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Molecular Characterization of Infectious Bronchitis Virus Variants and Development of an Effective Vaccine

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Molecular Characterization of Infectious Bronchitis Virus Variants and Development of an Effective Vaccine

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CERTIFICATE

The Department of Biological Sciences, Quaid-i-Azam University, Islamabad, accepts this dissertation by Zaheer Ahmed in its present form as satisfying the dissertation requirements for the Degree of Doctor of Philosophy in Biology (Microbiology).

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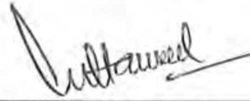

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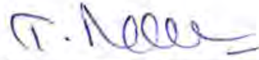

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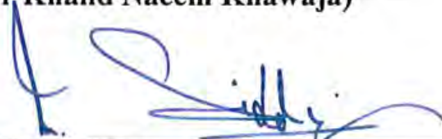

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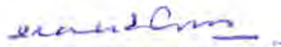

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Dated: 9/2 / 2007.

DEDICATION

I dedicate this effort to my loving parents

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LIST OF ABBREVATIONS

-	Negative
#	Number
%	Percent
+	Positive
AGPT	Agar Gel Precipitation Test
bp	Base Pairs
C	Celsius
d	Day
DEPC	Diethylpyrocarbonate
EID_{50}	Embryo Infective Dose
ELISA	Enzyme Linked Immunosorbant Assay
F.I.T.C	Fluorescence Isothiocynate Conjugate
Fig	Figure
g	Gravity
GKN	Glucose Potassium Chloride Sodium Chloride media
GMT	Geometric Mean Titre
HA	Haemagglutination Assay
HCl	Hydrochloric Acid
HI	Haemagglutination Inhibition
IB	Infectious Bronchitis
IBV	Infectious Bronchitis Virus
IFA	Immuno Fluorescence Assay
Ig G	Immunoglobulin G
IgA	Immunoglobulin A
IgM	Immunoglobulin M
ILT	Infectious Laryngotracheitis
KCl	Potassium Chloride
L	Liter
M	Molar
M	Marker
Min	Minute
ml	Milli Liter
N	Normal
N	Number
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ND	Not Done
NI	Neutralization Index
NK	Natural Killer cells
NRLPD	National Reference Lab for Poultry Diseases
O.C.T	Optimal Cutting Temperature
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEMS	Poult Enteritis and Mortality Syndrome

RBC	Red Blood Cells
RNA	Ribonucleic Acid
Rpm	Revolution per minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S.P.F	Specific Pathogen Free
Secs	Seconds
SNA	Serum Neutralization Assay
TBE	Tris Borate EDTA
ug	Microgram
ủl	Micro liter
UV	Ultra Violet
VNT	Virus Neutralization Test
VP	Viral Protein

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1. ABSTRACT

The current study reports the seroprevalence of infectious bronchitis virus (IBV) in commercial eggs and meat-type chickens in Pakistan. The objectives of the study were to monitor chicken flocks for the presence of various IBV strains, to determine their pathogenicity, to develop effective diagnostic measures for IBV strains and to develop and examine the efficacy of a killed IBV vaccine. Several commercial flocks (16 layers and 9 broilers) with a vaccination history against M-41 strain were bled and the serum samples were tested for the presence of antibodies against M-41, D-274, D-1466 and 4-91 IBV strains. The strain M-41 was found to be most prevalent (100% in layers flocks and 77% in broiler flocks) followed by D-1466 (52%), D-274 (40%) and 4-91 (8%) was the lowest. The Haemagglutination Inhibition (HI) titers were also determined and were found to be generally comparable between the layers and the broilers for a given IBV variant. The IBV antigen from clinically IBV suspect chickens was also found to be detectable using the indirect immunofluorescence assay (IFA). Lungs and trachea were the only organs tested with IFA in which 40% of lungs showed positive for strain M-41 whereas only 10% of tracheas showed positive. The IBV M-41 strain was readily detectable in homogenate of these tissues. The direct haemagglutination assay (HA) was least sensitive however its sensitivity improved significantly when the homogenates were pretreated with phospholipase C (1.3 % to 30.6%). In addition, agar gel precipitation test (AGPT) was also effective but detected only 5.3 % of the homogenate samples when tested for strain M-41 antigen. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was most sensitive and 57.3% of the tissue homogenate samples were found to be positive when tested against S1OLIGO5' and S1OLIGO3'neucleotide primers. A total of 43 out of 75 IFA positive

samples showed a viral PCR product around 1700 base pair. The tissue homogenates were passaged through the chicken embroynated eggs, which sequentially increased their teratological effects with each passage. The effects included dwarfing, curling, stunting and urate deposits in challenged embryos. These effects were effectively neutralized by using IBV variant specific antisera in a viral neutralization test. The IBV variants isolated and present in the embryonic fluid were used to prepare a formalin-killed IBV vaccine. This vaccine induced high levels of anti-IBV titers as determined by haemagglutination inhibition assay. The booster vaccine inoculation enhanced the titers as expected. The vaccine protective effects could not be tested in this study.

The results of this study which have shown the prevalence of various IBV variants in chickens suggest that despite an existing vaccine program, IBV infection is still prevalent in Pakistan. Nevertheless, by using proper diagnostic tools and reagents, the disease can be effectively and quickly diagnosed in affected flocks. In addition, indigenous vaccines representing the native IBV strains would be most effective since IBV is known to undergo significant antigenic changes overtime.

Key words: Infectious Bronchitis Virus, IBV strains, Seromonitoring, HA, HI, AGPT, RT-PCR, teratological effects, vaccine.

2. INTRODUCTION

Infectious bronchitis (IB) is an acute, highly contagious viral respiratory disease of chickens characterized by tracheal rales, coughing, and sneezing. In addition, the disease may affect kidneys, and in laying flocks there is usually a drop in egg production and egg quality. Mortality may occur in young chicks due to respiratory or kidney manifestations of the infection (reviewed by Cavanagh and Naqi, 1997). The respiratory infection is usually mild and self-limiting in the chickens. However, the economic importance of the disease is often complicated by IBV strains that cause kidney and oviduct damage as well as by secondary bacterial infection. Schalk and Hawn first described IB in 1931 in the United States, and initially, IB was recognized primarily as a respiratory disease of young chicks. Weight gain and feed efficiency were reduced in infected flocks. Later, the disease was observed in semi-mature and laying flocks, which experiences a decline in egg production and egg quality after the onset of typical respiratory illness. The infectious bronchitis virus (IBV) which belongs to the Coronaviridae family causes the disease. It is an enveloped virus with a diameter of 120 ηm (Davis and Macnaughton, 1979). The disease is prevalent worldwide with significance economic consequences. While effective vaccines are available and utilized routinely in commercial poultry production, the virus has a tendency of frequent mutations (Wang et al., 1993). There are more than 20 known serotypes within IBV recognized worldwide (Lee and Jackwood, 2000).

At present the poultry industry in Pakistan is facing great economic losses due to IBV in both layers and broilers. Drop in egg production in layers and mortality due to IBV or due to secondary complications contributes significantly towards such losses to the poultry

producers in Pakistan (Muneer et al., 1987). However, there are hardly any reports, which have attempted to document the incidence or economic losses in real monetary value. One report by Farooq et al. (2002) has attempted to tabulate losses due to various poultry diseases in Chakwal region of Pakistan. This report states mortality in layers in all phases, i.e., brooding, growing and laying periods. The mortality was highest (10 %) in the brooding period and minimum (2.4 %) during laying with an overall mortality of 6.35%. Although, the flocks in Pakistan are routinely vaccinated with M-41 strain of IBV, the problem still exists and the disease prevalence is routinely observed in vaccinated flocks (personal observation). Interestingly, the highest antibody titers in non-vaccinated flocks (8.7 %) are of that against Massachusettes (M-41) IBV strain (Muneer et al., 1987). In a serosurveillance study, Muneer et al (1987) found antibodies against Arkansas (2.6 %), Connecticut (2.2 %) type IBV as well no antibodies against JMK IBV variant. There are age and seasonal associations with the IBV infections reported in Pakistani flocks (Javed et al., 1991). The disease is more prevalent in 7 days to 5 weeks of age and the incidence is the highest (~67 %) in the winter time (Javed et al., 1991). Despite the fact that very limited reports are available on the incidence and severity of infectious bronchitis in Pakistan, the fact is that this is a serious problem which needs to be investigated and documented.

The current study was therefore designed to investigate the incidence of IBV in both commercial broiler and layer flocks of poultry. This survey-type study included seromonitoring of these flocks against M-41 as well as additional IBV strains not previously documented in Pakistani chickens. One additional avenue pursued in this study

was to develop IBV diagnostic and possibly control technologies, which can be used under poultry production and surveillance conditions in Pakistan. The specific objectives of this study were:

1. To conduct sero-surveillance of poultry flocks for IBV strain prevalence and possible identification and characterization of IBV strains.

2. To develop and improve disease diagnosis of IBV infection.

3. To undertake pathogenicity assessment of the field isolates of IBV.

4. To develop serotype specific killed vaccine using field IBV isolate(s) and test for vaccine response/ immunogenicity.

3. REVIEW OF LITERATURE

3.1 Historical Aspects

The first clinical description of an apparently new respiratory disease of chicks was reported by Schalk and Hawn in 1931 in North Dakota, USA. The disease occurred in chicks from 2 days to 3 weeks of age and was characterized by gasping and listlessness. Mortality rate as high as 40%-90% was observed. Necropsy findings included congestion and mucous exudate in bronchi and trachea and occasionally in the nasal passages. The disease was shown to be readily transmissible by contact exposure or by transfer of bronchial exudate to healthy chicks. The nature of the infectious agent was not determined at that time. Soon after, Bushnell and Brandly (1933) reported an essentially identical disease. They established that the causative agent was a filterable virus. Because the symptoms of respiratory distress were caused by a filterable virus, Bushnell and Brandly regarded the disease as a form of infectious laryngotracheitis (ILT). However, the identity of the causative virus was not determined. Bushnell and Brandly's report was considered by later research workers in avian respiratory diseases to actually describe further outbreaks of infectious bronchitis (IB). In 1930's source of confusion was that the name infectious bronchitis was used in some papers actually describing LT. This confusion was cleared few years later by Beach and Schalm (1936) who proved by cross-immunity studies in chickens that IB virus was distinct from ILT virus and also from coryza due to *Hemophilus gallinarum*. They also reconfirmed the filterability of the bronchitis virus and demonstrated that serum from IB-recovered birds was capable of neutralizing the infectivity of the virus in tracheal exudate suspensions.

Beaudette and Hudson (1937) were the first to cultivate the bronchitis virus in chick embryos inoculated via the chorioallontoic route. This enabled Van Roeckel et. al. (1942) to successfully use the serum neutralization test for bronchitis as a practical tool which employed embroynated eggs rather than chickens. A new appreciation of the economic impact of IB virus was realized when Delaplane and Stuart (1939) reported that IB was a common respiratory disease in semimature and older chickens in Rhode Island and could result in significant losses in egg production. Van Roeckel et. al., (1942) initiated a program of immunizing the growing chickens so that they were protected against the disease during the laying period.

Although this history ends at the point where commercial IB vaccines became available. However, within few years, a series of entirely new factors led the IB problem into new areas that still persist. The rapid growth of the poultry industry and the role of IB virus in chronic respiratory disease entirely changed the requirements for a successful disease control program. Just as important was the recognition by Jungherr et al., (1956) who noted that there were immunogenic differences in IB strains sufficient to prevent cross-protection.

3.2 Etiology

Infectious bronchitis virus belongs to the order of Nidovirales, the family of Coronaviridae, the genus Coronavirus, with an envelope and a diameter of about 120 ηm with club shape projections (spikes) about 20 ηm in length (Davies and Macnaughton. 1979). The spikes are not packed as closely as the rod-shaped spikes of the paramyxoviruses. Infectious

bronchitis virus virions contain three major virus-specific proteins, the spike (S) and membrane (M) glycoproteins, and the internal nucleocapsid (N) protein (Kant et. al. 1992).

3.3 Economic significance

Infectious bronchitis (IB) occurs worldwide. The flock management and the strain of virus involved play a major role in the impact of IBV infection. The principle losses are from production inefficiencies. The respiratory disease is debilitating, resulting in poor utilization of feed by young chicks, and hence poor weight gains. Condemnation at processing due to airsacculitis also contributes to production losses. Following an outbreak of IB, an estimated 3% to 8% of the broilers can be condemned at the processing plant, in comparison to flocks in which IBV is controlled, where condemnation can be below 1%. In layers and breeders, the main production losses are from non-realization of full egg-laying potential. This may be a result of the delayed maturity, decline in production during infection (estimated at between 3% and 50%) and a sub-optimal production after recovery. Additionally, losses are incurred due to downgrading of eggs (Bisgaard, 1976). In breeders, the fertility rate could be reduced during and after an outbreak. In cases of IB nephritis, in addition to losses from poor weight gains and downgrading of carcasses, losses from mortality may be in the order of 10% to 25%. In broiler flocks, morbidity is virtually 100%, whereas mortality is usually low. Early reports describe mortalities of 20% to 30%, and these were almost certainly due to mixed infections with other infectious agents such as *Escherichia coli* (*E. coli*) or Mycoplasma (McMartin, 1993). In younger chicks affected with IB nephritis, mortalities of up to 25% are common.

3.4 Transmission

The virus can be transmitted from clinically affected birds to susceptible flocks by direct contact and indirectly on fomites. Tracheo-bronchial exudate and feces of the infected birds are the major sources of virus. The virus spreads horizontally by aerosol or ingestion. The rate of spread is dependent upon the virulence of the virus and the immune status of the flock (Cumming, 1970). The virus is highly infectious, and under natural conditions respiratory signs will develop in contact birds within 36 hours. On a site, house-to-house spread will occur within two to three days, and between farms within three to four days. Feces and feed, and drinking water that have been contaminated by feces, are also sources of infection. The virus can survive for a considerable time in feces and is suspected to represent a continuing source of re-infection in the recovery phase of the disease. Contaminated litter, footwear, clothing, utensils, equipment and personnel are all potential sources of virus for indirect transmission and have been implicated in IBV spread over large distances.

3.5 Genome composition

IBV is among the largest RNA viruses with genome size of about 27.6 kb. The genetic map for IBV was generated based on the oligonucleotide fingerprinting and the in vitro translation of mRNA's (Stern and Kennedy, 1980, Stern and Selfton, 1984). In addition, the complete nucleotide sequence of IBV Beaudette strain has been determined (Boursnell et al., 1987). Overall, previous studies indicated that the gene located at 5'end of the genome RNA, about 20 kb, encodes viral RNA dependant RNA polymerase. Two open reading frames, 441 kDa and 300 kDa, respectively, overlapped by 42 nucleotides are

located at this region. Studies also suggested that a ribosomal frame-shifting mechanism might be employed for the translation of this region (Boursnell et al., 1987). The 3'end of the genome RNA, about 8kb, encodes nucleocapsid protein (N), membrane protein (M) and surface glycoprotein (S) sequentially forms the 3' to 5' (Siddell, 1995).

Wang et al., in 1993 first demonstrated the presence of five subgenomic mRNA's in the virion of IBV, which form a 3' coterminal set of mRNAs. The mRNAs have identical 3'ends but extend for different lengths in the 5' direction. Upon infection, the positive strand genomic RNA is translated to produce the RNA dependent RNA polymerase, which then transcribes the viral genome into complementary RNA. The negative strand RNA in turn serves as a template for the synthesis of genomic RNA. The viral or host enzymes that are involved in the replication are undefined. It is thought that precursor polypeptides and their end products may be involved in the replication process.

3.6 Surface glycoprotein (S) and its biological functions

The surface glycoprotein (S) is a precursor polypeptide of about 180 kDa that is cleaved into S1 and S2 polypeptides by host cellular protease in the Golgi apparatus or RER membranes (Stern and Selfton, 1982). S1 glycoprotein is located on the outer membrane and forms spikes, while S2 glycoprotein attaches S1 protein to the membrane (Cavanagh 1983a, b). The involvement of S protein in IBV attachment to cells has been demonstrated by Mockett et al., (1984) using monoclonal antibodies and by Cavanagh et al., (1984) using polyclonal antibodies raised against purified S protein to block the attachment of virus cell.

Moreover, Cavanagh et al., (1986) indicated that the removal of S1 by urea abolishes the infectivity of virus and qualitatively affects cell attachment.

The role of S protein on the tissue tropism and pathogenicity of the virus is not well defined. Studies by Kwon and Jackwood (1995) indicated that the S1 nucleotide sequences of JMK and Gray have 99% homology, through serologically they belong to different serotypes and the JMK strain mainly causes respiratory disease while Gray strain is nephrotropic. Six of the ten predicted amino acids differences located between residues 99-127 of S1 glycoprotein were assumed to be responsible for the different tissue tropism. However, the sequence analysis of nine nephropathogenic Australian strains of IBV failed to confirm the above assumption. In addition, no correlation was found between S1 amino acid sequences and nephropathogenicity of nine Australian strains of IBV (Sapats et al., 1996). Nevertheless, studies on Murine Hepatitis Coronavirus (MHV) do suggest that in some cases changes in virulence or tropism were paralleled by marked changes in S protein (Morris et al., 1989, La Monica et al., 1991).

There is strong evidence that S protein, especially S1 subunit, is a major inducer of protective immune response. IBV spike protein can induce both neutralizing and haemagglutination inhibition antibodies (Cavanagh et al., 1984).

Koch et al. (1990) identified two Mabs directed against S1 that moderately or strongly neutralized IBV at titers higher than $\log_{10}$. Studies by Karaca et al. (1990) indicated that Mabs, which exhibit serotype specificity, were all specific to S1 fractions of the

homologous serotype. IBV retaining S2 protein but not S1 can not induce virus neutralization and haemagglutination inhibition antibodies and fail to provide tracheal protection of chickens (Cavanagh et al., 1986)

3.7 Strain variations, serotypes and protectotypes

Strain variation of IBV was first reported by Jungherr et al. in 1956. They found that the Connecticut and Massachusetts isolates did not cross neutralize and fully cross protect each other, even though both cause similar disease in chickens. Since then, many different strains or serotypes of IBV have been identified based on the virus neutralization (VN) test. While most strains have a tissue tropism for the respiratory tract, both nephrotropic and enterotropic strains have been reported (Cumming, 1963; Cowen et al., 1987 and Karaca et al., 1990). Nephrotropic IBV strains can cause damage in kidney that is associated with high mortality, while enterotropic strains have not been reported to cause detectable clinical disease.

Similar to the other so called quasispecies RNA viruses, the genetic liability of IBV is partially attributable to the infidelity of RNA polymerase, and a lack of a proofreading mechanism. Interestingly, sequence analysis of structural protein genes among different serotypes of IBV indicated that the 5' end of S1 gene exhibited the highest variation rate, while S2, M and N genes were relatively conserved. Comparison of the S1 gene sequences of major North American isolates including strain M-41 (Niester et al., 1986), Ark-99, SE-17 and Pp-14 (Wang et al., 1993), CU-T2 (Jia et al., 1993), Gray and JMK (Kwon and Jackwood 1995), Holte and Iowa-609 (Wang et al., 1994) were performed. The results

revealed that some serotypes differ significantly from each other (about 20% S1 residues) such as JMK and Ark-99, while others have high homology (99%) like JMK and Gray. This suggested that a few amino acids change could lead to an antigenically different IBV strain. The homology analysis of S1 gene sequences of 13 different IBV strains within the same Massachusetts serotype indicated a higher variation rate in certain region of S1 gene is likely an indication of different serotype (Cavanagh et al., 1988, 1992a). Most recently, Callison et al., (1999) reported that variation in S2 sequence, even though small, may also contribute to strain variation of IBV. Analysis of Arkansas, Connecticut and Florida serotypes demonstrated that S2 was conserved within the same serotype, but different between serotypes, and it is consistent with the results of S1. They hypothesized that a small variation in S2 may result in different second structure of S1, therefore, affecting specific antibody binding of S1 subunit. Taken together, there is strong evidence that S1 gene variation is associated with serotypes.

Cavanagh et al., (1992b) for the first time proposed the recombination phenomenon within the Massachusetts serotype by comparing the nucleotide sequences of S genes. This was further confirmed by Wang et al., (1993, 1997) who examined both natural and experimental recombinant within S gene of different serotypes of IBV. Furthermore, Kottier et al, (1995) demonstrated that recombination could occur on the N gene and 3' untranslated region. In contrast, sequence analysis of the M glycoprotein gene and the intergenic region of several IBV serotypes suggested that strain variation is mainly due to base substitution and deletion/insertion rather than recombination of these regions (Cavanagh and Davis, 1988).

