MOLECULAR SEROTYPING OF *Avibacterium paragallinarum* AND DETECTION OF THE GENETIC DETERMINANTS OF ITS VIRULENCE FACTORS

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CERTIFICATE

Dr. SHAIK MOHAMMED UMAR, I.D. No: GVM/2018 – 031 *has satisfactorily prosecuted the course of research and that the thesis entitled* **"MOLECULAR SEROTYPING OF *Avibacterium paragallinarum* AND DETECTION OF THE GENETIC DETERMINANTS OF ITS VIRULENCE FACTORS"** *submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any university.*

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This is to certify that the thesis entitled "**MOLECULAR SEROTYPING OF *Avibacterium paragallinarum* AND DETECTION OF THE GENETIC DETERMINANTS OF ITS VIRULENCE FACTORS"** submitted in partial fulfillment of the requirements for the degree of "**MASTER OF VETERINARY SCIENCE"** of the **SRI VENKATESWARA VETERINARY UNIVERSITY, TIRUPATI**, is a record of the bonafide research work carried out by **Dr. SHAIK MOHAMMED UMAR, I.D. No: GVM/2018-031** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of the investigations have been duly acknowledged by the author of the thesis.

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Shaik Mohammed Umar

DECLARATION

I, SHAIK MOHAMMED UMAR hereby declare that the thesis entitled **"MOLECULAR SEROTYPING OF *Avibacterium paragallinarum* AND DETECTION OF THE GENETIC DETERMINANTS OF ITS VIRULENCE FACTORS "** submitted to Sri Venkateswara Veterinary University, Tirupati for the degree of **MASTER OF VETERINARY SCIENCE** is the result of original research work done by me. I also declare that the materials contained in this thesis have not been published earlier.

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ABSTRACT

Infectious coryza caused by *Avibacterium paragallinarum* is a highly contagious respiratory infection in poultry affecting the upper respiratory tract with the involvement of nasal passages, infra orbital and paranasal sinuses of chickens.

In the present investigation samples were collected from suspected cases of infectious coryza in commercial poultry farms and backyard poultry in East and West Godavari districts of Andhra Pradesh, for detection of *A. paragallinarum.* To detect *A. paragallinarum*, the samples were subjected to isolation by culture coupled with biochemical tests. Also, direct swab PCR test and culture PCR test were conducted

with the samples. Out of total 150 samples collected, 35 samples did not yield any result specific to *A. paragallinarum* as they were processed after six months of their collection due to COVID 19 national lockdown. The remaining 115 samples were processed immediately after collection to detect *A. paragallinarum*. A total of twelve samples (B1, B2, S1, S2, J1, J2, BA1, BA2, BA3, BA4, BA5 and BA6) were found to be positive for *A. paragallinarum* in cultural (with satellitism) and biochemical tests.

In both the culture PCR test and direct swab PCR test with oligonucleotide primers specific to *A. paragallinarum*, all the 12 isolates yielded a specific PCR product of 500 bp. The direct swab PCR test is found to be sensitive to detect *A. paragallinarum* in samples collected from infectious coryza cases, without the need of culture step, which is very helpful in rapid diagnosis.

In PCR test for detecting genetic determinant of putative virulence factor haemagglutinin (*hagA*) only 4 isolates of *A. paragallinarum viz.* B1, B2, J1 and J2 yielded specific PCR product of 900 bp. However, the genetic determinant of iron acquisition protein (*fur*) was not detected in all the 12 isolates of *A. paragallinarum*.

In molecular serotyping by multiplex PCR (mPCR) test using oligonucleotide primers specific to Page serotypes A, B and C, 8 isolates of *A. paragallinarum viz.* B1, B2, S1, S2, J1, J2, BA3 and BA4 were identified as serotype A with a PCR product of 372 bp, though the *A. paragallinarum* vaccine used as positive control in this study yielded PCR products of 372 bp and 800 bp. The remaining 4 isolates *viz.* BA1, BA2, BA5 and BA6 were identified as serotype B with a PCR product of 1100 bp. None of the *A. paragallinarum* isolates in this study were identified as serotype C.

The results of the present investigation with the molecular serotyping indicates that serotype A and B of *A. paragallinarum* are circulating in East Godavari and West Godavari Districts of A.P. However, further research is required with large number of samples to identify the circulating serotypes of *A. paragallinarum* in A.P.

LIST OF THE ABBREVIATIONS

BAHS	:	Basic Animal Husbandry Statistics
USD	:	United State Dollar
IC	:	Infectious coryza
hagA	:	Haemagglutinin gene
fur	:	Ferric uptake regulator gene
RTX	:	Repeat in toxin
NAD	:	Nicotinamide Adenine Dinucleotide
%	:	Percent
ºC	:	Degree Centigrade
M	:	Molar
mA	:	Milli ampere
Mm	:	Millimolar
pmole	:	picomole(s)
TBE	:	Tris borate EDTA buffer
bp	:	base pairs
et al	:	And associates or co workers
g	:	gram
ng	:	nano gram
DNA	:	Deoxyribo nucleic acid
PBS	:	Phosphate buffered saline
G-PBS	:	Glycerol- phosphate buffer saline
HI	:	Haemagglutination inhibition
µg	:	microgram
µl	:	microlitre
µm	:	micrometre
µg/ml	:	microgram per millilitre
mg	:	milligram
ml	:	millilitre
mm	:	millimeter
nm	:	nanometer
No.	:	Number
n	:	Number of isolates

PCR	:	Polymerase Chain Reaction
mPCR	:	Multiplex Polymerase Chain Reaction
rpm	:	Revolutions per minute
U.V.	:	Ultraviolet
V	:	Volts
viz.	:	*Videlicet*
etc.	:	Etcetera
i.e.	:	That is
EDTA	:	Ethylene diamine tetra acetic acid
CO_2	:	Carbon dioxide
ERIC	:	Enterobacterial Repetitive Intergenic Consensus
RFLP	:	Restriction Fragment Length Polymorphism
kbp	:	Kilobase pair
v/v	:	Volume per volume
w/v	:	Weight per volume
min	:	Minutes
sec	:	Seconds
spp.	:	Species
USA	:	United States of America
UK	:	United Kingdom
H_2O_2	:	Hydrogen peroxide

CHAPTER-I

INTRODUCTION

INTRODUCTION

The poultry industry in India is an exciting agribusiness sector. All India poultry breeders association reports that poultry contributes for USD 17.31 billion of total India's gross value. India ranks 3rd in egg production and 4th in meat production (BAHS 2019). In the poultry sector, broiler segment constitutes about 65.3% with the monthly turnover of 400 million chicks and layer segment constitutes about 34.7% with the monthly turnover of 8,400 million eggs (Kolluri *et al*., 2020). Both the intensive and backyard poultry farming is important in present settings in India. According to Department of Animal Husbandry and Dairying, Govt. of India (2019) current poultry population in India is 851.81 million.

Many bacteria and viruses are responsible for economically devastating diseases in poultry. Among different microbial infections that affect poultry, infectious coryza caused by the bacterium *Haemophilus paragallinarum* is a highly contagious respiratory infection affecting the upper respiratory tract including the involvement of nasal passages, infra orbital and paranasal sinuses (Blackall *et al*., 1990). Subsequently the *Haemophilus paragallinarum* was renamed as *Avibacterium paragallinarum* (Blackall *et al*., 2005). The infectious coryza disease is a major problem of commercial poultry and it is significant in broilers as well as in the layers. In developing countries like India, infectious coryza in poultry is usually complicated by the presence of a range of other secondary infections, resulting in severe disease and significant economic losses. Many reports are available on infectious coryza in Indian poultry (Ravikumar *et al*., 2020).

Infectious coryza causes major economic losses in the poultry industry due to increased culling rates and reduction in egg production up to 40% (Blackall and Vargas, 2020). Mortalities can be as high as 48% (Bland *et al.,* 2002). The chicken is

the natural host for *A. paragallinarum*. All ages of chickens can be infected, but the disease is usually more severe in older chickens.

A. paragallinarum is Gram negative, nonmotile, filamentous, pleomorphic and forms small grey colonies on chocolate agar (Mendoza-Espinoza *et al.,* 2008). Though nicotinamide adenine dinucleotide (NAD) supplement is required for the *in vitro* culture/growth of *A. paragallinarum*, growth of certain isolates without the requirement of NAD supplement is also reported (Garcia *et al.,* 2004, Rajurkar *et al.,* 2009 and Blackall *et al*., 2011). Page and Kume serotyping schemes are currently followed for serotyping of *A. paragallinarum* isolates (Blackall, 1999). In the Page agglutination scheme a total of three serovars namely serovars A, B and C are recognized (Page, 1962). In the Kume scheme seven serovars (HA1 to HA7) of *A. paragallinarum* were identified and were distributed into three serogroups (I, II and III) (Kume *et al.,* 1983). The procedures to serotype *A. paragallinarum* isolates by any of these two serotyping schemes are complicated and require the use of production of hemagglutinating antigens, reference antisera, and the use of chicken erythrocytes fixed with glutaraldehyde.

A. paragallinarum, is a slow growing haemophilic organism, requires NAD (V-factor) for growth in complex media and difficult to grow as pure culture *in-vitro*. To overcome such difficulties associated with conventional diagnostic methods, alternative approaches with molecular diagnostics such as polymerase chain reaction (PCR) test for diagnosis, multiplex PCR (mPCR) for serotyping (Sakamoto *et al*., 2012, Morales-Erasto *et al*., 2014), and '*hagA*' gene detection/sequencing (Miflin *et al*., 1995, Anjaneya *et al*., 2014a and Garcia-Sanchez *et al*., 2014) have been proved to be useful for specific and sensitive identification of *A. paragallinarum.*

The virulence factors of *A. paragallinarum* are not clearly understood. The role of capsule, hemagglutinin, hemocins, lipopolysaccharide, and iron acquisition proteins

RTX (repeat in toxin)-toxin, and cytolethal distending toxin in the virulence of *A. paragallinarum* was investigated. (Negrete Abscal *et al*., 2009, Liu *et al.*, 2016 and Chukiatsiri *et al*., 2012).

As far as Indian scenario is concerned, infectious coryza is one of the major problems affecting commercial poultry industry since its first report (Rao, 1958). However, only few studies had been conducted with regard to laboratory isolation and identification of *A. paragallinarum* from the morphoculturally related organisms from the clinical/field cases (Rajurkar *et al*., 2009). However, details about the serotypes of the circulating isolates of *A. paragallinarum* and the associated factors of its virulence are not widely studied by molecular characterization. Hence the present investigation is carried out with following objectives:

1. To isolate and identify *Avibacterium paragallinarum* from poultry.
2. To carry out molecular serotyping of *Avibacterium paragallinarum* isolates.
3. To identify the genetic determinants of selected virulence factors in *Avibacterium paragallinarum* isolates.
4. To analyze the association, if any, between selected virulence factors and identified serotypes of *Avibacterium paragallinarum*.

CHAPTER-II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.0 Poultry sector

The poultry sector is growing at a much faster rate than any other element of the agriculture and livestock sector. All India Poultry Breeders Association indicates that poultry contributes for USD 17.31 billion of total India's gross value. India ranks 3rd in egg production and 4th in meat production (BAHS 2019). In the poultry sector, broiler segment constitutes about 65.3% with the monthly turnover of 400 million chicks and layer segment constitutes about 34.7% with the monthly turnover of 8,400 million eggs (Kolluri *et al*., 2020). Both the intensive and backyard poultry farming is important in present settings in India. According to Department of Animal Husbandry and Dairying, Govt. of India (2019) current poultry population in India is 851.81 million. However, chickens are susceptible to many infectious diseases caused by bacteria, viruses and mycoplasma sometimes resulting in heavy mortality / production loss resulting in huge economic loss to poultry industry.

