



MSD

Animal Health



Universiteit Utrecht



9th INTERNATIONAL SYMPOSIUM ON AVIAN CORONA- AND PNEUMOVIRUSES

UTRECHT
THE NETHERLANDS
21-24 JUNE
2016

9th INTERNATIONAL SYMPOSIUM ON AVIAN CORONA- AND PNEUMOVIRUSES • 4th ANNUAL MEETING OF THE COST ACTION FA1207 • PROCEEDINGS



4th ANNUAL MEETING OF THE COST ACTION FA1207 ON CONTROLLING AVIAN CORONAVIRUSES



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THE NETHERLANDS
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Preface

The 9th International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens was held in combination with the 4th Annual Meeting of the Cost Action FA1207 “Towards control of avian coronaviruses: strategies for diagnosis, surveillance and vaccination” from June 21 till June 24, 2016. At a beautiful venue in the vicinity of Utrecht (ISVW, Leusden), The Netherlands, scientists from academia and industry gathered to discuss recent scientific advances on avian coronaviruses, avian metapneumovirus and complicating pathogens.

Due to the combination of both meetings a high number of scientists as well as veterinarians from the field, diagnostic laboratories, industry, and governmental authorities from over 20 countries from all over the world attended this meeting. This great interest indicated that these virus infections and especially avian bronchitis virus still are a constant threat for the poultry industry.

During the four days of the symposium new research data on all aspects of these virus infections were presented in 48 talks and discussed during a lively poster session, workshops, and at informal gatherings. Topics that were discussed, amongst others, were the typing of new IBV isolates, novel techniques to develop IBV vaccines, changes in the IBV viral genome, host pathogen and receptor interactions, the pathogenicity of coronaviruses for various avian species, and several studies on avian metapneumoviruses.

The organizing committee looks back at a very successful AvCoV2016 meeting. We would like to thank all participants for joining the meeting and in particular all speakers for their contributions. Our sincere gratitude also goes to the companies that financially supported the organization of the meeting. Last but not least, we would like to thank all people that helped before and during the meeting and thereby contributed to the success of this symposium.

The symposium on Avian Coronaviruses and Complicating Pathogens was organized since 1988 in the Castle of Rauischholzhausen in Germany by Dr. Ursula Heffels-Redmann, Prof. Dr. E. Kaleta, and Prof. Dr. Michael Lierz. After the 8th edition, in 2014, it was decided that the 9th edition would be organized in the Netherlands, in a joint effort between the Utrecht University (Faculty of Veterinary Medicine, Department Pathobiology) and the GD Animal Health, Deventer. We are convinced that we have maintained the spirit of this meeting and hope that many other

meetings will follow. We are therefore very happy that the next symposium will be held in Athens, Georgia, USA in 2018 and will be organized by Prof. Dr. Mark Jackwood, Department of Population Health at the University of Georgia. We hope to see you all there!

On behalf of the organizing committee,

Hélène Verheije
Sjaak de Wit

For more information, please visit the conference website www.AvCoV2016.org

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Serology & Immunology

Keynote lecture

Control of infectious bronchitis virus: What we need to know

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Abstract

A lack of cross protection among different infectious bronchitis virus (IBV) types and the continuous emergence of new strains makes control of this economically important poultry pathogen exceedingly difficult. A number of IBV strains have been used to develop type specific vaccines that induce protection through a local immune response (live attenuated vaccines) or a systemic response (killed vaccines). And, studies using combinations of those vaccines, the protectotype vaccination approach to control, have shown that enhanced protection can be obtained against some but not all unique strains of the virus. Difficulties in large scale vaccine delivery as well as short lived immunity and limited protection against unique and emerging strains remain as challenges to control. To truly control this disease, we need to do a better job of vaccinating against existing strains, and we need to respond quickly to the emergence of new strains. It is clear that vaccination can lower the amount of replicating virus, thus limiting the opportunity for mutations and recombination that lead to emergence of new strains. But, to develop superior tools for the control of IBV we need to know among other things, the 3D structure of spike, or at least the structure of the epitopes that induce neutralizing antibodies as well as those epitopes involved in attachment and entry into the host cell. We also need to know the viral and host cell genes and gene sequences involved in pathogenicity and the components of the immune response that are involved in cross-protection. That information will allow us to develop new tools that can be used to take a proactive approach to control.