Phylogenetic analysis of different strains of IBV in different geographic regions has been conducted. Some studies have shown that the geographic distribution of IBV strains is related to their antigenic variations (Kusters et al., 1987; Gelb et al., 1991a; Cavanagh and Davis, 1992). Therefore, the distinct evolutionary lineage of virus in different regions might be a means of virus mutation. However, different results have been obtained from other studies. For example, Cavanagh and Davis investigated the relationship of recent European IBV isolates using the method of limited proteolysis of the virion glycoproteins in 1987 and of the 1960s isolates in the U.K by sequence analysis in 1992. They found that the recent isolates (1980s) were unrelated to the isolates in the USA and also distinct form those of 1960s in European countries. King (1988) reported the identification of IBV isolates from layer flocks that has a history of reduced egg production and egg quality problems that are serologically different from currently used IBV vaccine strains. Overall, it appears that IBV may also evolve independently and there is no evidence to support that mutations occur in fixed progressive manner (Cavanagh et al., 1998). _Molecular epidemiological studies can further assist in phylogenetic analysis of IBV around various geographical regions. Huang et al (2004) compared an IBV strain isolated in 1964 and another 31 strains isolated from 1991 to 2003 in Taiwan. The N-terminal and S1 gene analysis were performed on selected strains (13 total) in terms of entire S1 and partial nucleocapsid (N) genes. Their studies indicated that Taiwanese IBV strains could be divided into two distinct lineages. Not surprisingly, S1 gene showed noticeably higher divergence than the N gene. The phylogenetic trees constructed from S1 and N genes indicated than intergenic recombination had occurred. This further suggests that developing vaccines from local strains is necessary for IBV control in various geographical regions.

Despite the genetic diversity and the distinct antigenicity of different serotypes defined by VN test, partial or complete cross-protection has been achieved by cross immunization and cross challenged studies (Darbyshire, 1980; Hofstad, 1981; Lambrecht et al., 1993). Hofstad (1981) studied the cross immunity and cross protection of seven IBV isolates, and different degrees of protection were obtained ranging from 0% to 80%. Darbyshire in 1985, demonstrated that vaccination of chicken with H120 resulted in a significant reduction in titer of the challenge virus Australian T strain, although no antigenic relationship was found based on VN test. Therefore, the term "protectotype" was proposed by Lohr (1988) to characterize some IBV isolates rather than as serotypes or variant strains. This concept was further supported by Cook et al., (1999) who showed that cross protection against heterlogous IBV serotypes induced by a vaccination regime using Massachusetts serotype for priming and heterologous serotype 4/91 for boost immunization was highly effective. Cavanagh et al. (1998) proposed that IBV mutation might occur more randomly. They concluded that there is little evidence to support the notion that IBV variant strain arises from immune pressure or immune selection by analyzing the S1 gene sequences of 28 IBV isolates isolated in France and Britain between 1985 and 1996.

3.8 Clinical signs and organ involvement

3.8.1 Respiratory infection: Replication of IBV in the respiratory tissues causes characteristic, but not pathogonomonic signs such as gasping, coughing, tracheal rales and nasal discharge. Occasionally, puffy, inflamed eyes and swollen sinuses may be seen. (Parsons et al., 1992; Capua et al., 1994). In uncomplicated cases these signs last for only 5 to 7 days and disappear within 10 to 14 days. The effected chickens also appear depressed,

and feed consumption and weight gains are significantly reduced from 3 days after infection (Otsuki et al., 1990). In uncomplicated cases, moralities are generally low and have been attributed to asphyxiation due to blocking of the lower trachea or bronchi by plugs of mucus. The upper respiratory tract is the main site of IBV replication, following which a viremia occurs and the virus gets widely disseminated to other tissues. The virus is epitheliotropic and enters the epithelial cells by viropexis. During the clinical phase of the disease, maximum virus titers are recorded in the trachea between 5 and 10 days post infection. (Otsuki et al., 1990; Ambali and Jones, 1990)

3.8.2 ***Reproductive effects:*** Infectious bronchitis can cause a severe decline in egg production in layers. Furthermore, the eggshell and internal quality deteriorates during the later period of laying (McDougall, 1968). Mild or no respiratory signs may accompany such effects (Muneer et al., 1986). Some strains produce only a loss in shell color (Cook, 1984). The severity of decline in egg production varies with the period of lay, the virulence of the virus involved and other non-specific factors. Egg production may start to increase after 2 to 3 weeks from the first appearance of clinical signs of the disease, but reaches only sub optimal levels. When laying is resumed, some eggs have soft shells, while others are mis-shape or rough shelled. Inspissated yolk material may be seen in the abdominal cavity of infected layers. IBV infection of female chicks of less than 2 weeks of age can cause permanent damage to the developing reproductive tract, resulting in "false layers" that do not lay normally at sexual maturity. The effect of IBV on the male reproductive tract has not been reported (Jones and Jordan, 1970).

3.9 Viral effects on other organs and functions

3.9.1 Nephritis: Although even those strains of IBV considered primarily to affect the respiratory tract such as strain M-41 can occasionally cause kidney damage nephropathogenecity has been associated only with certain strains (Jones, 1974). Viral replication in the kidney causes impaired fluid and electrolyte transport leading to acute renal failure (Afanador and Roberts, 1994). The kidneys infected with IBV are swollen and pale, with tubules and ureters distended with urates. The relative kidney weight and kidney asymmetries are increased (Winterfield and Albassam, 1984).

3.9.2 Intestinal involvement: Several strains of IBV have been isolated from cloacal swabs, feces and caecal tonsils (Alexander et al., 1978; Cook, 1984). The ability of the IBV strains to survive in the presence of low pH, digestive enzymes and bile salts may be relevant to enteric replication (Otsuki et al., 1990).

3.9.3 Pectoral myopathy: The important variant strain of IBV, 793/B, was recently isolated from a broiler breeder flocks where the affected birds had bilateral myopathy affecting both deep and superficial pectoral muscles (Gough et al., 1992). There are marked swelling and pallor of deep pectoral muscles together with the presence of occasional facial haemrorrahages and a layer of gelatinous edema over its surface (Dhinakar Raj and Jones, 1996).

3.9.3 Virus in other tissues: Infectious bronchitis virus has been isolated from the Harderian gland, (Toro et al., 1996) bursa of Fabricius, liver and spleen (Ambali and Jones, 1990). However, it has not been documented to be involved with any functional damage.

IBV has been isolated from semen and eggs of infected chickens (Cook, 1971), but vertical transmission appears to be of little importance.

3.10 Pathogenesis

The chicken is the only natural host of IBV that shows clinical disease. All ages of chickens are susceptible, but the disease is usually severe in 1-3 weeks old young chicks, and causes mortality (Cavanagh and Naqi, 1997). IB has a short incubation period of about 18-24 hrs, and is highly contagious among chickens in a flock (Cunningham, 1970). The characteristic clinical signs in chicks are coughing, sneezing and tracheal rales. The chicks appear depressed, the feed consumption and weight gain are significantly reduced. Older chickens usually recover in two weeks. In laying flocks the respiratory symptoms are mild, but egg production can be decreased upto 50% and the egg quality is also affected, resulting in misshapen, soft-shelled and infertile eggs. Experimental infection of one day old chicks with IBV can lead to permanent damage of the oviduct, therefore, the egg production and quality is reduced when the chickens come into lay (Crinion, 1972; Jones and Ambali, 1987). Infectious bronchitis virus can replicate in tissues of the respiratory tract, intestinal tract, kidneys, and the oviduct (Karaca et al., 1990). Commonly, IBV isolates, regardless of tissue of origin, readily infect the respiratory tract of the chickens and produce characteristic lesions in the trachea, airsacculitis develops because of secondary bacterial infections like *E. coli* and *Mycoplasma* infection. Combined infections cause mortality ranging from 14-82% (Cook et al., 1986). The highly egg adopted Beaudette strain is pathogenic, causes no detectable damage to the ciliated epithelium of the trachea and replicates predominantly in the subepithelial cells (Albassam et al., 1986).

In contrast, the virulent M-41 strain destroyed the ciliated epithelium prior to localization in the subepithelium. The Australian T strain is virulent and a known cause of mortality and kidney lesions. Viruses of other serotypes that are known to be nephropathogenic, but of less severity than T strain, include the United States strain Gray, and Holte, the Mass-Holland 52 strain, and the Belgian B1648 isolate (Pensaert and Lambrechts, 1994).

Virulence for the reproductive tract may also differ among IBV strains. Different IBV strains can produce a range of effects in susceptible layers varying from shell pigment changes with no production drop to production drops of 10 to 50% (Cook and Huggins 1986). Muscle pathology in broiler breeders has been described as pale and swollen deep pectoral muscle with occasional fascial hemorrhages and a layer of gelatinous edema over the surface of the muscle. Bilateral myopathy affected both deep and superficial pectoral muscles (Hopkins and Beard, 1985).

Studies on the pathogenicity of 25 strains of IBV isolated in Australia between 1961 and 1994 revealed that twelve strains were nephropathogenic and 10 respiratory, the other three being of mixed pathogenicity. The IBV strains identified as nephropathogenic induced clinical nephritis, gross and histological kidney lesions and mortality of 5-90%. The 10 respiratory strains caused histological lesions in trachea but not in the kidney, and did not induce clinical nephritis and mortality (Ignjatovic et al., 2002).

Histologically, the changes in the trachea are characterized as loss of cilia and desquamation of epithelial cells, followed by the infiltration of lamina propia with

heterophils, plasma cells and lymphocytes and regeneration of epithelial cells. The kidney lesions are primarily interstitial nephritis, characterized by massive infiltration of heterophils in the interstitium (Albassam et al.. 1986 and Riddell, 1987).

3.11 Diagnosis

The diagnosis can be confirmed by isolation and identification of the causal virus using egg inoculation and/or tissue culture techniques. Retrospective diagnosis is possible by demonstrating a significant rise in circulating antibody in paired acute and recovery-phase sera applying enzyme-linked immunosorbant assay (ELISA) or serum neutralization (SN) assay. Reverse transcriptase polymerase chain reaction (RT-PCR) is a reliable and sensitive method, which can be used to test field samples. The test can be tailored to detect all IBV strains by using primers for the conserved N protein or S1 glycoprotein of IBV (Smati et al., 2002; Zhou et al., 2003). However, RT-PCR is complex to perform and requires a level of expertise that may not be available in a diagnostic laboratory.

Virus isolation is usually performed using the 10-day old embroynated eggs, but adaptation may be needed for the initial isolation of the field samples. The characteristic changes are stunting, curling embryos and embryo death. Tracheal organ culture also had been used in virus isolation by assessing the movement of cilia after virus inoculation. IBV can always be detected by tracheal organ culture during the initial passage and no adaptation is usually required for this method (Sawaguchi et al.. 1985). Chicken embryo kidney cells and chicken kidney cells are the most often used primary cell cultures for IBV (Yamakami et al.. 1984).

Serology tests are routinely performed to monitor vaccine responses or are used as an indication of exposure to virus. Virus neutralization (VN) test is traditionally considered as the "Gold Standard" in terms of serotyping diagnosis. VN can be carried out in chicken embryos or tracheal organ culture by constant–serum variable virus method or constant-virus variable-serum method. VN has the disadvantage of being time-consuming, tedious and expensive. Haemagglutination inhibition (HI) test is able to measure the HI antibody response much faster and cheaper, but its application in serotyping is controversial. Studies of King and Hopkins (1983, 1984) demonstrated the ability of HI test in strain differentiation, especially following a single exposure. Other investigators indicated that the HI test is highly cross-reactive compared with the VN test (Lashgari and Newman, 1984), therefore, it is only suitable for monitoring immune status and rapid diagnosis. Agar gel precipitation (AGP) (Gough and Alexander, 1977), indirect immunofluorescence (IFA) (Chubb 1986), ELISA (Marquardt et al., 1981), and Western blot (Sneed et al., 1989) have been developed to detect antibody against IBV. However due to the cross reactivity, most serological tests, except VN, can not differentiate among different serotypes or strains of IBV. Even with the availability of monoclonal antibodies for some strains of IBV, which made the serology test serotype specific, their application is still very limited (Naqi et al., 1993).

Detection of IBV viral antigen in infected chickens using immunoperoxidase and immunofluorescence staining has been evaluated. Yagyu and Ohta in 1990 described the use of monoclonal antibodies specific for N protein of Massachusetts in immunofluorescence staining for the detection of viral antigen. By applying the technique

to tracheal smears or infected kidney cells, it was demonstrated to be suitable for early detection of virus. Similarly, Naqi (1990) reported a monoclonal antibodies based immunoperoxidase procedure for rapid detection of virus in infected chickens.

The reverse transcription-polymerase chain reaction (RT-PCR) test specific for IBV was first developed by Anderson et al., in 1991. Two synthetic primers chosen from the conserved Membrane and Nucleocapsid genes were used to amplify cDNA generated from reverse transcription of IBV genome RNA. Compared with the above methods, RT-PCR is much more sensitive, highly specific, but is expensive. With the availability of gene sequences of different strains of IBV, oligo primer pairs based on the S1 gene (Kwon et al., 1993) were designed and used in the RT-PCR. Since most of the oligo primers used in the RT-PCR were chosen from consensus sequences in the IBV genome, it can not differentiate various serotypes (Lin et al., 1991). A serotype specific RT-PCR of the S1 gene was reported by Keeler at el., (1998). In the future, with the advance of sequencing technology that makes the sequencing cost-effective, sequencing of the S1 glycoprotein gene is likely to become a popular diagnostic method, providing more accurate genetic information about virus isolates.

3.12 Immunity

It is believed that both cell-mediated immune response and humoral immunity play an important role against IBV infection. Immature breeding and commercial layer flocks are routinely vaccinated with a mild attenuated product (H-120, or Massachusetts or Connecticut or their combination) at 7days in drinking water or by aerosol (Gelb et al.,

1989). The vaccination is repeated at 30-40 days. The initial live vaccine should always be administered to susceptible breeder and layer flocks before 12 weeks of age to avoid possible damage to the developing reproductive tract of the pullet. Immunity in commercial layers can be boosted by administration of live attenuated vaccine in drinking water or as a coarse spray during the production period. Such commercial flocks receive inactivated IB vaccine as a booster, usually in the form of an injectable multivalent emulsion at the end of the rearing period and then at mid-cycle as considered necessary to maintain adequate maternal antibody transfer to progeny. Broilers in endemic areas are vaccinated by aerosol at day-old or subsequently by coarse spray or in drinking water at a suitable time (10-20 days) depending on maternal antibody transfer or pattern of field challenge. In some areas special IB vaccines are required to prevent clinical problems attributed to variant strains.

3.13 Immune responses of the chicken to IBV

3.13.1 Immunogenic proteins of IBV: IBV has three structural proteins. The spike 'S' glycoprotein is located at the surface of the virion, and consists of two subunits, S1 and S2, with molecular weights of 92 and 84 KD, respectively. The membrane 'M' glycoprotein is partially exposed at the surface of the virion with molecular weights ranging from 27 to 36 KD, and the nucleocapsid 'N' protein is internally located with a molecular weight of 52 KD (Wady and Westaway, 1981; Cavanagh, 1983. The S1 glycoprotein of IBV induces virus neutralization and haemagglutination inhibition (HI) antibodies and has been considered to most likely inducer of protection (Kant et al., 1992; Ignjatovic and Galli, 1994), but S2 and N proteins may also be important since they carried epitopes for induction of cross-reactive antibodies (Ignjatovic and Galli, 1995). A T-cell epitope has

been identified in the IBV 'N' protein (Boots et al., 1991) and has been shown to induce anti-viral responses (Boots et al., 1992). Cellular immune responses elicited by a live IBV vaccine have also been found to be cross-reactive and the responses varied in magnitude with the serotype of IBV used for *in vitro* stimulation (Dhinakar Raj and Jones, 1997b).

3.13.2 Innate Immunity: Immunity is considered to be either innate or acquired. Innate immunity comprises a collection of factors which resist invasion by external agents, such as physical barriers provided by skin and mucous membranes, soluble factors like lysozymes, complement an acute phase proteins, and cells such as granulocytes, macrophages and natural killer (NK) cells. In IBV-infected chickens, heterophils are the most numerous early inflammatory cells in respiratory lavage fluids (Fulton et al., 1993). The role of macrophages in IBV infection is unknown, while no alterations in NK cell activity have been found following IBV infection (Wakenell et al., 1995).

3.13.3 Acquired immunity: Acquired immunity results in the activation of antigen-specific effector mechanisms including B-cells (humoral), T-cells (cellular) activation and the production of memory cells. Upon receiving proper stimuli, B-cells differentiate into plasma cells to secrete antibodies either in the presence or absence of T-helper cells. Immunoglobulin G (IgG), the major circulating Ig, is the antibody detected by HI and an ELISA. Anti-IBV IgG can be detected as soon as four days post infection, reaches a peak at about 21 days but can remain in high titre in the serum for many weeks (Mockett and Darbyshire, 1981). This is the antibody measured in conventional serological test to monitor IBV infections or in response to a vaccine. Immunoglobulin M (IgM), present only

transitorily after infection, reaches peak concentrations about 8 days after IBV infection and levels then decline (Mockett and Cook, 1986).

Maternally derived antibodies can provide protection against IBV, but they are short lived (Darbyshire and Peters, 1985; Cook et al., 1991). Presence of maternally derived antibodies has no adverse effect on the efficacy of live IBV vaccines administered at one day of age (Davelaar and Kouwenhoven, 1977). Maternally derived IgG has been demonstrated in tracheal washes (Mockett et al., 1987). Chicks hatched with high levels of maternal antibody have been shown to have excellent protection (>95%) against IBV experimental challenge at one day of age. However, such protective effects are lost if challenged at seven days of age suggesting not only the protective role of the maternal antibodies but also that the maternal antibodies are degraded within the first week post-hatch (Mondal and Naqi, 2001).

Local immunity in the respiratory tract is of fundamental importance in protection against IBV (Hawkes et al., 1983). This has been exemplified by the use of an in vitro challenge model using tracheal organ culture from immunized chickens, for cross protection studies (Lohr et al., 1991). IBV specific IgA and IgG have been demonstrated in tracheal washes of infected chickens and antibody-secreting cells were shown in tracheal sections (Nakamura et al., 1991).

3.14 Control and Prevention

No treatment is available for IB, and antibiotics are usually used to prevent secondary bacterial infections. Recombinant chicken interferon has been examined as a potential anti-viral-agent, but disparate results were obtained and its application remains to be determined. Prevention is considered as the most extensively used strategy. This is obtained mainly be the massive vaccination of chickens with attenuated and inactivated vaccines.

Due to the highly transmissible nature of IB, Van Roekel (1951) tried to expose growing chickens to IBV under controlled condition to prevent decreased egg production and egg quality in layers and achieved some success. Subsequently, attenuated viruses obtained by serial passages in embroynated chicken eggs or primary chicken kidney cells have been used as live vaccines (Klieve and Cumming, 1988). Cold adapted virus has been assessed for protection. Gelb et al., (1991b) generated a cold adapted IBV Ark strain by passing virus in chicken embryos incubated at 28°C. It provided protection against homologous virus challenge, but its temperature sensitivity was lost after its administration to chickens.

Live vaccines are used for vaccination of broilers and for initial vaccination of breeders and layers. Good immune response can be achieved this way, but more evidence indicates that some attenuated vaccines increase their virulence by back passage in chickens, suggesting a threat of introducing pathogenic virus to chickens through the use of live virus vaccines. Hopkins and Yoder (1986) reported the reversion to virulence of chicken passaged IBV vaccine virus. Inactivated vaccines are also available and primarily used in layers to boost

immunization prior to laying eggs. It is not suitable for primary immunization. In addition, individual injection is needed. Therefore, the administration of inactivated vaccines is laborious and expensive.

The continuous emergence of new antigenic strains of IBV makes the prevention by live attenuated IBV vaccination difficult. Also, the *in vitro* virus neutralization results sometimes do not correlate well with cross protection (Raggi and Lee, 1975). To design a vaccination scheme in a region, one should be aware of all the existing IBV strains in the region, and also cross protection investigation has to be conducted to assist in selecting an appropriate vaccine. Since the Massachusetts strain is the initial isolate and distributed worldwide, Mass vaccine is used extensively without restraint. For other IBV vaccine strains to be used in a region, their prior existence has to be confirmed before its introduction into the region.

Vaccines using recombinant DNA technology are still being developed. No commercial recombinant vaccine is available yet. Most studies have focused on the surface glycoprotein S. Using vaccinia virus or baculovirus as a vector of immunogenic S or S1 protein genes, IBV specific antibody and protective immune responses were induced in immunized mouse or chickens (Tomley et al., 1987; Song et al., 1998). The major advantages of DNA vaccines over other recombinant vaccines are their ease of preparation and long lasting immunity. It is speculated that the presentation of antigen mimics that of natural infection because it results in *de novo* protein synthesis. For example, a DNA

vaccine based on the VP2 gene of Infectious Bursal Disease virus induced protective immunity against challenge with virulent IBDV strain (Fodor et al., 1999).

These observations in the literature clearly indicate that infectious bronchitis is a significant disease of important economic concern. The fact is that very few studies have documented the incidence of IB in Pakistan. A few studies which have reported the prevalence of IBV (Muneer et al., 1987; Farooq et al., 2002) warrant that a continuous seromonitoring needs to be done to get a better understanding and control of this disease in Pakistan.