2.1 Infectious coryza

Infectious coryza is a respiratory disease of chickens caused by the bacterium *Avibacterium paragallinarum*. This organism was initially named as *Haemophilus paragallinarum* and was reclassified as *A. paragallinarum* by Blackall *et al*., (2005), based on results obtained through 16S rDNA sequencing.

The first haemophilic organisms that caused upper respiratory tract disease in chickens were isolated by De Blieck, (1932) and was termed *Bacillus haemoglobnophilus coryza gallinarum*. The causative agent of infectious coryza was later renamed *Haemophilus gallinarum* (Elliot and Lewis, 1934). This organism was described as Gram negative, non-motile short rods as well as coccobacilli, by Beach and Schalm, (1936). It is a slow growing, fastidious organism that shows the tendency

to form filaments. Later it was renamed to *Haemophilus paragallinarum* due to being X-factor independent and V-factor dependent (Page, 1962 and Blackall and Yamamoto, 1989). In 2005 its name was changed again and termed *Avibacterium paragallinarum* (Blackall *et al*., 2005).

2.1.1 Classification of *Avibacterium paragallinarum*

Avibacterium paragallinarum belongs to the family *Pasteurellaceae* (Bisgaard, 1993). Blackall *et al*., (2005) conducted phylogenetic experiments on the 16S rDNA of the *Pasteurellaceae* family. The results showed that *H. paragallinarum, Pasteurella gallinarum, Pasteurella avium* and *Pasteurella volantium* formed a monophyletic group with 96.8% sequence. Based on these findings *H. paragallinarum* was reclassified into a new genus *Avibacterium* (Blackall *et al*., 2005). Phenotypic and genotypic testing supported the reclassification of the separate and distinct nature of this subcluster into the new genus *Avibacterium* (Blackall *et al*., 2005).

2.1.2 Effects of infectious coryza on poultry

Infectious coryza is an upper respiratory tract disease that occurs primarily in chickens. It is generally an acute, but can be a chronic disease (Yamamoto, 1984). Infectious coryza results in about 20-50% morbidity and 5-20% mortality in infected chickens (Chen *et al.,* 1993). This disease is of economic importance especially in the poultry industry as it causes a decrease in egg production and an increase in unthrifty chickens (Chen *et al*., 1996).

Blackall, (1999) reported that infectious coryza is transmitted through drinking water and is airborne over short distances. Replacement stocks are a major source of infection. Clinical symptoms associated with this disease include nasal discharge, facial swelling, lacrimation, anorexia and diarrhoea.

The disease characteristically has a short incubation period of 24 to 48 hours. Clinical signs in susceptible birds that were exposed to infected birds usually show

signs of the disease in 1 to 3 days. Birds of all ages are susceptible, with less severity in juvenile birds. In mature birds, especially laying hens, the incubation period is shortened, and the duration of the disease is longer. This results in growth retardation, weight loss and an increased number of culls (Yamamoto, 1984).

2.2 Isolation of *Avibacterium paragallinarum*

A. paragallinarum is a fastidious, slow-growing organism. Hence, it is often overgrown by other, faster-growing commensals. Biochemical characterization requires the availability of specialized, expensive media that can support the growth of NAD-dependent bacteria (Blackall, 1999).

Routine isolation and biochemical characterisation are often neglected in developing countries because it requires the availability of specialised complex media and difficult to obtain in pure cultures due to slow growth (Byarugaba *et al*., 2006). *A. paragallinarum* is commonly grown in an atmosphere of 5% carbon dioxide; however, carbon dioxide is not an essential requirement, because the organism is able to grow under reduced oxygen tension or anaerobically (Blackall and Vargas, 2020).

2.2.1 Bacteriological media and supplements

The reduced form of nicotinamide adenine dinucleotide (NAD) (NADH; 1.56–25 μg/mL medium or its oxidized form (20–100 μg/mL) is necessary for the *in vitro* growth of most isolates of *A. paragallinarum.* Sodium chloride (NaCl) (1.0–1.5%) is essential for growth of *A. paragallinarum*. Chicken serum (1%) is required by some strains whereas others merely show improved growth with this supplement (Blackall and Vargas, 2020). Brain heart infusion, tryptose agar, and chicken-meat infusion are some basal media to which supplements are added. Complex media are often used to obtain dense growth of organisms for characterization studies (Blackall and Vargas, 2020).

Thjotta and Avery, (1921) had introduced the term X and V factors for the growth requirement of *Haemophilus spp.* McGaughey, (1932), demonstrated that avian *Haemophili* strains required only V factor (nicotinamide adenine dinucleotide). In contrast, Delaplane *et al.*, (1938) reported that their isolates required both X (hemin) and V (NAD) factors. However, Page, (1962) found that none of the *Haemophilus* strains required hemin for growth, but all the strains required NAD for the growth. Biberstein and White, (1969) proposed that isolates from chickens that did not require haem for growth should be called *H. paragallinarum*. Blackall and Reid, (1982), Blackall and Yamamoto, (1989) and Yamamoto, (1991) also endorsed the requirement of NAD for the growth of *H. paragallinarum*.

The main characteristics that differentiate the NAD-independent from the NAD dependent *H. paragallinarum* are that the former does not have beta-galactosidase activity and does not ferment maltose (Mouahid *et al.,* 1992). Bragg *et al*., (1996) also vindicated in their studies that only some isolates causing infectious coryza disease in birds require V factor thereby reported that *H. paragallinarum* could be either V factor dependant or independent.

The NAD independent *H. paragallinarum* of serovar B was first reported by Garcia *et al.,* (2004). The isolates were NAD independent growing on blood agar without the need of a nurse colony as well as on a complex medium that lacked both NAD and chicken serum. Both isolates were pathogenic, causing the typical clinical signs of infectious coryza in susceptible chickens. One isolate was Page serovar B/Kume serovar B-1 and the other isolate was Page serovar C/Kume serovar C-2.

2.2.2 Culture conditions

Page *et al.,* (1963) reported that the minimum and maximum temperatures of growth are 25°C and 45°C respectively, the optimal range being 34 to 42°C. Tiny dew drop colonies up to 0.3 mm in diameter develop on suitable media. In obliquely

transmitted light, mucoid (smooth) iridescent and rough non iridescent and other intermediate colony forms have been observed (Sawata *et al*., 1983 and Blackall and Vargas, 2020).

Rajurkur, (2006) isolated *A. paragallinarum by using* haemophilus broth, haemophilus agar (HA) under reduced oxygen tension as a primary culture medium for preliminary isolation. Satellitism phenomenon was observed on blood agar with cross streaking of feeder culture of *S. aureus*. The colonies of *A. paragallinarum* were observed just near the feeder culture growth with shiny silver colour.

Hsu *et al*., (2007), Mendoza-Espiona *et al*., (2008) and Cabrera *et al*., (2011) used chocolate agar, blood agar and brain heart infusion (BHI) broth supplemented with serum and different concentration of NAD at 5% CO_2 for 18 to 24 hours for isolation of *A. paragallinarum.* Calderon *et al.,* (2010) collected samples from infraorbital sinuses of chickens and streaked on 10% sheep blood agar with *S. aureus* as a feeder colony, with incubation in candle jar at 37ºC for 18 hours. Cabrera *et al*., (2011) used *S. epidermidis* as a nurse colony for satellitism phenomenon.

Durairajan *et al.,* (2013) used culture media such as brain heart infusion broth, blood agar, Levinthals medium agar (LMA) (supplemented with 0.02% NAD and 2.5% Agar) for isolation and colony characterization of *A. paragallinarum*. Caseous, cheesy material was collected and inoculated on to the LMA and blood agar plate with *S. aureus*.

Anjaneya *et al*., (2014b) initially used blood agar (5% v/v sheep blood) with haemolytic *Staphylococcus* species, as feeder culture for preliminary isolation of *A. paragallinarum*. For further sub culturing and study, haemophilus agar, haemophilus broth and brain heart infusion (BHI) broth were used. Badouei *et al.,* (2014) used of Columbia lysed blood agar (CLBA) medium with and without *S. epidermidis* cross streak.

Patil *et al.,* (2016) used blood tryptose agar (BTA) cross streaked with *S. aureus* to observe satellitism phenomenon. Dew drop colonies with satellite growth were observed. The pure colonies of isolates were inoculated in brain heart infusion agar supplemented with 1% Sodium Chloride, 0.0025% (W/V) of reduced NAD and 1% (V/V) filter sterilized chicken serum and incubated under reduced O_2 tension for 48 hours. All the retrieved isolates from storage were maintained on test medium agar supplemented with chicken serum and NADH (TM/SN) (Patil *et al.,* 2017).

Balouria *et al.,* (2019) and Ahmed *et al.,* (2020) isolated *A. paragallinarum* by using Hemophilus test medium (HTM) broth and agar supplemented with 1% (v/v) filter-sterilized, heat-inactivated horse serum and 0.0025% (w/v) of reduced NAD. Feberwee *et al.,* (2019), Byukusenge *et al*., (2020), Mei *et al*., (2020), Muhammad *et al*., (2020) and Ruthra *et al*., (2020) reported isolation of *A. paragallinarum* on different selective and enriched bacteriological media.

2.3 Biochemical characterization of *A. paragallinarum*

Yamamoto, (1984) reported that *A. paragallinarum* could ferment fructose, glucose and mannose but not trehalose or galactose. However, Blackall, (1989) found that hydrogen sulfide and indole were not produced, gelatine was not liquefied, and litmus and methylene blue milk were not changed. *A. paragallinarum* can ferment maltose, D-mannitol and D-sorbitol. But the failure to ferment either galactose or trehalose and its lack of catalase activity clearly separates this organism from other members of the genus (Blackall and Yamamoto, 1998).

Prabhakar *et al*., (1998) carried out fermentation tests on sugars *viz*. fructose, glucose, mannose, galactose and trehalose by *A. paragallinarum* isolates. Blackall and Soriano, (2013) concluded that the failure of *A. paragallinarum* to ferment either galactose or trehalose and its lack of catalase clearly separate this organism from the other members of the genus.

Sameera *et al.,* (2001), Tongaonkar *et al*., (2003), Jaswinder-Kaur *et al*., (2004) Deshmuk *et al*., (2015) and Fedway *et al*., (2016) also reported about the fermentation activity of *A. paragallinarum* on carbohydrates and other specific biochemical activities.

2.4 Polymerase Chain Reaction (PCR) test for detecting *Avibacterium paragallinarum*

Confirmatory diagnosis of *A. paragallinarum* with conventional cultural and biochemical tests has become a difficult task due to the factors like co-occurrence of combined respiratory infections, occurrence of NAD independent strains, overgrowth of fast-growing bacteria that mask the growth of *A. paragallinarum*, and requirement of special media for culturing etc. Hence, nucleic acid-based techniques are the best alternative tools in the easy and rapid confirmatory diagnosis (Anjaneya *et al*., 2014a and Blackall and Vargas, 2020).

Chen *et al*., (1996) first described the application of the polymerase chain reaction (PCR) test for diagnosis of infectious coryza. The results of the study strongly suggested the PCR test as an alternative tool for the diagnosis of infectious coryza where Chen *et al*., (1998) further recommended the PCR test rather culture test for situations like already commencement of antibiotic treatment or difficulties in transport of samples.

