Isolation and Identification of Infectious Bronchitis Virus from Commercial Chickens

Momayez,*[1]§R., Pourbakhsh, S.A.,[1] Khodashenas, M.[2] and Banani, M.[1]

§: r momayies@rvsri.com

1. Avian Diseases Research & Diagnosis Dept., Razi Vaccine & Serum Research Institute, P.O.Box 11365-1558, Tehran, Iran

2. Electron Microscopy Unit, Razi Institute

Summary

Five infectious bronchitis viruses (IBV) were isolated from commercial chickens. One of them was isolated from a layer flock vaccinated with H_{120} on day old and the others were originated from broiler flocks without receiving the vaccine. All the isolates caused embryo mortality and or dwarfing, stunting, curling, clubbing of the down and urate deposits in the kidney. In tracheal organ cultures (TOCs), the isolates caused typical ciliostasis. Electron microscopy confirmed all the isolates have Coronavirus morphology. The isolates were also confirmed by dot-immunobinding assay using polyclonal antibody to IBV. In one-day-old specific pathogen free chicks, all the isolates could induce typical respiratory distress of IB at 36-48h postinoculation by intraocular route. Virus-neutralization test showed that two isolates were recovered from broiler flocks did not neutralize by monospecific Massachusetts (Mass) antiserum, indicating the presence of other serotypes in commercial chicken flocks in Iran.

Key words: infectious bronchitis viruses, isolation, identification, chicken

Introduction

Infectious bronchitis (IB) is a highly contagious acute viral disease that causes high morbidity in all ages of chickens and high mortality in chicks less than 6 weeks

old (Case *et al* 1983). The first incidence of IB virus (IBV) was reported in the USA by Schalk & Hawn (1931), since then it has been a cause of serious cause of IB economic loss in chickens. IB is known to be one of the major highly contagious diseases of the respiratory and urogenital tract of chickens (King *et al* 1991). IBV is belong to the *Coronaviridae* family, *Coronavirus* genus with more than 26 serotypes, (Cavanagh *et al* 1992, Cook 1983, Gelb *et al* 1991, Jia *et al* 1995). IBVs have been identified from all over the world and more may found in the future. Initially, it was believed all the isolates belong to a single prototype termed Massachusetts (Mass) serotype mostly isolated from commercial poultry (Cavanagh & Naqi. 1997). Subsequently, other serotypes were isolated and it is clear now that a considerable number of different serotypes with antigenic and pathogenic differences exist in different parts of the world (Gough *et al* 1992). Simultaneous infections such as Newcastle disease, avian influenza and avian adenovirus group 1, mycoplasma, and coliform bacteria may confuse diagnostic efforts (Gelb *et al.,* 1998).

The first isolation of IBV in Iran was reported by Aghakhan *et al* (1994). The isolate showed antigenic relationship to the Mass serotype. Vasfi Marandi and Bozorgmehri Fard (2000) were identified some IBV field strains suggested the presence of IBV variant (s) in Iran. In spite of regular vaccination with Mass strains, IB is still a serious problem in Iran, causing mortality and adverse effects on quantity and quality of egg production as well as renal failure in broilers and layers. The purpose of this study was conducted to isolate and identify IBVs from recent outbreaks in suspected commercial poultry flocks from several provinces in Iran. Subsequent studies are required to characterize of the IBV isolates.

Materials and Methods

Sample. Different organs including trachea, lung, kidney and cecal tonsils were removed aseptically from broiler and layer commercial poultry flocks of 2-18 weeks of age, which were suspected to IBV infection. The broiler flocks demonstrating a respiratory disorders, anorexia, loss of bodyweight, possibly associated with

increased mortality resulting from secondary bacterial infection, and the layer flocks showing aberrant egg production.

Chicken embryonated eggs. Specific pathogen free (SPF) emberyonated eggs (Valo, Lohmann, Cuxhaven, Germany) were used for inoculating the samples and preparing the tracheal organ cultures (TOCs) in order to isolate and detect the IB virus.

Chickens. Ten one-day-old chickens used for inoculation intraocularly of each IBV isolates were harvested from allantoic fluid of SPF stocks. The chickens were maintained under specific throughout the study.

Serology. A commercial ELISA kit from Kirkegaard & Perry Laboratories, Inc. (KPL), Gaithersburg, Maryland, USA, was used to determine IBV antibodies in sera of chickens, according to the manufacturer's instruction.

