Biological Characterization and Comparative Diagnostics of *Mycoplasma gallisepticum* Isolates

By

Saba Farooq

Department of Microbiology

Quaid-i-Azam University

Islamabad

2021

Biological Characterization and Comparative Diagnostics of *Mycoplasma gallisepticum* Isolates

A Thesis submitted to the
Department of Microbiology, Quaid-i-Azam University
Islamabad, Pakistan
In partial fulfillment of the requirements for the Degree of
Doctor of Philosophy in Microbiology

by

Saba Farooq

Department of Microbiology

Quaid-i-Azam University

Islamabad

2021

Author's Declaration

I **Ms. Saba Farooq** hereby state that my Ph.D. thesis titled **"Biological Characterization and Comparative Diagnostics of *Mycoplasma gallisepticum* Isolates"** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

At any time if my statement is found to be incorrect even after I Graduate, the University has the right to withdraw my Ph.D. degree.

Ms. Saba Farooq
Date: 26-05-2021

Plagiarism Undertaking

"Biological Characterization and Comparative Diagnostics of *Mycoplasma gallisepticum* Isolates" is solely my research work with no significant contribution from any other person. Small contribution / help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Quaid-i-Azam University towards plagiarism. Therefore I as an Author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

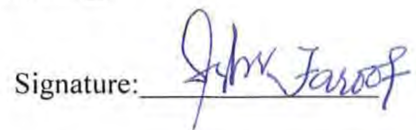
I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of Ph.D degree and that HEC and the University has the right to publish my name on the HEC/University Website on which names of students are placed who submitted plagiarized thesis.

Student / Author Signature:

Name: **Ms. Saba Farooq**

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled titled **"Biological Characterization and Comparative Diagnostics of *Mycoplasma gallisepticum* Isolates"** was conducted by **Ms. Saba Farooq** under the supervision of **Dr. Khalid Naeem (NARC, Islamabad)** and co-supervision of **Dr. Aamer Ali Shah.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

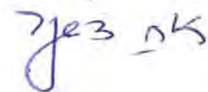
Student Name: **Ms. Saba Farooq** Signature: 

Examination Committee:

a) External Examiner 1: Signature:

Prof. Dr. Azra Khanum
University Institute of Biochemistry & Biotechnology
PMAS Arid Agriculture University
Murree Road, Rawalpindi

b) External Examiner 2: Signature:



Dr. Ijaz Ali
Associate Professor
Department of Biosciences
COMSATS University Islamabad Campus
Chak Shahzad, Islamabad

Supervisor Name: **Dr. Khalid Naeem** Signature:

Co-Supervisor Name: **Dr. Aamer Ali Shah** Signature:

Name of HOD: **Dr. Aamer Ali Shah** Signature:

DEDICATION

I dedicate this effort to my loving parents and teachers who have been torchbearer throughout this journey

CONTENTS

1 Introduction 1
1.1 Aims of the Study 6
1.2 Objectives of the Study 6
2 Literature Review 7
2.1 Classification 7
2.2 Signs and Symptoms 8
2.3 Transmission 8
2.4 Habitat 9
2.5 Host Range 9
2.6 Predisposing Factors of *M. gallisepticum* Infection 10
2.6.1 Age of Flock 10
2.6.2 Poultry Type 11
2.6.3 Seasonal Variation 11
2.6.4 Flock Size 12
2.7 Worldwide Epidemiology 12
2.8 Serological Status in Pakistan 13
2.9 Pathogenesis of *M. gallisepticum* 13
2.9.1 Infectivity and Pathogenicity 13
2.9.2 Intracellular Survival 14
2.9.3 Ability to Invade Non-phagocytic Cells 15
2.9.4 Systemic Infection 15
2.9.5 Interaction of *M. gallisepticum* with Host Airway Passage 16
2.9.6 Role of Co-infection in Disease Manifestation 16
2.10 Genome and Proteins 17
2.10.1 Haemagglutinin Multigene Family (*pMGA/vlhA*) 18
2.10.2 Organization of *pMGA/vlhA* genes 19
2.10.3 Variation in Expression of *pMGA/vlhA* 19
2.10.4 Variation in Trinucleotide length in 5' promoter region 19
2.10.5 Regulation of Gene Expression 20
2.10.6 Cytadherence Related Proteins 20
2.10.7 Phase Variable Protein (PvpA) 22
2.10.8 Membrane Associated Proteins 23
2.10.9 Antigenic Variation 23

2.11 Diagnostic Techniques 24
2.11.1 Serodiagnosis 24
2.11.1.1 Serum Plate Agglutination (SPA) Test 24
2.11.1.2 Enzyme Linked Immunosorbent Assay (ELISA) 25
2.11.2 Molecular Diagnostics 25
2.12 Genotyping 28
2.12.1 Restriction Fragment Length Polymorphism (RFLP) 29
2.12.2 Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) 30
2.12.3 Ribotyping 30
2.12.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 31
2.12.5 Sequence Analysis 31
2.13 Infectious Disease Control 32
2.13.1 Different Approaches to Control Infection 32
2.13.2 Use of Anti-*Mycoplasma* Antibiotics 32
2.13.3 Use of Vaccines 34
3 Materials and Methods 39
3.1 Study Area 39
3.2 Collection and Processing of Samples 39
3.2.1 Processing of Tracheal and Cloacal Swabs 39
3.2.2 Processing of Tissue Samples 41
3.3 Serodiagnosis of *Mycoplasma gallisepticum* 41
3.4 Molecular Diagnosis of *Mycoplasma gallisepticum* 42
3.5 Isolation of *Mycoplasma gallisepticum* 44
3.5.2 Preparation of *M. gallisepticum* Broth 44
3.5.3 Preparation of *M. gallisepticum* Agar 45
4 Sero-surveillance and Isolation of *Mycoplasma gallisepticum* from Field 48
4.1 Introduction 48
4.2 Materials and Methods 49
4.3 Results 55
4.4 Discussion 70
4.5 Conclusion 74
5 Biological and Molecular Characterization of Local *Mycoplasma gallisepticum* Isolates 75
A) Pathogenesis of Local *M. gallisepticum* Isolate Pak MG1 (ARL-1963)

5.1 Introduction 75
5.1.1 Aims and Objectives 76
5.2 Materials and Methods 76
5.2.1 Experimental Design 76
5.2.2 Clinical and Pathological Examination 77
5.2.3 Serology of Experimental and Control Group 77
5.2.4 Molecular Detection of Experimental and Control Group 77
5.3 Results 78
5.3.1 Clinical and Pathological Observations 78
5.3.2 Serological Evaluation 78
5.3.3 Detection of *M. gallisepticum* by PCR 78
5.4 Discussion 84
5.5 Conclusion 86
B) Molecular Characterization of Pakistani *M. gallisepticum* Isolates by Gene Target Sequence Analysis
5.6 Introduction 86
5.6.1Aims and Objectives 87
5.7 Materials and Methods 87
5.7.1 Primary Amplification 87
5.7.2 Sequence Analysis 87
5.7.3 Phylogenetic Analysis 88
5.8 Results 88
5.8.1 Sequence and Phylogenetic analysis of *gap A* gene 91
5.8.2 Sequence and Phylogenetic analysis of *lp* gene 98
5.8.3 Sequence and Phylogenetic analysis of *pvp A* gene 106
5.8.4 Sequence and Phylogenetic analysis of *mgc2* gene 113
5.9 Discussion 123
5.10 Conclusion 126
6 Development of Serum Plate Agglutination antigen using *Mycoplasma gallisepticum* Field Isolate 127
6.1 Introduction 127
6.1.1 Aims and Objectives 128
6.2 Materials and Methods 129
6.2.2 Serum Plate Agglutination Antigen Testing 129
6.2.3 Testing Plan for Field Samples 130

6.2.4 Sensitivity and Specificity of Antigen 130
6.2.5 Shelf Life Confirmation 130
6.3 Results 131
6.4 Discussion 134
7 Comparative Evaluation of Different PCR Based Diagnostic Techniques for etection of *Mycoplasma gallisepticum* 137
7.1 Introduction 137
7.1.1 Aims and Objectives 138
7.2 Materials and Methods 138
7.3 Results 145
7.4 Discussion 153
8 Evaluation of *Mycoplasma gallisepticum* Vaccine Efficacy 155
8.1 Introduction 155
8.2 Materials and Methods 156
8.2.2 Exposure Status in Vaccinated Flocks 157
8.3 Results 162
8.4 Discussion 173
Over all Conclusion of Study 175
Future recommendations/suggestions 176
REFERENCES 177

LIST OF TABLES

Sr. No	Description	Page No.
3.1	PCR mixture profile for *M. gallisepticum* detection Using Dream taq Green PCR master mix by Invitrogen	47
3.2	Polymerase Chain Reaction Profile for *Mycoplasma gallisepticum*	47
4.1	Year-wise number of sera samples tested for detection of anti- *Mycoplasma gallisepticum* antibodies	52
4.2	Sera Sample of backyard poultry and wild domestic birds from different provinces of Pakistan	52
4.3	Sera sample of backyard poultry and wild domestic birds deciphering different avian species	53
4.4	Year-wise samples data for detection of *M. gallisepticum* through PCR	54
4.5	Sample types used for detection of *M. gallisepticum* through PCR	54
4.6	Sero-surveillance of *M. gallisepticum* for detection of IgG in unvaccinated commercial poultry from 2015-2018	58
4.7	Sero-surveillance of *M. gallisepticum* for detection of IgM in unvaccinated commercial poultry from 2015-2018	59
4.8	Sero-monitoring of *M. gallisepticum* in breeder and layer bird types	60
4.9	Sero-monitoring of *M. gallisepticum* in backyard poultry and wild birds from different provinces of Pakistan	61
4.10	ELISA antibody titre of Backyard and wild birds against *M. gallisepticum*	62
4.11	Year-wise percentage of samples positive for *M. gallisepticum* through PCR	66
4.12	*M. gallisepticum* isolates selected for study	69
5.1	Post inoculation observations of pathological lesions in Trachea and Lungs of Experimental and Control group	80
5.2	Anti-*M. gallisepticum* antibody detection by SPA test and ELISA	81
5.3	Post Infection detection of *M. gallisepticum* from different organs of Experimental group by PCR	81
5.4	Primers and expected size of amplified product	89
5.5	Nucleotide and amino acid percentage similarity among Pakistani *M. gallisepticum* isolates	90
5.6	Similarity (percentage identity) of Pak MG1 (ARL-1963) *gapA* gene sequence with vaccine & reference strains and genotypes from different countries	94

5.7	Similarity (percentage identity) of Pak MG2 (ARL-2020) *gapA* gene sequence with vaccine & reference strains and genotypes from different countries	95
5.8	Similarity (percentage identity) of Pak MG3 (ARL-2668) *gapA* gene sequence with vaccine & reference strains and genotypes from different countries	96
5.9	Similarity (percentage identity) of Pak MG1 (ARL-1963) *lp* gene sequence with vaccine & reference strains and genotypes from different countries	101
5.10	Similarity (percentage identity) of Pak MG2 (ARL-2020) *lp* gene sequence with vaccine & reference strains and genotypes from different countries	102
5.11	Similarity (percentage identity) of Pak MG3 (ARL-2668) *lp* gene sequence with vaccine & reference strains and genotypes from different countries	103
5.12	Similarity (percentage identity) of Pak MG1 (ARL-1963) *pvpA* gene sequence with vaccine & reference strains and genotypes from different countries	108
5.13	Similarity (percentage identity) of Pak MG2 (ARL-2020) *pvpA* gene sequence with vaccine & reference strains and genotypes from different countries	109
5.14	Similarity (percentage identity) of Pak MG3 (ARL-2668) *pvpA* gene sequence with vaccine & reference strains and genotypes from different countries	110
5.15	Similarity (percentage identity) of Pak MG1 (ARL-1963) *mgc2* gene sequence with vaccine & reference strains and genotypes from different countries	116
5.16	Similarity (percentage identity) of Pak MG2 (ARL-2020) *mgc2* gene sequence with vaccine & reference strains and genotypes from different countries	117
5.17	Similarity (percentage identity) of Pak MG3 (ARL-2668) *mgc2* gene sequence with vaccine & reference strains and genotypes from different countries	118
5.18	Relatedness of Pakistani *M. gallisepticum* isolates on the basis of mgc2 nucleotide sequence	121
5.19	Accession numbers and designation of *M. gallisepticum* isolates submitted to GenBank	122
6.1	Comparative positivity between In-house and Imported SPA antigen	132
6.2	Statistical difference between In-house and Imported antigen	133
7.1	Real time-PCR Reaction mixture profile using Invitrogen SuperScriptTm One step qRT-PCR kit	142

7.2	Real time-PCR Primers and Probes used for the detection of *M. gallisepticum*	142
7.3	Real time-PCR thermal profile using Invitrogen SuperScriptTm One step qRT-PCR kit	142
7.4	Comparison of analytical sensitivity of iiPCR with qPCR and conventional PCR	147
7.5	Contingency table for the comparison of qPCR and conventional PCR assay for the detection of *M. gallisepticum*	148
7.6	Contingency table for the comparison of qPCR and iiPCR using POCKIT Central Nucleic Acid Analyzer for the detection of *M. gallisepticum*	148
7.7	Contingency table for the comparison of qPCR and iiPCR using Micro Plus Nucleic Acid Analyzer for detection of *M. gallisepticum*	149
7.8	Comparison of reliability of different PCR based diagnostic techniques for detection of *M. gallisepticum*	149
8.1	Farms and flock included in study	159
8.2	Post vaccination seroconversion against *M. gallisepticum* live vaccine	160
8.3	Post vaccination seroconversion against *M. gallisepticum* killed vaccine	161
8.4	Exposure Status of Flock IFH1.1 assessed by ELISA and PCR	165
8.5	Exposure Status of Flock IFT2.1 assessed by ELISA and PCR	166
8.6	Exposure Status of Flock IFT2.2 assessed by ELISA and PCR	167
8.7	Exposure Status of Flock KFB1.1 assessed by ELISA and PCR	168
8.8	Exposure Status of Flock KFB1.2 assessed by ELISA and PCR	169
8.9	Exposure Status of Flock KFQ2.1 assessed by ELISA and PCR	170
8.10	Exposure Status of Flock PFK1.1 assessed by ELISA and PCR	171
8.11	Exposure Status of Flock PFK2.1 assessed by ELISA and PCR	172

LIST OF FIGURES

Sr. No.	Description	Page No.
2.1	Classification of Mollicutes	7
3.1	Map of Pakistan showing selected study area	40
4.1	Samples evaluated for anti *M. gallisepticum*-IgG by ELISA	58
4.2	Samples evaluated for anti M. gallisepticum-IgM by Serum plate agglutination test	59
4.3	ELISA antibody titre against *M. gallisepticum* in backyard poultry and wild birds from different provinces of Pakistan	61
4.4	ELISA antibody titre against *M. gallisepticum* in backyard poultry and wild birds	62
4.5	Gel electrophoresis image of *M. gallisepticum* PCR products using 16S rRNA primer	65
4.6	Detection of *M. gallisepticum* through PCR from 2016-2019	66
4.7	Detection of *M. gallisepticum* through PCR during 2016	67
4.8	Detection of *M. gallisepticum* through PCR during 2017	67
4.9	Detection of *M. gallisepticum* through PCR during 2018	68
4.10	Detection of *M. gallisepticum* through PCR during 2019	68
4.11	*Mycoplasma gallisepticum* colony under 40X magnification of stereomicroscope	69
5.1	Tracheal lesions of experimentally infected chickens by PakMG1	82
5.2	Thoracic air sacs of control groups and of experimentally infected birds	83
5.3	Standard pattern of sequencing of Pakistani *M. gallisepticum* isolates	89
5.4	Phylogenetic tree showing gapA gene (295 bp) and inter-relationship among *M. gallisepticum* isolates from Pakistan, vaccine strains, and selected reference strains	93
5.5	Alignment of the gapA gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries	97
5.6	Phylogenetic tree showing lp gene (495 bp) and inter-relationship among *M. gallisepticum* isolates from Pakistan, vaccine strains, and selected reference strains	100
5.7	Alignment of the lp gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries	104-105
5.8	Phylogenetic tree showing pvpA (583 bp) gene and inter-relationship among *M. gallisepticum* isolates from Pakistan, vaccine strains, and selected reference strains	107

5.9	Alignment of the pvpA gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries	111-112
5.10	Phylogenetic tree showing mgc2 (621 bp) gene and inter-relationship among *M. gallisepticum* isolates from Pakistan, vaccine strains, and selected reference strains	115
5.11	Alignment of the mgcs gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries	119-121
5.12	Phylogenetic tree showing mgc2 gene (255 bp) and inter-relationship among *M. gallisepticum* isolates from Pakistan	121
6.1	Serum plate agglutination test using imported and in-house antigen	132
7.1	POCKIT Micro Plus Nucleic Acid Analyzer	143
7.2	Addition of Premix vial to the third well of transfer cartridge	143
7.3	Sample loading to the extraction cartridge	144
7.4	Cartridge loading to the POCKIT Central Nucleic acid analyzer	144
7.5	Agarose gel electrophoresis of *M. gallisepticum* PCR products of serial dilutions of reference strain	150
7.6	Standard pattern of real time PCR for detection of *M. gallisepticum*	150
7.7	Presentation of results on POCKIT Micro Plus Nucleic Acid Analyzer	151
7.8	POCKIT Central Nucleic Acid Analyzer	152
7.9	Presentation of results on POCKIT Central Nucleic Acid Analyzer	152
8.1	Vaccinated birds kept in chicken isolator chamber	159
8.2	Post vaccination antibody titre variation (live vaccine)	160
8.3	Post vaccination antibody titre variation (killed vaccine)	161
8.4	Variation in antibody titre of Flock IFH1.1 quantified by ELISA	165
8.5	Variation in antibody titre of Flock IFT2.1 quantified by ELISA	166
8.6	Variation in antibody titre of Flock IFT2.2 quantified by ELISA	167
8.7	Variation in antibody titre of Flock KFB1.1 quantified by ELISA	168
8.8	Variation in antibody titre of Flock KFB1.2 quantified by ELISA	169
8.9	Variation in antibody titre of Flock KFQ1.1 quantified by ELISA	170
8.10	Variation in antibody titre of Flock PFK1.1 quantified by ELISA	171
8.11	Variation in antibody titre of Flock PKF2.1quantified by ELISA	172

LIST OF ABBREVIATIONS

%	Percent
µm	Micron meter
ASp	Analytical Specificity
ASe	Analytical Sensitivity
BLAST	Basic Local Alignment Search Tool
CDS	Coding DNA Sequence
DNA	Deoxyribonucleic acid
Dpi	Day Post Infection
dsDNA	Double stranded Deoxyribo nucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
GMn	Geometric Mean Titer
HA	Haemagglutinin
HI	Haemagglutination inhibition
IiPCR	Insulated isothermal PCR
IBV	Infectious Bronchitis Virus
ICT	Islamabad Capital Territory
IgM	Immunoglobulin M
GTS	Gene Target Sequence analysis
IgG	Immunoglobulin G
KPK	Khyber Pakhtunkhwa
MEGA	Molecular Evolutionary Genetics Analysis
MLST	Multi Locus Sequence Typing
MG	*Mycoplasma gallisepticum*
MS	*Mycoplasma synovae*
NAD	Nicotinamide adenine dinucleotide
NJ	Neighbor-Joining
NARC	National Agricultural Research Center
NCBI	National Center for Biotechnology Information
NDV	Newcastle Disease Virus
NRLPD	National Reference Lab for Poultry Diseases
Pi	Post infection
Pak	Pakistan
PCR	Polymerase Chain Reaction
q-PCR	Quantitative Real-time PCR
Rpm	Revolutions per Minute
RFLP	Restriction Fragment Length Polymorphism
RAPD	Random Amplified Polymorphic DNA
SPA	Serum Plate Agglutination
SLST	Single Locus Sequence Typing

ACKNOWLEDGEMENTS

By the grace of Almighty Allah finally the day has arrived that I am writing this page of acknowledgements for my postgraduation thesis. I have numerous people to thank who were instrumental to make this day possible. First and foremost are my parents who have always been a source of unselfish devotion to my wellbeing under every circumstance. Their unconditional love, guidance and wishes were always with me to fulfill this dream. Echo of my late paternal and maternal grandparent's prayers was also driving force for me.

Professor Dr. Khalid Naeem, has been my supervisor and mentor, and an ispiration for me to keep working. I would like to express great appreciation and thanks for his great interest, never ending source of ideas and support regard to my academic and carrier and research. I highly appreciate his guidance and for providing with all necessary facilities during my graduate program especially his great help with the manuscripts and the thesis. He not only served as my research mentor but also opened his doors to me for all sorts of assistance, guidance, and discussions at any time during my stay in the laboratory as a doctoral student.

Dr. Aamer Ali Shah, Chairman of the department and co-supervisor, I would like to express the deepest appreciation, sincere thanks and gratitude for his persistent, valuable guidance, encouragement and help during entire period of graduate program. I never forget his endless support and contribution in academic and research. I highly appreciate his good and constructive advices and the friendly environment provided to me.

I am grateful to my colleagues at Allama Iqbal Open University, Islamabad including Dr. Hina Fatimah, Dr. Rizwana Kousar, Dr. Sadia Latif and Ms. Samar Naseer for continuous support and encouragement. Special thanks to Dr. Mahwish Siraj and Dr. Tooba Saleem for listening to me. In addition, this research was not possible without the support of Dr. Saba Rafique, Dr. Adeela Sharif, Dr. Athar Abbas and Dr. Naila Siddique from NRLPD, NARC.

Unlimited support was furnished by my sisters Sana and Huma, brothers Usman, Irfan and brother-in-law Waqar, my three little niece Sarah, Annayah and Bareera who charmed me in tough times. Much support and blessings by Madam Tabbasum and Mr. Ishfaq Ahmad

and their motivation in tough times helped me in achieving my goal. Continuous encouragement of and dear friends and family helped me in fulfilling the task.

Saba Farooq

ABSTRACT

Mycoplasma gallisepticum infections are of great economic importance for commercial poultry. Strategies to maintain *M. gallisepticum* free flock, include biosecurity practices to minimize likelihood of exposure, use of antibiotics in case of infected flocks to culminate spread of infection and reduce production losses or to use vaccines where exposure to infectious agent cannot be controlled. Continuous monitoring of commercial flocks is imperative for early control of disease. Highly infectious nature of organism is responsible for rapid spread of infection within a flock. Once the flock gets infected, management of infectious agent to suppress clinical infection is arduous for poultry farmer. The present study was undertaken to determine sero-surveillance, biological and molecular characterization of *M. gallisepticum* as well as to evaluate the effectiveness of lab diagnostic tests and vaccines used for the control of this disease in commercial and backyard poultry.

The serological study was designed to assess status of anti-mycoplasma antibodies in poultry, along with molecular detection of suspected and infected flocks. Attempts were made to recover field isolate from infected flocks. To evaluate seroprevalence of *M. gallisepticum* among commercial poultry, unvaccinated layer and breeder farms were investigated from year 2015-2018. Serum plate agglutination (SPA) test and Enzyme linked immunosorbent assay (ELISA) were performed to detect and quantify antibodies. Molecular prevalence of disease was assessed from 2016-2019 through polymerase chain reaction (PCR). Using modified Frey's media, isolation of *M. galllisepticum* from PCR positive field samples was done. High cumulative seropositivity was detected in backyard poultry (59.80%) than in commercial poultry (41.93%). Molecular detection rate of *M. galllisepticum* from selected study areas was 11.93% and isolation rate was 10.5%.

To unveil the biological behaviour of local *M. gallisepticum* isolates, pathogenesis of an isolate designated as Pak MG1 (ARL-1963) was studied. Experiment was setup to judge pathogenicity of isolate by determining level of tracheal lesions and air-sacculitis caused in experimental birds. In addition, dissemination of infection to internal body organs was also evaluated. It was found that PakMG1 (ARL-1963) was localized to upper respiratory tract and cause mild air sac lesions. No dissemination to internal body organs was noticed.

Molecular characterization of 03 Pakistani *M. gallisepticum* isolates i.e. Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) was carried out by gene target sequence analysis of 03 surface protein genes and 01 lipoprotein gene ((*gapA, lp, pvp A, mgc2*). The study provided an understanding about genetic relatedness of local isolates, Pak MG1(ARL-1963), and Pak MG3 (ARL- 2668) were more closely related to each other and distinct from Pak MG2 (ARL- 2020).

For cheap availability of rapid serological test to local farmers, a study was designed to develop SPA antigen using a local *M. gallisepticum* isolate (Pak MG1). Developed SPA antigen was tested for sensitivity, specificity and evaluated for shelf life. A comparison study was conducted using in house SPA antigen and commercially available antigen. It was found that in house SPA antigen can be a good alternative of commercially available imported antigen with 100% sensitivity and specificity.

Early and sensitive molecular detection of *M. gallisepticum* has been of foremost importance to initiate therapeutic management of disease. Validation of insulated isothermal PCR (iiPCR) was conducted in comparison with real-time PCR (qPCR) and conventional PCR (con-PCR). Analytical and diagnostic performance of assay was evaluated and compared with qPCR as gold standard. Detection limit of iiPCR was found comparable with that of qPCR. It was found that iiPCR can be a good alternative to qPCR.

To evaluate efficacy of commercially used *M. gallisepticum* vaccine, a longitudinal study was conducted on vaccinated breeder farms. The study was based on serological and molecular investigation of 08 breeder farms located in Islamabad, Khyber Pakhtunkhwa and Punjab region of Pakistan. For determination of antibody titers induced by vaccination, post vaccination baseline of live and killed *M. gallisepticum* vaccines was developed. High antibody titers with refernce to vaccination titers, were detected from all the farms included in the study from 2017-2019. This provided an evidence of incompatibility of *M. gallisepticum* vaccine used by farmers to the circulating strains in Pakistan.

1 Introduction

With the advent of commercial poultry in 1960s, there has been continuous and steadfast development in poultry industry. Based on production, Pakistan is ranked as 11th largest poultry producers, with more than Rs. 700 billion investments in this sector (GOP 2018-2019). Poultry is one of the most dynamic and organized sub-sector of livestock in Pakistan. This sector is responsible for provision of cheap source of animal protein and contribute about 1.4% in national GDP, and 7.8% in agricultural GDP (GOP 2018-2019). Regardless of a number of challenges, from 2000 to 2010 poultry industry in Pakistan achieved 127% growth in total production of birds, 126% growth is reported in meat production. Egg production increased up to 71% with 18 billion eggs produced per annum (Hussain et al., 2015; GOP 2018-2019). This sector contributes in bridging the gap between demand and supply of animal protein for human consumption.

In last couple of decades, in spite of excellent growth in poultry production, infectious disease outbreaks appeared as huge disaster for the industry. From 2003-2006, poultry industry endured outbreaks of high pathogenic avian influenza (HPAI) subtypes H5N1 and H7N3 and low pathogenic avian influenza (LPAI-H9N2) which were contained by strict biosecurity, vaccination and continuous surveillance programs (Naeem et al., 2007). Common infectious agents still prevailing in local poultry includes, LPAI-H9N2, infectious bronchitis virus (IBV), and New Castle disease virus (NDV) (Rehman et al., 2013; Usman et al., 2017). Other common viruses in circulation include avian adenoviruses (AAVs) (Shah et al., 2017), infectious bursal disease virus (IBDV), infectious laryngotracheitis virus (ILTV), avian metapneumovirus (aMPV). Various strains of IBV, reported in the country are D-274, D-1466, 4-91, and M-41 IBV strains (Ahmed et al., 2007). Colibacillosis, mycoplasmosis and salmonellosis are also common bacterial infections of local poultry.

Mycoplasmosis is caused by pathogenic mycoplasmas in poultry. Four avian mycoplasmas are commonly recognized as poultry pathogens: *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *Mycoplasma meleagridis* (MM) and *Mycoplasma iowae*

(MI). Infections caused by *M. gallisepticum* and *M. synoviae* are enlisted among notifiable diseases of terrestrial animals by World Organization of Animal health (Office International des Epizooties, OIE), with *M. gallisepticum* infections of prime economic importance in chicken (Kleven et al., 1988). *M. gallisepticum* infection once introduced in a poultry flock has ability to persist till life of the flock. Due to chronic and persistent nature of causative agent, decline in productivity both in terms of quantity and quality occurs. Efforts to culminate *M. gallisepticum* infections, and reduce clinical outcomes of disease, excessive and continuous of anti-mycoplasma antibiotics are used throughout the life of infected flock, which in turn not only increases production cost but can also poses threat of development of antimicrobial resistance (AMR).

The genus *Mycoplasma* contains about 110 recognized species, subspecies and candidate species. Members of genus *Mycoplasma* have wide host range including humans, cattle, birds, rodents and plants and are host and tissue specific. Mucous membranes of respiratory and urinogenital tract are preferable site of infection in humans and animals. Some of them are obligatory intracellular parasites, whereas all other mycoplasmas are facultative intracellular organisms. During evolution and adaptation to parasitic mode of life, the *Mycoplasmas* have developed various genetic systems providing a highly plastic set of variable surface proteins to evade the host immune system. In addition, these microorganisms have evolved molecular mechanisms needed to deal with host immune response, transfer to and colonization in a new host. These mechanisms include mimicry of host antigens, survival within phagocytic and non-phagocytic cells and generation of phenotypic flexibility (Razin et al., 1998).

Species isolated from chicken and turkeys include *M. gallisepticum, M. synoviae, M. meleagridis, M. gallinarum, M. iowae, M. iners, M. gallopavonis, M. gallinaceum, M. pullorum, M. lipofaciens, M. glycophilum, M. cloacale, Acholeplasma laidlawii, A. equifetale, M. imitans* and *Ureaplasma gallorale* (Stipkovits and Kempf, 1996).

Mycoplasma gallisepticum is considered aetiological agent of chronic respiratory disease (CRD) in poultry, with signs varying from subclinical to overt clinical signs of respiratory

infection including coryza, conjunctivitis, coughing, sneezing, and respiratory rales. CRD has persistent nature and is complicetd by secondary infections. *M. gallisepticum* infections have been reported in domestic as well as in commercial poultry, turkeys, pheasants, house finches and other bird spp. In commercial chicken, transmission of infection from *M. gallisepticum* infected parents to their offsprings through eggs (vertical transmission) is a major problem. In addition, infection can also transmit via contact, airborne dust or droplets (horizontal transmission). Losses attributed to mycoplasmosis, mainly *M. gallisepticum* infections, are due to decrease in egg production and egg quality, poor hatchability (high rate of embryonic mortality and culling of day-old birds), poor feed efficiency, increase in mortality and carcass condemnations, beside medication costs (OIE, 2018). Birds of all ages are at risk of developing disease but young birds are more susceptible to the infection than adults (Hossain et al*.,* 2010; Umar et al., 2017).

M. gallisepticum colonies appear as tiny, circular, translucent and smooth with a central dense area giving the colony characteristic “fried egg” shape with diameter ranging from 0.2–0.3 mm, and are visible under stereomicroscope. Unlike other bacterial spp. colony characteristics of *Mycoplasma* spp. play limited role in differentiating mix population.

Exclusivity of *Mycoplasmas* gained foremost interest after complete genome sequence analysis of *Mycoplasma genitalium,* a human pathogen which was second bacteria to be sequenced after *Hemophilus influenza* in 1995 (Fraser et al.,1995). Shortly, after that complete genome sequence of another human pathogen, *Mycoplasma pneumoniae* was available in 1996 (Himmelreich et al., 1996). Unusually reduced genome size, and exceptional genetic composition of mycoplasmas attracted attention of scientists to the smallest self-replicating organisms. *Mycoplasma* genome evolved from low G+C content gram positive bacteria by reductive evolution. During evolutionary process, minimum genes for survival in diverse niches were retained and most of non-essential genes, including those responsible for cell wall synthesis were lost. *Mycoplasma* spp. possess genomes with minimum genes required for survival (Bradbury, 2005).

Comparative genomics of *Mycoplasma* spp. indicated reduction in genes involved in biosynthetic mechanisms but maintaining those involved in genome replication and expression. Lack of genes regulating biosynthesis explains increased dependence of organisms on host nutrients. Whereas, genes associated with genome replication and expression are complete and well represented in genome (Razin,1998). *M. gallisepticum* R_{low} genome possess 966422 bp with overall 31 mol%, G+C content. Coding density of 91% is reported with 742 coding DNA sequences (CDSs) (Papazisi et al., 2003).

Core approaches to diagnose avian mycoplasma infections are based on isolation of organism, detection of immune response and molecular detection of the organism's nucleic acid by polymerase chain reaction (PCR) (Raviv and Kleven, 2009). Moderate to high level of seroprevalence in commercial poultry has been reported in Algeria, Bangladesh, Belgium, Egypt, India, Iran and Kuwait. In Pakistan, some selected studies have been carried out regarding *M. gallisepticum* (Umar et al., 2017). Seromonitoring data collected from the field indicated that in certain situations its prevalence may range from 44-76% among layers and breeding stocks (Haque, 2010; Mukhtar et al., 2012; Siddique et al., 2012). Some other studies comparing the sensitivity and specificity of different diagnostic tests including serum plate agglutination (SPA), Haemagglutination inhibition (HI), polymerase chain reaction (PCR) and real time PCR have been published indicating significance of different diagnostic tests while undertaking field diagnosis (Hanif and Najeeb, 2007; Rauf et al., 2013; Asif et al., 2015; Haque et al., 2015).

In some countries, avian mycoplasmosis has been successfully controlled by adoption of a policy of testing and culling of the infected parent stocks, however, in many parts of the world this practice is not possible to adopt because of the involvement of high cost of such test and slaughtering policy. Therefore, a number of vaccines have been introduced in many countries along with the adoption of strict biosecurity measures at the commercial farms. In Pakistan, the trend to regularly monitor the health status of Grand Parents (GPs) and Parent stocks has just begun. Furthermore, vaccination of commercial poultry against *M. gallisepticum* is in practice. However, despite the above efforts, there are reports of high incidence of *M. gallisepticum* in all types of poultry in this country. This would also include

the flocks vaccinated with *M. gallisepticum*. This may lead to believe that there is still a need to study the circulating isolates of *M. gallisepticum* in this country for developing and validating more sensitive & specific serological and molecular diagnostics against the circulating strains of *M. gallisepticum* in this country.

In the absence of any comprehensive nation-wide prevalence studies regarding *M. gallisepticum* in poultry, already conducted random studies indicate high rate of infection in commercial poultry. In spite of the availability of basic diagnostic tools and selective usage of *M. gallisepticum* vaccines, the disease is still not under control. It is therefore, hypothesized that different *M. gallisepticum* strains may be circulating in the field, locally. On the other hand, as most of *M. gallisepticum* infections are related to its vertical transmission from parent flock to day-old chicks, it is very important to develop very specific diagnostic tools and/or homologous vaccines based on the circulating strains of *M. gallisepticum* for launching an effective control strategy. In addition, due to recent issues of generation of antibiotic resistance in human pathogens on account of un-regulated usage of antibiotics among human & animals, it is need of hour to develop innovative diagnostics and vaccines for adopting appropriate preventive measures to control mycoplasma infections among chickens.

For this purpose, it would be logical to study the circulating strains of *M. gallisepticum* in this country, based on their biological, pathological and molecular characteristics. This information will lead to select more sensitive and specific diagnostic tools along with selecting most effective vaccines as a control measure. Molecular assays can help in identification of circulating strains of *M. gallisepticum* and pathogenesis studies can give insight to persistence of infectious agent, shedding, and route of transmission. In addition, assessment of protection induced by killed vaccine of *M. gallisepticum* is important to understand how the current economic losses can be minimized accordingly. Ultimately, for the detection of *M. gallisepticum* antibodies from field, use of locally developed plate agglutination antigen can be more effective. Due to fastidious nature and biological uniqueness, isolation of *M. gallisepticum* is laborious and very time-consuming practice. This problem has been resolved by PCR based molecular techniques for detection and

differentiation of *M. gallisepticum* strains. Comparative evaluation of molecular diagnostic techniques for early, rapid and economical detection of *M. gallisepticum* could help in control and prevention of infection. Therefore, it is anticipated that coordinated study determining molecular and pathogenesis versatility of *M. gallisepticum* will ultimately help in development of improved diagnostic reagent and control measures in this regard. Present study was carried out with following aims and objectives:

1.1 Aims of the Study

As, *M. gallisepticum* isolates from Pakistan have not been studied with reference to biological behaviour, and molecular characteristics. Following aims were devised:

1. Evaluation of pathogenic potential of selected *M. gallisepticum* isolates along with the immunological properties of available vaccines against them
2. Molecular characterization of local *M. gallisepticum* strains along with the development of specific and economical diagnostics

1.2 Objectives of the Study

To achieve the above-mentioned aims, following objectives were developed:

1. To determine serological status among high density areas of commercial poultry farming & to recover field isolates of *Mycoplasma gallisepticum*
2. To characterize selected *M. gallisepticum* isolates using biological and molecular tools
3. To develop and standardize serum plate agglutination (SPA) antigen using local isolate
4. To evaluate and compare various molecular diagnostic techniques for detection of *M. gallisepticum*
5. To evaluate efficacy of commercially used vaccines against *M. gallisepticum* infection

2 Literature Review

Mycoplasmosis was first described by Dodd in 1905 in England as "epizootic pneumoenteritis" in turkeys. Isolation of causative agent of the disease from chicken and turkey was reported in 1950s and isolated organisms were identified as members of pleuropneumonia group (*Mycoplasma* spp.).

2.1 Classification

Mycoplasmas belong to Class *Mollicutes*, the word is derived from Latin "molli" means soft and "cutes" means skin. Organisms included in this class are devoid of cell wall, cell membrane lack peptidoglycan, muramic acid, teichoic acid but is rich in sterol. Cellular structure consists of cell membrane, ribosomes and double stranded DNA molecule. Lack of cell wall render these organisms inherently resistant to Penicillin. On the basis of cellular architecture, *Mollicutes* are neither classified as gram negative nor gram positive. Previously, *Mollicutes* were placed in phylum Firmicutes by Garrity and Holt in 2001. Due to phenotypic and phylogenetic differences, they were excluded from phylum Firmicutes and classified in phylum Tenericutes. Class *Mollicutes* is further classified in four orders: *Mycoplasmatale, Entomoplasmatales, Acholeplasmatales, Anaeroplasmatales* (Fig.2.1).

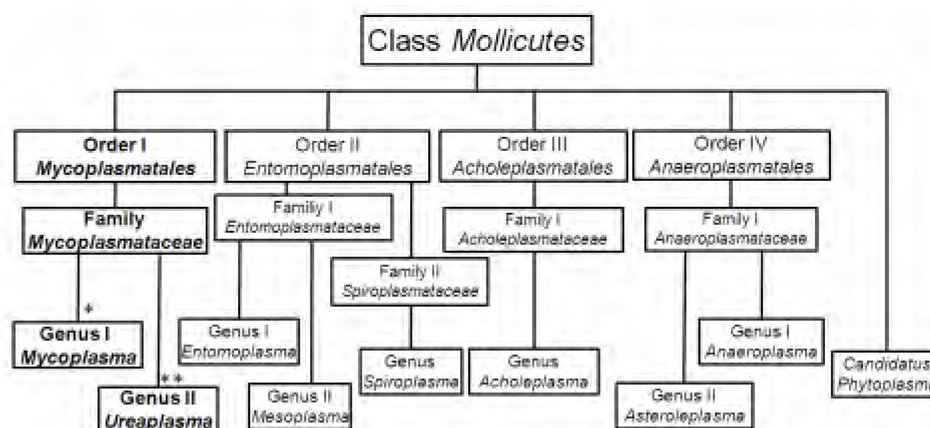


Fig. 2.1 Classification of *Mollicutes*

According to an evolutionary scheme proposed by Maniloff (2002), ancestral *Mycoplasma* originated 600 million years ago from *Streptococcus* phylogenetic branch with a probable genome size of 2000 kb. Mycoplasma branch further fragmented into two major branches one giving off the *Asteroleplasma*, *Anaeroplasma* and *Acholeplasma* the other branching to *Spiroplasma*, *Entomoplasma* and *Mycoplasma*. The phytoplasmas and *Ureaplasma* ascended from the *Acholeplasma* and *Mycoplasma* branch, respectively. Among both branches, degenerative evolution leading to genome reduction occurred independently. Thus, degenerate evolution of the *Acholeplasma-Anaeroplasma* branch, resulted in the *Phytoplasmas* with 600–1200 kb genomes. Second branch evolved to produce the *Spiroplasma* branch with 1000–2000 kb genomes and the *Entomoplasma*, *Mesoplasma*, *Mycoplasma* and *Ureaplasma* branches with 600–1200 kb genomes (Razin, 2006).

Acholeplasma and *Spiroplasma* species possess larger genome sizes than that of *Mycoplasma* and *Ureaplasma* species. Among *Mollicutes*, genome size variation is evident not only within same genera, but also among the strains of same species (Razin, 1998).

2.2 Signs and Symptoms

M. gallisepticum is highly infectious respiratory pathogen affecting commercial chicken and turkey. Signs and symptoms are more pronounced in turkeys than in chicken. Natural infections result in tracheal rales, nasal discharge, coughing, difficulty in breathing and sometimes conjunctivitis in chicken and turkeys. Body weight and feed consumption get reduce along with decline in productivity in laying flocks. In infected birds, trachea and lungs hyperemia, and severe airscculitis and in some cases encephalitis has been also observed (Charlton et al., 1999; Levisohn and Kleven, 2000; Feizi et al., 2013).

2.3 Transmission

Transmission of *M. gallisepticum* within a flock depends on the density of flock and biosecurity practices. Feathers, droppings, drinking water, and fomites can serve as vehicle in transmission of infection within a farm, from one flock to other or among different farms

(Marois et al., 2002; Racicot et al., 2011). In an infected farm, infection can transmit horizontally via respiratory droplets. Vertical transmission can occur in breeding flocks from infected parental flock. (Levisohn and Kleven, 2000; Bradbury, 2005; Nascimento et al., 2005; Machado et al., 2017)

2.4 Habitat

Mycoplasmas are parasites of almost all vertebrates including humans, mammals, reptiles, fish, arthropods, birds and plants. Nutritionally limited nature of these organisms defines their host specificity and parasitic mode of life. Primary habitats of human and animal mycoplasmas are the mucous membranes of the respiratory and urogenital tracts, eyes, alimentary canal, mammary glands, and joints (Razin et al., 1998). *M. pneumoniae* and *M. genitalium* are human respiratory and urogenital tract pathogens respectively. In some exceptions, isolation of *M. pneumoniae* has been reported from urogenital tract and *M. genitalium* from respiratory tract (Goulet et al., 1995).

2.5 Host Range

M. gallisepticum has been isolated from a number of bird species with either mild or no signs and symptoms at all. *M. gallisepticum* and *M. synovae* isolation from tracheal and cloacal samples of ducks showing no signs of infection was reported (Bencina et al., 1988; Buntz et al., 1986). Before 1994, they were considered as infectious agent of gallinaceous birds. Identification of *M. gallisepticum* as etiological agent of conjunctivitis and peri-orbital swelling in house finches (*Carpodacus mexicanus*) provided evidence of their extended host range in 1994, which further included American goldfinch (*Carduelis tristis*) in 1995 (Ley et al., 1996; Luttrell et al., 1996; Fischer et al., 1997). Experimental infection of house finches suggests rapid recovery but strong immune response on re-infection or recurrence of infection (Kollias et al., 2004; Sydenstricker et al., 2005; Sydenstricker et al., 2006).

Natural infections of *M. gallisepticum* have been reported in pheasants, chukar partridge, peafowl, bobwhite quail, and Japanese quail with successful isolation of the infectious agent (Reece et al., 1986; Cookson et al., 1994; Murakami et al., 2002; Bencina et al., 2003). They have also been isolated from a yellow-naped Amazon parrot and greater flamingos (El Shater, 1996). Pigeons and sparrows can also serve as intermittent steady carriers of *M. gallisepticum* without presenting seroconversion or sign of infection (Gharaibeh and Hailat, 2011).

2.6 Predisposing Factors of *M. gallisepticum* Infection

A number of contributing factors responsible for increased likelihood of disease have been reported in studies. These factors are described below.

2.6.1 Age of Flock

Seroprevalence studies conducted earlier, provided evidence of infections in young birds as compared to aged ones, also the presence of *M. gallisepticum* antibodies may not associate with clinical signs except in complicated cases. Rachida et al., (2013) revealed difference in rate of *M. gallisepticum* infection in pullets less than 18 weeks of age (woa) than in laying birds older than 18 woa as 70% and 41.2% respectively, in eastern Algeria. A similar study by Mukhtar et al., (2012) conducted in district Faisalabad, Pakistan described highest seroprevalence in pullets (54.84%), then adult 46.34% and old layers 44.44%.

Seroprevalence studies from Bhola Khulna, and Rajshahi district of Bangladesh presented pattern of infection and its relevance with age. In Bhola district, highest prevalence was found in pullets 60.63%, in adults 55.63% in old chicken it was 51.25% (Islam et al., 2014). Jalil and Islam, (2010) conducted a cross sectional study from August 2009 to July 2010 to assess seroprevalence of *M. gallisepticum* in Khulna district. It was reported that, in age group 21-56 weeks, highest prevalence 71.2% was found, followed by 66.8% in 8-20 woa group. In Rajshahi district, *M. gallisepticum* infection was reported to be highest in young poults 71.7% as compared to adult birds 50.4% (Hossain et al., 2010). Ahmad et al., (2008)

reported highest positive percentage (74.60%) of *M. gallisepticum* infection in breeding stock aging from 6 to 23 weeks while lowest (33.17%) in flocks of 60 to 76 woa.

2.6.2 Poultry Type

M. gallisepticum infections are of great importance in layer and breeding flocks. Infected parental broiler breeder flock, can transmit infection to progeny producing infected broilers. Information from various countries suggested presence of *M. gallisepticum* infection not only in commercial but also in backyard poultry.

Using serum plate agglutination (SPA) test Asif et al., (2015) reported the prevalence of *M. gallisepticum* in layer, broiler and breeder flocks as 67.2%, 60.5% and 68.2%, respectively. Serological evidence of *M. gallisepticum* and *M. synoviae* in Quetta, Pishin and Kuchlak districts of Balochistan, Pakistan was reported by Atique et al., (2012). The overall antibodies of *M. gallisepticum* in broiler were 13.16%, and in layers were 23.33%. Antibodies against *M. synoviae* were found to be 8.16%, 15.33%, in broiler and layer flocks, respectively. High rate of infection was reported in backyard poultry (62.5%) than in commercial poultry (53.61%) in Bhola district of Bangladesh. Another study reported that prevalence of *M. gallisepticum* antibodies was 53% in broiler and 73% in layer at Lohagara, whereas 46% in broiler and 60% in layer at Satkania Upazilla, Bangladesh (Barua et al., 2006).

2.6.3 Seasonal Variation

M. gallisepticum infections are related with seasonal variation. Association of *M. gallisepticum* infection with season reported high rate of infection in chickens reared in winter i.e. 60.42% than those reared in summer i.e. 51.25% (Islam et al., 2014). A study conducted in Eastern Algeria stated *M. gallisepticum* infections were higher in cold weather than warm weather *M. gallisepticum* (38.8% and 27.9%, respectively) (Rachida et al., 2013). Prevalence of *M. gallisepticum* was studied in Faisalabad district of Pakistan from January to December, 2010 (Mukhtar et al., 2012). Results indicated 40 flocks were positive for *M. gallisepticum* antibodies, representing 49.38% of flocks under respiratory

distress. Rate of infection was reported to be high in winters 45.13% than in summer 36.30%. Another study stated (Sikder et al., 2005) *M. gallisepticum* infections were higher in winter 61.45% than rainy season 51.74%.

2.6.4 Flock Size

Flocks having bird density equal or greater than 5000 showed high prevalence of *M. gallisepticum* infections as compared to small flocks. The study was carried in district Faisalabad of Pakistan from January to December, 2010 (Mukhtar et al., 2012). Another study reported *M. gallisepticum* infections and concurrent infection were highest in large flocks 68.6% and 17.1% as compared to small flocks 50.0% and 8.8% in Bangladesh (Hossain et al., 2010).

2.7 Worldwide Epidemiology

M. gallisepticum infections are endemic in many countries. Serological studies from different regions of Bangladesh reported high seroprevalence in Bhola district, Rajshahi district and Khulna districts (Hossain et al., 2010; Jalil and Islam 2010; Islam et al., 2014). A study to evaluate seroprevalence in Gaborone, Botswana, Mushi et al., (1999) reported 57.88% and 67.33% seropositive samples against *M. gallisepticum* and *M. synoviae*, respectively. Seroprevalence of *M. gallisepticum* and *M. synoviae* was evaluated in eight provinces of Eastern Algeria (Rachida et al., 2013). In addition, among broiler breeder farms of Tabriz-Iran, seroprevalence of *M. gallisepticum* in was reported as 19.09% (Feizi et al., 2013). Comparison of molecular and serological methods with cultural techniques for identification of *M. gallisepticum* in commercial broiler and layer farms of Kuwait reported 48% positive samples by Enzyme linked immunosorbent assay (ELISA), 58% by PCR and 14% with culture methods (Qasem et al., 2015). Another study conducted in Niger state, Nigeria to assess seroprevalence against *M. gallisepticum* / *M. synoviae* infections reported high seroprevalence i.e. 91.83% in indigenous chicken without obvious clinical signs (Ahmed et al., 2015). A report from India described 52.1% and 32.6% prevalence of *M. synoviae* and *M. gallisepticum* antibodies, respectively (Rajkumar et al., 2018).

2.8 Serological Status in Pakistan

Serological studies conducted in Pakistan reported use of SPA, and ELISA for detection of antibodies against *M. gallisepticum* (Atique et al., 2012; Mukhtar et al.,2012).

Prevalence of anti-mycoplasma IgG in unvaccinated breeding stock, in and around Lahore district reported high infection rate (Ahmad et al., 2008). The result further revealed that presence of *M. gallisepticum* antibodies may not associate with clinical signs except in complicated cases. Whereas, another study reported seroprevalence of *M. gallisepticum* in broilers 7.14%, broiler breeders 59.6%, and in layers 44.9% in Rawalpindi region (Shoaib et al., 2019).

2.9 Pathogenesis of *M. gallisepticum*

Pathogenesis of *M. gallisepticum* infections is still in the process of exploration, as it varies widely based on biosecurity status of farms, age of birds, and source of infection.

2.9.1 Infectivity and Pathogenicity

M. gallisepticum strains vary in pathogenicity, virulence and immunogenicity. To decipher variability exhibited by various strains, *in vivo* infections by aerosolization, intratracheal inoculation, intranasal inoculation and eye drop administration have been experimentally reproduced (Rodriguez and Kleven, 1980). Pathogenic potential of *M. gallisepticum* strain is defined with respect to its ability to produce tracheal and air sac lesions and ability of re-isolation of causative agent. *In vitro* evaluation of pathogenicity of *M. gallisepticum* strains has been done by using tracheal ring culture, to assess the extent of ciliostasis produced by the strain. Pathogenicity of F strain varies with route of inoculation, being less pathogenic when inoculated intratracheally or via eye drops, and considerably pathogenic in case of mixed infection and aerosol administration (Levisohn et al., 1986).

Pathogenicity of *M. gallisepticum* strains vary after multiple passages both *in vivo* and *in vitro*. Since the phenomenon is not generalised, reduced pathogenicity is observed in

passages of R_{low} to R_{high}, (164th passage), F strain and in A5969 strain. Strain S6 retains pathogenicity even after high passage. R_{low} presents highest degree of air sac lesions, followed by F_{low}, S_{high} and S_{low} showing moderate pathological changes. R_{high} and F_{high} and A5969 causes no changes in air sacs and trachea during 2 weeks. Strain A5969 results in least infectivity and no re-isolation. Serological response is directly related to the degree of infectivity of corresponding *M. gallisepticum* strains as well as organism's ability to re-isolate (Levisohn et al., 1986).

