Research Article

Isolation and Seroprevalence of Avian Infectious Bronchitis Virus Serotypes in Pakistan

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Abstract | The present work was conducted to investigate the isolation and seroprevalence of avian infectious bronchitis virus (IBV) serotypes in commercial poultry in Pakistan. In order to isolate IBV from clinically infected birds, specimens were processed and passaged in embryonated chicken eggs and in chicken embryo kidney cells (CEK). Out of twenty five suspected specimens six were found positive for IBV, on the basis of major lesions in embryonated chicken eggs on post in ovo inoculation and cytopathic effects (CPE) upon virus inoculation in CEKcell culture. None of the propagated sample was found positive through Agar Gel Precipitation Test (AGPT). Sero analysis of five hundred serum samples from six breeder and broiler flocks were screened against IBV serotypes, namely, D-274, D-1466, H-120,4/91 and M-41 using haemagglutination inhibition (HI) assay. During serological analysis seroconversion against IBV serotypes D-274 and D-1466were observed inPoultry Breeder farms at Abbotabad and Qalandarabad. Moreover, Poultry Breeder farms at Multan and Sihala (Islamabad) the seroconversion was observed against IBV serotype H-120 & 4/91, where as in Jumma Bazar Broilers, Poultry Breeder farms at Abbotabad and Chakwal the seroconversion against IBV serotypes M-41 was observed. In conclusion, presence of more than one serotypes of IBV reflects the circulation of several strains of IBV in commercial poultry of Pakistan.Further isolation and biological characterization of prevailing IBV is required to prepare the effective vaccine against IBV.

Keywords | Infectious bronchitis Virus, Seroprevalence, Agar gel precipitation test (AGPT), Poultry

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INTRODUCTION

Among many avian diseases prevalent in Pakistan, infectious bronchitis (IB) is one of the major disease causing heavy production losses throughout the life of the bird. Infectious bronchitis virus (IBV) which was first described in United States in 1930 (Schalk and Hawn, 1931) was identified all over the world by early 1960. The disease is of economic significance because infection in young chicks, broilers, laying and breeding flocks leads to mortality, poor growth performance, decrease in egg production and in egg quality. Clinical signs of an IBV infection are gasping, coughing, tracheal rales and nasal discharge (Cavanagh and Naqi, 1997) also seen are wet eyes and swollen sinuses. In addition nephropathogenic IBV strains causes besides the above respiratory signs, severe interstitial nephritis which can be lethal (Hofstad, 1984; Muneer et al., 1986; De Wit et al.,1992). The multiple serotypes of IBV may be associated with marked drop in egg production, laying of soft, misshapen and poor quality eggs for long periods and incomplete recoveryto rate of laying to pre-infection levels (Broadfoot et al., 1954). In addition IBV serotypes are the cause of poor weight gain and feed conversion in broilers (Broadfoot et al., 1954; Cavanagh

and Naqi, 1997; De Wit et al., 1998). Secondary bacterial infections may cause major losses by growth depression, mortality and condemnation (De Wit et al., 1992). Early work by Jungherr et al. (1956) suggested that more than one antigenic types of IBV existed. Since then a large number of IBV strains have been isolated and identified. In the more recent years, IBV has also been isolated from cloacal swabs (Picault et al., 1987; Lu et al., 1994) and a variant strain of IBV793/B was isolated from broiler breeder flock (Gough et al., 1992). Presently over 20 IBV serotypes have been identified in US and other poultry producing countries of the world (Picault et al., 1987; Lu et al., 1994) and new IB viruses regularly appear as a result of viral mutations and/or in vivo recombination (Meulemans and Berg, 1998).

Outbreaks of IB in vaccinated flocks are attributed to the emergence of different serotypes of IBV (Davelaar et al., 1984; Picault et al., 1986; Gelb et al., 1989; Cubillos et al., 1991; Parson et al., 1992; Cavanagh and Naqi 1997). Molecular studies have shown that a new IB serotype can emerge as a result of only a few amino acid changes in the spike 1(S1) part of the spike genome of the virus (Cavanagh et al., 1997). Mutagenic nature (Wang and Tsai, 1996) of IBV is considered an important factor for the development of antigenic variation and failure of vaccination programs. Since a broadspectrum vaccine giving protection against all strains of the virus has not been developed, it is necessary to isolate and characterize the strains and monitor the immune status of the vaccinated flocks, in any area where the disease is not adequately controlled. Many serological tests have been used for the detection of antibodies to avian IBV serotypes. However the HI test is most widely used for the diagnosis, vaccine testing and disease monitoring (Brown and Bracwell, 1985), because it is easy to perform, economical, rapid and serotype specific (King and Hopkins, 1984).