Virus isolation using embryonated SPF eggs. The samples were homogenized to give approximately 10% (w/v) suspension in tryptose phosphate broth (TPB) pH7.0-7.2 containing 10,000IU/ml penicillin, 10,000µg/ml streptomycin, and 250IU amphotericin B/ml. The homogenized samples were centrifuged at 1000g for 15min at 4°C. Contamination sometimes associated with virus isolation from cecal tonsil was avoided by passing the supernatant fluid through a 0.45µm filter membrane (Gelb *et al* 1998). The supernates was inoculated at 0.2ml via the chorioallantoic cavity of groups of ten 10-day-old SPF embryonated chicken eggs. Inoculated eggs were checked twice a day. Those that died within 24h after inoculation were discarded. Mortality between 2 and 7 days postinoculation (PI) were considered to be virus specific. The chorioallantoic fluid was harvested aseptically from embryos that died between 48 and 72h PI, if the fluid showed no haemagglutination (HA) activity. The harvested fluid was tested for the absence of bacteria and fungi. Dead embryos were examined for the presence of embryo stunting, curling, urate in the mesonephros, or focal necrosis in the liver. On day 3 PI, five live embryos were also removed from the incubator and were placed at 4°C for 24h and the chorioallantoic fluid of the embryos was collected for the next passage. Harvested fluid was made five blind passages in chicken embryos prior to being considered negative for IBV

isolation according to Wang *et al* (1996), Cavanagh & Naqi (1997), Gelb *et al* (1998). Samples being the only ones to affect embryos mortality without typical signs of infectious bronchitis suggesting that they may be having Newcastle disease or avian influenza viruses. The chorioallantoic fluid from each sample was examined for its ability to haemagglutinate chicken erythrocytes. The positive HA allantoic fluid of these samples were identified with NDV or H9N2 subtype of avian influenza virus antisera by haemagglutination inhibition (HI) test. The isolates were tested for possible contamination with other common avian viruses by electron microscopy.

Virus isolation using tracheal organ cultures. TOCs were prepared from 20-day-old SPF embryos as described earlier by Cook *et al* (1976). Suitable transverse sections or rings of the trachea were produced by tissue-chopper (Darbyshire *et al* 1978). The rings were about 0.5-1.0 mm thick, and were maintained in a medium consisting of Eagle's N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) in roller drums (15 rev/h) at 37°C. After an over night the media were drained from roller drums and they were inoculated with 0.1 ml of the supernatant fluid from samples and with the well characterized Mass 41 serotype of IBV, in the form of allantoic fluid. The media were changed after one hour of adsorption. The tracheal organ cultures were observed by low-power microscopy within 1-3 day PI for ciliostasis detection.

Electron microscopic (EM) examination. For EM examination the allantoic fluid of each isolate was harvested from weak embryoes about 48-72h after inoculation. The allantoic fluid of each passage was clarified by low-speed centrifugation (3000 rpm for 15 min) and the supernatant was placed on 200-mesh Formvar carbon-coated grids and stained with 3% phosphotungstic acid in pH7.3 for 30s. Grids were viewed in Philips 400 electron microscope at 100KV for the presence of IBV and other viruses (Wang *et al* 1996).

Dot-immunobinding assay. The positive isolates were tested by dot-immunobinding assay by using polyclonal antibody to IBV isolates that adsorbed on the nitrocellulose membrane. Rabbit anti-chicken IgG horseradish peroxidase (HRP) conjugate and hydrogen peroxide with 4-chloro-1-naphthol (HRP-color development

reagent) were applied for detecting antigen-antibody reaction. Normal chorioallantoic fluid and Mass IBV serotype were used as controls of the test (Mohammad *et al* 1988).

Virus neutralization. 10^3 mean embryo infectious dose (EID50) of the IBV isolates were neutralized by 10-20 antibody units of the reference Mass IBV antiserum for 60min at room temperature. Each sample was inoculated (0.2 ml inoculum) into five 10-day-old SPF embryonated eggs via the chorioallantoic sac. The eggs were incubated at 37°C and checked daily. At 7 days PI embryos were examined for IBV lesions such as stunting, curling and kidney urates. Field isolate that neutralized with the antiserum was considered to Mass serotype. The reference Mass IBV serotype was simultaneously used as control of the tests (Gelb *et al* 1998 and 1991).

Results

During a time period of almost one year (early 2000-2001) trachea, lung, kidney and cecal tonsils from 29 commercial chickens were examined, of which layers were experiencing aberrant egg production, broilers showing respiratory and nephritis infections were followed by mortality resulting from coli-septicaemia. Following passages in embryonated chicken eggs, 12 of the samples caused embryo mortality and/or dwarfing, stunting and curling of the embryos (Figure 1).