4. MATERIAL AND METHODS

4.1 Chicken Flocks: Source, Husbandry, and Sampling

This study was carried out on commercial chickens. The first source of chickens was commercial flocks located in various regions of Abbotabad, Manshera, Murree, Hafizabad, Arifwala and Rawalpindi regions of Pakistan. From these areas a total of 25 chicken flocks consisting of 16 layer and 9 broiler flocks were included in this study. The layer flocks were of varying ages ranging from 12 – 50 weeks of age whereas the broiler flocks ranged from 23 – 48 days of age. All flocks were recorded as being free from any apparent clinical disease. Vaccination history included all flocks been vaccinated against M-41 strain of Infectious Bronchitis Virus (IBV). These flocks served as a source of blood samples used in the serosurveillance study for the presence of various strains of IBV in these flocks. Twenty blood samples were collected from each of the 25 flocks. The samples were transported to the laboratory and the serum phase was collected and stored at $-20^{o}C$ for further analysis.

The second source of chickens used in these studies was the chickens brought to the Poultry Disease Diagnosis Laboratory by commercial farmers. These chickens (n = 150) of varying ages were brought as "suspect" cases from flocks exhibiting respiratory distress. These chickens were necropsied and lungs, trachea, kidneys and caecal tonsils were collected aseptically for tissue analysis, homogenate preparation and further analysis as described in subsequent sections.

The following flow chart describes the sequence of experimentation from samples obtained from both sources under this study:

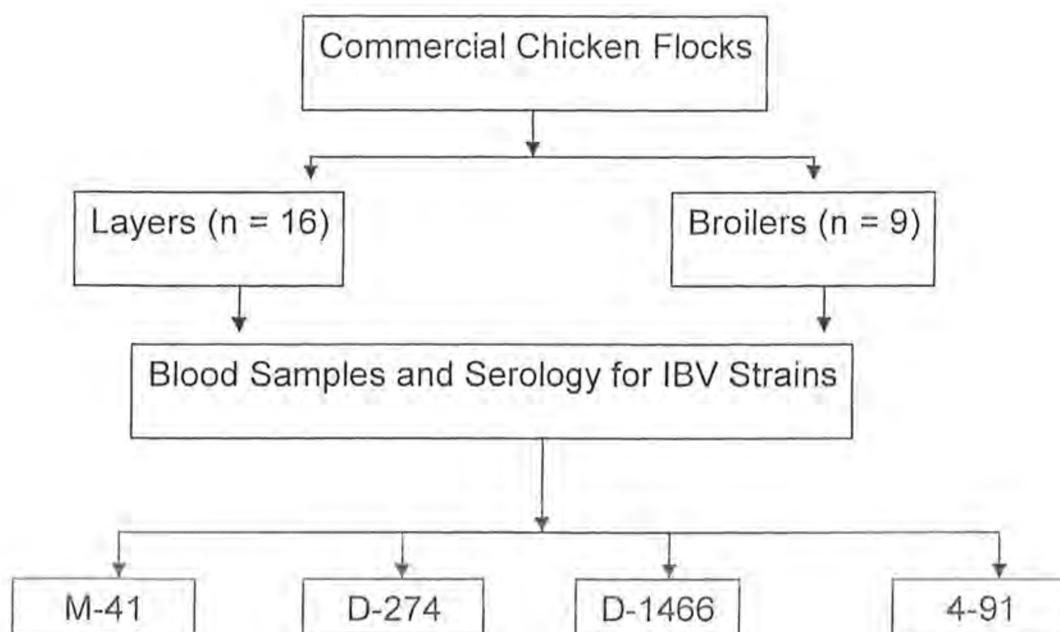


Figure 1A: Diagrammatic Illustration of the Experimental Design and Studies. The commercial flocks were M-41 strain vaccinated and were free from any clinical disease. Blood samples were collected from 20 chickens from each of the 25 flocks. The serology was carried out via haemagglutination inhibition assay against M-41, D-274, D-1466 and 4-91 IBV strains.

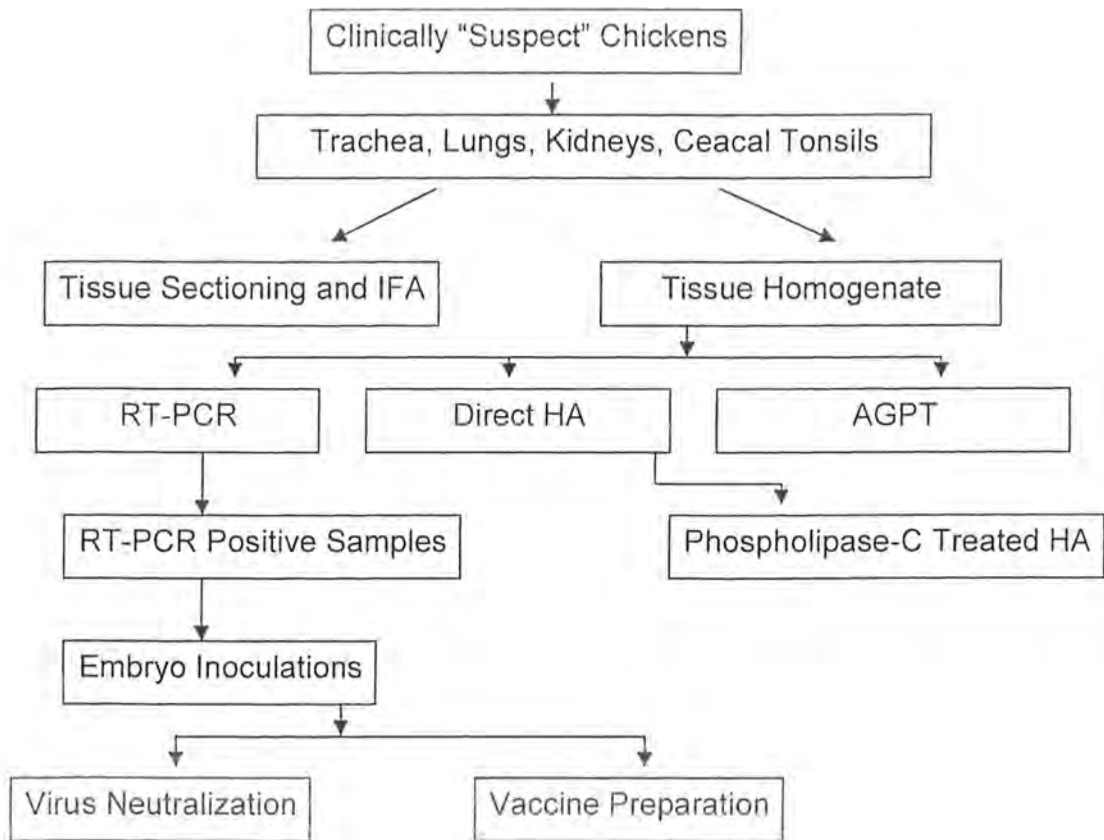


Figure 1 B: Diagrammatic Illustration of the Experimental Design and Studies. Clinically "suspect" chickens (n – 150) of varying ages were exhibiting respiratory distress upon arrival. Various tissue samples were then collected and processed for the detection of viral antigens, nucleic acid, as well as pathogenecity assays.

4.2 Serological Analysis for IBV Monitoring

Blood samples collected from the first group of chickens (i.e., 25 chicken flocks) were used to monitor the flocks status for the prevalence of various IBV strains. Specific antigens for four IBV strains, i.e., M-41, D-274, D-1466 and 4-91, were obtained from Gezondheidsdienst voor Dierenb.v.(G.D), Holland. All of these antigens were first tested by haemagglutination to determine the working antigen dilution for their subsequent use in the Haemagglutination Inhibition (HI) Assay. This was done as follows:

4.2.1 Haemagglutination (HA) Test: Two-fold serial dilutions of all four IBV antigens were prepared in round-bottom 96-well microtitre plates. This was done by adding 50 µl of the test antigen in a well containing 50 µl of 0.1M PBS (pH 7.2). The dilutions were made up to 1:4096 two-fold dilutions. Microtitre wells without antigens were also included in each plate for negative controls. To the diluted wells, 50 µl of 0.5% of chicken red blood cells (obtained from healthy in-house chickens) prepared in 0.1M PBS were added. The plates were agitated to ensure the even mixing of the well contents. The plates were then incubated at room temperature for 25 minutes and the HA end point was recorded. The Haemagglutination unit was defined as the reciprocal of the highest dilution of the virus which caused complete agglutination with an equal volume of appropriately diluted RBC. This end point was obtained for each of the viral antigen and was termed as 1HA unit. Based on the 1 HA unit information, the 4HA values for each of the viral antigens were calculated. The 4HA unit for each of the viral antigen was a given dilution which was a dilution in the fourth-well lower than the 1 HA unit where the last HA was observed.

4.2.2 Haemagglutination Inhibition (HI) Test: Haemagglutination inhibition (HI) assay was used to assess the Seroprevalence of IBV viral antigens in the serum samples as previously described (Beard 1970). Briefly, the serum samples were added in 96-well round bottom microtitre plates. The first working dilution for all serum samples was made as 1:5 in PBS in the first well, followed by a two-fold serial dilution up to the last well in each column. To each well, 25 µl of 4HA units as determined previously of IBV antigen was added. The plates were incubated at 37°C for 30-40 minutes. After this incubation period, 50 µl of 0.5% chicken RBCs suspension (diluted in PBS) were added into each well. The plate was agitated to ensure proper mixing of the well contents and reincubated at room temperature for up to 30-45 minutes or until a clean pattern of haemagglutination or haemagglutination inhibition (button formation) was observed. The maximum dilution of each serum sample causing inhibition of haemagglutination was used as the end point. The HI titre of each serum sample was expressed as reciprocal of the serum dilution (top to bottom). The results of HI titre of all the sera thus obtained were subjected to statistical analysis.

4.2.3 Statistical Analysis of the Titres: The Geometric Mean Titre (GMT) was calculated as described in appendix 1 by taking an average of well numbers showing HI activity from all serum samples within one flock. This well number average was then cross-checked against GMT values given in the Brug's table and were then reported as the GMT for a given flock against a particular IBV antigen.

4.3 Detection of IBV Antigens in "Clinically Suspect" Chickens

4.3.1 Tissue Analysis: The presence of M-41 IBV antigens as an evidence of IBV prevalence in commercial chickens was examined. M-41 strain was used as a "representative" of the IBV prevalence since the initial seroprevalence studies indicated that the highest incidence of IBV strains in commercial chicken population was of that of M-41 IBV strain (Layers = 100% and broilers = 67%; overall = 88%, Table 5) as compared with the rest of the IBV strains. To accomplish this, the internal organs namely trachea, lungs, kidneys and ceacal tonsils were collected aseptically from chickens submitted to the diagnostic laboratory as suspected cases of infectious bronchitis. These samples were transported back to the laboratory on ice. From each of the tissue under examination, a $4mm^3$ piece of infected tissue was embedded in the OCT embedding media (O.C.T. compound Miles, Inc., USA). The tissue blocks when frozen and hardened, were sectioned at 4 micron size by using the refrigerated microtome (Miles, USA) at -20°C.The section of the tissues were placed on properly labeled slides and kept at room temperature for about 15 minutes. The air-dried slides were immersed in ice cold acetone (-20°C) for tissue fixation 10 minutes. Slides were then stored at -20°C till tested for IFA.

4.3.2 Reagents and Standardization for Indirect Immunofluorescence Assay: The anti M-41 strain serum was obtained from commercial sources (Gezondheidsdienst voor Dievenb.v (G.D) Netherlands) for use in these studies. In addition, a batch of anti M-41 hyperimmune serum was also prepared against the vaccine strain (Intervet Int. The Netherlands) of M-41 IBV. This was accomplished by utilizing two weeks old unvaccinated broiler chicks raised under controlled environment. These chicks were tested

and found negative against IBV antibodies by Agar Gel Precipitation Test (AGPT). The M-41 vaccine strain was treated with 0.1 % formaline and the mixture was kept overnight at 4C. After 24 h, this mixture was blended with an equal volume of Complete Freund's Adjuvant (DIFCO Laboratories, Detroit, Michigan, USA). From the resulting suspension, six chicks were injected with 0.5 mL per bird subcutaneously and four chicks were kept as uninoculated controls. A week later, a second inactivated preparation was made similar to the first one, except that Incomplete Freund's Adjuvant was used. A booster injection employed 0.5 mL per bird injected subcutaneously. Blood samples were collected on intervals up to six weeks post boost. The sera were collected and tested for antibodies against IBV M-41 strain using AGPT and/or IFA. Finally, after six weeks, the positive birds were bled for serum collection. The blood was also collected from the uninoculated birds to serve as negative control for future use.

4.3.3 Standardization of IFA: The working dilution of the anti-M-41 antibodies and the FITC-labeled secondary (rabbit anti-chicken IgG, H&L, FITC labeled, Sigma) antibody was established in a preliminary trial in which specific pathogen free (SPF) chicks hatched from eggs obtained from Vety Care (Intervet) Islamabad, Pakistan were utilized. These day old chicks were reared in isolation and at 7 days of age, 4 chicks were inoculated with IBV, intranasal, at a dose of 10^{8} EID_{50}. Two SPF chicks were kept as negative control. At 48h post inoculation, the chicks were euthanized and infected lungs, trachea and kidneys were processed for sectioning and fixing as described earlier. For reagents standardization, two-fold dilutions of the hyper immune serum for IBV M-41 were made in PBS (pH 7.2) (1:10, 1:20 and 1:40). Each dilution was applied on three tissue slides, whereas one slide

acted as negative control. The serum was allowed to react for 30 min at 37°C in a moist chamber. The slides were washed thrice in PBS (pH 7.2) for 5 minute each. Dilution of FITC conjugate were also made in distilled water as 1:100, 1:200 and 1:400, which were applied on the slides in the way that each dilution of conjugate is applied on 3 dilution of the hyper immune serum (Table 1). The slides were incubated as above and washed similarly using PBS (pH 8.5). The slides were counter stained with Evan's blue and mounted. The results were recorded. The best combination of antisera and conjugate were selected on the basis of acceptable amount of background fluorescence of conjugate and prominent tissue structures. The serum samples from uninfected control chicks were used as negative controls and tested similar to the hyperimmune serum samples for conjugate titration as described in Table 1. None of the conjugate dilutions, i.e., 1:100, 1:200, and 1:400 produced a positive fluorescence and therefore was considered as background fluorescence relative to the test samples.

Table 1: Standardization of reagents for Indirect Immunofluorescence Assay.

DILUTION OF CONJUGATE	DILUTIONS OF HYPER IMMUNE SERUM			
	1:10	1:20	1:30	1:40
1:100	-	-	-	-
1:200	±	±	±	-
1:400	-	+	-	-

"+" = Satisfactory; "-" = Unsatisfactory.

4.3.4 Indirect Immunofluorescence Assay (IFA): Lung and Tracheal sections mounted on glass slides were flooded uniformly onto the tissue section area with hyperimmune 10μl of 1:10 diluted IBV hyper immune serum. The slides were incubated in a humidified chamber at 37°C for about 30-40 minutes. After this incubation, the slides were washed with PBS (pH 7.2), thrice for 5 minutes each. After the last wash, the slides were air dried and 25μl of 1:200 dilution of rabbit anti-chicken FITC- conjugated secondary antibody (IgG H&L) was added over the tissue section area and the slides re-incubated at 37°C for another 30-40 minutes in the humid chamber. The 1:10 dilution of hyper immune serum and 1:200 dilution of the FITC-conjugated secondary antibody was used to account for possible reduction in antibody activities during storage. Slides were again washed with PBS (pH 8.5), thrice for 5 minutes each. After the last wash, the slides were counter stained with Evan's blue stain for 1 minute and rinsed with distilled water. After drying, a drop of the FA mounting media was added to the section area and a glass coverslip was mounted on the section. The stained slides were refrigerated until viewing under a fluorescent microscope. The presence of fluorescence in the nucleus and/or cytoplasm of cells were considered as an indicator of a particular section being positive for IBV antigen. The caecal tonsils and kidney sections were not included in this study due to a limited amount of the reagents available for this analysis. Representative samples from each tissue type were also sham-treated with serum samples from control chicks to gauge the background level of fluorescence. Such fluorescence was found to be non-specific and was taken into consideration relative to the test sample results.

4.3.5 Preparation of tissue homogenate: Infectious bronchitis virus antigen-positive tracheae and lungs (n = 75) as determined by the IFA test were used for the preparation of tissue homogenates for use in subsequent analysis. Briefly, the tissues were minced with sterile scalpels and scissors in GKN buffer with antibiotics added (See Appendix 2 for composition). The resulting suspension was transferred in a sterile tube, washed once with GKN, and then minced again with a sterile syringe plunger. The resulting homogenate was frozen and thawed three times to release viruses from the cells. The preparation was then centrifuged at 1500 rpm for 15 minutes at 10°C. The supernatant was collected and pellet discarded. Finally the supernatant was filtered through 0.2 μm filter (S and S; USA), and the filtrate obtained was stored in aliquots of 2 ml at -20°C till used.

4.3.6 Detection of IBV in tissue homogenates: The tissue homogenates supernatants were tested for viral activity (presence) using direct HA, HA after treatment with phospholipase C, AGPT, and RT-PCR. The HA test was performed as described in the earlier section. The supernatants were diluted in two-fold dilutions and tested against chicken erythrocytes for direct HA activity. In order to examine the effects of enzymatic pretreatment on IBV HA activity present in the tissue homogenates, the homogenates were treated with phospholipase-C (Sigma). This was done by incubating 500 μl of tissue supernatants with 2 mL of 1M solution of Tris-HCl buffer (pH 6.5). After mixing, the samples were placed on an ice bath. Phospholipase-C type 1 enzyme was thawed and 35 μl were immediately added to the homogenate supernatant-Tris HCl suspension. The samples were then placed in a water bath at 37°C for two hours. These treated supernatants were

then tested for HA activity in order to see any change from the HA activity observed by using the direct HA test method.

4.3.7 Agar Gel Precipitation Test (AGPT): Agar Gel Precipitation Test (AGPT) used in this study was according to a previously described method (Crowle, 1973). The agar gel was prepared in Petri dishes (See Appendix 3). The wells in the agar gel were cut using a template capable of making seven satellite wells. The agar from the wells was removed by means of a needle and to the central well 30 µl of known IBV antisera (anti-M-41) was added, while in the peripheral wells 30 µl of test antigen (supernatant samples) were added. The plates were incubated at 37°C for 48 h in humidified chamber to avoid drying. The results were recorded after 24 to 48 h by observing the plates against an illuminated light source with a dark background. A white precipitin line between the antigen-antibody wells was considered as a positive result.

4.3.8 Detection of M-41 IBV in tissue homogenates supernatants by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR):

4.3.8.1 RNA Extraction: RT-PCR test was performed on supernatant samples against M-41 IBV strain. For RNA extraction from the homogenate supernatants, the samples (3 mL) were centrifuged at 14000 rpm for 5 min and the supernatants were collected for RNA extraction. To this sample, 200 µl of diethylpyrocarbonate (DEPC)-treated water was added. Then 600 µl of Trizol LS reagent was added, mixed by repeated pipetting and incubated for 5 min at room temperature. At this point, 200 µl of chloroform was added and mixed gently. This mixture was then incubated for 10 min. at room temperature and

then centrifuged at 14000 rpm at 4°C for 5 – 8 min. The supernatant fractions were collected into new micro centrifuge tubes. At this point, 300 µl of isopropyl alcohol were added and the reactions incubated at room temperature for 10 min. The preparation was then centrifuged at 14000 rpm for 10 min. The supernatant was discarded and 500 µl of 70% alcohol was added but not mixed. Tubes were then centrifuged at 14000 rpm for 5-8 min. The supernatant was discarded and the samples were air-dried under hood. The resulting pellets were suspended in 20 µl of DEPC-treated distilled water, incubated at 37°C for 20 min and then kept at –20°C. The extracted RNA was later quantified by spectrophotometer.

4.3.8.2 RT-PCR Protocol: A One-Step RT-PCR protocol was used by employing cMaster™ RT Kit (Eppendorf, International) and following the manufacturer's instructions. Briefly, a master mixture containing the primers, dNTPs, $RT_{plus,}$ PCR buffer and the enzymes was prepared following the manufacturer's instructions and kept on ice. The template RNA was added into each reaction sample individually and the final reaction volume was adjusted with RNase-free water. The reagents were thawed completely before use and kept on ice during working. The mixed reagents were spun down before setting up the reactions. A master mixture was prepared for each reaction depending on the number of samples to be tested (Table 2). The required master mixture 1 was dispensed into each 0.2ml PCR tube kept on ice. The desired volume of master mixture 2 (template RNA, max 10µl/50µl or 5µl/20µl reaction) was added to the master mixture 1 in each PCR tube. The caps were closed immediately and the mixture was spun down briefly to collect the reaction mixture at the bottom of the tubes. The reaction was placed in a thermal cycler.

equilibrated at the appropriate incubation temperature of the RT-PCR reaction step (Table 3) and the desired one step RT-PCR program was initiated. The amplified RT-PCR products were run on 0.8% agarose gel in 1X TBE following Davis and Sambrook (Davis et al., 1994, Sambrook et al., 1989). A total of 12 μl of the PCR product (10 μl of RT-PCR product plus 2 μl of loading dye), molecular weight marker, IBV RNA as positive control, and a normal cell RNA as negative control were run in parallel. The presence of marker and PCR product bands was visualized with a hand held UV light. The gel was documented with a photograph and the size of amplified fragment was compared with the markers.