Conventional PCR test targeting the hemagglutinin (HA) gene was developed specifically for identification of *A. paragallinarum*. The PCR, termed the HP-2 PCR, has been validated for use on colonies on agar or on mucus obtained from squeezing the sinus of live birds. This test yielded rapid results (within 6 hours) compared to conventional techniques that take days together. (Chen *et al.,* 1996, 1998). Direct PCR examination of sinus swabs outperformed traditional culture when used on routine diagnostic submissions. The HP-2 PCR is a robust test, sinus swabs from

A. paragallinarum infected birds stored for up to 180 days at 4°C or -20°C could also be detected positive in the PCR test (Chen *et al.,* 1998).

Khan *et al*., (1998) observed that Enterobacterial Repetitive Intergenic Consensus-based polymerase chain reaction (ERIC-PCR) technique was useful for typing and differentiation of *H. paragallinarum* isolates. The PCR test is specific and sensitive, able to accurately distinguish between *A. paragallinarum* and *Ornithobacterium rhinotracheale* (Miflin *et al*., 1999), also NAD^+-dependent and NAD^+-independent isolates of *A. paragallinarum* (Corney *et al*., 2008). Mendoza-Espiona *et al*., (2008) developed Restriction Fragment Length Polymorphism (RFLP) of 16S ribosomal genes amplified by a PCR, referred as Amplified 16S Ribosomal DNA Restriction Analysis (ARDRA), to diagnose infectious coryza.

Byarugaba *et al*., (2006) investigated the occurrence of *A. paragallinarum* in Uganda and reported that the PCR test detected more positive samples (16%) compared to cultural examination (7%). Further it was concluded that PCR is a better diagnostic tool compared to other methods. Blackall, (2011) also advocated use of PCR test as the best approach to diagnose the infectious coryza.

Blackall and Soriano, (2013) used DNA fingerprinting by restriction endonuclease analysis, which has been shown to be a suitable typing technique providing useful insights in epidemiologic studies of *A. paragallinarum,*

In India Anjaneya *et al*., (2014b), Muhammad and Sreedevi (2015), Patil *et al*., (2017), Dwivedi *et al*., (2018), Ravikumar *et al*., (2020) and Ruthra *et al*., (2020) identified *A. paragallinarum* by PCR based molecular techniques.

Corney *et al*., (2008) reported the use of Taq man probe based real-time PCR assay for diagnosis of *A. paragallinarum*. Feberwee *et al*., (2019) and Clothier *et al*.,

(2019) also reported about the significant advantages of this real time PCR test over conventional PCR test.

2.5 Serotyping of *A. paragallinarum* isolates

The serotyping of *A. paragallinarum* isolates is conducted by either conventional means using specific antisera (antibodies) or by molecular serotyping with PCR test using serotype specific oligonucleotide primers.

2.5.1 Conventional serotyping

2.5.1.1 Page scheme

Page, (1962) classified isolates of *H. paragallinarum* as serotypes A, B and C with the plate agglutination tests using whole cells and chicken antisera. Terozolo *et al.,* (1993) studied serological properties of *H. paragallinarum* isolates and serotyped according to page scheme. Out of twenty-four isolates ten isolates were serovar A, 11 were serovar B, one was serovar C, and the remaining two isolates could not be serotyped. Fernandez *et al*., (2000) conducted serotyping of all isolates of *A. paragallinarum* by the Page scheme and assigned 21 isolates to serogroup A, five isolates to serogroup B and 14 isolates to serogroup C.

In India Tongaonkar *et al*., (2003) conducted serotyping of *A. paragallinarum* isolates obtained from major poultry regions of India by Page scheme and reported that the isolates belong to either Page serovar A (18 isolates) or Page serovar C (18 isolates).

2.5.1.2 Kume scheme

An alternative serological classification of *H. paragallinarum* was developed by Kume *et al*., (1983) based on haemagglutination inhibition (HI) test using potassium thiocyanate treated and sonicated cells, rabbit hyper immune sera and gluteraldehyde fixed chicken erythrocytes. Many isolates that were non-typable in the Page scheme were easily typed using the Kume scheme (Eaves *et al*., 1989).

The modified Kume scheme consists of serogroups A, B and C which match the Page serovars A, B and C and the nine currently recognized Kume serovars are A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, and C-4 (Blackall *et al*., 1990). Soriano *et al.*, (2001) conducted a study to determine the occurrence of Kume serovars of *H. paragallinarum* in Mexico. Caberera *et al*., (2011) studied a total of 28 isolates of *A. paragallinarum* from Ecuador and serotyped them by the hemagglutinin scheme which recognized nine serovars. Wahyuni *et al*., (2018) conducted serotyping on three *A. paragallinarum* isolates according to the Kume's scheme by using antisera and showed that all three isolates were serovar B.

2.5.2 Molecular serotyping

Soriano *et al*., (2004) reported molecular serotyping of *A. paragallinarum*. A method developed by Sakamoto *et al*., (2012) by using multiplex PCR (mPCR)) and PCR-RFLP was successful in serotyping the isolates into the Page serovars A, B and C. Hyper variable region of the gene *HMTp210* that codes for haemagglutinating antigen was used as the target in both PCR assays. Both the mPCR and PCR-RFLP provided high accuracy for rapid serotyping of *A. paragallinarum*..

Han *et al*., (2016) conducted mPCR test for molecular serotyping of *A. paragallinarum*, with suitable modifications to the technique described by Sakamoto *et al*., (2012). Many reports are available about the application of mPCR test for molecular serotyping of *A. paragallinarum* (Morales-Erasto *et al*., 2014, Nouri *et al*., 2014, Fedway *et al*., 2016, Patil *et al*., 2017, Sarika *et al*., 2019 and Muhammad *et al*., 2020).

2.6 Virulence factors of *A. paragallinarum*

The study of *A. paragallinarum* genome and its virulence factors (hemagglutinin antigen, capsule, lipopolysaccharide, and *RTX* toxin) is important to better understand the pathogenesis of infectious coryza (Aguilar-Bultet *et al.*, 2013).

Usually, virulence factor-coding genes are located in genomic islands (GIs) comprising clusters of genes suspected to have a horizontal origin (integrons, transposons, integrative and conjugative elements, and prophages (Sui *et al*., 2009).

2.6.1 Detection of genetic determinants of virulence factors of *A. paragallinarum*

Negrete Abascal *et al*., (2009) cultured *A. paragallinarum* reference strain 0083 (serovar A) in an iron-restricted culture medium and compared with isolates grown in normal medium for the expression of the 60, 68 and 93 kDa outer membrane proteins. Expression of these proteins increased in isolates grown under iron restricted medium. Sera of chickens experimentally infected were recognized these iron restriction induced proteins, suggesting their expression *in vivo*. The three outer membrane proteins were identified as transferrin receptor and iron transport proteins by mass spectroscopy and are reported to be regulated by the *fur* protein in many bacteria.

Pan *et al.,* (2012) conducted whole-genome sequencing analysis and identified that *A. paragallinarum* strain H18 contains an *RTX* toxin–like operon with strong similarity to the *RTX* operons of other members of the *Pasteurellaceae*. Four genes, designated *avxIC, avxIA, avxIB, and avxID,* were found in this operon. A PCR test and sequencing analysis on field isolates of *A. paragallinarum* revealed that the *avxIA* gene was present in all isolates. Sera collected from chickens exposed to *A. paragallinarum* exhibited strong reactivity to the *avxIA* protein, which suggests that *avxIA* is immunogenic. Kung *et al*., (2013) reported that *avxA* is a common major virulence in all serotypes *A. paragallinarum*.

Anjaneya *et al*., (2014a) studied role of *A. paragallinarum* isolates in virulence and pathogenic mechanisms. *A. paragallinarum* possess the virulence determinant haemagglutinin outer membrane protein encoded (OMP) by '*hagA*' gene that functions as adhesion. A PCR assay was carried out using HA1 and HA2 primers to amplify 900

bp core region of OMP '*hagA*' gene. Liu *et al.,* (2016) identified a gene encoding the fimbrial protein *flfA* in *A. paragallinarum*. The *flfA*-deficient mutants of *A. paragallinarum* were found to be less virulent than their parental wild-type strains.

2.7 Incidence and prevalence of Infectious Coryza

Infectious coryza is a global disease that is present everywhere chickens are raised. However, it is considered as an exotic disease in New Zealand which is the only country that appears to be free from *A. paragallinarum* infection (Black, 1997).

2.7.1 Infectious coryza in abroad / other countries

Chen *et al*., (1993) isolated *H. paragallinarum* from outbreaks of infectious coryza in China. Terzolo *et al*., (1993) characterized *H. paragallinarum* recovered from 11 outbreaks of infectious coryza in layers and one case of swollen-head syndrome in broilers in Argentina. Poernomo *et al*., (2000) reported the cases of infectious coryza in indigenous Kampung chickens as well as in commercial poultry production systems from Indonesia. Byarugaba *et al*., (2006) investigated the occurrence of *A. paragallinarum* in Uganda. Calderon *et al.,* (2010) for the first time reported the isolation and identification of *A. paragallinarum* serovar B-1 from severe infectious coryza outbreaks in broiler breeders in Panama, Central America. Akter *et al*., (2014) reported *A. paragallinarum* infection in layer chickens of Gazipur, Bangaladesh between June 2012 to July 2013.

Fedway *et al.,* (2016) examined for phenotypic and genotypic characterisation of *A. paragallinarum* from different poultry farms in Egypt. Feberwee *et al.,* (2019) studied identification and characterization of 18 Dutch *A. paragallinarum* isolates from outbreaks of infectious coryza in layer flocks of The Netherlands. Mei *et al*., (2020) reported a concurrent infection of *A. paragallinarum* and fowl adenovirus in an infectious coryza like outbreak from Beijing, China.

2.7.2 Infectious coryza in India

The first isolation of *A. paragallinarum* (*H. paragallinarum*) from India was reported in 1950 (Rao, 1958, as cited by Adalkha, 1967) followed by a second report of isolation after 11 years by Saxena and Sawhney, (1961). Sobti *et al*., (2000) conducted the retrospective study of infectious coryza cases and reported that the highest incidence was observed during the months of January to April in a year.

Hanushi *et al.*, (2006) reported an outbreak of infectious coryza in Vanaraja poultry birds of Meghalaya. Murthy *et al*., (2008) isolated and identified *H. paragallinarum* along with *Ornithobacterium rhinotracheale* from respiratory illness of poultry. Rajurkar *et al*., (2009) recovered six *A. paragallinarum* isolates from 109 suspected samples of infectious coryza and opined that the secondary organisms complicate the disease condition along with *A. paragallinarum* infection.

Thenmozhi and Malmarugan, (2013) isolated and identified *A. paragallinarum* from air sacs and infra orbital sinus of Japanese quails in and around Namakkal region in Tamilnadu state and reported that out of the 53 samples collected, 8 were found to be positive for *A. paragallinarum* infections. Durairajan *et al.*, (2013) isolated *A. paragallinarum* from different districts of Himachal Pradesh and reported that out of 146 clinical samples processed, six revealed presence of organism culturally, morphologically, and biochemically. Anjaneya *et al.*, (2014b) reported infectious coryza from suspected cases of field samples from different geographical regions of the country *viz.* Bareilly (U.P), Pune (Maharashtra), Bangalore (Karnataka), Indore and Jabalpur (Madhya Pradesh).

Patil *et al.*, (2016) collected samples from different geographical locations of India during 2013 to 2015. A total of 65 samples, 17 field isolates were positive for *A. paragallinarum* and among 17 isolates, the serovar C was prevalent with 47% and serovar A with 27%, whereas serovar B was found to be only 11%. This was the first

confirmed report indicating presence of serovar B in Indian poultry isolates. Mohammad *et al.,* (2016) investigated six outbreaks of infectious coryza from Andhra Pradesh. Out of total 74 infra orbital sinus swabs tested, 27 (36.4%) isolates of *A. paragallinarum* were confirmed by PCR.

