Studies on Growth Kinetics of *Gallibacterium anatis* in Presence of Deuterium Oxide (D_2O, Heavy Water)

Thesis

Submitted to the
DEEMED UNIVERSITY
ICAR-Indian Veterinary Research Institute
Izatnagar - 243 122 (U.P.), India

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Veterinary Bacteriology)

2016

Dedicated to...

My Beloved Parents

and

Guide

भा.कृ.अ.प.–भारतीय पशु चिकित्सा अनुसंधान संस्थान
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Dated: 2016

Certificate

This is to be certified that the research work embodied in this thesis entitled "Studies on growth kinetics of *Gallibacterium anatis* in presence of deuterium oxide (D_2O heavy water)" *submitted by* **Dr. Shiv Varan Singh, Roll No. M-5547,** *for the award of* **Master of Veterinary Science** *Degree in* **Veterinary Bacteriology** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate herself under my supervision and guidance.*

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We the undersigned members of Advisory Committee of **Dr. Shiv Varan Singh, Roll No. M-5547,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Veterinary Bacteriology,** *agree that the thesis entitled* **"Studies on growth kinetics of *Gallibacterium anatis* in presence of deuterium oxide (D_2O, heavy water)"** *may be submitted in partial fulfillment of the requirement for the degree.*

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ACKNOWLEDGEMENTS

It gives me immense pleasure to acknowledge all the people who were responsible

for what I am today. I would like to express my sincere gratitude to all those who havemade my life beautiful so far.

First of all I express my first and foremost gratitude to my guide ***Dr. B.R. Singh****, Principal Scientist,Division of Epidemiology, IVRI, who have always been interested in my progress even in hisbusiest schedule. Thank you so much sir for accepting me and respecting all my opinionsthroughout my research.*

Words become insufficient, when I want to thank ***Dr. R.K. Agarwal****, Head,B&M for all the helps & advices.*

I would like to express sincere gratitude towards the members of my Advisory

Committee, ***Dr. Ravi Kant Agarwal, Dr. R. Sarvanan, Dr. Sameer Shrivastava*** *and* ***Dr. Vinodh Kumar. O.R*** *for their valuableadvice, guidance, constant encouragement and support throughout my study.*

*I am indebted to****Dr. Monika, Dr. Boby, Dr. Arijit, Dr. Swaraj,*** *who never said NO to whatever help I asked for.*

I pay sincere thanks to ***Dr. Mayankrawat, Dr. K. N. Viswas, Dr. Pallab Chaudhari, Dr .D. K. Sinha, Dr. K. P. Singh, Dr. Mithilesh, Dr. Pavan, Dr. S. Qureshi, Dr. Atira C. K, Dr. Aadeysha, Dr. Rohit Jaiswal, Dr. Abhinav, Dr. Manesh, Dr. Bardha Sankar Mohanti*** *for their help and support whenever needed.*

I am also very thankful of my supportive batchmate ***Dr. Shiva, Dr. Grace, Dr. Sushobhit, Dr. Shyam****,* ***Dr. Anukampa, Dr. Ankush, Dr. Vanta, Dr. Dipankar, Dr. Jugal****,* ***Sushil, Satyendre, Sanjeev, Ashutose.***

I reserve my special thanks to ***Dr. D.K. Sinha (Head of Epidemiology)*** *who has beena source of encouragement for me all these days. I am also indebted to all the scientists ofB&M who work in other divisions as they continue to be undividable part of B&M family.*

Words are insufficient for my seniors ***Dr. Ajit, Dr. Ravji, Dr. Ashu, Dr. Prasanna, Dr. Ankush, Dr. Ankita, Dr. Arun, Dr. Rekha, Dr. Kartik, Dr. Anshuja, Dr. Mamta, Dr. Jannudin, Dr. Kushbu, Dr. Saroj, Dr. Rajat, Dr. Stanzon, Dr. Mashook, Dr.***

Bhuvana, Dr. Madhu, Dr. Bina, Dr. Aparjita, Dr Arun, Dr Nitish, Dr. Nandi, Dr.Gangaram *for their incessant help and timely guidance.*

I will always remember the sincere advices and honest encouragement provided by my seniors ***Dr. Pawan, Dr. Nehra, Dr. Snehil, Dr. Messam Raja, Dr. Sudhir, Dr. Faneshwar****. Thank you so much for theprecious time you have dedicated for me.*

I would also like to acknowledge the help provided by the ***Dr. Ashok Kumar, Dr. Palanivelu. M, Dr. Pavan,****during my research.*

I thank particularly ***Mr. Laikurrahaman****for all their help.I am greatly thankful to* ***Karma, Chaavi, Prithvi, Nirupama, Vilal*** *for his great help during my research work.I am immensely thankful to my divisional batchmates* ***Shumila, Reshmi, Jitendra, Ishfaq, Sabita****for their whole hearted cooperation,joyful company and valuable help whenever required.*

I am also very muchthankful to my juniors specially ***Aakansha****,* ***Tania, Varsha, Balendari, Depika, Garima, Archana, Shikha, Dharmesh, Abhishek, Dhruv, Aarti,*** *for their all-round help and for all the lovely talks.I am highly thankful to staff of EPIDEMIOLOGY, SALMONELLA CENTER & WILD LIFE specially* ***Mr. H.C. Joshi****,* ***Mr. Arun, Mr. Mahesh, Mr. Haq and Mrs. Rupkalla, Mr. Kisanji, Mr. Jamal, Mr. Alim, Mr. Ashish, Mr. Bhatt, Mr. Haria, Mr. Hasgolla, Mr. Pant, Mr. Pandey ji, Mr. Mahesh babu****and other office staffor their co-operation throughout the course of study. I especially thank* ***Dr. D. K. Sinha****for all thepossible helps and constant encouragement during my degree.*

On the way to completion, I am delighted to acknowledge Dhanwantarihostel friends, ***Ashok, Ranjeet, Jayprakash, Pranav, Tanmay, Chandarbhan, Tamilmani, Bhupati, Parveen, Jay, Gopi sir*** *forthe blissful company.*

I thank my beloved friends and batchmates ***Rahul, Shashank, Shivendar, Vikram, Vyas, Sonu, Suyash, Rohitnani, Rohitpandit, Pradeep*** *and others for motivating me during my research.*

Being at IVRI has been a dream came true for me. I am indebted for life to this institute.I thank ICAR-IVRI for providing financial support in the form of sholership to study in this institute.

Its always been a joyful daily to express my gratitude to the most important people in my life, ***Mummy****,****Papa*** *and the entire family. They are my genesis and the reason of my existence. I would like to thank* ***Mr. Prasant, Kavita didi, Bunty bhiya,***

Sister Poonam, Niece Prisha, *for their unconditional love and for keeping me always close to their hearts. My special thanks to dear grandparents for taking care for me all these years.*

At last not least i would like to thank ***Mr. Dharmendra (Chachu), D.P., Honey*** *and* ***Kuldeep Bhai*** *for preparing this manuscript neetly and timely.*

To finish , finally I submit myself to the ***Lord Almighty*** *who listened and answered my prayers and made me feel his divine presence in places I go and people I met, you are my power and strength which makes things possible for me when all other hopes go in vain. Thank you so much for leading me even during intricate period of my life ...thank you.*

Shivvaran singh

Date: ***(Shiv Varan Singh)***

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ABBREVIATIONS

%	:	Percentage
°C	:	Degree Celsius
µ	:	Micron
µg	:	Microgram (s)
µl	:	Microliter
AE	:	Elution Buffer
AL	:	Lysis Buffer
AT	:	Aztreonam
ATL	:	Tissue Lysis Buffer
B	:	Button
BA	:	Blood Agar
BHI	:	Brain Heart Infusion
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
cAMP	:	Cyclic Adenosine Monophosphate
CHB	:	*Avibacterium species*
cfu	:	Colony forming unit
CLSI	:	Clinical and Laboratory Standards Institute
cm	:	Centimeter
CRDA	:	Congo Red Dye Assay
CRBA	:	Congo Red Binding Assay
CRDB	:	Congo Red Dye Binding
CWM	:	Cart Wheel Method
MCWM	:	Modified Cart Wheel Method
D_2O	:	Heavy water
DH5α	:	*E .coli* reference strain
DNA	:	Deoxyribonucleic acid
DPE	:	*E. coli* from Dog
dNTPs	:	Dinucleotide(s) triphosphate
E. coli	:	*Escherichia coli*
G. anatis	:	*Gallibacterium anatis*
EDTA	:	Ethylene diamine tetra acetic acid
ESBL	:	Extended spectrum β-lactmase
EtBr	:	Ethidium Bromide
ETP	:	Ertapenem
et al.	:	*et alii*
etc.	:	et cet·er·a

Fig.	:	Figure
g	:	Gram(s)
GIT	:	Gastro intestinal tract
H	:	Heavy water
h	:	Hour (s)
HA	:	Haemagglutination
H-H	:	Heavy Water to Heavy Water
HLY (HLY-O)	:	Parent *G. anatis* biovar Haemolytica from adult poultry
14H	:	*G. anatis* subculture in heavy water 14 times
17H	:	*G. anatis* subculture in heavy water 17 times
18H	:	*G. anatis* subculture in heavy water 18 times
21H	:	*G. anatis* subculture in heavy water 21 times
25H	:	*G. anatis* subculture in heavy water 25 times
36H	:	*G. anatis* subculture in heavy water 36 times
37H	:	*G. anatis* subculture in heavy water 37 times
38H	:	*G. anatis* subculture in heavy water 38 times
40H	:	*G. anatis* subculture in heavy water 40 times
51H	:	*G. anatis* subculture in heavy water 51 times
H_2S	:	Hydrogen sulfide
H-S	:	Heavy water to Simple water
1bs	:	*pounds*-force per square inch
i.e.	:	id est (that is)
IMViC	:	Indole Methyl-red Voges-Proskauer Citrate Test
IPM	:	Imipenem
IVRI	:	Indian Veterinary Research Institute
kDa	:	Kilo Dalton
KOH	:	Potassium hydroxide
LB	:	Luria-Bertani
M-97	:	*E.coli* from calf
A-290	:	*Pseudomonas aeruginosa*
M	:	MAT
mA	:	milliampere
MAT	:	Microagglutination test
MCA	:	MacConkey Agar
MDR	:	Multiple Drug Resistance
mg	:	Milligram
mg/l	:	Milligram Per Litre
MHA	:	Muller Hinton agar
MIC	:	Minimum Inhibitory Concentration
MIL	:	Motility Indole Lysine
min	:	Minutes

ml	:	Milliliter
mm	:	Millimeters
MSHA	:	Mannose sensitive haemagglutination
MRHA	:	Mannose resistance haemagglutination
m-PCR	:	multiplex-PCR
MR	:	Methyl Red
MRP	:	Meropenem
N-PAGE	:	Native Polyacrylamide gel electrophoresis
NCCLS	:	National Committee for Clinical Laboratory Standards
ng	:	nano gram
Non-adapted	:	Parent strain of *G. anatis* biovar haemolytica
NSS	:	Normal Saline Solution
OD	:	Optical density
6PH4	:	*G. anatis* biovar haemolytica from pigeon subcultured in heavy water.
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase Chain Reaction
PH4	:	*G. anatis* biovar haemolytica from Pigeon
PHLY	:	*G. anatis* biovar haemolytica subcultured in simple water
P51H	:	51H strain subcultured in simple water
pmole	:	Picomole (s)
QIAamp	:	DNA Stool Mini Kit
rpm	:	Revolution per minute
S	:	Simple water
SAM	:	*Staphylococcus aureus*
sec	:	Second
SDS	:	Sodium dodecyl sulphate
S-S	:	Simple water to Simple water
T	:	Tube
TBE	:	Tris borate EDTA buffer
0.01% TBC	:	Trypticase soya agar with 1.5% bile and 0.01% congo red dye
0.03% TBC	:	Trypticase soya agar with 1.5% bile and 0.03% congo red dye
0.01% TBnC	:	Trypticase soya agar with 0.01% congo red dye and without bile
0.03% TBnC	:	Trypticase soya agar with 0.03% congo red dye and without bile
TDP	:	Thermal Death Point
TDT	:	Thermal Death Time
TE	:	Tris-EDTA Buffer
TSA	:	Trypticase Soy Agar
TSB	:	Trypticase Soya Broth
TSI	:	Triple Sugar Iron Agar

U	:	Unit
USA	:	United State of America
UV	:	Ultra violet
V	:	Volts
viz.	:	Videlicet (namely)
VP	:	Voges Proskauer
W	:	Watt
WHO	:	World Health Organization
w/v	:	Weight per unit volume
β	:	Beta

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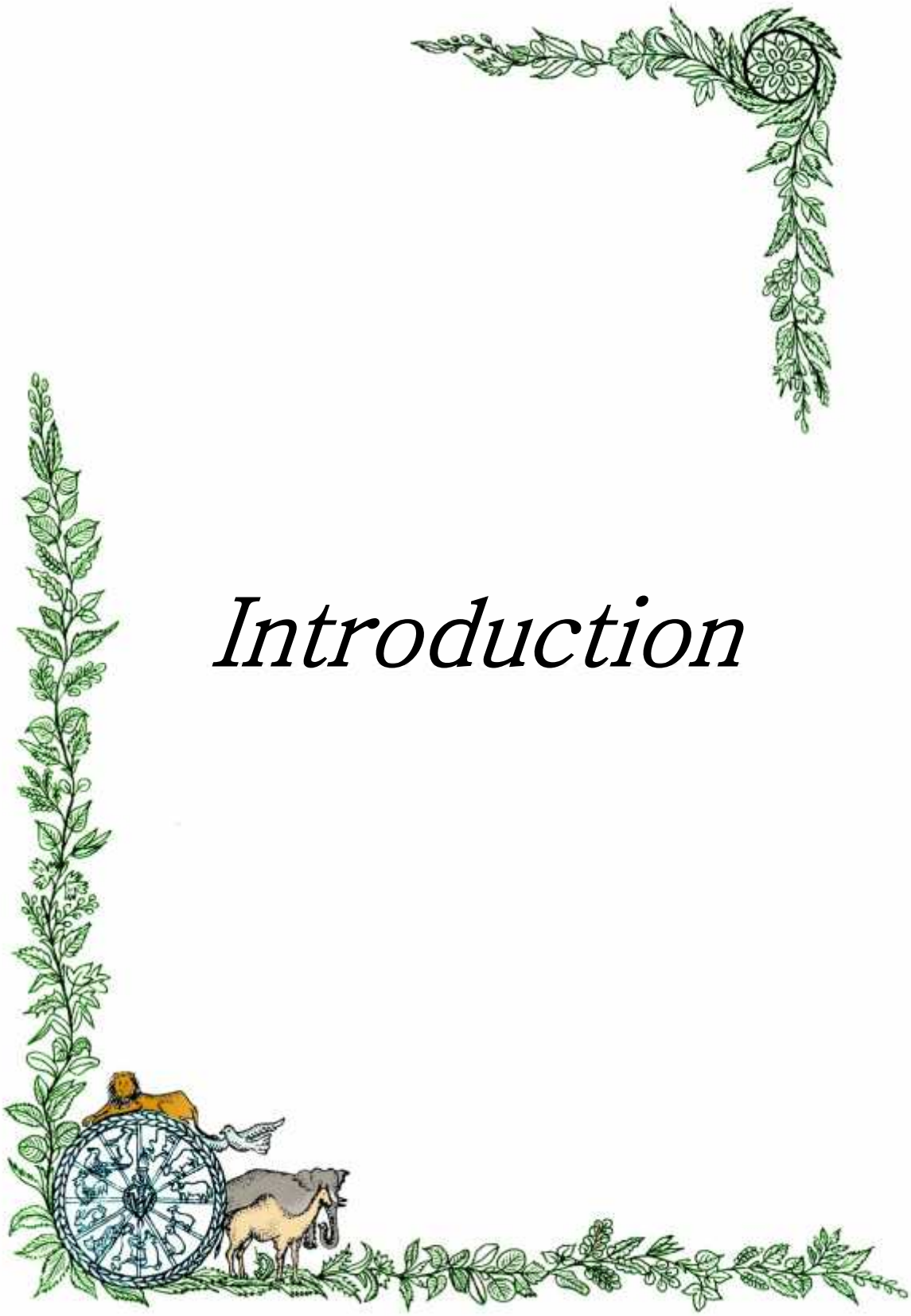


Introduction

1 INTRODUCTION

Global meat production is predicted to rise by 1.6% over the outlook period, with poultry becoming the largest meat sector by 2020 (AVEC, 2014) estimated to nearly triple by 2020. So besides paying attention towards important diseases of poultry need to focus also on emerging diseases of poultry. *Gallibacterium anatis* infection is an emerging disease of poultry. Growing concern about *G. anatis* is its poorly understood pathogenesis and growth kinetics. These knowledge gaps are inhibitory for taking countable step for control and prevention of *G. anatis* infections. *G. anatis* (earlier known as *Pasteurella anatis*) is resident in the upper respiratory tracts and the lower genital tracts of healthy chickens (Bisgaard, 1977; Mushin *et al*., 1980). It has been reported to be associated with bacteremia, oophoritis, follicle degeneration, salpingitis, peritonitis, hepatitis, enteritis, and respiratory tract diseases in chickens (Addo and Mohan, 1985; Bojesen *et al*., 2003b; Jordan *et al*., 2005; Neubauer *et al*., 2009; Kristensen *et al*., 2011).

Gallibacterium anatis is an important pathogen of intensively farmed poultry birds causing loss in production with heavy mortality in broiler chicken and drop in egg production in layers with increased mortality (Bojesen *et al*., 2008). *G. anatis* is also reported to infect turkeys, geese, ducks, pheasants, partridges, budgerigars, peacock, cage birds, wild birds, cattle and pig (Kjos-Hansen, 1950; Bisgaard, 1977; Mushin *et al*., 1980; Bisgaard, 1993; Christensen *et al*., 2003; Rzewuska *et al*., 2007; Bisgaard *et al*., 2009; Gregersen *et al*., 2010). Recently, it has also been associated with fatal bacteremia in an immunocompromized patient (Gautier *et al*., 2005; Aubin *et al*., 2013). The global distribution of *G. anatis* in Europe (Mráz *et al*., 1976; Bisgaard, 1977; Christensen *et al*., 2003; Bisgaard *et al*., 2009), Africa (Addo and Mohan, 1985), Asia (Suzuki *et al*., 1996), Australia (Gilchrist, 1963) and

the America (Hacking and Pettit, 1974; Shaw *et al*., 1990; Bojesen *et al*., 2007; Mendoza *et al*., 2014) indicated its worldwide prevalence. Its association with a variety of pathology makes it difficult to be diagnosed even after postmortem in absence of pathognomonic lesion and the disease often create a state of hocus and pocus with Fowl Coryza, New Castle disease and Bird Flu (Christensen *et al*., 2003).

Gallibacterium anatis is a gram-negative, rod shaped, non-motile, capsulated, facultative anaerobic bacteria, kept in family Pasteurellaceae by Pohl 1981 (Christensen *et al.*, 2003, Bisgaard *et al*., 2009). *G. anatis* has two biovar, i.e., a haemolytic bv.s haemolytica and a non-haemolytic bv. *anatis* (Christensen *et al*., 2003). Currently, *G. anatis* and *Gallibacterium* genomospecies 1 and 2 (Christensen *et al*., 2003), *Gallibacterium* genomospecies 3 and *Gallibacterium* group V are members of the genus *Gallibacterium* (Bisgaard *et al*., 2009).

Though the infection of *Gallibacterium* is treatable with antibiotics, the frequency of treatment failure of *Gallibacterium* seems to be a recurrent problem (Kehrenberg *et al*., 2006; Bortolaia *et al*., 2010). Ocurrence of multidrug resistant strains of *G. anatis* has been observed (Aarestrup *et al*., 2004; Schwarz *et al*., 2004; Kehrenberg *et al*., 2006; Bojesen *et al*., 2011) showing resistance towards novobiocin, tylosin, clindamycin, tetracycline and penicillin (Post *et al*., 1991; Watts *et al*., 1994; Mevius and Hartman, 2000; Malik *et al*., 2005; Berge *et al*., 2006; Hendriksen *et al*., 2008; Johnson *et al*., 2011; Jones *et al*., 2013). Resistance to sulpha drugs and streptomycin has also been reported in *Gallibacterium* strains a few years ago (Guo *et al*., 2009).

Deuterium (^{2}H or D), one of the two stables isotopes of hydrogen contains one neutron and one proton; it was first separated by Urey and coworkers (1932). The chemical properties of heavy water, oxide of deuterium (D_2O), and simple water (H_2O) are same but difference exists in physical properties of the two. Two independent physiological effects; (1) Isotope exchange effect and (2) Solvent isotope effect (Bass and Moore, 1973; Sugiyama, and Yoshioka, 2012) are the major reasons behind its biological effects on exposed living cells. Naturally D_2O is present in low concentration in natural water bodies (1 ppm) without any noticeable effect on life processes, but when its concentration goes above 20% its adverse health effects become evident specifically on higher life forms. It is due to 10 times stronger

C-D bond than C-H bond (Bass and Moore, 1973; Sugiyama, and Yoshioka, 2012) leading to structural tightening of biomolecules in turn causing, reduction in synthesis of proteins and nucleic acids, changes in kinetics of enzymatic reactions and morphological changes in cells (Katz *et al*., 1957; Thomson, 1960; Katz and Crespi, 1971; Ramakrishnaiah, 1990; Cioni and Strambini, 2002). However, microbes have been reported to adapt to grow in presence of even 100% D_2O. Adaptation to grow in D_2O is a multifactorial phenomenon, affecting cellular systems including biosynthesis of macromolecules, metabolism and transport functions of cells (Mosin and Ignatov, 2014). The ability of adaptation in heavy water is not same in all species of bacteria and may be varied in one frame of taxonomic family (Mosin *et al*., 1996a, 1996b). Deleterious effects of heavy water have been observed on luminous bacteria indicated by slow growth and low luminesces (Steinbach, 1949). Heavy water confer the stability to complex proteins when dissolved in it (Tomita *et al*., 1962). The data obtained confirm that adaptation to heavy water is a phenotypic phenomenon (Mosin *et al*., 1993) and may lead to morphological changes in macromolecules and thus in living cells (Walker and Syrett, 1959; Mosin, 2012). Heavy water is understood to affect stability and probably growth pattern of microbes. Thus, it may be exploited to increase shelf-life of microbes and their macro-molecules which may be used for development of thermostable vaccine, preservation of cells, organs and industrially useful enzymes. Moreover, through enhancing stability of pharmacologically important molecules biological efficacy of some of the drugs can be enhanced. Besides, if we can adapt the commercially important bio-molecule synthesizing microbes to D_2O they may be exploited for larger utility. Therefore, it is envisaged that growth kinetics, survival of adapted strain and parent strain, thermo-stability and antimicrobial drug resistance of *Gallibacterium anatis*, a fragile kind of bacterium difficult to maintain in laboratory under normal culture conditions, should be studied in presence of D_2O.

Therefore this study has been planned with the following objectives:

Objectives:

1. **To understand the growth kinetics and survivability of *Gallibacterium anatis* in presence of heavy water, and**
2. **Phenotypic characterization of D_2O adapted and parent (non-adapted) isogenic strain of *Gallibacterium anatis.***

Review of Literature

2 REVIEW OF LITERATURE

2.1 Classification of *Gallibacterium anatis* biovar haemolytica

Phylum**:** Proteobacteria, Class**:** Gammaproteobacteria, Family**:** Pasteurellaceae, Genus**:** *Gallibacterium*, Species**:** *anatis*, bv.**:** heamolytica and anatis (Mutters *et al*., 1985; Christensen *et al*., 2003).

2.2 History of naming *Gallibacterium*

First time the bacteria was isolated from cloaca of healthy chickens and described as haemolytic "cloaca bacterium" by Kjos-Hansen in 1950. The meaning of *Gallibacterium* is 'bacterium of chicken'. Being similar to *Pasturella* in several characters it was earlier known as *P. anatis*. The genus name *Gallibacterium* was first given by Bisgaard in the year (1982) on the basis of certain phenotypic characters used for identification of *Actinobacillus salpingitidis* and avian *P. haemolytica.* Christensen *et al*., (2003) established the genus *Gallibacterium* within the family of Pasteurellacae based on 16s rRNA (Christensen *et al*., 2003b) gene sequences. The genus includes the strains belonging to *G. anatis, Gallibacterium* genomospecies 1, 2, (Christensen *et al.,* 2003) 3 and un-named group V (Bisgaard *et al*., 2009).

Taxon 1 designated as a third group of strains by Bisgaard in 1982 and named *Pasteurella anatis* (Mutters *et al.,* 1985) was also found closely related to *A. salpingitidis* and avian *P. haemolytica*. However, with DNA-DNA hybridization they could not be clubbed into one genus (Piechulla *et al*., 1985; Bisgaard *et al.*, 2009). Confusion is still persistent in naming bv.s and may be a continued debate in coming years.

2.3 Epidemiology

2.3.1 Prevalence

Gallibacterium have been reported from many European countries like Switzerland, Denmark, Germany, Austria (Mráz *et al*, 1976; Bisgaard, 1977; Mirle *et al*., 1991; Neubauer *et al*., 2007), African countries like Nigeria (Addo and Mohan, 1985), Asian countries like Japan (Suzuki *et al.*, 1996), American countries like USA, (Shaw *et al*., 1990; Bojesen *et al*., 2007) and recently in India (Singh, 2015, IVRI annual Report 2015-16).

The factors related to host and environment also have been observed to nurture the infection of *G. anatis*. Host factors playing a role are hormonal influences (Kohlert, 1968; Gerlach, 1977), age (Janetschke and Risk, 1970; Bisgaard, 1977), stress (Rzewuska *et al*., 2007) and compromised immunological status (Bojesen *et al*., 2004). Important environmental factors are seasonal changes (Mirle *et al*., 1991) and cold stress (Matthes and Loliger, 1976) in the similar pattern reported for infections with other members of Pasteurellaceae (Gilchrist, 1963; Matthes *et al*., 1969; Shaw *et al*., 1990).

2.3.2 Habitat and Host range

Gallibacterium strains are known since long as common inhabitants of the respiratory tract and lower genital tract of healthy chickens (Bisgaard, 1977; Bisgaard *et al*., 1993; Bojesen *et al*., 2003b). *Gallibacterium anatis* has been isolated from chickens, ducks, geese, guinea fowl, turkeys, pheasants, psittacine birds, partridges, web-footed birds and budgerigars (Mushin *et al*., 1980; Bisgaard *et al*., 1993; Bojesen *et al*., 2003b). Especially bv. *haemolytica* has also been isolated from healthy (Harry, 1962; Bisgaard, 1977; Mushin *et al*., 1980; Bojesen *et al*., 2003b) as well as sick birds, cattle and pigs (Gerlach, 1977; Bisgaard, 1982, 1993; Christensen *et al*., 2003; Jordan *et al*., 2005; Bisgaard *et al*., 2009).

2.4 Cultural and Biochemical Characterization

The family of Pasteurellaceae comprises large group of facultatively anaerobic, gram-negative, non-sporing, chemo-organotrophic and fermentative bacteria including the genus *Gallibacterium*. All *Gallibacterium* species strains are non-motile have rod-shaped or pleomorphic cells occurring singly or in pairs. Colonies on bovine blood agar are strongly β-

haemolytic, smooth, greyish, non-transparent, shiny, circular and raised with an entire margins, 1-2 mm diameter after 24-48 h at 37 °C, glowing at the periphery having appearance of butyrous consistency (Bisgaard, 1982).

All strains are catalase, oxidase, and phosphatase positive, and reduce nitrate (Christensen *et al*., 2003). *Gallibacterium* genus can be differentiated from other genera of Pasteurellaceae with catalase, symbiotic growth, haemolysis, urease, indole, acid production from (+) D-xylose, (-) D-mannitol, (-) D-sorbitol, (+) D-mannose, maltose, raffinose, dextrin, ONPG and PNPG tests (Table 1) (Christensen *et al*., 2003).

Table 1. Phenotypic characterisation of *Gallibacterium* species by sugar fermentation

Characteristics	***G. anatis* bv.: haemolytica**	***G. anatis* bv.: anatis**	***Gallibacterium* genomospecies 1**	***Gallibacterium* genomospecies 2**
β-Haemolysis	+	-	+	+
Production of acid from:				
(-) D- Arabinose	(+)	-	(+)	D
(+) L- Arabinose	-	-	D	D
Mannitol	+		D	D
m-Inositol	D	D	-	D
(-) D- Sorbitol	D	D	-	-
(-) L- Fucose	(+)	-	+/(+)	D
Maltose	D	-	+	+
Trehalose	D	+	+	D
Dextrin	D	-	+/(+)	+

Note: Characters are scored as: +, ≥90% of strains positive within 1–2 days; (+), ≥90% of strains positive within 3–14 days; -, <10% of strains positive within 14 days; D, doubt (+/-) 11–89 % of strains positive. Besides these tests *G. anatis* was found to be positive for sugar D- Mannose, Melibiose, Raffinose, L-Rhamnose, Salicin, Sucrose and D-Xylose (GIDEON, Guide to Medically Important Bacteria).

2.5 Transmission

The common way of spreading infection is through horizontal dissemination. Infection of month old poultry bird is common and appears to be natural (Bisgaard, 1977). Trans-ovarian infection supporting the vertical transmission has been experimentally proved (Matthes and Hanscke, 1977) for *G. anatis*. Isolation of *G. anatis* from the egg yolk and detection of *G. anatis* in a 4-day-old chicken descended from a diseased parent also supported vertical

transmission hypothesis for the infection (Kohlert, 1968; Janetschke and Risk, 1970; Huangfu *et al*., 2012; Shapiro *et al*., 2013). Ascending infections appears to be the most probable route for the infection of reproductive organs (Bojesen *et al*., 2003b; Neubauer *et al*., 2009). Venereal transmission of infections also seems to be feasible as the bacterium has been detected in semen of infected cockerels (Paudel *et al*., 2014b). Under favourable circumstances, the bacterium can invade systemic circulation from its natural habitats in respiratory and reproductive tract (Hacking and Pettit, 1974; Shaw *et al*., 1990; Neubauer *et al*., 2009; Zepeda *et al*., 2010; Paudel *et al*., 2013).