Based on route of infection, colonization of R strain and F strain in chicken trachea show varied effects. R strain results in severe tissue damage with oedematous outcome, weight loss along with serological response as early as 3 days post infection. Whereas *M. gallisepticum* F strain infection show moderate expression in terms of tissue damage and oedema upto 14 days post infection with no humoral response. Pathogenic and immunogenic potential of *M. gallisepticum* R strain is greater than that of F strain. R_{low} strain and $S6_{low}$ strain retain pathogenic potential when inoculated via aerosols and intra tracheal route *in vivo*, and on tracheal ring culture *in vitro* (Rodriguez and Kleven, 1980; Levisohn et al., 1983).

M. gallisepticum strains which are difficult to isolate, show least pathogenicity and virulence, reduced transmissibility, are recognized as atypical or variant strains. Such strains are not only difficult to isolate but are least immunogenic and failed to generate antibody response detectable by SPA and HI. Re-inoculation of such variant strains in chicken and turkeys result in enhanced and detectable immune response. These characteristics can be due to altered antigenic profile (Yoder, 1986; Ferguson et al., 2003).

2.9.2 Intracellular Survival

First report of intracellular persistence of *Mycoplasmas* came from isolation of human pathogen *M. fermentans* from acquired immunodeficiency syndrome (AIDS) patient, followed by isolation of *M. penetrans* from AIDS patient showing intracellular survival capability both *in vivo* and *invitro* (Lo et al., 1991).

Among human mycoplasmas, *M. genitalium*, *M. pneumoniae*, and *M. penetrans* also possess, potential to invade non phagocytic cell (Vogl et al., 2008). On the basis of phylogenetic relatedness of *M. pneumoniae* and *M. gallisepticum*, *in vitro* cell invasion of latter was described by Winner et al., (2000). Also, *in vivo* and *in vitro* invasion of *M. gallisepticum* R_{low} in sheep and chicken erythrocytes has been described by Vogl et al., (2008).

2.9.3 Ability to Invade Non-phagocytic Cells

Invasion of *M. gallisepticum* in non-phagocytic cells not only provides advantage of hiding from host defence system, resisting antimicrobial therapy but also aid in its systemic spread to other organs and tissues leading to persistent and chronic infections. *M. gallisepticum* R_{low} is capable of invading *in vitro* cultured human epithelial cells (HeLa-229) and chicken embryo fibroblasts (CEF). High passaged R strain show adherence to eukaryotic cell surface but no invasion in both cell lines (Winner et al., 2000).

M. gallisepticum is able to invade RBCs, the property was demonstrated in *in vitro* as well as *in vivo* assays. This property to invade erythrocytes provide a system to transport infectious agent to all body tissues and organs (Vogl et al., 2008).

Influence of host factors also play an important role in facilitating adhesion and cell invasion of *M. gallisepticum*. Extracellular matrix (ECM) proteins of eukaryotic cell do exert positive effect on R_{low} and R_{high} adhesion and cell invasion of Hela cells and chicken RBCs. These ECM proteins involve fibronectin, Collagen type IV, fibronectin and plasminogen. Cholesterol depletion in HeLa cell membrane enhances adhesion of *M. gallisepticum* but reduces cell invasion (Fürnkranz et al., 2013).

2.9.4 Systemic Infection

M. gallisepticum is capable of crossing mucosal barrier of respiratory tract and systemic spread to internal body organs. Dissemination of pathogen to various body organs occur through blood stream. Experimental infection of chicken by R_{low} and R_{high} show systemic

spread of R_{low} to heart, brain, liver spleen and kidney. R_{high} remain limited to respiratory tract (Much et al., 2002).

2.9.5 Interaction of *M. gallisepticum* with Host Airway Passage

Pathology of *M. gallisepticum* infection in chicken is based on inflammatory response in trachea, air sacs and lungs. In the absence of conventional virulence factors, the mechanism involved in defining virulence of *Mycoplasmas* is ability of cytadherence and cell invasion consequently leading to inflammatory response of varying degree.

Lipid associated membrane proteins (LAMP) of virulent and avirulent *M. gallisepticum* strains upregulate inflammatory genes of tracheal epithelial cells (TEC) both *in vitro* and *ex vivo*. These genes include IL-1β, IL-6, IL-8, IL-12p40, CCL-20 and NOS-2. Virulent strains differentially regulate several unique genes, and lead to enhanced macrophage chemotaxis. Macrophages upon co-culture with *M. gallisepticum* exposed TECs up-regulated expression of IL-1β, IL-6, IL-8, MIP-1β, CXCL-13, CCL-20 and RANTES. More efficient gene up-regulation from macrophages occur in interaction of R_{low} with TECs as compared to R_{high} (Majumder, 2014).

2.9.6 Role of Co-infection in Disease Manifestation

Avian mycoplasmas cause disease after bacterial or viral infection and host weakness episode. Co-existence of pathogens may enhance consequences of disease due to increased virulence and same route and site of infection. In addition, age, breed and environmental conditions play an important role in establishment of infection (Bradbury, 1984; Yoder, 1991; Nascimento et al., 2005).

Turkeys develop severe air sacculitis and sinusitis in uncomplicated infection of *M. gallisepticum*. Respiratory tract infection in chickens, involving *M. gallisepticum* might not be severe as in the presence of co-infecting viral and bacterial agents. Co-infection of *M. gallisepticum* with New Castle disease virus (NDV), infectious bronchitis virus (IBV) and *E. coli* enhances the disease outcome. NDV and IBV co infection with *M. gallisepticum*

increase susceptibility to *E. coli* infection leading to air sacculitis, pericarditis and perihepatitis. Involvement of other respiratory pathogens including infectious laryngotracheitis virus (ILTV), reovirus, adenovirus, *Haemophilus gallinarum* along with *M. gallisepticum* infection has also been reported (Bradbury, 1984).

Experimental co-infection of *M. gallisepticum* followed by low pathogenic Avian Influenza (LPAI) H3N8 virus (A/mallard/Hungary/19616/07) infection in chicken resulted in severe signs and symptoms including tracheitis, bronchitis, air sacculitis, and pneumonia than infection by either the AIV H3N8 virus or *M. gallisepticum*. Infection of *M. gallisepticum* followed by H3N8 also reduces anti-mycoplasma antibody response. Since *M. gallisepticum* infection causes high morbidity but is rarely responsible for high mortality, mixed infections can lead to economic losses due to enhanced disease outcome (Stipkovits et al., 2012 (a); Stipkovits et al., 2012 (b)).

Co-infection of *M. gallisepticum* with field isolate of LPAI H9N2 from Pakistan exaggerated disease outcome. A non-pathogenic, field isolate of AIV H9N2 caused severe conjunctivitis, oedema, inflammation and necrotic lesions in lungs and kidneys in co-infection of H9N2 with field isolate of *M. gallisepticum* (Subtain et al., 2016).

2.10 Genome and Proteins

Complete genome sequence of *M. gallisepticum* R_{low}, provided deep insight about minimal genomic content of bacterium capable of carrying out complex and diverse functions. The genome of R_{low} is 996kb in size with 742 coding DNA sequences (CDSs), showing 91% coding density. Function has been assigned to 469 of the CDSs, while 150 are conserved hypothetical proteins and 123 remain as hypothetical proteins. Among CDSs 10% represent lipoproteins and approximately 20% contain multiple transmembrane domains. Membrane associated molecules constitutes a large percentage of *M. gallisepticum* genome, and about 17% genes are thought to be unique to *M. gallisepticum* (Papazisi et al., 2003).

Interaction between *M. gallisepticum* and host play a preliminary and critical role in disease establishment. The mechanism is regulated by cytadhesins and accessory proteins.

Investigations into the nature of homologous genes from related species of *Mycoplasmas* may provide insights into the conservation and/or modifications of genes essential for survival or pathogenesis throughout the process of degenerate evolution.

As our understanding of the complex mechanism of cytadherence increases, a more efficacious vaccine may also be designed for the control of *M. gallisepticum* disease, utilizing the cytadherence molecules as subunit antigens.

2.10.1 Haemagglutinin Multigene Family (*pMGA/vlhA*)

A multigene family *pMGA* which was renamed as *vlhA* in 2003, encodes major surface lipoproteins responsible for antigenic diversity in different strains of *M. gallisepticum*. Molecular cloning experiments unrevealed the complex nature and structural as well as functional diversity of members of this family. Host pathogen interaction, immune evasion and pathogenesis of *M. gallisepticum* infections depend upon the proteins encoded by variable lipoprotein and heamagglutinin (*vlhA*) gene family (Pflaum et al., 2016; Pflaum et al., 2018). Variation of expression within a strain can play important role in immune evasion, possibility of differential expression of *pMGA/vlhA* genes in different strains also exists (Markham et al., 1992; Markham et al., 1998; Papazisi et al., 2003).

All strains contain large *pMGA/vlhA* multigene family ranging in size from 32 to 70 genes (Ley, 2008). It second largest gene family after tRNA in prokaryotes (Bassegio et al., 1996). In S6 strain, it comprises of 33 genes and constitute about 7.7% of 1,030 kb genome (Glew et al., 1998). *M. gallisepticum* R_{low} possess 43 genes constituting a total of 103 kb or 10.4% of the genome (Papazisi et al., 2003). In spite of the presence of a large set of genes, each strain is capable of expressing only single member of *pMGA/vlhA*, others being transcriptionally silent or are transcribed at very low levels. The member expressed in strain S6 is *pMGA*1.1 (Markham et al., 1993; Markham et al., 1994; Glew et al., 1995).

2.10.2 Organization of *pMGA/vlhA* Genes

The *pMGA/vlhA* genes of *M. gallisepticum* R_{low} are present in clusters in the genome and are organized in same transcriptional orientation except *vlhA*1.05. The 43 *vlhA* genes are distributed among five loci containing 8, 2, 9, 12 and 12 genes, respectively (Glew et al., 1995, Papazisi et al., 2003). In *M. gallisepticum*, out of the 43 genes the signature *vlhA* gene features are present in 38 genes. These features include conserved regions flanking start codon and a GAA repeat motif 5' of a GTG start codon (Markham et al., 1994, Papazisi et al., 2003). These 38 genes represent sequence homology between 41 and 99 %. (Papazisi et al., 2003). The distance between the translational stop codon of one gene and the start codon of the adjacent gene is approximately 400 nucleotides (Markham et al., 1994, Glew et al., 1995). Transcription of *pMGA/vlhA* genes produce a monocistronic mRNA from a single transcriptional start site. In S6 strain of *M. gallisepticum*, transcription of many *pMGA/vlhA* genes occur simultaneously. Expression of *pMGA* 1.1 is 20 to 40-folds higher than any other *pMGA/vlhA* gene, resulting in production of the only detectable gene product (Glew et al., 1995).

2.10.3 Variation in Expression of *pMGA/vlhA*

In vitro expression of *pMGA*1.1 protein of *M. gallisepticum* S6 switches off when cultured in the presence of *pMGA*1.1 corresponding antibody MAb66. Loss of *pMGA* 1.1 is complemented by expression of another member of *pMGA* family i.e. *pMGA* 1.9. Reversal of expression of *pMGA*1.1 happens upon removal of respective antibody Mab 66 from the culture media (Markham et al., 1998). Further *in vivo* studies revealed, that switching of *pMGA* variable expression of surface protein is independent of pMGA antibodies and the process occurs naturally during infection process (Glew et al., 2000)

2.10.4 Variation in Trinucleotide Length in 5' Promoter Region

The transcriptional switching between *pMGA/vlhA* genes is shown unequivocally associated with changes in the length of a unique trinucleotide GAA repeat (Glew et al., 1998), a motif found to be common to all *pMGA/vlhA* genes. Specifically, a (GAA)12 motif

5' to a *pMGA*1.1 promoter appeared an obligate requirement for the expression of that gene (Glew et al., 1998). Changes in *pMGA/vlhA* gene expression occurred as a result of the inherent instability of GAA repeats in *M. gallisepticum* (Glew et al., 1998).

2.10.5 Regulation of Gene Expression

Vlh transcriptome study directly from tracheal swabs, during 7 days experimental infection (R_{low}) show global variation in expression of *vlhA* genes. *VlhA* 3.03 dominantly expressed on day 1 post infection (pi) followed by decrease till day 7 pi. Other *vlh* genes showing detectable expression include *vlhA* 4.07 and *vlhA* 5.05 which also decreased on day 7 pi. Changes in expression of *vlhA* genes are not regulated by adaptive immunity, instead pathological effects of infection can drive changes transcriptome in early infection. In recovery cultures, *vlhA* 2.02 was highly expressed, with single GAA trinucleotide in promoter region. Expression of *vlhA* 2.02 provided evidence that 12 GAA repeats might not be regulating factor for transcription of a particular gene (Pflaum et al., 2016)

Comparison of expression of two live attenuated vaccines Mg7 and GT5 (transformant with restored expression of *gapA*) showed increased expression of *vlhA* 3.03 on day one and day two pi, in contrary to the expression pattern of Rl_{ow} where *vlhA* 3.03 expression is reduced on day pi. In Mg7 increase in *vlhA* 2.02 occurred on day 1 and 2 pi. Both vaccines produced no significant tracheal lesions. *VlhA* 3.03 is not associated with colonization of respiratory tract or virulence as transposon inserted *vlhA* 3.03 mutant can produce pathological signs comparable to R_{low} infection. *VlhA* 3.03 mutant show increased expression of *vlhA* 4.07 at day 1 pi (Pflaum et al., 2018).

2.10.6 Cytadherence Related Proteins

Genes coding for cytadherence include *gapA crmA* operon, along with *crmB* and *crmC* (Papazisi et al., 2003). GapA (MGC1) is an important surface exposed, cytadherence protein **(**Keeler et al., 1996; Goh et al., 1998). Sequence homology exists between *P1* gene of *M. pneumoniae, MgPa* gene of *M. genitalium* and *gap A* gene of *M gallisepticum*. No such sequence homology occured with *M. synoviae*, *M. meleagridis*, *M. iowae*, or *M.*

gallinarum DNA (Dhoms et al., 1993; Goh et al., 1994; Gorton et al., 1995). A single copy of *gapA* gene exists in genome unlike *P1* and *MgPa* gene (Goh et al., 1998). Proline rich carboxy terminus of *gapA* regulate conformational changes in the region leading to topological arrangement of the cytadhesin (Razin & Jacobs, 1992).

Lack of *gapA* and *crmA* expression in R_{high} was reported by (Papazisi et al., 2000). In comparison with virulent R_{low}, a less virulent, laboratory attenuated strain R_{high} revealed lack of expression of three proteins in R_{high}. These proteins are GapA, the CrmA, and a component of a high-affinity transporter system, HatA (16 kDa and 45 kDa). Premature termination of translation occurred due to insertion of adenine at 105bp downstream of translational start codon (second adenine insertion 907bp) in R_{high}. Construction of transformants GT5 (with restored expression of GapA protein), SDCA (with restored expression of CrmA protein), showed cytadherence potential similar to parental strain R_{high}. Another transformant (GCA1), with complete gap A operon results in cytadherence comparable to R_{low}. Expression of both GapA and Crm A resulted in regain of virulence in GCA1 and could produce air sacculitis in chicken with no manifestation of tracheal lesions. (Papazisi et al., 2000; Papazisi et al., 2003). In addition, high frequency phase variation in expression of *gapA* gene occurs due to reversible point mutation in the beginning of *gap A* structural gene, effecting transcription of CrmA protein in return (mutant known as RCL2). Switching in expression of GapA and CrmA leads to variability in hemadsorption capability of *M. gallisepticum* (Winner et al., 2003).

Role of phase variation in Gap A expression has been investigated in R_{low}, R_{high}, RCL2 and HAD3 clonal variants. RCL2 lack Gap A and Crm A proteins due to a base substitution in *gapA* gene. HAD3 has been developed by insertion of Tn4001 in *crm A* gene, resulting lack of Crm A protein and reduced production of Gap A protein. Both RLC2 and HAD3 produced HA- phenotype. Both the mutants vary in virulence and colonization potential. RCL2 appeared efficient in colonization of upper and lower respiratory tract, slightly reduced than that of R_{low}. In spite of effective colonization, re-isolation of RCL2 is lower than R_{low} with reduced dissemination potential to other body organs.

2.10.7 Phase Variable Protein (PvpA)

PvpA is a phase variable non-lipid surface protein of *M. gallisepticum*, which undergo high-frequency phase variation. It also varies in size among different strains of *M. gallisepticum*, thus serving as a target of diagnostic and epidemiological importance.

The *pvp*A gene exists as a single copy and is not a member of multigene family. Sequence analysis of complete *pvpA* gene of R strain of *M. gallisepticum* indicates presence of N-terminal region with characteristic prokaryotic signal peptide sequences. The region lacks recognition site of signal peptidase, a characteristic feature of lipoproteins. Carboxy terminal region is surface exposed and rich in proline. C-terminal possess two identical direct repeat sequences consisting of 52 amino acids (aa), designated DR-1 and DR-2 (aa 224 through 275 and 301 through 352, respectively). Size variation in *pvp A* gene is due to deletions in C-terminal region and within DR-1 and DR-2. Unlike other variable proteins, *pvpA* gene does not undergo high frequency change in coding sequences. Proteins rich in proline residues are major immunogenic surface proteins, and are involved in host-pathogen interaction (Rosengarten et al., 1990; Yogev et al 1994; Boguslavsky et al., 2000; Liu et al., 2001).

M. gallisepticum strains vary in expression of PvpA protein. *M. gallisepticum* strain A5969 and R both possess complete *pvpA* gene, expression of *pvpA* product occur in R strain whereas in A5969 in spite of the transcription of gene, no PvpA protein has been detected using Mab 1E5. A probable explanation is nonsense mutation in DR-1 at nucleotide position 793 in strain A5969 might be responsible for premature termination of PvpA translation (Boguslavsky et al., 2000). Lack of recognition of protein by Mab 1E5 can be due to lack of C-terminal recognition site of the protein. Previous studies have shown differences in colonization and pathogenic abilities of strain A5969 and R resulting in reduced virulence of former and significant virulence of latter strain. Such variations in virulence can be related to the expression of PvpA protein (Levisohn et al., 1986; Boguslavsky et al., 2000).

PvpA protein of *M. gallisepticum* share similarities and differences with variable surface proteins of other Mycoplasmas. Earlier, three surface (VspA, VspB, and VspC) lipoproteins of *M. bovis* have been reported to spontaneously alter size and expression. Both *M. bovis* and *M. gallisepticum* share same epitope complementary to MAb 1E5. Variable surface proteins of mycoplasmas i.e Vsps of *M. bovis* and Vlps of *M. hyorhinis* are lipoproteins and the mechanism involved in surface anchoring is lipid modification of the said proteins. On the other hand, in PvpA hydrophobic regions serve as transmembrane domains (Rosengarten et al., 1990; Rosengarten et al, 1991; Rosengarten et al, 1994). Amino acid sequence shares 50% homology with another cytadhesin protein Mgc2 of *M. gallisepticum* (Boguslavsky et al., 2001). Cytadhesin proteins P30 and P32 of *M. pneumoniae* and *M. genitalium* share 54% and 52% homology with PvpA of *M. gallisepticum*. Another accessory protein HMW3 of *M. pneumoniae* involved in cytadherence is 49% similar to phase variable protein of *M. gallisepticum*.

2.10.8 Membrane Associated Proteins

A diverse range of membrane associated proteins is present in *M. gallisepticum*. These proteins include amino acid transport proteins (PotE), phosphate transport proteins (Pts) and those involved in protein translocation (SecA, SecE, SecY, YidC, trigger factor Tig and DnaK). Proteins involved in signal recognition pathway are FtsY and Ffh. Members of ABC (ATP binding cassettes) transporter family constitutes second major paralogous family of *M. gallisepticum* with 24 ATP-binding proteins out of total 75 proteins foreseen to be responsible for biomolecule transportation.

2.10.9 Antigenic Variation

Three immunogenic lipoproteins proteins p67, p72, and p75 along with PvpA play important role in antigenic variation. High-frequency phenotypic heterogeneity may arise due to a set of proteins including PvpA and pMGA along with other phase-variable proteins (i.e., p67, p72, and p75) (Yogev et al., 1994).

2.11 Diagnostic Techniques

Various diagnostic assays are being used for detection of antibodies and DNA of pathogen, in case of asymptomatic, acute and chronic infections of *M. gallisepticum*. SPA, i-ELISA and HI are of foremost importance. Due to reliability, specificity and sensitivity, SPA and i-ELISA are preferred. In addition, molecular assays based on polymerase chain reaction (PCR) are used for confirmation of infection. These diagnostic tests are internationally used at commercial level for screening and detection of infection.

SPA test is used for initial screening of flock, (Jalil and Islam 2010; Mukhtar et al, 2012; Islam et al., 2014), which may be followed by i-ELISA or HI for quantification of antibodies (Atique et al., 2012; Asif et al 2015; Ali et al., 2017). Serological monitoring of flock, in case of potential threat of infection by *M. gallisepticum* is preceded by attempts to isolate the causative agent and detection by PCR (Hanif and Najeeb, 2007; Heleili et al., 2011; Zute and Valdovska 2015; Rajkumar et al., 2018).

2.11.1 Serodiagnosis

Serological studies are conducted as a part of surveillance and monitoring programs for control of *M. gallisepticum* infections in commercial poultry. Serodiagnosis as a part of monitoring system, is based on SPA and ELISA.

2.11.1.1 Serum Plate Agglutination (SPA) Test

Screening tests are carried out to assess disease status in asymptomatic population or to evaluate probability of development of disease. After initial screening, positive population need further investigation by confirmatory tests (Maxim et al., 2014). SPA test is an important test for serological screening of flocks. Among different serological techniques for early, rapid and reliable detection of *M. gallisepticum* infection immune response, SPA test is reported to be more sensitive and less time consuming than ELISA and Heamagglutination Inhibition assay (HI).

A report by Lin and Kleven, (1982) explained serological difference among K503 and K730 strains from classic *M. gallisepticum* strain A5969. In the SPA test, birds singly infected with the variant strain had high antibody titers against the homologous antigen and a variable but lower response against the other antigens. It is well established that antigen differences between the hemagglutinin of the field strain and the diagnostic strains may lead to false negative results. Alteration in surface antigens can also result in varying serological response (Levisohn et al., 1995). This variability may function as a crucial adaptative mechanism, enabling the organism to escape from the host immune defence and to adapt to the changing host environment at different stages of a natural infection. Thus, diagnostic tools should be able to cope with a wide spectrum of antigen presentations.

2.11.1.2 Enzyme Linked Immunosorbent Assay (ELISA)

To assess serological response against *M. gallisepticum* infection as well as vaccination antibody titer, ELISA serves as most reliable serological assay. It is widely used for quantification of IgG produced in response to *M. gallisepticum* infection.

2.11.2 Molecular Diagnostics

Application of molecular techniques accelerated the detection of pathogens in field infections and outbreaks. PCR is one of the most important technique used for detection, diagnosis, and molecular typing of infectious agents. Its derivative techniques include random amplification polymorphic DNA (RAPD) or arbitrarily primed-PCR (AP-PCR) (Rawadi, 1998).

2.11.2.1 Polymerase Chain Reaction (PCR)

For detection of *M. gallisepticum* infection, use of PCR was reported by Nascimento et al., (1991). Simplicity and rapid detection of *M. gallisepticum*, *M. synoviae* and *M. iowea* PCR products has been reported by Garcia et al., (1996) using digoxigenin labelled oligonucleotide probes against variable region of 16S rRNA. PCR dot blot assay appeared to be more efficient and specific than gel electrophoresis. For prompt detection of

mycoplasmosis among commercial poultry, PCR is highly specific and sensitive, having potential of pathogen detection without isolation (Islam et al., 2011; Khalifa et al., 2013; Rauf et al., 2013).

Among two sets of rRNA genes (5S, 16S and 23S) present in *M. gallisepticum* genome, only one is organized as an operon. A unique 660-nucleotide intergenic spacer region (IGSR) is present between the 16S and the 23S rRNA genes. PCR targeted to the IGSR is valuable in discriminating a variety of *M. gallisepticum* laboratory strains, vaccine strains, and field isolates. Sequence analysis of *M. gallisepticum* IGSR appears to be a valuable single-locus sequence typing (SLST) tool for isolate differentiation, diagnostic as well as epizootiological studies (Raviv et al., 2007).

PCR assays also target the 16S rRNA gene, a highly conserved genomic region among bacteria for detection. *M. gallisepticum* infections among commercial and backyard poultry was detected, targeting 16S rRNA gene of *M. gallisepticum* by using PCR (Behban et al., 2005; Doosti et al., 2011; Rasoulinezhad et al., 2017; OIE, 2018). After targeting conserved regions for PCR, molecular study has been extended to surface protein (*LP* (*MGA*_0319), *gapA*, *pvpA* and *mgc2*) genes individually as well as collectively (Lui et al., 2001; Khumpim et al., 2015).

2.11.2.2 Real Time- Quantitative PCR (qPCR)

For prompt detection of avian mycoplasmas, molecular advancement leads to the development of real-time PCR. Concept of real-time PCR for detection of *M. gallisepticum* was introduced by Raviv et al., (2008). For detection of four different avian mycoplasma, real time Taq-man PCR assay was developed. Species specific assay was targeted against 16S-23S intergenic spacer region of *M. synoviae* and *M. meleadridis*, the upstream region to the 16S rDNA of *M. iowea*, and conserved region of the *mgc2* gene of *M. gallisepticum* (Mekkes and Feberwee, 2005; Raviv and Kleven, 2009). Multiplex real time PCR assay offers concurrent detection of 2 or more pathogens at the same time (Sprygin et al., 2010). The assay has been used for simultaneous detection of *M. gallisepticum* and *M. synoviae*

by Fraga et al., (2013) to detect mycoplasma infection among Brazilian commercial poultry.

A Taqman real-time PCR assay has been reported for detection of cytadhesin encoding surface protein (*mgc2*) gene of *M. gallisepticum* and haemagglutinin surface protein (*vlhA*) gene of *M. synoviae* by Ehtisham et al., (2015). Sensitivity of duplex RT-PCR is reported as 10^3 CFU/ml for *M. gallisepticum* and 10^2 CFU/ml for *M. synoviae* template DNA. Specificity of the assay is 100% for *M. synoviae* and *M. gallisepticum* specific probes.

2.11.2.3 Insulated Isothermal PCR (iiPCR)

A confined fluid layer when heated from below develop a buoyancy driven instability known as Rayleigh-Benard convection. This phenomenon has been used by Krishnan et al., (2002) to perform PCR. Steady circulation of energy by fluid density gradient leads to sequential development of denaturation, annealing and extension temperatures carried out in conventional process. The whole process has been deciphered by Chou et al., (2011) in developing capillary convective PCR (CCPCR). Natural convection, drive reagents in capillary to circulate through different temperature zones, corresponding to the PCR cycle, leading to the amplification of target DNA.

To overcome the influence of environmental temperature variations, a thermally baffled device has been developed by Chang et al., (2012). The insulated isothermal device (iiPCR) was further used by Tsai et al (2012(a)) to successfully demonstrate the diagnosis of white spot syndrome virus (WSSV). Amplicons were generated in 30-85 mins, in an iiPCR device, in which a special polycarbonate capillary tube (R-tubeTM) was heated isothermally by a copper ring attached to its bottom and shielded by a thermal baffle around its upper half. The iiPCR assay proved to be specific, sensitive, rapid and low cost. Initially, analysis of amplified products was carried out by agarose gel electrophoresis. Real time detection by using fluorescent dyes provide sensitive method of detection without post amplification processing. Tsai et al., (2012 (b)) integrated TaqMan probe for target specific detection of amplicons. Target specific TaqMan hydrolysis probes, with terminal reporter

dye and quencher dye, are designed to bind the template DNA at annealing step. During extension, hydrolysis of probe occurs separating reporter and quencher dye, which results in fluorescent emission. An optical detection system has been integrated in iiPCR device by for detection of fluorescent signals.

The assay is successfully used for detection of *Salmonella* from chicken meat samples employing TaqMan probes and PCR primers targeting *yrfH* gene (Tsen et al., 2013). A field deployable device, POCKIT^{TM} Micro Plus Nucleic Acid Analyzer has been used for detection of Canine distemper virus (CDV) (Wilkes et al., 2014), Equine influenza Virus (EIV) H3N8 (Balasuriya et al., 2014), Bluetongue virus in ruminants (Ambagala et al., 2015), classical swine fever virus (CSFV) (Lung *et al*., 2015), *Feline immunodeficiency virus* (FIV) (Wilkes et al., 2015), Foot-and-mouth disease (FMD) (Ambagala et al., 2016), *M. synoviae* (MS) (Kuo et al., 2016), malaria (Chua et al., 2016), rotavirus (Soltan et al., 2016), Feline leukaemia virus (FeLV) (Wilkes et al., 2017), *Vibrio parahaemolyticus* responsible for acute hepatopancreatic necrosis disease (AHPD) in shrimps (Chang et al., 2018), Dengue virus (DENV) infection and its serotypes (Go et al., 2016; Wang and Gubler 2018; Tsai et al., 2018, Tsai et al., 2019), Seneca Valley virus (SVV) (Zhang et al., 2019), and *Staphylococcus aureus* from food samples. Tsai et al., (2019) reported use of POCKIT Central Nucleic Acid Analyzer for detection of dengue virus in human serum and mosquitoes.

2.12 Genotyping

With the evolution of molecular methods, infectious disease investigations acquired new dimensions. Rapidly evolving molecular techniques are now being used to trace and track changes and variations in genetic makeup of infectious organisms responsible for economic losses and involved in spread to larger population both in animals and humans. Genotyping provides a reliable and authentic approach for elucidation of genetic changes and epidemiological background of infectious agent. For this purpose, these methods should be reproducible. Exploration of molecular biology of *M. gallisepticum* by Razin et al., (1998)

and whole genome sequencing by Papazisi et al., (2003) provided strong foundations for a number of modified techniques to be used for strain differentiation.

A number of different techniques have been employed for detection, and strain differentiation of *M. gallisepticum*. These comprise of polyacrylamide gel electrophoresis of cellular proteins, Southern blot analysis, RFLP of genomic DNA (Khan et al., 1987; Yogev et al., 1988; Kleven et al., 1988). Identification of *M. gallisepticum* strains and intraspecies variation is also carried out by RAPD (Geary et al., 1994; Fan et al., 1995a; Lui et al., 2001). PCR followed by high melting curve analysis also provides efficient and reliable means of genotyping of *M. gallisepticum* strains and isolates.

2.12.1 Restriction Fragment Length Polymorphism (RFLP)

Analysis of restriction digestion pattern using endonucleases is used for strain differentiation. It has been used for differentiation of mollicutes, including *Ureaplasmas, Spiroplasmas, Acholeplasmas and Mycoplasmas* (Bovè et al., 1982, Razin et al., 1983; Razin and Yogev, 1986). Restriction endonuclease analysis provided a pattern of relatedness and difference among North Carolina *M. gallisepticum* strains. Further studies included using rRNA gene specific probes in Southern blot hybridisation (Kleven 1988 a, b). For molecular typing, RFLP of *pvpA* gene was carried out by Lui et al., (2001).

M. gallisepticum pvpA gene, encoding phase variable protein is exploited to classify strains on the basis of variations in C-terminus-encoding region. PCR-RFLP could differentiate vaccinal strains from field isolates. Since, it can directly detect variations from clinical samples, the process is rapid and bypass the need to culture the organism. Based on assessment of divergence, sequence analysis and RFLP can be used for epidemiological studies (Lui et al., 2001)

2.12.2 Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP)

RAPD had been used to represent diversity among vaccinal strains and difference of vaccine strains from field and reference strains using commercially available kit (Charlton et al., 1999). The assay could discriminate vaccine and wild *M. gallisepticum* strains in turkeys (Kleven, 2004). RAPD and amplified-fragment length polymorphism (AFLP) have been used to assess genetic variability among house finch isolates of *M. gallisepticum* (Cherry et al., 2006).

The AFLP method can identify and differentiate both *M. gallisepticum* field strains from recent outbreaks, epidemiological relatedness, and source of mycoplasma infection. Vaccine strains can also be differentiated from other field strains. Discrimination potential of AFLP and RAPD and gene-targeted typing of gene cytadhesin-like protein encoding gene, *mgc*2 provided evidence of good co-relation among all the three assays, with AFLP analysis having a much higher discriminatory power and reproducibility (Hong et al., 2004). Genotyping of *M. gallisepticum* by AFLP and RAPD has been carried out to find inter species and intra species heterogeneity among 5 different avian mycoplasma spp. (Feberwee et al., 2005).

2.12.3 Ribotyping

Bacterial sub-species and intra-species variation can be assessed by ribotyping of rRNA genes (rDNA) targeting 23S, 5S and 16S genes. Intra species heterogeneity is reported by Razin & Yogev, (1986) targeting 16S rRNA gene. Technique for differentiating vaccine strain F from field isolates of *M. gallisepticum* has been demonstrated by Yogev et al., (1988). After the analysis of European, US and Japanese isolates of *M. gallisepticum*, Nagai et al., (1995) classified these isolates in 4 different phylogenetic groups.

2.12.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis has been done to differentiate protein banding pattern of vaccine strain (F), standard strains A5969, S6 and variant strain. and to differentiate between vaccine strains F and Ts-11(Khan et al., 1987).

2.12.5 Sequence Analysis

The sequencing of the *M. gallisepticum* IGSR (660 bp region between 16S and 23S rRNA gene) appears to be a valuable single-locus sequence typing (SLST) tool for *M. gallisepticum* isolate differentiation in diagnostic cases and epizootiological studies (Raviv et al., 2007).

A typing method based on the sequences of gene fragments from a number of different housekeeping loci [multilocus sequence typing (MLST)] proved to provide valuable epidemiological information about infectious disease. Gene-targeted sequencing (GTS) as a typing tool for differentiating *M. gallisepticum* strains is MLST. GTS analysis of *pvpA* gene, *gapA* gene, *mgc2*, and *lp* (MGA_0319), and RAPD analysis of 67 field isolates and 10 reference strains has been carried out by Ferguson et al., (2005). GTS analysis of individual genes, *gapA*, MGA_0319, *mgc2* and *pvpA*, identified 17, 16, 20 and 22 sequence types, respectively. Discriminatory power of GTS of multiple genes is greater than that of RAPD analysis.

M. gallisepticum infection in commercial and backyard turkey in Iran has been investigated by performing PCR targeted to 16SrRNA gene, followed by sequence analysis of *mgc2* gene. Sequence analysis of *M. gallisepticum* revealed high similarity to Pakistani (CK.MG.UDL.PK.2013.2(KF874280)) and Indian *M. gallisepticum* isolates (Rasoulinezhad et al., 2017)

2.13 Infectious Disease Control

Infectious diseases can be categorised into infections which can be consistently eradicated from the stock or population and others which cannot be. Effective biosecurity is the preferred method where elimination of infection can be achieved. Maintaining infection free flocks include practice of all-in all-out systems. In case of compromised biosecurity program, increased poultry concentration in small geographic area increases the probability of exposure. Multi-age poultry population in case of layers and in some cases of breeding flocks, also participates in transmission of infection. In such cases, antimicrobial medication or immunisation is the method of choice (Kleven et al., 1997; Machado et al., 2017).

2.13.1 Different Approaches to Control Infection

Mycoplasmosis is of significant importance among poultry diseases due to horizontal and vertical transmission. Since *Mycoplasmas* are vertically transmitted, mycoplasma free replacement flock is of foremost importance. Elimination of infection from breeder flocks is possible by breaking vertical transmission cycle. In countries with highly developed commercial poultry, breeder stocks are free of *M. gallisepticum* and *M. synoviae.* Eradication of *M. gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae* from genetic lines has been achieved by major poultry breeders in USA. Removal of *Mycoplasmas* include egg dipping, egg heating or egg injections with antibiotics. This procedure is followed by rearing and eliminating infected progeny. In case of infection of genetic lines or grandparent flocks with *M. gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae*, the major breeding companies eliminate infected flocks (Kleven, 2008).

2.13.2 Use of Anti-*Mycoplasma* Antibiotics

After the onset of infection, antibacterial medication can reduce growth and economic losses, as well as can control the severity of disease. In infected breeding stock, 5-7 days continuous treatment by medication is done. Controlling *M. gallisepticum* infection by medication also limits spread of disease. In commercial layers infected by *M.*

gallisepticum, antibiotic treatment is used to improve egg production and reduce vertical transmission via eggs (Kleven, 2008).

Due to lack of cell wall in *Mycoplasmas*, β-lactam antibiotics are ineffective for treatment. These include penicillins and cephalosporins. Macrolides, tetracyclines, fluoroquinolones are effective for control of Mycoplasmosis. In USA tylosin and tetracyclines are used to prevent respiratory infection in broilers and turkey. Highly effective antibiotics including enrofloxacin or tilmicosin, are not approved for use in poultry in the US (Kleven, 2008).

Tilmicosin, a broad-spectrum bacteriostatic synthesized from tylosin is the first choice for treatment of *M. gallisepticum* infection in many countries. Different doses have been described by Kempf et al., (1997) that significantly reduced growth losses and respiratory signs. Increased dose of tilmicosin resulted in decreased serologically positive, culture positive, experimentally infected birds. Number of *M. gallisepticum* shedding birds also reduced at high dose (Kempf et al., 1997; Roussan et al., 2006).

During *M. gallisepticum* outbreak in broilers, use of tilmicosin 20mg/kg body weight for successive five days is recommended (Garmyn *et al*., 2019). Tylosin and tilmicosin treatment in 35mg/kg or 100mg BW and 10mg/kg BW or 20 mg/kg BW respectively, have been efficacious in reducing pathological outcomes of disease. Comparative study about efficacy of different antibiotics in Pakistan showed promising effect by tilmicosin with MIC ranging from 0.78 µg/ml to 6.25 µg/ml. Enrofloxacin, erythromycin and tylosin showed moderate effect, tetracyclines (oxytetracycline and chlortetracycline) being least effective with highest MIC90 and MIC50 values (Khatoon et al., 2018).

In spite of the effectiveness of antibiotic therapy to decrease economic losses, to control vertical transmission, and to reduce pathological manifestation of disease, it plays no role in eliminating infection from the flock. Excessive use can lead to the development of resistance against antibiotics (Reinhardt et al., 2005). Emergence of antimicrobial resistance is a worldwide concern and a threat to human and animal health (Roth et al., 2019).

2.13.3 Use of Vaccines

In poultry industry, substantial level of protection against poultry pathogens is provided by vaccines. Administration of vaccines may vary depending upon individual administration to mass immunization. Preferred route of vaccination in case of bacterial and viral respiratory pathogens is via spray, to facilitate uptake by inhalation. Other routes include ocular, oral, intra muscular or subcutaneous (Bermudez, 2008).

Vaccination and immunization against *M. gallisepticum* are done where eradication of pathogen is not achievable, in case of multi age farms, and existence of unidentified reservoir of pathogen. Objective of vaccination include reduction in economic losses through drop in egg production, to get infection free progeny, and to limit the spread of pathogen through horizontal transmission (Fabricant, 1975; Whithear 1996; Kleven 2008; Umar et al., 2017)

Choice of vaccine or vaccine candidate strain is an important attribute effecting the flock health, control measures and subsequent impact on economic viability of the procedure. Noteworthy feature of vaccines is that it must be developed from highly potent and pure seed stock. In addition, a vaccine should be able to generate strong immune response by a single dose. Safety of vaccine strains vary depending upon the type of vaccine (Whithear, 1996). Oil-emersion bacterins, live vaccines and live attenuated vaccines possess concerns of adjuvant, potential to cause disease and reversion to virulent form. Commercially available vaccines include inactivated, oil-emulsion bacterins, live vaccines, or a recombinant live poxvirus vaccine containing and expressing key protective *M. gallisepticum* antigens (Kleven, 2008).

2.13.3.1 Bacterins

Bacterins contain inactivated/killed whole organism suspension in different adjuvants. A variety of adjuvants include oil emulsion, aluminium hydroxide-based adjuvants, liposomes, immune stimulating complexes (iscoms) (Kleven, 2008).

Bacterins are reported to slightly improve response against intratracheal infection challenge (Talkington et al., 1985). Although colonization of *M. gallisepticum* get reduced, it did not provide complete protection against infection of homologous as well as heterologous strains. Although bacterins offer protection against respiratory infection as well as vertical transmission, but lack of efficacy has also been reported (Khan et al., 1986; Kleven 1986; Yagihashi, 1986; Kleven, 2008; Machado et al., 2017). Inactivated/killed bacterin reduces the shedding of *M. gallisepticum* but there is no decrease in horizontal transmission in laying hens (Feberwee et al., 2006). Use of inactivated, oil emulsion bacterins is advantageous in field to reduce economic losses, without establishing the use of live vaccine strain. On the other hand, compromised protection and administration of vaccine to individual birds intramuscular or intravenous render it slightly less efficacious (Kleven, 2008).

2.13.3.2 Live Vaccines

Vaccination with live attenuated vaccines is an option for controlling *M. gallisepticum* infection when the prevention of exposure is impossible (Whithear, 1996; Shil et al., 2011).

Valuable properties of a live vaccine include, the ability of strain to induce strong, long term immune response without causing disease (limited to upper respiratory tract and restricted invasion to internal body organs) and limited transmissibility (horizontal transmission) (Whittlestone, 1976). Strong immune response is generated by pathogenic strains, such as *M. gallisepticum* F strain and R strain, but R strain appears to be more virulent. Use of virulent strains as vaccine is not recommended due to its disease-causing potential. Since *M. gallisepticum* possess tendency to interact with other pathogens that may cause severe infection. (Whittlestone 1976; Rodriguez and Kleven, 1980; Levisohn 1984; Levisohn et al., 1986; Whithear, 1990(b))

For use in layer chickens, three *M. gallisepticum* vaccine strains have been approved. These include 2 F-strain based live vaccines named as Poulvac Myco F, by FMG (Fort Dodge Animal Health, Ft. Dodge, IA) licensed by the USDA in 1988. The other is AviPro MG F

(Lohmann Animal Health Int., Winslow, ME) re-released in January 2011. Two other strains are TS-11 (Merial-Select, Gainesville, GA), developed from an Australian field strain in late 1980s and MYCOVAC-L, often referred to as 6/85 (Merck Animal Health, Millsboro, DE) approved for vaccination in 1990s (Purswell et al., 2012).

In *M. gallisepticum* strains, complex relationship exists in infectivity, pathogenicity and immunogenicity (Levisohn, 1984). Level of protection provided by a live vaccine is directly related to the virulence of vaccine strain (Lin and Kleven, 1984). Comparative efficacy of three live vaccines F-strain, ts-11 and 6/85 showed that F-strain provided better protection, produced stronger antibody response and persisted for longer time in trachea. Ts-11 and 6/85 strains show milder antibody response due to reduced virulence, and provide lower level of protection after aerosol challenge than F strain (Abdelmotelib and Kleven, 1993). However, administration via suggested routes (eye drop for ts-11 and fine aerosol for 6/85) resulted in significant level of protection by both vaccines (Noormohammadi and Whithear, 2019). Live *M. gallisepticum* vaccines to commercial layer chickens are recommended to be administered at less than 10 wk of age, and is oftenly carried out at 8 weeks of age (Purswell et al., 2012).

The prevention of exposure of flocks to wild-type challenge by intense biosecurity and biosurveillance via serological monitoring is considered the first choice in control practices (Stipkovits & Kempf, 1996).

Use of avirulent R_{high} strain with expression of GapA (protein) as live *M. gallisepticum* vaccine (GT5) is reported (Papazisi et al., 2002). GT5 induced immune response successfully neutralized the challenged R_{low} strain.

2.13.3.3 F-Strain Live Vaccine

Yamamoto and Adler described a naturally occurring *M. gallisepticum* strain, isolated in 1950s in US as F strain. Its use as vaccine was reported (Adler and Yamamoto, 1957; Carpenter et al., 1981; Levisohn et al., 1986). Use of F strain as live vaccine in commercial layers proved to be effective in providing protection from infection and in reducing

production losses. It appeared virulent for use in broilers and turkeys (Rodriguez et al., 1980 (a), Rodriguez et al., 1980 (b); Carpenter et al., 1981; Branton et al., 1985; Cummings et al., 1986). Egg transmission of F strain has been reported in chicken when challenged during production, whereas no transmission is reported when challenged before the onset of lay (Lin et al., 1982). F strain vaccines are available in lyophilized form and can be given in drinking water, eye drops, or by spray (Nascimento et al., 2006). Limited transmissibility and potential to displace field strains referred *M. gallisepticum* F strain a suitable candidate to be used in multi-aged flocks (Purswell et al., 2012). Live attenuated F strain could produce mild respiratory signs, which can be complicated by subsequent secondary pathogen.

2.13.3.4 6/85 Live Vaccine

M. gallisepticum vaccine strain 6/85 is developed from a U.S field isolate. The vaccine appeared to be safe and avirulent for both chickens and turkeys (Evans et al., 1992). Weak antibody response is generated by 6/85 vaccine immediately after vaccination, and subsequently increase to maximum level at 30 weeks post vaccination, accompanied by enhanced detection from upper respiratory tract. This may be due to its transmission among birds or enhanced colonization in respiratory tract (Noormohammadi and Whithear, 2019). However, the ability to replace challenge strain appeared to be limited (Kleven et al., 1998; Feberwee et al., 2006).

2.13.3.5 Ts-11 Live Vaccine

M. gallisepticum vaccine strain ts-11 has been developed from Australian field strain (80083) of moderate virulence by Soeripto (1987). The strain (80083) was attenuated to 50 repeated passages in broth and reported to induce less air sac lesions than parental strain. Complete avirulence is reported after 100 passages (Soeripto et al., 1989). Ts-11 is a temperature sensitive(ts) strain developed after chemical mutagenesis (100 µg/ml N-methyl-N-nitro-N-nitrosoguanidine) of field strain showing adequate growth at 33°C but reduced growth at 39.5°C. It is administered as single dose via eye drops at 4 weeks of age. Due to its

temperature sensitivity, ts-11 is restricted to upper respiratory tract and did not invade airsacs (Whithear et al., 1990 (a); Whithear et al., 1990 (b)). The immune response generated by vaccine is highly dependent on the dose of vaccine. In vaccinated flock, assessment of serological response has not been consistently detectable by SPA test (Whithear et al., 1990 (a); Shil et al., 2011).

Vaccine strain ts-11 lack primary cytadhesin GapA protein (Mudahi-Orenstein et al., 2003). Comparison of DNA sequence analysis of field strain 80083 and its derived vaccinal strain ts-11, provided evidence of presence of 20 bp repeat sequence. This sequence duplication due to frameshift masks the expression of GapA protein in ts-11(Kanci et al., 2004). A comparison of GapA+ *M. gallisepticum* ts-11 vaccine with commercially available Vaxsafe M. gallisepticum ts-11 vaccine is done (Shil et al., 2011). Challenge studies show persistent colonization, long term protective immunity and immunogenicity of GapA+ ts-11 vaccine than Vaxafe ts-11.

2.13.3.6 K Strain as Vaccine Candidate

Efforts to find and discover an efficacious vaccine are continuously taking place. Various strains have been tested as live vaccines, in order to further improve *M. gallisepticum* control by vaccination. K 5054 strain, isolated from turkeys experimentally infected by sinus exudates of asymptomatic, infected turkeys has been studied as potential vaccine candidate (Ferguson et al., 2003). The study reported K 5054 as avirulent and immunogenic strain which was consistently isolated from upper respiratory tract of vaccinated turkeys.

Three *M. gallisepticum* strains K3020, K4649A and K2101 have been evaluated as potential candidates of *M. gallisepticum* live vaccine. After initial screening based on pathogenicity and efficacy of live vaccine, re-isolate of K2101 (K5831B-19), designated as K strain for further testing. It has been found to transmit horizontally at low rate, show persistent colonization in upper respiratory tract, presented no increase in virulence, and no vertical transmission.

3 Materials and Methods

3.1 Study Area

The regions identified for study were based on commercial poultry rearing areas. These included Breeder Broiler farms located in Islamabad Capital Territory, Punjab Province (Lahore and Rawalpindi districts), Khyber Pakhtunkhwa (Mansehra district), and Layer hatcheries located in Lahore, Samundari, Kamalia and Chakwal districts of Punjab province (Figure 3.1).

3.2 Collection and Processing of Samples

During the study, samples including sera, tissue and swabs were collected and investigated from year 2015-2019. Commercially reared flocks from selected regions and domestic/ wild birds were sampled to assess anti-*Mycoplasma gallisepticum* antibodies. Blood samples were collected from subclavian vein of birds and transported to the laboratory following standard operating procedures (SOPs) (Tuck et al., 2009). Samples including tracheas, air sac exudates, turbinates, lungs of sick necropsied/dead birds were collected. Sample from live birds comprised of swabs from choanal cleft (as palatine fissure swab), trachea (as tracheal swabs) and cloaca and dipped in *Mycoplasma* broth/PBS (Annex-1) (OIE, 2018).

3.2.1 Processing of Tracheal and Cloacal Swabs

Swab samples from suspected birds/ flocks were collected and transported to the laboratory. Tubes containing swabs were vortexed at 1500 rpm for 2 minutes, swabs were squashed and removed. Residual material was again vortexed at 1500 rpm for 10 minutes and filtered through 0.45 µm syringe filters (Sartorius, Minisart, Germay). To the filtrate penicillin/ ampicillin was added to avoid contamination. Filtered samples were stored at -20°C or -70°C until further use (Rafique, 2018).

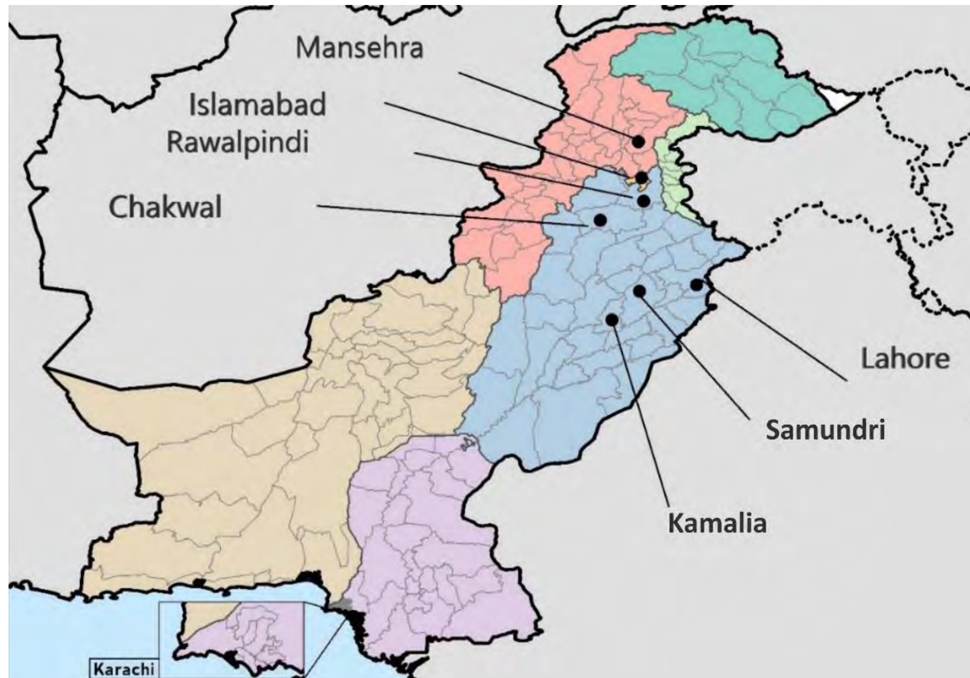


Fig. 3.1 Map of Pakistan showing selected study area

3.2.2 Processing of Tissue Samples

Tissue material was cut into fine pieces with the help of scissors and forceps in sterilized petri plate. To the samples, 2-3ml of PBS was added and suspended samples were then homogenized in stomacher (Biomaster, UK) at high speed for 30 seconds or medium speed for 1 minute. Suspended samples were then centrifuged at 2000 rpm for 10 minutes. Supernatant was collected in 5 ml collection tube and filtered through 0.45 µm syringe filter (Sartorius, Minisart, Germany). Processed and filtered samples were stored at -20°C or -70°C until further use.

