'yalova, G. and Timo Korpela, T. (2010). Adhesive organelles of Gram-negative pathogens assembled with the classical chaperone/usher machinery: structure and function from a clinical standpoint. *FEMS Microbiol Rev.* **34(3):** 317-378.

Zhang, Y.; Wulumuhan, N.; Gong, F. and Entomack, B. (2013). Construction and Characterization of an Acapsular Mutant of *Pasteurella multocida* Strain P-1059 (A:3). *J Vaccines Vaccin.* **4(4):** 1-6.

Zhao, X. and Hao, B. (2009). CVTree update: a newly designed phylogenetic study platform using composition vectors and whole genomes. *Nucleic Acids Res.* **37(Web Server issue):** W174-W178.

Zihler, A.; Le Blay, G.; de Wouters, T.; Lacroix, C.; Braegger, C. P.; Lehner, A.; Tischler, P.; Rattei, T.; Hachler, H. and Stephan, R. (2009). *In vitro* inhibition activity of different bacteriocin producing *Escherichia coli* against *Salmonella* strains isolated from clinical cases. *Letters in Applied Microbiology* **49:** 31-38.

Zurlo, J. J. (2000). *Pasteurella* species. In: Principles and Practice of Infectious Diseases (Mandell, G. L.; Bennett, J. E. and Dolin, R. Eds.) pp: 2402-2406. Churchill Livingstone, Philadelphia.

Zywietz, A.; Gohla, A.; Schmelz, M.; Schultz, G. and Offermanns, S. (2001). Pleiotropic effects of *Pasteurella multocida* toxin are mediated by Gq-dependent and -independent mechanisms. Involvement of Gq but not G11. *J. Biol. Chem.* **276:** 3840-3845.

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