Veterinaria Italiana, 2011, **47** (4), 461-468

Impact of competitive non-protective antigens in a booster killed vaccine on seroconversions to protective antigens of Newcastle disease virus in chickens

Elie K. Barbour[(1)], Hussein M. Halawi[(1)], Houssam A. Shaib[(1)], Lina S. Jaber[(1)] & Steve Harakeh[(2)]

Summary

This study was designed to examine the impact of competitive non-protective antigens in a bivalent killed vaccine of Newcastle disease virus and infectious bronchitis (IB) virus on seroconversions against protective fusion protein of Newcastle disease (ND) virus (NDV), in free-range layers primed by live ND-clone 30 and IB-H120 vaccines. The experimental design included two free-range layer farms in which sera of randomly chosen layers were collected on two occasions from each of the two farms namely: at the time of administration of the killed booster vaccine (23 weeks of age) and three weeks later. The Western immunoblotting technique was used to react the individual pooled sera collected at different times from each farm with antigens used in priming, namely those of the ND-clone 30 virus and the IB-H120 virus. The optical density bands formed by reactions were compared statistically between seroconverted antibodies at 23 weeks with those of layers aged 26 weeks. The killed booster vaccine offered a significant seroconversion on both farms to the non-protective L-protein (248.0 kDa) of NDV and on one of the two farms to the non-protective NDV-matrix protein (40.0 kDa) (p<0.05). However, seroconversion to the protective fusion protein of NDV (60 kDa) failed on both farms (p<0.05). In addition, on one farm, a statistical significance was revealed by the killed booster vaccine seroconversion to non-protective IBV-nucleocapsid protein (510 kDa) and, on the other farm, to another non-protective IBV-glycoprotein (28.0 kDa) (p<0.05). The impact of competitive seroconversions to non-protective antigens and seroconversion failures to low molecular weights of NDV protective fusion protein is discussed.

Keywords

Antigen, Chicken, Competition, Free-range chicken, Infectious bronchitis, Killed booster vaccine, Layer, Newcastle disease, Protective antigen, Seroconversion, Vaccine, Virus.

Impatto di antigeni non protettivi competitivi in un richiamo inattivato sulle sieroconversioni in antigeni protettivi del virus della malattia di Newcastle nei polli

Riassunto

Questo studio ha esaminato l'impatto di antigeni non protettivi competitivi in un richiamo bivalente inattivato del virus della malattia di Newcastle (NDV) e della bronchite infettiva (IB) sulle sieroconversioni dirette contro la proteina di

(1) Animal and Veterinary Sciences Department, Faculty of Agricultural and Food Sciences, American University of Beirut, PO Box 11-0236, Riad El Solh 1107-2020, Beirut, Lebanon
eb01@aub.edu.lb
(2) King Fahd Medical Research Center; King Abdul Aziz University, P.O.Box is 80200, Jeddah 21589; Saudi Arabia

fusione protettiva dell'NDV in galline ovaiole inoculate con i vaccini vivi ND Clone 30 e IB-H120. La sperimentale prevedeva il prelievo di siero da esemplari scelti con modalità random in due fattorie che praticano l'allevamento a terra, in due diverse occasioni da ciascuna delle due fattorie: al momento della somministrazione del richiamo inattivato (età: 23 settimane) e a distanza di 3 settimane. La tecnica di analisi Western blot è stata usata per indurre la reazione dei sieri raccolti in momenti diversi da ciascuna fattoria con gli antigeni utilizzati nel priming, ovvero quelli del virus ND Clone 30 e IB-H120. Sono state analizzate le bande di densità ottica formate dalle reazioni confrontando gli anticorpi sieroconvertiti a 23 settimane con quelli di esemplari di 26 settimane. In entrambe le fattorie, il richiamo ha permesso di ottenere una significativa sieroconversione nella L-proteina non protettiva (248,0 kDa) dell'NDV e, in una fattoria, nella proteina di matrice NDV non protettiva (40,0 kDa) (p<0,05). Tuttavia, la sieroconversione nella proteina di fusione protettiva dell'NDV (60 kDa) non è stata osservata in nessuna delle due fattorie (p<0,05). Inoltre, in una fattoria, la sieroconversione in proteina nucleocapside di IBV è risultata statisticamente significativa (510 kDa), mentre nell'altra fattoria è risultata significativa la sieroconversione in un'altra glicoproteina non protettiva dell'IBV (28.0 kDa). Questo contributo discute l'impatto delle sieroconversioni competitive in antigeni non protettivi e i fallimenti delle sieroconversioni a basso peso molecolare nella proteina di fusione protettiva dell'NDV.

