INTERACTION BETWEEN GENOMES OF INFECTIOUS BRONCHITIS AND NEWCASTLE DISEASE VIRUSES STUDIED BY REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

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Summary. – Reverse transcription–polymerase chain reaction (RT-PCR) with specific primers for the S1 gene of IBV and for the fusion protein cleavage site of NDV was used for detection of Infectious bronchitis virus (IBV, the family *Coronaviridae*) and Newcastle disease virus (NDV) genomes. The sensitivity of IBV and NDV RT-PCR was $10^{3.7}$ and $10^{3.0}$ EID_{50}, respectively. Although a multiplex RT-PCR could detect and differentiate NDV and IBV genomes present in the same sample, there was a slight inhibition of the IBV PCR if a high amount of NDV genome was present in the sample. To overcome this problem a separate PCR for each virus was used to assess the interaction between vaccine IBV and NDV either inoculated singly or together into chickens. In the group vaccinated with the Newcastle disease (ND) vaccine alone, the viral genome was detected on days 2, 4 and 7 post vaccination (p.v.), while in the chickens given the infectious bronchitis (IB) vaccine alone, the viral genome was detected only on day 4 p.v. In the group inoculated with both vaccine viruses there was a 10^3-fold reduction in the cDNA dilution factor on day 4 p.v. for both IBV and NDV genomes. This demonstrated clearly that when both these vaccines are administered there is a transient reduction in the replication of both viruses, probably due to their competition for the same target epithelial cells in the respiratory tract.

Key words: RT-PCR; Infectious bronchitis virus; Newcastle disease virus; virus interaction; cDNA dilution factor

Introduction

ND and IB are two of the most economically important diseases affecting chickens. In India, NDV infections are ubiquitous and chickens are vaccinated extensively using lentogenic, mesogenic or inactivated vaccine strains (Roy *et al*., 2000). Despite this, ND continues to be a major threat to the Indian poultry industry. In contrast, there are only stray reports of IB in India (Sukumar and Prabhakar, 1993; Rajeswar *et al*., 2002) and there is no information on the serotypes of IBV prevalent.

NDV is frequently isolated from the allantoic cavity of 9–10 day-old embryonated chicken eggs (ECE). Also IBV grows in same culture system. Preferred sites for sampling in chickens for NDV and IBV isolations are also similar, namely the trachea, lungs, caecal tonsils and kidneys. Thus there is likelihood of interaction between these two viruses, *in ovo* during isolation and also *in vivo* during mixed infections or during combined vaccinations. It has been reported that IBV interferes with NDV replication *in ovo* (Raggi *et al*., 1963; Hidalgo and Raggi, 1976) and also *in vivo* (Hanson *et al*., 1956; Thornton and Muskett, 1975). This inhibitory effect of NDV is also used in identifying IBV after initial isolations (Hidalgo *et al*., 1984; Culbillos *et al*., 1991).

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Abbreviations: APV = Avian pneumovirus; ECE = embryonated chicken eggs; HI = hemagglutination inhibition; IB = infectious bronchitis; IBV = Infectious bronchitis virus; ND = Newcastle disease; NDV = Newcastle disease virus; p.i. = post infection; PCR = polymerase chain reaction; p.v. = post vaccination; RT = reverse transcription

In conventional virology, separation of single virus from a mixture of two viruses requires neutralization of the other virus with specific antiserum. This is cumbersome, the exact quantity of the antiserum to be added has to be optimized and hence the entire procedure for field samples may not be practical. Interaction between Avian pneumovirus (APV, the family *Paramyxoviridae*) and IBV posed similar problems since both these viruses are assayed in tracheal organ cultures (Khehra and Jones, 1999). However, molecular techniques like PCR, by virtue of their exquisite specificity, are suitable for detection of viral genomes in their mixture and for studies of their interactions.