As far as the seroprevalence of the various infectious bronchitis strains in chickens is concerned, Muneer et al reported in 1999 that M-41 D-274 and D-1466 serotypes are common in various chicken flocks in Pakistan.

This study was designedtoisolate IBV from the suspected cases and to ascertain the seroprevalence of different serotypes of IBV (namely D-274, D-1466, H-120, 4/91 and M-41) among vaccinated and unvaccinated flocks. Findingswould help to establish a record about the prevalence of IBV serotype (s) circulating at different forms, so that an appropriate disease control program is devised for broilers layers and breeders.

MATERIALS AND METHODS

Viruses and Antiserum

Infected lungs and trachea homogenate was prepared in sterile (Glucose, KCl, NaCl) GKN C¯ Ab.(with antibiotics) pH 7.8. The suspension was freeze-thawed thrice and then centrifuged at 1500 rpm at 10°C for 15 minutes. The supernatant was filtered through 0.2µm filter (Sartorius, Germany) and the filtrate was stored at -20°C till used as virus isolate. The antisera against each serotype produced in specific pathogen free (SPF) chickens using the standard procedure (Harlow and Lane 1988).

Chicken Embryo Kidney Cell Culture

Primary chicken embryo kidney cells (CEK) monolayer was prepared using 18days old viable chicken embryonated eggs. The eggs were chilled at 4°C for 45 minutes and then blunt surface was wiped thoroughly with 70% alcohol before removing the embryos from the shell aseptically. Kidneys were taken out and placed in GKN solution before mincing finally and washing thrice with GKN solution containing antibiotics. Minced tissue was then transferred to conical flask,containing magnetic stirrer and 11% warm trypsin solution (10ml/embryo kidney) was added and stirred at 37°C for 40 minutes.

The trypsinized tissue was filtered through muslin cloth, added Eagle's Minimum Essential Medium (E-MEM) (1ml/10ml of trypsinized tissue filtrate) into the filtrate and centrifuge at 1200 rpm at 10°C for 15 minutes. The pellet obtained was suspended in the E-MEM with 10% foetal bovine serum (FBS) (5ml/1 embryo's trypsinized kidney tissues). Total cells were counted using neubar chamber by using cell counting method, (Rovozzo and Burke, 1973). The concentration of the cells in the original cell suspension was adjusted to 10^6cells/ml by diluting the cells with E-MEM containing 10% (v/v) FBS. Aliquots of resulting solutions containing cells after placing in tissue culture flask and plates were incubated at 37°C. Flask and plates containing confluent monolayer after 48 hours without any contamination were used further for inoculating the virus for passaging.

VirusInoculation in Chicken Embryo Kidney Cell Culture (CEK)

Virus isolates were further purified using limiting dilution method and then inoculated inCEK using the standard protocol (Burleson et al., 1992). The flasks were daily examined microscopically and when more than 75% cytopathic effects (CPE) observed, the contents of the flasks were freeze-thawedthrice to release the virus particles from the cells. The material was centrifuged, the supernatant was filtered through nitro-cellulose filterof 0.2µm and many aliquots of the filtrate were prepared and stored at -20°C for further use. A sample of this filtrate was tested by AGPT. This was referred to as passage-1 (P-1) of the virus in (CEK) culture.

Table 1: Cytopathic effects of IBV on inoculated embryonated chicken eggs

Name of the Farm	Passage-1	Passage -2	Passage-3	Passage-4	Passage-5	Passage-6
Alkaram P/F		+	++	+++	+++	+++
Chaudhry P/F	+	+	++	+++	+++	++++
Gillani P/F	+	+	++	++	+++	++++
Gilliat P/F		+	+	++	++	+++
Khalid P/F		+	++	++	++	++
Sayyed P/F	+	+	++	++	+++	+++

Table 2: In vitro passaging of IBV isolates

Status of passage	Cell culture type used	CPE on 2nd day PI	CPE on 3rd day PI	CPE on 4th day PI	CPE on 5th day PI
01	Chicken embryo kidney Cells	5%	15%	30%	50%
02		10%	20%	35%	55%
03		15%	25%	50%	65%
04		15%	30%	55%	75%
05		20%	35%	65%	80%