Parole chiave
Antigene, Antigene protettivo, Bronchite infettiva, Competizione, Galline ovaiole, Malattia di Newcastle, Polli di allevamento a terra, Popolazione aviaria, Richiamo inattivato, Sieroconversione, Vaccino, Virus.

Introduction

Newcastle disease (ND) and infectious bronchitis (IB) are the most common viral diseases that significantly affect the economy of egg-type commercial layer farming around the world (6, 20). The economic threat of ND and IB in free-range layer farming is of greater significance due to the increased level of exposure of these birds to various vectors in nature (3).

In the control of ND and IB, it is common to prime birds with live vaccines between 1 day to 60 days of age (2, 9), concluding the vaccination programme with a delivery at about 23 weeks of age of a killed booster vaccine containing whole antigens of ND virus (NDV) and IB virus (IBV).

Most evaluations of acquired immunity to NDV and IBV usually depend on tests for detection and quantitation of humoral antibodies specific to the whole proteins of the two viruses using commercial enzyme-linked immunosorbent immunoassays (ELISAs) (10, 17).

Such classical quantitation relied upon by most poultry specialists around the world can be misleading, in that non-protective-induced humoral antibodies cannot be differentiated from antibodies raised against protective antigens, thereby resulting in false inter-pretation of correlation of the antibody titres to protection, which in turn leads to failures of many costly programmes implemented to control ND and IB among chicken layers.

Research in the past two decades has focused on identifying the protective antigens in NDV and IBV (15, 16) that are involved in replication of the two viruses and in identifying their role in the pathogenesis of ND and IB (2, 9). Among the major protective antigens of NDV is the F-protein (60.0 kDa) that is involved in enabling cell fusion activity which leads to the penetration of NDV within the host cell (11). The L-protein (248 kDa) and the matrix M-protein (40.0 kDa) have been recognised as non-protective proteins that are present in the NDV (21, 26).

Researchers have also identified two non-protective antigens of IBV, among which are the nucleocapsid protein (N-protein) (51.0 kDa) which is a structural protein (8) and the membrane glycoprotein (GP 28.0 kDa) (23).

Unfortunately, most commercialised poultry diagnostics do not include in their developed kits selective quantitative procedures for acquired humoral immunity against specific protective antigens of aetiological agents (7).

This is the first study that presents a preliminary approach for the essential assessment of quantitative seroconversion to fusion protein of NDV in free-range layers, as affected by competitive immune responses to non-protective proteins present in NDV and IBV vaccines.

Materials and methods

Vaccination and sera collection

Two out of 60 free-range layer farms were randomly selected to study the impact of NDV and IBV non-protective antigens on sero-conversions against the protective F-protein of ND, induced by a booster-killed vaccine following a priming by live vaccines against ND and IB. The priming with live ND clone vaccine and IB-H120 was intraocular at 8 days of age, and repeated in drinking water at 25 days of age. The booster-killed vaccine (Mérial, Chignolo-Po) was delivered at 23 weeks of age, before the onset of egg production. This killed vaccine contained the whole antigens of NDV and IBV.

Paired serum samples were collected from the brachial vein of layers on both farms. Ten randomly chosen layers were bled at 23 weeks of age (booster time), and another 10 were bled at 26 weeks of age (seroconversion time) (1, 2), a sample size that represented the 500 birds that compose each of the free range flocks (22). The serum samples collected from each farm were pooled in equal volumes of 100 µl/individual sample, totalling 1 ml/farm/collection time, representing a pool of flock antibodies with various idiotype specificities (18). Each pooled serum sample was diluted 1:1, using sterile glycerol and stored at –20°C for use in Western immunoblotting to help identify the idiotype specificity to various antigens of NDV and IBV (1).