A slight disadvantage of PCR is that, until recently, it has been considered only a qualitative test. Nowadays, a quantitative competitive reverse transcription-PCRs has become common in the quantification of viral RNA either by designing an internal standard (Liu *et al.,* 1999) or by using a Taqman fluorogenic probe (Aldous *et al.,* 2001). Wang and Khan (2000) have semi-quantified relative amounts of IBV variants Mass 41 and JMK RNA in their mixture by performing RT-PCR-restriction fragment length polymorphism (RFLP), while Cavanagh *et al.* (1999) used the cDNA dilution factors to quantify the IBV RNA present in the swabs from broilers.

The aim of the present study was to examine the interaction between vaccine IBV and NDV *in vitro* or *in vivo* using multiplex or individual PCRs.

Materials and Methods

Chickens. One-day-old white leghorn chickens were obtained from the Poultry Research Station, Chennai, India. The birds were maintained under clean isolated conditions and fed and watered *ad libitum*. Chickens were randomly selected and checked for the presence of antibodies against IBV and NDV using ELISA and hemagglutination inhibition (HI), respectively.

Viruses. Commercially available IBV H120 and NDV LaSota vaccine strains were used. They were propagated in the allantoic cavity of 10–11 day old ECE obtained from local sources. Yolk samples randomly selected from several batches of eggs were tested by HI test for the presence of antibodies against NDV and by ELISA against IBV. All were found to be negative. Following inoculation, the allantoic fluids were harvested on day 5 post infection (p.i.) for NDV and on day 2 p.i. for IBV. The hemagglutination (HA) titer for NDV and the EID_{50}. titer, based on embryo lesions (stunting of growth and curling of toes, scored on day 5 p.i.), for IBV were calculated (Reed and Muench, 1938).

HI test and ELISA. A standard HI test using 4 HAU of NDV to assess NDV antibody titers on day 21 p.v. was employed. IBV antibody titers were assessed using a commercially available ELISA kit (FlockChek, Iddex, USA). The ELISA results were analyzed according to the manufacturer's instructions.

RT. Total RNA was extracted either from oropharyngeal swabs or from the pellets obtained from 25 ml aliquots of allantoic fluids processed by the method of Chomzynski and Sacchi (1987). RNA was dissolved in 3 µl of water and cDNA was synthesized in a 20 µl reaction mixture using the RevertAid RT kit and random primers (Fermentas, USA) following the manufacturer's instructions.

PCR for the IBV spike protein gene. The cDNA was amplified by PCR according to Cavanagh *et al*. (1999). Briefly, 3.0 µl of the RT reaction mixture was used in PCR (15 µl) consisting of 20 picomoles of positive sense (5'-CACTGGTAATTTTTCAGATGG-3') as well as negative sense (5'-CTCTATAAACACCCTTACA-3') primers, 1.5 µl of the 10x PCR buffer (22.5 mol/l $MgCl_2$, 0.4 mol/l dNTPs, and 0.5 U of Taq polymerase (AB gene, UK)). The PCR consisted of one cycle of 94°C/3 mins (initial denaturation), 30 cycles of 94°C/1 min (denaturation), 48°C/1.5 min (annealing), and 72°C/2 mins (extension), and final extension at 72°C for 7 mins. A 464 bp product in agarose gel (2%) electrophoresis indicated the presence of IBV genome in the sample.

PCR for the NDV fusion protein gene. A 362 bp portion of FPCS of the NDV fusion protein gene was amplified using the positive sense (5'-TTGATGGCAGGCCTCTTGC-3') and the negative sense (5'-GGAGGATGTTGGCAGCATT-3') primers (Kant *et al*., 1997). The PCR was performed in the reaction mixture mentioned above under the following conditions: one cycle of 94°C/3 mins (initial denaturation), 40 cycles of 94°C/1 min (denaturation), 50°C/1 (annealing), and 72°C/1 min (extension), and final extension at 72°C for 7 mins. A 362 bp product in agarose gel (2%) electrophoresis indicated the presence of NDV genome in the sample.

cDNA dilution factor. The cDNAs were initially used in the PCR reaction for single or both viral genomes. If the PCR was positive, original cDNA was diluted tenfold serially and the PCR was done with every cDNA dilution. The highest dilution of cDNA yielding a positive PCR was taken for the cDNA dilution factor.