Development of rationally attenuated live vaccines for effective control of infectious bronchitis

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Abstract

Live attenuated vaccines and inactivated vaccines are universally used in an attempt to control IBV but offer only short-lived protection and are poorly cross-protective between strains. Furthermore, live attenuated vaccines may revert to virulence or recombine with field strains to produce a novel pathogenic strain. It is clearly important that new and safer vaccines are developed for the control of IBV.

We have developed an IBV reverse genetics system based on the pathogenic isolate, M41, to identify genes that can be used for the rational attenuation of the virus. Our recombinant IBV, M41-K, replicated similarly to the parent virus, M41-CK, *in vitro* and was observed to be pathogenic in chickens.

We introduced a series of mutations in the non-structural proteins (nsp) of M41-K that resulted in an attenuated virus, M41-R. Mutations were introduced into nsp 14, an exoribonuclease (ExoN) involved in RNA cap-formation; nsp 16, also involved in RNA cap formation; nsp 10, a co-factor for nsp 14 and 16 that forms a heterodimer with both and stimulates ExoN and 2-O-MT activities; and nsp 15, an endonuclease. *In ovo* vaccination with M41-R of both commercial and SPF eggs protected against M41-CK challenge of 21-day old birds.

Chickens were infected with a panel of recombinant viruses expressing different combinations of mutations and nsp were shown to have varying effects on pathogenicity. Further analysis demonstrated that combined mutations nsp10 and nsp14 result in attenuation. In conclusion, engineered changes to nsp can be used to rationally attenuate IBV generating a safe vaccination, for use *in ovo*, without compromising efficacy.

Ability of Recombinant IBV expressing S1 subunits from M41 and QX to induce protection against homologous challenge

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Abstract

One major issue in the control of IBV is the continuing emergence of new serotypes. Vaccination can induce insufficient cross-protection against serotypes in the field and two or more antigenically different vaccines are often used in attempt to provide broader protection. Studies show that some combinations of vaccines can induce cross-protection against unrelated serotypes as discussed by De Wit *et al.* (2011). Amino acid differences in the major surface protein, have been revealed as a major cause of poor cross-protection and in particular the S1 domain has been shown to be an immunodominant part, shown by Ignjatovi & Galli (1994). The generation of a reverse genetic system for IBV has led to the development of a potential new generation of live vaccines. Previously it was shown by Hodgson *et al.* (2004) and Armesto *et al.* (2011), that replacement of the S gene of apathogenic Beaudette, with the S gene from a pathogenic strain to produce a recombinant IBV (rIBV) can act as a vaccine. The rIBV remained apathogenic, but was still immunogenic and protected SPF birds against challenge with both the homologous strain (from which the S gene was derived) and also a heterologous strain, by Hodgson *et al.* (2004) and Armesto *et al.* (2011). The focus of this experiment is to determine if rIBVs expressing S1 subunits derived from virulent strains (QX or M41) are able to protect against a homologous challenge and to investigate tissue tropism of rIBV and local cellular infiltration. IBV-antigen specific serum antibody titres were assessed by a commercial ELISA. At two weeks post-challenge, both vaccinated groups (BeauR-M41(S1) and BeauR-QX(S1)) reported high IBV-antigen specific antibody titres. Both viral load and tissue tropism were assessed in a selection of the head-associated lymphoid and respiratory lymphoid tissues by qRT-PCR and immunohistochemistry.

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Vaccine immune evasion by an infectious bronchitis virus field strain

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Abstract

The Arkansas (Ark) serotype/ArkDPI molecular type infectious bronchitis virus (IBV) is prevalent in the intensive broiler producing areas of the United States (Jackwood *et al.* 2005, Toro *et al.* 2006), in spite of vaccination with live-attenuated Ark serotype vaccines. Many factors may contribute to this, including selection of minor vaccine subpopulations in chickens (Gallardo *et al.* 2010, van Santen & Toro 2008, McKinley *et al.* 2008), resulting in persistence and reisolation of Ark vaccine virus (Jackwood *et al.* 2009). We addressed the question of how Ark serotype field strains can escape Ark vaccine-induced immunity, by vaccinating chickens and comparing their mucosal and systemic antibody responses against the vaccine strain and a field strain.