Table 2: Setting-up of One Step RT-PCR Reactions

Components	50μl reactions	20μl reaction	Final concentration in the reaction
MASTER MIX 1	40 μl	15 μl	-
RNase free water.	Upto 40μl	Upto 15 μl.	
RT plus PCR buffer with Mg ++	5 μl	2 μl	1x: 2.5mM Mg++
DNTP mix(10mM each)	1 μl	0.4 μl	200uM
C-Master RT enzyme	0.5 μl	0.25 μl	0.15U/ μl
C-Master PCR enzyme mix.	0.4-0.5 μl	0.2 μl	0.04-0.05U/ μl
Prime RNase inhibitor solution	0.5 μl	0.2 μl	0.01U/ μl
Forward primer	1 μl	1 μl	200-400nM.
Reverse primer	1 μl	1 μl	200-400nM
MASTER MIX 2	10 μl	5 μl	
Template RNA	1 μl	1 μl	1pg-1 μg of total RNA
RNase free water	Upto 10 μl	Upto 5 μl	

Table 3: Program Parameters for one-step RT-PCR

Cycles	Step	Temperature	Time	Description
1	1	42°C	55mins	Reverse Transcription
1	2	94°C	2mins	Initial Denaturation.
	3	94°C	15secs.	Template Denaturation.
	4	50°C	10-20secs.	Primer Annealing.
30	5	68°C	2mins.	Primer Extension/elongation.
1	6	72°C	2mins.	
1	7	4°C	10mins.	Until usage.

Table 4: Specific M-41 primers used for RT-PCR against IBV

	SEQUENCE (5' – 3')	BASES
IBV-1 (FORWARD)	CATAACTAACATAAGGGCA.	19
IBV-2 (REVERSE)	TGAAAACTGAACAAAAGACA.	20

From: Huang et al., 2004.

4.3.9 In Ovo Passaging of Tissue Homogenate Supernatants for IBV propagation and teratological effect studies:

Tissue homogenate supernatants found positive for M-41 IBV strain in RT-PCR analysis (n = 43) were passaged in embroynated eggs to determined the IBV-associated teratological effects as well as collecting allantoic and amniotic fluid as a source of IBV virus for further viral neutralization studies. For this purpose, 11 days old embroynated eggs were candled to check the viability of the embryos. The position of the embryo and air sac was also marked. The broader end of the eggs was disinfected with 70% ethanol. A hole was drilled in the middle of the eggshell at the broader end. With the help of a syringe fitted with 1 inch, 23-gauge needle, 0.2 ml of the viral inoculum was injected through the hole into chorioallontoic fluid. The hole was sealed with molten candle wax and the eggs were reincubated at 37°C. Eggs were candled after 24 hours post-inoculation and the eggs with dead embryos were discarded.

The eggs were reincubated for 8-9 days after which they were candled and chilled for 2 hours by placing them in refrigerator. The broader end of an egg was wiped with 70% ethanol and with the help of sterile scissors and forceps the shell was cut and removed. The membrane was pierced with the help of a disposable syringe and allantoic and amniotic fluid was collected. This set of passage was termed as Passage # 1. All embryos were opened to examine any teratological effects. The allantoic and amniotic fluids collected from passage # 1 were then inoculated into another set of embroynated eggs similar to the passage #1. This passage scheme was repeated for five times. At each passage, the allantoic and amniotic fluids were collected, and the teratological effects

examined. Any embryonic deformity such as dwarfness, stunting, curling, urates deposits, etc. were recorded on a scale of one "+" to four "++++" with four ++++ being the most severe teratological effect. The allantoic and amniotic fluids were stored at -20°C till further analysis.

4.3.10 Viral Neutralization Studies: Viral neutralization (VN) assay was used to determine the presence of IBV variants in the allantoic and amniotic fluids obtained from embryonic passages of the tissue homogenate supernatants. Only those supernatants were selected for VN studies_which had exhibited teratological changes (13 out of 43). Furthermore, since the starting material for the homogenate supernatants was tissues from clinically IBV suspect chickens which presumably would have multiple IBV variants, therefore, the VN studies were conducted against M-41 as well as against D-274, D-1466 and 4-91 IBV strains. For this purpose, the allantoic and amniotic fluid as a source of IBV was harvested from eggs with highest teratological effects. The viral neutralization of 13 such samples was examined by inoculating 11 days old embryos (five embryos per sample per each of the five, ten-fold dilutions) with varying viral dilutions pretreated with 1:5 diluted serum from various IBV strains (diluted virus , constant serum method). At seven days post inoculation, embryos were harvested and observed for IBV-typical lesions such as stunting, curling and urates. The absence of any teratological effect as an indication of virus neutralization was recorded as "+" sign and with a "-"sign to the contrary for the corresponding IBV variant.

A neutralization index (NI) was calculated by using the Embryo Infective Dose $_{50}$ (EID $_{50}$) procedure. This procedure was used to calculate the titre of infectious agent to the extent that the determined dose could affect 50% of the infected embryos. For this purpose 10-fold dilution of the virus were prepared in PBS (pH 7.0) ranging from 10^{-1} to 10^{-5}. Nine days old embroynated eggs were divided into 5 groups of six eggs each and were inoculated with 0.2ml of relevant dilution via allantoic route. Eggs were placed in an incubator and inspected periodically for embryonic viability. After 48 hours, eggs were harvested and allantoic fluid obtained aseptically and processed. HA was performed to determine viral presence. The number and group of eggs showing infectivity was recorded and EID_{50} was calculated following the method described by Reed and Muench (1939) by using the following data calculations.

- Proportionate distance = % infectivity at dilution next above 50% - 50% / % infectivity at dilution next above 50% - % infectivity at dilution next below 50%

- -ve log of EID $_{50}$ = -ve log of dilution above 50% infectivity + Proportionate distance factor

  = Log of EID $_{50}$ = calculated EID_{50}

(Please see Appendix 4 for additional details). The NI value of $\geq$ 3.0 was considered to be antigenically similar to the corresponding antisera, whereas the NI value of $\leq$ 2.0 was considered serotypically unrelated to the corresponding antisera.

4.3.11 Quantification of IBV Variants Antigenicity

4.3.11.1 Vaccine Preparation: In order to determine if the isolated variants of IBV were immunogenic (6 M-41 positive fluids, 3 D-274 positive, 3 D-1466 positive, and 1 4-91 positive amniotic fluids), the amniotic fluids with similar IBV variant reactivity were

pooled. A whole virus vaccine was then prepared by mixing two mL of each virus sample with 20 μl of 0.5% formaldehyde. The mixture was left at room temperature for 24 h on rotating platform to inactivate the virus. The alum-precipitated vaccine was then prepared by using potassium alum and sodium hydroxide. A 10% solution of potassium alum (6 mL) was mixed with 0.25 N NaOH (13.68 mL) added drop wise while vortexing. The solution was incubated at room temperature for 10 min. After centrifugation at 1000 x g for 10 min the supernatant was removed and discarded. To the pellet, 6 ml of sterilized distilled water was added and pellet was resuspended. The resultant solution (8.4-ml) was divided equally in 6 small test tubes. The tubes were centrifuged at 1000xg for 10 min. Supernatant was discarded. 0.75 ml each of formalin inactivated IBV serotype M-41, D-1466, D-274 and 4-91 was added to pellet in respective tube. Tubes were incubated at room temperature for 20 min. Alum precipitated antigen was stored at $-4^{o}C$ for over night. To confirm that all the viral polypeptides have precipitated with alum and that there is no polypeptide in unbound form, the precipitate solution was centrifuged at 16,700 x g for 10 min and supernatant was tested by AGPT along with control. After 48 hour no precipitation line was formed in test wells while precipitation line was formed between control wells. This confirmed that viral polypeptides had completely bound to alum precipitate and there was no unbound polypeptide left.

4.3.11.2 Vaccine Testing: The prepared alum-precipitated vaccines of various IBV strains were used to immunize 7 days old chicks. These chicks were pre-bled at one day and 7 days of age to assess the baseline anti-IBV titers. On day seven, chicks were randomly assigned to four groups of 30 chicks per group. Immunizations were carried out by

emulsifying the vaccines in incomplete Freund's adjuvant. Each chick was injected with 0.1 mL of the vaccine subcutaneously. The chickens were bled on 7th and 14th day post first injection and blood was collected from wing vein in small tubes. Tubes were kept for 30 minutes at room temperature to allow the blood to clot. The clot was dislodged from the surface of the tubes with fine needle. The tubes were then refrigerated overnight, centrifuged at 800 x g for 10 minutes at 10°C, and the sera was separated and stored at –20°C until used. The booster dose was given to the birds at 28 days of age (14 days post 1st injection). The blood samples were collected on 7th days post boost, sera separated and stored at –20°C till used. All the sera were tested for antibodies against corresponding IBV variant by HI assay.

5. RESULTS

Sero-prevalence of various infectious bronchitis virus variants in commercial layer and broiler flocks was examined and is reported in Table 5. A total of 25 flocks including 16 layer and 9 broiler were included in this study. Layer flocks ranged from 12 – 50 weeks of age whereas the broiler flocks were between the ages of 23 – 48 days. Blood samples collected from 20 chickens randomly selected from each of the 25 chicken flocks were then subjected to sero-analysis using Haemagglutination Inhibition (HI) assay. Infectious Bronchitis Virus (IBV) strain M-41 was found to be most prevalent in which this strain was present in 100% of layer flocks and in about 67% of broiler flocks with an overall combined incidence of 88% in all flocks tested (Table 5 and Figure 7). The incidence of D-274 IBV strain was about 44.0% in layers and 33.3% in broiler flocks with an overall percentage of 40% in all flocks tested (Table 5 and Figure 7). The incidence of IBV D-1466 strain was 50% in layers and 55.5% in broiler flocks with an overall percentage of 52% in all flocks tested (Table 5 and Figure 7). The incidence of IBV 4-91 strain was the lowest in which only 12.5% of the layer flocks tested were found to be sero-positive for strain 4-91. None of the broiler flocks were found to be sero-positive for IBV 4-91 serotype (Table 5 and Figure 7). The overall flocks sero-positively percentages indicated that the IBV strain M-41 had the highest incidence (88%) out of the four serotypes these flocks were tested for. The IBV strains D-274 and D-1466 were of intermediate prevalence in all chicken flocks tested (overall 40% positive for strain D-274 and 52% positive for strain D-1466). The IBV strain 4-91 was found to be of the lowest incidence (8%) amongst all flocks tested (Table 5). The Haemagglutination Inhibition (HI) titres as an indicator of the magnitude of infectious bronchitis virus exposure to these flocks were also recorded

against all four IBV serotypes. The HI titres represented as the Geometric Mean Titres (GMT) for IBV M-41 strain ranged from 65 – 115 in layer flocks and 55 – 110 in broiler flocks tested (Table 5). These titres were therefore very comparable between the layer and broiler flocks. The GMT range for HI titres against D-274 was 70 – 180 in layer flocks and 60 – 160 in broiler flocks (Table 5), apparently a little more intense sero-positivity in layers than in the broilers. The HI titres against D-1466 had the lowest range when compared to the rest of the IBV strains. The layer flocks had a range of 55 – 105 GMT whereas the GMT range for the broiler flocks was 40 – 80 (Table 5). Since none of the broiler chicken flocks were positive for IBV strain 4-91, no GMT was observed. However, the GMT range for the IBV strain 4-91 was 40 – 160 in the layer flocks which were sero-positive for strain 4-91 (Table 5). When comparing the HI GMT range of 40-160 of layers against IBV strain 4-91, it is apparent that some of the strongest HI response (i.e., 160) was observed in the layer flocks against IBV strain 4-91 as compared with the rest of the IBV strains (Table 5). Figure 2 is simply a graphic representation of the incidence of various IBV serotypes in all layer and broiler flocks. The data bars depict that the commercial flocks exhibited highest incidence of sero-positivity against IBV strain M-41 and the lowest incidence of IBV strain 4-91 whereas the sero-positivity against IBV strains D-274 and D-1466 was intermediate for both layer and broiler flocks.

Based on the sero-prevalence data reported in Table 5 and Figure 7 in which IBV strain M-41 was found to be the most prevalent serotype in chicken flocks. the presence of M-41 IBV antigen in lungs and tracheas of IBV suspected chickens were examined. The organs (lung, trachea, kidney and cecal tonsils) from IBV infected or suspected chickens submitted

through the poultry diagnostic lab were cryo-sectioned and subjected to indirect immunofluorescence assay. The anti M-41 (rabbit anti IBV M-41) antibody was employed for antigen detection in these sections. As shown in Table 6, 40% of lungs (60 out of 150 lung samples) from these IBV-suspect chickens were found to be positive for M-41 IBV antigen. On the contrary, a fewer numbers (i.e., 10%) of trachea (15 out of 150) were detected as positive for M-41 IBV antigen. The tissues were considered positive if the intensity of fluorescence in the test sections was specific and/or significantly greater than any observable background fluorescence in the control sections obtained from known healthy chicken tissues. Kidneys and ceacal tonsils were not tested for IBV antigen presence (due to the shortage of reagents) but were used in subsequent studies.

The lungs and trachea exhibiting positive reaction for M-41 antigen as determined by immunofluorescence assay (Fig. 4 & 5) were pooled and homogenized along with kidneys and ceacal tonsils from IBV suspected chickens. The supernatants were then subjected to confirmatory analysis via direct haemagglutination (HA) test and HA after treatment with phospholipase C enzyme. The results are shown in Table 7. Out of a total of 75 IBV-positive samples (as determined by immunofluorescence assay), the homogenates of only 1 sample was detected positive for M-41 antigen in the direct haemagglutination assay. This amounted to be 1.3% positivity. However, after treatment with the phospholipase C the detection limit for the direct haemagglutination assay increased significantly in that 23 out of 75 samples exhibited positive haemagglutination against M-41 antibody (Table 7). Therefore, the overall detection for M-41 antigen in 75 tissue homogenate samples increased from 1.3% to 30.6% (Table 7). Agar gel precipitation test was used as another

indicator of the presence of IBV M-41 antigen in the tissue homogenates as described in the material and methods section. In addition, the reverse transcriptase polymerase chain reaction (RT-PCR) was used as a molecular test to detect the M-41 IBV strain sequences in the tissue homogenates. For RT-PCR assay, the primers selected were representative of the S1 gene specific set of S1OLIGO5' and S1OLIGO3'. As shown in Table 8, only 4 out of 75 samples (5.3%) were found positive against M-41 antibody in the agar gel precipitation assay. However, when tested against M-41 specific primers in the RT-PCR assay, a significantly higher numbers of samples (43 out of 75; 57.3 %) showed a PCR product around 1700 base pair (Table 8). Figure 8 represents an example of RT-PCR positive samples in lanes 1 through 6. Lane 7 represents the positive control in which an M-41 vaccine strain of IBV was amplified using the same IBV primer sets. The lane "M" served as the molecular marker. The position of PCR products for the M-41 IBV strain in homogenate samples is shown by an arrow and was determined to be around 1700 base pairs.

Since tissue samples from 43 chickens were found positive for the presence of M-41 gene sequences with RT-PCR in the crude tissue homogenates, an experiment was conducted to see the effect of these homogenates on developing chicken embryos. This was based on the presumption that the positive RT-PCR product in these samples would be indicative of the presence of M-41 IBV strain. All 43 RT-PCR- positive homogenates were inoculated into 11-days old embryos (15 embryos per homogenate, 3 embryos per passage) via chorioallantoic cavity. The embryos were observed daily over a seven day period post inoculation. These embryos were then opened on days four or seven for any observable

effects on the growth and development of the embryos in response to the homogenate exposure. As indicated in Table 9, the exposed embryos from 13 out of 43 RT-PCR-positive samples exhibited significant teratological effects as evidenced by dwarfing, curling, stunting and urates deposits (Fig 6). The severity of these effects was also recorded upon visual observations. It was clear that as the embryonic passage numbers increased, the effect on embryonic development, such as dwarfing, curling and stunting became more pronounced. i.e., from a scale of one "+" positive to two "++" to three "+++" or to four "++++" positive (Table 9).

The allontoic fluid from embryos showing teratological effects was collected and examined for viral neutralization (VN) activity against various IBV strains (Table 10). Allantoic fluid as a source of IBV was harvested from eggs with highest teratological effects. The viral neutralization of 13 such samples was examined by inoculating 11-days old embryos (five embryos per sample per each of the five, ten-fold dilutions) with varying viral dilutions pretreated with 1:5 diluted serum from various IBV strains (diluted virus , constant serum method). At seven days post-inoculation, embryos were harvested and observed for IBV-typical lesions such as stunting, curling and urates. The signs "+" indicate the number of samples neutralized against the corresponding IBV strain antisera. Neutralization index (NI) was calculated by the method of Reed and Muench (1939) as described in the methods section. The NI value of ≥ 3.0 was considered to be antigenically similar to the corresponding antisera, whereas the NI value of ≤ 2.0 was considered serotypically unrelated to the corresponding antisera.

As expected, virus neutralization effects against M-41 antisera were maximum in which 6 out of 13 samples showed viral neutralization activity (Table 10). Viral neutralization activity against antisera from D-274, D-1466, and 4-91 was observed in 3, 3 and 1 sample, respectively. Virus neutralization index (NI) ranged from 3.6 to 4 for M-41 positive samples, 3.2 to 4 in D-274 positive samples, 3.2 to 3.8 in D-1466 positive samples and 3.6 in 4-91 positive samples. All VN-negative samples had minimal NI values, which ranged from 0.1 to 1.2 across various IBV strains antisera (Table 10).

The last experiment was conducted to see if the IBV strains present in allontoic fluid could be immunogenic if used as a vaccine after viral inactivation. The data are shown in Table 11. Allantoic fluid containing individual IBV strains (M-41 = pool of 6 samples; D-274 = pool of 3 samples; D-1466 = pool of 3 samples; and 4-91= pool of 1 sample) were mixed with 0.5% formalin and incubated for 24 hours at 37°C. At this point, 10% alum was added and allowed to mix by agitation for 2 hours. Broiler chicks were then injected subcutaneously with 0.1 cc per chick and bled at stated days for HI titers. Anti-IBV strain antibody titers were then monitored by using HI technique. The baseline constitutive levels (i.e., maternal) of anti-IBV strains were monitored for back ground purposes. The chickens tested positive against only M-41 strain antigen indicating a minimal level of back ground HI titers. None of the other strains tested positive for maternal antibody titers at day 1 and 7 of age (Table 11). The serum samples collected after 7 and 14 days of first injection (primary response) showed an elevated level of antibody titers against all IBV strains. Comparatively, anti-M-41 response was the highest with a GMT range of 55-68 and 104-274 at 7 and 14 days post-first immunization respectively. Response against D-274, D-

1466 and 4-91 was also appreciable and ranging collectively from 16-64 GMT at 7 days post first injection and from 64 – 119 GMT at 14 days post first injection (Table 11). As expected, the GMT levels against all strains were elevated significantly (as observed numerically since no statistical was applied to the data) when tested on 7 days post booster injection (Table 11). The highest GMT was observed against M-41 antigen with the GMT range of 388-630. Booster response against 4-91 was much lower (i.e, 128-194) as compared to the other two (i.e, D-274 = 168 – 315 GMT and D-1466 = 90.5 – 256 GMT). Taken together, these data clearly show that the IBV strains when used in a formalized vaccine state can induce a reasonable high antibody response through both the primary and secondary immunization phases.

Fig: 2. A photograph of chicken trachea infected with IBV. Tracheal exudates is prominent in the picture along with trachitis.

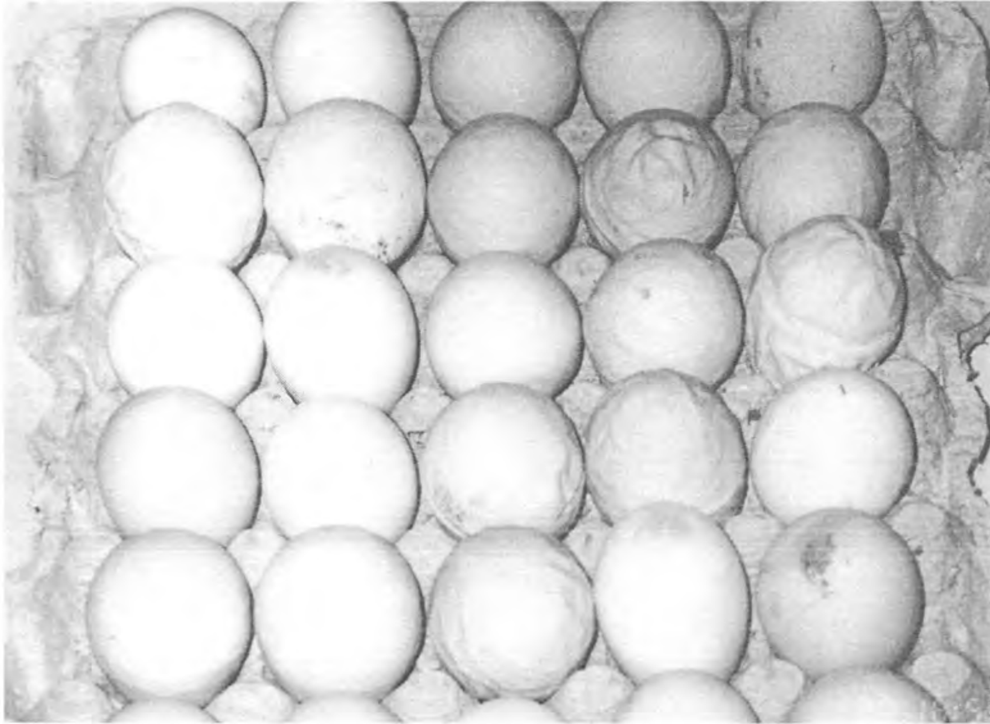


Fig: 3 A. A photograph showing eggs from an IBV infected layer flock. Misshapen eggs with thin shell are a prominent feature.

