COMPARATIVE STUDIES ON IMMUNO-PATHOGENETIC MECHANISM OF AVIAN INFECTIOUS CORYZA IN CHICKEN AND JAPANESE QUAIL

Thesis

Submitted to Guru Angad Dev Veterinary and Animal Sciences University

in partial fulfillment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY PATHOLOGY
(Minor Subject: Veterinary Microbiology)

By

Aadish Balouria
(L-2014-V-92-M)

Department of Veterinary Pathology
College of Veterinary Science

Ludhiana-141 004

2017

CERTIFICATE I

This is to certify that the thesis entitled, **"Comparative studies on Immuno-pathogenetic mechanism of avian infectious coryza in chicken and Japanese quail"** submitted for the degree of **M.V.Sc.**, in the subject of **Veterinary Pathology** (Minor subject: **Veterinary Microbiology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Aadish Balouria (L-2014-V-92-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

(Dr. Sidhartha Deshmukh)
Major Advisor
Assistant Veterinary Pathologist
Department of Veterinary Pathology
GADVASU, Ludhiana – 141004

CERTIFICATE II

This is to certify that the thesis entitled, **"Comparative studies on Immuno-pathogenetic mechanism of avian infectious coryza in chicken and Japanese quail"** submitted by **Aadish Balouria (L-2014-V-92-M)** to Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfillment of the requirements for the degree of **M.V.Sc.** in the subject of **Veterinary Pathology** (Minor subject: **Veterinary Microbiology**) has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

(Dr.Sidhartha Deshmukh)
Major Advisor

(Dr. Jyoti Misri)
External Examiner
Principal Scientist
Animal Health, Rajendra Prasad Road, ICAR, Krishi Bhavan,
New Delhi - 110001

(Dr. C.K. Singh)
Head of the Department

(Dr. Simrat Sagar Singh)
Dean, Postgraduate Studies

ACKNOWLEGDEMENT

First and foremost, I express my deepest gratitude to the Almighty and my Parents, by whose blessings I have been able to complete another coup of my life.

I express my sincere heartfelt gratitude to my major advisor ***Dr. Sidhartha Deshmukh,*** *Assistant Veterinary Pathologist, Department of Veterinary Pathology for his immense guidance, constant motivation and ready help rendered during the research work and thesis writing. I will be ever grateful and obligated to him for all his cooperation and timely advice.*

I feel privileged to express my gracious thanks to the respected members of my advisory committee ***Dr. Mudit Chandra*** *(Minor Advisor), Assistant Scientist, Department of Veterinary Microbiology* ***Dr. H. S. Banga****, Professor, Department of Veterinary Pathology,* ***Dr. C.K. Singh*** *(Dean PG's Nominee), Senior veterinary pathologist, Department of Veterinary Pathology, for their timely help, invaluable suggestion when-ever needed in the present study and enquiring the progress of work from time to time.*

I wish to extend my grateful thanks and venerations to all the respected faculty members of my department: ***Dr. R.S. Brar,*** *Professor,* ***Dr. B.S. Sandhu,*** *Professor,* ***Dr. N.K. Sood,*** *Sr. Veterinary Pathologist,* ***Dr. Amarjit Singh,*** *Professor,* ***Dr. Kuldip Gupta,*** *Professor,* ***Dr. A.P.S Brar,*** *Associate Professor,* ***Dr. N.D Singh,*** *Assistant Professor and* ***Dr. Vishal Mahajan****, Assistant Professor for letting me encroach upon their time and experiences freely in the form of technical help, answering my queries and moral support throughout my research programme. Heartfelt thanks to* ***Dr Sandip Sodhi*** *for her full cooperation and assistance at various instances.*

My vocabulary of praiseful adjectives is not sufficient to express the amount of appreciation for Dr. Gurleen Kour for rendering selfless assistance whenever and wherever needed. A special thanks to Dr. Ajaz for his willing co-operation, superb resourcefulness, motivation and moral support.

I would sincerely like to thank Dr. M M Chawak (PDRC, Pune), Dr Prashant Pawar, Dr Sodhi (Venkateshwara hatcheries, Ludhiana), Dr Shaktikant Dash (Assistant Professor, AGB), Mr. Panhale, Rakesh, Dr Dendore for their unselfish help and valuable suggestions.

I will always cherish the lighter moments spent with Dr(s) Simranjit, Karmenleen, Supreet, Meharans, Harankit, Rajat, Jatin, Ryan and my crew, and the good vibes shared with Jagmeet, Diptesh, Asma, Arati and other batchmates and juniors during my period of study.

The help rendered by the office staff (Mr. Sanjeev Kumar, Ms. Gagandeep and Mr. Rakesh) of Department of Veterinary Pathology is gratefully acknowledged. Thanks to Mr. Dilchain and Mr. Navdeep, who gave helpful laboratory assistance during my research work.

I owe my parents a tremendous respect and gratitude for their unconditional love and affection. Without their blessings, it would have been an impossible journey for me. The contribution of my mother, "Mrs. Renu Balouria" in my upbringing is immense and unfathomable. The moral support and confidence build-up provided by my father, "Mr. R.S. Balouria" has been pivotal during my entire life. All my endeavors are always dedicated to my beloved mother and father. I am grateful to the unending love and support of my loving brother "Mr. Aaditya Balouria", who have always been the bliss of my life.

Above all, I would like to acknowledge Dr. B.V Rao Poultry Research Foundation and SERB DST, Government of India, for providing financial support for this project.

I owe my heartfelt thanks to all those who supported this work directly or indirectly and helped me in making this dissertation possible.

All might not have been mentioned but none is forgotten.

Date:

Place: ***Aadish Balouria***

Title of Thesis:	:	Comparative studies on Immuno-pathogenetic mechanism of avian infectious coryza in chicken and Japanese quail
Name of the student and Admission No	:	Aadish Balouria L-2014-V-92-M
Major Subject	:	Veterinary Pathology
Minor Subject	:	Veterinary Microbiology
Name and Designation of Major Advisor	:	Dr. Sidhartha Deshmukh Assistant Veterinary Pathologist
Degree to be Awarded	:	M.V.Sc.
Year of award of Degree	:	2017
Total page of Thesis	:	100 + VITA
Name of the University	:	Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana- 141 004 (India)

ABSTRACT

Infectious coryza is almost a curse for poultry operation, especially when it occurs concurrently with other pathogens in causing swollen head syndrome. The pathogen responsible for infectious coryza is *Avibacterium paragallinarum*, inflicts an initial damage to the nasal and respiratory epithelium. This facilitates the progression of pathologic basis of disease across the nasal cavity, thereby providing a platform for other and similar kind of microbes to settle scores with the host. This study, has attempted to investigate comparative susceptibility as well as pathogenesis of infectious coryza in chicken and Japanese quail following *Av.paragallinarum* inoculation. The objective of the study included production of polyclonal antibody against *Avibacterium paragallinarum* to elucidate the early entrance/migration of the pathogen in chicken and Japanese quail. Secondarily, the interest was also to define the comparative histopathological changes along with cellular participation across the nasal cavity. *Avibacterium paragallinarum* being an upper respiratory tract pathogen invariably draws interest to work on mucosal immune response/pathogenesis as well as pathology pertaining to nasal cavity, a concept in which availability of information is scarce. In-house developed polyclonal antibody against pathogen revealed distinct specificity determined by counter immune-electrophoresis and immune dot blot. The utilization of the said polyclonal antibody has clearly demonstrated the differential migration pattern of the pathogen in chicken and Japanese quail, resultantly indicated chicken being more sensitive than Japanese quail in terms of pathogen load and subsequent tissue damage. Clinically, a similar indication was noted which was simultaneously buttressed by gross and histopathological findings. Biochemical estimation of erythrocytic lipid peroxidation and reduced glutathione suggested delayed accumulation of oxidative burden signifying possible systemic dissemination of the pathogen from the nasal cavity. Cell-wise distribution revealed initial participation of heterophilic cells replaced by massive presence of lymphoid cells, macrophages especially during the middle phase of infection. Terminal phase exhibited potential reparative attempt of the inflicted damage in both the species.

Keywords: *Av. paragallinarum*, Infectious coryza, Immunohistochemistry, Chicken, Japanese Quail, Oxidative stress, Experimental, Histopathology, Polyclonal antibody.

_____ _____

Signature of Major Advisor **Signature of Student**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Fig. No.	Title
31a.	Section middle turbinate (conchae): Only glandular hypertrophy noted. H&E X200
31b.	Section middle turbinate (conchae): Both glandular hypertrophy and hyperplasia observed with beginning of stratification (arrow) of glands. H&E X100
31c.	Section INM: Both glandular hypertrophy and hyperplasia observed with beginning of stratification of glands, goblet cell hyperplasia (arrow). H&E X200
32.	Heterophilic cell infiltration
32a.	Section middle turbinate (conchae): Scattered focal sub-epithelial infiltration in few places (arrows) confined to lamina propria, H&E X400
32b.	Section posterior turbinate: Widespread infiltration (arrow heads) with resultant spread to intra-epithelial region & sub mucosal region, within glandular lumen (arrow). H&E X200
32c.	Section middle turbinate (conchae): Dense infiltration across the lamina propria as well as in the epithelial region/sub mucosal zone. H&E x200
33.	Lymphoid cell infiltration
33a.	Section of middle turbinate (conchae): Scattered infiltration (arrow) in few places confined to lamina propria. H&E X100
33b.	Section of posterior turbinate: Widespread infiltration (arrows) with resultant spread out to intra-epithelial region and sub mucosal region replacing Bowman's gland. H&E X100
33c.	Section of middle turbinate: Dense infiltration/lymphoid aggregates (arrows) across the lamina propria as well as in the epithelial region/sub mucosal zone replacing glands. H&E X100
34.	Hemorrhages
34a.	Section of middle turbinate: Occasional to focal foci or thin foci of haemorrhages (arrows) especially around the blood vessels. H&E X200
34b.	Section of middle turbinate: Focal to large widespread areas of haemorrhages (arrows). H&E X100
34c.	Section of middle turbinate: Diffuse areas of haemorrhages (arrows). H&E X100
35.	NALT hyperplasia
35a.	35a: Section of middle turbinate: Expansion of lymphoid zone with haemorrhages. H&E X100
	35b: Section of INM: Expanded lymphoid zone (arrow) noted; often associated with encroachment to surrounding tissues. H&E X100
35b.	35c: Section of IOS: Profuse expansion of lymphoid zone (arrows) replacing the neighbouring tissue details. H&E X100
35c.	Section of IOS: Moderate brown immuno-staining noted at superficial & subepithelial region as particulate foci indicating bacterial migration. IHC, X200
36.	Section of IOS: Strong immuno-staining noted at epithelium as well as in surrounding blood vessels as diffuse distribution indicating expanded region of bacterial migration. IHC, X200
37.	Section of Middle turbinate: Very mild brown immuno-staining (arrows) noted in cilia & epithelium. IHC, X200

Fig. No.	Title
38.	Section of Middle turbinate: Mild brown immuno-staining (arrows) noted at cilia & upper epithelial surface. IHC, X400
39.	Section of Middle turbinate: Moderate immuno-staining noted at multifocal areas restricted to cilia & upper epithelial surface. IHC, X400
40.	Section of Middle turbinate: Very mild immuno-staining noted at superficial epithelium. IHC, X400
41.	Section of IOS (24h chicken): Moderate brown immuno-staining noted at superficial & subepithelial region as particulate foci indicating bacterial migration & engulfment (arrows) IHC, X400
42.	Section of INM (24h chicken): Moderate brown immuno-staining noted at superficial surface and particulate foci (arrows) indicating bacterial migration. IHC, X200
43.	Section of IOS (48h chicken): Strong brown immuno-staining of epithelial lining and particulate foci indicating bacterial migration. IHC, X200
44.	Section of MT (12h JQ): Strong brown immuno-staining noted at epithelial surface, cilia and mildly within glands. IHC, X400
45.	Section of AT (48h JQ): Moderate brown immuno-staining noted at the junction of anterior & middle turbinate (line). IHC, X100
46.	Section of AT (7d JQ): Moderate brown immuno-staining characterized by granular appearance lining modified keratinized epithelium. IHC, X400

LIST OF ABBREVIATIONS

%	Percent
@	at
µl	Microlitre
μM	Micromole
^{0}C	Degrees of centigrade
A.	*Avibacterium*
AP-1F	*Avibacterium paragallinarum* forward primer
AP-1R	*Avibacterium paragallinarum* reverse primer
ARDRA	Amplified 16s ribosomal DNA restriction analysis
ARP	Avian respiratory phagocytes
Av.	*Avibacterium*
BA	Blood agar
BALT	Bronchial-associated lymphoid tissue
bp	Base pair
BSA	Bovine serum albumin
CALT	Conjunctiva-associated lymphoid tissue
CFU	Colony forming unit
CIE	Counter immunoelectrophoresis
cm	Centimeter
Co.	Company
CO_2	Carbon dioxide
DAB	3,3'-diaminobenzidine
dl	Deciliter
DNA	Deoxyribose nucleic acid
DPI	Days post-infection
DPX	Distyrene, a plasticizer and xylene
DTNB	Ellman's reagent
DW	Distilled water
EDTA	Ethly-Dimethyl Triacetate
ERIC	Enterobacterial repetitive intergenic consensus
et al	and others
etc.	Etcetera
FARM	Free avian respiratory macrophages
FCA	Freund complete adjuvant

Fig.	Figure
g	Gram
GADVASU	Guru Angad Dev Veterinary and animal Sciences University
GC	Germinal center
GSH	Reduced gluatathione
h	Hours
H.	*Haemophilus*
HA	Haemophilus agar
HI	Hemagglutination inhibition
i.e.	That is
IAEC	Institutional animal ethics committee
IC	Infectious Coryza
IHC	Immunohistochemistry
INM	Inferior nasal meatus
IOS	Infraorbital sinus
JQ	Japanese quail
kbp	Kilobase pair
lb	Pound
Ltd.	Limited
MCT	Microcentrifuge tube
MDA	Malondialdehyde
MHC	Major histocompatibility complex
min	Minutes
mls	Mililtres
MR	Methyl red
MT	Middle turbinate
NA	Not appreciated
NAD	Nicotinamide dinucleotide
NALT	Nasal-associated lymphoid tissue
NBF	Neutral buffered formalin
ND	Nasal discharge
NFW	Nuclease free water
*n*g	Nanogram
NK	Natural killer cells
No.	Number
OD	Ocular discharge

ONPG	Ortho-Nitrophenyl-β-galactoside
PBS	Phosphate buffered saline
PCI	Phenol chloroform isoamyl alcohol
PCR	Polymerase chain reaction
PCR	Polymerase chain reaction
PDRC	Poultry Diagnostic & Research Centre
PES	Polyethylene sulphone
PG	Paranasal glands
PI	Post inoculation
PT	Posterior turbinate
PVDF	Polyvinylidene fluoride
RC	Redness of conjunctiva
RFLP	Restricted fragment length polymorphism
RNA	Ribonucleic acid
rpm	Rotation per minute
Rpm	Rotation per minute
rpm	Revolutions per minute
RPO	Redness of periorbital area
RT	Room temperature
SDS	Sodium dodecyl sulphate
SOIS	Swelling of infraorbital sinus
TBA	Thiobarbituric acid
TBE	Tris buffer EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline tween 20
URT	Upper respiratory tract
viz.	Namely (Latin Videlicet)
VP	Voges proskauer
µm	Micrometer

CHAPTER I

INTRODUCTION

Under intensive poultry operations, the infections affiliated to respiratory tract are innumerable and attribute enormous hindrance to the productive ability of birds. Their impact abrogates not only the commercial parameters of poultry industry, but has also downtrodden faith among poor farmers, thus discouraging their economic upliftment. Broadly considering, such incessant and uncontrollable respiratory tract infections in poultry operations are viewed as multi-factorial in nature (Jordan *et al* 2005). Complacent managemental practice(s), inadequate offering of quality feed stuffs, poor nutritional supplementation, poor housing facilities and bird's respiratory tract anatomy, all collectively and responsibly have steered towards specific infection patterns besides increasing the severity of conditions (Nighot *et al* 2002). It is paradoxical that respiratory tracts infections in poultry are clinically and etiologically indistinguishable as several pathogens present in concert, to deliver an overlapping clinical outcome with characteristic sneezing**,** gasping, facial swelling, nasal discharge and respiratory distress. Such varied infectious pathogenic insults have invariably affected our disease preventive strategies, thus resulting in significant disease occurrence and serious economic losses.

Treatment with antibiotics and chemical compounds in farm productions has been attempted earlier with success against all infectious diseases, however they were found to be short lived in efficacy with detrimental outcomes like genetic biotransformation and subsequent ecological bioaccumulation (Ispir *et al* 2009). Thus, a call for their replacement is warranted, with vaccine development and new vaccination strategies, which are currently underway as a master precautionary plan against the incidences of infectious diseases among birds and animals.

Recent experiences about recurrent infections with infectious coryza (IC) in numerous poultry farms, caused by *Avibacterium paragallinarum* have raised several concerns with context to rampant usage of antibiotics, which insinuate(s) inadequate efficacy of available antibiotics over continual changing molecular pattern of infectious pathogen(s) and its consequent meandering cum injurious impact over public health.

The pathogen *Avibacterium paragallinarum* (a Gram-negative bacterium) by its extant, considered as highly heterogeneous species not only with regards to virulence, but also at genetic and antigenic level, having little conferment of heterologus immunity, which accounts for poor host's immune protection (Bragg *et al* 1996). The pathogen is largely responsible for increased number of culls and fall in egg production (10% to over 40%) especially in multi-aged farms (Blackall 1999). The organism was previously classified under Family *Pasteurellaceae* as *Hemophilus paragallinarum* (Blackall *et al* 2005), is now rechristened as *Avibacterium paragallinarum.* Now a days, *Avibacterium paragallinarum* is rarely the sole etiological agent behind the development of clinical signs, it almost always occurs in conjunction with other pathogens thus making its clinical diagnosis as well as treatment very difficult (Glisson 1998). The term "Swollen head syndrome" which was earlier exclusively associated with infectious coryza can now be a manifestation of several infectious diseases like Mycoplasmosis, *Ornithobacterium rhinotrachaele* infections, or infections with infectious bronchitis virus. Incidentally, pathogen's strong ability to act as initial nidus of infection for other secondary invaders cannot be denied (Rajurkar *et al* 2009). The infection once set in, runs a rapid onset, with marked decrease in feed intake, occulo-nasal discharge, conjunctivitis, facial edema, swollen infraorbital sinus, dyspnea and lowered egg production/growth (Eaves *et al* 1989). Simultaneously, in recent times, reports related to disease occurrence in other species of poultry have also emerged, as noted in ornamental birds (Priya *et al* 2012) and Japanese quail (Thenmozhi and Malmarugan 2013) respectively.

Vaccination program against infectious coryza (IC) is frequently practiced in poultry worldwide and is mainly offered at 6-8 weeks of age in birds. Both killed and live vaccines are available; however, majority of them have lacked convincing immune protection against the pathogen and finally cued as complicity of crowded serovar(s) for its unsuccessful outcome (Sandoval *et al* 1994). The large number of empirical experiences pertaining to vaccine development study against this disease principally concentrated on showing reduction in clinical symptoms as the parameter for vaccine's efficacy without considering host's paramount and inherent intricate immune ability against the pathogen. In addition to it, a major quantum of research

work against this disease has principally deliberated over vaccine improvement rather than on understanding the execution of elements of immune system in conferring immunity against the normal course of infection, in addition to its pathogenesis. The classical pathological studies mainly focused on systemic tissue changes pertaining to the severity of cases and didn't involve recognizing pattern of histological changes confined to nasal passage in sequential and temporal manner, where the organism has propensity to establish initially. In our opinion, for any successful vaccination output it is necessary to asses initially, the invasion pattern being adopted by a pathogen to analyze its possible impact over host's immune system vis-a-vis host's qualitative response that governs mitigation of infectious assaults.

Avibacterium paragallinarum, an upper respiratory tract pathogen primarily affects the bird's nasal passage and its associated structure, was therefore mainly focused for understanding pathologic changes in nasal associated mucosal histo-architecture, immune relevant cell populations and its regulation(s) in temporal manner. This study also intended to understand the development of acquired nasal associated mucosal immunity as well as building up of infection pattern at an early age of chickens i.e. at 2.5 weeks, when ontogeny of localized immune system starts to begin (otherwise believed to get provoked clinically by 4^{th} weeks of age in chickens during *Av.paragallinarum* infection). Acquaintance of this knowledge would help us to underpin specific immunologic response exerting resistance or increased susceptibility among birds originated at localized area of mucosal membranes, where the pathogen (*Av.paragallinarum*) initially infects the hosts, besides gaining information about building of immunological memory in birds for longer duration, if only vaccination pre-poned by early 2-3 weeks of age. Further we speculate, variations in pathologic and immunologic process between chicken and Japanese quail, that might help us to build explanation for species specific deviations (in disease pathogenesis process /development of protective immunity), which can be explored for futuristic vaccine mucosal development/formulations. Furthermore, in order to simulate conditions of infectious coryza in small chicks, we decided to use Japanese quail as a possibility to replacement small chicks as the model vis- a-vis a possible study model for comparative pathological analysis for our future work.

Therefore, the objectives of this thesis were two-fold and were undertaken as follows:

1. To produce polyclonal antibody against infectious coryza organism for understanding the portal of entrance, differential pathogenetic process in chicken and Japanese quail respectively.
2. To evaluate the temporal participation of immune cell types as well as histological changes in different organs during different phases of infection in both chicken and Japanese quail.

CHAPTER II

REVIEW OF LITERATURE

2.1 Histo-anatomical descriptions of nasal passage (Poultry)

The respiratory system of birds shows many unique features when compared to that of mammals. The unique structures include air sacs, bronchial tree structure and breathing system. The respiratory system consists of nostrils leading to the nasal chamber, larynx, trachea, syrinx, bronchi, lungs and the air sacs (Getty1975).

Recently, Yan *et al* (2014) elaborated histo-anatomical description of poultry nasal cavity and explained the presence of nasal turbinate(s) occupying most of the space of chicken nasal cavity, which are mainly covered by stratified squamous epithelium more specifically at anterior nasal cavity (regio vestibularis). In the middle part of nasal cavity (regio respiratoria), the turbinates (concha nasalis media) are mainly lined by pseudo-stratified and ciliated columnar epithelium, where large number of simple and compound alveolar mucous glands containing goblet cells are located just beneath the epithelial lining. The surface of the posterior turbinate (concha nasalis caudalis) at posterior nasal cavity (regio olfactoria) is covered by simple epithelium consisting of flask shaped olfactory (Bowman) glands. The nasal associated lymphoid tissue (NALT) is present on both sides of choanal cleft, at the base of nasal septum as well as in the postmedian part of nasal cavity. Additionally, diffuse lymphoid tissues were found to be present under the epithelium of the concha nasalis media (middle turbinates) and the walls of nasal cavity. Similarly, Kang *et al* (2013) documented presence of large number of lymphoid cells under the epithelium of inferior nasal meatus. NALT (nasal-associated lymphoid tissue) present in nasal cavity are the first to encounter microorganisms that enter the body with inhaled air. Lymphocytes are also present in the paranasal glands and in their secretory ducts (Ohshima and Hiramatsu 2000).

Cevik-Demirkan *et al* (2007) studied the gross morphological features of nasal cavity of Japanese quail. Nasal cavity was found to be anatomically similar to that of chicken except for few differences. The size of nostril was noted to be considerably reduced due to well-developed operculum dorsally and vertical lamella ventrally at anterior nasal cavity. ‘C’-shaped anterior turbinate (rostral nasal concha) was found to impart complete obliterations of nasal cavity. The middle turbinate (middle nasal concha) was the largest of all and exhibited a scroll-like structure with

one-half turning ventro-laterally when seen in cross section. The posterior nasal turbinates (caudal nasal concha) was noted to be the smallest one, resembled a hemisphere in shape with a free cranial edge and the caudal border attaching to the olfactory region of the nasal cavity. Infraorbital sinus was found to be highly developed in the Japanese quails.

Kang *et al* (2014) characterized the distribution of NALT in the nasal cavity of ducks while studying the gross nasal anatomy and histology. Anterior nasal turbinate (concha nasalis rostralis) was observed to be less developed while rest of histological architecture was found to be similar to that of chickens except for few differences in distribution of NALT and the presence of keratinized epithelium in the anterior part of middle turbinate. An age-dependent increase in the presence of lymphoid aggregates in the nasal sub-epithelial layers was noted, with absolute absence in less than 3 weeks old ducks to a marked appreciable distribution in more than 2 months old ducks.

2.2 Infectious coryza and its clinical outcome

Infectious coryza (IC) is an acute respiratory disease of chickens caused by the bacterium known as *Avibacterium paragallinarum* and affects upper respiratory tract and para-sinuses of chickens (Blackall *et al* 2005) causing huge economic losses. The impact is so severe to the extent that eatable quality of broiler chickens becomes extinct and reduced egg production in layers becomes apparent (Mouahid *et al* 1989). As per earlier literature, the syndrome associated with infectious coryza was referred as roup, contagious or infectious catarrh, cold and coryza (Yamamoto 1972). The disease was named infectious coryza because it was infectious in nature and primarily affects the nasal passages (Beach and Schalm 1936).

Infectious coryza is known to occur in poultry birds of all age groups (Yamamoto 1991), and observed to be less effective in juvenile birds. However, Blackall (2011) suggested increasing susceptibility noted 4 weeks of age from hatching in chickens, a fact that calls for a timely and early vaccination. The incubation period is shortened, and the course of the disease tends to be longer in mature birds, especially hens with active egg production (Blackall & Soriano 2013).

The disease has been noted to occur more frequently as an insidiously progressing malady with a complicated infectious course in developing countries in contrast to developed countries where it occurs more commonly as an uncomplicated

disease (Deshmukh *et al* 2015). In the initial stages of infection, the main clinical sign includes sero-mucus discharge(s) oozes out from nostrils and eyes which later converts into caseous flake-like exudates and deposits in the nasal passages, around the nostrils as well as in eyes, consequently resulting in closure of eyes. Birds dying due to chronic infection may reveal similar flaky material as creamish white organized mass either unilaterally or bilaterally in the infra-orbital/supra-orbital sinuses (Deshmukh *et al* 2015). Besides primarily affecting the upper respiratory tract (URT), the infection may also descend to trachea, air sacs as well as cause pneumonia in very extreme cases. In layers, the organism may also affect the reproductive organs viz; ovary and oviduct and can lead to lowered egg quality and decreased egg production. In meat type birds, the disease can lead to poor growth rate and a decline in feed conversion efficiency due to inanition, leading to increased number of culls. (Deshmukh *et al* 2015).

The causative organism, previously known as *Hemophilus paragallinarum* was reclassified to its present name based upon 16s rRNA gene sequencing (Blackall *et al* 2005). Serotyping has been done using two different but similar schemes namely Page and Kume schemes (Blackall 1999). Using a plate or slide agglutination test, Page categorized *A. paragallinarum* in three serovars; serovars A, B and C (Page 1962). The three page serovars have been recognized as three distinct immunovars, as vaccine against one serovar does not provide protection against the other two (Blackall 1999). Kume serotyping scheme utilized hemagglutination-inhibition tests to recognize seven serovars (A1, A2, A3, B1, C1, C2, C3 and C4) which were organized into three serogroups (I, II and III) (Kume *et al* 1983).

2.3 Growth requirements and culture characteristics of *Avibacterium paragallinarum*

Nicotiniamide adenine dinucleotide (NAD) is necessary for the *in vitro* growth of most isolates of *A. paragallinarum* (Page 1962 and Rimler *et al* 1977) which may be added separately to the growth media. Apart from it, a feeder culture of *Staphylococcus aureus* can be streaked if the organism is being grown on blood agar (Quinn *et al* 1994). In addition to above, carbon dioxide (5-10%) was found to enhance the growth of bacterium (Schalm and Beach 1936).

As per cultural characteristics, the organism form tiny dew-drop like colonies on blood agar plates in the zone of hemolysis provided by a *Staphylococcus aureus* (Prasad *et al* 1999).

Prasad *et al* (1999) explored the morphological characteristics of *Avibacterium paragallinarum* in 58 isolates and found it to be Gram-negative non-motile bacterium, which appears as short rods or cocco-bacilli with polar staining in 24 hour cultures, with a tendency to form filaments over the time. The organism undergoes degeneration within 48-60 hrs, showing fragments and indefinite forms (Yamamoto 1991)

All species under the genus *Avibacterium* reduce nitrate to nitrite and also ferment glucose with no gas formation. Other biochemical properties that are common to all include positive oxidase activity, presence of alkaline phosphatase enzyme and no indole production or failure to hydrolyze urea (Blackall 1990). *A. paragallinarum* can be differentiated from other *Avibacterium* species by its failure to ferment galactose or trehalose and also from its lack of catalase activity (Prasad *et al* 1999).

2.4 Molecular characterization of pathogen (*Avibacterium paragallinarum*)

Chen *et al* (1996) developed a species-specific PCR (HPG-2 PCR) for the identification of *Avibacterium paragallinarum* which showed 100% agreement with the culturing method. It was also shown to be a reliable detection method when direct nasal swabs were used.

Hobb *et al* (2002) sequenced and cloned the hag A gene responsible for encoding hemagglutinin by *Avibacterium paragallinarum* thereby developing a recombinant *Avibacterium* recombinant hemagglutinin protein.

Soriano *et al* (2004) reported that an enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) could be used to identify Page serovars, but the analysis of results was deemed to be cumbersome since ERIC-PCR showed many patterns even within each serovar.

Espinoza *et al* (2008) employed ARDRA (amplified 16s ribosomal DNA restriction analysis) technique for the identification of *Avibacterium paragallinarum* from several field isolates in Peru. The technique involved amplification of 16s ribosomal DNA by PCR followed by restriction enzyme digestion (Dde I and Rsa I) and subsequent fragment analysis.

Sakamoto (2012) developed PCR to differentiate between serotypes A, B and C by targeting the hypervariable region of *HMTp210* gene which encodes for HA

antigen. Primer sets designed against the hypervariable region amplified 0.8, 1.1 and 1.6 kbp fragments for serovars A, B and C, respectively, thus providing a quick molecular basis of serotyping of *A. paragallinarum* isolates.

El-Sawah *et al* (2012) explored the utility of Western blot analysis and nucleic acid sequencing differences in differentiating between A and C Page serotypes and found that there were three SNPs (Single nucleotide polymorphism) between the two serotypes and one additional single nucleotide deletion on C serotype.

Anjaneya *et al* (2014) characterized the Indian *A. paragallinarum* serovars at molecular level based on the Restriction Fragment Length Polymorphism (RFLP) of 16S ribosomal gene amplified, amplified 16S ribosomal DNA restriction analysis (ARDRA), sequencing of haemeagglutinin antigen (hagA) gene and phylogenetic analysis. The 'hagA' encoding the haemeagglutinin antigen of *Av. paragallinarum* from all the field isolates exhibited homology between Australian A and C serovar strain but it could not be grouped based upon Page's scheme of classification for serovars.

Muhammad and Sreedevi (2015) standardized polymerase chain reaction (PCR) for the diagnosis of infectious coryza by using infectious coryza Killed vaccine as source of DNA of *A. paragallinarum*.

2.5 Epidemiological distribution, production losses due to infectious coryza.

2.5.1 At international level

Infectious coryza can be encountered almost everywhere where intensive poultry farming is practice although it has been reported in less intensive situations as well.

Chen *et al* (1993) estimated the economic impact of infectious coryza over a three years period (1986 to 1988) on China to be about 100 million Yuan (approximately $US16.5 million).

Akhtar *et al* (2001) observed the course and results of an infectious coryza outbreak in a white leghorns layer farm with total population of 20,000 in Arifwala, Pakistan. A production drop from 85 per cent to 60 per cent was noted in a span of 3 days.

Bland *et al* (2002) investigated an outbreak of the disease in older layer birds in California, which caused a total mortality of 48% and a drop in egg production from 75% to 15.7% over a 3-week period.

Chukiatsiri and Chansiripornchai (2008) documented several serovar A and C outbreaks of infectious coryza in industrial and native chickens in Thailand. In 2010, serovar B outbreak was also observed (Chukiatsiri *et al* 2010) thus marking the presence of all the known *Avibacterium paragallinarum* serotypes in Thailand.

Avibacterium paragallinarum serotype B was also isolated from a coryza outbreak in broiler breeders in Panama (Calderon *et al* 2010). Disease outbreaks were characterized by an up to 45% drop in egg production and increased mortality. Use of a commercial trivalent vaccine and an improved biosecurity program was shown to prevent outbreaks in susceptible flocks.