2.6 Virulence factor

2.6.1 RTX toxins are found in many members of Pasteurellaceae and are responsible for the haemolytic and leukotoxic properties in *G. anatis* (Frey and Kuhnert, 2002). *Gallibacterium anatis* bv. haemolytica produces haemolysin like GtxA which is a type of RTX-toxin (Kristensen *et al*., 2010). GtxA induces a strong leukotoxic effect on avian macrophages and is labelled as a most important virulence factor of *G. anatis* (Kristense *et al*., 2010, 2011). GtxA toxin has two domains: C-terminal with homology to other RTX toxin and N-terminal with no homology. C-terminal is responsible for haemolytic function but function of N-terminal is unknown, however, this domain is required for complete haemolytic activity (Kristensen *et al*., 2010). The four operon genes are responsible for RTX toxin and are transcribed in a order *i.e.*, *rtx*C, *rtx*A, *rtx*B, and *rtx*D. These toxins target the actin cytoskeleton of the host cells as their immune evasion strategy (Aktories *et al*., 2011).

2.6.2 *Gallibacterium anatis* have capability to adhere on the epithelial cells of chicken and other host cells (Klemm and Schembri, 2000; Lucio *et al*., 2012) by short fimbriae (Vaca *et al*., 2011). A number of fimbriae of different sizes and shapes have been defined belonging to the F17-like fimbriae and are grouped in 1-3 different fimbrial cluster (Kudirkiene *et al*., 2014). The *flfG* gene cluster is responsible for adhesion protein of F17 family (Bager *et al*., 2013a). One of the detected fimbriae seems to be type IV-like pili having bundle structure formed by thin filament like other pathogenic microorganisms evincing type IV pili (Craig *et al*., 2004). Type IV fimbriae are

appendages participating in intercellular motility, microcolony formation, colonization, and the secretion of proteases by host tissues (Craig and Li, 2008).

2.6.3 *Gallibacterium anatis* produces outer membrane vesicles (OMVs) *in vitro* (Bager *et al*., 2013b) similar to all other gram-negative bacteria. Beside, periplasmic components, components of cytoplasmic origin such as DNA have also been found as components contents in OMVs (Mashburn-Warren and Whiteley, 2006; Kulp and Kuehn, 2010; MacDonald and Kuehn, 2012).

2.6.4 *Gallibacterium* have capsule in some strain which may contain virulence properties as seen in *Pasteurella multocida* (Boyce and Adler, 2000). Capsule is a general structure made up of extracellular polysaccharide and has been reported both in gram-negative and gram-positive pathogens (Willis and Whitfield, 2013). The presence of a thin capsule on *G. anatis* has been observed with transmission electron microscopy (Bojesen *et al*., 2011). The presence of a capsule in primary culture and disappearance after sub-culture is common finding (Kjos-Hansen, 1950).

2.6.5 *Gallibacterium anatis* metalloproteases may be having role in infection process (Garcýa-Go mezl *et al*., 2005), are extracellular Zn containing enzymes (Hase and Finkelstein, 1993; Miyoshi and Shinoda, 2000; Garcýa-Go mez1 *et al*., 2005). However, their exact role is still to be understood.

2.6.6 Ability of a bacterium to form biofilm indicates its ability of adherence to surfaces and live tissues and plays role in pathogenesis of persistent and chronic infections with increased resistance to antimicrobials (Costerton *et al*., 1999; Donlan and Costerton, 2002). Biofilm forming ability of *G. anatis* may be weak, moderate and strong with conflicting reports (Johnson *et al*., 2013).

2.6.7 Some strains of *G. anatis* agglutinates avian erythrocytes (Ramirez *et al*., 2012; Zepeda *et al*., 2009). The presence of a potential haemagglutinin in OMVs released from *G. anatis* has been observed (Bager *et al*., 2013b; Johnson *et al*., 2013). Some of these haemagglutinins could be responsible for the observed agglutinating activity of some strains (Bager *et al*., 2013b; Bager *et al.*, 2014; Johnson *et al*., 2013).

2.6.8 Other potential factors involved in virulence:

2.6.8.1 Clustered regularly interspaced short palindromic repeats (CRISPRs) act as a bacterial defence system against foreign invasive DNA, such as DNA from phages and plasmids. CRISPRs have been shown to interfere with transformation, which could explain the difference in natural competence seen between strains of *G. anatis* (Horvath and Barrangou, 2010; Kristensen *et al*., 2012).

2.6.8.2 Integrative conjugative elements (ICE) are able to excise and integrate in the genome by genes encoded within these elements (Wozniak *et al*., 2009). ICEs have been identified in the genomes of *G. anatis* (Johnson *et al*., 2013). Antimicrobial resistance is widespread among isolates of *G. anatis* and might be associated with ICEs (Bojesen *et al*., 2011). The presence of mobile elements adjacent to genes encoding fimbrial clusters (flf) have also been identified (Kudirkiene *et al*., 2014).

2.6.8.3 Studies have described the presence of small colony variants (SCVs), especially observed in primary cultures of *Gallibacterium* (Harbourne, 1962; Janetschke and Risk, 1970; Greenham and Hill, 1962) showing differences in haemolytic activity (Greenham and Hill, 1962). The SCVs are reported to be associated with increased persistence, recurrent infections and increased resistance towards antimicrobials (Proctor *et al*., 2006).

2.7 Pathogenesis

Repeated isolation of *G. anatis* from the trachea and cloaca of healthy birds indicates its commensal status in the upper respiratory tract and lower genital tract of healthy chickens (Kjos-Hansen, 1950; Harry, 1962; Hacking and Pettit, 1974; Bisgaard, 1977; Mushin *et al*., 1980; Bojesen *et al*., 2003b). However, isolation of *G. anatis* in association with a wide range of different pathological lesions, including septicaemia, pericarditis, hepatitis, oophoritis, follicle degeneration, enteritis, upper respiratory tract lesions, salpingitis and peritonitis revealed its importance as an opportunistic pathogen (Greenham and Hill, 1962; Harbourne, 1962; Gilchrist, 1963; Kohlert, 1968; Janetschke and Risk, 1970; Pettit, 1974; Bisgaard, 1977; Hacking and Gerlach, 1977; Addo *et al*., 1985; Majid *et al*., 1986; Shaw *et al*., 1990; Mirle

et al., 1991; Suzuki *et al*., 1996). Recent investigations confirmed that *G. anatis* colonizes the upper respiratory tract without causing clinical signs, whereas it may cause severe lesions in the reproductive tract (Paudel *et al*., 2013). Studies established *G. anatis* as the most common single bacterial infection in chickens causing reproductive tract disorders (Mirle *et al*., 1991). Simultaneous infection with other microorganisms (Gilchrist, 1963; Matthes *et al*., 1969; Shaw *et al*., 1990), hormonal influences (Kohlert, 1968; Gerlach, 1977), age (Janetschke and Risk, 1970; Bisgaard, 1977), seasonal changes (Mirle *et al*., 1991), stress (Rzewuska *et al*., 2007), cold stress (Matthes and Loliger, 1976), and compromised immunological status (Bojesen *et al*., 2004) are a few predisposing factors nurturing the infection of *G. anatis*.

In experimental infections semen quality has been found to be reduced significantly due to decrease in sperm density, total motility with progressive motility and membrane integrity (Paudel *et al*., 2014b).

2.8 Diseases associated with *G. anatis* infection

Due to vast range of pathological manifestations of *G. anatis* infection it is difficult to decide the exact disease conditions caused by *G. anatis*. In diseased birds mortality might take place mainly due to salpingitis, oophoritis and peritonitis. Respiratory tract infections might be responsible for major economic losses due to the rise in treatment cost and losses due to higher condemnation rates and mortality. *Gallibacterium* may be causing primary or secondary infections leading to fatal bacteremia and septicaemia. The severity of clinical signs, duration of the disease and mortality rate are variable and influenced by environmental factors, such as poor hygiene, inadequate management ventilation, ammonia levels in poultry premises and concurrent diseases. Study on pathogen-specific genes of *Gallibacterium* populations (Johnson *et al*., 2013) suggested the ability of the pathogen to cause lesions in reproductive organs such as folliculitis, ruptured and haemorrhagic follicles as well as a drop in egg production in adult hens (Hacking and Pettit, 1974; Neubauer *et al.*, 2009; Jones *et al*., 2013; Paudel *et al*., 2014a).

Mirle *et al*. (1991) examined 496 hens with reproductive tract lesions and isolated *Gallibacterium* in pure culture from 23% of the diseased organs. Haemolytic *G. anatis* was

associated with infection in birds kept in intensive husbandry systems and suffering from reproductive disorders (Neubauer *et al*., 2009).

2.9 Clinical signs and lesions

Normally the finding and identification of signs and symptom of diseases caused by *Gallibacterium anatis* infection in chicken are not pathognomonic leading to creation of mumbo and jumbo condition between the different similar symptomatic diseases like Newcastle, fowl cholera and bird flu. Even though haemolytic isolates are primary disease causing bv. non-haemolytic strains might be associated with chronic cases of localized or generalized purulent peritonitis with *E. coli*. *Gallibacterium anatis* have also been found to cause acute septicaemia.

2.10 Diagnosis of *Gallibacterium* infection

Gallibacterium anatis infection can be confirmed only through agent isolation characterised by phenotypic and genotypic methods. The difference between the genomospecies 1 and 2 is possible only through genotypic methods due to the phenotypic heterogeneity among strains (Christensen *et al*., 2003). Presently the best way to identify the *Gallibacterium* is through phenotypic (Table 1) characterisation (Christensen *et al*., 2003) or by specific probe, GAN850 (Bojesen *et al*., 2003b). A number of genotypic methods have been established for identification of *Gallibacterium* (Bojesen *et al*., 2003a, 2007). The specificity of these methods, however, remains to be investigated (Bisgaard *et al*., 2009).

Specific ITS-PCR able to amplifies selectively *Gallibacterium* DNA and generating short fragments compared to other members of Pasteurellaceae (Leys *et al*., 1994; Fussing *et al*., 1998; Gu *et al*., 1998; Christensen *et al*., 2003b).

Gallibacterium has a relatively short internal transcribed 16S to 23S rRNA gene sequences compared to other members of Pasteurellaceae, based on the information the *Gallibacterium* specific PCR (Bojesen *et al*., 2007) targeting on 16s rRNA sequence are designed (Benson *et al*., 2004). The primer 1133fgal (5'-TATTCTTTGTTACCARCGG-3') and 114r (5'-GGTTTCCCCATTCGG-3') are often the chosen ones with specificity (Lane, 1991). Besides, AFLP typing method is found to be useful for distinguishing between

closely related *Gallibacterium anatis* clones, thus enabling recognition of specific pathogenic clonal lineages (Bojesen *et al*., 2003a).

Rapid and specific identification of individual bacterial cells can be achieved by the fluorescent *in situ* hybridization technique (FISH) based on fluorescent labelled oligonucleotides complementary to bacterial 16S rRNA. This method has advantages over the traditional culture based methods due to its ability to identify live/ intact cells as well as non-cultivable organisms (Bojesen *et al*., 2003a).

2.11 Prevention and control

A pan-genomic reverse vaccinology (RV) approach has been applied to identify novel and potentially broadly protective immunogens from *G. anatis* (Rappuoli, 2001; Medini, 2005; Bager *et al*., 2014). However, there is no potential vaccine in the market to control *G. anatis* infections. Only antimicrobial chemotherapy is the available method for containment of the disease caused by *G. anatis*. Besides, general hygienic measures can be taken in the way similar to control of other contagious diseases in poultry farms.

2.12 H_2O Vs D_2O- Physicochemical characteristics

Deuterium (^2H or D), an stable isotope of hydrogen, containing one neutron and one proton, was first separated by Urey *et al*. (1932). The O-H bonds are 0.5% longer than O-D bonds, marking the important difference between the molecular structures of ordinary and heavy water. Heavy water (D_2O) has completely the same chemical characteristics as H_2O, but it is quite different at physical front having higher melting point (3.82 °C), boiling point (101.72 °C), density (1.017 g/cm^3), temperature of maximum density (11.6 °C) and heat of vaporization (10,864 cal/mol). It has been accepted that two independent physiological effects occur when a living system is exposed to D_2O:

i) an isotope exchange effect on functional proteins

ii) a solvent isotope effect on ionic conductivity

These differences are responsible for lower mobility of monovalent cations, reduced to <20% (Bass and Moore, 1973; Sugiyama and Yoshioka, 2012). When H is replaced with

D in the biological molecule, the C-D bond is about 10 times stronger than the C-H bond, which means the C-D bond is more resistant than the C-H bond for dissociation conferring the increased stability to biomolecues (Sugiyama and Yoshioka, 2012).

2.13 Effect of D_2O on living cells/ bacteria

Chemical reactions proceed more slowly in deuterated water (Taylor *et al*., 1933) thus affecting all the biological processes which are the outcome of biochemical reactions. Oxygen consumption of luminous bacteria in water containing deuterium oxide slows down which shows the harmful effect of heavy water on living things (Taylor *et al*., 1933; Harvey and Taylor, 1934; Lewis., 1934). Excess of deuterated water causes reduction in synthesis of proteins and nucleic acids, disturbance in cell division, changes in enzymatic kinetic rates and cellular morphological changes (Katz *et al*., 1957; Katz and Crespi, 1971). For bacteria, different responses of deuterated water on growth ranging from no effect to inhibition and even stimulation have been reported (Lester, 1960). In the case of biochemical changes it has been observed that among citrate-positive organisms in the presence of D_2O, the utilization of citrate carbon was delayed and citrate utilizing *K. aerogenes* failed to do so in presence of D_2O (Ramakrishnaiah, 1990). Heavy water increases thermal stability of microorganisms and reduces disintegration of certain macromolecules (Crainic and Simpson, 1994; Kushner *et al*., 1996).

Though not in bacteria, heavy water is known to inhibit cellular processes in mammals at higher concentrations (Thomson, 1960). Consumption of high amounts of heavy water is toxic or even lethal to plants, animals, and humans (Katz and Crespi, 1966). The result of deuteration is cell dysfunction followed by death when > 50% of water is replaced by heavy water in higher organisms (Kushner *et al.*, 1999). A 4-5^oC reduction in storage temperature have been achieved in the presence of 95% D_2O compared to H_2O and 7-25% D_2O prevent protein denaturation in macromolecular pharmaceuticals such as vaccines. D_2O medium has been suggested for storing organs, tissues, tissue parts, proteins or enzymes, sera and blood preparations and for the perfusion of organs for transplants and also for longer storage of microorganisms and enzymes. Though D_2O stabilizes proteins and other macromolecules, it does not always make living cells more heat-stable, and may make them less so (Unno *et al*.,

1989). Making cells less stable may be due to loss of capability of forming heat shock proteins and other chaperons (Unno *et al*., 1987; Unno and Okada, 1991, 1994; Yokogaki *et al*., 1995). Toxic effects begin to appear after taking more than 20% D_2O content of blood, body fluids and in tissues of experimental animals (Thomson, 1963). Protozoa have ability to grow in 70-100% D_2O concentrations (Thomson, 1963). Bacteria showing growth in heavy water include *Escherichia coli, Pseudomonas putrefaciens, Serratia marcescens* (Thomson, 1963; Vanatulu *et al*., 1993) and extremely halophilic archae bacteria (Crespi, 1982). Mammals, such as rats, given heavy water to drink die after a week, at a time when their body water approaches about 50% deuteration (Thomson, 1960). The overall effect of heavy water is due to deuterium so it has been observed that at different concentration of deuterium the Na, K-ATPase activity have found to increase at low deuterium concentrations (Lobyshev *et al*., 1978, 1982). Heavy water is more toxic to malignant cells than normal cells but the concentrations needed are too high for regular use (Kushner *et al*., 1999). Luminous bacteria will grow slowly in 97 percent heavy water, though not grow as good as in ordinary water are not killed by long interaction with heavy water (Steinbach, 1949). These data suggest that adaptation to deuterium oxide is a multifactorial phenomenon, affecting many cellular systems, as biosynthesis of macromolecules, metabolism, and transport functions of cells (Mosin, and Ignatov, 2014).

2.14 Effect of D_2O on growth pattern/ kinetics of bacteria

After a period of adaptation ("lag phase"), growth resumes, but the rate is usually slower than in normal isotopic environment (Ernest and Rittenberg, 1960). Here the effects are coming mainly due to deuterium molecule which is responsible for changing in the physical properties of heavy water so outcome of deuterium enrichment may be the correct way to explain the outcome of heavy water. Indeed, literature suggests that the size and shape of the bacteria may get distorted when grown in a highly deuterated media, especially in the beginning of growth (Katz *et al*., 1957; Katz and Crespi, 1966, 1971). Lobyshev *et al*. (1978, 1982) studied the Na, K-ATPase activity at different concentration of deuterium and found it to increase at low deuterium concentrations, reaching maximum at 0.04-0.05%.

Prokaryotic organisms such as bacteria, which do not have the mitotic problems induced by deuterium, may be grown and propagated in fully deuterated conditions, resulting

in replacement of all hydrogen atoms in the bacterial proteins and DNA with the deuterium isotope (Trotsenko *et al.*, 1996).

Even at 50% D_2O, the maximum growth rate is only slightly (5%) lower than at normal conditions. Therefore, deuterium enriched medium is more correctly characterized as an unusual, rather than toxic, environment (Xie and Zubarev, 2014).

2.15 Study of drug resistance

Emergence of antimicrobial resistance has been observed among several organisms belonging to the Pasteurellaceae family (Aarestrup *et al*., 2004; Aarestrup, 2005) including *G. anatis* isolates. Resistance in chicken origin isolates of *Gallibacterium* is documented to novobiocin, tylosin, clindamycin, tetracycline antimicrobials and penicillin (Post *et al*., 1991; Watts *et al*., 1994; Mevius and Hartman, 2000; Lin *et al*., 2001; Malik *et al*., 2005; Hendriksen *et al*., 2008; Johnson *et al*., 2011). In contrast, Berge *et al*. (2006) reported rarity of tetracycline resistance in *G. anatis* of sheep and goat origin.

Materials and Methods

3 MATERIALS AND METHODS

The study was conducted in the Laboratory of Epidemiology Section, Indian Veterinary Research Institute (IVRI), Izatnagar-243122.

3.1. General experimental materials

3.1.1. Glasswares and Plasticwares

During the study, sterilized glasswares (Corning/Borosil grade) and plasticwares procured from Tarsons (India), Axygen (USA), Hi-media (Mumbai, India) and Nunc (Denmark) attuned with molecular biology work were used.

3.1.2. Media, Reference Stains, Chemicals, Buffers, Reagents etc.

The details of media, reference stains, chemicals, buffers and molecular biological reagents used in the study are given in the Appendix. General chemicals used were of analytical grade, procured from Sigma, USA or Merck, India. The media used in study were procured from Himedia, Mumbai, India and Difco (France). The Dream Taq Green, PCR MASTER MIX (2X) used from Thermo Scientific, Lithuania (Europe). The PCR primers were custom synthesized from Eurofin Pvt. Ltd. Bangaluru, India. All media for molecular biology and serological work were prepared in triple distilled water and for other works in single glass distilled water was used.

3.1.3. Equipments

Equipments used in the study were Autoclave (Scientific Equipments Works, New Delhi, India), Biomedical Refrigerator (BPL), Bio-safety cabinet (Kartos International, Noida, India), Cooling centrifuge (REMI Instruments Ltd., Mumbai, India), Deep freeze -20°C

(Vestfrost and Blue Star), Electronic balance (Mettler Toledo, Switzerland and Citizen), Gel documentation System (Bio-Rad, USA), SDS-PAGE Assembly (Bio-Rad and Tarson), Incubator (Khera Instruments Pvt., Ltd., New Delhi, India), Shaker Incubator (BIO-RED), Micro pipettes of variable volumes (Finnpipette), Water bath (M. B. Instrument, India), PCR thermal cycler (Eppendorf, Germany), U. V. IIluminator (Genei, Banglore, India), R8C Laboratory Centrifuge (REMI Instruments Ltd., Mumbai, India), Hot Air Oven (Scientific Equipments Works, New Delhi, India), Spectrophotometer (Laby, India) and Nano drop (Thermo fisher scientific, USA).

3.1.4. The Study strains

Gallibacterium anatis bv. haemolytica strain was the study strain in the present dissertation isolated from heart blood of dead poultry birds in the laboratory of Epidemiology, IVRI, IZATNAGAR, U.P., India, which was confirmed by morphological, staining, growth, biochemical characteristics and finally with amplification of specific PCR product using species specific primer.

3.1.4.1. Revival of strain of *Gallibacterium anatis* bv. haemolytica

To revive, the culture of *Gallibacterium anatis* was aseptically opened in laminar flow (Kartos international, Noida) in-front of flame and contents from glycerol broth were transferred to trypticase soya broth and thioglycollate media simultaneously and incubated overnight at 37°C. Loopful of broth culture was streaked on to blood agar (Blood agar base, Difco, USA with 5% defibrinated sheep blood) plates and incubated at 37°C for overnight. Isolated colonies typically greyish, non-transparent, smooth and shiny, circular, raised with an entire margin were picked to determine the identity of the culture using morphological, cultural and biochemical characteristics (Holt *et al*., 1994). The true and confirmed colonies were grown on blood agar plates and maintained at 4°C during the study for preparation of antigens used in serology and for different experiments.

3.2. METHODS

The samples collected from dead poultry were processed in two ways for identification of *Gallibacterium anatis*:

3.2.1. Identification of *Gallibacterium anatis* through conventional bacteriological method

The suspected colonies (small, pin point, greyish, glowing colonies on BA) were tested for catalase, oxidase and 10% KOH test. The colonies which were positive for catalase, oxidase and formed string in 10% KOH were re-streaked on Blood agar plate (Difco, France) and incubated for 24 h at 37°C. To further characterize the isolates, pure cultures were tested for biochemicals like citrate utilization, nitrate, reaction on triple sugar iron (TSI) slants, hydrolysis of tween-20, motility, indole production, lysine decarboxylation, MR-VP reaction and urease production. The colonies which failed to utilize citrate as sole source of carbon, reduced nitrate to nitrite, not decaroxylated lysine, not degraded urea and acidic triple sugar iron (TSI) agar slants, not hydrolysed tween-20, not produced indole and negative in MR-VP were identified as *G. anatis* bv. haemolytica. All the suspected isolates were further confirmed using species specific polymerase chain reaction (PCR) as described elsewhere. The isolated cultures were tested for *in vitro* antimicrobial susceptibility against several antimicrobial agents by the Disc diffusion method (CLSI, 2015) on Muller-Hinton (MHA) Agar (Himedia, Mumbai) plates.

The isolated culture was used as parent strain. The biochemical tests employed in identification of *G. anatis* as prescribed for gram negative organisms in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) were performed. The following tests were performed to characterize the culture:

Catalase test: This test was performed by taking 2-3 drops of 3% H_2O_2 on clean grease-free glass slide and single colony from BA plate was mixed with the help of a platinum wire loop. Immediate formation of gas bubbles was considered as positive test.

Oxidase test: Oxidase discs soaked in 1% NNN'N'–tetramethyl-p-phenylenediamine dihydrochoride were used to perform the test. The loopful culture from single colony was just touched on the disc using platinum loop. Development of purple colour within 15 s. was considered as positive test.

10% KOH test: This test was performed by taking a drop of 10% KOH on clean grease-free glass slide and mixing of bacterial colony from BA plate with the help of a wire

loop. The test is positive for gram negative bacteria, and the bacterial colony tested forms string on lifting of the culture mixed in KOH drop.

Nitrate reduction: A loopful of culture were added in peptone water containing 0.1 per cent potassium nitrate and then incubated at 37°C for 2 days. Presence of nitrite was detected by adding approximately 1.0 ml of sulfanilic acid and 1.0 ml of α-naphthylamine reagent to nitrate broth culture. Development of a distinct red colour (which may turn to brown rapidly) was considered as positive test. In case of negative test, a pinch of zinc dust was added into tube to confirm presence of nitrate or reduction of nitrite, development of red colour after adding zinc indicated no reduction of nitrate by the bacteria.

Urease test: Urea broth heavily inoculated with the test culture were incubated at 37°C and observed for 24 to 48 h. A negative reaction was observed with no colour change in the broth.

Triple sugar iron (TSI) agar: A single colony was inoculated on to TSI medium slant with wire loop which is first inserted in butt and then on to slant, incubated for 24 h at 37°C. Oxidative decarboxylation of peptone and fermentation of glucose and sucrose in the medium by *Gallibacterium* caused acidic reaction in the medium leading to development of yellow colour in butt and slant both.

Motility indole lysine media: MIL medium (Himedia Laboratories Ltd., Mumbai) was used for detection of motility, indole production, lysine decarboxylation and H_2S production. The loopful culture from single colony was stabbed into the tube and incubated at 37ºC. Non-motile organisms revealed growth along stabbed line while motile revealed diffused growth. Hydrogen sulphide production was indicated by blackening of the medium. All tests were negative for *Gallibacterium anatis.*

Methyl red (MR) and Voges Proskauer (VP) test: A loopful of culture was inoculated into a test tube containing 5ml MR-VP broth and incubated at 37°C for 48 h. First MR test was conducted through adding 1-2 drops of MR reagent, development of red colour indicates positive test. For VP test, in the same tube, 0.3 ml of 5% alpha naphthol in absolute ethyl alcohol and then 0.1 ml of 40% (w/v) KOH was added. A positive reaction was noticed

by development of bright red colour after 15 min and colour remaining yellow indicated negative reaction. Both the tests were negative for *Gallibacterium anatis*.

Tween-20 hydrolysis: Three ml of Tween 20 medium was heavily inoculated with test culture and incubated for 48 h, positive reaction is indicated by development of red colour. The isolated culture was found to be negative (no colour change).

Citrate utilization test: Simmon's citrate agar determines the ability of organism to metabolize citrate for energy. Simmon's citrate slant was inoculated with standard culture and incubated for 24 h. The citrate was not turned into blue colour (bromothymol blue as indicator), indicated alkaline byproducts shown the negative for *Gallibacterium anatis*.

Gelatinase test: A heavy inoculum from standard culture was stabbed into the gelatin media and incubated for 48 h to 2 week at 37°C. After incubation in refrigerator for approximately 30 min, the gelatin was not liquefied. It showed that *Gallibacterium anatis* did not produce gelatinase and found to be negative.

3.2.2. Identification of *Gallibacterium anatis* through molecular method: Genus and species specific PCR

3.2.2.1. Isolation of genomic DNA by Kit Method (QIAamp) from bacterial suspension cultures

The genomic DNA of isolated culture was extracted by QIAamp DNA Mini Kit (Qiagen India Pvt. Ltd., New Delhi, India).

1. The culture was prepared by inoculating the isolate in Luria-Bertani broth (LB-Broth; Hi-media, Mumbai) and incubating at 37°C for 24 h.
2. 1 ml bacterial culture was pipetted into a 1.5 ml microcentrifuge tube, and centrifuged for 5 min at 7500 rpm.
3. Buffer ATL (supplied in the QIAamp DNA Mini Kit) was added to a total volume of 180 µl.
4. 20 µl proteinase K was added, mix by vortexing and incubated at 56°C until the cells were completely lysed. Sample was dispersed by vortex during incubation in a shaking water bath.

5. 1.5 ml microcentrifuge was centrifuged briefly to remove drops from the inside of the lid.
6. 200 µl Buffer AL was added to the sample, by mixing with pulse-vortexing for 15 s, and incubate at 70°C for 10 min.
7. 200 µl ethanol (96–100%) was added to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
8. Mixture was carefully applied to Step 7 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Cap was closed and centrifuged at 8000 rpm for 1 min. QIAamp Mini spin column was placed in a clean 2 ml collection tube, and discared the tube contained the filtrate.
9. QIAamp Mini spin column was opened carefully and added 500 µl Buffer AW1 without wetting the rim. Cap was closed and centrifuged at 8000 rpm for 1 min. QIAamp Mini spin column was placed in a clean 2 ml collection tube, and discared the collection tube contained the filtrate.
10. QIAamp Mini spin column was opened carefully and added 500 µl Buffer AW2 without wetting the rim. Cap was Closed and centrifuged at full speed (14,000 rpm) for 3 min.
11. Centrifuged at full speed for 1 min additionally to eliminate the chance of possible Buffer AW2 carryover.
12. QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and discared the collection tube contained the filtrate. QIAamp Mini spin column was opened carefully and added 200 µl Buffer AE and Incubated at room temperature for 1 min, and then centrifuged at 8000 rpm for 1 min.
13. Bacterial genomic DNA was stored at -20°C.

3.2.2.2. Quantification of DNA

The concentration and purity of the DNA extracted from the overnight culture in LB broth were measured by Nano-drop after initialization with distilled water and wiping it with a tissue paper and DNA concentration with the corresponding purity was determined with help

of the attached computer. DNA from each sample was measured after cleaning the optical lens with tissue paper at each reading. DNA samples with sufficient amount of concentration and purity indicated by 260/280 reading in 1.6 to 1.8 ranges with Nano-drop were used as template for PCR reaction.

3.2.2.3. Standardization of PCR for *Gallibacterium anatis*

The genomic DNA and snap chilled supernatant used as template DNA for standardization of PCR for *G. anatis*. The identification of *G. anatis* carried out by using universal primer as control and genus specific primers in PCR and duplex PCR in thermal cycler (Eppendorf, Germany), (Bojesen *et al*., 2007).

Primers: Two sets of specific primers were designed from available universal primer to amplify the 161-bp fragment of all prokaryotic 16S rRNA sequence (Table 2) while *Gallibacterium anatis* genus specific primers were designed from 16S rRNA gene and 23S rRNA gene (Table 3). All the primers were custom synthesized from Eurofin Pvt. Ltd. Bangaluru, India. Standared Quantity, concentration and thermal cycler condition were used (Table 4 & 5).