3.2.3 Processing of Blood Samples

Blood samples were collected from subclavian vein of suspected/experimental birds using sterilized syringes. Collected blood samples were immediately transferred to collection tubes and centrifuged at 1500 rpm for 5 min. After settlement of blood cells at the bottom of the tube, serum was collected from the upper layer. In case of haemolysis, serum sample was discarded and not used.

3.3 Serodiagnosis of *Mycoplasma gallisepticum*

Sero-surveillance was carried out to check the status of anti-*M. gallisepticum* antibodies in unvaccinated commercial flocks and backyard poultry. Techniques used for antibody detection included Serum plate agglutination (SPA) test and Enzyme linked immunosorbent assay (ELISA).

3.3.1 Serum Plate Agglutination Test (SPA)

For sero-monitoring of *M. gallisepticum*, SPA was performed by using commercially available *M. gallisepticum* antigen (Charles River Laboratories) within 72 hrs of serum collection. Test sera and SPA antigen were brought to room temperature at 20-25°C. On a clean white tile, each test serum was added in 0.25ml, followed by addition of 0.25ml *M. gallisepticum* stained antigen. Serum and antigen were mixed using a wooden stick/ stirring rod and tile was swirled to increase surface area of reaction. Observations were made

within made within two minutes of addition of antigen to the sera (Wanasawaeng et al., 2015; OIE, 2018). Positive and negative controls were included in the test.

3.3.2 Enzyme Linked Immunosorbent Assay (ELISA)

Indirect enzyme linked immunosorbent assay (ELISA) was performed using commercially available Idexx *M. gallisepticum* ELISA kit. Test sera and reagents were brought to ambient temperature and the assay was carried out at room temperature (20-25°C).

Test sera were diluted by adding 1µl to 500 µl of the diluent provided with the kit. Undiluted negative and positive controls, 100 µl were loaded in duplicate in first four wells (A1-A4) of antigen coated plate respectively. Each test sample i.e. 100 µl was loaded in the ELISA plate and position was recorded. Antigen coated plate with test samples was incubated for 30 minutes at room temperature. After 30 minutes, washing of each well was carried out by deionized water thrice. Anti-MG horseradish peroxidase enzyme (provided in kit) was added in a quantity of 100 µl as conjugate, and incubated for 30 minutes at room temperature. After incubation, the plate was washed thrice, using deionized water. TMB substrate (provided in kit) was added in a quantity of 100µl to each well, and incubated at room temperature for 15 minutes followed by addition of 100 µl of stop solution to each well. X-check software was used to record results, by taking absorbance at 650nm using ELISA reader.

3.4 Molecular Diagnosis of *Mycoplasma gallisepticum*

3.4.1 DNA Extraction

DNA extraction was carried out by using Favorgen nucleic acid extraction kit (Favorgen, Taiwan). For DNA extraction 150 µl of sample was taken in a sterile, appropriately labelled 1.5ml microcentrifuge tube. To each sample tube 570 µl of VNE buffer was added, and incubated at room temperature for 10 mins after mixing. After incubation, 570 µl of 96-100% ethanol was added to the sample and mixed by pulse vortexing. VNE column was placed on collection tube, and after appropriate labelling 700 µl of sample was loaded on

VNE column. Test samples were centrifuged for 1 min at 8000 x g. Fluid in collection tube was discarded, rest of the sample was loaded on VNE column and centrifuged at 8000 x g for 1 minute. Flow-through was discarded and VNE column was combine with a new collection tube. 500µl of Wash buffer 1 was added to each column, centrifuged at 8000 x g for 1 minute, and flow through was discarded. After combining VNE columns with collection tubes, 750 µl Wash buffer 2 was added to each column and centrifuged at 8000 x g. Flow through was discarded and the step was repeated after combining VNE column and collection tube. To remove residual fluid, VNE column was centrifuged at 18000 x g, flow through was discarded along with collection tube. VNE column was combined with elution tube. To the central membrane of VNE column, 50 µl pre-heated RNase-free water was added and allowed to stand for 2 minutes, followed by centrifugation for 2 minutes. DNA collected in elution tube was stored -20 till further use.

3.4.2 Nucleic Acid Quantification

For DNA quantification, NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific) was used. To the lower measurement pedestal 1 µl of autoclaved nuclease free water (Gibco, Invitrogen, USA) was added, and upper arm was lowered for spectral measurement. Thermo Software IQ program was run to measure the optical density of blank. Upper arm was raised and lower pedestal was wiped. DNA sample, 1 µl was added to the lower pedestal, upper arm was lowered and after assigning ID to the sample, reading was recorded using the software at A260/A280 ratio.

3.4.3 Polymerase Chain Reaction

DNA polymerase chain reaction was performed using Dream Taq Green PCR Master Mix (2X) (Invitrogen, Cat. No. K11081), using 16SrRNA primers (MG-14F: 5’-GAG-CTAATCTGTAAAGTTGGTC3’ and MG-13R: 5’GCTTCCTTGCGGTTAGCAAC-3’) (OIE, 2018) following the protocol mentioned below.

All the components and reagent were vortexed gently after thawing. Placed the thin walled PCR tube on ice stand and 50ul reaction was setup by adding 25ul of 2x master mix, 10ul

DNA template, 10ul RNase free water, 1ul forward and reverse primer each according to the concentrations mentioned below. To homogenize the mixture, PCR tubes were vortexed again (Table 3.1). PCR was performed in a thermal-cycler (Eppendorf, Germany) using the profile recommended by (OIE, 2018).

The tubes were then placed in thermal cycler for the following cycles: 40 cycles: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 1 cycle (final extension): 72°C for 5 minutes and soak at 4°C given in (Table 3.2) (OIE, 2018).

3.4.4 Gel Electrophoresis

Amplified products were visualised by gel electrophoresis, using 1% agarose gel (Annex-IV). Briefly, 1 gm of Invitrogen Ultra-pure Agarose (16500-500) was dissolved in 100 ml of 1X TBE buffer (Annex-II), and boiled till clear. Ethidium bromide (Vivantis lot # 0298B236) (Annex-V) was added from 6-10 µl and mixed. The mixture was poured in casting tray with comb inserted in it and allowed to solidify. Upon solidification, 10 µl of amplified PCR (Annex-VI) product was loaded along with DNA step ladder (Gene ruler Thermo Scientific SM0313) (Annex-VII). The gel was run for 40 mins at 170V and viewed in gel documentation system.

3.5 Isolation of *Mycoplasma gallisepticum*

3.5.2 Preparation of *M. gallisepticum* Broth

M. gallisepticum broth base (Oxoid-CM0403) 2.04 g was dissolved in 80 ml of distilled water. Broth was mixed using magnetic stirrer, and autoclaved at 121℃ for 20 mins. Supplement G (Oxoid SR0059) was dissolved in 20 ml of distilled autoclaved water. The broth was allowed to cool at 50℃, and mixed with reconstituted supplement G aseptically.

Alternatively, *M. gallisepticum* broth was prepared as recommended in (OIE, 2018) with slight modification of Frey’s media (Ley, 2008).

Part A: *M. gallisepticum* broth base (Oxoid-CM0403) 17.85 g was dissolved in 700 ml of distilled water. The solution was mixed by using magnetic stirrer and autoclaved at 121 ℃

for 15 mins at 15lb pressure. Media was allowed to cool till 56℃ before addition of component B.

Part B was composed of horse serum (heat inactivated) 150 ml, 100ml of 25% (w/v) yeast extract, 10 ml of 10% (w/v) glucose solution, 10ml of 5% (w/v) thallous acetate (Alfa Aesar-11842, Germany), 20ml of 0.1 % solution of phenol red was added as indicator, 5ml of Ampicillin (200,000 International Units (IU)/ml), 5ml of 1% L. cysteine (Daejung 2611-4425, Korea) was added to the solution. Nicotinamide adenine dinucleotide (NAD) was omitted form the media composition. pH was adjusted to 7.8 and the solution was filtered by syringe filtration (Minisart, Sartorius Germany). Both component A and B were mixed aseptically and *M. gallisepticum* broth was stored at 4°C.

3.5.3 Preparation of *M. gallisepticum* Agar

M. gallisepticum agar base (Oxoid-CM0401) 2.84 g was dissolved in 80 ml of distilled water. Broth was mixed using magnetic stirrer, and autoclaved at 121℃ for 20 mins. Supplement G (Oxoid-SR0059) was dissolved in 20 ml of distilled autoclaved water. *M. gallisepticum* broth was allowed to cool at 50℃, and mixed with reconstituted supplement G. Agar media was poured in petri plates aseptically.

Alternatively, *M. gallisepticum* agar was prepared as recommended in (OIE, 2018) with slight modification of Frey's media (Ley, 2008).

Part A: *M. gallisepticum* agar base (Oxoid-CM0401) 24.8 g was dissolved in 700 ml of distilled water. The solution was mixed by using magnetic stirrer and autoclaved at 121℃ for 15 mins at 15lb pressure Media was allowed to cool till 56℃ before addition of component B.

Part B was composed of horse serum (heat inactivated) 150 ml, 100 ml of 25% (w/v) yeast extract, 10 ml of 10% (w/v) glucose solution, 10ml of 5% (w/v) thallous acetate (Alfa Aesar-11842, Germany), 20 ml of 0.1 % solution of phenol red was added as indicator, 5 ml of Ampicillin (200,000 International Units (IU)/ml), 5 ml of 1% L. cysteine (Daejung 2611-4425, Korea) was added to the solution. Nicotinamide adenine dinucleotide (NAD) was omitted form the media composition. pH was adjusted to 7.8 and the solution was

filtered by syringe filtration (Minisart, Sartorius Germany). Mixing of component, A and B was done carefully to avoid bubble production. Media was poured in sterilized glass petri plates, plates were sealed and stored at 4°C upto14 days (Ley, 2008).

3.5.3 Inoculation of Samples

Processed tissue and swab samples were inoculated after filtration through 0.45µm syringe filter (Minisart, Sartorius Germany). *M. gallisepticum* broth was inoculated with appropriate amount of sample and incubated at 37°C for 5-7 days. Samples were observed daily for change in colour of the broth from red to orange yellow. Field samples were maintained by subsequent passaging till 20-25 days. Positive *M. gallisepticum* broth culture was further streaked on *M. gallisepticum* agar. Agar plates were incubated in moisture and CO_2 rich environment for 5-7 days. Inoculated plates were daily checked under stereomicroscope (Labomed-CSM2) for 7-14 days.

Table 3.1: PCR mixture profile for *M. gallisepticum* detection Using Dream taq Green PCR master mix by Invitrogen

Sr.	Components	Final Concentraton	Volume/50µl
1	2X Reaction Mix	2X	25 µl
2	Template DNA	10pg-1	5-10 µl
3	Primer (Sense) (10µM)	0.2µM	1 µl
4	Primer (Anti-sense) (10µM)	0.2µM	1 µl
5	Autoclaved distilled water	--	10-12 µl
	Total reaction mixture		50 µl

Table 3.2: Polymerase Chain Reaction Profile for *M. gallisepticum*

Sr.	Steps for PCR	Temperatures	Time	Cycles
1.	Denaturation	94ºC	30 seconds	X 40
2.	Annealing	55ºC	30 seconds	
3.	Extension	72ºC	60 seconds	
4.	Final Extension	72ºC	05 min	
5.	Storage	4ºC	until used	

4 Sero-surveillance and Isolation of *Mycoplasma gallisepticum* from Field

4.1 Introduction

Serology is an inexpensive and rapid procedure to assess development of antibodies against infectious agent. Sero-epidemiology of different human and animal diseases has not only been helpful in identifying population exposed to the pathogen of concern but also in delineating determinants associated with diseased state. Continuous monitoring of highly contagious diseases is necessary for control and preventive measures.

M. gallisepticum infections are endemic in poultry rearing countries. Such infections present variety of symptoms ranging from sub-clinical infection to chronic respiratory disease (CRD). Significant economic losses occur due to reduced productivity of *M. gallisepticum* infected poultry. At subclinical or asymptomatic stage, due to lack of apparent symptoms of infection, transmission rate within a flock could be high and disease can spread rapidly (Ley, 2008). Seromonitoring studies, play a pivotal role in tracing and evaluating subclinical infections. These studies could help regulatory authorities and poultry farmers to maintain infection free flocks by implementing control measures either by medication or vaccination. In past, Pakistan had successfully controlled, outbreaks of low and high pathogenic avian influenza by adopting active surveillance programs (National Program for Control and Prevention of Avian Influenza, 2006).

Serological surveillance provides knowledge about the extent of spread and control of a number of poultry infections. Also, it could be helpful in developing a profile of infections in unvaccinated poultry. Vaccination of commercial poultry against *M. gallisepticum* is practiced in the country. Backyard poultry is based on birds reared for household purpose in rural areas of Pakistan. Due to lack of regulated system of poultry rearing in rural area, backyard poultry population mostly remains unvaccinated. In Pakistan backyard poultry and wild birds had not been studied to evaluate seropositivity against *M. gallisepticum*. In order, to have a clear understanding of *M. gallisepticum* infections in commercial and backyard poultry it is logical to conduct sero-surveillance studies. Most common

techniques used for serodiagnosis are serum plate agglutination test (SPA) and enzyme linked immunosorbent assay (ELISA). Molecular detection using polymerase chain reaction (PCR) and isolation of organism from seropositive flock further confirm the presence of infectious agent. The present study was designed to investigate antibody status against *M. gallisepticum* in unvaccinated commercial poultry and backyard poultry population. Serologically positive flocks were confirmed by PCR for *M. gallisepticum* infection and attempts were made to recover field isolate by standard cultivation procedures.

4.1.1 Aims and Objectives:

The aim of study was to assess status of *M. gallisepticum* infection in local poultry. Following were specific objectives of the study:

1. To evaluate serological status of *M. gallisepticum* infections in commercial poultry
2. To evaluate serological status of *M. gallisepticum* infections in backyard poultry
3. To detect *M. gallisepticum* infections using PCR from suspected/ infected poultry farms
4. To recover *M. gallisepticum* isolates from infected/suspected poultry

4.2 Materials and Methods

4.2.1 Serological evaluation of commercial poultry samples

To evaluate presence of anti *M. gallisepticum* antibodies total 4,167 sera were tested to detect IgM using serum plate agglutination (SPA) test (as mentioned in section 3.3.1) and 2,881 sera were tested to check presence of IgG using commercially available *M. gallisepticum* Idexx indirect ELISA (i-ELISA) kit (as mentioned in section 3.3.2) (Table 4.1). Inclusion criteria for serum collection was symptomatic or asymptomatic, unvaccinated poultry for *M. gallisepticum*. Exclusion criteria was poultry vaccinated for

M. gallisepticum. For this purpose, 18 layer farms and 4 breeder farms were investigated. A total 4,167 sera were tested by SPA test, and 2,881 were investigated by ELISA. Among 2,881 sera 348 were collected from breeder flocks and 2,533 were collected from layer flocks. The study was conducted from year 2015 to 2018.

4.2.2 Serological Evaluation of Wild birds/Backyard Poultry Samples

To evaluate presence of anti-*M. gallisepticum* antibodies, 1,020 blood samples were collected from backyard poultry/wild domestic and fancy birds. Among total 1,020 blood samples for serological evaluation 150 were collected from Gilgit Baltistan, 100 from Islamabad Capital Territory (ICT), 200 from Khyber Pakhtunkhwa (KPK), 320 from Punjab, and 250 blood samples were collected from Sindh (Table 4.2). Out of total 1,020 sera sample, 900 were from backyard poultry. Remaining 120 samples were from wild domestic and fancy birds (Table 4.3). All samples were tested for presence of IgG using commercially available *M. gallisepticum* i-ELISA kit (Idexx).

4.2.3 Molecular Detection of *M. gallisepticum*

Processed swab and tissue samples collected from suspected and morbid birds were confirmed for presence of *M. gallisepticum* by PCR. Swab and tissue samples from 15 breeder and 15-layer farms were collected and processed. Two broiler flocks with clinical signs and symptoms were also sampled for PCR detection and isolation.

4.2.3.1 Clinical Samples:

During the study from year 2016 to 2019, total 2,025 (1359 swabs and 666 tissue) samples were collected from commercial poultry for *M. gallisepticum* detection by PCR. Samples were processed as mentioned in section (3.2.1 and 3.2.2). During 2016 total 350 samples, in 2017 total 347 samples, in 2018 & 2019 total 775 and 553 samples were tested to detect *M. gallisepticum*, respectively. In 2016, tested samples comprised of 199 swabs, 151 tissue, in 2017 swab and tissue samples tested were 230 and 117, respectively. In 2018, 550 swab

sample, and 225 tissue samples were received. In 2019, total samples comprised of 380 swabs and 173 tissues samples (Table 4.4 & 4.5).

4.2.4 Isolation of *M. gallisepticum*

Samples collected from *M. gallisepticum* suspected/ infected birds including tracheal, cloacal swabs, and tracheal tissue homogenates, already confirmed by PCR were used for isolation of *M. gallisepticum*. Total 110 swab and 70 tissue samples inoculated on *M. gallisepticum* broth and *M. gallisepticum* agar.

Table 4.1 Year-wise number of sera samples tested for detection of anti-*M. gallisepticum* antibodies

	Year (2015-2018)			
Bird Type	**2015**	**2016**	**2017**	**2018**
		Sera samples for ELISA		
Breeder	88	75	58	127
Layer	275	907	428	923
Total	353	982	486	1050
		Sera sample for serum plate agglutination (SPA) test		
Breeder	485	620	502	410
Layer	380	795	495	480
Total	865	1415	997	890

Table 4.2 Sera Sample of backyard poultry and wild domestic birds from different provinces of Pakistan

Province of Pakistan	**No of Sera Sample**
Gilgit Baltistan	150
ICT	100
Punjab	320
Sindh	250
KPK	200
Total	1020

Table 4.3 Sera sample of backyard poultry and wild domestic birds deciphering different avian species

	Avian Species	**No. of Sera Sample**
Backyard Birds	Desi	470
	Golden	345
	Peacock	65
	Pheasant	35
Wild Domestic Birds	Fancy	35
	Duck	15
	Pigeon	15
	Turkey	5
	Rhode Island Red	35
	Total samples	1020

Table 4.4 Year-wise samples data for detection of *M. gallisepticum* through PCR

	Months	2016	2017	2018	2019
1st Quarter	**Jan-March**	72	85	105	73
2nd Quarter	**April-June**	61	66	201	110
3rd Quarter	**July-Sept**	91	87	178	120
4th Quarter	**Oct-Dec**	126	109	273	250
	Total	350	347	775	553

Table 4.5 Sample types used for detection of *M. gallisepticum* through PCR

Sample Type	2016	2017	2018	2019
Swabs	199	230	550	380
Tissue	151	117	225	173
Total	350	347	775	553

4.3 Results

4.3.1 Sero-monitoring of Unvaccinated Commercial Poultry

To evaluate seroprevalence of *M. gallisepticum* serum samples collected from unvaccinated *M. gallisepticum*, commercial poultry were tested for presence of anti-mycoplasma antibodies by serum plate agglutination (SPA) test and enzyme linked immunosorbent assay (ELISA). Sera samples collected from year 2015 to 2018 were evaluated. A total 4,167 sera were tested by SPA test, 326 were found positive representing total 7.8% positivity (Table 4.7). Out of 2881 sera tested by ELISA, 1208 were found positive representing 41.93% positive samples (Table 4.6).

4.3.1.1 Status of IgM and IgG against *M. gallisepticum* in 2015

During 2015, total 865 sera were tested for detection of IgM against *M. gallisepticum* by SPA, among total tested sera 56 were found positive representing (6.47%) positive samples. None of these birds was previously vaccinated for *M. gallisepticum* (MG). For detection of IgG, ELISA of 363 samples was done. Sera samples were collected from unvaccinated birds. Results revealed total 131 positive samples, representing 36% of total sera samples.

4.3.1.2 Status of IgM and IgG against *M. gallisepticum* in 2016

During 2016, total 1415 sera were tested for detection of IgM against *M. gallisepticum* by SPA. Among total tested sera 117 were found positive representing (8.2%) positive samples. None of these birds was previously vaccinated for *M. gallisepticum.* For evaluation of IgG against *M. gallisepticum*, ELISA of 982 sera samples was carried out. None of the birds was vaccinated for *M. gallisepticum*. Results revealed total 364 positive samples, representing 37% of total sera samples.

4.3.1.3 Status of IgM and IgG against *M. gallisepticum* in 2017

During 2017, total 997 sera were tested for detection of IgM against *M. gallisepticum* by SPA. Among total tested sera 58 were found positive representing (5.8%) positive samples.

None of these birds was previously vaccinated for *M. gallisepticum.* For evaluation of IgG against *M. gallisepticum*, ELISA of 486 sera samples was carried out. None of the birds was vaccinated for *M. gallisepticum*. Results revealed total 258 positive samples, representing 53.1% of total sera samples.

4.3.1.4 Status of IgM and IgG against *M. gallisepticum* in 2018

During 2018, total 890 sera were tested for detection of IgM against *M. gallisepticum* by SPA. Among total tested sera 95 were found positive representing (10.7%) positive samples. None of these birds was previously vaccinated for *M. gallisepticum.* For evaluation of IgG against *M. gallisepticum*, ELISA of 1,050 sera samples was carried out. None of the birds was vaccinated for *M. gallisepticum*. Results revealed total 455 positive samples, representing 43.3%% of total sera samples.

4.3.2 Sero-monitoring of *Mycoplasma gallisepticum* in backyard poultry and wild birds

From 2015 to 2018, total 1,020 sera samples of backyard poultry and wild birds collected from different provinces of Pakistan were evaluated for anti-*M. gallisepticum* antibodies. Results revealed 610 samples were positive by *M. gallisepticum* ELISA, representing 59.80% seropositivity. Samples collected from Khyber Pakhtunkhwa (KPK), Punjab and Sindh represented 75% (150/200), 71.88% (230/320) and 47.5% (95/250) seropositivity, with high *M. gallisepticum* ELISA titer 12773, 24916 and 26126, respectively. Gilgit Baltistan (G.B) and Islamabad Capital Territory (ICT) showed 73.33% (110/150) and 25% (25/100) positive samples with i-ELISA titer 8387 and 7281, respectively (Table 4.9, Fig 4.3).

Among 915 sera samples of backyard poultry, 600 sera were positive representing 65.57% seropositivity with min-max antibody titer of 45-26126. Based on bird type, 315/470 sera collected from Desi birds were positive showing (67%), from Golden breed 260/345 (75.36%) were positive, Peacock 10/65 (15.3%), Pheasants 15/35 (42.85%). From total 105 samples of wild/fancy birds, 10 samples were positive by ELISA representing 9.5%, with min-max titer range 52-3821. Among wild/fancy birds only 10/35 fancy birds showed

seroconversion with titer range of 86-3821. Sera samples of Ducks, Pigeons, Turkeys and Rhode Island red were negative for seroconversion (Table 4.10, Fig. 4.4).

Table 4.6 Sero-surveillance of *M. gallisepticum* for detection of IgG in unvaccinated commercial poultry from 2015-2018

Year of Sampling	Total no. of Serum samples	No. of samples positive for *M. gallisepticum* antibodies	No. of samples negative for *M. gallisepticum* antibodies	Percentage of Positive Samples
2015	363	131	232	36%
2016	982	364	618	37%
2017	486	258	228	53.1%
2018	1050	455	595	43.3%
Total	2,881	1,208	1,673	41.93%

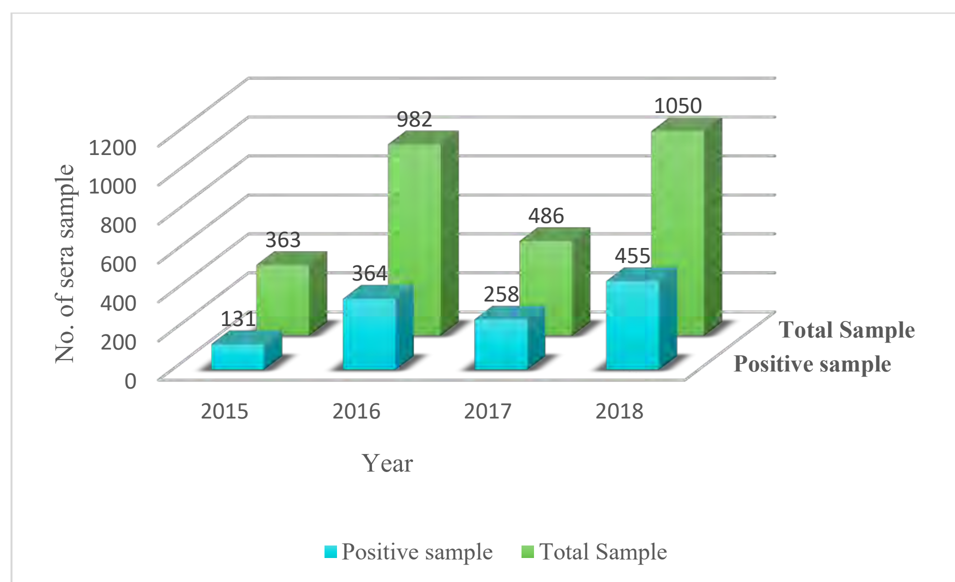


Fig. 4.1 Samples evaluated for anti *M. gallisepticum*-IgG by ELISA

Table 4.7 Sero-surveillance of *M. gallisepticum* for detection of IgM in unvaccinated commercial poultry from 2015-2018

Year of Sampling	Total no. of Serum samples	No. of samples positive for *M. gallisepticum* antibodies	No. of samples negative for *M. gallisepticum* antibodies	Percentage of Positive Samples
2015	865	56	809	6.4%
2016	1415	117	1298	8.2%
2017	997	58	939	5.8%
2018	890	95	795	10.7%
Total	4,167	326	3,841	7.8%

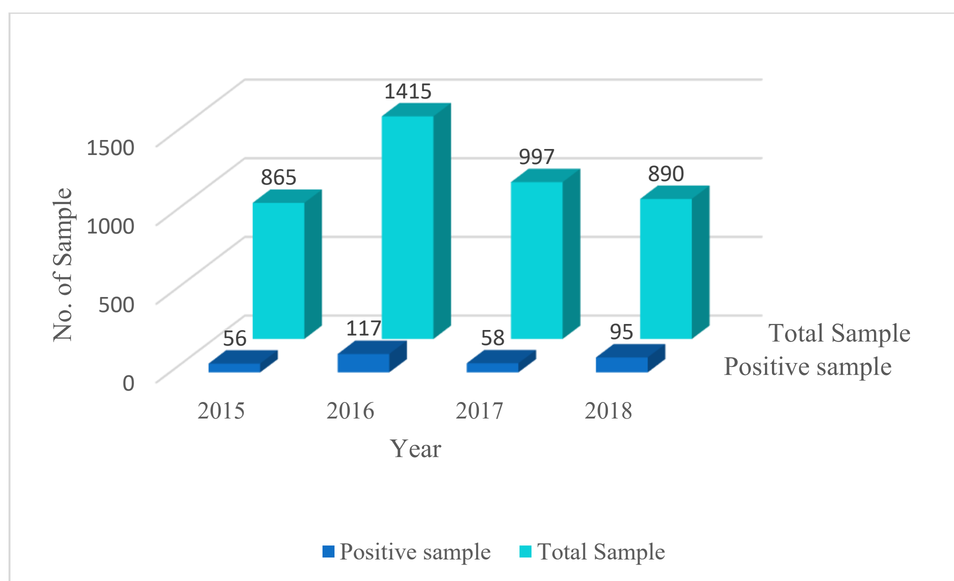


Fig. 4.2 Samples evaluated for anti *M. gallisepticum*-IgG by Serum plate agglutination test

Table 4.8 Sero-monitoring of *M. gallisepticum* in breeder and layer bird types

Year (2015-2018)								
Bird Type	**2015**		**2016**		**2017**		**2018**	
	Total No. of Samples	**No. of Positive Samples**	**Total No. of Samples**	**No. of Positive Samples**	**Total No. of Samples**	**No. of Positive Samples**	**Total No. of Samples**	**No. of Positive Samples**
Breeder	88	05	75	30	58	20	127	54
Layer	275	126	907	334	428	238	923	401
Total	353	131	982	364	486	258	1050	455

Table 4.9 Sero-monitoring of *M. gallisepticum* in backyard poultry and wild birds from different provinces of Pakistan

Province	Total No of Samples	No. of Positive Samples	Min Titer	Max Titer
Gilgit Baltistan	150	110	65	8387
ICT	100	25	45	7281
Punjab	320	230	34	24916
Sindh	250	95	52	26126
KPK	200	150	99	12773
Total	1020	610		

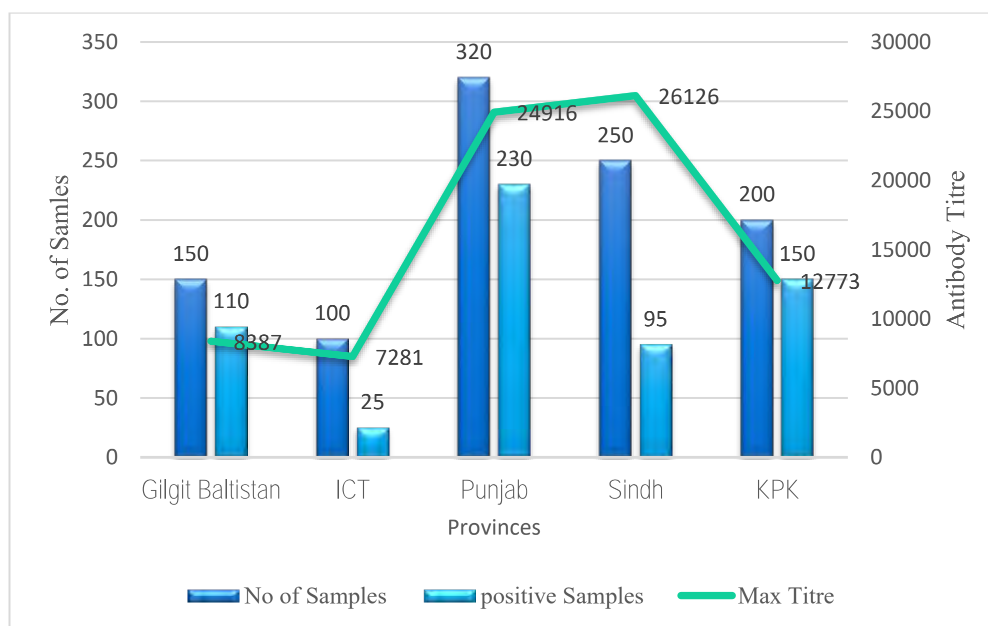


Fig. 4.3 ELISA antibody titer against *M. gallisepticum* in backyard poultry and wild birds from different provinces of Pakistan

Table 4.10 ELISA antibody titer of Backyard and wild birds against *M. gallisepticum*

Type of Birds		Total No. of Sera	Positive	Min-Max Titer
Backyard Poultry	Desi	470	315	99-26126
	Golden	345	260	34-24916
	Peacock	65	10	309-7551
	Pheasant	35	15	45-2693
Wild Birds	Fancy	35	10	86-3821
	Duck	15	0	160-270
	Pigeon	15	0	52-741
	Turkey	5	0	431-528
	Rhode Island Red	35	0	99-182
Total		1020	610	

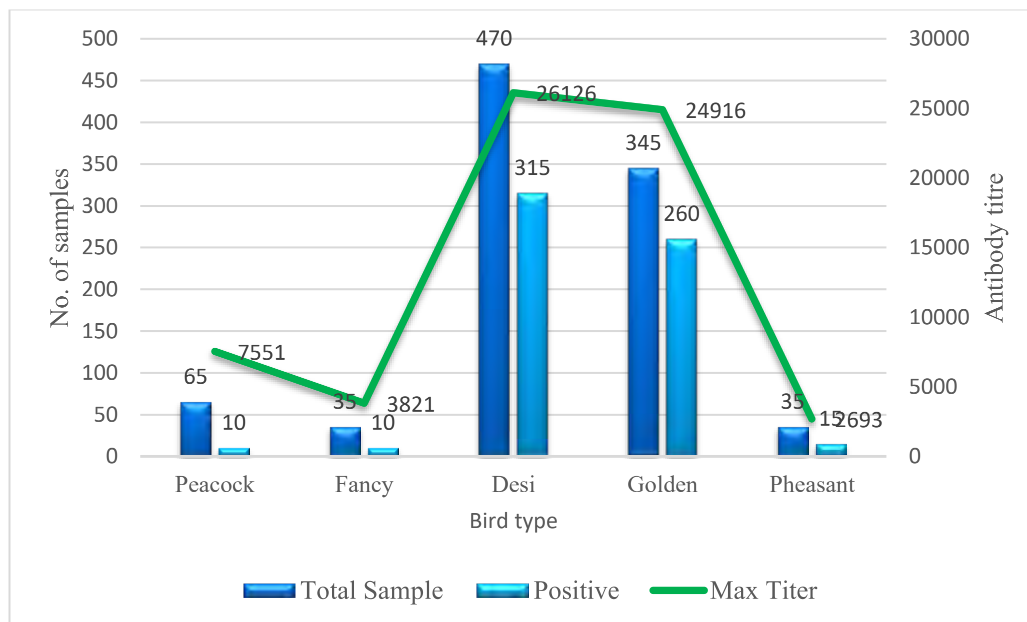


Fig. 4.4 ELISA antibody titer against *M. gallisepticum* in backyard poultry and wild birds

4.3.3 Detection of *M. gallisepticum* from clinical samples through PCR

Molecular detection frequency of *M. gallisepticum* was found during 2016-2019 by PCR. During this investigation, samples yielding a band of 185bp upon agarose gel electrophoresis were considered positive (Fig. 4.5). Out of 2,025 total tissue and swab samples, 242 were positive by PCR representing 11.95% of total samples. Samples yielding ambiguous results were repeated for detection of *M. gallisepticum* by PCR.

4.3.3.1 *M. gallisepticum* detection through PCR in 2016

During 2016, a total of 45/350 samples were detected through PCR. Among these samples, maximum positive samples i.e. 15/75 (20.8%) were detected during months of January to March, followed by 11/61 (18.03%) detections in months of April to June. A decline was observed in months of July to September and October to December representing only 10/91 (10.9%) and 09/126 (7.14%) detections, respectively.

4.3.3.2 *M. gallisepticum* detection through PCR in 2017

During 2017, a total of 47/347 samples were positive for *M. gallisepticum* by PCR. Among these samples, maximum positive samples i.e 17/85 (20%) were detected during months of January to March, followed by 13/66 (19.7%) detections in months of April to June. A decline was observed in months of July to September and October to December representing only 09/87 (10.3%) and 08/109 (7.34%) detections, respectively.

4.3.3.3 *M. gallisepticum* detection through PCR in 2018

During 2018, a total of 84/775 (10.84%) samples were positive for *M. gallisepticum* by PCR. Among these samples, 26/105 (24.76%) were detected during months of January to March, followed by detections 21/201 (10.45%) in months of April to June. In the months of July to September 18/178 (10.11%) detections were made and in October to December 19/273 (6.96%) samples were positive for *M. gallisepticum*.

4.3.3.4 *M. gallisepticum* detection through PCR in 2019

During 2019, a total of 66/553 (11.93%) samples were positive for *M. gallisepticum* by PCR. Among these samples, 23/73 (31.50%) were detected during months of January to

March, followed by 12/110 (10.90%) detections in months of April to June. In the months of July to September 16/120 (13.33%) detections were made and in October to December 15/250 (6.0%) samples were positive for *M. gallisepticum*.

4.3.4 Isolation of *M. gallisepticum*

For isolation of *M. gallisepticum*, swab and tissue samples were inoculated in *M. gallisepticum* broth and further on *M. gallisepticum* agar. Total 180 samples were inoculated, out of which 19 samples were positive for *M. gallisepticum* growth on agar, yielding fried egg like colonies (Fig. 4.11). Isolation rate was found to be 10.5%. Three isolates coded as Pak MG1 (ARL-1963), Pak MG2 (ARL-2020) and Pak MG3 (ARL-2668) were selected for further studies. Two selected isolates i.e. Pak MG1 (ARL-1963) and Pak MG2 (ARL-2020) were isolated from breeder broiler farms, whereas third isolate Pak MG3 (ARL-2668) was isolated from broiler farm.

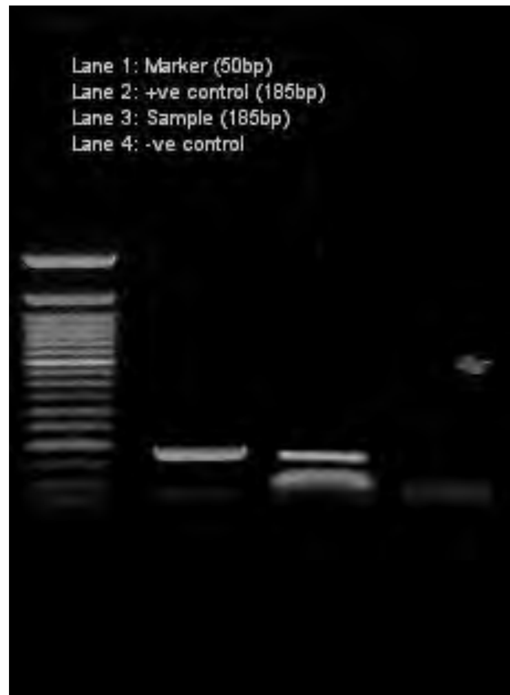


Fig. 4.5 Gel electrophoresis image of *M. gallisepticum* PCR products using 16S rRNA primer, lane 1: DNA step ladder, lane 2: Positive control of *M. gallisepticum*, lane 3: Field sample, lane 4: Negative control

Table 4.11 Year-wise percentage of samples positive for *M. gallisepticum* through PCR

Months	2016	2017	2018	2019
Jan-March	20.8%	20%	24.76%	31.50%
April-June	18.03%	19.7%	10.45%	10.90%
July-Sept	10.9%	10.3%	10.11%	13.33%
Oct-Dec	7.14%	7.34%	6.96%	6.0%
Total	12.86%	13.5%	10.84%	11.93%

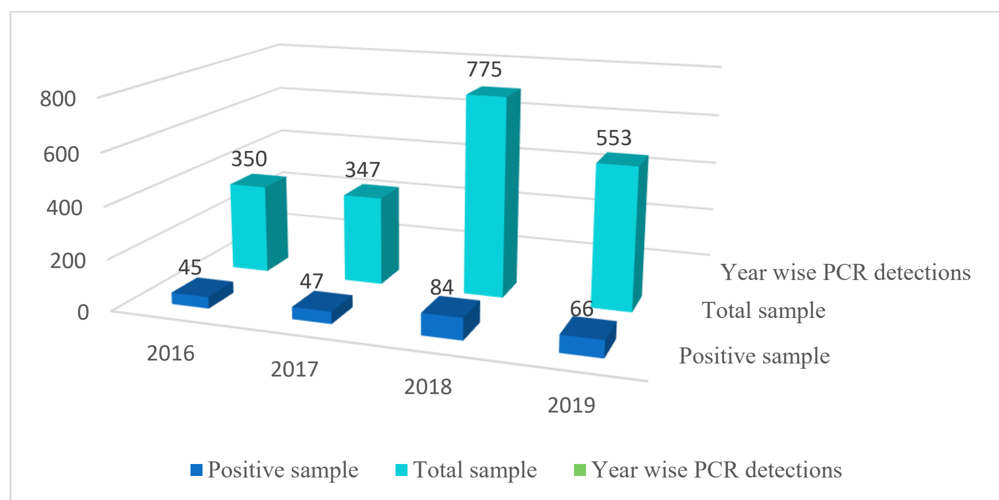


Fig. 4.6 Detection of *M. gallisepticum* through PCR from 2016-2019

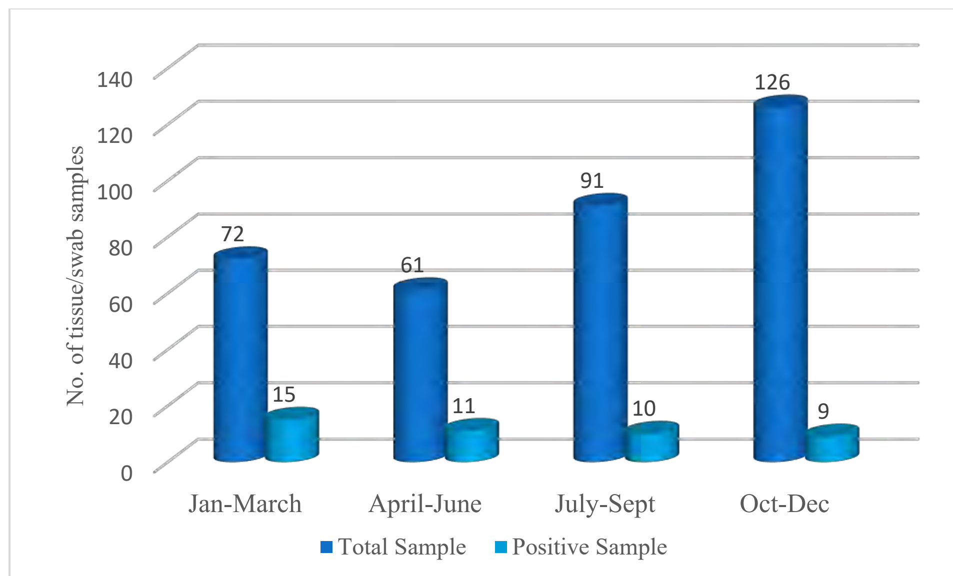


Fig. 4.7 Detection of *M. gallisepticum* through PCR during 2016

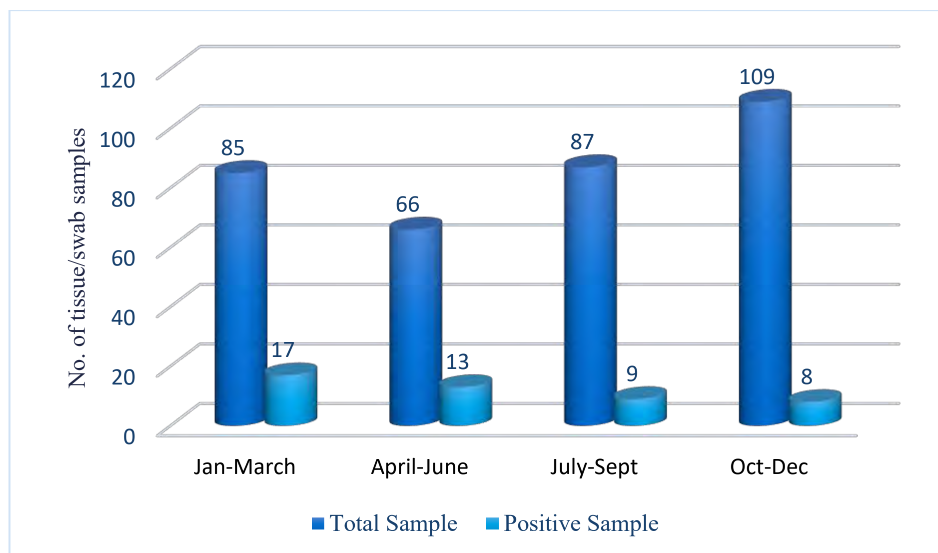


Fig. 4.8 Detection of *M. gallisepticum* through PCR during 2017

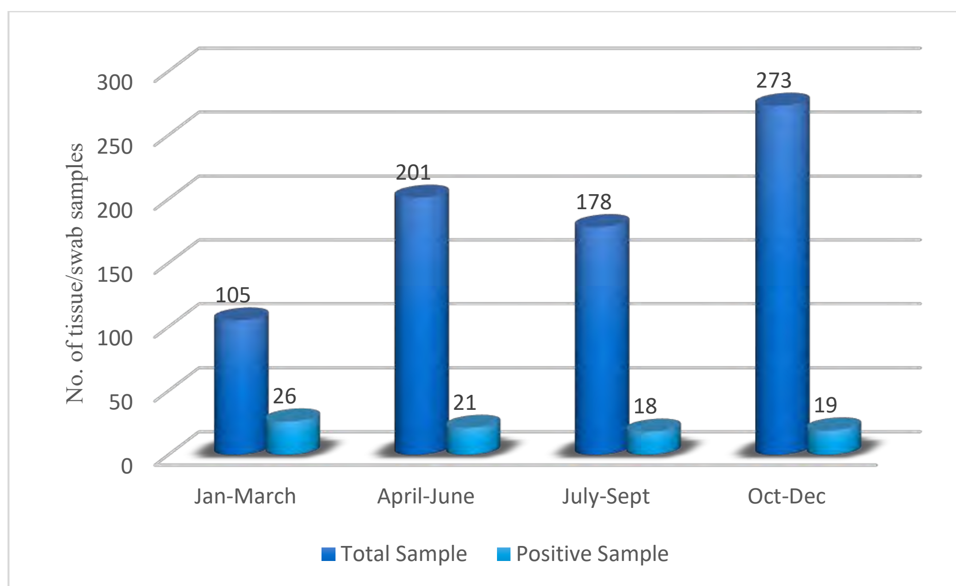


Fig. 4.9 Detection of *M. gallisepticum* through PCR during 2018

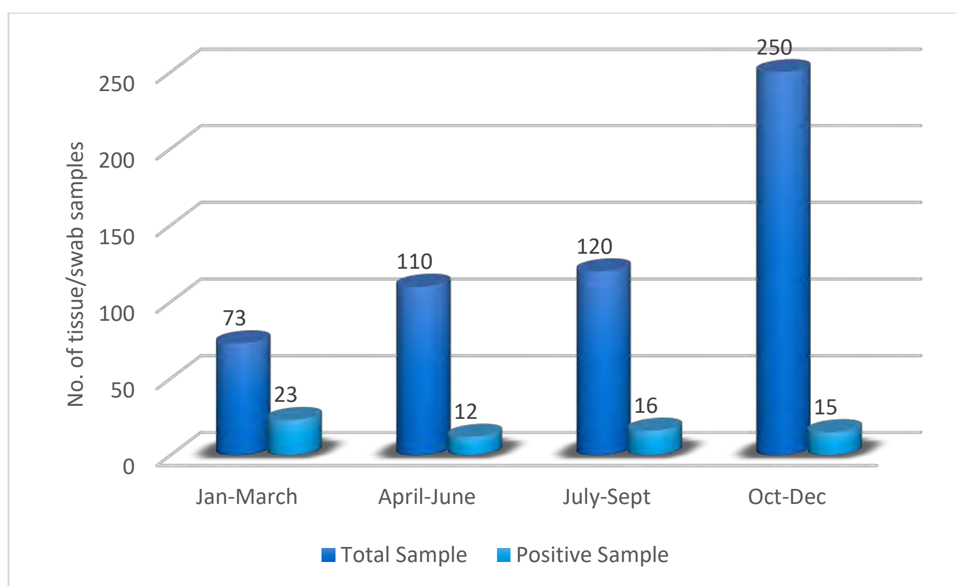


Fig. 4.10 Detection of *M. gallisepticum* through PCR during 2019

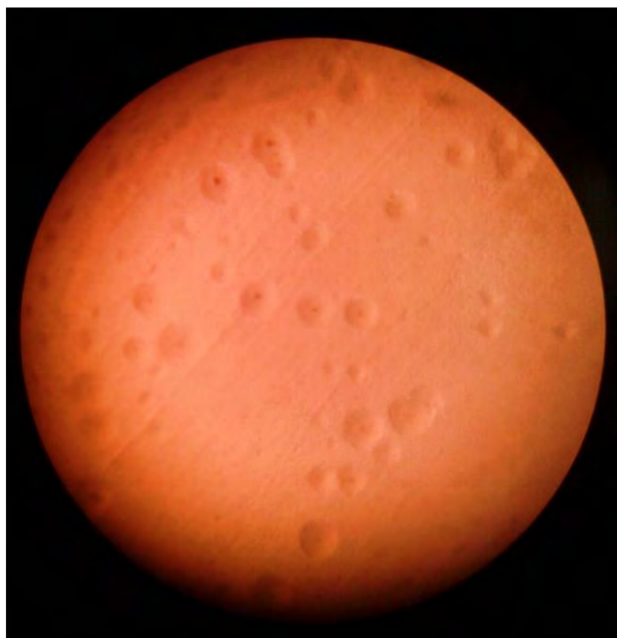


Fig. 4.11 *M. gallisepticum* colony under 40X magnification of stereomicroscope

Table 4.12 *M. gallisepticum* isolates selected for study

Sample ID	Type of Sample	Type of Bird	Region	Age
Pak MG1 (ARL-1963)	Swabs and Tissues	Breeder Broiler	Islamabad	1 week
Pak MG2 (ARL-2020)	Swabs	Breeder Broiler	Islamabad	16 wk
Pak MG3 (ARL-2668)	Swabs	Broiler	Islamabad	21 days

4.4 Discussion

Serological status of unvaccinated commercial poultry, breeder and layer farms located in selected study area was determined by assessing antibody levels through various serological assays, most commonly serum plate agglutination (SPA) and enzyme linked immunosorbent assay (ELISA). Keeping in view, the inclusion and exclusion criteria, breeder farms vaccinated with *M. gallisepticum* were not included in serological study. In this regard, 18-layer farms and 04 breeder farms were studied. Cumulative seropositivity among commercial unvaccinated flocks studied from 2015 to 2018 was found to be 41.93% (Table 4.6, Fig. 4.1) by indirect ELISA (i-ELISA) and 7.8% by using SPA (Table 4.7, Fig. 4.2). Since, most of the breeder farms were vaccinated for *M. gallisepticum*, 12.07% of total samples were collceted from breeder flocks and 87.92% samples were collected from layer flocks. Prevalence of IgG among breeder flocks was found to be 31.32% and among layer flocks it was found to be 43.38% (Table 4.8). Our study is supported by earlier published research by Hussain et al., (2018), who employed i-ELISA to find out 53.4% seroprevalence of *M. gallisepticum* in breeder farms located in Punjab province of Pakistan. A similar study was carried out by Ahmad et al., (2008) on breeder flocks located in Lahore district. The study reported 33.17% sera samples positive for IgG by ELISA. A relevant investigation was carried out by Atique et al., (2012) on layer poultry and reported 31.66% seropositivity in Balochistan province of Pakistan. SPA detects level of IgM, signifying early infection. Assessment of IgM against *M. gallisepticum* has been reported by (Shoaib et al., 2019). The study reported 44.9% and 59.6% seropositivity by using SPA test, among poultry farms located in Rawalpindi region.