Welchman *et al* (2010) found the presence of disease in multi-age hobby flocks of pure breed and hybrid chickens in Southern England. A mortality of 4.5% was recorded and a positive response to antibiotics was also observed.

Akter *et al* (2014) collected 122 samples from different areas of Bangladesh for the period of March 2011 to February 2014. The higher rate of occurrence of *A. paragallinarum* was found in Sylhet (66.66%) and lowest in Dhaka (43.75%). Higher incidence rate was found in winter season (52.26%) in comparison with summer season (1.85%).

Han *et al* (2016) conducted a serological survey in South Korea and found the rate of cases exhibiting clinical signs in the upper respiratory tract and decrease in egg production to be high, with positivity rates of 86.4% in 2009, 78.9% in 2010, 70.0% in 2011, and 69.6% in 2012, all the isolated strains being Page serovar A.

Interestingly, New Zealand was documented to be the only country in the world to be free from infectious coryza (IC), thanks to their stringent quarantine system, where it is considered as an exotic disease (Vargas and Terzolo, 2004).

2.5.2 At National level

Avibacterium paragallinarum Page serovars A and C have been identified to be prevalent in India (Tongaonkar *et al* 2002). Similarly, Haunshi *et al* (2006) reported the presence of infectious coryza in Vanaraja poultry, a local indigenous breed of chicken from Meghalaya.

Priya *et al* (2012) isolated and identified *Avibacterium paragallinarum* from a commercial flock of thirty (30) ornamental birds in Thrissur, Kerala highlighting the presence of the disease beyond commercial poultry.

Anjaneya *et al* (2014) investigated freshly dead and ailing birds from thirty-four (34) commercial farms with the history of respiratory distress, facial swelling and conjunctivitis, from four (4) different states of India viz., Uttar Pradesh, Karnataka, Maharashtra and Madhya Pradesh from May 2010-August 2011.

Muhammad and Sreedevi (2015) studied five (5) outbreaks of infectious coryza from Andhra Pradesh and tested a total of fifty six (56) infra orbital sinus swabs and twenty two (22) nasal swabs by PCR. Of fifty six (56) infra orbital sinus swabs tested, forty seven (47) were positive (83.9%) and nine (9) nasal swabs (40.9%) out of twenty two (22) tested were found to be positive for infectious coryza.

Recently, Patil *et al* (2016) collected fifteen (15) field isolates of suspected *Avibacterium paragallinarum* from major poultry rearing states of India including Punjab, Haryana, Maharashtra, Andhra Pradesh and Tamil Nadu, therefore confirmed and serotyped them through molecular techniques. Out of fifteen (15), five (5) isolates were serotype A, two (2) were serotype B whereas one (1) isolate were found to be serotype C.

2.6 Disease transmission

The diseases transmission is more severe where multi age flocks are kept. The intense rearing practice not only accentuates the problem of mixed respiratory tract infections, but also galvanizes the occurrence of singular pathogen led infection to acquire more serious outcomes.

Clark and Godfrey (1961) reported the spread of disease in multi-aged farm from infected older birds to the susceptible younger flock (1-6 weeks) following their movement from brooder house to the proximity of older groups of infected birds.

Yamamoto & Clark (1966) suggested transmission of *A. paragallinarum* by airborne route in isolated farms. So far in-ovo transmission has not been seen or reported in case of infectious coryza infection.

In general, chronic or healthy carrier birds have long been recognized as the main reservoir of infectious coryza (IC) infection. Blackall *et al* (1990) confirmed

the role of carrier birds in disease transmission by the application of molecular fingerprinting techniques.

The occurrence of infectious coryza has been observed to be more frequent in winter and fall, but such findings need to be separately studied after dissociating them from other factors like seasonal change in managemental practices e.g., introduction of susceptible replacement pullets onto farms where infectious coryza is prevalent (Blackall & Soriano, 2013).

2.7 Pathogenesis

Iritani *et al* (1981) explained that crude polysaccharide derived from *A. paragallinarum* as a responsible factor for the elicitation of adverse clinical signs following administration of bacterin to chickens.

Various factors have been associated with the pathogenicty of *Av.paragallinarum*, the most important being the haemeagglutinin (HA) antigen. Studies conducted by Sawata and Kume, (1983) and Yamaguchi *et al* (1993) observed that the HA antigen acts significantly in colonization of the organism on to the mucosal surface. In addition, capsule has also been implicated to play an important role in colonization of bacteria (Sawata and Kume, 1983; Sawata *et al* 1985) and also protecting the bacteria from the bactericidal activity of chicken serum (Sawata *et al* 1984).

According to Kume *et al* (1984) a toxin released from capsular zone of bacteria during multiplication within chicken embryo was responsible for the clinical disease development. Colonization of the nasal mucosa by encapsulated *A. paragallinarum* is suggested to be necessary to initiate morphological events in the nasal mucosa (Sawata *et al* 1985).

Rocha *et al* (2006) conducted an electron microscopy based study and described release of extracellular membrane vesicles (MVs), containing immunogenic proteins, proteases, putative RTX proteins, hemagglutinin, and nucleic acids by *A. paragallinarum* which might play role in the pathogenicty of disease process.

2.8 Lesions (pathology) associated with infectious coryza (IC)

2.8.1 Gross lesions

Gross lesions caused by *A. paragallinarum* include an acute catarrhal inflammation of mucous membranes of nasal passages and sinuses along with

catarrhal conjunctivitis and subcutaneous edema of face and wattles. Pneumonia and air-sacculitis can be a rare finding, however, it has been observed in some broiler outbreaks where infected cases showed increased condemnation due to air-sacculitis, particularly in absence of any other recognized viral or bacterial pathogens (Droual *et al* 1990 and Hoerr *et al* 1994).

Anjaneya *et al* (2014) noted the development of gross lesions over a period of 10 days in 7 weeks old chickens experimentally infected with *A. paragallinarum*. The infected birds showed nasal cavities filled with grayish white thick exudates with congestion and edematous swelling of mucus membranes within 48 hours after infection. Presence of exudate was seen in infraorbital sinuses cavities along with edema of periorbital subcutaneous tissue by 72 hours of infection. Spread to lower respiratory tract and in some cases, involvement of airsacs was observed particularly during later periods of infection.

Akter *et al* (2013) examined infectious coryza cases and found the presence of hemorrhages in mucus membranes of nasal passage and trachea in two out of four (4) cases of infectious coryza.

Ramachandran *et al* (2013) studied the pathogenicity of field isolates of *A. paragallinarum* by experimentally inoculating three (3) seven weeks old chickens and observed the accumulation of yellowish white inflammatory exudates in the trachea and congestion tracheal membrane as the main post-mortem findings.

2.8.2 Microscopic lesions

Fujiwara and Konno (1965) studied histopathological alterations due to *A. paragallinarum* infection in chickens at 12 hours to 3 months post intra-nasal inoculation. Sloughing, disintegration, hyperplasia of mucosal and glandular epithelial cell, edema as well as hyperemia and infiltration of heterophils in the tunica propria of the mucous membranes were the main changes noted in the nasal cavity, besides involvement of infraorbital sinuses, and trachea. The initiation of pathologic changes was first observed at 20 hours post inoculation which reached its maximal severity by 7-10 days post infection, and a gradual repair was noted by 14-21 days post infection. Acute catarrhal bronchopneumonia with heterophilic infiltration and cellular debris in the lumen of secondary and tertiary bronchi; hyperplasia of epithelial cells of air capillaries primarily prevailed in birds showing lower respiratory tract infection.

In addition, Sawata *et al* (1985) noted profound mast cell infiltration in the lamina propria of the mucous membrane of the nasal cavity. Various products released by mast cells, heterophils, and macrophages were implicated in causing severe vascular changes and cellular damages resultantly produced infectious coryza infections.

Akter *et al* (2013) illustrated acanthosis, congestion, and mucoid gland cell hyperplasia, besides hyperplasia of nasal sinus epithelium and even parakeratosis of nasal epithelial lining in positive cases of infectious coryza

Ali *et al* (2013) studied pathogenicty of local *A. paragallinarum* isolates from Bangladesh by experimentally inoculating two weeks old chickens and observed acanthosis and congested blood vessels of nasal passage, pneumonic lesion of lung, focal hepatitis of liver and fatty change and lipid nodules in macrophages of heart as an important microscopic pathology.

Anjaneya *et al* (2013) tested the pathogenicity of Indian field isolates by experimentally inoculating four field isolates in seven weeks old chickens and examining them for disease progression over a period of seven to ten days in a time-based manner. The microscopic examination of mucosal, epithelial and glandular cells of middle, and rostral nasal sinuses revealed hyperplasia and hyperactivity with intense infiltration of heterophils and mononuclear cells in lamina propria after three days of infection. Simultaneously, sloughing of mucosal and glandular epithelial cells was noted in nasal mucosa. Other changes included hyperplasic epithelial cells along with heterophilic and lymphocytic infiltration in lamina propria along with similar changes in the infraorbital sinus.

2.9 Oxidative stress during respiratory tract infection

Placer *et al* (1966) estimated the levels of malondialdehyde in tissue lysates by using thiobarbituric acid (TBA) and alkaline pyridine-butanol reagent which lead to the formation of a colored trimethine substance which was spectrophotometrically measured.

Emerit and Chaudiere (1989) described the potential role of lipid peroxidation and products of peroxidation in the degenerative changes associated with aging and also in the pathogenesis of several clinical diseases.

Esterbauer *et al* (1991) noted that lipid peroxidation often occurs in the body in response to oxidative stress, which leads to the formation of a variety of aldehyde compounds, MDA (malondialdehyde) being one of the reactive aldehydes.

Gutteridge (1995) noted that altered balance between the generation of various reactive oxygen species such as superoxide, hydrogen peroxide, hypochlorous acid and hydroxyl radicals; and antioxidant defense machinery against them leads to oxidative stress, which precipitates tissue damage by releasing prooxidative forms of reactive iron that are able to cause lipid peroxidation and by depleting protective antioxidants.

Zhao *et al* (2003) documented an increase in the serum MDA content of Beijing ducks infected with different strains of duck hepatitis virus.

Georgieva *et al* (2005) monitored the levels of MDA, SOD (superoxide dismutase) and catalase in birds infected with *Eimeria tenella* and found increase in the MDA and catalase in the infected birds and a decrease in the levels of SOD suggesting impaired antioxidant mechanism in the infected birds.

Keles *et al* (2009) infected white Lohmann hens with Marek's disease virus and found the MDA to be elevated and GSH (Reduced glutathione) to be decreased in the infected group of birds.

Wang *et al* (2011) investigated the changes in serum oxidative index in eighty (80) 15-day-old chickens experimentally infected with infectious bronchitis virus and found the levels of MDA to be significantly higher in the infected group, especially at 6^{th} day and 9^{th} day after infection.

Sodhi *et al* (2017) observed an increase in the lipid peroxidation in erythrocytes and liver of chickens in the form of elevated level of MDA, upon administration of bromadiolone, a rodenticide. A simultaneous decrease in the levels of erythrocytic reduced glutathione was also noted.

2.10 Participation of immune system in response to respiratory tract infections.

The respiratory system harbors the most extensive and thinnest surface across which the body is exposed to the external environment. Due to this characteristic, a vast array of proteins and pathogens are challenging this system on a daily basis. To cope with these pathogens, birds have well-developed defense mechanisms.

2.10.1 Innate immune response

The first line of defense to prevent the pathogens from invading the body includes the nasal and tracheal epithelium along with multiple mucous glands which thus traps the bacteria and excrete it via sneezing and coughing (Koch 1991 and

Sharma 2003) or by the action of various antibacterial enzymes which prevents the colonization of organism. The pathogens that manage to enter the body are then attacked by the cells of innate immune mechanism like macrophages, heterophils, and natural killer (NK) cells. Macrophages are active in innate as well as acquired immunity (Qureshi *et al* 2000). The avian respiratory tract is more easily attacked by pathogens as compared to mammalian respiratory tract due to its large proportion and much thinner tissue barrier (Nganpiep and Maina 2002). Another defense system i.e. avian respiratory phagocytes (ARP) (includes macrophages and polymorpho-nuclear leukocytes such as heterophils) occur on the surface of avian lungs. Paradoxically, the level of ARPs is very low in normal, steady-state as compared to the infectious state where heavy inflow of ARP occurs in response to non-specific infections caused by the bacteria and other pathogens (Ficken *et al* 1986; Qureshi *et al* 1994; Klika *et al* 1996; Qureshi *et al* 2000; Toth 2000). Though these ARPs are present extensively in mammalian respiratory tract, the scarcity or even absence of ARPs in birds make them more susceptible to the respiratory infections.

However, studies done by Nganpiep and Maina (2002) showed that a composite defense weapon protecting respiratory system has additionally developed in avian species which includes highly lytic upper airway epithelium equipped with lysosomes, ARPs, and efficient translocation of sub-epithelial macrophages onto the respiratory surface (Nganpiep and Maina 2002). Clearance of pathogens from air sacs which are thin walled and lack ciliated epithelium is accomplished by phagocytic cells (Nganpiep and Maina 2002; Reese *et al* 2006).

2.10.2 Adaptive Immune response of respiratory tract

The upper respiratory tract has various associated structures, such as Harderian gland (HG), conjunctiva-associated lymphoid tissue (CALT) and paranasal glands (PG), which participate in local mechanisms of the mucosal immunity. (Smialek *et al* 2011).

Aggregates of lymphoid tissue in the nasal mucosa (NALT - nasal-associated lymphoid tissue) are the foremost portion which encounters inhaled pathogens. Cellular composition of lymphoid nodules includes B cells with frequently developed germinal centres (GC), with a periphery of $CD4^+$ cells, whereas $CD8^+$ cells are found in the mucosal layer and in the lamina propria of nasal epithelium. Trachea has been shown to be reactive to pathogenic insult despite of

lack of lymphoid tissue. B cells secrete antibodies (IgM, IgY or IgA) upon contact with an immunogen (Smialek *et al* 2011).

Bronchus-associated lymphoid tissue (BALT) is the major hub of bronchial immune processes and its morphological and functional profile in birds is age-dependent.

Macrophages make up the first line of defense mechanisms followed by polymorphonuclear cells mainly heterophils. Phagocytic cells in lungs known as FARM (free avian respiratory macrophages) are considerably several times lower in number in chickens and turkeys when compared to rodents (Smialek *et al* 2011).

In general adaptive immunity includes both cell mediated and humoral response, activation of both are regulated by the T lymphocytes (T cells), maturation of which occurs in thymus, a feature shared with mammalian species (Arstila *et al* 1994). The antigen has to be presented by host cells in the context of their major histocompatibility complex (MHC) molecules, only then T cells can initiate the immune response to a pathogen. Chicken MHC is mainly defined by reaction of erythrocytes with antibodies specific to MHC class I (BF) and MHC class IV (BL) antigens (Fulton *et al* 2006).

The chicken MHC is known as the B locus because it was first identified as a serological blood group locus (Briles *et al* 1950) encoding the polymorphic, and highly immunogenic, BG antigen. The BG antigen has been found to be highly expressed on blood cells; its mammalian equivalent has not been encountered. In contrast to the large mammal MHC, the chicken B locus is minute and only two copies each of class I (BF) and class IIβ (B/Lβ) genes are found in the chicken B locus.

Unlike mammals, birds have an exceptional organ, the bursa of Fabricius, where the B lymphocytes (B cells) develop which thus differentiate into plasma cells secreting antigen-specific antibodies thus generating humoral immunity. Both cell-mediated and humoral immunity can be stimulated but the type of immunity activated vary with the organism encountered (Vandaveer *et al* 2001; Sharma 2003 and Erf 2004).

2.10.3 Immunological response to infectious coryza infection

Immunological response against infectious coryza in terms of protection from development of clinical symptoms in chickens is well studied (Kume *et al*

1980; Sawata *et al* 1982), and protection is observed due to development of Haemeagglutination inhibition (HI) antibodies in hosts. However, over the period of years, some conflicting evidences had evolved out from some empirical experience(s) which demonstrated lack of participation by HI antibodies, in conferring immune protection to birds (Garcia *et al* 2008) and were further reaffirmed by Sakamoto *et al* (2013). Simultaneously, on an another note, host specific serum resistance to pathogen's infectivity was also studied (Byarugaba *et al* 2007), which elaborated increased pathogen's (*Av. Paragallinarum*) sensitivity upon interaction with species specific serum that indirectly conveyed an information about the presence of some unknown innate immune factor in host's serum that perhaps imparted immune protection in phylogenetically related avian species.

The birds that recover from the active infections caused by the infectious coryza possess varying amount of immunity to re-exposure. Resistance to re-exposure develops as early as 2 weeks after initial exposure by the intra-sinus route (Sato and Shifrine 1964).

Boucher *et al* (2014) investigated the regulation of immune mechanism by the number of genes activated during the chickens' response to *A. paragallinarum* serovar C3 infection. An up-regulation of TLR7 which recognizes viral-like particles was observed which magnified upon the presence of virus like prophages reported in the genome of *A. paragallinarum* (Roodt *et al* 2012). Significant down-regulation of metabolic pathways was noticed, which possibly pointed towards the activation of host's oxidative stress pathways as an initial immune response.

Boucher *et al* (2015) concluded that immune response initiation against *Avibacterium paragallinarum* infection is via TLR4, which leads to a Th2 dominant type response. Additionally, TLR4 was found to activate the MyD88-dependent pathway, resulting in early onset of NF-kβ leading to the production of inflammatory cytokines.

2.11 Production of polyclonal antibody against respiratory pathogens of chickens

York and Fahey (1988) raised polyclonal antibodies against Infectious laryngotracheitis virus in rabbits by subcutaneous and intramuscular administration of purified virus in conjunction with Freund's complete or incomplete adjuvant.

Thornton and Blackall (1984); Espinoza *et al* (2008) raised antisera against *Avibacterium paragallinarum* in rabbit by using overnight grown culture and administering it subcutaneously with Freund's incomplete adjuvant. The generated antibodies were mainly used for serotyping of *Avibacterium paragallinarum* isolates. To our knowledge, so far it's usage to demonstrate localization of antigen/ pathogen in tissue through immuno-histochemistry was never been carried out.

Jirjis *et al* (2001) raised polyclonal antiserum in rabbits against the Avian pneumovirus (APV) by mixing sucrose gradient purified APV with Freund adjuvant and injecting it subcutaneously over a period of 11 weeks. The raised antiserum was successfully used for immunohistochemical demonstration of APV in formalin-fixed tissues.

York and Fahey (1988) raised polyclonal antibodies against Infectious laryngotracheitis (ILT) virus in rabbits by subcutaneous and intramuscular administration of purified virus in conjunction with Freund's complete or incomplete adjuvant. The raised antibody was used as a primary antibody for the ELISA-based detection of the virus and the results were compared to that of agar gel precipitation test and fluorescent antibody test to determine the relative efficacy of ELISA for diagnosis of ILT.

Lehmann and Reis (1978) raised polyclonal antisera against alkaline extract of *Aspergillus fumigatus* mycelium extract and found it to be specific for a wide range of Aspergillus antigens. The raised antisera were shown to be of diagnostic value in the detection of antigen in the serum and urine of infected humans and rabbits.

Fouchier *et al* (2005) described the raising of polyclonal antiserum against various strains of Avian influenza virus. Rabbits were immunized by the HA (hemagglutinin) and NA (Neuraminidase) obtained from specific strains of Avian influenza virus in combination with Specol, and injected subcutaneously. The resultant hyperimmune antisera were used for the HI-based characterization of various strains.

2.12 Control and preventive measure against infectious coryza

The disease is largely controlled through suitable preventive measure i.e. by vaccination, however, in amidst of serious disease outbreak; the disesaes is

effectively contained through antibiotic therapy. A majority of work pertaining to antimicrobial sensitivity against this pathogen have been originated from Asian countries (Akter *et al* 2013; Noonkhokhetkong *et al* 2013; Akter 2014). The pathogen was found to be sensitive to penicillin and few new generation penicillin compounds like amoxicillin, ampicillin etc, with some degree of partial sensitivity towards macrolide antibiotics such as erythromycin and third generation fluoro-quinolones i.e. enrofloxacin. Most of the Asian isolates were resistant to sulphonamides, amino glycosides and tetracycline compounds with rare sensitivity to gentamycin and oxytetracycline compounds.

Vaccine(s) and vaccination strategies have always protected the animal health and public health domain from ill effects of antibiotics. The development and emergence of new bio variants of pathogen(s) are the results of indiscriminate usage of antibiotics that has created inappropriate pressure on the survivality of pathogen(s) and ensures them to adapt new biogenerative potential to emerge as new pathogenic insults. The history of development of vaccines against infectious coryza dates back to long ago, when this latest concept of new bioaccumulation was never thought of. Plenty of works pertaining to vaccine improvement and superior vaccination strategies against this disesaes were undertaken and several innovative directions were followed that begins from classical research on whole cell killed vaccines/ bacterin(s) to latest recombinant protein technology.

Iritani *et al* (1980) and Blackall and Reid (1987) have attempted both subcutaneous and intramuscular routes of immunization and found impressive imparting protection against *Avibacterium paragallinarum* infection.

Injection of the *Avibacterium* bacterin into the leg muscle was noted to give better protection than when injected into the breast muscle (Iritani *et al* 1984). The intranasal route for immunization with the regular bacterin against infectious coryza was reported to be not effective (Blackall and Reid 1987). Oral delivery of an IC bacterin although effective, required 100 times higher dose of cells as with the parenteral route (Nakamura *et al* 1994).

Infectious coryza bacterin(s) are generally injected in birds between 10 and 20 weeks of age (Blackall and Soriano 2013).

2.12.1 Killed vaccines

Matsumoto and Yamamoto (1975) suggested that bacterin(s) must contain at least 10^8 CFU/ml to be effective.

Jacobs *et al* (2003) tested the efficacy of a tetravalent vaccine which contained an additional field isolate over the contemporary trivalent vaccine and found the tetravalent vaccine to be significantly superior in preventing disease outbreaks, thereby highlighting the necessity to add local immunotypes for better vaccine efficacy.

Killed or inactivated vaccines are prepared from microorganisms that have been killed via physical or chemical processes. These kinds of vaccines are incapable of causing diseases. Several studies (Chukiatsiri *et al* 2009; Wambura 2010) indicate that chickens can be protected via autogenous vaccines. The commercially available vaccines currently are only killed whole cell vaccines although alternate approaches are being actively researched. Mineral oil and aluminium adjuvant vaccines are known to provide superior protection (Chukiatsiri *et al* 2009; Wafaa and El-Ghany, 2011).

2.12.2 Live vaccines

Efforts are underway to develop a live infectious coryza vaccine. The major difficulty in the development of live vaccine against IC revolves around the notoriously fastidious nature of the bacteria. Vaccination trials against infectious coryza have mainly utilized live cultures from chicken embryonated eggs (Tennison and Siddle 1961). Live vaccines have the potential to provide cross protection among serovars, which has been lacking in traditional killed vaccines.

2.12.3 Subunit vaccines/ recombinant vaccines

Subunit vaccines differ from the killed whole-cell vaccines only in the fact that subunit vaccines contain only the antigenic part of the pathogen, rather than the whole pathogen. These antigenic parts are necessary to evoke a protective immune response.

Hsu *et al* (2007) elucidated the immunological potential of a recombinant haemagglutinin protein (rHagA) derived from a Taiwan isolate strain A9 as the immunogen for vaccination. The rHagA subunit vaccine protected 71% of immunized chickens against a dose of 10^{10} CFU/ml dose of A9 strain. Haemagglutination (HA)

of serovars A and C was not affected by the presence of rHagA specific antiserum.

Wu *et al* (2011) located the hypervariable region in the HA proteins of strains of serovars A and C of *A. paragallinarum* and showed that it was more immunogenic than the other regions of HA protein. Inclusion of the hypervariable region in the recombinant vaccine resulted in 83-100% protection against clinical disease.

Sakamoto *et al* (2012) demonstrated the effectiveness of recombinant fusion peptide derived from HMTp210 (region 2 of serotype A and C) in producing effective and safe vaccines against infectious coryza in chickens.

2.12.4 Mucosal vaccines

In recent times, a wave for development of mucosal vaccination strategies has emerged, in which many modified microbes/pathogen(s) were found to be effective in elicitation of localized immune response. This phenomenon can only be utilized in those disesae(s) conditions where microbes/pathogen enters mainly through mucosal membranes. Excitement of robust localized immune response mechanism at mucosal surface through tampered pathogen/ microbes would undoubtedly ensures less colonization of wild pathogen across the surfaces thereby may reduces the chances of disease development and its associated complication. Recently, several experimental researches performed to this directions has documented promising outcomes in either mice or rat model of disease(s) (Eko *et al* 2003) without involving the actual host(s) for their efficacious outcomes. Our concern lies to the fact that actual host's originated excitement of immune response mechanism would slightly vary from the other model host(s), may yield incongruous results or outcome and may also deliver clinical and vaccine oriented discrepancies. We believe, this problem could easily be substituted and addressed in our research domain where the actual host(s) (i.e. chicken) can play a significant role in understanding the intricacies of immunologic process held directly by implicating host-pathogen interactive studies. Like infectious coryza in chicken, a prominent disease of human affecting mainly children of lower age caused by *Hemophilus influenza* (a bacterium) was studied for development of mucosal vaccines that provided significant observation on elicitation of primary immune response in rats could acts as a torch bearer for our further studies in chicken.

CHAPTER – III

MATERIALS AND METHODS

3.1 Place of Work

All the Experimental (infection) studies and the major laboratory work was carried out at the Department of Veterinary Pathology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana. Few initial steps of microbiological isolation and PCR (Polymerase Chain Reaction) studies were undertaken at the Department of Veterinary Microbiology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana with its prior approval/collaboration from the concerned officials.

3.2 Ethical Approval

The ethical approval related to the work involving participation of chicken and Japanese quail as subject in the experimental studies was principally accorded by local Institutional Animal Ethical Committee (IAEC), Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana (vide Memo. No. GADVASU/2014/IAEC/23/006 IAEC/2014/125-53 dated 13-08-2014).

3.3 General Consideration

The majority of the culture media, biochemical kits relevant to the microbiological work were obtained from M/s. Hi-Media Laboratories Pvt. Ltd, Mumbai. The chemicals used for the preparation of buffers, solutions and reagents were of molecular biology grade. Plastic wares including micro-centrifuge tubes, micropipette tips, cryovials, Petri-dishes, syringe filters were procured from M/s. Tarsons Products Pvt. Ltd., Kolkata. Utmost care has been taken to prepare culture media, buffers, supplements and other biochemical reagents in distilled water with relevant pH value.

3.4 Media preparation

A) *Hemophilus agar (HA)*: HA was prepared from Haemophilus test agar base (Product Catalogue no. M1259; Hi Media Laboratories Pvt. Ltd, Mumbai) as per the manufacturer's instructions (Hi-Media Laboratories Pvt. Ltd, Mumbai) with certain modifications. Briefly, Haemophilus test agar base was measured (21.50 g then dissolved in 500 ml distilled water), heated and autoclaved at 15psi at

121°C for 15 min. HA was cooled to 45^0C and horse serum (Product catalogue No. RM10436; Hi Media Laboratories Pvt. Ltd, Mumbai) was added (@ 1:20) after filtration through a 25mm (0.2µ) Polyethylene sulphone made (PES) syringe filter (Product catalogue No. SFGFPE 25R; Axiva Sichem Biotech, Delhi). Similarly, Nicotinamide dinucleotide (NAD solution, Product catalogue no. RM391-5G; Hi Media Laboratories Pvt. Ltd, Mumbai) ready to use Hemophilus test supplement (Product catalogue no. FD117; Hi Media Laboratories Pvt. Ltd, Mumbai) were filtered through 25mm (0.2µ) PES syringe filter and added (@1:100) to partially cooled agar medium and then gently mixed before pouring in to plates.

B) *Blood agar (BA)*: BA was prepared as per manufacturer's (Hi Media Laboratories Pvt. Ltd, Mumbai) instructions. Briefly, BA base (Product catalogue no.M144A; Hi Media Laboratories Pvt. Ltd, Mumbai) was measured (21.50 g then dissolved in 500 ml of distilled water), autoclaved at 15 psi at 121^0C for 15 min, cooled to 45^0C and then defibrinated sheep blood added at the rate 5% v/v to the molten agar base, mixed gently and poured on plates.

   At every occasion, the finally prepared media plates were allowed to solidify and incubated at 37^0C for 24h to check for its ultimate sterility.

C) *Hemophilus Broth (HB)*: HB was prepared from Hemophilus test agar base by dissolving the base in distilled water (DW) as according to manufacturer's instructions followed by filtering the solution through Whattman's filter paper No. 4 (Product catalogue no.1004-125; GE Healthcare UK Ltd.). The filtrate obtained was autoclaved at 15psi at 121^0C for 15 min, subsequently cooled to 45^0C and was added with filtered horse serum (@1:20), filtered NAD solution and Hemophilus test supplement (@1:100) each respectively.

3.5 Source of isolates

Avibacterium paragallinarum isolate(s) were obtained from Poultry Disease Research Centre (PDRC), Pune. The isolate(s) were streaked on freshly made Hemophilus Agar (HA) media plates and were transported in a candle jar that provides nearly 5% CO_2.

The isolates brought to the laboratory were immediately inoculated on newly made Hemophilus Agar plates and Blood Agar (BA). Streaking on BA was done in a

zig-zag manner and perpendicularly overlaid with singular streak of *Staphylococcus aureus* organism. The inoculated plates were incubated at 37ºC for 24-48 hours in a candle jar with 5% CO_2 (Dousse *et al* 2008).

3.6 Identification of bacterial isolates

The isolated bacterial colonies after incubation were subjected to Gram's staining for identification and biochemical tests. The final confirmation of organism was relied upon using molecular techniques i.e. PCR (Chen *et al* 1996)

3.6.1 Gram's staining

Suspected pure colonies from HA and BA were stained using the Gram's staining protocol (Adderson 2008). Briefly, smears were prepared on glass slides by mixing 0.9% normal saline and loop full of bacterial colonies, which were then air dried and heat fixed. Fixed smears were stained with Crystal violet and permeabilized with Gram's iodine as a mordant, decolorized with acetone alcohol and counter stained with Safranin and then morphological characteristics were examined under the microscope using oil immersion lens (i.e. at 1000X magnifications).

3.6.2. Biochemical characterization

All isolate(s) with confirming phenotypic characteristics of *Avibacterium paragallinarum* species were characterized biochemically using ready-to-use Hi Nisseria® Identification Kit as per manufacturer's instructions (Catalogue No. KB008; Hi Media Laboratories Pvt. Ltd, Mumbai). The suspected bacterial colonies were tested for oxidase, urease, catalase, nitrate reduction and their ability to ferment some sugars i.e., glucose, sucrose, mannose, maltose, lactose and fructose (Byarugaba *et al* 2007; Wambura 2010). Most of the biochemical parameters utilized for identification of *Nisseria* organism resemble the biochemical parameters of *Avibacterium paragallinarum* species, with few exceptions, thus facilitated the use of Hi Nisseria® Identification Kit.

3.6.3. Molecular characterization

3.6.3.1. DNA extraction

DNA was extracted from bacterial colonies supposedly of *Avibacterium paragallinarum* by suspending a loop full of pure colonies in 200 µl of Nuclease Free Water (Catalogue No-P1193; Hi Media Laboratories Pvt. Ltd, Mumbai) in 1.5

ml capacity micro-centrifuge tubes (MCT). These were then placed in boiling water for 10 minutes, after which they were immediately placed in crushed ice for 10 minutes. MCTs were then centrifuged at 5000 rpm for 5 minutes. The supernatant was collected and stored at -20ºC until further use as DNA template.

3.6.3.2. DNA extraction from Commercial Vaccine against *Avibacterium paragallinarum*

Bacterial DNA from Commercial vaccine was extracted and employed as positive control template for polymerase chain reaction (PCR). The following steps were used:

1. Around 560µl of *Avibacterium Paragallinarum* vaccine (Inactivated and oil adjuvanted manufactured by Venkateshwara Hatcheries Pvt. Ltd. Pune) was taken in a 2ml capacity micro-centrifuge tube (MCT).
2. To this, 30µl each of 10% SDS (Sodium Dodecyl Sulphate, Catalogue No. GRM6218; HiMedia) and 3µl Proteinase K-200µg/ml, (Catalogue no- RM2957; HiMedia) was added to make a lysate. The mixture was incubated in a water bath preset @ 37°C for 1h.
3. After one hour, equal volume of phenol, choloroform and iso-amyl alcohol (PCI) (@25:24:1) was added to the lysate and the two phases were mixed by frequent vortex followed by centrifugation @13,000 rpm (Eppendorf Co.) for 10 minutes at 4°C.
4. The supernatant was then collected carefully in a fresh micro-centrifuge tube and to this, equal volume of PCI (@25:24:1) was added again, mixed by repetitive vortex and centrifuged @13,000 rpm for 10 minutes and the supernatant was collected in another fresh micro centrifuge tube.
5. To this supernatant, 2.5 volumes of chilled absolute ethanol and 3M Sodium acetate (pH 5.2) equal to one-tenth of the volume of supernatant was added, mixed by continual vortex and kept at -20°C for overnight incubation.
6. Following incubation, the supernatant was again centrifuged @13,000 rpm for 10 minutes and pellet was collected after discarding the supernatant. The pellet was washed twice with 500µl of 70% ethanol and centrifuged @13,000rpm for 10 minutes.
7. The supernatant was decanted and the residual ethanol was removed carefully by inverting the tubes and left for 15-30 minutes for the evaporation of ethanol.