Sodium-dodecylsulfate-polyacrylamide gel electrophoresis in banding Newcastle disease virus and infectious bronchitis viral antigens

The ND clone 30 and IB-H120 vaccines (Nobilis®, Intervet International BV, Boxmeer) were subjected to sodium-dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to band their major antigens (4, 14). Briefly, the lyophilised content of 1 000 doses-vial of NDV and IBV vaccines were each suspended in 2 ml of sterile distilled water, yielding a protein level of 36.8 mg/ml and 20.3 mg/ml, respectively, using the Bio-Rad reagents for protein determination (Bio-Rad Laboratories, Hercules, California). Each suspension was diluted 1:2 in SDS-reducing sample buffer. This diluent was prepared by mixing 25 µl of β-mercaptoethanol and 475 µl stock sample buffer. The concentrations of the components of the stock sample buffer were 1.2 ml of 0.5 M Tris-HCL, 1 ml glycerol, 2 ml of 10% SDS, 0.5 ml of 0.1% bromophenol blue, with the addition of 4.8 ml of distilled water. The 1:2 dilution of each vaccine suspension was then heated at 95°C for 5 min. A volume of 20 µl of each reduced vaccine was applied on each of 4 lanes at the top of a 4% stacking gel that was located over a 12% separating gel. The 5th lane was loaded with 5 µl of Kaleidoscope prestained molecular weight markers, with a broad range of 6.5-200 kDa (Bio-Rad Laboratories). The Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories) was run at 200 volts and 120 mA for a period of 45 min. The two gels were kept at 4°C in a transfer buffer, to be used later in Western immunoblotting. One gel contained the quadruplicate lanes of banded antigens of NDV and the fifth lane of the banded molecular weight markers, while the second gel contained the quadruplicate lanes of banded IBV antigens, and the fifth lane of the banded markers.

Western immunoblotting

The Western immunoblotting technique was used to react the collected sera from the two free-range farms with the banded antigens of ND clone 30 and IB-H120 vaccines that were both used in priming the layers during the rearing stage. Briefly, each of the two gels was sandwiched to a nitrocellulose membrane (NCM) and set to a Transblot Cell (Bio-Rad Laboratories) that was already filled with a transfer buffer. The composition of the transfer buffer was as follows: 6 g Tris-base, 28.8 g glycine, 400 ml methanol, adjusted in volume

to 2 000 ml with distilled water, and to a pH of 8.4. The transfer time was 1 h at 100 V and 25 mA. Each lane on the NCM was separated from the others by cutting, followed by blocking with 5% gelatin in Tris buffer saline. The molecular weight markers were stained with the Ponceau S Stain (Sigma, St Louis, Missouri), while the others containing NDV or IBV antigens were reacted to each of the pooled sera (two paired pooled sera samples on farm 1 and two paired pooled sera samples on farm 2). Each pooled serum sample was diluted 1:100 before reaction with the antigens on the cut lane of the NCM, using a 1% gelatine in Tris-Tween-buffer saline diluent as described by Towbin *et al.* (25). The reaction of the sera and antigens lasted 2 h at 37°C. The conjugate used to detect the captured chicken antibodies was a goat anti-chicken IgG (H+L chains), labelled with peroxidase enzyme (Sigma, St Louis), used in a 1:1 000 dilution and reacted to NCM lanes at 37°C for 30 min. The substrate used was 3,3′-diaminobenzidine (DAB) peroxidase substrate solution (Sigma, St Louis) reacted to NCM for 30 min at 37°C.

Documentation and quantitative assessment

The documentation of the colour of bands that developed due to the reaction of specific antibodies in the pooled paired sera on each farm to the specific antigens of NDV and IBV was accomplished using a GelDoc 2000 System (Bio-Rad Laboratories) as shown in Figures 1 and 2.

The quantitation of captured antibodies reacting to each banded antigen on the NCM was measured by reading the optical densities (OD) of each band using a computerised program namely, the quantity One 1-D Analysis Software (Bio-Rad Laboratories). Briefly, three areas on each band were randomly selected and the mean OD values were obtained for the band.

Statistics

The one-way analysis of variance (ANOVA) was applied to compare the mean optical density of the band capturing the seroconverted antibodies in sera collected 3 weeks post administration of the booster (layers at 26 weeks of age) in comparison to the acquired primed antibodies specific to the same molecular weight band, obtained at the administration time of the booster (layers at 23 weeks of age). This comparison is done for each farm and for each specified antigen in NDV and IBV.