To estimate the seroprevalence of *M. gallisepticum* in backyard and wild birds, samples from different provinces of Pakistan were collected, and tested by indirect ELISA. Results showed high seroconversion titers in backyard poultry against *M. gallisepticum*. Present study revealed cumulative seropositivity of 59.80% in backyard/wild birds which is high in comparison to that observed in commercial poultry, viz., 41.93%. This finding is supported by the study of Islam et al., (2014), who reported high rate of infection i.e. 62.5% in backyard poultry than 53.61% in commercial poultry. Seroconversion rate of 75% was

observed in samples received from KPK province marking the highest rate with antibody titers ranging from 45-12733. From Punjab 71.88% samples showed maximum antibody titer of 24916 and from Sindh 47.5% samples were serologically positive showing a titer up to 26126. Samples from Gilgit-Baltistan showed high seropositivity than Punjab with antibody detection rate of 73.33%, but with maximum antibody titer of 8387. From ICT 25% sera samples were positive with maximum antibody titer of 7281 (Table 4.9, Fig. 4.3). Among different bird types, 65.57% was found seropositive among backyard poultry which included different breeds with variable prevalence, such as Desi (67%), Golden (75.36%), Peacock (15.3%) and Pheasants (42.85%). Here it can be clearly seen that a reasonable number of Desi, Golden and Pheasants were positive for *M. gallisepticum* antibodies. Maximum antibody titers in Desi & Golden were 26126 & 24916, respectively, indicating *M. gallisepticum* exposure titers. Whereas, in Peacocks and Pheasants the recorded titers were 7551 and 2693, respectively (Table 4.10, Fig. 4.4). Based on these figures, Desi and Golden bird types can be considered as a reservoir for *M. gallisepticum* in Pakistan. Only a single study (Rehman et al., 2018), based on serological evaluation of backyard poultry against *M. gallisepticum* from Dera Ismail Khan and Tank districts of Khyber Pakhtunkhwa (KPK), Pakistan reported 80% and 56% sero-prevalence, respectively. The significantly high seroprevalence described in that study can be due to very small sample size, comprising of 50 samples from each district. Sero-prevalence study in Brazil by De silva et al., (2015) declared *M. gallisepticum* infection endemic in backyard poultry with 53.33% positive. In Southern Mozambique, 48.8% seroprevalence was reported by Messa Junior et al., (2017). These studies provide a strong evidence of existence of *M. gallisepticum* in non-commercial poultry. The study is supported by the fact described by Dhondt et al., (2014) that *M. gallisepticum* infections might be widespread geographically and occur without apparent signs and symptoms. Also, there is possibility of introduction of *M. gallisepticum* infection from backyard to wild birds. Diverse *M. gallisepticum* strains might be present and maintained in backyard poultry. The *M. gallisepticum* infections are not limited to commercial poultry, as reports of occurrence of *M. gallisepticum* in wild house sparrow from India is dated back to 1971, also in wild tree sparrows from Japan it dates back to 1979. In 1993-94, an epidemic of *M. gallisepticum* conjunctivitis in house

finches was reported in Maryland, USA, which later became endemic in the region. The outbreak was first evidence of spread of *M. gallisepticum* infection in wild birds on larger scale (Fischer et al., 1997, Dhondt et al., 2003). (Dhondt et al., 2014) has been of the view point that possibility of circulation of *M. gallisepticum* in wild birds worldwide, even before the outbreak in house finches in North America, cannot be ruled out.

Diverse host range of *M. gallisepticum*, include Pheasants, chukar partridge, peafowl, bob white quail, Japanese quail, yellow-naped Amazon parrot and greater flamingos with successful isolation of the infectious agent has been described by various studies (Reece et al., 1986; El Shater et al., 1996; Cookson et al., 1994; Murakami et al., 2002; Bencina et al., 2003). Partial susceptibility of pigeon and sparrows for *M. gallisepticum* was demonstrated by Gharaibeh and Hailat, (2011). The study concluded both bird types can serve as temporary biological carriers or vectors for transmission of *M. gallisepticum*, without maintaining stable carrier state. Seroprevalence of *M. gallisepticum* in backyard and fancy breed poultry was studied by Haesendonck et al., (2014) in Belgium, and found 36.7% positive rate.

The current report is first study of *M. gallisepticum* seroprevalence in backyard and wild poultry in Pakistan as most of the earlier studies focused on commercial poultry. Further studies, based on molecular detection can provide stronger evidence regarding circulation of *M. gallisepticum* in backyard poultry.

For diagnosis of *M. gallisepticum* infections, isolation of causative agent from infected flock is considered a “gold standard”. To culture *M. gallisepticum* is laborious and time-consuming process, as it may take 7 to 21 days in case of field samples. For clinical diagnosis, sero-diagnosis can be supported by confirmatory tests such as molecular assays. Molecular detection of *M. gallisepticum* from clinical samples is commonly carried out through PCR, which is a rapid and reliable method of detection of *M. gallisepticum* nucleic acid (Islam et al., 2011; Khalifa et al., 2013; Rauf et al., 2013).

In the present study, molecular detection of 2,025 clinical samples collected from suspected/infected flocks from commercial poultry rearing areas of Pakistan were tested through PCR from 2016 to 2019. For this purpose, 15 layer and 15 breeder farms were

studied. Cumulative detection rate of *M. gallisepticum* was found to be 11.95% through PCR. Year wise detection rate in 2016 was 12.86%, in 2017 it was 13.5%, in 2018 it was 10.84%, and in 2019 detection rate was11.93%. Statistical analysis revealed no significant difference in the rate of detection among different years. However, in these 4 years pattern of detections by PCR was evaluated in different quarters of a year. Highest rate of detection was found during first quarter (Jan-Mar) of the year and gradually low in next quarters (Table 4.11, Fig. 4.6). This pattern was same in all years included in this study. In 2016, detection rate was highest during first quarter (Jan-Mar) i.e. 20.8% and lowest in last quarter (Oct-Dec) i.e. 7.14%. Detection rate recorded in 2017 was 13.5% with highest during first quarter (Jan-Mar) i.e. 20% and lowest during last quarter (Oct-Dec) i.e. 7.34%. During 2018, a total of 10.84% samples were positive for *M. gallisepticum* by PCR. The highest detection rate of 24.7% was recorded in Jan-Mar and the lowest of 6.96% was determined during Oct-Dec. During 2019, a total of 11.93% samples were positive for *M. gallisepticum* by PCR. Here the highest detection rate was found in first quarter (Jan-Mar) as 31.50% and the lowest was reported in last quarter (Oct-Dec) as 6.0%. Detection rate of *M. gallisepticum* by PCR is reported by Muhammad et al., (2018) as 23% by PCR in a study during 2015-2016. According to this report the prevalence of *M. gallisepticum* infection was higher in winters.

Similar study was conducted by Rajkumar et al., (2018), who reported 11.65% molecular prevalence of *M. gallisepticum* in different geographical regions of India from 2013-2014. Hassan et al., (2014) reported significant higher prevalence of *M. gallisepticum* in winters as compared to summers. Detection rate in commercial poultry including breeder broiler and layer flocks might be affected by therapeutic measures taken to suppress disease outcomes and economic losses. Seasonal stress including low temperature due to lack of temperature controlling measures may contribute to the variation in infection outcome (Heilili et al., 2011; Mukhtar et al., 2012). Earlier a study by Qasem et al., (2015) referred PCR as accurate method for detection and diagnosis of *M. gallisepticum* infection. Although molecular tests detect nucleic acid and not live particles, diagnosis can be made on clinical status and serological investigations coupled with molecular detection by PCR. For detection of *M. gallisepticum*, PCR is considered as a dependable molecular diagnostic

technique worldwide, (Behbahan et al., 2005; Khalifa et al., 2013), which was first reported by Nascimento et al., (1991). Different genes and genomic regions have been targeted for detection of *M. gallisepticum* and differentiation of field isolates from live vaccine strains in countries where use of live vaccine to control *M. gallisepticum* infection is in practice. The technique is well exploited in molecular characterisation of *M. gallisepticum* using primers specific to 16S rRNA, IGSR and mgc2 gene (Kleven, 2004; Rasoulinezhad et al., 2017), in assessing pvpA gene variability and for the analysis of virulence genes (*pvpA*, *gapA* and *mgc2*) (Khumpim et al., 2004).

Isolation of *M. gallisepticum* was successful from 11 different, vaccinated and unvaccinated layer, breeder and broiler farms. Three isolates designated as PakMG1(ARL-1963), Pak MG2 (ARL-2020), and Pak MG3 (ARL- 2668) were selected for further studies.

4.5 Conclusion

Present study revealed cumulative seropositivity against *M. gallisepticum* was (59.80%) in backyard/wild birds which is high in comparison to that observed in commercial poultry (41.93%). Due to continuous and repeated medication of infected flock's detection rate through PCR was found to 11.95%. *M. gallisepticum* isolation was made from samples collected during acute/initial infection and isolation rate was 10.5%.

5 Biological and Molecular Characterization of Local *Mycoplasma gallisepticum* Isolates

A) Pathogenesis of Local *M. gallisepticum* Isolate Pak MG1 (ARL-1963)

5.1 Introduction

Among various poultry pathogens responsible for respiratory tract infections, *Mycoplasma gallisepticum* is incriminated as an organism having the ability to persist for long duration once it has infected the host. Infections caused by *M. gallisepticum* ranges from mild respiratory illness to chronic respiratory disease when complicated with other co-infecting pathogens (Levisohn and Kleven, 2000; Ley 2008). Host and environmental factors play an important role in regulating pathogenesis of the disease. Exposition of invasion process of *M. gallisepticum* in non-phagocytic cells including chicken embryo fibroblast and HeLa-229 and ability to survive in intracellular spaces, provided an insight about the mechanism of evasion of host defences, anti-mycoplasma therapy and potential to cause systemic infection (Winner et al., 2000; Fürnkranz et al., 2013).

Respiratory tract infections caused by *M. gallisepticum* involves colonization of upper respiratory tract, which can further lead to inflammation of trachea and air sacculitis. Earlier studies reporting arthritis, salpingitis, conjunctivitis, meningoencephelopathy in chicken and turkeys suggested that the organism is not restricted to respiratory tract only. Experimental infection by pathogenic *M. gallisepticum* R strain provided evidence of systemic spread to the heart, brain, liver, spleen, and kidneys, unveiling the potential of *M. gallisepticum* to cross mucosal barrier of respiratory tract and dissemination to internal body organs (Much et al., 2002; Vogl et al., 2008; Ramadan, 2019).

Marked differences have been observed in infectivity potential of different strains of *M. gallisepticum*. In case of experimental infections, it varies with route of inoculation, type of *M. gallisepticum* strain and number of passages of the strains used for challenge (Levisohn and Kleven, 2000). Experimental co-infection of *M. gallisepticum* and Low

Pathogenic Avian Influenza Virus (LPAIV) H9N2 exaggerated disease outcome (Subtain et al., 2016).

Persistent nature of the organism and potential of vertical as well as horizontal transmission render *M. gallisepticum* infections as one of the most important infections of poultry, causing significant economic losses. Even in the absence of apparent clinical infection of *M. gallisepticum*, co-infecting bacterial or viral pathogens can exacerbate disease condition. Pakistani *M. gallisepticum* isolates have not been studied in terms of infectivity and predilection sites of infection. The present study was designed to assess preferred sites of colonization and infection of *M. gallisepticum* isolate recovered from a vaccinated flock.

5.1.1 Aims and Objectives

The aim of present study was to appraise pathogenesis of *M. gallisepticum* isolate Pak MG1(ARL-1963). Following were the specific objectives of the study:

1. To determine pathogenic potential of *M. gallisepticum* isolate Pak MG1(ARL-1963) by evaluating tracheal lesions and air sacculitis in experimental infection
2. To study dissemination of Pak MG1(ARL-1963) to internal organs of experimental birds
3. To assess development of serological response in experimental birds against Pak MG1(ARL-1963) infection

5.2 Materials and Methods

5.2.1 Experimental Design

To determine the predilection sites of *M. gallisepticum*, 48 day-old chicks were reared at animal house facility of National Reference Lab of Poultry Diseases (NRLPD), National Agricultural Research Centre, Islamabad, Pakistan.

At the age of 10 days, birds were randomly divided in experimental and control groups, each having 32 and 16 birds, respectively. Experimental group was inoculated intratracheally with 0.5ml of *M. gallisepticum* culture (1x10^6 CFU/ml) using hypodermic needle. Control group was sham inoculated with sterile *M. gallisepticum* broth

intratracheally. Both groups were separately placed in glove port chicken isolator chambers (Alternative Design Manufacturing). Blood samples were withdrawn from each group randomly at day 5, 10, 15, 20 post-infection (pi). At day 3, 5, 7, 9, 11, 15, 19, 21 pi, 4 experimental birds and 2 control birds were sacrificed.

5.2.2 Clinical and Pathological Examination

Experimental and control groups were subjected to daily observations for development of respiratory signs and symptoms. Trachea of each necropsied bird was examined for development of lesions as described by Machado et al., (2017). Lesions were graded as mentioned: 0= Normal, 1= hyperemic/ petequiae with mucous, 2= considerable mucous, 3= excess mucous, 4= excess mucous with tracheal thickening. Air sacs of each sacrificed bird were examined for gross lesions and scored as described (Evans and Hafeez 1992; Gaunson et al., 2006). 0= clear air sacs with no lesions; 1= slightly cloudy with little thickness; 2= cloudy and foamy, 3= thickened, cloudy and opaque with reasonable amount of caseous material attached, and 4= thickened with extensive cloudiness and with caseous depositions (Much et al., 2002; Gaunson et al., 2006).

5.2.2.1 Statistical Analysis

Tracheal lesion scores and air sac lesions scores were compared between the birds necropsied at different dpi by using Kruskal–Wallis test. A P value ≤ 0.05 was considered significant.

5.2.3 Serology of Experimental and Control Group

Blood samples were withdrawn from each group randomly at day 5, 10, 15, 20 pi. Serum samples were subjected to SPA test and indirect ELISA as described in (section 3.2.1 and 3.2.2).

5.2.4 Molecular Detection of Experimental and Control Group

Swab samples collected from different organs after necropsy were collected according to plan. Detection for presence of *M. gallisepticum* was made through PCR (section 3.4).

5.3 Results

5.3.1 Clinical and Pathological Observations

Respiratory distress was observed in experimental group from day 6 pi onwards. Signs and symptoms included tracheal rales and sneezing, which intensified from day 9 till day 21 pi. No such signs were apparent in sham inoculated group.

Tracheal lesions and air sac lesions were recorded macroscopically after necropsy. Statistical analysis revealed no significant difference among tracheal lesions recorded on day 7, 9 and 21 pi. Similarly, no significant difference was found in tracheal lesions recorded on day 11, 15 and 19 pi. Tracheal lesion scores recorded on day 11, 15 and 19 pi differed significantly from those recorded on days 7, 9 and 21 pi. Air sac lesion scores recorded on day 11 and 15 pi differed significantly from the air sac lesions recorded on day 3, 5, 7, 9, 19 and 21 pi. No birds in control group develop tracheal or air sac lesions (Table 5.1).

5.3.2 Serological Evaluation

Seroconversion was observed from day 5 pi by SPA test. Number of positive samples remained low as 2 out of 5 (2/5) and 3 out of 5 (3/5) on day 10 and 15 pi. By day 20 pi all samples were positive for SPA test. For detection of IgG, ELISA was conducted. On day 10, 15, 20 pi 2/5, 4/5, 3/4 samples were positive showing antibody titer range of 223-1509, 114-5860, and 628-6192, respectively. No seroconversion was observed in control group either by SPA or ELISA (Table 5.2).

5.3.3 Detection of *M. gallisepticum* by PCR

Swabs from organs of necropsied birds were collected including trachea, lungs, liver/spleen and cloaca (Table 5). PCR was done to detect *M. gallisepticum* in swab samples collected from designated organs. Tracheal swabs were positive by PCR from day 7 to 21 pi. On 7, 9, 19 and 21 dpi 2/4 i.e. 50% of tracheal swabs were positive. On day 11 and 15 pi, 4/4 i.e. 100% of tracheal swabs were positive for *M. gallisepticum*. Swabs collected from lungs were positive for *M. gallisepticum* on day 9 till 21 pi (Table 5.3). No swab sample collected

from liver/spleen and cloaca was positive. In control group all samples were negative for *M. gallisepticum*.

Table 5.1 Post inoculation observations of pathological lesions in Trachea and Lungs of Experimental and Control group

	Day 3	Day 5	Day 7	Day 9	Day 11	Day 15	Day 19	Day 21
				Experimental group				
Tracheal lesions^{ab}	0/4(0.0)^{A}	0/4(0.0)^{A}	3/4(0.75)^{B}	3/4(0.75)^{B}	4/4(2.0)^{C}	4/4(2.0)^{C}	3/4(1.5)^{C}	2/4(0.5)^{B}
Air sac lesions^{ac}	0/4(0.0)^{A}	0/4(0.0)^{A}	0/4(0.0)^{A}	1/4(0.2)^{A}	2/4(0.5)^{B}	2/4(0.5)^{B}	1/4(0.0)^{A}	1/4(0.0)^{A}
				Control Group				
Tracheal lesions	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}
Air sac lesions	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}	0/2(0.0)^{A}

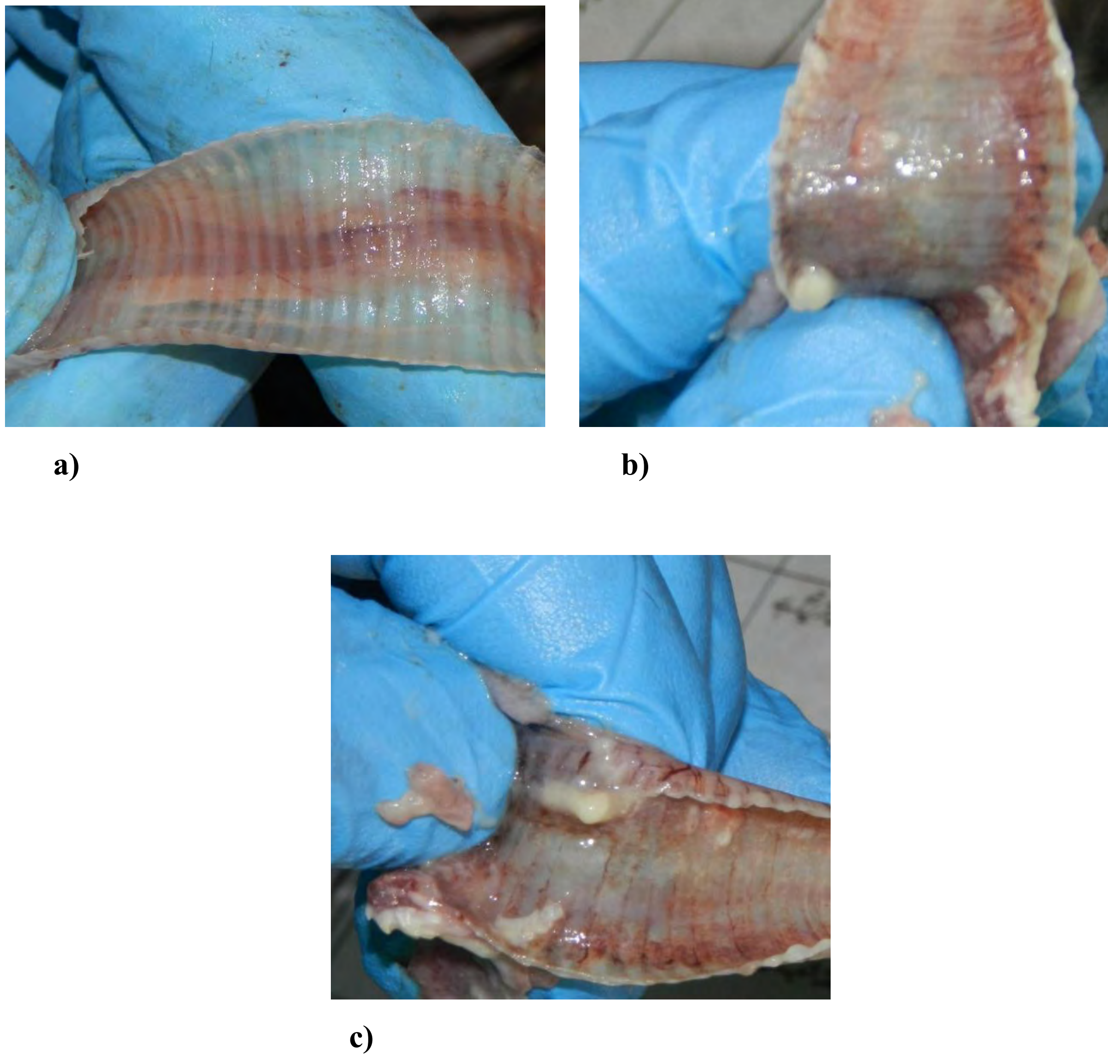
Values within a row with a different uppercase, superscripted letter are significantly different ($P \leq 0.05$).
[a]No. of positive samples/No. of tested samples
[b]Mean tracheal lesion score (macroscopically scored from 0 to 4)
[c]Mean air sac lesion score (macroscopically scored from 0 to 4)

Table 5.2 Anti-*M. gallisepticum* antibody detection by SPA test and ELISA

Post Infection	Day 5	Day 10	Day 15	Day 20
		Experimental Group		
ELISA	0/5	2/5+ve	4/5+ve	3/4+ve
Titer Range		223-1509	114-5860	628-6192
SPA	1/5+ve	2/5+ve	3/5+ve	4/4+ve
		Control Group		
ELISA	0/5	0/5	0/5	0/4
SPA	0/5	0/5	0/5	0/4

Table 5.3 Post Infection detection of *Mycoplasma gallisepticum* from different organs of Experimental group by PCR

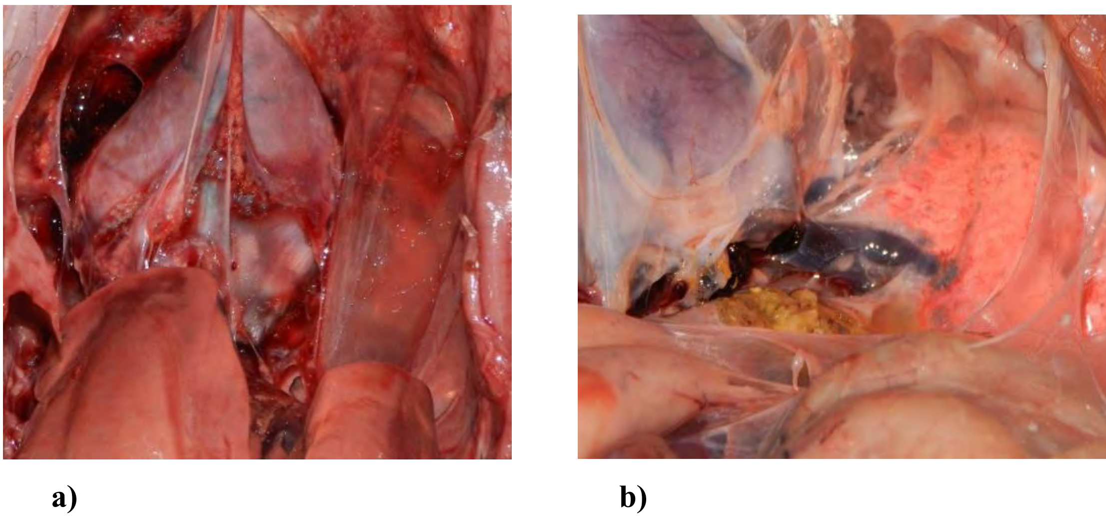
Organs	Day 3	Day 5	Day 7	Day 9	Day 11	Day 15	Day 19	Day 21	Total PCR positive
Trachea	0/4	0/4	2/4	2/4	4/4	4/4	2/4	2/4	16/32
Lungs	0/4	0/4	0/4	¼	2/4	2/4	2/4	1/4	08/32
Liver/ Spleen	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/32
Cloaca	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/32



a) b)

c)

Fig. 5.1 Tracheal lesions of experimentally infected chickens by Pak MG1 a) Control group with no tracheal lesions and no mucous accumulation b) Hyperemic trachea with presence of mucous c) Hyperemic trachea with petechial haemorrhages and considerable mucous



a) **b)**

Fig. 5.2 a) Thoracic air sacs showing thin walls with glossy appearance in control groups b) Thoracic air sacs showing thickened and hazy appearance with flacks of pus in experimentally infected birds

5.4 Discussion

Currently, in Pakistan *M. gallisepticum* infection has been on the rise in commercial poultry, despite the fact that number of drugs are used to control such infections along with using *M. gallisepticum* vaccines. This study was our first attempt to isolate and characterize Pakistani *M. gallisepticum* field isolate on biological and molecular basis. The goal of study was to investigate, biological behaviour of Pakistani *M. gallisepticum* isolate designated as PakMG1 (ARL-1963) by studying its predilection sites in commercial chickens, and to assess genetic relatedness with known reference strains.

For this purpose, *M. gallisepticum* field isolate was recovered from the field among serologically *M. gallisepticum* positive breeder flock. Since *M. gallisepticum* is transmitted via horizontal as well as vertical route, assessment of locally circulating field isolate with regard to its localization to upper respiratory tract and further dissemination to other organs and persistence in any specific organ was considered for evaluation. Development of symptoms of disease initiated on day 6 post infection (pi), which included slight sneezing and rales and intensified with nasal discharge and difficulty in breathing till day 21 pi. It is earlier reported that *M. gallisepticum* infections in the field presents a wide spectrum of disease from mild infection in the presence of a single infectious agent to severe clinical disease complicated with other respiratory pathogens (Siddique et al., 2012; Feiziet al., 2013). Seroconversion against field isolate of *M. gallisepticum* was detected by SPA test and ELISA. Results revealed positive SPA from day 5 till 20 pi. Commercially available SPA test antigen prepared from *M. gallisepticum* strain A5969 was used here (Stipkovits and Kempf, 1996). On the other hand, indirect ELISA was conducted to assess IgG antibodies against *M. gallisepticum*. The results revealed development of moderate antibody titers from day 10 to 20 pi (Ahmad et al., 2008). In the present study, development of mild respiratory symptoms coincided with the positive SPA test. This is in line with the earlier reported observation that serological response developed against *M. gallisepticum* is directly related to the degree of infectivity of corresponding strains (Levisohn and Kleven, 2000). To evaluate infectivity of field isolate, birds from experimental and control groups were sacrificed according to plan and tracheal as well as air sac lesions were

observed macroscopically. Tracheal lesion scores recorded on day 7, 9 and 21 pi differed significantly from tracheal lesion scores recorded on day 11, 15, and 19 pi. Our results vary from the previous study, which reported development of severe tracheal lesions 2 to 3 weeks after challenge which eventually subsided slowly (Sanei et al., 2007). Maximum severity observed in air sac lesions was from day 11 to day 19 pi, which significantly differed from air sac lesions noted during study. As earlier reported by Majumder, (2014) pathology of *M. gallisepticum* infection in chicken is based on inflammatory response in trachea, air sacs and lungs.

Persistence of *M. gallisepticum* in upper respiratory tract and dissemination of infection to internal body organs was detected by PCR through swabbing of organs after necropsy (Rauf et al., 2013; Haque et al., 2015; Spickler, 2018). Tracheal swabs were positive from day 7 till 21 pi with 100% positivity on day 11 and 15 pi. Swabs from lungs were positive from day 9 to 21 pi with 50% detection on day 11, 15 and 19 pi. No detection by PCR was made from liver and cloacal swabs. Although moderate morbidity was observed, no mortality or severe infection occurred during this experiment. Lack of *M. gallisepticum* detection in the cloacal swabs could be due to poor potential of this isolate to persist and/or shed after infection. *M. gallisepticum* infections in the field are complicated by some co-infecting organisms as well as due to any environmental stress. Concurrent infection of LPAIV H3N8, and H9N2 with *M. gallisepticum* have been investigated previously and provided evidence of exaggerated disease condition than infections caused by a single pathogen (Sprygin et al., 2011; Sid et al., 2016; Subtain et al., 2016; Canter, 2019). Different strains may differ in biological properties, including attachment and destruction of epithelial lining. Role of surface exposed cytadhesins GapA and CrmA in effective colonization to upper and lower respiratory system but reduced dissemination potential to other body organs is well documented (Indiková et al., 2013).

The study demonstrated upper respiratory tract as preferred site of infection of *M. gallisepticum* local isolate with moderate infection of lungs. There is high probability of *M. gallisepticum* strains circulating in Pakistan with diverse biological characteristics. Due to limitations, multiple *M. gallisepticum* isolates were not used in the study.

5.5 Conclusion

The present study provided an insight about predilection sites of *M. gallisepticum* isolate field. During the course of experiment, infection was limited to respiratory tract and no dissemination to internal organs was found. Appearance of symptoms of infection coincided with development of serological response. The isolate used in study was moderately pathogenic. It is anticipated that such biological characterization of the local isolates would help in better understanding of circulating *M. gallisepticum* strains.

B) Molecular Characterization of Pakistani *M. gallisepticum* Isolates by Gene Target Sequence (GTS) Analysis

5.6 Introduction

Mycoplasmas are unique in their small size and simplest cellular architecture. In addition, distinctively small genome size with loss of biosynthetic genes and conservation of genes involved in DNA replication, transcription and translation proved their minimal nature for survival. A number of molecular techniques have been used for strain identification, epidemiological tracking of infections and differentiation of live vaccine strains from field strains. These techniques involved restriction length polymorphism (RFLP), ribotyping, and use of strain specific primers. Random amplified polymorphic DNA (RAPD) proved to be a promising technique in differentiating field strain from vaccinal strains.

Since *M. gallisepticum* lack classical virulence factors as present in other bacterial spp., successful attachment and colonization of mucous membranes play vital role in establishment of infection. Such processes are regulated by surface proteins of *M. gallisepticum*. Gene target sequence analysis (GTS) of surface protein genes for typing of *M. gallisepticum* was first described by Ferguson et al., (2005), in comparison with RAPD to describe discriminatory power of both techniques. The technique has also been used for assessment of surface protein genes in molecular typing of *M. gallisepticum*, as well as in describing epidemiological relationship between isolates from different areas and outbreaks.

Surface protein genes comprised of *gap A* gene which encodes a cytadhesin protein (Goh et al., 1998), *mgc2* gene encodes secondary cytadhesin protein, *pvpA* gene encodes a size variable, putative cytadherence protein (Boguslavsky et al., 2000; Liu et al., 2001). A gene encoding conserved lipoprotein was also studied (Nascimento et al. 1991). Since there is no data available with reference to molecular characterization of Pakistani *M. gallisepticum* isolates, the current study was designed to assess molecular characteristics and relatedness of selected isolates.

5.6.1 Aims and Objectives

The aim of study was to characterize local *M. gallisepticum* isolates on molecular basis. Following were specific objectives of study:

1. To study genetic relatedness of local *M. gallisepticum* isolates i.e. Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) with reference strains
2. To study genetic relationship of local *M. gallisepticum* isolates i.e. Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) with other field isolates of different countries
3. To study genetic relatedness of local *M. gallisepticum* isolates (Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) with each other

5.7 Materials and Methods

5.7.1 Primary Amplification

Primary PCR amplification of selected genes (*gapA, lp, pvp A, mgc2*) of three isolates Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) was carried out using primers as described by Ferguson et al., (2005) (Annex-VIII) as mentioned in (section 3.4). thermal profile was optimized for amplification of each gene (Table. 5.4).

5.7.2 Sequence Analysis

Purification and sequencing of (*gapA, lp, pvp A, mgc2*) genes of Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) was done by Novagen company (China).

5.7.3 Phylogenetic Analysis

All sequence data was compiled and analyzed using BioEdit 7.0.5 software. Gene sequences of the selected *M. gallisepticum* isolates were subjected to Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to retrieve relevant sequences. Reference gene sequences of (*gapA, lp, pvp A, mgc2*) were selected from the Genbank on the basis of sequence identity. Multiple nucleotide and amino acid sequence alignments for the genes under study were performed using Clustal W. Phylogenetic tree was made with the help of neighbor-joining analysis in the Mega -X and were evaluated by bootstrap of 1000 replicate (Kabiri et al., 2019).

5.8 Results

The Pakistani *M. gallisepticum* (*gapA, lp, pvp A, mgc2*) genes of selected isolates were partially amplified, in order to get an insight into the evolutionary status and possible origin of the Pakistani *M. gallisepticum* isolates. The sequences obtained were further analyzed to deduce phylogenetic relationship and percentage homology with reference strains, vaccine strain and isolates from other countries.

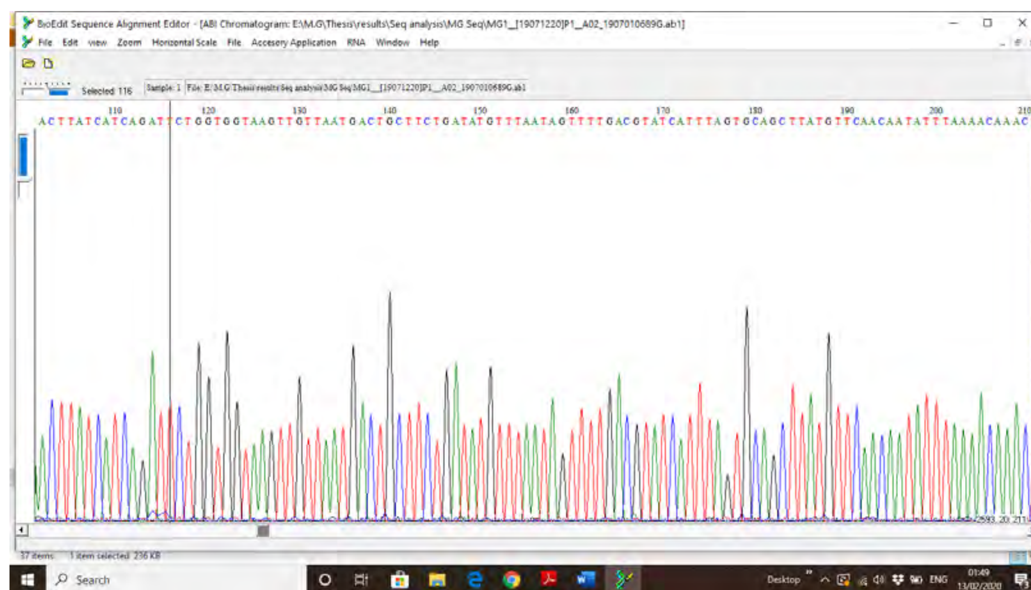


Fig. 5.3 Standard pattern of sequencing of Pakistani *M. gallisepticum* isolates

Table 5.4 Primers and expected size of amplified product

Gene specific Primers	Expected bp size	Reference
lp	590 bp	(Ferguson et al., 2005, Raviv et al., 2007, Armour, 2014)
gapA	332 bp	
pvpA	702 bp	
mgc2	824 bp	

Table 5.5 Nucleotide and amino acid percentage similarity among Pakistani *M. gallisepticum* isolates

	Pak MG1 (ARL-1963)		**Pak MG2 (ARL-2020)**		**Pak MG1 (ARL2668)**	
	Ntd %	A.a %	Ntd %	A.a %	Ntd %	A.a %
Pak MG1 (ARL1963)-*gapA*			94.5	95.3	99.2	98.4
Pak MG2 (ARL2020)-*gapA*	94.5	95.3			93.7	90.6
Pak MG3 (ARL2668)-*gapA*	99.2	98.4	93.7	90.6		
Pak MG1 (ARL1963)-*lp*			96.8	94.4	97.0	
Pak MG2 (ARL2020)-*lp*	96.8	94.4			97.0	92.3
Pak MG3 (ARL2668)-*lp*	97.0	92.8	97.0	92.3		
Pak MG1 (ARL1963)-*pvpA*			84	77.1	100	100
Pak MG2 (ARL2020)-*pvpA*	84	77.1			84	77.5
Pak MG3 (ARL2668)-*pvpA*	100	100	84	77.5		
Pak MG1 (ARL1963)-*mgc2*			97.1	91.8	98.2	94.5
Pak MG2 (ARL2020)-*mgc2*	97.1	91.8				
Pak MG3 (ARL2668)-*mgc2*	98.2	94.5	97.8	95.4	97.8	95.4

5.8.1 Sequence and Phylogenetic analysis of *gap A* gene

Phylogenetic analysis was carried out on the basis of nucleotide sequences of *gap A* gene of *M. gallisepticum* available in GenBank. Partial sequencing of *gap A* gene of three isolates Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) was done. Alignment and comparison of partial nucleotide sequence of *gap A* gene revealed 93.7% to 99.2% relatedness among Pakistani isolates. Amino acid analysis of *gap A* revealed 90.6%-98.4% identity (Table 5.5).

For multiple sequence alignment analysis, *M. gallisepticum gap A* gene sequences were retrieved from GenBank database. Selected sequences included vaccine strains *M. gallisepticum* ts-11, F strain and 6/85, reference strains R strain, A5969, and S6 strain. Field strain sequences originating from USA, Australia, Russia and South Africa were used (Fig.5.5). A comparative analysis of *gapA* gene sequences retrieved from NCBI and sequences of Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) isolates, was performed using the MEGA-X Sequence Alignment Program. Phylogenetic tree was constructed using MEGA-X (Fig. 5.6). A detailed analysis of sequence alignment showed few common patterns throughout the genes. Deletion of a G at nt-5162 was observed in both Pak MG1(ARL-1963), and Pak MG2(ARL- 2020). In Pak MG3 (ARL-2668) deletion of a T at ntd-5404 and 5413 was observed with changes at A5153 (T), A5162 (G), A5195 (G). The *gapA* gene sequences were submitted to GeneBank, accession numbers given in (Table. 5.19)

5.8.1.1 Nucleotide and Amino acid Similarity of *gap A* gene

Nucleotide and amino acid similarity were computed by using MEGA-X. Pak MG1 (ARL-1963) g*apA* gene exhibited 90.04% to 97.09% nucleotide homology and 84.80% to 94.74% amino acid homology with retrieved sequences. Maximum sequence homology of Pak MG1 (ARL-1963) g*apA* gene was 97.09% with MG-RV-2 (Israel), and 96.75% with K435TK73 (USA) shown in (Table 5.6). Least relatedness was 90.04% with BRT14 (Israel). Pak MG2 (ARL-2020) *gapA* gene showed 91.54% to 99.19% nucleotide homology and 89.03% to 98.54% amino acid homology with retrieved sequences.

Maximum sequence homology of Pak MG2 (ARL-2020) *gapA* gene was 99.19% with *M. gallisepticum* 140905 (Russia). Least relatedness was 91.54% with BRT14 (Israel) (Table 5.7). Pak MG3 (ARL-2668) *gapA* gene presented 90.41% to 99.65% nucleotide homology and 82.56% to 98.68% amino acid homology with retrieved sequences. Maximum nucleotide sequence homology of Pak MG3 (ARL-2668) *gapA* gene was 99.65% with K435TK73 (USA). Least relatedness observed was 93.4% with BRT14 (Israel) (Table 5.8).

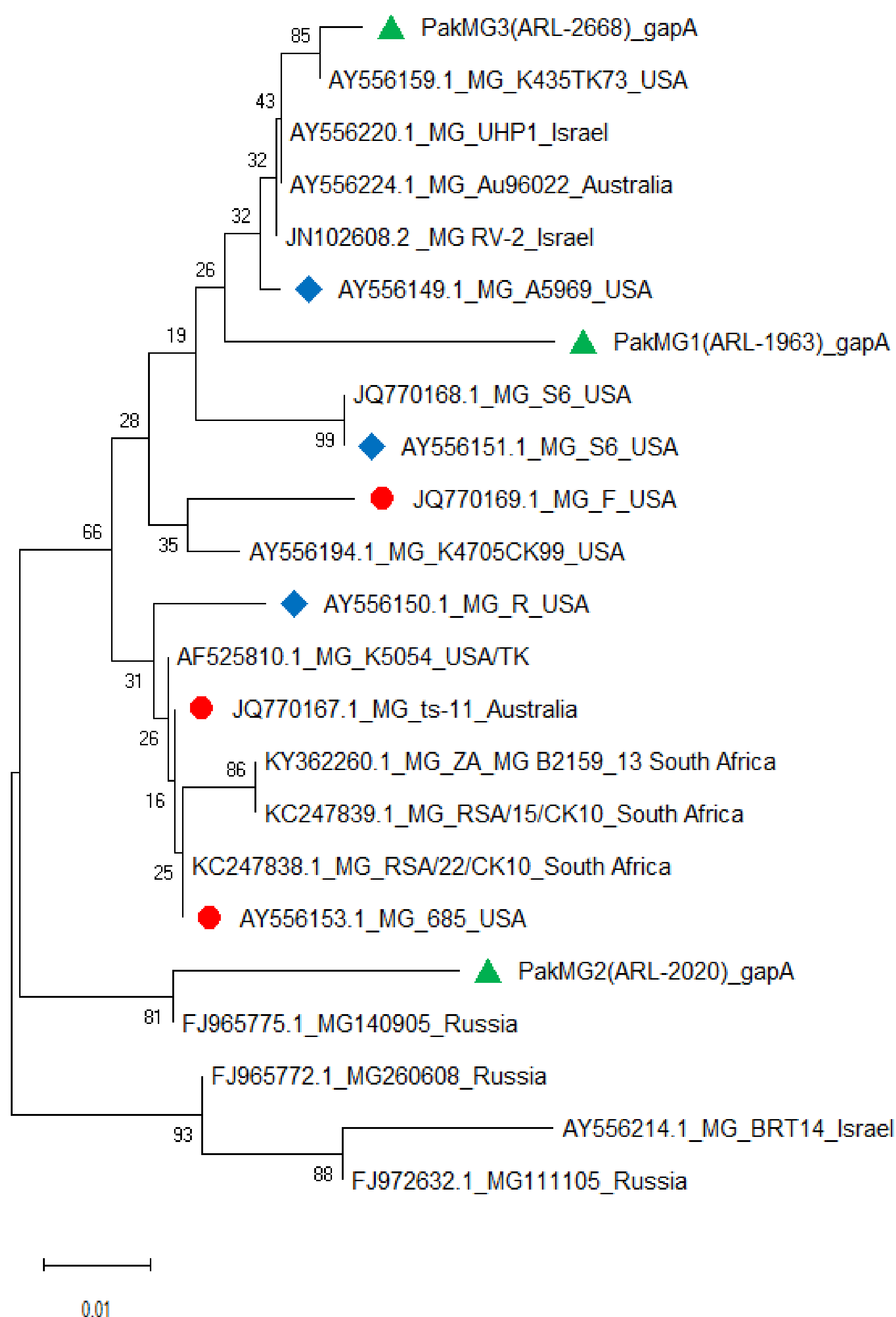


Fig. 5.4 Phylogenetic tree showing *gapA* gene (295 bp) and inter-relationship among *M. gallisepticum* isolates from Pakistan (Green triangle), vaccine strains (Red circle) and selected reference strains (Blue colour)

Table 5.6 Similarity (percentage identity) of Pak MG1 (ARL-1963) *gapA* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no	Isolate/strain	Country	Nucleotide	Amino acid
JN102608.2	RV-2	Israel	97.09	94.74
AY556159.1	K435TK73	USA	96.75	96.08
FJ965775.1	140905	Russia	96.68	95.49
AY556224.1	Au96022	Australia	96.37	94.74
AY556220.1	UHP1	Israel	96.37	94.74
AY556149.1	A5969	USA	95.99	93.38
JQ770167.1	ts-11	Australia	95.95	94.59
AF525810.1	K5054 /TK	USA	95.95	94.59
FJ965772.1	260608	Russia	95.80	90.76
KY362260.1	ZA_MG_B2159_13	South Africa	95.26	92.00
JQ770168.1	S6	USA	95.25	92.00
AY556194.1	K4705CK99	USA	95.25	93.38
AY556151.1	S6	USA	95.25	92.00
KC247839.1	RSA/15/CK10	South Africa	95.24	92.00
FJ972632.1	111105	Russia	95.06	89.30
AY556153.1	685	USA	94.52	92.00
KC247838.1	RSA/22/CK10	South Africa	94.50	92.00
JQ770169.1	F	USA	93.75	90.60
AY556150.1	R	USA	93.74	90.60
AY556214.1	BRT14	Israel	90.04	84.80

Table 5.7 Similarity (percentage identity) of Pak MG2 (ARL-2020) *gapA* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no	Isolate/strain	Country	Nucleotide	Amino acid
FJ965775.1	140905	Russia	99.19	98.54
FJ965772.1	260608	Russia	97.09	92.48
FJ972632.1	111105	Russia	96.74	92.59
JQ770167.1	ts-11	Australia	94.77	96.03
AY556153.1	685	USA	94.77	96.03
AF525810.1	K5054/TK	USA	94.77	96.03
KC247838.1	RSA/22/CK10	South Africa	94.75	96.03
KY362260.1	ZA_MG_B2159_13	South Africa	94.00	96.03
AY556150.1	R	USA	94.00	94.67
KC247839.1	RSA/15/CK10	South Africa	93.98	96.03
AY556194.1	K4705CK99	USA	93.18	93.29
JN102608.2	RV-2	Israel	92.41	93.29
AY556224.1	Au96022	Australia	92.41	93.29
AY556220.1	UHP1	Israel	92.41	93.29
AY556149.1	A5969	USA	92.39	93.29
JQ770169.1	F	USA	92.39	89.03
JQ770168.1	S6	USA	92.00	91.89
AY556159.1	K435TK73	USA	91.99	91.89
AY556214.1	BRT14	Israel	91.54	89.03

Table 5.8 Similarity (percentage identity) of Pak MG3 (ARL-2668) *gapA* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no	Isolate/strain	Country	Nucleotide	Amino acid
AY556159.1	K435TK73	USA	99.65	98.68
AY556224.1	Au96022	Australia	99.29	97.33
AY556220.1_ _	UHP1	Israel	99.29	97.33
JN102608.2	RV-2	Israel	99.27	97.30
AY556149.1	A5969	USA	98.93	95.97
AY556194.1	K4705CK99	USA	98.21	95.97
KY362260.1	ZA_MG_B2159_13	South Africa	97.48	94.59
KC247839.1	RSA/15/CK10	South Africa	97.48	94.59
JQ770169.1	F	USA	96.76	91.78
KC247838.1	RSA/22/CK10	South Africa	96.75	94.59
AY556153.1	685	USA	96.75	94.59
JQ770168.1	S6	USA	96.73	91.78
JQ770167.1	ts-11	Australia	96.60	94.36
AF525810.1	K5054/TK	USA	96.60	94.36
FJ965775.1	140905	Russia	96.20	93.75
FJ965772.1	MG260608	Russia	95.31	88.79
AY556150.1	R	USA	95.25	90.34
FJ972632.1	111105	Russia	94.57	87.28
AY556214.1	BRT14	Israel	90.41	82.56

Fig. 5.5 Alignment of the *gapA* gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries

5.8.2 Sequence and Phylogenetic analysis of *lp* gene

Phylogenetic analysis was carried out on the basis of nucleotide sequences of *Lp* gene of *M. gallisepticum* available in GenBank. Partial sequencing of *lp* gene of three isolates Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) was done. Alignment and comparison of partial nucleotide sequence of *lp* gene revealed that Pakistani isolates were 96.8% to 97% related. Amino acid analysis of *lp* showed (92.3%-94.4% identities (Table 5.5).

For multiple sequence alignment analysis, *M. gallisepticum lp* gene sequences were retrieved from GenBank database. Selected sequences included vaccine strains *M. gallisepticum* F strain and 6/85, reference strains included R strain, A5969, and S6 strain (Fig. 5.7). Field strain sequences originating from Australia, Israel, South Africa and USA were used. A comparative analysis of *lp* gene sequence of Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) isolates was performed using the MEGA Sequence Alignment Program. Phylogenetic tree was constructed using MEGA-X (Fig. 5.6).

5.8.2.1 Nucleotide Sequence and Amino acid similarity of *lp* gene

Pak MG1 (ARL-1963) *lp* gene displayed 95.97%-98.14% nucleotide homology and 92.17%- 96.54% amino acid homology with retrieved sequences. Maximum sequence homology of Pak MG1 (ARL-1963) *lp* gene was 98.14% with 685 (USA). Least relatedness was observed with RSA/21/CK10 (South Africa) i.e. 95.97% (Table 5.9).

Pak MG2 (ARL- 2020) *lp* gene presented 96.40%-99.59% nucleotide homology and 991.42%-98.62% amino acid homology with retrieved sequences. Maximum sequence homology of Pak MG2 (ARL- 2020) *lp* gene was 99.59% with K5054 (USA). Least relatedness observed was 96.40 % with F (USA) (Table 5.10). PakMG3 (ARL-2668) *lp* gene exhibited 95.03%-97.08 % nucleotide homology and 91.36%-95.15% amino acid homology with retrieved sequences. Maximum sequence homology of PakMG3 (ARL-

2668) *lp* gene was 97.08% with 685 (USA). Least relatedness was observed with RSA/21/CK10 (South Africa) i.e. 95.03% (Table 5.11).