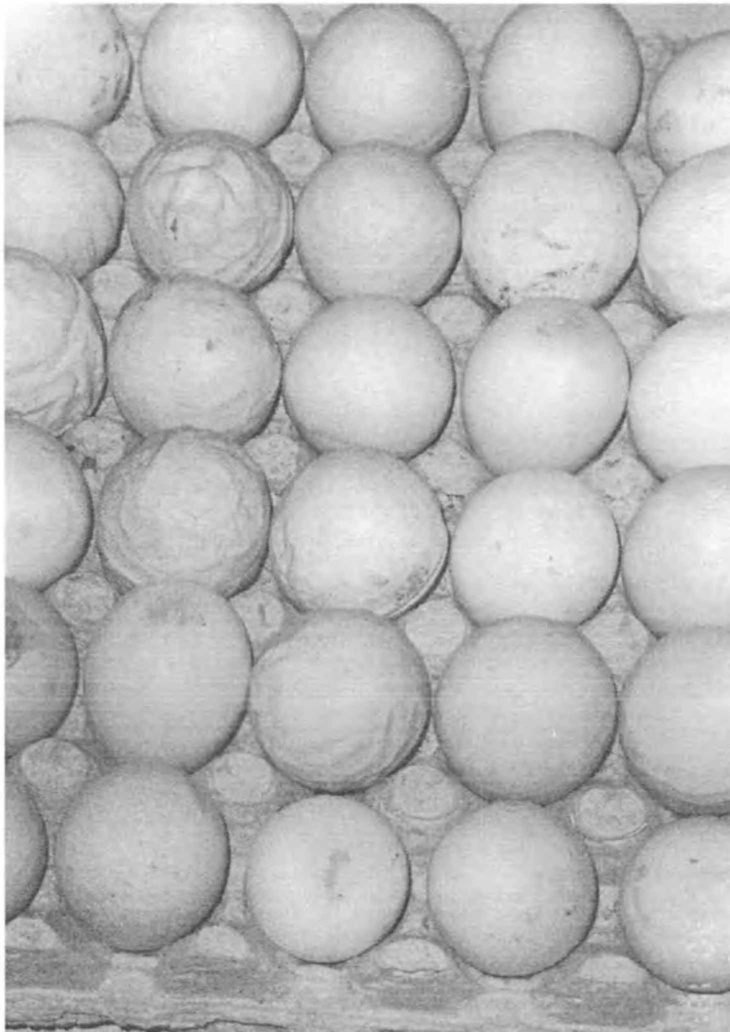


Fig: 3 B. A photograph showing eggs from an IBV infected layer flock. Misshapen eggs with thin shell are a prominent feature.

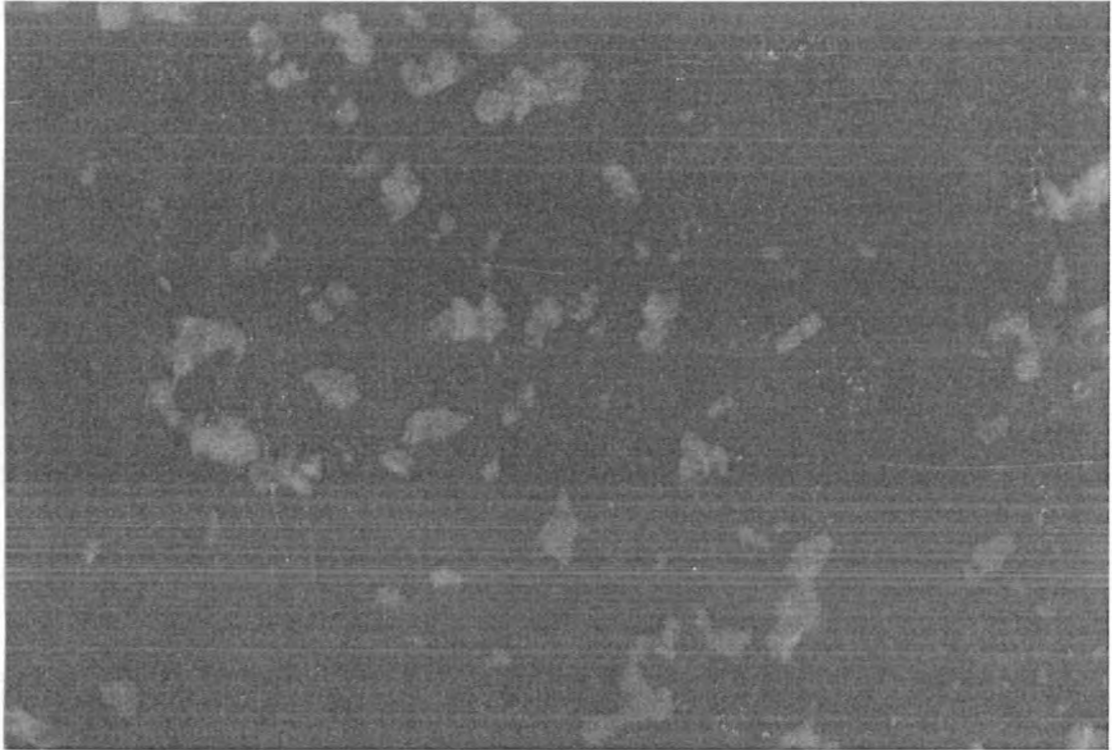
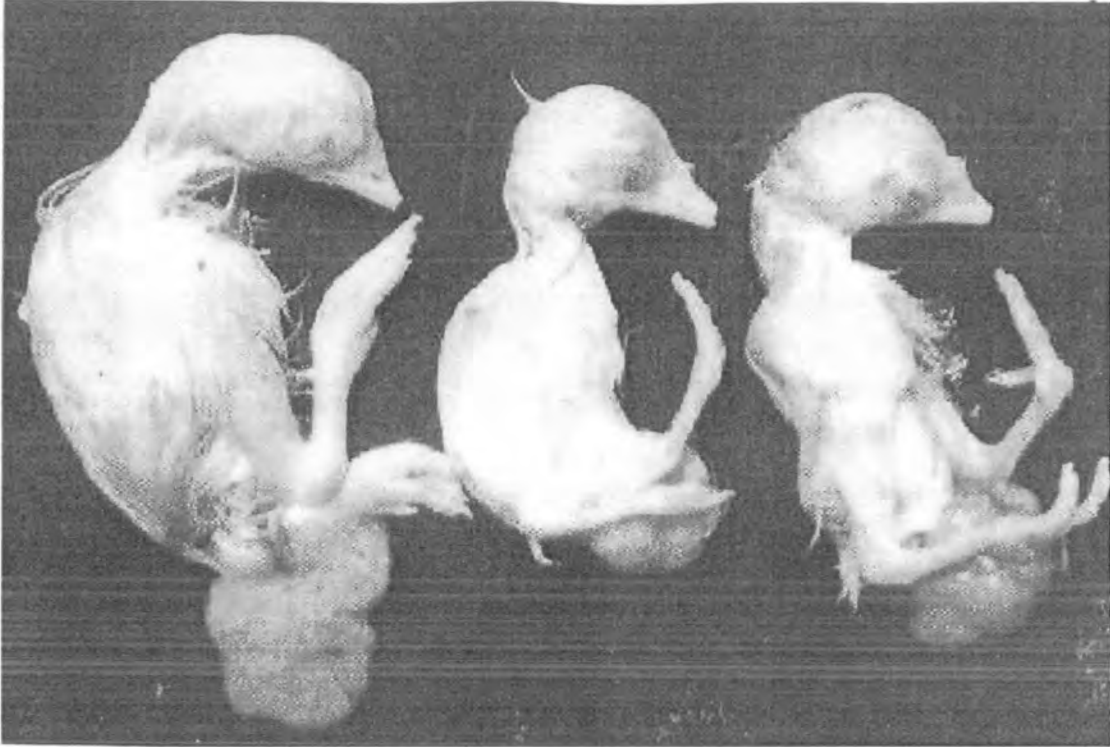


Fig: 4. A photograph showing IFA positive IBV infected chick lungs. Fluorescent cells showing presence of IBV in the tissue (100x).

Fig: 5. A photograph showing IFA positive IBV infected chick trachea. Fluorescent cells showing presence of IBV in the tissue (100x).



NORMAL EMBRYO (1L) IBV INFECTED EMBRYOS (2C, 3R)

Fig: 6. A photograph of a 14 day old chicken embryo infected with IBV in comparison with the normal 14 day old chicken embryo. The infected embryo is showing signs of dwarf ness and curling.

(L = Left, C = Center and R = Right)

Table 5: Seroprevalence of Infectious Bronchitis Virus variants in commercial chicken flocks vaccinated with Massachusetts 41 strain of Infectious Bronchitis Virus. [1]

Strain M- 41			Strain D-274		Strain D-1466		Strain 4-91	
Flocks tested (#)	Flocks Positive (#)	HI Titre[2]	Flocks Positive (#)	HI Titre[2]	Flocks Positive (#)	HI Titre[2]	Flocks Positive #	HI Titre[2]
Layer 16	16 (100)[3]	65-115	7 (43.7)[3]	70-180	8 (50)[3]	55-105	2 (12.5)[3]	40-160
Broiler 9	6 (66.6)[3]	55-110	3 (33.3)[3]	60-160	5 (55.5)[3]	40-80	None (0)[3]	-
25	22 (88)[4]		10 (40)[4]		13 (52)[4]		2 (8)[4]	-

1. Twenty random blood samples from chickens in each of the 25 chicken flocks were collected and the sera tested for the presence of antibodies against the indicated strain antigens by using Haemagglutination Inhibition (HI) assay. Layer flocks ranged from 12 – 50 weeks of age whereas the broiler flocks were between the ages of 23 – 48 days.

2. Values in these columns represent the range of HI titres calculated as the grand Geometric Mean of 320 blood samples for layers and 180 blood samples for broilers (total 500 samples).

3. Values in parenthesis represent percent incidence of a given serotype in the corresponding flock type.

4. These values indicate combined percentage incidence in all 25 flocks tested.

Figure 7: Sero-prevalence of IBV variants in commercial chicken flocks

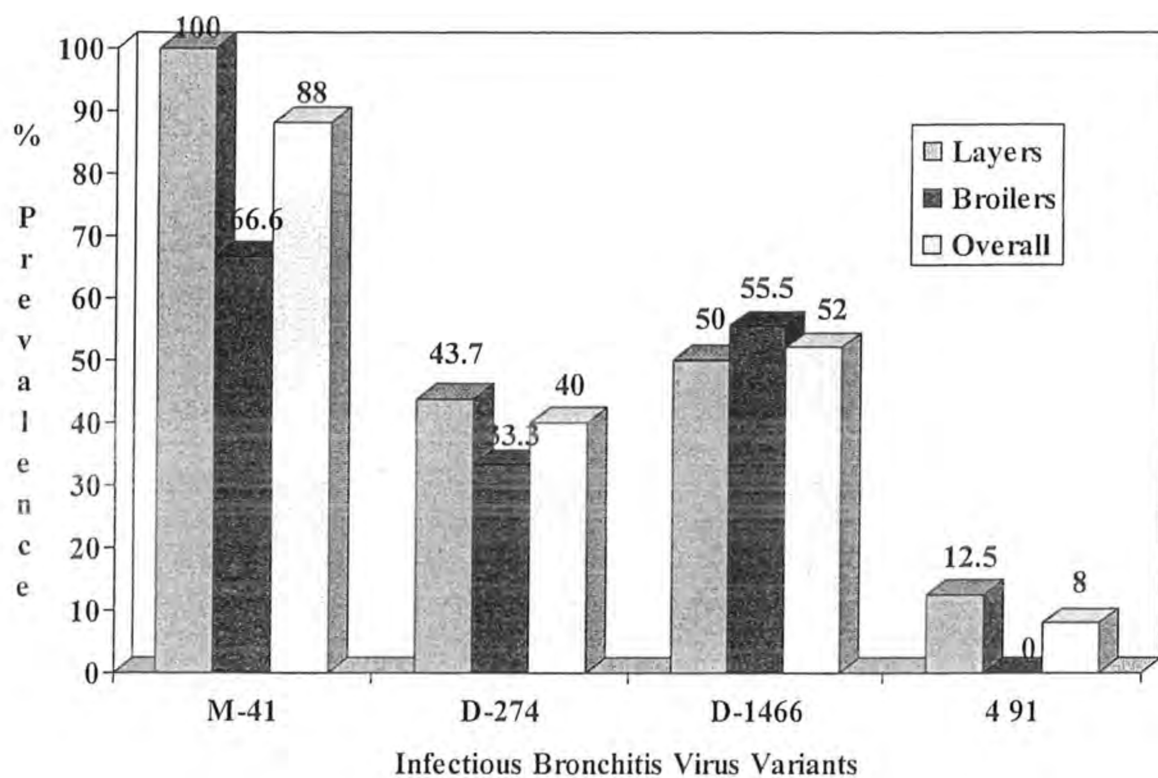


Table 6: The detection of Massachusetts-41 IBV strain in organs from commercial chickens submitted to the diagnostic laboratory as Infectious Bronchitis- suspect cases. [1]

Organs	N	IFA Positive	% Positive
Lung	150	60	40
Trachea	150	15	10
Kidney	150	ND[2]	ND
Cecal Tonsil	150	ND[2]	ND

[1] Internal organs were aseptically removed from IBV infected (suspected) chickens submitted to poultry diagnostic laboratory in Rawalpindi. These organs from 150 individual chickens (broilers and layers combined) were cryo-sectioned and the sections subjected to indirect immunofluorescence assay (IFA) against anti-M41 IBV rabbit antibody.

[2] ND = Not done.

Table 7: The detection of M-41 strain of Infectious Bronchitis Virus in homogenates from organs of IBV positive commercial chickens [1]

Homogenate	DIRECT HA		HA AFTER PHOSPHOLIPASE C TREATMENT	
Lungs, Trachea, Kidney and Cecal Tonsils	+	-	+	-
	1	74	23	52
Total 75 samples	**1.3 %**		**30.6 %**	

[1.] Organs from IBV "positive" chickens (n= 75 as determined by the fluorescence antibody staining) were pooled and homogenized. The homogenate was centrifuges and the supernatant further subjected to confirmatory analysis via direct haemagglutination (HA) test and HA after treatment with phospholipase C enzyme.

Table 8: The detection of M-41 strain of Infectious Bronchitis Virus in homogenates from organs of IBV positive commercial chickens [1]

Homogenate	AGPT		RT-PCR	
Lungs, Trachea, Kidney and Cecal Tonsils	+	-	+	-
	4	71	43	32
Total 75 samples	5.3 %		57.3 %	

[1]. Organs from IBV "positive" chickens (n= 75 as determined by the fluorescence antibody staining) were pooled and homogenized. The homogenate was centrifuges and the supernatant further subjected to confirmatory analysis via agar gel precipitation test (AGPT), and reverse transcriptase polymerase chain reaction (RT-PCR).

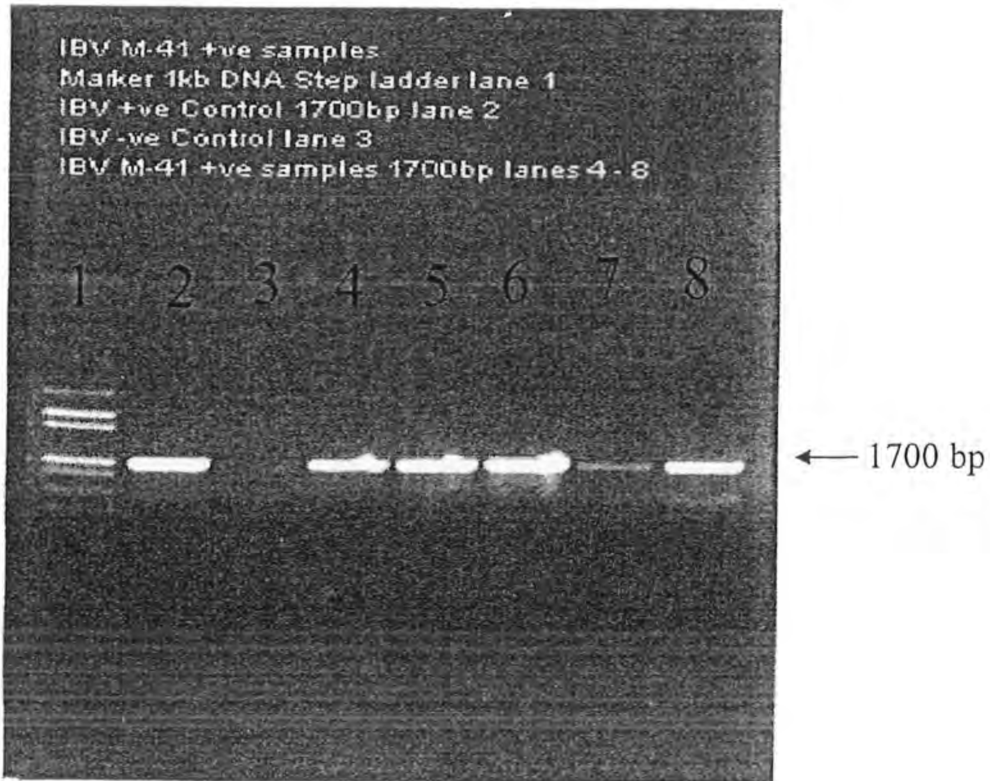


Figure 8: An example of RT-PCR product of S1 gene of the IBV from homogenate samples reported in Table 3. Lane 1 = marker; lane 2 = M-41 vaccine strain IBV used as a positive control; lane 3 used as negative control; lanes 4-8 = homogenate samples. The position of IBV S1 gene product amplified with oligonucleotide primers S1OLIGO5' and S1OLIGO3' is indicated around 1700 base pairs.

Table 9: Egg inoculation and Teratological effects studies of RT-PCR-Positive IBV homogenates: Effects of passage on induction of embryonic effects.[1]

Homogenate Number	Passage #1	Passage#2	Passage#3	Passage#4	Passage#5
Embryonic Development Effects (Dwarfing, Curling, Stunting, and Urates)					
1	+[2]	+	++	+++	++++
2	+	+	++	++	+++
3	-	+	+	+++	+++
4	+	+	++	+++	+++
5	+	+	++	++	++
6	+	+	++	+++	++++
7	+	+	++	+++	+++
8	-	+	+	++	++
9	+	+	++	++	+++
10	+	+	++	+++	++++
11	-	+	++	++	+++
12	+	+	++	+++	+++
13	-	+	+	++	+++

1. The IBV RT-PCR-positive organs homogenate samples (n=43) were inoculated via chorioallantoic cavity into the 11 days old embryos (three embryos, 0.1 cc per embryo per sample per passage). Embryos were observed four to seven days post inoculation for any teratological effects. Only 13 out of 43 RT-PCR-positive samples exhibited IBV-associated teratological effects over various passages as indicated.

2. "+" indicates live embryo with minimal signs of dwarfing relative to controls. "++", "+++" and "++++" indicate gradually progressive teratological effect on subsequent passages. "-" indicates no observable effect.

Table 10: Viral neutralization studies on allantoic fluid from RT-PCR-positive embroynated eggs-passaged samples.[1]

Allantoic fluid from embryos showing +++ to ++++ Teratological Changes (#)	VN against M-41 Antisera		VN against D-274 antisera		VN against D-1466 antisera		VN against 4-91 antisera	
	+	-	+	-	+	-	+	-
13	6	7	3	10	3	10	1	12
Neutralization Index (NI) Range[2]	3.6 to 4.0	0.1 to 1.0	3.2 to 4.0	0.4 to 1.2	3.2 to 3.8	0.2 to 1.1	3.6	0.2 to 1.2

[1.] Allantoic fluid as a source of IBV was harvested from eggs with highest teratological effects. The viral neutralization of 13 such samples was examined by inoculating 11 days old embryos (five embryos per sample per each of the five, ten-fold dilutions) with varying viral dilutions pretreated with 1:5 diluted serum from various IBV strains (diluted virus , constant serum method). At seven days post inoculation, embryos were harvested and observed for IBV-typical lesions such as stunting, curling and urates. The signs "+" indicate the number of samples neutralized against the corresponding IBV strain antisera.

