Differential Diagnosis of *Chlamydia psittaci* and Avian Influenza H9N2 in Poultry of Pakistan

By

Noor ul Ain

Department of Biochemistry

Faculty of Biological Sciences

Quaid-i-Azam University,

Islamabad, Pakistan

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This dissertation is submitted in candidature to the Department of Biochemistry in partial fulfillment of the requirement for the degree of Master of Philosophy.

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In the name of Allah, The most beneficent, The most merciful

"Read! In the Name of your Lord who has created (all that exists). He has created man, from a clot (a piece of thick coagulated blood). Read! And your Lord is the Most Generous. Who has taught the writing by the pen? He has taught the man that which he knew not"

[Quran, 96: 1-5]

AUTHORSHIP STATEMENT

I, Noor ul Ain, declare and affirm on oath that I myself have authored this M.Phil. thesis with my own work and means, and I have not used any further means except those I have explicitly mentioned in this report. All items copied from internet or other written sources have been properly mentioned in quotations marks and with a reference to the source of citation.

Noor ul Ain

DEDICATION

Every challenging work needs self-efforts as wells as guidance of elders especially those who were very close to our heart.

My humble effort, I dedicate to my sweet and loving

Father and Mother,

Whose affection, love, encouragement and prayers of day and night made me able to attain such success

I wish you all the comforts in your life.

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NOOR UL AIN

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Gross Domestic Product	(GDP)
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Avian Chlamydiosis	(AC)
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Newcastle Disease Virus	(NDV)
Avian Influenza	(AI)
Nuclear Proteins	(NP)
Matrix Proteins	(M)
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Neuraminidase	(NA or N)
Low Pathogenic Avian Influenza	(LPAI)
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Specific-Pathogen-Free	(SPF)

ABSTRACT

ABSTRACT

Poultry contributes 2.41% to total GDP of Pakistan and is affected by various viral and bacterial infections. Avian influenza (AI) pathotype H9N2 and *Chlamydia psittaci* (*C. psittaci*) infect wide variety of poultry and result in influenza like symptoms with acute respiratory distress and loss in egg production. It is also known for the zoonotic importance. Viral-bacterial cooccurrence increases disease intensity. *C. psittaci* are known to enhance the AIV H9N2 infection by suppressing the host immunity. The present study was conducted on poultry of Pakistan for isolation and detection of H9N2 and *C. psittaci,* and to determine their co-occurrence. Total 550 samples containing 491 tissue and swab samples, and 247 serum samples were processed for isolation of H9N2 by hemagglutination (HA) and virus neutralization (VN). Conventional PCR and real time PCR were used for the detection of both AIV H9N2 and *C. psittaci.* Seroprevalence of serum samples for AIV and *Chlamydia psittaci* were checked by ELISA and confirmed by hemagglutination inhibition (HI). A total of 184 samples showed positive HA and 132 were confirmed as H9N2 by VN; 175 samples showed seroconversion for H9N2. Maximum seroconversion was observed from commercial poultry of KPK in spring season. 148 samples were found positive for H9N2 by PCR and maximum detections were from commercial poultry of Sindh in winter. Only 52 sera samples were found positive by ELISA for *C. psittaci* belonging to domesticated birds of Punjab in summer. Similarly, only 60 samples showed *C. psittaci* detection by PCR mostly from domesticated poultry of Punjab in summers. Coinfection of both was observed in 32 tissue and swabs, and 26 serum samples. It was also found that PCR was more specific as compared to *in-ova* inoculations. It is concluded that the chlamydial infection prevails in Pakistan alone and along with coinfection of H9N2, thus adversely affecting poultry. Proper vaccination and preventive measures must be taken to curtail their growth.

INTRODUCTION

1. INTRODUCTION

1.1 Poultry

Poultry is represented by domesticated birds such as ducks, geese, chickens and turkeys *etc*. that are used as a protein source worldwide, in the form of meat and eggs (Mifflin, 2000). Poultry contributes around 24.1% to the annual gross domestic product (GDP) and plays a pivotal role in growth of agriculture sector in Pakistan (Hussain *et al.*, 2015). Yearly production of the breeders and layers is 2-5 million and 0.5 million respectively. A total of 80% of breeding stocks are nurtured within northern areas of country, with temperature ranges of 2°C to 25°C, as compared to the high summer temperature ranging from 35°C to 45°C in rest of the areas. Ecologically controlled sheds are made for breeders in warmer regions (Naeem *et al.*, 1999). Although poultry is a vibrant and established domain of livestock, however its production can be affected by various bacterial and viral infections.

Avian influenza virus (AIV) infection in poultry was first time reported in 1995 in Pakistan (Naeem *et al.*, 1999). Besides poultry, AIV causes infection in zoos and wild birds as well. However, the virus is less pathogenic in domestic fowl as compared to the highly pathogenic form commonly known as fowl plaque that occur in poultry farms (Swayne, 2009).

Avian chlamydiosis (AC) is a recurring bacterial infection in commercial or domesticated poultry. The infection is mainly characterized by digestive or respiratory disorder and can either be subacute, acute or chronic in nature. AC has been detected in more than 460 avian species with pigeons and turkeys being the commonly infected groups. This disease has caused considerable economic losses worldwide (Andersen *et al.*, 2000).

1.2 Avian Influenza

Some of the most important infectious viruses include Avian Influenza Virus (AIV), Infectious Bronchitis Virus (IBV) and Newcastle Disease Virus (NDV). Avian Influenza (AI), also known as bird flu, belongs to the family Orthomyxoviridae and is highly infectious viral disease originated by any of the subtypes of influenza virus particularly by a type A influenza virus (Beard *et al.*, 1980; El-Baky, 2013; Sarwar *et al.*, 2013). “Influenza” is promptly denoted as epidemics of severe, rapidly spreading

catarrhal fevers of humans. The Orthomyxo viruses cause infections in upper respiratory tract of human, domestic pigs, horses and many birds specie (El-Baky, 2013). AI comprises of many strains and emerged as a leading cause of viral infections in poultry resulting in extensive economic losses globally. Highly susceptible bird species include; chickens, turkeys, pheasants, quails, ducks, geese, migratory waterfowls and pet birds. Chicken flocks including broiler, layers and layer breeders are among the strains affected by the AI virus (Beard *et al.*, 1980; Cheema *et al.*, 2011; Abid *et al.*, 2017). Domestic poultry infected with avian influenza virus exhibited syndromes extending from asymptomatic infections to respirational diseases and decreased egg production to the acute systemic infections with nearly 100 per cent mortality (El-Baky, 2013).

1.2.1 Classification of Influenza Viruses

Avian and mammalian influenza viruses consist of different genera such as influenza type 'A', 'B' and 'C' based on the antigenic differences of their nuclear proteins (Ghorbanpoor *et al.*) and matrix proteins (M) (Kamps *et al.*, 2006; Cheung *et al.*, 2007; Cheema *et al.*, 2011). Anomalous behaviour of influenza A viruses with respect to other groups attributes to the fact that they are highly infection causing pathogens in a variety of mammals and birds. Only type A is zoonotic, which means it can infect animals and infection can transmit to humans as well, whereas Type B infect human and causes mild disease. Influenza A virus is categorised into subtypes on account of antigenic properties of 2 major proteins of surface known as glycoproteins, Hemagglutinin (HA) and Neuraminidase (NA). So far, 16 HA and 9 NA subtypes have been identified (Yassine *et al.*, 2008; Fouchier *et al.*, 2009; Abid *et al.*, 2017).

1.2.2 Virulence and Severity of Avian Influenza Virus (AIV)

Avian influenza virus strains infecting poultry are generally categorized based on disease severity: Low pathogenic avian influenza (LPAI) strains and Highly pathogenic avian influenza (HPAI) strains. LPAI show fewer or no clinical signs resulting in a milder, primarily respiratory infection in poultry. Sometimes they might be exacerbated by environmental factors or any other infection resulting in a serious disease while highly pathogenic strain is highly virulent with severe clinical signs and potentially high mortality rates sometimes as high as 100% in poultry. They include the subtypes H5 and H7 (Alexander, 2000; Andersen *et al.*, 2008). Understanding about the disease severity has resulted from long-term surveillance studies, performed in

various areas of the world (Fouchier *et al.*, 2009). The severity of infection in poultry depends upon both the infecting virus and the host species (Naeem *et al.*, 1999).

1.2.3 Structure of Influenza Virion

Type A influenza virus is enveloped and pleiomorphic, roughly spherical, or longitudinally oriented, filamentous in shape with size fluctuating from 80–120 nm with single-strand genome. The negative-sense RNA contains eight genome segments that code 10 to 11 proteins depending upon the isolate (Kamps *et al.*, 2006; Spackman, 2008).

A protein matrix covers the nucleo-capsid with helical symmetry. M2, *i.e.* less abundance ion channel, is involved in un-coating and HA (Hemagglutinin) maturation. Protein matrix or M1 protein is main morphological constituent of virion which acts as an adaptor molecule between lipid envelope and internal RNP particles and it initiates virus budding. The surface of nucleo-capsid consists of two types of antigenic determinants *i.e.* glycoprotein projections or spikes. HA and NA can perform immune actions that are subtype specific but only partially protective across different subtypes and are involved in cell entry and exit, respectively (Portela *et al.*, 2002; Kamps *et al.*, 2006; Cheema *et al.*, 2011).

Haemagglutinin (HA or H) possesses 2-3 glycosylation sites and having a molecular weight of about 76,000. It traverses through lipid membrane to display its main part at the outer surface. The main part possesses at least 5 antigenic domains. HA binds with sialic acid (N-acetyl-neuramsinic acid) that acts as a receptor and through membrane fusion causing dispersion of core of the virus particle. Like HA, neuraminidase (NA or N) are present as protuberance on the surface of virus with an average molecular mass of about 220,000 bearing a tetrameric structure.

The NA molecule possesses a small cytoplasmic tail. They are present on the lipid layer, displaying out their main part. NA also plays enzymatic role by cleavage of sialic acid from the HA, glycoproteins, surface glycolipids and from other NA molecules. They bring about virus penetration in respiratory epithelium via mucin layer and act as an important antigenic site. Influenza A virus are grouped in 16 pathotypes based on antigenicity of these glycoproteins (Kamps *et al.*, 2006).

Influenza A virus produces HA0 as progenitor HA which after post-translational cleavages (by host proteases and virus particles) becomes virulent. The LPAI for

poultry have HA0 with a basic amino acid positioned at −3 or −4 from the cleavage sites and a single arginine at cleaving site. LPAI virus are restricted from cleavage by any host proteases like trypsin and their presence in respiratory and intestinal tracts restrict the replication at those sites. HA0 of HPAI virus have numerous basic amino acids like arginine and lysine present at their cleaving spots and easily cleavable by intracellular ubiquitous proteases. HPAI viruses can replicate throughout the birds, and damage important tissues and organs resulting in disease and ends in death (Alexander, 2007).

1.2.4 Signs and Symptoms

Several disease symptoms are obvious after AIV infection ranging from asymptomatic infection to respiratory disorders. Clinical signs of AIV include inflammation in periorbital tissues and sinuses, lack of appetite, lethargy, severely hindered breathing, nasal discharge, coughing, sneezing, drops in egg production, soft-shelled or abnormal shaped eggs to extreme systemic infection with almost 100% mortality. Although AIV symptoms resemble other respiratory infections; however, in influenza an abrupt onset of fever is accompanied with muscle pain and weakness. Sometimes birds affected with Avian flu show varied symptoms ranging from undetectable to a mild drop in egg production and subsequent high mortality. Sudden death with no symptoms, uncoordinated diarrhoea, variety of lesions such as swelling on head, eyelids, cyanosis of comb and wattles, occurrence of Purple discoloration at wattles, combs, and legs can also be evident in infected birds.

These viruses normally infect the lower intestinal tract of various bird species and transmit through faecal-oral route. Intensity of disease in poultry and genetic diversity determines the origin of virus either from LPAI or HPAI. LPAIV strains causes mild symptoms when transmitted from avian reservoir hosts to extremely prone poultry species for instance chickens and turkeys. Among the LPAI viruses, the subtype H9N2 is considered to spread most rapidly within the domestic poultry industries. H9N2 LPAI virus has been prevailing in multiple avian species resulting in considerable economic losses in the form of moderate to high mortality, respiratory infections and drop in egg production. Moreover, they are receptor specific like humans and transmissibility to humans and other mammalian species raises public health concerns. Zoonosis of H9 has also been reported and such cases continue to raise public health concern (Beard *et*

al., 1980; Potter, 2001; Nili *et al.*, 2003; Kamps *et al.*, 2006; Parker *et al.*, 2012; Lee *et al.*, 2013; Munir *et al.*, 2013).

1.2.5 Host Range

Avian influenza is a contagious viral infection known for infecting wide array of warm-blooded species which includes domestic and wild birds. Influenza viruses (IV) can cause infection in domestic ducks, geese, chickens, turkeys, free-living birds, captive birds, and other many domestic poultry. AI may occasionally infect mammals, including humans. The type A viruses may also infect other mammals such as horses, mink, pigs and swine. However, the virus inhabits natural hosts in the wild like waterfowl, gulls, and shorebirds. AI virus infects the poultry of all ages with mortality ranging from 30% to 100%. AI has emerged as a disease with devastating potential and adversely affects commercial poultry resulting in extensive losses. All of the strains of poultry including broiler, layers and layer breeders are affected with the AI virus (Suarez *et al.*, 2000; Fouchier *et al.*, 2005; Holmes *et al.*, 2005; Alexander, 2007; Cheema *et al.*, 2011; Park *et al.*, 2011).

1.2.6 Re-assortment Phenomenon

Influenza viruses are distinguished because they are constantly changing and evolving by undergoing genetic variations through re-assortment or mutation. Following H5N1 influenza virus subtype, another influenza virus subtype H9N2, became panzootic and has been reported worldwide from variety of terrestrial poultry. Both surface antigens of the influenza A viruses can modify in two distinct manners either antigenic drift/point mutations or antigenic shift/genetic. Antigenic drift is more common as compared to antigenic shift. Antigenic drift carries some subtle changes in the HA and NA, these mutations occurred due to lack of proofreading activities of viral RNA polymerase, whereas antigenic shift involves major changes in these molecules as a result of re-arrangement of the gene segment. During antigenic shifting, novel strains are produced to which humans have no immunity thus causing severe global outbreak of human influenza. Influenza type A virus undergo both type of changes and undergo abrupt mutation. These viruses have the potential to produce antigenic changes as sudden and impulsive mutations. Antigenic drift and shift add new variants to inter species transmission and evade immunity of host to propagate infection. Therefore there is no chance of acquired immunity as in case of other viral diseases (Webster, 1998; Li *et al.*, 2003; Trampuz *et al.*, 2004; Holmes *et al.*, 2005). The assortment of one

or more genes between AIV and humans mostly occur in intermediate host that are usually pigs (Zhou *et al.*, 1999). Mutation of genes takes place when two different and separate viruses simultaneously infect a cell and exchange their genome to assemble a virus with new genome (Zhou *et al.*, 1999; Mehle *et al.*, 2012).

1.2.7 Bird influenza virus subtype H9N2

Birds influenza A viruses (AIV) pathotypes *i.e.* H9, H7 and H5 through adaptations cause severe infection in domestic poultry. However, only H9 subtype exists as LPAIV (Munir *et al.*, 2013). Infections with H9 subtype of AI are common among poultry. During 1994-1999, AIV of H9N2 pathotype has turned out to be enzootic in chickens, domestic ducks and turkeys across the world resulting in slight respiratory infections with drop in egg manufacturing and ultimate significant economic losses (Alexander, 2000; Trampuz *et al.*, 2004; Park *et al.*, 2011; Jonas *et al.*, 2018).

1.2.8 Zoonotic Impact of H9N2

Beside economic losses, humans living in vicinity of these are at high risk of zoonotic transmission and evolution of infectious entities, public health being at threat with AI subtype H9N2. In 1999, these viruses were described sporadically for first time in public; however, they could not produce widespread infections in humans (Trampuz *et al.*, 2004; Munir *et al.*, 2013; Abid *et al.*, 2017). The AIV subtype H9N2 with the ability to infect humans must be observed for their ability to switch over the hosts because of frequent genetic variation. The AIV H9N2 has gained a considerable importance during the recent years because their infections are reaching the panzootic points (Abid *et al.*, 2017). On the surface of AIV H9N2, an important glycoprotein, NA is present which carries out the enzymatic activity required for viral infection and evolution. It plays important functions by assisting the entry and releases of virus from infection points. In respiratory tract, NA brings about the spread of the progeny virions. The penetration of virus into the epithelium of respiratory tract is initiated by cleavage in sialic acid by NA in mucosa of respiratory tract and prevents inactivation of the virus (Abid *et al.*, 2017).

The H9 subtypes are considered to cause mild conjunctivitis and respiratory disorders in human beings. The AIV H9N2 was frequently reported from China. In northern areas of Pakistan, AI subtype H9N2 was isolated from a poultry flocks and antibodies against serotype H9N2 were verified in seven wild bird species while human cases were

reported in Rawalpindi and Islamabad. Cross reaction of AIV H9N2 and antibodies against Influenza A H3 and H1 infect human being. The high percentage of seropositivity against H9N2 AIV was reported in districts of Toba Tek Singh, Multan and Haripur (Trampuz *et al.*, 2004).

1.2.9 Interspecies Transmission

Manifestation of cross species transmission was done by comparison between pathogenic and genetic characteristics of H1 AIVs in Korea. Despite the fact that no relationship is observed between swine and avian viruses, some kind of correlation between viral genes of human and swine was there. The antigenic and phylogenetic investigation of Korean H1 AIV produced signs of genetic resemblance between viruses infecting wild birds and those passing infection to domestic ducks. Comparatively high viral shedding in chickens was observed. Virus titers in chicken and domestic ducks were higher as compared to that of wild birds. However, replication of both viruses was poor in ducks. In case of swine viruses, the replication rate was negligible in domestic ducks and in chickens, but six viruses showed clinical signs and high titers in case of mice. These results suggested that Korean AIV H1 subtype have the potential to produce infection in mammals (Kang *et al.*, 2014).

Avian influenza H9N2 interspecies transmission depends on the host and pathological genomic factors. Transmission of viruses within mammals and birds is hindered by several barriers. These barriers act at numerous stages of viral duplication cycle. They comprise interactions of virus surface glycoproteins, NA and HA, by means of some precise glycan receptors, that vary among individual species within both tissue distribution, structure and in the recruitment of cellular factors needed for efficient replication. Lastly, viruses has to cope with species-specific differences in native immune system, as well as cellular and humoral immunities (Starick *et al.*, 2011).

1.2.10 Pathogenicity

Systemic spread of variant less pathogenic bird's influenza virus was examined by isolating the functional virus H7N1 from brain and lungs of chicken and the RNA load of virus present organs is measured by testing different pathotypes of H9 (H9N2), H7 (H7N7, H7N1) and H5 (H5N2, H5N1). The viral RNA from various organs such as liver, brain, heart, kidney, intestine, lungs, peripheral blood, spleen and mononuclear cells was isolated from poultry suffering from low pathogenic avian influenza viruses

like H5N2, H7N1, H7N2 or H9N2 and examined by PCR. It was determined that chicken isolated LPAIV were spreading, irrespective of the strains (Post *et al.*, 2013).

Different lineages of H9N2 viruses are found distributed in poultry from China, Central Asia, and Europe (Peiris *et al.*, 2001). Genetic investigation revealed similarity among H9N2 isolates from Hong-Kong and Pakistan causing infections in human and the strains causing infection in Pakistan were also recorded from China in late 1990s (Munir *et al.*, 2013). The subtype H9N2 of AIV has become endemic among various terrestrial chickens in Eurasia. Genetic and epidemiological manifestations exposed those three separate lineages of AIV H9N2 responsible for disease outbreaks. AIV subtype H9N2 were reported only in quails, domestic ducks, and chickens through surveillance between 1976 and 1980. In 2000, another outbreak occurred in domestic ducks.

Molecular epidemiological researches on AIV H9N2 virus isolates during 2000/2005 indicated the viral occurrence in quail. Moreover, the quail isolates from AIV H9N2 were as well periodically reported within chickens and many other poultry during an investigation program in southern China. AIV H9N2 also have recorded zoonosis from poultry to mammals such as humans and pigs. In 2003, genomic analyses suggested that human isolate of influenza virus H9N2 was a new re-assortment and most probably directly originated from live local poultry markets. The current transmission events within species propose that existing AIV H9N2 variants are still having high zoonotic potential with two-way interspecies transmission as revealed by genetic analyses. Phylogenetic analysis of different types of poultry suggest that the longstanding co-circulation of various viruses lineages such as H9N2 and H5N1 viruses assist the frequent re-assortments responsible for the excessive genetic variation in both H5N1 and H9N2 (Xu *et al.*, 2007).

1.2.11 Prevalence of AIV H9N2

The first isolated subtype H9N2 AIVs of low pathogenicity were recorded from the USA in 1966 and is notable because of their extensive spread in domestic poultry extending from the Far to the Middle East. LPAIV subtype H9N2 has also been recorded from poultry of many Asian and Middle Eastern countries since late 1990s including South Africa, Hong Kong, South Korea, mainland China, North America, the Middle East, and Europe. AI of pathotype H9N2 have been frequent to cause infections

in poultry during 1994-1999. H9N2 subtype of AI outbreaks occurred in poultry in Italy in 1994, ostriches in USA in 1995, pheasants in Ireland in 1996 and chickens in Korea in 1997, ostriches in South Africa 1995 and chicken, turkeys and domestic ducks in Germany between 1995-98. Recently, pathotype H9N2 has been isolated in connection with extensive and infections in commercial chickens from Pakistan and Iran. The perpetual circulation of pathotype H9N2 virus in poultry reveals ability of rapid evolution and high virulence of these viruses. Besides mild viral infections among terrestrial birds, they occasionally can cause severe outbreaks in poultry (Alexander, 2000; Park *et al.*, 2011; Munir *et al.*, 2013; Abid *et al.*, 2017).

First outbreak of AI from northern Pakistan was reported in 1994 to 1995, caused by an H7N3 type A influenza viruses, known to have high mortality in the field and high pathogenicity in the laboratory (Naeem *et al.*, 1995). Second AI outbreak took place in northern Pakistan in 1998, caused by mild pathogenic type A influenza virus *i.e.* H9N2. It resulted in drop-in egg production, respiratory lesions and daily mortality of 2 to 3% (Naeem *et al.*, 1999; Naeem *et al.*, 2003). Since 1995, there have been five severe influenza outbreaks in Pakistan caused by either of the subtypes H5, H7 or H9 of AIVs. During these outbreaks, primary focus was on the diagnosis and control by strict observation of biosecurity measures and strategic vaccinations. The understanding of genetic and biological characteristics of the H9N2 viruses largely remained unexplored (Munir *et al.*, 2013).

During 1998-2001, an epidemic of AIV H9N2 took place in broiler chicken farms in Iran with mortality rate of 20% to 60%. Co-infections of influenza viruses with any other respiratory pathogens, mostly *Mycoplasma gallisepticum* and infectious bronchitis virus, were known to be the causative agents for increase mortality (Nili *et al.*, 2003). In August 1998, the H9N2 viruses were isolated in Hong Kong from pigs which raised concern about their zoonosis. H9N2 viruses from five patients with influenza-like infection were isolated in the Guangdong province of China during July and August, 1999. Also in southern China, circulation of these viruses in domestic birds continued to threaten public health (Lin *et al.*, 2000).

1.2.12 Diagnosis of subtype H9N2

Avian influenza virus pathotype H9N2 has been rooted among domestic poultry, with recorded clinical signs, pathological lesions and drop in egg production which can be

diagnosed by different laboratory techniques like virus isolation (VI), identification, detection and other serological examination (Spackman *et al.*, 2002; Jonas *et al.*, 2018). AIV subtype H9N2 can be isolated from embryonated eggs of chickens (Pourbakhsh *et al.*, 2000) and serological identification is done for the detection of virus-specific antibodies in hemagglutination inhibition (HI) tests and Enzyme-linked immunosorbent assays (ELISA) which is another tool to detect antibodies against influenza A (Spackman *et al.*, 2002; Chaharaein *et al.*, 2006). Over the last few decades, the use of molecular assays and PCR based techniques have enabled the precise detection of AIV, even at a very low pathogen count. By using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), detection and subtyping of AIV has been precisely reported. Studies have revealed an excellent concordance of RT-PCR with the VI and serological analyses of influenza viruses (Jeffery *et al.*, 1997; Chaharaein *et al.*, 2006).

1.2.13 Comparison of Diagnostic Techniques

It was experienced in some studies that HI assays accompany some demerits such as being tedious to perform, and tough to incorporate into automatic procedures. It also needs a non-stop supply of appropriate erythrocytes. On contrary, quick identification of virus holds significant clinical, epidemiological and economic applications (Chaharaein *et al.*, 2006). Although, the egg inoculation method has been successfully used for identification of the circulating AIV in poultry but the RT-PCR is more sophisticated due to its ability to detect the higher number of AIV from the healthy flocks that could otherwise be easily skipped by routine laboratory techniques (Siddique *et al.*, 2008) as implied by recent researches reporting that Polymerase Chain Reaction (PCR) assay is speedy with more sensitivity for the detection of influenza virus. PCR gives better recognition of virus from clinical samples which might appear negative due to inappropriate sampling or due to loss of infection during shipment (Vabret *et al.,* 2000). The VI technique only detects the live influenza viruses determined by HA, but a high concentration of viral particles are needed in the sample while RT-PCR detect a single minute virus particle, either inactive or active (Siddique *et al.*, 2008). The comparative effectiveness and performance of VI, ELISA and RT-PCR suggested that the degree of efficacy of ELISA and VI were irrespective of patient's age, showing that ELISA was much efficient and reliable technique for the identification of virus in infants and young children while VI and RT-PCR were most suitable in case of older patients (Steininger *et al.*, 2002).

1.3 Bacterial Infections

Bacterial pathogens are also pivotal for triggering respiratory diseases in poultry besides virus and fungus. Ecological factors also enhance these pathogens for obvious clinical symptoms (Glisson, 1998). Contaminated poultry meat and eggs are important reservoirs of infection (Humphrey, 2006). Infectious bacteria *Mycoplasma gallisepticum* cause respiratory tract disease, drop in egg production, as well as debilitation and carcass condemnation in domestic poultry (Benskin *et al.*, 2009). *Pasteurella multocida* (PM), cause fowl cholera in chickens, quail, ducks, and turkeys (Glisson, 1998). *Escherichia coli* are present in host mucosal surfaces and are specific in their virulence. Avian Pathogenic *E. coli* is responsible for extra-intestinal infections, mainly of breathing tract or systemic infections, resulting in various infections and severe economic loss (Dho-Moulin *et al.*, 1999).

1.3.1 Avian Chlamydiosis

Halberstaedter and von Prowazek were the first to describe the chlamydial organisms in 1907. They identified the intra-cytoplasmic inclusions encompassing great number of microorganisms from the cells of conjunctival scrapings of humanoid patients suffering from trachoma, and from orangutans injected with the material from trachoma cases. These organisms were considered to be protozoans and named ``chlamydozoa'' after “chlamys” for mantle, in which the red EBs were found embed. Psittacosis disease was recognized by Ritter in 1879 (Longbottom *et al.*, 2003). In 1893, flu-like symptoms were observed in Paris after the reported spread of infection causing agent from the parrots to humans and the infection was named psittacosis, after the Latin word uses for parrot, psittucus (Morange, 1895). It was first reported in parrots, other psittacine birds, and humans associated with psittacine birds, thus got the name of psittacosis (Madan *et al.*, 2011).