Recently, infectious coryza cases in poultry were reported from Kerala (Sarika *et al.,* 2019), Hyderabad (Ravikumar *et al*., 2020) and Tamilnadu (Ruthra *et al*., 2020 and Vadivoo *et al.,* 2020).

CHAPTER-III

MATERIALS & METHODS

MATERIALS AND METHODS

3.0 Samples for investigation

Total one hundred and fifty (n=150) samples were collected from different age groups of chickens suspected for infectious coryza, based on clinical signs and post-mortem examination. All the samples were collected from the commercial poultry farms and backyard poultry in East Godavari and West Godavari Districts of Andhra Pradesh.

All the samples from chickens were collected from January 2020 to 3rd week of March 2020 and from October 2020 to December 2020. Due to COVID 19 national lockdown, samples were not collected in the period from 4th week of March 2020 to the end of September 2020.

3.1 Collection of samples

Samples were collected in duplicate from live birds and post-mortem specimens (Table No. 1).

3.1.1 Live birds

In the farm / flock each bird was first inspected visually for any clinical signs of infectious coryza (facial swelling, nasal discharge). The bird was then held while gentle pressure was exerted on each infra-orbital sinus. The mucosal discharges oozing out were collected using sterile cotton swabs (Figure No. 1, 2, 3, 4 and 5).

3.1.2 Post-mortem samples

The suspected birds showing post-mortem lesions like infectious coryza (swollen infra-orbital sinuses, nasal discharge) were identified and the caseous, cheesy exudates in infra orbital sinuses and trachea were aseptically collected with sterile cotton swab (Figure No. 6).

3.1.3 Transportation of the samples

Nasal swabs and eye swabs from live birds and infra orbital sinus cavity exudates from dead birds were collected with sterile cotton swabs and transported in 30% glycerol phosphate buffer saline (G-PBS) to the laboratory of Dept. of Veterinary Microbiology, NTR College of Veterinary Science, Gannavaram, under cold chain conditions.

3.2 Glassware and chemicals

In the present study glassware such as test tubes, conical flasks, petri plates from Borosil (India) & Duran (Germany), and routinely used chemicals from Sisco Research Labs (SRL), India were used. Bacteriological media and cotton swabs manufactured by Oxoid (UK), Himedia (India) and Merck (Germany) were used. Master mix for PCR test from New England Biolabs (NEBL), USA and DNA markers from Sisco Research Labs (SRL), India were used. Plasticware like microcentrifuge tubes (1.5 ml and 2 ml), 0.2 ml PCR tubes from Tarsons (India) and filtered or aerosol barrier microtips from Genaxy scientific private limited (India) were used. Carbohydrate discs used in this study were procured from Himedia Laboratories Pvt. Ltd. (Mumbai). Some of the important equipment used in the present study is given in the Table No. 2.

3.2.1 Preparation of bacteriological media and reagents

Chocolate Agar was prepared by suspending dehydrated nutrient agar powder in distilled water, autoclaved at 121ºC for 15 minutes and allowed to cool at 45-50ºC. Then 5-7% aseptically collected sheep blood, was added, and kept in water bath at 80ºC with continuous stirring for lysis of red blood cells. Just before pouring the molten agar into petri plates, 0.0025% nicotinamide adenine dinucleotide (NAD) and 1% sterilised (by 22nm membrane filter) heat inactivated chicken serum were added and allowed to solidify at room temperature.

Blood agar was prepared by suspending dehydrated nutrient agar powder in distilled water, autoclaved at 121ºC for 15 minutes and allowed to cool at 45-50ºC. Then 5-7% sheep blood and 0.0025% NAD were added, mixed gently, poured into petri plates and allowed to solidify.

3.3 Isolation and cultural characterization of *Avibacterium paragallinarum*

Each sample was directly swabbed on chocolate agar medium and incubated at 37°C for 48 hours in anaerobic jar by using HiAnaerogas packs. The isolates were observed for colony morphology. Selected colonies were further streaked on fresh chocolate agar medium plate and incubated at 37°C for 48 hours in anaerobic conditions to obtain pure culture. Single colony of the isolates so obtained was used for further identification by biochemical tests and other cultural studies.

3.4 Biochemical characterization of *A. paragallinarum* isolates

The *A. paragallinarum* isolates were characterized by biochemical tests *viz*., catalase test, oxidase test and fermentation of different carbohydrates *viz.*, galactose, maltose, mannitol, trehalose, arabinose.

3.4.1 Catalase test

This test was performed by mixing single identified bacterial colony with 2 to 3 drops of 3% hydrogen peroxide (H_2O_2) on a sterile glass slide with the help of wire loop. Immediate formation of gas bubbles was considered as positive reaction (Salasia *et al*., 2004).

3.4.2 Oxidase test

Standard oxidase discs were used to perform the oxidase test. The discs were just touched on to the single colony and checked for development of blue colour within ten seconds (Carter and Cole, 1990).

3.4.3 Carbohydrate fermentation tests

Carbohydrate fermentation tests were conducted with five carbohydrates *viz.,* galactose, maltose, mannitol, trehalose and arabinose.

Phenol red broth base and carbohydrate solutions were prepared separately, and carbohydrate solution was added to the broth base at 1% concentration. Then medium inoculated with the selected bacterial colony by using inoculation loop and incubated anaerobically for 48 hours at 37˚C. Andrade's indicator was added to detect acid production by observing colour change (Anjaneya *et al*., 2014b).

3.5 Satellitism phenomenon

After the preliminary growth of the selected bacterial colony on chocolate agar, the developed colonies were streaked on blood agar plates containing 0.0025% NAD with cross-streaking of *Staphylococcus aureus* (characterized isolate of *S. aureus* from the of Dept. of Veterinary Microbiology) as feeder culture, incubated for 24 to 48 hours at 37°C in anaerobic conditions and observed for the satellitism phenomenon (Balouria *et al.,* 2019).

3.6 Preparation of bacterial DNA template for polymerase chain reaction (PCR) test

Infectious coryza inactivated vaccine (CORYVAX, Batch No. IC1119, Indovax Pvt. Ltd.) was used as positive DNA control in the PCR test. Culture PCR test and direct swab PCR test were employed for detecting *A. paragallinarum* in the collected samples.

3.6.1 Extraction of DNA from inactivated vaccine

The DNA of infectious coryza inactivated vaccine (CORYVAX) was extracted as per the method described by Antony *et al.,* (2006). Briefly,

1. Two ml of vaccine mixed with 8ml of isopropanol in a 15 ml conical centrifuge tube and vortexed for 5 minutes.
2. Then centrifuged at 5200 rpm for 12 minutes.
3. Both the oil phase and aqueous phase in supernatant were discarded without disturbing the pellet.
4. Then pellet was washed twice with PBS (pH 7.2), suspended in 100 µl of Milli-Q water, mixed thoroughly and transferred to 1.5 ml microcentrifuge tube.
5. The contents in the microcentrifuge tube were boiled at 100ºC for 10 minutes followed by freezing at -20ºC for 30 minutes.
6. The microcentrifuge tubes with the contents were centrifuged at 5200 rpm for 10 minutes.
7. Supernatant was collected and used as template DNA for PCR test.

3.6.2 Extraction of DNA from bacterial colony for culture PCR test

The DNA from bacterial colonies on chocolate agar culture plates was extracted for culture PCR test as per the method described by Badouei *et al.*, (2014). Briefly,

1. Loopful of bacterial culture was suspended in 300 µl of Milli-Q water in a 1.5 ml microcentrifuge tube and subjected to vigorous shaking.
2. The suspension was boiled for 10 minutes followed by rapid chilling for 5 minutes.
3. The microcentrifuge tubes are centrifuged at 12000 X g for 2 minutes.
4. The supernatant was carefully collected and used as DNA template in culture PCR test.

3.6.3 Extraction of DNA from the sample collected by swabs for direct PCR test

All the collected samples by swabs were subjected to direct PCR test by extracting template DNA from the sample collected swabs as described by Badouei *et al*., (2014). Briefly,

1. Swabs were soaked in 400 µl PBS in 15 ml conical centrifuge tube for 1 hour at room temperature.
2. The total volume (400 µl) was transferred into 1.5 ml microcentrifuge tube.
3. The tubes were centrifuged at 2000 rpm for 5 minutes to settle the blood or debris.
4. Supernatant was then transferred into a 1.5 ml microcentrifuge tube and centrifuged at 13000 rpm for 15 minutes.
5. Supernatant was discarded and pellet was suspended in lysis buffer {10-20 µl of 1X PCR buffer containing 0.5% (v/v) of Nonidet P-40, 0.5% (v/v) of TWEEN 20 and 200 µg/ml of proteinase-K}.
6. The tubes were then incubated at 56ºC for 1 hour in a water bath, subsequently heated at 98ºC for 10 minutes to inactivate Proteinase-K, followed by chilling for 10 minutes.
7. The processed sample was used as a source of template DNA.

3.6.4 Quality checking and quantification of DNA

The quality and purity of extracted bacterial deoxy ribonucleic acid (DNA) was checked by Nanodrop (Thermo Scientific).

3.7 PCR test for detecting *Avibacterium paragallinarum* and its genetic determinants of putative virulence factors in the collected samples

The *A. paragallinarum* in the collected samples was detected in the PCR test by using of species-specific oligonucleotide primers (Chen *et al*., 1996, Table No.5). The genetic determinants of putative virulence factors of *A. paragallinarum* were detected in PCR test using specific oligonucleotide primers to putative virulence factors haemagluttinin (*hagA*) and iron acquisition (*fur*) (Anjaneya *et al.,* 2014a and Negrete Abascal *et al.,* 2009, Table No. 5).

The oligonucleotide primers were synthesized at Eurofins Genomics (India) Pvt. Ltd, Bangalore, India. The PCR tests were carried out in Proflex PCR system, Applied biosystems (USA). All the reactions were carried out in a volume of 25 µl in 0.2 ml PCR tubes. The composition of Master Mix (2x) is shown in Table No.3.

The PCR amplicons were analyzed by electrophoresis on a 1.5 % agarose gel stained with 0.5 µg of ethidium bromide / ml in Tris Borate EDTA (TBE) buffer. Electrophoresis was carried out at 70 V for 50 minutes in submarine gel electrophoresis unit (BIORAD, UK) and the PCR products were visualized in BIORAD molecular imager XR+, UK. The sizes of PCR products were verified by comparison with quantitative DNA ladder (SRL). Negative controls i.e, distilled water was maintained in PCR tests.

3.8 Molecular serotyping of *Avibacterium paragallinarum* isolates

All the isolates that were confirmed as *A. paragallinarum* were subjected to molecular serotyping as described by Sakamoto *et al.,* (2012) with suitable modifications by Han *et al.,* (2016). Multiplex PCR (mPCR) test was conducted with a common forward primer (ABC) and serotype specific (A/B/C) reverse primer as presented in the Table No. 5. PCR products were analysed by agarose gel electrophoresis as described in 3.7.

