Short communication

Serotype Identification of Recent Iranian Isolates of Infectious Bronchitis Virus by Type-Specific Multiplex RT-PCR

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Summary

Fourteen isolates of infectious bronchitis virus (IBV) from Iran in 2001 were typed by a type-specific multiplex RT-PCR. The RT-PCR reaction has been designed to detect and differentiate strains of Massachusetts, D274 and 4/91 (793/B) types. Based on the DNA band produced in RT-PCR, twelve isolates were classified in the type Massachusetts and two isolates (13/2001 and 14/2001) in the type 4/91. The identity of isolate 14/2001, as being from the type 4/91, was also confirmed by sequence analysis of its RT-PCR product. The result of this study shows the presence of at least two types of IBV in Iran.

Key words: infectious bronchitis virus, RT-PCR, sequence, serotype

Introduction

Infectious bronchitis (IB) is an acute and highly contagious viral disease of chickens. Respiratory signs including tracheal rales, coughing and sneezing characterize the disease in young chickens. The kidneys may also be affected and in laying flocks, a drop in egg production and egg quality may occur (Cavanagh & Naqi 1997). Infectious bronchitis virus (IBV), as a member of *Coronaviridae*, has the ability to mutate or change its genetic make up readily. As a result, several new IBV serotypes or variants have been detected, particularly in areas of more intensive poultry

or variants have been detected, particularly in areas of more intensive poultry breeding. Since the currently available vaccines may not provide a sufficient protection against the heterologous serotypes, serotype identification of field strains is necessary in selecting an appropriate vaccine strain for use in future.

So far the antigenic nature of IBV in Iran has not been defined. Aghakhan *et al* (1994) showed a serologic evidence of the presence of Dutch variants of IBV but they did not isolate the virus. By isolation and serologic identification of some IBV field strains, Vasfi Marandi (2000) suggested the presence of IBV variant(s) in Iran, but the antigenic type of the virus(es) was not determined. The present study describes for the first time, the serotype identification of fourteen recent Iranian isolates of IBV, by a type-specific multiplex RT-PCR.

Materials and Methods

Viruses. History of the isolates used in this study is indicated in table 1. The viruses have been all isolated at the faculty of veterinary medicine of Shahid Chamran University, during the year 2001 (Seyfi Abad Shapouri *et al*, in preparation).

Table 1. *History of IBV isolates*

IBV isolate	Source of isolation	Age of chicks (day)	Vaccination with H-120
1/2001	Boushehr	30	yes
2/2001	Ilam	30	?
3/2001	Ilam	50	yes
4/2001	Khorasan	44	yes
5/2001	Sistan	?	?
6/2001	Hormozgan	26	?
7/2001	Kordestan	?	?
8/2001	Hamedan	32	no
9/2001	Markazi	43	yes
10/2001	Markazi	?	?
11/2001	Markazi	30	yes
12/2001	Khouzestan	29	no
13/2001	Markazi	49	yes
14/2001	Khouzestan	38	no

Isolations have been made from the trachea of broiler flocks, with a respiratory disease characterized by coughing, sneezing, tracheal rale, nasal discharges and

elevated moralities. At necropsy, the chickens had shown a light to a moderate hyperemia and exudates in the trachea. In this study H120 (Razi Institute) and 4/91 (Intervet) live attenuated vaccines were also used as the controls.

Multiplex RT-PCR. The viral RNA was extracted from 300µl of IBV-positive allantoic fluids by Tripure (Roche), a commercial RNA extraction solution. The RNA pellet was then dissolved in 4µl of DEPC (Sigma) treated double distilled water and processed for RT-PCR reaction. RNAs extracted from H120 and 4/91 live attenuated vaccines were also used as the controls. A type-specific multiplex RT-PCR was used, as described by Cavanagh *et al* (1999). Oligonucleotide primers used in the reaction were included MCE1+, DCE1+ and BCE1+, respectively specific for a hypervariable region in the S1 genes of serotypes Massachusetts, D274 and 4/91 (793/B), along with primer XCE3-, common for the three serotypes (Adzhar *et al* 1997, Cavanagh *et al* 1999). The primers have been designed to generate cDNAs of 295, 217 and 154bp for the serotypes Massachusetts, D274 and 4/91 (793/B), respectively. The reaction of RT-PCR was performed by a one-tube RT-PCR kit (Titan kit, Roche). Primers were used at the concentration of 40pmol each and cDNA was synthesized at 50°C for 30min. PCR amplification was performed for 35 cycles (94°C 1min, 48°C 1.5min and 68°C 2min) using a Corbet Research thermal cycler. The RT-PCR products were visualized by electrophoresis of 5 to 10µl of each product in a %2 agarose gel, containing ethidium bromide, followed by UV transillumination.

Sequencing. The RT-PCR product of one isolate (14/2001) was also analysed by sequencing. The DNA band of this isolate was purified from the gel by a DNA extraction kit (Roche), dissolved in water at the concentration of 5µg/µl and then sent to the seqlab company (Germany) for sequencing. Sequencing was performed with the same primers used in the RT-PCR.

Results and Discussion

As indicated in figure 1a (lane 2) and 1b (lane 3), reaction of multiplex RT-PCR on the Massachusetts and 4/91 control viruses resulted in the generation of the expected 295 and 154bp DNA bands (Cavanagh *et al* 1999), respectively.