Table 2: Primer used as control by amplify the all prokaryotic 16S rRNA sequences

Name of primers	Sequence 5'-3'	Product length (bp)	References
161UF	CCTACGGGAGGCAGCAG (17)	161-bp	Bojesen *et al*., 2007
161UR	ATTACCGCGGCTGCTGG (17)		

Table 3: Genus specific Primer based on 16S rRNA and 23S rRNA genes of *Gallibacterium anatis*

Name of primers	Sequence 5'-3'	Product length (bp)	References
1133Fgal	TATTCTTTGTTACCARCGG (19)	789 bp,	Bojesen *et al*., 2007
114R	GGTTTCCCCATTCGG (15)	985bp, 1032 bp	

Note: (F) = Forward primer; (R) = Reverse primer

Table 4: Cycling parameters for PCR

Initial denaturation	Denaturation	Annealing	Extension	Final extension	Total no. of cycles
95°C 10 min	94 °C 1.00 min	55 °C 1..00 min	72 °C 1.00 min	72 °C 10 min	35

*For uniplex (single amplicon) PCR reaction was carried out in 25 µl

Table 5: Quantity and concentration of various components used for uniplex and multiplex PCR

S1. No	Component	Vol. Per reaction	Final Conc.
1	PCR Master Mix (2X)	12.5 µl	1X
2	Forward Primer (10 pmol/ µl	1 µl	10 pmole/ µl
3	Reverse Primer (10 pmol/ µl	1 µl	10 pmole/ µl
4	Template DNA	4 µl	-
5	Nuclease Free Water (NFW)	6.5 µl	-
6	**Total Volume**	**25 µl**	

3.2.2.4. Agarose gel electrophoresis

The following reagents were used for agarose gel electrophoresis

A. Agarose

B. Tris Borate EDTA (TBE) buffer, pH 8.3 (10X)

C. Ethidium bromide (1%)

To confirm the targeted PCR amplification, 10 µl of PCR product from each tube was loaded in well of prepared 1.5 per cent agarose gel along with 100bp plus DNA Ladder (Gene Ruler 100 bp plus, Thermo Scientific) and stained with ethidium bromide (1 per cent solution at the rate of 5 µl/100 ml) at constant 80 V for 30 min in 1X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

The PCR was performed three times to ensure the repeatability of the technique and to make sure that isolates were correctly assigned to respective patterns.

3.3. Plasmid isolation

The standard culture and field isolate were subjected for plasmid isolation via Thermo Scientific (Gene JET Plasmid Miniprep Kit, Thermo scientific). The cultures were inoculated in 3 ml tryptic soya broth (TSB), incubated overnight at 37°C.

Plasmid DNA was purified by using centrifuges:

1) The 2 ml of bacterial culture was centrifuged at 8,000 rpm for 2 min at room temperature.
2) After centrifugation, Pelleted cells were resuspended in 250 µl of the resuspension solution and transfer it to a microcentrifuge tube with proper vortexing or pipetting up and down until no cell clumps remain.
3) Thereafter, add 250 µl of Lysis Solution and mix thoroughly by inverting the tube 4 to 6 times until the solution becomes viscous and slightly clear.
4) Then 350 µl of the Neutralization Solution was added and mixed immediately by inverting the tube 4 to 6 times.
5) The mixture was centrifuged at 13,000 rpm for 5 min to pellet cell debris and chromosomal DNA.
6) The supernatant was transferred to the Gene JET spin column without disturbing or transferring the white precipitate.
7) The supernatant in the Gene JET spin column was centrifuged at 13,000 rpm for 1 min.
8) Then 500 µl of the Wash Solution was added to Gene JET spin column and centrifuged at 13,000 rpm for 30-60 seconds. After that discard the flow-through and spin column was placed back into the same collection tube.
9) Above procedure was repeated again using 500 µl of the Wash Solution.
10) Additional centrifugation at 13,000 rpm for 1 min, was required to remove residual Wash Solution and ethanol in plasmid preps.
11) Gene JET spin column was transfered into a fresh 1.5 ml microcentrifuge tube and add 50 µl of the Elution Buffer to the center of Gene JET spin column to elute the plasmid DNA.

12) After addition of Elution Buffer incubation was recommended at room temperature for 2 min.

13) Then centrifugation at 13,00 rpm for 2 min was performed.

14) The Plasmid DNA eluded with Elution Buffer was stored at -20 ^{o}C till further use.

3.4. Adaptation

Adaptation of *Gallibacterium anatis* strain to grow in 99% D_2O instead of normal water was carried out through growing the bacteria in increasing concentrations of D_2O (10%, 20%, 30%, 50%, 75%, 90% and 99%) and measuring optical density (OD) at 590 nm wavelength to monitor the growth in broth medium. For the adaptation, fresh culture growing in 99% D_2O medium was inoculated in TSB made in D_2O incubated at 37^{o}C for 24h and from the same tube reinoculated and incubated till the 51 passage as under (Table 6).

Table 6: Two loop full cultures were transferred from one step to next step of adaptation as under mention

	Concentration of D_2O in Percentage	**H_2O Combination**	**D_2O Combination**
1	10% D_2O	$H_2O \rightarrow H_2O$	$D_2O \rightarrow D_2O$
2	20% D_2O	$H_2O \rightarrow H_2O$ $D_2O \rightarrow H_2O$	$D_2O \rightarrow D_2O$
3	30% D_2O	$H_2O \rightarrow D_2O$ $D_2O \rightarrow H_2O$	$H_2O \rightarrow D_2O$ $D_2O \rightarrow H_2O$
4	50% D_2O	$H_2O \rightarrow H_2O$ $D_2O \rightarrow H_2O$	$D_2O \rightarrow D_2O$ $H_2O \rightarrow D_2O$
6	75% D_2O	$H_2O \rightarrow H_2O$ $D_2O \rightarrow H_2O$	$50\%D_2O \rightarrow 50\%D_2O$ $50\%D_2O \rightarrow 75\%D_2O$ $H_2O \rightarrow 75\%D_2O$
7	90% D_2O	$H_2O \rightarrow H_2O$ $D_2O \rightarrow H_2O$	$75\%D_2O \rightarrow 75\%D_2O$ $75\%D_2O \rightarrow 90\%D_2O$ $H_2O \rightarrow 90\%D_2O$
8	99% D_2O	$H_2O \rightarrow H_2O$ $D_2O \rightarrow H_2O$	$90\%D_2O \rightarrow 90\%D_2O$ $90\%D_2O \rightarrow 99\%D_2O$ $H_2O \rightarrow 99\%D_2O$

After adaptation in 99% D_2O, strain was again subcultured in 99% D_2O to generate 51 passaged D_2O adapted strain of *G. anatis* bv. haemolytica.

3.5. Thermal death Point (Thermal death time)

The thermal death point (TDP) is the temperature at which an organism is killed in 10 min while the thermal death time is a concept used to determine how long it takes to kill a specific bacteria at a specific temperature. Determination of thermo-tolerance of adapted and parent strain of *G. anatis* at 55°C and 60°C and determination of thermal death time using standard colony forming unit (cfu/ml) counting method **(**Miles and Misra, 1938).

3.5.1. Strain used

Parent *Gallibacterium anatis* bv. haemolytica designated as HLY (parent) and adapted strain of *G. anatis* bv. haemolytica in heavy water for 51 passages designated as 51H.

3.5.2. Method

1) Both strains were inoculated in 10 ml Trypticase soya broth (TSB) at 37°C for 24 h separately.
2) Bacterial suspension was centrifuged at 5,000 rpm for 15 min.
3) Supernatant was discarded and washed twice with PBS, made in heavy water and also with equal volume of simple PBS made in triple distilled water.
4) Bacterial pellet was resuspended in PBS of equal volume of initial broth culture and mixed through vortexing for 1 min.
5) Resuspension was divided in 5 (1 ml in each) tubes with equal volume according, one set for each time parameter (Combination).
6) The stand was kept in water bath, set at 55°C or 60°C as per test.
7) Experiment was performed at 55°C and 60°C with different time combination in min like:

3.5.2.1 Time combination of 60°C

a) 0 min, 5 min, 10 min, 15 min, 20 min, 30 min, 40 min, 50 min, 60 min
b) 0 min, 2 min, 4 min, 5 min, 7 min, 10 min, 15 min

c) 0 min, 1 min, 2 min, 3 min, 4 min, 5min

3.5.2.2. Time combination of 55°C

a) 0 min, 5 min, 10 min, 15 min, 20 min, 30 min, 40 min, 50 min, 60 min

b) 0 min, 1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min, 10 min, 11 min, 12 min, 13 min, 14 min, 15 min, 16 min

8) After completion of experiment, stand was kept at 4°C untill cfu/ml count.

9) **Serial Dilution Protocol**

a) Counting of the appropriate plate was determined (30 – 300 cfu/ml).

b) Dilution factor for the dilution tubes was determined and each tube with 10-fold dilution starting from the undiluted culture. The first tube had 1:10 dilution, the second a 1:100, the third a 1:1000 and so on, dilution factor for first, second and a 3rd tube will be 10, 100, 1000 and so on.

c) Number of live bacteria in each tube was determined through plating 10 µl of the contents in triplicate.

10) Then cfu counts per ml were determined by Miles and Misra (1938) method for different time and temperature combinations separately.

Total Count Calculation (standard formula) cfu/ml:

$$\frac{\text{Colony counted (CFUs) on an agar plate} \times 1000 \times \text{Dilution factors}}{\text{Volume plated in µl}}$$

11) Result was observed after incubation at 37°C for 24 h to 48 h.

12) After cfu/mL count thermal death point was determined by graphical method.

Note: In parent strain washing was performed by only simple PBS made in triple distilled water.

3.6. Growth Kinetics and Growth Curve

Understanding the growth kinetics and growth curves for D_2O adapted and parent strain of *G. anatis* bv. haemolytica in presence and in absence of D_2O at 10°C, 25°C, 37°C and 45°C were determined through OD_{580} and cfu/ml count methods.

The experiment for 25°C, 37°C and 45°C were performed in water bath (LABMAN, INDIA) at room temperature and for 10°C in refrigerated incubator (REMI, INDIA).

3.6.1. Method

1) TSB tubes (2 ml in each) were prepared with 0% D_2O and 99% D_2O.
2) Culture was grown in TSB media at 37°C for 18-24 h separately for adapted and parent strain in 10 ml TSB.
3) 100 µl of culture was inoculated in each TSB tube.
4) OD_{580} reading were taken with spectrophotometer up to 48 h at each 2 h interval.
5) Experiment was conducted in triplicate and mean value was calculated at10°C, 25°C, 37°C and 45°C.
6) Cfu/ml counts were determined a described earlier through serial dilutions and plating on trypticase soya agar plates incubated at 37°C for 24-48h.

3.7. Determination of survival of D_2O adapted and parent stain

Determination of survival of D_2O adapted and parent strain of *G. anatis* bv. haemolytica in presence and in absence of D_2O in storage medium at 4°C, 37°C and 45°C was done through cfu/ml count at weekly interval for two months**.**

3.7. 1. Method

1) TSB tubes were prepared in small 2 ml autoclaved plastic culture tubes.
2) Each experimental combination was performed in triplicate.
3) Different combinations of TSB tube were prepared for each strain with different percentage of heavy water in medium (Table 7).

Table 7: Combination chart at different D_2O concentration for each temperature at 4°C, 37°C and 45°C

	D_2O concentration in medium	Adapted strain (51H)	Parent strain (HLY)
1	0%	T1, T2, T3	T1, T2, T3
2	20%	T1, T2, T3	T1, T2, T3
3	75%	T1, T2, T3	T1, T2, T3
4	90%	T1, T2, T3	T1, T2, T3
5	99%	T1, T2, T3	T1, T2, T3

Note: T- tube

4) Culture was grown in TSB media at 37°C for 18-24 h separately for adapted and parent strain in 10 ml glass tube.

5) 100 µl Culture was inoculated in to each 2 ml TSB tube.

6) cfu counting was done as described earlier.

3.8 Method for virulence characterization of *G. anatis* bv. haemolytica

3.8.1. Determination of *Gallibacterium anatis* bv. haemolytica antibodies in Poultry, Pig and Goat serum

3.8.2. Standardization of Micro-agglutination test (MAT)

3.8.3. Preparation of antigen for Micro-agglutination test (MAT) (Talwar and Gupta, 1992)

3.8.4. Strain used in Antigen preparation: Parent *Gallibacterium anatis* bv. haemolytica and adapted strain of *G. anatis* bv. haemolytica to grow in heavy water.

3.8.5. Preparation of O-Antigen (boiled antigen) and formalized antigen (for proteinaceous surface antigens)

3.8.5.1. Preparation of O-antigen

Pure culture was streaked on blood agar plate, incubated at 37 °C for 24 h.

Then 3-5 smooth colonies were picked up and inoculated in 5 ml LB broth and incubated for 16-24 h at 37 °C in shaker incubator at 160 rpm.

1) Broth culture of *G. anatis* was inoculated on to trypticase soy agar (Becton Dickson and company, sparks USA) made in Roux flasks and incubated for 24-48 h at 37°C.

2) Harvesting: Growth was collected from two Roux flasks in total volume of 25 ml of normal saline solution.

3) Purity: Harvesting culture was streaked on blood agar plate and incubated at 37 °C for 24- 48 h. If pure growth was observed on blood agar plate with haemolysis then further processing was done otherwise fresh harvests were made.

4) Boiling: Bacterial suspension was placed in boiling water bath by fixing at 80 °C for two and a half hours.

5) Sterility: Sterility of bacterial suspension was confirmed by streaking 0.1 ml on blood agar plate and observed for growth for 48h at 37 °C.

6) Centrifugation: Bacterial suspension was centrifuged at 1400×g for 30 min and supernatant was discarded.

7) Resuspension: The pellet was resuspended in 0.5% formalinized saline (5ml).

8) Concentration of bacterial suspension was adjusted according to the desired value by using a spectrophotometer. The stock antigen was diluted in 0.5% formal saline to have an OD_{620} of 0.5.

9) Stock antigen was store at 4 °C in refrigerator.

3.8.6. Preparation of Formalinized Bacterial Suspension

Pure culture was streaked on blood agar plate, incubated at 37°C for 24 h and smooth colonies were inoculated in 5 ml LB broth for 16-24h growth at 37°C in shaker incubator at 160 rpm.

1) One ml LB broth culture of *G. anatis* was inoculated in trypticase soy broth (500 ml) (Becton Dickson and company, Sparks USA) in two conical flasks (each have 500 ml TSB) and incubated for 16-24 h at 37°C on orbital shaker incubator with speed of 160 rpm.

2) Purity: After visible growth culture was streaked on blood agar plate and incubated at 37°C for 24-48 h. If pure and typical to *G. anatis* bv. haemolytica growth was observed on blood agar plate with haemolysis then processed further.

3) 1% formalinized saline solution was added in to each flask and kept at room temperature for three days.

4) Sterility: Sterility of bacterial suspension was checked after three days by streaking on blood agar plate as for O antigen.

5) Rest of the stpes were same as for the 'O' antigen described earlier

3.8.7. MAT Procedure

The Micro-agglutination test on poultry, pig and goat sera was performed in V-bottom (for formalinized antigen) and U-bottom (for O antigen) 96-well microtitre plates (Tarson, India), using 100 µl serum, serially 2-fold diluted in 100 µl NSS, pH 7.01 (final dilution 1: 2). The last row in each plate was used as negative control. To each well, 100 µl of *G. anatis* bv. haemolytica antigen was added. Micro-titre plates were incubated in a humid chamber to prevent drying of the plates and incubated for 24-48 h at 37°C. Plates were read against a dark back-ground after 48h (Boot *et al*., 1993). The highest dilution up to which MAT formation was there, considered as positive titre.

3.8.8. Preparation of 3% (v/v) RBCs

Blood from young cow, ox, sheep, goat, horse, pig, dog, guinea pigs, rat, fish, turkey, and healthy humans (blood groups AB^+ and B^+) was collected in Alsever's solution (pH-6.1), centrifuged at 2000 × g for 10 min to collect erythrocytes (RBCs). Erythrocytes were washed twice with phosphate buffered saline (PBS, pH 7.2) and finally re-suspended in PBS (3 % v/v) and stored at 4°C until used. To obtain tanned erythrocytes, one volume of 3 % (v/v) RBCs was treated with equal volume of 0.003 % (w/v) tannic acid for 10 min at 37°C, then RBCs were collected as above and washed twice before suspending to 3 % (v/v) in PBS for use (Singh and Sharma, 2000).

3.8.9. Haemagglutination (HA) test

3.8.9.1. Preparation of bacterial suspension

1) For preparation of bacterial suspension, culture was grown in BHI agar (Brain heart infusion agar) supplemented with with 10% sheep blood and TSB (Trypticase soya broth).

2) Culture grown on BHI agar was kept for 48 h at 37°C was harvested in normal saline.

3) TSB culture as well as BHI agar harvested cultures were centrifuged at 5000 × g for 10 min, separately.

4) The supernatant was discarded and the bacterial cell pellets were suspended in PBS (1×) to a final concentration of about 1×10^{11} cfu (colony forming units)/ ml i.e., working solution of bacterial suspension for HA had OD_{620} equal to 0.5.

3.8.9.2. Method

A 100 µl of aliquot of the bacterial suspension was mixed with equal volume of the 3% RBCs with and without 1 % D-mannose. The second last and last row of the microtitre plate were kept as bacterial suspension control and RBC control which contained 100 µl of PBS (1×) and 100 µl of 3% RBCs, respectively. Agglutination plates were gently shaken for 20-30 min on a shaking platform, at room temperature (25°C) results were read thereafter. Haemagglutination with tanned RBCs was done in the same way (Old and Adegbola 1983).

3.8.10. Congo Red Dye Assay (CRDA) **or Congo Red Binding Assay**

To have an indication of the invasivness of *Gallibacterium anatis* bv. haemolytica, affinity to Congo red dye was measured through colony colour on medium containing Congo red dye.

CR agar was trypticase soy agar (BD) supplemented with 0.01% or 0.03% of Congo red dye (Sigma Chemical Co., St. Louis, Mo.) with or without 10% bile salts (Difco, France). Congo-red-Positive *G. anatis* were identified by the appearance of red/ brown colonies. The reaction in the colonies was best seen after 24 hours of incubation at 37 °C, and then by leaving them at room temperature for an additional 2 days (not to exceed 4 days). Congo-red-negative *G. anatis* colonies did not bind the dye (white colonies). To help in preliminary identification of *G.anatis* bv. haemolytica, blood agar was used simultaneously with a CR medium as a selective medium (Berkhoff and Vinal, 1986).

3.8.11. Detection of efflux pump mediated resistance for antibiotics

The ethidium bromide (EtBr)-agar cart wheel method which involves preparation of trypticase soy agar (TSA) with EtBr ranging concentration from 1 mg/L, 2 mg/L, 3 mg/L. The TSA plates was prepared fresh on previous day of the experiment and kept protected from light. The TSA plates were divided into sectors by radial lines forming a cart wheel pattern and modified cart wheel method (spotting). The test cultures were grown in LB medium at 37°C with overnight incubation, the test cultures were swab inoculated along the radial lines marked on the EtBr-TSA plates starting from the centre of the plate to the rim. Each plate included a reference efflux pump negative strains (DH5α) as a control. The TSA plates are incubated at 37°C for 16-24 h and examined under gel imaging system/UV transiluminator. Fluorescence of growth was indicator of accumulation of EtBr in bacteria i.e., no activity of efflux pump while the growth without fluorescence was the indicator of presence of active efflux pump in the strain.

3.8.12. Antimicrobial drug resistance of the *Gallibacterium anatis* bv. haemolytica

3.8.12.1. Disc diffusion assay

The bacterial isolates were subjected to *in vitro* antibiotic sensitivity test by disc diffusion assay (CLSI, 2015) on Muller-Hinton agar (MHA) plates (Bauer *et al.*, 1966). The antibiotic discs were obtained from BD. Isolates were tested against commonly used antibiotics viz., Penicillin(10 µg), Ampicillin (10 µg), Gentamicin (10 µg), Tetracycline (30 µg), Azithromycin (30 µg), Chloramphenicol (30 µg), Cotrimoxazole (10 µg), Nitrofurantoin (300 µg), Ciprofloxacin (5 µg), Imipenem (10 µg), Meropenam (10 µg), Ertapenem (10 µg), Aztreonam (30 µg) and also EDTA (10 µg).

Isolates were grown in LB broth overnight and lawn cultures were prepared on MHA plates. The plates were allowed to dry. Antibiotic discs were placed on agar surface at about two cm apart. The plates were incubated at 37°C overnight and diameter of the zones of inhibition was measured. The measurements were compared with zone size interpretative chart furnished by the manufacturer and the zones were graded as sensitive, intermediate and resistant.

3.8.12.2. Broth dilution / E-strip methods:

The bacterial isolates were subjected to *in vitro* antibiotic sensitivity test by broth dilution method (CLSI, 2015) in Muller-Hinton broth (Bauer *et al.,* 1966). E-strip was used for determining the MIC.

3.8.12.3. Determination of Minimal Inhibitory Concentration (MIC) by broth tube dilution Method:

The tube dilution test is the standard method for determining levels of resistance to an antibiotic. Serial dilutions of the antibiotic was made in a liquid medium which is inoculated with a standardized number of organisms and incubated for a prescribed time. The lowest concentration (highest dilution) of antibiotic preventing appearance of turbidity was considered as minimal inhibitory concentration (MIC).

3.8.13. Protein profiling:

Protien profiling was performed to know the any appreciable effect of D_2O on *G. anatis* bv. haemolytica by comparing the adapted and parent strain by NATIVE-PAGE and SDS-PAGE.

3.8.13.1. Bacterial Protein Extraction Protein Extraction

1) Pellet of 10ml broth was resuspend in 1ml lysis buffer.

2) Lysis buffer was used 100 mM NaCl; 25 mM TrisHCl; pH 8.0.

3.8.13.2. Sonication:

1) The suspended cells were sonicated for 5 min in ice using a Soniprep 150 sonicator (MSE UK Ltd.) at 12 µm amplitude. The sequence followed for sonication was 12 µm for 15 sec followed by 10 sec gap. The cycle was repeated for 10 times (lysis was completed when the cloudy cell suspension becomes translucent.

2) Centrifugation was performed at 13,000 rpm for 5 min in temperature maintaining machine at 4°C. Soluble proteins (supernatant) was separated from insoluble or inclusion bodies proteins (pellet) and used for next step.

3.8.13.3. Native PAGE

Native PAGE uses the same discontinuous chloride and glycine ion fronts as SDS-PAGE to form moving boundaries that stack and then separate polypeptides by charge to mass ratio. Protein solution was prepared in a non-reducing non-denaturing sample buffer, which maintains the proteins secondary structure and native charge density. Therefore, multiple bands from the native PAGE gel can be seen for a single protein if target proteins were present in polymerized forms in the sample.

3.8.13.3.1. Gel running protocol

1. Separating gel was prepared in appropriate amount having a small beaker. AP and TEMED were gently added with swirling of the beaker to ensure a sufficient mixing. Gel solution was pipetted into the gap between the glass plates of gel casting (don't fully fill). Fill the rest space with water (isopropanol alternatively). Allowed for 20-30 min for a complete gelation.
2. Stacking gel was prepared in appropriate amount in a beaker and mix with 10% AP and 1% TEMED. Water was poured out in the first step and pipetted the stacking gel solution into the gap and the comb was inserted. Solution was allowed to gelate for 20-30 min.
3. Sample buffer was mixed properly with sample without any heating.
4. Sample was loaded and set at an appropriate voltage to run the electrophoresis.
5. Coomassie-blue dye and silver stain were used for staining purpose.

3.8.13.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was carried out as per the protocol described by Laemmli (1970) with slight modifications. The gel casting platforms was assembled and sealed with agarose. The stacking (5%) and separating gels (12%) were prepared as per protocol. About 20 µl of cell lysate was mixed with equal volume of 2 × sample loading buffer, heated at 95°C for 10 min and then loaded into wells along with protein molecular weight marker. The gel was electrophoresed at 90 V till the tracking dye traversed the stacking gel. The voltage was then maintained at 120 V till the dye reached bottom of the separating gel. The gel was then stained

with Coomassie blue staining. Gel with clear discrete protein bands was analyzed with the help of silver light illuminator in gel doc and was photographed.

3.8.14. Protocol for Silver Staining

1. Gel was put in a large glass tray with about 1 liter volume capacity; enough of each solution was used at each step to cover gel; agitate gently during steps; gel was touched with a glass rod only or not at all until staining is finished and large amount of water was used for each washing step to give clean results.
2. Fixation of protein was performed in gel by using fixative for overnight.
3. Reductant was used for 1 min for completion of reduction.
4. Washing was carried out for 30 sec in water at least for three times.
5. After that gel was kept in silver stain for 20 min.
6. Washing was repeated again (like step 4).
7. After that gel was kept in developer for developing band until bands become visible (fresh developer should be prepared).
8. Washing was repeated again (like step 4).
9. Stop solution was used finally to stop reaction otherwise bands become dark black in colour.

3.8.15. Protocol for Coomassie Blue Staining

1. Gel was placed in staining solution and shaking for 1h to overnight.
2. Staining solution was replaced with de-staining solution and shaken slowly for 30 min.
3. Destaining Solution was removed and replaced with new destaining solution for three times at least.
4. Cracking was minimized by adding 1% glycerol to the last destaining solution before drying the gel.

Results

4 RESULTS

4. Phenotypic characters of adapted (51H) and parent stain of *Gallibacterium anatis* bv. haemolytica

4.1. Morphological and Cultural Characteristics

Total 10 strain of *Gallibacterium anatis* bv. haemolytica from dead poultry birds and one strain from piegon were isolated with similar biochemical test. *Gallibacterium anatis* bv. haemolytica included in this study was isolated from heart blood in pure culture from broiler birds dying *en masse* in May-June 2015 at CARI and near by villages in Bareilly (Singh, 2015, IVRI annual Report 2015-16). Colonies on sheep blood agar of parent strain were strongly haemolytic, greyish, non-transparent, circular with a butyrous consistency after incubation at 37°C for 18-24 h while adapted strain had weak haemolyticless transparent peripheral area, less greyish, compact, and more shiny colonies (Fig. 1a, 1b & 2). The change in adapted strain was observed at 36 passage onward in medium containing D_2O (Fig. 3a & 3b). After back passaging of adapted strain in H_2O some colony had large haemolytic zone like parent while in most of the colonies had haemolytic zone remained restricted to the centre of colony (Fig. 4a & 4b). The normal haemolytic zone of parent strain appeared on blood agar within 18-24 h at 37°C but of adapted strain was very small at the same time (Fig. 5). The microscopic examination of the Gram stained parent culture revealed that the organisms were Gram negative bacilli and adapted strain has not shown more change in shape (Fig. 6b). However, on subculturing change in parent strain have observed from bacilli to coccobacilli after the passaging in H_2O (Fig. 6a). The organisms did not grow on MacConkey agar.

4.2. BIOCHEMICAL CHARACTERISTICS

The cultures of *G. anatis* bv. haemolytica were found positive for catalase, oxidase, KOH and nitrate reduction test whereas negative reactions were observed for citrate utilization, methyl red and Voges-Proskauer tests. It fermented mannitol, trehalose, maltose and sucrose. Adapted strain had similar characteristics except weak string formation with 10% KOH. Parent strain grew aerobically while the adapted strain had ability to grow anaerobically too in thioglycollate medium (Fig. 7).

4.3. MOLECULAR CHARACTERIZATION OF *G. anatis* bv. haemolytica

4.3.1. Isolation of Genomic DNA and PCR

The genomic DNA with high purity (260/280 : 1.6-1.8) was used as template for *G. anatis* bv. haemolytica specific PCR using genus specific primer. The genome of bacteria was confirmed by bacterial specific PCR using16S rRNA universal primer (Fig. 8). Specificity of *G. anatis* PCR using 16S rRNA and 23S rRNA gene primers was established by amplification of three specific amplicons (Fig. 9) of ~789 bp, 985bp,1032 bp indicating presence of specific internal transcribed spacer (ITS) regions (Fig. 10) confirmed by sequencing.

4.4. Growth Kinetics

Heavy water in growth medium increased growth time of *G. anatis* irrespective of adaptation at all growth temperatures and all concentrations of D_2O in medium.

However, in medium with 99% D_2O parent strain had longer lag phase than adapated strain when grown at 10°C, 25°C, 37°C and at 45°C (Table 8, 9, 10 & 11) (Graph 1-7).