Results

The qualitative seroconversions by the booster-killed vaccine in birds of each of the two free-range layer farms, against the protective and non-protective antigens of NDV, is documented in the image presented in Figure 1. The priming by the live ND clone 30 on both farms was capable of maintaining antibodies in the sera until the booster time, which were captured by three banded antigens of NDV located at 248.0 (L-protein), 60.0 (F-protein) and 40.0 (M-protein) kDa, as shown in lanes 2 (farm 2) and lane 4 (farm 1). Reacting the sera collected at three weeks following the booster (layers at 26 weeks of age), some NDV

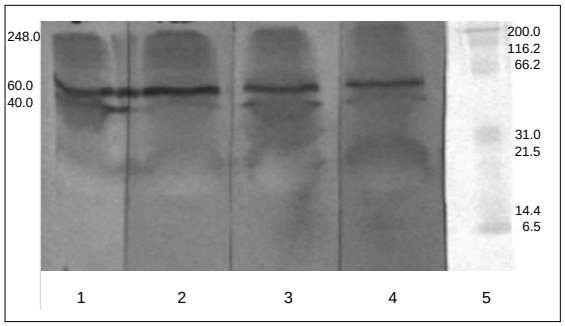

Figure 1
Qualitative seroconversions to a booster-killed vaccine on two free-range layer farms against Newcastle disease virus antigens present in Newcastle disease clone 30 vaccine (L-protein: 248 kDa, F-protein: 60 kDa and matrix M-protein: 40 kDa)
Lane 1: Reaction of 10 pooled sera from layers on farm 2 collected 3 weeks post administration of killed vaccine booster
Lane 2: Reaction of 10 pooled sera from layers on farm 2 collected at the time of booster administration
Lane 3: Reaction of 10 pooled sera from layers on farm 1 collected 3 weeks post administration of killed vaccine booster
Lane 4: Reaction of 10 pooled sera from layers on farm 1 collected at the time of booster administration
Lane 5: Molecular weight markers (kDa)
Banded antigens are those of live Newcastle disease clone 30 vaccine

bands were more intensified in colour, while others faded (lane 1 for farm 2 and lane 3 for farm 1), which required a quantitative assessment for this seroconversion to antigens of NDV, as documented in Table I. The statistical analysis of the quantitative seroconversions on each farm, to each of the three antigens of NDV, revealed consistent and significant seroconversion in birds of both farms to the non-protective L-protein of NDV (248.0 kDa) ($p<0.05$) and inconsistent seroconversion to the non-protective M-protein (40.0 kDa) on farm 2 (significant seroconversion, $p<0.05$) and farm 1 (insignificant seroconversion, $p>0.05$) and a complete failure of seroconversion to the protective F-protein of NDV (60.0 kDa) in birds from both farms ($p>0.05$).

The qualitative seroconversion by the booster-killed vaccine in birds of each of the two free-range layer farms against the IBV antigens, is documented in the image presented in Figure 2. The priming by live IB-H120 vaccine was able to maintain specific antibodies in the sera of the birds from the two farms at 23 weeks of age, the age at which the booster-killed vaccine was administered. This is shown in lanes 4 and 2 of farms 1 and 2, respectively. These antibodies were captured onto the non-protective antigens of the IBV namely, the nucleocapsid protein (51.0 kDa) and the

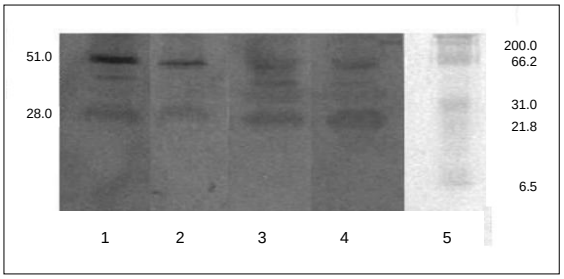

Figure 2
Qualitative seroconversions to a booster-killed vaccine on two free-range layer farms against infectious bronchitis virus antigens present in H120 vaccine (nucleocapsid protein: 51 kDa and membrane glycoprotein: GP 28 kDa)
Lane 1: Reaction of 10 pooled sera from layers on farm 2 collected 3 weeks post administration of killed vaccine booster
Lane 2: Reaction of 10 pooled sera from layers on farm 2 collected at the time of booster administration
Lane 3: Reaction of 10 pooled sera from layers on farm 1 collected 3 weeks post administration of killed vaccine booster
Lane 4: Reaction of 10 pooled sera from layers on farm 1 collected at the time of booster administration
Lane 5: Molecular weight markers (kDa)
Banded antigens are those of live infectious bronchitis H120 vaccine