8. The pellet was then reconstituted in 50µl of Nuclease Free Water (NFW).

3.6.3.3. Confirmation of DNA isolation:

The presence of the isolated DNA was confirmed by running the DNA on a 1.5% agarose gel by mixing the 5µl of DNA with 1µl 6X gel loading dye.

3.6.3.4 Polymerase chain reaction (PCR)

3.6.3.4.1 Species specific PCR

Identification of *Avibacterium paragallinarum* organism was carried out using a PCR based assay by employing species-specific DNA primers (Table 1), N1 and R1 (Chen *et al* 1996). The 25µl reaction mixture contained 12.5µl of Master Mix (Promega, USA), 1µl of 25 pmol/ul of each forward and reverse primers (Xcelris Genomics, Gujarat), 5µl of template DNA and the reaction volume was made to 25µl by adding nuclease free water (Promega, USA) (Table 2).

PCR was performed on a thermo cycler (Veriti, Applied Biosystem, USA) with the following conditions; an initial denaturation at 94^{o}C for 4 minutes followed by 35 cycles each of denaturation at 94^{o}C for 1 minute, annealing at 63^{o}C for 1 minute and extension at 72^{o}C for 1 minute. This was followed by a final extension at 72^{o}C for 10 minutes (Table 3) with an expected amplification result of approximately 500 bp.

Table 1: Primer sequence(s) used for genus-specific identification of *Avibacterium paragallinarum*

S. No.	5' to 3'	Amplicon Size (bp)	Annealing Temp (°C)	Reference
1.	F:TGAGGGTAGTCTTGCACGCGAAT R:CAAGGTATCGATCGTCTCTCTACT	500	63	Chen *et al* (1996)

Table 2: PCR reaction mixture for the identification of *Av. paragallinarum*

S. No.	Components	Volume (25 µl)
1	Master mix	12.5
2	Primer F (25pmol/µl)	1
3	Primer R (25pmol/µl)	1
4	Template DNA	5
5	Nuclease Free Water (NFW)	5.5

Table 3: PCR program used for the detection of *Avibacterium paragallinarum*

Stage	Step	Temperature (^{o} C)	Duration	No. of cycles
1	Initial denaturation	94	4 min	1
	Denaturation	94	1 min	
2	Annealing	63	1 min	35
	Extension	72	1min	
3	Final extension	72	10 min	1

3.6.3.4.2 Serotype specific PCR

In order to further identify the serotype, another PCR was run by employing serotype-specific primers (Sakamoto *et al* 2011) (Table 4) on DNA templates which showed amplified PCR products corresponding to 500 bps. The 25µl reaction mixture contained 12.5µl of Master Mix (Promega, USA), 3µl of 25 p mol/ul of forward primer and 1µl each of reverse primers corresponding to serotype A, B and C (Xcelris Genomics Pvt. Ltd, Gujarat), 5µl of template DNA and the final reaction volume was made to 25µl by adding nuclease free water (Promega, USA) (Table 5).

PCR was performed on a thermocycler (Veriti, ABI, USA) with the following conditions; an initial denaturation at 98^{o}C for 1 minutes followed by 30 cycles each of denaturation at 98^{o}C for 10 seconds, annealing at 56^{o}C for 10 seconds and extension at 72^{o}C for 2 minute. This was followed by a final extension at 72^{o}C for 7 minutes (Table 6) with expected amplification result of approximately 0.8 kbp in case of serotype A, 1.1 kbp in case of serotype B and 1.6 kbp in case of serotype C.

Table 4: Primer sequence(s) used for serotype-specific detection of *Avibacterium paragallinarum*

S. No.	Primer	Fragment length	5' to 3'	Reference(s)
1.	ABC Forward A reverse	0.8 kbp	GGCTCACAGCTTTATGCAACGAA CGCGGGATTGTTGATTTTGTT	
2.	ABC Forward B Reverse	1.1 kbp	GGCTCACAGCTTTATGCAACGAA GGTGAATTTCACCACACCAC	Sakamoto *et al* (2011)
3.	ABC Forward B Reverse	1.6 kbp	GGCTCACAGCTTTATGCAACGAA TAATTTTCTTATTCCCAGCATCAATACCAT	

Table 5: PCR reaction mixture used for serotype specific identification of *Avibacterium paragallinarum*

S. No.	Components	Volume (25µl)
1	Master Mix	12.5
2	Primer F (25pmol/µl)	3
3	Primer A reverse (25pmol/µl)	1
4	Primer B reverse (25pmol/µl)	1
5	Primer C reverse (25pmol/µl)	1
6	Template DNA	5
7	Nuclease Free Water (NFW)	1.5

Table 6: PCR program used for the serotype detection

Stage	Step	Temperature (^{o} C)	Duration	No. of cycles
1	Initial denaturation	98	1 min	1
	Denaturation	98	10 sec	
2	Annealing	56	10 sec	30
	Extension	72	2 min	
3	Final extension	72	7 min	1

3.6.4 DNA confirmation by Agarose Gel Electrophoresis

The DNA extracted was also run on a 1.5% agarose gel to observe its consistency.

Equipment used:

a. Weighing balance (Denver Instruments, USA)

b. Horizontal electrophoresis apparatus with power pack (Cleaver Scientific Ltd.)

c. Microwave oven (BPL 600T)

d. Gel documentation System (Alpha Imager, Innotech)

Reagents utilized:

a. Agarose (Promega, USA)

b. Tris Borate EDTA buffer (10X, pH 8.2) (Promega, USA)

The concentration was made 1X before use by adding DW.

d. Ethidium bromide (10 mg/mL) (Promega, USA)

Procedure:

Gel Casting

Molecular grade agarose (1g) was melted by heating in microwave oven and subsequently made to dissolve in 100 mL of 1X Tris Borate EDTA (TBE) buffer. Prior to casting the gel, the molten agarose was allowed to cool to about 50°C, and 2.5 µl of ethidium bromide was added to make a final concentration of 0.5 mg/ml. It was mixed thoroughly and the gel was casted on a gel casting tray fitted with acrylic comb and was allowed to solidify. After solidification of the gel, the comb was carefully removed from the gel and the gel casting tray was submerged along with the gel in an electrophoresis tank containing 1X TBE buffer.

Gel Loading

10µl of the PCR product from both tested DNA templates as well as from positive control template i.e. Vaccine DNA template were loaded in the wells of submerged agarose gel. In one of the wells, 5µl of 100 bp DNA molecular weight marker mixed with 1µlof 6X gel loading dye (Promega, USA) was loaded. Electrophoresis was carried out at 5V/cm until the tracking dye had reached the other end of the gel.

Gel documentation

Following electrophoresis, DNA bands were visualized and the images were captured by using Gel Documentation System (Alpha Imager, Innotech)

3.7 Generation of polyclonal antisera against *Avibacterium paragallinarum*

Polyclonal antisera against *Avibacterium paragallinarum* was raised as per the procedure given by Sangdee *et al* (2012) with slight modification.

3.7.1 Animal used

Two (2) healthy male Soviet chinchilla rabbits, aged > 6 months of age were used in the present study and was obtained after the completion of an experiment, in which both were acted as naïve control from Department of Veterinary Physiology and Biochemistry, GADVASU, Ludhiana.

3.7.2 Preparation of formalinized *Avibacterium paragallinarum* antigen (Ag)

1. Around 10 ml of Haemophilus broth was inoculated with *Avibacterium paragallinarum* and incubated in a candle jar at 37°C for 48hrs.

2. Following incubation, the broth was transferred to a 15ml centrifuge tube and centrifuged at 5000 rpm for 10 minutes.
3. The pellet obtained was washed three times with Phosphate Buffered Saline (PBS) and then re-suspended in 10ml PBS (pH 7.4).
4. Approximately 50µl of commercially available formaldehyde and 25mg Sodium thiosulphate were added to the washed suspension to make it 0.5% formalinized antigen following which it was stored at 4°C, until further use.

3.7.3 Administration of 0.5% formalinized antigen

0.5 ml of formalinized *Avibacterium paragallinarum* antigen (Ag) in conjunction with 0.5 ml Freund's complete adjuvant (FCA) was administered bilaterally through subcutaneous route on the 1st day of application. The schedule for administering antigen in conjunction with adjuvant, route of administration and serum collection are given in the table (Table 7) as follows:

Table 7: Administration schedule

Days of dosing /sampling	**Antigen dosing, Route of administration and Serum collection**	
	Adjuvant (Rabbit)	**Antigen (Rabbit) (0.5% Formalinized Antigen)**
0 day	Collected blood for serum isolation (Stored at -20^{0}C)	Collected blood for serum isolation (Stored at -20^{0}C)
1st day	0.5ml Freund's Complete Adjuvant (FCA), Subcutaneously	0.5ml Formalin inactivated *A. paragallinarum* Ag + 0.5ml Freund's Complete Adjuvant (FCA), Subcutaneously
6th day		0.5ml Formalin inactivated *A. paragallinarum* Ag + 0.5ml Incomplete Freund's Adjuvant Subcutaneously
9th day		1ml Formalin inactivated *A. paragallinarum* Ag + 0.5ml Incomplete Freund's Adjuvant Subcutaneously
14th day	Collected blood first for serum collection (Stored at -20^{0}C)	Collected blood first for serum collection (Stored at -20^{0}C) 1ml Formalin inactivated *A. paragallinarum* Ag + 0.5ml Incomplete Freund's Adjuvant Subcutaneously

Days of dosing /sampling	Antigen dosing, Route of administration and Serum collection	
	Adjuvant (Rabbit)	**Antigen (Rabbit) (0.5% Formalinized Antigen)**
19th day		1ml Formalin inactivated *A. paragallinarum* Ag + 0.5ml Incomplete Freund's Adjuvant Subcutaneously
21st day	Collected blood for serum collection (Stored at -20^{0}C) 0.5ml Freund's Complete Adjuvant (FCA), Subcutaneously	Collected blood for serum collection (Stored at -20^{0}C)
28th day	Collected blood for serum collection (Stored at -20^{0}C)	Collected blood first for serum collection (Stored at -20^{0}C) 1.5 ml Formalin inactivated *A. paragallinarum* Ag Without Incomplete Freund's Adjuvant Intravenously
35th day (1st bleeding)		Collected blood for serum isolation (Stored at -20^{0}C)
42nd day (2nd bleeding)		Collected blood for serum isolation (Stored at -20^{0}C)
48th day (3rd bleeding)		Collected blood for serum isolation (Stored at -20^{0}C)

3.7.4 Collection of blood and serum

Approximately, 2ml blood was taken out from the ear vein using 2ml sterile syringe at the indicated time interval (Table 7) and collected in a vial without any anticoagulant. The collected blood was kept in slant position for a while and thereafter transported on ice packs and immediately centrifuged at 6500 rpm for 10minutes and the serum was transferred to sterile vials, labelled and stored at -20°C, until further use.

3.7.5 Determination of specificity of raised polyclonal antisera against *Avibacterium paragallinarum*

3.7.5.1 Immuno dot blot assay

3.7.5.1.1 Preparation of Antigen:

1. *Avibacterium paragallinarum* was grown in Haemophilus broth for 48 hours and was the washed thrice with PBS @ 5000rpm for 10 minutes.

2. The washed cells were then resuspended in PBS.
3. Then, one drop (5µl) 1% SDS was added per milliliter of antigen.
4. This was then sonicated thrice using 30s pulses/burst with 15s pause for cooling.
5. The antigen was preserved at -20°C.

3.7.5.1.2 Procedure for Immuno dot blot

1. The grid lines were drawn on the PVDF membrane to indicate the region of blotting and to the center of this grid, 2µl of sample was placed.
2. The membrane was then allowed to dry for 1hour at 37°C.
3. The non-specific sites were thus blocked by soaking 2% BSA in TBST for 2 hours followed by washing with TBS-T buffer for 5 mins.
4. The primary antibody (raised sera) prepared in 0.1% BSA was added to it and incubated for 1 hour at room temperature or overnight at 4°C followed by washing with TBST thrice for 5 minutes and washed finally with TBS.
5. Then, 1% H_2O_2 was used for washing followed by final wash with TBS-T for 5 minutes.
6. Secondary antibody conjugated with HRP (1:2000) was added and incubated for 1 hour at room temperature followed by washing with TBS-T thrice and once with TBS (5 minutes).
7. After one hour, DAB solution was added over it and observed for the development of brown colour.

3.7.5.2 Counter immunoelectrophoresis (CIE)

Counter Current Immunoelectrophoresis is a modification of immunoelectrophoresis in which antigen and antibody move in opposite directions and form precipitates in the area between the cells where they meet in concentrations of optimal proportions.

Procedure:

1. A volume of 10 ml of 1.0% Agarose (0.1 g/10 ml) was prepared in 1X Assay Buffer by heating slowly.
2. The ends of a glass slide were marked as ‘+ve’ and ‘-ve’, and the slide was placed on a horizontal surface.

3. Approximately, 5 ml of agarose was placed onto the glass slide and allowed to solidify for 15 minutes.
4. The wells were then cut using gel puncher with the distance between the two wells not exceeding 0.5 cm.
5. The slide was placed in the electrophoresis tank with the '+ve' mark facing towards anode and the '-ve' mark facing towards cathode. The tank was filled with 1X electrophoresis buffer till the buffer covered the gel surface.
6. Then, 10µl of antigen was added in each of the two wells towards cathode (Negative electrode) and 10µl of positive control antiserum and test antisera in wells towards anode (Positive electrode).
7. The power cord was connected to the electrophoretic power by applying 50 V for about 45 minutes and the precipitin lines between the antigen and antisera wells were thus observed.
8. The formation of precipitin line indicates the presence of antibody for the antigen in the test sera whereas the absence of the precipitin line indicates the absence of any antibody for the antigen in the test sera.

3.7.6 Colony forming unit (CFU) estimation

1. Around 10 ml of Haemophilus broth was inoculated with *Avibacterium paragallinarum* culture and kept for incubation at 37^0C in a candle jar for at least 24 hours.
2. To this, 0.9 ml PBS (pH 7.4) was added to nine 1.5ml micro-centrifuge tubes.
3. Then, 100µl of inoculated broth was added to the first micro-centrifuge tube and thereafter tenfold serial dilutions were made in the subsequent tubes.
4. Haemophilus agar plate was divided into zones from the outside with the help of a marker and each zone was allotted to one level of dilution.
5. 10µl each of a dilution were then placed in their respective allotted zone in duplicate and allowed to dry and repeated for every dilution.
6. Then, HA plate was kept for incubation at 37^0C in a candle jar for 24 hours.
7. Colony forming units (CFU) were calculated after counting the number of distinct visible *Avibacterium paragallinarum* colonies from the lowest dilution zone showing countable colonies and taking average of the paired readings obtained from that particular dilution.

8. CFU/ml was then calculated using the following formula:

$$\text{CFU/ml} = \frac{\text{Average no. of colonies}}{\text{Level of dilution} \times \text{Volume of dilution plated}}$$

3.7.7 Preparation of McFarland's Nephalometer tubes

1. Initially, 1% solution of each barium chloride ($BaCl_2$) and sulfuric acid (H_2SO_4) was prepared respectively.
2. The two solutions were then combined and mixed to form a turbid suspension and barium sulphate ($BaSO_4$) was also added in a specific proportion for each McFarland's turbidity standard as shown in Table 8.
3. The final mixture was stored in Aluminium foil covered with screw capped tubes to avoid any oxidative changes.

Table 8: McFarland's turbidity standards

S. No.	McFarland's turbidity standard No.	0.5	1	2	3	4
1	1% barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
2	1% sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
3	Approx. Cell density ($1*10^8$ CFU/ml)	1.5	3	6	9	12

3.8 Serial passages in chickens:

Avibacterium paragallinarum isolate(s) were passaged in chicken, at least for three times in a row to make the isolate(s) host adapted. Every time, approximately 10^8 CFU/ml were injected intra-sinus in each bird. In the initial two passages, the birds did not show development of clinical signs. However, from the third passage onwards, mild degree of clinical signs was noticed which was characterized by nasal and ocular discharges with mild subcutaneous swelling around infraorbital region. This observation ensured us to utilize the isolate(s) for our subsequent experiment as well as for the production of polyclonal antibody.

3.8.1 Re-isolation attempts:

Birds inoculated with *Avibacterium paragallinarum* isolate(s) were sacrificed at various time points post infection and their heads were collected in a sterile petri plate. The head samples collected were then transported to the laboratory on ice packs. Before proceeding with re-isolation, the skin above the infraorbital sinus was seared with a heated stainless-steel spatula following which an incision was made on

the seared skin with a Bard-Parker blade No. 11. A sterilized stainless-steel inoculation loop was introduced in the infraorbital sinus and the collected exudate was streaked on an HA agar plate. Similarly, streaking was also done on a BA plate and streaked lines were perpendicularly overlaid with a single streaked line of *Staphyloccus aureus* bacteria. The streaked plates were then incubated at 37°C in a candle jar with 5% CO_2 and bacterial growth was observed after 24 hours and 48 hours of incubation.

3.9. Pilot experiment:

Pilot experiment was conducted once in several groups of chickens to determine infective dose (i.e. CFU^{-1}ml of bacteria) that can exhibit development of clinical signs in birds. Serial dilutions of organism(s) were carried out from HA both that was inoculated with bacteria for 24 hrs. The maximum no. of organism present in undiluted broth was 10^8 CFU^{-1}ml and out of which four (4) dilutions were selected i.e. 10^8, 10^7, 10^6 and 10^5 CFU^{-1}ml for pilot infection experiment. Birds from 10^8 CFU^{-1}ml dilution (i.e. undiluted broth) revealed some degree of clinical signs among birds noted over a period of five (5) days. However, none of the birds from rest of the selected dilutions exhibited any form of clinical signs. Thus, decided to consider 10^8 CFU^{-1}ml as infective dose for final experiment.

3.10 Final experiment:

3.10.1 Preparation of experiment rooms:

The experimental room was thoroughly cleaned and fumigated with a solution of potassium permanganate and formaldehyde solution (40 gm in 1 litre) prior to introduction of birds. The bird cages were thoroughly scrubbed and washed with potassium permanganate solution before the introduction of birds for experiment.

3.10.2 Introduction of day old chicks:

Day old chickens and Japanese quails were obtained from Venkateshwara hatcheries, Ludhiana and Central Poultry Development Organisation, Chandigarh respectively.

3.10.3 Rearing of birds:

Birds were provided with *ad libitum* filtered water and commercial poultry feed (Layer ration). Bedding was periodically changed (initially twice a day and

after two weeks every alternate day) and utmost care was taken to ensure no movement from the infected room to the control room.

3.10.4 Grouping of chicks for experiment:

At the age of 2.5 weeks, 28 chickens and 28 Japanese quails were separated. Both chickens and Japanese quails were grouped into 2 groups each i.e. a 'sterile broth group' consisting of 5 birds and an 'Infected group' containing 23 birds. 'Infected group' was further sub-divided into technical duplicates and sampling was done as biological triplicates on each time points.

3.10.5 '0 day' sacrifice (Naïve birds):

Two chickens and two Japanese quails were sacrificed as a part of Naïve control group (i.e. no inoculation done). Requisite organs were collected and stored and gross findings were noted.

3.10.6 Inoculation of birds for experiment:

Each bird was inoculated with a fixed volume of haemophilus broth (Table 9). The infected birds were inoculated with broth containing 1.5 $x10^8$ CFU/ml whereas the 'Sterile broth' birds were inoculated with the same volume of sterile haemophilus broth instead.

Table 9: Volume and site of broth inoculated per bird

Species	Volume of 1.5 $x10^8$ CFU/ml broth inoculated per bird	Distribution of inoculum
Chicken	1ml	0.5ml right intra-sinus 0.2ml left nasal passage 0.2ml left eye 0.1ml right eye
Japanese quail	0.5ml	0.25ml right intra-sinus 0.1ml left nasal passage 0.1ml left eye 0.05ml right eye

3.10.7 Evaluation of clinical signs:

Birds inoculated with broth were evaluated for the development of clinical signs at 12hrs, 24hrs, 48hrs, 5days and 7 days post-infection respectively. Based upon the clinical signs development in the pilot experiment, scoring criteria was

developed to evaluate various clinical signs. Parameters for scoring included ocular discharges, nasal discharges, swelling of infraorbital sinus, redness of conjunctival surface and redness around periorbital sinuses. Number of birds at a given time point showing the above signs, the area affected i.e. unilateral or bilateral and the severity of clinical signs (mild, moderate or profuse/marked) was recorded.

3.10.8 Sacrifice of birds and recording of gross lesions:

Birds were sacrificed according to the sacrifice schedule (Table 10). Random sampling was employed in selecting birds for sacrifice which involved picking up of either male or female birds alternatingly from each box.

Based upon the gross lesions observed in various areas in the pilot experiment, scoring criteria was developed. Parameters for scoring included redness of nasal passage, mucus accumulation, Harderian gland enlargement/discoloration and conjunctival hemorrhages. Number of birds at a given time point with the above lesions and the severity of lesions (mild, moderate or severe/marked) was recorded. Descriptive information about the trachea, lungs, liver, kidneys and reproductive organs was also noted.

Table 10: Number of birds sacrificed for sample collection at each time point

Timepoint (Hrs/Days post infection)	Infected Chicken	Infected Japanese Quail	Sterile broth chicken	Sterile broth Japanese quail
12hrs	n=2	n=2	n=1	n=1
24hrs	n=5	n=5	n=1	n=1
48hrs	n=5	n=5	n=1	n=1
5 days	n=5	n=5	n=1	n=1
7 days	n=5	n=5	n=1	n=1

3.10.9 Collection and storage of samples

Organs/Tissues were collected from both chicken and Japanese quail, were later subjected to histopathological processing. The important organs like nasal turbinate, conjunctiva, orbital sinuses (subcutaneous tissues), trachea, lungs, liver, kidney and the reproductive tract were collected in 10% neutral buffered formalin (NBF).

3.11 Infection challenge at 6 weeks age

The remaining naïve birds from the first infection challenge experiment were reared till 6 weeks age and a second infection challenge experiment was performed on the same lines. A sacrifice schedule was prepared (Table 11). Due to limited number of birds, no sample was collected on the 5th day post infection. Clinical signs and gross lesions were recorded in the same way as in first infection experiment. Additionally, blood samples were also collected in heparinized vials for oxidative stress studies.

Table 11: Number of birds from various groups sacrificed at different time points during infection challenge at 6 weeks age.

Timepoint (Hrs/Days post infection)	Infected Chicken	Infected Japanese Quail	Sterile broth chicken	Sterile broth Japanese quail
12hrs	n=2	n=2	n=1	n=1
24hrs	n=5	n=5	n=1	n=1
48hrs	n=5	n=3	n=1	n=1
7 days	n=4	n=4	n=1	n=1

3.12 Oxidative stress assay

3.12.1 Lipid peroxidation activity

Erythrocytic lipid peroxidation was estimated by the method of Placer *et al* (1966). The principle of this method is that malondialdehyde (MDA), a final product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to give a pink colored trimethine complex which can be spectrophotometrically measured at 548nm.

Reagents

i) 0.2 M Tris-0.16 M KCl buffer (pH 7.4): 2.422 g Tris and 1.192 g KCl were dissolved in double distilled water and pH was adjusted to 7.4.

ii) 7% Perchloric acid: 7ml perchloric acid was taken in a measuring cylinder and the volume was made upto 100ml with double distilled water.

iii) 1N sodium hydroxide (NaOH)

iv) Thiobarbituric acid (TBA) reagent

v) Pyridine-n-butanol reagent (3:1, v/v): 1ml n-butanol was diluted with 3ml pyridine and reagent was stored in amber colored bottle

Procedure:

A. Hemoglobin estimation: Haemoglobin (g/dl) was estimated by using Sahli's hemometer by following the manufacturer's instructions (Himedia India Pvt Ltd, Mumbai).

B. Preparation of hemolysate: Heparinized vials containing were marked at the upper meniscus and the samples were centrifuged at 2500-3000 rpm for 20minutes. After separating plasma, RBCs pellet were washed thrice with normal saline solution. Finally, hemolysates were prepared by adding distilled water slowly with constant stirring to initial levels marked.

C. Test and control were prepared as follows:

i. 1.4ml Tris-KCl buffer was added to 0.1ml of hemolysate.
ii. Test solution as incubated at 37°C for 30mins. Control solution was not incubated.
iii. 1.5ml of TBA reagent was added to all the tubes (test and control).
iv. Test solution was then heated in boiling water bath for 10mins.
v. Test solution was allowed to cool.
vi. Control solution was neither heated nor cooled.
vii. 3ml of pyridine/n-butanol reagent (3:1, v/v) and 1ml 1N NaOH was added in test and control solutions.
viii. Finally, the absorbance of test and control was read against blank at 548nm.

Calculation:

$$\text{Erythrocytic lipid peroxidation (nmol MDA produced/g Hb)} = \frac{A_t - A_c}{X} \times 46 \times 1000$$

Where, '*X'* is hemoglobin concentration in g/0.1ml hemolysate.

3.12.2 Reduced glutathione activity

Erythrocytic reduced glutathione (GSH) was estimated by the method of Hafeman *et al* (1974).

Reagents:

1. 5% Trichloroacteic acid
2. 0.4M Sodium phosphate buffer (pH 7.1) containing 4×10^{-4}M EDTA.
3. DTNB reagent: Prepared by dissolved 40mg DTNB in 100ml aqueous 1% Tri-sodium citrate

Procedure:

a. To 0.5ml hemolysate, 0.5ml of 5% Trichloroacetic acid was added.

b. Solution was mixed thoroughly and centrifuged at 3000rpm for 20mins.

c. Supernatant was collected for GSH estimation.

d. To 0.2ml of supernatant, 1.25ml of 0.4M sodium phosphate buffer containing EDTA was added followed by addition of 0.015ml DTNB reagent.

e. Solution was mixed thoroughly and absorbance was recored at 420nm.

f. Standard curve was then used to calculate the GSH concentrations of test samples. μg/ml was converted to mM , concentration in mg/ml was divided by molecular weight of reduced glutathione (307.3235 g/mole) and multiplied by 1000.

3.13 Decalcification of Nasal turbinates and head section

Nasal turbinates and head section collected in 10% NBF during sampling were subjected to decalcification using Osteosoft (Merck KGaA, Germany), a soft decalcification solution. The turbinates were kept in decalcification solution for 14-16 hours. Extent of decalcification was judged by pricking the bony tissue with a pin.

3.14 Processing of samples

The formalin preserved samples were then washed in running water, dehydrated in ascending grades of alcohol and acetone then cleared in benzene and embedded in paraffin at 58^0C.

3.15 Histopathology

Tissue sections (4-5μ) were placed on the glass slides and stained using routine Haematoxylin & Eosin (H&E) staining technique for routine histopathology (Luna 1968).

3.16 Immunohistochemistry

For immunohistochemical studies, tissue sections were mounted on Poly-L-Lysine coated slides. Slides were cleaned with 0.1N HCl and were placed in Poly-L-Lysine 0.1% (w/v) solution (Sigma-Aldrich) which was diluted to 1:10 with distilled water for 5 minutes, drained and then dried in oven at 60^0C for one hour.

Materials required:

1. **Tris Buffered Saline (TBS) pH 7.6 (0.05 M Tris, 0.15M NaCl)**

   Tris Base 6.06 g

   NaCl 8.76 g

   Dissolve Tris Base and NaCl in 800 ml of distilled water (DW).

   Adjust pH to 7.6 using HCl

   Add H_2O to 1000 ml.

2. **Tris-EDTA Buffer (10Mm Tris Base, 1mM EDTA, pH 9.0)**

   Tris Base 1.21 g

   EDTA 0.37 g

   Dissolve in 1000 ml of distilled H_2O.

   Check and adjust pH to 9.0

3. **1% Bovine Serum Albumin (BSA) in TBS with 0.02% Sodium Azide NaN_3**

   BSA 1 g

   TBS 100 ml

   NaN_3 100 µl

   Dissolve BSA in TBS and add NaN_3

4. **2% Bovine Serum Albumin (BSA) in TBS with 0.02% Sodium Azide NaN_3**

   BSA 2 g

   TBS 100 ml

   NaN_3 100 µl

   Dissolve BSA in TBS and add NaN_3.

5. **Primary antibody**

Primary antibody against known *Av.paragallinarum* isolates used in this study were raised in rabbit as per Sangdee *et al* (2012) with slight modification.

Figure 1: Protocol for Immunohistochemistry

STEP 1: DEPARAFFINIZATION:

Xylene I - 5 minutes

↓

Xylene II -5 minutes

↓

Absolute Alcohol I- 3 mins

↓

Absolute Alcohol II- 3 mins

↓

96% Alcohol I - 3 mins

↓

70% Alcohol I – 3 mins

↓

TBS – 2 mins

↓

1.5% H_2O_2 in TBS – 10mins

↓

Wash in **tap water**- 5 mins

STEP 2: ANTIGEN RETRIEVAL

Put slides in **Tris EDTA buffer** (Blue coupling jar) – Heat- 400w – 3*5 mins (Make sure to **refill** with dH_2O as buffer evaporates; Tissues must not dry out)

↓

Cool at room temperature till slides come to **normal temperature**

↓

Wash 5 mins in **tap water**

↓

Transfer the slides to **TBS** at room temperature

STEP 3: BLOCKING & PRIMARY ANTIBODY INCUBATION

Cover tissues with **2% BSA** in TBS at room temperature **using tip** and incubate- 10 mins

↓

Tap off excess BSA

↓

Cover tissues with **Primary Antibody** and incubate at 4^0C **O/N**

STEP4: SECONDARY ANTIBODY INCUBATION & DAB

Rinse off Primary Ab with TBS using **Spray bottle**

↓

Incubate in **TBS** at room temp for 5 mins

↓

Tap off excess TBS

↓

Cover tissues with **Secondary antibody** and incubate for **45 mins** at RT

↓

Incubate in **TBS** – 2 mins at RT

↓

Wash in **tap water** – 2 mins

↓

Incubate in **DAB** until **color** develops

↓

Wash 2-5 mins **in tap water**

↓

Put slides in **dH_2O** at RT

STEP5: COUNTER STAINING & MOUNTING

Rinse slides in **deionoized** water

↓

Cover tissue area with **Mayer's Hematoxylin** for 50 sec

↓

Gently rinse slides in **tap water**

↓

Dehydrate in **70% Alcohol II** – 3 mins

↓

96% Alcohol II - 3 mins

↓

Absolute Alcohol III - 3 mins

↓

Xylene III – 5 mins

↓

Xylene IV – 5 mins

↓

Mount in **DPX**

3.17 Statistical analysis

The data generated during study was subjected to normal distribution analysis followed by non-parameteric tests including Kruskal-Wallis test (non-parametric ANOVA test). In case of two sample variance study, Mann-Whitney test (non-parametric t-test) was employed. In all the analyses, preference was given to two-tailed analysis. Statistical significance limit was kept at $p < 0.005$ (95%). GraphPad Prism 6.0 (GraphPad Software, inc) was used for above statistical analyses.

CHAPTER – IV

RESULTS AND DISCUSSION

Infectious coryza, an infectious respiratory disease of poultry has always remained a cynosure of malady for poultry industry/operation as a whole, due to its concurrent involvement with other pathogens. Current vaccines as well as vaccination strategies have failed to cope up with the infection pressure, possibly either due to poor understanding of host's immune system as well as anatomical complexities or due to scarce knowledge about its propensity towards host's tissues in particular. The organism, being tagged as an upper respiratory pathogen has never been dealt with its real-time involvement in the nasal mucosal surface, which perhaps is the initial homing site for its colonization and replication with in host's system. Consequently, this work was undertaken to elucidate the pathological process being initiated as a function of the infection in two different avian species i.e. chicken and Japanese quail. Further, our speculation about species-specific differences entails us to elaborate more on the directions of pathogen's migration across the host system. Therefore, the present investigation was taken to study the portal of entrance, differential pathogenetic process in chicken and Japanese quail following infection with *Av. paragallinarum*, the causal agent of Infectious coryza, and to produce polyclonal antibody against the organism. In addition to this, temporal participation of immune cell types as well as histological changes in different organs during different phases of infection in both chicken and Japanese quail were studied.