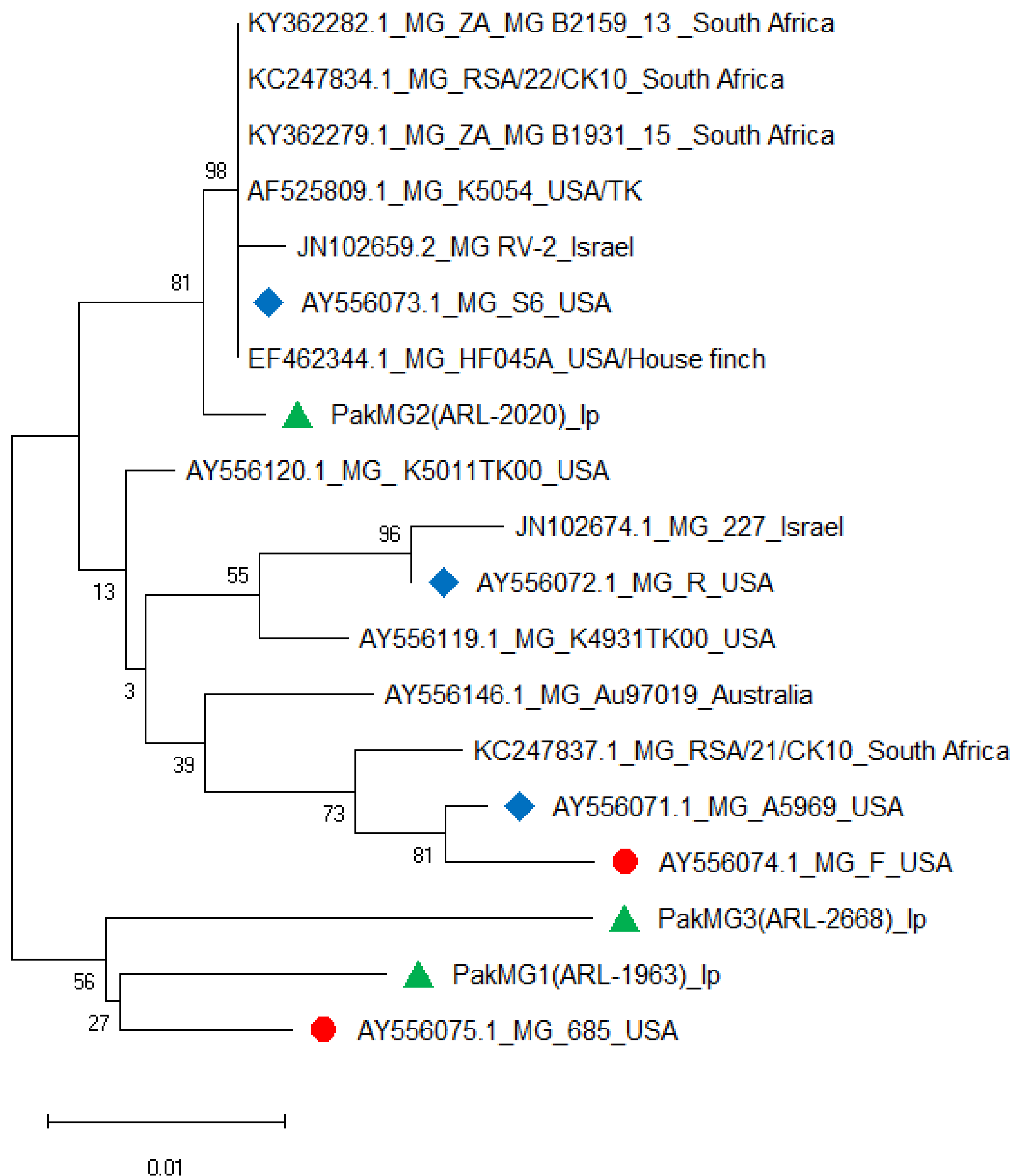


Fig. 5.6 Phylogenetic tree showing *lp* gene (495 bp) and inter-relationship among *M. gallisepticum* isolates from Pakistan (Green triangle), vaccine strains (Red circle) and selected reference strains (Blue colour)

Table 5.9 Similarity (percentage identity) of Pak MG1 (ARL-1963) *lp* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no.	Isolate/strain	Country	Nucleotide	Amino acid
AY556075.1	685	USA	98.14	96.54
AY556120.1	K5011TK00	USA	97.71	95.83
AF525809.1	K5054 /TK	USA	97.50	94.40
AY556073.1	S6	USA	97.50	94.40
EF462344.1	HF045A /House_finch	USA	97.50	94.40
AY556146.1	Au97019	Australia	97.50	93.68
KY362279.1	ZA_MG_B1931_15	South Africa	97.49	95.09
KY362282.1	ZA_MG_B2159_13	South Africa	97.49	95.09
KC247834.1	RSA/22/CK10	South Africa	97.49	95.09
JN102659.2	RV-2	Israel	97.29	93.68
AY556119.1	K4931TK00	USA	96.87	93.68
AY556071.1	A5969	USA	96.86	93.68
AY556072.1	R	USA	96.66	92.22
AY556074.1	F	USA	96.44	91.48
JN102674.1	227	Israel	96.23	91.48
KC247837.1	RSA/21/CK10	South Africa	95.97	92.17

Table 5.10 Similarity (percentage identity) of Pak MG2 (ARL-2020) *lp* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no.	**Isolate name**	**Country**	**Nucleotide**	**Amino acid**
AF525809.1	K5054 /TK	USA	99.59	98.62
AY556073.1	S6	USA	99.59	98.62
EF462344.1	HF045A /House_finch	USA	99.59	98.62
KY362279.1	ZA_MG_B1931_15	South Africa	99.58	98.59
KY362282.1	ZA_MG_B2159_13	South Africa	99.58	98.59
KC247834.1	RSA/22/CK10	South Africa	99.58	98.59
JN102659.2	RV-2	Israel	99.38	97.92
AY556120.1	K5011TK00	USA	98.54	95.80
AY556072.1	R	USA	98.34	95.09
AY556146.1	Au97019	Australia	98.33	96.52
JN102674.1	227	Israel	97.92	94.36
AY556119.1	K4931TK00	USA	97.70	93.64
KC247837.1	RSA/21/CK10	South Africa	97.66	95.71
AY556075.1	685	USA	97.27	93.64
AY556071.1	A5969	USA	96.83	93.64
AY556074.1	F	USA	96.40	91.42

Table 5.11 Similarity (percentage identity) of Pak MG3 (ARL-2668) *lp* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no.	Isolate/strain	Country	Nucleotide	Amino acid
AY556075.1	685	USA	97.08	95.15
AF525809.1	K5054 /TK	USA	96.65	94.44
AY556073.1	S6	USA	96.65	94.44
EF462344.1	HF045A /House finch	USA	96.65	94.44
AY556120.1	K5011TK00	USA	96.63	94.44
KY362279.1	ZA_MG_B1931_15	South Africa	96.59	94.32
KY362282.1	ZA_MG_B2159_13	South Africa	96.59	94.32
KC247834.1	RSA/22/CK10	South Africa	96.59	94.32
JN102659.2	RV-2	Israel	96.43	93.73
AY556146.1	Au97019	Australia	96.41	92.28
AY556119.1	K4931TK00	USA	95.77	92.28
AY556071.1	A5969	USA	95.76	92.28
AY556072.1	R	USA	95.56	90.81
AY556074.1	F	USA	95.33	90.06
JN102674.1	227	Israel	95.12	90.06
KC247837.1	RSA/21/CK10	South Africa	95.03	91.36

Fig. 5.7a Alignment of the *lp* gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries

Fig. 5.7b Alignment of the *gapA* gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries

5.8.3 Sequence and Phylogenetic analysis of *pvp A* gene

Phylogenetic analysis was carried out on the basis of nucleotide sequences of *pvp A* gene of *M. gallisepticum* available in GenBank. Partial sequencing of *pvp A* gene of three isolates Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) was carried out. Alignment and comparison of partial nucleotide sequence of *pvp A* gene revealed that Pakistani isolates were 80.9% to 99.8% related. Amino acid analysis of *pvp A* showed (100%-77.1%) identities.

For multiple sequence alignment analysis, *M. gallisepticum pvpA* gene sequences were retrieved from GenBank database after BLAST search. Selected sequences included vaccine strains ts-11 and 6/85, reference strains PG31 and S6 strain. Field strain sequences originating from, Australia, China, Germany, Iran, Israel and USA were used (Fig-5.9). A comparative analysis of *pvp*A gene sequence of Pak MG1(ARL-1963), Pak MG2 (ARL-2020) and Pak MG3 (ARL- 2668) isolates was performed using the MEGA Sequence Alignment Program. Phylogenetic tree was constructed using MEGA-X (Fig. 5.8).

5.8.3.1 Nucleotide Sequence and Amino acid Similarity of *pvp A* gene

Pak MG1(ARL-1963) and Pak MG3 (ARL-2668) *pvpA* genes were identical in nucleotide sequence. Both presented 93.78% -100% nucleotide homology and 80.36%-100% amino acid homology with retrieved sequences. Pak MG1 (ARL-1963) and Pak MG3 (ARL-2668) *pvpA* genes exhibited 100% sequence homology with IRMG13PC01 (Iran). Relatedness to K5054 (USA), 1608/2/11/TK (Germany) and S6 (ATCC_15302) (USA) was 99.2%, 98.2% and 97.6%, respectively. Nucleotide homology of Pak MG1(ARL-1963) and Pak MG3 (ARL-2668) *pvpA* gene JC6 (China) was 93.78% (Table 5.12 & 5.14). Pak MG2 (ARL-2020) *pvpA* gene displayed 84.36%-96.63% nucleotide homology and 80.3%-94.87% amino acid homology with retrieved sequences. Maximum sequence homology of Pak MG2 (ARL-2020) *pvpA* gene was 96.63% with R strain (USA). Least relatedness observed was 84.36% with K5054 (USA) (Table 5.13).

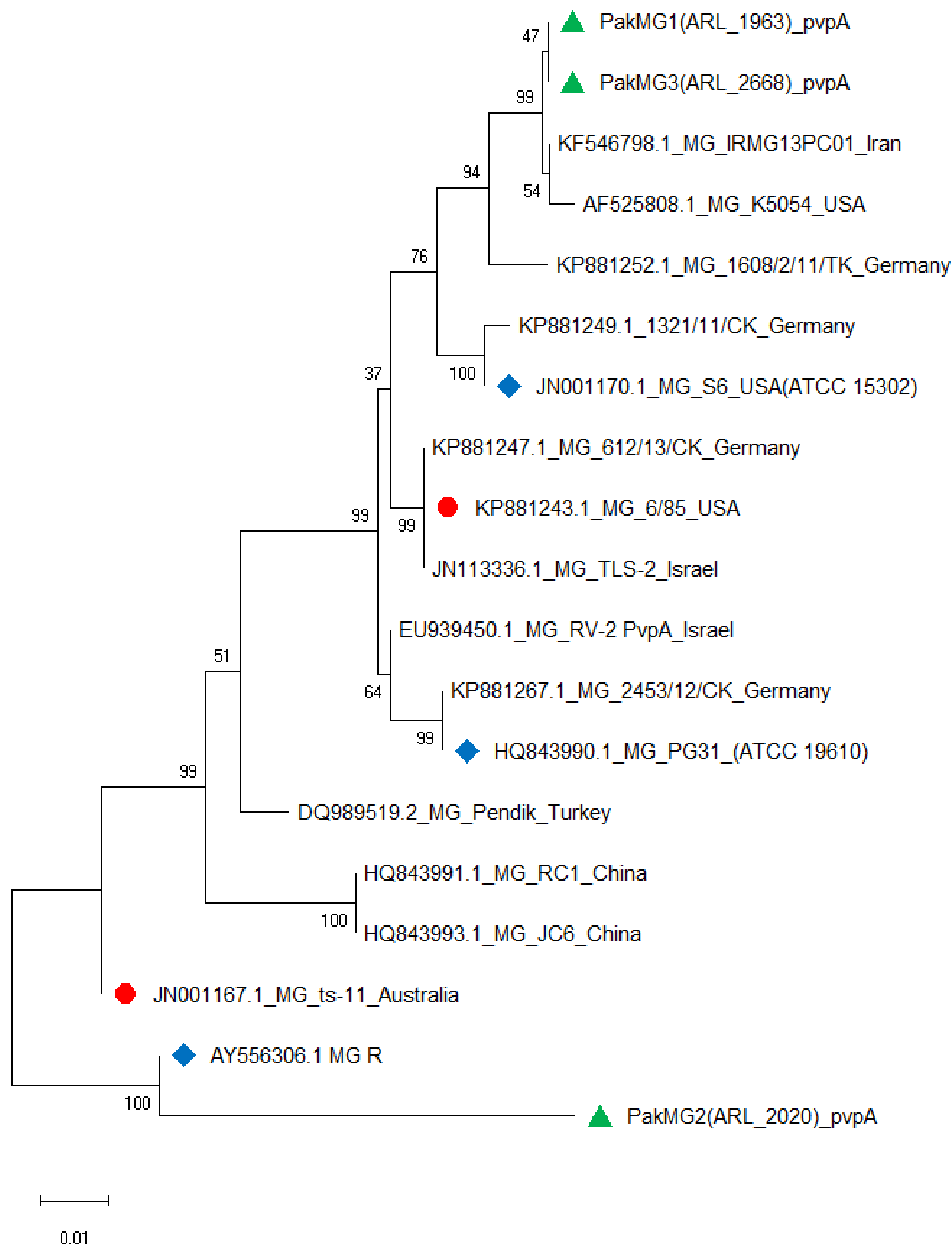


Fig. 5. 8 Phylogenetic tree showing *pvpA* (583 bp) gene and inter-relationship among *M. gallisepticum* isolates from Pakistan (Green triangle), vaccine strains (Red circle) and selected reference strains (Blue colour)

Table 5.12 Similarity (percentage identity) of Pak MG1 (ARL-1963) *pvpA* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no.	Isolate name	Country	Nucleotide	Amino acid
KF546798.1	IRMG13PC01	Iran	100.00	100.00
AF525808.1	K5054	USA	99.23	100.00
KP881252.1	1608/2/11/TK	Germany	98.25	96.78
JN001170.1	S6 (ATCC_15302)	USA	97.65	94.80
EU939450.1	RV-2_PvpA	Israel	97.40	95.05
KP881249.1	1321/11/CK	Germany	97.25	94.80
KP881247.1	612/13/CK	Germany	97.06	95.47
KP881243.1	6/85	USA	97.06	95.47
JN113336.1	TLS-2	Israel	97.06	95.47
KP881267.1	2453/12/CK	Germany	95.84	94.14
HQ843990.1	PG31(ATCC19610)	China	95.84	94.14
DQ989519.2	Pendik	Turkey	95.37	91.36
JN001167.1	ts-11	Australia	94.82	91.41
AY556306.1	R	USA	94.22	93.04
HQ843991.1	RC1	China	93.78	91.41
HQ843993.1	JC6	China	93.78	91.41

Table 5.13 Similarity (percentage identity) of Pak MG2 (ARL-2020) *pvpA* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no.	Isolate name	Country	Nucleotide	Amino acid
AY556306.1	R	USA	96.63	94.87
JN001167.1	ts-11	Australia	90.94	87.52
DQ989519.2	Pendik	Turkey	87.61	83.55
HQ843991.1	RC1	China	87.45	81.77
HQ843993.1	JC6	China	87.45	81.77
EU939450.1	RV-2_PvpA	Israel	85.75	81.08
JN001170.1	S6 (ATCC_15302)	USA	85.07	81.13
KP881247.1	612/13/CK	Germany	84.95	79.72
KP881243.1	6/85	USA	84.95	79.72
JN113336.1	TLS-2	Israel	84.95	79.72
KP881249.1	1321/11/CK	Germany	84.82	81.13
KF546798.1	IRMG13PC01	Iran	84.68	80.22
KP881267.1	2453/12/CK	Germany	84.47	78.16
HQ843990.1	PG31 (ATCC_19610)	China	84.47	78.16
KP881252.1	1608/2/11/TK	Germany	84.38	77.21
AF525808.1_ _	K5054	USA	84.36	80.36

Table 5.14 Similarity (percentage identity) of Pak MG3 (ARL-2668) *pvpA* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no	Isolate name	Country	Nucleotide	Amino acid
KF546798.1	IRMG13PC01	Iran	100.00	100.00
AF525808.1	K5054	USA	99.23	100.00
KP881252.1	1608/2/11/TK	Germany	98.25	96.78
JN001170.1	S6 (ATCC_15302)	USA	97.65	94.80
EU939450.1	RV-2_PvpA	Israel	97.40	95.05
KP881249.1	1321/11/CK	Germany	97.25	94.80
KP881247.1	612/13/CK	Germany	97.06	95.47
KP881243.1	6/85	USA	97.06	95.47
JN113336.1	TLS-2	Israel	97.06	95.47
KP881267.1	2453/12/CK	Germany	95.84	94.14
HQ843990.1	PG31 (ATCC_19610)	China	95.84	94.14
DQ989519.2	Pendik	Turkey	95.37	91.36
JN001167.1	ts-11	Australia	94.82	91.41
AY556306.1	R	USA	94.22	93.04
HQ843991.1	RC1	China	93.78	91.41
HQ843993.1	JC6	China	93.78	91.41

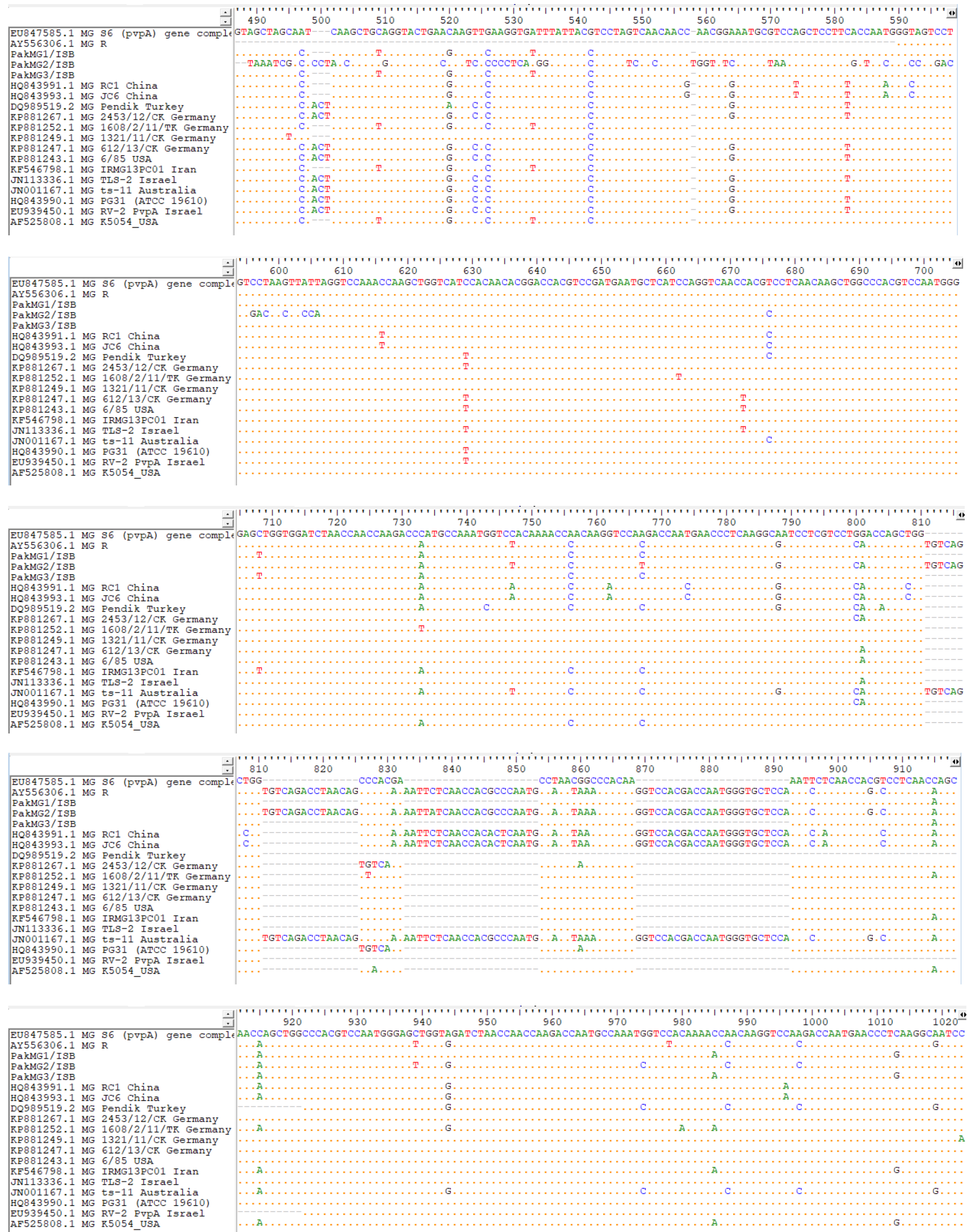


Fig. 5.9 a Alignment of the *pvpA* gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries

```
                                 1020      1030      1040      1050      1060      1070      1080      1090      1100      1110      1120
EU847585.1 MG S6 (pvpA) gene comple AGGCAATCCTCGTCCTCAACCAGCTGGTGTCAGACCTAACAGCCCACAAGCTAACCAGCCAGGACCACGTCCAACGCCAAATAATCCTCAAGGACCACGGCCAATGGGTCCA
AY556306.1 MG R                    ....G.........................----------------------------------------------------------------------------------
PakMG1/ISB                          ........................................................--------------------------------------------------------
PakMG2/ISB                          ........................................................--------------------------------------------------------
PakMG3/ISB                          ........................................................--------------------------------------------------------
HQ843991.1 MG RC1 China             ........................................................--------------------------------------------------------
HQ843993.1 MG JC6 China             ........................................................--------------------------------------------------------
DQ989519.2 MG Pendik Turkey         ....G...................................................--------------------------------------------------------
KP881267.1 MG 2453/12/CK Germany    ........................................................--------------------------------------------------------
KP881252.1 MG 1608/2/11/TK Germany  ........................................................--------------------------------------------------------
KP881249.1 MG 1321/11/CK Germany    ........A...............................................--------------------------------------------------------
KP881247.1 MG 612/13/CK Germany     ........................................................--------------------------------------------------------
KP881243.1 MG 6/85 USA              ........................................................--------------------------------------------------------
KF546798.1 MG IRMG13PC01 Iran       ..................................................--------------------------------------------------------------
JN113336.1 MG TLS-2 Israel          ........................................................--------------------------------------------------------
JN001167.1 MG ts-11 Australia       ....G...................................................--------------------------------------------------------
HQ843990.1 MG PG31 (ATCC 19610)     ........................................................--------------------------------------------------------
EU939450.1 MG RV-2 PvpA Israel      ........................................................--------------------------------------------------------
AF525808.1 MG K5054_USA             .....................................................C.A--------------------------------------------------------
```

Fig. 5.09 b Alignment of the *pvpA* gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries

5.8.4 Sequence and Phylogenetic analysis of *mgc2* gene

Phylogenetic analysis was carried out on the basis of nucleotide sequences of *mgc2* gene of *M. gallisepticum* available in GenBank. Partial sequencing of *mgc2* gene of three isolates Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) was done. Alignment and comparison of partial nucleotide sequence of *mgc2* gene revealed that Pakistani isolates were 98.2 % to 97.8% homologous. Amino acid analysis of *mgc2* showed (91.8%-95.4%) identities.

For multiple sequence alignment analysis, *M. gallisepticum mgc2* gene sequences were retrieved from GenBank database. Selected sequences included vaccine strains *M. gallisepticum* ts-11 and F strain, reference strains A5969 and S6 strain. Field strain sequences originating from Australia, Brazil, Egypt, India, South Africa and USA were used (Fig. 5.11). A comparative analysis of *mgc2* gene sequence of Pak MG1(ARL-1963), Pak MG2 (ARL- 2020) and Pak MG3 (ARL- 2668) isolates was performed using the MEGA Sequence Alignment Program. Phylogenetic tree was constructed using MEGA-X (Fig. 5.10).

5.8.4.1 Nucleotide Sequence and Amino acid Similarity of *mgc2* gene

Pak MG1(ARL-1963)-*mgc*2 gene showed 94.7%-98.25% nucleotide homology and 94.46% to 90.8% amino acid homology with retrieved sequences. Maximum sequence homology of Pak MG1(ARL-1963) *mgc2* was 98.2% with AHRU2009CU2006.1/2015 (Thailand) and K435TK73 (USA), 98% with UFMG2 (Brazil), S6 (USA), A5969 and MG_Eis10-17 (Egypt). Least relatedness observed was 94.7% with MG F strain.

Pak MG2(ARL-2020) *mgc2* showed 95.7%-100% nucleotide homology and 100% to 92.93% amino acid homology with retrieved sequences. Maximum sequence homology of Pak MG2(ARL-2020) *mgc2* was 100% with ts-11 (vaccine strain), K621D, K6112B (vaccine derived strains), 99.9% and 99.7% with B2771_14 (South Africa), B2159_13 (South Africa). Least relatedness observed was 95.7 % with MG F strain.

Pak MG3 (ARL-2668) *mgc2* showed 96.19%-100% to nucleotide homology and 94.5% -100% amino acid homology with retrieved sequences. Maximum sequence homology of Pak MG3 (ARL-2668) *mgc2* was 100% with AHRU2009CU2006.1/2015 (Thailand) and K435TK73 (USA), 99.7% with UFMG2 (Brazil), S9 (India). Least relatedness observed was 96.1% with MG F strain. Pakistani sequences were retrieved and studied in terms of relatedness. (Table 5.18) summarizes the relationship among local isolates (Fig. 5.12).

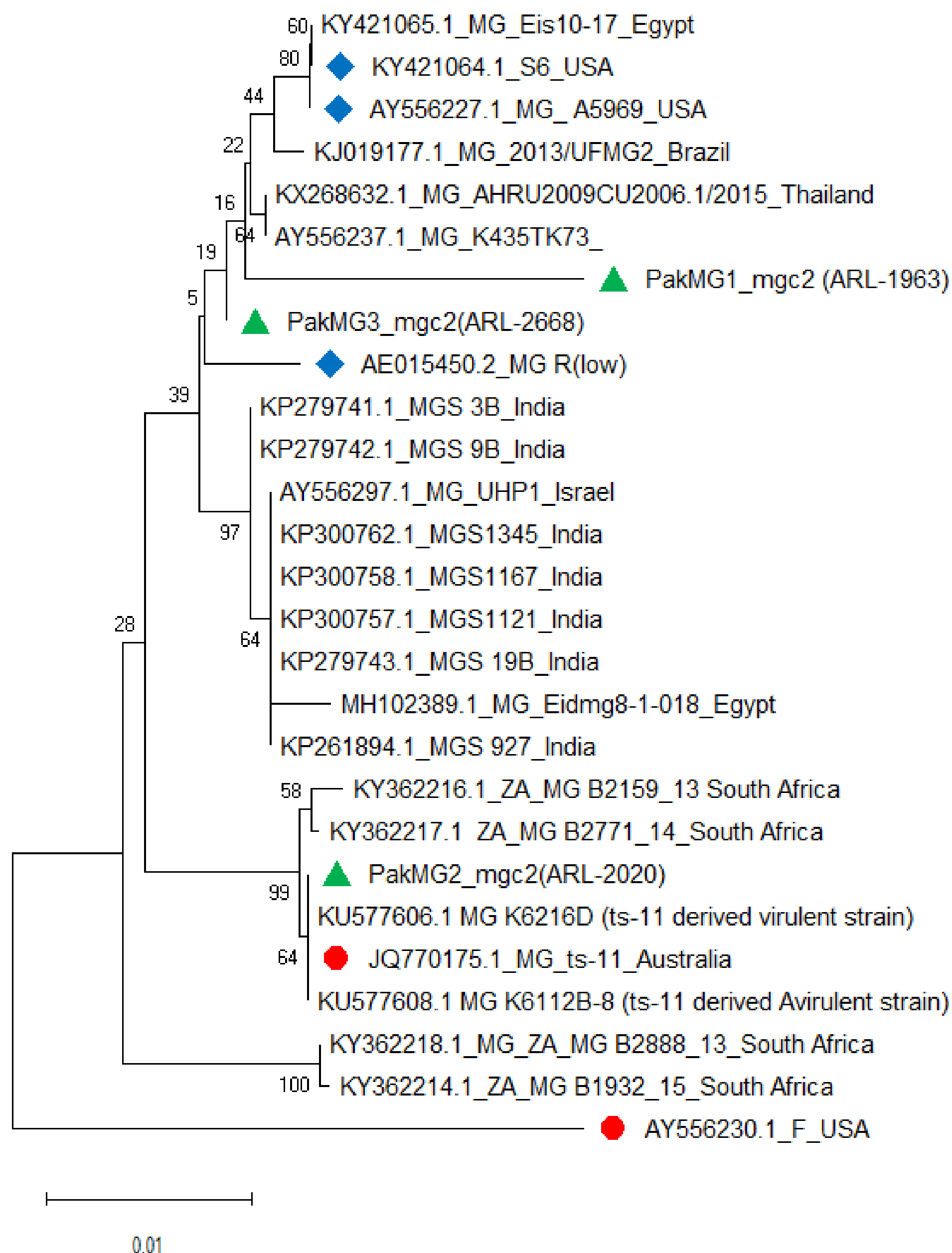


Fig. 5.10 Phylogenetic tree showing *mgc2* (621 bp) gene and inter-relationship among *M. gallisepticum* isolates from Pakistan (Green triangle), vaccine strains (Red circle) and selected reference strains (Blue colour)

Table 5.15 Similarity (percentage identity) of Pak MG1 (ARL-1963) *mgc2* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no.	Isolate name	Country	Nucleotide	Amino acid
KX268632.1	AHRU2009CU2006.1/2015	Thailand	98.25	94.46
AY556237.1	K435TK73		98.25	94.46
KJ019177.1	2013/UFMG2	Brazil	98.04	93.97
KY421065.1	Eis10-17	Egypt	98.04	95.00
KY421064.1	S6	USA	98.04	94.97
AY556227.1	A5969	USA	98.03	94.97
AE015450.2	R(low)	USA	97.93	94.13
KP279742.1	MGS_9B	India	97.83	93.45
KP279741.1	MGS_3B	India	97.83	93.45
KP300758.1	MGS1167	India	97.72	92.93
KP300757.1	MGS1121	India	97.72	92.93
KP279743.1	MGS_19B	India	97.72	92.93
KP261894.1	MGS_927	India	97.72	92.93
KP300762.1	MGS1345	India	97.72	92.89
AY556297.1	UHP1	Israel	97.71	92.89
MH102389.1	Eidmg8-1-018	Egypt	97.40	91.34
KU577606.1	K6216D (ts-11 derived)	USA	97.18	91.83
KU577608.1	K6112B-8 (ts-11 derived)	USA	97.18	91.83
JQ770175.1	ts-11	Australia	97.17	91.83
KY362217.1	ZA_MG_B2771_14	South Africa	97.07	91.83
KY362218.1	ZA_MG_B2888_13	South Africa	9706	92.36
KY362218.1	ZA_MG_B2888_13	South Africa	96.89	94.13
KY362214.1	ZA_MG_B1932_15	South Africa	96.87	93.78
AY556230.1	F	USA	94.7	90.76

Table 5.16 Similarity (percentage identity) of Pak MG2 (ARL-2020) *mgc2* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no.	Isolate name	Country	Nucleotide	Amino acid
JQ770175.1	ts-11	Australia	100.00	100.00
KU577606.1	K6216D (ts-11 derived)	USA	100.00	100.00
KU577608.1	K6112B-8 (ts-11 derived)	USA	100.00	100.00
KY362217.1	ZA_MG_B2771_14	South Africa	99.90	100.00
KY362216.1	ZA_MG_B2159_13	South Africa	99.70	99.51
KP279742.1	MGS_9B	India	98.79	96.53
KP279741.1	MGS_3B	India	98.79	96.53
KP300762.1	MGS1345	India	98.69	96.02
KP300758.1	MGS1167	India	98.69	96.02
KP300757.1	MGS1121	India	98.69	96.02
KP279743.1	MGS_19B	India	98.69	96.02
KP261894.1	MGS_927	India	98.69	96.02
AY556297.1	UHP1	Israel	98.68	96.02
KX268632.1	AHRU2009CU2006.1/2015	Thailand	98.58	95.51
AY556237.1	K435TK73	USA	98.58	95.51
AE015450.2	R(low)	USA	98.50	96.31
KJ019177.1	2013/UFMG2	Brazil	98.48	95.51
MH102389.1	Eidmg8-1-018	Egypt	98.37	94.48
KY421065.1	Eis10-17	Egypt	98.37	96.02
KY421064.1	S6	USA	98.37	96.02
AY556227.1	A5969	USA	98.37	96.02
KY362218.1	ZA_MG_B2888_13	South Africa	98.06	96.33
KY362214.1	ZA_MG_B1932_15	South Africa	97.90	95.92
AY556230.1	F	USA	95.74	92.93

Table 5.17 Similarity (percentage identity) of Pak MG3 (ARL-2668) *mgc2* gene sequence with vaccine & reference strains and genotypes from different countries

Accession no.	Isolate name	Country	Nucleotide	Amino acid
KX268632.1	AHRU2009CU2006.1/2015	Thailand	100.00	100.00
AY556237.1	K435TK73	USA	100.00	100.00
KJ019177.1	2013/UFMG2	Brazil	99.67	98.92
KY421065.1	Eis10-17	Egypt	99.56	99.46
KY421064.1	S6	USA	99.56	99.46
AY556227.1	A5969	USA	99.56	99.46
AE015450.2	R(low)		99.30	98.27
KP279742.1	MGS_9B	India	99.67	98.92
KP279741.1	MGS_3B	India	99.67	98.92
KP300758.1	MGS1167	India	99.56	98.38
KP300757.1	MGS1121	India	99.56	98.38
KP279743.1	MGS_19B	India	99.56	98.38
KP261894.1	MGS_927	India	99.56	98.38
KP300762.1	MGS1345	India	99.56	98.38
AY556297.1	UHP1	Israel	99.56	98.38
MH102389.1	Eidmg8-1-018	Egypt	99.23	96.74
KU577606.1	K6216D_(ts-11_derived)	USA	98.67	96.18
KU577608.1	K6112B-8_(ts-11_derived)	USA	98.67	96.18
JQ770175.1	ts-11	Australia	98.67	96.18
KY362217.1	ZA_MG_B2771_14	South Africa	98.56	96.18
KY362216.1	ZA_MG_B2159_13	South Africa	98.44	96.18
KY362218.1	ZA_MG_B2888_13	South Africa	98.34	98.28
KY362214.1	ZA_MG_B1932_15	South Africa	98.28	97.78
AY556230.1	F	USA	96.19	94.50

```
         200       210       220       230       240       250       260       270       280
AE015450.2 MG R(low)   ATCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTAGGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATG
JQ770177.1 MG S6 USA
PakMG1 mgc2
PakMG2 mgc2
PakMG3 mgc2
AY556230.1 MG F USA
JQ770175.1 MG ts-11 Australia
AY556227.1 MG  A5969 USA
KU577606.1 MG K6216D (ts-11 derived virulent strain
KU577608.1 MG K6112B-8 (ts-11 derived Avirulent str
KX268632.1 MG AHRU2009CU2006.1/2015 Thailand
KJ019177.1 MG 2013/UFMG2 Brazil
KY362218.1 ZA MG B2888 13 South Africa
KY362216.1 ZA MG B2159 13 South Africa
KY362214.1 ZA MG B1932 15 South Africa
KY362217.1 ZA MG B2771 14 South Africa
KP300762.1 MGS1345 India
KP300758.1 MGS1167 India
KP300757.1 MGS1121 India
KP279743.1 MGS 19B India
KP261894.1 MGS 927 India
KP279742.1 MGS 9B India
KP279741.1 MGS 3B India
AY556237.1 MG K435TK73 USA
AY556297.1 MG_UHP1_Israel
```

```
      320       330       340       350       360       370       380       390       400       410
AE015450.2 MG R(low)   GATGGTTGAATCCCTTGGAATAATTGAAGAACAAAATAAAACAGAAGCGATTGAGCCAACTG---------------AAGAAGTTAATACTCAAGAAC
JQ770177.1 MG S6 USA
PakMG1 mgc2
PakMG2 mgc2
PakMG3 mgc2
AY556230.1 MG F USA
JQ770175.1 MG ts-11 Australia
AY556227.1 MG  A5969 USA
KU577606.1 MG K6216D (ts-11 derived virulent strain
KU577608.1 MG K6112B-8 (ts-11 derived Avirulent str
KX268632.1 MG AHRU2009CU2006.1/2015 Thailand
KJ019177.1 MG 2013/UFMG2 Brazil
KY362218.1 ZA MG B2888 13 South Africa
KY362216.1 ZA MG B2159 13 South Africa
KY362214.1 ZA MG B1932 15 South Africa
KY362217.1 ZA MG B2771 14 South Africa
KP300762.1 MGS1345 India
KP300758.1 MGS1167 India
KP300757.1 MGS1121 India
KP279743.1 MGS 19B India
KP261894.1 MGS 927 India
KP279742.1 MGS 9B India
KP279741.1 MGS 3B India
AY556237.1 MG K435TK73 USA
AY556297.1 MG_UHP1_Israel
```

```
  420       430       440       450       460       470       480       490       500       510
AE015450.2 MG R(low)   CAACCAGCTGGTGTTAATGTAGCTAATAACCCTCAGATGGGGATCAATCAACC---------------TCAGATTAATCCGCAATTTGGTCCTAATCC
JQ770177.1 MG S6 USA
PakMG1 mgc2
PakMG2 mgc2
PakMG3 mgc2
AY556230.1 MG F USA
JQ770175.1 MG ts-11 Australia
AY556227.1 MG  A5969 USA
KU577606.1 MG K6216D (ts-11 derived virulent strain
KU577608.1 MG K6112B-8 (ts-11 derived Avirulent str
KX268632.1 MG AHRU2009CU2006.1/2015 Thailand
KJ019177.1 MG 2013/UFMG2 Brazil
KY362218.1 ZA MG B2888 13 South Africa
KY362216.1 ZA MG B2159 13 South Africa
KY362214.1 ZA MG B1932 15 South Africa
KY362217.1 ZA MG B2771 14 South Africa
KP300762.1 MGS1345 India
KP300758.1 MGS1167 India
KP300757.1 MGS1121 India
KP279743.1 MGS 19B India
KP261894.1 MGS 927 India
KP279742.1 MGS 9B India
KP279741.1 MGS 3B India
AY556237.1 MG K435TK73 USA
AY556297.1 MG_UHP1_Israel
```

```
520       530       540       550       560       570       580       590       600       610
AE015450.2 MG R(low)   CAAAGAATTAACCCACAGTGCTTTGGTGGCCCAATGCAACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCACAAATGGGAGGAAT
JQ770177.1 MG S6 USA
PakMG1 mgc2
PakMG2 mgc2
PakMG3 mgc2
AY556230.1 MG F USA
JQ770175.1 MG ts-11 Australia
AY556227.1 MG  A5969 USA
KU577606.1 MG K6216D (ts-11 derived virulent strain
KU577608.1 MG K6112B-8 (ts-11 derived Avirulent str
KX268632.1 MG AHRU2009CU2006.1/2015 Thailand
KJ019177.1 MG 2013/UFMG2 Brazil
KY362218.1 ZA MG B2888 13 South Africa
KY362216.1 ZA MG B2159 13 South Africa
KY362214.1 ZA MG B1932 15 South Africa
KY362217.1 ZA MG B2771 14 South Africa
KP300762.1 MGS1345 India
KP300758.1 MGS1167 India
KP300757.1 MGS1121 India
KP279743.1 MGS 19B India
KP261894.1 MGS 927 India
KP279742.1 MGS 9B India
KP279741.1 MGS 3B India
AY556237.1 MG K435TK73 USA
AY556297.1 MG_UHP1_Israel
```

Fig. 5.11 a Alignment of the *mgc2* gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries

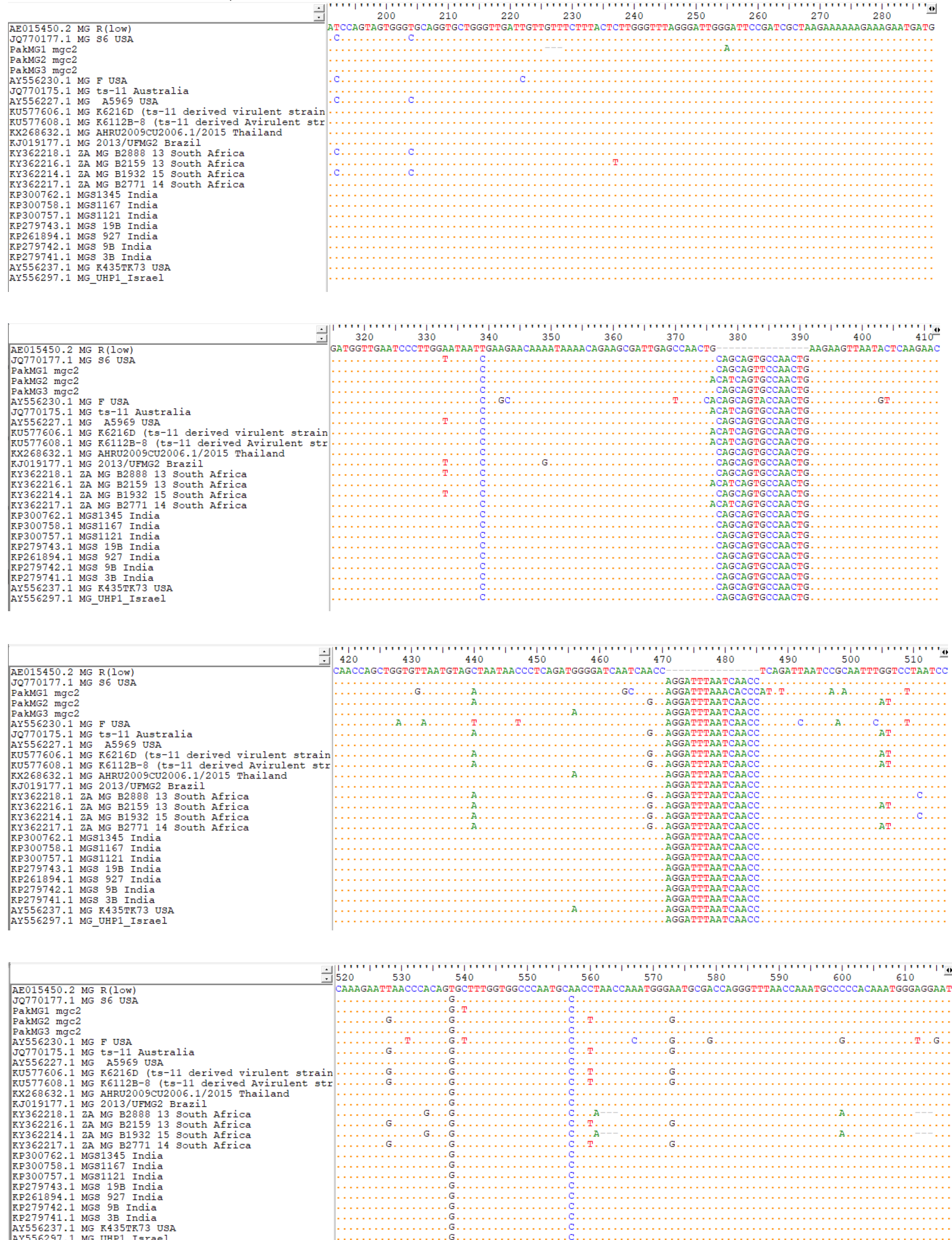


Fig. 5.11 b Alignment of the *mgc2* gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries

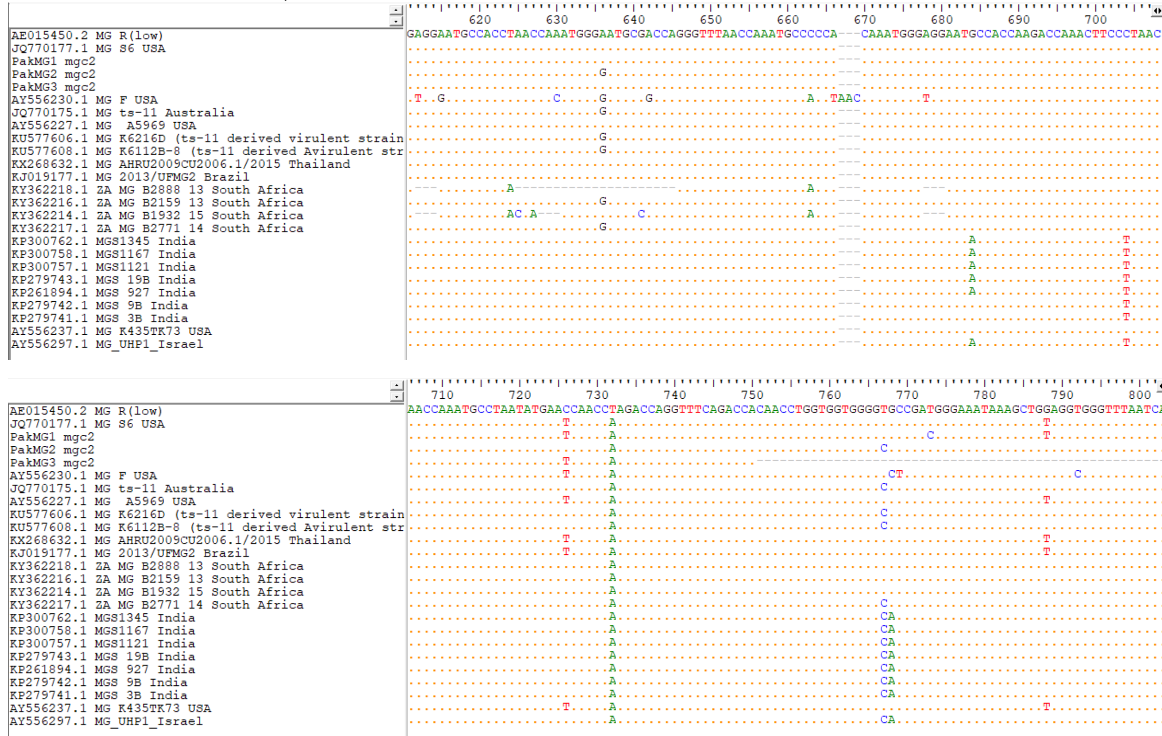


Fig. 5.11 c Alignment of the *mgc2* gene sequences of Pakistani *M. gallisepticum* isolates with reference strains, vaccine strains and isolates from various countries

Table 5.18 Relatedness of Pakistani *M. gallisepticum* isolates on the basis of *mgc2* nucleotide sequence

Accession No.	Isolate Name	Pak MG1 (ARL-1963)	Pak MG2 (ARL-2020)	Pak MG3 (ARL-2668)
FJ395202.1_	EgPk1UAF08	96.32	96.75	97.98
KF874283.1	CK.MG.UDL.PK.2013.1	96.80	96.39	98.42
KF874279.1	CK.MG.UDL.PK.2013.4_	96.79	96.38	98.41
KF874281.1	CK.MG.UDL.PK.2013.9	97.19	96.38	98.41
KF874278.1	CK.MG.UDL.PK.2013.1a	95.96	95.55	97.61
KF874280.1	CK.MG.UDL.PK.2013.2	98.41	97.21	100.00

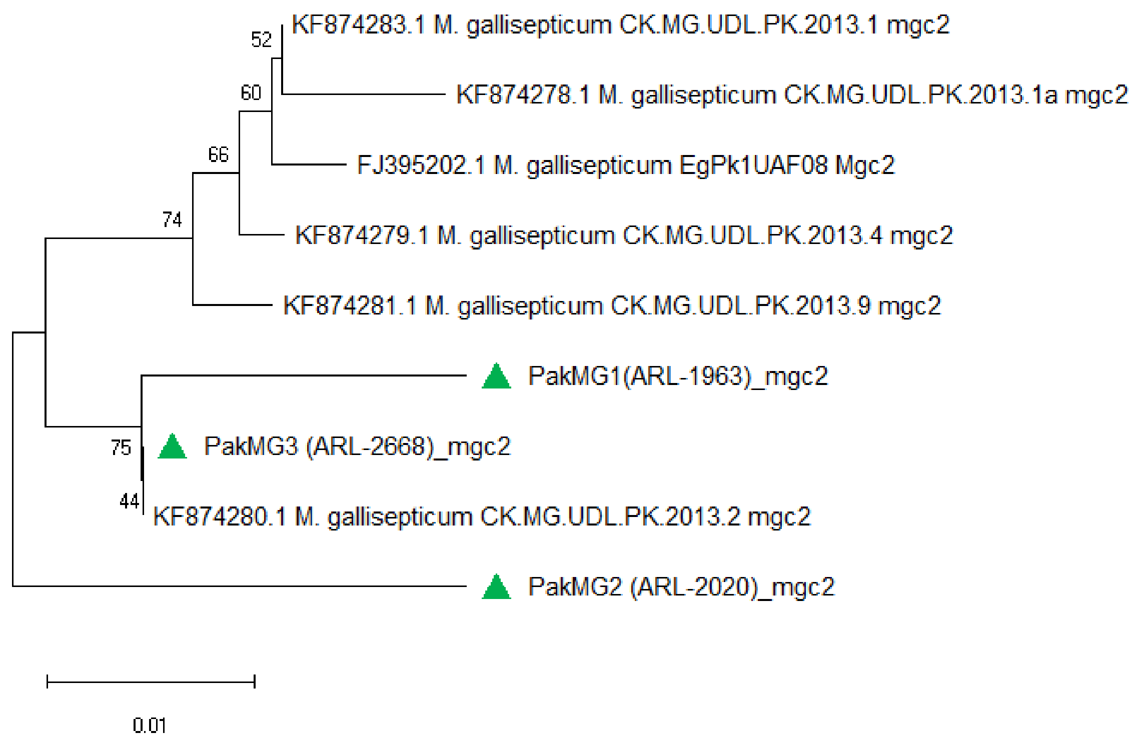


Fig. 5.12. Phylogenetic tree showing *mgc2* gene (255 bp) and inter-relationship among *M. gallisepticum* isolates from Pakistan

Table 5.19 Accession numbers and designation of *M. gallisepticum* isolates submitted to GenBank

Isolates	*gapA* Accession No.	*mgc2* Accession No.
Pak MG1 (ARL-1963)	--	MT349656
Pak MG2 (ARL-2020)	MT349659	MT349657
Pak MG3 (ARL-2668)	MT349660	MT349658

5.9 Discussion

Biological characteristics of organisms largely depend on molecular makeup and organization. Molecular biology of Pakistani *M. gallisepticum* isolates remain un-investigated till to date. For characterization of Pakistani *M. gallisepticum* isolates, gene target sequence (GTS) analysis of 04 virulence genes of three isolates Pak MG1(ARL-1963), Pak MG2 (ARL-2020), and Pak MG3 (ARL- 2668) was done. Four genes selected for GTS analysis included, three surface protein genes and a conserved lipoprotein gene. Similar study was reported by Khumpim et al., (2015), who stated presence of virulence genes (*lp, gapA, pvpA, mgc2*) in Thai isolates. Another study was conducted by Kleven et al., (2007) targeting above mentioned genes to trace *M. gallisepticum* outbreak in turkeys in USA. Authors reported 97.6% to 100% homology of 5 studied isolates with *M. gallisepticum* live vaccine strain 6/85. In spite of close genetic relatedness clinical trials proved biological behaviour (respiratory signs and antibody response) of field isolates was different from that of 6/85 strain.

Analysis of *gapA* gene showed that Pakistani *M. gallisepticum* isolates are 93.7% to 99.2% similar. No two sequences were 100% homologous to each other. On comparison with *gapA* sequences of selected reference and field isolates, Pak MG1 (ARL-1963) shared maximum 97.4% similarity with RV-2 (Israel), Pak MG2 (ARL-2020) shared 99.19% similarity with 140905 (Russia) and Pak MG3 (ARl-2668) shared 99.65% sequence similarity with K435TK73 (USA). *M. gallisepticum gapA* gene share sequence homology with cytadhesin genes of human mycoplasmas, 45% homology to *M. pneumoniae* P1 gene, 46% homology to *M. genitalium* MgPa gene and 47% homology with *M. pirum* P1-likeprotein gene (Goh et al., 1998). Significance of GapA protein in association with CrmA protein is well established in colonization and cytadherence of *M. gallisepticum* consequently determining the pathogenesis and virulence of organism. Mutants lacking Gap A protein show reduced adherence and low pathogenicity. Subsequent passages of *M. gallisepticum* can result in loss of *gapA* gene, which alter the colonization properties of passaged isolate (Papazisi et al., 2000; Papazisi et al., 2002; Indikova et al., 2013). Study

reported by Fujisawa et al., (2019) targeting *gapA* gene for sequence analysis reported no relationship of field isolates of *M. gallisepticum* from Myanmar with vaccine strains.

On the basis of sequence analysis of *pvpA* gene, isolates Pak MG1 (ARL-1963), Pak MG2 (ARL-2020), and Pak MG3 (ARL- 2668) share 81% to 100% nucleotide homology with each other. Nucleotide sequence of *pvpA* gene of Pak MG1(ARL-1963) and Pak MG3 (ARL- 2668) were identical and share 81% similarity with that of were Pak MG2 (ARL-2020). In our study of *M. gallisepticum pvpA* gene, nucleotide deletions of 60 bp were observed in Pak MG1 (ARL-1963) and Pak MG3 (ARL- 2668) in proline rich, carboxy terminal of the gene. These two samples shared deletions with vaccine strain 6/85 (USA), reference strain S6 and PG31 and isolates from Iran (IRM13PC01), Israel (TSL-2), and USA (K5054). Pak MG1(ARL-1963) and Pak MG3 (ARL- 2668) *pvpA* gene appeared to be 100% similar with *M. gallisepticum* Peacock isolate (IRM13PC01), of Iranian origin. Pak MG2(ARL-2668) *pvpA* gene sequence was found to be 96.6% related to that of *M. gallisepticum* R strain. The present study can be related to the work of (Yasmin et al., 2018) who demonstrated uniqueness of Malaysian *M. gallisepticum* isolates based on molecular characterization of *pvpA* and *pMGA* genes.

(Yogev et al., 1994) published characteristics of phase variable protein, PvpA. Molecular characterization of *M. gallisepticum pvpA* gene, as reported by Boguslavsky et al., (2000) described size variation in the respective gene ultimately expressing PvpA protein of varying size. Based on size variation due to deletion in 3' end of *pvp A* gene, (Liu et al., 2001) described its diagnostic application for strain typing and epidemiological study of *M. gallisepticum* isolates. Although, number of Pakistani *M. gallisepticum* isolates analysed was limited, size polymorphism in *pvpA* gene suggests that there might be molecular diversification among local isolates. (Sprygin et al., 2010) published utility of single-locus sequence typing (SLST) of *pvpA* gene. According to authors, sequence analysis of variable locus of *pvpA* gene of *M. gallisepticum* of Russian isolates, proved to be a successful method of typing with discriminatory index of 0.975. Size polymorphism in *pvpA* gene and relatedness of 6 Iranian *M. gallisepticum* isolates with house finch isolate (USA) and S6 strain was described in a recent report by Kabiri et al., (2019).

Surface protein genes had been extensively studied in the past to delineate pathogenesis of disease and role of each gene in virulence of organism. In addition, variation in genes had been explored for molecular typing of *M. gallisepticum* isolates.