Sensitivity of RT-PCR for each viral genome. The titrated viruses were diluted tenfold serially in sterile phosphate-buffered saline pH 7.2 (PBS), RNA was extracted from each virus dilution and subjected to RT-PCR. The virus dilution that gave positive RT-PCR was related to the infectivity titer and the sensitivity of RT-PCR was assessed.

Experimental design. cDNA dilution factors. Three groups of 9-11 day-old ECE were inoculated with 10^6 EID_{50} of NDV, with 10^3 EID_{50} of IBV, and with a sham inoculum, respectively. The eggs of each group were chilled at 24 hrs intervals for up to 5 days p.i. The allantoic fluids for each interval were harvested and pooled, and 25 ml aliquots were centrifuged at 35,000 rpm for 2.5 hrs at 4°C in a 70 Ti rotor of Beckman (L765) ultracentrifuge. RNA was extracted from the pellets and used for cDNA synthesis. The cDNAs at different dilutions were subjected to the PCR to assess the cDNA dilution factors.

Experimental design. Interaction between vaccine NDV and IBV in vitro. For simultaneous detection of both viral genomes in the same sample various parameters such as cross-reactivity of primers for the other viral genome, reaction conditions and interaction between different amounts of cDNAs were studied. Cross-reactivity of primers was checked by using the cDNA prepared from IBV RNA with FPCS gene primers of NDV and by performing PCR. Similarly the cDNA obtained from NDV RNA was mixed with the S1 gene primers of IBV and PCR was performed. To determine optimum conditions for PCR that could amplify both

viral genomes various combinations of annealing temperature, amount of the enzyme, polymerase extension time, primer ratio, concentrations of RNA template and $MgCl_2$ were tested. NDV and IBV cDNAs of known cDNA dilution factors were mixed together in various concentrations and the PCR was performed.

Experimental design. Interaction between vaccine NDV and IBV in vivo. One-day-old layer chickens were divided into four groups with minimum of 15 birds in each group. Group I chickens were inoculated with 10^6 EID_{50} of NDV LaSota strain vaccine in 100 µl, Group II with 10^3 EID_{50} of IBV Mass 41 strain vaccine, Group III with 10^6 EID_{50} of NDV LaSota strain vaccine and with 10^3 EID_{50} of IBV Mass 41 strain vaccine. Group IV chickens were kept as an uninoculated control. The vaccines were given intraocularly and intranasally on day 4 days of age. Oro-pharyngeal swabs from all the vaccinated and control birds were collected on days 2, 4, 7, 10, and 14 p.v. Ten swabs from each group were pooled and RNA was extracted. After RT the cDNAs were subjected to PCR as follows. The cDNA from Group I was used in the PCR for NDV and those from Group II in the PCR for IBV. The cDNA from Group III was used in the PCRs for IBV and NDV individually. The cDNA from Group IV was also used in the PCRs for IBV and NDV individually. The cDNA dilution factor was determined only for PCR-positive samples. The extracts from swabs collected on day 4 and 7 p.v. from Group III was inoculated into 10-day-old ECE. The allantoic fluids were collected on day 3 p.i. and used after ultracentrifugation in the PCR for IBV and NDV individually. The birds were bled on day 21 p.v. and the samples were tested for NDV and IBV antibodies using the HI test and ELISA, respectively.