Table No. 1 Samples collected

S. No.	Place of sample collected	No. of samples collected
1	Bhimavaram, West Godavari Dist.	14
2	Samalkota, East Godavari Dist.	20
3	Jaggampeta, East Godavari Dist.	18
4	Balabhadhrapuram, East Godavari Dist.	23
5	Balabhadhrapuram, East Godavari Dist.	24
6	Balabhadhrapuram, East Godavari Dist.	29
7	Animal Disease Diagnostic Laboratory, Dept. of Animal Husbandry, Kakinada, East Godavari Dist.	22
	Total	**150**

Table No. 2 Important laboratory equipment used

S. No	Equipment	Details
1	Thermal cycler	Proflex PCR system, Thermo, USA
2	Gel documentation system	Biorad molecular imager XR+, UK
3	Submarine gel electrophoresis Unit	Biorad, UK
4	Refrigerated Centrifuge	Thermo Fisher Scientific Soravall ST 8R, USA
5	Centrifuge	Remi R 8C, INDIA
6	Water bath	Kemi KWB 210 A, INDIA
7	Nanodrop	Thermo Scientific 2000C, USA
8	pH	Eutech, USA

Table No. 3 Composition of components for PCR reaction

S No	Reagents	Quantity (µl)	Final concentration
1	2x Master Mix (Appendix)	12.5	2x
2	Forward primer	1.0	20 pmole/ µl
3	Reverse primer	1.0	20 pmole/ µl
4	DNA template	4.0	150 ng
5	Distilled water	6.5	-------
	Total	**25**	-------

Table 4 Composition for Multiplex PCR (mPCR) test reaction

S No	Reagents	Quantity (µl)	Final concentration
1	2x Master Mix (Appendix)	12.5	2x
2	ABC Forward primer	3.0	20 pmole/ µl
3	A Reverse primer	1.0	20 pmole/ µl
4	B Reverse primer	1.0	20 pmole/ µl
5	C Reverse primer	1.0	20 pmole/ µl
6	DNA template	4.0	150 ng
7	Distilled water	2.5	--------
	Total	**25**	--------

Table No. 5 Oligonucleotide primers used in PCR tests

S. No	Primer / Gene	Sequence (5′- 3′)	PCR Product size(bp)	Initial denaturation		Denaturation		Annealing		Extension	
				Temp	Time	Temp	Time	Temp	Time	Temp	Time
1	*AP*	F - TGA GGG TAG TCT TGC ACG CGA AT R - CAA GGT ATC GAT CGT CTC TCT ACT	500	94ºC	4 min	94ºC	1 min	63ºC	1 min	72ºC	1 min
2	*hagA*	F - TGT AGC TCA AGC TCC ACA AG R - TCA AGC GAT AAG TGC TTT ACG ACC	900	94ºC	5 min	94ºC	1 min	47ºC	1 min	72ºC	1.5 min
3	*fur*	F – ATG TCT GAA GAA AAT RYW AAA R – TTA ATC TTT TTT ATC TTT TG	453	95ºC	5 min	95ºC	30 sec	43ºC	1 min	72ºC	1 min
4	*HMTp210*	ABC F - GGC TCA CAG CTT TAT GCA ACG AA A Reverse - CGC GGG ATT GTT GAT TTT GTT B Reverse - GGT GAA TTT CAC CAC ACC AC C Reverse - TAA TTT TCT TAT TCC CAG CAT CAA TAC CAT	372 / 800 1100 1600	94ºC	2 min	94ºC	20 sec	56ºC	12 sec	72ºC	1 min

PCR test was run for 35 cycles for the above S.No.1, 2, and 3 primer sets but for the S. No. 4 the PCR test was run for 30 cycles. The final elongation/extension step is at 72°C for 10 min for S.No.1, 2, and 3 primer sets but for the S. No.4 the final elongation/extension step is at 72°C for 3 min**.** (Chen *et al.,* 1996, Anjaneya *et al.,* 2014a, Negrete Abascal *et al.,* 2009 and Han *et al.,* 2016).

Figure No. 1 Collection of ocular swab from infectious coryza suspected live bird.

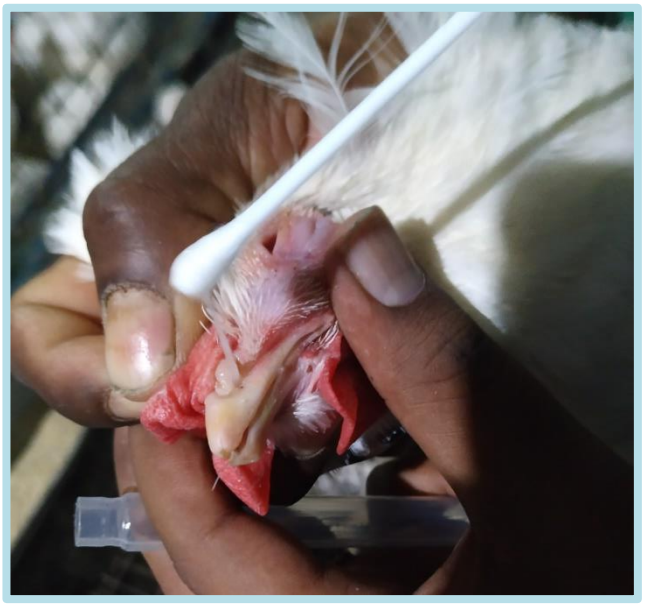


Figure No. 2 Collection of nasal swab from infectious coryza suspected live bird.

Figure No. 3 Infectious coryza suspected bird showing facial swelling due to exudates filled in infraorbital sinus.

Figure No. 4 Infectious coryza suspected bird showing nasal and ocular discharges.

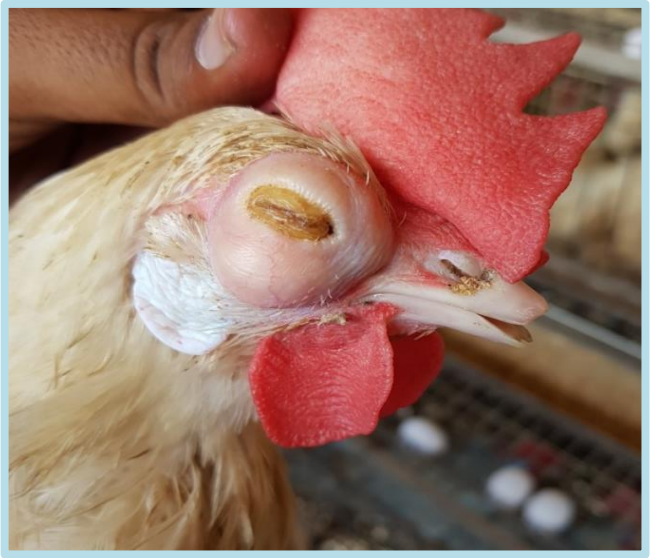


Figure No. 5 Infectious coryza suspected bird showing severe unilateral facial edema and swelling of infraorbital sinus.

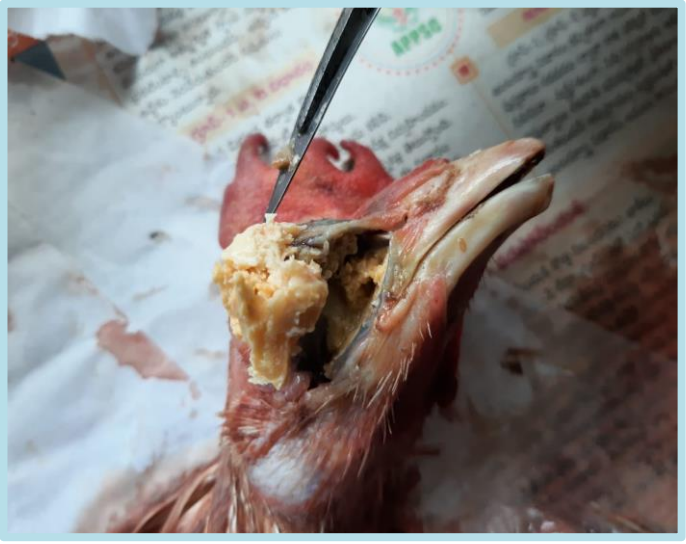


Figure No. 6 Accumulation of purulent material in the infraorbital sinus cavity of infectious coryza suspected bird.

CHAPTER-IV

RESULTS

RESULTS

4.1 Collection of samples

Out of the 150 samples collected, 35 samples that were collected in the early third week of March 2020 could not be immediately processed due to closure of the laboratory/college from 3rd week of March 2020 to September 2020 as per the COVID-19 national lockdown guidelines. Though these 35 samples were stored in deep freezing conditions, they didn't yield any result specific to *A. paragallinarum* when they were processed for isolation and parallelly subjected to direct PCR and culture PCR tests in October 2020. The remaining 115 samples (that were collected much early to lockdown or after reopening of college / lab after September 2020) were processed immediately after collection for isolation and parallelly subjected to direct swab PCR and culture PCR tests to detect *A. paragallinarum*. A total of twelve (n=12) samples were found to be positive for *A. paragallinarum* by isolation, direct PCR and culture PCR tests.

4.2 Cultural and biochemical characterization of the isolates

A total of twelve isolates were isolated on the selective medium chocolate agar with NAD supplement.

4.2.2 Morphological and biochemical characterization of *A. paragallinarum*

The samples that showed smooth, dew drop like, tiny and transparent colonies were provisionally identified as *A. paragallinarum* (Figure No.7). The bacterial cultures of isolates were identified as Gram-negative coccobacilli in the microscopic morphology by Gram's staining (Figure No.8).

The biochemical characterization of these isolates by carbohydrate fermentation tests was presented in the Table No.6. All the 12 isolates that were provisionally identified as *A. paragallinarum* fermented the carbohydrates maltose and

mannitol, but did not ferment the carbohydrates galactose, trehalose and arabinose. All these 12 isolates were negative for catalase and oxidase tests. In the test for satellitism phenomenon all the 12 isolates that were provisionally identified as *A. paragallinarum* showed significant growth adjacent to *S. aureus* culture line and appeared as transparent, dewdrop like tiny colonies (Figure No.12).

4.3 PCR test for detection of *A. paragallinarum*

4.3.1 Purity of the extracted DNA

The DNA extracted for both the direct swab PCR test and culture PCR test was found to have A 260/280 ratio of about 1.8 by Nanodrop measurement.

4.3.2 Culture PCR test and direct swab PCR test for detection of *A. paragallinarum*

Results of the direct PCR test and culture PCR test with the provisionally identified *A. paragallinarum* isolates on the culture plates using species specific oligonucleotide primers is presented in the Table No.7. All the 12 provisionally identified *A. paragallinarum* isolates on the culture plates yielded a specific PCR product of 500 bp in the PCR test with species specific oligonucleotide primers (Figure No.13). Further, all these 12 samples also produced identical result of 500 bp PCR product in the direct swab PCR test with the species-specific oligonucleotide primers.

The inactivated vaccine (CORYVAX) also yielded 500 bp PCR product in the PCR test with the species-specific oligonucleotide primers.

4.4 Detection of putative virulence factors of *A. paragallinarum* by PCR test

Results of all the 12 isolates of *A. paragallinarum* tested for the presence of genetic determinants of putative virulence factors haemagluttinin (*hagA*) and iron acquisition protein (*fur*) in the PCR test with specific oligonucleotide primers is presented in the Table No. 7. The *hagA* gene was detected in 4 isolates of

A. paragallinarum viz. B1, B2, J1 and J2 with a PCR product size of 900 bp. The *hagA* gene was also detected in the vaccine that was used as positive control (Figure No.14). However, the *fur* gene was not detected either in field isolates or in the vaccine strain.