Table 3: Disease and Vaccination of Broiler Breeder Flocks

Poultry Farm	Flock Size	Age of birds (weeks)	Samples collected	Clinical history of Poultry farm	Vaccination done up to sampling
Poultry Breeders Farm1 (Abottabad)	30,000	35 weeks	100	Respiratory signs plus 40% drop in production with poor quality eggs	IB (H-120) 6 shots (5 live and 1 killed)
Poultry Breeders Farm 2 (Chakwal)	20,000	25 weeks	70	Respiratory signs	IB (H-120) 5 shots. (4 live and 1 killed)
Poultry Breeders Farm 3 (Multan)	20,000	20 weeks	70	Respiratory signs	IB 4 shots (live)
Poultry Breeders Farm 4 (Qalandarabad)	40,000	40 weeks	140	Respiratory signs along with mild drop in egg production	IB 8 shots (6 live and 2 killed)
Poultry Breeders Farm 5 (Islamabad)	25,000	20 weeks	80	Mild respiratory signs	IB 4 shots (live)
Jumma Bazaar (broilers)	Unknown	6-7 weeks	50 Two flocks (25+ 25)	Apparently no clinical signs	IB 1 shot live

Virus Inoculation in Embryonated Chicken Eggs

To study in ovo pathogenicity of virus, the cell culture propagated virus and lung trachea homogenate (earlier prepared) was inoculated and passaged in the embryonated SPF chickeneggs via allantoic fluid (AF) route and yolk sac (YC) route following the standard protocol (Clarke JK et al.,1972). The fluids obtained were tested by AGPT for the detection of IBV and embryos were also examined for any lesions.

Serological Evaluation

Serological analysis was conducted following the standard protocols of Agar Gel Precipitation Test (AGPT) (Crowle, 1973) and Haemagglutination Inhibition (HI) test (Alexander and Chettle, 1977). For HI analysis reference antigens of IBV serotypes D-274, D-1466, 4/91, H-120 and M-41 were used.

RESULTS

Embryonated Chicken Egg Inoculation

Out of twenty five isolates (Broilers=16, Broiler Breeder=9) ten were found positive on passage-1 in embryonated chicken eggs.These embryos were dwarfed size,2cm less than the normal and had hemorrhages, more prominent on the head and on the lateral side of the body.

All 25 passage-1 isolates were found negative by AGPT. After third passage the isolates which were found negative for IB, were discarded and remaining six isolates were serially passaged upto six passage and their results were given

Table 4: Haemagglutination inhibition IBV antibody titers in sera of various flocks

Name of the farm	No. Of serum samples	Antibody Titers Against									
		D-274		D-1466		H-120		4/91		M-41	
		GMT	Range	GMT	Range	GMT	Range	GMT	Range	GMT	Range
Poultry Breeders Farm 1 (Abottabad)	100	86	40-100	80	40-160	NA	NA	NA	NA	92	40-160
Poultry Breeders Farm 2 (Chakwal)	70	NA	NA	NA	NA	NA	NA	NA	NA	98	40-160
Poultry Breeders Farm 3 (Multan)	70	NA	NA	NA	NA	113	80-320	106	40-320	NA	NA
Poultry Breeders Farm 4 (Qalandarabad)	140	92	40-230	92	40-160	NA	NA	NA	NA	NA	NA
Poultry Breeders Farm 5 (Islamabad)	80	NA	NA	NA	NA	113	80-640	98	40-320	NA	NA
Jumma Bazaar (broilers)	50	NA	NA	NA	NA	106	80-320	121	80-640	NA	NA

in Table 1.

Inoculation of the Samples on Chick Embryo Kidney Cells

Out of 25 samples 06 showed significant CPE including rounding of cells,syncytial formation followed by necrosis were inoculated on multi-well tissue culture (CEK) plate. These six isolates were passaged six times successfully in monolayer of CEK with increasing CPE on successive passaging as given in Table 2.

But always found negative when tested by AGPT.Since CEK cell culture showed better results than other tissues and organ cultures (mentioned in materials and methods) CEK was routinely used and results are presented here.

The disease and vaccination history of various flocks included in this investigation for serology is presented in Table 3.

A total of 500 serum samples were collected from the birds of various age groups,which had different disease incidences and vaccination histories as shown in Table 3. It is important to note that vaccination against IB variants (D-274,D-1466 and IB 4/91) was not used at any of these flocks.

The detail of haemagglutination inhibition (HI) IBV antibody titers in the sera of various flocks is given in Table 4. These results clearly indicate high antibody titers against each serotype.