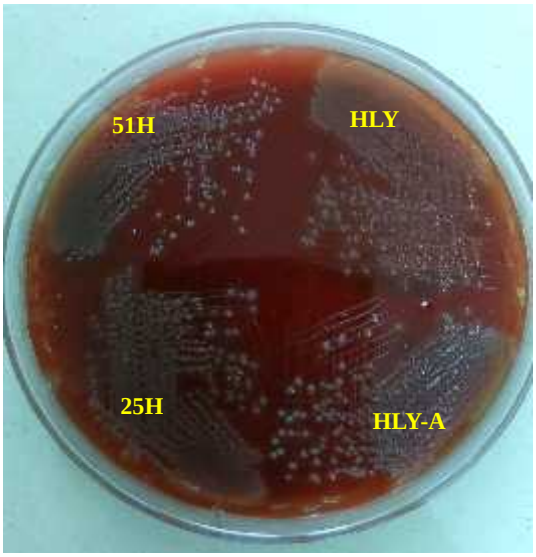


Fig. 1a: Morphology of colony in 99% D_2O at different passage time with parent strain of *G. anatis* bv. haemolytica on sheep blood agar plate incubated at 37 ^{0}C for 24 h

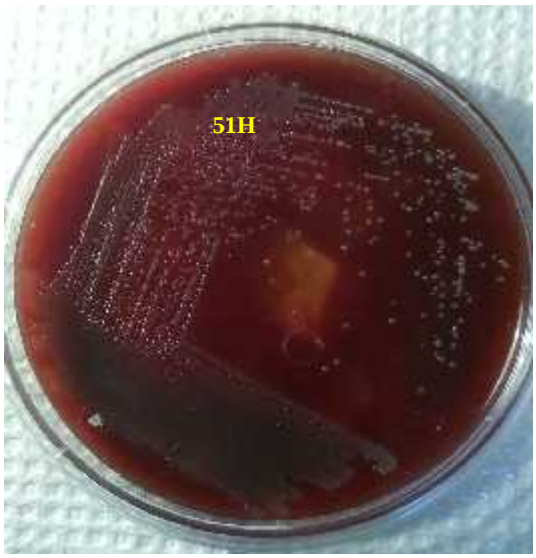


Fig. 1b: Morphology of colony after 51 time passage in 99%D_2O of *G. anatis* bv. haemolytica on sheep blood agar plate incubated at 37 ^{0}C for 24 h

Fig. 2: Difference in Colony size after 17 and 36 passage in 99% D_2O of *G. anatis* bv. haemolytica on sheep blood agar plate incubated at 37^{o}C for 24 h

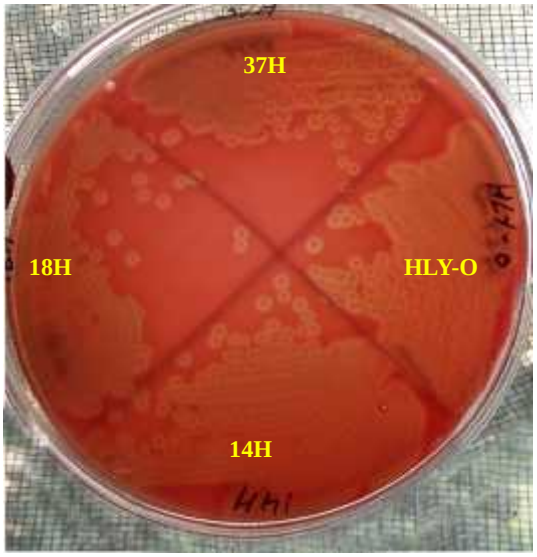


Fig. 3a: Change observed in haemolytic zone in 99% D_2O at different passage of *G. anatis* bv. haemolytica on sheep blood agar plate incubated at 37oC for 24 h

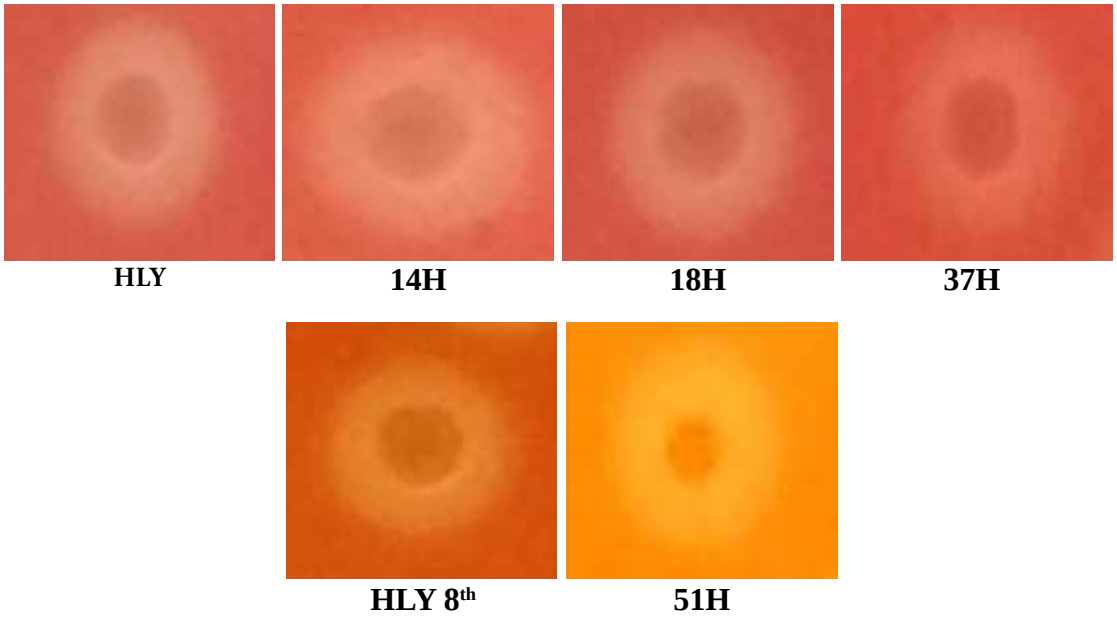


Fig. 3b: Pattern of strong to weak haemolytic zone diameter at different passage strain in 99% D_2O of *G. anatis* bv. haemolytica

HLY : Parent strain of *G. anatis* bv. haemolytica
14H : 14 time passage in 99% D_2O of *G. anatis* bv. haemolytica
18H : 18 time passage in 99% D_2O of *G. anatis* bv. haemolytica
37H : 37 time passage in 99% D_2O of *G. anatis* bv. haemolytica
51H : Adapted strain of *G. anatis* bv. haemolytica after 51 time passage in 99% D_2O

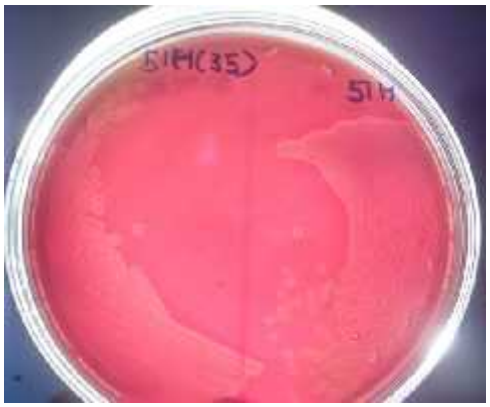


Fig. 4a : Difference in heamolytic zone after back passage of 51H strain of *G. anatis* bv. haemolytica on sheep blood agar plate incubated at 37°C for 24 h

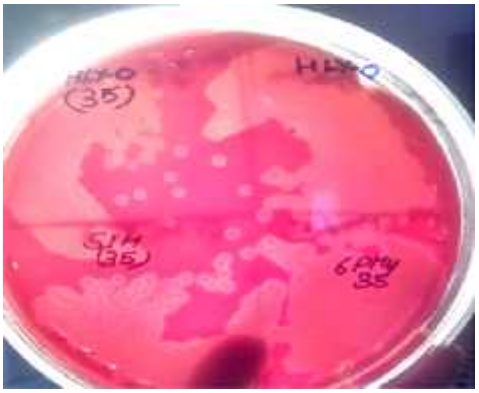


Fig. 4b : Difference in heamolytic zone of *G. anatis* bv. haemolytica after passage in H_2O on sheep blood agar plate incubated at 37°C for 24 h

HLY **:** Parent strain of *G. anatis* bv. haemolytica
51H **:** Adapted strain of *G. anatis* bv. haemolytica after 51 time passage in 99% D_2O
51H (35) **:** Adapted strain after 35 time back passage in H_2O
6PH4 (35) **:** *G. anatis* bv. haemolytica after 35 time passage in H_2O

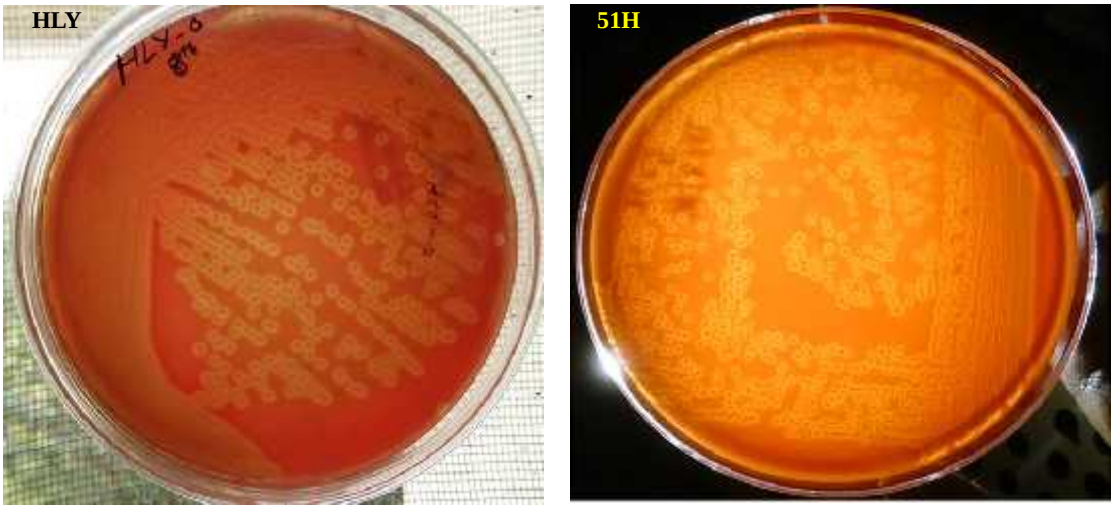


Fig. 5 : Differences in haemolytic zone of parent (HLY) and adapted strain (51H) of *G. anatis* bv. haemolytica on sheep blood agar plate incubated at 37°C for 24 h

HLY : Parent strain of *G. anatis* bv. haemolytica
51H : 51 time passage strain in 99% D_2O of *G. anatis* bv. haemolytica

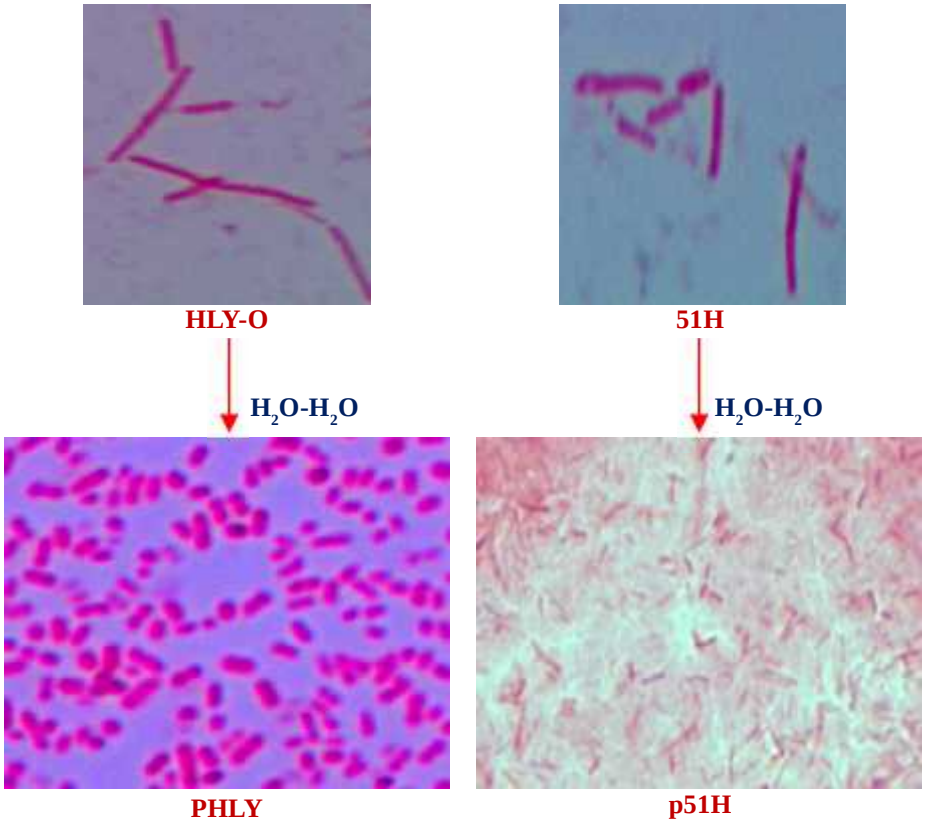


Fig. 6: (a) Parent *G. anatis* bv. haemolytica after passage in H_2O under microscope (Gram's staining, 1000X)

(b) 51H strain of *G. anatis* bv. haemolytica after passage in H_2O under microscope (Gram's staining, 1000X)

HLY : Parent strain of *G. anatis* bv. haemolytica

PHLY : Parent strain of *G. anatis* bv. haemolytica after 51 time passage in H_2O

51H : Adapted strain of *G. anatis* bv. haemolytica after 51 time passage in 99% D_2O

P51H : Adapted strain of *G. anatis* bv. haemolytica after 51 time passage in H_2O

Fig. 7: Shifting of parent strain from aerobic to anaerobic after 51th passage in 99% D_2O

HLY : Parent strain of *G. anatis* bv. haemolytica

51H : Adapted strain of *G. anatis* bv. haemolytica after 51 time passage in 99% D_2O

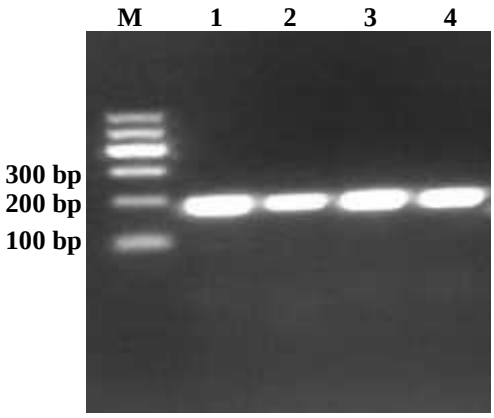


Fig 8: Confirmation of bacterial DNA on the basis of 16S rRNA gene universal primers
Lane M : 100 bp DNA ladder
Lane 1 : *E. coli* genomic DNA
Lane 2 : *Brucella species* genomic DNA
Lane 3 : Poultry isolated *G. anatis* bv. haemolytica genomic DNA
Lane 4 : Pigeon isolated *G. anatis* bv. haemolytica genomic DNA

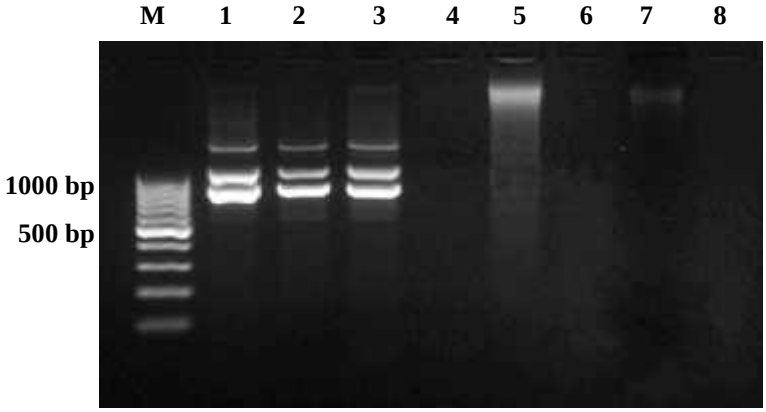


Fig 9: Specificity of genus specific primer of *Gallibacterium* targeting 16S rRNA and 23S rRNA gene
Lane M : 100 bp ladder
Lane 1 : Positive control (*G. anatis* bv. haemolytica genomic DNA)
Lane 2 : Parent strain of *G. anatis* bv. haemolytica genomic DNA
Lane 3 : Pigeon isolated *G. anatis* bv. haemolytica genomic DNA
Lane 4 : *Mycoplasma bovis* genomic DNA
Lane 5 : *E. coli* genomic DNA
Lane 6 : *E. coli* genomic DNA
Lane 7 : *Pasturella multocida* genomic DNA
Lane 8 : Negative control

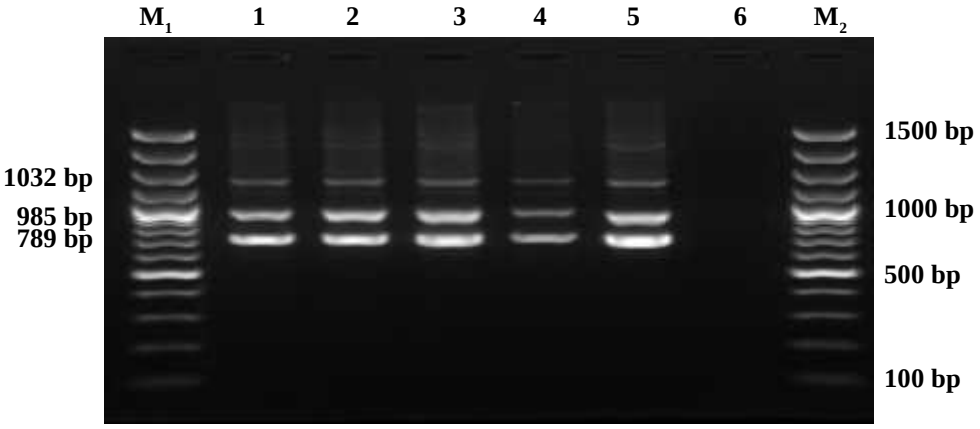
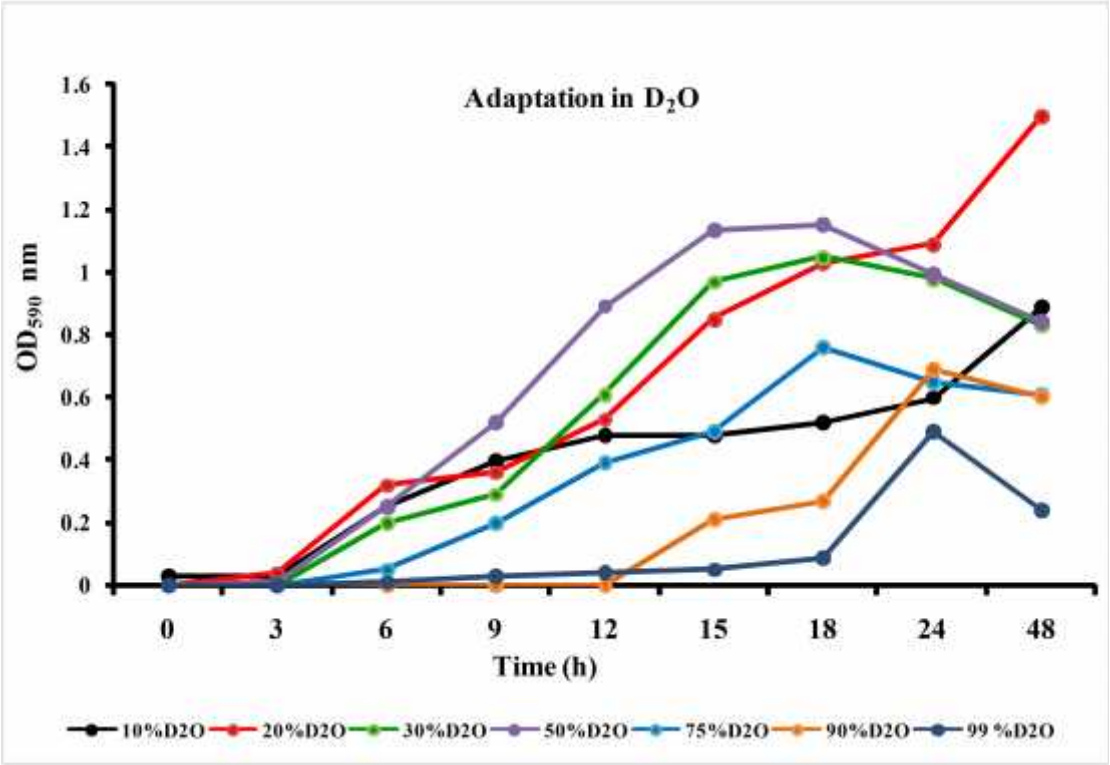
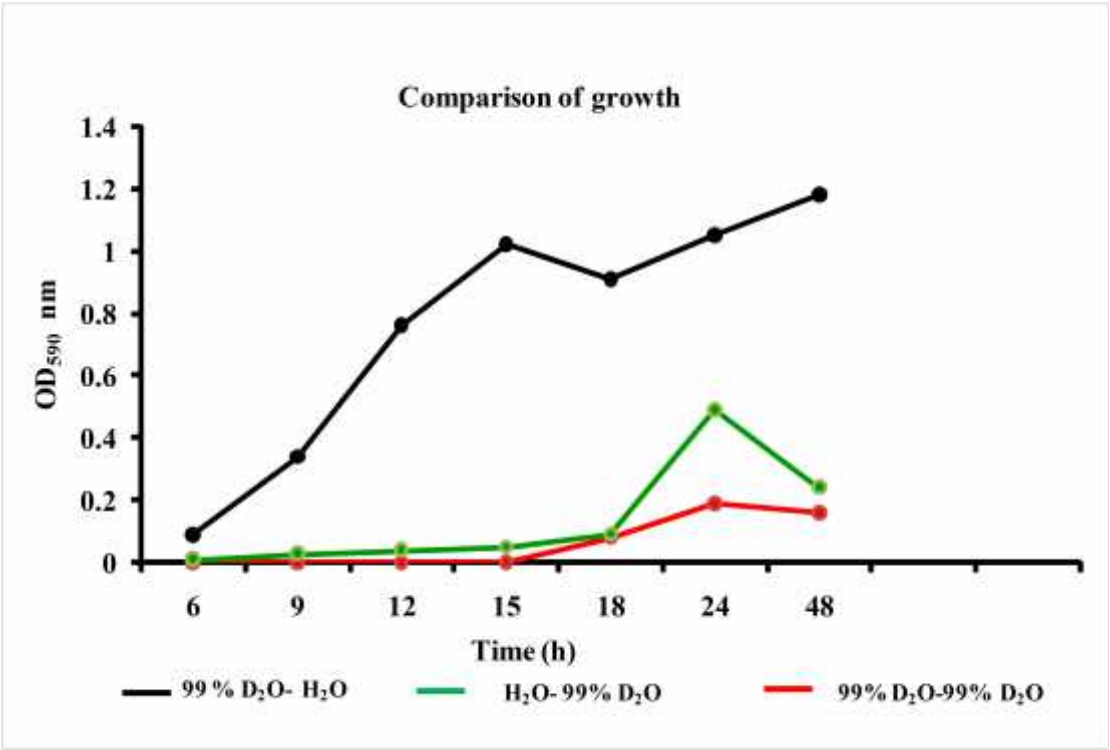


Fig. 10 : Genus specific PCR targeting 16S rRNA and 23S rRNA gene

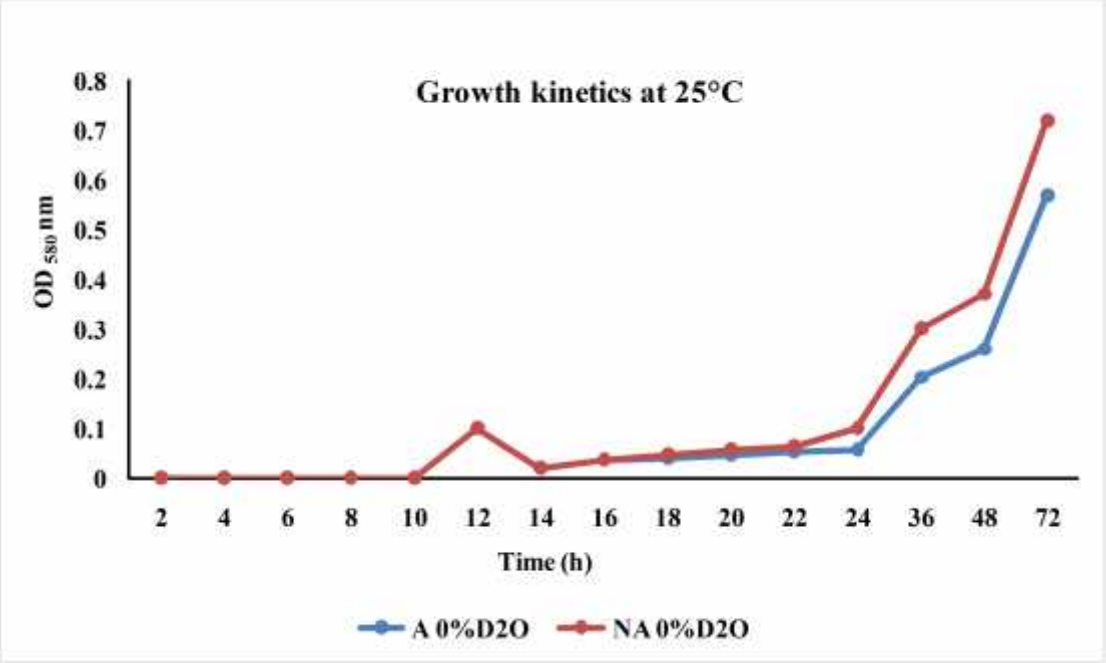
Lane M_1 &M_2 : 100 bp plus ladder
Lane 1 **:** Positive control (*G. anatis* bv. haemolytica genomic DNA)
Lanes 2-3 **:** Parent strain (*G. anatis* bv. haemolytica genomic DNA)
Lanes 4 -5 **:** Pigeon isolated parent strain (*G. anatis* bv. haemolytica genomic DNA)z
Lane 6 **:** Negative control



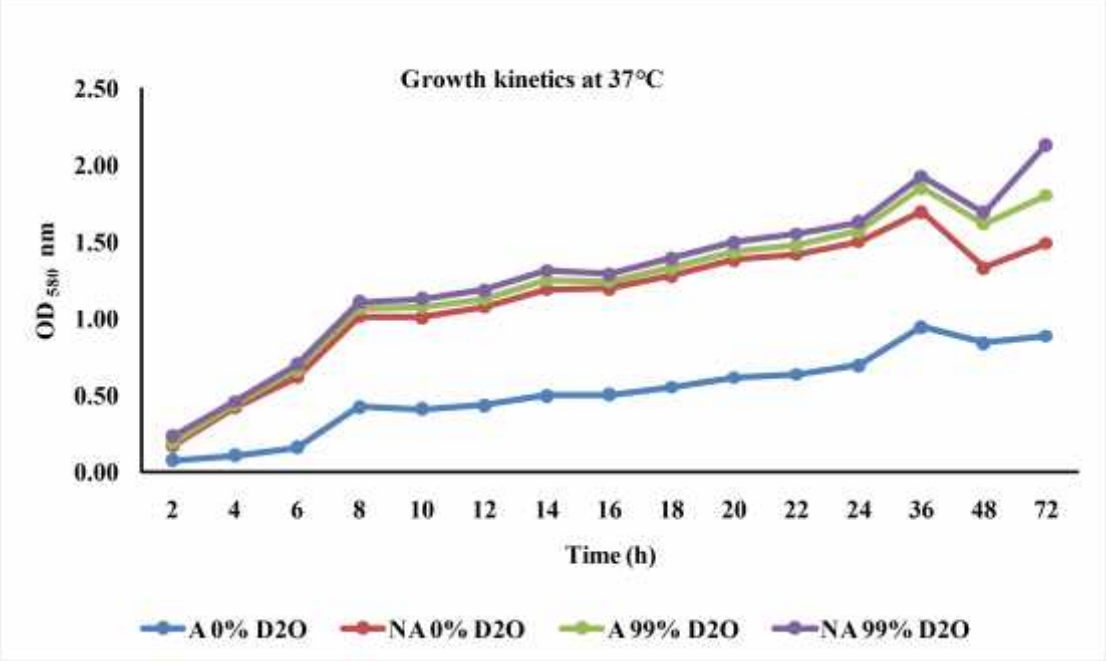
Graph 1: Adaptation of *G. anatis* bv. haemolytica in different concentration of D_2O



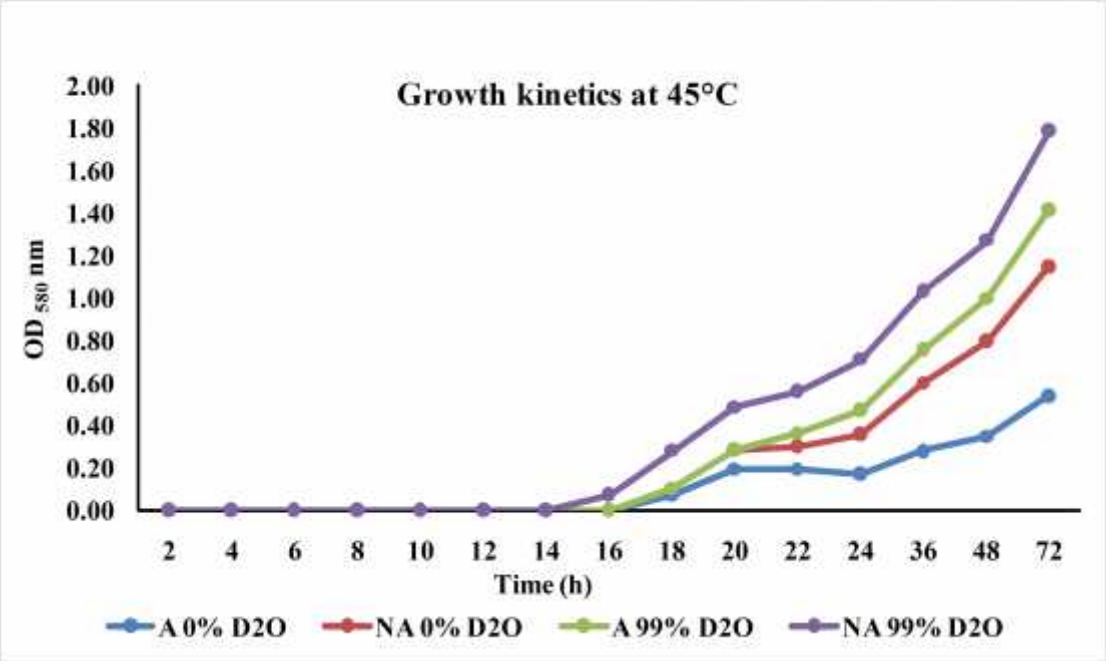
Graph 2: Comparsion of growth of adaptated strain of *G. anatis* bv. haemolytica in H_2O and 99% D_2O