Table I
Quantitative seroconversions to a booster-killed vaccine on two free range layer farms against NDV antigens (L-protein: 248 kDa, F-protein: 60 KDA and matrix M-protein: 40 kDa)

Antigens of Newcastle disease virus antigens (kDa)	Farm No.(a)	Mean optical density of bands(b) resulting from reaction of 10 pooled sera to specific antigens of Newcastle disease clone 30 when sera were collected upon administration of booster and 3 weeks later		SEM(c)
		At vaccination (23 weeks of age)	3 weeks post vaccination (26 weeks of age)	
L-protein	1	3.92	4.76	0.19*
(248.0 kDa)	2	3.47	4.95	0.34*
F-protein	1	4.66	4.74	0.05
(60.0 kDa)	2	5.55	4.73	0.20
Matrix protein	1	3.71	3.85	0.06
(40.0 kDa)	2	3.46	4.23	0.20*

(a) 10 sera were randomly collected from 10 layers on each of two farms, at vaccination time (23 weeks of age) and 3 weeks later (26 weeks of age)
Equal volumes of sera from each collection on each farm were pooled together.

(b) optical densities were averaged from pixels read at three different randomly selected areas of each band

(c) SEM: the standard error of mean is derived from statistical comparisons of the mean optical density of the two respective bands obtained from sera collected on the same farm at 23 versus 26 weeks of age

* indicates a significant difference in the mean optical density of the bands compared in a row ($p<0.05$

membrane glycoprotein (28.0 kDa). The seroconversion on both farms also resulted in antibodies that were captured to the same non-protective antigens, as shown for farms 2 and 1 in lanes 1 and 3, respectively (Fig. 2). Again, the quantitative comparison of the colour intensity (OD) values is presented in Table II. The seroconversion by the booster-killed vaccine to each of the two non-protective antigens of IBV was inconsistent between the birds from the two farms; more specifically, birds on farm 1 revealed significant seroconversion to the nucleocapsid protein (51.0 kDa) ($p<0.05$) but not birds from farm 2 ($p>0.05$); however, birds from farm 2 showed significant seroconversion to the glycoprotein (28.0 kDa) ($p<0.05$) but not those from farm 1 ($p>0.05$).

Discussion

The consistent and significant seroconversion of birds from both farms to the non-protective L-protein of NDV (248.0 kDa) ($p<0.05$), the respective inconsistent seroconversion on the two farms to the non-protective M-protein (40.0 kDa) and the complete failure of seroconversion to the protective F-protein (60.0 kDa) on both farms, was most likely due to the difference in antigenicity of these three proteins of NDV. The high molecular weight of the L-protein will most likely provide high antigenicity by the booster killed vaccine compared to the other two molecular weights of the F- and M-protein (27), with expected higher competitiveness of the L-protein to the immune system of layers (29).

Future investigations should emphasise the inclusion of higher quantities of the protective F-proteins in booster-killed vaccine to be able to compete with the highly antigenic non-protective L-protein to obtain significant consistent seroconversion to such a protective antigen of NDV (24). Another reason is most probably the difference in the antigenic nature of the fusion protein that is used in priming (ND clone vaccine) to that of the F-protein that is used in boosting (oil emulsion killed vaccine). Indeed, priming with live ND clone vaccine provides immunity to NDV H and N-proteins but not to F-protein (19, 28). The importance of obtaining a significant seroconversion to the F-protein is due to its role in the pathogenesis of ND, in which the F-protein assists the virus to be involved in cell fusion leading to penetration of the host cell, a prerequisite for the multiplication mechanism (11). Acquiring a significant seroconversion to the F-protein of NDV is expected to improve the protection of birds against pathogenic field strains of NDV (13).