During serological analysis seroconversion against IBV serotypes D-274 and D-1466 were observed in Poultry Breeder farms at Abbotabad and Qalandarabad. Moreover, Poultry Breeder farms at Multan and Sihala (Islamabad) the seroconversion was observed against IBV serotype H-120 & 4/91, whereas in Jumma Bazar Broilers, Poultry Breeder farms at Abbotabad and Chakwal the seroconversion against IBV serotypes M-41 was observed.

DISCUSSION

The study reported here was planned to isolate IBV from suspected cases of IB and to study the seroprevalence of different serotypes of IBV. In this regard Hofstad (1984) earlier reported that diagnosis of IB must be based on the isolation of virus or by demonstrating on ascending antibody titer against a known strain of IBV preferably other than Massachusetts serotype.

Presence of IBV in the embryonating chicken eggs following inoculation is somewhat difficult to discern in the initial passage since there is little change in most of the embryos inoculated. Dwarfing of few embryos with survival of 90% through the 19th day of incubation is the characteristic of IBV field material upon initial inoculation in 10 to 11 day old embryonated chicken eggs. Embryo mortality and dwarfism increases as the number of serial passages increases (Cavanagh and Naqi, 1997).

The results in Table 1 revealed that out of 6 isolates of IBV, 3 were found positive at the first passage. This might be due to the low pathogenicity of these 3 strains of IBV, which were not well adapted in chick embryo.

Furthermore, all the samples tested by AGPT were found negative for IBV which might be due to relative insensitivity of the test. Jordan (1991) and De Wit et al. (1997) reported the similar results. Since the precipitating antibodies disappear within afew weeks, therefore, a positive would only be possible if the sample is tested from a recently infected flock.

In this study attempts were also made to passage the IBV in different cell cultures we found significant growth of field avian IBV isolatesin chicken embryo kidney (CEK) cell culture. Otsuki et al. (1979) has also reported similar growth for 10 various strains of IBV on CEK cell culture. Zhang et al. (1995) reported on the growth of nephropathogenic avian IBV in CEK cell culture. Bhattacharjee and Jones (1997) have also reported the growth of M-41 and 10 other strains of IBV isolated from intestine on the proventriculus, bursa and kidney tissue culture. Almost similar significant antibody titers were found against all five serotypes of IBV in different flocks in different localities. In Pakistan, vaccination of IBV is mainly done with H-120 or M-41 serotypes of IBV. We found significant antibody titers against D-274, D-1466 and 4/91 against which no vaccination is done in broilers and mostly of the breeding stock in Pakistan. Muneer et al. (1999) has earlier reported the presence of antibodies against IBV serotypes D-271 and D-1466.

Because of the cross reactions, the presence of haemagglutinating antibodies against a certain IBVserotype in chickens is not necessarily a proof of an infection caused by that serotype. Therefore, one should be very cautious in concluding that a (new) serotype is present in aregion only on the basis of detecting haemagglutinating antibodies to it. To resolve this to certain extent attempts were made to isolate the virus from the diseased cases. In this study the isolation of six strains through cell culture and egg passage indicates the presence of IBV serotypes in the field. The virus isolation in most of the cases was possible after 2nd or 3rd passage of the specimens. It has been previously reported that the field IBV are not egg adapted and do not

produce any typical IBV infected embryo signs on the 1st or 2ndembryo passage (Cavanagh and Naqi, 1997; Song et al., 1998).

Other methods for determining the presence of IBV in the unknown samples includes Dot Immunobinding Assay (DIA), antigen capture Enzyme Linked Immunosorbent Assay (ELISA), Immunoflourecence assay(IFA) and Polymerase Chain Reaction(PCR) (Song et al., 1998; Wang et al., 1999).

Our findings clearly indicate the presence of more than one serotype of IB in Pakistan in different poultry farms at different areas. Based on this data it is believed that vaccination against IB should also include D-274, D-1466 and 4/91 along with M-41. However, it would still remain to be seen if some other serotype of IBV exists in Pakistan. Further biological characterization of IB isolates and more isolation of IBV strains are needed. So that we come to know that which strain (s) is prevalent and vaccination should be done against that serotype or polyvalent vaccines to be made.

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CONFLICT OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

AUTHORS CONTRIBUTION

AR, KN and AH conceived the study. KN & AR designed the experiments. KN, NS and MAA provided consultation and MAA arranged reagents and materials. AR performed experimentation and AA & SR helped in experimentation. KN, NS & MAA helped in data analysis. AR wrote the manuscript. All the authors reviewed the manuscript.

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