Avian Chlamydiosis (AC) outbreak caused considerable economic losses in psittacine birds and domestic poultry and is caused by bacterium *Chlamydophila psittaci* which is the pathogen avian chlamydiosis and human ornithosis (formerly psittacosis). They infect lower respiratory tract and results in systemic, sometimes fatal disease in birds (Tong *et al.*, 1993; Andersen *et al.*, 2003; Geigenfeind *et al.*, 2010; Smith *et al.*, 2011). *C. psittaci* is a heterogeneous group with representative isolates both from mammalian and avian origin. Attempts were carried out to differentiate among members of *C. psittaci* specie, resulting in identification of four separate Geno species by marking the

substantial variation among *C. psittaci* group through DNA-DNA re-association studies (Denamur *et al.*, 1991). *Chlamydia psittaci* is a key aetiological agent in avian respiratory infections and considerable zoonotic infections (Yang *et al.*, 2007; Lagae *et al.*, 2014).

1.3.2 Structure of *Chlamydia psittaci*

*Chlamydia*ceae are Gram-negative obligate intracellular bacteria with bacterial characters such as cell wall composition, presence of both DNA and RNA, and asexual reproduction by binary fission and viral character of obligate parasitism and replicate only within living cells, like tissue culture, embryonated eggs and lab animal (Schachter *et al.*, 1973; Shewen, 1980; Joseph *et al.*, 1986; Vanrompay, Andersen*, et al.*, 1993; Pannekoek *et al.*, 2010).

Chlamydia psittaci exhibit single spherical chromosome of about 1.172 Mb with a single plasmid of 7553 bp and chromosome having 967 coding sequences (CDSs) and a plasmid is known to have almost 8 coding sequence, from which 26% of CDSs encode hypothetical products. They have 38 tRNA and a single rRNA operon (Voigt *et al.*, 2012). Avian strains of *C. psittaci* are categorized in fifteen outer membrane protein A (OmpA) geno-types, each one associated with respective bird species (Heddema, Van Hannen*, et al.*, 2006; Hulin *et al.*, 2015).

1.3.3 Host Range

Chlamydia psittaci have a huge impact on avian species and have also been isolated from mammalian hosts. Birds are their natural host however, they can also infect vertebrate hosts such as pigs, cattle, goats, sheep, horses, swine and crocodiles (Vanrompay, Andersen*, et al.*, 1993; Voigt *et al.*, 2011; Voigt *et al.*, 2012; Chu *et al.*, 2017). *C. psittaci* infection in birds is very common worldwide and has been reported in approximately 465 species with 30 different orders (Kaleta *et al.*, 2003; Lagae *et al.*, 2014). *C. psittaci* infects wild birds, pet birds and poultry but mostly infects commercial poultry and occasionally humans. Therefore, precautionary measures must be observed while handling contaminated material and infected specimens (Andersen *et al.*, 2003; Geigenfeind *et al.*, 2010; Opota *et al.*, 2015). Among caged birds, *C. psittaci* infections are most common in pigeons and doves (Smith *et al.*, 2011).

1.3.4 Signs and Symptoms

Infections caused by *chlamydia* are often confused with other respiratory pathogens due to similar symptoms (Tomić, 2013). Psittacosis is occasionally fatal depending upon host susceptibility, severity, and disease duration (Madan *et al.*, 2011). In case of chronic infection by *C. psittaci*, birds don't show any clinical signs while influenza-like symptoms are typically obvious after infection with *C. psittaci* and occasionally lead to severe pneumonia and non-respiratory complications. Some clinically ill birds show symptoms such as swollen eyelids, conjunctivitis, rhinitis, ruffled feathers, respiratory distress, abnormal excretions, nasal and eye discharge, sneezing, anorexia, dyspnoea, polyuria, anorexia, lethargy, dullness, diarrhoea, yellowish droppings, reduced egg production, emaciation and even death. Clinical signs are generally non-specific varying from non-apparent to severe depending on the species, age, stress level and health status of host and Chlamydial strain. Affected birds' necropsy reveals typical findings such as multifocal hepatic necrosis, liver and spleen enlargement, pericarditis and enteritis. In humans, ornithosis is characterised by mild influenza-like symptoms ranging from fever, cough and headache to severe pneumonia, myocarditis, endocarditis, blindness, urogenital or reproductive disorders, abortions and encephalitis. Infections caused by *C. psittaci* can be treated with optimum concentrations of tetracycline, chloramphenicol and erythromycin. However, if left undetected and untreated, death may raise to as high as approx. 15% (Schachter *et al.*, 1973; Andersen *et al.*, 2000; Andersen *et al.*, 2003; Longbottom *et al.*, 2003; Smith *et al.*, 2005; Fenga *et al.*, 2007; Geigenfeind *et al.*, 2010; Xu *et al.*, 2010; Smith *et al.*, 2011; Jiang *et al.*, 2013; Lagae *et al.*, 2014; Hulin *et al.*, 2015).

1.3.5 Pathogenicity

Chlamydia psittaci (*C. psittaci*) have many strains isolated from avian and mammalian hosts including man, that differ considerably in their infectivity (Xu *et al.*, 2010). Serotyping of mammalian *C. psittaci* isolates have been reported and associated with different hosts with different pathogenicity (Vanrompay, Ducatelle*, et al.*, 1993). Eight serotypes of *C. psittaci* were identified by the monoclonal antibodies against major outer membrane protein A (ompA) including A to F, WC and M56. Serotypes WC and M56 were entirely associated with mammals and serotype A, B, C, D, E and F were isolated from different bird species. Genotypes A and genotype B are associated to psittacine birds including parakeets, cockatoos, pigeons and parrots. The C genotype is

linked with geese and duck, while D genotype was mainly reported from turkeys. Genotype E has considerable host diversity and isolated from ratites, pigeons, turkeys, ducks and occasionally humans. Psittacine birds and turkeys are also infected with genotype F while genotype E or genotype B have mainly been isolated from the ducks. Genotypes WC and genotype M56 were reported from epizootics in muskrats and cattle. Genotypes B, C, D, F, and E/B have been reported in chickens. Genotypes B and D were frequently isolated from broiler although chickens show more strong clinical signs infected with the highly virulent genotype D strain (Van Lent *et al.*, 2012; Madani *et al.*, 2013; Yin *et al.*, 2013). In turkeys, pathogenicity is caused by genotypes A and D and is comparatively higher than genotype B because the longer incubation period delay maximal replication in genotype B (Van Buuren *et al.*, 1994; Beeckman *et al.*, 2010).

Comparing pathogenicity of isolated *C. psittaci* reference strains from turkeys in the USA with that from Europe resulted in the identical serovar (D) and with 2 other European *C. psittaci* strain from pigeon and parakeets that belonged to dissimilar serovars (B and A, respectively). All of the 4 strains of *C. psittaci* observed as pathogenic for the SPF turkeys but differ in virulence as revealed by variations in their experimental and gross histopathological lesions. Lesions observed were mostly from respiratory organs. Some serotypes of *C. psittaci* are preferentially connected with certain hosts. Serovar A strain revealed as the most pathogenic to turkeys as they display acute clinical sign and the most widespread lesions. Serovar A strain are generally related with psittacine birds (Vanrompay *et al.*, 1994; Vanrompay *et al.*, 1995).

1.3.6 Prevalence

In order to determine the prevalence of *C. psittaci*, number of surveys were conducted including isolation of pathogens and serological studies (Wills *et al.*, 1988). Research on chlamydiosis was instigated in 1948 after several cases of psittacosis were reported among the workers of a Texas poultry processing plant (Irons et al, 1951) followed by several infections reported in turkeys from the USA (Vanrompay *et al.*, 1994).

In many European cities, it was revealed that *Chlamydophila psittaci* inhabit pigeons and the feral rock dove (*Columbia livia*) living in human vicinity also causing zoonotic disease. (Heddema, ter Sluis*, et al.*, 2006). An outbreak of extreme respiratory infection

diseases occurred within mixed poultry flocks in 2005 that happened to be infected with *C. psittaci* and resulted in the spread of infection to at least 100 minor poultry farms in eleven districts of the Central Germany (Gaede *et al.*, 2008)**.** Serological identification reported Chlamydial infection of birds in Iran (Madani *et al.*, 2013) with psittacosis outbreaks being reported worldwide including the US, China, India, Australia and Europe in last three decades (Lagae *et al.*, 2014). Ornithosis and psittacosis are among the notable disease in birds of Switzerland with five to ten annual cases of captive birds (Zweifel *et al.*, 2009).

Chlamydia psittaci has been as an important avian pathogen in the United Kingdom associated with the import of psittacine birds and with domestic poultry (Tong *et al.*, 1993; Pennycott *et al.*, 2009). *C. psittaci* has been found in Australian wild and domesticated psittacine species including reports of presence of *chlamydia* also in Australian commercial duck flocks (McElnea *et al.*, 1999). More often *C. psittaci* has been revealed in chickens from countries like China, Germany, France, Belgium, and Australia, with recent isolation of C. *psittaci* in Belgian chicken slaughterhouse and hatchery including both high and low virulent strains of *C. psittaci* (De Boeck *et al.*, 2015).Trachoma, a sight-threatening condition is caused by infections of conjunctiva with *Chlamydia*e and being main reason of infectious blindness worldwide. In Pakistan, highest prevalence of trachoma was reported in the province of Punjab including children with trachoma infected by *C. trachomatis* in district of Attock. Until 2006, no case of infection with *Chlamydia psittaci* were reported (Goldschmidt *et al.*, 2006). However, previously few cases of *C. psittaci* was reported in poultry of Faisalabad (Siraj *et al.*, 2018).

1.3.7 Zoonotic Impact

Birds infected with *Chlamydia* pose a considerable health hazard to humans and other mammals. *C. psittaci* not only infects poultry and pet birds but also infect humans causing psittacosis and is a leading cause of preventable blindness, respiratory infections, typical pneumonia and have also been implicated in cardiovascular diseases after zoonotic transmission (Shewen, 1980; Longbottom *et al.*, 2003; Smith *et al.*, 2011; Chu *et al.*, 2017).

Persons prone to domestic and companion birds are at a high risk of getting chlamydiosis with persons in specific occupations like laboratory and wildlife workers,

veterinarians, poultry farmers, or bird breeders are also at a major risk. Turkey feather mites and chicken lice also harbour infective organisms with psittacine birds, parrots, chickens, ducks, turkeys, seagulls, whilst wild and domestic songbirds contribute in zoonotic transmissions. *Chlamydia* are mostly shed in lacrimal and nasal secretions and faeces and remain viable for several months. Respiratory exposure is known as the primary route of infection whereas oral infection being the secondary (Shewen, 1980; Xu *et al.*, 2010; Smith *et al.*, 2011; Voigt *et al.*, 2012; Opota *et al.*, 2015).

1.3.8 Diagnosis and Detection of *Chlamydia*

Accurate diagnosis of Chlamydial infection is linked to the isolation and identification from blood, discharges, faeces, or post mortem tissues of infected animals and birds. The isolation was carried out in specific-pathogen-free (SPF) chicken embryos, mice eggs, cell culture monolayers and in McCoy cell lines, and can be identified as *Chlamydia*e by different assays. The yolk sac is mostly preferred for culturing due to its growth components for all known chlamydial strains (Shewen, 1980; Madan *et al.*, 2011; Yang *et al.*, 2011). The serological detection of *C. psittaci* is performed with ELISA (Lublin *et al.*, 1997; de Freitas Raso *et al.*, 2002). Various standard and real-time PCR techniques are applied by targeting different genes for detection, identification and/or quantification of *chlamydia psittaci* (Ehricht *et al.*, 2006; Ménard *et al.*, 2006).

1.3.9 Comparison of Diagnostic Techniques

Despite the health concerns of *Chlamydia,* reliable and efficient tools for laboratory diagnosis are not available. The conventional approaches for isolation of *C. psittaci* include inoculation of embryonated chickens or mice eggs but using mice has many demerits such as high cost, difficulty in housing, safety risks and tedious process while growth in chicken egg is also a poor method with certain disadvantages. Therefore, an effective system is required for isolation of *C. psittaci* from suspected cases within domestic poultry and exotic birds (Bevan *et al.*, 1978; Tong *et al.*, 1993). However, commercial serological test *i.e.* ELISA for monitoring antibody activity to *C. psittaci* is more effective (de Freitas Raso *et al.*, 2002). Compared with conventional PCR, real-time (RT)-PCR is more fast, sensitive and specific for diagnosis of intracellular pathogens (Opota *et al.*, 2015).

1.3.10 Differential Diagnosis

Numerous infectious agents have been known to contribute to AIV H9N2 and *Chlamydia psittaci (C. psittaci).* Isolation of infectious strain from pigeon farms show severe respiratory signs and is also known as a zoonotic pathogen in humans with health hazards. In addition to C. *psittaci*, AIV H9N2 also causes respiratory distress in birds with co-infection leading to 30% mortality in chickens (Chu *et al.*, 2017). Although only three case reports of co-infection have been observed, viruses such as AIV, NDV and IBV most frequently affect the respiratory tract in broilers, and thus might interact to *C. psittaci* (De Boeck *et al.*, 2015; Zhu *et al.*, 2018).Viral-bacterial co-occurrence is often considered to increase disease intensity in both animals and humans causing huge economic losses. Differential diagnosis of *C. psittaci* should especially be considered when AIV H9N2 and *Chlamydia psittaci* are isolated from poultry with same respiratory disease. Primarily *C. psittaci* infection enhances the infection of AIV H9N2 by the suppression of immunity and trigger adaptive immunity in chickens. The *C. psittaci* infiltration to target organs like lungs and air sacs along with H9N2 contribute to increase mortality as well as lesion expansion suggesting that *C. psittaci* assists the survival of H9N2 by lowering the INF-γ secretion and contributes to the high mortality compared to the *C. psittaci* or H9N2 alone (Ou *et al.*, 2014; Opota *et al.*, 2015; Chu *et al.*, 2016; Chu *et al.*, 2017).

1.4 Summary

Re-emerging pathogens now-a-days in poultry has become a matter of significant importance due to their pathogenic potential and their zoonotic impact. Timely diagnosis of these professional pathogens is necessary to prevent economic losses. Therefore, differential diagnostic techniques are employed for point of care detection and in-depth characterization of these emerging pathotypes.

1.4 Aim of the Study

Endeavour of the present research study was the evaluation of differential diagnosis of Birds Influenza virus strain H9N2 and *Chlamydia psittaci* in poultry of Pakistan.

Objectives

Objectives of this study were:

1. To isolate Avian Influenza subtype H9N2 and *Chlamydia psittaci* from poultry samples.
2. To detect H9N2 and *Chlamydia psittaci* within poultry samples by Conventional PCR.
3. To employ Real-Time PCR for recognition of H9N2 and *Chlamydia psittaci* from poultry samples.
4. To determine the co-occurrence of H9N2 and *Chlamydia psittaci* among poultry samples.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

The present analysis was aimed to determine the differential diagnosis of *Chlamydia psittaci* and AIV H9N2 within poultry during the year 2018-19. This examination was performed in National Reference Laboratory for Poultry Diseases (NRLPD), National Agricultural Research Council (NARC), Islamabad from July 2018 to June 2019. This lab is SAARC Regional leading lab (RLDL) for diagnosis of HPAI and other zoonotic diseases.

2.1 Source and Collection of Specimens

Samples from various organs like lungs, trachea, spleen, tonsils, caecal and kidneys were taken from infected poultry with symptoms, from different locations of Pakistan. Other samples included swabs from nasal area, throat, trachea, cloaca and faeces along with serum from suspected birds.

Serological diagnosis was done using serum samples while tissues and swabs were obtained for molecular investigation. For this purpose, 550 samples were obtained from poultry of various ecological zones including Punjab, KPK, Sindh and Islamabad Capital Territory. Shipment of samples was done in icebox to laboratory for timely diagnosis by keeping them at 0°C to -20°C until used.

2.2 Virus Isolation

Test samples, in transport medium were received as tracheal or cloacal swabs (Appendix I) as preserved or fresh tissues or as bird specimen for post-mortem examination (Numan *et al.*, 2008; group, 2012; Thiermann, 2015) . Unpacking of the specimens was done using biosafety cabinet.

Processing of Swabs

- Tracheal and cloacal swabs were obtained in about 2ml of shipment medium.

- The transport medium from each swab sample was shifted to sterilized ampoule having 0.25 ml of stock solution of antibiotics.
- The ampoules were sealed and incubated for 15 mints at temperature of 37 °C in isolation room or refrigerated for 24 hrs at 4°C.
- Centrifugation of the transport medium was done at about 2000 rounds/mints for ten mints and supernatant was filtered across 0.2 µm filter paper before inoculation in case of heavy bacteriological contamination (Cheema *et al.*, 2011).

Processing of Tissues

- Tissue samples from trachea, lungs, spleen, liver and intestine were isolated from the suspected birds in a sterile petri dish.
- About one gram of tissue from each sample was poured off in petri dish and labelled with sample ID.
- Tissue was then triturated using sterilized scissor and forceps and placed in a stomacher bag.
- The material in stomacher bag was then diluted using PBS with specific antibiotics, kept in a stomacher machine and blended for half a minute.
- The tissue homogenate was then shifted to a sterile tube of 1.5ml or 25ml universal bottle and were centrifuged at 2000rpm for ten minutes.
- Supernatant was then shifted to a sterile tube of 4ml for inoculation in embryonated eggs and occasionally filtered before inoculation through a 0.2 µm filter in case of heavy bacterial contamination (Slorach, 2006).

2.2.1 Virus Isolation *via* Egg Inoculation

a) Candling of Eggs

Candling of the 9 days old embryonated chicken eggs were done for detecting viability in embryos checked by marking the blood vessels and air sacs and discarded if no movement was detected. Fertile chicken eggs were then inoculated in bio-safety cabinet (Alexander, 2000; Bayvel, 2004; group, 2012; Thiermann, 2015).

b) Inoculation of Eggs

- The inoculation point of each egg was wiped with 70 percent ethanol, air dried and an electronic gun was used to drill a hole in egg shell.
- Eggs were inoculated (in the chorio-allantoic membrane located beneath the air sacs) with each sample with almost 0.2ml of total inoculum injected into each egg using 3ml syringe, and syringes were discarded after inoculation.
- The punched end of each egg was disinfected with 70% ethanol, air dried and sealed with liquid gum (UHU) or heated wax.
- The inoculated eggs were kept at temperature of 37°C for 48 hours (Swayne, 1998).

Figure 2.1: Drilling of eggs for inoculation

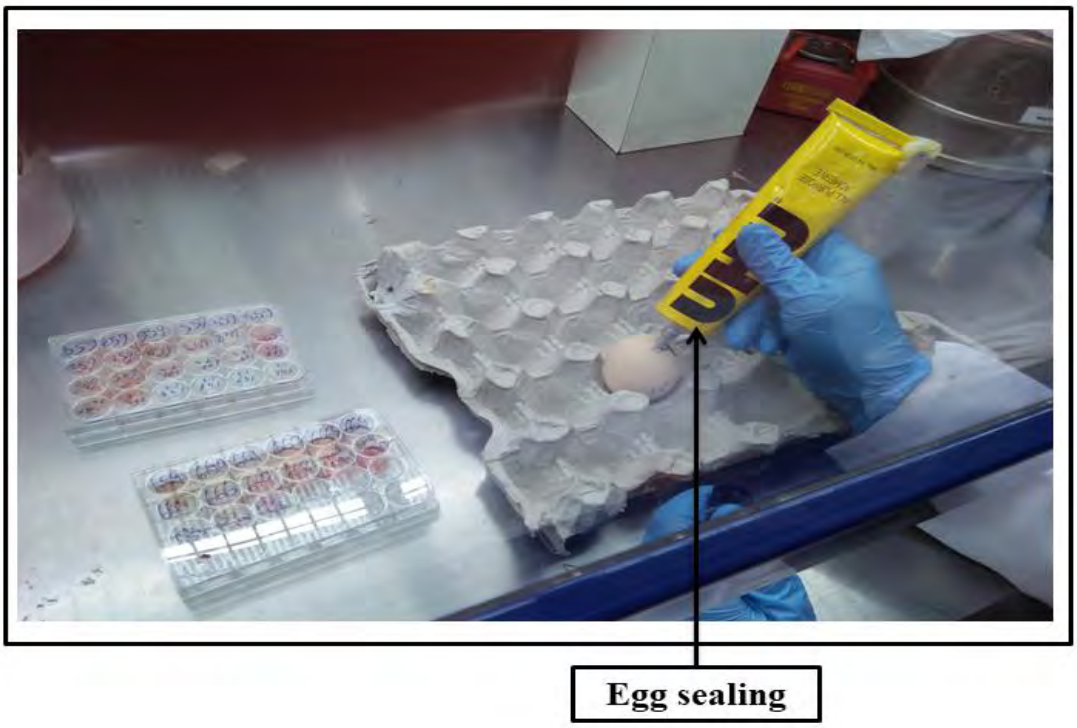

Figure 2.2: Sealing of eggs after inoculation

c) Harvesting

- The viability of eggs was confirmed daily by candling and embryos which died day after inoculation were discarded and declared unspecific deaths while deaths subsequent to this period were observed for hemagglutination.
- If during 24 hrs all eggs from a group died, then 0.22µm filter was used for inoculum filtration to remove bacterial contaminations and new eggs were inoculated, further processed after two days and were chilled at freezing temperature *i.e.* -20°C for 2 hrs and then harvested.
- Opaque shell membrane was eradicated and allantoic fluid (AF) was collected with a 21-gauge blunt ends needle using a sterile syringe.
- AF from all surviving eggs were examined for occurrence of hemagglutinating agent using HA test and further confirmation was done using Virus neuralization test (VN).
- Hemagglutination negative allantoic fluids from dead eggs were pooled with AF from live eggs of same batch and again 0.2 ml was inoculated in embryonated eggs' allantoic sac for 2nd passage (Thiermann, 2015).

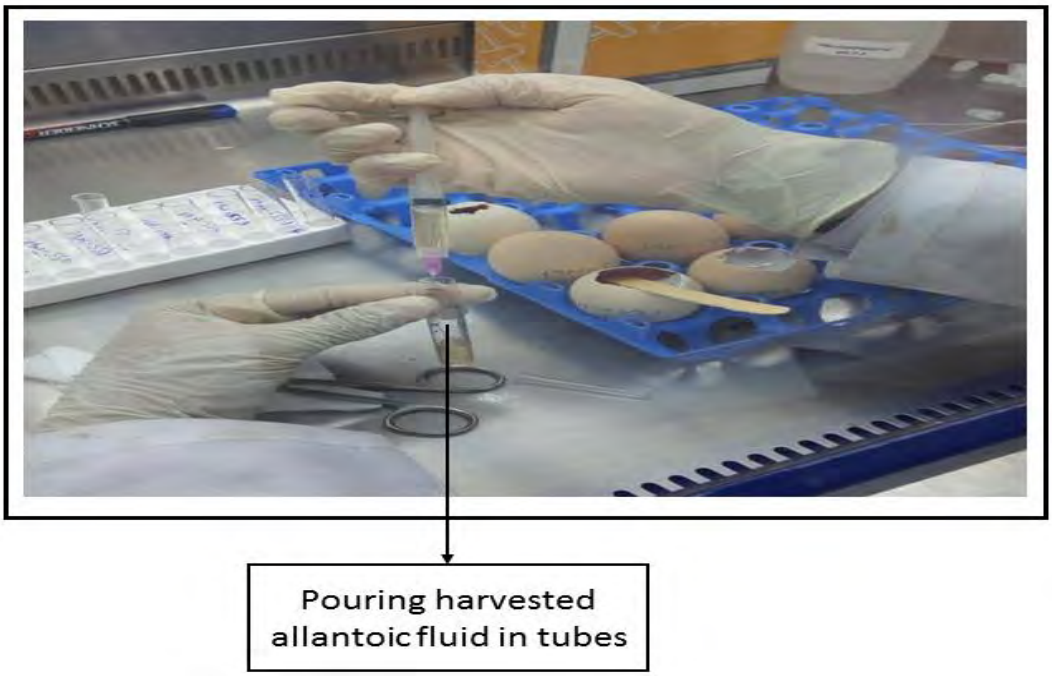

Figure 2.3: Standard protocol of pouring allantoic fluid in testing tubes after eggs harvesting

Interpretation of Results

Eggs that died within 24 hours of inoculation were discarded and those died after that were then tested for the occurrence of hemagglutinating agents while dead eggs without any hemagglutination were negative (-ve) for occurrence of particular hemagglutinating viruses. However eggs that did not show any hemagglutination after two passages were declared negative for hemagglutinating viruses (Thiermann, 2015).

2.3 Serological Evaluation

Manifestation of seroprevalence was done using Enzyme Linked Immunosorbent Assay (ELISA) and Hemagglutination Inhibition (HI) tests for antibodies detection against AI in processed samples and the determinations of antibody titters and subtyping of ELISA positive serums samples respectively.

Processing of Serum Samples

Serum samples were obtained for serological analysis and stored at 4°C after labelling; however, whole clotted blood was also occasionally obtained, centrifuged at about 2000 rpm at 10 °C temperature in refrigerated centrifuge for 10 mints for isolation of sera

(Martin *et al.*, 2006; Slorach, 2006; Cheema *et al.*, 2011). Identification of AIV was carried out by evaluating serum samples, for which 253 serum samples were obtained from various locations and initial analysis of AIV was done using ELISA.

2.3.1 Enzyme Linked Immuno-Sorbent Assays (ELISA)

ELISA test was carried out with IDEXX AIV antibody detection kits (group, 2012; Thiermann, 2015).

Test Method

- Reagents after reaching room temperature (18-26°C) were mixed by inverting and swirling. The dilution of test samples was done five hundred times (1:500) with sample diluents.

- Antigen coated plate was present in kit and positions of each sample was identified on ELISA work sheet.
- 100µl of concentrated negative and positive controls were loaded in relevant identified wells *i.e.* A1, A2 and A3, A4 and loaded plates were then incubated at temperature range 18-26°C for approx. 30 mints followed by rinsing of antigen plate with autoclaved distilled water 3-5 times.
- 100µl conjugate of anti-chicken horse reddish peroxide was added within each well and incubated again for half an hour at room temperature, washed again with distilled water 3-5 times and then 100µl of TMB substrate solution was added into the wells and incubated for 15 mints at room temperature.
- Incubation period was followed by dispensation of 100µl stop solution in the wells for stopping the reaction.
- Then at 650 nm absorbance values of the samples were calculated and noted using applicable software by Multi scan ELISA reader.

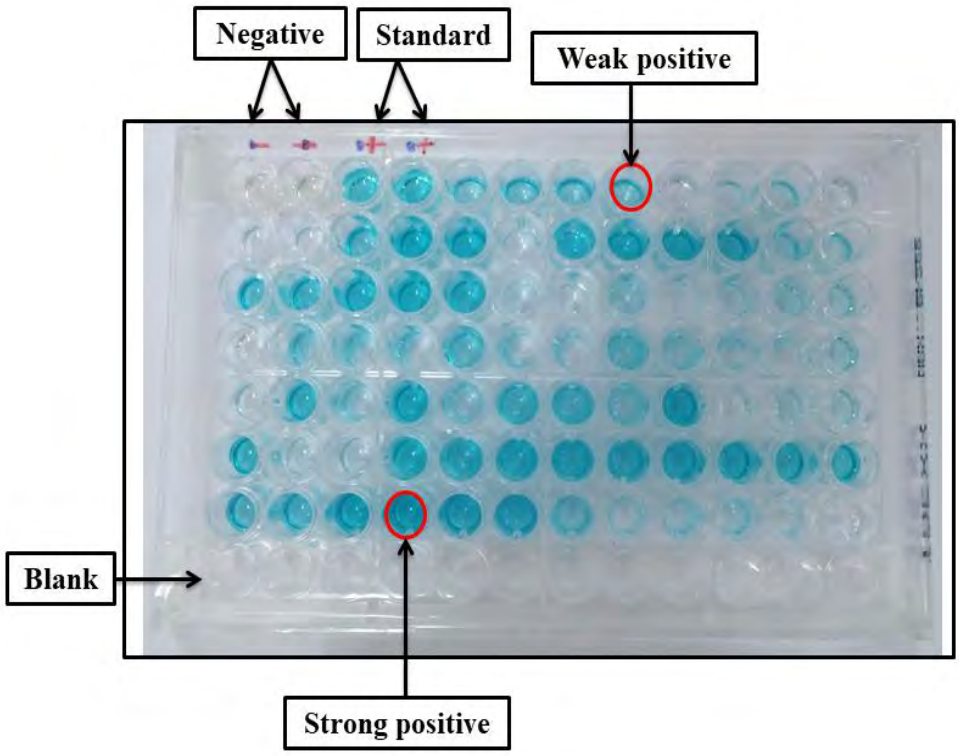

Figure 2.4: Standard pattern of ELISA

Results

- For the precision of results, difference between means of both negative controls and positive controls should be greater (>) than 0.075 with negative control means absorbance should be less than or almost equivalent (≤) to 0.150.
- The sample to positive (S/P) ratio is used for calculating the relative level of antibodies within unknown samples.