4.5 Molecular serotyping of *A. paragallinarum* isolates

Results of the molecular serotyping of all the 12 isolates of *A. paragallinarum* using Page serotypes A, B and C specific oligonucleotide primers in the mPCR test are presented in the Table No.8. Out of the 12 *A. paragallinarum* isolates tested, 8 isolates *viz.* B1, B2, S1, S2, J1, J2, BA3 and BA4 were identified as serotype A with a PCR product size of 372 bp and the remaining four isolates *viz.* BA1, BA2, BA5 and BA6 were identified as serotype B with a PCR product size of 1100 bp with a faint band detected in the gel. (Figure No. 15 and Figure No. 16). None of the *A. paragallinarum* isolates reacted with serotype C specific oligonucleotide primers in the mPCR test.

The *A. paragallinarum* vaccine (V1 and V2) used as positive control in this study yielded PCR products of 372 bp and 800 bp in mPCR test (Figure No. 16). The BLAST primer analysis with Page serotype A specific oligonucleotide primers was performed and presented in Figure No.17.

Only serotype A was detected in vaccine (positive control) by molecular serotyping. Conventional serotyping was not done for all the isolates of *A. paragallinarum* including vaccine.

Table No. 6 Biochemical characterization of *A. paragallinarum* isolates

S. No	Sample No	Biochemical tests		Carbohydrate fermentation test				
		Catalase	Oxidase	Galactose	Maltose	Mannitol	Trehalose	Arabinose
1	B1	-	-	-	+	+	-	-
2	B2	-	-	-	+	+	-	-
3	S1	-	-	-	+	+	-	-
4	S2	-	-	-	+	+	-	-
5	J1	-	-	-	+	+	-	-
6	J2	-	-	-	+	+	-	-
7	BA1	-	-	-	+	+	-	-
8	BA2	-	-	-	+	+	-	-
9	BA3	-	-	-	+	+	-	-
10	BA4	-	-	-	+	+	-	-
11	BA5	-	-	-	+	+	-	-
12	BA6	-	-	-	+	+	-	-

Table No.7 Detection of A. *paragallinarum* and its genetic determinants of putative virulence factors by PCR test

S. No	Sample no	*A. paragallinarum*		Genetic determinant	
		Direct PCR	Culture PCR	*hagA*	*fur*
1	B1	+	+	+	-
2	B2	+	+	+	-
3	S1	+	+	-	-
4	S2	+	+	-	-
5	J1	+	+	+	-
6	J2	+	+	+	-
7	BA1	+	+	-	-
8	BA2	+	+	-	-
9	BA3	+	+	-	-
10	BA4	+	+	-	-
11	BA5	+	+	-	-
12	BA6	+	+	-	-
13	V1	+	-	+	-
14	V2	+	-	+	-

Table No.8 Molecular serotyping of *A. paragallinarum* isolates in mPCR test.

S.No	Sample no	Serotype		
		A	**B**	**C**
1	B1	+	-	-
2	B2	+	-	-
3	S1	+	-	-
4	S2	+	-	-
5	J1	+	-	-
6	J2	+	-	-
7	BA1	-	+	-
8	BA2	-	+	-
9	BA3	+	-	-
10	BA4	+	-	-
11	BA5	-	+	-
12	BA6	-	+	-
13	V1	+	-	-
14	V2	+	-	-

Figure No. 7 Colonies of *A. paragallinarum* showing smooth, tiny dew drop like moist colonies on chocolate agar.

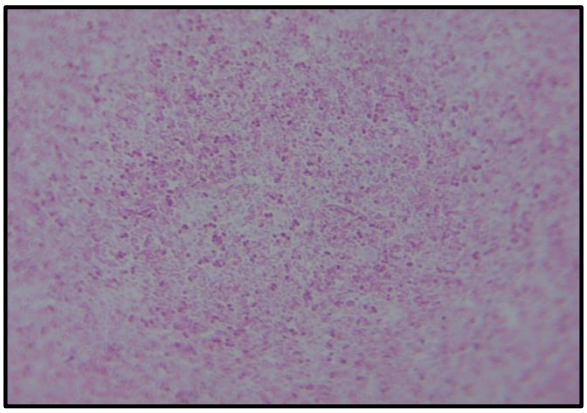


Figure No. 8 Gram negative coccobacilli (*A. paragallinarum*)

Figure No. 9 Left: *A. paragallinarum* negative for catalase test. Right: catalase positive control *(S. aureus)*.

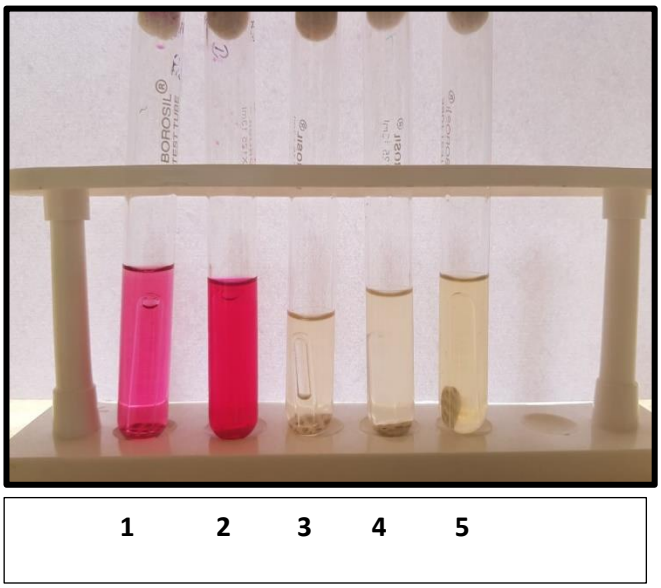


Figure No. 10 Carbohydrate fermentation tests with *A. paragallinarum* isolates positive for maltose and mannitol fermentation (tube 1 and 2).

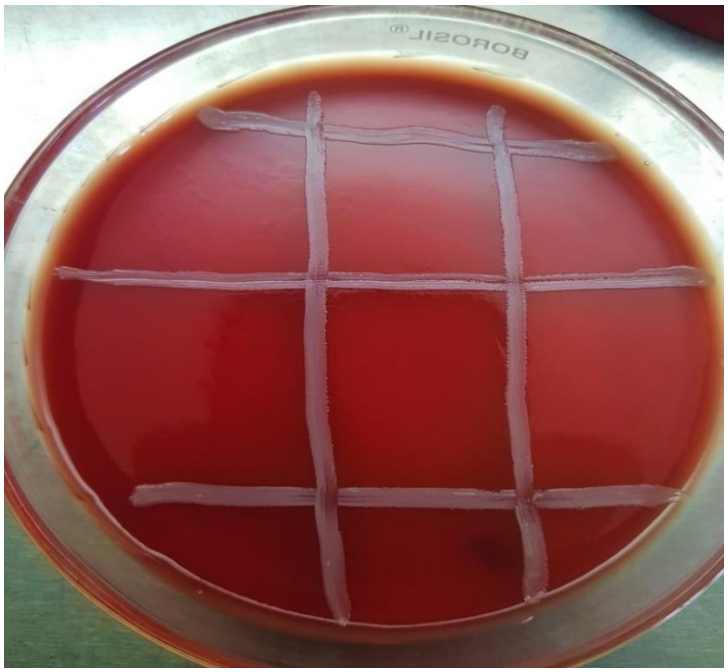
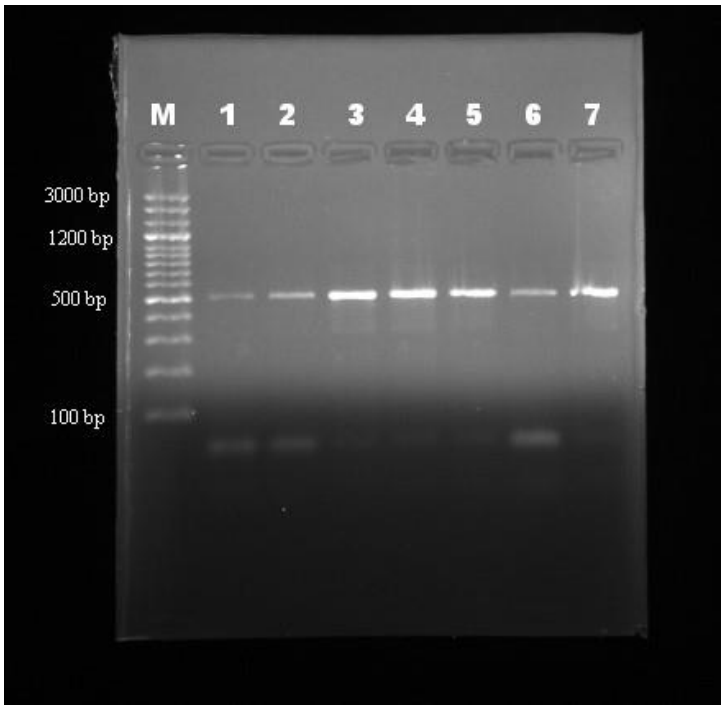


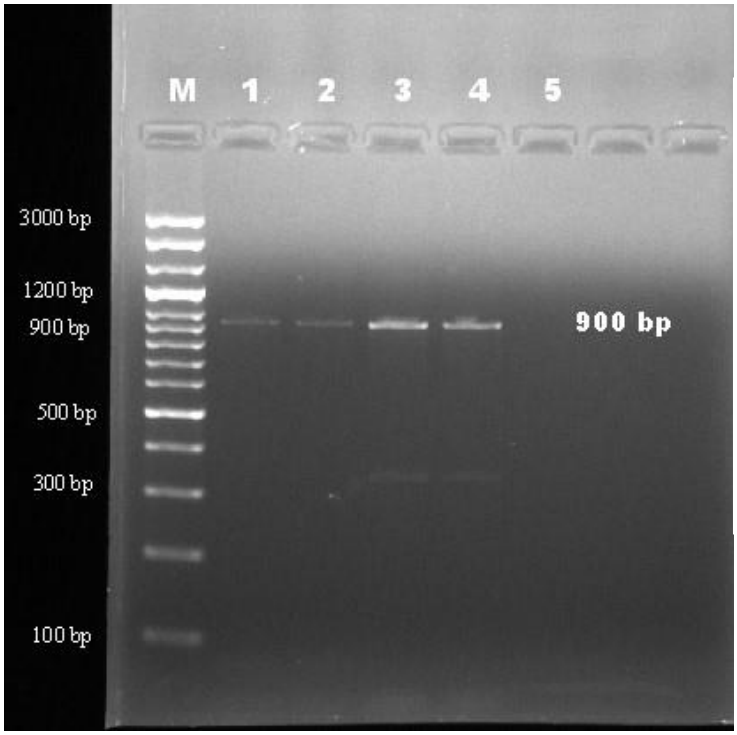
Figure No. 11 Haemolysis of *Staphylococcus aureus* on blood agar.

Figure No. 12 Colonies of *A. paragallinarum* on blood agar, cross streaked with *S. aureus* culture showing satellitism phenomenon.