4.1 Isolation and identification of *Av.paragallinarum*

4.1.1 Identification by Culturing and Biochemical tests

Confirmation of isolate(s) procured from PDRC of Venkateshwara Hatcheries, Pune, India as *Av. paragallinarum* was done on the basis of cultural, morphological and biochemical characters. The colonies on the blood agar appeared as typical dew drop like colonies in the β-zone of hemolysis created by perpendicular streak of *Staphylococcus* organism (Fig.2). Similarly, greyish-white dew drop like colonies were observed on the Hemophilus test agar (Fig.3). Gram's staining revealed Gram negative cocco-bacilli organisms (Fig.4) indicative of *Av.paragallinarum* (100x). These findings agreed with those reported earlier by several workers (Yamamoto 1991; Sameera *et al* 2001; Kaur *et al* 2004).

The battery of biochemical tests *viz.*, urease, ONPG, Voges-Proskauer, oxidase, catalase, nitrate reduction, and fermentation of sugars like glucose, maltose,

lactose, sucrose, fructose and mannose all confirmed it to be an *Av. paragallinarum* isolate. Specifically, the biochemical tests like catalase and oxidase activity were considered to be an important marker of pathogen's identification (Rimler 1979; Piechulla *et al* 1985). In this study, the isolate(s) typically revealed negative catalase reaction and positive oxidase activity; with the additional phenomenon of positive nitrate reduction test (Fig.5a & 5b) which confirming its bacteriological identity. Kaur *et al* (2004) suggested the lack of catalase activity by any suspected isolates obtained from upper respiratory tract of birds would confirm it to be *Av. paragallinarum* isolate. Nitrite production from nitrate was also found to be a qualitative biochemical parameter for *Av. paragallinarum* isolate (Rimler 1979) and in this case, the isolates showed a positive nitrite production.

4.1.2 Molecular diagnosis of *Avibacterium paragallinarum* by PCR

To instil more confidence about the authenticity of isolates to be used for the experiment, polymerase chain reaction for *Avibacterium* spp was carried out by utilizing species specific primers (Table 1) and standard PCR conditions (Table 3) pertaining to species which yielded an amplified band at 500bp (Fig.6) correspondingly similar to known vaccine isolates containing *Av. paragallinarum*. This observation concurs with the recording made by Chen *et al* (1996). Further, serotype specific PCR was carried out to confirm the serotype involved which confirmed the isolate to be *Av. paragallinarum* serotype B based upon the band amplification at 1.1 Kbps (Fig.7). These results were in agreement with the results provided by Sakamoto *et al* (2011).

4.2 Determination of specificity of raised polyclonal antisera

The rabbit designated as negative control for antibody production was found sero negative by immuno dot blot techniques as no typical brown staining was observed upon application of DAB solution. The successive anti-sera collected from the rabbit injected with *Avibacterium* antigen at various time points, exhibited development of intense brown colour limited to the zone of spotted antigenic sites of PVDF membrane (Fig.8). A similar but with slightly different objectives of immuno dot blot technique was undertaken by Blackall *et al* (1990) to authenticate the suitability of monoclonal antibodies raised for *Avibacterium paragallinarum* serotyping.

Similarly, from counter-immuno-electrophoresis (CIE) procedure, an increased sensitivity of detection of antiserum's specificity was observed (Fig.9). A

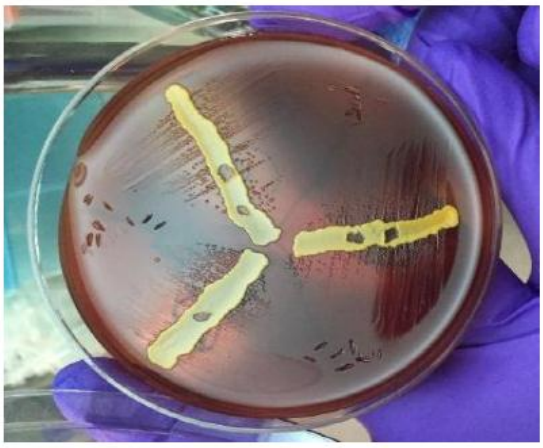

Fig. 2: Blood agar plate showing typical dew-drop like colonies of *A. paragallinarum* in the β-zone of hemolysis created by perpendicular streak of *Staphylococcus* organism.

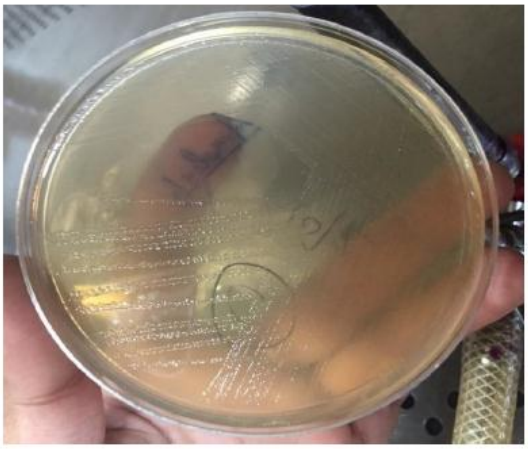

Fig. 3: Hemophilus test agar showing characteristic dew-drop like grayish-white colonies of *Av.paragallinarum*.

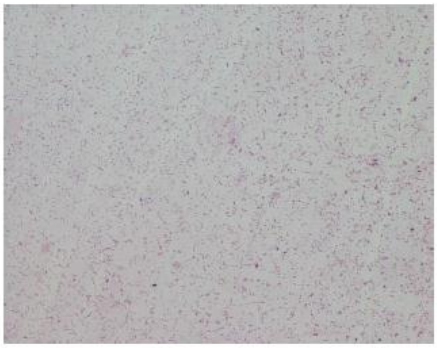

Fig. 4: Smear showing Gram negative cocco-bacilli organisms indicative of *Av. paragallinarum* (100x).

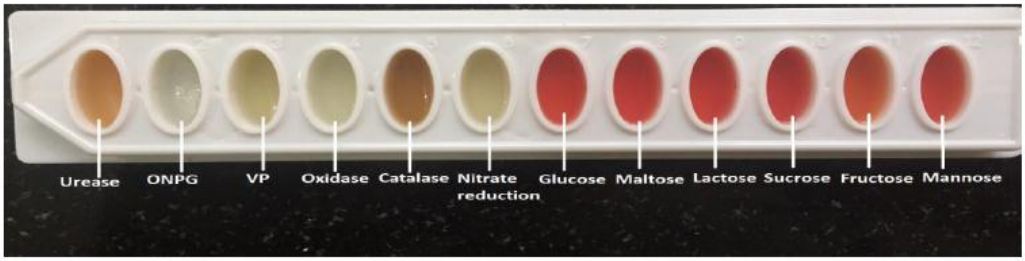

Figure 5a: Uninoculated biochemical identification strip

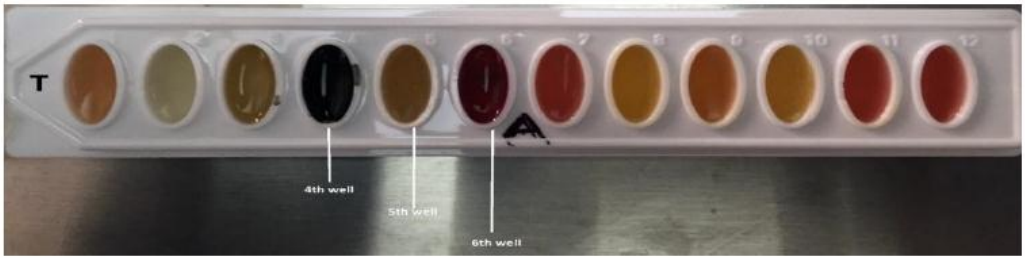

Figure 5b: (From L to R) 4th well from showing positive oxidase reaction as deep purple colour development, 5th well showing negative catalase activity as absence of effervescence and 6th well showing positive nitrate reduction as pinkish red colour development confirming *Avibacterium paragallinarum*.

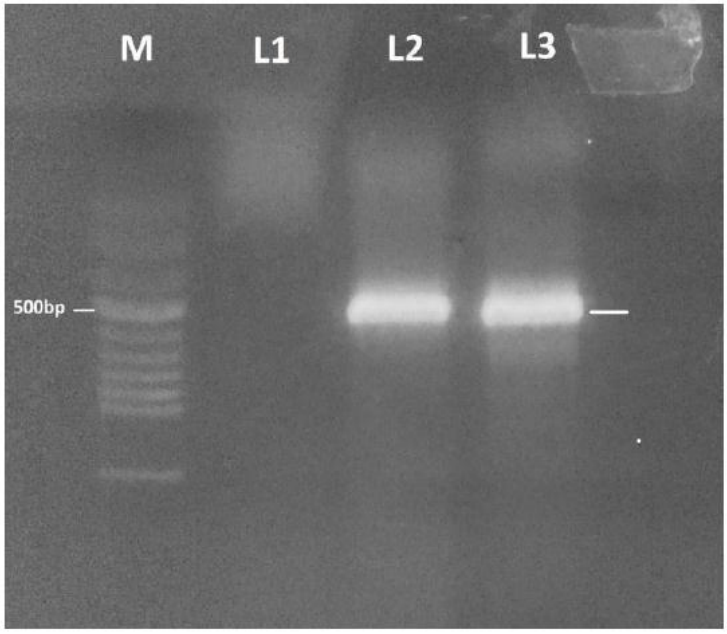

Figure 6: Molecular identification of *Avibacterium paragallinarum*. M: 100bp DNA ladder; L1: Nuclease free water as negative control template; L2: test sample (*Av.paragallinarum* isolate); L3: Positive control template (Killed coryza vaccine, source: Venky's India Ltd)

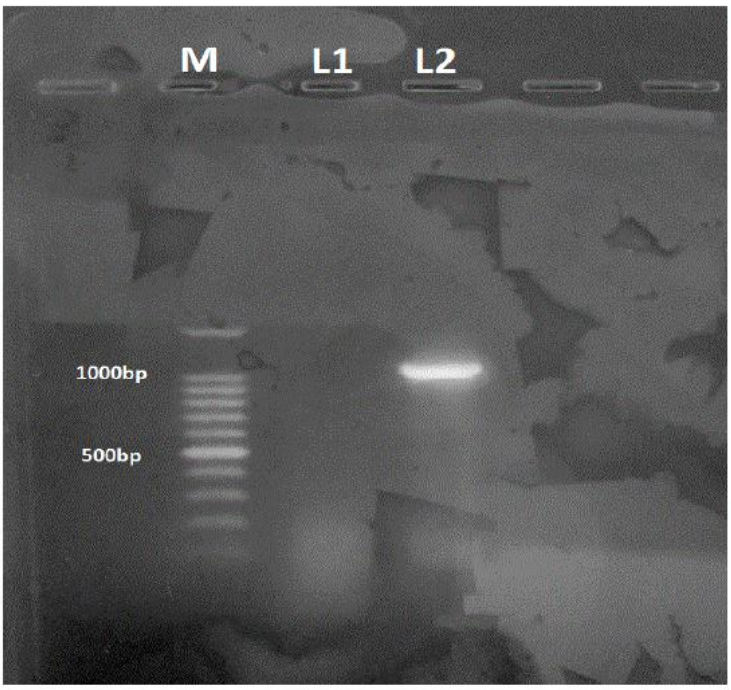

Figure 7: Serotype specific molecular identification of *Av.paragallinarum*. M: 100bp DNA ladder; L1: Non-specific migration/indistinct migration; L2: test sample (*Av.paragallinarum* serotype B).

modest precipitin band between the two closely-held punched holes (antigen-antibody) was indicative of a true positive reaction and was observed from all the serum samples collected periodically from the same rabbit. Similar quality of precipitin band was noticed from the positive control specimen. This observation has improved the possibilities of using CIE as one of the rapid method for detection of *Av. paragallinarum* infection from chicken's sera, which was never explored before. CIE has always remained a valuable, sensitive method for the early detection of infections like *Haemophilus influenza* in human subjects (Artman *et al* 1980) and still continues to hold an important position as a technique for rapid and early identification of other important septicemic diseases in both humans and animals. The pathogen *Av. paragallinarum* used in this study has a close phylogenetic relationship with the genus *Haemophilus* and was earlier grouped in same genus as *Haemophilus paragallinarum* which can possibly explain the reason for it tendency to undergo the unique electrophoretic phenomenon.

4.3 Determination of infectious dose for raising of polyclonal antisera and challenge experiment.

In order to raise polyclonal clonal antibody in rabbits, the optimum challenge dose was calculated by ten-fold serial dilutions and plate count method (Figure 10). Normally, for each dilution, paired colony forming units were estimated and calculated as per calculation method the given below.

$$\text{CFU/ml} = \frac{\text{Average no. of colonies}}{\text{Level of dilution} \times \text{Volume of dilutions plated}}$$

For polyclonal antibody production, an estimated 4.6 x 10^8 CFU/ml of bacterial suspension was used, while for infection challenge experiments performed at 2.5 weeks of age, the infective inoculum dose was 1.5 x 10^8 CFU/ml. For the second infection challenge experiment, the dose was maintained at 1.7 x 10^8 CFU/ml. The estimation of colony-forming unit for raising antibody was done for future appraisal, as one can utilize the similar dose for raising polyclonal antisera in rabbit against this pathogen under the stated conditions. The similarity in the inoculum dosage at both the experiments was to assure uniformity in the conduct of our experiment and to avoid the variation bias. Almost similar dose of bacteria was utilized by Byarugaba *et al* (2007), when conducting a study to evaluate the virulence of isolates from Uganda. Unlike others, Anjaneya *et al* (2013) used a higher dose of 10^{11} CFU/ml as

experimental inoculation dose to study the pathogenicity of various field isolates of *Avibacterium paragallinarum*, because of their varied experimental set up i.e. they employed in contact challenge method to induce clinical signs in chickens. Patil *et al* (2017) used 10^8 CFU/ml as the dose for intra-sinus inoculation for virulence studies on *Avibacterium paragallinarum* field isolates. Therefore 10^8 CFU/ml, was also backed by literature as a suitable infective dose for intra-sinus method of inoculation.

4.4 Final experiment:

4.4.1 Clinical signs development:

Birds were noted for the development of clinical signs at 12 hrs, 24 hrs, 48 hrs, 5 days and 7 days post-infection respectively. The clinical signs were evaluated on the basis of self-employed scoring criteria (Table 12). The criteria were developed based upon observations made during pilot experiment.

Table 12: Scoring criteria for the intensity of various clinical signs

Criteria	Score value	Descriptions
1. Nasal discharge		
Profuse/Marked	3	Flowing out of secretions from nasal passages without pressing.
Moderate	2	Sticking of fluid within nasal passages.
Mild	1	Pressing resulting in secretions from nasal passages.
2. Ocular discharge		
Profuse/Marked	3	Flowing out of secretions from ocular area without pressing.
Moderate	2	Sticking of fluid within orbital area.
Mild	1	Handling/pressing resulting in ocular discharge.
3. Swelling of infraorbital sinus		
Profuse/Marked	3	Both side/unilateral with raised bumps.
Moderate	2	Bilateral/unilateral swelling of sinus with resultant raised surface.
Mild	1	Bilateral/unilateral swelling of triangular fossa
4. Redness of conjunctival surface		
Profuse/Marked	3	Near dark red discolouration.
Moderate	2	Orange red discolouration.
Mild	1	Mild red to pinkish discoloration.
5. Redness around periorbital sinuses		
Profuse/Marked	3	Near dark red discolouration.
Moderate	2	Orange red discolouration.
Mild	1	Mild red to pinkish discoloration.

Figure 8: Specificity of raised polyclonal antisera confirmed by Immuno blot (development of brown color upon addition of DAB) (Negative control – no antigen)

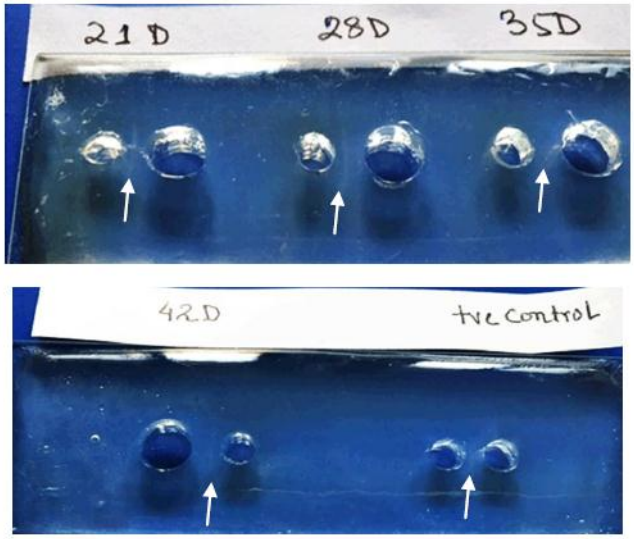

Figure 9: Specificity of raised polyclonal antisera confirmed by counter immuno-electrophoresis. Formation of precipitation lines between antigen and antibody wells (indicated by white arrows) confirmed specificity.

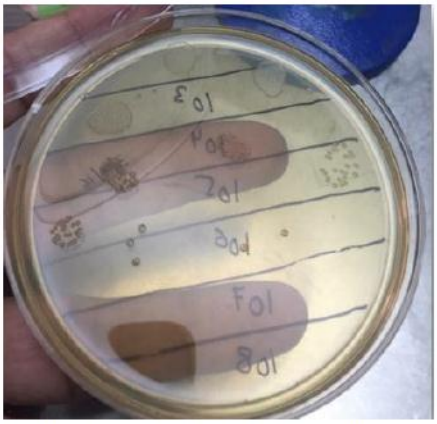

Figure 10: HA plate showing distinct colonies of *Avibacterium paragallinarum.* Colony count in respective zones of dilution used to calculate CFU/ml.

4.4.1.1

4.4.1.2 Clinical signs development at 2.5 weeks:

The clinical signs developed as early as 12 hrs post-infection in chickens, with characteristics occurrence of milder degree of nasal discharges, ocular discharges, swelling of infraorbital sinus, redness of conjunctiva and peri-orbital areas. Majority of birds exhibited unilateral presence of the symptoms in the beginning of infection and by 24hrs; there was a prominent presence of bilateral nasal discharge (Fig. 11 & Fig. 12). On the contrary, Japanese quails were comparatively resistant in expression of clinical signs and showed only milder degree of redness of conjunctiva throughout the period of experimental studies. In the earlier stages of infection, nasal discharge was the most consistent and pronounced clinical sign, followed by redness of conjunctiva. By 5 days and onwards, drastic decline in the presence of clinical signs was noted. A statistically significant association of clinical signs with species particularly to chicken has been noted (Table 13).

4.4.1.3 Clinical signs development at 6 weeks' age:

The clinical signs in chicken started to appear by 12 hrs post-infection with milder degree of unilateral nasal discharges as the prominent clinical outcome at 24 hrs (Fig. 13) and 48 hrs post-infection. Both swelling of intra-orbital sinus (Fig. 14) and redness of peri-orbital sinus grew by 5 days post-infection and subsided by 7 DPI. Conversely, Japanese quail by this age appeared to be strong and resistant in exhibiting clinical signs and looked clinically healthy with occasional to rare occurrence of ocular discharges in very few birds. Nasal discharges were significantly associated with chicken at this age (Table 14).

No clinical sign was noticed in any bird injected with sterile broth in both the experiments.

4.4.2 Comparison of clinical signs between two experiments

Nasal discharge was the prominent clinical sign in the infected chickens of both the ages. Presence of clinical signs was found to be more in the birds infected at 2.5 weeks. Maximum occurrence was noted at the early time points i.e. at 12 hrs and 24 hrs PI in the first experiment. Birds infected at 6 weeks age showed lesser presence of clinical signs. The development of clinical signs was also slower in the second experiment with peak occurrence noted at 5^{th} day PI.

As such, clinical signs development in Japanese quails were transient and fluctuating in both the experiments. In contrast to chickens, nasal discharge and swelling of infraorbital sinus were not appreciated in any Japanese quail during any of the trial. All the clinical signs noticed in Japanese quails were in the ocular region.

Our observation of clinical signs development in birds were in concurrence to those of earlier workers (Page *et al* 1962, Blackall 1989 and Ali *et al* 2013), who conducted the experimental studies at various places involving varied age groups of birds with different geographically-isolated pathotypes. Anjaneya *et al* (2013) also observed the presence of nasal discharge, swelling of infraorbital sinus and conjunctivitis mainly at 24hrs, 48 hrs and 3 days post-inoculation in seven weeks old broilers. A disappearance of the nasal discharge was noted by 5^{th} day PI and the other clinical signs gradually resolved after 10^{th} day PI.

Similarly, Akter *et al* (2016) reported the disappearance of nasal discharge by 5^{th} day PI, but noted the persistence of other signs till the last day of observation (7^{th} day PI) in birds infected at two weeks age. In our study, a similar timeline on the initiation of clinical signs was observed but the resolution of signs was recorded around 7^{th} day PI. This may be attributed to the moderate virulence of the strain used in our study. Zhao *et al* (2010) reported the onset of clinical signs by 24 hrs of inoculation when given by intra-sinus route while the development of visible disease was delayed by 3-5 days during the natural in-contact method of infection. These results also matched with our findings where early development of signs upon intra-sinus inoculation was observed. Patil *et al* (2017) described the onset of clinical signs at around 5^{th} day PI in seven weeks old chickens infected with *Av. paragallinarum* serotype B via in-contact method of transmission and noted the clinical signs to persist till 15^{th} day PI. In our study, the intra-sinus route of inoculation along with the milder virulence of the *Av. paragallinarum* serotype B strain may have been the reason for the early development and early resolution of the clinical signs.

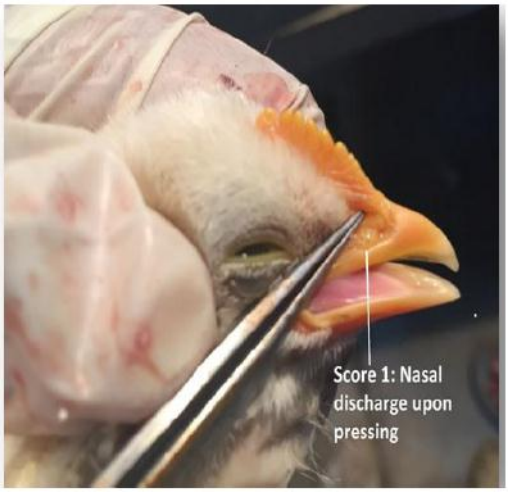

Figure 11: Chicken showing nasal discharge upon pressing (Score 1) based on the scoring criterion described before, (24 hours post-infection) at 2.5 weeks of age.

Figure 12: Chicken showing nasal discharge without pressing (Score 2) based on the scoring criterion described before, (24 hours post-infection) at 2.5 weeks of age.

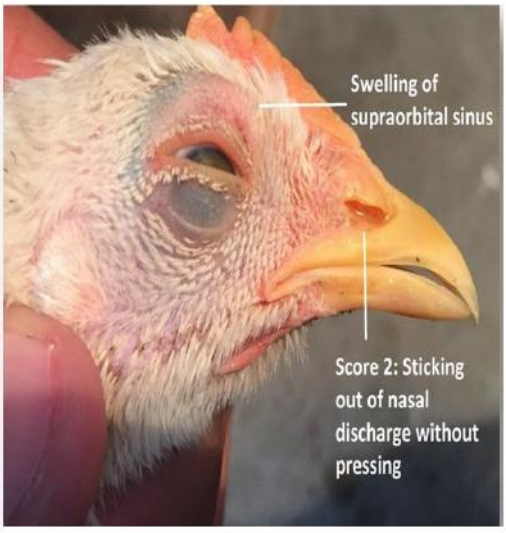

Figure 13: Chicken showing nasal discharge without pressing (Score 2) and mild swelling of supraorbital sinus, based on the scoring criterion described before (24 hours post-infection) at 6 weeks of age.

Figure 14: Chicken showing nasal discharge within the nasal cavity without pressing (Score 2) and swelling of the triangular fossa (Infraorbital sinus) (Score 1), based on the scoring criterion described before, at 5 days post-infection at 6 weeks of age.

Table 13: Number of birds showing different clinical signs of variable intensity at various time intervals following infection with *A. paragallinarum* at 2.5 weeks of age

Type of clinical signs	Intensity	12hrs (n= 23 C;23 J)						24hrs (n=21 C; 21 J)						48hrs (n=14C;14 J)						5DPI (n=10 C;9 J)						7DPI (n=4 C;4 J)					
		Number of birds showing clinical signs of variable intensity																													
		U		Bi		Total		U		Bi		Total		U		Bi		Total		U		Bi		Total		U		Bi		Total	
		C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J
OD	Mild	2	0	1	0	3	0	1	0	0	0	1	0	3	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
	Moderate	0	0	0	0			0	0	0	0			0	0	0	0			0	0	0	0			0	0	0	0		
	Severe	0	0	0	0			0	0	0	0			0	0	0	0			0	0	0	0			0	0	0	0		
ND	Mild	11^{c}	0	5^{b}	0	19^{c}	0	4^{a}	0	8^{c}	0	17^{c}	0	4	0	4	0	8^{b}	0	2	0	1	0	3	0	0	0	0	0	0	0
	Moderate	0	0	3	0			2	0	3	0			0	0	0	0			0	0	0	0			0	0	0	0		
	Severe	0	0	0	0			0	0	0	0			0	0	0	0			0	0	0	0			0	0	0	0		
SOIS	Mild	2	0	0	0	4	0	0	0	1	0	1	0	0	0	2	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
	Moderate	2	0	0	0			0	0	0	0			2	0	0	0			0	0	0	0			0	0	0	0		
	Severe	0	0	0	0			0	0	0	0			0	0	0	0			0	0	0	0			0	0	0	0		
RC	Mild	3	0	1	0	4	0	4	2	10^{b}	1	15^{c}	3	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0
	Moderate	0	0	0	0			0	0	0	0			0	1	0	0			0	0	0	0			0	0	1	0		
	Severe	0	0	0	0			0	0	1	0			0	0	0	0			0	0	0	0			0	0	0	0		
RPO	Mild	11	0	0	0	11	0	1	0	2	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Moderate	0	0	0	0			1	0	0	0			0	0	0	0			0	0	0	0			0	0	0	0		
	Severe	0	0	0	0			0	0	0	0			0	0	0	0			0	0	0	0			0	0	0	0		

Number of birds in each infected group=23. DPI=Days post infection; OD= Ocular discharge; ND= Nasal discharge; SOIS= Swelling of infra-orbital sinus; RC= Redness of conjunctiva; RPO= Redness of peri-orbital area; U= Unilateral appearance of clinical signs; Bi= Bilateral appearance of clinical signs; Total= Total no. of birds showing specific clinical signs at specific time interval. C= Chicken; J= Japanese quail; Significant association of clinical signs development noted within species as specified by subscript a $P<0.05$, b $P<0.01$, c $P<0.001$, and was made through Chi square ($\chi 2$) test.

Table 14: Number of birds showing different clinical signs of variable intensity at various time intervals following infection with *A. paragallinarum* at 6 weeks of age.

Type of clinical signs	Intensity	12hrs (n= 22 C;14 J)						24hrs (n=18 C; 12 J)						48hrs (n=12C;7 J)						5DPI (n=8 C;9 J)						7DPI (n=4 C;4 J)					
		Number of birds showing clinical signs of variable intensity																													
		U		Bi		Total		U		Bi		Total		U		Bi		Total		U		Bi		Total		U		Bi		Total	
		C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J	C	J
OD	Mild	1	1	0	0	1	1	0	1	0	0	0	1	1	0	0	0	1	0	0	-	0	-	1	0	0	0	0	0	0	0
	Moderate	0	0	0	0			0	0	0	0			0	0	0	0			1	-	0	-			0	0	0	0		
	Severe	0	0	0	0			0	0	0	0			0	0	0	0			0	-	0	-			0	0	0	0		
ND	Mild	5	0	2	0	7	0	4	0	3	0	7[a]	0	2	0	2	0	5	0	1	-	0	-	3	0	0	0	0	0	0	0
	Moderate	0	0	0	0			0	0	0	0			1	0	0	0			1	-	1	-			0	0	0	0		
	Severe	0	0	0	0			0	0	0	0			0	0	0	0			0	-	0	-			0	0	0	0		
SOIS	Mild	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	-	1	-	4	0	0	0	0	0	0	0
	Moderate	0	0	0	0			0	0	0	0			0	0	0	0			1	-	1	-			0	0	0	0		
	Severe	0	0	0	0			0	0	0	0			0	0	0	0			0	-	0	-			0	0	0	0		
RC	Mild	1	0	1	0	2	0	0	0	0	0	0	0	1	0	1	0	2	0	0	-	0	-	0	0	0	0	0	0	0	0
	Moderate	0	0	0	0			0	0	0	0			0	0	0	0			0	-	0	-			0	0	0	0		
	Severe	0	0	0	0			0	0	0	0			0	0	0	0			0	-	0	-			0	0	0	0		
R PO	Mild	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	1	-	2	-	3	0	0	0	1	0	1	0
	Moderate	0	0	0	0			0	0	0	0			0	0	0	0			0	-	0	-			0	0	0	0		
	Severe	0	0	0	0			0	0	0	0			0	0	0	0			0	-	0	-			0	0	0	0		

Number of birds in infected groups=23 chicken and 14 Japanese quail i. DPI=Days post infection; OD= Ocular discharge; ND= Nasal discharge; SOIS= Swelling of infra-orbital sinus; RC= Redness of conjunctiva; RPO= Redness of peri-orbital area; U= Unilateral appearance of clinical signs; Bi= Bilateral appearance of clinical signs; Total= Total no. of birds showing specific clinical signs at specific time interval. C= Chicken; J= Japanese quail; Significant association of clinical signs development noted within species as specified by subscript a $P<0.05$, and was made through Chi square ($\chi 2$) test.

4.4.3 Gross pathological examination:

In the present study, the gross lesions observed included the redness of turbinate, mucus accumulation in nasal passage, enlargement or discoloration of Harderian gland and conjunctival haemorrhages along with descriptive changes in the trachea and lungs. Scoring criteria for the gross lesions to be observed from head/nasal area were developed (Table 15) based upon the observations made in the pilot experiment.

Table 15: Scoring criteria for the evaluation of various gross lesions

Criteria	Score value	Descriptions
1. Redness of turbinates		
Severe/Marked	3	Dark intense red discolouration with haemorrhagic impression.
Moderate	2	Red discoloration without haemorrhagic impression.
Mild	1	Red tinge or pinkish colouration of turbinate.
2. Mucus accumulation		
Severe/Marked	3	Copious accumulations of watery to sticky mucus secretions.
Moderate	2	Sticky mucus accumulation.
Mild	1	Extremely scanty with just sticky mucus adherence.
3. Harderian gland discoloration, if any/ swelling		
Severe/Marked	3	Red to dark red discoloration of gland with enlargement/swelling.
Moderate	2	Dark red discoloration of the gland.
Mild	1	Only redness with no evident swelling.
4. Conjunctival haemorrhages		
Severe/Marked	3	Diffuse to patchy, intense red discoloration of surface with haemorrhagic impression.
Moderate	2	Diffuse to patchy redness of surface with or without haemorrhagic impression.
Mild	1	Localized areas of redness with or without scattered Petechial haemorrhages.

4.4.3.1 Gross pathological observation at 2.5 weeks:

Majority of chickens revealed redness of turbinates and nasal passage along with mucus accumulation within the nasal passages immediately at 12 hrs post-infection with varying severity (score level 3-1) that continued until final day of observation i.e. 7 days PI. The nasal turbinates were either characterized as intense red turbinate folds with haemorrhagic foci/blotches to red or pinkish discoloration of turbinate folds. At 24 hrs post-infection, majority of chickens showed redness of turbinates (score level 2). The severity of lesion gradually waned down to pinkish discoloration of turbinates (i.e. milder intensity; score level 1) from 5^{th} day post infection onwards. However, increased appearance of mucus accumulation within nasal passages was noted by 48 hrs post-infection (score level 3) that persisted for a day with subsequent decline in amount of secretions noted as scanty mucus with adherence to turbinates (score level 1). Conjunctival haemorrhages appeared infrequently in chickens and were consistent on 48 hrs post-infection with diffuse to patchy redness of conjunctival surface comprising haemorrhagic foci (score level 3). The Japanese quail, in contrast to chickens only exhibited milder degree of redness of turbinate and nasal passage (score level 1) that prevailed only at 48 hrs post-infection. A statistically significant difference in the mean gross lesion score was noted among groups ($p<0.0005$) over the period of experiment with substantial degree of changes noted beginning 24 hrs and 48 hrs post-infection between chicken and Japanese quail (Fig. 15). On an average, the mean gross lesions score in chickens at 48 hrs post-infection were significantly higher than those noted in Japanese quails.