[2.] Neutralization index (NI) was calculated by the method of Reed and Muench as described in the methods section. The NI value of ≥ 3.0 was considered to be antigenically similar to the corresponding antisera, whereas the NI value of ≤ 2.0 was considered serotypically unrelated to the corresponding antisera.

Table 11: Efficacy of formalized vaccine prepared from IBV egg-propagated strains.[1]

Challenge	Testing Days					
	1 d Baseline Age = 1 d	7d Baseline Age = 7d	7d Post 1st Inject. Age = 14d	14d Post 1st Inject. Age = 21d	7d Post boost Age = 28d	n
	Haemagglutination Inhibition Antibody Titers (GMT Log_2 Range)					
M-41	18-26	4.6 – 14.9	55-68	104-274	388-630	30
D- 274	0	0	32-52	64-97	90.5-256	30
D-1466	0	0	52-64	90-119	168-315	30
4-91	0	0	16-52	64-90.5	128-194	30

[1.] Allantoic fluid containing individual IBV strains (M-41 = pool of 6 samples; D-274 = pool of 3 samples; D-1466 = pool of 3 samples; and 4-91= pool of 1 sample) were mixed with 0.5% formalin and incubated for 24 hours at 37C. At this point, 10% alum was added and allowed to mix by agitation for 2 hours. Broiler chicks were then injected subcutaneously with 0.1 cc per chick and bled at stated days for HI titers.

6. DISCUSSION

The present study was conducted to monitor the incidence if infectious bronchitis in commercial chickens in Pakistan. As stated earlier, this disease is of significance economic concern for poultry producers in Pakistan (Muneer et al., 1987) and around the globe (De Wit, 2000). The disease is known to cause severe respiratory distress, as well as reproductive and production losses (poor hatchability, loss of egg production, poor egg quality, etc.). The causative agent of the disease, the infectious bronchitis virus (IBV), is prevalent world-wide and in all countries with an intensive poultry industry, the incidence of infection approaches 100 % in most locations (Ignjatovic and Sapats, 2000). Pakistan is no exception to most viral and bacterial poultry diseases. There is limited evidence in the literature which describes the prevalence of poultry disease in Pakistan. One report describes the analysis of 900 serum samples from 23 flocks in Pakistan and reported an incidence of three strains of IBV (M-41, Arkansas, Connecticut) in Pakistan (Muneer et al., 1987). Therefore, the current study attempted to monitor IBV variants in commercial chicken flocks, with particular emphasis on strains not reported before in Pakistan (i.e. D-274, D-1466 and 4-91). In addition, attempts were also made to develop diagnostic tools as well as a possible vaccine against IBV. The assumption was that such technology development will enable poultry researchers to properly identify and manage this disease in Pakistan.

As is the case with most viruses, the IBV virus also has multiple serotypes. More than 20 serotypes within IBV have been recognized worldwide (Lee and Jackwood, 2000). These new strains arise due to rapid recombination, insertions, deletions or point mutation events,

predominantly in the S1 (the spike protein gene) gene resulting in the generation of Mass-like and Ark-like IBV strains (Wang et al., 1993). The commercial chicken flocks included in our study were vaccinated with the Massachusetts 41 (M-41) IBV strain, a common practice to vaccinate against IBV in Pakistan. This is based on the fact that M-41 strain is known to be most prevalent IBV strain in Pakistan (Muneer et al., 1987). This study also employed serotype identification of IBV field isolates because vaccines are selected based on the serotypes of field isolates in a given geographical area. As shown in Table 5, both layer and broiler flocks exhibited the presence of IBV with most flocks showing seroconversion against M-41. This was expected since the flocks used in the current study had vaccination history against M-41 IBV strain. Surprisingly, not all broiler flocks were positive for M-41. The 3 out of 9 negative M-41 flocks might have been either poor immune responders or perhaps (more than likely) due to vaccine failure. The integrity of vaccine may have been compromised due to temperature fluctuations or quality, both of which are very difficult to control under the given field conditions of poultry production in Pakistan. Furthermore, the integrity of blood/serum samples during transportation from the field to the laboratory may also be a factor of observed negativity in broiler flocks. It is interesting to note that three previously not reported strains of IBV, i.e., D-274, D-1466 and 4-91 in Pakistan were clearly shown to be present, i.e., seropositive, both in layers and broilers as documented in this study. While the conclusive evidence of pathogenicity of these new variants cannot be established, it is assumed that these three IBV variants may also be involved in causing infectious bronchitis in Pakistan. Two additional IBV variants were also detected subsequent to this study. These included D-8888 and D-386 variants. This detection was made on field samples received in our NRLPD laboratory. The GMT

log2 titers ranged from 16 – 128 and 32 – 256 for the D-8888 and for D-386, respectively (data not shown). These observations suggest that there is a possibility of the presence of several other IBV strains in Pakistan. This is perhaps limited only due to the lack of available reagents for sero-detection and sero-typing of IBV in commercial poultry in Pakistan. The detection capabilities are also hindered due to the fact that the poultry production in Pakistan is also not very regulated. While large scale commercial broiler and layer operations are fairly well established, there is no restriction on raising poultry as a back-yard flock. The "domestic" poultry therefore goes completely un-noticed and does not fall under any "formal" or "official" epidemiological surveillance program.

Rabbit antisera prepared against avian M-41infectious bronchitis virus is known to have cross reactive antibodies against other IBV strains such as T, Holte, Connecticut, Beaudette, or H120 (Collins and Alexander, 1987). While absorption of concentrated M-41 with various IBV strains removes almost all cross reactivity, the resulting IBV M-41 antisera still maintains multiple precipitin lines suggesting significant antigen heterogeneity. This may be one of the reasons why several flocks even though vaccinated with M-41 exhibited significant levels of detectable antibodies (as measured by the HI assay) against D-274, D-1466 and 4-91 IBV strains (Table 5). However, it is most likely that the flocks used in this study were also exposed naturally to various IBV variants under commercial management practices. What is surprising is the fact that the levels of antibodies (reported as GMT range; Table 5) against non M-41 strains were also very high. However, none of the flocks under our sero-monitoring study exhibited any IBV-representative clinical signs. It is well recognized that the outbreaks of Infectious

Bronchitis in vaccinated flocks occur quite frequently, perhaps due to inappropriate vaccination or rapid emergence of new strains (Smati et al., 2002).

In addition, the environmental factors (such as heat, mycotoxins, etc.) which can induce significant stress on birds have the potential to make the birds immunosuppressed thereby increasing the chances of viral infections. For example, Miller and Qureshi (1991, 1992a, b) have shown that chicken macrophages exposed to incremental increase in temperature (i.e., 1 – 5 Celsius above the physiological temperature) exhibit the induction of several heat shock proteins (an indicator of a stress response). When macrophages exposed to elevated temperatures were tested for effector functions such as phagocytosis, it was shown that this non-specific first line of defense was down regulated in heat-stressed macrophages (Miller and Qureshi, 1991, 1992 a). While the Fc-mediated phagocytosis which employs the Fc-portion of the antibody molecule coating an opsonized antigenic target was not affected by the heat stress, the phagocytic potential for the unopsonized antigenic targets reverted to normal levels once the heat stress was removed. This suggests that the effect of heat stress may be transient on macrophage functions. Similar effects on macrophage functions were seen *in vivo* when chickens were exposed to elevetad temperature such that their core physiological temperature was elevated above the normal levels. In addition to the phagocytic function changes, Miller and Qureshi (1992b) also reported a reduced tumoricidal activity in macrophages after heat shock. Similarly, mycotoxins such as aflatoxin has been shown to suppress cell-mediated immune response. Dietert et al (1985) showed that chicks hatched after embryonic exposure to aflatoxin-B1 were suppressed in graft vs host response as well as exhibited reduced cutaneous basophilic hypersensitivity

response. Both of these would affect an arm of the immune response (i.e., cell mediated immune response) which is crucial against viral infections. In addition, Neldon-Ortiz and Qureshi (1991) have shown reduced macrophage functions after aflatoxin-B1 exposure where as Qureshi and Hagler (1992) have reported cytotoxicity to macrophages as well as reduced tumoricidal activity by macrophages after exposure to fumonisin-B1. These examples which show that heat stress as well as mycotoxin exposure compromises immune response demonstrate that chickens exposed to these "environmental" factors will be more prone to viral infections.

One aspect of disease outbreaks which is often not paid a great deal of attention is the health status of the birds with regards to the latent viruses such as the chicken infectious anemia virus. These "latent carrier" states can undoubtedly make the birds more prone to infections with respiratory viruses such as IBV or avian influenza, just to name a few. Furthermore, such latent viral exposures which might go undetectable may serve as a basis of immunocompromise leading to secondary infections with bacterial strains (Heggen-Peay et al., 2002). A case in point is the recent outbreak in the 1990's in turkey poults called "poult enteritis and mortality syndrome" or PEMS. This disease caused losses worth millions of dollars in the United States. While a clear etiological agent was not identified, several "novel"enteric viruses such as a turkey variant of an astrovirus and a turkey variant of a reovirus were implicated (Qureshi et al., 2000; Heggen-Peay et al., 2002). These studies also indicated that PEMS infected poults exhibited reduced bursal development, thymic atrophy, altered macrophage-mediated cytokine profile, reduced lymphoproliferative response, and even reduced antibody response. While the novel

PEMS-turkey reo and astro viruses were shown to induce disease, the death was usually observed or enhanced when PEMS-poults were infected with secondary bacterial infections such as with *E. coli*. These reports again suggest that in practical poultry production, while viral infections can have a primary pathological impact, such effects can become worst or even more pronounced in the presence of bacterial infections.

The poultry producers seek assistance for disease diagnosis through a limited network of poultry diagnostic laboratories. Clinically "suspect" birds for IBV are routinely received in these laboratories. We utilized some of these birds to monitor the prevalence of M-41 serotype. This is because M-41 is considered to be the most prevalent IBV strain in commercial chickens. We found that indirect immunofluorescence (IFA) can be of good value to asses the level of IBV involvement and presence in various tissues of the suspect birds. The IFA has been shown to be of value in detecting IBV antigen in infected chicken kidney cells and tracheal smears (Yagyu and Ohata, 1990). In our study we found that IBV antigen can be detected via IFA in lungs and trachea. Not all organs tested were found positive and it was not possible to clearly associate the presence (or absence) of IBV antigen with the observance of clinical signs of infectious bronchitis. We did not test kidneys and caecal tonsils for IBV antigen. The limiting factor in this decision was the limited supply of our IFA reagents. What was interesting though was the fact that the M-41 antigen was much more readily detectable in lungs in which 40% of the samples were found to be positive (Table 6) whereas tracheal samples were only minimally positive (15%). The question to be asked is that is it because the infectious bronchitis virus immediately translocates to the lungs and uses it as a preferential site of replication or these

samples were collected at a stage of infection which was more chronic. One would expect that tracheal tissue will harbor significant amount of the virus by being the source of entry through the upper respiratory tract. Nevertheless, one could argue that with improved diagnostic technologies such as RT-PCR the incidence reported based on detection by non-molecular techniques may be vastly different. This is supported by our own study that only 4 out of 71 samples were found positive for M-41 antigen by using the agar gel precipitation assay (Table 8) where as out of the same samples the positive percentage increased from 5.3% to 57.3% when the RT-PCR technique was used (Table 8).

During the course of this study, we were also interested to see which technique will be most rapid and sensitive for the detection of IBV or its antigen in suspect or clinical samples. There are several commonly used methods for such detection namely HA (Lashagari and Newman, 1984), HAI (King and Hopkins, 1983), AGPT (Lohr, 1980 and 1981) and RT-PCR (Kwon et al., 1993). In our study, we tested the homogenate supernatants from M-41 positive lungs and tracheal samples. Only 1.3% of the samples were positive with direct HA. However, treatment with phospholipase C enzyme increased the detection limit, presumably by exposing (and dissolving the spike protein S1) the proteins responsible for haemagglutination of erythrocytes (Lashagari and Newman, 1984). This increase was from 1.3 % in direct HA to 30.6 % with phospholipase pretreatment. The agar gel precipitation was not very sensitive for detecting IBV from tissue homogenates since only 5.3 % of the samples were detectable. However, the RT-PCR was most sensitive in which 57.3% of the tissue samples exhibited the specific viral product representing IBV against the specific primers used. These experiments clearly indicate that

pretreatment with phospholipase C and then doing an HA or HAI will be most cost effective and practically applicable under the diagnostic conditions in Pakistan. However, if RT-PCR can be established as a routine diagnostic test, it would by far be the most sensitive diagnostic assay.

The only limiting factor is the cost which becomes prohibitive considering the budgetary limitations in Pakistan. Nevertheless, our RT-PCR experiment clearly demonstrates that IBV M-41 strain is readily detectable in homogenate samples from clinically suspect infectious bronchitis virus infected chickens. The uniformity of the RT-PCR product at 1700 bp further indicates that the primer sets used for this experiment were very efficient in detecting the IBV genome. It would be interesting to see the prevalence of other IBV variants in Pakistan using this sensitive RT-PCR technique. While RT-PCR may not be a "field friendly" diagnostic test, it certainly would be a very reliable tool in accurately estimating the epidemiological incidence of infectious bronchitis in Pakistan. While the primer set used for RT-PCR experiment was supposedly M-41 IBV strain specific, there always is a possibility that such a primer set may serve possibly as a "universal" primer set thereby detecting IBV strains other than M-41 as well. It is therefore, imperative, that the findings of the current study be considered in the light of such a cross-reactive possibility. Due to the importance of this disease for the poultry industry in Pakistan as well as the need to establish reliable data on epidemiological, diagnostic and preventive aspects of IB, one recommendation will be to start a "core" research group to investigate all aspects of this disease. This most certainly will involve facilities and resources as well as the

supporting infrastructure to utilize PCR and other modern technologies (i.e., information from organismal genome sequences etc) to tackle important poultry diseases in Pakistan.

One additional test which involves using embryonic model employs the ability of anti-IBV antibodies to neutralize viral effects on embryonic development. It is well known that tissue homogenate samples are not very virulent to embryos upon first supernatant collection (Darbyshire et al., 1975; Yachida et al., 1979). However, when passaged through embroynated eggs, the virulence of the virus as well as the teratological effects increases significantly. Why the virulence of certain viruses changes upon passaging is largely unknown and variable depending upon the type of the virus. For example, influenza viruses are known to undergo mutational changes when adapted through mouse. Brown et al (2001) have shown that a group of 11 mutations can convert an avirulent virus to a virulent variant that can kill at a minimal dose. Thirteen of the 14 amino acid substitutions detected among the clonal isolates were likely instrumental in adaptation because of their positive selection, location in functional regions of the virus, and /or independent occurrence in other virulent influenza viruses. Mutation in virulent variants repeatedly involved nuclear localization signals and sites of protein and RNA interaction, implicating them as novel modulators of virulence. Mouse adapted influenza variants with the same hemagglutinin mutations possessed different pH optima of fusion, indicating that fusion activity of hemagglutinin can be modulated by other viral genes. Therefore, analysis of viral adaptation by serial passage appears to provide the identification of biologically relevant mutations which in the case of the present study seem to have increased the teratological effects (Table 9).

The RT-PCR positive IBV homogenates of this study were therefore passaged through the embroynated eggs and the effect of each passage in embryonic development was recorded. The sequential passages of the IBV containing supernatants most certainly increased the pathogenicity of virus in the supernatants (Table 9). The observed teratological effects included dwarfing, curling, stunting and urates which were similar to as previously reported for IBV (Clark et al., 1972; De Wit, 2000). It is interesting to note that only 13 out of 43 RT-PCR positive samples showed teratological effects. While this may indicate differential pathogenicity of IBV strains (for M-41 strain) for chick embryos, the fact that our starting homogenate material was not one but perhaps a mixture of IBV strains may confound our observations.

Virus neutralization (VN) was used to neutralize the IBV-associated teratological effects By using antisera from all four IBV variants, the effects of IBV variants present in allantoic fluid were neutralized. It is interesting to note that although the homogenate supernatants used in the embryonic inoculation studies were pre-selected as being RT-PCR positive for M-41, the allantoic fluids still showed activity for other IBV strains, namely D-274, D-1466 and 4-91 (Table 10). This is possibly due to the fact that the original homogenate must have more than M-41 infecting serotypes present in the original tissues. Furthermore, there is evidence (as stated earlier) of cross-reactivity of strain M-41 antigens with other IBV serotypes (Collins and Alexander, 1987). Infectious bronchitis viruses are well known for their cross reactivity which might be the reason as to why infectious bronchitis is not as critical as the avian influenza in terms of disease outbreaks linked to spontaneously arising mutational variants almost on daily basis. For example, the "H" strain of infectious

bronchitis was one of the earliest live attenuated IBV vaccine to be developed and has continued to be used in most parts of the world for almost 20 years (Bijlenga et al., 2004). This vaccine has been popular because of its ability to provide heterologous cross-protection against a number of IBV viruses of different serotypes and has proven to be one of the most widely used live attenuated IBV vaccines. In fact, the H120 vaccine is possibly the most widely used live attenuated IBV vaccine globally to this date (Bijlenga et al., 2004). However, the use of live attenuated vaccines has declined significantly over the years, especially for the Infectious bronchitis dues to the availability of more safe and highly efficacious inactivated IBV vaccine.

Nevertheless, the VN test as reported previously by other investigators (Cowen and Hitchner, 1975; Wooley et al., 1976) was considered as a reliable test to monitor for the presence of IBV strains in the embryonic fluids or tissue homogenates. As shown in Table 10, the allantoic fluid selected for VN activity monitoring was collected from embryos showing the highest levels (+++ or ++++) of teratological changes. Although all samples were pretreated with equal volume and dilution of antibodies representing various IBV serotypes, yet, the VN effect observed was quite variable, i.e., not all samples were neutralized effectively despite pretreatment with the corresponding antibody (Table 10). The possible reasons for such differential viral neutralizations could be many. For example, the IBV strains present in the selected allantoic fluid samples may indeed have variable virulence although they all had the highest levels of observable teratological effects. Another possibility could be the affinity or the avidity of the antibody used in the neutralization experiment against the corresponding IBV variant.

The last series of experiments in this study involved preparation of a formalized vaccine by using various IBV strains obtained from embryonic fluids. Chicks challenged with these variants vaccines seroconverted very well. The response was much pronounced upon booster injection. It is interesting to note that the strain M-41 vaccine exhibited the highest titers in challenged chicks (Table 11). However, the HI titers were quite reasonable for D-274, D-1466 and 4-91 as well. It must be emphasized that the technique employed in preparing the formalized vaccine as well as the resulting vaccine trials employed very minimal numbers of samples as well as a rather crude protocol. What is impressive though is the fact that the IBV variants present in the allantoic fluid were indeed inactivated with this formalin treatment and the product worked exactly the way a putative vaccine should. This is evident by the kinetics of the antibody response as shown in Table 11. The sampling done after 7 days of the first injection was indicative of a true inductive phase response with the HI titers ranging from 16-68 across all IBV variants. By day 14 after the first injection the HI titers went up and were higher than the day 7 post immunization titers. However, after second or booster injection the antibody response was truly indicative of a "secondary" antibody response. This suggests that all formalized vaccine preparations were able to induce "immunological memory" as evident from an enhanced secondary antibody response against all variants. This is encouraging because no matter how crude or experimental the methodology might have been, these pilot studies indicate that one can certainly up-scale these efforts for a larger scale vaccine production and testing, perhaps at a filed level. Based on differential Hi response one can speculate that some IBV strains or isolates simply are better at inducing an immune response. Although we have measured only the antibody-based response, it is known that a strong cell mediated immune response

may be important in cross protection because cross-reactive cytotoxic T lymphocytes can be found after immunization of chickens with a DNA vaccine against IBV (Seo et al., 1997). Furthermore, the route of vaccination may also be a factor. Infectious bronchitis vaccine is also administered via a coarse spray or in drinking water. For example, Jackwwod et al., (2003) have shown that regardless of the mechanism of protection, an in-house prepared IBV vaccine "70 GA98" when given at 1 day of age by coarse spray and at 14 days of age in the drinking water, can protect against a homologous GA98 challenge as well as provide good protection against related IBV viruses. We could not perform the IBV challenge in chicks immunized with our formalin-killed IBV variant vaccines. This needs to be done to further verify that the induced immune response is indeed a "protective" immune response. Nevertheless, previous studies have shown that vaccines prepared in similar way induce significant protection in challenged chicks regardless of the IBV variant used (Winterfield et al., 1978; Box et al., 1988; Gough et al., 1981).