Materials and Methods

3-week-old SPF white leghorn chickens were vaccinated ocularly with 3×10^5 EID₅₀ of a commercial ArkDPI vaccine strain. The resulting antibody responses against the vaccine strain and an Ark serotype field isolate, AL/4614/98, were compared. This field isolate has 96% S1 amino acid identity with the vaccine strain and virus neutralization tests indicate 92% antigenic similarity (Toro *et al.* 2006). Antibody responses against the vaccine strain and the field strain were compared by ELISPOT to enumerate virus-specific IgA-secreting cells in head-associated lymphoid tissue (HALT), comprised of a mixture of lymphocytes from Harderian gland and conjunctiva-associated lymphoid tissue (CALT), and virus-specific IgG- and IgA-secreting cells in the spleen for each virus 8, 10, and 12 days post-vaccination as described (van Ginkel *et al.* 2009), using nitrocellulose backed, 96-well microplates coated with 100 μ l of 5 μ g/ml heat-inactivated IBV (Ark-DPI vaccine strain or field isolate AL/4614/98). Endpoint titers for virus-specific IgA in tears and IgG and IgA in plasma were determined for each virus by ELISA as described (Orr-Burks *et al.* 2014) two weeks post vaccination. Hemagglutination inhibition (HI) titers were determined against each virus two weeks post vaccination using four hemagglutinating units of receptor-destroying enzyme-treated virus. Finally, IgA and IgG responses to linear epitopes in

S1 of each virus were determined in plasma pooled from five immunized and boosted chickens two-weeks post-boosting using a peptide array ELISA. The peptide array contained 17-mers representing the first 535 amino acids of each S1, overlapping by 10 amino acids. Background of absorbance from binding of plasma from unimmunized chickens was subtracted for each peptide and values greater than two times the background, obtained using no plasma, were considered positive.

Results

Ocular vaccination resulted in 68% fewer IgA-secreting cells in HALT recognizing the field strain compared to the vaccine strain 8-12 days post-vaccination (Fig. 1a). This indicates that a significant proportion of antibody secreting cells induced in these mucosa-associated lymphoid tissues generate IgA antibodies that recognize the vaccine virus but not the field IBV strain. Consistent with this, IgA antibody titers in tears against the field strain were statistically significantly lower than against the vaccine strain (Fig. 1b). Thus a significant proportion of IBV-specific IgA antibodies in tears recognize the vaccine virus but not the field IBV strain. Differences in the systemic antibody response were less apparent. Numbers of IgA and IgG secreting cells in the spleen producing antibodies to the field strain and to the vaccine strain were similar, and although titers of IgA and IgG antibodies in plasma recognizing the field strain were lower than those recognizing the vaccine strain, differences were not statistically significant. Plasma HI titers were lower against the field strain than against the vaccine strain, suggesting a reduced ability to neutralize the field strain virus, but the difference was not statistically significant.

The peptide array ELISA identified four linear epitopes on the vaccine S1 protein recognized by IgG antibodies in plasma of immunized and boosted chickens, and four recognized by plasma IgA antibodies. Importantly, plasma antibodies did not recognize peptides derived from the corresponding positions in the field strain S1 for two of the IgG epitopes and one of the IgA epitopes.

Discussion

Our results show that S1 amino acid differences in field strains of the same serotype as vaccine strains can result in decreased antibody recognition in vaccinated chickens. This reduction is most evident in the immune response at mucosal sites, emphasizing the importance of IgA antibodies produced at these sites for controlling entry of IBV into its host. Thus, selection of mutations observed in field isolate S proteins may be driven in part by host immunity after vaccination.