Gross lesions observed in the lungs included occasional unilateral or bilateral brown patchy discoloration in both the species. Majority of the sacrificed birds at all the time points post-infection revealed grossly normal trachea and lungs. This may be attributed to the possible absence of a concurrent respiratory pathogen which prevented the spread of the infection to the lower respiratory tract (Blackall 1989).

Other visceral organs including liver, kidney and reproductive tract were found to be normal except for occasional pale discoloration of liver in some cases, which was deemed to be non-specific. This observation may be explained by the fact the coryza is mainly a disease of upper respiratory tract and it rarely only affects the lower respiratory tract and visceral organs when complicated by secondary pathogens (Anjaneya *et al* 2013).

4.4.3.2 Gross pathological observation at 6 weeks:

The only and early major gross lesions present in chickens were copious accumulation of mucus and adherence of mucus to the turbinates (score level 3-2) that was noted by 12 hrs and 48 hrs post-infection. Gradually, the intensity of mucus accumulation subsided from 5th day post-infection onwards with infrequent evidences of copious accumulations (score level-3) in few birds up to 7 DPI. Redness of turbinates, observed as a pinkish discoloration was noted as an intermittent occurrence up to 7 DPI. The Japanese quails on the other hand, showed rare to occasional occurrence of mucus accumulation and redness of turbinates (score level-l). None of the sterile broth injected chicken or Japanese quail evinced any prominent lesions and displayed normal coloration of turbinates without any additional mucus formation and no conjunctival congestion/haemorrhages. A statistically significant difference ($p<0.0340$) in mean gross lesion score among groups was noted across the time intervals (Fig. 16).

Majority of the sacrificed birds at all the time points post-infection revealed grossly normal trachea and lungs. This may be due to the possible absence of a concurrent respiratory pathogen which prevented the spread of the infection to the lower respiratory tract (Blackall 1989).

Other visceral organs including liver, kidney and reproductive tract were found to largely normal. This observation possibly rules out the absence of an overlapping infection by a secondary pathogen. (Anjaneya *et al* 2013).

4.4.4 Differences between gross lesions development during both the experiments

Higher mean gross lesion score was noted in the birds infected at 2.5 weeks age, with the score peaking at 48 hrs post-infection during this experiment. During the second experiment, maximum gross lesion score value was observed at 24 hrs post-infection. In both the experiments, gross lesions were present in the birds sacrificed at 7th day PI, as opposed to clinical signs which were rarely noticed at this time point.

The occurrence of gross lesions as well as the pattern of development of lesions were more or less similar to other studies (Yamamoto 1972; Blackall 1989; Blackall and Soriano 2013). Anjaneya *et al* (2013), Ali *et al* (2013) and Akter *et al* (2016) documented the presence of nasal exudate and mucus membrane congestion at 48 hrs, 72 hrs and 5th day PI while noting the involvement of lower respiratory tract during later time points after infection. Our results followed a similar trend despite of

the usage of different Avibacterium serotypes. This observation led to a hypothesis that even the isolates/ serotypes of the pathogen of different geographical origin may result in the formation of similar lesions by employing similar trend in pathologic basis of disease development. Incidentally, in our study, the evidence of gross lesion subsided to almost negligible by 7 day PI in both the species, despite of using intra-sinus route of administration. We assume that a moderate degree of virulence of isolate used could have led to an early resolution of the infection.

Av. paragallinarum, a pathogenic microbe, mainly affects the upper respiratory tract of birds and rarely results in lower respiratory tract infections. This report is further attested based upon the similar pattern of lesion development in our study. Both of our infection challenge experiments have resulted in similar development of lesions localised to the upper respiratory tract, arguably due to the fact that the experimental facility maintained during experiments was free from any major secondary pathogen, thus preventing co-infections. Involvement of the lower respiratory tract during infectious coryza is mainly attributed to the synergistic presence of various other respiratory pathogens (Blackall 1989), which thereby explains the scarcity of lesions in lower respiratory tract like trachea, bronchi and alveolar region of lungs. None of the sampled trachea and lungs during either of the experimental set-up evinced any prominent gross lesions which could be considered as a result of *Av. Paragallinarum* infection. The other organs collected like liver, kidney and reproductive tract were devoid of any significant gross lesions in both of the experimental studies.

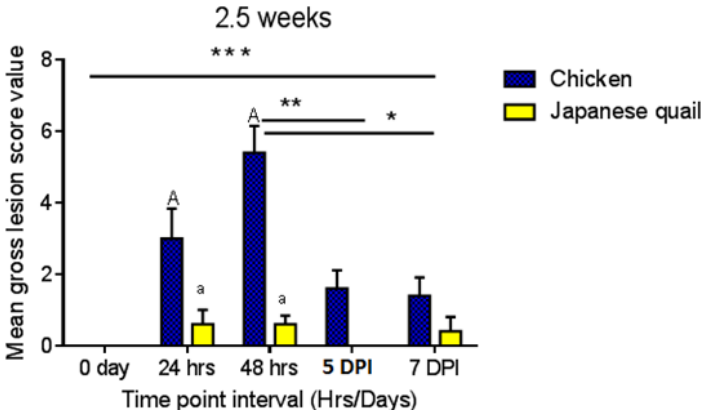

Fig. 15: Comparison of mean gross lesion score value of 2.5 weeks old infected chicken and Japanese quail at various time points post-infection.

* indicates $p<0.05$ level of significance, ** indicates $p<0.01$, *** indicates $p<0.001$ level of significance.

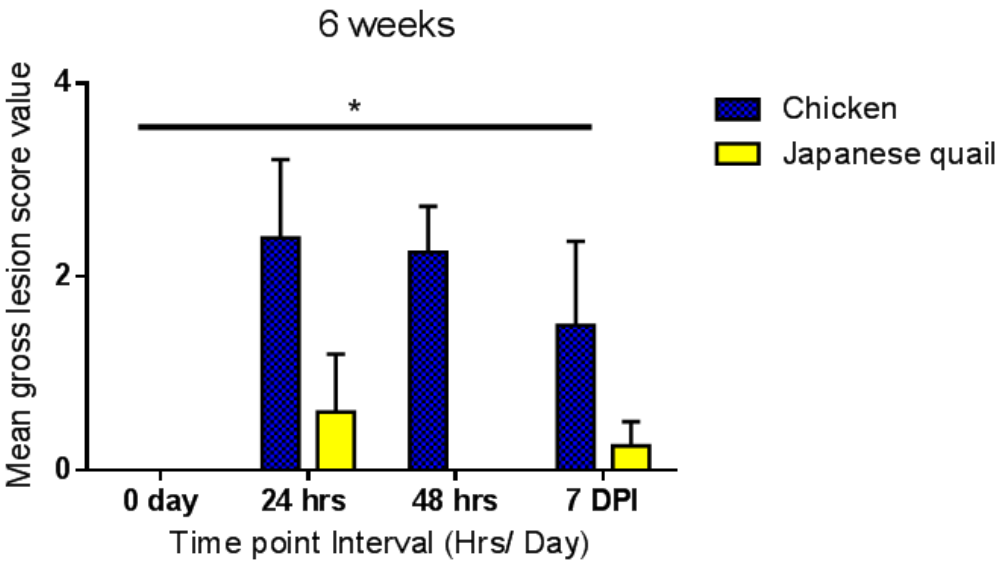

Fig. 16: Comparison of mean gross lesion score value of 6 weeks old infected chicken and Japanese quail at various time points post-infection.

* indicates $p<0.05$ level of significance (Graphpad® 6.0 software, USA)

4.5 Oxidative stress studies

4.5.1 Erythrocytic lipid peroxidation (MDA concentration)

The oxidative stress analysis was performed only on the blood samples collected during the infection challenge conducted on 6 weeks old birds.

Prior to infection, the lipid peroxidation in the form of malondialdehyde (MDA) concentration was found to be minimal with non-significant difference between groups. Following bacterial challenge, there was a gradual increase in the concentration of MDA observed (in chickens and Japanese quail) either at 24 hrs and 48 hrs post-infection respectively (compared to naïve chicken and Japanese quail), which further rose significantly at 7 DPI. Between chicken and Japanese quail, the latter exhibits higher magnitude of lipid peroxidation in the form of higher MDA concentration with significant difference ($p < 0.0001$) at 7 DPI (Fig.17).

4.5.2 Reduced glutathione:

The reduced glutathione didnot show a stark difference within the two species after infection; however, a trend of gradual decline was noted over the time period. Significant difference was noted between the GSH values of naïve control chicken and both infected Japanese quail and chicken following infection challenge (Fig. 18).

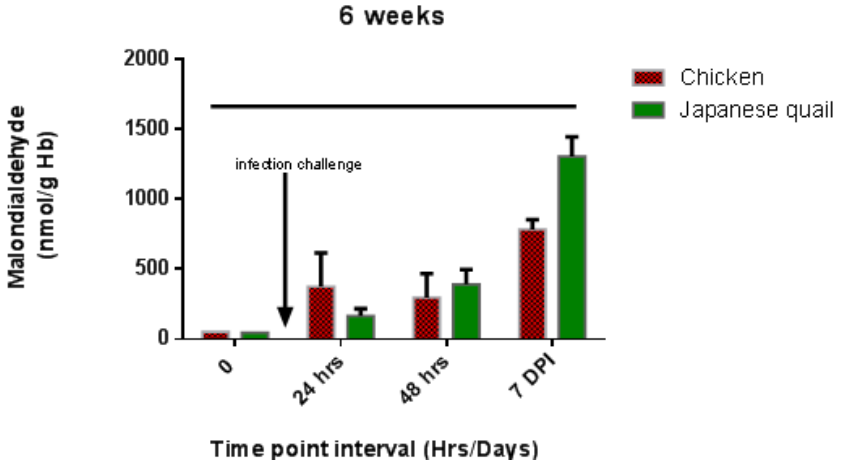

Fig. 17: Comparison of erythrocytic lipid peroxidation, MDA (nmol/g Hb) in chicken and Japanese quails at various time points post-infection.

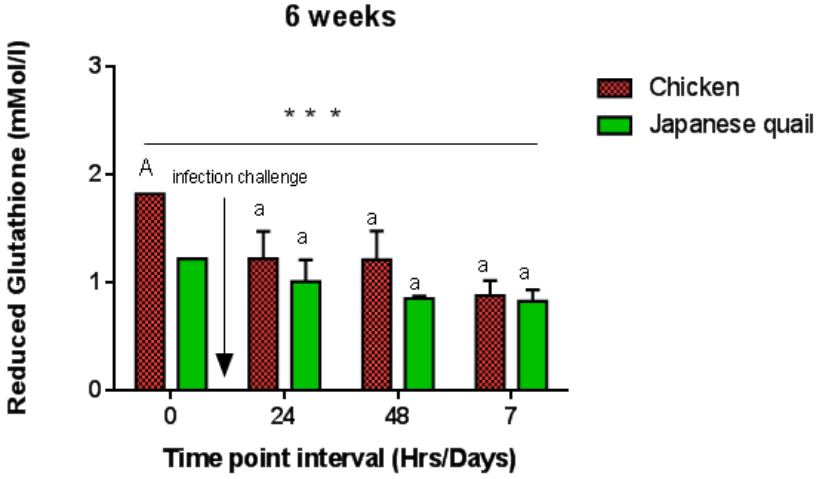

Fig. 18: Comparison of reduced glutathione (nmol/l) in chicken and Japanese quails at various time points post-infection.

*** indicates $p<0.001$ level of significance.

Our survey of literature suggested no evidence of work on lipid peroxidation assay pertaining to infectious coryza infections in birds, however work related to other respiratory tract pathogen as well as known economically distressing diseases was found available (Zhao *et al* 2003; Georgieva *et al* 2005; Keles *et al* 2009 and Wang *et al* 2011). In our study, we attempted the estimation of circulatory level of malondialdehyde concentration which gradually increased from 0 day to 7th day post-infection in both the species. We hypothesise that the sluggish trend of increasing erythrocytic lipid peroxidation could be the result of a delayed systemic entrance of the pathogen. As explained before, due to the modest nature of pathogen used, its causal ability to produce a systemic disease at an early stage of infection might have

been hindered strongly by host immune arsenal thus resulted in gradual rise of MDA by 7th day post infection. Incidentally, Boucher *et al* (2014) suggested from their experimental study that the host may utilize the antioxidant mechanisms as an initial response strategy to avoid pathogenic insults from *Av. Paragallinarum*. Their observation was based on transcriptional profiling studies performed with the help of in-silico analysis.

Reduced glutathione (GSH), a cellular antioxidant, gets utilized by glutathione peroxidase enzyme for the neutralization of reactive oxygen species (ROSs) that generally emanate from host-pathogen interaction. The estimation of GST concentration revealed depleted level in the current study which corresponded well with the elevated level of MDA concentration. This suggested a pivotal role being played by certain class of mononuclear cells towards mitigation of pathogenic damage caused by systemically gained pathogen.

Interestingly, Japanese quail in comparison to chicken has shown higher MDA concentrations possibly indicating a case of well-conceived protective mechanism being adopted by host against this pathogen. Low grade clinical disease in Japanese quail despite having higher lipid peroxidation values may be due to higher tolerance of this species towards oxidative stress, conjointly may also be inferred about other possibilities other than oxidative stress-dealing mechanisms in conferring resistance to Japanese quails.

4.6 Re-isolation attempts:

4.6.1 2.5 weeks age experiment:

Organism was successfully re-isolated from the chickens sacrificed at 24 hrs and 5 days post-infection respectively. However, from infected Japanese quails, re-isolation attempt resulted successful retrieval on 7th day post-infection. No organism was isolated from sterile broth inoculated birds and naive birds at any stage of experiment.

4.6.2 6 weeks age experiment:

Similarly, at this age, the successful retrieval of organism from chickens was possible at 24 hrs, 5th day & 7th day post-infection respectively. In Japanese quail, the

successful re-isolation of organism was done on 24 hrs & 7^{th} day post-infection respectively. Sterile broth groups were found completely free from the pathogen at each respective point of sacrifice. Similar to our study, Matsumoto and Yamamoto (1972) isolated *Av. paragallinarum* from the nasal sinus of infected birds after five to six days after intra-sinus exposure. Ali *et al* (2013) and Akter *et al* (2016) re-isolated the bacteria from the nasal passage of experimentally infected chickens seven days after the infection. This shows that viable bacteria may persist in the nasal cavity of infected birds, even after the resolution of clinical disease, thereby suggesting an attempt of providing immunological ensconce to pathogen by the host's system. On similar grounds, Japanese quails may not have displayed any clinical sign but harboured reliable bacteria in the nasal cavity.

4.7 Microscopic lesions:

4.7.1 In nasal region

Histopathological analysis of tissues revealed several changes which were more pronounced in the nasal region. Nasal tissue sections of naïve birds were examined to verify the normal histoarchitecture (Fig 20 & 21). Based upon the observations, scoring criteria (Table 16) was developed to evaluate the degree of various pathomorphological alterations in the nasal turbinates (middle turbinate and posterior turbinate) and associated structures (inferior nasal meatus and infraorbital sinus).

Table 16: Microscopic score based on the self-made scoring criterion:

S. No.	Types of microscopic lesions & its description	Intensity	Score
A	Loss of mucosal epithelial cells **(Fig. 30)**		
1	Focal to multifocal marginal loss/ degenerative to necrotic damage of the epithelial cells involving upper 1/3rd of mucosal lining.	+ (Mild) (Fig. 30a)	1
2	Widespread loss involving a stretch of mucosal lining in the form of desquamation (sloughing)/necrotic damage, leaving aside partial denudation of lamina propria.	++ (Moderate) (Fig. 30b)	2
3	Complete denudation leaving aside exposed lamina propria, often accompanied by haemorrhages and cellular infiltration	+++ (Severe) (Fig. 30c)	3
B	**Loss of cilia/deciliation**		
1	Focal to multifocal loss of cilia from epithelium or fur like arrangement of cilia at some places	+ (Mild)	1
2	Widespread loss involving a stretch of mucosal lining in the form of sloughing	++ (Moderate)	2
3	No traces of cilia noted	+++ (Severe)	3
C	**Alveolar mucoid gland activity (Fig. 31)**		
1	Only glandular hypertrophy noted with resultant secretions.	+ (Mild) (Fig. 31a)	1
2	Both glandular hypertrophy and hyperplasia observed with beginning of stratification of glands	++ (Moderate) (Fig. 31b)	2
3	Massive presence of glands noted with resultant stratification	+++ (Severe) (Fig. 31c)	3
D	**Heterophilic cell infiltration (Fig. 32)**		
1	Scattered infiltration in few places confined to lamina propria	+ (Mild) (Fig. 32a)	1
2	Widespread infiltration with resultant spread out to intra-epithelial region and sub mucosal region	++ (Moderate) (Fig. 32b)	2
3	Dense infiltration across the lamina propria as well as in the epithelial region/sub mucosal zone	+++ (Severe) (Fig. 32c)	3

S. No.	Types of microscopic lesions & its description	Intensity	Score
E	**Lymphoid cell infiltration (Fig. 33)**		
1	Scattered infiltration in few places confined to lamina propria	+ (Mild) (Fig. 33a)	1
2	Widespread infiltration with resultant spread out to intra-epithelial region and sub mucosal region	++ (Moderate) (Fig. 33b)	2
3	Dense infiltration across the lamina propria as well as in the epithelial region/sub mucosal zone	+++ (Severe) (Fig. 33c)	3
F	**Vascular congestion/ Hyperaemic changes**		
1	Only vascular congestion noted without dilatation of blood vessels.	+ (Mild)	1
2	Vascular congestion noted with marked dilatation of blood vessels, along with probable increase in its number	++ (Moderate)	2
3	Marked dilation of blood vessels; often accompanied with haemorrhages	+++ (Severe)	3
G	**Haemorrhages (Fig. 34)**		
1	Occasional to focal foci or thin foci of haemorrhages especially around the blood vessels.	+ (Mild) (Fig. 34a)	1
2	Focal to large widespread areas of haemorrhages	++ (Moderate) (Fig. 34b)	2
3	Diffuse areas of haemorrhages	+++ (Severe) (Fig. 34c)	3
H	**(Nasal associated lymphoid tissue) hyperplasia (Fig. 35)**		
1	Expansion of lymphoid zone with mild vascular congestion and haemorrhages	+ (Mild) (Fig. 35a)	1
2	Expansion of lymphoid zone noted with or without germinal centre formation; often associated with encroachment to surrounding tissues.	++ (Moderate) (Fig. 35b)	2
3	Profuse expansion of lymphoid zone replacing the neighbouring tissue details	+++ (Severe) (Fig. 35c)	3

Table 17: Histopathological changes in the nasal structures at various time points after infection with *Av. paragallinarum* in 2.5 weeks old birds

Time point (Hrs/Days post infection)	Species	12hrs	24hrs	48hrs	5th day	7th day
Histopathological changes						
1. Hemorrhages	Chicken	Mild	Mild to moderate	Mild to moderate	Mild to moderate	Mild to moderate
	JQ	Mild	Mild	Mild	Mild	Mild
2. Loss of cilia	Chicken	Very mild	Moderate	Moderate to marked	Moderate to marked	Mild to moderate (Epithelium regeneration)
	JQ	Mild	Mild	Mild to moderate	Mild (fibrotic changes)	Very mild (Regenerative changes)
3. Loss of epithelium	Chicken	Very mild	Mild to moderate	Mild to moderate	Moderate (Fibroplasia)	Mild to moderate (Fibroplasia)
	JQ	Very mild	Mild	Mild	Mild	Very mild
4. Alveolar mucoid gland activity	Chicken	Absent	Moderate	Mild	Very mild	Very mild
	JQ	Absent	Mild	Mild	Mild	Very mild
5. Lymphoid cell infiltration	Chicken	Absent	Mild	Moderate	Moderate	Moderate
	JQ	Very mild	Mild	Mild	Mild	Very mild
6. Heterophilic infiltration	Chicken	Very mild	Moderate	Very mild	Very mild	Mild to moderate
	JQ	Absent	Mild	Very mild	Absent	Absent
7. Heterophilic infiltration	Chicken	Absent	Mild	Mild to moderate	Mild to moderate	Moderate
	JQ	Absent	Mild	Mild to moderate	Mild to moderate	Moderate

Table 18: Histopathology of Inferior nasal meatus of chicken & Japanese quail inoculated with *A. Paragallinarum* at 2.5 weeks of age.

Time points	*A. paragallinarum* inoculated Chicken							*A. paragallinarum* inoculated Japanese quail						
Hrs/DPI[A]	Lc[B]	Le[C]	Ga[D]	He[E]	Ly[F]	Hm[G]	NLT[H]	Lc	Le	Ga	He	Ly	Hm	NLT
12 hrs	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	1.0	1.0	0.0	0.0
24 hrs	3.0	1.0	1.3	1.3	2.3	1.6	2.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0
48 hrs	2.5	2.2	2.0	0.2	2.0	1.5	1.7	1.0	1.0	0.5	0.5	1.0	1.0	0.0
5 DPI	1.6	2.2	0.6	0.6	1.6	2.0	2.4	Nd	Nd	Nd	Nd	Nd	Nd	Nd
7 DPI	1.0	1.6	1.0	0.0	2.6	2.0	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total														

DPI[A] Days post infection.

Lc[B] Loss of cilia or Deciliation.

Le[C] Loss/damages of epithelium either in the form of necrosis or degenerative changes.

Ga[D] Alveolar Gland activity in inferior nasal meatus.

He[E] Heterophilic infiltration in tunica propria of inferior nasal meatus.

Ly[F] Lymphocytic infiltration in tunica propria of inferior nasal meatus.

Hm[G] Haemorrhages in tunica propria of inferior nasal meatus.

NLT[H] Nasal associated lymphoid tissue hyperplasia.

Nd Not determined due to non-availability of sufficient tissue sections.

Mean of severity index [I] = (0, 1, 2, and 3 which is normal, mild, moderate and severe intensity of lesion based on Table __, respectively in 3 birds each time.

Table 19: Histopathology of Middle turbinates of chicken & Japanese quail inoculated with *A. Paragallinarum* at 2.5 weeks of age.

Time points	*A. paragallinarum* inoculated Chicken							*A. paragallinarum* inoculated Japanese quail						
Hrs/DPI[A]	Lc[B]	Le[C]	Ga[D]	He[E]	Ly[F]	Hm[G]	NLT[H]	Lc	Le	Ga	He	Ly	Hm	NLT
12 hrs	0.0	0.0	0.0	0.5	0.0	0.0	0.0	1.0	0.0	0.0	0.0	1.0	1.0	0.0
24 hrs	2.6	1.6	1.8	1.8	1.2	1.2	1.0	1.4	0.8	1.6	0.8	1.0	1.0	0.0
48 hrs	1.2	1.4	1.4	0.4	1.8	1.2	0.8	2.0	0.7	0.7	1.2	1.5	0.5	0.0
5 DPI	2.6	1.6	1.2	0.6	1.8	1.2	1.0	1.3	0.6	1.0	0.0	1.0	1.0	0.0
7 DPI	1.6	1.3	0.6	1.6	1.6	1.3	1.6	0.7	0.2	0.5	0.2	0.5	1.5	0.0

DPI[A] Days post infection.

Lc[B] Loss of cilia or Deciliation.

Le[C] Loss/damages of epithelium either in the form of necrosis or degenerative changes.

Ga[D] Alveolar Gland activity in Middle turbinates (conchae nasalis medialis).

He[E] Heterophilic infiltration in tunica propria of Middle turbinates (conchae nasalis medialis).

Ly[F] Lymphocytic infiltration in tunica propria of Middle turbinates (conchae nasalis medialis).

Hm[G] Haemorrhages in tunica propria of Middle turbinates (conchae nasalis medialis).

NLT[H] Nasal associated lymphoid tissue hyperplasia

Nd Not determined due to non-availability of sufficient tissues sections.

Mean of severity index [I] = (0, 1, 2, and 3 which is normal, mild, moderate and severe intensity of lesion based on Table __, respectively in 3 birds each time.

Table 20: Histopathology observation in Infra orbital sinus of chicken and Japanese quail inoculated with *A. Paragallinarum* at 2.5 weeks of age.

Time points	*A. paragallinarum* inoculated Chicken							*A. paragallinarum* inoculated Japanese quail						
Hrs/DPI[A]	Lc[B]	Le[C]	Ga[D]	He[E]	Ly[F]	Hm[G]	NLT[H]	Lc	Le	Ga	He	Ly	Hm	NLT
12 hrs	1.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0	1.0	0.0	0.0
24 hrs	2.5	2.0	0.0	1.2	1.5	2.7	0.2	1.8	0.75	0.2	0.0	1.6	0.6	0.0
48 hrs	3.0	2.5	0.7	0.0	1.5	1.5	0.2	1.3	0.6	1.0	0.3	0.6	0.3	0.0
5 DPI	3.0	2.8	0.4	0.8	2.2	1.6	0.8	2.0	1.3	0.0	0.3	1.3	0.0	0.3
7 DPI	3.0	2.3	0.0	0.0	2.6	2.0	1.0	1.0	0.5	0.0	0.2	0.2	0.0	0.0

DPI[A] Days post infection.

Lc[B] Loss of cilia or Deciliation.

Le[C] Loss/damages of epithelium either in the form of necrosis or degenerative changes.

Ga[D] Alveolar Gland activity in infra-orbital sinus / maxillary sinus.

He[E] Heterophilic infiltration in tunica propria of infra-orbital sinus / maxillary sinus.

Ly[F] Lymphocytic infiltration in tunica propria of infra-orbital sinus/ maxillary sinus.

Hm[G] Haemorrhages in tunica propria of posterior (dorsal) turbinates.

NLT[H] Nasal associated lymphoid tissue hyperplasia

Nd Not determined due to non-availability of sufficient tissue sections.

Mean of severity index [I] = (0, 1, 2, and 3 which is normal, mild, moderate and severe intensity of lesion based on Table __, respectively in 3 birds each time.

Table 21: Histopathology of Posterior turbinates of chicken & Japanese quail inoculated with *A. Paragallinarum* at 2.5 weeks of age.

Time points	*A. paragallinarum* inoculated Chicken							*A. paragallinarum* inoculated Japanese quail						
Hrs/DPI[A]	Lc[B]	Le[C]	Ga[D]	He[E]	Ly[F]	Hm[G]	NLT[H]	Lc	Le	Ga	He	Ly	Hm	NLT
12 hrs	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0
24 hrs	2.2	0.8	0.0	1.0	1.2	1.2	0.0	1.0	0.6	0.0	0.0	0.6	0.0	0.0
48 hrs	2.3	0.6	0.0	0.6	1.3	1.3	0.0	1.5	0.7	0.0	0.0	1.0	0.7	0.0
5 DPI	3.0	2.5	0.0	0.0	2.0	2.5	0.0	Nd	Nd	Nd	Nd	Nd	Nd	Nd
7 DPI	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.0	0.0	0.0	0.0	0.3	0.0	0.0

DPI[A] Days post infection.

Lc[B] Loss of cilia or Deciliation.

Le[C] Loss/damages of epithelium either in the form of necrosis or degenerative changes.

Ga[D] Alveolar Gland activity in posterior (dorsal) turbinates.

He[E] Heterophilic infiltration in tunica propria of posterior (dorsal) turbinates.

Ly[F] Lymphocytic infiltration in tunica propria of posterior (dorsal) turbinates.

Hm[G] Haemorrhages in tunica propria of posterior (dorsal) turbinates.

NLT[H] Nasal associated lymphoid tissue hyperplasia

Nd Not determined due to non-availability of sufficient tissue sections.

Mean of severity index [I] = (0, 1, 2, 3 which is normal, mild, moderate and severe intensity of lesion based on Table ____, respectively in 3 birds each time.

4.7.1.1 Comparative assessment of histopathological parameters of nasal cavity

For easy comprehension of histopathological changes pertaining to nasal cavity in both chicken and Japanese quail, we endeavoured to highlight the pathological impact from individual parameters selected from our self-employed scoring criteria (Table 17). The entire observation was made on five histo-anatomical regions. In order to make this observation more meaningful, we also abstracted semi-quantitative evaluation of the stated parameters (Table 18-21). This particular attempt was made from samples collected during 2.5 weeks of experiment. The following descriptions, has been casted in the form of differential elaboration noted between two species. In each of the following histological explanation, chicken-associated tissue changes have preceded the Japanese quail.

a) *Haemorrhages:* Mild to moderate haemorrhages were a consistent feature observed at all time points after infection in chicken, plateauing at 24 hrs PI (Fig. 22) and continuing till the last day of observation i.e. 7th day of experiment. Milder degree of haemorrhages were noted in Japanese quail at each of the stated point of sampling. A possible reason for the early vascular changes in the given study could be due to the direct inoculation of the pathogen in the infraorbital sinus region. The purpose behind direct instillation of the pathogen suspension in the infraorbital sinus is to ensure a uniform delivery of the pathogen load to each and every bird in the experiment. We consider that this approach has provided uniformity in the assessment of our pathological changes restricted to nasal cavity.

b) *Loss of cilia:* A gradual increase in the loss of cilia was observed in chickens which started as a mild deciliation at 12 hrs PI, gradually peaking at 48 hrs and 5^{th} day PI in the form of moderate to marked deciliation. A decrease in the intensity was noted at 7^{th} day PI which also coincided with an observation of epithelium regeneration. Infected Japanese quails showed milder intensity of deciliation (Fig. 23) which reached to a moderate degree at 48 hrs PI. A subsequent decline in the occurrence of this lesion was observed, which coincided with initial fibroblastic changes at 5^{th} day PI and epithelium regeneration noted at 7^{th} day PI only leaving behind occasional mild areas of cilia loss. The observed early loss of cilia in either of the species indicated an attempt

of initial defensive action by the host against pathogen which is normally characterised by mucociliary purgation of pathogen from nasal tract. Similar, but for a different etiological reason, Nakamura *et al* (1991) reported mucociliary loss of tracheal epithelium against infectious bronchitis virus which indicated a phenomenon of degenerative impact over mucosal epithelial cells.

c) *Loss of epithelium*: Loss of epithelium, an effect which can be considered as a concatenating phenomenon of deciliation was observed with an almost identical trend, peaking at 5^{th} day PI with moderate intensity. This outcome apparently declined by 7^{th} day PI, giving an indication towards an attempt of regeneration (Fig. 28). This observation was largely seen in case of chicken. Interestingly, towards the end of 5^{th} day PI, a makeover of damaged epithelium as well as its surrounding tissue was also appreciated in Japanese quail (Fig. 27) which was favoured by transient fibroblastic changes. Prominent reparative changes/regenerative attempt was noted in both species by 7 days PI. Unlike chicken, Japanese quail had mild to moderate loss of epithelium initially which was subsequently restructured by 7^{th} day PI (Fig. 29). Similar loss of epithelium was also noted by Anjaneya *et al* (2013) from more or less a usage of similar isolates in chicken.

d) *Alveolar mucus gland activity:* Alveolar mucus gland activity was found to be maximum at 24 hrs PI in chicken and showed a subsequent decline from there on till the 7^{th} day PI where it was rarely observed. In Japanese quail, mild degree of mucus gland activity was noted during 24 hrs, 48 hrs (Fig. 25) and 5^{th} day PI, with rare occurrence at 7^{th} day PI. Host's mucus gland hyperactivity can be viewed as a strategy to debar the pathogen from interacting with deeper areas of the tissues. This kind of defensive action has been noted in previous studies conducted on infectious coryza (Kaur *et al* 2004; Nakamura *et al* 1991; Akter *et al* 2013; Anjaneya *et al* 2013).

e) *Lymphoid cell infiltration:* There was a gradual increase in the lymphoid cell infiltration till 48hrs PI (Fig. 24). The degree of infiltration was noted to be moderate to marked at 48 hrs, 5^{th} day (Fig. 26) and 7^{th} day PI, with the formation of germinal centres on and around 7^{th} day PI. In Japanese quail, the lymphoid cell

infiltration was milder as compared to chicken which was common in 24 hrs, 48 hrs and 5th day PI. This infiltration waned to a negligible number by 7th day PI. The evidence of germinal center formation suggests a continual presence of antigenic insults resulting into modification and activation of immune cells for higher degree of immune-protection. Surprisingly to our knowledge, none of the retrieved literature on pathogenesis of infectious coryza has reported the formation of germinal centres at submucosal region of nasal turbinates. A sincere effort has been made to recluse NALT from this characteristic observation.

f) *Heterophilic infiltration:* Heterophils, in general, are an initial arsenal against any bacterial infection in avian species. Following *Avibacterium* infection, heterophilic infiltration in the sub-epithelial region of nasal turbinates predominated at 24 hrs PI in chickens. Number of heterophils subsequently dwindled at later time points and were replaced by more number of lymphoid cells and specialised cells like macrophages and plasma cells. Interestingly, a small-sized re-infiltration of heterophils around tunica propria and submucosal vascular channels was seen at 7th day PI thus explaining re-action against putative immunologically evaded pathogen with reparative outcome. Comparatively, in Japanese quail, only mild infiltration was noted at 24 hrs PI which was largely absent at other time points. Similar observations on heterophilic infiltration were also reported in infectious coryza and other respiratory tract pathogens (Fujiwara and Konno 1965, Nakamura *et al* 1991; Kaur *et al* 2004, Akter *et al* 2013; Anjaneya *et al* 2013).

g) *NALT hyperplasia:* A gradual increase in the size of NALT, mainly in the inferior nasal meatus was noted which traversed full length of mucosal thickness encroaching the surrounding histo-architecture at 7th day PI. Remarkably, Japanese quail revealed a similar degree of NALT hyperplasia at the respective sites with a similar trend. Due to the absence of lymph nodes in avian species, NALT plays pivotal role in the primary immune response and antigen uptake in the nasal cavity (Kang *et al* 2013). A hyperplasia of NALT observed at 7 days PI may be in response to persistent presence of bacterial antigen which can be attested by the isolation of bacteria from infected birds as late as 7 days PI.