Another gene of Pakistani field isolates of *M. gallisepticum*, investigated in present study was *mgc2*. Isolates Pak MG1 (ARL-1963), Pak MG2 (ARL-2020), and Pak MG3 (ARL-2668) share 97.1%-98.2% nucleotide homology with each other. Maximum nucleotide homology of PakMG1 (ARL-1963) and Pak MG3 (ARL-2668) *mgc2* gene was with Thai (AHRU2009CU2006) and USA isolates (K435TK73). Pak MG2 (ARL-2020) *mgc2 gene* shared 100% sequence similarity with ts-11 (Vaccine strain) and ts-11 derived strains (K621D, K6112B). All three isolates shared minimum sequence homology of *mgc2* gene with that of MG F strain. Cytadhesin gene *mgc2*, is the only gene which has already been studied with reference to Pakistani isolates (Haq, 2010). Relatedness of Pakistani isolates was investigated on the basis of 255bp of mgc2 gene. Sequence similarity among sequences was found from 95.5% to 100%. *Mgc2* gene is believed to belong to conserved cytadhesin gene family of Mycoplasmas, which share similarity with cytadhesin P30 gene of *M. pneumoniae* and P32 of *M. genitalium* (Hnatow et al., 1998). (Loolmani et al., 2014) reported genetic heterogeneity based on sequence analysis of *mgc2* gene among Iranian *M. gallisepticum* isolates from broiler breeder flocks. Findings of (Rajkumar et al., 2018), supported our present study, who described molecular typing of 13 field isolates based on *mgc2* sequence analysis and grouped isolates in 4 clusters with nucleotide homology among 4 groups ranging from 94.3%-99.2%. Isolates in described study were indistinguishable, on the basis of disease severity and clinical stage. *Mgc2* gene is the most extensively used for molecular characterization. Study by Gharaibeh et al., (2011) described Jordanian isolates classified in 2 groups, one sharing identity with *M. gallisepticum* F strain and the other group sharing 91% to 94% similarity with *M. gallisepticum* F strain based on sequence analysis of mgc2 gene, 16S-23S rRNA IGSR. Authors concluded that similarity of *M. gallisepticum* isolates from non-vaccinated birds may be due to use of *M. gallisepticum* live F vaccine which was transmitted to non-vaccinated poultry. Comparable study reported by Khalifa et al., (2014) demonstrated classification of 4 Egyptian isolates from breeder broiler flocks. Sequence analysis of *mgc2*

and IGSR grouped 3 isolates as wild type and 1 shared identical sequence similarity with *M. gallisepticum* F strain. Eissa et al., (2011) stated potential of *mgc2* gene analysis to differentiate wild type and vaccine strains. The study described molecular characterization of 5 *M. gallisepticum* strains, 4 were isolated from chickens and 1 from turkey.

Genetic diversity of Pakistani *M. gallisepticum* isolates, is unknown yet. Up to our knowledge, this is the first study reporting GTS of *M. gallisepticum* isolates.

5.10 Conclusion

Size polymorphism was observed in *pvpA* gene of PakMG1 (ARL-1963) and Pak MG3 (ARL-2668) with deletion of approximately 59-60 bp. Size polymorphism in *pvpA* gene suggests that there might be molecular diversification among local isolates. *M. gallisepticum gapA* and *lp* genes show less variability. Cytadhesin gene *mgc2*, is the only gene which has already been studied with reference to Pakistani isolates (Haq, 2010). Relatedness of Pakistani isolates was investigated on basis of 255bp of *mgc2* gene. Sequence similarity among isolates was found from 95.5% to 100%. PakMG1 (ARL-1963) and Pak MG3 (ARL-2668) isolates share deletion in *pvpA* gene with vaccine strain 6/85 (USA), reference strain S6 and PG31 and isolates from Iran (IRM13PC01), Israel (TSL-2), and USA (K5054). Although, number of isolates used for study was limited, but these isolates depict variation with each other. Pak MG1 (ARL-1963) and Pak MG3 (ARL-2668) share genetic relatedness with each other and Pak MG2 (ARL-2020) was found to be genetically distinct.

6 Development of Serum Plate Agglutination antigen using *Mycoplasma gallisepticum* Field Isolate

6.1 Introduction

Main approaches to diagnose avian mycoplasma infections are based on isolation of organism, detection of immune response and molecular detection of the organism's nucleic acid by polymerase chain reaction (PCR) (Raviv and Kleven, 2009).

Isolation of organism is laborious, so emphasis is laid on serological screening of potentially infected flock. In addition, vaccine response is also assessed by serological testing. Serological techniques used for screening includes serum plate agglutination (SPA) test and enzyme linked immunosorbent assay (ELISA). Screening tests are used to assess the probability of disease, in asymptomatic population. Such tests are not used to diagnose the disease, further confirmation by defined diagnostic tests is required (Maxim et al., 2014).

Seromonitoring data collected from the field indicated that prevalence of *M. gallisepticum* may range from 44%-76% among layers and breeding stocks in Pakistan. In broilers recorded seroprevalence is 7.14%-37.23%, in broiler breeders it is 59.6%, whereas in layers the rate is 44.9% (Mukhtar et al., 2012; Siddique et al., 2012; Khatoon et al., 2018). Moderate to high level of seroprevalence in commercial poultry has been reported in Algeria, Bangladesh, Belgium, India, Iran and Kuwait (Heleili et al., 2011; Rachida et al., 2013; Feiziet al., 2013; Ali et al., 2015; Qasem et al., 2017; Rajkumar et al., 2018)

SPA test and ELISA can detect positive antibodies in experimental infections as earlier as 10 days post infection. SPA detects significantly more positive samples than ELISA during initial infection (Kempf and Gesbertr, 1998; Atique et al., 2012). Among different serological techniques for early, rapid and reliable detection of *M. gallisepticum* infection immune response, SPA test is reported to be more sensitive and less time consuming than ELISA and Heamagglutination Inhibition assay (HI) (Hanif and Najeeb, 2007; Asif et al., 2015). In Pakistan, commercially available SPA antigens used in different laboratories

include Nobilis *M. gallisepticum* antigen S6 strain and Charles River *M. gallisepticum* antigen based on *M. gallisepticum* A5969 strain. Differences exists in sensitivity of two SPA antigens used for the detection of serological response against *M. gallisepticum* strains K503 and K730 having low virulence. Highest sensitivity was observed against homologous antigen. Whereas, among heterologous antigens S6 was more sensitive in assessment of serological response than A5969. Infection of *M. gallisepticum* strains R, F and S6 was equally well detected by using any SPA antigen e.i A5969, S6, K503 and K730 (Lin and Kleven, 1982).

Lack of sensitivity, or poor reactivity of heterologous antigen used for detection can be due to variations in surface antigen among different *M. gallisepticum* strains (Kleven et al., 1988; Markham et al., 1992; Levisohn et al., 1995). Other studies reported inability of antigens used in *M. gallisepticum* serological assay to completely detect all types of antibodies in seroconverted chickens (Noormohammadi et al., 2002).

Keeping in view the variation among *M. gallisepticum* strains, it can be expected that such property may lead to incomplete detection of its antibodies upon using imported antigens as compared to the antigen prepared from indigenous isolate during SPA testing. Also, using native isolate as SPA antigen, could offer cheap screening test for poultry farmers. To address this, SPA test antigen was to be developed from local *M. gallisepticum* isolate followed by its standardisation to detect early infection in the field.

6.1.1 Aims and Objectives

Aim of the present study was to develop a rapid serological test for detection of early infection. Following were specific objectives:

1. To develop serum plate agglutination (SPA) antigen using local field isolate of *M. gallisepticum* PakMG1 (ARL-1963)
2. To assess sensitivity and specificity of in-house SPA antigen
3. To compare diagnostic performance of in-house SPA antigen with commercially available SPA antigen

6.2 Materials and Methods

6.2.1 Preparation of *M. gallisepticum* antigen

Using field isolate, SPA test antigen was prepared by following a published protocol (Arefin et al., 2011; Rasool et al., 2017) with slight modifications. In this regard *M. gallisepticum* field isolate Pak MG1 (ARL-1963) was inoculated in 5 ml of *M. gallisepticum* broth (Oxoid-CM0403) and incubated at 37 °C for 2 to 3 days. For culture enrichment, 1.5 ml of log culture was then transferred to 5 ml of *M. gallisepticum* broth and incubated at 37 °C for 1-2 days. This culture was transferred to 90 ml of fresh *M. gallisepticum* broth (Oxoid-CM0403), incubated at 37 °C for 7 days. For inactivation of live culture, 0.5% phenol was added to *M. gallisepticum* broth, and kept for 2 hours. Inactivated culture was then centrifuged at 14000 rpm for 30 minutes. The supernatant was discarded and pellet was washed twice at 14000 rpm for 10 minutes using phosphate buffer saline (PBS). Pellet was suspended in PBS to get concentration of 100 µg/ml. To the final suspension, 1% crystal violet was added. Thiomerosal sodium (0.01%) was added as preservative. Stained antigen was vortexed thoroughly for homogenization and stored at 4°C till further use.

6.2.2 Serum plate agglutination antigen testing

In house developed SPA antigen and imported antigen were tested using serum from PCR positive cases of *M. gallisepticum*. Following OIE recommendations such agglutination tests were performed within 72 hours of serum collection. Test sera were added on the white ceramic tile in a quantity of 0.025 ml, followed by the addition of same amount of stained antigen. Antigen and test sera were mixed by sterile wooden stick, tile was swirled and results were recorded within 2 minutes at room temperature. Positive and negative controls were tested along with field sera. Agglutination is indicated by flocculation of the antigen within 2 minutes as shown in (Fig. 6.1). Same process was repeated using imported *M. gallisepticum* plate antigen (Charles River, USA) (OIE, 2018).

6.2.3 Testing Plan for Feld Samples

In house developed antigen along with imported, commercially available antigen was tested against 300 sera collected from the suspected flocks which were later confirmed by PCR during a routine flock monitoring activity taking place at 6-week interval in the flocks registered for this study, located in Islamabad region of Pakistan. Sera were first tested undiluted, positive sera were retested after being heated at 56 ℃ and diluted 1: 4 with phosphate-buffered saline pH 7. Sera that still reacted when diluted were considered positive. Sera that reacted only when undiluted were recorded as suspicious (Nascimento et al., 2005; Wanasawaeng et al., 2015). Percentage of postive and negative sera was calculated. Statistical analysis was carried out by using Fisher's exact test.

6.2.4 Sensitivity and Specificity of Antigen

In house developed antigen was assessed for its sensitivity and specificity. Sensitivity and cut-off value were evaluated by using reference *M. gallisepticum* antiserum (GD Laboratories, 11314). Aliquots of reference sera, and a panel of dilutions i.e 1:2, 1:4, and 1:8 dilutions of reference sera were used to assess sensitivity of in-house antigen. In house antigen was used to check cross reactivity against positive sera of *Salmonella pullorum* (SP), *Pasteurella multocida* (PM) and *Mycoplasma synoviae* (MS). In addition, reference sera against various respiratory viruses such as infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), Reovirus, infectious laryngotracheitis virus (ILTV) (Synbiotics Corporation, USA) were used to check cross reactivity (Wanasawaeng et al., 2015). Using the same testing protocol described for SPA assay, 15 positive sera of each bacterial and viral pathogen were used to assess cross reactivity as a determinant of specificity of antigen.

6.2.5 Shelf Life Confirmation

For shelf-life confirmation, *M. gallisepticum* reference positive sera were tested using in house antigen after 14 days interval for 4 months.

6.3 Results

6.3.1 Comparative Evaluation of In-house and Imported Antigen

Among 300 total sera, 227 (75.6%) were found positive upon testing with in-house developed antigen and 210 (70%) sera were positive when tested using, commercially available imported antigen. Positive sera were retested using both antigens after heating and dilution. After heat treatment, 6/227 (2%) and 2/210 (0.9%) sera gave negative results with the in-house antigen and imported antigen, respectively. Upon 1: 4 dilution 16/227 (5.3%) sera became negative when tested using in-house antigen and 10/210 (4.7%) appeared negative when tested using imported antigen and classified as suspicious samples. This gave an overall 68.3% (205/300) of positive samples using in-house antigen and 66% (198/300) positive samples using imported antigen as represented in (Table. 6.1). No significant difference was observed (P = 1.000) in detection of anti-*M. gallisepticum* antibodies by local and imported antigen by Fisher's exact test (Table 6.2).

6.3.2 Sensitivity and Specificity

No cross reaction was observed with sera against respiratory pathogens including *Salmonella pullorum*, *Pasteurella multocida* (PM) and *Mycoplasma synoviae*, infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), Reovirus, infectious laryngotracheitis virus (ILTV). Specificity of in-house developed antigen was assessed on basis of lack of cross reactivity. In-house developed antigen was found to be 100% sensitive and 100% specific.

6.3.3 Shelf Life

For shelf life confirmation, known positive sera of *M. gallisepticum* were tested using in house antigen after 14 days interval for 4 months. Antigen stably detected agglutinating antibodies against *M. gallisepticum* throughout the 4 months period.

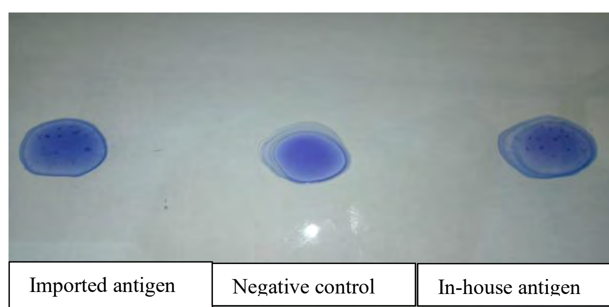


Fig. 6.1 Serum plate agglutination test using imported and in-house antigen

Table 6.1 Comparative positivity between In-house and Imported SPA antigen

Sera Samples	Total Number	In House *M. gallisepticum* Ag		
		Positive	Negative	Suspicious
Pre heating	300	227 (75.6%)	73 (24.3%)	0
Post heating and dilution	227	205 (68.3%)	06 (2%)	16 (5.3%)
		Imported *M. gallisepticum* Ag		
Pre heating	300	210 (70%)	90 (30%)	0
Post heating and dilution	210	198 (66%)	02 (0.9%)	10 (4.7%)

Table 6.2 Statistical difference between In-house and Imported antigen

Antigen	No. of Sera Samples	Post-heat treatment		P value
		Positive	Negative	
In house *M. gallisepticum* Ag	300	205	79	**1.000**
Imported *M. gallisepticum* Ag	300	198	92	

6.4 Discussion

Continuous flock monitoring is a pre-requisite for developing an effective disease response and control program. In the presence of clinical signs of infection, serological assays may provide preliminary diagnosis, which can be further confirmed by isolating the infectious agent or by its molecular detection in a sophisticated laboratory setting. One of the most common screening tests employed for sero-monitoring in Pakistan is serum plate agglutination (SPA) assay. It is rapid, cheaper than ELISA and easy to perform than HI in the absence of trained or skilled labour. Here detection of early seroconversion is achievable by testing the presence of antibody of IgM subtype using SPA, which is detectable only at the early stage of *M. gallisepticum* infection. On the other hand, for more specific detection of *M. gallisepticum* infection, the detection of IgG antibodies has been recommended by using an ELISA or HI based tests (Ahmad et al., 2008; Atique et al., 2012; Rasool et al., 2017). However, lack of specificity can be a matter of concern in conducting a reliable SPA assay, which would require the inclusion of multiple controls while setting up an SPA assay.
In the present study, conventional method of isolation of *M. gallisepticum* was used, coupled with molecular detection. Field isolate of *M. gallisepticum* was used to develop SPA in-house antigen as recommended by (OIE, 2018). Development of local antigen using field isolates is already reported for *M. gallisepticum* in different countries and for *M. synoviae* in Pakistan (Rasool et al., 2017).

To be used for diagnostic purpose, optimization of locally developed antigen was done by assessing its sensitivity, specificity and shelf life as recomended by (OIE, 2018). Sera for antigen testing were collected from the flocks located in Islamabad region of Pakistan, initially suspected of *M. gallisepticum* infection and later confirmed by PCR. The present study indicates that no significant difference was found between sensitivity and specificity of locally developed antigen and imported *M. gallisepticum* antigen from Charles River (USA) with comparable detection rate of 68.3% and 66%, respectively. Non-specific agglutination reactions present in the sera can limit the specificity of a assay. These can be either due to cross reacting proteins belonging to some other pathogens in sera or found in

birds using of inactivated vaccines. To avoid some of these false positive results, it is recommended to pre-heat the sera prior to test. Alternatively, non-specific reactions can be avoided by diluting test sera as 1:4 to 1:8. This is used to differentiate specific and nonspecific reactions in the plate agglutination tests (Ley 2008; Rasool et al., 2017).

Variation in sensitivity and specificity of antigen may lead to false positive results (Kleven et al., 1988). Sensitivity and specificity of immunological assays depends largely on the strain infecting the flock and the strain used as an antigen to detect related antibodies. In this regard, earlier studies have reported lack of sensitivity of *M. gallisepticum* A5969 strain used as antigen in HI and SPA. Similarly, variation in antigenic profile was evident in detection of antibody response after vaccination with *M. gallisepticum* ts-11 strain. It was, therefore, recommended to improve sensitivity of serodiagnosis by using homologous antigen instead of heterologous antigen (Ley, 2008).

For evaluating the cross reactivity of *M. gallisepticum* antigen, standard antisera of *Salmonella pullorum*, *Pasteurella multocida*, *Mycoplasma synoviae*, and infectious bronchitis virus (IBV), has been previously reported (Wanasawaeng et al., 2015). Upon following the same pattern of testing, using the newly prepared *M. gallisepticum* antigen under this study, no cross reactivity with the listed antisera of bacterial and viral pathogens was observed in this study. In addition to this, the developed antigen was repeatedly assessed for determining its shelf life. This in-house prepared antigen was found to be compatible to the imported antigen for upto 4 months testing period. Optimization of SPA using local antigen provided an opportunity to replace imported antigen for early screening of *M. gallisepticum* infections in local flocks. Similar studies have already been reported elsewhere (Arefin et al., 2011; Rasool et al., 2017).

6.5 Conclusion:

In conclusion, development and standardization of SPA antigen using local field isolate of *M. gallisepticum* may provide an opportunity to replace imported antigen, and facilitate in the provision of cheap antigen for initial screening of *M. gallisepticum* infection in poultry flocks. Furthermore, the closer compatibility of such antigen prepared from locally

prevalent strain of *M. gallisepticum* would result in offering low probability of giving false negative reactions in SPA assay.

7 Comparative Evaluation of Different PCR based Diagnostic Techniques for Detection of *Mycoplasma gallisepticum*

7.1 Introduction

M. gallisepticum infections cause high morbidity, when complicated by secondary pathogens. In the absence of secondary infections, *M. gallisepticum* infections may be asymptomatic and can be overlooked. In addition, initial signs of respiratory infections could not be differentiated from other bacterial and viral respiratory infections. To reduce *M. gallisepticum* outbreaks and to maintain biosecurity, routine monitoring of poultry farms is recommended. Detection of *M. gallisepticum* is most successful during acute phase, when high number of organisms are present in trachea (Gaunson et al., 2006). Early detection of *M. gallisepticum* infections and differentiation from other respiratory pathogens could aid in designing and implementing therapeutic or preventive approaches to maintain disease free flock.

World organization of animal health (OIE) recommended isolation, serology and polymerase chain reaction (PCR) for diagnosis of *M. gallisepticum* infections in poultry. PCR provided a sensitive, and rapid method of *M. gallisepticum* detection, as to culture the organism need specialized media reagents and is time consuming (Nascimento et al., 1991; Nascimento et al., 1993; Kleven 2008). Isolation of *M. gallisepticum* may also be compromised in case of complicated infections, due to presence of competing microorganisms, overgrowth of non-pathogenic mycoplasma spp. and low infectious dose of organism under investigation (Ley, 2008; Kleven 2008). Real time PCR proved to be more sensitive, with higher detection limit than conventional PCR (con PCR). However, it needs specialized laboratory setup and trained manpower to conduct the test. To facilitate in-time and rapid detection of *M. gallisepticum*, a sensitive, specific and user friendly molecular diagnostic assay is need of the hour. In developing countries like Pakistan, benefit of poultry farmer lies in early and cheap detection of pathogen so that economic losses due to disease outbreaks, medication costs and production losses can be minimized by adopting appropriate and in time measures.

Use of insulated isothermal PCR (iiPCR) for detection of various human and animal pathogens has been reported. The assay is based on Rayleigh-Bernard convention (Krishnan et al., 2002), which operates on the principle of heat transfer due to movement of fluids. POCKIT^{TM} Nucleic Acid analyzer by (POCKIT^{TM}; GeneReach, Taichung, Taiwan), claimed to provide a rapid and sensitive procedure for diagnostic of different pathogens (Tsai et al., 2012). Application of iiPCR using POCKIT^{TM} Nucleic Acid analyzer could provide sensitive and rapid detection of *M. gallisepticum* from poultry in Pakistan. For this purpose, the analytical and diagnostic performance criteria of iiPCR (POCKIT Central Nucleic Acid Analyzer and POCKIT Micro Plus Nucleic Acid Analyzer) was evaluated and comparison between different PCR based diagnostic techniques was made.

7.1.1 Aims and Objectives

The aim of present study was to validate iiPCR as reliable technique for detection of *M. gallisepticum* from field samples. Following are the specific objectives of study:

1. To evaluate analytical sensitivity and specificity of iiPCR for detection of *M. gallisepticum*
2. To determine diagnostic sensitivity of iiPCR for detection of *M. gallisepticum*
3. To compare conventional PCR, real time PCR and iiPCR for detection of *M. gallisepticum*

7.2 Materials and Methods

7.2.1 Real-time / Quantitative Polymerase Chain Reaction (Q-PCR)

To evaluate analytical and diagnostic performance of iiPCR, real-time PCR (qPCR) was performed as a standard assay. *M. gallisepticum* F (live vaccine) strain was used as a positive standard along with three laboratory isolates including Pak MG1 (ARL-1963), Pak MG2 (ARL-2020), and Pak MG3 (ARL-2668). Each sample was optimised at concentration range of 10^0–10^7 CFU/ml by using 10-fold dilutions of *M. gallisepticum* broth. DNA extraction was carried out as mentioned in (section 3.4.1), and qPCR was done

as mentioned below. In addition, 95 clinical samples, already tested by conventional PCR (con-PCR) were subjected to qPCR. Reactions with a threshold cycle (Ct) number value 35 were considered positive.

The reaction was performed using Invitrogen Super ScriptTm One step QRT-PCR kit following manufacturer's protocol. All the components and reagents were vortexed gently after thawing. Reaction was set up in 96 well PCR plate, placed the plate on ice stand and components of reaction mixture were added for 50 ul reaction each (Table 7.1 & 7.2). The 96-well plate was vortexed again to make the mixture homogenous. The qPCR was performed in a thermal-cycler (ABI 7500 Real time PCR Plus) using the temperature parameters as mentioned in (Table 7.3) provided by the manufacturer.

7.2.2 Validation of Insulated Isothermal Polymerase Chain Reaction (iiPCR)

For validation of iiPCR, POCKIT Central Nucleic Acid Analyzer and POCKIT Micro Plus Nucleic Acid Analyzer both with automatic nucleic acid extraction procedures were appraised for detection of *M. gallisepticum*. Analytical performance criteria of assay, comprising of analytical sensitivity (ASe) and analytical specificity (ASp) were estimated. Diagnostic performance of assay was evaluated by comparison with *M. gallisepticum* qPCR using clinical samples.

7.2.2.1 Insulated Isothermal PCR (iiPCR) using Micro Plus Nucleic Acid Analyzer (Field deployable POCKIT^{TM} device)

Test samples were transferred to the lysis buffer vial. Vials with lysis buffer and sample were capped and mixed by vigorous shaking for 30 times. Drop-n-Go cassette provided with the kit was unscrewed, to load the sample. A single sample was loaded to each cassette. 60 µl of sample (pre-treated with lysis buffer) was transferred to the sample well with the help of V-dropper. Fibre disc was gently pressed and sample was incubated for 5-10 secs. Two drops of wash buffer were added to the sample well and incubated for 10 mins to air dry the fibre disc. Fibre disc was transferred from sample well to elution buffer vial and mixed 30 times. Nucleic acid extract of sample was used to reconstitute the premix

tube. Constituted premix was transferred to respective R-tube and tube was capped tightly. R-tubes for each sample were placed in POCKIT^{TM} device and "RUN" button was pressed. The Micro Plus Nucleic Acid Analyzer was run on a default program set by the manufacturer, with a total run time of 45 min (Fig. 7.1).

7.2.2.2 Insulated Isothermal PCR (iiPCR) using POCKIT Central Nucleic Acid Analyzer

For each sample, 1 transfer cartridge and 1 extraction cartridge was prepared. Transfer cartridge was labelled with the sample ID, and premix vial was added to the well no. 3 of the respective cartridge. Extraction cartridge was labelled with sample ID, and 200µl sample was added to the first well of cartridge (Fig. 7.2 & 7.3). Both cartridges were loaded to the POCKIT Central Nucleic Acid Analyzer, and the program was initiated (Fig. 7.4). The sequence information of the iiPCR primers and probes, and the components of the assay were proprietary to GeneReach Taiwan.

7.2.2.3 Analytical Performance of iiPCR

Analytical Specificity (ASp) of iiPCR using POCKIT Central Nucleic Acid Analyzer and POCKIT Micro Plus Nucleic Acid Analyzer was assessed by testing live vaccine of *M. gallisepticum* F strain as reference, laboratory isolates Pak MG1 (ARL-1963), Pak MG2 (ARL-2020), and Pak MG3 (ARL-2668), along with reference antigens of other poultry pathogens MS, AIV H9N2, NDV and SP. Analytical Sensitivity (ASe) and limit of detection of iiPCR by Micro Plus Nucleic Acid Analyzer and POCKIT Central Nucleic Acid Analyzer was assessed by using a range of 10-fold diluted concentrations in *M. gallisepticum* broth (10^0–10^7 CFU/ml) of *M. gallisepticum* F (live vaccine), Pak MG1 (ARL-1963), Pak MG2 (ARL-2020), and Pak MG3 (ARL-2668). Each sample/dilution was tested by iiPCR and qPCR, and con-PCR in triplicates.

7.2.2.4 Diagnostic Performance of iiPCR

Diagnostic performance of iiPCR to detect *M. gallisepticum* in clinical samples was determined by comparing it with qPCR as reference. In total 95 clinical samples, randomly

selected from con-PCR positive and negative samples were used in the study. Test samples were used for detection of *M. gallisepticum* by both qPCR and iiPCR (POCKIT^{TM} Micro Plus Nucleic Acid Analyzer and POCKIT Central Nucleic Acid Analyzer) by following the protocol mentioned above.

7.2.3 Statistical Analysis

Percentage agreement between different PCR based techniques was calculated and statistical analysis was done to assess reliability by using Cohen's kappa (Carossino et al., 2016).

Table 7.1 Real time-PCR Reaction mixture profile using Invitrogen SuperScriptTm One step qRT-PCR kit

Components	Volume/50µl	Final Concentraton
Reaction Mix (2X)	25µl	1X
Template DNA	2 µl	10pg-1
Sense primer (10µM)	1 µl	0.2µM
Anti-sense Primer (10µM)	1 µl	0.2µM
Platinum TaqMix	1 µl	
Autoclaved distilled water	10 µl	1X
Total	50 µl	

Table 7.2 Real time-PCR Primers and Probes used for the detection of *M. gallisepticum*

Reagent name	Sequence	Company/code
Probe	5’-(6-FAM)-GTTGCCAAACG (Tamra-Q)-3’	Operon
Forward Primer	5’-CCTAGCCACTATTATATG TGGG-3’	Operon
Reverse Primer	5’-CTGGATGYTGACCTTATG TAGCAG-3’	Operon

Table 7.3 Real time-PCR profile using Invitrogen SuperScriptTm One step qRT-PCR kit

Steps of q-pcr	Temperatures /time	Cycles
Initial Denaturation	94 ºC /02min	X1
Denaturation	95 ºC /15 sec	X40
Annealing	58 ºC /1 min	X1

Fig 7.1 POCKIT Micro Plus Nucleic Acid Analyzer

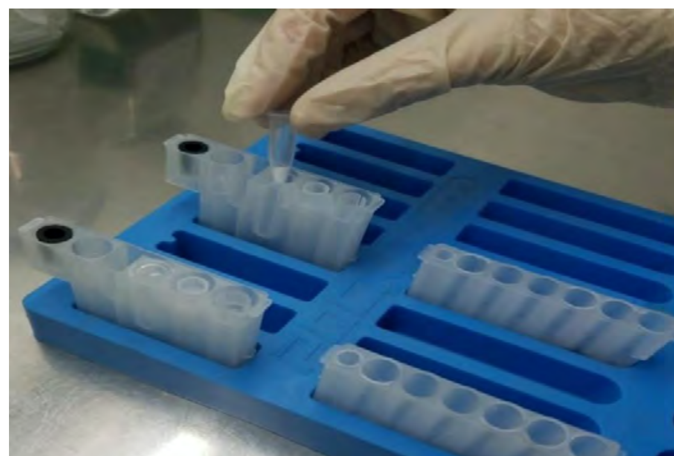


Fig 7.2 Addition of Premix vial to the third well of transfer cartridge

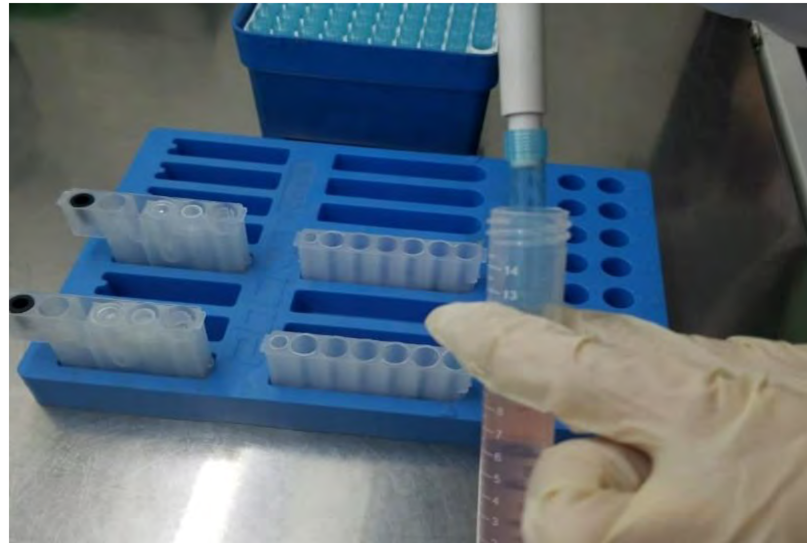


Fig. 7.3 Sample loading to the extraction cartridge

Fig 7.4 Cartridge loading to the POCKIT Central Nucleic Acid Analyzer

7.3 Results

7.3.1 Real Time/Quantitative PCR for detection of *M. gallisepticum*

Quantitative PCR of *M. gallisepticum* F strain, and three field isolates i.e Pak MG1 (ARL-1963), Pak MG2 (ARL-2020), and Pak MG3 (ARL-2668) was carried out. Recorded threshold cycle value for $1x10^7$ CFU/ml, $1x10^5$ CFU/ml, $1x10^5$ CFU/ml was in range of 24.50-25.55, for $1x10^4$ CFU/ml it was 26.95 to 28.65, for $1x10^3$ CFU/ml it was 31.05 to 32.50, for $1x10^2$ CFU/ml it was 35.05 to 35.45 (Table 7.4).

Among 95 clinical samples subjected to qPCR, 70 were already declared positive and 25 were declared negative by con-PCR. However, qPCR revealed 72 positive and 23 negative samples with Ct ranging from 18-35. Samples showing Ct value greater than 35 were considered negative, based on this cut-off value 23 samples were negative.

7.3.2 Analytical sensitivity (Ase) of iiPCR for detection of *M. gallisepticum*

Analytical sensitivity (Ase) or limit of detection of iiPCR was established by using concentrations of *M. gallisepticum* F strain, Pak MG1 (ARL-1963), Pak MG2 (ARL-2020), and Pak MG3 (ARL-2668) corresponding to CFU ranging from 1×10^7/ml to 10^0/ml. Minimum concentration giving 100% positive results by POCKIT Central Nucleic Acid Analyzer and POCKIT Micro Plus Nucleic Acid Analyzer was found to be $1x10^3$ CFU/ml. Lack of uniformity in test results was found at $1x10^2$ CFU/ml with similar trend being recorded by qPCR as shown in (Table 7.4). Comparison of con-PCR using 16S rRNA primers (OIE, 2018) and iiPCR in terms of sensitivity revealed con-PCR using showed 100% detection with pure culture, 10^{-1}, 10^{-2}, 10^{-3}, 33.33%-66% detection was recorded with 10^{-4} and 10^{-5} was negative (Fig. 7.5).

7.3.3 Analytical specificity (Asp) of iiPCR for detection of *M. gallisepticum*:

For detection of *M. gallisepticum*, iiPCR was found to be very specific. No cross reactivity was recorded with other respiratory pathogens while testing for *M. gallisepticum* on POCKIT Central Nucleic Acid Analyzer and POCKIT Micro Plus Nucleic Acid Analyzer. Results were negative for *M. synoviae* (MS), New Castle disease virus (NDV), Avian

influenza virus (AIV H9N2) and *Salmonella pullorum* (SP) which were used as negative control. On the other hand, *M. gallisepticum* F strain, Pak MG1 (ARL-1963), Pak MG2 (ARL-2020), and Pak MG3 (ARL-2668) were positive for *M. gallisepticum* iiPCR by both analyzers. Analytical specificity of iiPCR using both analyzers was found to be 100%.

7.3.4 Diagnostic performance of iiPCR for detection of *M. gallisepticum*:

A panel of clinical samples containing positive and negative samples already tested by con-PCR and by qPCR, was used to assess diagnostic performance of iiPCR using POCKIT Central Nucleic Acid Analyzer and POCKIT Micro Plus Nucleic Acid Analyzer for detection of *M. gallisepticum*. For this purpose, DNA extraction of designated samples was carried out (section 3.4) and qPCR was done as mentioned. Out of total 95 tested samples by qPCR, 72 were positive with Ct ranging from 18-35 and 23 were negative. Results of ii PCR using POCKIT Central Nucleic Acid Analyzer showed 71 positive and 22 negative samples with one false positive and one false negative sample (Table 7.6). Same samples were tested by iiPCR Micro Plus Nucleic Acid Analyzer, results showed 70 samples were positive, and 21 were negative for *M. gallisepticum* with one false positive and 02 false negative results (Table 7.7). Statistical analysis revealed almost perfect reliability of both techniques for detection of *M. gallisepticum* from clinical samples with percentage agreement of 95% -97% (Table 7.8)

Table7.4 Comparison of analytical sensitivity of iiPCR with qPCR and conventional PCR

	MG CFU/ml	Micro plus Nucleic acid Analyzer			POCKIT Central Nucleic acid Analyzer			Q-PCR C_T			Con-PCR
***M. gallisepticum* F strain**	1×10^7	+	+	+	+	+	+	18.50	19.05	18.28	3/3 +ve
	1×10^6	+	+	+	+	+	+	22.03	22.38	22.50	3/3 +ve
	1×10^5	+	+	+	+	+	+	24.88	25.05	25.50	3/3 +ve
	1×10^4	+	+	+	+	+	+	26.95	27.50	27.20	3/3 +ve
	1×10^3	+	+	+	+	+	+	31.05	31.20	32.50	2/3 +ve
	1×10^2	-	-	+	-	+	+	Negative	35.52	35.09	-ve
	10	-	-	-	-	-	-	Negative	Negative	Negative	
Pak MG1 (ARL-1963)	1×10^7	+	+	+	+	+	+	19.06	19.30	19.28	3/3 +ve
	1×10^6	+	+	+	+	+	+	22.29	22.14	22.02	3/3 +ve
	1×10^5	+	+	+	+	+	+	24.68	25.25	24.90	3/3 +ve
	1×10^4	+	+	+	+	+	+	27.22	26.80	26.95	3/3 +ve
	1×10^3	+	+	+	+	+	+	31.15	31.20	31.55	1/3 +ve
	1×10^2	-	+	-	-	+	-	Negative	35.35	Negative	-ve
	10	-	-	-	-	-	-	Negative	Negative	Negative	
Pak MG2 (ARL-2020)	1×10^7	+	+	+	+	+	+	19.20	19.50	19.35	3/3 +ve
	1×10^6	+	+	+	+	+	+	22.08	23.05	22.35	3/3 +ve
	1×10^5	+	+	+	+	+	+	25.55	25.25	25.50	3/3 +ve
	1×10^4	+	+	+	+	+	+	27.30	27.55	28.25	3/3 +ve
	1×10^3	+	+	+	+	+	+	31.45	32.20	32.50	1/3 +ve
	1×10^2	+	-	-	-	+	-	Negative	35.45	Negative	-ve
	10	-	-	-	-	-	-	Negative	Negative	Negative	
Pak MG3 (ARL-2668)	1×10^7	+	+	+	+	+	+	19.16	19.05	19.30	3/3 +ve
	1×10^6	+	+	+	+	+	+	22.35	22.07	22.15	3/3 +ve
	1×10^5	+	+	+	+	+	+	24.50	24.75	25.15	3/3 +ve
	1×10^4	+	+	+	+	+	+	27.92	27.80	28.65	3/3 +ve
	1×10^3	+	+	+	+	+	+	32.30	31.45	32.10	2/3 +ve
	1×10^2	-	+	-	+	+	-	37.95	38.05	Negative	-ve
	10	-	-	-	-	-	-	Negative	Negative	Negative	

Table 7.5 Contingency table for the comparison of qPCR and conventional PCR assay for the detection of *M. gallisepticum*

		Q-PCR		
		Positive	Negative	Total
Con PCR	Positive	70	0	70
	Negative	02	23	25
	Total	72	23	95

Table 7.6 Contingency table for the comparison of qPCR and iiPCR using POCKIT Central Nucleic Acid Analyzer for the detection of *M. gallisepticum*

		Q-PCR		
		Positive	Negative	Total
iiPCR (POCKIT Central Analyzer)	Positive	71	01	72
	Negative	01	22	23
	Total	72	23	95

Table 7.7 Contingency table for the comparison of qPCR and iiPCR using Micro Plus Nucleic Acid Analyzer for detection of *M. gallisepticum*

		Q-PCR		
		Positive	Negative	Total
iiPCR (Micro Plus Analyzer)	Positive	71	01	72
	Negative	01	22	23
	Total	72	23	95

Table 7.8 Comparison of reliability of different PCR based diagnostic techniques for detection of *M. gallisepticum*

Tests in Comparison	**Test by Test agreement**		
	Percentage of agreement	**Kappa**	**Agreement**
Q-PCR vs Con-PCR	97.89%	0.94	Almost perfect
Q-PCR vs iiPCR POCKIT Central Analyzer	97.89%	0.94	Almost perfect
Q-PCR vs Micro Plus Analyzer	95.7%	0.88	Almost perfect

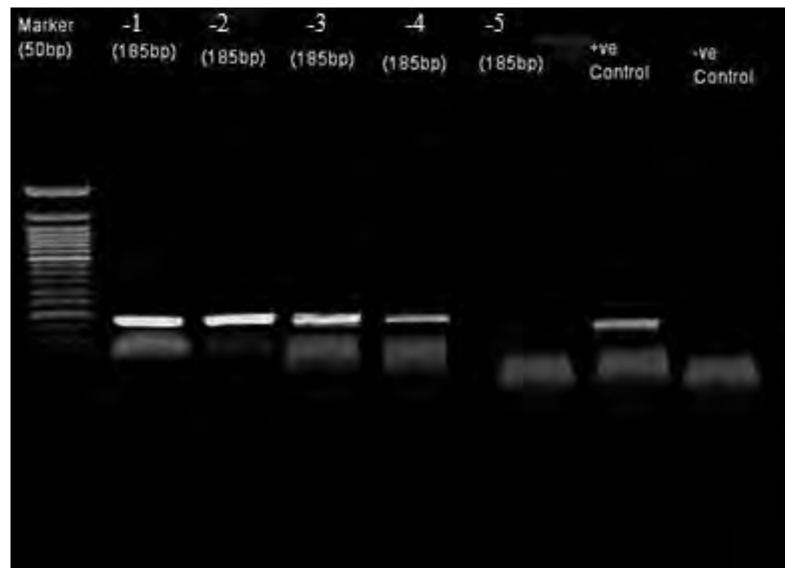


Fig. 7.5 Agarose gel electrophoresis of *M. gallisepticum* PCR products of serial dilutions of reference strain

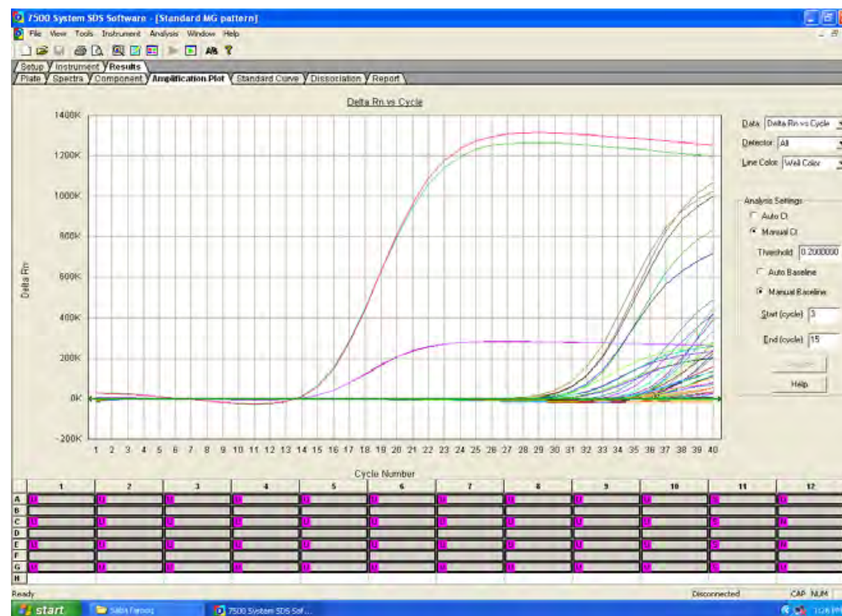


Fig. 7.6 Standard pattern of real time PCR for detection of *M. gallisepticum*

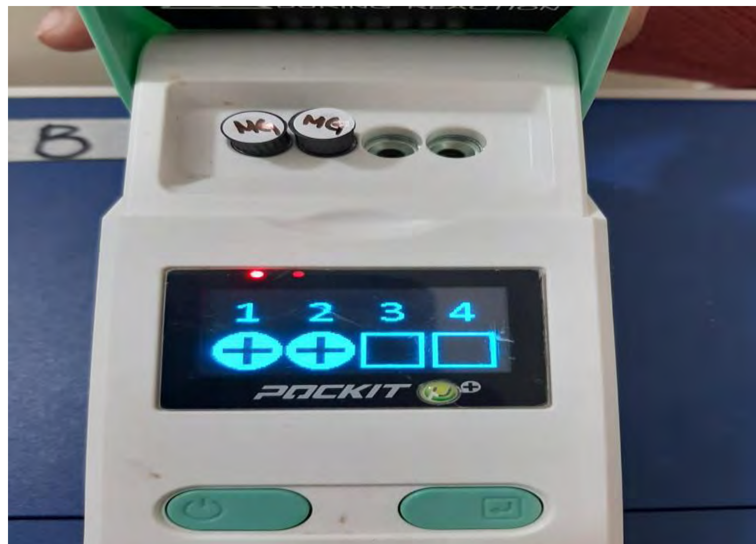


Fig 7.7 Presentation of results on POCKIT Micro Plus Nucleic Acid Analyzer

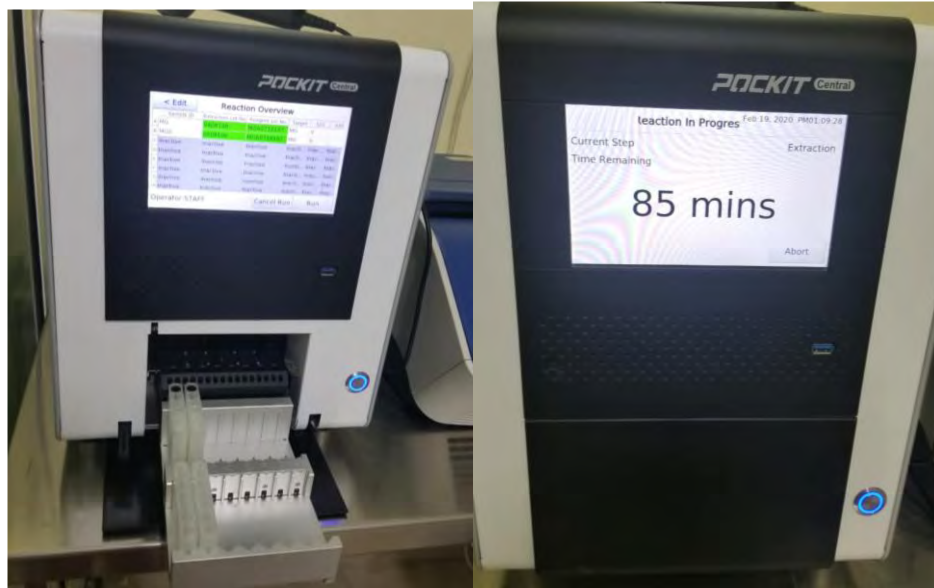


Fig. 7.8 POCKIT Central Nucleic Acid Analyzer

Fig. 7.9 Presentation of results on POCKIT Central Nucleic Acid Analyzer

7.4 Discussion

Molecular diagnostic techniques are rapidly evolving. A diagnostic assay after development, optimization and standardization is validated to ensure its fitness for intended purpose. Validation is based on determination of analytical and diagnostic performance of the said assay. In the present study, iiPCR test was assessed for its analytical sensitivity and specificity as well as diagnostic sensitivity and specificity for testing *M. gallisepticum* antigen. iiPCR was developed based on the principle of convective heat transfer. Heat convection is a phenomenon of heat transfer due to motion of fluid. After initial development of iiPCR based on Rayleigh-Benard convection, by Krishnan et al., (2002) the technique has undergone a number of user-friendly modifications during last couple of decades. In its present form, it is now available as all-in-one version with minimum run time of 45 mins for 4 to 8 samples as a field portable device i.e. Micro Plus Nucleic Acid Analyzer as well as in the form of laboratory diagnostic device i.e. POCKIT Central Nucleic Acid Analyzer. Incorporation of Taqman probe and optical detection module has further improved target specific detection (Tsai et al 2012 (b), Tsen et al., 2013). *M. gallisepticum* specific iiPCR assay has shown to have first-rate analytical sensitivity and specificity. Analytical specificity of the assay was found to be 100% as no cross reaction was observed with reference antigens of various pathogens such as AIV H9N2, NDV, MS and SP, along with 100% detection of a *M. gallisepticum* F live vaccine strain and three laboratory isolates of *M. gallisepticum* i.e Pak MG1 (ARL-1963), Pak MG2 (ARL-2020), and Pak MG3 (ARL-2668). Analytical sensitivity and limit of detection of *M. gallisepticum* iiPCR was found to be 1×10^3 CFU/ml, which is comparable to *M. gallisepticum* qPCR and far better than conventional PCR assay conducted for routine diagnosis. In order to assess reliability of iiPCR to produce intended results, a comparative study was conducted with qPCR as a standard molecular method for the detection of *M. gallisepticum*. Using real time PCR (qPCR) as reference standard technique, diagnostic performance of different techniques was evaluated and compared. Comparison of reliability of different PCR based diagnostic techniques revealed 97.89% (Cohen’s Kappa value, 0.94) agreement between qPCR and conventional PCR, also between qPCR and iiPCR POCKIT Central Analyser. qPCR and POCKIT Micro Plus Nucleic Acid Analyser shared 95.7% (Cohen’s Kappa

value, 0.88) agreement. Statistical analysis to assess reliability, of iiPCR in comparison to qPCR elucidated compatibility of both techniques for use in diagnosis of *M. gallisepticum*. The samples showing false positive results were processed tissue samples, since iiPCR assay is based on direct detection from sample, tissue exudates can be the reason of hinderance in appropriate detection. Our study is supported by already published work of (Kuo et al., 2016). The study reported 97.8% agreement of iiPCR with real time PCR for detection of *M. synoviae* infection in poultry by using filed deployable POCKIT^{TM} device for timely detection on suspected farm. Insulated isothermal PCR has found wide application in prompt detection of a number of bacterial and viral pathogens for effective and swift control of infections. Reported studies included application of iiPCR in diagnosis of all serotypes of dengue virus, rotavirus (Soltan et al., 2016), equine arteritis virus (Carossino et al., 2016) having an agreement limit of more than 90% with the standard assays. (Yin et al., 2019) has reported practical application of POCKIT^{TM} device in detection of *Staphylococcus aureus* in processed food. In addition to molecular detection of early infection, post treatment monitoring of infected flock is of prime importance in regulation of therapeutic treatment. Sensitive detection limit of iiPCR make it useful for evaluation of effectiveness of therapeutic measures. The study described iiPCR as a relatively cheap and sensitive alternative of qPCR for the detection of *M. gallisepticum* from field samples.

7.5 Conclusion

Insulated isothermal PCR (iiPCR) using Micro Plus Nucleic Acid Analyzer and POCKIT Central Nucleic Acid Analyzer showed same results and both were good alternate of qPCR for detection of *M. gallisepticum*. The result showed a high sensitivity 90.9% and 100% of Micro Plus Nucleic Acid Analyzer and POCKIT Central Nucleic Acid Analyzer keeping, qPCR as gold standard. Detection limit of iiPCR was found to be comparable with qPCR. No non-specific detections were observed.

8 Evaluation of *Mycoplasma gallisepticum* Vaccine Efficacy

Development of post-vaccination baseline against *M. gallisepticum* in vaccinated birds

8.1 Introduction

Maintenance of *M. gallisepticum* free flock is of foremost importance for poultry producers. Approaches commonly adopted to manage such infections are based on efforts to curtail exposure to the pathogen, reduce economic losses by medication and to immunize the flock where risk of potential exposure cannot be minimized (Umar et al., 2017). Maintenance of mycoplasma free flocks can be achieved by intense biosecurity measures. However, increase in poultry concentration in limited geographic area increased the risk of exposure. Environmental survival and formation of biofilms by *M. gallisepticum* and *M. synoviae* for extended period of time also enhances risk of indirect exposure. Surveillance and constant monitoring is vital for control practices, and for early detection of exposure in a flock. Appropriate management of exposed flock reduces risk of horizontal as well as vertical transmission (Stipkovits & Kempf, 1996; Kleven 1997; Kleven 2008, OIE, 2018).

In developed countries, poultry improvement programs oversee *M. gallisepticum* control in commercial poultry. These programs include National Poultry Improvement Plan in the USA (Kleven 2008), National Sanitary and Hygienic Control Programme in France (Stipkovits & Kempf, 1996), and the National Avian Sanitary Program in Brazil (Villa, 1998). In spite of all efforts, *M. gallisepticum* outbreaks continue to occur in different countries.

Use of live and killed vaccine to control *M. gallisepticum* infections, is in practice in different countries world-wide. Approved live vaccines F strain vaccine with commercial name of Poulvac Myco F, by FMG (Fort Dodge Animal Health) and AviPro MG F (Lohmann Animal Health Int.), an Australian temperature sensitive strain ts-11 (Merial-Select, Gainesville, GA) and 6/85 strain (Merck Animal Health, Millsboro, DE). Killed vaccine (bacterin) is based on *M. gallisepticum* F strain. In Pakistan, *M. gallisepticum* F

strain killed vaccine is used. Despite vaccination *M. gallisepticum,* outbreaks and infections have been reported in poultry. Use of killed/inactivated vaccines do not provide consistent protection against *M. gallisepticum* infections (Abd-el-Motelib & Kleven, 1993). Due to economic constrains, Pakistani poultry farmer cannot adopt culling and slaughtering of *M. gallisepticum* infected flock as done in USA and European countries, to maintain disease free flocks. Furthermore, to reduce production losses irrational use of anti-mycoplasma antibiotics is common in local poultry. Excessive use of antibiotics in poultry to control *M. gallisepticum* infections could lead to development of antimicrobial resistance (AMR) in animals and humans. A comparative study to evaluate post vaccination baseline antibody titers of *M. gallisepticum* vaccines (live and killed) was designed. Using, vaccination titers as reference, different breeder farms vaccinated for *M. gallisepticum* were studied.