Interpretation of Results

- S/P ratios ≤ 0.5 were declared negative.
- S/P ratios > 0.5 were positive with indications of AI exposure.

Sera samples positive from ELISA were further subjected towards the Hemagglutination Inhibition (HI) Assay for subtyping by first performing Hemagglutination Test (HA) to know the titer of antigen present in sample.

2.3.2 Hemagglutination Test (HA) / Antigen titration

Hemagglutination Test was carried out according to procedure suggested by guideline manual of OIE (Beard, 1980; Bayvel, 2004; group, 2012; Thiermann, 2015) for which 0.5% red blood cells of chickens were used.

0.5 % chicken RBC suspension preparation

- 3ml of blood sample was collected by sterile syringe from cephalic vein of chicken having almost equal quantity of Alsevere's solution (Appendix II) followed by gentle shaking to avoid blood clotting.
- Blood was shifted to 15ml pointed graduated test tube and then centrifugation was performed at the rate of 1200 rpm for about 10 min at 10°C temp.
- Supernatant having serum and whitish buffy layer was isolated with great care by using sterilized syringe. The 0.1M PBS (Appendix III) was used to wash RBC's present in the pellet.
- Supernatant showing clear and transparent appearance were removed while the volume of remaining packed and washed Red Blood Cell's were measured and 25% stock solution of RBC's were prepared by adding 3 folds more Alsevere's solution and was refrigerated until used or up to three days.
- Up to 0.5% dilution of working solutions of RBC's for HA, VN and HI tests were prepared with 0.1M PBS of pH 7.2 measured before use.

Hemagglutination Test Procedure

- Using multi-channel pipette, 25µl PBS having 7.2 pH was loaded into each well from 1 to 12 of A to H rows of 96 wells plastic U-bottomed micro titration plate, followed by addition of 25µL of avian influenza virus antigen for H9 in the first well *i.e.* A1.
- Two-folds successive virus suspension dilutions from well 1 to 12 (column 1 to 12) were carried out and 25µL was discarded from the last well (well 12) while 25µL of PBS was further put into each well.

- 25µl of v/v 1% chicken RBC's were poured within each well, plate was agitated for the settlement of RBC's at room temperature *i.e.* 20 °C for about 40 min at or for half hour to 1 hour at 4°C in case of high ambient temperature.
- Negative control *i.e.* 50µl PBS + 25 µl RBCs and positive control *i.e.* Reference antigen titration were also added to the test samples and results being recorded.

Results Interpretation

The plates were examined for the HA with highest dilution being consider as end-point, where there was absolute agglutination whereas in negative cases at the bottom of plate wells RBCs resulted in compact circular button with no hemagglutination while haziness indicated hemagglutination. Maximum dilution with haziness in a well, right before the compact button of RBC's represented 1 HA unit (HAU) and considered as titre of the hemagglutinating virus (Beard, 1980).

Calculation of Hemagglutination Units (HAU)

One HA unit (1 HAU) is defined as reciprocal of highest dilutions of viruses that causes complete agglutination/clumping of RBC's when treated with properly diluted RBC's of equal volume.

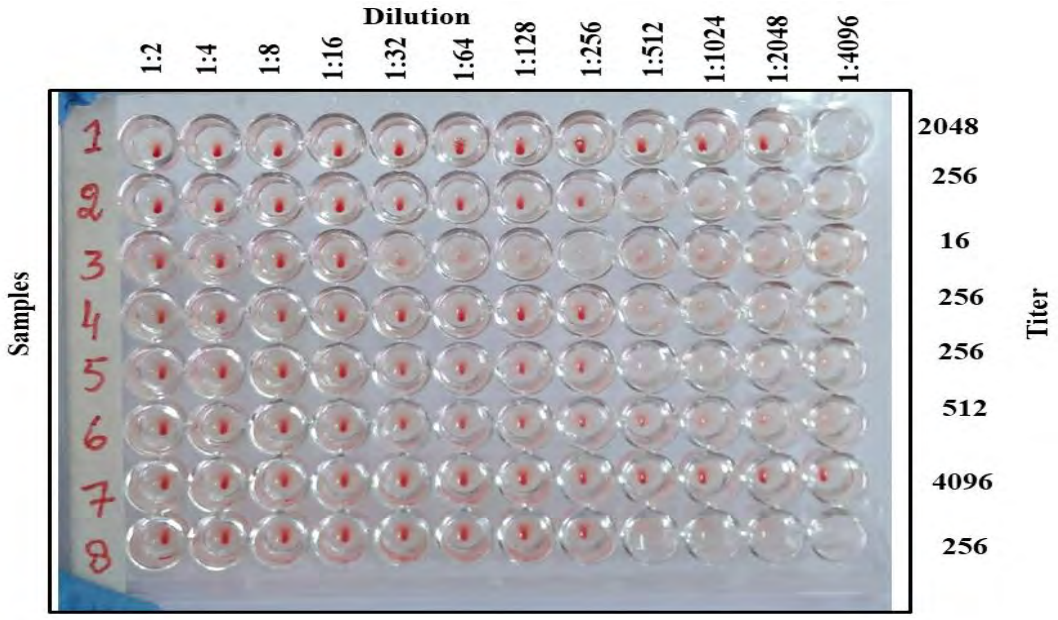

Figure 2.5: Standard Hemagglutination Test (HA) Pattern

2.3.3 Hemagglutination-Inhibition Assay

Hemagglutination-inhibition test was performed according to recommended procedure by the OIE guideline manual 2012 (Cheema *et al.*, 2011).

4HAU Preparation

In order to perform HI assay, serum samples were tested contrary to 4 HAU of virus. Formation of 4 hemagglutinating units (4HAU) of working dilution of virus/ antigen, calculation of the total antigen volume was needed to check all sera under investigation. Formation of the 4HAU antigen solution or working dilution required dividing end point titration by the number 4.

For example, if titration end-point was at distance of 1:512, working dilution of this antigen preparation was 1:128 (*i.e.* 512/4=128). Then dilutions of antigen for HI assay with 4-HAU was 1:128. Hence, for test performance we used: 1ml of Antigen in 127ml PBS and in 63.5ml of PBS we used 0.5ml antigen.

Back-titration

Back-titration of 4HAU of the antigen/working dilution was carried out and showed presence of 4 HAU of virus before adding sera which involved:

- Addition of 25µl PBS (Appendix III) into all wells within row A of microtitre plate except the 1st well *i.e.* from A2 to A12.
- Dispensation of 25µl 4HAU working dilution of antigen containing into 1st and 2nd wells from row A *i.e.* A1-A2.
- Making of two-folds sequential dilutions from wells A2 to A12 and 25µl from the last well was discarded to get 4, 2, 1, 0.5, 0.25, 0.125……. HAU.
- Wells volume was raised up to 50µl by addition of 25µl PBS solution to all wells of microtiter plate row A (A1 to A12).
- Addition of 25µl RBCs suspension (1%) in all wells from A1 to A12.
- Agitation of the plate and allowing RBCs to settle at about 20 °C *i.e.* room temp for 40 mints and results were recorded.

Interpretation of Results

- Complete hemagglutination in the 1st three well *i.e.* A3 to A1, containing 1, 2, 4 HAU respectively.
- Partial/incomplete formation of hemagglutinating agents (minute spot at well's bottom) in the well A4 means it contain 0.5 HAU.
- Total inhibition of hemagglutination in A5-A12 wells having 0.250, 0.125 and so on ……HAU respectively.

Procedure for HI Assay

The microtiter plates number was calculated based on sum of test samples, test parameters and controls, by listing of which the microtiter plates were marked with permanent markers (Smith *et al.*, 2015).

- 25µl PBS was poured within all the 96 wells present in microtiter plate.
- 25µl of testing sera as well as controls were added in first wells of 1st column of microtitration plates in accordance with the respective marking.
- Two-folds sequential dilutions of both sera and control samples was performed from the first well till last of each row *i.e.* A to H and 25µl from the last well were discarded with the help of a multichannel micropipette.
- 25µl of antigen containing 4HAU/ working dilution was then added throughout the microtitration plate.
- Plates were then covered and left for incubation for 30 min at room temp from 20 °C to 24 °C or at 4 °C for about 60 minutes followed by pouring of 25µl of 1% RBCs suspension in all of 96 wells plate.
- Plates were again covered and incubated at same temp for almost 30 mints or for 60 min at 4°C.

Results Interpretation

Results were recorded once the control RBCs were settled, by examination of the plates for absence or presence of hemagglutinating agents. Occurrence of hemagglutination activity of RBCs was considered negative for HI, whereas restricted activity of HA by the respective antibodies or absence of buttons formation at wells bottom was

considered HI positive, which was done by inclining and noticing for the presence or absence of small tear shaped streaming of RBC's at same rate as control wells containing RBCs alone.

HI titer is maximum serum dilution resulting from total suppression of 4HAU of antigen. The sample was declared negative when the HI titer was $\leq$ 1:8 while considered positive when the HI titer was $\geq$ 1:16.

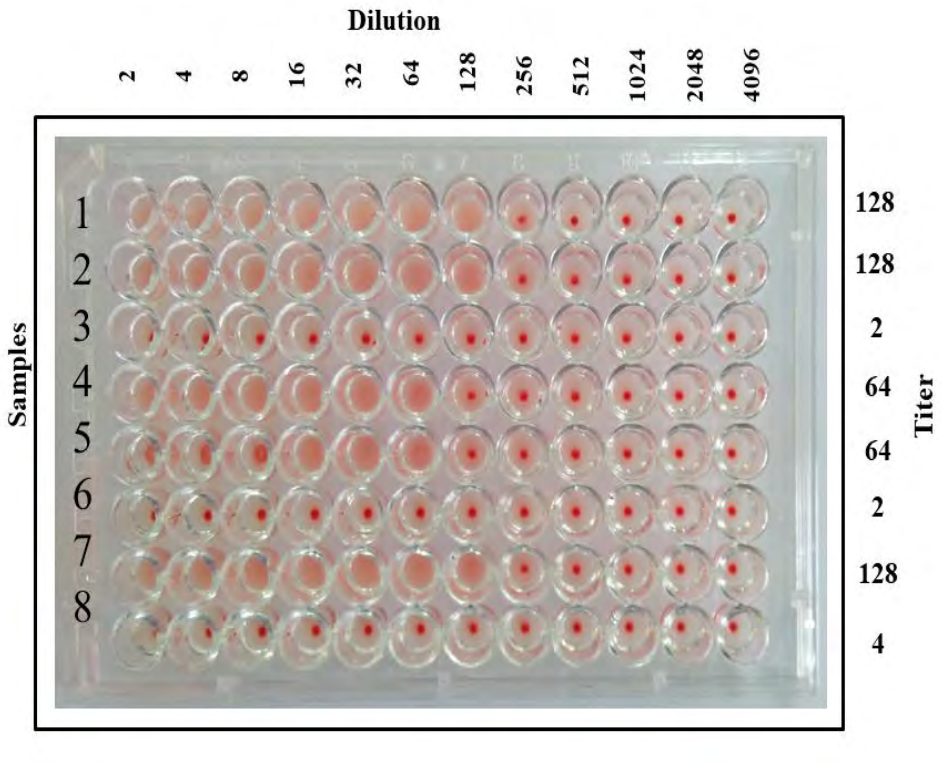

Figure 2.6: Standard pattern of Hemagglutination Inhibition Test (HI)

2.3.4 Subtype Confirmation of Avian Influenza Viruses: Virus Neutralization Test for H- Typing (VN)

The allantoic fluid with positive HA activity, were classified using specific reference antisera in the virus neutralization test conferring to protocol defined within OIE 2012 manual.

- 4HA of each antigen/ virus was prepared according to the standard procedure.
- 25µl of 0.1M Phosphate Buffer Saline having 7.2 pH was dispensed in all wells *i.e.* from 1 to 12 of A to H rows of U-shaped bottom 96 wells micro-titre plates using multichannel pipette.

- 25µl of each of the prepared antigen was then added to first well from A to H rows of micro-titre plate followed by the addition of 25µl of reference antisera
- against various subtypes of avian influenza to the first well of each marked row.
- Adding antisera was followed by making of two-fold serial dilution up to the 11th well of plate and ice incubation was done for half an hour followed by addition of 50µl chicken RBC suspension (0.5%) in all wells.
- Results were recorded after micro-titration plate was incubated for 20-30 minutes at 37 °C in the form of clear button (bead) formation as positive and the neutralization of that with corresponding antiserum was declared viral HA subtype.

2.4 Molecular Diagnosis

- Presence of Avian Influenza was confirmed after the samples being processed for virus isolation using molecular techniques like RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) and qRT-PCR (Real Time Polymerase Chain Reaction).
- Processed swabs and tissues samples were then subjected to extract RNA. Extraction of RNA was also done from Allantoic fluids (negative and positive through HA).
- All samples were processed within the biological safety cabinet from class II.

Controls

- Positive Control: Known Antigen/ Virus (propagated)
- Negative Control: PBS/Normal Saline/ water
- Note: The RNA extraction of positive control was also performed.

Influenza viral genome is established by single stranded negative RNA that can be isolated from number of samples like tissues, swabs, organs, blood, faeces, *etc* of diseased birds. Key factors to be taken in account in RNA handling is chemical stability which is lower than in Deoxyribonucleic acid (DNA), hence must be handled with great care to prevent any damage/degradation so handling is crucial besides storage.

2.4.1 Extraction of RNA by using Qiagen Kit

Ribonucleic Acid (RNA) was extracted from clinical samples by QIAamp Viral RNA mini kit with strict accordance to constructor's guide lines (QIAamp Viral RNA mini kit, QIAGEN, USA CAT# 52906). The process included the steps shown below:

- In 1.5 ml microcentrifuge tube 560µl of prepared buffer AVL having carrier RNA was added.
- Buffer AV having carrier RNA was poured with 140µl viral sample in micro centrifuge tube following pulse vortexing for 15 sec for mixing.
- Tubes containing buffer and sample were kept for incubation for 10 min at 15-25°C (room temp) followed by short period of microcentrifugation for removal of drops from inside.
- Within sample 560µl of 96-100% ethanol was added and mixed using pulse-vortexing at least for 15 sec and again briefly spin in the micro-centrifuge for removal of drops.
- From the last step 630µl of the sample was added to QIAamp Mini spin columns (in a 2ml collection tubes) without moistening the rim, the caps were then closed and microcentrifuge at 6000 × g (8000rpm) for a minute. QIAamp spin columns was then shifted into new collection tube of 2ml. Also, tube having filtrate present was trashed.
- Open the QIAamp Mini spin columns and the previous step was repeated.
- At that point 500µl of Buffer (washing buffers) AW1 was added, caps of spin columns were again closed, and centrifugation was done at 6000 × g (8000rpm) for a min and QIAamp Mini spin columns was then kept in clean collection tube of 2ml and trash tube having filtrate.
- QIAamp Mini spin columns were opened with care and 500µl of washing Buffer 2 (AW2) was added and this time centrifuged at about full speed (20,000 × g; 14000rpm) for 3 min.
- QIAamp Mini spin columns were then fixed on a fresh, clean 2ml collection tube and the old tubes having filtrate in them were trashed followed by another centrifugation round of QIAamp Mini spin column within a fresh collection tube (2ml) for a minute at a full speed.

- QIAamp spin columns again reopened with high care and 60µl of Elusion Buffer AVE was added and equilibrated to room temp followed by closing their cap and incubation for a min at room temp and centrifugation at again 6000 × g (8000rpm) for a min.
- Mini spin column was discarded, and the extracted RNA was stored at -20°C.

Table 2.1: One-step RT-PCR Reaction Mixture by Invitrogen Kit

Components	Volume / 50 µl	Volume / 25 µl
2X Reaction Mix	25µl	12.5µl
Platinum/ RT Taq mix	1µl	0.5µl
Sense Primers (25 µM)	2µl	1µl
Anti-Sense Primers (25 µM)	2µl	1µl
Autoclaved distilled water	15µl	5µl
Template RNA	5µl	5µl
Total Volume	50µl	25µl

2.4.2 Protocol for RT-PCR (Reverse Transcription PCR)

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was done using kit, Invitrogen SuperScript^{Tm} One step RT-PCR with Platinum Taq Cat. No 10928-042 following the manufacturer's procedure as follows:

- Reaction mix was made by the addition of 25µl 2X Reaction Mixture. PCR Buffer and dNTPs were present in the 2X master mix.
- The reaction was performed in autoclaved 0.2ml PCR tubes with all the vials and tips being properly autoclaved.
- 3µl of the template RNA previously extracted was then added in the reaction mix followed by addition of 1µl forward (sense) primer and 1µl of reverse (anti-sense) primer.

- Afterwards, 1µl of Platinum RT-Taq polymerase and 21µl nuclease free water (GIBCO, Invitrogen, USA) were added in the above-mentioned reaction mixture and make the total volume of 50 µl (Table 2.1).

A concentration (conc.) of around 20 pmol/µl of every gene specific primer was used for amplification of target gene of the Avian Influenza Viruses. Initially we used the M-Gene primers to screen AIV positive isolates as M-gene is highly specific for almost all the subtypes of Avian Influenza Viruses. M-Gene positive isolates were then tested for the subtyping of AIV by the use of specific primers for AIV-H9 (Rashid, 2010). The subsequent positive H-type was further proceeded to test the N-type by using the specific primers. One step RT-PCR was carried out within a thermo-cycler (Thermal cycler Veriti, Applied Biosystems) using specific temperature profile for RT-PCR (Table 2.2) provided by manufacturers (Invitrogen, USA). Optimized temperature program was 45°C for about 25 minutes (Reverse Transcription/ cDNA synthesis), 94°C for almost 15 sec, annealing occurs at 58°C for 30 sec and extension at 70 °C for about 10 min. This was finally stored/kept at 4°C until further use.

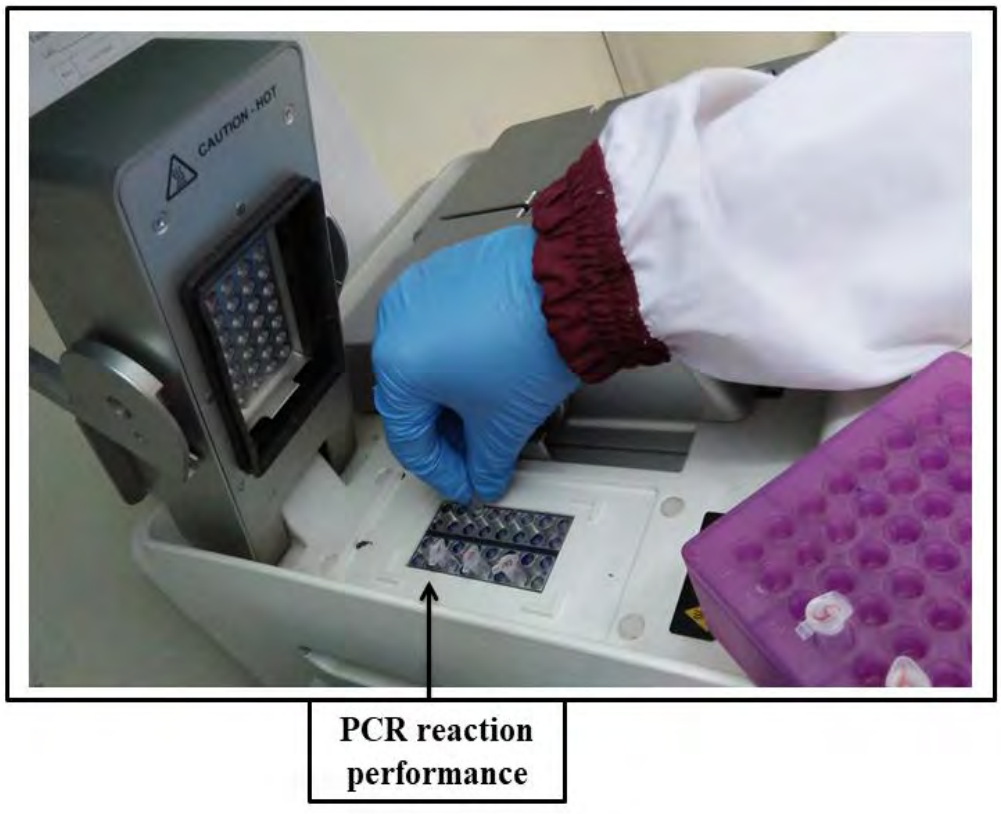

Figure 2.7: Performing PCR Reaction

Table 2.2: Temperature Profile for RT-PCR with Invitrogen SuperScript™ One Step RT-PCR Kit

Sr.no	RT-PCR steps	Temperatures	Cycles
1	Reverse Transcription	45°C for 25 minutes	1
2	Initial denaturations	94°C for 2 minutes	1
	PCR CYCLE		
1	Denaturation	94°C for 15 seconds	X40
2	Annealing	58°C for 30 seconds	
3	Extension	70°C for 1 minute	
4	**Final extension**	72°C for 10 minutes	1
5	**Storage**	4°C until used	1

2.4.3 Electrophoresis of Agarose Gel

- The amplified/magnified PCR amplicons run on the agarose gel (Appendix IV) and ethidium bromide (EthBr) was added in gel, allowed to cool down for 30 min and comb was lodged inside gel casting trays.
- After cooling, the molten agarose gel having EthBr was poured into a gel casting tray, ensuring that no air bubble was trapped and tray was then kept at room temp (20°C to 24°C) for gel to solidify, for 25-30 min and after solidification of gel, comb was removed with care.
- Solidified gel was kept in the electrophoresis chamber having 1X TBE buffer (Appendix V).
- A sum of 10µl amplified PCR product of all the tubes were kept in micro centrifuge tubes followed by mixing of 2µl loading dye (Appendix VI).
- Only 2µl DNA ladder and 10µl sterile H_2O were mixed in ladder vials (Appendix VII).

- Mixture of PCR product and dye was then loaded into wells of solidified gel, along with the marker.
- After closing chamber, 100V voltage was applied for about 60 min to 1.5 hour.
- After gel running process was completed, the gel was then taken from electrophoresis chamber and placed inside the gel-documentation system and PCR bands were detected by comparing them with ladder bands and was photographed in BioCapt Software (Vilber- Lourmat, France) (Sambrook, 1989).

Table 2.3: Sequence of Primers use for Detection of M-Genes, AIV H9, And N2 by RT-PCR

Primer Name	Primer's oligonucleotide Sequence	Product (bp)	References
AIV			Dr. R. J. Webster (SJCRH, memphis, TN).
M-WSN-F	5'-AAT GGA AAA TGT TCA TAG ACG-3'	1023	
M-1023-R	5'-CAT AGA ACC TTG TTA ATT ACA T-3'		
Pathotype H9			
H9-D-F	5'-CAG ACA CAA TCT GGA ATG TGAC-3'	471	NRLPD, NARC
H9-D-R	5'-TCA GTC TGG CAT TGC ACT AC-3'		
Subtype H9			
H9-E F	5'-TCA GCA GTA AAT GGA ACG TG-3'	574	NRLPD, NARC
H9-E R	5'-TCA GTC TGG CAT TGC ACT AC-3'		
N2			
N2-87 F	5'-AGCAACGGATATGACACTAC-3'	720	WHO
N2-807 R	5'-TGACAGTGGACTAATGTGGAC-3'		

(Rashid, 2010)

Interpretation of Results

The results were interpreted and manifested for the existence of bands having specified base pairs (bps or bp) size and positive results were shown as follows:

- AIV matrix gene (1023 bp)
- AIV- H9 (471 bp & 574 bp)
- N- Typing for N2 (720 bp)
- No bands or bands showing non-specific base pair sizes represented as negative results (Seifi *et al.*, 2010; Sarwar *et al.*, 2013).

2.5 Isolation of *Chlamydia psittaci*

Processing of swabs and tissue samples was done for *Chlamydia psittaci* isolation and identification Clinical samples were handled with great care during shipment and storage to prevent any loss of *chlamydia*. Before inoculation contaminated samples were pre-treated and different antibiotics were used but they had no inhibitory effect on *chlamydia*.

Three common methods were used such as:

- ✓ Treatment/handling with different antibiotics,
- ✓ Action of antibiotics followed by centrifugation at low speed (Andersen & Vanrompay, 2008),
- ✓ treatment of antibiotics followed by filtration (Dufour-Zavala, 2008).
- ✓ Samples were homogenised in the PBS, having pH 7.2, and containing following antibiotics in maximum amount: gentamicin (50– 200µg/ml), streptomycin and vancomycin (100µg/ml each). nystatin (50µg/ml each) or Amphotericin B were added for controlling yeast and fungal growths. Usually addition of chloramphenicol, tetracycline and penicillin were avoided for they are known to inhibit the growth of *Chlamydia*.

2.5.1 Isolation of Chlamydia psittaci by In-ova Propagation/ Egg Inoculation Technique

d) Candling of Eggs

For the isolation of *chlamydia* chicken embryos were used. Candling of 6-7 days old chicken embryonated eggs was done for checking embryos viability. If there was no movement observed inside egg it was discarded (Authority *et al.*, 2018). Fertile eggs of chicken were transferred for the inoculation, to a biological safety cabinet (Dufour-Zavala, 2008).

e) Inoculation of Eggs

- 70% ethanol was used to wipe the broader inoculation site of eggs, left for air drying and then a small hole was punched inside egg shell with electronic gun.
- A total of about 0.5ml of each sample was inoculated within each egg by 3ml syringe and needle. Both syringe and needle were discarded after use.
- After inoculation the hole was sealed with a melted wax or by some other sealant like UHU liquid gums.
- Eggs after inoculation were transferred to incubator and incubated for 48 hrs, at 39°C rather than at less temp like 37°C because *chlamydia* multiplication/duplication markedly increased at higher temperatures (Authority *et al.*, 2018).