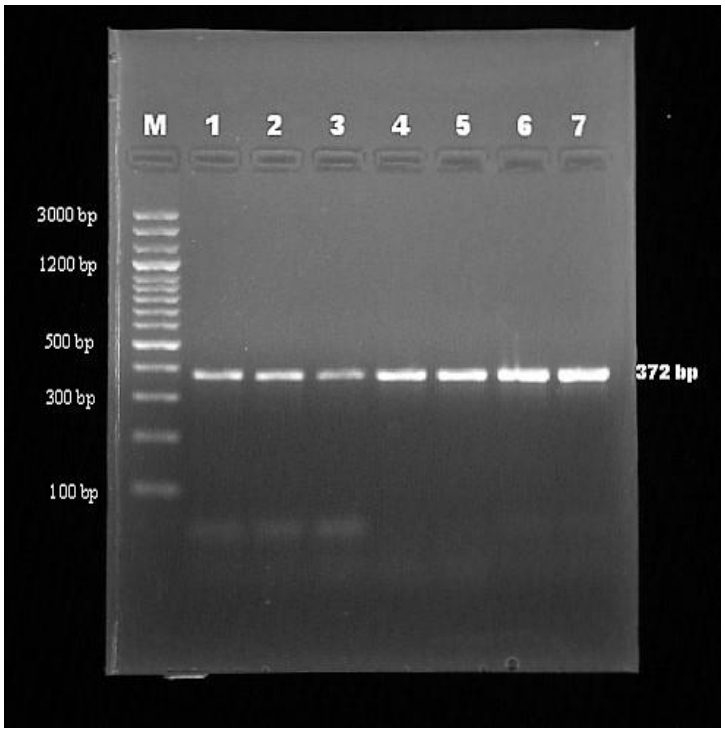
Lane M : 3000 bp marker.
Lane 1 : B1
Lane 2 : S1
Lane 3 : J2
Lane 4 : BA3
Lane 5 : BA5
Lane 6 : V1 (positive control)

Figure No. 13 PCR test for detecting *A. paragallinarum* with species specific oligonucleotide primers.



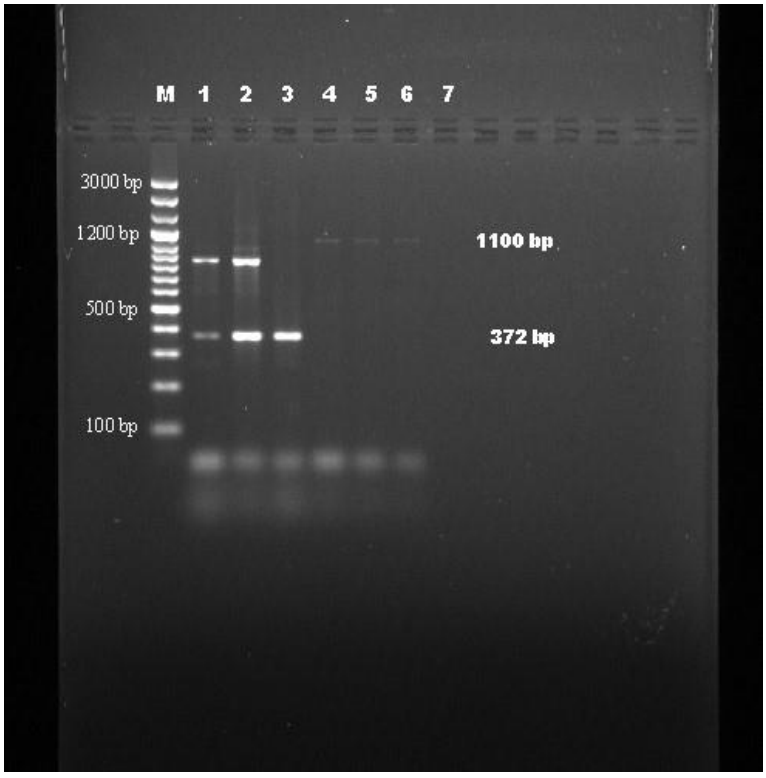
Lane M : 3000 bp marker
Lane 1 : J1
Lane 2 : J2
Lane 3 : B1
Lane 4 : B2
Lane 5 : Negative control

Figure No. 14 PCR test for detecting *hagA* gene of *A. paragallinarum* isolates.



Lane M : 3000 bp marker
Lane 1 : B1
Lane 2 : B2
Lane 3 : S1
Lane 4 : S2
Lane 5 : J1
Lane 6 : BA3
Lane 7 : BA4

Figure No. 15 Multiplex PCR (mPCR) test for molecular serotyping of *A. paragallinarum* isolates – detection of serotype A.



Lane M : 3000 bp marker
Lane 1 : V1
Lane 2 : V2
Lane 3 : J2
Lane 4 : BA1
Lane 5 : BA2
Lane 6 : BA6
Lane 7 : Negative control

Figure No. 16 Multiplex PCR (mPCR) test for molecular serotyping of *A. paragallinarum* isolates – detection of serotype B.

>KJ930422.1 Avibacterium paragallinarum strain VRDC/Avpg/WZ/H35 hemagglutinin gene, partial cds

product length = 840

Forward primer 1 GGCTCACAGCTTTATGCAACGAA 23
Template 3019 3041
Reverse primer 1 CGCGGGATTGTTGATTTTGTT 21
Template 3858 3838

>KJ930420.1 Avibacterium paragallinarum strain VRDC/Avpg/WZ/h31 hemagglutinin gene, partial cds

product length = 840
Forward primer 1 GGCTCACAGCTTTATGCAACGAA 23
Template 3022 3044
Reverse primer 1 CGCGGGATTGTTGATTTTGTT 21
Template 3861 3841

>KJ930418.1 Avibacterium paragallinarum strain VRDC/Avpg/H15 hemagglutinin gene, partial cds

product length = 840
Forward primer 1 GGCTCACAGCTTTATGCAACGAA 23
Template 3019 3041
Reverse primer 1 CGCGGGATTGTTGATTTTGTT 21
Template 3858 3838

>KJ930417.1 Avibacterium paragallinarum strain VRDC/Avpg/SZ/H10 hemagglutinin gene, partial cds

product length = 840
Forward primer 1 GGCTCACAGCTTTATGCAACGAA 23
Template 3016 3038
Reverse primer 1 CGCGGGATTGTTGATTTTGTT 21
Template 3855 3835
>KJ930416.1 Avibacterium paragallinarum strain VRDC/Avpg/WZ/H6 hemagglutinin gene, partial cds

product length = 840
Forward primer 1 GGCTCACAGCTTTATGCAACGAA 23
Template 2950 2972
Reverse primer 1 CGCGGGATTGTTGATTTTGTT 21
Template 3789 3769

>KJ930415.1 Avibacterium paragallinarum strain VRDC/Avpg/H41 hemagglutinin gene, partial cds

product length = 840
Forward primer 1 GGCTCACAGCTTTATGCAACGAA 23
Template 3019 3041
Reverse primer 1 CGCGGGATTGTTGATTTTGTT 21
Template 3858 3838

>MT050506.1 Avibacterium paragallinarum strain TW13 hemagglutinin (hmtp210) gene, complete cds

product length = 372
Forward primer 1 GGCTCACAGCTTTATGCAACGAA 23
Template 3229 3251
Reverse primer 1 CGCGGGATTGTTGATTTTGTT 21
Template 3600 .A...C..... 3580

>MT050502.1 Avibacterium paragallinarum strain TW08 hemagglutinin (hmtp210) gene, complete cds

product length = 372
Forward primer 1 GGCTCACAGCTTTATGCAACGAA 23
Template 3226 3248
Reverse primer 1 CGCGGGATTGTTGATTTTGTT 21
Template 3597 .A...C..... 3577

>KJ867498.1 Avibacterium paragallinarum strain TW07 Hmtp210 (hmtp210) gene, complete cds

product length = 372
Forward primer 1 GGCTCACAGCTTTATGCAACGAA 23
Template 3229 3251
Reverse primer 1 CGCGGGATTGTTGATTTTGTT 21
Template 3600 .A...C..... 3580

Figure No. 17 BLAST analysis with oligonucleotide primers specific to Page serotype A of *A. paragallinarum* showing PCR products of about 800 bp and 372 bp for serotype A.

CHAPTER-V

DISCUSSION

DISCUSSION

Among the infectious diseases that affect poultry, infectious coryza is one of the major diseases affecting the commercial poultry industry in India (Ravikumar *et al*., 2020). Infectious coryza is an upper respiratory disease of chickens caused by *A. paragallinarum.* Considering the significance of the disease, the present investigation was undertaken with the aim to detect *A. paragallinarum* from the suspected cases of infectious coryza in commercial poultry farms and backyard poultry in East and West Godavari districts of Andhra Pradesh. Conventional cultural, and biochemical characterization with collateral confirmation by PCR test was conducted for the isolates obtained from all the samples. Further, PCR test with specific oligonucleotide primers was conducted for the detection of genetic determinants of putative virulence factors of *A. paragallinarum.* Also, molecular serotyping of the *A. paragallinarum* isolates was done by mPCR test using Page serotype A, B, and C specific oligonucleotide primers.

In the present study swollen infraorbital sinuses, nasal and ocular discharges were observed in the *A. paragallinarum* infected birds. These findings confirm the earlier reports of Ravikumar *et al*., (2020) about the clinical features of infectious coryza in the affected birds. However, in the present study similar clinical features were also observed in certain chickens, which were later turned out to be negative for *A. paragallinarum* by conventional and molecular diagnostics. As reported by Blackall and Vargas, (2020) the disease infectious coryza is observed most commonly in adult chickens, including in the backyard poultry.

Perhaps this is the first investigation on molecular characterization of *A. paragallinarum* isolates from Andhra Pradesh, for the selected characters. All the samples from chickens were collected from January 2020 to 3rd week of March 2020

and from 2nd week of October 2020 to December 2020. Due to COVID 19 national lockdown, samples were not collected in the period from 4th week of March 2020 to the end of September 2020.

Out of the 150 samples collected, 35 samples that were collected in the early third week of March 2020 could not be immediately processed due to closure of the laboratory/college from 3rd week of March 2020 to September 2020 as per the COVID-19 national lockdown guidelines. Though these 35 samples were stored in deep freezing conditions, they didn't yield any result specific to *A. paragallinarum* when they were processed for isolation by culture and parallelly subjected to direct swab PCR and culture PCR tests in October 2020. This might be due to storage of samples for more than 6 months without any scope for monitoring the sample preservation by this researcher or other laboratory staff. The remaining 115 samples (that were collected much early to lockdown or after reopening of college / laboratory after September 2020) were processed immediately after collection for isolation and parallelly subjected to direct swab PCR and culture PCR tests to detect *A. paragallinarum*. A total of twelve (n=12) samples were found to be positive for *A. paragallinarum* by isolation, cultural and biochemical characterization, also by direct swab PCR and culture PCR tests.

All the 12 samples *viz*. B1, B2, S1, S2, J1, J2, BA1, BA2, BA3, BA4, BA5 and BA6 showed smooth, tiny dew drop like, moist and transparent colonies on chocolate agar culture (Figure No.7). In microscopic morphology with Gram's staining, the bacterial culture appeared as Gram negative coccobacilli (Figure No.8). These cultural and staining characters are in accordance with the findings of Hsu *et al*., (2007), Akter *et al*., (2014), Thenmozhi and Malmarugan, (2013), Feberwee *et al.*, (2019), Byukusenge *et al*., (2020) and Blackall and Vargas, (2020).

The 12 isolates of *A. paragallinarum* were found to be negative in catalase and oxidase tests (Table No. 6 and Figure No. 9). In carbohydrate fermentation test with the carbohydrates galactose, maltose, mannitol, trehalose and arabinose, all the 12 isolates fermented only maltose and mannitol, but not galactose, trehalose and arabinose (Table No. 6 and Figure No. 10). These results concur the earlier findings of Anjaneya *et al.*, (2014b) and Blackall and Vargas, (2020). Failure of *A. paragallinarum* to ferment either galactose or trehalose and its lack of catalase clearly separate this organism from the other members of the genus. (Blackall and Yamamoto, 1998 and Blackall and Soriano 2013). Absence of catalase activity is a unique feature of *A. paragallinarum* (Anjaneya *et al.*, 2014b).