Acknowledgements

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The effect of modulation by food components on the chicken innate immune system and the responsiveness to infectious bronchitis virus

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Infectious diseases in poultry are mostly prevented by vaccination and use of antibiotic drugs, the latter posing a risk for the induction of antibiotic resistance. Therefore, improving the immune status is important. We investigated modulation by food components of innate immune responses in the gut and if better immune response at mucosal sites and enhanced protection against viral infections are achieved.

We selected compound X by screening its effects on the function of chicken innate immune cells using the chicken macrophage cell line HD11, and primary natural killer (NK) cells. It was added to a commercial chicken feed. In the *in vivo* experiment, day-old chicks received either standard feed (control group) or feed supplemented with compound X (compound X group) for a period of 18 days. At day 19, chickens were inoculated with infectious bronchitis virus (IBV) M41. Before, and at 1 and 4 days post infection, chickens were sacrificed and lungs, ileum and blood were collected, lymphocytes were isolated and analyzed by flowcytometry.

Chickens from the compound X group showed enhanced activation of gut and lung NK cells and in the lungs increase in IFN mRNA. A significant decrease in B-cells in the gut, and in the lung a change in NK cell subsets, and a lower percentage of T cells and CD4+ T cells were observed.

Upon IBV infection of controls, IBV mRNA was detected in lungs and gut, paralleled by an increase in NK cell activation. IBV infection of chickens from the compound X group did not result in further increase of NK cell activation. IBV mRNA levels were similar to the control group.

In conclusion, compound X enhances the function of NK cells in the gut and the lung, but protection against IBV infection, based on IBV mRNA levels, is not enhanced.

Identification of immunogenic epitopes on the S1 protein of infectious bronchitis virus strains, M41 and QX

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Abstract

One of the major issues facing IBV control is the emergence of new serotypes. Vaccination can induce insufficient cross-protection against serotypes in the field and two or more antigenically different vaccines are often used in attempt to provide broader protection. Studies show that some combinations of vaccines can induce cross-protection against unrelated serotypes as discussed by De Wit et al. (2011). Amino acid differences in the major surface protein, have been revealed as a major cause of poor cross-protection and in particular the S1 domain has been shown to be immunodominant by Ignjatovic & Galli (1994). The generation of a reverse genetic system for IBV has led to the development of a potential new generation of live vaccines. Previously it was shown by Hodgson et al. (2004) and Armesto et al. (2011) that replacement of the S gene of apathogenic Beaudette, with the S gene from a pathogenic strain to produce recombinant IBV (rIBV) can act as a vaccine. The rIBV remained apathogenic, but was still immunogenic and protected SPF birds against challenge with both the homologous strain (from which the S gene was derived) and also a heterologous strain, shown by Hodgson et al. (2004), Armesto et al. (2011). Here, we attempt to address the issue of poor and unpredictable cross-protection of IBV vaccines in the field by using a novel epitope mapping technology to map immunogenic epitopes presented on S1 proteins from two IBV strains, M41 and QX. To allow identification of S1 epitopes, a homologous vaccination-challenge with rIBV viruses expressing the S1 subunits of M41 and QX was conducted with SPF chickens. At 14 days post-challenge, both vaccinated groups, BeauR-M41(S1) and BeauR-QX(S1) reported high IBV-specific antibody titres and this serum was subjected to epitope mapping. Briefly, a library of surface-immobilised conformationally CLIPs-constrained peptides (looped and linear series) covering the S1 sequences of two strains, M41 and QX, was constructed. The antibody binding affinity to each peptide construct was determined by ELISA. Preliminary results from the epitope mapping will be discussed.

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Efficacy of the H120 infectious bronchitis virus vaccine against a Nigerian IBADAN06 virus in the chicken model

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Abstract

Infectious bronchitis viruses (IBV) are causing great economic losses to the poultry industry worldwide including in West Africa where poultry is not systematically vaccinated against the pathogen. Available commercial IBV vaccines are the same in Africa and in Europe, despite differences in IBV strains in circulation. To date, the IBV genotype IBADNA06 has only been detected in Nigeria and in Niger. The full genome sequence NGA/A116E7/2006 (IBADAN06 genotype) has a genetic distance of 9.7-16.4 % at the nucleotide level with all available fully sequenced strains. The antigenic relatedness of NGA/A116E7/2006 was compared with strains of other serotypes: a low level cross-reactivity was observed between NGA/A116E7/2006 and ITA/90254/2005 (QX-like) but the Nigerian strain did not cross-react with antisera against IT02, M41, D274, Connecticut or 793/B strains in virus neutralization assays (Ducatez *et al.*, 2009). We therefore studied the efficacy of the Massachusetts H120 vaccine, the most commonly used IBV vaccine in West Africa, against NGA/A116E7/2006 in the chicken model.