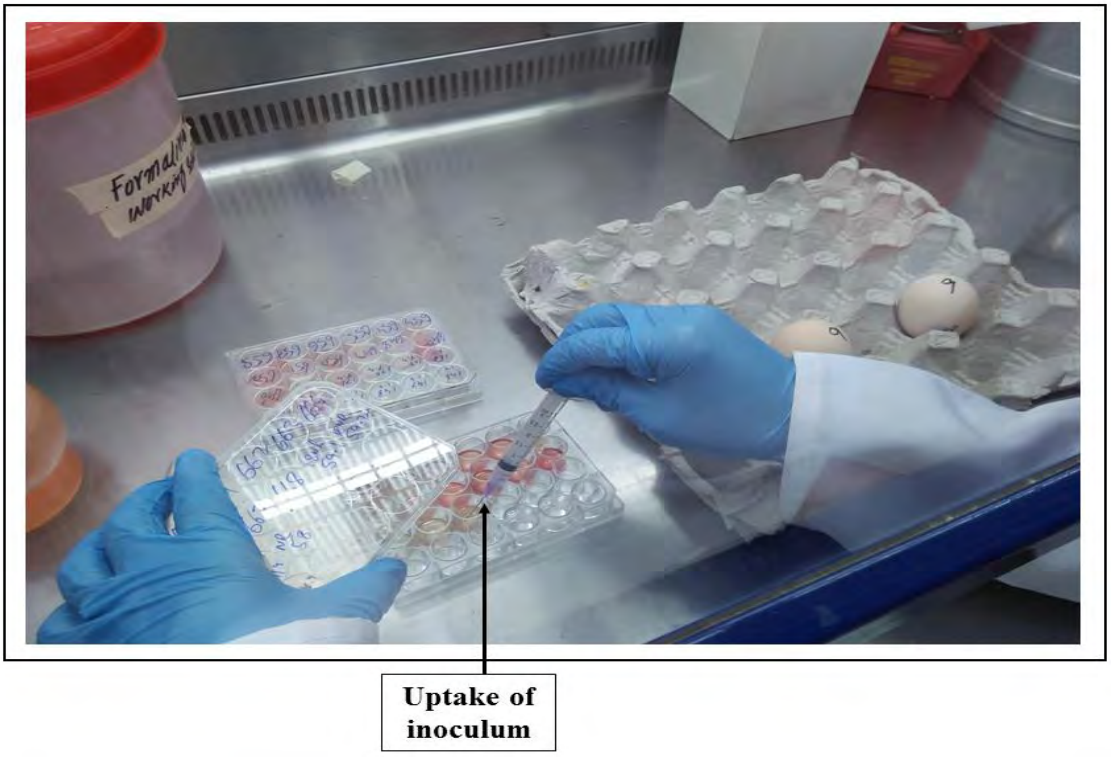

Figure 2.8: Uptake of inoculum by standard procedure

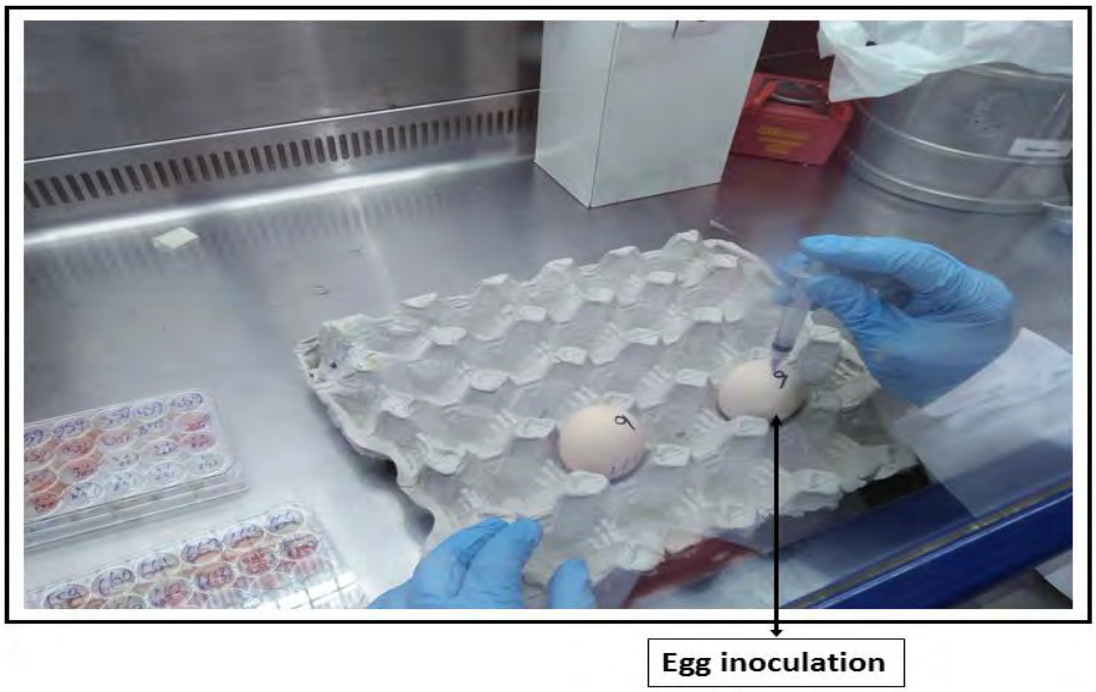

Figure 2.9: In-ova Inoculation by standard procedure

f) Harvesting

- To check the viability of inoculated eggs candling was done on daily bases.
- Embryo deaths with in a day after inoculation were discarded and considered as non-specific deaths. Whereas deaths subsequent to this period were further checked for chlamydial presence.
- If after a day all the eggs from any single group were found dead after inoculation, inoculum was then filtered to remove bacteria that cause contamination, through a 0.22µm filter and fresh eggs were then again inoculated.
- After 2 days of inoculation further processing of surviving eggs was performed and chilled for 120 min at freezing temp *i.e.* -20°C prior to harvesting.
- Allantoic fluid of survived egg was then checked for chlamydial presence through serological test (Authority *et al.*, 2018).

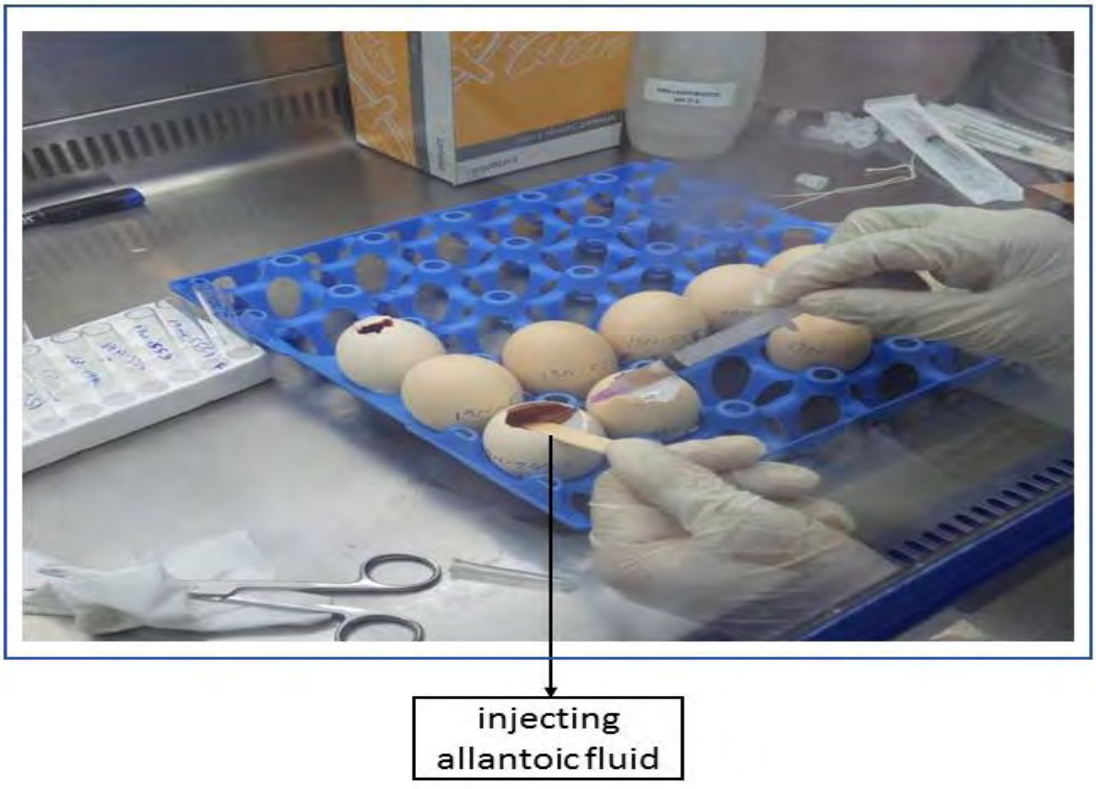

Figure 2.10: Standard procedure of In-ova harvesting

Interpretation of Results

Discard the eggs that died within the first day after inoculation and eggs died after 24 hrs period were further checked for *Chlamydia psittaci* presence. Eggs that died but did not show antigens were negative for occurrence of *Chlamydia psittaci*. If eggs showed no antigen after two passages, samples were considered as negative for the *Chlamydia psittaci* (Authority *et al.*, 2018).

2.6 Serological Evaluation

Seroprevalence of chlamydial antibodies was determined by the main serological method that is ELISA. The sera samples after processing were subjected towards ELISA, that was performed for the detection of antibiotics against *Chlamydia psittaci*.

2.6.1 ELISA (Enzyme Linked Immunosorbent Assay)

Enzyme linked immunosorbent assay (ELISA) was performed by ImmunoComb test kit (Biolage laboratories, Kibbuz Baled, Israel designed Avian Chlamydophila Antibody Test Kit Cat # 50ACP301) (Phalen, 2001). IgG antibody titer to *Chlamydia* psittaci were analyzed from avian serum samples using this kit.

Test Procedure

- The solid phase dot ELISA technology is the basic principle of ImmunoComb ELISA test.
- There are 12 comb shaped plastic cards for testing up to 10 testing samples, a positive and negative controls, and also antigens were applied onto the solid phase.
- Testing samples were mixed with diluents in first row of wells of the multi-chamber developing plates and then incubated. During incubation, specific IgG antibodies present in the testing samples will bind with antigens at test spots.
- Comb after incubation was transferred to the place where already unbounded antibodies were washed from antigen spots.

- Comb was then allowed to react with an antigen avian IgG alkaline phosphate conjugate, resulting in the formation of antigen-antibody complexes at test spots.
- Comb was moved towards last well after two more washes where due to enzymatic reaction, color developed.
- Color intensity of test spots directly corresponded with the antibody level in the tested samples.

Table 2.4:Results Interpretation

Color	Comb Scale Values	Results	Result Interpretation
White color	0	Negative	No antibodies of *C. psittaci*
Trace of Grey	>0 – 1	Suspicious	Variable, according to bird types
Light Grey	>1 – 2	Low positive	low antibody titer to *C. psittaci*
Medium Grey	3 – 4	Positive	Moderate level of antibodies to *C. psittaci*
Dark Grey	4 – 5	Highly positive	High antibody titer to *C. psittaci*

2.7 Molecular Diagnosis

- After chlamydial isolation these samples were further subjected to molecular biology techniques to confirm the presence of *Chlamydia psittaci i.e.* Conventional PCR (RT-PCR) and real time PCR (qRT-PCR) (Lagae *et al.*, 2014).
- DNA from processed swabs and tissue samples was extracted.
- All samples were processed in class II biological safety cabinet.

Controls

- Positive Control: Known Antigen/ *Chlamydia psittaci* (propagated)
- Negative Control: PBS/Normal Saline/ water
- The DNA extraction of controls was also performed.

Chlamydia psittaci is a double stranded DNA that can be extracted from conjunctival, pharyngeal, or cloacal swab samples or tissues of infected birds. Handling and storage of DNA is an important factor. Chemical stability of DNA is the most important factor. Therefore, it must be handled carefully to avoid any degradation.

2.7.1 DNA Extraction by Favorgen Kit

DNA extraction of clinical samples was done with FavorPrep^{TM} Nucleic Acid Extraction Kit I based on manufacturer's instruction (FavorPrep^{TM} Nucleic Acid Extraction Kit I, FAVORGEN CAT# FAVNK 001). Following steps were employed in this procedure:

- 150µl testing sample and 570µl of lysis VNE Buffer were dispensed in a microcentrifuge tube and mixed well by vortexing and left for incubation for 10 min at room temp.
- Within the sample mixture additional 570µl of 96-100% Ethanol was added and then mixed well by pulse vortexing.
- After that VNE columns were combined with collecting tubes and then 700µl of sample mixture was transferred to VNE column. Then collection tube was centrifuged for 1 min at about 8000 x g. After centrifugation, flow-through was discarded.
- After that remaining sample was transferred into VNE column and centrifuged again for a minute at 8000 x g. After centrifugation flow-through along with collection tubes were discarded and VNE column was combined to new collection tubes.
- Then 500µl Washing buffer 1 was dispensed in VNE columns and it was centrifuged at 8000 x g for about 1 min and flow-through from column was again discarded and combine VNE column with used collection tube.
- Next to first washing 750µl of washing buffer 2 was poured to VNE column and again centrifugation for a min was performed at 8000 x g and the flow-through from column was again discarded and VNE column was again combined with used collection tube.

- The previous step was repeated and washing with the washing buffer 2 was performed. Then it was centrifuged at the full speed (..18,000 x g) for extra 3 min to dry VNE columns. Flow-through and collection tubes were discarded.
- VNE column was again combined to an elution tube and 50µl of pre-heated RNase-free H_2O was added to membrane centre of VNE column and incubated for two min.
- After incubation it was centrifuged for 2 min to elute DNA and extracted DNA was then stored at -70°C.

2.7.2 Quantification of DNA

DNA concentration was assessed using Nandrop (thermoscientific). DNA quantity was measured directly by the software and the purity ratio was assessed by measuring the absorption spectra at wavelength 260/280.

2.7.3 Protocol for Conventional PCR

Conventional PCR was performed by using ThermoScientific DreamTaq Green PCR Master Mixture (2X), #K1071 adopting manufacturer's protocol as shown below: (Gennari *et al.*, 2011; Lagae *et al.*, 2014)

- DreamTaq Green PCR Master Mixture contained following components: DreamTaq DNA polymerases, dNTPs 0.4mM (dCTP, dATP, dTTP and dGTP) each, 2X DreamTaq Green buffer and 4mM $MgCl_2$. Tips and vials used in the reaction were already autoclaved.
- After DreamTaq Green PCR Master Mixture (2X), was briefly vortexed and then centrifuged for short time and 25µl was added in thin walled 0.2ml autoclaved PCR tube placed on ice.
- Then already extracted 5µl template DNA was dispensed to this reaction mixture followed by addition of 1µl of forward (sense) primers and 1µl reverse (anti-sense) primers into reaction mixture tubes.
- Nuclease free H_2O was then added with in reaction mixture and total volume was made up to 50µl (Table 4) and samples were then gently vortexed and spun down.

Concentration of 20 pmol/ µl of specific gene primer was used that amplified the targeting genes of *Chlamydia psittaci*. It was difficult to differentiate between chlamydial species only on the basis of 16s rDNA therefore we identified *C. psittaci* by targeting a specific 23s rDNA. Outer membrane protein (*omp*A) was also one of the major target gene in *C. psittaci* because of its importance as a constituent of chlamydial membrane and a principal antigen. Inclusion protein (*Inc*A) plays an important role in homotypic fusion. One step DNA-PCR was carried out inside a thermo-cycler (Thermalcycler Veriti, Applied Biosystems) with the temp parameters recommended for conventional DNA-PCR (Table 2.5) by the manufacturers (TermoScientific DreamTaq Green PCR). The optimized temperature was 95°C for 1-3 min for denaturation, annealing at about 5°C less than the melting temp of primer for 30 seconds and optimum extension temp for DreamTaq DNA polymerases at 70-75 °C for almost 10 min. This DNA PCR product was then finally stored at 4°C until used.

2.7.4 Electrophoresis of Agarose Gel

- On the 1% agarose gel (Appendix IV) PCR amplified amplicon were run.
- Comb inserted in agarose gel was removed after solidification of gel with great care and solidified gel was then placed into an electrophoresis chamber in which 1X TBE buffer (Appendix V) was already added.
- Total amount of 10µl amplified PCR products from each tube were loaded in the wells of solidified gel and 7µl of 100 or 50 bp DNA ladder was loaded in ladder wells.
- 100V voltage was applied for approximately 1-1.5 hours after the chamber was closed.
- When the gel running process was completed it was removed from electrophoresis chamber and was then placed in gel documentation system. The bands were detected by comparing them with ladder bands (Kaltenboeck *et al.*, 1991).

Interpretation of Results

The results were confirmed based on the presence of bands of specified base pairs size (specific bands), positive results were shown as follows:

- Band size for *Chlamydia* 1200 & 1100 bp
- Band size for subtype *C. psittaci* 76 & 393 bps
- No bands or bands observed are of non-specific base pair sizes represented negative results.

Table 2.5: Conventional PCR Reaction Mixture by DreamTaq Green Kit

Components	Volume / 50µl	Volume / 25µl
DreamTaq Green PCR Master Mix (2X)	25µl	12.5µl
Forward Primer (25 µM)	2µl	1µl
Reverse Primer (25 µM)	2µl	1µl
Nuclease free Water	15µl	5µl
DNA template	5µl	5µl
Total	50µl	25µl

Table 2.6: Temperature Profile for Conventional-PCR using ThermoScientific DreamTaq Green PCR Master Mix (2X)

Sr.no	RT-PCR steps	Temperatures	Cycles
1	Initial denaturation	95°C temp for 1-3 mint	1
2	Denaturation	95°C temp for 30 sec	X40
3	Annealing	Tm-5°C for about 30 sec	
4	Extension	72°C temp for 1 min	
5	**Final extension**	72°C temp for 5-15 min	1
6	**Storage**	4°C until used	1

Table 2.7: Sequence of Primers used for Detection of *chlamydia psittaci* by RT-PCR

Primers Name	Primer's oligonucleotide Sequence	Product (bp)	References
Chlamydia			
NRL-70R2r	5'- ATG AAA AAA CTC TTG AAA TCG G -3'	1200	(Sachse *et al.*, 2008)
NRL-70R2f	5'- TCC TTA GAA TCT GAA TTG AGC -3'		
NRL-70R3r	5'- ATGAAAAAACTCTTGAAATCGG -3'	1100	(Yang *et al.*, 2011)
NRL-70R3f	5'- CAAGATTTTCTAGACTTCATTTTGTT -3'		
Subtype C. psittaci			
NRL-69O1r	5'- CTGCGCGGATGCTAATGG -3'	76	OIE Terrestrial manual 2018
NRL-69O1f	5'- CACTATGTGGGAAGGTGCTTCA -3'		
NRL-69R2r	5'- ACTTGTTCCGCAGTTTGTTCCATC -3'	393	(Opota *et al.*, 2015)
NRL-69R2f	5'- ATGATTAACACAGCTATCGGC -3'		

2.8 Real time RT-PCR Reaction Mix

qRT-PCR reaction mixture was prepared using Invitrogen SuperScript ^{TM} III Platinum^{R} One Step Quantitative RT-PCR System Cat. No. 11732-020. Already extracted RNA/DNA were used to carry out the procedure.

- Reaction took place on ice, components of 50 µl reaction are given in table 7.
- The PCR plate was with microfilm and centrifuged briefly on centrifugation machine. Plate was then placed in the Real Time PCR machine for analysis.

- Plate document was prepared on 7500 Real Time PCR equipment. PCR profile was adjusted on Real Time PCR equipment for the Matrix, H9 and *Chlamydia psittaci* genes (Ménard *et al.*, 2006) according to the specified temperature program

Table 2.8: One-step Real Time RT-PCR TaqMan Probe Reaction Mix by Invitrogen Kit

Components	**Volume/50 µl**	**Volume/25 µl**
2X Reaction Mix	25.0µl	12.5 µl
RT/Platinum Taq mix	1.0 µl	0.5 µl
ROX reference Dye (Optional)	1.0 µl	0.5 µl
Sense Primer (10 µM)	2.0 µl	1.0 µl
Anti-Sense Primer (10 µM)	2.0 µl	1.0 µl
Probe (5 µM)	2.0 µl	1.0 µl
RNase free water	12.0 µl	3.5 µl
RNA template	5.0 µl	5.0 µl
Total	50.0 µl	25.0 µl

Note: template RNA and NTC (negative template control) added at the end in a separate clean area and positive control was also added in separate clean area to avoid template cross contamination

Table 2.9: Temperature Profile for Matrix Gene

Reverse Transcription and pre-denaturation	Temperatures	Time	Cycles
cDNA synthesis	50°C	30 min	1
Pre-denaturation	95°C	15 min	1
PCR AMPLIFICATION			
Denaturation	95°C	10 sec	X40
Annealing	60°C	30-60 sec	

Table 2.10: qRT-PCR Primers for the detection of M-gene of AIV

Primer Name	**Primer's Oligonucleotide Sequence**
Sense primer	5'-AGA TGA GTC TTC TAA CCG AGG TCG-3'
Antisense primer	5'-TGC AAA AAC ATC TTC AAG TCT CTG -3'
Probe	5'-FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3'

Table 2.11 : Temperature Profile for AIV H9

Reverse Transcription And pre-denaturation	Temperatures	Time	Cycles
cDNA synthesis	50°C	20 min	1
Pre-denaturation	95°C	15 min	1
PCR AMPLIFICATION			
Denaturation	95°C	45 sec	X40
Annealing	54-60°C	30-45 sec	

Table 2.12: qRT-PCR Primers for the detection of H9N2 subtype of AIV

Primer Name	**Primer's Oligonucleotide Sequence**
Sense primer	5'-ATG GGG TTT GCT GCC-3'
Antisense primer	5'-TTA TAT ACA AAT GTT GCA YCTG -3'
Probe	5'-FAM-TTC TGG GCC ATG TCC AAT GG-TAMRA-3'

Table 2.13: Temperature Profile for *Chlamydia psittaci*

Reverse Transcription And pre-denaturation	Temperatures	Time	Cycles
cDNA synthesis	50°C	20 min	1
Pre-denaturation	95°C	15 min	1
PCR AMPLIFICATION			
Denaturation	95°C	45 sec	X40
Annealing	54-60°C	30-45 sec	

Table 2.14: qRT-PCR Primers for the detection of *Chlamydia psittaci*

Primer Name	**Primer's Oligonucleotide Sequence**
Sense primer	5'- CTG AAA CCA GTA GCT TAT AAG CGGT -3'
Antisense primer	5'- ACC TCG CCG TTT AAC TTA ACT CC -3'
Probe	5'-FAM-CTC ATC ATG CAA AAG GCA CGC CG-TAMRA -3'

(Ehricht *et al.*, 2006)

Primer Name	Primer's Oligonucleotide Sequence
Sense primer	5'- CTG CGC GGA TGC TAA TGG -3'
Antisense primer	5'- CAC TAT GTG GGA AGG TGC TTCA -3'
Probe	5'- FAM-CGC TAC TTG GTG TGAC-TAMRA -3'

OIE Terrestrial manual 2018

Primer Name	Primer's Oligonucleotide Sequence
Sense primer	5'- CGG CGT GCC ACT TGA GA -3'
Antisense primer	5'- GCC ATC ATG CTT GTT TCG TTT -3'
Probe	5'-FAM-TCA TTG TCA TTA TGG TGA TTC AGG A-NFQ-MGB -3'

(Ménard *et al.*, 2006)

Result Interpretation

Results were observed by the fluorescence pattern in fluorogram, samples above the baseline were positive, while samples below the base line were negative. No or very low florescence depicted negative results. Moreover, ct values were also the indicative of results. Following measures were taken to ensure quality of product.

- Positive and negative controls were run to check the validity of reaction.
- The specificity of M, H9 and *chlamydia* primers and probes were accurate. Primer and probes cross reactivity was nil/zero.
- Excessive freeze/thaw cycles were avoided for primers and other temperature sensitive reagents.

2.9 Acceptance Criteria

- The positive control (strong positive) gave good ct values between 10-25.
- The negative samples were negative and had no cross contamination.
- Samples with ct values > 35 were consider negative.

- In case of weak positive samples with ct values > 30, reaction was repeated.
- Template contamination was avoided.

2.10 Precautions

- All the chemicals required careful handling. Gloves were used while working.
- Hazardous chemicals were labelled and placed at designated place.
- Working place were always decontaminated before and after working.

RESULTS

3. RESULTS

3.1 Ecological Zones for Surveillance of AIV H9N2 and *Chlamydia psittaci*

Different sera. swab and tissue samples from various bird types including commercial poultry, backyard poultry, wild domestic and wild migratory birds were collected from poultry-populated ecological regions of Pakistan during July 2018 to June 2019. These zones comprised of following areas except Baluchistan, as no sampling was done here during this time

i. Khyber Pakhtoon Khawa (KPK)
ii. Punjab
iii. Sindh
iv. Along with the Islamabad Capital Territory (Fernández-Aguilar *et al.*)

In Khyber Pakhtoon Khawa samples were received from different cities including Bannu, Mansehra, Peshawar, Karak and Gilgit Baltistan. While from Punjab province sampling was done from Sahiwal, Rawalpindi, Gaggo mandi, Pakpattan, Arif wala, Sargodha and Lahore. Moreover, in Sindh province samples were received from Karachi.

Figure 3.1: Ecological zones of Pakistan for AIV H9N2 and Chlamydia psittaci diagnosis

3.2 Area wise Sampling Data during July 2018 - June 2019

Total 550 samples from various districts of Pakistan were collected that were subjected to different tests for finding AIV H9N2 and *Chlamydia psittaci* during July 2018 to June 2019. Out of 550 samples, 285 (51.81%) samples were collected from KPK, 131 (23.27%) were collected from Punjab province, 69 (11.63%) samples were collected from Sindh province and no sampling was done in Baluchistan during this period. From Islamabad Capital Territory 73 (13.27%) samples were received for diagnosis as shown below in pie chart:

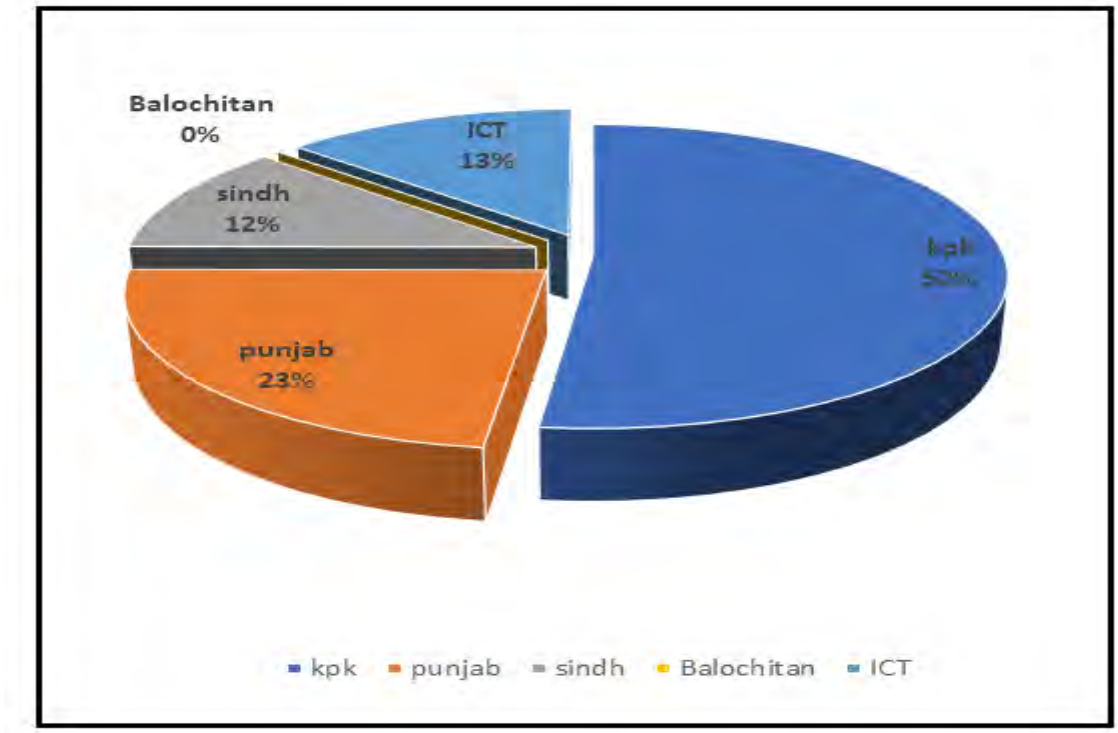

Figure 3 2: Pie Chart showing percentage of collected samples from various regions of Pakistan for AIV H9N2 and Chlamydia psittaci diagnosis

3.3 Month wise Sampling Data

From total 550 samples, the highest sample number were collected in January whereas the lowest number of samples were collected in December. 158 out of 550 samples were collected in summers, (June - August), followed by 91 in autumn (September - November), 134 in winter (December - February) while maximum samples 167, were collected during spring season (March - May).