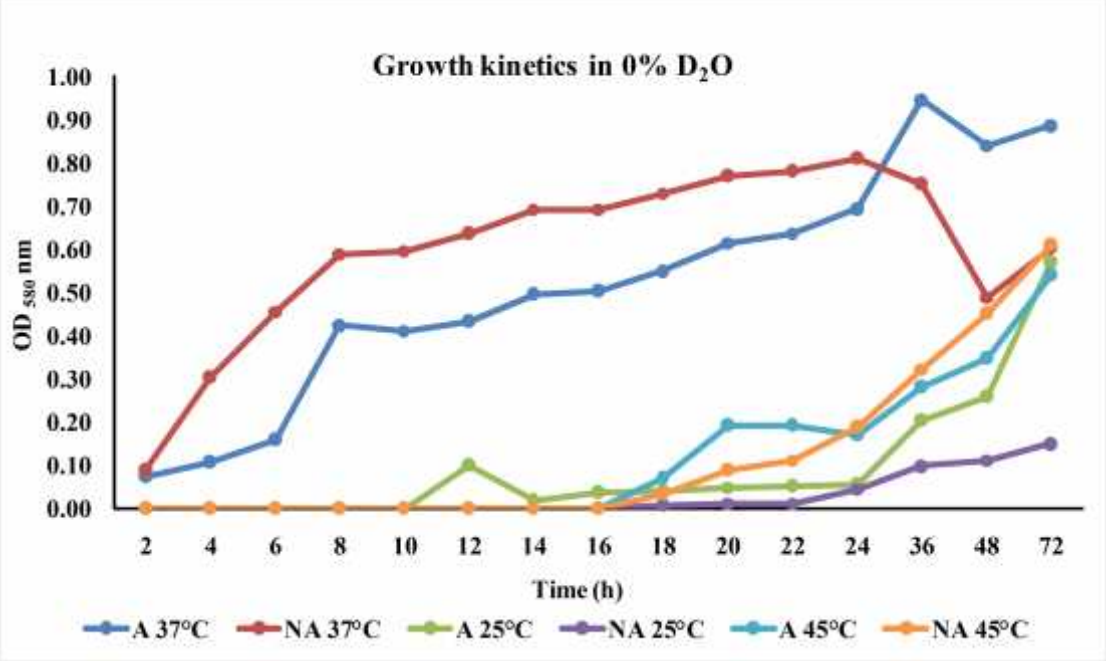
Graph 3: Growth kinetic curve of *G. anatis* bv. haemolytica at 25˚C



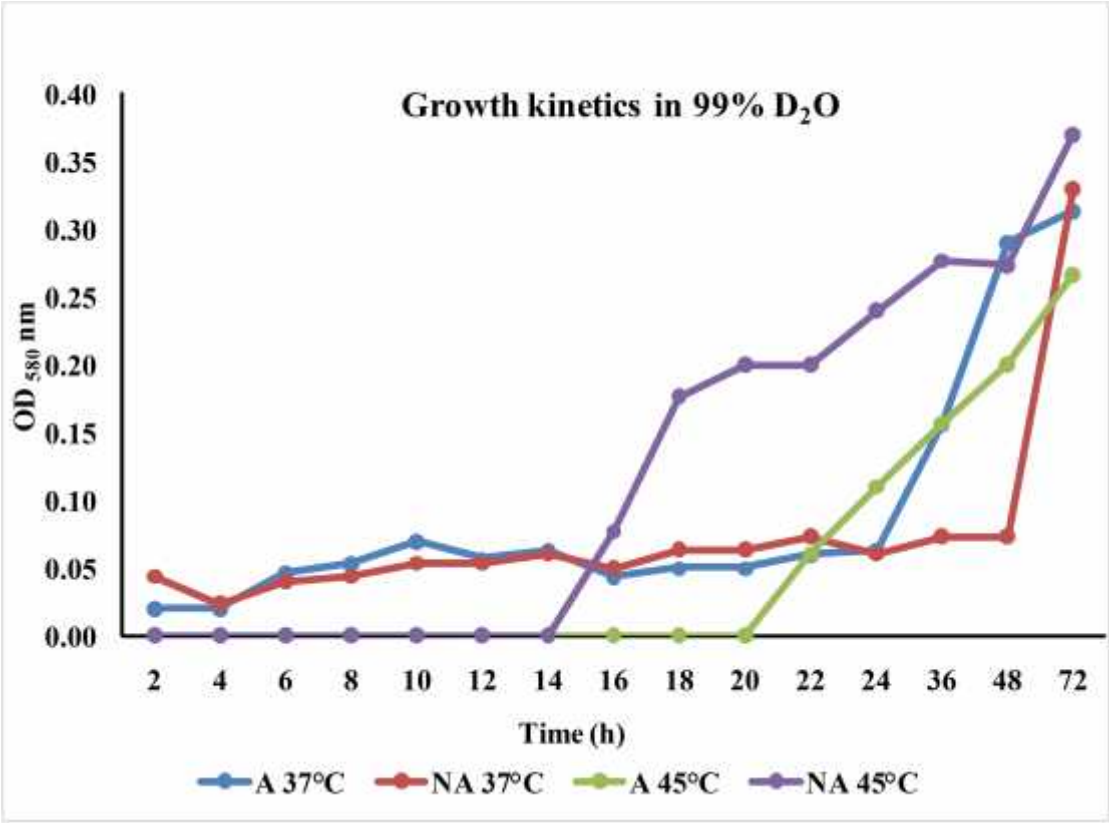
Graph 4: Growth kinetic curve of *G. anatis* bv. haemolytica at 37˚C



Graph 5: Growth kinetic curve of *G. anatis* bv. haemolytica at 45˚C



Graph 6: Growth kinetic curve in 0% D_2O of adapted and non-adapted strain of *G. anatis* bv. haemolytica



Graph 7: Growth kinetic curve in 99% D_2O of adapted and non-adapted strain of *G. anatis* bv. haemolytica

Table 8 : Growth kinetics of *G. anatis* biovar haemolytica at 10°C (Averge Log $_{10}$ count ±SD value)

Time (h)	0% D_2O		99% D_2O	
	Adapted	Non-adapted (Parent)	Adapted	Non-adapted (Parent)
0	7.45±0.16	7.47±0.15	7.48±0.07	7.36±0.14
24	7.31±0.07	7.16±0.07	7.63±0.37	7.06±0.05
48	6.49±0.18	6.74±0.22	6.83±0.55	6.30±0.72
72	7.15±0.67	6.70±0.23	5.83±0.07	7.07±0.28
96	6.52±0.31	6.55±0.20	6.72±0.79	5.26±0.05
120	7.29±0.71	6.60±0.48	6.99±0.66	4.90±0.12
144	7.29±0.68	6.35±0.68	6.77±0.61	4.55±0.06
168	7.53±0.87	6.32±0.78	6.31±0.57	4.55±0.03
192	7.59±0.89	6.26±1.00	6.06±0.69	4.47±0.82
216	7.26±0.94	6.01±1.13	5.56±0.87	3.28±0.10

Table 9 : Growth kinetics of *G. anatis* biovar haemolytica at 25°C (Averge Log $_{10}$ ±SD value)

Time (h)	0% D_2O		99% D_2O	
	Adapted	Non-adapted (Parent)	Adapted	Non-adapted (Parent)
0	7.47±0.18	7.47±0.15	7.39±0.19	7.36±0.14
6	7.28±0.12	7.86±0.04	0.00±0.00	7.82±0.03
12	6.51±0.06	6.70±0.07	4.52±2.89	5.92±0.08
18	6.25±0.06	6.81±0.02	0.00±0.00	4.52±2.89
24	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
36	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
48	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
72	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 10 : Growth kinetics of *G. anatis* biovar haemolytica at 37°C (Averge Log $_{10}$ ±SD value)

Time (h)	0% D_2O		99% D_2O	
	Adapted	**Non-adapted (Parent)**	**Adapted**	**Non-adapted (Parent)**
0	7.47±0.18	7.47±0.15	7.39±0.19	7.36±0.14
6	8.67±0.04	8.73±0.08	6.59±0.17	5.77±0.58
12	8.75±0.03	8.66±0.05	5.03±0.24	5.26±0.93
18	8.92±0.12	8.63±0.14	5.39±0.58	5.12±0.60
24	8.95±0.10	8.59±0.14	7.08±1.19	5.31±0.26
36	8.98±0.09	8.72±0.17	7.33±0.27	5.13±0.18
48	9.47±0.30	8.14±0.06	8.26±0.03	8.23±1.26
72	8.75±0.14	8.29±0.10	7.88±0.81	7.92±0.20

Table 11 : Growth kinetics of *G. anatis* biovar haemolytica at 45°C (Averge Log $_{10}$ ±SD value)

Time (h)	0% D_2O		99% D_2O	
	Adapted	**Non-adapted (Parent)**	**Adapted**	**Non-adapted (Parent)**
0	7.39±0.28	7.51±0.20	7.39±0.19	7.36±0.14
6	6.04±0.20	5.05±0.57	6.33±0.10	7.32±0.29
12	5.56±0.87	6.29±0.16	6.23±0.18	5.53±0.39
18	7.07±0.76	7.03±0.49	6.80±0.40	7.30±0.24
24	7.10±0.06	7.13±0.32	7.87±0.41	7.09±0.05
36	7.46±0.05	7.32±0.17	8.09±0.26	7.29±0.16
48	7.76±0.04	7.45±0.12	7.80±0.08	7.35±0.13
72	7.74±0.13	7.47±0.10	8.04±0.15	7.45±0.06

4.5. Survival

Survival of parent as well as adapted strain in storage medium having 0%, 20%, 75%, 90% and 99% D_2O was best at 37°C, (112-119 days) less at 45°C (35-56 days) and the least (14-28 days) at 4°C. However, survival was better when D_2O was added in storage medium irrespective of adaptation to grow in presence of D_2O (Table 12, 13, 14, 15, 16 & 17) (Graph 8-12).

Table 12: Survival of Adapted *G. anatis* biovar haemolytica at 4°C (Averge Log_{10} ±SD value)

Time (days)	0% D_2O	20% D_2O	75% D_2O	90% D_2O	99% D_2O
0	7.24±0.13	7.29±0.20	7.26±0.12	7.28±0.16	7.26±0.24
7	7.03±0.20	7.40±0.16	7.28±0.12	0.00±0.00	0.00±0.00
14	6.12±0.08	6.85±0.04	6.51±0.06	4.52±2.89	0.00±0.00
21	6.48±0.03	6.43±0.04	6.25±0.06	0.00±0.00	0.00±0.00
28	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
35	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 13. Survival of Non-adapted (Parent) *G. anatis* biovar haemolytica at 4°C (Averge Log_{10} ±SD value)

Time (days)	0% D_2O	20% D_2O	75% D_2O	90% D_2O	99% D_2O
0	7.41 ± 0.17	7.39±0.08	7.33±0.31	7.31 ±0.27	7.28±0.33
7	8.00 ± 0.06	7.93±0.07	7.86±0.04	7.82±0.03	0.00 ± 0.00
14	6.16 ± 0.12	7.00±0.11	6.70±0.07	5.92±0.08	0.00 ± 0.00
21	0.00 ± 0.00	6.94±0.03	6.81±0.02	4.52±2.89	0.00 ± 0.00
28	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
35	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 14: Survival of Adapted *G. anatis* biovar haemolytica at 37°C (Averge Log $_{10}$ ±SD value)

Time (days)	0% D_2O	20% D_2O	75% D_2O	90% D_2O	99% D_2O
0	5.55±0.17	5.52±0.10	5.49±0.18	5.44±0.13	5.40±0.14
7	7.91±0.09	7.83±0.16	7.91±0.06	8.00±0.11	7.87±0.06
14	7.67±0.07	7.27±0.18	7.75±0.05	7.62±0.07	7.59±0.10
21	6.99±0.07	7.00±0.02	7.00±0.04	7.00±0.05	7.00±0.08
28	6.95±0.04	7.00±0.06	7.00±0.03	6.52±0.14	7.00±0.11
35	6.99±0.11	7.00±0.07	6.81±0.06	6.88±0.04	6.99±0.11
42	7.67±0.09	7.43±0.07	7.64±0.09	7.44±0.15	7.43±0.09
49	7.51±0.13	7.58±0.18	7.41±0.24	7.22±0.26	7.51±0.79
56	7.09±0.21	7.39±0.15	7.58±0.14	7.60±0.07	7.80±0.08
63	6.75±0.12	6.40±0.06	7.00±0.09	6.88±0.11	6.95±0.07
70	6.00±0.04	6.87±0.08	6.75±0.20	6.60±0.15	7.00±0.12
77	6.24±0.09	6.80±0.14	7.00±0.13	7.00±0.08	7.00±0.11
84	6.70±0.04	6.87±0.05	7.00±0.24	6.52±0.14	7.00±0.06
91	6.05±0.24	6.87±0.04	7.00±0.05	6.70±0.11	7.00±0.12
98	7.00±0.08	6.92±0.08	7.00±0.17	7.00±0.04	7.00±0.15
105	7.00±0.09	6.82±0.03	6.66±0.07	6.36±0.05	6.87±0.09
112	0.00±0.00	6.04±0.08	5.60±0.24	0.00±0.00	6.46±0.19
119	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
126	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 15. Survival of Non-adapted (parent) *G. anatis* biovar haemolytica at 37°C (Averge Log $_{10}$ SD value)

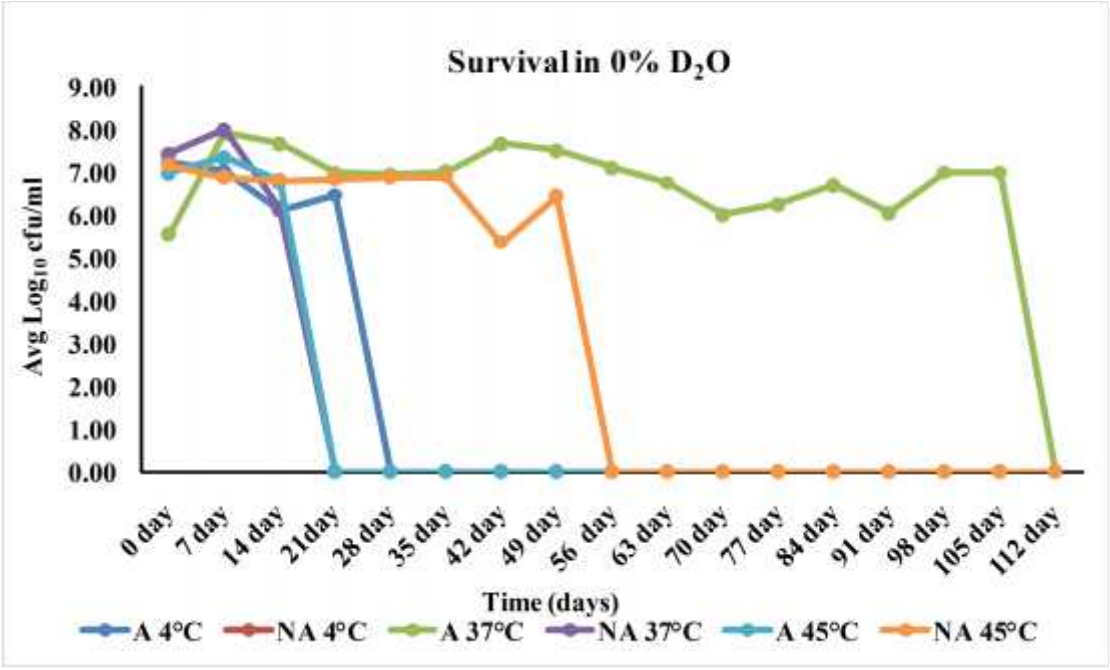
Time (days)	0% D_2O	20% D_2O	75% D_2O	90% D_2O	99% D_2O
0	5.64±0.09	4.98±0.18	5.49±0.17	5.43±0.06	5.38±0.16
7	8.00±0.07	7.87±0.14	7.94±0.05	7.96±0.10	8.00±0.03
14	6.98±0.08	7.02±0.08	6.95±0.06	7.01±0.07	6.90±0.15
21	6.88±0.04	6.52±0.11	7.00±0.04	6.22±0.15	6.64±0.06
28	7.00±0.04	7.00±0.06	6.75±0.09	6.80±0.06	7.00±0.13
35	6.62±0.23	7.00±0.15	6.93±0.07	6.87±0.10	6.88±0.13
42	6.56±0.14	7.00±0.03	6.75±0.05	6.88±0.29	6.70±0.17
49	6.25±0.20	7.00±0.03	6.98±0.11	6.54±0.25	6.85±0.04
56	7.07±0.11	6.95±0.10	6.93±0.08	6.95±0.08	6.75±0.13
63	6.74±0.11	6.73±0.30	6.79±0.19	6.10±0.05	6.60±0.22
70	6.60±0.09	7.00±0.07	6.75±0.03	6.52±0.18	7.00±0.04
77	6.70±0.22	7.00±0.23	7.00±0.21	7.00±0.11	7.00±0.14
84	6.97±0.14	6.87±0.13	6.64±0.07	7.00±0.08	7.00±0.14
91	6.97±0.14	6.87±0.11	6.92±0.15	7.00±0.15	7.00±0.27
98	7.00±0.16	7.00±0.08	6.62±0.16	6.85±0.12	6.87±0.23
105	6.99±0.13	6.89±0.06	6.53±0.15	6.85±0.09	6.95±0.13
112	0.00±0.00	5.98±0.18	0.00±0.00	0.00±0.00	6.22±0.04
119	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
126	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 16: Survival of Adapted *G. anatis* biovar haemolytica at 45°C (Averge Log $_{10}$ ±SD value)

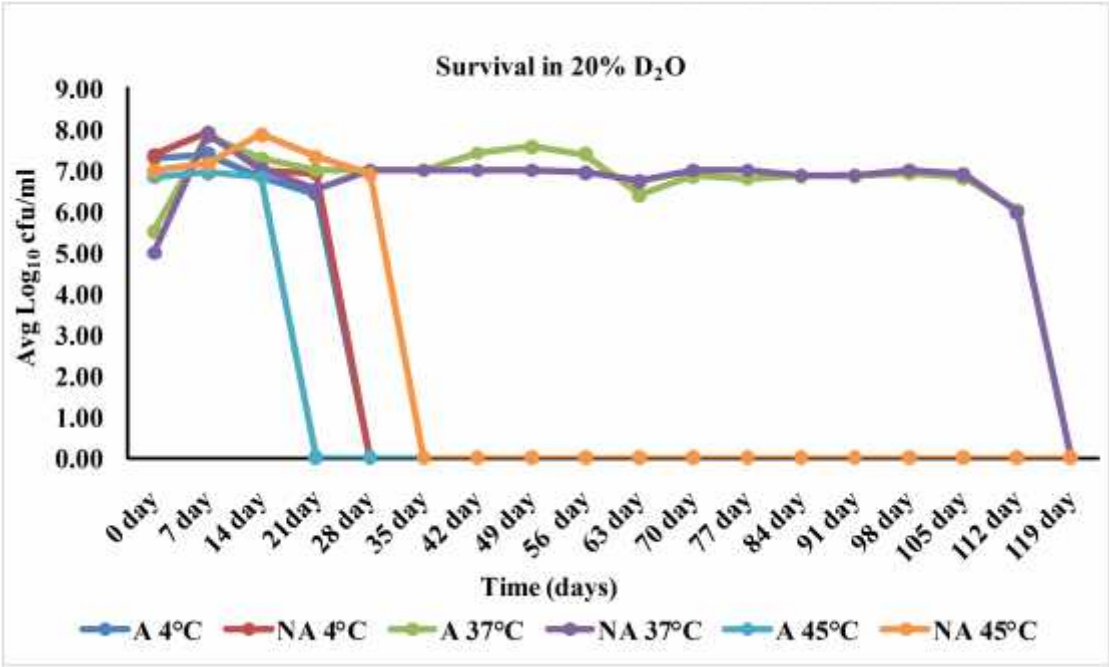
Time (days)	0% D_2O	20% D_2O	75% D_2O	90% D_2O	99% D_2O
0	7.00 ±0.04	6.85±0.22	6.82±0.13	6.87±0.29	6.90±0.31
7	7.36 ± 0.16	6.95±0.10	7.70±0.18	7.43±0.18	7.19±0.15
14	6.79 ± 0.05	6.85±0.02	0.00 ± 0.00	6.76±0.02	6.40±0.03
21	6.48 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.03±0.15
28	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.94±0.09
35	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.81±0.06
42	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.34±0.12
49	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.64±0.22
56	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
63	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
70	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 17: Survival of Non-adapted (Parent) *G. anatis* biovar haemolytica at 45°C (Averge Log $_{10}$ ±SD value)

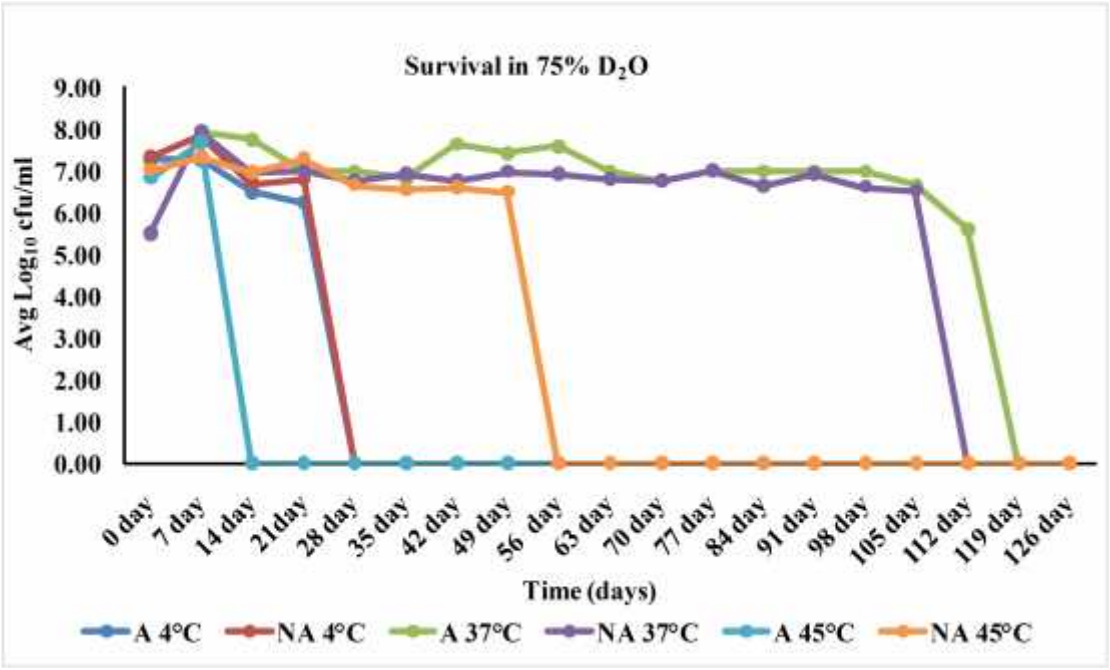
Time (days)	0% D_2O	20% D_2O	75% D_2O	90% D_2O	99% D_2O
0	7.18±0.16	6.97±0.19	7.01±0.23	6.87±0.22	7.00±0.15
7	6.88±0.06	7.16±0.07	7.33±0.04	7.52±0.02	7.03±0.11
14	6.82±0.11	7.87±0.18	6.95±0.10	7.12±0.22	6.87±0.07
21	6.85±0.29	7.31±0.07	7.30±0.16	7.26±0.08	7.29±0.32
28	6.88±0.06	6.90±0.07	6.66±0.07	6.84±0.05	6.75±0.09
35	6.91±0.15	0.00±0.00	6.54±0.16	6.98±0.19	6.82±0.09
42	5.37±0.10	0.00±0.00	6.59±0.04	5.73±0.33	6.93±0.12
49	6.45±0.12	0.00±0.00	6.50±0.14	6.68±0.34	6.73±0.07
56	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
63	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
70	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00



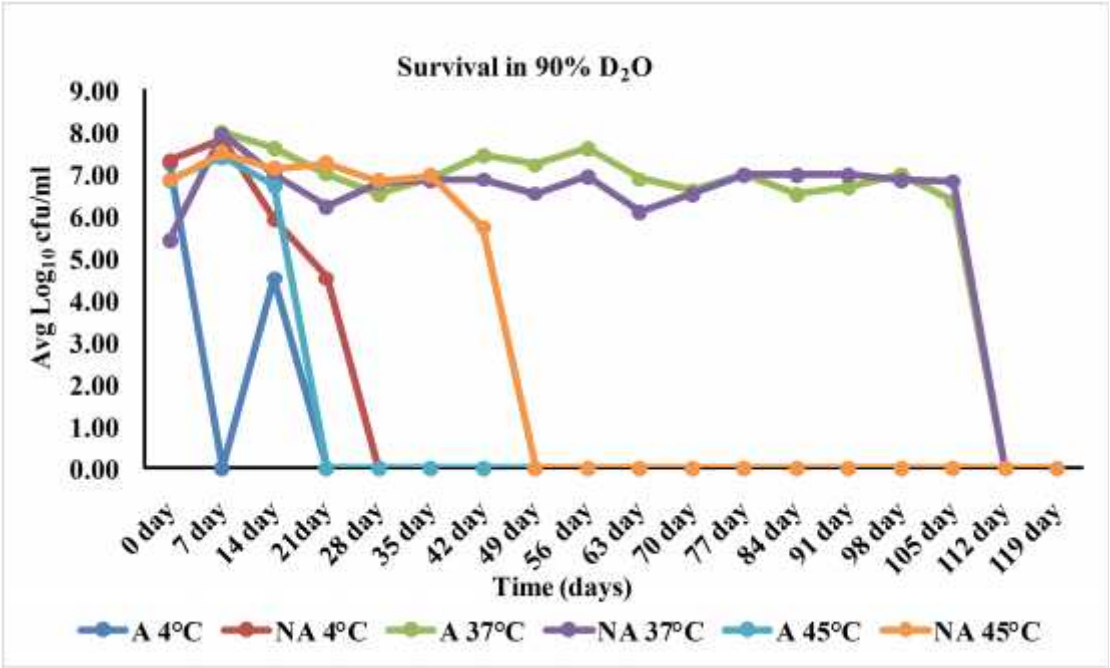
Graph 8: Survival of adapted and non-adapted strain of *G. anatis* bv. haemolytica in 0% D_2O at different temperature



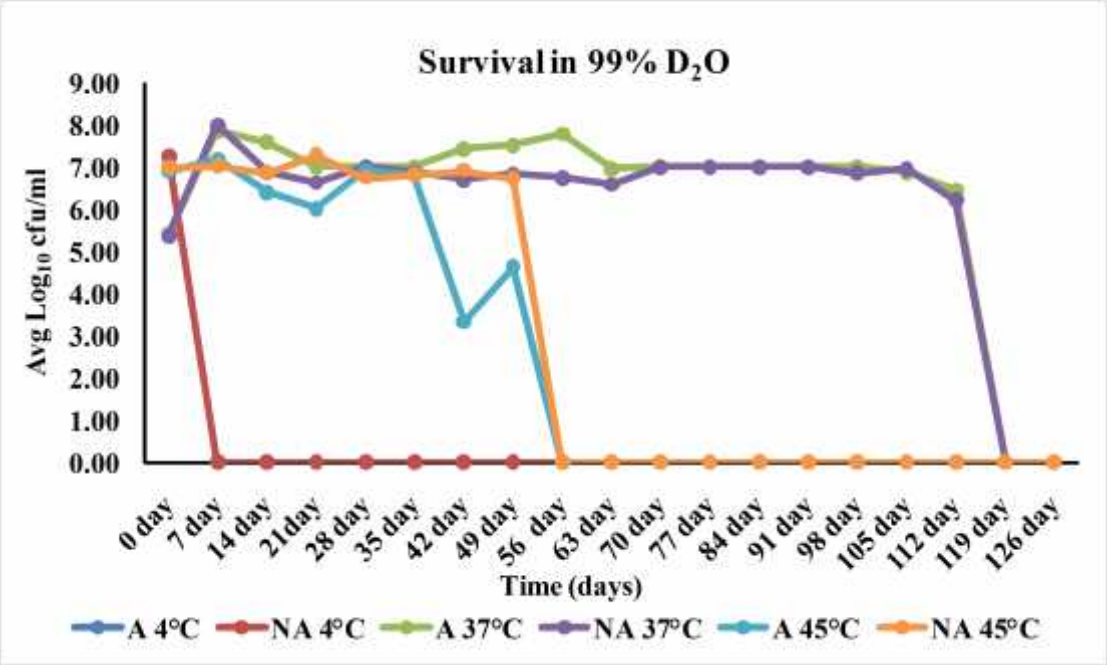
Graph 9: Survival of adapted and non-adapted strain of *G. anatis* bv. haemolytica in 20% D_2O at different temperature



Graph 10: Survival of adapted and non-adapted strain of *G. anatis* bv. haemolytica in 75% D_2O at different temperature



Graph 11: Survival of adapted and non-adapted strain of *G. anatis* bv. haemolytica in 90% D_2O at different temperature



Graph 12: Survival of adapted and non-adapted strain of *G. anatis* bv. haemolytica in 99% D_2O at different temperature

4.6. Phenotypic characteristics and virulence markers of parent and D_2O adapted isogenic strain of *G. anatis* bv. haemolytica

4.6.1. Congo Red Dye Binding (CRDB) Assay

Of the two Congo red dye concentrations (0.01% and 0.03%) employed, 0.03% permitted the better discrimination of strains included in study for CRDB ability (Fig. 11). Parent strain was negative in CRDB assay producing colourless colonies while 51H (adapted strain) of *G. anatis* bv. haemolytica formed brown to dark brown colonies on CRD agar plates having 0.01% and 0.03% of dye, respectively (Fig. 12). Addition of bile salt in CRD medium inhibited growth of parent strain totally but a few red colonies could be seen of adapted strain (Table 18).