Figure 1. *Comparison of normal 15-day-old embryo (right) and 3 curled stunted, infected embryos*

Following passage in TOCs they also caused ciliostasis of trachea within 1-3 days. Only five IBVs were isolated from examined chickens. The history and the results of VN tests were shown in table 1. The isolated viruses were found to be IBV that confirmed by electron microscopy to have Coronavirus morphology (Figure 2). After 5 to 7 passages in SPF embryonated eggs the embryo mortalities became stable. The dead embryos showed stunting, curling, clubbing of the down and urate deposits in the mesonephros of the kidney or focal necrosis in the liver. These isolates produced ciliostasis in TOCs in the form of allantoic fluid.

Table 1. *History of infectious bronchitis virus isolates*

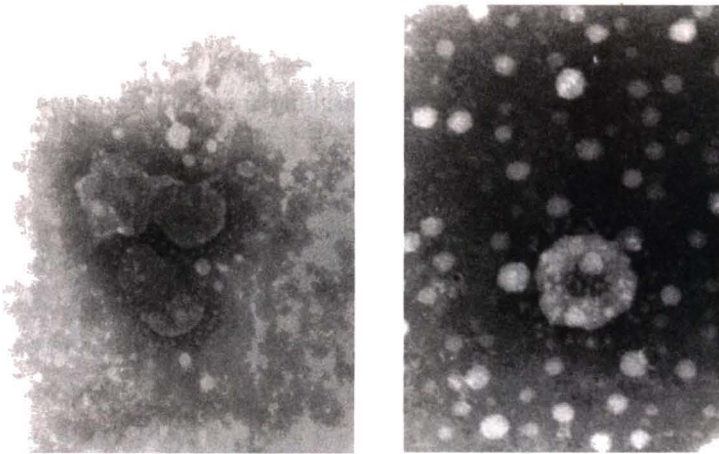| Type of birds	Age (day)	Province	IBV vaccine	Isolation		VN against mass. monospeccific Ab
				Source	Year	
Layer	24	Tehran	Mass$_{(H120)}$	Trachea	2000	Mass.
Broiler	26	Tehran	None	Trachea, Cecal tonsil	2000	Mass.
Broiler	30	Fars	None	Trachea, Cecal tonsil	2001	Mass.
Broiler	24	Ardebil	None	Trachea, Cecal tonsil	2001	-
Broiler	17	Gilan	None	Trachea, Cecal tonsil	2001	-

Figure 2. *Electron micrograph of IBV isolates (left x218000, right x200000)*

The one-day-old susceptible chickens inoculated with IBV isolates were showed respiratory distress 36-48h PI and the viruses could be reisolated from these chickens 3 to 4 days after exposure. The sera of the inoculated chickens revealed high level of

IBV antibodies two weeks PI. The isolates showed positive reaction to polyclonal IBV antibody by dot-immunobinding assay (Figure 3).
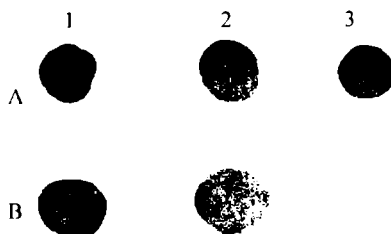

Figure 3. _Dot-immunobinding patterns of polyclonal antibody against IBV isolates. A₁, A₂, A₃ and B₁ : field isolated; B₂ control (+); B₃ control (-)_

Discussion

In the present study attempts were made to isolate IBV from commercial chicken flocks in different provinces of Iran submitted to Razi Institute. All of the flocks suspected to IB, suffering from respiratory disorders. Most of the suspected flocks showed high level of antibody titers to IBV by ELISA technique. Only five IBV isolates were recovered from flocks with respiratory signs. By virus-neutralization test using monospecific Massachusetts's antiserum three of the isolates were shown to belong to Mass serotype, whereas two of them did not neutralize with Mass antiserum. This test determined that in spite of Mass serotype, the presence of other serotypes is inevitable in poultry flocks in Iran. Probably the break of IB vaccination with Mass strains for prevention the IB disease that frequently occurred in chickens could partly explained the presence of other serotypes in Iran. One of the isolates recovered from a layer flock exhibited respiratory disorders with the mortality about 5%. This flock received live H_{120} vaccine strain at one day old, thus the possibility of the vaccine strain isolation cannot be excluded. The other four isolates were recovered from broiler flocks with typical signs of IB and the mortality ranged from 5-10% without receiving the IB vaccine. In some of the vaccinated flocks (H_{120} strain) although showing clinical signs and raised antibody titers to IBV, it was not possible to isolate the virus, however were considered positive for IBV. It could be postulated that new emerging variant strain nearly different from Mass strains was