All the 12 isolates of *A. paragallinarum* streaked on blood agar showed significant growth adjacent to *Staphylococcus aureus* culture line and appeared as transparent, dew drop like tiny colonies. This typical satellitism phenomenon observed is in conformity with the earlier reports of Akter *et al*., (2016), Durairajan *et al.*, (2013), Khatun *et al*., (2016) and Balouria *et al.,* (2019) (Figure No.11 and 12).

Both the culture PCR and direct swab PCR tests were conducted to detect the *A. paragallinarum* in the collected samples. The quality and purity of extracted bacterial deoxy ribonucleic acid (DNA) was checked by Nanodrop (Thermo Scientific) and the A260/280 ratio is about 1.8 for the DNA extracted for each type of PCR test, which is highly satisfactory for use in PCR test (Dwivedi *et al.,* 2018).

In both the culture PCR test and direct swab PCR test with oligonucleotide primers specific to *A. paragallinarum*, all the 12 samples yielded a specific PCR product 500 bp. These results are in accordance with the findings of (Badouei *et al*., 2014). Further, results of direct swab PCR test indicate that it is sensitive to detect *A. paragallinarum* samples collected from infectious coryza cases, without the need of

culture step, which is very much helpful in rapid diagnosis of infectious coryza. In this study results with the direct swab PCR test were obtained within 6 to 8 hours after receiving the sample at the laboratory. The results of the direct swab PCR test for detection of *A. paragallinarum* further confirmed the significance of this test for rapid diagnosis as described by (Dwivedi *et al.,* 2018). Different components of lysis buffer for the direct swab PCR test include Nonidet P-40 (4-Nonylphenyl-polyethylene glycol) that is a non-ionic & non-denaturing detergent, TWEEN 20 that lyses mammalian cells and Proteinase K (a serine protease) that digests proteins and removes contamination from nucleic acid preparations (Badouei *et al*., 2014). The present investigation further proved that direct swab PCR is highly useful to screen large number of samples.

The genetic determinant of putative virulence factor haemagglutinin (*hagA*) was detected in only four isolates of *A. paragallinarum viz.* B1, B2, J1 and J2 with a PCR product size of 900 bp. The *hagA* gene was also detected in vaccine that was used as positive control (Table No. 7, Figure No. 14). These results are in accordance with the earlier reports of Anjaneya *et al.,* (2014a), Garcia-Sanchez *et al*., (2014) and Umar *et al*., (2020). However, the genetic determinant of iron acquisition protein (*fur*) was not detected in all the 12 isolates of *A. paragallinarum*. The *fur* gene was also not detected in *A. paragallinarum* vaccine that was used as positive control (Table No. 7). Since it was reported that *fur* gene was expressed only in *A. paragallinarum* that was cultured in iron deficient medium (Negrete Abascal *et al*., 2009), it is surmised that the *fur* gene could not be detected in the *A. pargallinarum* isolates of the present investigation as they were cultured on chocolate agar enriched with lysed erythrocytes that released iron rich haem. However, it needs further investigation.

All the 12 isolates that were confirmed as *A. paragallinarum* in PCR test were subjected to molecular serotyping by mPCR test using oligonucleotide primers specific

to Page serotypes A, B and C as described by Sakamoto *et al*., (2012) and Han *et al.*, (2016). Only 8 isolates (B1, B2, S1, S2, J1, J2, BA3 and BA4) were identified as serotype A with a PCR product size of 372 bp (Table No. 8 and Figure No. 15). However, Sakamoto *et al*., (2012), Han *et al*., (2016) and Patil *et al*., (2017) reported a PCR product 800 bp under the same mPCR test conditions. It is interesting to note that the vaccine (V1 and V2) used as positive control in this study yielded PCR products of 372 bp and 800 bp in mPCR test (Table No. 8 and Figure No. 16). The BLAST primer analysis with Page serotype A specific oligonucleotide primers was performed and presented in Figure No.17 that showed both the PCR products of about 800 bp and 372 bp as probable PCR products. Therefore, it may be surmised that the oligonucleotide primers specific to Page serotype A of *A. paragallinarum* used in the mPCR test of this study might have bound complementary regions of another region/sub-region of the *HMTp210* gene which might have resulted in a PCR product of 372 bp in field isolates of *A. paragallinarum.* However, the PCR product of 372 bp along with 800 bp was found in *A. paragallinarum* vaccine samples (positive controls). But further detailed investigation such as sequencing of the PCR products and their analysis is required for confirmation.

In mPCR test for molecular serotyping of *A. paragallinarum,* the remaining four isolates *viz.* BA1, BA2, BA5 and BA6 were identified as serotype B with a PCR product size of 1100 bp (with a faint band detected in the gel). These results are in conformity with the earlier reports of Sakamoto *et al*., (2012), Han *et al*., (2016) and Patil *et al*., (2017). Page serotype C of *A. paragallinarum* was not identified for any sample in molecular serotyping in this study.

Since antisera/ antibodies to the Page serotypes A, B and C could not be obtained commercially, conventional serotyping was not conducted for the *A. paragallinarum* isolates in the present investigation.

The results of the present investigation with the molecular serotyping indicates that serotype A and B of *A. paragallinarum* are circulating in East Godavari and West Godavari Districts of Andhra Pradesh. Previous reports on molecular serotyping of *A. paragallinarum* are not available from Andhra Pradesh to support the findings of the present investigation. To further confirm the serotypes of *A. paragallinarum* circulating in Andhra Pradesh, a large number of samples have to be collected, characterized and subjected to molecular serotyping. Due to COVID 19 national lock down from the 4th week of March 2020 to the end of September 2020 and restriction on movements / entry into commercial poultry farms during unlock period from October 2020, it had become very difficult to collect samples from the poultry farms. In addition, certain farmers got used to start treatment with antibiotics quickly as soon as they suspect infectious coryza or observe symptoms in the birds in their farms / nearby farms. This has greatly reduced the chances of detecting *A. paragallinarum* from the collected samples by both conventional and molecular diagnostics. Therefore, a detailed investigation with large number of samples from infectious coryza cases, may be taken up for future studies.

CHAPTER-VI

SUMMARY

SUMMARY

The poultry sector is growing at a much faster rate than any other element of the agriculture and livestock sector. The growth potential of this sector is bright due to regular flow of income throughout the year in the rural economy of the India. Among the infectious diseases that affect poultry, infectious coryza caused by *Avibacterium paragallinarum* is one of the major infectious diseases affecting the commercial poultry industry in India. The present investigation is carried out to detect the *A. paragallinarum* in suspected samples of infectious coryza by cultural and molecular tests, also to detect genetic determinants of its putative virulence factors in polymerase chain reaction (PCR) test and serotypes in multiplex PCR (mPCR) test.

In the present study, out of the 150 samples collected from suspected infectious coryza cases, 35 samples that were collected in the early third week of March 2020 could not be immediately processed due to closure of the laboratory/college from 3rd week of March 2020 to September 2020 as per the COVID-19 national lockdown guidelines. Though these 35 samples were stored in deep freezing conditions, they didn't yield any result specific to *A. paragallinarum* when they were processed for isolation and parallelly subjected to direct swab PCR and culture PCR tests in October 2020.

The remaining 115 samples (that were collected much early to lockdown or after reopening of college / laboratory after September 2020) were processed immediately after collection for isolation by culture and parallelly subjected to direct swab PCR and culture PCR tests to detect *A. paragallinarum*. A total of twelve (n=12) samples were found to be positive for *A. paragallinarum* by isolation, direct swab PCR and culture PCR tests.

All the twelve isolates *viz*. B1, B2, S1, S2, J1, J2, BA1, BA2, BA3, BA4, BA5 and BA6 that were isolated on the selective medium chocolate agar with NAD supplement showed characteristic smooth, tiny dew drop like, moist and transparent colonies. In microscopic morphology with Gram's staining, the bacterial culture appeared as Gram negative coccobacilli. In biochemical characterisation, of all the 12 isolates of *A. paragallinarum* were found to be negative in catalase and oxidase tests and fermented only maltose and mannitol, but not galactose, trehalose and arabinose. All the 12 isolates of *A. paragallinarum* streaked on blood agar showed significant growth adjacent to *Staphylococcus aureus* culture line and appeared as transparent, dew drop like tiny colonies which indicated typical satellitism phenomenon. Hence, all the 12 isolates were confirmed as NAD dependent *A. paragallinarum.* All these 12 samples also produced a specific PCR product of 500 bp in culture PCR and direct swab PCR tests with *A. paragallinarum* specific oligonucleotide primers. The inactivated vaccine of *A. paragallinarum* (CORYVAX) was used as positive control in PCR tests throughout this study

In PCR test, the genetic determinant of putative virulence factor haemagglutinin (*hagA*) was detected in four isolates of *A. paragallinarum viz.* B1, B2, J1 and J2 with a PCR product size of 900 bp. However, in PCR test the genetic determinant of iron acquisition protein (*fur)* was not detected in all the 12 field isolates, since the *fur* gene is reported to be expressed only in *A. paragallinarum* that is cultured in iron deficient medium.

All the 12 isolates that were confirmed as *A. paragallinarum* in PCR test were subjected to molecular serotyping by multiplex PCR (mPCR) test using oligonucleotide primers specific to Page serotypes A, B and C. Eight isolates (B1, B2, S1, S2, J1, J2, BA3 and BA4) were identified as serotype A with a PCR product size of 372 bp, though the *A. paragallinarum* vaccine used as positive control in this study

yielded PCR products of 372 bp and 800 bp. The BLAST primer analysis with Page serotype A specific oligonucleotide primers was performed, which revealed both the PCR products of about 800 bp and 372 bp as probable PCR products.

The remaining four isolates *viz.* BA1, BA2, BA5 and BA6 were identified as serotype B with a PCR product size of 1100 bp (with a faint band detected in the gel). None of the *A. paragallinarum* isolates reacted with serotype C specific oligonucleotide primers in the mPCR test.

The results of the present investigation indicate that all the isolates of *A. paragallinarum* are NAD dependent and belong either serotype A or B. However, to further confirm the serotypes of *A. paragallinarum* circulating in Andhra Pradesh, a large number of samples have to be collected, characterized and subjected molecular serotyping

Due to COVID 19 national lock down from the 4th week of March 2020 to the end of September 2020 and restriction on movements during unlock period from October 2020, it had become very difficult to collect samples from the poultry farms. In addition, certain farmers got used to start treatment with antibiotics quickly as soon as they suspect infectious coryza or observe symptoms in the birds in their farms / nearby farms. This has greatly reduced the chances of detecting *A. paragallinarum* from the collected samples by both conventional and molecular diagnostics.

CHAPTER-VII

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LITERATURE CITED

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CHAPTER-VIII

APPENDIX

APPENDIX

1X PBS (Phosphate buffered saline) pH 7.2 - 1000ml:

Sodium chloride	8 g
Potassium chloride	0.2 g
Potassium dihydrogen orthophosphate	0.24 g
Disodium hydrogen orthophosphate	1.133 g
Double distilled water	1000 ml

30 % G-PBS (Glycerol Phosphate buffered saline)

PBS (pH 7.2)	70 ml
Glycerol	30 ml

1x TBE Electrophoresis Buffer:

Tris base	10.8 g
Boric acid	5.5 g
EDTA disodium salt	0.74 g

Make up to 1000 ml with triple distilled water.

Ethidium bromide (1%)

Ethidium bromide	10 mg
1x TBE Electrophoresis Buffer	1.0 ml

Lysis Buffer for PCR

Proteinase K,	200 µg/ml
TWEEN 20,	0.5%
Nonidet P 40	0.5%

TE buffer pH 8.0 - 8.2

Tris HCL	10mM
EDTA	1mM