The total histological score given to a bird was divided with the number of histological regions to give a mean score. The mean scores of all the birds of a species at a given time point were added to yield the overall nasal histology score. The overall nasal histology scores of chickens and Japanese quails were then compared (Fig. 19).

4.7.1.2 Immune cell distribution in nasal turbinates and its associated structures

The ensuing paragraph endeavoured to undertake special elaboration of immune cell distribution across the nasal cavity in response to the infection with *Av. paragallinarum* and was simultaneously tabulated to make it more informative (Table 22 & Table 23). Further attempt has been done to highlight chicken specific cellular distribution in precedence to Japanese quail.

a. *Heterophilic infiltrations:* There was moderate to marked heterophilic infiltration noted around 24 hrs post-infection in tunica propria of almost all the examined turbinates, followed by its sharp decline in all the regions by 48 hrs PI. Interestingly, resurgence of heterophils in the tunica propria of middle turbinate was seen at 7th day post-infection barring all other regions. This pattern of cellular infiltration signifies a re-attempt to resolve the left over or immunologically-evaded pathogen following a regenerative effort specific to middle turbinates. Similarly, Japanese quails evinced milder degree of heterophilic infiltration in the tunica propria of middle turbinate at 12 hrs PI occasional presence of moderate infiltration, especially in the tunica propria of middle turbinate and infraorbital sinus respectively was noted at 24 hrs PI. Thereafter, there was a disappearance of this cell type from all the relevant histo-anatomical sites except for occasional mild infiltration in the tunica propria of infraorbital sinus, clearly indicating its early defensive role against the pathogen. Heterophils, a counterpart of neutrophils in mammalian species (Genovese *et al* 2013), were instigated to participate in the early process of pathogenetic mechanism by host, to remove the pathogen load.

b. *Macrophages:* At the initial phase of infection particularly around 12 hrs PI, macrophages were observed near the sub-epithelial layer of infraorbital sinus and their presence gradually increased to an extensive degree by 5^{th} and 7^{th} day post infection. In addition to the presence near infraorbital sinus, inferior nasal meatus showed considerable number of macrophages towards the fag end of experimental sampling i.e. 7^{th} day PI. In comparison, Japanese quails revealed occasional to

moderate level of infiltration in the lamina propria mainly confined to the middle turbinate. This observation was obtained from one infected Japanese quail sacrificed at 5 days PI. Macrophages being a first line of immunological defense (Qureshi *et al* 2000) were likely employed by the host to counter the bacterial load. The insignificant presence of macrophages in Japanese quail following infection may signify the presence or implementation of alternative mechanisms of immune protection, which requires further scientific exploration.

c. *Lymphocytes:* Resident lymphocytes were observed in the nasal sections of chickens at 12 hrs PI. At 24 hrs and 48 hrs PI, there was a moderate presence of lymphocytes (marked in some cases) in the sub-epithelium of almost all the regions. By 5th day PI, lymphocytes were extensively present in the epithelium and in the lamina propria, involving entire length of mucosal lining of in some cases. In addition, plasma cells were observed in the sub-epithelium of inferior nasal meatus of infected chickens at 5 days after infection. On the 7th day PI, marked presence of lymphocytes was noted which was occasionally mixed with heterophils. Germinal center formation in the lymphoid follicles was also observed at this time point. In infected Japanese quails, mild infiltration of mononuclear cells in the sub-epithelial layers was observed at all the time points post-infection. Similar to chicken, a mixed population of lymphocytes and heterophils was occasionally noted at the 7^{th} day PI. Strong presence of lymphocytes in the tissue sections of birds sacrificed at 5 days and 7 days PI along with re-appearance of heterophils can be attributed to an active presence of pathogen even during later phases of infection. Furthermore, the successful re-isolation of the organism from the nasal cavity of birds sacrificed at 7 days PI further supports this hypothesis.

Table 22: Immune cell distribution in nasal turbinates & other associated changes in chicken & Japanese quail at 12 hrs. 24 hrs and 48 hrs following infection with *Av. paragallinarum*

Time point/Cells	Species	12hrs	24hrs	48hrs
Heterophils	Chicken	Mild in tunica propria of epithelial lining limited to MT-2*	Marked (all regions-1); Mild (not in PT-1); Moderate (less in PT-1); Moderate (absent in PT & INM-1) Mild (all regions-1)	Mild (all regions-2); Absent-2
	Japanese quail	Mild (in IOS-2)	Absent-1; Moderate (MT-1); Mild (MT-1)	Absent -3; Absent (mild in IOS-1)
Macrophages	Chicken	Not appreciated	Moderate presence mainly in IOS-1	Moderate presence in and near IOS-1
	Japanese Quail	Not appreciated	Not appreciated	Not appreciated
Plasma cells	Chicken	Not appreciated	Not appreciated	Not appreciated
	Japanese quail	Not appreciated	Not appreciated	Not appreciated
Lymphoid cells	Chicken	Resident lymphoid cells noted	Marked (absent in IOS-1); Mild to moderate (more in MT-1); Mild (not in IOS); Marked (all regions-2)	Moderate (more in IOS-1); Marked (all regions-1) Moderate (less in PT-1); Mod-1
	Japanese quail	Mild (in IOS-2)	Mild (Marked in IOS-1); Moderate (MT-1); Moderate-1	Mild (inter epithelial-1); Moderate to marked-1; Absent (mild in MT-1); Absent (very mild in MT-1)

Time point/Cells	Species	12hrs	24hrs	48hrs
Epithelial Hyperplasia	Chicken	Absent-2	Marked (MT & INM-1); Absent-1; Very Mild (PT-1); Absent (mild in MT-1); Absent-1	Absent (mild in MT-2); Absent (mod in MT-1); Absent-1
	Japanese Quail	Absent-2	Mild (Marked in IOS-1); Mild-2	Mild to Moderate (in MT-1); Absent-2; Marked (only in MT-1)
Oedema	Chicken	Absent-2	Moderate (MT & INM-1); Moderate (more in MT-1); Moderate (Less in PT-1); Marked (All regions-2)	Mild (more in IOS-1); Mild (more in MT-1); Moderate (less in PT-1); Moderate-1
	Japanese Quail	Absent-2	Absent -1; Marked (in MT-1); Mild-1	Absent (Mild in MT-1); Absent (mild in IOS-1); Absent-2
Exudate	Chicken	Absent-2	Moderate (in IOS-1); Moderate (in MT-1); Moderate (in MT-1); Marked (less in PT-1); Mild eosinophilic (more in IOS-1)	Marked (absent in IOS-1); Massive in INM-1 Marked (in MT-1); Marked
	Japanese quail	Absent-2	Absent-1; Marked in IOS-1; Absent-1; Moderate in MT-1	Absent-3; Mild in MT-1

Number after hyphen (-) represents the number of birds showing the respective lesion. MT- middle turbinate, PT- Posterior turbinate, IOS- Infraorbital sinus, INM- Inferior nasal meatus

Table 23: Immune cell distribution in nasal turbinates and other associated changes in chicken & Japanese quail at 5 days and 7 days following infection with *Av. paragallinarum*

Time point/Cells		5 days	7 days
Heterophils	Chicken	Mild (absent in MT-1); Absent (mild in MT-3) Absent (moderate in INM & IOS-1)*	Moderate (only in MT mixed with mononuclear cells-1) Absent-2
	Japanese quail	Absent-2; Absent (mild in IOS-1)	Absent-3; Very Mild-1
Macrophages	Chicken	Extensive in INM & IOS-1	Marked presence in IOS & MT-1
	Japanese quail	Moderate presence in MT	Not appreciated
Plasma cells	Chicken	Occasionally observed mainly in INM	Not appreciated
	Japanese quail	Not appreciated	Not appreciated
Lymphoid cells	Chicken	Moderate (less in MT-1); Marked-2 Marked (less in MT & PT-1); Marked (more in PT-1)	Marked (germinal centre formation-1); Moderate to marked (fibroplasia in lamina propria-1); Moderate-1
	Japanese quail	Mod-1; Mild-2	Mild-1; Mild (only in PT-1); Mild (mixed with heterophils-1); Absent-1
Epithelial Hyperplasia	Chicken	Absent -3; Absent (Fibroplasia in MT-1); Absent (Fibroplasia in IOS-1)	Absent; Absent (mild in MT); Moderate (regeneration)
	Japanese quail	Mild (in MT with fibroplasia-1); Moderate (in MT with regeneration of epithelium-1); Absent (marked fibroplasia in tunica propria-1)	Marked (regeneration & fibroplasia-1); Mild (fibroplasia in lamina propria-1); Absent-1; Marked (normal epithelium restored-1)

Time point/Cells		5 days	7 days
Oedema	Chicken	Mild (more in IOS-1); Mild (marked in IOS-1); Mod-2 Absent (mod in INM & IOS-1)	Absent-3
	Japanese quail	Mild-1; Absent-2	Absent-5
Exudate	Chicken	Moderate (more in IOS-1); Mild-1; Moderate (muco-hemmorhagic, absent in PT-1); Moderate-1; Massive (mucopurulent with RBCs-1)	Absent (mild in MT-1); Absent; Absent (sloughing)
	Japanese quail	Mild-1; Absent-1; Mild bloody (marked mucoid in IOS-1)	Absent-1; Mild (haemorrhagic-1); Mild to moderate (haemorrhagic-1) Absent-1

Number after hyphen (-) represents the number of birds showing the respective lesion. MT- middle turbinate, PT- Posterior turbinate, IOS- Infraorbital sinus, INM- Inferior nasal meatus

4.7.2 Histopathological changes in other organs

a. *Lungs:* Histopathological alterations were limited to varied degrees of non-inflammatory vascular changes including congestion of blood vessels, pulmonary congestion and hyperemia, and accumulation of haemorrhagic exudate within the parabronchial lumen. Fresh bleeding in the respiratory tract was observed in many of the birds immediately after cervical dislocation which was employed as a method for sacrifice which correlates with the microscopic findings. Absence on any significant inflammatory changes in the lungs may be due to the restriction of the infection in the upper respiratory tract which can be attributed to absence of any concurrent respiratory pathogen (Blackall 1989) or due to the moderate virulence of the *Av. paragallinarum* isolate used in the experiment.

b. *Conjunctiva:* Loss of epithelial lining with depletion of mucoid glands along with haemorrhages and heterophilic infiltration in the sub epithelial region was noted by 24 hrs post infection. At 48 hrs post infection, mucus gland hyperplasia was seen, with the cellular population mainly consisting of mononuclear cells. Mononuclear cell infiltration further intensified by 5^{th} day PI and hyperplasia of goblet cells was also appreciated. By 7 days post-infection, return to normal architecture was observed in most of the cases while few others showed mild mononuclear cell infiltration and mild epithelial cell hyperplasia. The microscopic changes in the conjunctiva of infected birds follow an almost similar trend as that of the nasal cavity. The microscopic findings as well as the clinical signs suggest that the organism has propensity towards the conjunctival region.

c. *Trachea***:** Vascular changes in the form of congestion in submucosa along with sloughing of mucosal lining and mild goblet cell hyperplasia were seen as early as 12 hrs post infection in chickens. Vascular changes predominated in Japanese quail at the same time point. By 24 hrs, loss of epithelium became more evident in chicken along with congestion and edema in the lamina propria. Similar changes were also noted in Japanese quails at 24 hrs PI. Loss of epithelium, mucus gland hyperplasia and increased activity were consistently seen in chickens till 7^{th} day post-infection. Japanese quails showed almost similar changes but majority of the lesions were resolved by 7th day post-infection.

d. *Liver:* Histopathological changes in the liver mainly consisted of occasional mild congestion and haemorrhages. Vacuolar degeneration and fatty degeneration

were observed in some birds sacrificed 48 hrs post infection and onwards. But this finding was considered to be non-specific as no pattern of occurrence could be established and similar changes were also noted in some birds from the sterile broth group.

e. *Kidney:* No major renal changes were noted in most of the sacrificed birds. Mild degree of haemorrhage and congestion in the parenchyma was occasionally seen in the renal section of some birds. Hydropic degeneration and edematous changes were inconsistently observed in some birds with no seeming pattern of occurrence in relation to time after infection challenge.

f. *Harderian gland:* The most frequently observed microscopic change in Harderian gland was the infiltration of mononuclear cells around the periductular area. This observation was almost constant in all the time points in infected chickens and was also infrequently seen in sterile broth chicken and sterile broth Japanese quails. In infected Japanese quail, the degree of mononuclear cell infiltration was milder as compared to infected chickens, in addition occasional mild congestion was noted. Sloughing and degeneration of the excretory duct was observed in few infected Japanese quails at 5^{th} day post-infection.

g. *Reproductive tract:* Reproductive tract (ovaries as well as testes) was observed to be largely unaffected. Mild scattered haemorrhages in the ovaries and mild degeneration of seminiferous tubules were a rare finding.

Similar microscopic findings were documented by several investigators (Fujiwara and Konno 1965; Reid and Blackall 1984; Blackall 1989; Kaur *et al* 2004; Akter *et al* 2013). Anjaneya *et al* (2013) noted similar changes including epithelial hyperplasia, infiltration of heterophils and mononuclear cells in the sub-epithelium, sloughing and edema of mucosal layers but these signs were observed at 3 days PI. Ali *et al* (2013) reported the involvement of lungs, liver and heart along with hyperplasia of epithelium and mucus glands among other changes in the nasal sections. In our study, an earlier occurrence of the microscopic changes i.e. by 12-24 hrs PI may be due to the intrasinus route of infection employed in our study. Furthermore, little to no specific changes were observed in the lungs and other visceral organs in our study which could be attributed to the low virulence of the strain used.

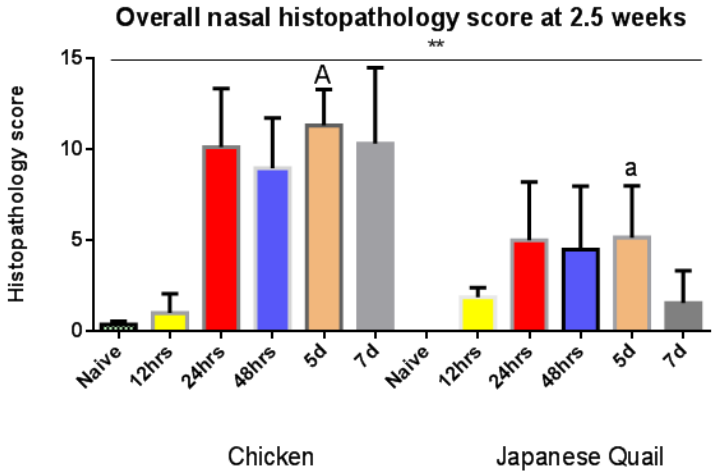

Fig 19: Overall nasal histology score of chickens and Japanese quails at various time points during the infection challenge at 2.5 weeks age.

** indicates $p<0.01$ level of significance**.**

4.8 Immunohistochemistry

Labelled slides were observed at x200 and x400 magnifications, using a Nikon light microscope fitted with DS fi2 camera. Five histoanatomical regions namely anterior turbinate (rostral nasal concha), middle turbinate (middle nasal concha), posterior turbinate (caudal nasal concha), infraorbital sinus and inferior nasal meatus were examined in every section at the intensity & distribution of immunostaining was noted (Table 24).

Following infections with *Av. paragallinarum*, the bacterial antigen was noted in the nasal passage of both chicken and Japanese quail at 12 hrs post-infection. In case of Japanese quail, the presence of bacterial immuno-staining was predominantly noticed in all the five (5) histo-anatomical regions of the nasal cavity. The immuno-staining reactions varied from strong brown reaction to moderate one, spanning larger areas of the nasal cavity with few isolated regions showing mild reaction. Unlike Japanese quail, chickens' nasal tissue exhibited moderate immuno reactions that were limited to anterior turbinates and middle turbinates. The reactions were observed microscopically as thick brown granular-like deposits appearing to diffuse within the superficial layer of modified keratinized epithelium of anterior nasal turbinates as well as in respiratory epithelium of middle turbinates. Interestingly, a strong positive reaction was noted in the anterior turbinates of Japanese quail when compared to chicken at 12 hrs post inoculation (Fig. 45). Gradual decline in immuno-reactivity against the bacterial antigen was noticed in the middle, posterior turbinates and other

areas like infra-orbital sinuses and inferior nasal meatus in Japanese quail. The reaction with mild intensity was observed in some of the alveolar mucoid glands of middle turbinates as well as in Bowman's gland of posterior turbinates respectively.

By 24 hrs post-inoculation, chicken tissue revealed strong immune reaction to the bacterial antigens confined to modified keratinized epithelium of anterior turbinates and the ciliated zone of respiratory epithelium of middle turbinates. Posterior part of nasal cavity as well as the lower nasal region i.e. inferior nasal meatus and infra-orbital sinus showed moderate reaction (Fig. 36). Evidences of migration of bacteria were noted in the form of strong pin-point like particulate antigen across the epithelium and tunica propria and were more common in sub-epithelial region of inferior nasal meatus (Fig. 43) and infra-orbital sinus (Fig 42). Some of the areas just beneath the epithelium, particularly of infra-orbital sinus were reportedly found to be populated with macrophages engulfing bacterial antigen within their cytoplasm. The major luminal surfaces of turbinates were also found to contain desquamated cells with exudates containing phagocytic cells laden with bacterial antigen. In contrast, mild immuno-reactivity was noticed at anterior turbinates of Japanese quail. Mild to moderate immune reaction specific to areas of superficial epithelium and ciliated zone was observed in the rest of turbinates (Fig. 39).

At 48 hrs post inoculation, chicken nasal sections continued to display strong immuno-reactivity (Fig. 37 & Fig. 44) at all the epithelial surfaces of major histo-anatomical sites barring posterior turbinates with concurrent damages to the cilia and epithelial surfaces. The brown staining reaction was diffused across the epithelium and several pin-points; discrete brown particulate like staining was common. Strong to moderate staining intensity was also found near vascular channels of sub-epithelial regions and were prominent in all major compartments, thus describing the propensity of bacteria to become systemic within hosts. The bacterial immuno- reactivity was also noticed in the areas of pectum occuli, a specialized area responsible for providing nutrition to the retina of eyes. However, at this time point in Japanese quail, the frequency and strength of antigenic immuno-reactivity rather revived to a moderate level with multi-focal distribution (Fig. 40 & Fig. 46), mainly affecting the ciliated zone and superficial epithelial surfaces. This feature of immuno-histochemical reaction continued in the Japanese quail until 5 days post-inoculation of pathogen, restricted to ciliated surfaces without any major involvement of epithelial surface. The

bacterial antigen homing was comparatively lesser seen in chicken by 5 days post-inoculation which indicated either absorption of pathogen within the systemic circulation or resolution of infections by the host immune arsenals at localized region and its dissipations by certain innate immune factors restricted to nasal passages. Only mild immunostaining was observed at some places with total elimination of bacterial antigen from anterior turbinates.

At 7 days post inoculation, focal, scattered mild immuno-reactivity was seen (Fig. 38 & Fig. 41) in all the major designated histo-anatomical regions thus probably explaining the complete recovery of infections in both species. One bird from Japanese quail group displayed moderate immuno-reactivity in the anterior turbinate (Fig. 47) during IHC which may possibly be link to a case in which a recovery of pathogen from the bird was successful.

In this study, we used our own in-house generated polyclonal antibody raised against *Av. paragallinarum* to demonstrate its presence and migration across the nasal tissues of both chicken and Japanese quail following inoculations. To our knowledge, the availability of commercial antibody against this pathogen is very scarce, probably because of difficulty in raising against the wide diversity of proteins and different immunological subtypes, besides stringent ethical laws. The raised polyclonal antibody in our case was against one particular serotype i.e. Serotype B. This anti-serum when tested for its specificity against the pathogen, revealed unique conformity and was rapidly detected through immuno dot blot techniques and counter-immuno electrophoresis (CIE). Therefore, through this attempt, we are able to demonstrate the possibilities of detecting the *Av. paragallinarum* antibodies in chicken flock through CIE techniques. CIE has always remains a valuable, sensitive method for the early detection of infections like *Haemophilus influenza* in human subjects (Artman *et al* 1980) and still continued to hold an important position as technique for rapid and early identification of other important septicemic disesaes in both humans and animals. The pathogen *Av. paragallinarum* used in this study has some close phylogenetic relationship with genus *Haemophilus* and was earlier grouped in same genus as *Haemophilus paragallinarum*, possibly explain the reason behind its tendency to undergo unique electrophorectic phenomenon. The qualitative immuno dot blot technique revealed a trend of increase in the staining reactions following incubation in all the successive sera samples obtained from rabbit thus buttressed its

further specificity and authenticity. A similar but with slightly different objectives of immuno dot blot technique was undertaken by Blackall *et al* (1990) to authenticate the suitability of monoclonal antibodies raised for *Avibacterium paragallinarum* serotyping.

In order to distinctly discern the migration pattern of *Av. paragallinarum* through immunohistochemistry (IHC) technique, an infection challenge experiment was conducted in both chicken and Japanese quail at an early age i.e. 2.5 weeks of age. The tender age was chosen to appreciate ability of the respective species absorb the antigen or to promote the pathogen's invasion across nasal mucosal lining (turbinates) and its subsequent migration to other distant places thus may assist in understanding the differential immuno-pathologic process as a result of *Av. paragallinarum* either towards protective immune mechanism or as increased diseased outcome. In other words, this observation may help us in corroborating direct interplay of pathogen with the host tissue in regulation of immune arsenals at an early age, thus may find a future advocation of nasal mucosal vaccination practice with putatively modified *Av. paragallinarum* antigen in birds, at an early age. Additionally, an attempt was also made to describe the early pathogenesis of host pathogen interaction, until 1st week of post exposure by employing immunohistochemistry.

Following initial inoculation of pathogen *Av. paragallinarum*, a positive immune reactivity was noticed in the anterior turbinates of both chicken and Japanese quail respectively. The persistence of brown immuno staining was more in Japanese quail as compared to chicken at 12 hrs post-inoculation and was more localized to the initial site of inoculation. In our opinion, the initial deposits of bacterial antigen in chicken nasal cavity may have extended/diffused uniformly across the early part of nasal passage i.e. anterior turbinates, resultantly producing lesser intensity of immuno-reactivity in them. It's not uncommon to visualize prolonged presence of extraneous objects including bacterial pathogen/antigen in the early region of anterior turbinates, because of strong, impervious covering of nasal epithelium. This covering is so robust which may in fact disallow any particulate or soluble article attach to the surface/mucosal coverings. Uniquely, the Japanese quail's anterior nasal cavity has very complex and well-developed rostral nasal concha (anterior turbinates) with near obliterations of nasal passages (Cevik-Demirkan *et al* 2007), besides, an extended modified stratified epithelium of rostral nasal concha to the early part of middle nasal

concha (turbinates) from the nasal septum, which may have offered a strong physical barrier to the initial permeability of bacterial entrance across the nasal cavity. Unlike quail, chicken has a less complex coiling of nasal turbinates and no extension of modified stratified complex epithelium in to the middle nasal conchae, which may have provided sufficient time to the bacterium to adhere to the epithelial surface and travel from rostral nasal concha (anterior turbinates) to middle nasal concha (middle turbinates). Apparently, the bacteria used in this study, showed less ability to penetrate across the epithelial surface in both the tested species at an early time point, and was found to be restricted to the superficial surfaces of the epithelium as strong brown staining. This implies the presence of some innate defense molecules or factors at the top superficial layer of epithelium across all the turbinates exerting defensive action against pathogen's invasive ability. This needs to be verified further in subsequent study either by employing specific immunostaining techniques or gene expression studies. Further we have not ruled out the fact that the pathogen used in the study was of mild to moderate virulence, which may not have exerted strong pathogenic damages to histo-architecture of tissue involved.

At 24 to 48 hrs post inoculation, the amount of brown immuno-staining gradually increased particularly at the junction between anterior turbinates and middle turbinates and was more pronounced in chicken compared to Japanese quail. This probably indicated gaining of multiplicative (proliferative) time by few bacteria after an initial lodgment to the epithelial surface by one or the other/several mechanisms thus enabling a firm establishment. There were differences between species at this time point, where Japanese quail revealed mild to moderate brown immuno-reactivity of tissues suggesting a possible suppression of initial bacterial establishment and proliferation, when compared to chicken. There was trans-epithelial migration of bacteria in the form of pin-point particulate immunostaining within mucosal lining and connective tissues and was surrounded by demonstrable presence of lymphoid like cells. This observation indicated the initiation of host's ability to neutralize the bacterial invasion in mucosal surface and other sub epithelial region. It was more pronounced in chicken than Japanese quail. Earlier, observation affiliated to *Avibacterium paragallinarum* infections in chicken indicated poor mounting ability of immune response in less than 4 weeks of age, therefore considered as a disease of growing birds more than 5 weeks of age (Blackall *et al* 1999), which prompted us to

undertake this study in small age chickens and small sized and aged Japanese quail respectively. Unlike *Haemophilus influenza*, the migration pattern of *Avibacterium paragallinarum* (*earlier Haemophilus paragallinarum*) with in brain or cranial cavity appeared negligible and no specific immunostaining was demonstrated at any stages of infection in both chicken and Japanese quail. Accidentally, we noticed bacterial antigenic expression in pectum occuli, a spiral loop like structure that confers nutritive supplementation to retina of eyes in chicken. This observation should be further explored in to future studies to assess the impact of pathogen migration on visual ability of birds. The waning intensity of bacterial antigen immunostaining at 5 DPI post-inoculation was predominantly noticed in chickens suggests possible bacterial clearance. Comparative assessment indicated a delayed elimination of the pathogen in Japanese quail, unequivocally pointing out longer lodgment of bacteria owing to highly intricate tissue architecture of the anterior turbinates leading to decreased access to subsequent regions of nasal cavity. This has probably brought about a delayed implementation of the immune response mechanism in Japanese quail. Additionally, this has also solidified the fact the chickens are sensitive and fast to show clinical signs development and to clear the bacteria through regulation of mucosal immunity. It is noteworthy, that there was a further decline in immunostaining intensity observed in both the species by 7 days post-inoculation, even so with sluggish but effective immunologic response seen in Japanese quail. This findings has gone well in agreement to one of our segments of analysis, where strong lipid peroxidation activity and decreasing content of erythrocytic reduced gluthaione level, a cellular antioxidant responsible for mitigation of formed reactive oxygen species following lipid peroxidation reaction in Japanese quail was noted particularly by 7 days post exposure (data not shown). Boucher *et al* (2014) suggested possible implementation of oxidative stress mechanism by hosts as an initial immune response strategy against *Av. paragallinarum* infections in chicken. However chickens in our study, revealed a lesser degree of lipid peroxidation activity as well as delayed, reciprocated erythrocytic reduced glutathione level, when estimated in conjunction with blood samples taken from Japanese quail by employing Malonaldialaldehyde assay (MDA) and reduced glutathione estimation assay.

Table 24: Intensity and distribution of Immunohistochemical staining at various time points following infection with *Av. Paragallinarum* in Chicken & Japanese quails

Time point (hrs/days)		12h PI		24h PI		48h PI		5d PI		7d PI	
		Chi	JQ	Chi	JQ	Chi	JQ	Chi	JQ	Chi	JQ
Region											
Anterior turbinate	Intensity	Moderate	Strong	Strong	Mild	Strong	Moderate	NA	Moderate	No staining	Moderate
	Distrib.	Keratinized Epithelium	Keratinized Epithelium	Keratinized Epithelium	Turbinate junction	Keratinized Epithelium	Turbinate junction	-	Multifocal, kerat. layer	-	Desquam. epithelium
Middle turbinate	Intensity	Moderate	Strong	Strong	Mild to moderate	Strong	Moderate	Moderate	Moderate	Very mild	Very mild
	Distrib.	Multifocal	Cilia & glands	Cilia	Cilia & epithelium	Epithelial lining	Cilia & epithelium	Multifocal, cilia	Cilia	Cilia & epithelium	Superficial epithelium
Posterior turbinate	Intensity	No expression	Moderate	Mild	No staining	No staining	Moderate	NA	Moderate	NA	NA
	Distrib.	-	Cilia & glands	Turbinate junction	-	-	Multifocal, epithelium	-	Cilia	-	-
Inferior nasal meatus	Intensity	Mild	Moderate	Mild to mod	Moderate	Strong	Moderate	Moderate	NA	Very mild	NA
	Distrib.	Cilia	Cilia & epithelium	Percolated to sub epith.	Focal (intact epith.)	Damaged cilia	Cilia	Multifocal	-	Focal, subepith.	-
Infra orbital sinus	Intensity	NA	Moderate	Mild to mod	Moderate	Strong	Mild	NA	Mild	Very mild	Very mild
	Distrib.	-	Entire epithelium	Percolated to sub epi.	Superficial epithelium	Epithelial lining	Superficial epithelium	-	Exudate	Exudate	Focal, epithelium

CHAPTER V

SUMMARY AND CONCLUSIONS

Avibacterium paragallinarum isolates were obtained from PDRC, Pune and maintained in on Haemophilus agar and Blood agar plates. The microbiological confirmation of the organism was done based upon cultural characteristics, microscopic appearance and biochemical tests profile. Molecular method based identification and serotyping of the organism was done using PCR which confirmed the isolates to be *Avibacterium paragallinarum* Serotype B.

An inoculum containing known bacterial dose (colony-forming unit per ml (CFU/ml) of *Av. paragallinarum* was formalinized and subcutaneously injected in conjunction with Freund adjuvant in adult rabbits over a period of 28 days. Serum thus raised (containing anti-*Av.paragallinarum* antibodies) was collected from the rabbit on a weekly basis for seven weeks after the initial administration of antigen and was stored at -80°C. The specificity of the raised antiserum against *Avibacterium paragallinarum* was confirmed by immune dot blot technique as well as by counter immune-electrophoresis.

The bacterial isolates were passaged thrice in live chickens to enhance the virulence of the strain. Further, a pilot experiment was conducted to determine the suitable infective dose of the bacteria for the final infection challenge which was determined to be 10^8 CFU/ml.

Day old disease-free chickens and Japanese quails with no vaccination against infectious coryza were procured and reared till 2.5 weeks age in hygienic conditions. At the age of 2.5 weeks, 28 chickens and 28 Japanese quails were separated. Twenty-three birds from each species were inoculated with Haemophilus broth containing 1.5 x 10^8 CFU/ml of *Avibacterium paragallinarum* via intra-sinus, intranasal and ocular route. The remaining five birds from each species were inoculated with a sterile Haemophilus broth. The development of clinical signs and presence of gross lesions was recorded at 12 hrs, 24 hrs, 48 hrs, 5 days and 7 days post-infection (PI) and scored based upon self-employed scoring criteria. Maximum development of clinical signs including nasal discharge, redness of conjunctiva and swelling of face was noted at 24 hrs and 48 hrs PI with decline in the occurrence of clinical disease by 7^{th} day post-infection in chickens. Similarly, gross lesions including redness of turbinates,

presence of exudate in turbinates and redness on conjunctiva were found to be maximum at 48 hrs post-infection and gradually declined to minimum by 7 days PI. Japanese quails were found to be significantly more resistant to the development of a clinical disease, although viable organisms were isolated from infected Japanese quail as late as 7th day after infection. An identical experimental challenge was performed using 6 weeks old birds which included oxidative stress estimation as an additional study.