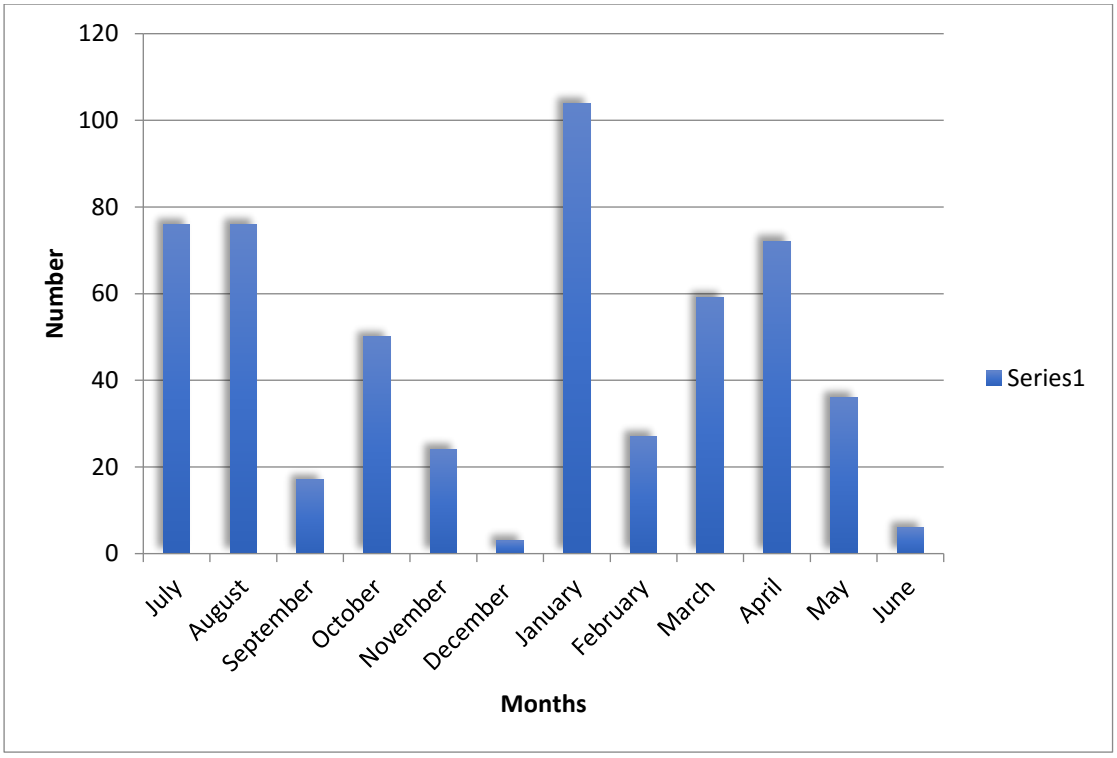

Figure 3.3: A bar graph representing yearly collected samples for AIV H9N2 and Chlamydia psittaci diagnosis

3.4 Bird Type wise Sampling Data

The samples containing serum, swabs and tissues for the differential diagnosis of AIV H9N2 and *Chlamydia psittaci* were collected from different bird types belonging to four major categories including commercial poultry, backyard poultry, wild domestic or fancy birds and wild migratory birds as shown in table 3.1

Table 3.1: Samples collected from different bird species

Commercial poultry		**Backyard poultry**		**Wild domestic/ Fancy**		**Wild migratory**		**Total**
Broiler	360 (65.45%)	Desi	19 (3.45%)	Peacock	36 (6.54%)	Crow	5 (0.909%)	
		Missri	4 (0.72%)	Pigeon	4 (0.72%)	Raj Hans	2 (0.36%)	
		Nacked Neck	34 (6.18%)	Turkey	1 (0.18%)	Flamingo	1 (0.18%))	
		Mix Breed	12 (2.18%)	Quail	1 (0.18%)			
		Golden	67 (12.18%)	Duck	2 (0.36%)			
		Guinea Fowal	1 (0.18%)	Black Swan	1 (0.18%)			
	360 (65.45%)		**137 (24.90%)**		**45 (8.18%)**		**8 (1.45%)**	**550 (100%)**

Out of 550 samples, 360 (65.54%) were collected from commercial poultry containing Broilers only. While backyard poultry included 137 (24.90%) samples and contained Desi, Missri, Golden, Nacked Neck, Guinea Fowal and Mix Breed. Almost 45 (8.18%) samples from wild domestic and fancy birds that included Peacock, Pigeon, Turkey, Quail, Duck and Black Swan were collected. Crow, Raj Hans and Flamingo belong to wild migratory bird category and only 8 (1.45%) samples were reported from this category.

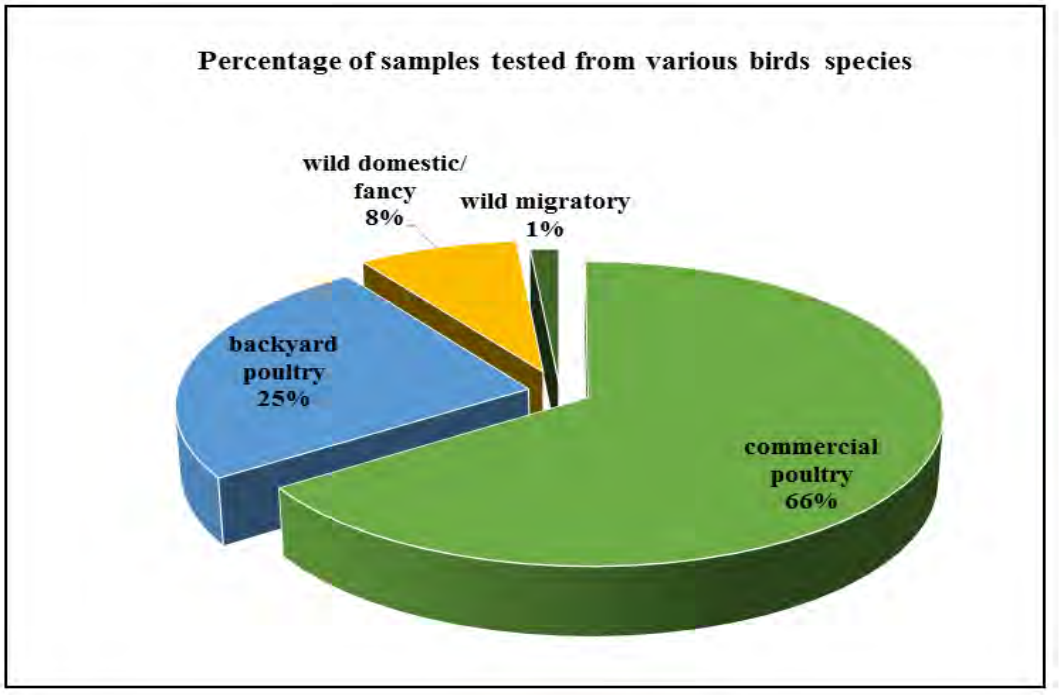

Figure 3.4: Pie graph showing percentage of different birds' species used for AIV H9N2 and chlamydia psittaci differential diagnosis

3.5 In-ova Inoculation for Identification and Isolation of Avian Influenza Virus

Total 491 tissue and swab samples were processed for the virus and bacterial isolation through in-ova inoculation techniques. Only 184 samples showed positive Hemagglutination activity. Positive samples were further subjected to VN test for the confirmation of AIV subtype H9N2 confirmation and only 132 samples were found positive for AIV H9N2 by virus neutralization.

3.5.1 Seasonal Isolations of AIV H9N2 from July 2018 to June 2019

From 184 samples detected positive for AIV only 132 were found positive for the subtype H9N2 by VN. Highest number of isolations 80 (60.60%) were carried out during the winter (December - February) season followed by 40 (30.30%) during spring (March - May) season. Only 12 (9.09%) isolations were done in summer (June - August) while no isolation was performed in the autumn (September - November). Figure 3.5

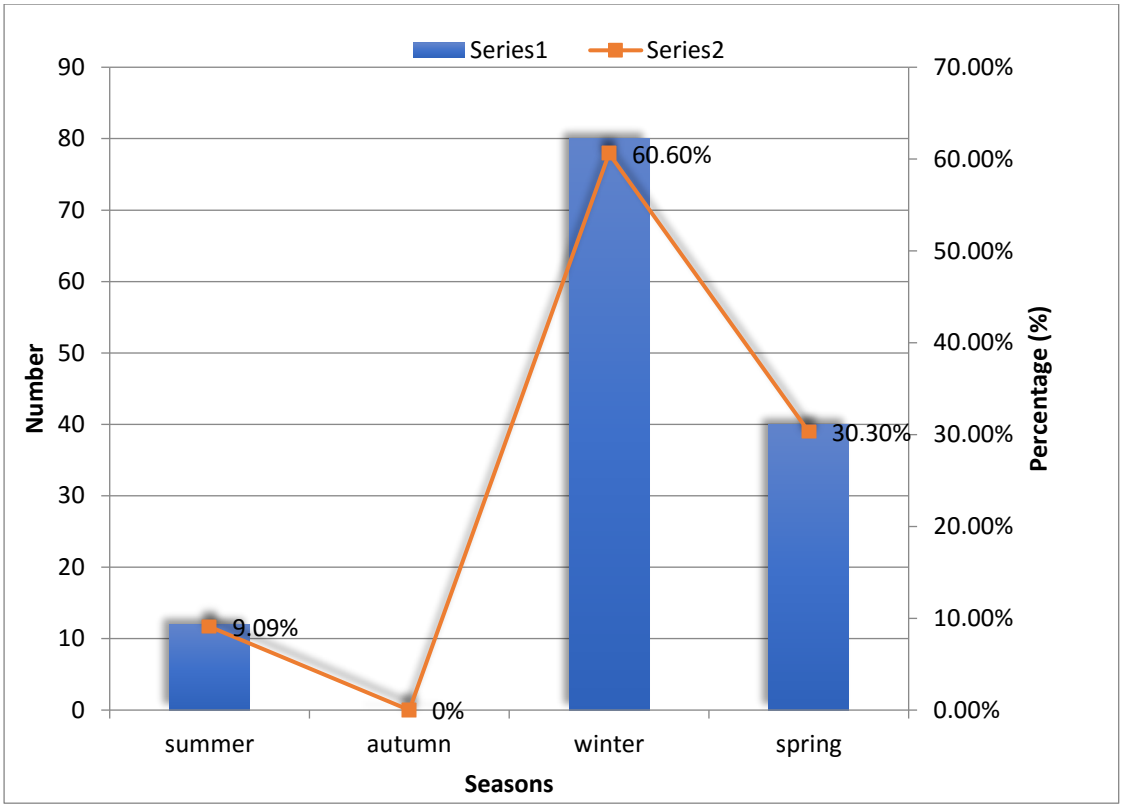

Figure 3.5: Seasonal isolation of AIV H9N2

3.5.2 Bird Type wise Isolation of AIV H9N2 from July 2018 to June 2019

During July 2018 to June 2019 out of 132 samples positive for AIV isolation, 100 (75.75%) belonged to commercial poultry while 20 (15.15%) belonged to backyard poultry. 8 (6.06%) positive samples belonged to wild domestic and only 4 (3.03%) were referred to as wild migratory birds.

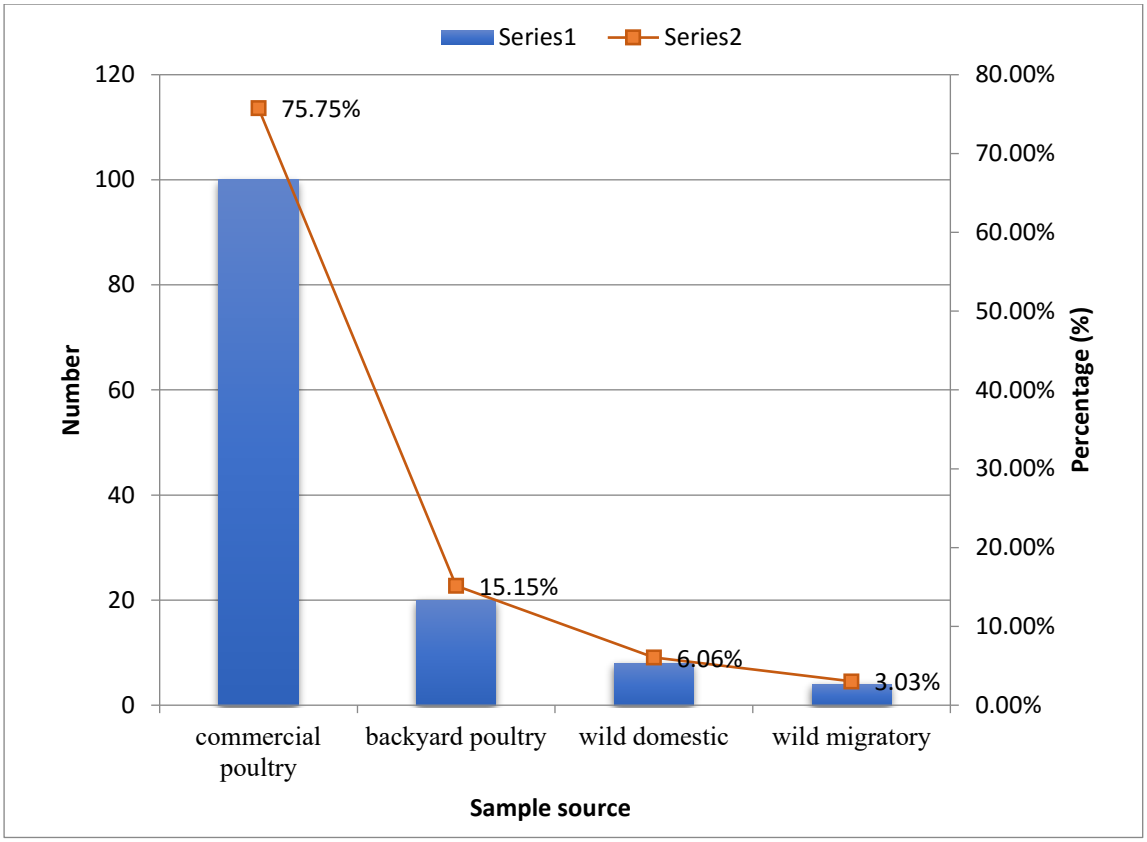

Figure 3.6: Bird type wise isolation of AIV H9N2

3.6 Serological Examination of AIV H9N2

Out of 550 samples 247 samples had sera. Initially ELISA was performed to screen the samples for AIV antibody prevalence in these samples.

Serological Evaluation of Sera Samples through Enzyme Linked Immunosorbent Assay (ELISA)

3.6.1 Seasonal Seroprevalence of AIV in Pakistan

In 175 positive sera maximum seroconversion was observed. Approximately 49 serum samples showed seroprevalence in May while no seroprevalence was observed in August, September and November, December. It was observed in this study that maximum seroprevalence was in spring season with 105 (60%) positive sera, then in winter season with 49 (28%) positive sera. Whereas only 14 (8%) sera were positive in early summers and in autumn 7 (4%) seroconversions were recorded.

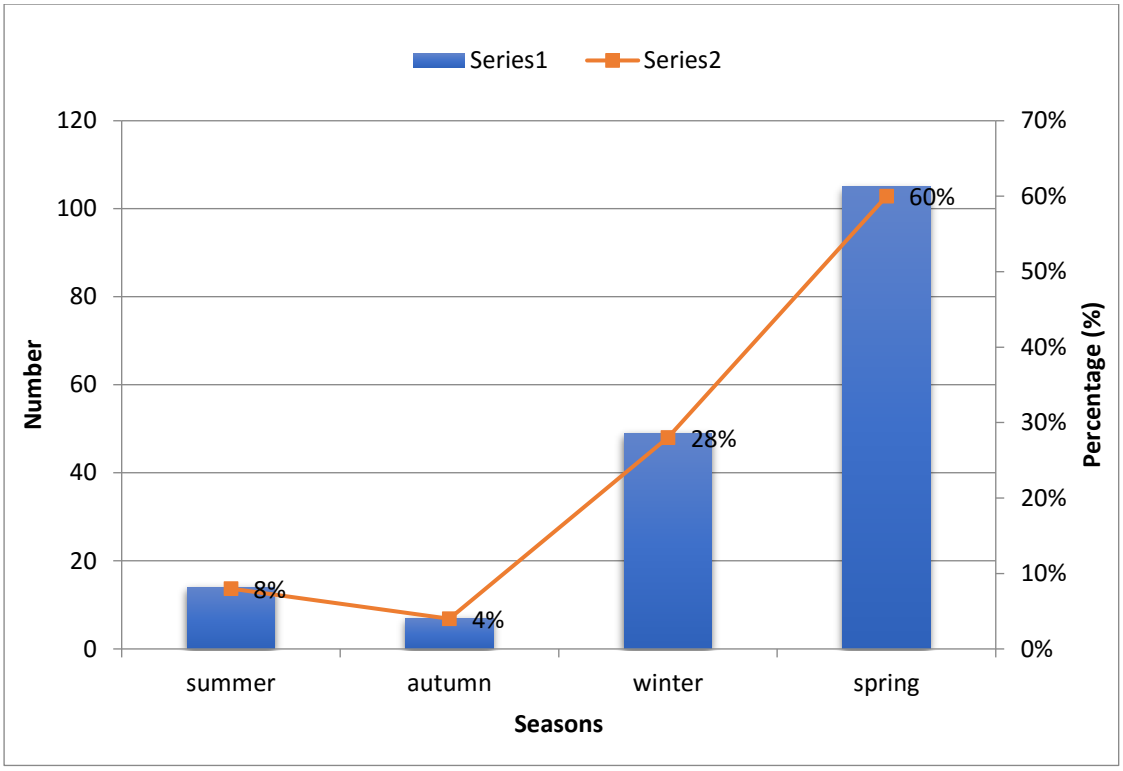

Figure 3.7: Seasonal seroprevalence of AIV H9N2

3.6.2 Area wise Seroprevalence of AIV in Pakistan

Out of 175 positive sera from ELISA test, highest seroprevalence was recorded in KPK 56 (32%) followed by 49 (28%) in Punjab, while 28 (16%) in Sindh and 42 (24%) in ICT.

Table 3.2: Area wise seroconversion of AIV from July 2018 to June 2019

Province	**Punjab**		**KPK**		**Sindh**	**ICT**	**Total**
Cities	Rawalpindi	Sahiwal	Mansehra	Peshawar	Karachi	Islamabad	
Positive samples	42	7	7	49	28	42	175
Positive samples %	24%	4%	4%	28%	16%	24%	100
Total	**49 (28%)**		**56 (32%)**		**28 (16%)**	**42 (24%)**	**175 (100%)**

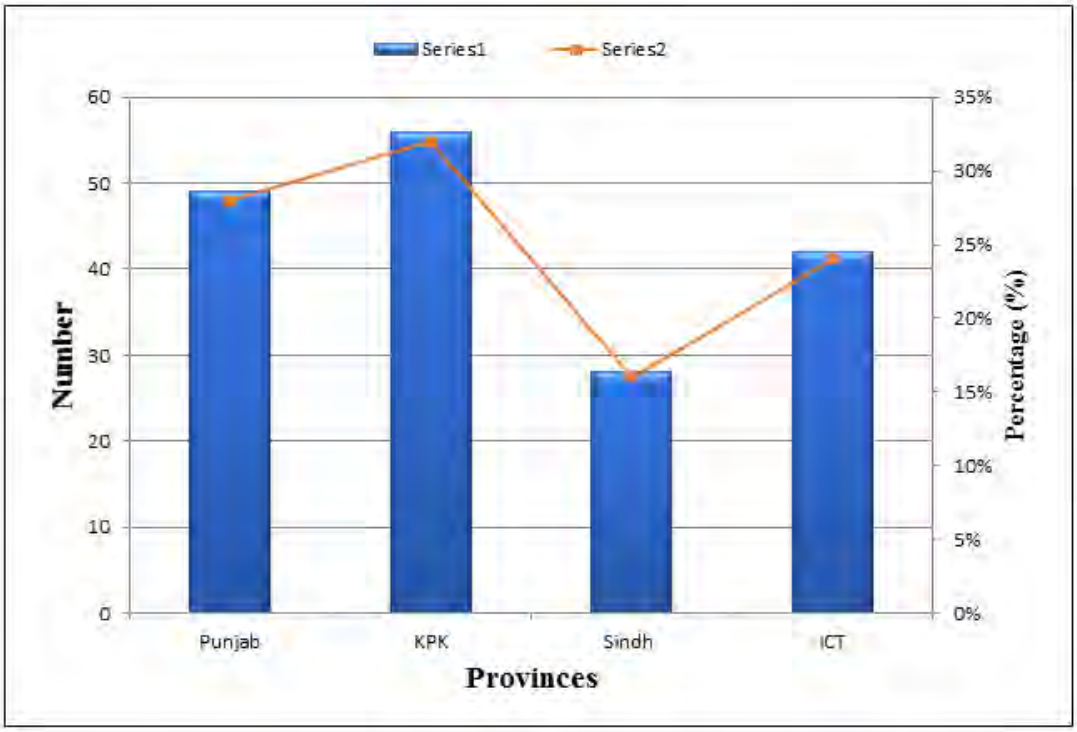

Figure 3.8: Province wise seroprevalence of AIV H9N2

3.6.3 Bird Type wise Seroconversion of AIV during July 2018 to June 2019

In this study 247 sera samples were received out of which 175 sera were detected positive for AIV. From 175 positive sera, 63 (36%) belongs to commercial poultry. From backyard poultry 56 (32%); 21 (12%) were positive from wild migratory; whereas, 35 (20%) sera sample showed seroconversion from wild domestic class.

Table 3. 3: Bird Type wise Seroprevalence of AIV from July 2018 to June 2019

Number of samples	Commercial poultry	Backyard poultry	Wild Migratory	Wild Domestic	Total positive samples
235	63	56	21	35	175
Percentage	36%	32%	12%	20%	100%

3.6.4 Hemagglutination Inhibition Assay

Positive serum for AIV confirmed from ELISA were further subjected to HI for the detection of subtype H9N2 and their antibody titers (MT log 2) The HI was performed as described earlier and H9 antibody titers of positive sera are shown in table

Table 3. 4: Area wise seroconversion recorded against AIV H9N2 in MT log 2

Ecological zones	Seroconversion recorded against AIV H9N2 in MT log 2
Punjab	**3.44- 7.22**
Sindh	**0.00 -8.33**
KPK	**1.00 – 10.70**
ICT	**4.00-7.50**
Total	**0.00-10.70**

3.7 Molecular Detection of AIV H9N2

All the 491 samples containing swabs and tissues were subjected to RNA extraction for the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Out of total extracted samples 220 samples were found positive for the Matrix gene and 148 were found positive for the AIV subtype H9N2.

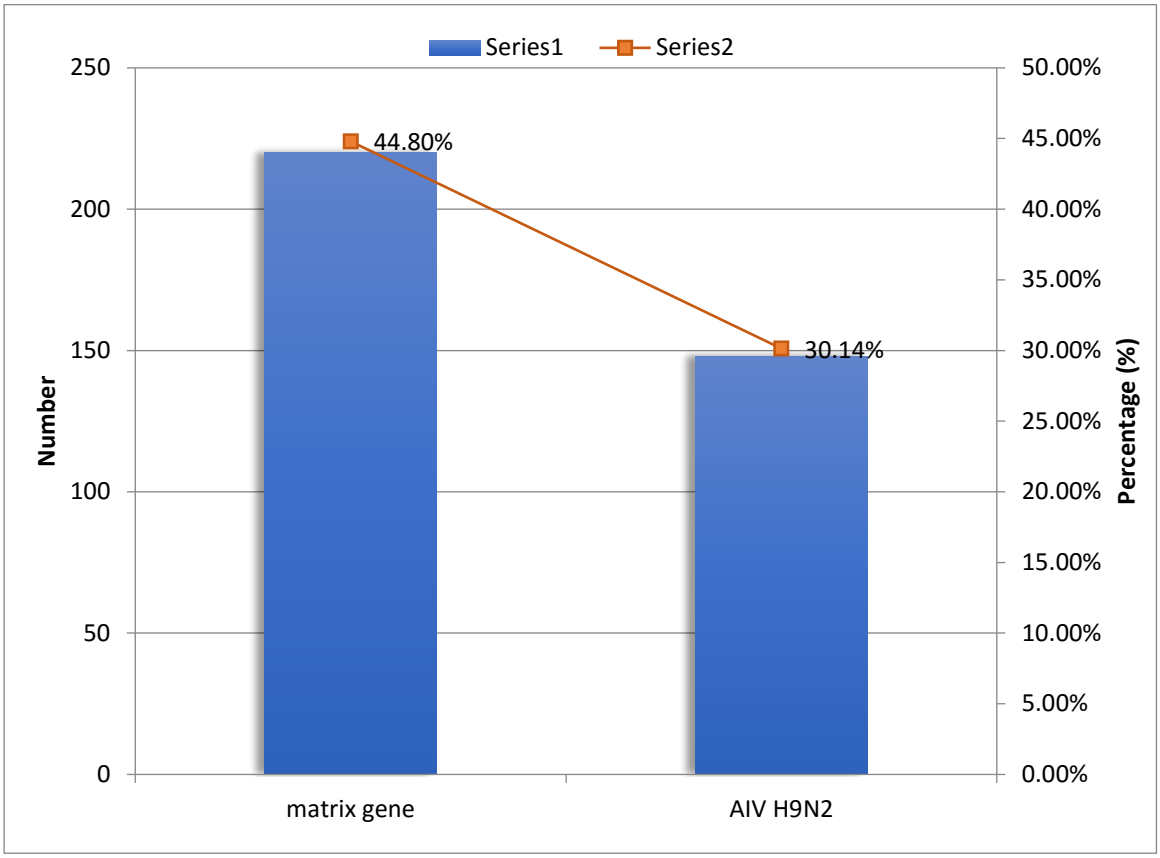

Figure 3.9: AIV subtype H9N2 prevalence during July 2018 to June 2019

Table 3.5: Detection by RT-PCR

No. of samples	Matrix gene (positive)	Hemagglutinin Gene (positive)		N-Typing	
		AIV H9	Other Subtypes	Isolates Tested	N2
491	220	148	72	148	148

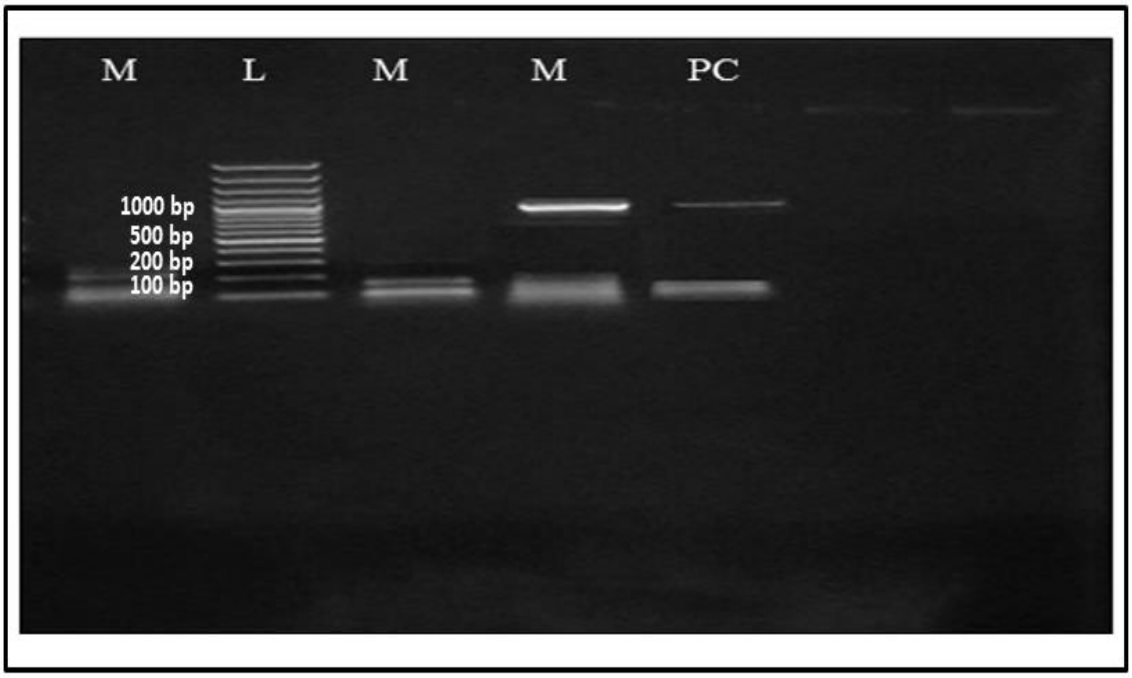

Figure 3.10: Agarose Gel Electrophoresis of RT-PCR amplified Matrix Gene. Lane 1: negative for Matrix gene 1023 bp; Lane 2: Marker 100 bp DNA ladder; Lane 3: negative for Matrix gene 1023 bp; Lane 4: Matrix gene positive sample 1023 bp; Lane 5: Matrix gene positive control