Results

A 464 bp product of a part of the S1 spike protein gene of IBV was amplified by RT-PCR from the allantoic fluids harvested from ECE inoculated with IBV (Fig. 1, lane 1). A 362 bp part of FPCS of the NDV fusion protein gene was amplified by RT-PCR from the allantoic fluids harvested from ECE inoculated with NDV (Fig. 1, lane 2). The detection limit of individual RT-PCRs for NDV and IBV

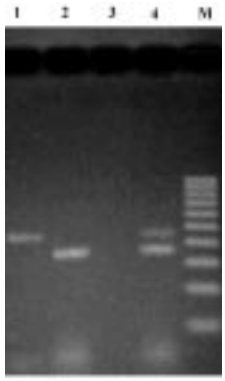

Fig. 1

Agarose gel electrophoresis of PCR products

IBV genome band of 464 bp in the IBV-specific PCR (lane 1), NDV genome band of 362 bp in the NDV-specific PCR (lane 2), both IBV and NDV genome bands in the multiplex PCR (lane 4), negative control (lane 3), and a 100 bp ladder.

Table 1. cDNA dilution factors of the cDNAs prepared from the RNAs isolated from the allantoic fluids of ECE inoculated with NDV or IBV

Days p.i.	cDNA dilution factors	
	ECE inoculated with NDV	ECE inoculate with IBV
1	10^{-3}	10^{-2}
2	10^{-3}	10^{-3}
3	10^{-4}	10^{-3}
4	10^{-4}	10^{-2}
5	10^{-5}	ND

ND = not done.

determined in relation to the virus titers was $10^{3.0}$ EID_{50} and $10^{3.6}$ EID_{50} respectively.

cDNA dilution factors

In order to ascertain whether the cDNA dilution factor is a true reflection of viral RNA present in the sample, the cDNA dilution factors were estimated for allantoic fluids collected at 24 hr intervals from NDV- and IBV-infected ECE. A 362 bp band for NDV was obtained from the allantoic fluids collected on all five days and a 464 bp band for IBV was obtained from the allantoic fluids collected on all four days (the collection on the 5th day was not done). The cDNA dilution factors of all the positive samples for both NDV and IBV are shown in Table 1. The highest cDNA dilution factor of 10^{-5} was obtained for NDV on day 5 p.i. and 10^{-3} for IBV on days 2 and 3 p.i. The PCR for NDV is about 100 times more sensitive than that for IBV.

Interaction between NDV and IBV vaccines in vitro

The IBV and NDV genome primers did not cross-react i.e. the IBV primers did not amplify any part of NDV genome and *vice versa*. The primers were specific for their respective genomes in a mixture of IBV and NDV genomes. Positive bands (Fig. 1, lane 4) for IBV and NDV genomes were obtained in the multiplex PCR when the following PCR conditions were used: 3 µl of RT reaction mixture containing cDNA with 20 picomoles of both primer pairs, and 1.5 µl of 10x PCR buffer in a total volume of 15 µl. The cycling conditions consisted of one cycle of 94°C/3 mins (initial denaturation), 35 cycles of 94°C/1 min (denaturation), 48°C/1 min (annealing), and 72°C/1.5 min (extension) and final extension at 72°C for 7 mins. Table 2 shows the results obtained with different quantities of cDNA in terms of cDNA dilution factors mixed together in the multiplex PCR. Only with the NDV cDNA amount 100-fold lower than that of IBV cDNA the multiplex PCR gave both bands of equal intensity (Fig. 3, lane 4).

Table 2. Effect of various concentrations of NDV cDNA and IBV cDNA on the intensity of the respective PCR bands in multiplex PCR

cDNA dilution factors		Intensity of the respective bands in multiplex PCR	
IBV cDNA	NDV cDNA	IBV	NDV
10^{-3}	10^{-3}	–	+++
10^{-3}	10^{-3}	+	+++
10^{-3}	10^{-2}	++	+++
10^{-3}	10^{-1}	+++	+++

(–) = no PCR product.
(+) = faint band.
(++) = band of medium intensity.
(+++) = band of strong intensity