In conclusion, the current study presents evidence that IBV variants are prevalent in various meat and egg-type commercial chickens in Pakistan. The incidence of the disease is not well documented since there is no such mechanism at the national level. This might present a huge challenge in itself since the poultry industry in Pakistan is not well integrated. In addition to a select few operation, which can be considered "large-scale", there are numerous "family" or "back-yard" poultry production operations, which never enter into any statistics. Furthermore, the animal health, disease surveillance, guidelines for vaccination etc are either no mandated or nor well overseen. This may present even a larger concern considering the recent outbreaks of avian influenza world wide. It must be

realized, based on the flue example, that animal health is directly linked to the human health as well. There indeed is a potential that viruses such as avian influenza and even infectious bronchitis which express intrinsic tendency of mutations can cross the "species barriers" and infect human populations. Nor are the economic effects of infectious bronchitis are well documented. It would be nice if this can be done by using a well defined longitudinal epidemiological study over a longer period of time. One factor which may ensure a reduction in the incidence of IBV perhaps could be strict enforcement of vaccination of all poultry flocks in Pakistan. Along with vaccination, it is imperative to continuously monitor the existence of various serotypes so that the vaccines used can be customized depending upon the prevalence of particular serotypes in a given geographical area. There is an increasingly tendency by the poultry and livestock producers to "import" vaccines from other countries for use in Pakistan. While this may work in cases where the antigenic stability of the vaccine strain is unquestionable, one would expect that the prevalence of a particular disease causing strain may be variable across the world or even within one particular country or geographical area. One recommendation could be to initiate a massive effort to isolate and catalogue the viral (and even bacterial) agents which are implicated in various live stock or poultry disease in Pakistan. One can certainly build a program on the lines of a "type-culture" collection as being done in several other countries.

The current study has certainly identified and utilized several techniques which will be helpful in seromonitoring and disease diagnosis. Although IBV vaccines are very efficacious, the best countermeasure against IBV or for that matter any other poultry disease is the implementation of strict biosecurity measures. The application of various

commercial anti-viral disinfectants has been found to be useful (personal observation). In addition simple commonsense measures such as limit people movement, avoid visitors, controlling site traffic to a minimum, spray vehicles especially wheels, use of appropriate clothing and boots, use of foot dips and hand washing facilities, etc. can most certainly assist in disease prevention.

Based on the study presented here, the following could serve as "broad" recommendations:

1. While certain serotypes of the infectious bronchitis virus have been identified in this study, a detailed survey should be done to document all possible variants of infectious bronchitis virus in Pakistan.

2. Once isolated and identified, the infectious bronchitis variants should be tested for their antigenicity and pathogenicity. This information will be helpful in deciding and selecting the possible infectious bronchitis strain to be used as a vaccine strain in Pakistan. It may even be necessary to use a "multi-strain" vaccine but this can only be determined if a clear picture emerges regarding the prevalence of pathogenic infectious bronchitis variants in poultry in Pakistan.

3. Strict implementation of biosecurity protocols be carried out both for the commercial as well as for the domestic poultry in Pakistan. For this to happen, the prerequisite would be to register or document such poultry operations in Pakistan. While it is not easy to

"monitor" or "enforce" such guidance or regulations, one could at least expect that such regulations do exist and the farmers are "expected" to comply with such regulations.

4. General improvements in allied parameters which are required for efficient poultry production must be made. For example, it is necessary to have adequately trained poultry veterinarians who can assist in providing expert services in both preventive and treatment phases of animal/poultry health and disease. One aspect of poultry production which is often assumed to be without any problems is the poultry feed. It is well known that poultry feed which is contaminated with mycotoxins can not only affect the performance characteristics (such as growth) but can severely compromise the integrity of the immune system. In fact, there are reports in the literature that even the progeny chicks hatched from breeders which have consumed a mycotoxin-adulterated diet are severely immunocompromised upon hatch and soon thereafter. Such situations will make the chicks more susceptible not only to the viral disease agents encountered soon after hatch but also to subsequent bacterial or secondary infections. Therefore, strict criteria of feed testing be established and enforced to avoid mycotoxin-based problems for animal health.

5. A "standardized" panel of diagnostic test must be developed and used for monitoring the infectious bronchitis in poultry. While classical tests such as haemagglutination inhibition, virus neutralization, etc. work very well and are relatively simple to conduct, they may not be very sensitive to distinguish amongst various genetics-based serotypes of the virus. Therefore, it is necessary to adapt molecular tests such as RT-PCR as tested and used in this study.

6. Poultry extension veterinarians be encouraged to develop a national "road-map" for poultry diseases surveillance and monitoring. As a starting point, it is important to organize stakeholder meetings and workshops to identify the issues related to poultry health in Pakistan. The "stakeholders" including the producers, animal health personals as well as the government authorities and regulators should all be on board for such efforts to materialize. It is extremely important to have a clear understanding and vision of where the poultry industry is in Pakistan. One must identify the situation, opportunities, and associated threats to this important agriculturally important economic commodity. The recent surge in avian influenza at a global level may have tremendous impact on poultry industry practices in Pakistan. Furthermore, the avian influenza is one of several examples which have a potential of being an animal-to-human transfer. The diseases such as bronchitis are quite common in humans and animals alike. It may not be too far fetched to consider infectious bronchitis virus also on the same lines as a possible human threat just like the avian influenza. The similarities between these two viruses include multiple strains and high rate of mutation. Therefore, in order for the Pakistan poultry industry to be able to meet the local demands as well as to be a possible export player at the regional or local level, it is very important that this industry has a clear bill of health to compete. The "road-map" can identify such issues and priorities.

7. REFERENCES

1. Afanadaor, G. and Roberts, J. R., 1994. Effect of nephropathogenic infectious bronchitis virus on renal function in male broiler chickens. Br. Poul. Sci., 35: 445-456.
2. Albassam, M.A., Winterfield, R.W. and Thacker, H. L., 1986. Comparison of the nephropathogenicity of four strains of infectious bronchitis virus. Avian Dis., 30:468-476.
3. Alexander, D.J., Gough, R.E. and Pattison, M., 1978. A long-term study of the pathogenesis of infection of fowls with three strains of avian infectious bronchitis virus. Res. Vet. Sci., 24:227-233.
4. Ambali, A.G. and Jones, R.C., 1990. Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. Avian Dis., 34:809-817.
5. Anderson, J. R., Jackwood, M.W. and Hilt, D.A., 1991. Polymerase chain reaction amplification of the genome of infectious bronchitis virus. Avian Dis., 35:216-220.
6. Beach. J. R., and Schalm, O.W., 1936. A filterable virus, distinct from that of laryngotracheitis, the cause of a respiratory disease of chicks. Poult. Sci., 15:199-206.
7. Beard, C.W., 1970. Demonstration of type specific antibody in mammalian and avian sera by immunodiffusion. Bull. World Health. Org., 42: 779-785.
8. Beaudette, F.R. and Hudson, S.B.,1937. Cultivation of the virus of infectious bronchitis. J. Amer. Vet. Med. Assoc., 90:51-58.
9. Bijlenga, G., Cook, J. K. A., Gelb, J. and de Wit, J. J., 2004. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. Avian Pathol., 33:550-557.
10. Bisgaard M., 1976. Influence of infectious bronchitis virus on egg production, fertility, hatchability and mortality rate in chickens. Nord. Vet. Med., 28:368-376.

11. Boots, A. M. H., Benaissa-Trouw, B.J., Hesselink, W., Rijke, E., Schrier, C. and Henson, E.J., 1992. Introduction of antiviral immune responses by immunization with recombinant DNA encoded avian coronavirus nucleocapsid protein. Vaccine., 10:119-124.

12. Boots, A.M.H., Kusters, J.G., Van Noort, J.M., Zwaagstra, K.A., Rijke, E., Van der Zeijst, B.A.M. and Henson, E.J., 1991. Localization of a T-cell epitope within the nucleocapsid protein of the avian corona virus. Immunology., 74: 8-13.

13. Boursnell, T.P., Brown, T.D., Foulds, I.J., Green, P.F., Tomley, F.M. and Binns, M.M., 1987. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J. Gen. Virol., 68:57-77.

14. Box, P.G., Holmes, H.C., Finney, P.M. and Froymann, R., 1988. Infectious bronchitis in laying hens: the relationship between haemagglutination inhibition antibody levels and resistance to experimental challenge. Avian Pathol., 17: 349-361.

15. Brown, E. G., Liu, H., Kit, L. C. and Nesrallah, M., 2001. Pattern of mutation in the genome of influenza A virus on adaptation to increased virulence in the mouse lung: identification of functional themes. Proc. Natl. Acad. Sci., 98:6883-6888.

16. Bushnell, L. D. and Brandly, C.A., 1933. Laryngotracheitis in chicks. Poult. Sci., 12:55-60.

17. Callison, S.A., Jackwood, M.W. and Hilt, D.A., 1999. Infectious bronchitis virus S2 gene variability may affect S1 subunit specific antibody binding. Virus Genes., 19:143-151.

18. Capua, I., Gough, R. E., Mancini, M., Casaccia, C. and Weiss, C., 1994. A novel infectious bronchitis strain-infecting broiler chickens in Italy. J. Vet. Med. Sr.B., 41:83-89.

19. Cavanagh, D. and Naqi, S. A., 1997. Infectious bronchitis. In diseases of Poultry 10th Edition, pp 511-526. Ames, Iowa, USA: Iowa State University Press.

20. Cavanagh, D., 1983a. Coronavirus IBV glycoproteins: size of their polypeptide moieties and nature of their oligosaccharides. J. Gen. Virol., 64:1187-1191.

21. Cavanagh, D., 1983b. Coronavirus IBV: further evidence that the surface projections are associated with two glycoproteins. J. Gen. Virol., 64: 1787-1791.

22. Cavanagh, D. and Davis, P. J., 1987. Coronavirus IBV: relationship among recent European isolates studied by limited proteolysis of the virion glycopolypeptides. Avian Pathol., 16: 1-13.

23. Cavanagh, D. and Davis, P. J., 1988. Evolution of avian coronavirus IBV: sequence of the matrix glycoprotein gene and intergenic regions of several serotypes. J. Gen. Virol., 69:621-629.

24. Cavanagh, D. and Davis, P. J., 1992. Sequence analysis of strains of avian infectious bronchitis coronavirus isolated during the 1960s in the U.K. Arch. Virol., 130:471-476.

25. Cavanagh, D. and Naqi, S. A., 1997. Infectious Bronchitis. In: Calnek, B. W. Barnes, H. J., Beard, C. W., McDougald, L. R., and Saif, Y. M. (eds) "Diseases of Poultry". Iowa State University Press, Ames, IA. Pp 511-526.

26. Cavanagh, D., Darbyshire, J.H., Davis, P. and Peters, R. W., 1984. Induction of humoral neutralizing and haemagglutination-inhibiting antibody by the spike protein of infectious bronchitis virus. Avian Pathol., 13: 573-583.

27. Cavanagh, D., Davis, P. J. and Cook, J.K.A., 1988. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res., 11: 141-150.

28. Cavanagh, D., Davis, P. J. and Cook, J.K.A., 1992b. Infectious bronchitis virus: Evidence for recombination within Massachusetts serotype. Avian Pathlo., 21: 401-408.

29. Cavanagh, D., Davis, P. J., Cook, J.K.A., Li, D., Kant, A. and Koch, G., 1992a. Location of amino acid differences in the spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. Avian Pathol., 21: 33-43.

30. Cavanagh, D., Davis, P. J., Darbyshire, J.H. and Peters, R. W., 1986. Coronavirus IBV: virus retaining spike glycopeptide S2 but not S1 is unable to induce virus neutralizing or haemagglutination inhibiting antibody or induce chicken tracheal protection. J. Gen. Virol., 67: 1435-1442.

31. Cavanagh, D., Mawditt, K., Adzhar, A., Gough, R.E., Picault, J.P., Naylor, C.J., Haydon, D., Shaw, K. and Britton, P., 1998. Does IBV change slowly despite the capacity of the spike protein to vary greatly? Adv. Exp. Med. Biol., 440: 729-734.

32. Chubb, R. C., 1986. The detection of antibody to avian infectious bronchitis virus by the use of immunofluorescence with tissue sections of nephritic kidneys. Aust. Vet. J., 63:131-132.

33. Clarke, J.K., McFerran, J.B. and Gay, F.W., 1972. Use of allantoic cells for the detection of avian infectious bronchitis virus .Archiv. fur die Gesamte Virusforschung., 36:62-72..

34. Collins, M.S. and Alexander, D.J., 1987. Strain specific antibodies revealed by immuno absorption studies with avian infectious bronchitis virus. Vet. Res. Commun., 11(2): 109-118.

35. Cook, J. K. A., 1971. Recovery of infectious bronchitis virus from eggs and chicks produced by experimentally inoculated hens. Jr. of Comp. Path., 81:203-211.

36. Cook, J. K. A., 1984. The classification of new serotypes of infectious bronchitis virus isolated from poultry flocks in Britain between 1981 and 1983. Avian Path., 13:733-741.

37. Cook, J. K. A., Huggins, M. B. and Ellis, M. M., 1991. Use of an infectious bronchitis virus and *E. coli* model infection to assess the ability to vaccinate successfully against infectious bronchitis in the presence of maternally derived immunity. Avian Pathol., 20:619-626.

38. Cook, J.K.A. and Huggins, M.B., 1986. Newly isolated serotypes of infectious bronchitis virus. Their role in disease. Avian Pathol., 15:129-138.

39. Cook, J.K.A., Orbell, S.J., Woods, M.A. and Huggins, H.B., 1999. Breadth of protection of the respiratory tract provided by different live attenuated infectious bronchitis vaccines against challenge with IBV of heterologous serotypes. Avian Pathol., 28: 477-485.

40. Cook, J.K.A., Smith, H.W. and Huggins, M.B., 1986. Infectious bronchitis immunity: Its study in chickens experimentally infected with mixture of infectious bronchitis virus and *E. coli*. J. Gen Virol., 67:1427-1434.

41. Cowen, B.S. and Hitchner, S.B., 1975. Serotyping of avian infectious bronchitis virus by the virus neutralization test. Avian Dis., 19: 583-595.

42. Cowen, B.S., Wideman, R.F.Jr., Braune, M.O. and Owen, R.L., 1987. An infectious bronchitis virus isolated from chickens experiencing a urolithiasis outbreak. I. In vitro characterization studies. Avian Dis., 31:878-883.

43. Crinion, R.A.P., 1972. Egg quality and production following infectious bronchitis virus exposure at one day. Poul. Sci., 51:582-585.

44. Crowle, A.J., 1973. Immunodiffusion. Academic Press, New York, NY.

45. Cumming, R. B., 1970. Studies on Australian infectious bronchitis virus IV. Apparent farm-to-farm airborne transmission of infectious bronchitis virus. Avian Dis., 14:191-195.

46. Cumming., R.B., 1963. Infectious avian nephrosis (uraemia) in Australia. Aust. Vet. J., 45:200-203.

47. Cunningham, C.H., 1970. Avian infectious bronchitis. Adv. Vet. Sci. Comp. Med., 14: 105-148.

48. Darbyshire, J. H. and Peters, R.W., 1985. Humoral antibody response and assessment of protection following primary vaccination of chicks with maternally derived antibody against avian infectious bronchitis virus. Res. In Vet. Sci., 38:14-21.

49. Darbyshire, J.H., 1980. Assessment of cross immunity in chickens to strains of avian infectious bronchitis virus using tracheal organ cultures. Avain Pathol., 9: 179-184.

50. Darbyshire, J.H., Cook, J.K.A. and Peters, R.W., 1975. Comparative growth kinetic studies on avian infectious bronchitis virus in different system. Jr. of Comp. Pathol., 85: 623-630.

51. Davelaar, F. G. and Kouwenhoven, B., 1977. Influence of maternal antibodies on vaccination of chicks of different ages against infectious bronchitis virus. Avian Pathol., 6:41-50.

52. Davies, H. A. and Macnaughton, M. R., 1979. Comparison of the morphology of three corona viruses. Arch Virol., 59:25-33.

53. Davies, L.G., Kuehl, W.M. and Batty, J.F., 1994. Basic methods in Molecular biology. In: Appelton and Long (Eds). East Norwak Connect., Pp 159-161.

54. De Wit, J. J., 2000. Detection of infectious bronchitis virus. Avian Pathol., 29:71-93.

55. Delpaplane, J. P. and Stuart, H.O., 1939. Studies of infectious bronchitis. Rhode Island Agricultural Experimental Station, RI. Bulletin., 273.

56. Dhinakar Raj, G. and Jones, R.C., 1996. Immunopathogenesis of infection in SPF chicks and commercial broilers of a variant infectious bronchitis virus of economic importance. Avian Path., 25:481-501.

57. Dhinakar, R. and Jones, R.C., 1997b. Cross reactive cellular immune responses in chickens vaccinated with live infectious bronchitis vaccine. Avian Pathol., 26:641-649.

58. Dietert, R. R., Qureshi, M. A., Nanna, U. C. and Bloom, S. E., 1985. Embryonic exposure to aflatoxin-B1: Mutagenicity and influence on development and immunity. Environ. Mutagenesis., 7:715-725.

59. Farooq, M., Milan, M. A., Durrani, F. R. and Syed, M., 2002. Prevalent diseases and mortality in egg type layers under subtropical environment. Livestock Research for Rural Develop., 14: 1-8.

60. Fodor, I., Horvath, E., Fodor, N., Nagy, E., Rencendorsh, A., Vakharia, V.N. and Dube, S.K., 1999. Induction of protective immunity in chickens immunized with plasmid DNA encoding infectious bursal disease virus antigens. Acta. Vet. Hung., 47:481-492.

61. Fulton, R.M., Reed, M. and Thacker, H.L., 1993. Cellular response of the respiratory tract of chickens to infection with Massachusetts 41 and Australian T infectious bronchitis viruses. Avian Dis., 37:951-960.

62. Gelb, J. Jr., Rosenberger, J.K., Fries, P.A., Cloud S.S., Odor, E.M., Dohms, J.E.and Jaeger, J.S., 1989. Protection afforded infectious bronchitis virus-vaccinated sentinal chickens raised in a commercial environment. Avian Dis., 33:764-769.

63. Gelb, J.Jr., Lernt, R.L., Metz, A.L. and Fries, P.A., 1991b. Attenuation of avian infectious bronchitis virus by cold adaptation. Avian Dis., 35:847-853.

64. Gelb. J.Jr., Wolff. J.B. and Moron, C.A., 1991a. Variant serotypes of IBV isolated from commercial layers and broiler chickens. Avian Dis., 35:82-87.

65. Gough, R.E. and Alexander, D.J., 1977. Comparison of serological tests for the measurement of the primary immune response to avian infectious bronchitis vaccines. Vet. Microbiol., 2:289-301.

66. Gough, R.E., Randall, C.J., Dagless, M., Alexander, D.J., Cox, W.J. and Pearson, D., 1992. A new strain of infectious bronchitis virus infecting domestic fowl in Great Britain. Vet. Rec.,130:493-494.

67. Gough, R.E., Wyeth, P.J., and Bracewell, C.D., 1981. Immune response of breeding chickens to trivalent oil emulsion vaccines: responses to infectious bronchitis. Vet. Rec., 108(5): 99-101.

68. Hawkes, R.A., Darbyshire. J.H., Peters, R.W., Mockett. A.P.A. and Cavanagh, D., 1983. Presence of viral antigens and antibody in the trachea of chickens infected with avian infectious bronchitis virus. Avian Pathol., 12:331-340.

69. Heggen-Peay, C. L., Qureshi, M. A., Edens, F. W., Sherry, B., Wakenell, P. S., O'Connell, P. H. and Schat, K. A., 2002. Isolation of a reovirus from poult enteritis and mortality syndrome and its pathogenicity in turkey poults. Avian Dis., 46: 32-47.

70. Hofstad, M.S., 1981. Cross immunity in chickens using seven isolates of avian infectious bronchitis virus. Avian Dis., 25: 650-654.

71. Hoopkins, S.R. and Beard, H. W., 1985. Studies on methods for determining the efficacy of oil emulsion vaccines against infectious bronchitis virus. J Am Vet Med Assoc., 187:305.

72. Hopkins, S.R. and Yoder, H.W. Jr., 1986. Reversion to virulence of chicken passaged infectious bronchitis virus. Avian Dis., 30:221-223.

73. Huang, Y-P., Lee, H-C., Cheng, M-C. and Wang, C-H., 2004. S1 and N gene analysis of avian infectious bronchitis viruses in Taiwan. Avian Dis., 48: 581-589.

74. Ignjatovic, J. and Galli, L., 1995. Immune responses to structural proteins of avian infectious bronchitis virus. Avian Pathol., 24:313-332.

75. Ignjatovic, J. and Galli, L., 1994. The S1 glycoprotein but not the N or M protein of avian infectious bronchitis virus induces protection in vaccinated chickens. Archives of Virol., 138:117-134.

76. Ignjatovic, J. and Sapats, S., 2000. Avian infectious bronchitis virus. Rev. Sci. Tech., 19(2): 493-508.

77. Ignjatovic, J., Ashton, D.F., Reece, R., Scott, P. and Hooper, P., 2002. Pathogenicity of Australian strains of avian infectious bronchitis virus. J. Comp Pathol., 126:115-123.

78. Jackwood, M. W., Hilt, D. A. and Brown, T. P., 2003. Attenuation, safety, and efficacy of an infectious bronchitis virus GA98 serotype vaccine. Avian Dis., 47:627-632.

79. Javed, T., Siddique, M. and Hameed, A., 1991. Persistence and morpho-pathological studies on infectious bronchitis in chickens in Pakistan. Assiut Vet. Medical Jour., 25:216-228.

80. Jia, W., Karaca, K., Parrish, C.R. and Naqi, S.A., 1993. Sequences of the spike protein gene of IBV strains Arkansas 99 (Genbank Accession Number L 10384) and CU-T2 (Genbank Accession Number U 04739).