8.1.1 Aims and Objectives

The aim of the study was to determine the efficacy of *M. gallisepticum* vaccine used by local farmers to prevent infection. Following were specific objectives of study:

1. To develop post vaccination baseline using *M. gallisepticum* killed vaccine
2. To develop post vaccination baseline using *M. gallisepticum* live vaccine
3. To evaluate exposure antibody titers in poultry farms located in Islamabad Capital Territory (ICT)
4. To evaluate exposure antibody titers in poultry farms located in Khyber Pakhtunkhwa (KPK) province
5. To evaluate exposure antibody titers in poultry farms located in Punjab province

8.2 Materials and Methods

8.2.1 Post Vaccination Base Line against *M. gallisepticum* Live and Killed Vaccine

8.2.1.1 Live Vaccine Trial

Total 40-day old layer pullets were obtained from local hatchery and placed at animal house facility at National Reference Laboratory for Poultry Diseases (NRLPD), NARC, Islamabad. To assess maternal antibody status, 08 birds were randomly bled and tested by indirect-ELISA (Idexx) (mentioned in section 3.3.2). Imported live vaccine strain F (F vax-MG by Intervet) was used for vaccination of 30 experimental birds at the age of 9 week as recommended. Control group was unvaccinated and comprised of 10 birds. Vaccine was administered via intranasal route (10^6 CFU /dose) 0.2ml/ bird. Both groups were placed in separate chicken isolator chambers. Birds from experimental and control groups were bled at recommended interval of 10 to 15 days, till 32nd week of age (woa). Serology was done to detect the level of antibody by indirect *M. gallisepticum* ELISA using commercially available Idexx i-ELISA kit (section 3.3.2).

8.2.1.2 Killed Vaccine Trial

Total 40-day old layer pullets were obtained from local hatchery and kept at animal house facility at NRLPD. To assess maternal antibody status, 08 birds were randomly bled and tested by ELISA. Imported killed vaccine (MG-Bac by Zoites) was used for vaccination of 30 experimental birds at the age of 5 to 6 woa. Vaccine was administered in a recommended dose of 0.5ml/ bird subcutaneously in the lower neck region. Second shot of vaccine was administered at 16th woa. Control group was unvaccinated and comprised of 10 birds. Both groups were placed in separate chicken isolator chambers. Birds from experimental and control groups were bled at recommended interval of 10-15 days till 42nd woa. Serology was done to detect the level of antibody using commercially available Idexx i-ELISA kit (section 3.3.2).

8.2.2 Exposure Status in Vaccinated Flocks

A prospective longitudinal study was carried out from February 2017 to April 2019, on breeder farms located in Islamabad Capital territory (ICT), Punjab and Khyber Pakhtunkhwa (KPK). The study was designed to monitor designated farms and flocks for post vaccination exposure to *M. gallisepticum* infection. Minimum two different farms from each region were included in the study. To maintain confidentiality, each farm and

flock was given a unique code. Flocks of each farm were vaccinated for *M. gallisepticum* at 6-8 woa and second shot was given at 16 woa. Depending on flock density, sample from 10% of the birds were collected as scheduled and received at Averose Laboratories, Rawalpindi. Samples included sera samples for serological study and swab samples for PCR. Sera samples were subjected to *M. gallisepticum* i-ELISA (section 3.3.2) and swab samples were investigated by PCR (section 3.4). From ICT, 02 different farms were selected for study and coded as IFH and IFT. A single flock was studied from IFH and 02 flocks from IFT were studied in different time durations as shown in (Table. 8.1). From KPK, 02 farms KFB and KFQ were selected, 02 flocks from former and 01 from later was included in study. From Punjab, 02 selected farms were PFK1 and PFK2, single flock from each farm was included in study.

Table 8.1 Farms and flock included in study

Region	Time period	Farm Id	Flock Id	Age of Flock (Week)
Islamabad	Feb 2017- Oct 2017	IFH	IFH1.1	14-47
	May 2017-Mar 2018	IFT	IFT2.1	09-44
	Jun 2018-Mar 2019	IFT	IFT2.2	07-51
KPK	Jun 2018-Apr 2019	KFB	KFB1.1	08-51
	Aug 2018-Apr 2019	KFB	KFB1.2	14-52
	Sep 2018-Apr 2019	KFQ	KFQ1.1	14-47
Punjab	Oct 2017-Sep 2018	PFK1	PFK1.1	16-58
	Apr 2018-Apr 2019	PFK2	PFK2.1	05-57

Fig. 8.1 Vaccinated birds kept in chicken isolator chamber

Table 8.2 Post vaccination seroconversion against *M. gallisepticum* live vaccine

Age (week)	1	9	11	12	14	16	18	21	25	28	32
Min titer	1	277	401	188	2306	2114	2549	1725	1050	1423	927
Max titer	307	6783	5976	2909	4723	10690	12190	10424	8559	6674	5486
GMT	17	1203	2451	1004	3685	4084	7408	4329	3751	2776	3212
CV	82	100.5	58.9	73.2	22.1	63.3%	37.7	53.5	53.8	66.3	74.3

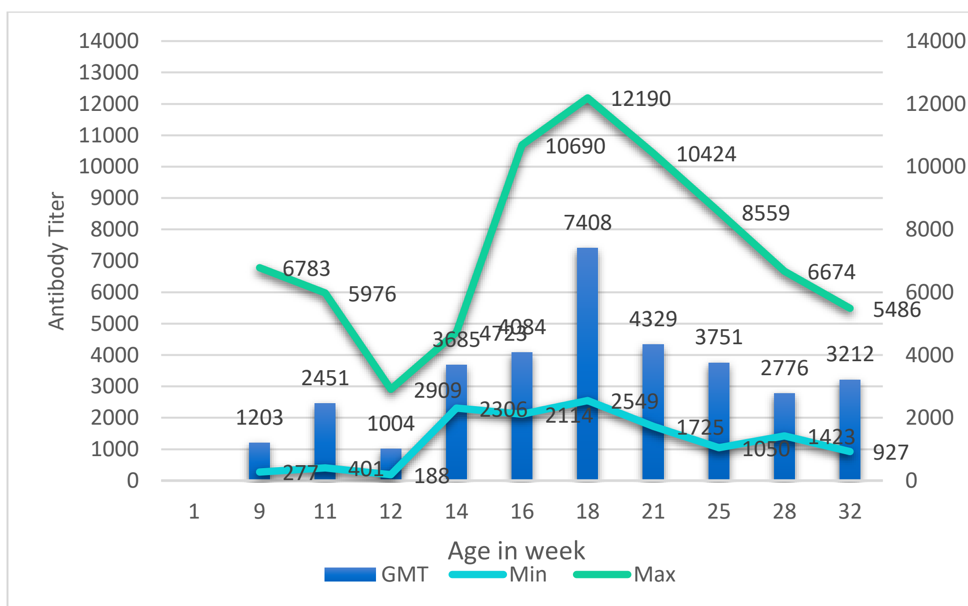


Fig. 8.2 Post vaccination antibody titer variation (live vaccine)

Table 8.3 Post vaccination seroconversion against *M. gallisepticum* killed vaccine

Age (Week)	DOC	3	6	10	16	20	25	32	42
Min	242	95	1	581	548	3288	1327	1640	1095
Max	605	343	250	6344	8819	12615	12405	11895	10322
GMT	435	189	63	2128	2575	7401	7283	5241	4151
CV	19.3	29.7	63.6	49.4	66.1	31.9	34.6	45.5	53.3

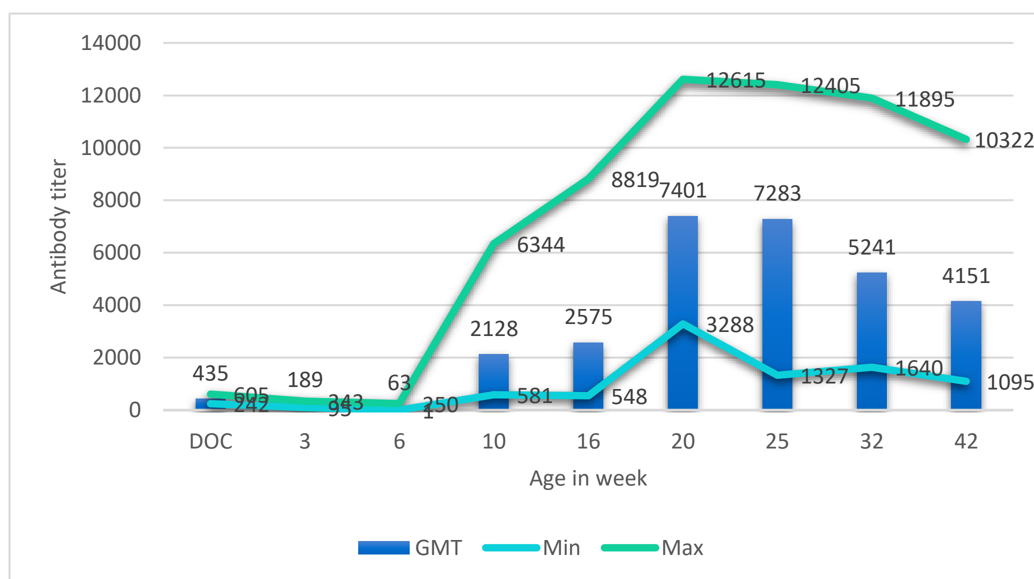


Fig. 8.3 Post vaccination antibody titer variation (killed vaccine)

8.3 Results

Post vaccination assessment of live vaccine titer as mentioned in (Table 8.2) displayed rise in antibody from 14th week, with maximum titer observed at 18th woa as 2549-12190 (min-max titer). Decline in antibody was recorded after 18th woa which sustained till 32nd woa with antibody titer of 927-6674 (min-max titer). Killed vaccine was administered at 6th woa, and subsequent serology presented rise in antibody titer from 10th woa which reached at highest range on 20th woa with 3288-12615 (min-max titer). Killed vaccine, maintained titer range of 1095-10322 (min-max titer) till 42nd woa (Table 8.3).

8.3.1 Evaluation of Exposure Status of Vaccinated Flocks

Breeder farms located in ICT, KPK and Punjab were monitored for post vaccination exposure to *M. gallisepticum* infection.

8.3.1.1 Study of Farms Located in ICT

From Islamabad region, two farms IFH and IFT were assessed. In total, 1 from flock IFH (IFH2.1) 2 flocks from IFT (IFT2.1, IFT2.2) and were evaluated serologically and by PCR. A single flock (IFH2.1) from second breeder farm located in Islamabad region was assessed for antibody against *M. gallisepticum* exposure. Samples were collected from the designated flock of IFH farm on 14th, 21st, 27th, 32nd, 40th, and 47th woa. The antibody titer range at 14th woa was 97-7496 (min-max titer), which increased at 27th, 32nd to 40th woa showing 1211-17599, 1143-31989, 1638-28384 (min-max titer), respectively. Decrease trend in antibody titer was observed on 47th woa with 1163-13566 (min-max titer). PCR of swabs collected from suspected birds for detection of *M. gallisepticum* was negative on 14th, 21st and 47th woa, and positive from 27th to 40th woa (Table 8.4, Fig.8.4).

During monitoring of first flock (IFT2.1) from May 2017 to March 2018, antibody titers were recorded at 9th, 16th, 21st, 25th, 30th and 51st woa. At 9th woa recorded antibody titer was 371-5475. The flock showed reasonably high post vaccination titer on 16th, 21st and 25th woa having 8434-17836, 766-15597, 913-16541 (min-max titer), respectively. From

30th woa antibody titer raised, which continued to increase till 37th and 51st woa having 1762-19064, 2341-20894, 2988-20397 (min-max titer), respectively. Rise in titer from 16th week onwards indicated exposure to *M. gallisepticum* infection. *M. gallisepticum* was detected by PCR on 16th and 25th woa only (Table 8.5, Fig.8.5).

Second flock (IFT.2) was monitored at 7th, 17th, 26th, 30th, 37th, and 44th woa. Results of serological monitoring of second flock (IFT.2) at 7th woa showed antibody titer ranging from 01-2268 (min-max titer). High post vaccination titer was recorded on 17th and 26th woa having 11820-31049, and 7237-21663 (min-max titer). From 30th woa antibody titer declined, which continued to decline at 37th and 44th woa having 5534-13647, 1604-16212, 4256-18978 (min-max titer), respectively. PCR for *M. gallisepticum* was positive on 17th, 30th and 37th woa (Table 8.6, Fig. 8.6).

8.3.1.2 Study of Farms Located in KPK Province

From KPK province two different farms i.e KFB and KFQ were studied to assess exposure to *M. gallisepticum* infection after vaccination from June 2018 to April 2019. Two flocks from KFB farm and 1 from KFQ farm were monitored to detect anti *M. gallisepticum* antibodies. First flock (KFB1.1) of KFB showed high post vaccination titer on 15th, 24th, 26th and 31st woa having 11635-23996, 7485-29218, 5881-26716 and 7554-29020 (min-max titer), respectively. On 45th woa antibody titer declined (1301-15220), which again raised at 52nd woa having 4861-21335 (min-max titer). In spite of high antibody titer, no detection of *M. gallisepticum* was made from the flock during entire period (Table 8.7, Fig. 8.7).

Second flock (KFB1.2) showed low post vaccination titer on 14th woa i.e 1582-4792, which increased on 23th, 25th and 30st woa having 2393-23608, 1575-26465, and 2484-25035 (min-max titer), respectively. On 44th woa antibody titer declined (2845-17018), which again raised at 51st woa having 2144-23133 (min-max titer). In spite of high antibody titer, no detection of *M. gallisepticum* was made from the flock during entire period (Table 8.8, Fig. 8.8).

A single flock (KFQ 2.1) from second breeder farm located in KPK province was assessed for antibody against *M. gallisepticum* exposure. Results showed moderately high post vaccination titer at 8th woa with a range of 328-12845 (Min-Max titer), high antibody titer on 14th and 22nd woa having 4877-21915, 3634-25181. On 30th and 40th woa antibody titer declined 869-12695, 2845-17018 (min-max titer), respectively, and raised slightly on 47th woa i.e 3931-19105 (min-max titer). No detection of *M. gallisepticum* by PCR was made from the flock during entire period (Table 8.9, Fig. 8.9).

8.3.1.3 Study of Farms Located in Punjab Province

From Punjab province two different farms i.e PFK1 and PFK2 were studied to assess exposure to *M. gallisepticum* infection after vaccination from November 2017 to August 2018 and August 2018 to April 2019, respectively. One flock from each farm was studied. First flock (PKF1.1) showed high post vaccination titer on 15th, 24th, 26th and 31st woa having 11635-23996, 7485-29218, 5881-26716 and 7554-29020 (min-max titer), respectively. On 45th woa antibody titer declined (1301-15220), which again raised at 52nd woa having 4861-21335 (min-max titer), No detection of *M. gallisepticum* by PCR was made from the flock during entire period (Table 8.10, Fig. 8.10).

Second flock (PKF2.1) showed high post vaccination titer on 20th, 24th, 31st, and 38th woa having 7783-32501, 6125-26370, 3910-27754, and 7717-32893 (min-max titer), respectively. Slight decrease in antibody titer was detected on 46th woa i.e. 2333-15068 (min-max titer), which again rise on 54th and 57th woa. No detection of *M. gallisepticum* by PCR was made from the flock during entire period (Table 8.11, Fig. 8.11).

Table 8.4 Exposure Status of Flock IFH1.1 assessed by ELISA and PCR

Date	Feb-17	Apr-17	May-17	Jul-17	Aug-17	Oct-17
Age (Week)	14	21	27	32	40	47
Min	97	285	1211	11463	1638	1163
Max	7496	11983	17599	31989	28384	13566
GMn	1096	1505	8964	18980	7048	4975
C.v	102	105	44	26.3	76.6	55.8
Detections by PCR	--	--	++	++	++	--

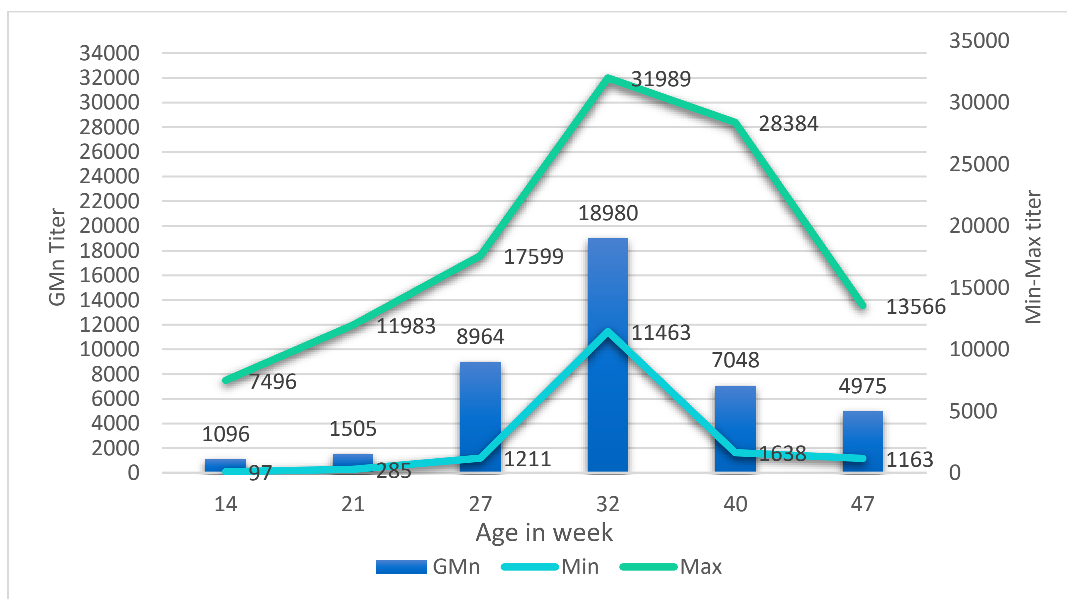


Fig. 8.4 Variation in antibody titer of Flock IFH1.1 quantified by ELISA

Table 8.5 Exposure Status of Flock IFT2.1 assessed by ELISA and PCR

Date	May-17	Jul-17	Aug-17	Sep-17	Oct-17	Dec-17	Mar-18
Age (week)	09	16	21	25	30	37	51
GMn	1149	13501	6397	3289	9069	10961	7483
C.v	69.8%	21.80%	55.60%	86%	46.80%	60.60%	54.10%
Min	341	8434	766	913	1762	2341	2988
Max	5475	17836	15597	16541	19064	20894	20397
Detections by PCR	–	++	_	++	_	--	--

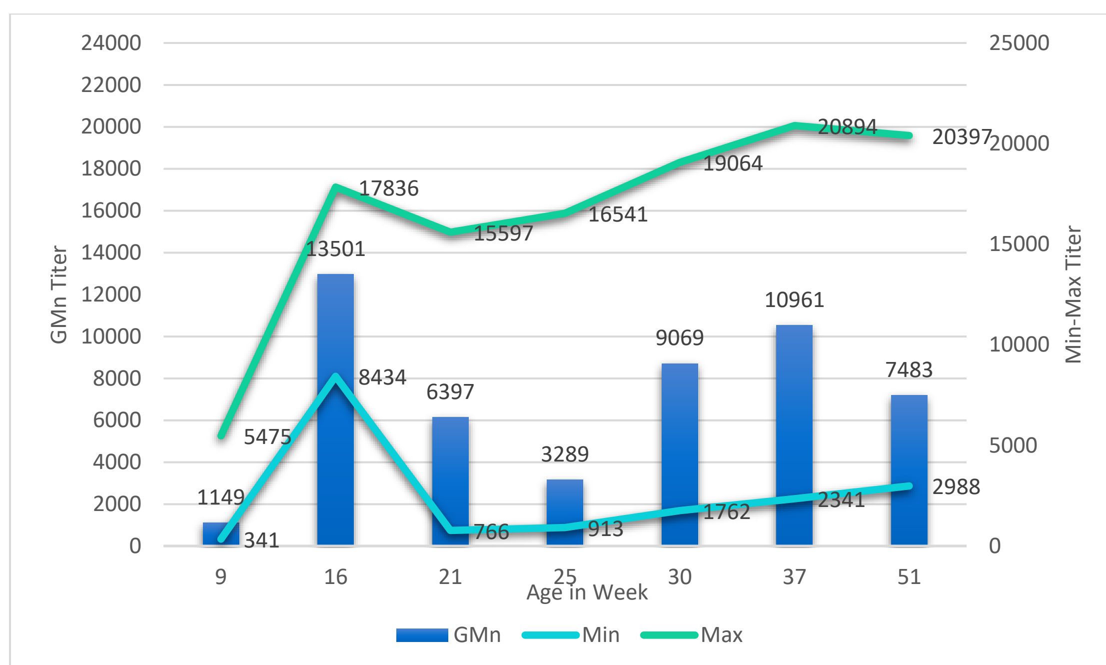


Fig. 8.5 Variation in antibody titer of Flock IFT2.1 quantified by ELISA

Table 8.6 Exposure Status of Flock IFT2.2 assessed by ELISA and PCR

Date	Jun-18	Aug-18	Sep-18	Oct-18	Dec-19	Jan-19	Mar-19
Age (week)	07	17	26	30	37	44	51
Min	1	11820	7237	5534	1604	4256	4752
Max	2268	31049	21663	13647	16212	18978	17998
GMn	312	20721	12192	8595	9191	9938	10213
C.v	98%	20.10%	29.90%	26.40%	32%	30.80%	31%
Detections by PCR	--	++	--	++	++	--	_

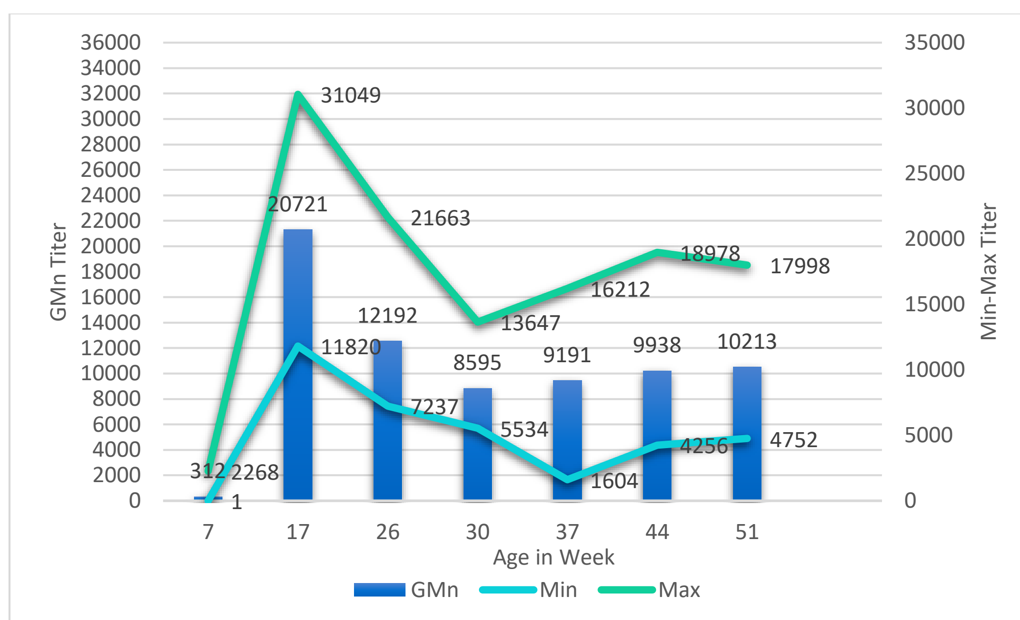


Fig. 8.6 Variation in antibody titer of Flock IFT2.2 quantified by ELISA

Table 8.7 Exposure Status of Flock KFB1.1 assessed by ELISA and PCR

Date	Jun-18	Aug-18	Oct-18	Oct-18	Nov-18	Feb-19	Apr-19
Age (week)	08	15	24	26	31	45	52
Min	160	11635	7485	5881	7554	1301	4861
Max	908	23996	29218	26716	29020	15220	21335
GMn	404	18605	16684	13400	16178	9082	13377
C.v	51.3	23.70%	33%	30%	28.50%	32.70%	28.90%
Detections by PCR	--	--	--	--	--	--	--

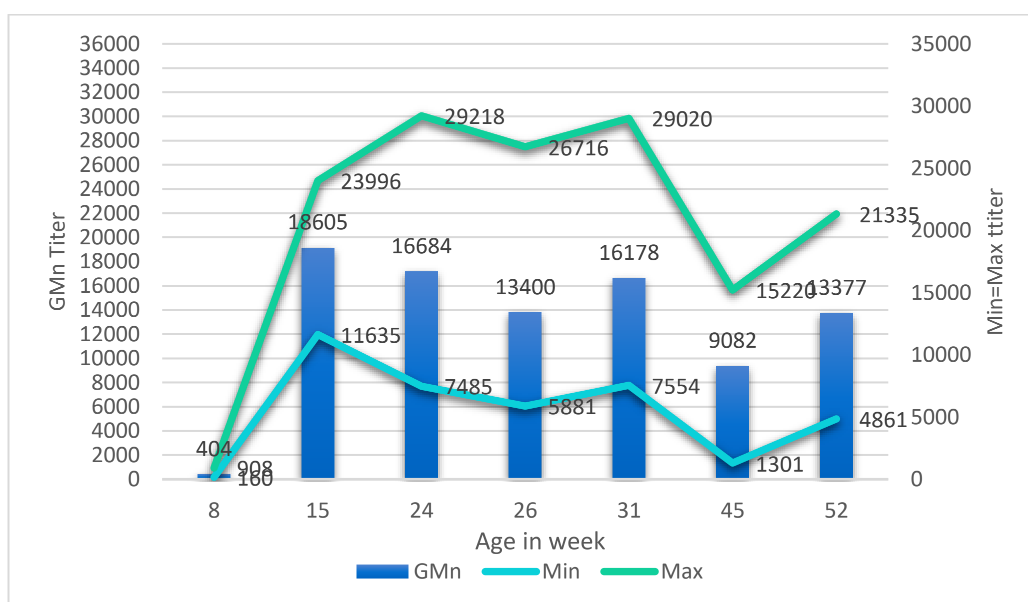


Fig. 8.7 Variation in antibody titer of Flock KFB1.1 quantified by ELISA

Table 8.8 Exposure Status of Flock KFB1.2 assessed by ELISA and PCR

Date	Aug-18	Oct-18	Oct-18	Nov-18	Feb-19	Apr-19
Age (Week)	14	23	25	30	44	51
Min	1582	2393	1575	2484	2845	2144
Max	4792	23608	26465	25035	17018	23133
GMn	2896	12705	12587	13069	9880	11443
C.v	31.70%	33%	35%	40.20%	31.10%	33.80%
Detections by PCR	--	--	--	--	--	--

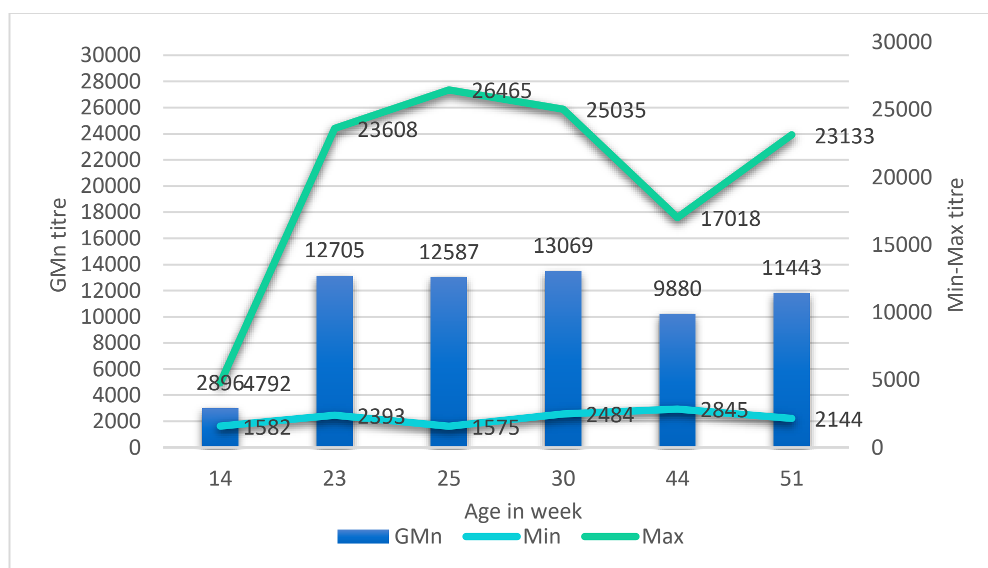


Fig. 8.8 Variation in antibody titer of Flock KFB1.2 quantified by ELISA

Table 8.9 Exposure Status of Flock KFQ2.1 assessed by ELISA and PCR

Date	Jul-18	Sep-18	Nov-18	Dec-18	Mar-19	Apr-19
Age (Week)	08	14	27	30	40	47
Min	328	4877	3634	869	2845	3931
Max	12845	21915	25181	12695	17018	19105
GMn	2696	13402	11242	6054	8839	8172
C.v	88.8	24.90%	38%	43%	41.80%	31.20%
Detections by PCR		++	--	--	--	--

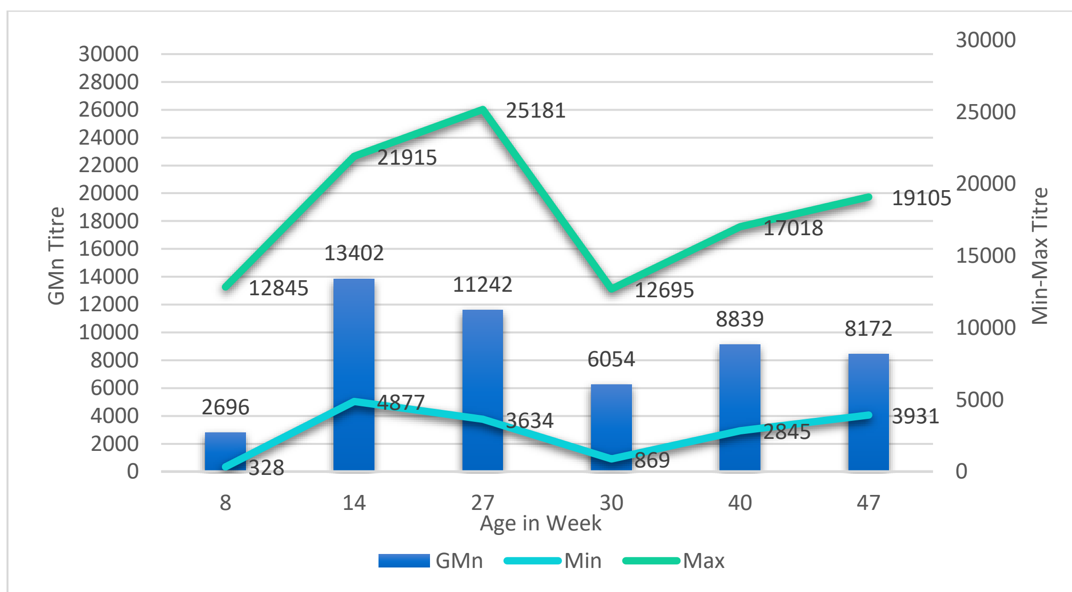


Fig. 8.9 Variation in antibody titer of Flock KFQ1.1 quantified by ELISA

Table 8.10 Exposure Status of Flock PFK1.1 assessed by ELISA and PCR

Date	Oct-17	Nov-17	Jan-18	Mar-18	May-18	Jul-18	Sep-18
Age (Week)	16	20	27	35	47	53	58
Min	277	4532	3545	1142	10	846	1258
Max	8821	21278	26470	24576	21996	27622	24697
GMn	2548	12357	13006	7046	3353	6891	7095
C.v	61.8	39.9	45	59	91	69.2	72.7
Detections by PCR		--	--	--	--	--	--

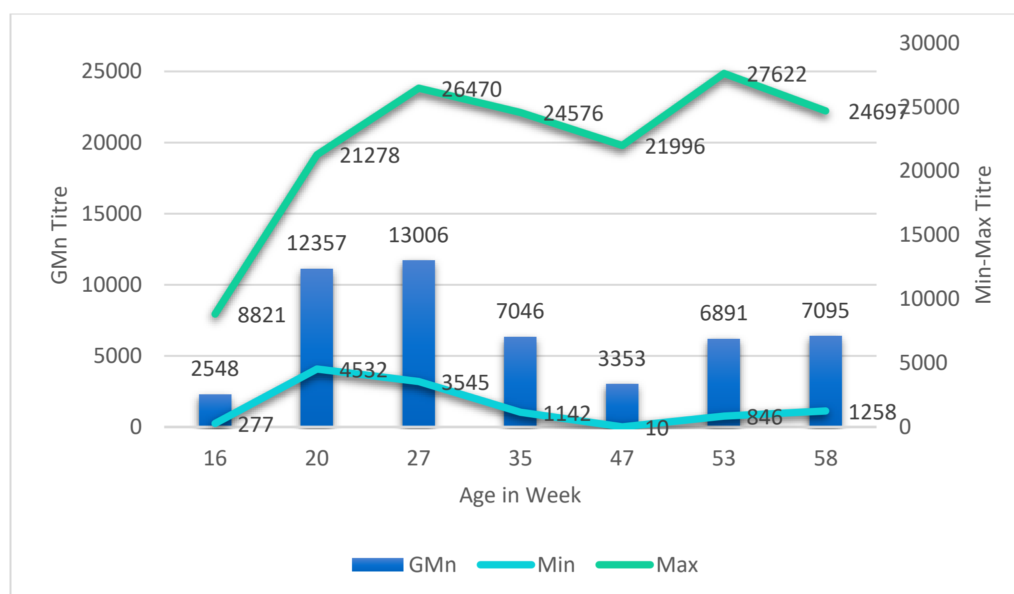


Fig. 8.10 Variation in antibody titer of Flock PFK1.1 quantified by ELISA

Table 8.11 Exposure Status of Flock PFK2.1 assessed by ELISA and PCR

Date	April-18	Aug-18	Sep-18	Oct-18	Nov-18	Jan-19	Mar-19	Apr-19
Age (Week)	05	20	24	31	38	46	54	57
Min	217	7783	6125	3910	7717	2333	3118	3126
Max	684	32501	26370	27754	32893	15068	25537	28516
GMn	373	15642	13636	12485	21376	8583	13821	17050
C.v	32.8	20.6	36.5	45.5	28.3	39	35	35.2
Detections by PCR	-ve	--	--	--	--	--	--	--

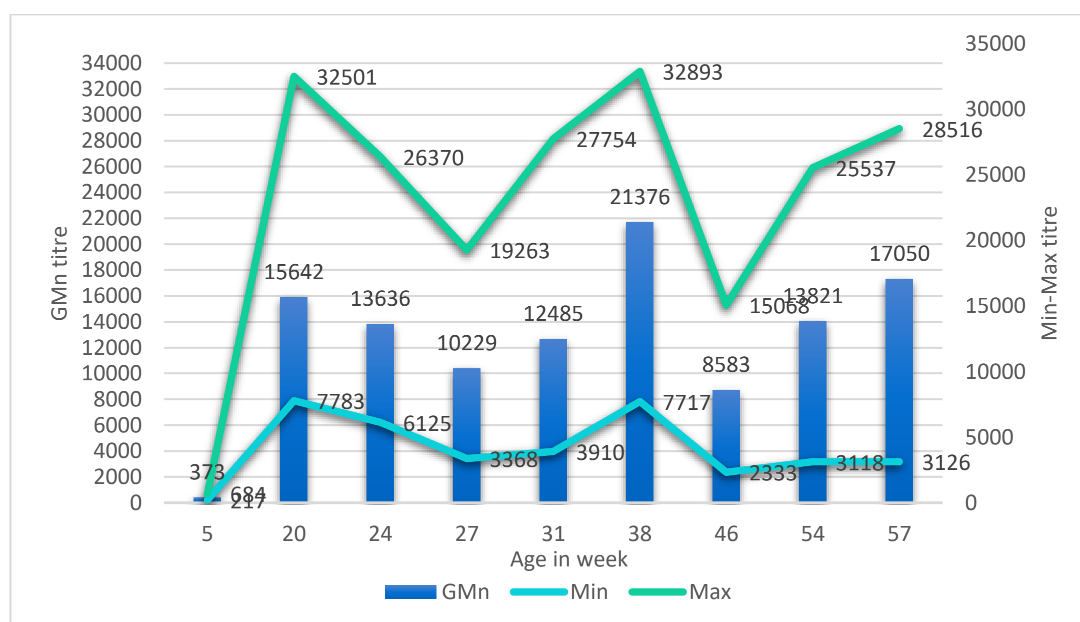


Fig. 8.11 Variation in antibody titer of Flock PKF2.1 quantified by ELISA

8.4 Discussion

In Pakistan, *M. gallisepticum* F strain killed vaccine is used for the control of *M. gallisepticum* infections. However, isolation of *M. gallisepticum* from vaccinated flock, raised concerns about the protective efficacy of such vaccines. To address this, it was considered a top priority to develop baseline antibody titer of vaccine (killed and live). Using these titers, total 08 breeder farms i.e. 03 from Islamabad Capital territory (ICT), 03 from Khyber Pakhtunkhwa (KPK) and 02 from Punjab were studied for the detection of protective and exposure antibody titers. Here the commercially available live *M. gallisepticum* vaccine named F-vax was used to assess level of protective antibody. Maximum antibody titer of 12190 was recorded at 18thweek of age, which declined to 5486 at 32nd week of age. In case of killed vaccine, the highest antibody titers achieved in this study were 12615 and 12405 at 20th and 25th week of age. Comparative assessment of breeder farms from ICT revealed, a maximum antibody titer of 31989, which is significantly higher than the anticipated post-vaccination titers against *M. gallisepticum* vaccine. Rise in antibody titers was recorded as earlier as from 16th week of age in case of 02 farms. From farms located in KPK, maximum antibody titer of 29218 was recorded. Infectious antibody titers from all 03 farms included in the study were detected from 15th week of age. Maximum antibody titer recorded from breeder farms located in Punjab was 32501. Rise in antibody titer was recorded from 18th to 20th week of age. In spite of vaccination of breeder flocks at the age of 6-8 week and second shot at 16-17 week of age, outcomes of the study provided evidence of prevailing infectious titers in the flocks from ICT, KPK and Punjab. With reference to post vaccination antibody titers, all the flocks included in this study were infected/exposed to *M. gallisepticum* during the study time period. As antibody titers recorded during this study were higher than the established post vaccination titers. These findings suggested failure of *M. gallisepticum* F-strain (killed vaccine) to protect flocks against locally prevalent strain of *M. gallisepticum.* Lack of efficacy of F-vaccine in control of *M. gallisepticum* infection in breeder farms has already been reported (Khalifa et al., 2014). Efforts have been made to develop better *M. gallisepticum* vaccine and to evaluate different candidate vaccine strains for use in breeder and layer farms (Ferguson-Neol and Williams, 2014).

Effectiveness of vaccines vary from country to country. For successful control of diseases, the biological and antigenic properties of the circulating strains of various pathogens have to be taken into account. Nucleic acid sequence analysis and other molecular biology techniques have paved the way to adopt new approaches in developing vaccines of better efficacy. Such vaccines can lead to reduce the use of antibiotics for the treatment of infections in livestock sector including poultry. Since antimicrobial resistance (AMR) has been occurring due to over use of antibiotics, development and usage of vaccines against animal pathogens could reduce non-judicial usage of antibiotics, eventually resulting in the control of AMR spread in humans as well. The eventual outcome of this research would be to provide better understanding of farmers and veterinarians for introducing sensitive and specific diagnostics for *M. gallisepticum* infection, practice judicial usage of the anti-mycoplasma drugs in poultry rearing, and select *M. gallisepticum* vaccines homologous to the pathogenic strains circulating in the field. This information would further lead to the development of more potent vaccines using local field isolates.

8.5 Conclusion

In spite of vaccination of breeder flocks at the age of 6-8 week and second shot at 16-17 week of age, outcomes of the study provided evidence of prevailing infectious titers in the flocks from ICT, KPK and Punjab. Based on post vaccination antibody titers, all studied flocks were infected/exposed to *M. gallisepticum* during the study time period. Antibody titers recorded during study were higher than the established post vaccination titers. These findings suggested failure of *M. gallisepticum* F-strain (killed vaccine) to protect flocks against the infection.

Over all Conclusion of Study

- The data regarding serology show high *M. gallisepticum,* antibody titres in backyard poultry than in commercial poultry. This recognizes the role of backyard poultry as a potential reservoir of *M. gallisepticum,* without manifesting clinical signs of disease. Despite the fact that in this country around 60-70% of breeder flocks are vaccinated for *M. gallisepticum*, still the disease is endemic in this region.
- Though biological characterization of *M. gallisepticum* isolate recovered from the field represents mild pathogenicity in this study, molecular evaluation shows diverse genetic organization with no homology with MG-F strain of vaccine being used in this country.
- Development of in-house diagnostic antigen proved to be a successful screening tool for initial screening of *M. gallisepticum* in poultry. Dissemination of such locally developed antigen can provide cheap diagnostics to the local poultry farmer.
- From the diagnostic persptective, this study reported comparable detection limit of iiPCR assay as compared to real-time PCR assay for *M. gallisepticum* detection, former being cheap as compared to latter. It can also be used effectively for the evaluation of efficacy of anti-Mycoplasma drug used for treatment in affected flocks.

Future recommendations/suggestions

Prevailing *M. gallisepticum* infections in commercial poultry contribute to economic burden on poultry farmer. Here are few suggestions in the light of present study:

- A highly sensitive and specific diagnostic tests at the early age of chicks is to be employed for the detection of *M. gallisepticum* infection for introducing appropriate control measures.
- Monitoring of cost treatment effectiveness of anti-mycoplasma drugs used in the infected flocks for proper selection, dose adjustment and regulation of duration of drug administration. Sensitive and rapid post treatment molecular diagnostics could regulate the usage of such therapeutics. Irrational use of anti-mycoplasma medication poses threat of development of anti-microbial resistance (AMR), which in turns is a threat for human food safety and must be minimized.
- In the present circumstances, use of preventive approach rather than therapeutic approach seems logical in the control of *M. gallisepticum* infection. Field data suggests incompatibility of the killed vaccine strain of *M. gallisepticum* in use at commercial level with the circulating field strains of *M. gallisepticum*. Development of homologous vaccine (tailor-made vaccine) of *M. gallisepticum*, may offer better protection, control even leading to its eradication from commercial poultry

REFERENCES

Abd-El-Motelib, T.Y. and Kleven, S.H. (1993) A comparative study of *Mycoplasma gallisepticum* vaccines in young chickens. *Avian Dis.* 37: 981-987

Adler, H., and Yamamoto, R. (1957) Pathogenic and nonpathogenic pleuropneumonia-like organisms in infectious sinusitis of turkeys. *Am. J. Vet. Res.* 18(68): 655.

Ahmad, A., Rabbani, M., Yaqoob, T., Ahmad, A., Shabbir, M.Z., Akhtar, F. (2008) Status of Igg antibodies against *Mycoplasma gallisepticum* in non-vaccinated commercial poultry breeder flocks. *J Ani Plant Sci.* 18: 2-3.

Ahmed, Z., Naeem, K., Hameed, A. (2007) Detection and seroprevalence of infectious bronchitis virus strains in commercial poultry in Pakistan. *Poult Sci*. 86: 1329–35.

Ahmed, J.S., Lawal, S.M., Fatihu, M.Y., Moses, D.G., Barde, I.J., Kumbish, P.R. and Oladele, S.B. (2015) Isolation and serological detection of mycoplasma gallisepticum and mycoplasma synoviae using a combined mg/ms enzyme-linked immunosorbent assay kit in indigenous chickens in Niger State, Nigeria. *Afr. J. Cell. Path. 4*(6): 70-73.

Ambagala, A., Fisher, M., Goolia, M., Nfon, C., Furukawa-Stoffer, T., Ortega Polo, R. and Lung, O. (2017a) Field-Deployable Reverse Transcription-Insulated Isothermal PCR (RT-ii PCR) Assay for Rapid and Sensitive Detection of Foot-and-Mouth Disease Virus. *Transbound. Emerg. Dis. 64*(5): 1610-1623.

Ambagala, A., Pahari, S., Fisher, M., Lee, P.Y., Pasick, J., Ostlund, E.N., Johnson, D.J. and Lung, O. (2017b) A rapid field-deployable reverse transcription-insulated isothermal polymerase chain reaction assay for sensitive and specific detection of bluetongue virus. *Transbound Emerg Dis. 64*(2): 476-486.

Arefin, M., Begum, J.A., Parvin, R., Rahman, M.M., Khan, M.A.H.N.A. (2011) Development of slide agglutination test for the rapid diagnosis of *Mycoplasma* infection in the chicken. *The Bangladesh Veterinarian.* 28 (2): 80-84.

Armour, N.K. (2014) An evaluation of the molecular epidemiology and transmission of avian *Mycoplasma* genotypes in commercial poultry. PhD dissertation. University of Georgia, Athens

Asif, M., Khalid, S., Sarfaraz, I., Rahman, S. U. and Rehman, A. (2015) Comparative Efficacy of Rapid Serum Agglutination and Haeagglutination Inhibition Tests In The Seroprevalence Study Of *Mycoplasma gallisepticum* In Poultry Birds. *Sci.Int.* 27(5), 4317-4319.

Atique, M.A., Abbas, F., Awan, M.A., Tariq, M.M., Ahmed Z., Ali, I. and Alam, M.A. (2012) Identification of avian *Mycoplasma* species in commercial broilers and layers with respiratory symptoms in Balochistan. *Afr J. of Biotech.* 11:16557-16559

Bagheri, H., Doosti, A. and Arshi, A. (2011) Detection of Mycoplasma gallisepticum in Chaharmahal Va Bakhtiari province poultry using PCR. *Glob. Vet. 7*(1): 54-59.

Balasuriya, U.B., Lee, P.Y.A., Tiwari, A., Skillman, A., Nam, B., Chambers, T.M., Tsai, Y.L., Ma, L.J., Yang, P.C., Chang, H.F.G. and Wang, H.T.T. (2014) Rapid detection of equine influenza virus H3N8 subtype by insulated isothermal RT-PCR (iiRT-PCR) assay using the POCKIT™ Nucleic Acid Analyzer. *J. virol. methods. 207*: 66-72.

Barua, S.R., Prodhan, A.M., Islam, S. and Chowdhury, S. (2006) Study on Mycoplasma gallisepticum in chickens in selected areas of Bangladesh. *Bangl. J. Vet. Med. 4*(2): 141-142.

Baseggio, N., Glew, M.D., Markham, P.F., Whithear, K.G. and Browning, G.F. (1996) Size and genomic location of the pMGA multigene family of Mycoplasma gallisepticum. *Microbiology* 142(6): 1429-1435.

Bencina, D., Mrzel, I., RoJs, O.Z., Bidovec, A., and Dovc, A. (2003) Characterisation of Mycoplasma gallisepticum strains involved in respiratory disease in pheasants and peafowl. *Vet Rec.* 152:230–234.

Bencina, D., Tadina, T. and Dorrer, D. (1988) Natural infection of ducks with Mycoplasma synoviae and Mycoplasma gallisepticum and mycoplasma egg transmission. *Avian Pathol.* 17:441–449.

Bermudez, A. J. (2008) Principles of disease prevention: Diagnosis and control. In; *Diseases of Poultry* (12th ed.) Y. M. Saif, (ed.), pp 3–42, Blackwell Publishing Professional, Ames, IA.

Boguslavsky, S., Menaker, D., Lysnyansky, I., Liu, T., Levisohn, S., Rosengarten, R., Garcia, M. and Yogev, D. (2000) Molecular characterization of the Mycoplasma gallisepticum pvpA gene which encodes a putative variable cytadhesin protein. *Infect. Immun. 68*(7):3956-3964.

Bradbury, J.M. (1984) January. Avian mycoplasma infections: prototype of mixed infections with mycoplasmas, bacteria and viruses. *Ann. Microbiol*. (Inst. Pasteur). 135(1A): 83-89.

Bradbury, J.M. (2005) Poultry mycoplasmas: sophisticated pathogens in simple guise. *Br. poult. sci. 46*(2): 125-136.

Buntz, B., Bradbury J.M., Vuillaume, A., and Rousselot-Paillet, D. (1986). Isolation of *Mycoplasma gallisepticum* from geese. *Avian Pathol.* 15:615–617.

Chang, H.F.G., Tsai, Y.L., Tsai, C.F., Lin, C.K., Lee, P.Y., Teng, P.H., Su, C. and Jeng, C.C. (2012) A thermally baffled device for highly stabilized convective PCR. *Biotechnol. J.l 7*(5): 662-666.

Charlton, B.R., Bickford, A.A., Walker, R.L. and Yamamoto, R. (1999) Complementary randomly amplified polymorphic DNA (RAPD) analysis patterns and primer sets to differentiate *Mycoplasma gallisepticum* strains. *J. Vet. Diagn. Invest. 11*(2): 158-161.

Cherry, J.J., Ley, D.H. and Altizer, S. (2006) Genotypic analyses of Mycoplasma gallisepticum isolates from songbirds by random amplification of polymorphic DNA and amplified-fragment length polymorphism. *J. Wildl. Dis. 42*(2): 421-428.

Chin, R.P., Daft, B.M., Meteyer, C.U. and Yamamoto, R. (1991) Meningoencephalitis in commercial meat turkeys associated with Mycoplasma gallisepticum. *Avian Dis.* 35(4): 986-993.

Chou, W.P., Chen, P.H., Miao, J.M., Kuo, L.S., Yeh, S.H. and Chen, P.J. (2011) Rapid DNA amplification in a capillary tube by natural convection with a single isothermal heater. *Biotechniques. 50*(1): 52-57.

Chua, K.H., Lee, P.C. and Chai, H.C. (2016) Development of insulated isothermal PCR for rapid on-site malaria detection. *Malar. J. 15*(1): 134.

Cookson, K.C. and Shivaprasad, H.L. (1994) *Mycoplasma gallisepticum* infection in chukar partridges, pheasants, and peafowl. *Avian Dis.* 38:914–921.

Dohms, J.E., Hnatow, L.L., Whetzel, P., Morgan, R. and Keeler Jr, C.L. (1993) Identification of the putative cytadhesin gene of Mycoplasma gallisepticum and its use as a DNA probe. *Avian Dis.* 37(2): 380-388.

Haque, E., S., Rahman, S., Khan, M., Younus, M., Awais, M. and Nasir, A. (2015) A simplified duplex real-time PCR incorporating TaqMan minor groove binder (MGB) probes and an exogenous internal positive control for the simultaneous detection of *Mycoplasma gallisepticum* and Mycoplasma synoviae cultures. *Vet Medicina*. *60*(5): 268-273.

El Shater, S.A.A. 1996. Mycoplasma infection in greater flamingo, grey Chinese goose and white pelican. *Vet Med J Giz.* 44:31–36.

Evans, R.D., and Hafez, Y.S. (1992) Evaluation of *Mycoplasma gallisepticum* Strain exhibiting reduced virulence for prevention and control of poultry Mycoplasmosis. *Avian Dis.* 36:197-201.

Fabricant, J. (1975) Immunization of chickens against *Mycoplasma gallisepticum* infection. *Am. J. Vet. Res*. 36(2): 566.