Table II
Quantitative seroconversions to a killed vaccine booster on two free-range layer farms against infectious bronchitis virus antigens (Nucleocapsid protein: 51 kDa and membrane glycoprotein: GP 28 kDa)

Infectious bronchitis virus antigens (kDa)	Farm No.[a]	Mean optical density of bands[b] resulting from reaction of 10 pooled sera to specific antigens when sera were collected upon administration of booster and 3 weeks later		SEM[c]
		At vaccination (23 weeks of age)	3 weeks post vaccination (26 weeks of age)	
28.0	1	4.57	4.35	0.06
	2	2.81	3.21	0.09*
51.0	1	4.22	4.45	0.06*
	2	3.11	3.76	0.19

(a) 10 sera were randomly collected from 10 individual layers on each of two farms, at vaccination time (23 weeks of age) and 3 weeks later (26 weeks of age)
Equal volumes of sera from each collection on each farm were pooled together

(b) optical densities were averaged from pixels read at three different randomly selected areas of each band

(c) SEM: the standard error of mean derived from statistical comparisons of the mean optical density of the two respective bands obtained from sera collected on the same farm at 23 versus 26 weeks of age

* indicates a significant difference in the mean optical density of the bands compared in a row ($p<0.05$)

The birds were not sampled beyond the three weeks post booster administration due to the fact that such sampling was not expected to result in seroconversion to the F-protein of NDV, since most research in poultry has proved that maximum response is usually obtained at three weeks post booster, the interval that we respected in the paired sampling (1, 5). The haemagglutination-inhibition test measures only the humoral immunity to the haemagglutinin protein (H). In previous work, most flocks seroconverted to H-protein, with mean HI titres exceeding 1:100 dilution, titres that are sufficient to protect against lentogenic strains of NDV but not against their velogenic counterparts (12).

Regarding the IBV, both non-protective antigens capturing the acquired antibodies due to priming with IB-H120 live vaccine were of low molecular weight namely, the 51.0 kDa-nucleocapsid protein and the 28.0 kDa-glycoprotein. This fact could also explain the inconsistent significance in results of seroconversions between the birds from the two farms to these low antigenic proteins of IBV (Table II).

It is recommended in the future to develop recombinant vaccines that express only the protective antigens of the aetiological agents to avoid competition from the non-protective proteins, thus helping to achieve higher seroconversions to the virulence factors of aetiological agents of birds. In addition, the use of the Western-immunoblotting will enable a quantitative evaluation of such new vaccines, or the indirect ELISA could be modified to include coating of its well walls with only the protective antigens.

References

1. Alexander D.J. 1998. Newcastle disease and other avian paramyxoviruses. *In* A laboratory manual for the isolation and identification of avian pathogens, 4th Ed. (D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson & W.M. Reed, eds). American Association of Avian Pathologists, Kennett Square, 156-163.
2. Alexander D.J. 2003. Newcastle disease. *In* Diseases of poultry, 11th Ed. (Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald & D.E. Swayne, eds.). Iowa State University, Ames, Iowa, 64-87.
3. Alexander D.J. & Gough R.E. 1986. Isolations of avian influenza virus from birds in Great Britain. *Vet Rec*, **118**,537-538.
4. Barbour E.K., Newman J.A., Sasipreeyajan J,. Caputa A.C. & Muneer M.A. 1989. Identification of the antigenic components of the virulent *Mycoplasma gallisepticum* in chickens: their role in differentiation from the vaccine strain (F). *Vet Immunol Immunopathol*, **21**, 197-206.
5. Barbour E.K., Sagherian V., Talhouk S., Talhouk, R., Farran M.T., Sleiman F.T. & Harakeh S. 2004. Evaluation of immunopotentiation and performance in broiler chickens exposed to live viral vaccines and administered *Calendula officinalis* extract. *Med Sci Mon*, **10** (8), 281-285.
6. Beard C.W. & Hanson R.P. 1984. Newcastle disease. *In* Diseases of poultry, 8th Ed (M.S. Hofstad, H.J. Banes, B.W. Calnek, W.M. Reid & H.W. Yoder, eds.). Iowa State University, Ames, Iowa, 452-470.
7. Belli S.I., Mai K., Skene C.D., Gleeson M.T., Witcombe D.M., Katrib M., Finger A., Wallach M.G. & Smith N.C. 2004. Characterisation of the antigenic and immunogenic properties of bacterially expressed, sexual stage antigens of the coccidian parasite, *Eimeria maxima*. *Vaccine*, **22**, 4316-4325.
8. Cavanagh D. 1981. Structural polypeptides of coronavirus IBV. *J Gen Virol*, **53**, 93-103.
9. Cavanagh D. & Naqi S.A. 2003. Infectious bronchitis. *In* Diseases of poultry (Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald & D.E. Swayne, eds.). Iowa State University, Ames, Iowa, 101-120.
10. De Wit J.J. 2000. Detection of infectious bronchitis virus. *Avian Pathol*, **29**, 71-93.
11. Gravel K.A. & Morison T.G. 2003. Interacting domains of the HN and F proteins of NDV. *J Virol*, **77**, 11040-11049.
12. Halvorson D.A., Shaw D., Sivanandan V., Barbour E.K., Maheshkumar S., Newman J.A. & Newman L. 1991. Serological response in broiler chicks to different commercial Newcastle disease and infectious bronchitis vaccines. *Avian Dis*, **35**, 978-981.
13. Kapczynski D.R. & Tumpey T.M. 2003. Development of a virosome vaccine for Newcastle disease virus. *Avian Dis*, **47**, 578-587.

14. Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, **227**, 173-185.
15. Maas R.A., Komen M., Van Diepen M., Oei H.L. & Claassen I.J. 2003. Correlation of haemagglutinin-neuraminidase and fusion protein content with protective antibody response after immunisation with inactivated Newcastle disease vaccines. *Vaccine*, **21**, 3137-3142.
16. Maas R., Venema S., Kant A., Oei H. & Claassen I. 2004. Quantification of infectious bursal disease viral proteins 2 and 3 in inactivated vaccines as an indicator of serological response and measure of potency. *Avian Pathol*, **33**, 126-132.
17. Makkay A.M., Krell P.J. & Nagy E. 1999. Antibody detection-based differential ELISA for NDV-infected or vaccinated chickens versus NDV HN-subunit vaccinated chickens. *Vet Microbiol*, **66**, 209-222.
18. Mathew T. 2002. Modern concepts of immunology in veterinary medicine: poultry immunology. Thajema Publishing, West Orange, New Jersey,456 pp.
19. Morgan R.W., Gelb Jr, J., Schreurs C., Lutticken D., Rosenberger J.K. & Sondermeijer P. 1992. Protection of chickens from Newcastle and Marek's diseases with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion protein. *Avian Dis*, **36**, 858-870.
20. Perrotta C., Furtek C., Wilson R.A., Cowen B.S. & Eckronde R.J. 1988. A standardized enzyme-linked immunosorbent assay for infectious bronchitis virus: comparison with hemagglutination-inhibition and virus-neutralization assays for measuring protective antibody levels in chickens. *Avian Dis*, **32**, 451-460.
21. Randall R.E., Young D.F. & Southern J.A. 1988. Immunization with solid matrix-antibody-antigen complexes containing surface and internal virus structural proteins protects mice from infection with the paramyxovirus, simian virus 5. *J Gen Virol*, **69**, 2517-2526.
22. Steel R.G.D., Torrie J.H. & Dickey D.A. 1997. Principles and procedures of statistics: a biometrical approach. McGraw Hill, Boston, Massachussetts, 666 pp.
23. Stern D.F., Burgess L., Sefton B.M. 1982. Structural analysis of virion proteins of the avian coronavirus infectious bronchitis virus. *J Virol*, **42**, 208-219.
24. Taylor J., Edbauer C., Rey-Senelonge A., Bouquet J.F., Norton E., Goebel S., Desmettre P. & Paoletti E. 1990. Newcastle disease virus fusion protein expressed in a fowlpox virus recombinant confers protection in chickens. *J Virol*, **64**,1441-1450.
25. Towbin H., Stachelin T. & Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci* USA, **76**, 7350-4354.
26. Umino Y., Kohama T., Sato T.A. & Sugiura A. 1990. Protective effect of monoclonal antibodies to Newcastle disease virus in passive immunization. *J Gen Virol*, **71**, 1199-1203.
27. Wang C., Hsu C.J., Chen H.J., Chulu J.L. & Liu H.J. 2008. Development of a reliable assay protocol for identification of diseases (RAPID)-bioactive amplification with probing (BAP) for detection of Newcastle disease virus. *Vet Microbiol*, **130**, 28-36.
28. Yan Y. 2008. Role of noncoding regions in Newcastle disease virus replication and pathogenesis. PhD thesis, University of Maryland, College Park, Maryland, 124 pp (drum.lib.umd.edu/bitstream/1903/8838/1/umi-umd-5866.pdf accessed oon 7 December 2011).
29. Zimecki M. & Webb D.R. 1978. The influence of molecular weight on immunogenicity and suppressor cells in the immune response to polyvinylpyrollidone. *Clinl Immunol Immunopathol*, **9**, 5-79.