81. Jones, R.C. and Jordan, F.T.W., 1970. The exposure of day-old chicks to infectious bronchitis and subsequent development of the oviduct. Vet Rec., 87: 504-505.

82. Jones, R.C., 1974. Nephrosis in laying chickens caused by Massachusetts-type of infectious bronchitis virus. Vet. Rec., 95:319.

83. Jones, R.C. and Ambali, A.G., 1987. Re-excretion of an enterotropic infectious bronchitis virus by hens at point of lay after experimental infection at day old. Vet. Rec., 120:617-620.

84. Jungherr , E.I., Chomiak, T.W. and Luginbuhl, R.E., 1956. Immunologic differences in strains of infectious bronchitis virus. In: Proc. 60th Annual Meeting U.S. Livestock Sanitary Association., 203-209.

85. Kant, A., Koch, G., Van Roozelaar, D.J., Kusters, J.G., Poelwijk, F.A.J. and Van der Zeilst, B.A.M., 1992. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. Journal of Gen. Virol., 73:591-596.

86. Karaca, K., Naqi, S. A., Palukatis, P. and Lucio, B., 1990. Serological and molecular characterization of three enteric isolates of infectious bronchitis virus of chickens. Avian Dis., 34:899-904.

87. Keeler, C.L., Reed, K.L., Nix, W.A. and Gelb, J.Jr., 1998. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S1) gene. Avian Dis., 42:275-284.

88. King, D.J., 1988. Identification of recent infectious bronchitis virus isolates that are serologically different from current vaccine strains. Avian Dis., 32:362-364.

89. King, D.J. and Hopkins, S.R., 1983. Evaluation of the haemagglutination inhibition test for measuring the responses of chickens to avian infectious bronchitis virus vaccination. Avian Dis., 27:100-112.

90. King, D.J. and Hopkins, S.R., 1984. Rapid serotyping of infectious bronchitis virus isolates with the haemagglutination inhibition test. Avian Dis., 32:362-364.

91. Klieve, A.V. and Cumming, R.B., 1988. Immunity and cross protection to nephritis produced by Australian infectious bronchitis virus used as vaccines. Avian Pathol., 17: 829-839.

92. Koch, G., Hartog, L., Kant, A. and Van Roozelaar, D.J., 1990. Antigenic domains on the peplomer proteins of avian infectious bronchitis virus: correlation with biological functions. J. Gen. Virol., 71: 1929-1935.

93. Kottier, A.S., Cavanagh, D. and Britton, P., 1995. Experimental evidence of recombination in coronavirus IBV. Virol., 213: 569-580.

94. Kusters, J.G., Niesters, H. G. M., Bleumink-Pluym, N. M. C., Davelear, F. G., Horzinck, M.C. and Van der Zeijst, B.A.M., 1987. Molecular epidemiology of infectious bronchitis virus in the Netherlands. J. Gen. Virol., 68: 343-352.

95. Kwon, H. M. and Jackwood, M.W., 1995. Molecular cloning and sequence comparison of the S1 glycoprotein of the Gray and JMK strains of avian infectious bronchitis virus. Virus genes., 9: 219-229.

96. Kwon, H.M., Jackwood, M.W. and Gelb, Jr., 1993. Differentiation of infectious bronchitis virus using the polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis., 37:194-202.

97. La Monica, N., Banner, L. R., Morris, V.L. and Lai, M.M.C., 1991. Localization of extensive deletions in the structural genes of two neurotropic variants of murine coronavirus JHM. Virol., 182: 883.

98. Lambrechts, C., Pensaert, M. and Ducatelle, R., 1993. Challenge experiments to evaluate cross protection induced at the tracheal and kidney level by vaccine strains and Belgian nephropathogenic isolates of avian infectious bronchitis virus. Avian Pathol., 22: 577-590.

99. Lashgari, M.S. and Newman, J.A., 1984. Serological comparison and antigenic relationships of seven serotypes of infectious bronchitis virus using haemagglutination inhibition test. Avian Dis., 28:435-443.

100. Lee C.W. and Jackwood, M. W., 2000. Evidence of genetic diversity generated by recombination among avian coronavirus IBV. Archiv. Virol., 145:2135-2148.

101. Lin, Z., Kato, A., Kudou, Y. and Ueda, S., 1991. Typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length polymorphism. Virol., 116:19-31.

102. Lohr, J. E., 1981. Diagnosis of infectious bronchitis (IB) by examination of tracheal mucous for IB-precipitating antigens. Avian Dis., 25:1058-1064.

103. Lohr, J.E., 1980. Infectious bronchitis agar gel precipitation test- use of infected allantoic fluid as antigen. Avian Dis., 24(2): 463-467.

104. Lohr, J.E., 1988. Differentiation of IBV strains. Proceedings of the 1st international symposium on infectious bronchitis. Rauischolzhausen, Germany, pp. 199-207.

105. Lohr, J.E., Hinze, V. and Kaleter. M., 1991. Immunological relationship between the New Zealand A and the Australian T strains of infectious bronchitis viruses measured by cross immunization tests in tracheal organ cultures from immunized birds. New Zealand Vet. Jr., 39: 113-114.

106. Marquardt, W.W., Snyder, D.B. and Schlotthober, B.A., 1981. Detection and quantification of antibodies to infectious bronchitis virus by enzyme-linked immunosorbent assay. Avian Dis., 25:713-722.

107. McDougall, J.S., 1968. Infectious bronchitis in laying fowls: its effect upon egg production and subsequent egg quality. Vet Rec., 83: 84-86.

108. McMartin, D.A., 1993. Infectious bronchitis. In: virus infections of birds. Elsevier Science Publishers, Amsterdam, pages 249-274.

109. Miller, L. and Qureshi, M. A., 1991. Induction of heat-shock proteins and phagocytic function of chicken macrophage following in vitro heat exposure. Vet. Immunol. Immunopathol., 30:179-191.

110. Miller, L. and Qureshi, M. A., 1992a. Molecular changes associated with heat-shock treatment in avian mononuclear and lymphoid lineage cells. Poultry Sci., 71:473-481.

111. Miller, L. and Qureshi, M. A., 1992b. Comparison of heat-shock-induced and lipopolysaccharide-induced protein changes and tumoricidal activity in a chicken mononuclear cell line. Poultry Sci., 71:979-987.

112. Mockett, A.P.A. and Cook, J.K.A., 1986. The detection of specific IgM to infectious bronchitis virus in chicken serum using an ELISA. Avian Pathol., 15:437-446.

113. Mockett, A.P.A. and Darbyshire, J.H., 1981. Comparative studies with an enzyme linked immunosorbent assay for antibodies to avian infectious bronchitis virus. Avian Pathol., 10:1-10.

114. Mockett, A.P.A., Cavanagh, D. and Brown, T.D.K., 1984. Monoclonal antibodies to the S1 spike and membrane proteins of avian infectious bronchitis coronavirus strain Massachusetts M-41. J. Gen. Virol., 65: 2281-2286.

115. Mockett, A.P.A., Cook, J.K.A. and Huggins, M.B., 1987. Maternally derived antibody to infectious bronchitis virus: its detection in chick trachea and serum and its role in protection. Avian Pathol., 16:407-416.

116. Mondal, S. P. and S. A. Naqi., 2001. Maternal antibody to infectious bronchitis virus: its role in protection against infection and development of active immunity to vaccine. Vet. Immunol. Immunopathol., 79:31-40.

117. Morris, V.L., Tieszer, C., Mackinnon, J. and Percy, D., 1989. Characterization of coronavirus JHM variants isolated from Wistar Furth rats with a viral induced demyelinating disease. J. Gen. Virol., 169: 127.

118. Muneer, M. A., Newman, J. A., Goyal, S. M. and Ajmal, M., 1987. Antibodies to avian infectious bronchitis virus in Pakistani chickens. Poult. Sci., 66:765-767.

119. Muneer, M.A., Halvorson, D.A., Sivanandan, V., Newman, J.A. and Coon, C.N., 1986. Effects of infectious bronchitis on laying chickens. Avian Dis., 30: 644-647.

120. Nakamura, K., Cook, J.K.A., Otsuki, K., Huggins, M.B. and Frazier, J.A., 1991. Comparative study of respiratory lesions in two chicken lines of different susceptibility, infected with infectious bronchitis virus: histology, ultra structure and immunochemistry. Avian Pathol., 20:245-261.

121. Naqi, S.A., 1990. A monoclonal antibody based immunoperoxidase procedure for rapid detection of infectious bronchitis virus in infected tissues. Avian Dis., 34:893-898.

122. Naqi, S.A., Karaca, K. and Bauman, B., 1993. A monoclonal antibody based antigen capture enzyme-linked immunosorbent assay for identification of infectious bronchitis virus serotypes. Avian Pathol., 22:555-564.

123. Neldon-Ortiz, D. L. and Qureshi, M. A., 1991. Direct and microsomal activated aflatoxin B1 exposure and its effects on turkey peritoneal macrophage functions in vitro. Toxicology and Applied Pharmacol., 109:432-442.

124. Niester, H.G.K., Lenstra, J.A., Spaan, W.J.M., Zijderveld, A.J., Bleumink-Pluym, N. M.C., Hong, F., Van Scharrenburg, G. J.M., Horzinek, M.C. and Van der Zeijst, B.A.M., 1986. The peplomer protein sequence of the M-41 strain of coronavirus IBV and its comparison with Beaudette strain. Virus Res., 5: 253-263.

125. Otsuki, K., Huggins, M.B. and Cook, J.K.A., 1990. Comparison of the susceptibility of avian infectious bronchitis virus infection of two inbred lines of white leghorn chickens. Avian Pathol., 19:467-475.

126. Parsons, D., Ellis, M.M., Cavangh, D. and Cook, J.K.A., 1992. Characterization of an infectious bronchitis virus isolated from vaccinated broiler breeder flocks. Vet. Rec.,131: 408-411.

127. Pensaert, M. and Lambrechts, C., 1994.Vaccination of chickens against a Belgian nephropathogenic strain of infectious bronchitis virus B 1648 using attenuated homologous and heterologous strains. Avian Pathol., 23:631-641.

128. Qureshi, M. A. and Hagler, Jr. W. M., 1992. Effect of fumonisin-B1 exposure on chicken macrophage functions in vitro. Poultry Sci., 71:104-112.

129. Qureshi, M. A., Yu, M. and Saif, Y. M., 2000. A novel small round virus inducing poult enteritis and mortality syndrome and associated immune alterations. Avian Dis., 44:275-283.

130. Raggi, L.G. and Lee, G.G., 1975. Relationship between immunity and serum neutralization titers in chickens inoculated with three infectious bronchitis vaccines. Am. J. Vet. Res., 18: 740-742.

131. Reed, L. J. and Muench, H., 1939. A simple method of estimating fifty percent end points. Amer. J. Hyg., 27:493-497.

132. Riddell, C., 1987. Avian Histopathology. American Association of Avian Pathology. Kennett Square, PA.

133. Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. Molecular cloning, a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring, NY.

134. Sapats, S.I., Ashton, F., Wright, P.J. and Ignjatovic, J., 1996. Sequence analysis of the S1 glycoprotein of infectious bronchitis virus-identification of a novel genotypic group in Australia. J. Gen. Virol., 77: 413-418.

135. Sawaguchi, K., Tachida, S., Aoyama, S., Takahashi, N., Iritai, Y. and Hayashi, Y., 1985. Comparative use of direct organ culture of infected chicken tracheas in isolating avian infectious bronchitis virus. Avian Dis., 29: 546-551.

136. Schalk, A.F. and M.C. Hawn., 1931. An apparently new respiratory disease of chicks. J. Am. Vet. Med. Assoc., 78:413-422.

137. Seo, S. H., Wang, L., Smith, R. and Collisson, E. W., 1997. The carboxyl-terminal 120-residue polypeptide of infectious bronchitis virus nucleocapsid induces cytotoxic T lymphocytes and protects chickens from acute infection. J. Virol., 71:7889-7894.

138. Siddell, S.G. (edn)., 1995. The Coronaviridae. Plenum Press, New York, USA.

139. Smati, R., Silim, A., Guertin, C., Henrichon, M., Marandi, M., Arella, M. and Mersouki, A., 2002. Molecular characterization of three new avian infectious bronchitis virus (IBV) strains isolated in Quebec. Virus Gen., 25:85-93.

140. Sneed, L.W., Butcher, G.D., Parr, R., Wang, L. and Collisson, E.W., 1989. Comparison of the structural proteins of avian IBV as determined by western blot analysis. Viral Immunol., 2:221-227.

141. Song, C.S., Lee, Y.J., Lee, C.W., Sung, H.W., Kim, J.H., Mo, I.P., Izumiya, Y., Jang, H.K. and Mikami, T., 1998. Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by a recombinant baculovirus. J. Gen. Virol., 79: 719-723.

142. Stern, D.F. and Kennedy, S.I., 1980. Coronavirus multiplication strategy. II. Mapping the avian infectious bronchitis virus intracellular RNA species to the genome. J. Virol., 36: 440-449.

143. Stern, D.F. and Selfton, B.M., 1982. Coronavirus proteins: Biogenesis of avian infectious bronchitis virus virion proteins. J. Virol., 44: 794-803.

144. Stern, D.F. and Selfton, B.M., 1984. Coronavirus multiplication. Locations of genes of virion proteins on the avian infectious bronchitis virus genome. J. Virol., 50: 22-29.

145. Tomley, F.M., Mocker, A.P.A., Boursnell, M.E.G., Binns, M.M., Cook, J.K.A., Brown, T.D.K. and Smith, G.L., 1987. Expression of the infectious bronchitis virus spike protein by recombinant vaccinia virus and induction of neutralizing antibodies in vaccinated mice. J.Gen.Virol., 68: 2291-2298.

146. Toro, H., Godoy, V., Larenas, J., Reyes, E. and Kaleta, E.F., 1996. Avian infectious bronchitis-viral persistence in the Harderain gland and histological changes after eye-drop vaccination. Avian Dis., 40: 114-120.

147. Van Rockel, H., Clarke, M.K., Bullis, K.L., Olesiuk, O.M. and Sperling, F.G., 1951. Infectious bronchitis. Am. J. Vet. Res., 12: 140-146.

148. Van Roeckel, H., Bullis, K.L., Flint, O.S. and Clarke, M.K., 1942. Poultry disease control service. Massachusetts Agricultural Experimental Station, MA. Annual Report. Bul., 388:99-103.

149. Wadey, C.N. and Westaway, E.G., 1981. Structural proteins and glycoproteins of infectious bronchitis virus particles labeled during growth in chick embryo cells. Int. Jr. Virol., 15:19-27.

150. Wakenell, P.S., Sharma, J.M. and Slocombe, R.F., 1995. Embryo vaccination of chickens with infectious bronchitis virus-histologic and ultrastructural lesion response and immunological response to vaccination. Avian Dis., 39:752-765.

151. Wang, L., Junker, D. and Collisson, E.W., 1993. Evidence of natural recombination within the S1 gene of infectious bronchitis virus. Virol., 192: 710-716.

152. Wang, L., Junker, D., Hock, L., Ebiary, E. and Collisson, E.W., 1994. Evolutionary implications of genetic variations in the S1 gene of infectious bronchitis virus. Virus Res., 34: 327-338.

153. Wang, L., Xu, Y. and Collisson, E.W., 1997. Experimental confirmation of recombination upstream of the S1 hypervariable region of IBV. Virus Res., 49: 139-145.

154. Winterfield, R.W. and Albassam, M.A., 1984. Nephropathogenicity of infectious bronchitis virus. Poul. Sci., 63:2358-2363.

155. Winterfield, R.W., Hoerr, F.J. and Fadley, A.M., 1978. Vaccination against infectious bronchitis and the immunosuppresive effects of infectious bursal disease. Poul. Sci., 57: 386-391.

156. Wooley, R.E., Brown, J., Davis, R.B., Blue, J.L. and Lukert, P.D., 1976. Comparison of a microneutralization test in cell culture and virus neutralization test in embroynated eggs for determining infectious bronchitis virus antibodies. J. Clin. Microb., 3(2): 149-156.

157. Yachida, S., Iritani, Y. and Katagiri, K., 1979. Effect of incubation temperature on infectivity titration of mouse brain passaged avian infectious bronchitis virus in laboratory host systems. Acta. Virol., 23: 398-402.

158. Yagyu, K. and Ohta, S., 1990. Detection of infectious bronchitis virus antigen from experimentally infected chickens by indirect immunofluorescent assay with monoclonal antibody. Avian Dis., 34:246-252.

159. Yamakami, T., Doi, M., Koimaru, H., Jde, S., Yoshimuru, M. and Masu, S., 1984. Modifications of three avian viruses passaged in Chinese hamster lung cell (Don) in pathogenesis to chicken embryos. Avian Dis., 28:532-535.

160. Zhou, J.Y., Wu, J.X., Cheng, A.Q., Zheng, X-J., Gong, H., Shang, S.B. and Zhou, E.M., 2003. Expression of immunogenic S1 glycoprotein of infectious bronchitis virus in transgenic potatoes. J. Virol., 77:9090-9093.

8. APPENDIX

8.1 APPENDIX 1

Geometric Mean Titre (GMT)

The GMT was calculated by the following two methods;

Tube/Well Method:

Example:

2 samples with end point in well number 3

3 samples with end point in well number 4

4 samples with end point in well number 5

1 samples with end point in well number 6

Average of well number

2x3=6,3x4=12,4x5=20,1x6=6

Grand Total=44.

Number of samples=10

Average well number=44/10=4.4

Observe 4 under column of 1/5 and 0.4 on the right side of the Brug's table. The GMT was 53. This was the titre if starting dilution was 1:5

Log Method:

Example:

6 samples with titre of 5 (1:5)

7 samples with titre of 20 (1:20)

5 samples with titre of 40 (1:40)

2 samples with titre of 80 (1:80)

Log of titre was multiplied by numbers of samples.

Log of 5= 0.698

Log of 20=1.301

Log of 40=1.602

Log of 80=1.903

0.698 x 6 = 4.19

1.301 x 7 =9.11

1.602 x 5 = 8.01

1.903 x 2 =3.81

Products are added and divided by total number of samples as,

4.19 + 9.11 + 8.01 + 3.81/6 + 7 + 2 + 5

=25.12/20

=1.26

Antilog of 1.26 will be equal to GMT.

i.e. 18.20

Titre in individual well was recorded as:

Well/Tube No.	Titers
1	5
2	10
3	20
4	40
5	80
6	160
7	320
8	640

8.2 APPENDIX 2

G.K.N (1Litre)

Glucose	1g
NaCl	8 g
KCl	0.2 g
Phenol red 1%	2 ml
De-ionized distilled water	900 ml

Adjust pH to 7.8 with $NaHCO_3$ solution or HCl solution. Then make volume up to 1L with distilled water. Autoclave at $15Lb^2$ for 10 minutes at 121°C and store at 4°C. Add antibiotics at the following rates:

Pen-Strep (150ug /ml)	2ml
Amphoteracin B (250ug/ml)	4ml
Gentamycin (1ug/ml)	2ml

8.3 APPENDEX 3

AGPT MEDIUM (for 5 plates)

Nobel agar	1g
NaCl	8g
Phosphate buffer pH 7.2(0.2 M)	100ml

Autoclave at 15lb^2 pressure and 121°C temperature for 10 minutes.

8.4 APPENDIX 4

ARRANGEMENTS OF DATA USED IN CALCULATION OF EID $_{50}$

				Accumulated Value			
Virus Dilution	Infectivity Ratio Ratio	Infected	Non-Infected	Infected	Non-Infected	Ratio	Percentage
A	B	C	D	E	F	G	H
10^{-1}	5/6	5	1	15	1	15/16	94%
10^{-2}	5/6	5	1	10	2	10/12	83%
10^{-3}	4/6	4	2	5	4	5/9	56%
10^{-4}	1/6	1	5	1	9	1/10	10%
10^{-5}	0/6	0	6	0	15	0/15	0%

CALCUALTIONS: EID $_{50}$ (Reed and Muench Method).

Proportionate distance = % infectivity at dilution next above 50% - 50%

%infectivity at dilution next above 50% - % infectivity at dilution next below 50%.

= 83-50/83-56.

= 1.2

-ve log of EID $_{50}$ = -ve log of dilution above 50% infectivity + Proportionate distance factor

= - 3.00 -1.2

= - 4.2

Log of EID $_{50}$ = $10^{4.2}$

The neutralization index (NI) was determined by subtracting the log titre of the virus negative control serum mixture (or log titre virus alone) from the log titre of the serum virus mixture representing the standard reference antisera. A NI $\geq$ 3.0 indicates that the

unknown field isolate is antigenically related to the reference serotype used to produce the antiserum used in the VN test. NI values 2.0-3.0 suggest a somewhat lesser degree of similarity and NI $\leq$ 2.0 indicates that the unknown isolate is serotypically unrelated to the standard reference antiserum.

Calculation of Neutralization Index:

Log titre of negative control serum-virus mixture	=	6.0
Log titre of unknown virus-serum mixture	=	2.0
Neutralization Index	=	4.0