Microscopically, the nasal turbinates revealed marked heterophilic infiltration in the tunica propria at 24 hrs PI and a re-appearance at 7 days PI. Lymphocytic population in the sub-epithelium gradually peaked at 5 days PI and sustained a high number at 7th day PI with the presence of germinal center formation, signifying continuous presence of the pathogen. Tissue architecture showed moderate to marked damage at 24 hrs and 48 hrs PI and the reparative changes ensued at 5 days and 7 days PI. Conjunctiva from the infected birds showed epithelial damage, mucus gland hyperplasia and vascular changes which reverted to normal by 7 days PI. Japanese quails in comparison showed only mild degree of heterophilic and lymphocytic infiltration and revealed extensive coverage by keratinized epithelium which may have conferred resistance against a serious infection.

Immunohistochemical studies revealed the sound presence of the bacteria in the nasal sections as early as 12 hrs PI. In chicken, the bacteria were found to be initially located in the anterior and middle turbinate and was strongly visualized in all the nasal regions by 24 hrs PI and 48hrs PI. Migration across the epithelium and interaction with macrophages was also seen at 24hrs and 48 hrs PI with indications of systemic spread of the bacteria. These findings coincided with the clinical signs and gross lesion findings. There was a decline in the presence of bacteria at 5 days PI which diminished even further by 7 days PI. Japanese quails showed omnipresence of bacterial antigen at 12 hrs PI, and the immunostaining peaked at 48 hrs PI. There was a decline in the staining by 7th day PI even though one bird showed moderate staining explaining the successful isolation of bacteria from Japanese quails at 7th day PI.

. Spectrophotometric estimation of the levels of malondialdehyde in the blood using thiobarbituric acid reagent revealed a gradual increase in the erythrocytic lipid peroxidation till 7th day PI in both the species which may possibly be due to

subsequent systemic dissemination. A simultaneous decrease in erythrocytic reduced glutathione levels supported the finding of increased oxidative burden in both the species.

Thus, from the study it can be concluded that,

1. Chicken(s) less than or equal to 2.5 weeks of age were found to be susceptible to induced/experimental *A. paragallinarum* infection, resultantly reflected participation of local mucosal defense system in mitigating disease process.
2. Pathological phenomenon was demonstrated as increased mucus gland activity, vascular compromise, cellular infiltrations, epithelial cell hyperplasia and some degree of fibroplasia at later stages, indicating involvement of local immune system in both the species with lesser severity in Japanese quail.
3. Japanese quails appeared to be less susceptible than chicken possibly owing to its inherent nasal anatomical complexity.
4. Raised polyclonal antisera against *Avibacterium paragallinarum* in rabbit was shown to successfully locate bacterial antigen via immunohistochemistry.
5. Middle turbinates being close to anterior turbinates demonstrated the maximum pathologic damage & immunological response, when infection was given through nasal route.
6. Infraorbital sinus & inferior nasal meatus showed maximum damage when infection was given through intra-sinus method.
7. Maximum lodging of pathogen was found to be in the anterior turbinate in both species with larger quantum in Japanese quail.
8. Scoring criteria developed (gross pathology, clinical signs and microscopic changes) can be used to in future vaccine efficacy related studies.
9. Oxidative stress possibly plays a significant role in the precipitating the clinical severity of the disease.

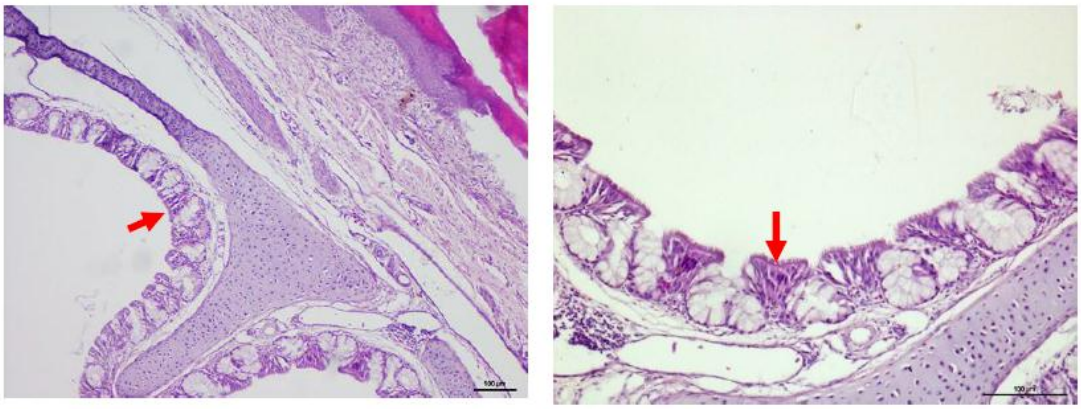

Fig 20: Naïve Birds Chicken 0 day. (LS, MT),showing intact respiratory epithelium and its associated structure. H&E. 10X (Left) 20X (Right).

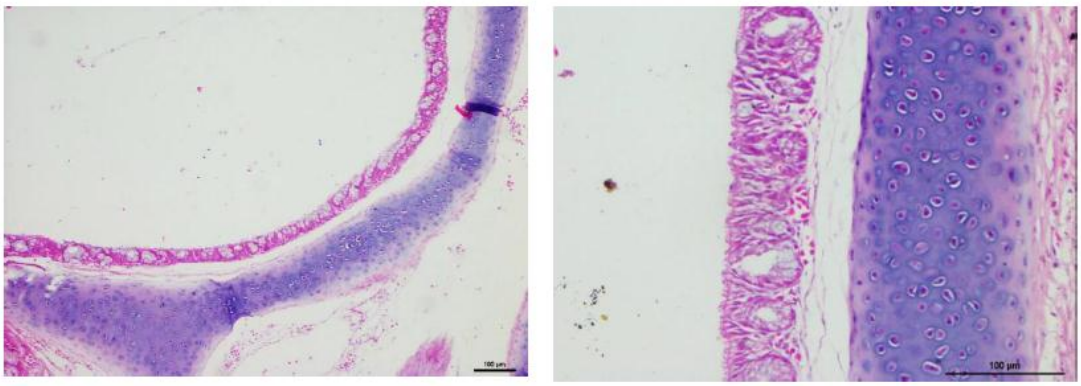

Fig. 21: Japanese quail 0 day, (LS, MT & PT), complete respiratory epithelium and olfactory epithelium in middle as well as dorsal turbinate. H&E. 10X (Left) 20X (Right)

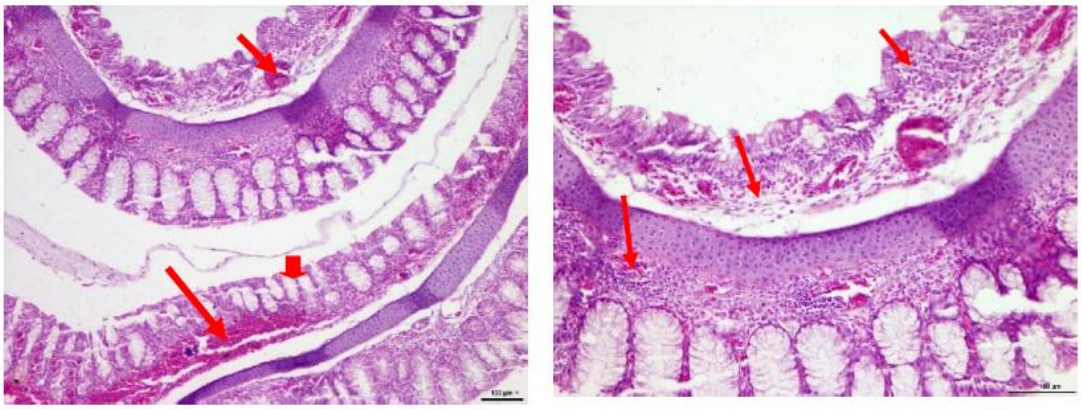

Fig. 22: Chicken 24hrs post-infection, (CS, MT), showing acute vascular changes (arrow), inflammatory changes (small arrow) and increased mucoid gland activity (arrow head) in the turbinates. H&E. 10X (Left) 20X (Right).

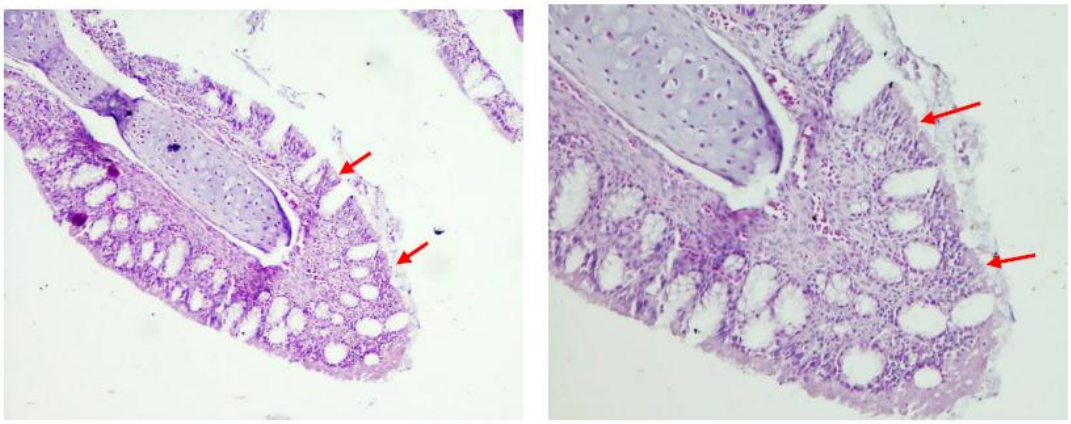

Fig. 23: Japanese quail 24hrs PI (CS, MT & AT), showing mild deciliation of epithelium (arrow), mild necrotic changes and mild vascular changes. H&E. 10X (Left) 20X (Right)

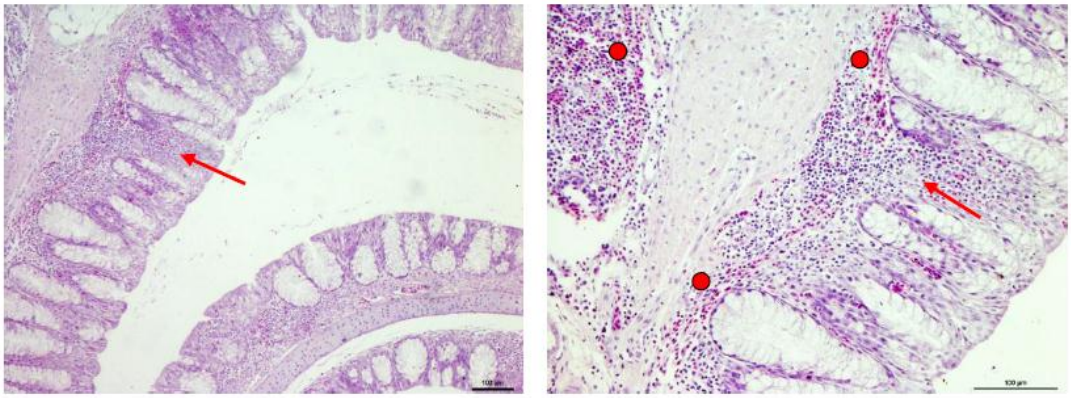

Fig 24: Chicken 48hrs PI, (CS, MT) showing thickening of epithelial lining at all places due to cellular infiltration (arrow), few small areas of accumulated hemorrhages (circles) & excess mucoid gland activity (small arrow). H&E. 10X (Left) 20X (Right)

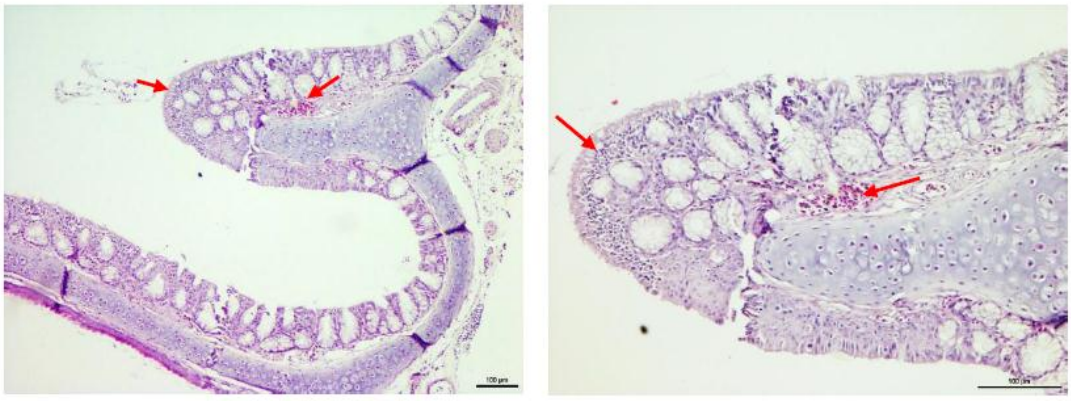

Fig. 25: Japanese quail 48hrs p.inf.,(CS, MT & AT), showing moderate cellular infiltration (arrow), moderate vascular changes (small arrow) and increased mucoid gland activity. H&E. 10X (Left) 20X (Right)

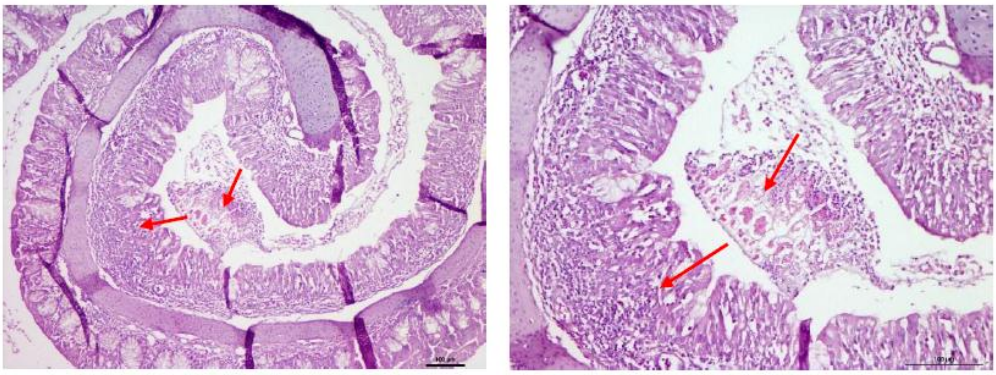

Fig. 26: Chicken 5th day PI, (CS, MT) Showing marked cellular infiltration (arrow) and excess mucoid gland activity besides overwhelming mixed exudative contents in luminal surface (small arrow). H&E. 10X (Left) 20X (Right).

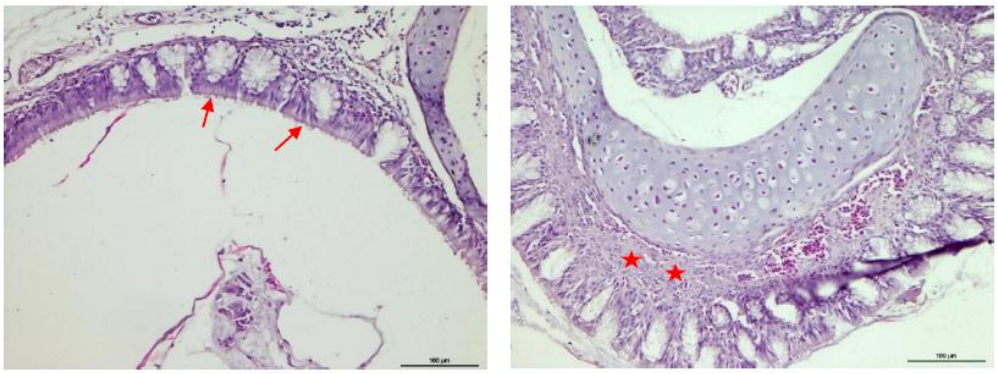

Fig. 27: Japanese quail 5th day PI, (CS, MT), Showing epithelial cell regeneration (arrow heads), increased dense connective tissue (*), increased glandular activity, in addition to hemorrhagic changes in lamina propria. H&E. 10X (Left) 20X (Right)

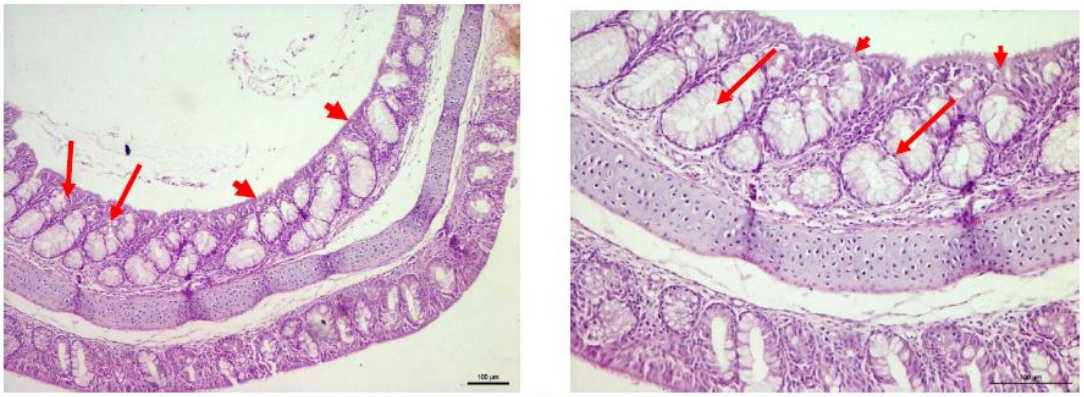

Fig.28 : Chicken 7days PI, (CS, MT) Showing hyperplasia/regeneration of epithelium(arrow head) along with moderate mucoid gland activity (arrow) H&E. 10X (Left) 20X (Right)

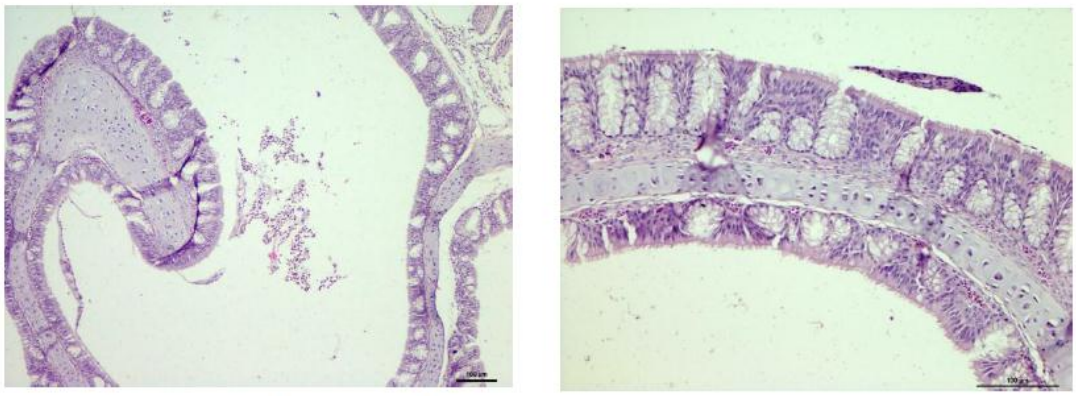

Fig. 29: Japanese quail 7days PI (CS, MT), Showing regeneration and return to normalcy of histo-architecture with moderate mucus gland activity. H&E. 10X (Left) 20X (Right)

Fig. 30: Loss of mucosal epithelial cells

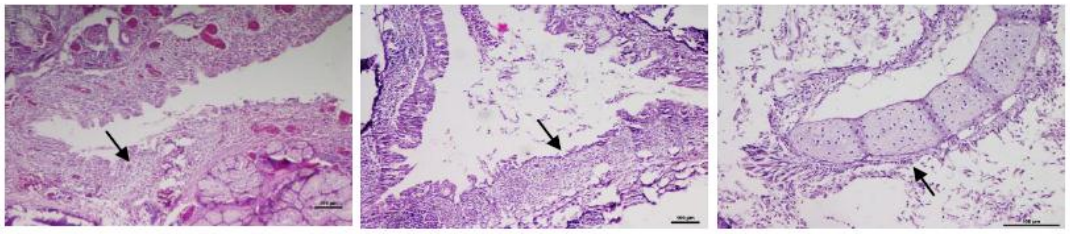

Fig.30a : **Section IOS:** Focal to multifocal marginal loss/ degenerative to necrotic damage (arrow) of the epithelial cells involving upper 1/3rd of mucosal lining, H&E X100

Fig.30b : **Section IOS:** Widespread loss involving a stretch of mucosal lining in the form of desquamation (sloughing) (arrow) / necrotic damage, leaving aside partial denudation of lamina propria. H&E X100

Fig.30c : **Section middle turbinate (conchae):** Complete denudation (arrow) leaving aside exposed lamina propria; often accompanied by haemorrhages and cellular infiltration. H&E X200

Fig. 31: Alveolar mucoid gland activity

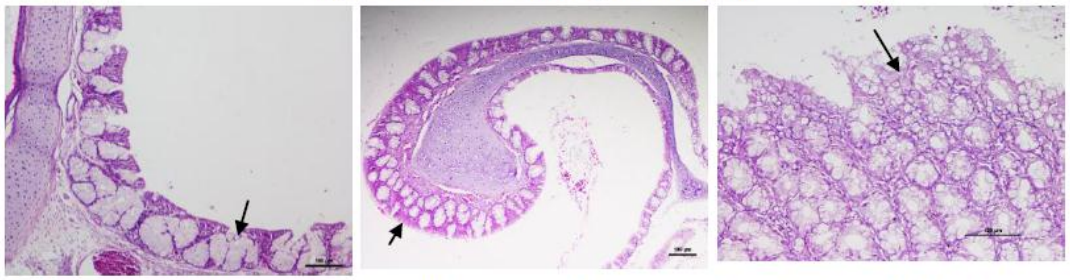

Fig.31a: **Section middle turbinate (conchae):** Only glandular hypertrophy noted. H&E X200

Fig. 31b: **Section middle turbinate (conchae):** Both glandular hypertrophy and hyperplasia observed with beginning of stratification (arrow) of glands. H&E X100

Fig. 31c: **Section INM:** Both glandular hypertrophy and hyperplasia observed with beginning of stratification of glands, goblet cell hyperplasia (arrow). H&E X200

Fig. 32: Heterophilic cell infiltration

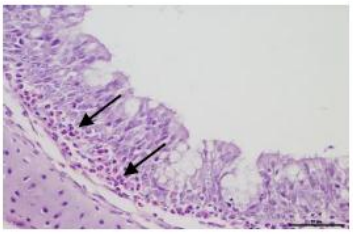

Fig. 32a: **Section middle turbinate (conchae):** Scattered focal sub-epithelial infiltration in few places (arrows) confined to lamina propria, H&E X400

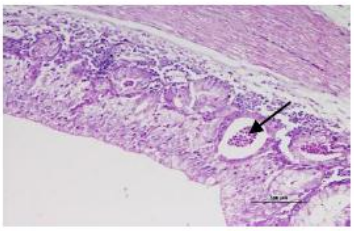

Fig. 32b: **Section posterior turbinate:** Widespread infiltration (arrow heads) with resultant spread to intra-epithelial region & sub mucosal region, within glandular lumen (arrow). H&E X200

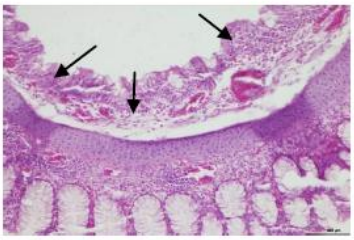

Fig. 32c:

Section middle turbinate (conchae): Dense infiltration across the lamina propria as well as in the epithelial region/sub mucosal zone. H&E x200

Fig. 33: Lymphoid cell infiltration

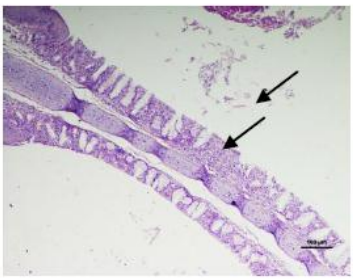

Fig. 33a: **Section of middle turbinate (conchae):** Scattered infiltration (arrow) in few places confined to lamina propria. H&E X100

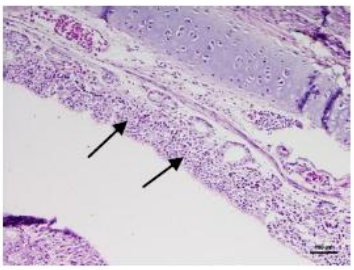

Fig. 33b: **Section of posterior turbinate :** Widespread infiltration (arrows) with resultant spread out to intra-epithelial region and sub mucosal region replacing Bowman's gland. H&E X100

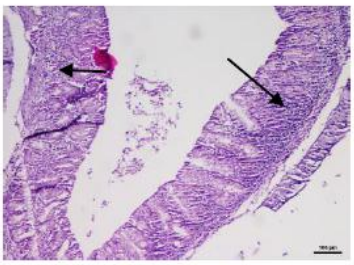

Fig. 33c: **Section of middle turbinate:** Dense infiltration/lymphoid aggregates (arrows) across the lamina propria as well as in the epithelial region/sub mucosal zone replacing glands. H&E X100

Fig. 34: Hemorrhages

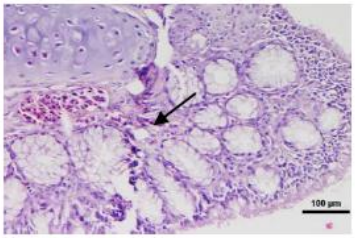

Fig. 34a: **Section of middle turbinate: Occasional to focal foci or thin foci of haemorrhages (arrows) especially around the blood vessels. H&E X200**

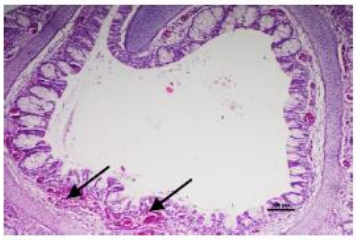

Fig. 34b: **Section of middle turbinate: Focal to large widespread areas of haemorrhages (arrows). H&E X100**

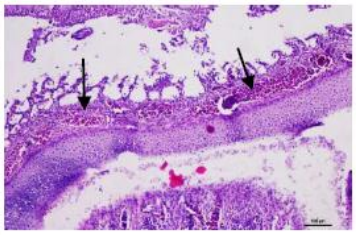

Fig. 34c: **Section of middle turbinate: Diffuse areas of haemorrhages (arrows). H&E X100**

Fig. 35: NALT hyperplasia

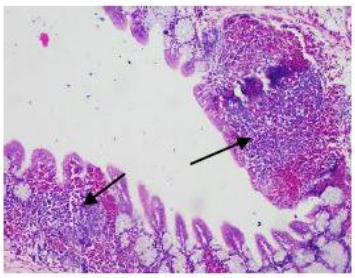

Fig.35a : **Section of middle turbinate:** Expansion of lymphoid zone with haemorrhages. H&E X100

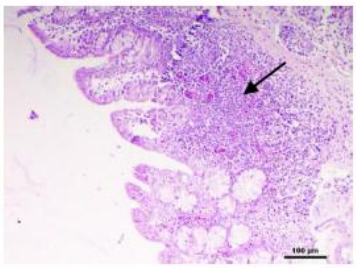

Fig. 35b: **Section of INM:** Expanded lymphoid zone (arrow) noted; often associated with encroachment to surrounding tissues. H&E X100

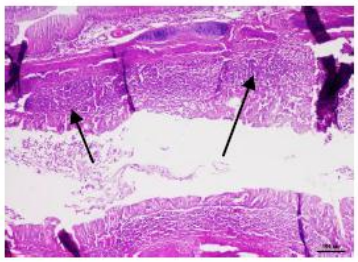

Fig. 35c: **Section of IOS:** Profuse expansion of lymphoid zone (arrows) replacing the neighbouring tissue details. H&E X100

Species/ time point

24 hours **48 hours** **7 days**

Chicken

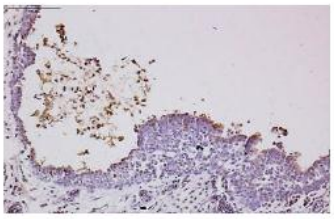

Fig. 36: **Section of IOS:** Moderate brown immuno-staining noted at superficial & subepithelial region as particulate foci indicating bacterial migration. IHC, X200

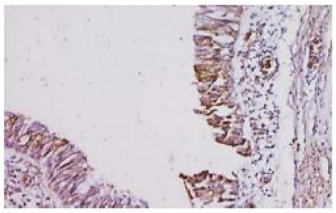

Fig. 37: **Section of IOS:** Strong immuno-staining noted at epithelium as well as in surrounding blood vessels as diffuse distribution indicating expanded region of bacterial migration. IHC, X200

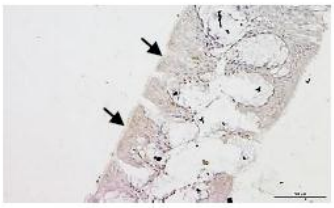

Fig. 38: **Section of Middle turbinate:** Very mild brown immuno-staining (arrows) noted in cilia & epithelium. IHC, X200

Japanese quail

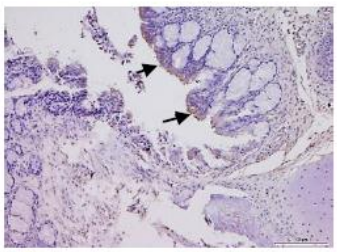

Fig. 39: **Section of Middle turbinate:** Mild brown immuno-staining (arrows) noted at cilia & upper epithelial surface. IHC, X400

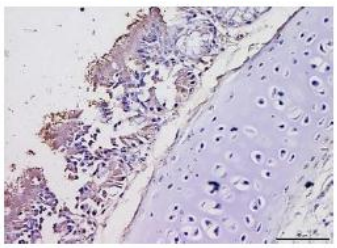

Fig. 40: **Section of Middle turbinate:** Moderate immuno-staining noted at multifocal areas restricted to cilia & upper epithelial surface. IHC, X400

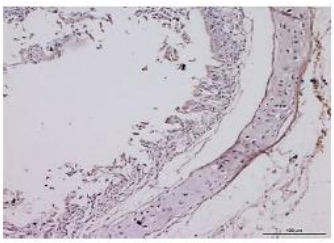

Fig. 41: **Section of Middle turbinate:** Very mild immuno-staining noted at superficial epithelium. IHC, X400

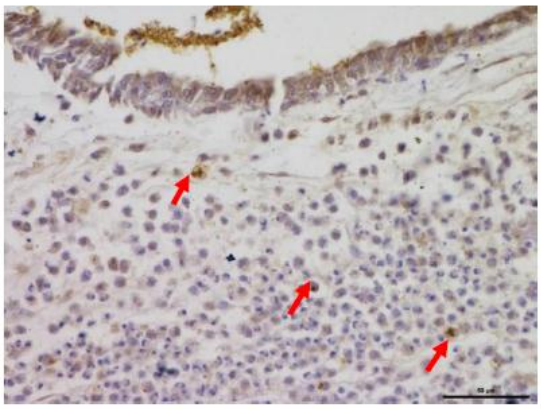

Fig. 42: **Section of IOS (24h chicken):** Moderate brown immuno-staining noted at superficial & subepithelial region as particulate foci indicating bacterial migration & engulfment (arrows) IHC, X400

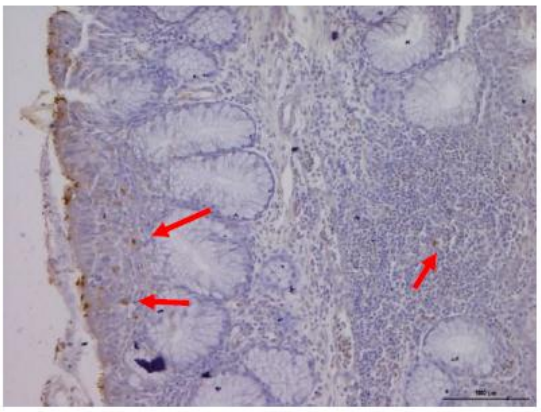

Fig.43 : **Section of INM (24h chicken):** Moderate brown immuno-staining noted at superficial surface and particulate foci (arrows) indicating bacterial migration. IHC, X200

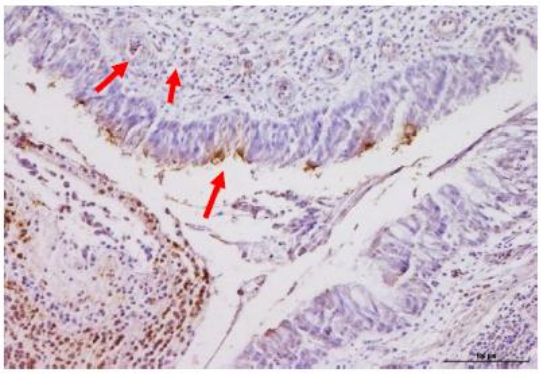

Fig. 44: **Section of IOS (48h chicken):** Strong brown immuno-staining of epithelial lining and particulate foci indicating bacterial migration. IHC, X200

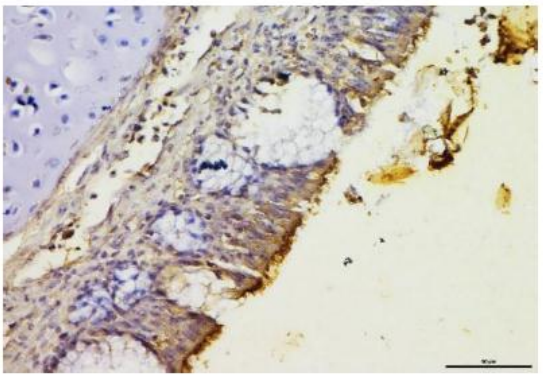

Fig. 45: **Section of MT (12h JQ):** Strong brown immuno-staining noted at epithelial surface, cilia and mildly within glands. IHC, X400

REFERENCES

Adderson E E. 2008. *Avibacterium paragallinarum* In: Goldman E and Green L H (Eds.) *Practical Handbook of Microbiology,* CRC press pp. 519-31.