Material and methods

Twenty-four one-day-old specific pathogen free chicks were purchased from the Plateforme d'Infectiologie Expérimentale (INRA, Nouzilly, France) and divided into three groups: (i) vaccinated and challenged, (ii) non vaccinated and challenged, (iii) non-vaccinated and non-challenged. Vaccinated birds received two doses of H120 vaccine (at 1 day and 2 weeks of age) and the challenge took place three weeks post-boost with NGA/A116E7/2006. Clinical signs were recorded daily. Virus shedding was measured by real-time-RT-PCR in oropharyngeal swabs from day 2 to day 13 post-infection. Blood was collected before each vaccination and two weeks post challenge to look for anti-IBV antibodies.

Results

As previously reported (Ducatez *et al.*, 2009), no clinical signs were observed after challenge irrespective of the vaccine regimen. H120 vaccine did not prevent NGA/A116E7/2006 virus replication but control (unvaccinated) birds shed slightly more virus than their vaccinated counterparts (the difference was not statistically significant) and virus clearance was slightly delayed (day 10 versus day 13 for vaccinated and unvaccinated birds, respectively). Anti-IBV antibodies were detected after prime and boost vaccinations and after challenge as expected. Control birds were seronegative throughout the experiment.

Discussion

Our study suggests that a low genetic similarity and low antigenic similarity between virus and vaccine is not necessarily indicative of low protection and that H120 vaccine is likely to provide minimal protection to birds against IBADNA06. The present vaccine study is difficult to assess as no mortality and no morbidity were observed in unvaccinated challenged birds. Correlates of protection per se could therefore not be evaluated. We were just able to compare virus shedding both in duration and intensity in vaccinated and unvaccinated birds and we showed a slight benefit of using H120 vaccine against IBADAN06 virus.

We chose to test the efficacy of H120 vaccine because it is the most common IBV vaccine available in West Africa. We then tried to predict the best vaccines to use against NGA/A116E7/2006 by looking at IBV sequence identities. The first candidates were a 4/91-vaccine (sequence accession number: KF377577), a D274 vaccine (sequence accession number: X15832), a QX vaccine, and an H120 vaccine (sequence accession number: FJ888351). These results are dependent on full genomes available and would likely change if more sequence data were available. It would, however, be interesting to repeat the vaccine challenge study with the 4/91 vaccine strain and compare the virus shedding with what was observed after vaccination with H120.

Acknowledgments

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Evaluation of cross-protection against IBV 793B strain after combined vaccination with Massachusetts type and Brazilian variant vaccines at one-day-old

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Abstract

Epidemiological investigations in Brazil showed a strong prevalence of Brazilian variant (BR-I) and Massachusetts type Infectious Bronchitis Viruses (IBV), although the presence of a new variant genetic group (BR-II group; Fraga *et al.*, 2013) and, for a very limited period and geographical distribution, 793B type viruses were also reported (Villarreal *et al.*, 2010). Mass-type IB vaccines, which have been exclusively used in Brazil provide only partial protection to the Brazilian variant field strains, therefore a homologous live vaccine was developed and registered to improve protection in the field. The aim of our study was to check the level of protection provided by the simultaneous application of a Mass-type vaccine (B48 strain) and the BR-I type vaccine strain against 793B challenge. Day-old SPF chickens were vaccinated and then challenged at 28 days of age. Efficacy of vaccination was evaluated by scoring the extent of ciliostasis and histological lesions, and by measuring the IBV RNA load in the trachea using a one-step real-time RT PCR method. Results in vaccinated chickens indicated marked reduction of challenge virus replication and, as a consequence, significant protection against macroscopic and microscopic lesions in the trachea. Based on this finding, a vaccination program comprising Mass-type and BR-I type IB vaccines is able to provide strong protection against 793B type IBV, reducing the risk of the introduction and spread of this genotype in Brazil.