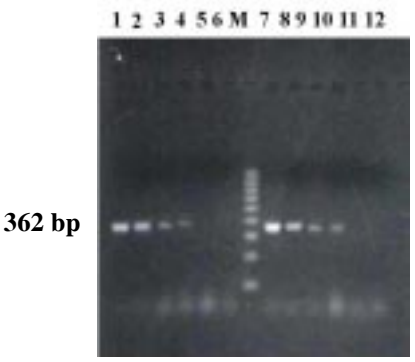

Fig. 2

Determination of cDNA dilution factors

Agarose gel electrophoresis. The 362 bp PCR product from undiluted, diluted 10^{-1}, 10^{-2} ,10^{-3} ,10^{-4} and 10^{-5} NDV cDNA (lanes 1–6) prepared from the RNA extracted from the allantoic fluid collected on day 1 p.i. from ECE inoculated with NDV. The 362 bp PCR product from undiluted, diluted 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} NDV cDNA (lanes 7–12) prepared from the RNA extracted from the allantoic fluid collected on day 2 p.i. from ECE inoculated with NDV. A 100 bp ladder (lane M).

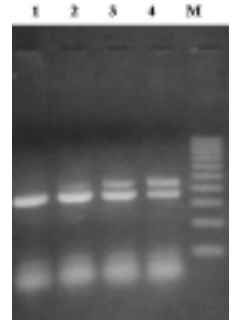

Fig. 3

Effect of the interaction between the quantities of IBV cDNA and NDV cDNA on the intensity of the respective bands in the multiplex PCR

See also Table 1. Agarose gel electrophoresis. Dilution factors of 10^{-3} for IBV cDNA and 10^{-4} for NDV cDNA (lane 1). Dilution factor of 10^{-3} for IBV cDNA as well as NDV cDNA (lane 2). Dilution factors of 10^{-3} for IBV cDNA and 10^{-2} for NDV cDNA (lane 3). Dilution factors of 10^{-3} for IBV cDNA and 10^{-1} for NDV cDNA (lane 4). A 100 bp ladder (lane M). Note bands of equal intensity in lane 4.

Interaction between vaccine NDV and IBV in vivo

The interaction between IBV and NDV was studied *in vivo* by either single or simultaneous inoculation of NDV and IBV vaccines into 4-day-old chickens. At the time of inoculation the random samples of sera collected from chickens showed a mean $\log_2$ HI titer ± SD of 1.25 ± 0.43 for NDV and a negative ELISA titer for IBV.

The PCR results along with the cDNA dilution factors for the PCR positive samples of the *in-vivo* experiment are shown in Table 3. A positive PCR band for NDV was obtained from Group I birds on days 2, 4 and 7 but not on days 10 and 14 p.v. A positive PCR band for IBV was obtained only on day 4 p.v. from Group II birds. All the other day's samplings were negative. In Group III birds, using PCR for NDV, positive bands were obtained only on days 2 and 7 p.v., while no positive band was obtained for IBV on any of the days tested.

Table 3. PCR results of individual swabs collected from chickens vaccinated with NDV and IBV either alone or in combination

Groups		Days p.v.				
		2	4	7	10	14
Group I (NDV alone)		+ (10^{-3})	+ (10^{-3})	+ (10^{-3})	–	–
Group II (IBV alone)		–	+ (10^{-3})	–	–	–
Group III (IBV + NDV)	IBV	–	+[a]	–	–	–
	NDV	+ (10^{-3})	+[a]	+ (10^{-2})	–	–
Group IV (NDV PCR + IBV PCR)		–	–	–	–	–

[a]Positive only when the swabs were passaged in ECE and the allantoic fluids collected on day 3 p.i. were used in PCRs. PCR amplification using swab samples directly was negative.
The cDNA dilution factors are shown in parentheses.

Table 4. Seroconversion (mean ± SD) against NDV and IBV using HI test and ELISA in chickens vaccinated either individually or in combination

	Group I (NDV alone)	Group II (IBV alone)	Group III (NDV + IBV)	Control
HI titer for NDV[a] (n = 10)	4.25 ± 1.18	< 1.0	3.43 ± 0.49	< 1.0
ELISA titer for IBV[b] (n = 7)	38.67 ± 27.7	524 ± 199.5	481.7 ± 212.1	77.33 ± 35.21

[a]$\log_2$ HI titer using 4 HAU of NDV.
[b]ELISA titer calculated using the Iddex software (USA)

No positive bands were obtained either for IBV or NDV from the control chickens on any day.