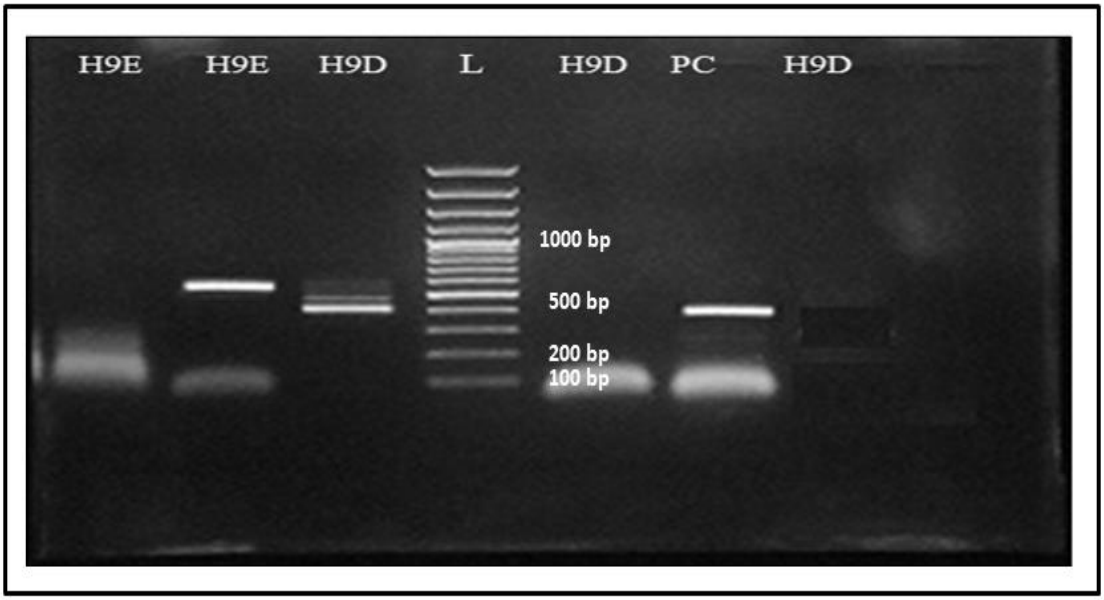

Figure 3.11: Agarose Gel electrophoresis of RT-PCR amplified subtype H9 genes. Lane 1: H9 negative sample 574 base pair; Lane 2: H9 positive sample 574 base pair; Lane 3: H9 positive sample 471 base pair; Lane 4: Marker 100 bp DNA ladder; Lane 5: H9 negative sample 471 base pair; Lane 6: Positive control of H9 471 base pair; Lane 7: Negative Control of H9 471 base pair

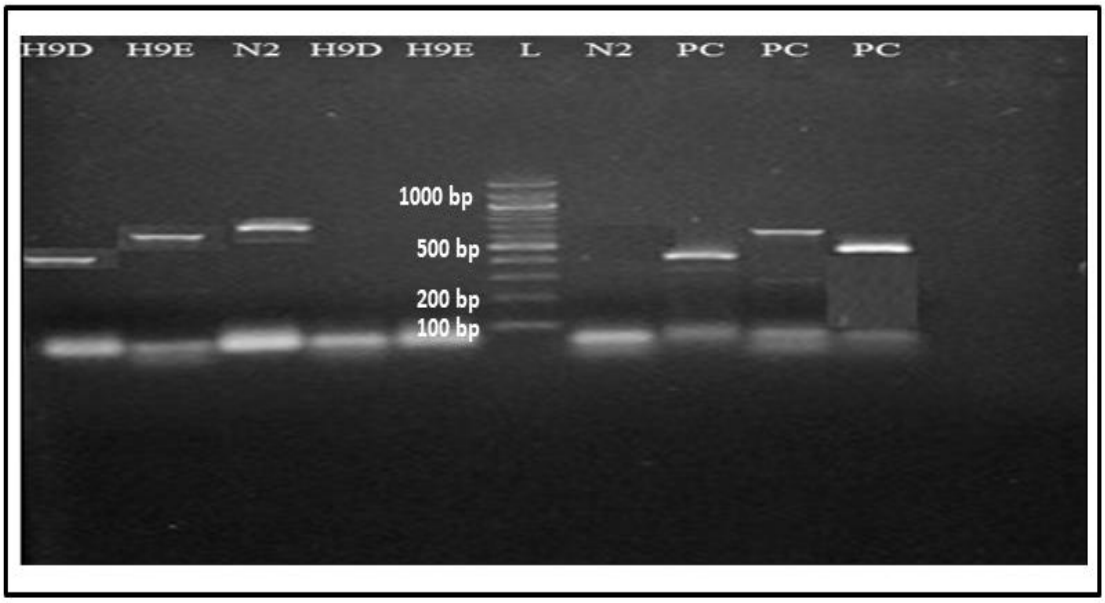

Figure 3.12: Agarose Gel electrophoresis of RT-PCR amplified subtype AIV H9N2. Lane 1: H9 positive sample 471 bp; Lane 2: H9 positive sample 574 bp; Lane 3: N2 positive sample 720 bp; Lane 4: H9 negative sample 471 bp; Lane 5: H9 negative sample 574 bp; Lane 6: Marker 100 bp DNA ladder; Lane 7: N2 negative sample 720 bp; Lane 8: Positive control of 471 bps; Lane 9: Positive control of 720 bps; Lane 10: Positive control of 574 bps

3.7.1 Detection through Real Time PCR for Sensitivity Check of H9 Gene

Some random AIV H9 positive samples from qRT-PCR were tested on RT-PCR by the standard protocols. Samples above the threshold base line indicated positive results whereas samples below the threshold line were considered negative for AIV H9. The threshold cycles (Fernández-Aguilar *et al.*) values are also shown in the following table:

Table 3.6: Threshold cycles (Fernández-Aguilar *et al.*, 2017) values of Real Time PCR

Molecular Techniques	Sample tested (randomly selected)	AIV H9 positive results	Ct-values RT PCR
qRT-PCR	50	50	15.47 to 17.73
RT-PCR		50	

Figure 3.13: A graph showing amplification of subtype AIV H9 genes

3.7.2 Comparison of Different Techniques

The sensitivity and specificity of different techniques were also compared. Results indicated that virus isolation by in-ova inoculation 184 (37.47%) was less specific as compared to the PCR technique 220 (44.80 %) as shown in the figure 3.14:

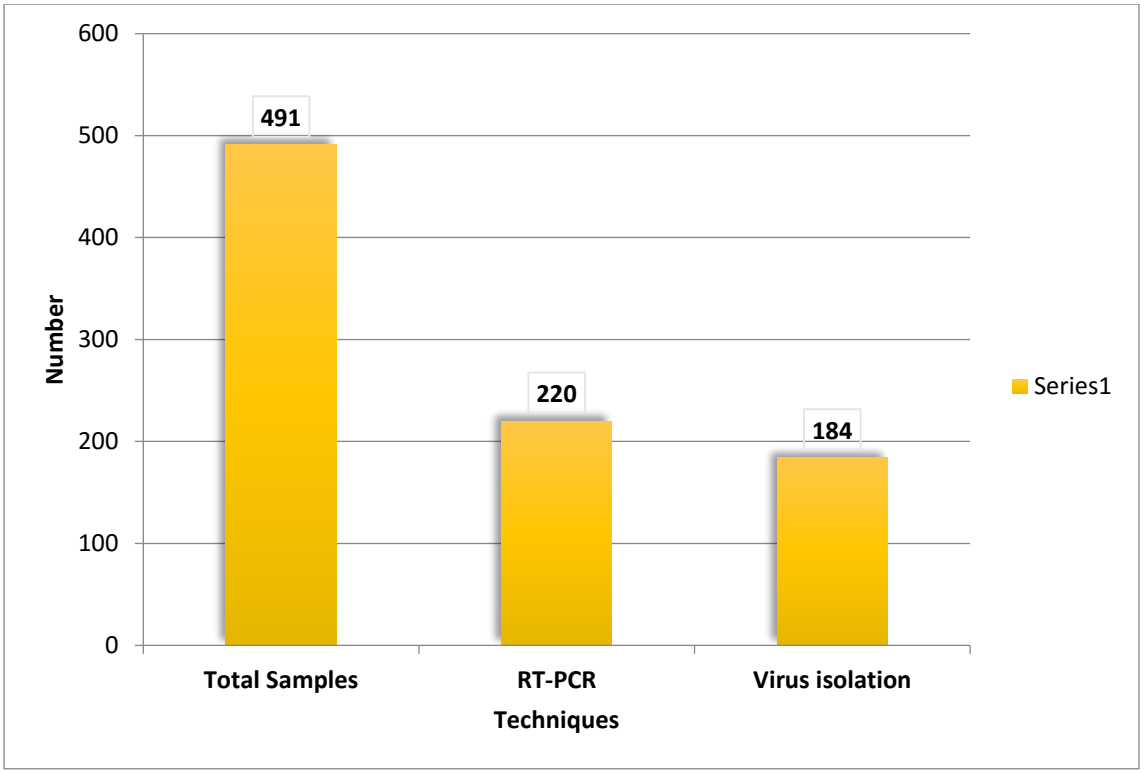

Figure 3.14: Comparison of different techniques used in AIV detection

3.7.3 Seasonal Prevalence of H9N2 during July 2018 to June 2019

During the period from July 2018 to June 2019 total 148 samples were detected positive for AIV H9N2 by RT-PCR. Maximum seasonal prevalence was observed during winter (December-February) *i.e.* 84 (56.75%) were found positive. Followed by 52 (35.13%) in spring and 12 (8.1%) in summer whereas no prevalence was observed in autumn.

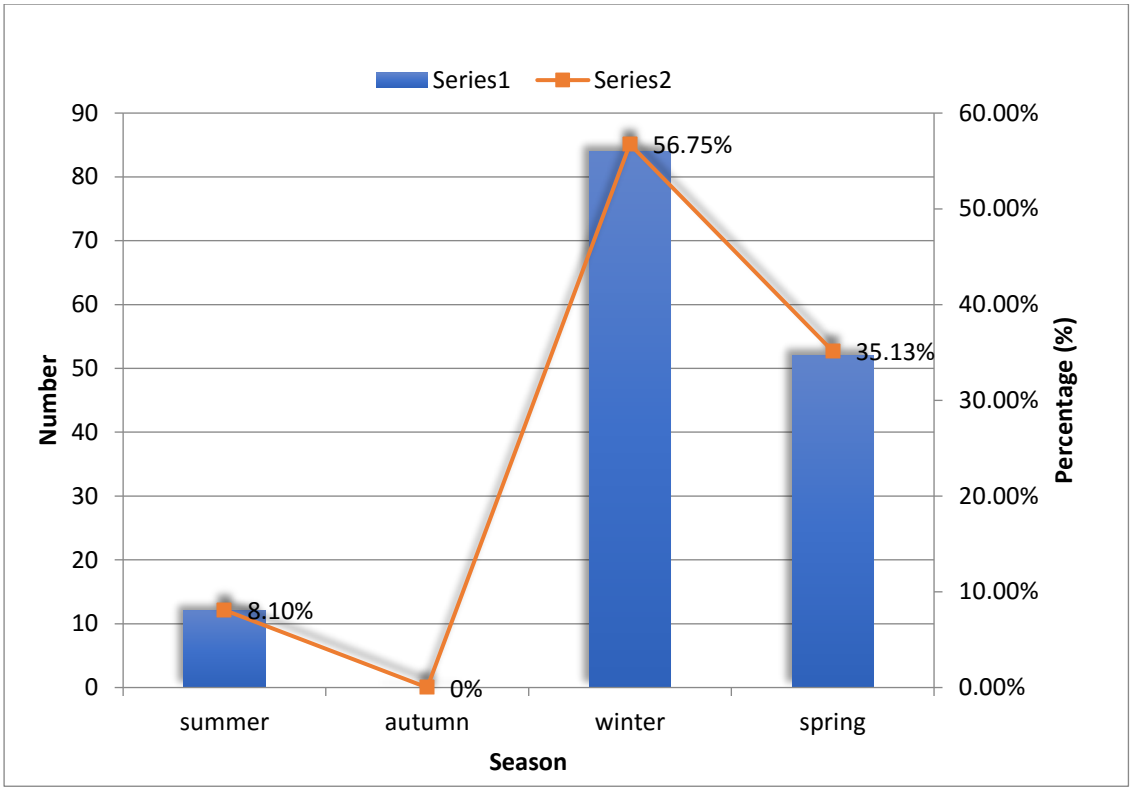

Figure 3.15: Seasonal prevalence of AIV H9N2

3.7.4 Province wise Prevalence of AIV H9N2 from July 2018 to June 2019

From total 148 detections of AIV H9N2 by RT- PCR, maximum number of detections were from Sindh 56 (37.83%) followed by 52 (35.13%) in KPK, from Punjab 32 (21.62%) and only 8 (5.40%) from ICT.

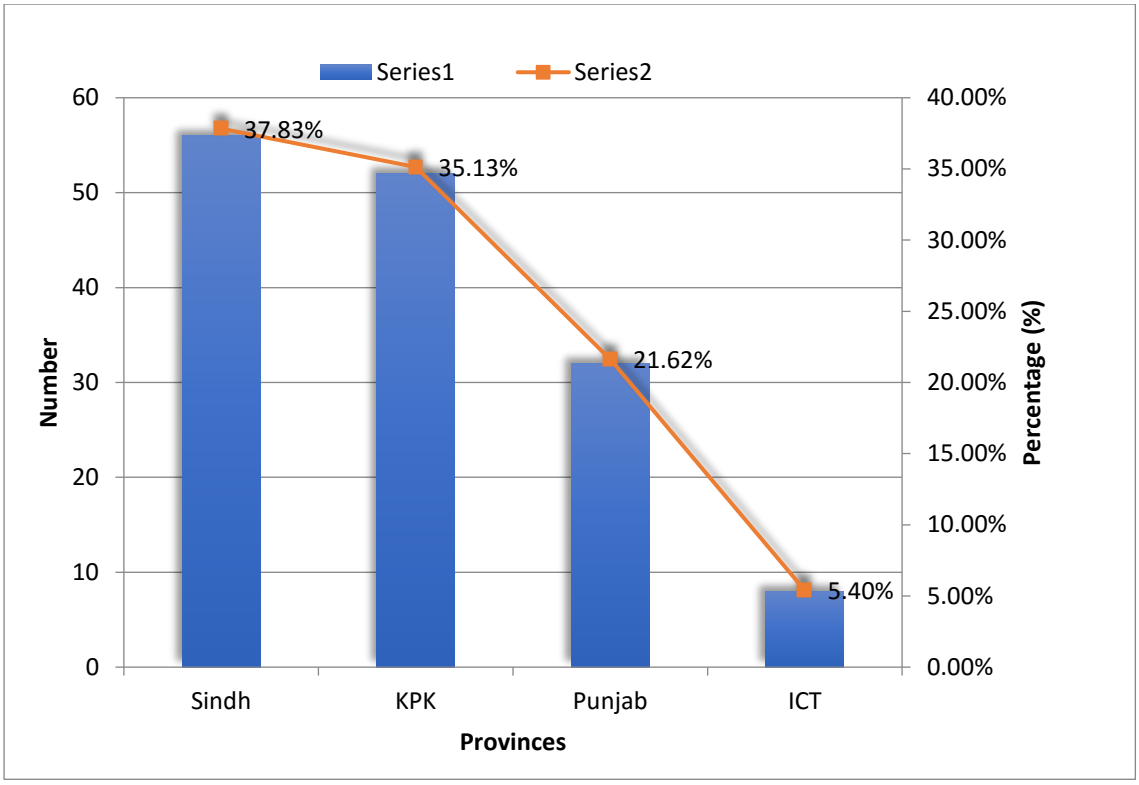

Figure 3.16: Province wise detections of AIV H9N2 by RT-PCR

3.7.5 Bird Type wise Prevalence of AIV H9N2 from July 2018 to June 2019

Out of 146 samples detected positive for AIV H9N2 by RT-PCR 108 (72.97%) were positive from commercial poultry (broilers), while 24 (16.21%) were positive from backyard poultry, 12 (8.10%) were positive from wild domestic birds whereas only 4 (2-70%) were detected positive from wild migratory birds as shown in the figure 3.17.

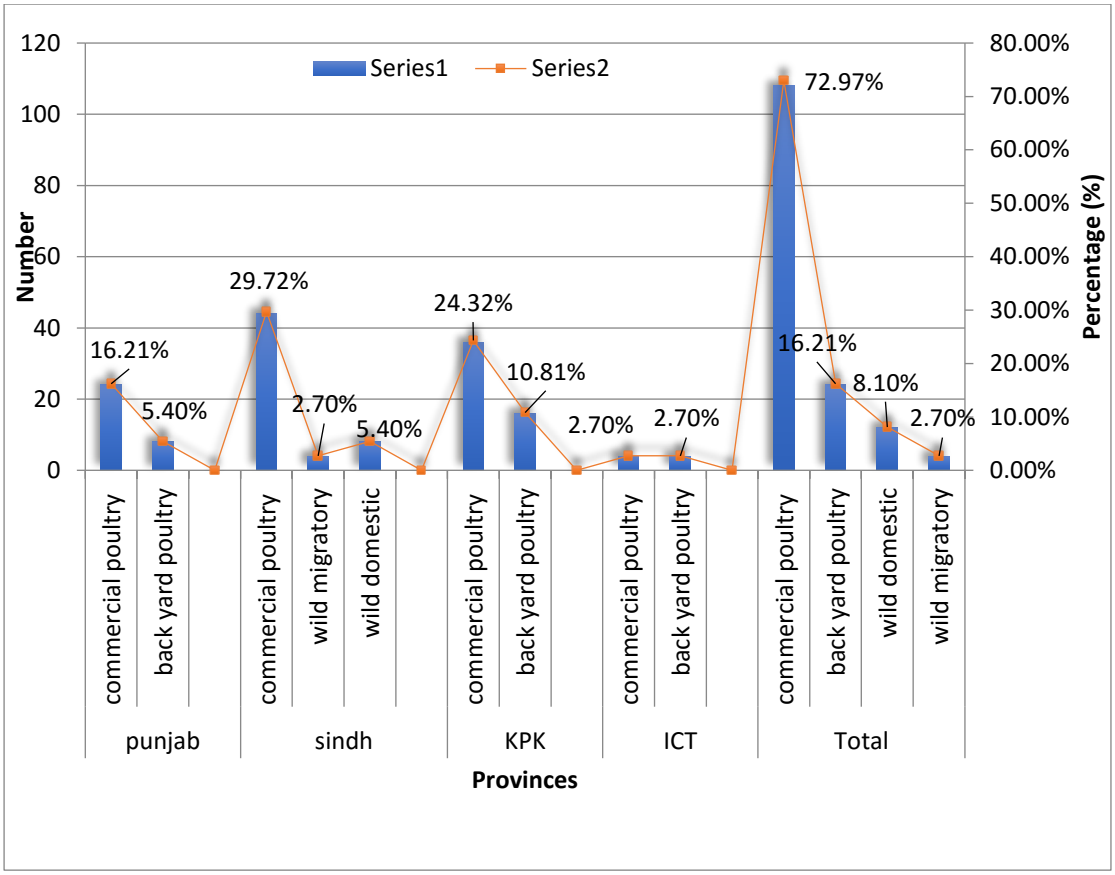

Figure 3.17: Bird type wise detection of AIV H9N2 by RT-PCR

3.8 *In-ova* Inoculation for Identification and Isolation of *Chlamydia*

Total 491 tissue and swab samples were processed for bacterial isolation through *in-ova* inoculation techniques. Only 184 samples showed positive results and were checked further for the *chlamydia* presence by PCR.

3.9 Serological Examination of *Chlamydia*

From total 550 samples, 247 sera samples were collected from various species.

Serological Evaluation of Sera Samples through Enzyme Linked Immunosorbent Assay (ELISA)

Out of 247 serum samples received from different areas of Pakistan 52 (21.03%) samples were found positive for the *Chlamydia psittaci.*

3.9.1 Seasonal Seroprevalence of Chlamydia psittaci from July 2018 to June 2019

Out of 52 samples showing seroprevalence, 32 (61.50%) from summer, 13 (25%) from winter and 7 (13.46%) from spring showed seroprevalence while no seroprevalence was observed in autumn.

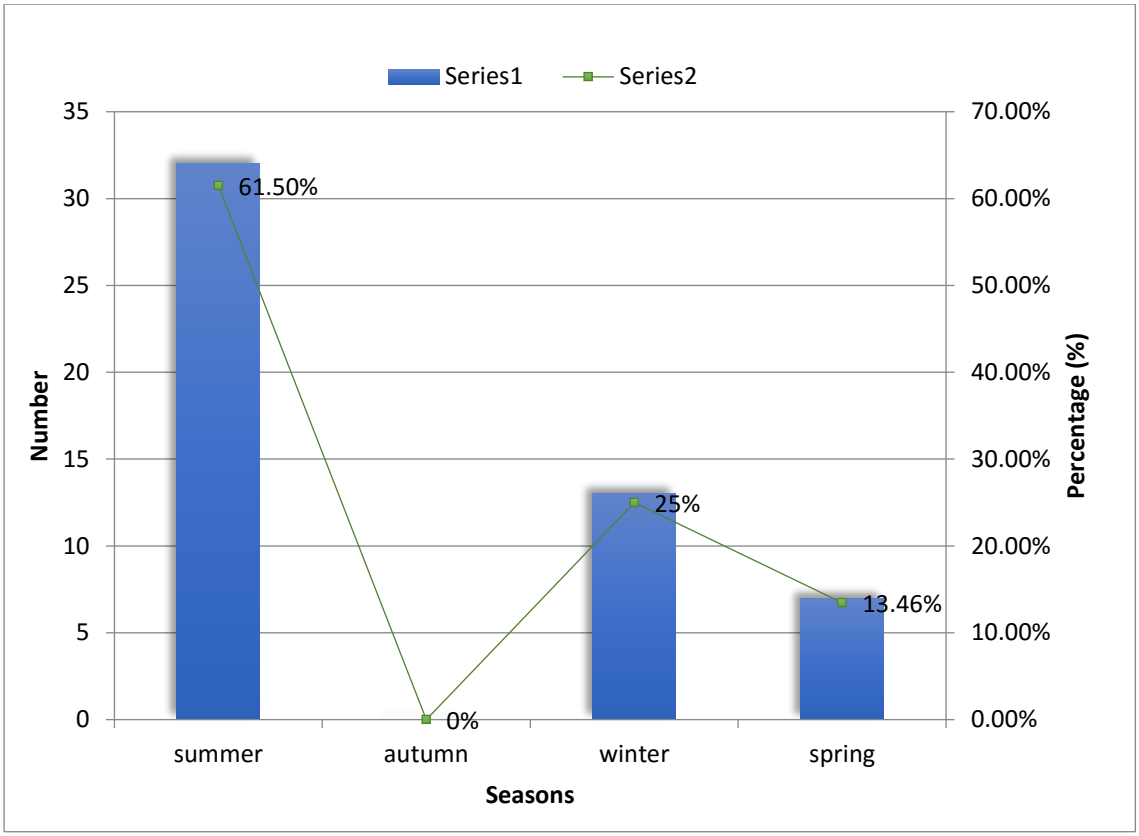

Figure 3.18: Seasonal seroprevalence of Chlamydia psittaci

3.9.2 Province wise Seroprevalence *Chlamydia psittaci* from July 2018 to June 2019

Out of 52 sera detected positive by ELISA for *Chlamydia psittaci* maximum number 36 (69.2%) was observed from Punjab, followed by KPK showing 8 (15.3%), while 4,4 samples each with a percentage of 7.69% were found positive in Sindh and ICT.

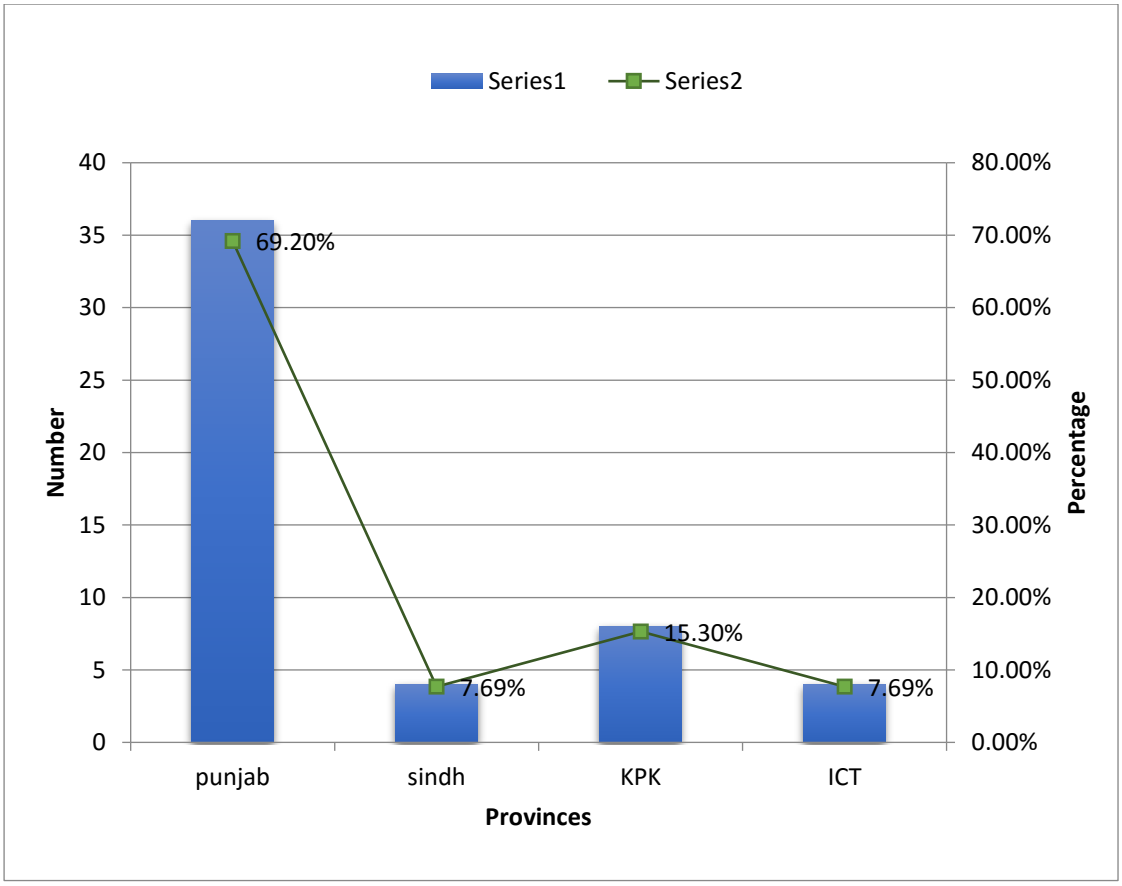

Figure 3.19: Province wise seroprevalence of Chlamydia psittaci

3.9.3 Bird Type wise Seroconversion of *Chlamydia psittaci* during July 2018 to June 2019

From 52 positive sera samples, 16 (30.7%) samples were positive from commercial poultry and 12 *i.e.* 23% samples were found positive in backyard poultry. Wild domesticated poultry showed maximum positive samples *i.e.* 24 (46.1%) whereas no sample was found positive in wild migratory birds.

