ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 6 (12): 866-870, 2007
ISSN 1682-8356
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Quantification of Teratological Effects of Infectious Bronchitis Virus Isolated from Commercial Poultry in Pakistan

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Abstract: In continuation of our studies with the Infectious Bronchitis prevalence in Pakistan, the current study was conducted to determine the teratological effects of the Infectious Bronchitis Virus (IBV) on developing chicken embryo. The tissues collected (Trachea and Lungs) from clinically positive IB chickens were tested with the indirect immunofluorescence assay against M-41 IBV strain (reported previously). The positive samples were further tested with the RT-PCR against the IBV primers (reported previously). The tissue homogenates from 43 such RT-PCR-positive samples were serially passaged through 11 days old embryos and the effects on the embryonic development in terms of dwarfing, curling, stunting and urates deposits were recorded. The data showed that the passaging sequentially increased the IBV teratological effects. Only 13 out of 43 samples exhibited IBV-associated teratological effects over various passages. These effects were effectively neutralizes by using IBV variant specific antisera in a viral neutralization test. These studies imply that IBV variants isolated from Pakistan are no different in producing teratological effects considered representative of the IBV.

Key words: Chickens, infectious bronchitis virus, teratological effects, Pakistan

Introduction

Infectious Bronchitis (IB) is an acute, highly contagious viral respiratory disease of chickens (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 Infectious Bronchitis Virus (IBV) strains that cause kidney and oviduct damage as well as by secondary bacterial infection. The disease is prevalent worldwide with significant 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). The Infectious Bronchitis Virus (IBV) which belongs to the *Coronaviridae* family causes this disease. It is an enveloped virus with a diameter of 120 çm (Davies and Macnaughton, 1979). There are more than 20 known serotypes within IBV recognized worldwide (Lee and Jackwood, 2000).

We have recently reported the seroprevalence of M-41, D-274, D-1466 and 4-91 strains of IBV in Poultry in Pakistan (Ahmed *et al*., 2007). M-41 strain was most prevalent in the 88% of layers and broiler flocks tested. Indirect immunofluorescence (IFA) analysis on lungs and tracheal tissues from 150 clinically positive commercial chickens revealed 60 lungs and 15 trachea positive for M-41 IBV. These 75 IFA-positive samples were homogenized and subjected to RT-PCR analysis which revealed 43 samples showing M-41 IBV-specific PCR product. In the current study, these RT-PCR-positive M-41 IBV-positive homogenates were tested for teratological effects upon embryonic inoculation. In addition, the allantoic fluid collected from these embryos was also tested for IBV antigen presence via the viral neutralization test. Our assumption was that the IBV variants prevalent in Pakistan as reported in our previous study (Ahmed *et al*., 2007) will resemble in pathogenicity to known IBV isolates currently prevalent around the world.

Materials and Methods

Source of Chickens and Tissue Homogenate: The chickens used in this study were commercial layers and broilers brought to our laboratory as suspect positive for infectious bronchitis. Tracheal and lung tissues from 150 such chickens of varying ages were tested for the presence of M-41 IBV antigen by indirect immunofluorescence against M-41 antisera (Gezondheidsdienst voor Dieren B. V. Animal Health Service, Deventer, Netherlands). A total of 60 lungs and 15 trachea were determined positive (Ahmed *et al*., 2006). These tissues were homogenized and subjected to RT-PCR analysis as described previously (Ahmed *et al*., 2007). The homogenates from a total of 43 samples which showed M-41-specific RT-PCR products were used for *in ovo* inoculation to quantitate the teratological effects.

***In Ovo* Passaging of Tissue Homogenate Supernatants for IBV propagation and teratological effects studies:** Tissue homogenate supernatants found positive for M-41 IBV strain in RT-PCR analysis (n = 43) were

passaged in embryonated 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 embryonated 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 inoculum was injected through the hole into chorioallantoic 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 at 4°C. 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 embryonated 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.

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 7.2) ranging from 10^{-1} to 10^{-5}. Nine days old embryonated eggs were divided into 5 groups of six eggs each and were inoculated with 0.2 mL 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). The NI value of ≥ 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.

Results and Discussion

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 (Ahmed *et al*., 2007), 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 1, 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. 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 1). 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 embryonated eggs, the

Table 1: 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 deposits)				
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, 0.2 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 2: Viral neutralization studies on allantoic fluid from RT-PCR-positive embryonated eggs-passaged samples[1]

Total # of Allantoic fluid from embryos showing +++ to ++++	VN against M-41 Antisera		VN against D-274 antisera		VN against D-1466 antisera		VN against 4-91 antisera	
Teratological Changes (#)	+	-	+	-	+	-	+	-
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 $\geq$ 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

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 1).

The observed teratological effects included dwarfing, curling, stunting and urates deposits which were similar to as previously reported for IBV (Clarke *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.

The allantoic fluid from embryos showing teratological effects was collected and examined for Viral Neutralization (VN) activity against various IBV strains (Table 2). 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.

As expected, virus neutralization effects against M-41 antisera were maximum in which 6 out of 13 samples showed viral neutralization activity (Table 2). 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 2).

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 2). 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 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 due 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 2, 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 2). 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.

In conclusion, this study suggests that the IBV variants isolated from commercial poultry flocks in Pakistan represent "typical" IBV types (in terms of pathogenicity) prevalent elsewhere in the world. Although, the poultry flocks in Pakistan are routinely vaccinated with Massachusetts-41 (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 M-41 strain as well (Muneer *et al*., 1987) thereby suggesting the presence of this and possibly other IBV strains in Pakistan. In a sero-surveillance study, Muneer *et al*. (1987) found antibodies against Arkansas (2.6% positive flocks) and Connecticut (2.2% positive flocks) type IBV whereas no flock was found with antibodies against JMK IBV variant. There are age and seasonal associations with the IBV infections reported in Pakistani flocks (Javed *et al*., 1991). For example, 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.

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