The swabs collected on days 4 and 7 p.v. were inoculated into ECE in order to amplify any virus present in those samples. The allantoic fluids collected on day 3 p.i. were used in the PCR for NDV and IBV separately. The PCR for NDV was positive with samples collected on days 4 and 7 p.v., while the PCR for IBV was positive only with the sample collected on day 4 p.v.

The seroconversion to NDV and IBV measured by HI test and ELISA are shown in Table 4. No significant differences ($p > 0.05$) were seen in HI and ELISA titers between groups vaccinated either singly or together for the respective vaccine.

Discussion

The present study was undertaken as it was felt that the number of IBV isolations in India is low probably due to concomitant presence of NDV in chickens. During isolation procedures NDV always emerges as a dominant virus especially after a couple of passages in ECE. This occurs despite the fact that IBV inhibits NDV replication. Hence it was thought that the genome detection methods such as PCR could be the answer for detection of both viral genomes individually. Such a method would reveal the true extent of IBV infections in India.

The IBV PCR used in this study amplified the S_1 part of the spike protein gene, which is the major immunogenic protein that gives serotype specificity to IBV. For NDV, FPCS was amplified as a sequence that determines the pathotype of NDV. Both these PCRs were optimized for individual viruses, giving PCR products of 464 bp and 362 bp for IBV and NDV genomes, respectively. The sensitivity of the RT-PCR for IBV and NDV was $10^{3.6}$ and $10^{3.0}$ respectively. The sensitivity reported for NDV PCR in an earlier study by Gohm *et al*. (2000) was $10^{2.7}$ EID_{50}. The sensitivities measured in terms of quantity of RNA were found to be 5–10 pg for IBV and 100 pg for NDV (Pang *et al*., 2002; Wang and Khan, 1999). The sensitivity of PCR is largely governed by the sequence of the primers (Kim *et al*., 2001). In this study the primer choice was governed largely by the region to be amplified that is S1 gene of IBV and FPCS of NDV.

The estimation of cDNA dilution factor is a relatively simple technique to quantify the amount of RNA present in the initial sample, which in turn reflects the presence of virus (although non-infectious viruses would also be positive by PCR). This method has been successfully used to quantify the amount of IBV and avian pneumovirus-specific RNA from vaccinated chickens (Cavanagh *et al*., 1999). Other sophisticated methods such as real time PCR using Taqman probe (Aldous *et al*., 2001) have now transformed the PCR technique into a quantitative method.

In IBV-inoculated eggs, the IBV genome was detected on all days with maximum cDNA dilution of 10^{-3} on days 2 and 3. This is in accordance with the earlier reports stating that for propagation of IBV the allantoic fluids have to be harvested at 36 to 48 hrs p.i. as virus titers are highest at this time (Darbyshire *et al*., 1975) despite the fact that embryo lesions are detectable only at day 5 p.i. With NDV, the maximum cDNA dilution factor (10^{-5}) was obtained on day 5 p.i. This again corresponds to the routine procedure of collecting allantoic fluids following NDV inoculation on that day. Thus the determination of cDNA dilution factor appears to be a true reflection of the amount of virus-specific RNA and in turn the number of virus particles.

In the multiplex RT-PCR, optimization of the annealing temperature, amount of enzyme, polymerase extension time, primer ratio, and concentrations of $MgCl_2$ and cDNA template are necessary for obtaining positive PCR products for the genomes to be detected. In general, it has been found that the sensitivity of multiplex PCR is lower than that of individual PCR (Johnson *et al*., 2000). This is because of the compromise made with respect to the above parameters, e.g. thermal cycling conditions.