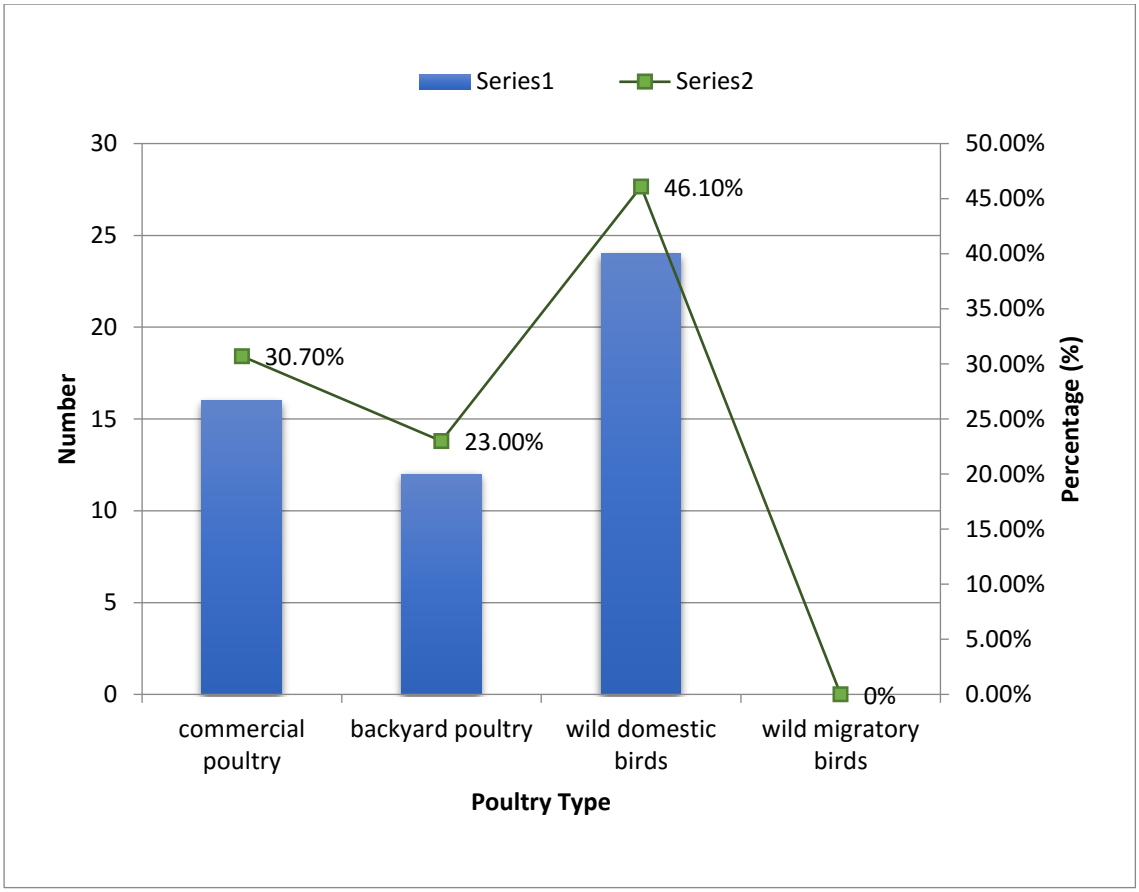

Figure 3.20: Bird type wise seroprevalence of Chlamydia psittaci

Table 3.7: Bird Type wise Seroprevalence of AIV from July 2018 to June 2019

Number of samples	Commercial poultry	Backyard poultry	Wild Migratory	Wild Domestic	Total positive samples
247	16	12	0	24	52
Percentage	30.7%	23%	0%	46.1%	100%

3.10 Molecular Detection

After DNA extraction, nano drop was used for measuring the amount of DNA and it was observed that different samples showed different concentrations ranging from 115.6 to 119.8 ng/µl.

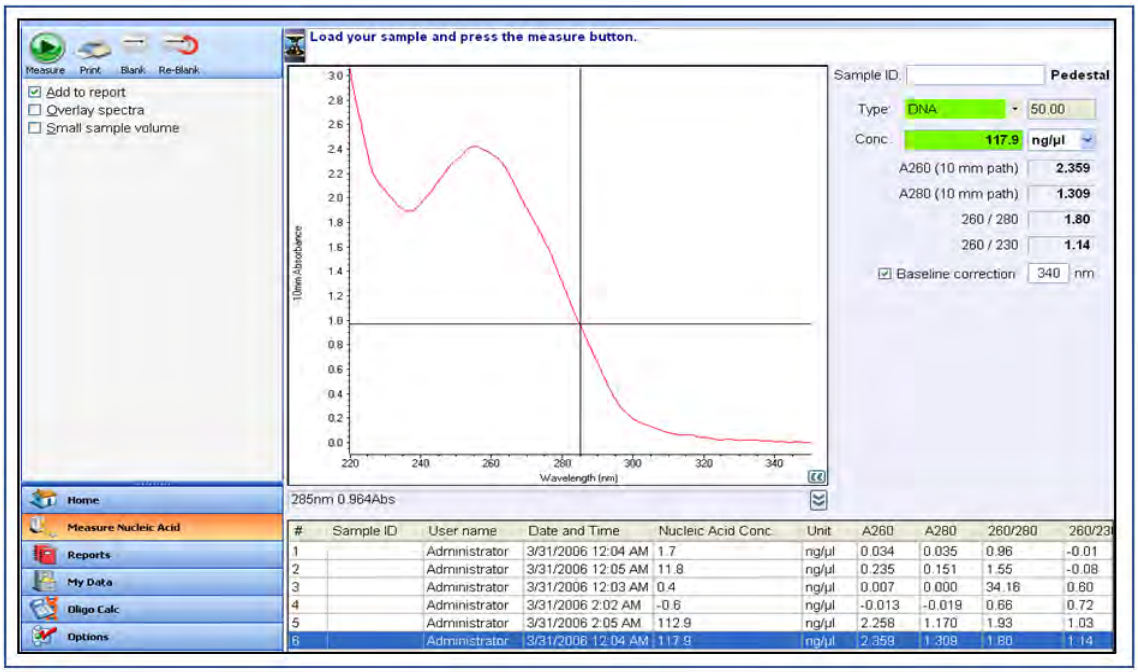

Figure 3.21: Nano-dropping showing DNA concentration

All the 491 swab and tissue samples were also subjected to DNA extraction for the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and real-time PCR (qRT-PCR). Out of total extracted samples, 80 (16.29%) samples were found positive for the *chlamydia* and only 60 (12.21%) samples belonged to *C. psittaci.*

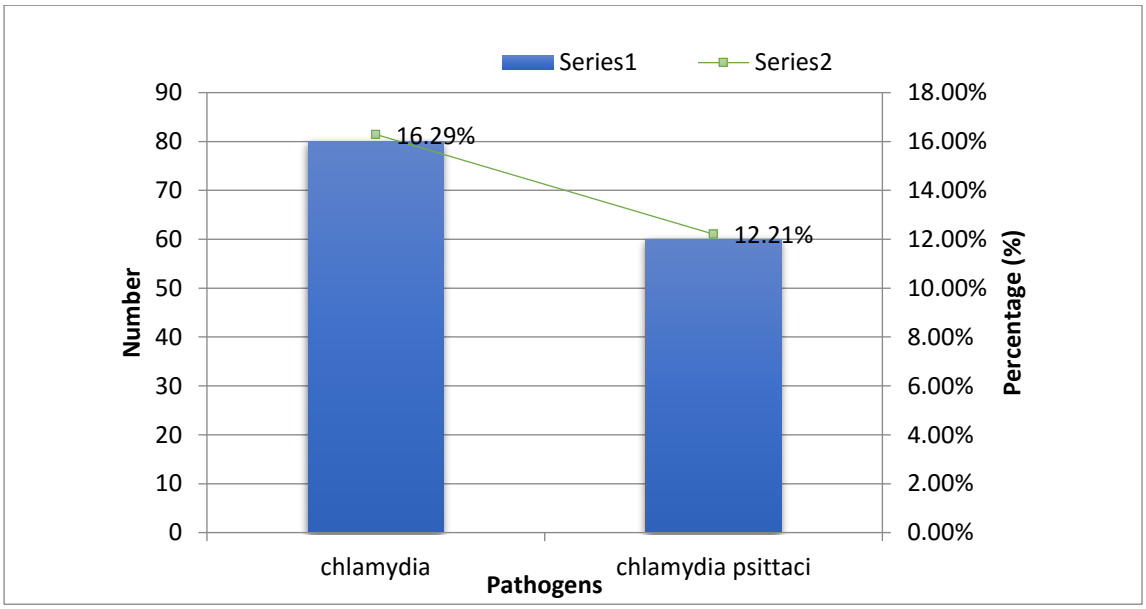

Figure 3.22: Chlamydia psittaci Prevalence during July 2018 to June 2019

Table 3.8: Detection by RT-PCR

No. of samples	*Chlamydia* general (positive)	*Chlamydia psittaci* (positive)
491	80 (16.29%)	60 (12.21%)

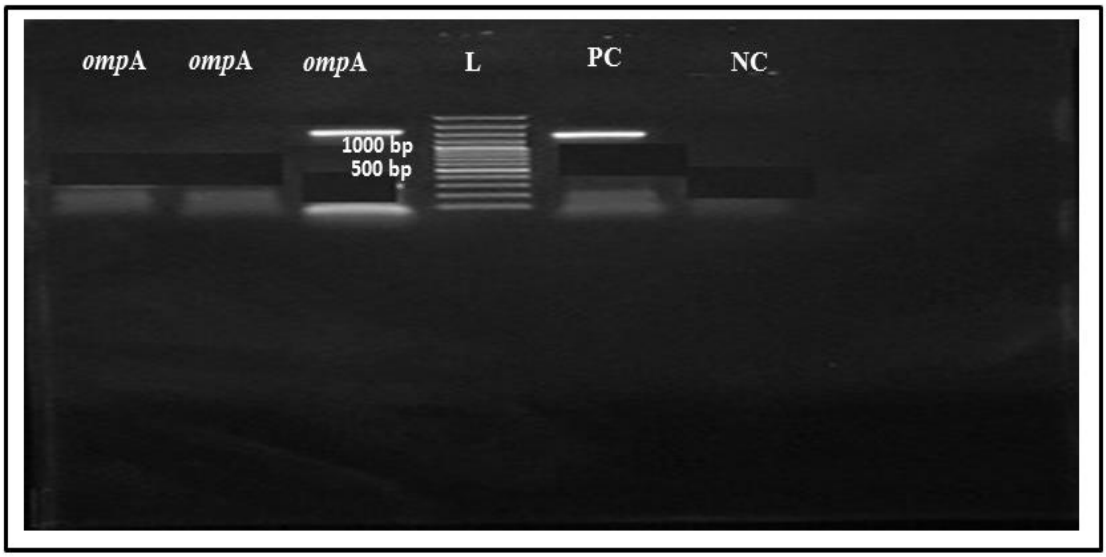

Figure 3.23: Agarose Gel Electrophoresis of RT-PCR amplified ompA *Chlamydia* general gene. Lane 1: negative sample for *omp*A gene 1200 bp; Lane 2: negative for *omp*A gene 1200 bp; Lane 3: positive for *omp*A gene 1200 bp; Lane 4: Marker 100 bp DNA ladder; Lane 5: *omp*A gene positive control; Lane 6: *omp*A gene negative control

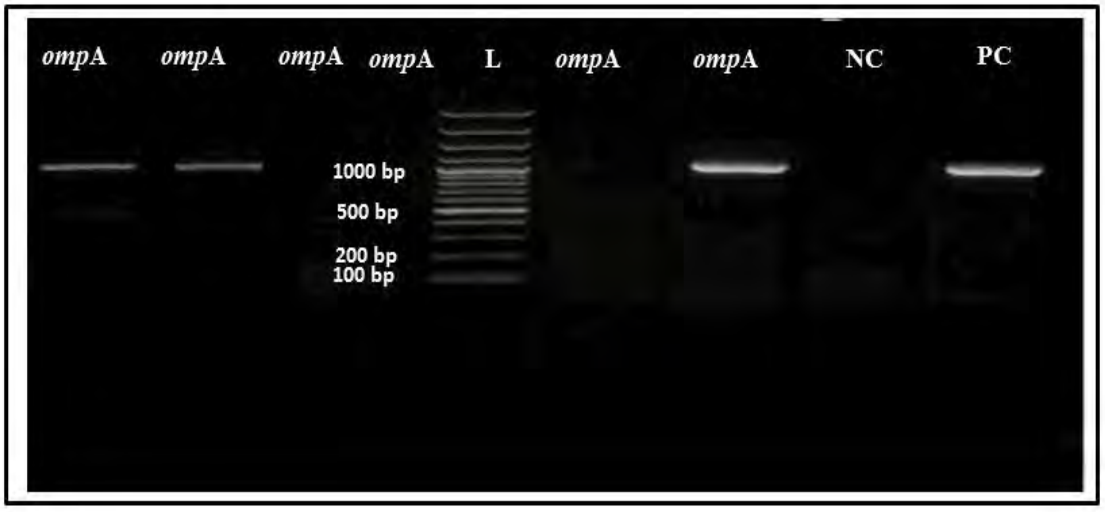

Figure 3.24: Agarose Gel Electrophoresis of RT-PCR amplified ompA *Chlamydia* general gene. Lane 1: Positive sample for *omp*A gene 1100 bp; Lane 2: Positive sample for *omp*A gene 1100 bp; Lane 3: negative sample for *omp*A gene 1100 bp; Lane 4: negative *omp*A for gene 1100 bp; Lane 5: Marker 100 bp DNA ladder; Lane 6: negative *omp*A for gene 1100 bp; Lane 7: Positive sample for *omp*A gene 1100 bp; Lane 8: negative control for *omp*A gene 1100 bp; Lane 9: positive control for *omp*A gene 1100 bp

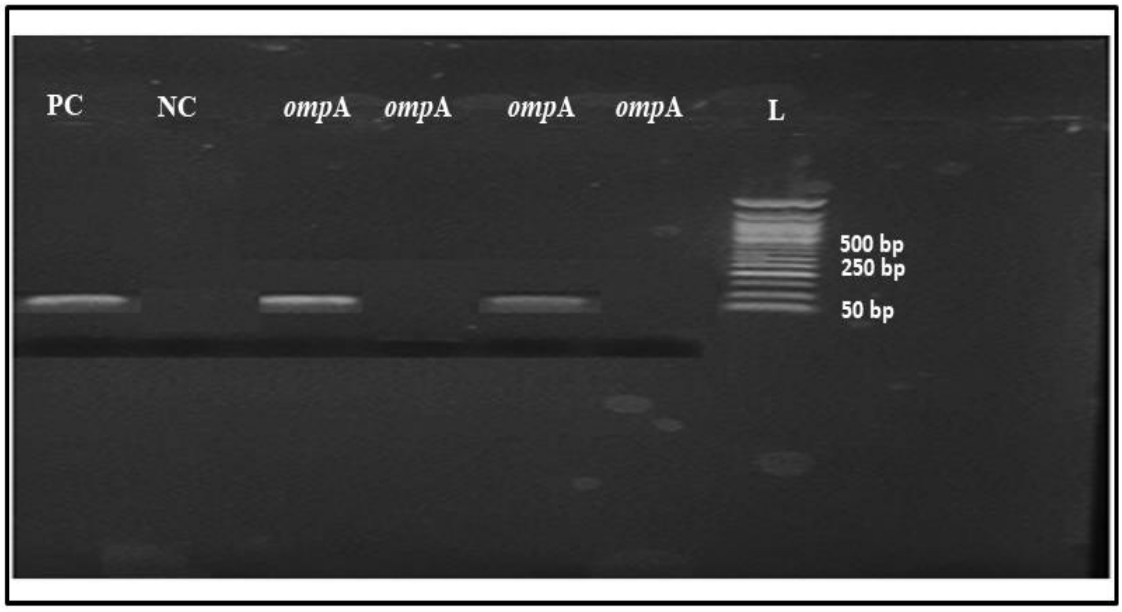

Figure 3.25: Agarose Gel Electrophoresis of RT-PCR amplified ompA *Chlamydia psittaci* specific gene. Lane 1: Positive control *omp*A 76bp; Lane 2: Negative control *omp*A 76bp; Lane 3: Positive sample for *omp*A 76bp; Lane 4: *omp*A gene negative sample 76 bp; Lane 5: Positive sample for *omp*A 76bp; Lane 6: *omp*A gene negative sample 76 bp; Lane 7: 50 bp DNA Marker

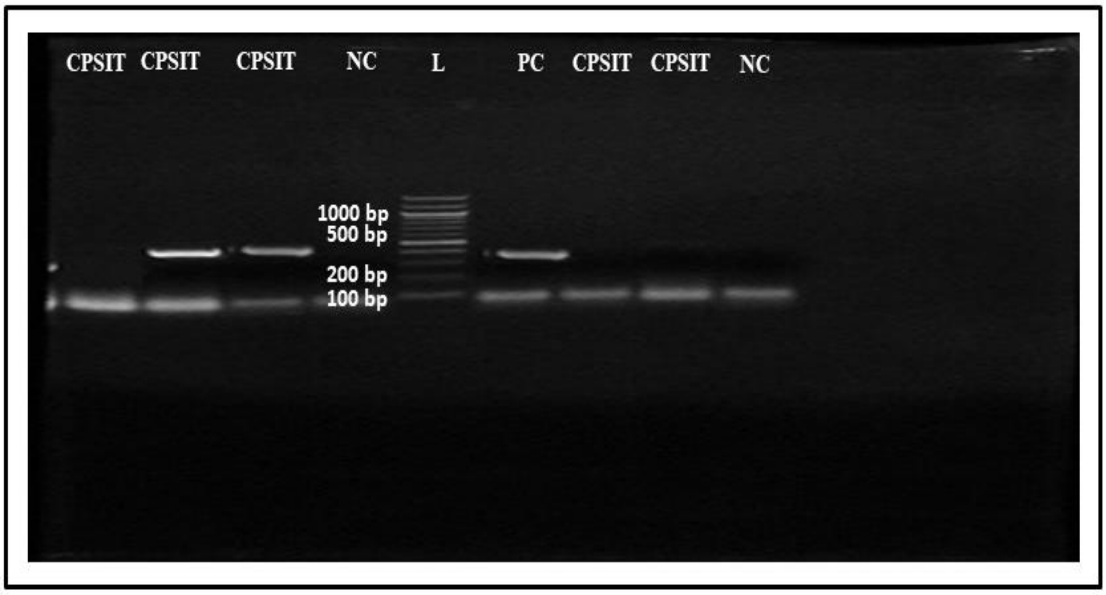

Figure 3.26: Agarose Gel Electrophoresis of RT-PCR amplified CPSIT_0607 *Chlamydia psittaci* specific gene. Lane 1: negative sample for CPSIT_0607 gene 393 bp; Lane 2: CPSIT_0607 gene positive sample 393 bp; Lane 3: CPSIT_0607 gene positive sample 393 bp; Lane 4: negative control CPSIT_0607 gene 393 bp; Lane 5: Marker 100 bp DNA ladder; Lane 6: Positive control CPSIT_0607 gene 393 bp; Lane 7: negative sample for CPSIT_0607 gene 393 bp; Lane 8: negative sample for CPSIT_0607 gene 393 bp; Lane 9: negative control CPSIT_0607 gene 393 bp

3.10.1 Detection through Real Time PCR for *Chlamydia psittaci*

Positive samples were further checked by qRT-PCR based on standard protocols. Samples above the threshold base line indicated positive results whereas samples below the threshold line were considered negative for *C. psittaci*. The threshold cycles (Fernández-Aguilar *et al.*) values are also shown in the table 3.9 below:

Table 3.9: Threshold cycles (Fernández-Aguilar *et al.*) values of Real Time PCR

Molecular Techniques	Sample tested (randomly selected)	*C. psittaci* positive results	Ct-values RT PCR
qRT-PCR	80	60	10.43 to 15.63
RT-PCR		60	

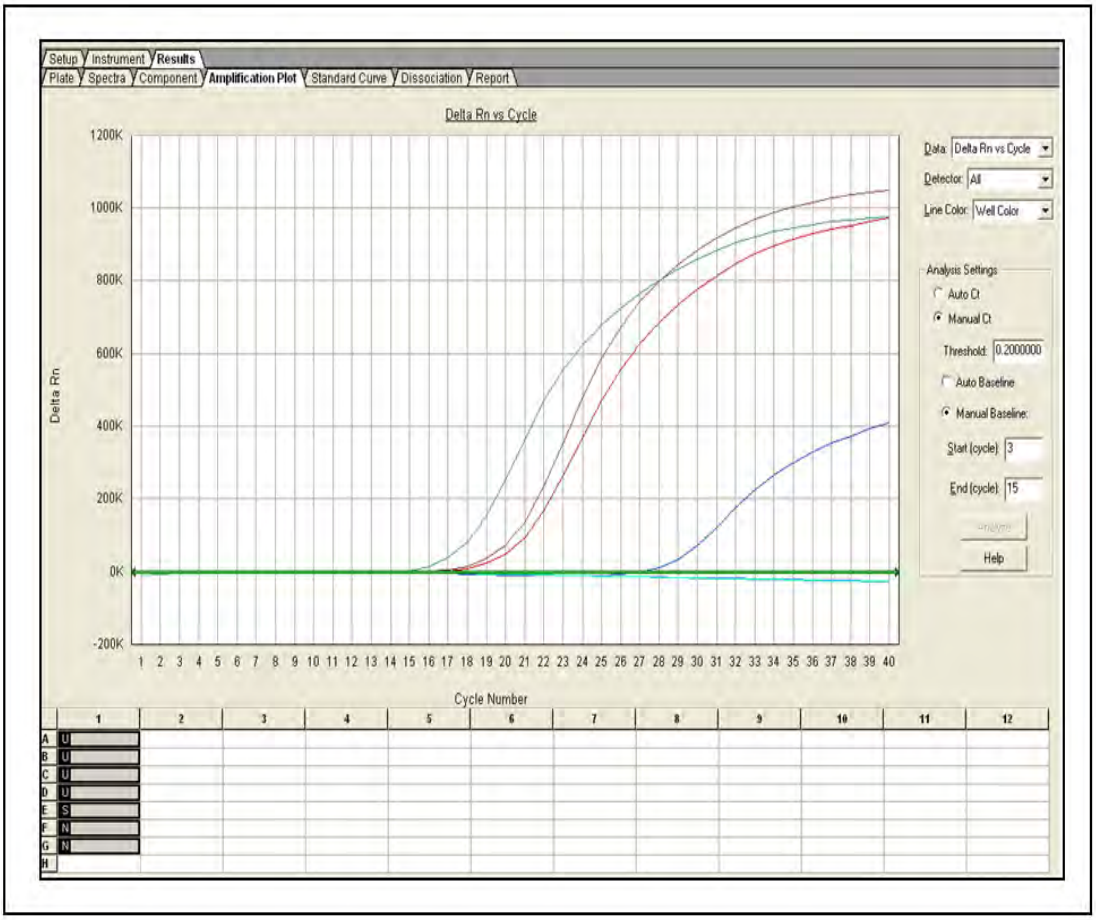

Figure 3.27: A graph showing amplification of Chlamydia psittaci genes

3.10.2 Comparison of Different Techniques

The sensitivity and specificity of different techniques such as *in-ova* inoculation and PCR were also compared. Results indicated that 184 samples were suspected to have Chlamydia in them by *in-ova* inoculation technique showing 37.47% specificity towards results. When these suspected samples were tested by PCR for chlamydial confirmation it showed 43.47% positive samples out of the total suspected samples hence proving to be more specific than *in-ova* inoculation method. The specificities of *in-ova* inoculation and PCR are shown in the figure 3.28.

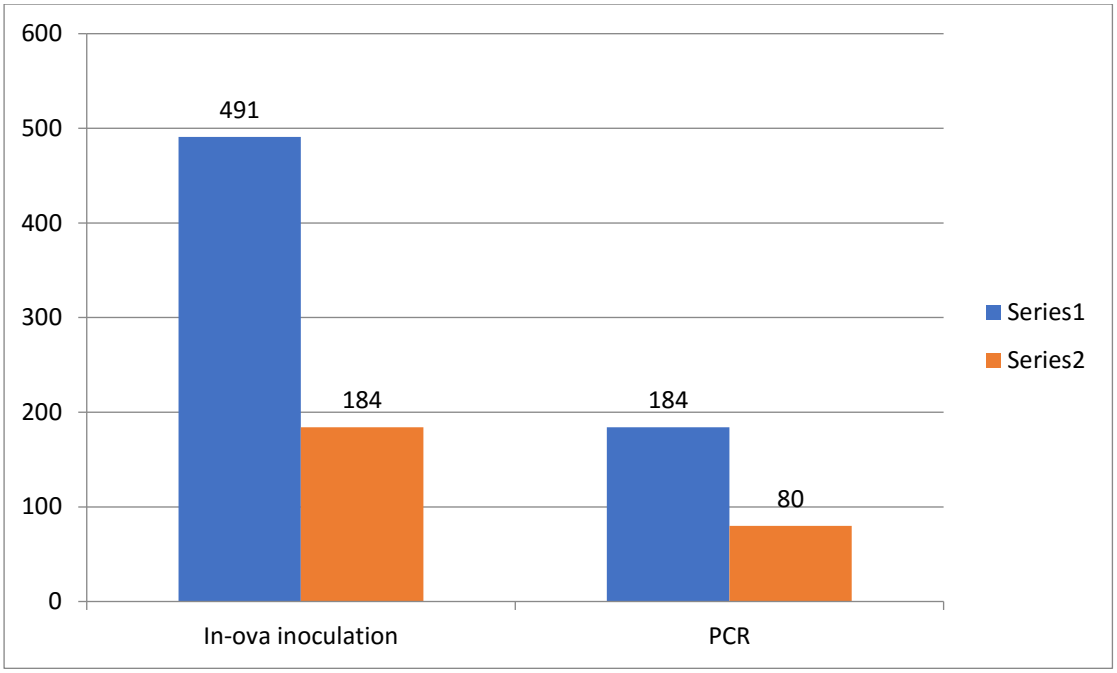

Figure 3.28: Comparison of different techniques used in Chlamydia detection

3.10.3 Seasonal Prevalence of *Chlamydia psittaci* during July 2018 to June 2019

Out of 60 positive samples for *C. psittaci* highest prevalence was observed during summer *i.e.* 39 (65%), 15 samples were found positive from winter showing 15% prevalence, and 6 (10%) samples were found positive from spring season and 0% prevalence were observed in autumn.