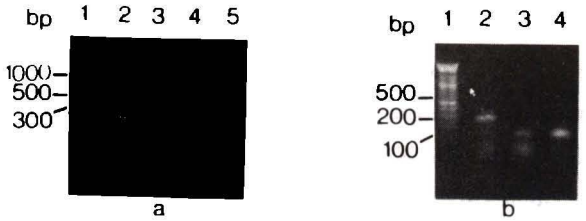

Figure 1. *Multiplex RT-PCR to serotype IBV isolates. In a, lane 1 contains DNA size markers. Lanes 2, 3, 4 and 5 show the results of RT-PCR reaction with RNA from H120 vaccine and isolates 1/2001, 2/2001 and 3/2001 respectively. In b, lane 1 contains DNA size markers and lanes 2, 3 and 4 show RT-PCR reaction with RNA from H120 vaccine, 4/91 vaccine and isolate 14/2001 respectively*

Amplification of the expected bands from the control viruses confirmed that the reaction has been performed correctly. Among the tested isolates, 12 (1/2001-12/2001) isolates produced a DNA band of 295bp, similar to that of the Massachusetts type. Therefore, these were considered as belonging to this serotype. Isolates 13/2001 and 14/2001 generated a 154bp band corresponding to that of the 4/91 type. Hence, these were identified as the isolates of 4/91 type. The RT-PCR products of three isolates of Massachusetts's type and one isolate of 4/91 type are indicated in Figure 1a and b, respectively. None of the isolates produced a DNA band corresponding to that (217 bp) of D274 type. To confirm the identity of isolate 14/2001, as being a virus of 4/91 type, the RT-PCR product of the virus was sequenced and compared with the corresponding sequences in serotypes 4/91 (Adzhar *et al* 1997), D274 (Jordi *et al* 1989), D1466 (Kusters *et al* 1989) and Massachusetts (Cavanagh *et al* 1988). The sequence consisted of 114 bases (primers sequences were excluded) (Fig 2). Comparison of this sequence with the corresponding region in the S1 gene of other serotypes revealed a highest degree of homology with the type 4/91 (Fig 2 and Table 2), confirming the identity of the isolate 14/2001, as a virus of 4/91 type.

The results of this study indicate the presence of 4/91 serotype of IBV in Iran, clearly. It also indicates that among our isolates, Massachusetts serotype is the dominant type of IBV. However, the Massachusetts type isolates could be a result of

the reisolation of vaccine strain. It is also possible that they are mutants of vaccine strains. Further studies are necessary to support this conclusion.

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14/2001 TCTGATTTCA TGTATGGGTC TTATCATCCA AAGTGTAATT TTAGACCAGA  50
4/91    .........T. ..........  A....C...C. ....T..... ..........  50
H-120   .....A.....T. ..........A... .....C..... ..GT..... .....T.....  50
D274    .........AT. .......... .....C...C..... ..GT.....G.. .....T.....  50
D1466   .........T.. CT.....T.... .........AAG GCT..... .....T.....  50

14/2001 GAATATTAAT AATGGCTTAT GGTTTAATTC ATTATCTGTG TCACTTACTT 100
4/91    .......... .......... .......... .......... .......... 100
H-120   A..C..... .....T..G.. .......... ..C..T...A....T .....A....G..... 100
D274    A..C..... .....T...G. .....C..... ..C..T...C.....C .....T.....GG... 100
D1466   A..G..... .....T.....A T.....A.. T.....AG.....T .....TA.....GC.. 100

14/2001 ACGGACCCAT TCAA  114
4/91    .......... .....  114
H-120   .....T..TC. .....  114
D274    .....T..... C.....  114
D1466   ..T.....AC. ..A..G  114
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Figure 2. *Comparison of nucleotide sequence of the RT-PCR product of isolate 14/2001 with the corresponding region in the types 4/91, H-120, D274 and D1466. The sequence for isolate 14/2001 is shown in full and only differences from this sequence shown for the other types*

Table 2. *Comparison of nucleotide sequence of the RT-PCR product of isolates 14/2001 with the corresponding region in the types 4/91, H-120, D274 and D1466*

Type	Percent of difference	Percent of identity
4/91	4.38	95.62
H-120	17.54	82.46
D274	19.29	81.71
D1466	26.31	73.69

The 4/91 type of IBV was first isolated in 1985 in France (Cavanagh *et al* 1998). Then, it spread to many other countries in Europe and was also detected in swabs collected from Saudi Arabia, Japan, Thailand and Mexico (Cook *et al* 1996, Cavanagh *et al* 1999). The pathogenicity of the virus for chickens was first shown in 1990/1991 in Britain (Gough *et al* 1992). As demonstrated by Parson *et al* (1992), Massachusetts type vaccines such as H120 are only partially effective against 4/91. Actually, 4/91 is the major type of IBV in some countries such as the UK. It has been suggested that a 25% amino acid difference between the S1 gene of 4/91 strains and that of the H120 vaccinal strain, commonly used in these country, has contributed to the persistence of the 4/91 serotype (Adzhar *et al* 1997). Hence, 4/91 type vaccine has been commercialized in some countries in Europe.

In Iran, like many other parts of the world, Massachusetts type vaccines are the only live attenuated IBV vaccines in use. If 4/91 type of the virus which can break through immunity induced by Massachusetts vaccines persist in the field, appropriate vaccines and vaccination programs may be necessary. In fact, for preventing the IBV induced losses to the poultry industry, disease surveillance efforts must increase and serotype of the new IBV isolates must regularly be determined. Actually we are virologically studying on many samples obtained from the IB suspected respiratory diseases of broilers. The final results of this investigation will help us to decide for the vaccination strategy against IBV in Iran.

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