Table 18 : Growth and colour of colonies on trypticase soy agar

Tested bacteria species	With 0.01% Congo red dye		With 0.03% Congo red dye	
	On TBC	On TBnC	On TBC	On TBnC
G. anatis bv. haemolytica (HLY) from Poultry	NG	Little	NG	Little
G. anatis bv. haemolytica (PH4) from Pigeon	NG	Little	NG	Little
Adapted *G. anatis* bv. haemolytica (51H)	Few red colonies	Brown colonies	Few red colonies	Dark brown colonies
Staphylococcus aureus (SAM)	Slight Red	Slight Red	Slight Red	Slight Red
Pseudomonas aeruginosa (A-290)	White	White	White	White
Avibacterium species. (CHB)	NG	NG	NG	NG
Escherichia coli (DPE) from Dog	Slight Red	Slight Red	Slight Red	Slight Red
Escherichia coli (M-97) from calf	Red colonies	Red colonies	Red colonies	Red colonies

Note : NG- No growth, TBC- Trypticase soya agar with 10 % bile and congo red dye
TBnC- Trypticase soya agar with congo red dye

4.6.2. Efflux pump activity in adapted and parent strain of *Gallibacterium anatis* bv. haemolytica

Of the 5 strain tested including parent and adapted strain of *G. anatis* bv. haemolytica named HLY, 14H, 25H, 51H and PH4 (6PH4) growth of first three was non-fluorescent i.e. having active efflux pump while the last two strains formed fluorescent growth indicating loss of efflux pump activity (Fig. 13, 14 & 15).

4.6.3. Antibodies for *Gallibacterium anatis* bv. haemolytica:

Results for 70 serum samples of poultry (Table 19), 8 of pig (Table 20) and goat (Table 21) each revealed presence of antibodies in serum of 23 birds, 7 pigs and 8 goats for formailinized antigen and in 15, 5 and 8 serum samples for O antigen, respectively.

Table 19: Microagglutination test in Poultry

Species	Total Sample (N)	MAT titre for 'O' antigen Total Positiv sample		MAT titre for 'formalinized' antigen Total Positive sample	
		No. of Sample	Titre	No. of Sample	Titre
Poultry	**70**	13	1:2	3	1:2
		2	1:4	2	1:4
				5	1:8
				5	1:16
				6	1:32
				1	1:64
				1	1:128

Table 20: Microagglutination test in Pig

Species	Total Sample (N)	MAT titre for 'O' antigen Total Positiv sample		MAT titre for 'formalinized' antigen Total Positive sample	
		No. of Sample	Titre	No. of Sample	Titre
Pig	**8**	1	1:2	2	1:8
		3	1:4	1	1:16
		1	1:8	2	1:32
				2	1:64

Table 21: Microagglutination test in Goat

Species	Total Sample (N)	MAT titre for 'O' antigen Total Positiv sample		MAT titre for 'formalinized' antigen Total Positive sample	
		No. of Sample	Titre	No. of Sample	Titre
Goat	**8**	5	1:8	2	1:8
		2	1:16	2	1:16
		1	1:32	2	1:32
				1	1:64
				1	1:128

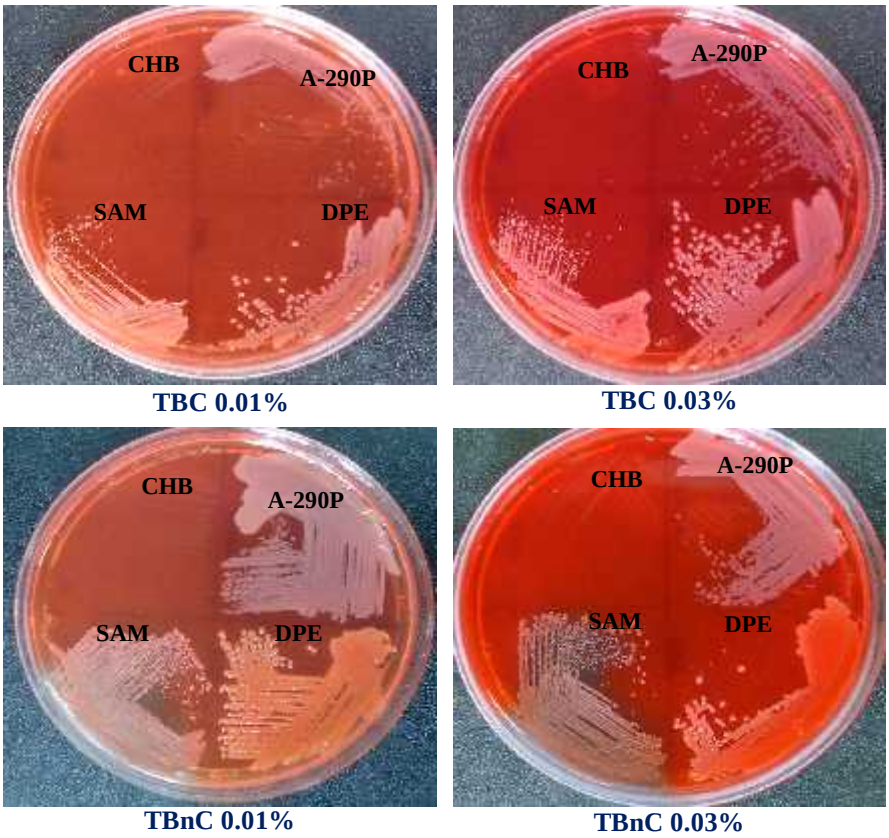


Fig. 11: Growth and colour of colonies of strain CHB, SAM, A-290P and DPE on trypticase soya agar in Congo red binding assay

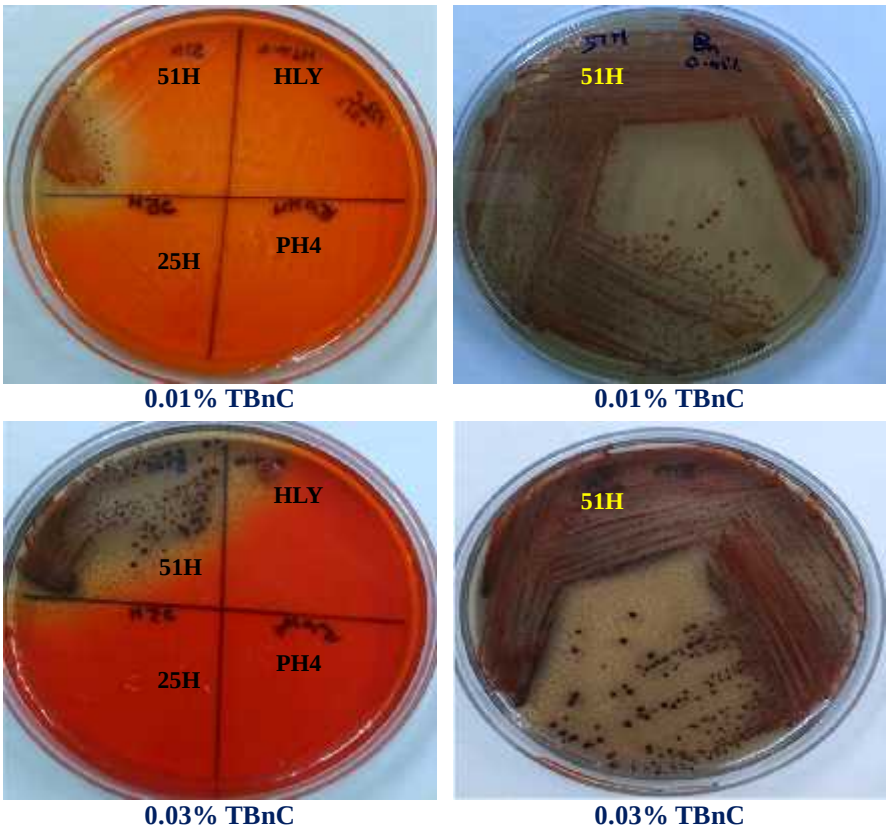


Fig. 12: Growth and colour of colonies of HLY, 25H, 51H and PH4 strain of *Gallibacterium anatis* bv. haemolytica on trypticase soya agar in Congo red binding assay

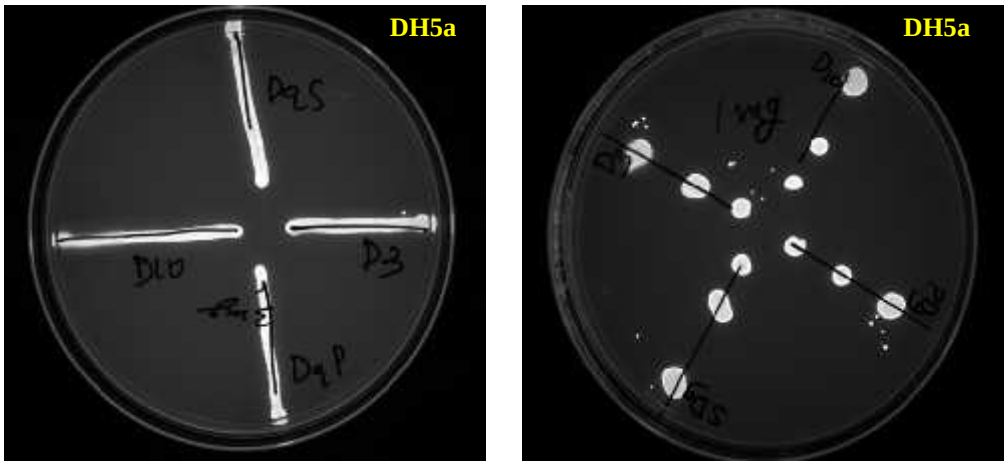


Fig. 13: Control strain DH5~:of *E. coli* without efflux pump showing fluorescence on Ethidium bromide plate

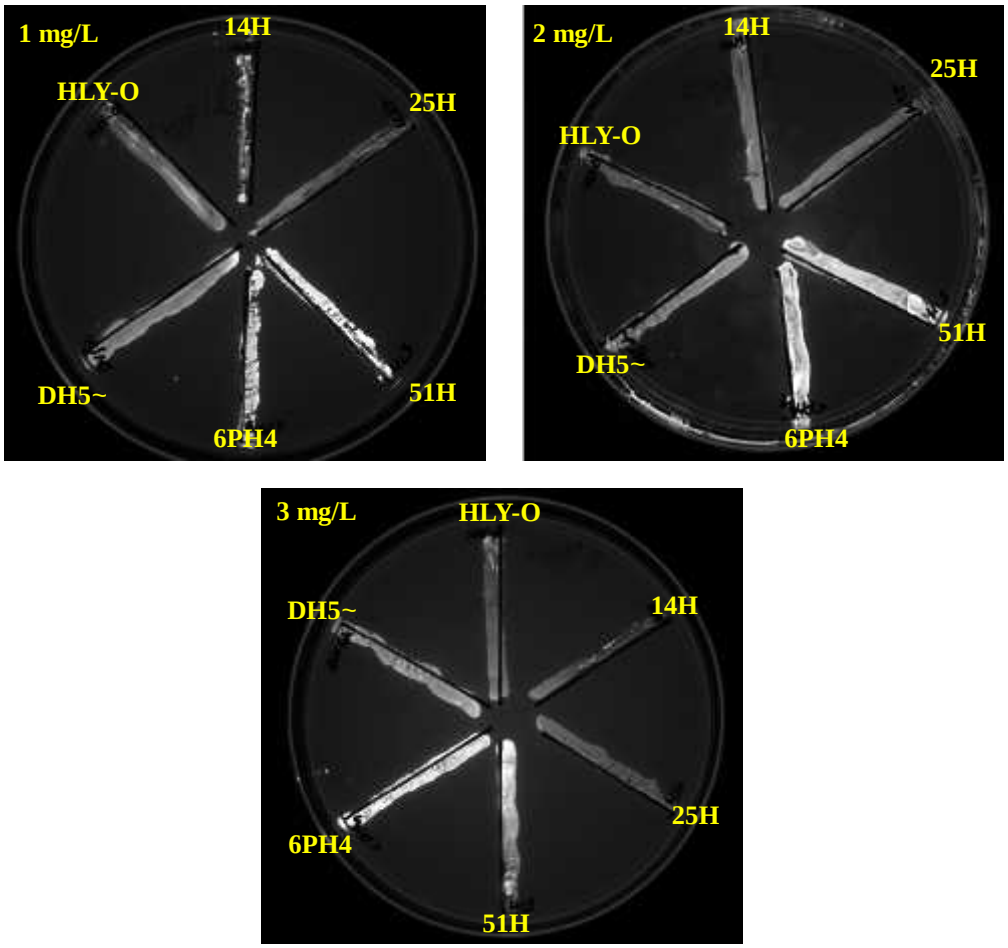


Fig. 14: Efflux pump activity at different concentration of Ethidium bromide plate in HLY, 14H, 25H, 51H and 6PH4 strain of *G. anatis* bv. haemolytica by Cart wheel method
Fluorescence : Efflux pump inactive
Non- fluorescence : Efflux pump active
HLY : Parent strain of *G. anatis* bv. haemolytica
14H : 14 time passage in 99% D_2O of *G. anatis* bv. haemolytica
25H : 25 time passage in 99% D_2O of *G. anatis* bv. haemolytica
51H : Adapted strain of *G. anatis* bv. haemolytica after 51 time passage in 99% D_2O
6PH4 : 37 time passage in 99% D_2O of *G. anatis* bv. haemolytica
DH5~ : Control strain DH5α (*E. coli)*

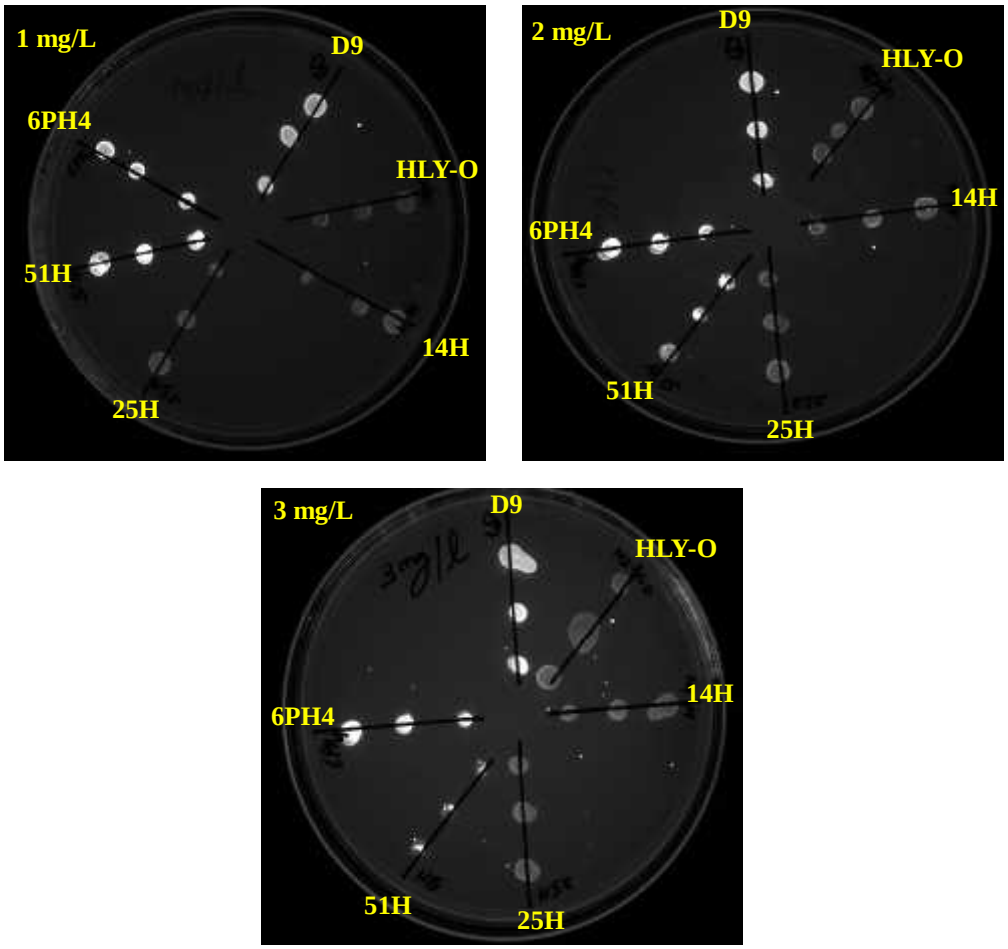


Fig. 15: Efflux pump activity at different concentration of modified Ethidium bromide plate in HLY, 14H, 25H, 51H and 6PH4 strain of *G. anatis* bv. haemolytica by Cart wheel method
Fluorescence : Efflux pump inactive
Non- fluorescence : Efflux pump active
HLY : Parent strain of *G. anatis* bv. haemolytica
14H : 14 time passage in 99% D_2O of *G. anatis* bv. haemolytica
25H : 25 time passage in 99% D_2O of *G. anatis* bv. haemolytica
51H : Adapted strain of *G. anatis* bv. haemolytica after 51 time passage in 99% D_2O
6PH4 : 37 time passage in 99% D_2O of *G. anatis* bv. haemolytica
DH5~ : Control strain DH5α (*E. coli)*

4.6.4. Haemagglutination (HA)

The results of HA patterns of *Gallibacterium anatis* bv. haemolytica shown in Table 22 indicated that there was difference in HA pattern of the parent and adapted strains.

Table 22: Hemagglutinating activity of Parent (HLY) and Adapted (51H) strain of *G. anatis* bv. haemolytica

Source of Erythrocytes	Agglutination of fresh RBCs with parent (HLY)		Agglutination of tanned RBCs with adapted strain 51H	
	Mannose resistant	Mannose sensitive	Mannose resistant	Mannose sensitive
Pig	-	+	-	-
Dog	-	-	-	+
Rat	+	+	-	-

4.6.5. Antibiotic sensitivity and MIC of parent and adapted strain

The results revealed (Table 23) that the parent strain was more sensitive to Gentamicin, Chloramphenicol, Azithromycin, Nitrofurantoin, Ampicillin, Imipenem, Meropenem, Ertapenem and EDTA while adapted strain was more sensitive for Penicillin, Gentamicin, Chloramphenicol, Azithromycin, Nitrofurantoin, Ampicillin, Imipenem, Meropenem, Ertapenem and EDTA. The MIC results indicated that adaptation of *G. anatis* bv. haemolytica to grow in D_2O containing medium affected the MIC of different drugs differently. The adpated strain had more MIC for ampicillin and ciprofloxacin while less for Azithromycin and Chloramphenicol than its parent (Table 24).

Table 23: Antibiotic sensitivity test of parent and adapted strain of *G. anatis* bv. haemolytica

Drug	Parent (HLY)	Inhibition zone (mm)	Adapted (51H) Inhibition zone (mm)
1	Penicillin	14	18
2	Tetracycline	12	10
3	Co-trimoxazole	0	0
4	Gentamicin	18	24
5	Chloramphenicol	30	32
6	Azithromycin	17	24
7	Nitrofurantoin	23	28
8	Ciprofloxacin	7	8.5
9	Ampicillin	20	26
10	Imipenem	24	35
11	Meropenem	30	25
12	Ertapenem	30	33
13	EDTA	24	28

Table:24 MIC determination of selective drug in parent and adapted strain of *G. anatis* bv. haemolytica

Drug	Parent (HLY) (µg/mL)	Adapted (51H) (µg/mL)
Ampicillin	16	32
Azithromycin	64	32
Ciprofloxacin	64	128
Chloramphenicol	16	8

4.6.6. Protein profiling:

NATIVE-PAGE of parent (HLY) and adapted (51H) strains under study revealed presence of more than 6 distinct bands in Coomassie blue staining (Fig. 16) and 8-10 in silver staining (Fig. 17) of NATIVE-PAGE. The SDS-PAGE profile revealed the presence of proteins by use of 10 to 180 kDa ladder with lysate of the *G. anatis* bv. haemolytica strains in the study. Each of the two strains (parent and adapted) had at least one distinct band different from the other in NATIVE-PAGE while in SDS-PAGE protein band between 51 kDa to 71 kDa was missing in adapted strain (Fig. 18).

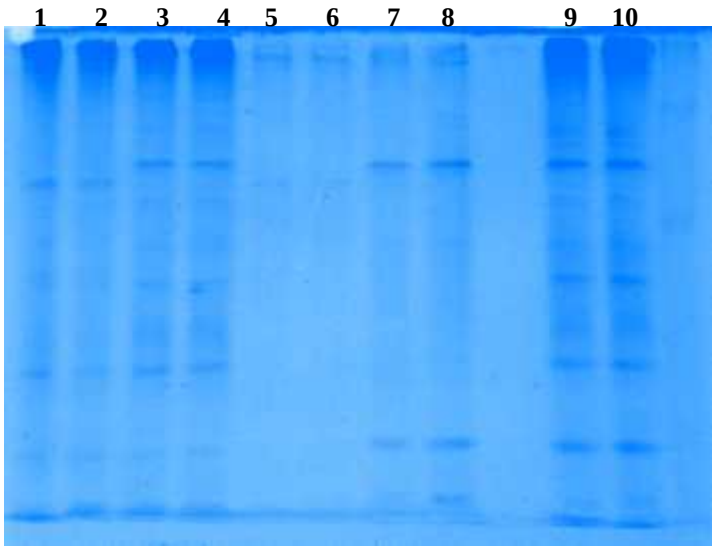


Fig. 16: NATIVE-PAGE profile of HLY, 51H, PHLY AND P51H by Coomasie brilliant blue staining

Lanes 1-2 : 51H
Lanes 3-4 : HLY
Lanes 5-6 : P51H
Lanes 7-8 : PHLY
Lanes 9-10 : Positive control HLY

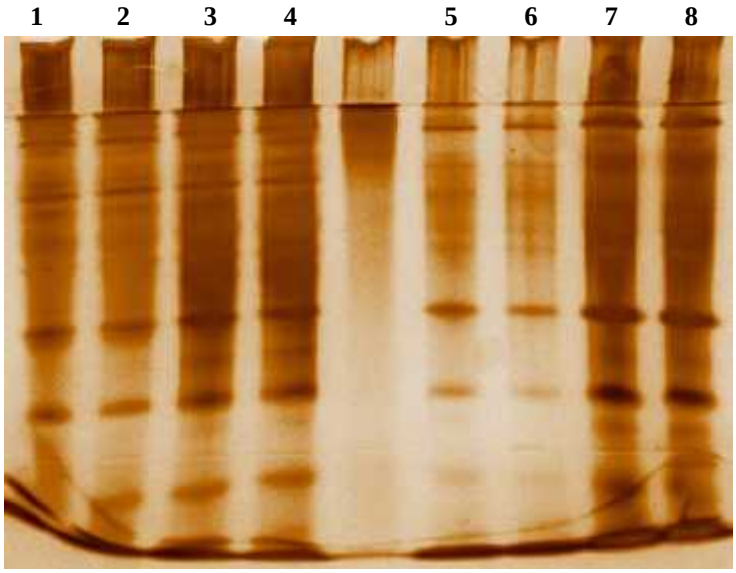


Fig. 17: NATIVE-PAGE profile of HLY, 51H, PHLY AND P51H by Silver staining

Lanes 1-2 : 51H
Lanes 3-4 : HLY-O (Positive control)
Lanes 5-6 : P51H
Lanes 7-8 : PHLY
HLY : Parent strain of *G. anatis* bv. haemolytica
PHLY : Parent strain of *G. anatis* bv. haemolytica after 51 time passage in H_2O
51H : Adapted strain of *G. anatis* bv. haemolytica after 51 time passage in 99% D_2O
P51H : Adapted strain of *G. anatis* bv. haemolytica after 51 time passage in H_2O

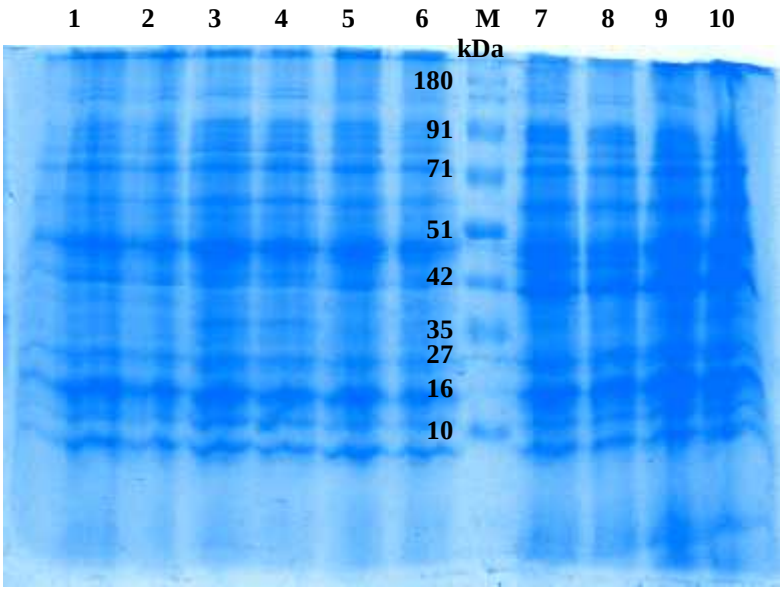


Fig. 18: SDS-PAGE profile of HLY-O, 14H, 25H, 51H and 6PH4 by Coomasie brilliant blue staining

Lanes 1-2 : HLY-O
Lanes 3-4 : 14H
Lanes 5 -6 : 25H
Lane M : Protein molecular weight marker in kDa
Lanes 7-8 : 51H
Lanes 9-10 : 6PH4
HLY-O : Parent strain of *G. anatis* bv. haemolytica
14H : 14 time passage in 99% D_2O of *G. anatis* bv. haemolytica
25H : 25 time passage in 99% D_2O of *G. anatis* bv. haemolytica
51H : Adapted strain of *G. anatis* bv. haemolytica after 51 time passage in 99% D_2O
6PH4 : *G. anatis* bv. haemolytica isolated from Pigeon

Discussion

5 DISCUSSION

5.1. Growth kinetics

In presence of D_2O increase in lag phase was evident for both adapated and parent strains of *Gallibacterium. anatis* bv. haemolytica. However, in case of *E. coli* strain there appeared to be no significant effect on lag phase but growth was significantly reduced as in case of *G. anatis* bv. haemolytica strains. Similar results have been reported for *E. coli* in earlier studies (Anthony salvagno, 2013). The difference between *E. coli* and *G. anatis* bv. haemolytica strains might be due to the inherent difference in the two bacteria (Katz *et al*., 1957; Thomson, 1960; Katz and Crespi, 1971; Ramakrishnaiah, 1990; Cioni and Strambini, 2002). In the present study *G. anatis* bv. haemolytica strains grew better at 37^{o}C than at 10^{o}C, 45^{o}C. It is a known fact that different species of microbes have a specific temperature for optimal growth. The adapted strain when was re-grown in medium made in normal water, it grew as efficiently as the parent and in instances even better. However, earlier observations report that lag phase is often longer on change of isotopic environment (Ernest and Rittenberg, 1960). This might be due to some specific growth characteristics of *G. anatis* bv. haemolytica, not explored in the study.

5.2. Survival

Though there was no marked difference in survival of D_2O adapted and isogenic parent strains of *G. anatis* bv. haemolytica when stored in presence or in absence of D_2O in storage medium at different temperatures of storage, presence of D_2O in storage medium

increased the survival of the bacterium irrespective of adaptation. The increased survival might be associated with increased stability of structural proteins in presence of D_2O reported earlier (Moner, 1972). Stability of pathogens has been reported to change with addition of D_2O in medium at different temperatures depending on concentration of D_2O in the medium (Wu *et al*., 1995; Ikizler *et al*., 2002; Kushner *et al.*, 1999). In the present study too, survival was the best at 99% of D_2O concentration in the storage medium that too at 37°C.

5.3. Congo Red dye Assay

In the present study parent strain *of Gallibacterium anatis* biovar haemolytica failed to produce red or brown colonies on Congo red agar while adapted strain (51H) formed brown or dark brown colonies. The colour of colonies get darker with increase in CR dye in the growth medium. Congo red dye binding assay (CRBA) is established method to determine virulence traits of several bacterial species (Kay *et al*., 1985; Cangelosi *et al*., 1999; Kimizuka *et al.*, 2009). Congo red binding (CRB) cells produce intense red colonies and non-CRB cells produce colourless colonies (Qadri *et al*., 1988), this property has been shown to be associated with cell surface hydrophobicity (CSH) in many of the bacteria colonizing the skin and various mucosal surfaces (Doyle & Rosenberg, 1990; Goulter *et al*., 2010). Though, *G. anatis* bv. haemolytica is known to colonise in upper respiratory and lower reproductive tract (Bisgaard, 1977; Mushin *et al*., 1980). CRB does not appears to be an important virulence marker for *G. anatis* bv. haemolytica as the CR binding increased on subculturing of the bacteria which is known to cause decrease in virulence (Cruickshank *et. al*., 1968).

Though the parent strain of *G. anatis* bv. haemolytica didn't grow in presence of bile salt, growth of D_2O adapted strain in presence of bile salt indicated some physiological change in the bacteria. Similar effect of bile has been reported on over expression of fimbriae and increase in bacterial adhesion in *E. coli* and *B. fragilis* (de Jesus *et al*., 2005; Pumbwe *et al*., 2007).

5.4. Effulx pump

Efflux-mediated drug resistance has been reported in bacteria (Li and Nikaido, 2009). Out of 5 strains tested including parent and adapted strain of *G. anatis* bv. haemolytica, HLY,

14H and 25H had active efflux pump forming non-fluorescent colonies on EtBr medium while 51H and PH4 (6PH4) strains growth showing fluorescence indicated loss of efflux pump activity. However, studies on drug resistance and MIC patterns of adapted and parent strains didn't corroborated with the activity of efflux pump in the study. It might be due to the fact that all kinds of drug resistance in not mediated through efflux pum activity (Kumar and Singh, 2013). Although tetracycline resistance determinant (tet31) *G. anatis* is reported to be mediated through efflux pump activity (Bojesen *et al*., 2011), in the present study tetracycline resistant *G. anatis* bv. haemolytica strain remained resistant to tetracycline even after complete loss of efflux pump in adapted strain. This might be due to maintenance of other tetracycline resistance trait by *G. anatis* bv. haemolytica as reported earlier in many other bacteria (Kumar and Singh, 2013).

5.5. *Gallibacterium anatis* bivovar haemolytica antibodies in birds and animals

The study revealed presence of antibodies for formalinized antigen (probably fimbrial antigens) and O antigen of *G. anatis* bv. haemolytica in serum of birds as well as goat and pigs. It indicated that though *G. anatis* bv. haemolytica was isolated for the first time in India, it may not be a novel pathogen in Indian environment. It necessitate the study to understand the prevalence of the pathogen in different birds and animals in India. Similar studies in other parts of the world has revealed prevalence of *G. anatis* bv. haemolytica infection in many parts and animals of different continents (Wang *et al*., 2011; LI *et al*., 2012).