Introduction

Infectious bronchitis virus (IBV) is one of the major poultry pathogens worldwide. Depending on the pathogenicity of the virus, age of chickens at infection, the presence of co-infection with other pathogens (e.g. *Mycoplasma sp.*, *E. coli*, Low Pathogenic Avian Influenza Virus), and the rearing conditions, manifestation of IBV infection shows a wide range of clinical signs from mild respiratory signs to severe respiratory and/or renal disease accompanied by significant mortality. Control of the disease relies on vaccination and strict biosecurity measures.

IBV can be characterized with great genetic and antigenic heterogeneity among the strains due to the high mutation rate and recombination events (Jackwood & de Wit (2013)). Due to this high variability several genetic and antigenic variants are present, which show different geographical distribution. Some of them are widespread, like the QX-type, Q1-type and 793B-type strains, others show restricted geographical distribution, such as the Brazilian variants (e.g. BR-I group) and different groups of US variants, for example Delaware 072 (Cook (2015), Jackwood (2012)). In most cases there are different genetic groups of IBV coexisting in the same country or region and vaccination programs should provide appropriately broad protection. Although there are homologous vaccines against certain variants to improve the efficacy of vaccination (793B-type; QX-type; D274-type; Var-2-type; DE-type, Ark-DPI, GA08-type etc) these do not cover all the variants present in the field. As reported previously, broad cross-protection can be achieved by the combination of certain vaccines representing different genogroups of IBV mainly involving the combination of Massachusetts-type (Mass-type) vaccine with 793B-type vaccine (Cook (1999), de Wit (2011), Jackwood & de Wit (2013)). Although this combination showed good efficacy against a wide range of variant IBVs, most of the national authorities restrict the use of vaccines to those strains belonging to a genetic group already existing in the concerned country, in order to better control the genetic variability of field IBV strains.

In Brazil, apart from the Mass-type strains, unique Brazilian variants (BR-I) are widely present. These variants have been isolated from flocks either with respiratory signs or reproductive disorders. The pathogenicity of several isolates belonging to this group was analysed under experimental conditions, and showed variable pathogenicity for the respiratory system (ciliostatic effect) compared to the IBV M41 reference strain (Chacón (2014a, 2014b), Cook (1999), De Wit (2015)) and for the kidney (Chacón (2014), de Wit (2015), Fernando (2015)). Most recent investigations have demonstrated the predominance of BR-I strains in the three most important poultry producing regions (Balestrin (2014), Chacón (2011), Colvero (2015), Felipe (2010), Fraga (2013), Montassier (2012), Villarreal (2007)). The effect of IBV infection on the Brazilian poultry industry was analysed by Assayag *et al.* (2012) and Colvero *et al.* (2015), who demonstrated its measurable economic impact.

Mass-type vaccines provide limited protection against BR-I-type IBV isolates, and this has been demonstrated both by controlled studies (Cook (1999)) and the frequent isolation of these variant viruses from flocks with IB clinical signs vaccinated exclusively with Mass-type vaccines (Balestrin (2014), Chacón (2011), Villarreal (2010)). To provide better protection against the BR-I field strains and fulfil

the expectation of using live vaccines which match the genetic groups of field IB viruses, a live BR-I-type vaccine has been developed and registered in Brazil.

Several authors have shown that the great majority of IBV strains belong to either the Mass or BR-I group, however, there are publications reporting the characterization of two 4-91-type (793B-type) IBV isolates (Villareal (2010)), and the emergence of a new Brazilian genotype (BR-II, Fraga (2013)).

The aim of our study was to investigate the range of protection achievable by combining a Mass-type vaccine with the BR-I-type vaccine. Here we report the vaccine efficacy test results following challenge with 793B IBV in day-old SPF chickens.