Akhtar S, Batti R A and Muhammad K. 2001. Clinico-therapeutic observations on an outbreak of infectious coryza. *International Journal of Agriculture and Biology* **03**(4): 531-32.

Akter M R, Khan M S R, Rahman M M, Kabir S L and Khan M A S. 2016. Epidemic behavior of the etiological agent of infectious coryza in layer chicken of Bangladesh with isolation, identification and pathogenicity study *Asian Journal of Medical and Biological Research* **2**: 82-94.

Akter M R, Khan M S R, Rahman M M, Khan M S and Kabir S L. 2013. Investigation on infectious coryza of layer chicken in Bangladesh with isolation, identification and antibiogram study. *Scientific Journal of Veterinary Advances* **2**(6): 83-89.

Akter S, Saha S, Khan K A, Amin M M and Haque M E. 2014. Isolation and Identification of *Avibacterium paragallinarum* from Layer Chickens in Gazipur, Bangladesh *Microbes and Health* **3**(1): 9-11.

Ali M, Hossain M S, Akter S, Khan M A H N A and Hossain M M. 2013. Pathogenesis of infectious coryza in chickens (*Gallus gallus*) by Avibacterium paragallinarumn isolate of Bangladesh. *The Agriculturists* **11**: 39-46.

Anjaneya, Singh S D, Dhama K, Gowthaman V and Chawak M M. 2013. Pathogenicity study of the field isolates of Avibacterium paragallinarum in experimentally infected birds. *Indian Journal of Veterinary Pathology* **37**: 13-17.

Anjaneya, Singh S D, Dhama K, Wani M Y, Gowthaman V and Chawak M M. 2014. Molecular characterization of *Avibacterium paragallinarum* isolated from poultry flocks of India. *Asian Journal of Animal and Veterinary Advances* **9**(7)*:* 440-51.

Arstila T P, Vainio O and Lasilla O. 1994. Centrale role of CD4+ T cells in avian immune responses. *Poultry Science* **73**: 1019-26.

Artman M, Weiner M and Frankl G. 1980. Counterimmunoelectrophoresis for early detection and rapid identification of Haemophilus influenzae type b and *Streptococcus pneumoniae* in blood cultures *Journal of clinical Microbiology* **12**: 614.

Beach J R and Schalm O W. 1936. Studies of the clinical manifestations and transmissibility of infectious coryza of chickens. *Poultry Science* **15**(6): 466-72.

Blackall P J and Soriano E V. 2013. Infectious coryza and related bacterial infectious *Diseases of Poultry*, pp. 859-74.

Blackall P J, Christensen H, Beckenham T, Blackall, L L and Bisgaard M. 2005. Reclassification of *Pasteurella gallinarum*, [Haemophilus] paragallinarum, Pasteurella avium and *Pasteurella volantium* as *Avibacterium gallinarum* gen nov, comb nov, *Avibacterium paragallinarum* comb nov, *Avibacterium avium* comb nov and *Avibacterium volantium* comb nov. *International journal of Systematic and Evolutionary Microbiology* **55**(1): 353-62.

Blackall P J, Eaves L E and Rogers D G. 1990. Proposal of a new serovar and altered nomenclature for Haemophilus paragallinarum in the Kume hemagglutinin scheme. *Journal of Clinical Microbiology* **28**(6): 1185-87.

Blackall P J, Yamaguchi T, Iritani Y and Rogers D G. 1990. Evaluation of two monoclonal antibodies for serotyping *Haemophilus paragallinarum. Avian Diseases* **34**(4)*:* 861-64.

Blackall P J. 1989. The avian haemophili. *Clinical Microbiology Reviews* **2**(3): 270-77.

Blackall P J. 2011. An update on the diagnosis and prevention of fowl cholera and infectious coryza. In: The *Proceedings of 22nd Latin American Poultry Congress*, Argentina.

Blackall P J and Reid G G. 1987. Further efficacy studies on inactivated, aluminum-hydroxide-adsorbed vaccines against infectious coryza. *Avian Diseases* **31**(3)527-32.

Blackall, P J, Morrow C J, McInnes A, Eaves L E & Rogers D G. 1990. Epidemiologic studies on infectious coryza outbreaks in northern New South Wales, Australia, using serotyping, biotyping, and chromosomal DNA restriction endonuclease analysis. *Avian Diseases* **34**(2)267-276.

Blackall, P J. 1999. Infectious coryza: overview of the disease and new diagnostic options *Clinical Microbiology Reviews* **12**(4): 627-32.

Bland M P, Bickford A A, Charlton B R, Cooper G C, Sommer, F and Cutler G. 2002. Case report: a severe infectious coryza infection in a multi-age layer complex in central California. In: *51st Western Poultry Disease Conference/XXVII ConvencionAnual ANECA*. PeurtoVallajarta, Mexico, pp. 56-57.

Boucher C E, Theron C W, Hitzeroth A C and Bragg R R. 2015. Regulation of chicken immunity-related genes and host response profiles against *Avibacterium paragallinarum* pathogen challenge. *Veterinary Immunology and Immunopathology* **167**(1): 70-74.

Boucher C E, Theron C W, Jansen A C and Bragg R R. 2014. Transcriptional profiling of chicken immunity-related genes during infection with *Avibacterium paragallinarum*. *Veterinary Immunology and Immunopathology* **158**: 135-142.

Bragg R R, Coetzee L and Verschoor J A. 1996. Changes in the incidences of the different serovars of *Haemophilus paragallinarum*in South Africa: A possible explanation for vaccination failures. *Onderstepoort Journal of Veterinary Research* **63**: 217-26.

Briles W E, Mc Gibbon W H and Irwin M R .1950. On multiple alleles effecting cellular antigens in the chicken *Genetics* **35:** 633-40.

Byarugaba D K, Minga U M, Gwakisa P S, Katunguka-Rwakishaya E, Bisgaard M and Olsen J E. 2007. Virulence characterization of *Avibacterium paragallinarum* isolates from Uganda. *Avian Pathology* **36:**35-42.

Calderon E N, Thomas K, Morales-Erasto V, Salgado-Miranda C and Soriano-Vargas E. 2010. Identification of *Avibacterium paragallinarum* serovar B-1 from severe infectious coryza outbreaks in Panama. *Avian Diseases* **54**(3): 1095-97.

Cevik-Demirkan Ç A, Haziroğlu R M and Kürtül I .2007. Gross morphological and histological features of larynx, trachea and syrinx in Japanese quail. *Anatomia, histologia, Embryologia* **36**: 215-19.

Chen X, Miflin J K, Zhang P and Blackall P J.1996. Development and Application of DNA probes and PCR tests for *Haemophilus paragallinarum. Avian Diseases* **40**: 398-407.

Chen X, Chen Q, Zhang P, Feng W and Blackall P J. 1998. Evaluation of a PCR test for the detection of Haemophilus paragallinarum in China. *Avian Pathology* **27**: 296- 300.

Chen X, Zhang P, Blackall P J and Feng W. 1993. Characterization of *Haemophilus paragallinarum* isolates from China. *Avian Diseases* **37**(2): 574-576.

Chukiatsiri K and Chansiripornchai N. 2008. An outbreak of infectious coryza in a layer farm. *Journal of Thailand Veterinary Medical Assoc*iation **58**(3): 98-107.

Chukiatsiri K, Chotinun S and Chansiripornchai N. 2010. An outbreak of *Avibacterium paragallinarum* serovar B in a Thai layer farm. *The Thai Journal of Veterinary Medicine* **40**(4): 441-44.

Chukiatsiri K, Sasipreeyajan J, Neramitmansuk W and Chansiripornchai N. 2009. Efficacy of autogenous killed vaccine of *Avibacterium paragallinarum. Avian Diseases* **53**(3): 382-86.

Clark D S and Godfrey J F. 1961. Studies of an inactivated Hemophilus gallinarum vaccine for immunization of chickens against infectious coryza. *Avian Diseases* **5**(1): 37-47.

Deshmukh S, Banga H S, Sodhi S and Brar R S. 2015. An update on avian infectious coryza: it's re-emerging trends on epidemiology, etiologic characterization, diagnostics, therapeutic and prophylactic advancements. *Journal of Dairy Veterinary and Animal Research* **2**: 00037.

Dousse F, Thomann A, Brodard I, Korczak B M, Schlatter Y, Kuhnert P and Frey J. 2008. Routine phenotypic identification of bacterial species of the family Pasteurellaceae isolated from animals. *Journal of Veterinary Diagnostic Investigation* **20**: 716-24.

Droual R, Bickford A A, Charlton B R, Cooper G L & Channing S E. 1990. Infectious coryza in meat chickens in the San Joaquin Valley of California. *Avian Diseases* **34**(4): 1009-1016.

Eaves L E, Rogers D G and Blackall P J. 1989. Comparison of hemagglutinin and agglutinin schemes for the serological classification of *Haemophilus paragallinarum* and proposal of a new hemagglutinin serovar. *Journal of Clinical Microbiology* **27**(7): 1510-13.

Eko F O, W Lubitz L M, Ramey K, Moore T T, Ananaba G A, Lyn D, Black C M, and Igietseme J U. 2003. Recombinant *Vibrio cholerae* ghosts as a delivery vehicle for vaccinating against *Chlamydia trachomatis*. *Vaccine* **21**: 1694-1703.

El-sawah YA, Soliman and Shafey S M. 2012. Molecular characterization of Avibacterium paragallinarum strains used in evaluation of coryza vaccine in Egypt. *Journal of American Science* **8**(3): 253-263.

Emerit J and Chaudiere J. 1989. Free radicals and lipid peroxidation in cell pathology In: Miquel J, Quintanilha A T and Weber H (Eds.) *CRC Handbook of Free Radicals and Antioxidants in Biomedicine.* Boca Raton, FL, CRC Press. pp-177-85.

Erf G F. 2004. Cell-mediated immunity in poultry. *Poultry Science* **83:** 580-90.

Espinoza A M, Koga Y and Amparo I. 2008. Zavaleta. *Avian Diseases* **52**(1): 54-58.

Espinoza A M, Ysabelkoga A I and Zavaleta. 2008. Amplified 16s Ribosomal DNA Restriction Analysis for identification of Avibacterium paragallinarum. *Avian Diseases* **52**: 54-58.

Esterbauer H, Schaur R J and Zollner H. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine* **11**: 81-128.

Ficken M D, Edwards J F and Lay J C. 1986. Clearance of bacteria in turkeys with Bordetellaavium-induced tracheitis. *Avian Diseases* **30**: 352-57.

Fouchier R A, Munster V, Wallensten A, Bestebroer T M, Herfst S, Smith D and Osterhaus A D. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *Journal of Virology* **79**: 2814-2822.

Fujiwara H & Konno S. 1965. Histopathological studies on infectious coryza of chickens I findings in naturally infected cases. *National Institute of Animal Health Quarterly* **5**: 36.

Fulton J E, Juul-Madsen H R, Ashwell C M, McCarron A M, Arthur J A, O'Sullivan N P and Taylor R L. 2006. Molecular genotype identification of the Gallus gallus major histocompatibility complex. *Immunogenetics* **58***:*407-21.

Garcia A, Romo F, Ortiz A M and Blackall P J. 2008. The vaccination-challenge trial: the gold standard test to evaluate the protective efficacy of infectious coryza vaccines. *Avian pathology* **37***:* 183-86.

Genovese K J, He H, Swaggerty C L and Kogut M H. 2013. The avian heterophil. *Developmental and Comparative Immunology* **41**(3): 334-40.

Georgieva N V. 2005. Oxidative stress as a factor of disrupted ecological oxidative balance in biological systems—a review *Bulgarian Journal of Veterinary Medicine* **8:** 1-11.

Getty R. 1975. Aves respiratory system. *Anatomy of domestic animals 5th Ed.*1884-1917.

Gutteridge J M. 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clinical Chemistry* **41**: 1819-28.

Glisson J R. 1998. Bacterial Respiratory Diseases of Poultry. *Poultry Science* **77**:1139–1142.

Hafeman D G, Sunde R A and Hoekstra W G. 1974. Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. *The Journal of Nutrition* **104:** 580-87.

Han M S, Kim J N, Jeon E O, Lee H R, Koo B S, Min K C and Jang H S. 2016. The current epidemiological status of infectious coryza and efficacy of Poul Shot Coryza in specific pathogen-free chickens, *Journal of Veterinary Science.* **17**(3): 323-30.

Haunshi S, Dutta B and Saxena S C. 2006. An outbreak of infectious coryza in Vanaraja poultry of Meghalaya. *Indian Journal of Veterinary Pathology* **30**(1): 55-55.

Hobb R I, Tseng H J, Downes J E, Terry T D, Blackall P J, Takagi M and Jennings M P. 2002. Molecular analysis of a haemagglutinin of *Haemophilus paragallinaruma. Microbiology* **148**(7): 2171-79.

Hoerr F J, Putnam M, Rowe-Rossmanith, S, Cowart W and Martin J .1994. Case report: Infectious coryza in broiler chickens in Alabama In *Western Poultry Disease Conference (USA).*

Hsu Y M, Shieh H K, Chen W H, Shiang J H and Chang P C. 2007. Immunogenicity and haemagglutination of recombinant Avibacterium paragallinarum HagA. *Veterinary Microbiology* **122**: 280-89.

Iritani Y, Iwaki S and Yamaguchi T. 1981. Biological activities of crude polysaccharide extracted from two different immunotype strains of *Hemophilus gallinarum* in chickens. *Avian Diseases* **25**(1): 29-37.

Iritani Y, Katagiri K and Arita H. 1980. Purification and properties of *Haemophilus paragallinarum* hemagglutinin. *American Journal of veterinary Research.* **41**(12): 2114-18.

Iritani Y, Kunihiro K, Yamaguchi T, Tomii T and Hayashi Y. 1984. Difference of immune efficacy of infectious coryza vaccine by different site of injection in chickens. *Journal of the Japanese Society of Poultry Diseases (Japan)* 20: 182-85.

Ispir U, Gokhan H B, Ozcan M, Dorucu M and Saglam N. 2009. Immune response of rainbow trout (*Oncorhynchus mykiss*) to selected antigens of Yersinia ruckeri. *Acta Veterinaria Brno* **78**(1): 145-50.

Jacobs A A, van den Berg K and Malo A. 2003. Efficacy of a new tetravalent coryza vaccine against emerging variant type B strains. *Avian Pathology* **32:** 265-69.

Jirjis F F, Noll S L, Halvorson D A, Nagaraja K V and Shaw D P. 2001. Immunohistochemical detection of avian pneumovirus in formalin-fixed tissues. *Journal of Veterinary Diagnostic Investigation* **13**(1): 13-16.

Jordan F, Pattison M, Alexander D, Faragher T, Textbook of Poultry Diseases. In Bradbury JM (2005). Fifth Edition, eds. W.B. Saunders, London, UK, 178–193.

Kang H, Yan M, Yu Q and Yang Q. 2013. Characteristics of nasal-associated lymphoid tissue (NALT) and nasal absorption capacity in chicken *PloSone*. **8**(12): 840-97.

Kang H, Yan M, Yu Q and Yang Q .2014. Characterization of Nasal Cavity-associated lymphoid tissue in ducks. *The Anatomical Record* **297***:* 916-24

Kaur J, Sharma N S, Gupta K and Singh A. 2004. *Indian Journal of Animal Sciences* **74**: 462-65.

Keles H, Fidan A F, Cigerci I H, Kucukkurt I, Karadas E and Dundar Y. 2009. Increased DNA damage and oxidative stress in chickens with natural Marek's disease. *Veterinary Immunology and Immunopathology* **133**: 51-58.

Klika E, Scheuermann D W, Groodt-Lasseel D, Bazantova I, and Switka A. 1996. Pulmonary macrophages in birds (barn owl, *Tyto tyto alba*), domestic fowl (*Gallus gallus domestica*), quail (*Coturnix coturnix*), and pigeons (*Columbia livia*). *The Anatomical Record* **246**(1): 87-97.

Koch G. 1991. The immune system in poultry. *Magazine for Animal Healing* **116**(14): 728-34.

Kume K, A Sawata, and T Nakai. 1984. Clearance of the challenge organisms from the upper respiratory tract of chickens injected with an inactivated *Haemophilus paragallinarum* vaccine. *The Japanese Journal of Veterinary Science* **46**:843-50.

Kume K, Sawata A, Nakai T and Matsumoto M. 1983. Serological classification of *Haemophilus paragallinarum* with a hemagglutinin system. *Journal of Clinical Microbiology* **17**(6): 958-64.

Kume K, Sawata A and Nakase Y. 1980. *Haemophilus* infections in chickens 3 Immunogenicity of serotypes 1 and 2 strains of *Haemophilus paragallinarum. Japanese Journal of Veterinary Science* **42**: 673-80.

Lehmann P F and Reiss E. 1978. Invasive aspergillosis: antiserum for circulating antigen produced after immunization with serum from infected rabbits. *Infection and Immunity* **20**: 570.

Luna L G. 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology. *American Registry of Pathology*. 3rd Edn. pp. 258.

Matsumoto M and Yamamoto R. 1975. Protective quality of an aluminum hydroxide-absorbed broth bacterin against infectious coryza. *American Journal of Veterinary Research* **36**: 579-82.

Matsumoto M and Yamamoto R. 1971. A broth bacterin against infectious coryza: immunogenicity of various preparations. *Avian Diseases* **15**: 109-17.

Mouahid M, Bouzoubaa K and Zouagui Z. 1989. Chicken infectious coryza in Morocco: epidemiological study and pathogenicity trials. *Proceedings of the Agronomic and Veterinary Institute Hassan II* **9**: 11-16.

Muhammad T N and Sreedevi B. 2015. Detection of *Avibacterium paragallinarum* by Polymerase chain reaction from outbreaks of Infectious coryza of poultry in Andhra Pradesh. *Veterinary World* **8**(1): 103.

Nakamura K, Cook J K, Otsuki K, Huggins M B and Frazier J A. 1991. Comparative study of respiratory lesions in two chicken lines of different susceptibility infected with infectious bronchitis virus: histology, ultrastructure and immunohistochemistry. *Avian Pathology* **20**(2): 241-57.

Nakamura T, Hoshi S, Nagasawa Y and Ueda S. 1994. Protective effect of oral administration of killed *Haemophilus paragallinarum* serotype A on chickens. *Avian Diseases* **38**(2): 289-292.

Nganpiep L N and Maina J N. 2002. Composite cellular defence stratagem in the avian respiratory system: functional morphology of the free (surface) macrophages and specialized pulmonary epithelia. *Journal of Anatomy* **200**(5): 499-516.

Nighot P K, Kolte G N and Ghalsasi G R. 2002. Physiopathology of avian respiratory diseases-Respiratory diseases are often complex and multi-factorial but understanding the important factors can help in disease control. *Poultry International* **41**(9): 24-29.

Noonkhokhetkong T, Chukiatsiri K, Sasipreeyajan J and Chansiripornchai N. 2013. Determination of antimicrobial susceptibility, antimicrobial resistance genes and *in vivo* testing of antimicrobial susceptibility of *Avibacterium paragallinarum*. *The Thai Journal of Veterinary Medicine* **43**: 525.

Ohshima K and Hiramatsu K. 2000. Distribution of T-cell subsets and immunoglobulin-containing cells in nasal-associated lymphoid tissue (NALT) of chickens. *Histology and Histopathology* **15**(3): 713-20.

Page L A. 1962. Haemophilus infections in chickens I Characteristics of 12 Haemophilus isolates recovered from diseased chickens. *American Journal of Veterinary Research* **23**: 85.

Patil V V, Mishra, D N and Mane D V. 2016. Isolation, Characterization and Serological Study of *Avibacterium paragallinarum* field isolates from Indian Poultry. *Journal of Animal and Poultry Sciences* **5**(1): 13-20.

Patil V, Mishra D and Mane D. 2017. Virulence pattern of Avibacterium paragallinarum isolates studied from Indian field condition *International Journal of Livestock Research.* **7:** 201-207.

Piechulla K, Hinz K H and Mannheim W. 1985. Genetic and phenotypic comparison of three new avian Haemophilus-like taxa and of Haemophilus paragallinarum Biberstein and White 1969 with other members of the family Pasteurellaceae Pohl 1981. *Avian Diseases* **29**: 601-12

Placer Z A, Cushman L L and Johnson B C .1966. Estimation of product of lipid peroxidation (malonyldialdehyde) in biochemical systems. *Analytical Biochemistry* **16**: 359-64.

Prasad V, Murthy K and Murthy P. 1999. Antibiotic sensitivity studies on *Haemophillus paragallinarum* isolated from chickens. *Indian Veterinary Journal* **76**(3): 253-54.

Priya P M, Krishna S, Kumar V, Mini M. 2012. Isolation and characterization of Avibacterium paragallinarum from ornamental birds in Thrissur, Kerala. *International Journal of Life Sciences* **1**(3): 87-88.

Quinn P J, Carter M E, Markey B K and Carter G R. 1994. *Clinical Veterinary Microbiology* 2nd Edn. pp. 273-77, Wolfe, USA.

Qureshi M A, Heggen C L and Hussain I. 2000. Avian macrophage: effector functions in health and disease. *Developmental & Comparative Immunology* **24**(2): 103-19.

Qureshi M A, Marsh J A, Dietert R R, Sung Y J, Nicolas-Bolnet C and Petitte J N. 1994. Profiles of chicken macrophage effector functions. *Poultry Science* **73**(7): 1027-34.

Rajurkar G, Roy A, & Yadav M M. 2009. An overview on Epidemiologic investigations of Infectious coryza. *Veterinary world* **2**(10): 401-03.

Ramachandran D, Kalimuthu V and Sharma M. 2013. Pathogenicity Study of Field Isolates of *Avibacterium paragallinarum* in Seven Week Old Chicks. *Tamilnadu Journal Veterinary and Animal Sciences* **9**: 259-263.

Reese S, Dalamani G and Kaspers B. 2006. The avian lung-associated immune system: a review. *Veterinary Research* **37**(3): 311-24.

Reid G G and Blackall P J. 1984. Pathogenicity of Australian isolates of *Haemophilus paragallinarum* and *Haemophilus* avium in chickens. *Veterinary Microbiology* **9**: 77-82.

Rimler R B and Davis R B. 1977. Infectious coryza: in vivo growth of *Haemophilus gallinarum* as a determinant for cross protection. *American journal of Veterinary Research* **38**(10): 1591-93.

Rimler R B. 1979. Studies of the pathogenic avian haemophili. *Avian Diseases* **23**: 1006-18.

Rocha R M O, Garcia-Gonzalez O, Perez-Mendez A, Ibarra-Caballero J, Perez-Marquez V M, Vaca S and Negrete-Abascal E. 2006. Membrane vesicles released by *Avibacterium paragallinarum* contain putative virulence factors. *FEMS Microbiology Letters* **257**(1): 63-68.

Roodt Y, Bragg R R and Albertyn J. 2012. Identification of prophages and prophage remnants within the genome of *Avibacterium paragallinarum* Bacterium *Sequencing.* 1-5.

Sakamoto R, Baba S, Ushijima T, Kino Y, Honda T, Mizokami H and Sakaguchi M. 2013. Development of a recombinant vaccine against infectious coryza in chickens. *Research in Veterinary Science* **94**: 504-09.

Sakamoto R, Kino Y and Sakaguchi M. 2011. Development of a multiplex PCR and PCR-RFLP method for serotyping of *Avibacterium paragallinarum*. *Journal of Veterinary Medical Science* **74**(2): 271-73.

Sameera A, Asif R and Muhammad. 2001. Clinico-Therapeutic Observations on an outbreak of Infectious Coryza. *International Journal Agriculture Biology* **3**: 531-32.

Sandoval V E, Terzolo H R and Blackall P J. 1994. Complicated infectious coryza outbreaks in Argentina. *Avian Diseases* **38**: 672-78.

Sangdee K, Thummabenjapone P and Sangdee A. 2012. Evaluation of Antigen Preparation Methods for Polyclonal Antibody Production against *Streptomyces* spp. *British Microbiology Research Journal* **2**:137-45.

Sato S and Shifrine M.1964. Serologic response of chickens to experimental infection with *Hemophilus gallinarum* and their immunity to challenge. *Poultry Science* **43(**5): 1199-1204.

Sawata A, & Kume, K. 1983. Relationships between virulence and morphological or serological properties of variants dissociated from serotype 1 Haemophilus paragallinarum strains. *Journal of Clinical Microbiology* **18**(1): 49-55.

Sawata A, Kume K and Blackall P J. 1982. Hemaglutinin of *Haemophilus paragallinarum* serotype 2 organisms: occurrence and immunologic properties of hemagglutinin. *American Journal of Veterinary Research* **43**: 1311-14.

Sawata A, Kume K and Nakai T. 1984. Relationship between anticapsular antibody and protective activity of a capsular antigen *of Haemophilus paragallinarum. The Japanese Journal of Veterinary Science* **46**(4): 475-86.

Sawata A, Nakai T, Kume K, Yoshikawa H and Yoshikawa T. 1985. Intranasal inoculation of chickens with encapsulated or nonencapsulated variants of *Haemophilus paragallinarum*: electron microscopic evaluation of the nasal mucosa. *American Journal of Veterinary Research* **46**(11): 2346-53.

Sawata A, Nakai T, Kume K, Yoshikawa H and Yoshikawa T. 1985. Intranasal inoculation of chickens with encapsulated or nonencapsulated variants of Haemophilus paragallinarum: electron microscopic evaluation of the nasal mucosa. *American Journal of Veterinary Research* **46**(11): 2346-53.

Schalm O W and ARBeach J R. 1936. Studies of infectious coryza of chickens with special reference to its etiology. *Poultry Science* **15**(6): 473-82.

Sharma J M. 2003. *Diseases of Poultry*. 11th Edn. pp 5-16. Iowa State Press, Iowa, USA.

Smiałek M, Tykałowski B, Stenzel T and Koncicki A. 2011. Local immunity of the respiratory mucosal system in chickens and turkeys. *Polish Journal of Veterinary Sciences.* **14***:*291-297

Smiałek M, Tykałowski B, Stenzel T and Koncicki A. 2011. Local immunity of the respiratory mucosal system in chickens and turkeys. *Polish Journal of Veterinary Sciences* **14:** 291-97.

Sodhi S, Brar R S, & Banga H S. 2017. Bromadiolone Induced Oxidative Stress and Cytological Damage in Layer Birds. *International Journal of Advanced Veterinary Science and Technology* **6**(4): 334-339.

Soriano V E, Tellez G, Hargis B M, Newberry L, Salgado-Miranda C and Vazquez J C. 2004. Typing of *Haemophilus paragallinarum* strains by using

enterobacterial repetitive intergenic consensus-based polymerase chain reaction. *Avian Diseases* **48**(4): 890-95.

Tennison L B and Siddle P J. 1961. Limited study with Haemophilus cultures. *Avian Diseases* **5**(3): 352-54.

Thenmozhi V and Malmarugan S. 2013. Isolation, identification and antibiogram pattern of *Avibacterium paragallinarum* from Japanese quails. *Tamilnadu Journal of Veterinary and Animal Sciences* **9**: 253-58.

Thornton A M and Blackall P J. 1984. Serological classification of Australian isolates of *Haemophilus paragallinarum*. *Australian Veterinary Journal* **61**: 251-53.

Tongaonkar S, Deshmukh S and Blackall P J. 2002. Characterisation of Indian isolates of *Haemophilus paragallinarum*. *Proceedings of the 51st Western Poultry Disease Conference*, pp. 58

Toth T E. 2000. Nonspecific cellular defense of the avian respiratory system: a review. *Developmental & Comparative Immunology* **24**(2): 121-39.

Vandaveer S S, Erf G F and Durdik J M. 2001. Avian T helper one/two immune response balance can be shifted toward inflammation by antigen delivery to scavenger receptors. *Poultry Science* **80**(2): 172-81.

Vargas E S and Terzolo H R. 2004. *Haemophilus paragallinarum*: Etiology of infectious coryza. *Veterinarni Mexico* **35**(3): 245-59.

Wafaa A and El-Ghany A. 2011. Evaluation of autogenous *Avibacterium paragallinarum* bacterins in chickens *International Journal of Poultry Science* **10**(1): 56-61.

Wambura P N. 2010. Preparation and use of autogenous vaccine from *Avibacterium paragallinarum* (strain Tan 1-05) in layer chickens. *Tropical Animal Health and Production* **42**(3): 483-86.

Wang H F, Zhong X H, Shi W Y and Guo B. 2011. Study of malondialdehyde (MDA) content, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in chickens infected with avian infectious bronchitis virus. *African Journal of Biotechnology* **10**: 9213-17.

Welchman D D B, King S A, Wragg P, Wood A M, Irvine R M, Pepper W J and de Wit J J. 2010. Infectious coryza in chickens in Great Britain. *The Veterinary Record* **167**(23): 912-16.

Wu J R, Wu Y R, Shien J H, Hsu Y M, Chen C F, Shieh H K & Chang P C. 2011. Recombinant proteins containing the hypervariable region of the haemagglutinin protect chickens against challenge with Avibacterium paragallinarum. *Vaccine* **29**(4): 660-67.

Yamaguchi T, Kobayashi M, Masaki S and Iritani Y. 1993. Isolation and characterization of a *Haemophilus paragallinarum* mutant that lacks a hemagglutinating antigen. *Avian Diseases* **37**(4): 970-76.

Yamamoto R. 1972. Infectious coryza In: Hofstad M S, Calnek B W, Helmboldt C F, Reid W M and Yoder W H (Eds.) *Diseases of Poultry*, 6th Edn. Iowa State University Press, Ames, Iowa, pp. 272-81.

Yamamoto R. 1991. Infectious coryza: In: Hofstad M S, Calnek B W, Helmboldt C F, Reid W M and Yoder W H (Eds.) *Diseases of Poultry,* Iowa State University Press, Ames, Iowa.

Yamomoto R and Clark G T. 1966. Intra-and interflock transmission of *Haemophilus gallinarum*. *American Journal of Veterinary Research* **27**(120): 1419.

Yan Meng-fei, Kang Hai-hong, Yang Qian. 2014. Histological characteristic of chicken nasal cavity and the distribution of nasal-associated lymphoid tissue. *Acta Veterinaria Et Zootechnica Sinica* **45**(12): 2043-49.

York J J and Fahey K J. 1988. Diagnosis of infectious laryngotracheitis using a monoclonal antibody ELISA. *Avian Pathology* **17**(1): 173-82.

Zhao H Q, Wang B Y, Feng J, Wu P Y and Wang K. 2003. Influence of DHV on activities of SOD and GSH-Px in livers of Ducks. *Chinese Journal of Preventive Veterinary Medicine* **25**: 44-46.

Zhao Q, Sun Y N, Zhang X X, Kong Y B, Xie Z J, Zhu Y L and Jiang S J. 2010. Evaluation of two experimental infection models for *Avibacterium paragallinarum*. *Veterinary microbiology* **141**: 68-72.

VITA

Name of the student	:	Aadish Balouria
Father's name	:	Mr. R.S. Balouria
Mother's name	:	Mrs. Renu Balouria
Nationality	:	Indian
Date of birth	:	25-03-1990
Permanent home address	:	H.B.No.302, Defence colony, Pathankot, PO Jandwal, Teh & Distt. Pathankot 145001, Punjab.

EDUCATIONAL QUALIFICATION

Bachelor degree	:	B.V.Sc. and A.H.
University	:	GADVASU-Ludhiana
Year of award	:	2015
OGPA/OCPA/% marks	:	7.89/10.00
Master's degree	:	M.V.Sc.
OCPA	:	8.20/10.00