5.6. Haemagglutination patterns (fimbriae)

Parent as well as adapted isogenic strain of *Gallibacterium anatis* biovar. haemolytica failed to agglutinate erythrocytes of most of the animals except rat, pig and dog. Adapted strain failed to induce mannose resistant haemagglutination (MRHA) of rat erythrocytes and mannose sensitive HA (MSHA) of pig erythrocytes but it started to induce MSHA of dog erythrocytes. This might be due to change in binding epitopes of fimbriae resulting in changed affinity to different erythrocytes. This kind of change might be associated with change in protein folding or tightening associated with growth in presence of D_2O (Katz *et al.*, 1957; Thomson,

1960; Katz and Crespi, 1971; Ramakrishnaiah, 1990; Cioni and Strambini, 2002). The haemagglutinating ability of bacteria for different erythrocytes has been used to screen for the presence of the different colonization factor or fimbrial antigens of bacteria (Evans *et al*., 1979). Either the change in HA pattern observed in the present study was associated or not with colonization ability of adapted strain needs further studies.

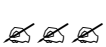
5.7. Minimum Inhibitory Concentration and antimicrobial drug resistance

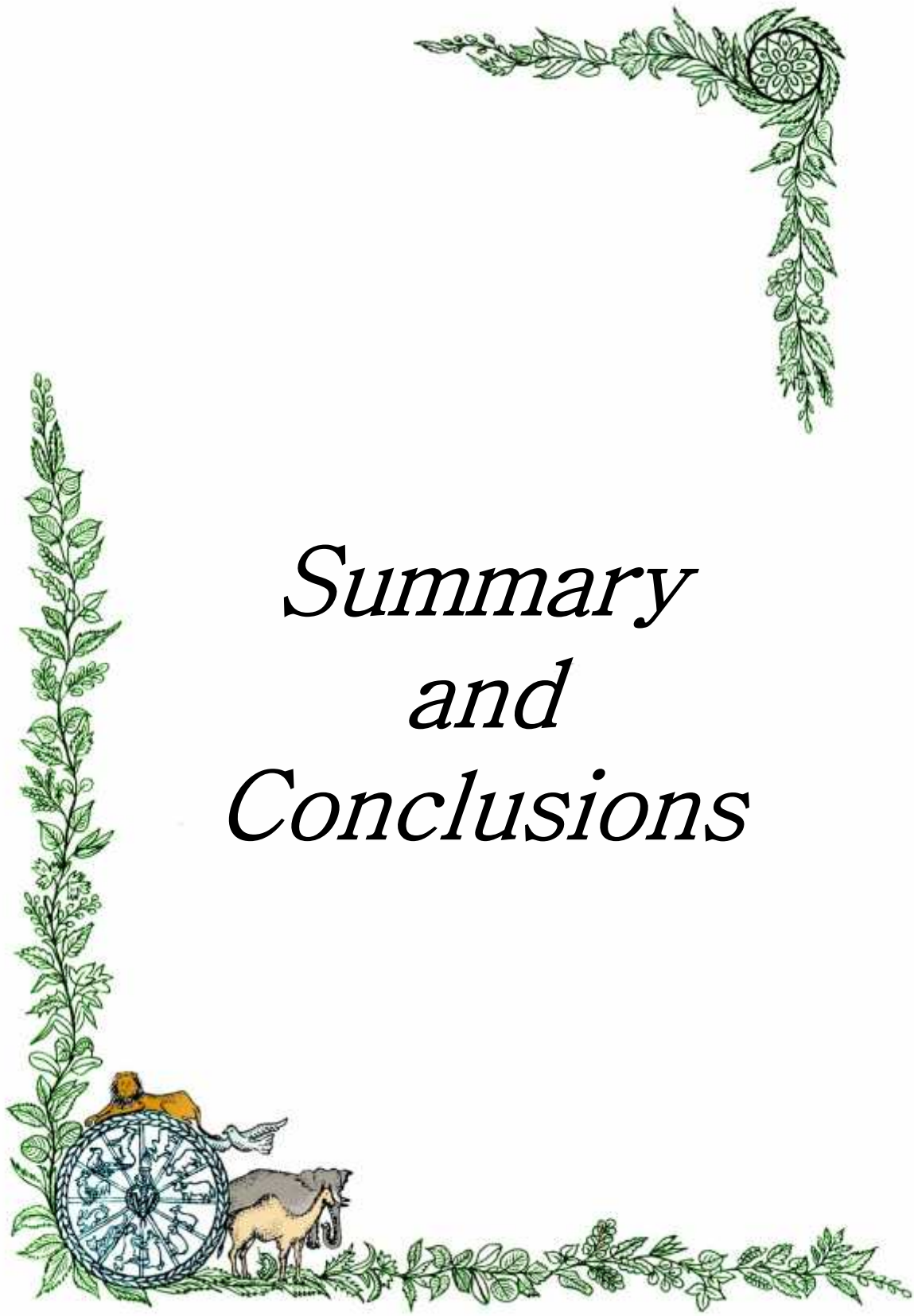
The study strains (adapted and parent) both were resistant to penicillin, tetracycline, cotrimoxazole and ciprofloxacin. Though the inhibition zone for penicillin was more (18 mm) for adapted strain than the parent (14 mm), it cannot be marked sensitive. The observation corroborate well with the earlier reports of resistance in *Gallibacterium* strains of poultry origin (Post *et al*., 1991; Watts *et al*., 1994; Mevius and Hartman, 2000; Lin *et al*., 2001; Malik *et al*., 2005; Hendriksen *et al*., 2008 and Johnson *et al*., 2011). Emergence of antimicrobial resistance has been observed among several organisms belonging to the Pasteurellaceae family (Aarestrup *et al*., 2004; Aarestrup, 2005) including *G. anatis* isolates. No difference in adapted and parent strain with respect to antibiotic sensitivity revealed stability in the trait which might be associated with several mechanisms of resistance than efflux pump (Kumar and Singh, 2013). The antibiotic sensitivity assay results were further confirmed by MIC studies of the two strains. The study revealed two fold decrease in sensitivity on adaptation for ampicillin (32 mcg/ ml) and ciprofloxacin (128 mcg/ ml), it may not be the simple effect of subculturing as on subculturing studies has indicated icrease in sensitivity rather decrease (Kumar and Singh, 2013). It can be explained with the changed molecular structure of bacteria on adaptation to grow in presence of D_2O leading to important changes in molecular structure of macromolecules some of them might be associated with decrease or altered receptors for the drugs (Katz *et al*., 1957; Thomson, 1960; Katz and Crespi, 1971; Ramakrishnaiah, 1990; Cioni and Strambini, 2002). The adapted strain had less MIC than parent strain for azithromycin and chloramphenicol, which can be attributed as effect of sub-culturing during adaptation procedure as reported earlier (Kumar and Singh, 2013).

5.8. Protein profiles of adapted and parent strain

Results of protein profiling revealed changes in proteins of the two strains. The change might be due to just change in macromolecular structure, protein folding or tightening of the protein molecules altering the migration of the proteins or it might be due to induction of synthesis of new protein molecules to cope the stress of D_2O or might be due to stoppage of expression of some proteins of parent strain. However, the dilemma can only be cleared through more studies at molecular level. Heavy water is known to make changes in molecular folding of biological macromolecules (Milstien *et al*., 1997; Newman *et al*., 1995; Sen *et al.*, 2009) and changes in gene expression (Hohlefelder *et al*., 2013) in several life forms leading to overexpression of certain heat shock proteins (Unno *et al*., 2005).

The observation of the study indicated that *G. anatis* bv. haemolytica though difficult to isolate and grow in general laboratory was quite able to adapt the stress and grow even in presence of 99% D_2O. Further, there was no much effect of adaptation of *G. anatis* bv. haemolytica to grow in D_2O medium specifically on survivability, growth rate and antimicrobial drug resistance. However, there was appreciable effect detected in protein profile of the two strains and also with respect to haemagglutination and Congo red dye binding, which might affect its pathogenic potential and warrant more targeted studies. The addition of D_2O in storage medium was helpful in long term maintenance of the pathogen even at 37°C which is an important finding and can be utilized for thermostable packaging of live vaccines. The study concludes that the *G. anatis* bv. haemolytica infection might also be common in India and needs further studies to establish the true prevalence of the infection.





Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

India is a country where agriculture and animal husbandry sectors are the important pillars of economy. In the livestock industry, poultry sector has emerged the fastest as an eco-business in world. Due to significant losses due to diseases conditions caused *Gallibacterium anatis* infection; it has been identified as one of the important emerging bacterial diseases in Asian countries. Due to its fastidious nature, commensal status and with no pathgnomonic lesions in diseased birds *G. anatis* infection often remains obscure for diagnosis. The outbreaks of the disease are reported all over the world and recently in northern India (Singh, 2015, IVRI Annual Report 2015-16). However, little is understood about the pathogen with respect its adaptation to changing environment, expression of its virulence factors, diagnosis of the infection and utility/ availability of good vaccine for control of the outbreaks. The present study was designed to understand the growth kinetics, survival, thermal death point, multiple drug resistances and phenotypic characteristics, changes in protein profile in presence of D_2O using an D_2O adapted and parent strain. The genotypic confirmation of *G. anatis* was done with PCR through observation of three band of different molecular weight by gel electrophoresis due to presence of internal transcribed spacer region (ITS) separating the 16s and 23 gene. *Gallibacterium anatis* growth at different levels of D_2O in growth medium and differences in growth of D_2O adapted and parent strains were observed with reference to colony morphology, haemolytic zone, growth patterns in liquid thioglycollate medium, and microscopic morphology of bacteria.

In presence of heavy water in growth medium *G. anatis* took longer time to grow than without D_2O. On increasing the concentration of the heavy water (10%, 20%, 30%, 50%, 75%, 90% and 99%) prolongation of lag phase was evident. However, when 50% water contents of medium were replaced by D_2O growth was much better than at other concentration of D_2O in growth medium. Even D_2O adapted strain grew better in medium without D_2O.

Both adapted as well as non-adapted *G. anatis* strains survived at 60°C for 2 minutes but not for 3 minutes, while at 55°C for 15 minute but not for 16 minute, irrespective of adaptation to grow in D_2O.

Irrespective of adaptation to grow in D_2O survival at 37°C was for the longest periods (119days) in comparison to other temperatures as at 45°C, 25°C and 10°C. On storage in presence of D_2O, survival was better at 37°C than at 45°C and 4°C suggesting that addition of D_2O in storage medium might be helpful to increase the life of bacteria.

Phenotypic differences in parent and adapted strain of *G. anatis* biovar haemolytica were observed with respect to colony morphology, haemolysis zone, Congo red dye binding, haemagglutination patterns, drug resistance, ability to grow in anaerobic environment and presence of Congo red dye and bile salt, and in activity of efflux pump. At molecular level effect of adaptation was evident in protein profiles of the two strains.

With the increase passage level of the parent strain in D_2O inactivation of efflux pump was more and more evident and finally got inactivated. Congo red dye binding assay was negative for parent strain but brown to dark brownish colonies of adapted strain (51H) was observed. Mannose sensitive haemagglutination (MSHA) of pig erythrocyte was detected with parent *G. anatis* strain while MSHA tanned dog erythrocyte was detected with adapted *G. anatis* strain only. Antibodies against boiled and formalized antigen of *G. anatis* were detected in poultry, pig and goat serum with agglutination titres ranging from <2 to 128.

6.1. Conclusion

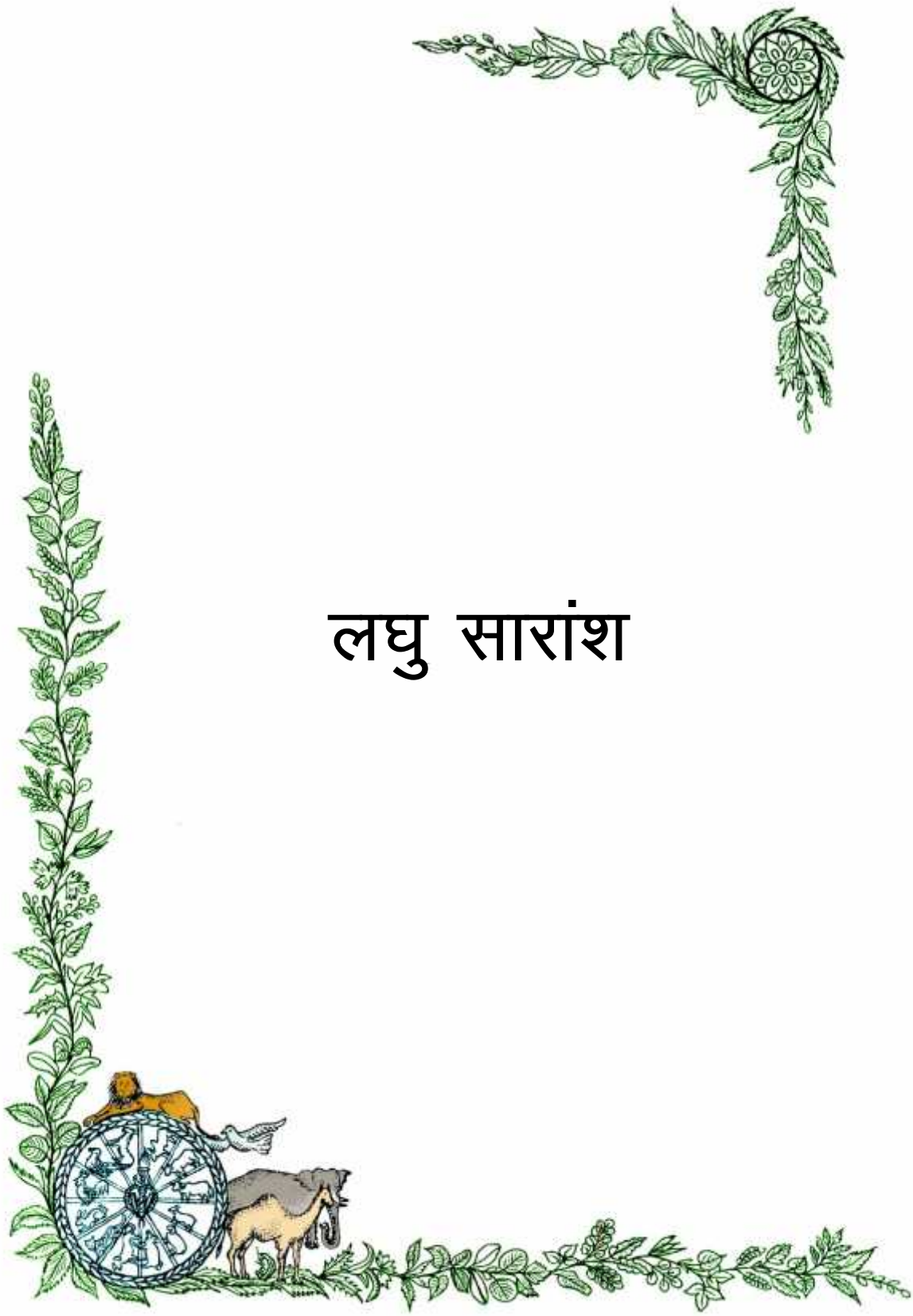
Heavy water induced changes in phenotypic characteristics of *G. anatis* both at macroscopic and molecular levels. Detection of *G. anatis* antibodies in serum of healthy

poultry birds (23/70), Pig (8/7) and Goat (8/8) indicated prevalence of infection in birds and animals in Bareilly region. The study revealed that *G. anatis* can adapt easily to grow in D_2O and probably in other stressful environment too. Adaptation to grow in presence of D_2O not affected survival of *G. anatis* at different storage temperature, however, caused reduction in MIC of azithromycin, chloramphenicol and increase in MIC of ampicillin and ciprofloxacin. Though growth of *G. anatis* was slower in presence of D_2O irrespective of adaptation or no-adaptation, adapted strain grew much faster than parent strain in presence of D_2O, similarly in presence of 0.01% to 0.03% Congo red dye in growth medium. The adapted strain of *G. anatis* also grew in presence of bile salt while parent strain failed to grow. Protein profiling of adapted and parent strain of *G. anatis* revealed that some of the protein were missing in adapted while few were missing in parent strain indicating that D_2O causes either changes in expression or in molecular weight of protein or in folding of protein molecules affecting migration of protein in NATIVE-PAGE and SDS-PAGE. Back passage of adapted strain in medium without D_2O helped the bacteria to regain the parental protein profile. Study on HA of fresh as well as tanned RBCs with adapted and parent strain indicated that adaptation to grow in presence of D_2O causes change in HA pattern i.e., expression of fimbriae. Survival of *G. anatis* was always better in presence of D_2O (20% to 99%) irrespective of prior exposure of D_2O (adaptation) or storage temperature.

Mini Abstract

7 MINI ABSTRACT

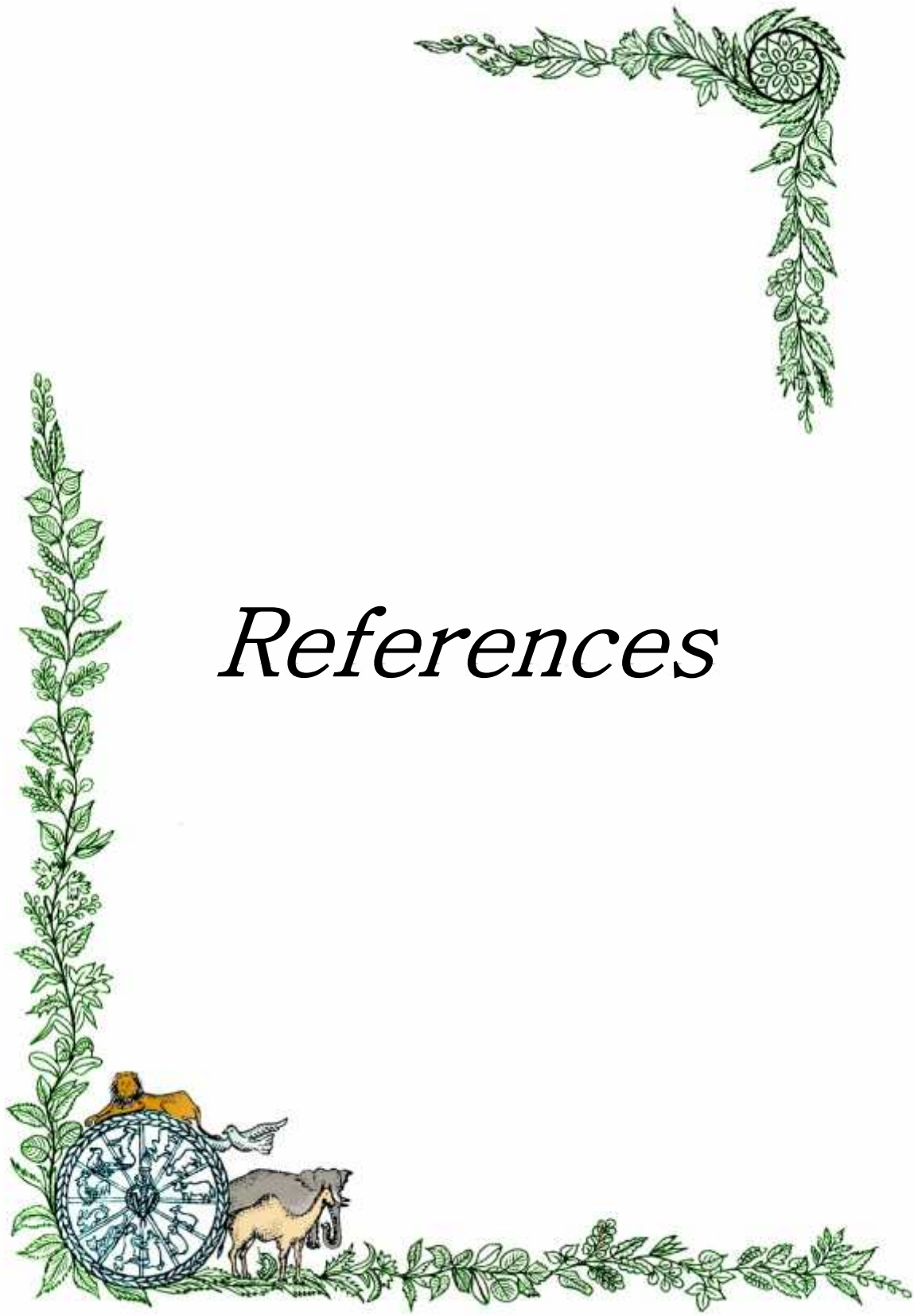
Gallibacterium anatis is an important pathogen of intensively farmed poultry birds causing loss in production with heavy mortality in broiler chicken and drop in egg production in layers with increased mortality. *Gallibacterium anatis* (earlier known as *Pasteurella anatis*) is resident in the upper respiratory tracts and the lower genital tracts of healthy chickens. It has been reported to be associated with bacteremia, oophoritis, follicle degeneration, salpingitis, peritonitis, hepatitis, enteritis, and respiratory tract diseases in chickens. Due to its fastidious nature, commensal status and with no pathognomonic lesions in diseased birds *G. anatis* infection often remains obscure for diagnosis. Poor understanding of its growth kinetics, environmental survival, epidemiology, virulence factors and pathogenesis work on development of effective vaccine get obscured. However, once entered it is difficult to get rid of *G. anatis* infection on affected poultry farms. Thus to understand more detailed studies on its virulence factors, growth kinetics and phenotypic characterization is necessary. This study using conventional bacteriological, serological and molecular method (PCR, cloning and sequencing) revealed the potential of *G. anatis* to survive and grow under adverse environment. *Gallibacterium anatis* biovar haemolytica included in this study was isolated from heart blood in pure culture from broiler birds dying *en masse* in May-June 2015 Izatnagar, Bareilly and in nearby villages (Singh, 2015, IVRI Annual Report 2015-16). The *G. anatis* biovar haemolytica isolate got adapted to grow in 99% heavy water (D_2O, Deuterium oxide). On comparison of heavy water adapted strain with parent strain (non-adapted) for survival, growth kinetics, thermal death point, multiple drug resistances and phenotypic characteristics revealed that *G. anatis* die at 60°C in 3 minutes and in 16 min at 55°C, irrespective of adaptation However, adaptation to D_2O lead to reduction in minimum inhibitory concentration (MIC) of azithromycin and chloramphenicol and increase in MIC of ampicillin and ciprofloxacin. Irrespective of adaptation, *G. anatis* survival at 37°C was the best than at 4°C and 45°C . However survival of adapted strain at 45°C in 99% D_2O was better than parent strain. Growth of parent strain at 37°C was faster than the adapted strain but with similar growth pattern. The growth of parent strain was slower than the adapted strain in media made in 99% heavy water. On subculture, the adapted strain had more consistent morphology and maintained its bacillary form while parent strain acquired coco-bacillary form. Delayed formation of string in KOH, better anaerobic growth in thioglycollate medium, reduced colony size and haemolytic zone, ability to grow in presence of bile salt and Congo red dye after adaptation to D_2O were some important changes observed. Besides, appreciable difference in protein profile and haemagglutination pattern of D_2O adapted and parent strains were also evident effects of adaptation to grow in presence of D_2O. The study concluded that *G. anatis* adaptation to grow in presence of D_2O may cause changes in morphology, growth and virulence factor expression probably through altering gene expression which needs to be explored further for explicit understanding of the effect of D_2O. However, this study has lucidly shown that for storage of *G. anatis* at 37°C in presence of D_2O was much better and may be a used for storage of live vaccines to enhance their thermo-stability.



लघु सारांश

8 लघु सारांश

सघन कुक्कुट उत्पादन प्रक्षेत्रों में *गैलीबैक्टेरियम एनाटिस (जी. एनाटिस)* एक महत्वपूर्ण रोग जनक जीवाणु है, जिसके कारण ब्रायलर कुक्कुटों में अधिक मृत्यु एवं मुर्गियों में अण्डे के उत्पादन में कमी के साथ मृत्युदर में वृद्धि होती है। *गैलीबैक्टेरियम एनाटिस* को पहले *पाश्चुरेल्ला एनाटिस* के नाम से जाना जाता था। यह जीवाणु स्वस्थ कुक्कुटों के श्वसन तंत्र के उपरी एवं जननांग के निचले भागों में मुख्यतः पाया जाता है तथा कुक्कुटों में जीवाणुरक्तता, डिंबग्रंथि शोथ, पुटक अपजनन, डिंबवाहिनी–शोध, पर्युदर्या शोथ, यकृत शोथ, आंत्र शोथ एवं श्वसन तंत्र की बीमारियाँ उत्पन्न करता है। इसकी दुराराध्य एवं सहभोजी प्रकृति तथा रोग ग्रस्त कुक्कुटों में विशिष्ट व्याधिज्ञापक विक्षति के कमी के कारण *जी. एनाटिस* संक्रमण के निदान के साथ अस्पष्टता बनी रहती है। इस जीवाणु के वृद्धि बलगतिकी, पर्यावरण अस्तित्व, जानपदकीय ज्ञान, उग्रता कारकों एवं रोग जनन की अल्प जानकारी के कारण प्रभावी टीके का विकास कर पाना संदेहपूर्ण है। गैलीवैक्टेरियम एनाटिस का संक्रमण किसी कुक्कुट प्रक्षेत्र में एक बार हो जाने पर इससे छुटकारा पाना मुश्किल है प्रस्तुत अध्ययन में पारम्परिक जीवाणुविक, सीरमीय एवं आणविक (पीसीआर, क्लोनिंग एवं अनुक्रमण) विधियों का उपयोग कर *जी एनाटिस* की संभावित अतिजीवन और प्रतिकुल वातावरण में जीवित रह सकने की क्षमता का पता लगाया गया है। इस अध्ययन में *जी. एनाटिस* जैव भेद हिमोलिटिका का उपयोग किया गया है, जो मई–जून 2015 में इज्जतनगर, बरेली, और आस–पास के गाँवों के ब्रायलर कुक्कुटों के हृदय रक्त से पृथक किया गया था जिनसे ब्रायलरों में अधिक मृत्यु हो रही थी। *जी. एनाटिस* को 99 प्रतिशत भारी पानी (D_2O ड्येटेरियम ऑक्साइड) में विकसित करने के लिए अनुकुलित किया गया। भारी पानी में अनुकुलित उपभेद की तुलना गैर अनुकुलित उपभेद से अस्तित्व, वृद्धिबलगतिकी, ताप मृत्यु बिन्दु, दवा प्रतिरोध, दृश्य प्ररूपी विशेषताओं के लिए किया गया। *जी. एनाटिस* (अनुकुलित एवं गैर अनुकुलित) 4 एवं 45ः सेल्सियस की तुलना में 37^0 सेल्सियस पर सबसे ज्यादा वृद्धि पायी गई तथ 55^0 से.ग्रे. पर 16 मिनट एवं 60^0 सेल्सियस पर 3 मिनट में मर जाता है। 99 प्रतिशत भारी पानी में अनुकुलित *जी. एनाटिस* उपभेद मूल उपभेद से 45^0 सेल्सियस पर सबसे ज्यादा अस्तित्व में रहता है। भारी पानी में अनुकुलन के कारण एजीथ्रोमाइसीन एवं क्लोरोफेनीकाल के एम.आई.सी. में कमी परन्तु एम्पीसीलिन एवं सीप्रोफ्लोक्सासिन के एम.आई.सी. में वृद्धि पायी गई। 99 प्रतिशत भारी पानी युक्त संवर्धन माध्यम में अनुकुलित *जी. एनाटिस* उपभेद मूल उपभेद से ज्यादा पायी गयी। अध' संवर्धन पर, अनुकुलित उपभेद का बैसीलरी आकार बना रहा। जबकि मूल उपभेद ने उपभेद का आकार बना रहा जबकि मूल उपभेद ने कोको–बैसीलरी रूप ले लिया। भारी पानी में अनुकुलित उपभेद में पोटेशियम हाइड्रोक्साइड स्टिंग की देरी से गठन, थायोग्लाइकोलेट माध्यम में बेहतर आवायवीय विकास, कॉलोनी आकृति एवं रक्तसंलीय जोन में कमी, पित्त लवण और कांगों लाल रंग की उपस्थिती में विकास करने की क्षमता इत्यादि महत्वपूर्ण परिवर्तन मिला। भारी पानी अनुकुलित उपभेद एवं मूल उपभेद के प्रोटीन पार्श्व दृश्य एवं रक्ताणु–समूहण पैटर्न में पर्याप्त अंतर पाया गया। इस अध्ययन के परिणामों से यह निष्कर्ष निकला कि *जी. एनाटिस* का भारी पानी में संवर्धन करने से उसके आकृति वृद्धि एवं उग्रता की अभिव्यक्ति में परिवर्तन शायद जीन अभिव्यक्ति में अंतर आने के कारण हुआ। भारी पानी की उपस्थिती में *जी. एनाटिस* का भंडारण 37^0 सेग्रे. पर अति उपयुक्त पाया गया। अतः पारी पानी / जल, निर्मित जीवित टीका का उपयोग ताप–स्थिरता बढ़ाने के लिए भंडारण हेतु किया जा सकता है।



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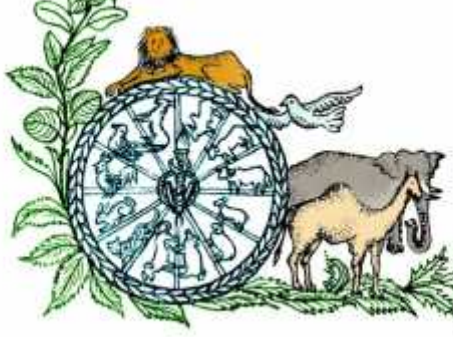
Appendix



APPENDIX

Bacteriological media

BLOOD AGAR

Yeast extract and agar in BHI (Himedia)

Sheep blood 5-10%

Blood agar base first sterilized by autoclaving at 15 psi (1.05Kg/cm2) for minutes and after cooling to 50°C, blood should be added.