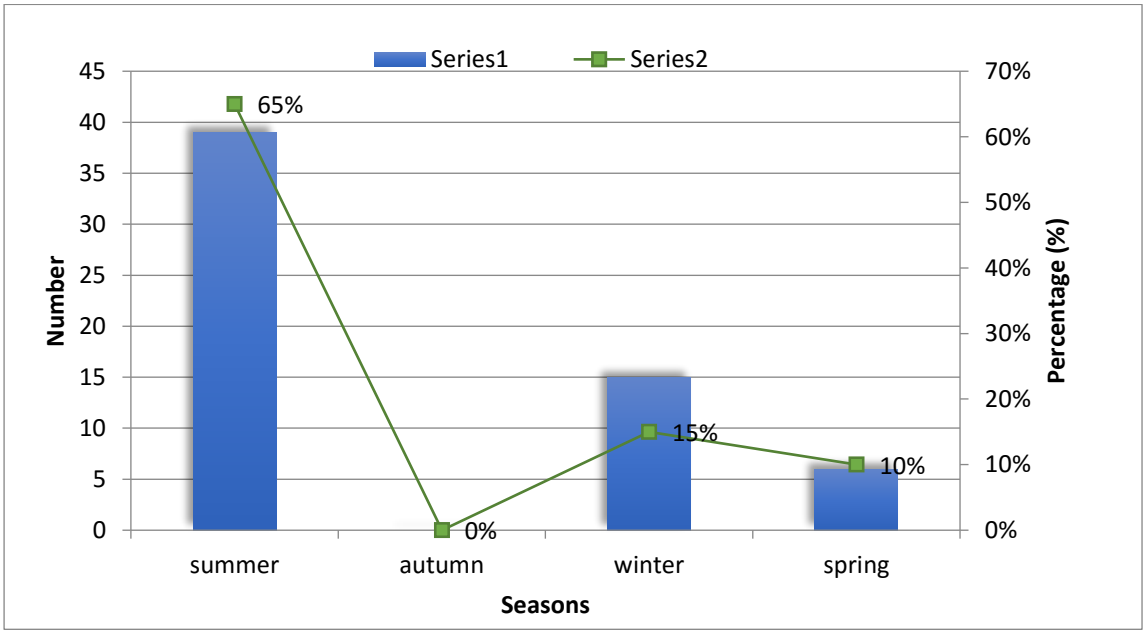

Figure 3.29: Seasonal Prevalence of Chlamydia psittaci by RT-PCR

3.10.4 Province wise Prevalence of *Chlamydia psittaci* from July 2018 to June 2019

From total 60 detections of *C. psittaci* by RT- PCR highest detections were from Punjab 44 (73.33%), while in Sindh only 4 (6.66%) samples were detected positive, in KPK 8 (13.33%) positive samples and in ICT 4 (6.66%) samples were positive for *C. psittaci.*

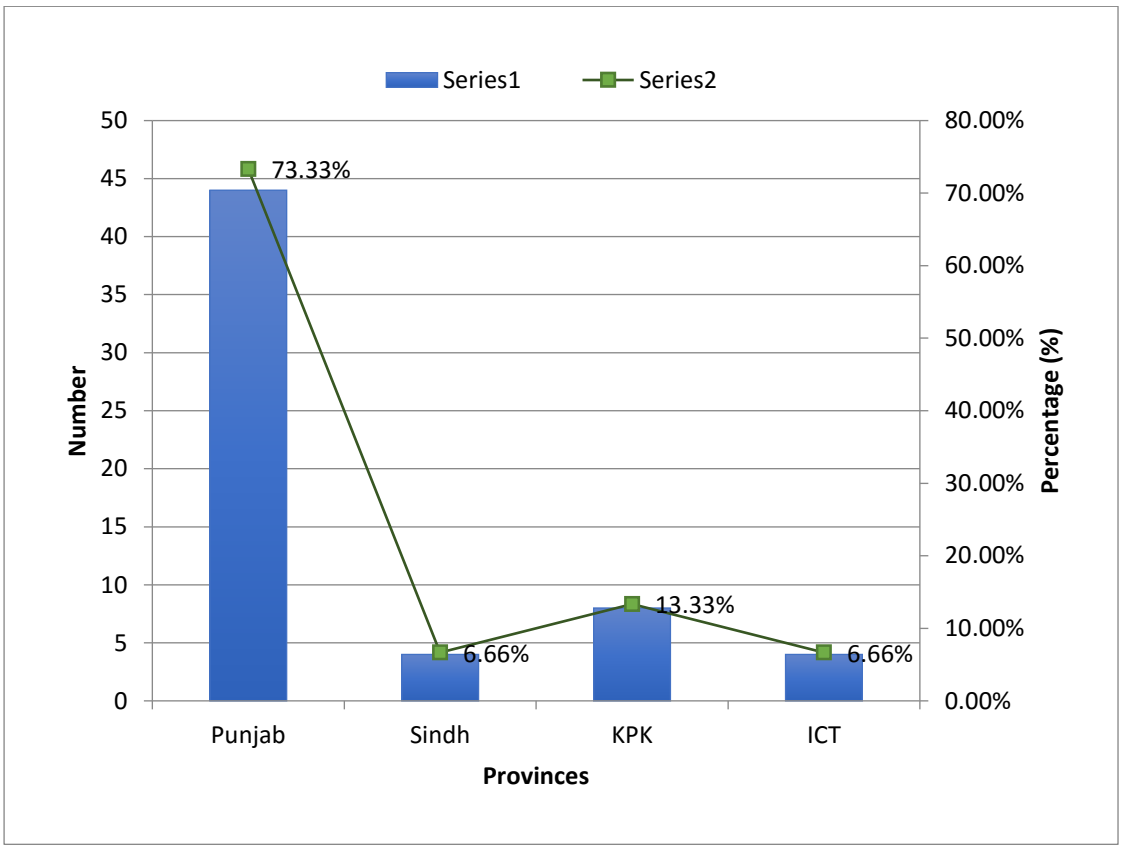

Figure 3.30: Province wise detections of Chlamydia psittaci by RT-PCR

3.10.5 Bird Type wise Prevalence of *Chlamydia psittaci* from July 2018 to June 2019

Out of 60 *C. psittaci* positive by RT-PCR, 20 (33.33%) samples were found positive from commercial poultry, 12 (20%) from backyard poultry, while the maximum *i.e.* 28 (46.66%) samples were detected positive from the wild domestic birds and no prevalence was observed in wild migratory birds.

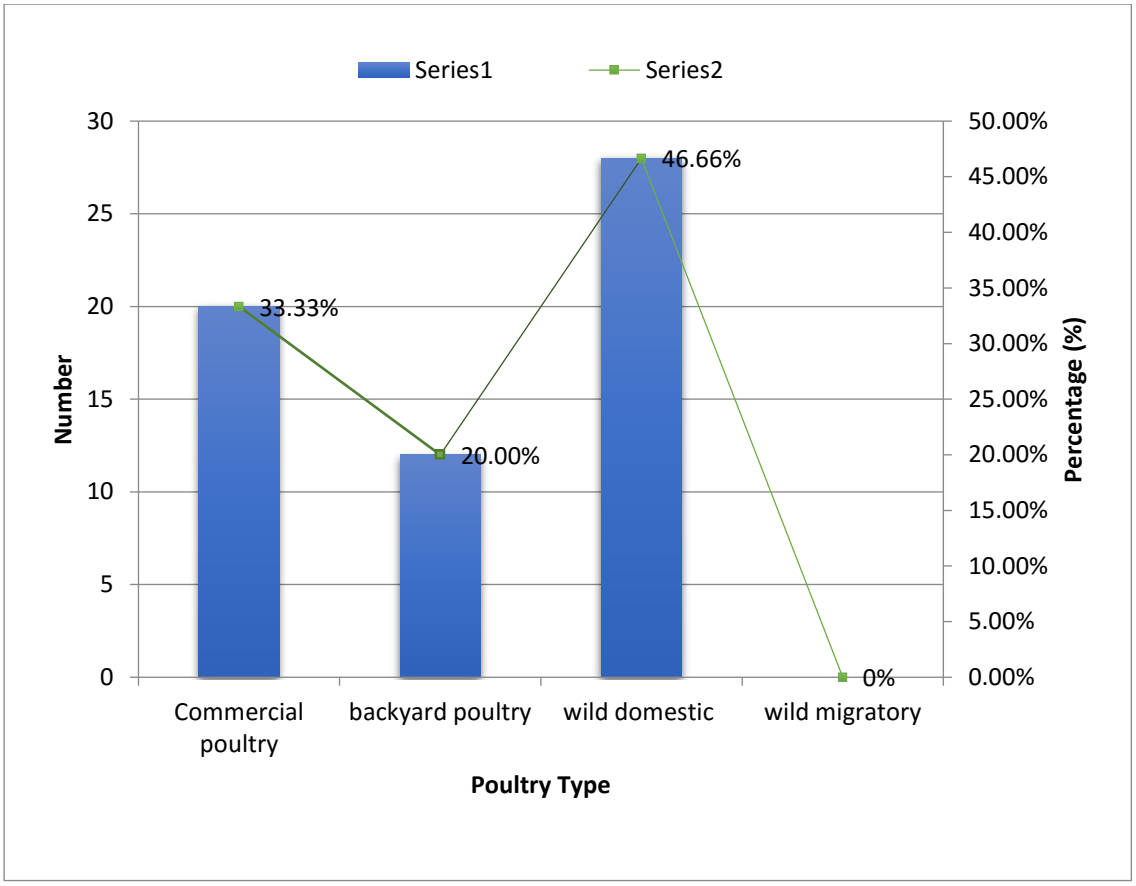

Figure 3.31: Bird type wise detection of Chlamydia psittaci by RT-PCR

3.11 Co-infection of *Chlamydia psittaci* and AIV H9N2

From the 491 tissue and swab sample collected from the different regions of Pakistan from July 2018 to June 2019, 147 (29.9%) samples were found positive for AIV H9N2 and 60 (12.21%) showed positive results for *Chlamydia psittaci*. Among the total 207 positive detections 32 (15.45%) samples showed the coinfection of both AIV H9N2 and *Chlamydia psittaci*. Approximately same ratios were found in serum samples. From the 247 serum samples 175 (70.85%) were found positive for AIV, 52 (21.05%) were positive for *Chlamydia psittaci* and 26 (11.45%) samples were found to have a coinfection of AIV H9N2 and *Chlamydia psittaci*.

Table 3. 10: Tissue samples showing positive results of AIV H9N2 and *Chlamydia psittaci* and their coinfection

Infection Type	Total tissue samples	AIV H9N2	*Chlamydia psittaci*	Coinfection of AIV H9N2 and *C. psittaci*
Number	491	147	60	32
Percentage %	100%	29.9%	12.21%	15.45%

Table 3. 11: Serum samples showing positive results of AIV H9N2 and *Chlamydia psittaci* and their coinfection

Infection Type	Total serum samples	AIV H9N2	*Chlamydia psittaci*	Coinfection of AIV H9N2 and *C. psittaci*
Number	247	170	52	26
Percentage %	100%	70.85%	21.05%	11.45%

DISCUSSION

4. DISCUSSION

Poultry (chickens, geese, ducks, turkey, quail, and pigeons etc.) is a vibrant factor of agriculture industry in Pakistan, responsible for both egg and meat production. Poultry is a good source of nutritious and palatable meat contributing approximately 19% of total meat production and 1.3% in nation GDP (Ghafoor *et al.*, 2010; Hussain *et al.*, 2015). Various infectious diseases are caused by both viruses and bacteria, and are not only a threat to poultry industry but also responsible for huge economic losses (Abbas *et al.*, 2015).

Avian influenza is an important threat to the poultry industry because of its significant characteristic of enormous variability and there are several factors that contribute to the variability (Webster *et al.*, 1997). Wild birds belonging to orders Charadriiformes (shorebirds, gulls and terns) and Anseriformes (geese, swans and ducks) are the natural source of avian influenza viruses (Webster *et al.*, 1992; Fereidouni *et al.*, 2010). First outbreak of Avian Influenza virus in Pakistan was reported in 1995 (Shahid *et al.*, 2009). In 1998, Pakistani poultry industry was highly infected by avian influenza subtype H9N2 and resulted in a severe decline in egg production (Naeem *et al.*, 2006; Bashashati *et al.*, 2013). Avian influenza subtype H9N2 outbreaks were also observed in chickens, domesticated ducks and turkeys (Alexander, 2000). AIV H9N2 is a low pathogenic virus and usually shows mild signs but exceptionally high morbidity rate, severe diarrhoea and reduced egg production. It also infects the respiratory system of poultry worldwide (Sarwar *et al.*, 2013) and as a zoonotic infection also is a serious threat to public health (Peiris *et al.*, 1999; Lin *et al.*, 2000). AI is a threat to the economy of poultry industry; therefore, continuous surveillance and proper vaccinations are the major factors help in controlling the disease. (Jakhesara *et al.*, 2014). Surveillance have been helpful against the initial threatening signs of avian influenza virus present in the poultry as well as in human population (Capua *et al.*, 2000; Alexander, 2003; Afzal *et al.*, 2012).Avian chlamydiosis (AC) also known as psittacosis (in psittacine birds) or ornithosis (in non-psittacine birds), is caused by the *Chlamydia psittaci*. AC naturally occurs in wild as well as domestic poultry worldwide (Grimes, 1994; Sachse *et al.*, 2015). *Chlamydia psittaci* strains were isolated from turkeys, ducks, chickens, pigeons, quails and mammals (Andersen *et al.*, 2000; Longbottom *et al.*, 2003; Andersen *et al.*,

2008). Zoonotic impact of *chlamydia* was also observed due to contact with psittacine birds, wild birds, chickens, pigeons etc (Eidson, 2002; Sachse *et al.*, 2015). *Chlamydia psittaci* infections show great variability in clinical symptoms in birds such as respiratory infections, conjunctivitis, mucopurulent discharge from eyes and nose, cough, greenish to greyish faeces whereas in humans chlamydiosis mainly show respiratory tract infections, difficulty in breathing, low pulse, diarrhoea etc. (Beeckman *et al.*, 2009; Sachse *et al.*, 2015). *Chlamydia psittaci* infects the poultry industry (Dhama *et al.*, 2008).

The current study was directed to isolate the Avian Influenza Virus (AIV) subtype H9N2 and *Chlamydia psittaci* from various samples of commercial and backyard poultry as well as from the wild domestic and wild migratory bird species in Pakistan from July 2018 to June 2019. This study further focused on the co-occurrence of *Chlamydia psittaci* and AIV H9N2 from these samples.

Low pathogenic avian influenza virus H9N2 show high mortality and morbidity along with acute respiratory signs and significant decrease in egg production (Bano *et al.*, 2003). From July 2018 to June 2019, total 550 (491 tissues and swab, and 247 serums) samples were collected from different ecological zones of Pakistan. During the study period, from the collected 491 tissues and swab samples tested through *in-ova* inoculation, 184 samples showed positive hemagglutination activity and only 132 (27%) were confirmed as AIV H9N2 by virus neutralization test. Out of 132 AIV H9N2 isolates, 80 (61%) samples were positive during the winter season whereas in spring 40 (30%) isolation were diagnosed. During the summer season 12 (9%) AIV H9N2 was recorded while no isolation was found in autumn. Similar results like maximum prevalence of AIV H9N2 was observed in winter approximately 75% by Kim *et al., 2018* in his studies because during cold temperatures virus dissemination increases (Kim *et al.*, 2018).

An increased detection rate (12/16 positive cases; 75%) was observed during cold season, while the prevalence rate (4/16 positive cases; 25%) of AIV subtype H9 was generally decreased during warm season, indicating that the prevalence of the H9 virus infection in the winter was higher than that in the summer. It was also observed that from the 132 positive samples for AIV H9N2, 100 (76%) samples were from commercial poultry, 20 (15%) from the backyard poultry, wild domesticated birds

contribute 8 (6%) and only 4 (3%) samples were positive from wild migratory birds. Also high prevalence of AIV H9N2 was observed by Kim and his cowerkers in commercial poultry than in backyard (Kim *et al.*, 2018). Similar findings were also reported by Ali and his colleagues in Pakistan where the prevalence of H9N2 was 6.7% and 2.7% in commercial and backyard poultry, respectively (Ali *et al.*, 2017).

For the current study, 247 sera samples from various ecological zones of country were collected and subjected to ELISA for initial screening of AIV antibodies detection. 175 (70.85%) samples showed positive sera from July 2018 to June 2019. Whereas in the previous study conducted in 2018 by Kausar and colleagues only 53% serum samples were found positive (Kausar *et al.*, 2018). Different factors such as sample size, immunological factors, weather conditions and geographical zones influence the outcomes of different studies. In another examination conducted by Fereidouni and his woorkers different seroprevalence rates were observed during different years; 35.5% in 2003 and 2004, 21% in 2005 and 57.4% during 2007 (Fereidouni *et al.*, 2010). The seroprevalence observed in 2007 was close to our study.

Seasonal seroprevalence in present study revealed that highest prevalence was recorded in spring season *i.e.* 105 (60%) followed by 49 (28%) in winter season. However, 14 (8%) prevalence cases were observed in summer and least 7 (4%) were reported in autumn. Approximately similar results were observed in study conducted by Kausar *et al.,*2018 where highest prevalence was observed in spring with 35%, followed by 32% in summers, 23% in winter and only 10% seroprevalence was observed in autumn (Kausar *et al.*, 2018).

During the period from July 2018 to June 2019, the maximum seroprevalence was reported in KPK *i.e.* 56 (32%), 49 (28%) in Punjab, 28 (16%) in Sindh while 42 (24%) from ICT. In 2018 Kausar and colleagues revealed different seroprevalence percentages in different zones of Pakistan with maximum 48% in Sindh, followed by 24% in KPK and only 2.4% in Punjab (Kausar *et al.*, 2018). The difference in the seroprevalence percentages in different studies is most probably because of sample size collected from different areas. Out of 175 positive sera samples, 63 (36%) were recorded from commercial poultry, 56 (32%) from backyard poultry, 21 (12%) from wild migratory birds while 35 (20%) wild domesticated birds showed positive seroconversion for AIV antibodies. According to work undertaken by Hadipur, 2011, the seroprevalence of

H9N2 was 78.4% in wild domestic and 62.9% in backyard poultry (Hadipour, 2011). And 40.6% of commercial poultry was found positive for H9N2 in 2013 by Ghaniei and coworkers (Ghaniei *et al.*, 2013). For the detection of subtype AIV H9, positive sera samples from ELISA were further subjected to another assay *i.e.* HI for antibody titer detection. The mean HI titer against AIV H9 was 10.70. In comparison of this study, mean HI titer reported by Hadipour *et al.,*2011 was 7.3 which was slightly differ from this study (Hadipour *et al.*, 2011).

Out of 148 recorded positive samples by RT-PCR for AIV subtype H9N2, maximum seasonal prevalence was 84 (57%) from winter season while during summers 12 (8%) samples were detected positive for the subtype H9N2. However, 52 (35%) samples were detected positive for AIV H9N2 by RT-PCR during spring season and no detection of AIV H9N2 was observed during autumn. Other studies revealed same results such as high detection of AIV H9N2 approximately 75% during winter and 25% in the summer season, (Kraidi *et al.*, 2017), and 71.6% in winter and 28.4% in summers, (Awad *et al.*, 2015) thus supporting the theory of increased AIV H9N2 activity in low temperature as the mortalities and lesions increase during winter season than in summer season. In the present analysis, maximum number of AIV H9N2 samples were positive from Sindh *i.e.* 56 (38%), KPK 52 (35%), Punjab 32 (22%) and from ICT 8 (5%). Contrary to our studies,it was observed by Kausar *et al.,*in 2018 different percentages from different ecological zones of Pakistan with the

maximum *i.e.* 69% positive samples detected from Punjab, 19% from KPK and 12% from Sindh (Kausar *et al.*, 2018). The difference between the results is probably because of different number of samples collected from the different areas at different time period. From the AIV H9N2 146 positive samples, highest number of samples were contributed from commercial poultry *i.e.* 108 (73%) whereas backyard poultry contributed 24 (16%) to the positive samples. It was also recorded during this study that wild domestic birds contributed 12 (8%) and wild migratory birds contributed 4 (3%) to the positive H9N2 recorded samples. Similar results were also reported by Bahari where 59.3% of the RT-PCR positive samples were isolated from commercial poultry (Bahari *et al.*, 2015). In contrast to this study prevalence results revealed by Srawar *et al.,* 2013 showed that 0.012% samples were positive from commercial poultry in Lahore (Sarwar *et al.*, 2013).

In the on-going study, out of 491 tissue and swab samples 37% virus isolations of AIV were recorded by *in-ova* inoculation technique and HA activity from the time between July 2018 to June 2019. By hemagglutination inhibition test only 27% were confirmed as AIV H9N2 while 30% detections of AIV H9N2 were recorded by reverse transcriptase PCR during this time. The difference between the results of both techniques showed that PCR is highly sensitive than in-ova inoculation technique. Siddique *et al.,* 2008 proposed in their study that additional 63% samples detected positive for AIV H9N2 through the RT-PCR that were missed during in-ova inoculation (Siddique *et al.*, 2008). It was also quoted in various studies that number of isolated samples differ from those detected by RT-PCR because viral titer is not sufficient for detection by HA while PCR is more sensitive and amplify viral RNA very efficiently even if present in very minute quantity. In-ova inoculation technique require two to three days incubation period so that virus can grow in embryonated eggs where as in RT-PCR no incubation period is required and is less time taking. Another disadvantage of in-ova inoculation is that only live virus can be isolated while PCR not only detect very minute quantity of virus but can also detect active or even inactive virus. Therefore, PCR is considered more advantageous over in-ova inoculation (Vabret *et al.*, 2000; Kausar *et al.*, 2018; Kim *et al.*, 2018).

Avian chlamydiosis is caused by the bacteria *Chlamydia psittaci* and results in psittasois or ornithosis. Depending on the chalamydial species they can cause systematic to fatal diseases along with the severe respiratory stress (Rešidbegović *et al.*, 2006; Lagae *et al.*, 2014). During July 2018 to June 2019, 550 samples were collected from different regions of Pakistan, including 491 tissue and swab samples and 247 serum samples and these samples were tested for the presence *Chlamydia psittaci.* All the 491 samples (tissue and swabs) were inoculated within embryonated eggs and tested for the chlamydial presence by the *in-ova* inoculation method. 184 (37.47%) samples showed positive results, and these were confirmed for chlamydial presence through the PCR technique. According to studies conducted Siraj and coworkers *Chlamydia* was effectively isolated at the rate of 52.94% - 72.55% and 54.17% - 79.17 % from feaces and conjunctival swabs, respectively (Siraj *et al.*, 2018). The difference in the isolations is due to difference in the size of the samples collected and areas of the samples collection.

During the study period, 247 sera samples were collected from different regions and subjected to ELISA to check the seroprevalence of *Chlamydia psittaci.* 52 samples *i.e.* 21.03% samples showed seroprevalence. However 77.8% samples were found positive by ELISA in the study performed by Yang and his colleagues (Yang *et al.*, 2007). The differences in the results might be due to host species, maintenance (cage or free), physiological status and severity symptoms or not. Also, geographical area and sampling technique affects the results. From 52 samples showing seroprevalence, maximum seroconversion was observed during summers with 32 (61.50%) positive samples, followed by 13 (25%) from winter and only 7 (13.46%) from spring showed seroprevalence whereas no seroprevalence was observed during autumn. Similarly Vanrompay *et at.,* 1997 observed in their study that samples collected during summers showed high seroprevalence rates as compared to those collected in winter season (Vanrompay *et al.*, 1997).

Different seroprevalence rates were observed in the different provinces. From the 52 positive sera samples for *Chlamydia psittaci* highest seroprevalence was observed in Punjab *i.e.* 36 (69.2%) samples were found positive followed by KPK with 8 (15.3%) positive samples, whereas only 4 (7.69%) samples were found positive in Sindh and ICT. Also in the study conducted in 2018 by Siraj different seroprevalence was observed *i.e.* 32.43%, 33.33%, 26.47%, 30.23% and 32.60% in different areas of Punjab (Siraj *et al.*, 2018). As compared to this, low seroprevalence was observed in other areas of Pakistan because of difference in the temperature conditions and other environmental and regional factors.

It was also observed that the maximum seroprevalence was in wild domesticated birds with 24 (46.1%) positive samples followed by 16 (30.7%) samples positive from commercial poultry and 12 (23%) from the backyard poultry. No seroprevalence was observed during this time from wild migratory birds. Similar results were also found in other studies showing maximum seroprevalence in ducks 29.62% and pigeons 44.64% whereas in chickens the seroprevalence rate was 9.75% (Siraj *et al.*, 2018) thus supporting our results where highest seroprevalence was observed in wild domesticated or fancy birds including ducks and pigeons. In another study performed by Cong and coworkers in 2013 seroprevalence rate range from 34.9% to 50.4% in domesticated birds (Cong *et al.*, 2013). Other studies conducted by Madan *et al.,*2011 and Feng *et*

al., 2016 also showed high prevalence in domesticated birds than any other poultry species (Madan *et al.*, 2011; Feng *et al.*, 2016).

From the 491 tissue and swab samples collected from different regions of Pakistan during July 2018 to June 2019, 80 (16.29%) samples were detected positive for *Chlamydia* and from them only 60 (12.21%) were found positive for *Chlamydia psittaci* by the PCR methods. In contrast to this, in a study conducted by Guo *et al.,* 2016, 26.2% samples were found positive from the poultry samples by PCR method (Guo *et al.*, 2016). The difference in the detections might be due to the number of samples collected and due to the type of samples collected during this time period. Out of 60 positive samples for *C. psittaci* maximum prevalence was observed in summer *i.e.* 39 (65%), 15 samples with 15% prevalence in winter, 6 (10%) samples during spring season and 0% prevalence in autumn.

According to the studies performed by Vanorompay *et al.,* 1997 and Siraj *et al.,* 2018 it was observed that *Chlamydia psittaci* are likely to grow in summers when the temperature are usually high (Vanrompay *et al.*, 1997; Siraj *et al.*, 2018). Maximum detections of *Chlamydia psittaci* were from Punjab with 44 (73.33%) followed by KPK with 8 (13.33%) and 4 (6.6 %) prevalence was observed in Sindh and ICT. In 2018 Siraj along with colleagues observed the maximum detections of *Chlamydia psittaci* from different areas of the Punjab that supports our findings (Siraj *et al.*, 2018).

From the 60 positive samples for *C. psittaci,* highest 28 (46.66%) samples were from wild domesticated birds followed by 20 (33.33%) from commercial poultry, 12 (20%) from backyard poultry and no sample was detected positive from wild migratory birds. Similarly it was shown in 2016 by Guo and coworkers that *Chlamydia psittaci* is common in all the four poultry types but high prevalence was observed in domesticated birds including pigeons and ducks (Guo *et al.*, 2016). In 2014 Legae along with colleagues showed that 5.4% prevalence rate was observed in commercial poultry (Lagae *et al.*, 2014).

From the observed results, the sensitivity and specificity of different techniques was also checked, and it was seen that *in-ova* inoculation was specific though PCR methods were more sensitive as 184 (37.74%) samples were supposed to have a *Chlamydia* in them and only in 80 (16.29%) samples *Chlamydia* was detected by PCR. It was also documented by Ghorbanpoor *et al.,* 2015 and Balsamo *et al.,* 2017 that PCR is highly

sensitive method for the detection of *C. psittaci* and is more advantageous over other testing techniques. PCR is more rapid and also nucleic acid amplification has increased availability and reliability. Additionally, to being specific and sensitive for *C. psittaci,* nucleic acid-based tests are also used for strain genotyping (Ghorbanpoor *et al.*, 2015; Balsamo *et al.*, 2017).

From the tested 491 tissue and swabs 147 (29.9%) showed AIV H9N2 and 60 (12.21%) showed *Chlamydia psittaci* whereas in 32 (15.45%) coinfection of both the virus and bacteria was observed. Similar values were observed in sera samples as 175 (70.85%) were found positive for AIV H9N2 and 52 (21.05%) samples showed *Chlamydia psittaci* and 26 (11.45%) showed coinfection. In 2016 Chu and his coworkers showed in their study that AIV H9N2 along with *Chlamydia psittaci* contribute in the mortality as well as in lesion severity and effect the survival rates (Chu *et al.*, 2016).

4.1 Conclusion

The prevalence rate of low pathogenic avian influenza subtype H9N2 was recorded as 27% isolations and 30.14% detections in poultry from July 2018 to June 2019. Total 71% seroconversion was observed during this period and high HI antibody titers (MT log 2) were detected against AIV H9. Similarly, *Chlamydia psittaci* was detected in 12.21% samples and 21.03% seroconversion was observed during this time in poultry. Also, 15.38% coinfection was observed from tissue and swabs and 11.45% coinfection was observed in serum samples. Results revealed the persistent exposure of H9N2 and occasional occurrence of *C. psittaci* among the poultry and they appeared as a looming threat in the live bird market of Pakistan. Therefore, vaccination campaigns are required to control the disease. Both AIV H9N2 and *C. psittaci* have zoonotic impact therefore; there is the need for systematic sampling and rapid detection of infected poultry for the eradication of contagious viral and bacterial diseases. Other preventive strategies including strict biosecurity measures, awareness programs among layman for disease vaccination and proper monitoring of poultry and wildlife is of prime importance. Molecular techniques detect pathogens that would otherwise have been missed by routine laboratory methods or incorrectly diagnosed and treated undesirably. There is further need for the rapid and advanced techniques to diagnose various respiratory pathogens.

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5. REFERENCES

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