responsible for this disease. Similar findings were also reported previously by Jia *et al* (1995) who showed that point mutations might lead to the generation of IBV variants in the field. However, both circumstantial and experimental evidences suggest that the main mechanism of generation of variant strains of IBV is also by recombination (Cavanagh *et al* 1992, Jia *et al* 1995). Therefore the Massachusetts vaccine strains may not be fully efficacious and this could be promoted by the use of more than one strain of IBV for vaccination. Bacteriological examination showed most of the infected chickens contaminated with *E coli*. However, it is worth mentioning that other avian viral pathogens including avian influenza virus and avian adenovirus with bacteria such as *E.coli*, mycoplasma or other bacteria isolated from some of the flocks were sometimes associated with IBV were contributing to severity the disease problems observed in the field. Although some failures to isolate IB virus may be explained either by the presence of high levels of neutralizing antibody at the time of the virus isolation or by simultaneous other avian viral infections and its poor growth in culture systems. So it is recommended to use the particular biological techniques such as RT-PCR, immunoperoxidase assay (IPA) and indirect immunofluorescent assay (IFA) for rapid detection of IBV (De Wit 2000). The simultaneous presence of vaccine and variant field strains of IBV in the poultry population complicate the diagnostic procedures, efficient and highly sensitive diagnostic tools to distinguish between various strians of IBV is required. RT-PCR, IPA and IFA with monoclonal antibodies have proved useful in the detection and differentiation of IBV strains and evaluating the rapid methods for the diagnosis.

References

Aghakhan, S.M., Abshar, N., Rasoul Nejad Fereidouni, S., Marunesi, C and Khodashenas, M. (1994). Studies on avian viral infections in Iran. *Archives de L,Institute Razi* 44/45:1-10.

Case, J.T., Ardans, A.A., Bolton, D.C. and Reynolds, B.J. (1983). Optimization of parameters for detecting antibodies against infectious bronchitis virus using an enzyme-linked immunosorbent assay: temporal response to vaccination and challenge with live virus. *Avian Diseases* 27:196-210.

Cavanagh, D., Davis, P.J. and Cook, J.K.A. (1992). Infectious bronchitis virus: evidence for recombination within the Massachusetts serotype. *Avian Pathology* 21:401-408.

Cavanagh, D., Naqi, S.A. (1997). Infectious Bronchitis. In: B.W. Calnek, J.H. Barnes, C.W. Beard, L.R. Mcdougald and Y.M. Saif, Jr (Eds), *Diseases of Poultry.* (10th edn.), Pp:511-526. Ames, Iowa, Iowa State University Press.

Darbyshire, J.H., Cook, J.K.A. and Peters, R.W. (1978). Growth comparisons of avian infectious bronchitis virus strains in organ cultures of chicken tissues. *Archives of Virology* 56:317-325.

De Wit, J.J. (2000). Technical review: Detection of infectious bronchitis virus. *Avian Pathology* 29:71-93.

Gelb, J.J., Jackwood, M.W. (1998). Infectious bronchitis. In: D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson and W.M. Reed (Eds), *A Laboratory Manual for the Isolation and Identification of Avian Pathogens* (4rd edn), Pp:169-174. Kennet Square, PA: American Association of Avian Pathologists.

Gelb, J., Wolff, J.B. and Moran, C.A. (1991). Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. *Avian Diseases* 35:82-87.

Mohammad, A.M., Newman, J.A., Sivanandan, V. and Halvorson, D.A. (1988). A dot-immunobinding assay for Infectious bronchitis virus. *Avian Diseases* 32:137-139.

Jia, W., Karaca, K., Parrish, C.R. and Naqi, S.A. (1995). A novel variant of avian infectious bronchitis virus resulting from recombination among three different strains. *Archives of Virology* 140:259-271.

King, D.J., Cavanagh, D. (1991). Infectious Bronchitis. In: B.W. Calnek, H.J. Barnes, C.W. Beard, W.M. Reid and H.W. Yoder, Jr (Eds), *Diseases of Poultry*. (9th edn.), Pp:471-484. Ames, Iowa: *Iowa State University Press*.

Vasfi Marandi, M., Bozorgmehri Fard, M.H. (2000). Isolation and identification of infectious bronchitis viruses in chickens in Iran. *Proceedings of the World's Poultry Congress* 2000, Montreal, Canada/August 20-25.

Wang, C.H., Hsieh, M.C. and Chang, P.C. (1996). Isolation, pathogenicity and H120 protection efficacy of infectious bronchitis viruses isolated in Taiwan. *Avian Diseases* 40:620-625.