BRAIN HEART INFUSION AGAR (BHI Agar) (BD, Difco)

Calf Brains, Infusion from 200g	7.7 g
Beef Heart, Infusion from 250g	9.8 g
Proteose Peptone	10.0 g
Dextrose	2.0 g
Sodium Chloride	5.0 g
Disodium Phosphate	2.5 g
Agar	15.0 g
Distilled water	1000 ml

Dissolve the ingredient by boiling, sterilize by autoclaving at 121°C at 15 psi (1.05 Kg/ cm^2) for 20 minutes and the solution was stored at 4°C.

EOSIN METHYLENE BLUE (EMB) AGAR (BD, Difco)

Peptic digest of Animal tissue	10.0 g
Di-potassium phosphate	2.0 g
Lactose	5.0 g
Sucrose	5.0 g
Eosin-y	0.4 g
Methylene blue	0.065 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.2

LURIA-BERTANI BROTH (LBB) (BD, Difco)

Tryptone	10.0 g
Yeast Extract	5.0 g
Sodium Chloride	10.0 g
Distilled water	1000 ml
pH	7.0

MacCONKEY AGAR (BD, Difco)

Pancreatic digest of gelatin	17.0 g
Peptic digest of animal tissue	1.5 g
Casein enzymic hydrolysate	1.5 g
Lactose	10.0 g
Bile salts	1.5 g
Sodium chloride	5.0 g
Neutral red	0.03 g
Crystal Violet	0.001 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.2

MULLER HINTON AGAR (BD, Difco)

Beef Extract Powder	2.0 g
Acid Digest of Casein	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	1000 ml
pH	7.3

MULLER HINTON BROTH (BD, Difco)

Beef Extract Powder	2.0 g
Acid Digest of Casein	17.5 g
Starch	1.5 g
Distilled water	1000 ml
pH	7.3

NUTRIENT AGAR (BD, Difco)

Beef extract	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	20.0 g
Distilled water	1000 ml
pH	7.2

OXGALL (BD, Difco)

Dehydrated Fresh Bile

100g/ 1000 ml Distilled water

TRYPTIC SOYA AGAR (BD, Difco)

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean	5.0 g
Sodium Chloride	5.0 g
Distilled water	1000 ml
pH	7.3

TRYPTICASE SOYA BROTH (BD, BBL)

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean	3.0 g
Sodium Chloride	5.0 g
Dipotassium Phosphate	2.5 g
Dextrose	2.5 g
Distilled water	1000 ml
pH	7.3

SOLUTIONS BUFFERS FOR AGAROSE GEL ELECTROPHORESIS

a) 0.5 M EDTA (pH 8.0)

EDTA (Disodium dihydrate salt)	18.60 g
Double distilled water	80 ml

The pH was adjusted to 8.0 with NaOH pellets. The volume was made up to 100 ml.

b) 0.5 M EDTA (pH 8.0)

EDTA (anhydrous acid free EDTA (Molecular Weight 292.24)	14.61 g
Double distilled water	80 ml

The pH was adjusted to 8.0 with NaOH pellets. The volume was made up to 100 ml.

c) Tris-borate-EDTA (TBE) buffer 5X

Tris-base	54 g
0.5 M EDTA (pH 8.0)	20 ml
Boric acid	27.5 g
DW	900 ml

Add DW to make the final volume upto 1000 ml. A working solution of 0.5X used.

d) Ethidium bromide stock solution (10 mg/ml)

Ehidium bromide	100 mg
Distilled water	10 ml

The solution was mixed and stored at 4°C. A concentration of 0.5 mg/ml was used

e) DNA ladder marker

100bp Plus ladder: It contains 12 bands ranging from 100bp to 1500 bp

100bp ladder: It contains 6 bands ranging from 100 bp to 600 bp

f) Isopropanol

Molecular biology grade stored at 4°C

g) 70 % Ethanol

Dehdrated ethanol	70 ml
TDW	30 ml

Store at 4

MISCELLANEOUS BUFFERS AND SOLUTIONS

AlSEVER'S SOLUTION

Dextrose	2.05 g
Sodium citrate	0.80 g
Sodium Chloride	0.42 g
Distilled Water	100 ml

pH adjusted to 6.1 with 10% citric acid before autoclaving at 10 1bs for 15 minutes. Filter before used with syringe filter and stored at 4°C until used.

PHOSPHATE BUFFER SALINE (PBS; 0.85 M, pH-7.2)

Sodium chloride (NaCl)	8 g
Disodium hydrogen phosphate	1.16 g
Potassium chloride	0.2 g
Potassium dihydrogen orthophosphate	0.2 g
Distilled water	1000 ml

Sterilized at 151bs pressure for 20 min and stored at 4°C until used.

NORMAL SALINE SOLUTION (NSS; 0.85 M, pH-7.00)

Sodium chloride (NaCl)	8.5 g
Distilled water	1000 ml

SOLUTIONS FOR ESTIMATION OF PROTEIN

Modified Lowry's Method (for 0.1 to 1 mg / ml protein)

Solution A:1% $CuSo_4.5H_2O$	1 g/ 100 ml distilled water
Solution B: 2% sodium potassium tartarate	1 g/ 50 ml distilled water
Solution C: 0.2 M, NaOH	0.8 g/100 ml
Solution D: 4% Na_2CO_3	4 g/100 ml

Reagent Mixtures

Solution E: 49 ml each of solution C and solution D and 1 ml each of solution A and solution B were mixed prior to use.

Standard solutions

1% bovine albumin	0.1 g in 10 ml distilled water

Folin Reagent: To 10 ml Folin Ciocalteau, add 10 ml Distilled water and use it as reagent F.

Procedure: To 0.5 ml sample containing up to 0.5 mg protein, mix 2.5 ml of reagent E and keep for 10 min then add 0.25 ml of reagent F and incubate for 30 min at RT. Measure absorbance at 750 nm.

Lysis Buffer

NaCl	100 mM
TrisHCl	25 mM
pH	8.0

REAGENTS AND SOLUTIONS FOR SDS-PAGE

1. **30% Acrylamide- Bisacrylamide mix**

Acrylamide	29.0 g
Bis-acrylamide	1.0 g

Make volume upto 100 ml using distilled water. Mix by boiling and filter. Store at 4°C.

2. **0.5M Tris**

Tris	6.05 g

Dissolve in 65 ml of distilled water by gently heating. pH adjusted to 8.8 using 1N HCl and volume made up to 100 ml with distilled water. Autoclave and store at 4°C

3. **1.5M Tris (PH 8.8)**

Tris	18.15

Dissolve in 65 ml of distilled water by gentle heating. pH adjusted to 8.8 using 1N HCL and volume made up to 100 ml with distilled water. Autoclave and store at 4°C.

4. **10% SDS**

Sodium dodecyl sulphate	10 g

Made volume upto 100 ml with distilled water

5. **10% APS**

Ammonium per sulphate	100 mg

Make volume up to 100 ml with distilled water. Mix and store at 4°C

6. **Tris glycine buffer (5X) (electrode buffer)**

Tris base	15.1 g
Glycine	54.0 g
DW	900 ml
10% SDS	50 ml

Adjust volume to 1000 ml and adjust pH to 8.2

7. **Sample loading buffer (2X)**

Tris HCl (1M, pH 6.8)	3.12 ml
Glycerol	5 ml
10% SDS	10 ml
2-Mercapto ethanol	1 ml
Bromophenol blue	1 mg

Adjust the volume to 25 ml using DW. Mix and store at 4°C

8. **Staining solution**

Coomassie brilliant blue	1.0 g
Methanol	250 ml

Mix by stirring

Acetic acid	50 ml

Mix and filter through whatman filter paper no. 1 and store at amber coloured bottle.

9. Destaining solution

Methanol	150 ml
Glacial acetic acid	50 ml
DW	300 ml

10. GEL COMPOSITION FOR SDS-PAGE

COMPONENT	RESOLVING GEL (12%)	STACKING GEL (5%)
DW	4.9 ml	3.4 ml
1.5 M Tris (pH 8.8)	3.8 ml	-
0.5 M Tris (pH 6.8)	-	630 µl
30% Acrylamide-Bisacrylamide mix	6.0 ml	830 µl
10% SDS	150 µl	50 µl
10% APS	150 µl	50 µl
TEMED	6 µl	5 µl

11) PROTEIN coloured marker for SDS-PAGE (LONZA)

9 colour bands ranging from 11 kDa to 184 kDa or 10-180 kDa.

REAGENTS AND SOLUTIONS FOR NATIVE-PAGE:

Buffers:

For a 5ml native PAGE stacking gel:

1.	0.375 M Tris-HCl pH=6.8	4.275 ml
2.	Acrylamide/Bis-acrylamide(30%/0.8% w/v)	0.67 ml
3.	10% (w/v) ammonium persulfate (AP)	0.05 ml
4.	TEMED	5 µl

For a 10ml native PAGE separating gel:

	Acylamide percentage	**10%**
1.	Acrylamide/Bis-acrylamide(30%/0.8% w/v)	3.4 ml
2.	0.375M Tris-HCl (pH=8.8)	6.49 ml
3.	10% (w/v) ammonium persulfate (AP)	100 µl
4.	TEMED	10 µl

Sample Buffer for 10 ml (2X):

1.	62.5 mM Tris-HCl, pH 6.8	0.1 g
2.	25% glycerol	2.5 ml
3.	1% Bromophenol Blue	0.1 g

Running Buffer (For 1Litre):

1.	25 mM Tris	3.9 g
2.	192 mM glycine	14.41 g

Note: running buffer should be~ pH 8.3. Do not adjust the pH.

A) Silver staining solution

Fixative:

40% methanol; 10% acetic acid; 50% water

Wash Sol:

30% ethanol in water

Water:

Single and Triple d.i. water

Reductant:

200 mg sodium thiosulfate in 1 liter water

Silver stain:

2 g silver nitrate; 200 µl formaldehyde in 1 liter of water. Wrap bottle in aluminum foil to prevent light from getting in.

Developer:

30 g sodium carbonate; 5 mg sodium thiosulfate; 500 µl formaldehyde in 1 L water

Stop Sol:

5% acetic acid in water

VITAE

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Veterinary Science & Technology

Singh, et al., J Veterinar Sci Techno 2016, 7:3
http://dx.doi.org/10.4172/2157-7579.1000324

Review Article Open Access

Gallibacterium anatis: An Emerging Pathogen of Poultry Birds and Domiciled Birds

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Rec date: Feb 09, 2016; **Acc date:** Mar 16, 2016; **Pub date:** Mar 18, 2016

Abstract

Gallibacterium anatis though known since long as opportunistic pathogen of intensively reared poultry birds has emerged in last few years as multiple drug resistance pathogen causing heavy mortality outbreaks not only in poultry birds but also in other domiciled or domestic birds. Due to its fastidious nature, commensal status and with no pathgnomonic lesions in diseased birds *G. anatis* infection often remains obscure for diagnosis. Poor understanding of its epidemiology, virulence factors and pathogenesis work on development of effective vaccine obscured its importance; however, it is difficult to get rid of *G. anatis* infection on affected poultry farms. The present review summarises the current knowledge on *G. anatis* and its infections.

Keywords: *Gallibaterium*; *Pasteurella anatis*; Egg drop; Mortality outbreaks; Multiple drug resistance; Vaccine

Introduction

Global meat production is predicted to rise by 1.6% over the outlook period, with poultry becoming the largest meat sector by 2020 [1]. *Gallibacterium anatis* infection is an emerging disease of poultry. Growing concern about *G. anatis* is its poorly understood growth kinetics, virulence markers, pathogenesis and vaccine(s) to control. *Gallibacterium anatis* (earlier known as *Pasteurella anatis*) is commensal in upper respiratory tracts and the lower genital tracts of healthy chickens [2,3]. It has been reported to be associated with bacteremia, oophoritis, follicle degeneration, salpingitis, peritonitis, hepatitis, enteritis, and respiratory tract diseases in chickens [4-8]. *Gallibacterium anatis* mostly affects intensively farmed poultry birds causing loss in production with heavy mortality in broiler chicken and drop in egg production in layers with increased mortality [9]. *Gallibacterium anatisis* also reported to infect turkeys, geese, ducks, pheasants, partridges, budgerigars, peacock, cage birds, wild birds, cattle and pig [2,3,10-15]. Recently, it has also been associated with fatal bacteremia in immune-compromised patient [16]. *Gallibacterium anatis* causing disease in birds has been reported from all continents [2,4,12,17,18]. Its association with a variety of pathology makes it difficult to be diagnosed even after post-mortem in absence of pathgnomonic lesion(s) and the disease is often confused with Fowl Coryza, New Castle disease and Bird Flu [12].

Gallibacterium anatisis, a Gram-negative, rod shaped, non-motile, capsulated, facultative anaerobic bacteria was classified in family Pasteurellaceae by Pohl [11,12]. *Gallibacterium anatis* has two biovars i.e., a haemolytic biovar, Haemolytica and a non-haemolytic biovar, Anatis [12]. Currently, *G. anatis* and *G. genomospecies* 1, 2 [12], *G. genomospecies* 3 and *Gallibacterium* group V are the defined members of the genus *Gallibacterium* [11].

Though the infection of *G. anatis* is treatable with antibiotics, the frequency of treatment failure is an emerging and recurrent problem. Multidrug resistant strains of *G. anatis* [5,19] have shown resistance to sulpha drugs, novobiocin, tylosin, clindamycin, tetracycline and penicillin [20-28]. Concerns have been shown for biosecurity measures towards control of disease, handling of pathogen and prevention of spread. *Gallibacterium* should be handled at biosafety level 2 (BSL-2) facilities, it has a little potential personnel and environmental risk, and however, more is too understood for its exact categorisation to some risk group.

Taxonomy of *G. anatis*

It belongs to phylum, Proteobacteria; class, Gammaproteobacteria; family, Pasteurellaceae; genus, *Gallibacterium* and has two biovars, heamolytica and anatis [12,29]. First time in 1950 the bacteria was isolated from cloaca of healthy chickens and was described as haemolytic "cloaca bacterium" by Kjos-Hansen. The meaning of *Gallibacterium* is 'bacterium of chicken'. Being similar to *Pasturella* in several characters it was earlier known as *P. anatis*. The genus name Gallibacterium was first given by Bisgaard in the year 1982 on the basis of certain phenotypic characters used for identification of *Actinobacillus salpingitidis* and avian *P. haemolytica* [2,10,11,30,31]. Christensen et al. [32] established the genus *Gallibacterium* within the family of Pasteurellacae based on 16s rRNA gene sequences. The genus includes the strains belonging to *G. anatis*, *G. genomospecies* [1-3,12] and un-named group V [11]. Taxon 1 designated as a third group of strains, by Bisgaard in 1982 [30] and named Pasteurella anatis [29], was also found closely related to *A. salpingitidis* and avian *P. haemolytica*.

Besides this, comparison of whole genome by protein profiling [33]and amplified fragment length polymorphism [34] uncover the unique properties of five groups and assigned the proposal of new name to these three novel species of *Gallibacterium* naming, *G. melopsittaci* sp. nov. (Type strain F450T 5CCUG 36331T 5CCM 7538T), *G. trehalosi* fermentans sp. nov. (Type strain 52/S3/90T 5CCUG 55631T 5CCM 7539T) and *G. salpingitidis* sp. nov. (Type strain F150T 5CCUG 15564T 5CCUG 36325T 5NCTC 11414T).

However, with DNA -DNA hybridization they could not be clubbed into one genus [11,35]. Confusion is still persistent in naming biovars and may be a continued debate in coming years.

Epidemiology

Gallibacterium have been reported from many European countries viz., Switzerland, Denmark, Germany, Austria [2,36-38], African countries viz., Nigeria [4], Asian countries including China, India and Japan [17], American countries viz., USA [18,39,40], and several other countries like Colombia, Taiwan, Norway, Australia, Syria, England, Sweden and Czech Republic and recently in India. The epidemiological outcome strongly depends upon strain, route of inoculation and secondary factor [9]. Host and environmental factors have been observed to nurture the infection of *G. anatis*. Host factors playing a role are hormonal influences [41-43], age [2,44], stress [15], and compromised immunological status [45]. Important environmental factors are seasonal changes [23], and cold stress [46] in the similar pattern reported for infections with other members of Pasteurellaceae [38,47,48].

Habitat and host range

Gallibacterium strains are known since long as common inhabitants of the respiratory tract and lower genital tract of healthy chickens [2,10,49]. *Gallibacterium anatis* has been isolated from chickens, ducks, geese, guinea fowl, turkeys, pheasants, psittacine birds, partridges, web-footed birds, cattle egrets and budgerigars [3,10,49]. Especially biovar haemolytica has also been isolated from healthy [2,3,49,50] as well as sick birds, cattle and pigs [10-12,30,42,51].

Cultural and biochemical characteristics

The family Pasteurellaceae comprises large group of facultative anaerobes, gram-negative, non-sporing, chemo-organotrophic and fermentative bacteria including the genus *Gallibacterium*. All *Gallibacterium* species strains are non-motile have rod-shaped or pleomorphic cells occurring singly or in pairs. Colonies on bovine blood agar are strongly β-haemolytic, smooth, greyish, non-transparent, shiny and circular, raised, with entire margins, 1-2 mm diameter after 24-48 h at 37°C, and glowing at the periphery having appearance of butyrous consistency [8,9]. All strains are catalase, oxidase, and phosphatase positive, and reduce nitrate [12]. *Gallibacterium* genus can be differentiated from other genera of Pasteurellaceae with catalase, symbiotic growth, hemolysis, urease, indole, acid production from (+) D-xylose, (-) D-mannitol, (-) D-sorbitol, (+) D-mannose, maltose, raffinose, dextrin, ONPG and PNPG tests [18].

Transmission of *G. anatis* infection

The common way of spreading infection is through horizontal dissemination. Infection of month old poultry birds common and appears to be natural [2]. Vertical transmission though not common in Pasteurellaceae family, trans-ovarian infection supporting the vertical transmission has been experimentally proved for *G. anatis* [52]. Isolation of *G. anatis* from the egg yolk and detection of *G. anatis* in a 4-day-old chicken descended from a diseased parent also supported vertical transmission hypothesis for the infection [43,45,53]. Ascending infections appears to be the most probable route for the infection of reproductive organs [38,50]. Venereal transmission of infections also seems to be feasible as the bacterium has been detected in semen of infected cockerels [54]. Under favourable circumstances, the bacterium can invade systemic circulation from its natural habitats in respiratory and reproductive tract [38,39,55-57].

Virulence factors

RTX toxins are found in many members of Pasteurellaceae and are responsible for the haemolytic and leukotoxic properties in *G. anatis* [58]. *Gallibacterium anatis* biovar haemolytica produces haemolysin like GtxA which is a type of RTX-toxin [60]. GtxA induce a strong leukotoxic effect on avian macrophages and is labelled as a most important virulence factor of *G. anatis* [7,43,59]. GtxA toxin has two domains, C-terminal with homology to other RTX toxin and N-terminal with no homology. C-terminal is responsible for haemolytic function but function of N-terminal is unknown, however, this domain is required for complete haemolytic activity [59]. The four operon genes are responsible for RTX toxin and are transcribed in an order i.e., rtxC, rtxA, rtxB, and rtxD [43]. MARTX (multifunctional autoprocessing RTX) toxins bind and regulate the actin protein of cytoskeleton by which microorganism making a path of immune evasion strategy [60].

Gallibacterium anatis have capability to adhere on the epithelial cells of chicken and other host cells [61,62] by short fimbriae [63]. A number of fimbriae of different sizes and shapes have been defined belonging to the F17-like fimbriae and are grouped in 1-3 different fimbrial cluster [64]. The flfG gene cluster is responsible for adhesion protein of F17 family [65]. One of the detected fimbriae seems to be type IV-like pili having bundle structure formed by thin filament like other pathogenic microorganisms evincing type IV pili [66]. Type IV fimbriae are appendages participating in intercellular motility, microcolony formation, colonization, and the secretion of proteases by host tissues [67].

Gallibacterium anatis produces outer membrane vesicles (OMVs) *in vitro* [65] similar all other Gram-negative bacteria. Virulence properties of OMVs shown by microorganism are adherence, colonization, binding and removal of antibacterial substances along with antibiotics which envisage the survival of microorganism [68,69]. Beside, periplasmic components, compounds of cytoplasmic origin such as DNA have also been found as components contents in OMVs [69,70].

Gallibacterium have capsule in some strain which may contain virulence properties as seen in *Pasteurella multocida* [71]. Capsule is a general structure made up of extracellular polysaccharide and has been reported in both Gram-negative and Gram-positive pathogens [72]. The presence of a thin capsule on *G. anatis* has been observed by transmission electron microscopy [73]. The presence of a capsule in primary culture and disappearance after sub-culture is common finding [14].

Gallibacterium anatis metalloproteases may be having role in infection process [74] are extracellular Zn containing enzymes [74-76], however, their exact role is still to be understood.

Ability of a bacterium to form biofilm indicates its ability of adherence to surfaces and live tissues and plays role in pathogenesis of persistent and chronic infections with increased resistance to antimicrobials [77,78]. Biofilm forming ability varies between isolates of *G. anatis* and categorises as weak, moderate and strong biofilm producer [79].

Some strains of *G. anatis* agglutinate avian erythrocytes [80,81]. The presence of a potential haemagglutinin in OMVs released from *G. anatis* has been observed [79,82]. The activity of haemagglutinating (HAs) to RBC of host are of mainly depend on two type adhesins naming filamentous adhesins and non-filamentous adhesins of pathogens [83]. The type identity of adhesins of *G. anatis* involve in HAs are still unknown [81]. Some of these haemagglutinins could be responsible for the observed agglutinating activity of some strains [79,82-84].

Clustered regularly interspaced short palindromic repeats (CRISPRs) consider as a defence system of bacteria against foreign invasive DNA, such as DNA from phages and plasmids. The difference of natural competence have been explicate between strains of *G. anatis* by CRISPRs interfere in the process of transformation [85,86].

Integrative conjugative elements (ICE) have genes which are present within these elements are capable to excise and integrate in the genome [87]. Identification of ICEs have been reported in the genomes of *G. anatis* [79]. As we know that antimicrobial resistance have been reported in the large number of isolates of *G. anatis* with the possibilities of association to ICEs [19]. Genes encoding fimbrial clusters (flf) have been reported with adjacent mobile elements [64].

Small colony variants (SCVs) have been observed with differences of haemolytic activity [88], in primary cultures of *Gallibacterium* [45,88,89]. Increased persistence, recurrent infections and increased resistance towards antimicrobials have been observed in association with SCVs [8].

Pathogenesis

Repeated isolation of *G. anatis* from the trachea and cloaca of healthy birds indicates its commensal status in the upper respiratory tract and lower genital tract of healthy chickens [2,14,49,50,55,90]. However, isolation of *G. anatis* in association with a wide range of different pathological lesions, including septicaemia, pericarditis, hepatitis, oophoritis, follicle degeneration, enteritis, upper respiratory tract lesions, salpingitis and peritonitis revealed its importance as an opportunistic pathogen [2,17,36,39-43,45,47,55,88,89,91,92]. Recent investigations confirmed that *G. anatis* colonizes the upper respiratory tract without causing clinical signs, whereas it may cause severe lesions in the reproductive tract [54,56]. Studies established *G. anatis* as the most common single bacterial infection in chickens causing reproductive tract disorders [36]. Simultaneous infection with other microorganisms [39,47,48], hormonal influences [42,43], age [2,45], seasonal changes [36], stress [15], cold stress [47], and compromised immunological status [46] are a few predisposing factors nurturing the infection of *G. anatis*. In experimental infections semen quality has been found to be reduced significantly due to decrease in sperm density, total motility with progressive motility, and membrane integrity [54].

Disease associated with *G. anatis* infection

Due to vast range of pathological manifestations of *G. anatis* infection it is difficult to decide the exact disease condition caused by *G. anatis*. Incidences of infection to chicken increase during the peak and late phases of production period [41]. In diseased birds' mortality might take place mainly due to salpingitis, oophoritis and peritonitis. Respiratory tract infections might be responsible for major economic losses due to the rise in treatment cost and losses due to higher condemnation rates and mortality.

Gallibacterium may be causing primary or secondary infections leading to fatal bacteremia, septicaemia and acute septicaemia [41]. The severity of clinical signs, duration of the disease and mortality rate are variable and influenced by environmental factors, such as poor hygiene, inadequate management ventilation, ammonia levels in poultry premises and concurrent diseases. Study on pathogen-specific genes of *Gallibacterium* populations [79] suggested the ability of the pathogen to cause lesions in reproductive organs such as folliculitis, ruptured and haemorrhagic follicles as well as a drop in egg production in adult hens [22,38,55,90]. Haemolytic *G. anatis* was associated with infection in birds kept in alternative husbandry systems and suffering from reproductive disorders [38].

Clinical sign and lesion on *G. anatis* infection

Normally the signs and symptom of diseases caused by *G. anatis* infection in chicken are not pathgnomonic leading to creation of confusion between the different similar symptomatic disease like Newcastle, fowl cholera and bird flu. The clinical sign are unspecific but include depression, diarrhoea, pasting around the vent and loss of egg production take place around peak of lay [41]. Mirle et al. [36] examined 496 hens with reproductive tract lesions and isolated *Gallibacterium* in pure culture from 23% of the diseased organs. Even though haemolytic isolates are primarily diseased causing biovar but non-haemolytic strains might be associated with chronic cases of localized or generalized purulent peritonitis with *E. coli* [41].

Diagnosis of *Gallibacterium* infection

Gallibacterium anatis infection can be confirmed only through agent isolation characterised by phenotypic and genotypic methods. The difference between the genomospecies 1 and 2 is possible only through genotypic methods due to the phenotypic heterogeneity among strains [12]. Presently the best way to identify the *Gallibacterium* is its phenotypic (Table 1) characterisation [12] or with GAN850, a *G. anatis* specific probeat position between 850-867 of 16S rRNA [93]. A number of genotypic methods have been established for identification of *Gallibacterium* [12,18,49]. The specificity of these methods, however, remains to be investigated [11]. *Gallibacterium* has a relatively short internal transcribed 16S to 23S rRNA gene sequences compared to other members of Pasteurellaceae, based on the information the *Gallibacterium* specific PCR [18] targeting on 16s rRNA sequence are designed [94]. These Specific ITS-PCR able to amplifies selectively *Gallibacterium* DNA and generating short fragments compared to other members of Pasteurellaceae [95-97]. Primers chosen with specificity for *G. anatis* are 1133fgal (5`-TATTCTTTGTTACCARCGG-3`) and 114r (5`-GGTTTCCCCATTCGG-3`) [18,98]. Besides this, AFLP typing method and pulsed-field gel electrophoresis are found to be useful for distinguishing between closely related *G. anatis* clones, thus enabling recognition of specific pathogenic clonal lineages [99-101].

Rapid and specific identification of individual bacterial cells can be achieved by the fluorescent in situ hybridization technique (FISH), based on fluorescent labelled oligonucleotides complementary to bacterial 16S rRNA. This method has advantages over the traditional culture based methods due to its ability to identify live/intact cells as well as non-cultivable organisms [49]. Rapid and accurate identification of related organism can be made along with *G. anatis* in human being with help of gene identified like soda gene [102].

Serological studies also have been found to be helpful in detection of *G. anatis* specific antibodies in infected tested sera by latex agglutination test and enzyme-linked immunosorbent assay (ELISA) [41].

Characteristics	G. anatis biovar haemolytica	G. anatis biovar anatis	G. genomospecies 1	G. genomospecies 2	G. genomospecies 3
B-Haemolysis	+	-	+	+	-
Production of acid from:					
(-) D- Arabinose	(+)	-	(+)	D	D (+/-)
(+) L- Arabinose	-	-	D	D	D (+/-)
Mannitol	+		D	D	
m-Inositol	D	D	-	D	
(-) D- Sorbitol	D	D	-	-	D (+/-)
(-) L- Fucose	(+)	-	+/(+)	D	D (+/-)
Maltose	D	-	+	+	
Trehalose	D	+	+	D	-
Dextrin	D	-	+/(+)	+	-

Table 1: Phenotypic characters of *Gallibacterium* species [12]. Note: Characters are scored as: +, ≥ 90% of strains positive within 1–2 days; (+), ≥ 90% of strains positive within 3-14 days; -, <10% of strains positive within 14 days; d, 11–89% of strains positive, D, doubt (+/-) variation from strain to strain.

Prevention and control of *G. anatis* infection

A pan-genomic reverse vaccinology (RV) approach has been applied to identify novel and potentially broadly protective immunogens from *G. anatis* [84,103-105]. Screening approaches of reverse vaccinology have been applied to identify potential immunogens for 71 proteins in which only one protein contain immunization properties [106]. FlfA and GtxA-N have potential to induce a protective immunity in the homologous strain of *G. anatis* [83]. Although, some commercial vaccine are available for more prevalent three biovar [40] but there is no potential vaccine globally present in the market to control *G. anatis* infection and still protection under field condition remains to be investigated against commercial vaccine [40]. Only antimicrobial chemotherapy is the available method for containment of the disease caused by *G. anatis*, however, it is also proven that *G. anatis* rapidly acquires resistance [40]. Besides, general hygienic measures can be taken in the way similar to control of other contagious diseases in poultry farms.

Antimicrobial drug resistance in *Gallibaterium*

Emergence of antimicrobial resistance has been observed among several organisms belonging to the Pasteurellaceae family [5,106] including *G. anatis* isolates. Though the infection of *Gallibacterium* is treatable with antibiotics, the frequency of treatment failure of *Gallibacterium* seems to be a recurrent problem [107,108]. Resistance in chicken origin isolates of *Gallibacterium* is documented to novobiocin, tylosin, clindamycin, spectinomycin, tetracycline antimicrobials and penicillin [20-26,52,109]. In contrast, Berge et al. reported rarity of tetracycline resistance in *G. anatis* of sheep and goat origin [27].

Conclusions

Gallibacterium anatis especially biovar haemolytica has emerged as an important pathogen of broilers and layers in several countries. However, in lack of elaborate scientific understanding of the pathogen and vaccine(s), efforts to control outbreaks and prevent the disease is a big challenge for poultry scientists and microbiologists.

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