Feberwee, A., Dijkstra, J.R., von Banniseht-Wysmuller, T.E., Gielkens, A.L. and Wagenaar, J.A. (2005) Genotyping of *Mycoplasma gallisepticum* and M. synoviae by amplified fragment length polymorphism (AFLP) analysis and digitalized random amplified polymorphic DNA (RAPD) analysis. *Vet. Microbiol.* 111(1-2): 125-131.

Feberwee, A., Landman, W.J.M., Banniseht-Wysmuller, T.V., Klinkenberg, D., Vernooij, J.C.M., Gielkens, A.L.J. and Stegeman, J.A. (2006) The effect of a live vaccine on the horizontal transmission of *Mycoplasma gallisepticum*. *Avian Pathol.* 35(5): 359-366.

Feizi, A., Kaboli, K., Reza, A., Moggadam, J., Ali, M.J. and Hosseini, H. (2013) Evaluation of *Mycoplasma gallisepticum* infection in Tabriz broiler breeder farms by RSA. *Euro J Zool Res.* 2(4): 60-62.

Feizi, A., Khakpour, M., Nikpiran, H., Kaboli, K., Reza, A., Moggadam, J., Bijanzad, P. and Hosseini, H. (2013) Study on clinical signs and gross lesions of *Mycoplasma gallisepticum* in broiler breeder farms. *Eur. J. Exp. Biol. 3*(2): 387-390.

Ferguson, N.M., Hermes, D., Leiting, V.A. and Kleven, S.H. (2003) Characterization of a naturally occurring infection of a *Mycoplasma gallisepticum* house finch-like strain in turkey breeders. *Avian Dis.* 47(3): 523-530.

Ferguson, N.M. (2003) The evaluation of a live mycoplasma gallisepticum vaccine candidate and dna sequence analysis in the molecular epidemiology of *Mycoplasma gallisepticum*. PhD dissertation. University of Georgia, Athens.

Ferguson, N.M., Hepp, D., Sun, S., Ikuta, N., Levisohn, S., Kleven, S.H., García, M. (2005) Use of molecular diversity of Mycoplasma gallisepticum by gene-targeted sequencing (GTS) and random amplified polymorphic DNA (RAPD) analysis for epidemiological studies. *Microbiology* 6: 1883-1893. doi: 10.1099/mic.0.27642-0. PMID: 15941996.

Ferguson-Noel, N.M. and Williams, S.M. (2015) The efficacy of *Mycoplasma gallisepticum* K-strain live vaccine in broiler and layer chickens. *Avian Pathol.* 44: 75-80

Fischer, J.R., Stallknecht, D.E., Luttrell, P., Dhondt, A.A., and Converse K.A. (1997) Mycoplasmal conjunctivitis in wild songbirds: the spread of a new contagious disease in a mobile host population. *Emerg Infect Dis* 3:69–72.

Fraga, A.P., Vargas, T.D., Ikuta, N., Fonseca, A.S.K., Celmer, Á.J., Marques, E.K. and Lunge, V.R. (2013) A Multiplex real-time PCR for detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in clinical samples from Brazilian commercial poultry flocks. *Braz. J. Microbiol. 44*(2): 505-510.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., Bult, C.J., Kerlavage, A.R., Sutton, G., Kelley, J.M., Fritchman, R.D., Weidman, J.F., Small, K.V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T.R., Saudek, D.M., Phillips, C.A,, Merrick, J.M., Tomb, J.F., Dougherty, B.A., Bott, K.F., Hu, P.C., Lucier, T.S., Peterson, S.N., Smith, H.O., Hutchison, C.,A., Venter,

J.C. (1995) The minimal gene complement of *Mycoplasma genitalium*. *Science*. 20;270(5235):397-403. doi: 10.1126/science.270.5235.397. PMID: 7569993.

Fujisawa, S., Murata, S., Takehara, M., Katakura, K., Hmoon, M.M., Win, S.Y., Ohashi, K. (2019) Molecular detection and genetic characterization of *Mycoplasma gallisepticum*, *Mycoplama synoviae,* and infectious bronchitis virus in poultry in Myanmar. *BMC Vet Res.* 15 (1): 261. doi:10.1186/s12917-019-2018-2

Fürnkranz, U., Siebert-Gulle, K., Rosengarten, R. and Szostak, M.P. (2013) Factors influencing the cell adhesion and invasion capacity of *Mycoplasma gallisepticum*. *Acta Vet Scand. 55*(1): 1-8.

Garcia, M., Jackwood, M.W., Head, M., Levisohn, S. and Kleven, S.H. (1996) Use of species-specific oligonucleotide probes to detect *Mycoplasma gallisepticum*, *M. synoviae*, and *M. iowae* PCR amplification products. *J. Vet. Diagn. Invest. 8*(1): 56-63.

Gaunson, J.E., Philip, C.J., Whithear, K.G., & Browning, G.F. (2006). The cellular immune response in the tracheal mucosa to *Mycoplasma gallisepticum* in vaccinated and unvaccinated chickens in the acute and chronic stages of disease. *Vaccine*. 24(14): 2627–2633. doi: 10.1016/j.vaccine.2005.12.008

Gharaibeh, S. and Hailat, A. (2011) *Mycoplasma gallisepticum* experimental infection and tissue distribution in chickens, sparrows and pigeons. *Avian Pathol*. 40(4):349-54. doi: 10.1080/03079457.2011.582480. PMID: 21812712.

Gharaibeh, S., Laibinis, V., Wooten, R., Stabler, L., Ferguson-Noel, N. (2011) Molecular characterization of Mycoplasma gallisepticum isolates from Jordan. *Avian Dis*. 55(2):212-216. doi: 10.1637/9526-091510-Reg.1. PMID: 21793435.

Garmyn, A., Vereecken, M., De Gussem, K., Depondt, W., Haesebrouck, F. and Martel, A. (2019) Efficacy of Tylosin and Tilmicosin Against Experimental Mycoplasma gallisepticum Infection in Chickens. *Avian Dis.63*(2): 359-365.

Glew, M.D., Baseggio, N., Markham, P.F., Browning, G.F. and Walker, I.D. (1998) Expression of the pMGA genes of Mycoplasma gallisepticum is controlled by variation in the GAA trinucleotide repeat lengths within the 5′ noncoding regions. *Infect. Immun*. 66(12): 5833-5841.

Glew, M.D., Browning, G.F., Markham, P.F. and Walker, I.D. (2000) pMGA Phenotypic Variation in Mycoplasma gallisepticumOccurs In Vivo and Is Mediated by Trinucleotide Repeat Length Variation. *Infect. Immun.* 68(10): 6027-6033.

Glew, M.D., Markham, P.F., Browning, G.F. and Walker, I.D. (1995) Expression studies on four members of the pMGA multigene family in Mycoplasma gallisepticum S6. *Microbiology*. 141(11): 3005-3014.

Go, Y.Y., Rajapakse, R.J., Kularatne, S.A., Lee, P.Y.A., Ku, K.B., Nam, S., Chou, P.H., Tsai, Y.L., Liu, Y.L., Chang, H.F.G. and Wang, H.T.T. (2016) A pan-dengue virus reverse transcription-insulated isothermal PCR assay intended for point-of-need diagnosis of dengue virus infection by use of the POCKIT nucleic acid analyzer. *J. Clin. Microbiol. 54*(6): 1528-1535.

Goh, M.S., Forsyth, M.H., Gorton, T.S. and Geary, S.J. (1994) Cloning and sequence analysis of a Mycoplama gallisepticum cytadhesin gene, pp. 669-670. In *Proceedings of the 10th International Congress of the International Organization for Mycoplasmology.* International Organization for Mycoplasmology, Bordeaux, France.

Goh, M.S., Gorton, T.S., Forsyth, M.H., Troy, K.E. and Geary, S.J. (1998) Molecular and biochemical analysis of a 105 kDa Mycoplasma gallisepticum cytadhesin (GapA). *Microbiology. 144*(11): 2971-2978.

Goh, M.S., Gorton, T.S., Forsyth, M.H., Troy, K.E. and Geary, S.J. (1998) Molecular and biochemical analysis of a 105 kDa Mycoplasma gallisepticum cytadhesin (GapA). *Microbiology. 144*(11): 2971-2978.

GOP (Government of Pakistan). Economic Survey of Pakistan, Ministry of Finance, Government of Pakistan. (2018-19), Islamabad pp:26-33.

Gorton, T.S., Goh, M.S. and Geary, S.J. (1995) Physical mapping of the Mycoplasma gallisepticum S6 genome with localization of selected genes. *J. Bacteriol. 177*(1): 259-263.

Goulet, M., Dular, R., Tully, J.G., Billowes, G. and Kasatiya, S. (1995) Isolation of Mycoplasma pneumoniae from the human urogenital tract. *J. Clin. Microbiol. 33*(11): 2823-2825.

Hanif, A. and Najeeb, M.I. (2007) Comparison of Conventional Bacterial isolation, Rapid Slide Agglutination and Polymerase Chain Reaction for Detection of *Mycoplasma gallisepticum* in Breeder Flocks. *Pak. j. life soc. sci*. 5(1-2): 1-5

Hnatow, L.L., Keeler Jr., C.L., Tessmer, L.L., Czymmek, K., Dohms, J.E. (1998) Characterization of MGC2, a *Mycoplasma gallisepticum* cytadhesin with homology to the *Mycoplasma pneumoniae* 30-kilodalton protein P30 and *Mycoplasma genitalium* P32. *Infect. Immun.* 66: 3436-3442

Haque, S.E. (2010). Diagnostic and Molecular Epidemiological Investigations of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in poultry. PhD Thesis, Deptt. Vet. Microbiol., Univ. Agri (U.A.F), Faisalabad. Pakistan.

Heleili, N., Mamache, B. and Chelihi, A. (2011) Incidence of avian mycoplasmosis in the région of Batna, Eastern Algeria. *Vet. World 4*(3): 101.

Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B. C., & Herrmann, R. (1996) Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. *Nucleic acids Res.* 24(22):4420–4449.

Hong, Y., García, M., Levisohn, S., Savelkoul, P., Leiting, V., Lysnyansky, I., Ley, D.H. and Kleven, S.H. (2005) Differentiation of Mycoplasma gallisepticum strains using amplified fragment length polymorphism and other DNA-based typing methods. *Avian Dis. 49*(1): 43-49.

Hossain, K.M.M., Hossain, M.T. and Yamato, I. (2010) Seroprevalence of Salmonella and Mycoplasma gallisepticum infection in chickens in Rajshahi and surrounding districts of Bangladesh. *Int. J. Biol.* 2(2).

Hussain, J., Rabbani, I., Aslam, S., & Ahmad, H. A. (2015) An overview of poultry industry in Pakistan. *Worlds Poult Sci J.* 71(4):689-700. https://doi.org/10.1017/S0043933915002366

Indiková, I., Much, P., Stipkovits, L., Siebert-Gulle, K., Szostak, M.P., Rosengarten, R. and Citti, C. (2013) Role of the GapA and CrmA cytadhesins of *Mycoplasma gallisepticum* in promoting virulence and host colonization. *Infect. Immun. 81*(5): 1618-1624.

Islam, A., Aslam, A., Chaudhry, Z.I., Ahmed, M.U.D., Rehman, H.U., Saeed, K. and Ahmed, I. (2011) Pathology of *Mycoplasma gallisepticum* in naturally infected broilers and its diagnosis through PCR. *Int. J. Agri. Biol.* 13(4): 835-837.

Islam, M., Hassan, J. and Khan, M.S.R. (2014) Seroprevalence of *Mycoplasma gallisepticum* infection in backyard and commercial layer chickens in Bhola district, Bangladesh. *J. Adv. Vet. Anim. Res. 1*(1): 11-15.

Jalil, M.A. and Islam, M.T. (2010) A cross-sectional study for *Mycoplasma gallisepticum* antibodies in non-vaccinated commercial layer birds in Khulna district. *BJVM. 8*(2): 93-96.

Jordan, F. T. and M. M. Amin. 1980. A survey of Mycoplasma infections in domestic poultry. *Res Vet Sci.* 28:96–100.

Kabiri, N., Bashashati, M., Vasfi M. M., Banani, M., Sabouri, F. (2019). Genetic analysis of Iranian *Mycoplasma gallisepticum* isolates using gene-targeted sequencing of complete pvpA gene. *Turk J Vet Anim Sci.* 43. 399-407. 10.3906/vet-1901-40.

Kanci, A., Browning, G.F., Geary, S.J., Papazisi, L., Gorton, T.S., Markham, P.F. (2004) Is an unstable repeated sequence responsible for the attenuation of the Mycoplasma gallisepticum vaccine strain ts-11. In 15th Congress of the International Organisation for Mycoplasmology, p. 118. Athens, GA.

Keeler, C.L., Hnatow, L.L., Whetzel, P.L. and Dohms, J.E. (1996) Cloning and characterization of a putative cytadhesin gene (mgc1) from *Mycoplasma gallisepticum. Infect. Immun. 64*(5): 1541-1547.

Kempf, I., Reeve-Johnson, L., Gesbert, F. and Guittet, M. (1997) Efficacy of tilmicosin in the control of experimental *Mycoplasma gallisepticum* infection in chickens. *Avian Dis.*41(4): 802-807.

Khalifa, K.A., Sidahmed Abdelrahim, E., Badwi, M. and Mohamed, A.M. (2013) Isolation and Molecular Characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in Chickens in Sudan. *J. vet. Med. 2013*.

Khalifa, R., Eissa, S., El-Hariri, M., and Refai, M. (2014) Sequencing analysis of *Mycoplasma gallisepticum* wild strains in vaccinated chicken breeder flocks. *J Mol Microbiol Biothechnol*. 24(2): 98–104. Doi: 10.1159/000357733

Khan, M.I., Kirkpatrick, B.C. and Yamamoto, R. (1989) *Mycoplasma gallisepticum* species and strain-specific recombinant DNA probes. *Avian Pathol. 18*(1): 135-146.

Khan, M.I., Lam, K.M. and Yamamoto, R. (1987) *Mycoplasma gallisepticum* strain variations detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Avian Dis.*31(2): 315-320.

Khan, M.I., McMartin, D.A., Yamamoto, R. and Ortmayer, H.B. (1986) Observations on commercial layers vaccinated with *Mycoplasma gallisepticum* (MG) bacterin on a multiple-age site endemically infected with MG. *Avian Dis* 30: 309-312.

Khatoon, H., Afzal, F., Tahir, M.F., Hussain, M. and Khan, S.U. (2018) Prevalence of Mycoplasmosis and antibiotic susceptibility of *Mycoplasma gallisepticum* in commercial chicken flocks of Rawalpindi division, Pakistan. *Pak Vet J. 38*(4): 446-448.

Khumpim, P., Tampaisarn, R., Sasipreeyajan, J. and Pakpinyo, S. (2015) Analysis of Virulence Genes and Pathogenicity of Thai *Mycoplasma gallisepticum* Isolates. *The Thai J. Vet. Med. 45*(2): 213-220.

Kleven, S.H. (1997) Changing expectations in the control of *Mycoplasma gallisepticum*. *Acta Vet. Hung. 45*(3): 299-305.

Kleven, S.H., Browning, G.F., Bulach, D.M., Ghiocas, E., Morrow, C.J. and Whithear, K.G. (1988) Examination of *Mycoplasma gallisepticum* strains using restriction endonuclease DNA analysis and DNA-DNA hybridisation. *Avian Pathol. 17*(3): 559-570.

Kleven, S.H., Fulton, R.M., Garcia, M., Ikuta, V.N., Leiting, V.A., Liu, T., Ley, D.H., Opengart, K.N., Rowland, G.N. and Wallner-Pendleton, E. (2004) Molecular characterization of *Mycoplasma gallisepticum* isolates from turkeys. *Avian Dis. 48*(3): 562-569.

Kleven, S.H., Morrow, C.J. and Whithear, K.G. (1988) Comparison of *Mycoplasma gallisepticum* strains by hemagglutination-inhibition and restriction endonuclease analysis. *Avian Dis.* 32: 731-741.

Kollias, G. V., Sydenstricker, K.V., Kollias, H.W., Ley, D.H., Hosseini, P.R., Connolly, V., and Dhondt, A.A. (2004) Experimental infection of house finches with *Mycoplasma gallisepticum*. *J Wildl Dis* 40:79–86.

Krishnan, M., Ugaz, V.M. and Burns, M.A. (2002) PCR in a Rayleigh-Benard convection cell. (Biochemistry). *Science 298*(5594): 793-794.

Kuo, H.C., Lo, D.Y., Chen, C.L., Tsai, Y.L., Ping, J.F., Lee, C.H., Lee, P.Y.A. and Chang, H.F.G. (2016) Rapid and sensitive detection of *Mycoplasma synoviae* by an insulated isothermal polymerase chain reaction-based assay on a field-deployable device. *Poul. Sci. 96*(1): 35-41.

Levisohn, S. and Kleven, S.H. (2000) Avian mycoplasmosis (*Mycoplasma gallisepticum*). *Rev sci tech. (International Office of Epizootics) 19*(2): 425-442,

Levisohn, S., Dykstra, M.J., Lin, M.Y. and Kleven, S.H. (1986) Comparison of in vivo and in vitro methods for pathogenicity evaluation for *Mycoplasma gallisepticum* in respiratory infection. *Avian pathol. 15*(2): 233-246.

Levisohn, S., Dykstra, M.J., Lin, M.Y. and Kleven, S.H. (1986) Comparison of in vivo and in vitro methods for pathogenicity evaluation for *Mycoplasma gallisepticum* in respiratory infection. *Avian Pathol. 15*(2): 233-246.

Levisohn, S., Yegana, Y., Hod, I. and Herz, A. (1983) A correlative in vivo study of the surface morphology and colonisation of the chicken trachea infected by *Mycoplasma gallisepticum* strains R and F. *Avian Pathol. 12*(2): 247-261.

Ley, D. H. *Mycoplasma gallisepticum* infection. In: Saif YM (editor). Diseases of poultry. 12th ed. Ames, Iowa, USA: Blackwell publishing; 2008. pp. 807-812.

Ley, D. H., D. S. Sheaffer, and A. A. Dhondt. (2006) Further western spread of *Mycoplasma gallisepticum* infection of house finches. *J Wildl Dis* 42:429–431.

Ley, D. H., J. E. Berkhoff, and J. M. McLaren. (1996) *Mycoplasma gallisepticum* isolated from house finches (*Carpodacus mexicanus*) with conjunctivitis. *Avian Dis.* 40:480–483.

Ley, D. H., S. J. Geary, J. E. Berkhoff, J. M. McLaren, and S. Levisohn. (1998). Mycoplasma sturni from blue jays and northern mockingbirds with conjunctivitis in Florida. *J Wildl Dis* 34:403–406

Lin, M.Y. and Kleven, S.H. (1982) Egg transmission of two strains of *Mycoplasma gallisepticum* in chickens. *Avian Dis.* 26(3): 487-495.

Liu, T., Garcıa, M., Levisohn, S., Yogev, D. and Kleven, S.H. (2001) Molecular Variability of the Adhesin-Encoding Gene *pvpA* among *Mycoplasma gallisepticum* Strains and Its Application in Diagnosis. *J. Clin. Microbiol. 39*(5): 1882-1888.

Lo, S.C., Hayes, M.M., Kotani, H., Pierce, P.F., Wear, D.J., Newton, P.B., Tully, J.G. and Shih, J.W. (1993) Adhesion onto and invasion into mammalian cells by *Mycoplasma penetrans*: a newly isolated mycoplasma from patients with AIDS. *Modern pathol*. 6(3): 276-280.

Lo, S.C., Hayes, M.M., Wang, R.H., Pierce, P.F., Kotani, H. and Shih, J.K. (1991) Newly discovered mycoplasma isolated from patients infected with HIV. *The Lancet 338*(8780): 1415-1418.

Loolmani, F.S., Pourbakhsh, S.A., Banani, M., Charkhkar, S. (2014) Phylogenetic analysis of mgc2 gene of *Mycoplasma gallisepticum* isolates from broiler breeder flocks in Tehran province, Iran. *Euro J Zool Res*. 3 (2):37-42

Lung, O., Pasick, J., Fisher, M., Buchanan, C., Erickson, A. and Ambagala, A. (2016) Insulated Isothermal Reverse Transcriptase PCR (ii RT-PCR) for Rapid and Sensitive Detection of Classical Swine Fever Virus. *Transbound. Emerg. Dis. 63*(5): e395-e402.

Luttrell, M.P., Fischer, J.R., Stallknecht, D.E, and Kleven, S.H. (1996) Field investigation of *Mycoplasma gallisepticum* infections in house finches (Carpodacus mexicanus) from Maryland and Georgia. *Avian Dis.* 40:335–341.

Machado, L.D.S., Abreu, D.L.D.C., Lemos, M., Tortelly, R., Pimentel, J.C., Sesti, L., Pereira, V.L.D.A. and Nascimento, E.R.D. (2017) Performance, serologic and tracheal responses of laying hens exposed to F strain of *Mycoplasma gallisepticum*. *Arq. Inst. Biol*. 84:1-4

Majumder, S. (2014) Role of *Mycoplasma gallisepticum* and host airway epithelial cell interaction in inflammation, Ph.D. thesis. University of Connecticut, Storrs, U.S.

Maniloff, J., McElhaney, R. N., Finch, L. R., and Baseman, J. B., (eds.) (1992) *Mycoplasmas, Molecular Biology and Pathogenesis,* American Society for Microbiology, Washington DC, USA.

Markham, P.F., Glew, M.D., Brandon, M.R., Walker, I.D. and Whithear, K.G. (1992) Characterization of a major hemagglutinin protein from *Mycoplasma gallisepticum*. *Infect. Immun. 60*(9): 3885-3891.

Markham, P.F., Glew, M.D., Browning, G.F., Whithear, K.G. and Walker, I.D. (1998) Expression of two members of the pMGA gene family of *Mycoplasma gallisepticum* oscillates and is influenced by pMGA-specific antibodies. *Infect. Immun.* 66(6): 2845-2853.

Markham, P.F., Glew, M.D., Sykes, J.E., Bowden, T.R., Pollocks, T.D., Browning, G.F., Whithear, K.G. and Walker, I.D. (1994) The organisation of the multigene family which encodes the major cell surface protein, pMGA, of *Mycoplasma gallisepticum*. *FEBS letters*. 352(3): 347-352.

Markham, P.F., Glew, M.D., Whithear, K.G. and Walker, I.D. (1993) Molecular cloning of a member of the gene family that encodes pMGA, a hemagglutinin of *Mycoplasma gallisepticum*. *Infect. Immun. 61*(3): 903-909.

Marois, C., Dufour-Gesbert, F. and Kempf, I. (2002) Polymerase chain reaction for detection of *Mycoplasma gallisepticum* in environmental samples. *Avian pathol. 31*(2): 163-168.

Maxim, L.D., Niebo, R. and Utell, M.J. (2014) Screening tests: a review with examples. *Inhal. Toxicol. 26*(13): 811-828.

Much, P., Winner, F., Stipkovits, L., Rosengarten, R., Citti, C. (2002) *Mycoplasma gallisepticum*: influence of cell invasiveness on the outcome of experimental infection in chickens. FEMS Immunol. Med. Microbiol. 34:181–186.

Mudahi-Orenstein, S., Levisohn, S., Geary, S.J., Yogev, D. (2003) Cytadherence-deficient mutants of *Mycoplasma gallisepticum* generated by transposon mutagenesis. *Infect Immun* 71: 3812-3820.

Mukhtar, M., Awais, M. M., Anwar, M. I., Hussain, Z., Bhatti, N., and Ali, S. (2012) Seroprevalence of *Mycoplasma gallisepticum* Among Commercial Layers in Faisalabad, Pakistan. *J. of Bas & App Sci*. 8: 183-186.

Murakami, S., Miyama, M., Ogawa, A., Shimada, J., and Nakane, T. (2002) Occurrence of conjunctivitis, sinusitis and upper region tracheitis in Japanese quail (Coturnix coturnix japonica), possibly caused by *Mycoplasma gallisepticum* accompanied by Cryptosporidium sp. infection. *Avian Pathol.* 31:363–370.

Mushi, E.Z., Binta, M.G., Chabo, R.G., Mathaio, M. and Ndebele, R.T. (1999) Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* antibodies in the sera of indigenous chickens by rapid serum agglutination test at Mmopane, Gaborone, Botswana. *J. Vet. Res. 66: 333-334.*

Naeem, K., Siddique, N., Ayaz, M., Jalalee, M.A. (2007) Avian influenza in Pakistan: outbreaks of low- and high-pathogenicity avian influenza in Pakistan during 2003-2006. *Avian Dis*. 51(1):189-93. doi: 10.1637/7617-042506R.1. PMID: 17494552.

Nagai, S., Kazama, S. and Yagihashi, T. (1995) Ribotyping of *Mycoplasma gallisepticum* strains with a 16S ribosomal RNA gene probe. *Avian Pathol.* 24(4): 633-642.

Nascimento, E.R., Pereira, V.L.A., Nascimento, M.G.F. and Barreto, M.L. (2005) Avian mycoplasmosis update. *Rev. Bras. Cienc. Avic*. 7(1): 1-9.

Nascimento, E.R., Yamamoto, R., Herrick, K.R. and Tait, R.C. (1991) Polymerase chain reaction for detection of *Mycoplasma gallisepticum*. *Avian Dis.* 35(1): 62-69.

Noormohammadi, A.H. and Whithear, K.G. (2019) Comparison of the short-term and long-term efficacies of the *Mycoplasma gallisepticum* vaccines ts-11 and 6/85. *Avian Pathol.* 48(3): 238-244 doi: 10.1080/03079457.2019.1572103

OIE, 2018. Avian Mycoplasmosis. In: manual of diagnostic tests and vaccines for terrestrial animals 2018 (OIE, Biological, Standard, Commission, eds). 2018 Ed, OIE, Paris, pp:845-859.

Papazisi, L., Gorton, T.S., Kutish, G., Markham, P.F., Browning, G.F., Swartzell, S., Madan, A., Mahairas, G. and Geary, S.J. (2003) The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain Rlow. *Microbiology.* 149(9): 2307-2316.

Papazisi, L., Troy, K.E., Gorton, T.S., Liao, X. and Geary, S.J. (2000) Analysis of Cytadherence-Deficient, GapA-Negative *Mycoplasma gallisepticum* Strain R. *Infect. Immun.* 68(12): 6643-6649.

Pflaum, K., Tulman, E.R., Beaudet, J., Canter, J. and Geary, S.J. (2018) Variable lipoprotein hemagglutinin A gene (vlhA) expression in variant Mycoplasma gallisepticum strains in vivo. *Infect. Immun.* 86(11): e00524

Pflaum, K., Tulman, E.R., Beaudet, J., Liao, X. and Geary, S.J. (2016) Global changes in Mycoplasma gallisepticum phase-variable lipoprotein gene vlhA expression during in vivo infection of the natural chicken host. *Infect. immun.* 84(1): 351-355.

Purswell, J.L., Evans, J.D., Leigh, S.A., Collier, S.D., Olanrewaju, H.A., Kim, E.J., Pharr, G.T., Peebles, E.D., Branton, S.L. (2012) *Mycoplasma gallisepticum* transmission: comparison of commercial F-strain vaccine versus layer complex-derived field strains in a tunnel ventilated house. *Poult Sci.* 91(12):3072-3079. doi: 10.3382/ps.2012-02619. PMID: 23155015.

Qasem, J.A., Al-Mouqati, S.A., Al-Ali, E.M. and Ben-Haji, A. (2015) Application of molecular and serological methods for rapid detection of Mycoplasma gallisepticum infection (Avian mycoplasmosis). *Pak J Biol Sci.* 18(2): 81-87.

Qadir, M.F, Quratulain, Ashok, S. (2020) Current Status of *Mycoplasma gallisepticum* in Chickens and Associated Risk Factors in Pakistan. *Acta Sci. Microbiol* 3.1: 120-123.

Rachida, A., Omar, B., Elhacène, B., Rachid, K. and Cherif, A.M. (2013) Serological investigation on avian mycoplasmosis in laying hen farms in Eastern Algeria. *IJAVMS*. *7*(5): 170-177.

Racicot, M., Venne, D., Durivage, A. and Vaillancourt, J.P. (2011) Description of 44 biosecurity errors while entering and exiting poultry barns based on video surveillance in Quebec, Canada. *Prevent. vet. med.* 100(3-4): 193-199.

Rafique, S. (2018) Seroprevalence and molecular characterization of Infectious bronchitis virus variants from poultry in Pakistan. PhD thesis (published). Deptt of Microbiology, Quaid-i-Azam University, Islamabad.

Rajkumar, S., Reddy, M.R. and Somvanshi, R. (2018) Molecular prevalence and seroprevalence of *Mycoplasma gallisepticum* and *M. synoviae* in Indian Poultry Flocks. *Indian J. Anim. Res. 8*(1): 15-19.

Rasool, A., Anjum, A.A., Rabbani, M., Lateef, M., Nawaz, M. (2017) Preparation of *Mycoplasma Synoviae* antigens and evaluation by Rapid Slide Agglutination and Enzyme Linked Immunosorbent Assay. *The J. Ani & Pl Scs.* 27(3): 841-847.

Rasoulinezhad, S., Bozorgmehrifard, M.H., Hosseini, H., Sheikhi, N. and Charkhkar, S. (2017) Molecular detection and phylogenetic analysis of *Mycoplasma gallisepticum* from backyard and commercial turkey flocks in Iran. In *Vet. Res. Forum* 8(4): 293-298.

Rauf, M., Chaudhary, Z.I., Younus, M., Anjum, A.A., Ali, M.A., Ahmad, A.N. and Khan, M.U.R. (2013) Identification of Mycoplasma gallisepticum by Polymerase Chain Reaction and Conventional diagnostics from White Leghorn Layer flocks. *The J of Ani & Pla Sci.* 23(2): 393-397

Raviv, Z. and Kleven, S.H. (2009) The development of diagnostic real-time TaqMan PCRs for the four pathogenic avian mycoplasmas. *Avian Dis.53*(1): 103-107.

Raviv, Z., Callison, S., Ferguson-Noel, N., Laibinis, V., Wooten, R. and Kleven, S.H. (2007) The Mycoplasma gallisepticum 16S–23S rRNA intergenic spacer region sequence as a novel tool for epizootiological studies. *Avian Dis. 51*(2): 555-560.

Razin S. (2006) The Genus *Mycoplasma* and Related Genera (Class Mollicutes). In: *The Prokaryotes* (4th edn), Dworkin M., Falkow S., Rosenberg E., Schleifer KH. and Stackebrandt E. (eds), pp.836-904, Springer, New York, USA.

Razin, S. and Jacobs, E. (1992) Mycoplasma adhesion. *Microbiology* 13*8*(3): 407-422.

Razin, S., Yogev, D. (1986) Genetic relatedness among Ureaplasma urealyticum serotypes (serovars). *Pediatr. Infect. Dis. J.* 5(6 Suppl): S300-4.

Razin, S., Yogev, D. and Naot, Y. (1998) Molecular biology and pathogenicity of mycoplasmas. *Microbiol.Mol. Biol. Rev.* 62(4): 1094-1156.

Reece, R.L., Ireland, L., and Barr, D.A. (1986) Infectious sinusitis associated with Mycoplasma gallisepticum in game-birds. *Aus. Vet. J.* 63:167–168.

Rehman, H., Fawad, N., Abbas, G., Naheed, G., Siddique, B., Afzal, F. (2013). Surveillance of poultry diseases in Punjab province, Pakistan; special reference to Newcastle disease. *Res J Vet Pract.* 1:1–4.

Rehman, A., Shah, A.H. and Rahman, S. (2018) Seroprevalance of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in Commercial Broilers and Backyard Poultry in Five Districts of Khyber Pakhtunkhwa-Pakistan. *Pak Vet J*. 38: 149-152. doi:10.29261/pakvetj/2018.047.

Reinhardt, A.K., Gautier-Bouchardon, A.V., Gicquel-Bruneau, M., Kobisch, M. and Kempf, I. (2005) Persistence of Mycoplasma gallisepticum in chickens after treatment with enrofloxacin without development of resistance. *Vet. Microbiol.* 106(1-2): 129-137.

Rodriguez, R. and Kleven, S.H. (1980) Pathogenicity of two strains of Mycoplasma gallisepticum in broilers. *Avian Dis.*4(4): 800-807.

Rosengarten, R. and Wise, K.S. (1990) Phenotypic switching in mycoplasmas: phase variation of diverse surface lipoproteins. *Science.* 247(4940): 315-318.

Rosengarten, R. and Wise, K.S. (1991) The Vlp system of *Mycoplasma hyorhinis*: combinatorial expression of distinct size variant lipoproteins generating high-frequency surface antigenic variation. *J. Bacteriol.* 173(15): 4782-4793.

Rosengarten, R., Behrens, A., Stetefeld, A., Heller, M., Ahrens, M., Sachse, K., Yogev, D. and Kirchhoff, H. (1994) Antigen heterogeneity among isolates of *Mycoplasma bovis* is generated by high-frequency variation of diverse membrane surface proteins. *Infect. Immun.* 62(11): 5066-5074.

Roth, N., Käsbohrer, A., Mayrhofer, S., Zitz, U., Hofacre, C. and Domig, K.J. (2019) The application of antibiotics in broiler production and the resulting antibiotic resistance in *Escherichia coli*: A global overview. *Poul. Sci.* 98(4): 1791-1804.

Roussan, D.A., Abu-Basha, E.A. and Haddad, R.R. (2006) Control of *Mycoplasma gallisepticum* infection in commercial broiler breeder chicken flocks using tilmicosin (Provitil Powder®) oral formulation. *Int. J. Poult. Sci. 5*(10): 949-954.

Shah, M.S, Ashraf, A., Khan, M.I., Rahman, M., Habib, M., Chughtai, M.I., Qureshi, J.A. (2017) Fowl adenovirus: history, emergence, biology and development of a vaccine

against hydropericardium syndrome. *Arch Virol*. 162(7):1833-1843. doi: 10.1007/s00705-017-3313-5. PMID: 28283816.

Shoaib, M., Riaz, A., ul Hassan, M., Yousaf, A., Rehman, S.U., Zafar, M.A., Kamran, M., Amir, R.M. and Malik, A.M. (2019) Sero-Prevalence and Associated Risk Factors of *Mycoplasma Gallisepticum, Mycoplasma Synoviae* and *Salmonella Pullorum/Gallinarium* in Poultry. *Pak Vet J.* 40(2): 253-256.

Shil, P.K., Kanci, A., Browning, G.F., Marenda, M.S., Noormohammadi, A.H., Markham, P.F. (2011) GapA+ Mycoplasma gallisepticum ts-11 has improved vaccine characteristics. *Microbiology*. 6:1740-1749. doi: 10.1099/mic.0.046789-0. Epub 2011 Feb 10. PMID: 21310786.

Siddique, A.B., Rahman, S.U., Hussain, I., Muhammad, G. (2012) Frequency distribution of opportunistic avian pathogens in respiratory distress cases of poultry. *Pak Vet J*. 32 (3): 386-389.

Sikder, A.J., Islam, M.A., Rahman, M.M. and Rahman, M.B. (2005) Seroprevalence of *Salmonella* and *Mycoplasma gallisepticum* infection in the six model breeder poultry farms at Patuakhali district in Bangladesh. *Int. J. Poult. Sci.* 4(11): 905-910.

Soeripto, W., Whithear, K.G., Cottew, G.S. and Harrigan, K.E. (1989) Virulence and transmissibility of *Mycoplasma gallisepticum*. *Aust Vet J.* 66: 65-72.

Soltan, M.A., Tsai, Y.L., Lee, P.Y.A., Tsai, C.F., Chang, H.F.G., Wang, H.T.T. and Wilkes, R.P. (2016) Comparison of electron microscopy, ELISA, real time RT-PCR and insulated isothermal RT-PCR for the detection of Rotavirus group A (RVA) in feces of different animal species. *J. Virol. methods.* 235: 99-104.

Stipkovits, L. and Kempf, I. (1996) Mycoplasmoses in poultry. *Rev Sci Tech.* 15(4): 1495-1526.

Stipkovits, L., Egyed, L., Palfi, V., Beres, A., Pitlik, E., Somogyi, M., Szathmary, S. and Denes, B. (2012 a) Effect of low-pathogenicity influenza virus H3N8 infection on *Mycoplasma gallisepticum* infection of chickens. *Avian Pathol.* 41(1): 51-57.

Stipkovits, L., Glavits, R., Palfi, V., Beres, A., Egyed, L., Denes, B., Somogyi, M. and Szathmary, S. (2012 b) Pathologic lesions caused by coinfection of *Mycoplasma*

gallisepticum and H3N8 low pathogenic avian influenza virus in chickens. *Vet. Pathol.* 49(2): 273-283.

Subtain, S.M., Manzoor, S., Khan, F.M., Hussain, Z. and Mukhtar, M. (2016) Study on coinfection of *Mycoplasma gallisepticum* and low pathogenic avian influenza virus H9 in broilers. *J. Antivir. Antiretrovir*. 8: 95-99.

Sydenstricker, K.V., Dhondt, A.A., Ley, D.H., and Kollias, G.V. (2005) Re-exposure of captive house finches that recovered from *Mycoplasma gallisepticum* infection. *J. Wildl. Dis*. 41: 326-333. Doi: 10.7589/0090-3558-41.2.326.

Sydenstricker, K.V., Dhondt, A.A., Hawley, D.M., Jennelle, C. S., Kollias, H.W. and Kollias, G.V. (2006) Characterization of experimental *Mycoplasma gallisepticum* infection in captive house finch flocks. *Avian Dis* 50:39–44.

Talkington, F.D. and Kleven, S.H. (1985) Evaluation of protection against colonization of the chicken trachea following administration of Mycoplasma gallisepticum bacterin. *Avian Dis.*29: .998-1003.

Tsai, J.J., Liu, L.T., Lin, P.C., Tsai, C.Y., Chou, P.H., Tsai, Y.L., Chang, H.F.G. and Lee, P.Y.A. (2018) Validation of the Pockit dengue virus reagent set for rapid detection of dengue virus in human serum on a field-deployable PCR system. *J. Clin. Microbiol.* 56(5): e1865.

Tsai, J.J., Liu, W.L., Lin, P.C., Huang, B.Y., Tsai, C.Y., Chou, P.H., Lee, F.C., Ping, C.F., Lee, P.Y.A., Liu, L.T. and Chen, C.H. (2019) An RT-PCR panel for rapid serotyping of dengue virus serotypes 1 to 4 in human serum and mosquito on a field-deployable PCR system. *PloS one* 14(3): e0214328.

Tsai, Y.L., Lin, Y.C., Chou, P.H., Teng, P.H. and Lee, P.Y. (2012a) Detection of white spot syndrome virus by polymerase chain reaction performed under insulated isothermal conditions. *J. Virol. Methods.* 181(1): 134-137.

Tsai, Y.L., Wang, H.T.T., Chang, H.F.G., Tsai, C.F., Lin, C.K., Teng, P.H., Su, C., Jeng, C.C. and Lee, P.Y. (2012 b) Development of TaqMan probe-based insulated isothermal PCR (iiPCR) for sensitive and specific on-site pathogen detection. *PloS one* 7(9): e45278.

Tsen, H.Y., Shih, C.M., Teng, P.H., Chen, H.Y., Lin, C.W., Chiou, C.S., Wang, H.T.T., Chang, H.F.G., Chung, T.Y., Lee, P.Y. and Chiang, Y.C. (2013) Detection of Salmonella in chicken meat by insulated isothermal PCR. *J. Food Prot. 76*(8): 1322-1329

Tuck, M.K., Chan, D.W., Chia, D., Godwin, A.K., Grizzle, W.E., Krueger, K.E., Rom, W., Sanda, M., Sorbara, L., Stass, S. and Wang, W. (2009) Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. *J proteome Res. 8*(1): 113-117.

Umar, S., Munir, M.T., Ur-Rehman, Z., Subhan, S., Azam, T. and Shah, M.A.A. (2017) Mycoplasmosis in poultry: update on diagnosis and preventive measures. *World's Poultry Sci J.* 73(1): 17-28.

Usman, M., Sarwar, F., Umar, S., Subhan, S., Sohail, M.L., Younus, M. (2017) A clinical survey of viral respiratory diseases of commercıal poultry in Pakistan. *J Anim Plant Sci*. 27:2086–9.

Vogl, G., Plaickner, A., Szathmary, S., Stipkovits, L., Rosengarten, R. and Szostak, M.P. (2008) *Mycoplasma gallisepticum* invades chicken erythrocytes during infection. *Infect. Immun.* 76(1): 71-77.

Wang, W.K. and Gubler, D.J. (2018) Potential point-of-care testing for dengue virus in the field. *J. Clin. Microbiol*. *56*(5): e203.

Wanasawaeng, W., Chaichote, S., Chansiripornchai, N. (2015) Development of ELISA and serum plate agglutination for detecting antibodies of *Mycoplasma gallisepticum* using strain of Thai isolate. *Thai J Vet Med.* 45 (4): 499-507.

Whithear, K.G., Soeripto, Harrigan, K.E., Ghiocas, E. (1990a). Immunogenicity of a temperature sensitive mutant *Mycoplasma gallisepticum* vaccine. *Aust Vet J.* 67: 168-174.

Whithear, K. G., Soeripto, Harringan, K. E. & Ghiocas, E. (1990b). Safety of temperature sensitive mutant Mycoplasma gallisepticum vaccine. *Aust Vet J.* 67: 159-165.

Whithear, K.G. (1996). Control of avian mycoplasmoses by vaccination. *Rev Sci Tech* 15:1527-1553.

Whitford, H.W., Rosenbusch, R.F. and Lauerman, L.H. (1994) *Mycoplasmosis in animals: laboratory diagnosis*, Iowa State University Press, USA.

Wilkes, R.P., Anis, E., Dunbar, D., Lee, P.Y.A., Tsai, Y.L., Lee, F.C., Chang, H.F.G., Wang, H.T.T. and Graham, E.M. (2018) Rapid and sensitive insulated isothermal PCR for point-of-need feline leukaemia virus detection. *J. Felin. Med. Sur. 20*(4): 362-369.

Wilkes, R.P., Tsai, Y.L., Lee, P.Y., Lee, F.C., Chang, H.F.G. and Wang, H.T.T. (2014) Rapid and sensitive detection of canine distemper virus by one-tube reverse transcription-insulated isothermal polymerase chain reaction. *BMC Vet. Res. 10*(1): 213.

Winner F, Rosengarten R, Citti C. (2000) In Vitro Cell Invasion of *Mycoplasma gallisepticum. Infect. Immun.* 68: 4238–4244

Winner, F., Markovà, I., Much, P., Lugmair, A., Siebert-Gulle, K., Vogl, G., Rosengarten, R. and Citti, C. (2003) Phenotypic switching in *Mycoplasma gallisepticum* hemadsorption is governed by a high-frequency, reversible point mutation. *Infect. Immun. 71*(3): 1265-1273.

Yasmin, F., Ideris, A., Omar, A.R., Bejo, M.H., Islam, R., Wei, T.S., Giap, T.C., Ahmad, K. (2018) Molecular characterization of field strains of *Mycoplasma gallisepticum* in Malaysia through *pMGA* and *pVPA* genes sequencing. *Cogent Biol.* 4:1. doi: 10.1080/23312025.2018.1456738

Yoder Jr, H.W. (1986) A historical account of the diagnosis and characterization of strains of *Mycoplasma gallisepticum* of low virulence. *Avian Dis.* 30(3):510-518.

Yoder Jr, H.W. (1991) Mycoplasmosis. In: Diseases of poultry, Calnek, B.W., Burnes, H.J., Beard, C.W. and Yoder Jr, H.W. (eds), pp. 196-198. Ames: Iowa State University Press, Ames, Iowa, USA.

Yogev, D., Levisohn, S., Kleven, S.H., Halachmi, D. and Razin, S. (1988) Ribosomal RNA gene probes to detect intraspecies heterogeneity in Mycoplasma gallisepticum and M. synoviae. *Avian Dis.* 32(2): 220-231.

Yogev, D., Menaker, D., Strutzberg, K., Levisohn, S., Kirchhoff, H., Hinz, K.H. and Rosengarten, R. (1994) A surface epitope undergoing high-frequency phase

variation is shared by Mycoplasma gallisepticum and Mycoplasma bovis. *Infect. Immun. 62*(11): 4962-4968.

Yogev, D., Rosengarten, R., Watson-McKown, R. and Wise, K.S. (1991) Molecular basis of Mycoplasma surface antigenic variation: a novel set of divergent genes undergo spontaneous mutation of periodic coding regions and 5′ regulatory sequences. *The EMBO Journal.* 10(13): 4069-4079.

Zhang, J., Nfon, C., Tsai, C.F., Lee, C.H., Fredericks, L., Chen, Q., Sinha, A., Bade, S., Harmon, K., Piñeyro, P. and Gauger, P. (2019) Development and evaluation of a real-time RT-PCR and a field-deployable RT-insulated isothermal PCR for the detection of Seneca Valley virus. *BMC Vet. Res. 15*(1): 1-11.

ANNEXURE-1

Phosphate Buffer Saline (0.01M) pH 7.3

NaCl	80.0g
KCl	2.0g
Sodium phosphate, dibasic anhydrous (NaH_2PO_4)	11.5g
Sodium phosphate, monobasic anhydrous ($Na_2HPO_4.H_2O$)	21.7g (substituted)
DdH_2O	10Liter

ANNEXURE -II

5X – Tris Borate EDTA Buffer (TBE)

Stock solution of 5X/liter was prepared by adding the following:

TRIS Pure (Research Organics, Cat # 30950T)	54 gm
Boric Acid (Fisher Scientific, Cas # 10043-35-3)	27.5 gm
0.5M EDTA (pH 8.00) (MP Biomedicals, Cat# 195173)	20 ml

The above mentioned chemicals were dissolved in 980 ml of pure distilled water.

Working solution was prepared as follows:

1X TBE was prepared by dissolving 200 ml of 5X Stock solution in 800 ml of distilled water.

ANNEXURE-III

0.5M EDTA pH 8.00 (Ethylene Diamine Tetra Acetic Acid)

EDTA	186.1 gm
Water	80 ml

186.1 gm of EDTA (MP Biomedicals, Cat# 195173) was added to 80ml of pure water. It was stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.00 with NaOH

(Merck) pellets (20 gm of NaOH pellets). The solution was dispensed in aliquots after being autoclaved at 121°C for 15 minutes.

ANNEXURE-IV

Agarose Gel (1%) for Electrophoresis

Agarose	1 gm
1X TBE Buffer	100 ml
Ethidium Bromide	6-8µl

1gm of Agarose was dissolved in 100 ml of 1X TBE buffer and 6-8µl of ethidium bromide. The mixture was heated until it boiled and was poured in a mould to set with a gel comb.

ANNEXURE-V

Ethidium Bromide (10mg/ml)

Ethidium Bromide	1gm
Water	100ml

1 gm of ethidium bromide was added to 100ml of water. Mixture was stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The container was wrapped in aluminium foil or transferred to a dark bottle and stored at 4 C.

ANNEXURE- VI

Loading Solution for Agarose Gel Electrophoresis

PCR product	10 µl
Bromophenol Blue	2 µl

ANNEXURE- VII

DNA step ladder

Distilled Water	10 µl
Bromophenol Blue	2 µl

DNA Step ladder 2 µl

ANNEXURE-VIII

Primer sequences	**Sequence (5' to 3')**
lp F	GGATCCCATCTCGACCACGAGAAAA
lp R	CCAGGCATTTAAAAATCCCAAAGACC
gapA F	TTCTAGCGCTTTAGCCCTAAACCC
gapA R	CTTGTGGAACAGCAACGTATTCGC
pvpA F	GCCAMTCCAACTCAACAAGCTGA
pvpA R	GGACGTSGTCCTGGCTGGTTAGC
mgc2 F	GCTTTGTGTTCTCGGGTGCTA
mgc2 R	CGGTGGAAAACCAGCTCTTG

Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2020.079

RESEARCH ARTICLE

Biological Characterization of Locally Circulating *Mycoplasma gallisepticum* in Poultry

Saba Farooq[1,2], Aamer Ali Shah[1], Naila Siddique[2], Saba Rafique[2], Adeela Sharif[2], Muhammad Athar Abbas[2] and Khalid Naeem[2*]

[1]Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad; [2]National Reference Lab for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Centre, Islamabad, Pakistan
*Corresponding author: naeem1955@gmail.com

ARTICLE HISTORY (20-237)

Received: May 08, 2020
Revised: August 29, 2020
Accepted: August 30, 2020
Published online: September 13, 2020

Key words:
Air sacculitis
Dissemination
Dissemination pattern
Seroconversion
Tracheal lesions
Virulence

ABSTRACT

Mycoplasma gallisepticum (MG) is a pathogen of concern for poultry. Present study was conducted to determine the biological characteristics of a field isolate of MG, recovered from an MG-affected flock. This isolation was made through conventional method of MG cultivation, using modified Frey's media after confirming the isolate by polymerase chain reaction (PCR). A total of 48 birds were segregated into experimental group (32 birds) and the control group (16 birds). To appraise primary site of infection, MG broth propagated culture containing $1x10^6$ CFU/ml was inoculated intratracheally to each bird in the experimental group, whereas the control group was sham inoculated by uninoculated broth. The clinical signs and symptoms were recorded daily from day 1 to 21 post-infection (p.i.). Seroconversion monitoring was carried out, at day 5, 10, 15, 20 p.i. by Serum Plate Agglutination test (SPA) and Enzyme Linked Immunosorbent Assay (ELISA). To determine the dissemination pattern of MG, birds were sacrificed according to plan, swabbed from various organs and subjected to MG-specific PCR. Tracheal lesions and air sac lesions were scored after necropsy. Clinically, mild signs of respiratory discomfort were observed on day 5 p.i., which intensified on day 9 to 21 p.i. in the experimental group. PCR of tracheal swab samples was positive from day 7 to 21 p.i., and the swabs collected from lungs were positive for MG from day 9 to 21 p.i. The study concluded that, MG isolate from field showed limited dissemination pattern and is restricted to respiratory tract.

©2020 PVJ. All rights reserved

To Cite This Article: Farooq S, Shah AA, Siddique N, Rafique S, Sharif A, Abbas MA and Naeem K, 2020. Biological characterization of locally circulating *Mycoplasma gallisepticum* in poultry. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2020.079

INTRODUCTION

Among various poultry pathogens responsible for respiratory tract infections, *Mycoplasma gallisepticum* (MG) is incriminated as an organism having the ability to persist for long duration once it has infected the host.

Infections caused by MG ranges from mild respiratory illness to chronic respiratory disease when complicated with other co-infecting pathogens (Levisohn and Kleven, 2000). Host and environmental factors play an important role in regulating pathogenesis of the disease. Exposition of invasion process of MG in non-phagocytic cells including chicken embryo fibroblast and HeLa-229 and ability to survive in intracellular spaces, provided an insight about the mechanism of evasion of host defences, limited effect of anti-mycoplasma therapy and potential to cause systemic infection (Winner *et al.*, 2000; Fürnkranz *et al.*, 2013).

Respiratory tract infections caused by MG involves colonization of upper respiratory tract, which can further lead to inflammation of trachea and air sacculitis. Earlier studies reporting arthritis, salpingitis, conjunctivitis, meningoencephelopathy in chicken and turkeys suggested that the organism is not restricted to respiratory tract only. Experimental infection by pathogenic MG R strain provided evidence of systemic spread to the heart, brain, liver, spleen, and kidneys, unveiling the potential of MG to cross mucosal barrier of respiratory tract and dissemination to internal body organs (Much *et al.*, 2002; Vogl *et al.*, 2008; Ramadan, 2019).

Marked differences have been observed in infectivity potential of different strains of MG. In case of experimental infections, it varies with route of inoculation, type of MG strain and number of passages of the strains used for challenge (Levisohn and Kleven, 2000).