It was found that only when the amount of NDV cDNA given into PCR was 100 times lower than that of IBV, both the IBV and NDV bands were equal in intensity. When both cDNAs were in equal amounts, only a very faint IBV band was seen. This would also probably reflect the slightly lower sensitivity of the IBV PCR. In other words, in a multiplex PCR using the above set of primers and PCR conditions, the NDV detection overwhelmed the IBV detection even if equal amounts of NDV and IBV RNA were present. It means

that there was an inhibition of IBV PCR if a high amount of NDV genome was present in the sample. To overcome this problem, individual PCRs were performed to assess their interactions *in vivo*. Further studies are needed to reduce the inhibition of NDV genome in detection of IBV by testing different sets of primers before the application of multiplex PCR for simultaneous detection of both the viral genomes in field samples.

In the *in vivo* experiment pooled swabs from 10 birds were used for RNA extraction followed by RT-PCR. Since the birds were 4-days-old at the time of inoculation, it was difficult to collect tracheal swabs. Hence oro-pharyangeal swabs were collected. This method has been successfully used for IBV genome detection by Cavanagh *et al*. (1999) and for APV genome detection by Cook *et al*. (2001).

In the group I inoculated with NDV vaccine alone PCR was positive on days 2,4 and 7 p.v. Similar times of virus isolation following infection of chickens with NDV have been reported (Parede and Young, 1990). Following IBV vaccination (Group II), PCR was positive only on day 4 p.v. Longitudinal field studies on broilers using PCR showed that NDV Mass 41 was present only during the first week of the bird's life although not in all the flocks (Cavanagh *et al*., 1999). Normally, the isolation of IBV following experimental infection was limited to the first 7 days p.i. (Raj and Jones, 1997). Combined vaccination did not yield any PCR products for IBV while NDV gave positive bands on days 2 and 7 p.v. The swabs collected on days 4 and 7 were passaged once in ECE and PCR done with allantoic fluids. This would have amplified the virus present in the original swabs. The allantoic fluid gave positive PCR for both NDV and IBV on day 4 p.v., which reflects the presence of NDV and IBV (or viral RNAs) below the detection limit of PCR in the original swab samples.

It is interesting to note that on day 4 p.v. in Group III, the NDV PCR (before egg passage) was negative while NDV cDNA had a dilution factor of 10^{-3} on the same day in Group I. This clearly indicates a transient inhibition of NDV replication in Group III, although virus (viral RNA) was present following propagation in ECE on that day. Even the IBV PCR was negative on day 4 p.v. in the combined group, while the 10^{-3} cDNA dilution factor was observed for IBV alone (Group II). Again IBV (RNA) was positive on this day when the swabs were propagated in ECE. Thus it appears that NDV as well as IBV replication was reduced below the threshold of detection limit of the respective PCR on day 4 in the combined group since both the viruses were probably competing for same target cells. Moreover, IBV is a positive-strand RNA virus, while NDV is a negative-strand RNA virus. Hence it is possible that IBV starts replicating first and inhibits the NDV replication, as has been reported earlier (Thornton and Muskett, 1975). However, because of the slightly lower sensitivity of the IBV PCR compared to the NDV PCR both the viral genomes were not detected on day 4 p.v. in the combined vaccinated group.

In an earlier study by Schrier and Cornelissen (1998) it has been shown that IBV H120 strain and NDV 30 strain, when used as a combined vaccine, protected against IBV challenge not sufficiently. Thirty % of the birds showed clinical signs post challenge and 42% birds showed ciliostasis. Following NDV challenge, only 83% of birds were protected. However, birds vaccinated with IBV Ma 5 strain and NDV clone 30 combined vaccine showed 100% protection to both IBV and NDV challenge. This further emphasizes the fact that any two different virus strains or different viruses cannot be used for combined live vaccines unless they are compatible.

The seroconversion results revealed no significant reduction in HI titer for NDV and ELISA titer for IBV in the group inoculated with combined vaccine as compared with the respective individual vaccinated groups. This may indicate that although the propagation of both vaccine viruses was reduced on day 4 p.v. the propagation was still sufficient to elicit immune responses to the level of single vaccination group. Also Cook *et al*. (2001) have observed that in combined APV and IBV vaccination IBV reduced APV antibody titers although this reduction did not affect protective immunity.

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