Materials and methods

Viruses

Day-old vaccination was performed with two commercial live vaccines containing either IBV B48 strain or IBV BR-I strain.

Challenge was performed with IBV 793B strain.

Chickens

Day-old SPF layer-type chickens were used for the experiment. The birds were kept in animal rearing rooms equipped with HEPA filtered air in- and outflow, in positive pressure throughout the experiment. Different groups were isolated. Water and feed was provided ad libitum.

Experimental design

Day-old SPF layer chicks were assigned to two groups. Group 1 was immunized at day-old with IBV B48 and IBV BR-I strains simultaneously (1 dose each, eye drop application). Group 2 served as non-vaccinated controls. At 28 days of age, 20 birds from the vaccinated and 10 from the control group were submitted to challenge infection with IBV 793B strain. Challenge virus was applied at a dose of $5.0 \log_{10} \text{EID}_{50}$ /chicken intratracheally and intraocularly. Additionally, on the day of challenge, 10 vaccinated and five control birds were euthanized and tracheal samples were collected for the measurement of ciliary activity and vaccine-virus persistence. The remaining five birds in the control group were used as negative controls for the post-challenge sampling. The efficacy of vaccination was evaluated at five days post-challenge on the basis of respiratory signs, ciliary activity, histological lesions and IBV load in the trachea. The experimental design is summarized in Table 1.

Clinical observation

Five days post-challenge respiratory symptoms were scored on a scale of 0 to 3 (normal breathing (0) to severe tracheal rales (3); see details in Tatár-Kis (2012)).

Post-challenge sampling

Five days after challenge the birds were euthanized, and the whole trachea was removed and put into pre-warmed MEM-H medium. Three portions of trachea were collected for the ciliary activity test (upper, middle and lower part) one portion for histological examination and one portion for qRT-PCR.

Ciliary activity test

Ciliary activity testing was performed on 10 tracheal rings from each bird. Evaluation was done by two methods. In case of the quantitative method, all rings were evaluated on a scale from 0 to 5 (from lack of ciliostasis (0) to total ciliostasis (5)), the scores were summed for each bird and the groups were compared statistically. The qualitative method was performed according to the European Pharmacopoeia (04/2013:0442), when each ring showing at least 50% vigorous ciliary movement was evaluated as protected and birds with at least nine protected rings out of the 10 examined proved to be protected. The percentage of protected birds was calculated.

Histological examination

Trachea samples were fixed in 10% buffered formaldehyde and the prepared sections were examined after haematoxylin-eosin staining. Presence and severity of (i) epithelial deciliation, (ii) epithelial metaplasia, (iii) epithelial degeneration, (iv) lymphocyte infiltration in the lamina propria and mucosal thickening, were scored on a scale from 0 to 3 (0 = Normal, 1 = Mild lesions, 2 = Moderate lesions, 3 = Severe lesions). The sum of the obtained scores was used for the description of the severity of IB specific lesions in the trachea for each bird.

Quantification of IBV load in the trachea by quantitative one-step real-time RT-PCR (qRT-PCR)

Trachea samples were stored at -80°C until processing. After thawing, the organ sample was homogenized in 1 ml sterile PBS using Tissue Lyser II (Qiagen). Viral RNA from samples was extracted using QIAxtractor Virus Kit (QIAGEN) according to the manufacturer's instructions. Two microliter RNA extract was used as a template for the real-time one-step RT-PCR (Rotor-Gene Probe RT-PCR Kit, Qiagen). The primers and probes used for the amplification and detection of a fragment of 5' UTR were described previously (Callison et al. (2006)). Appropriate controls were included in each run to allow comparison of IBV load based on Ct values. Positivity limit was set

at 35 Ct, samples above this limit or showing lack of amplification were included in the calculations with 40 Ct value.

Statistical analysis

The results of qRT-PCR (Ct), clinical scores, histology scores and sum ciliostasis scores were compared between the vaccinated and the positive control groups using One-way ANOVA (Statgraphics software) at 95 % significance level.

Results

Results are summarized in Table 2.

Clinical observation

Mild-moderate respiratory signs were observed in all control chickens at 5 days post-challenge (dpch). In contrast, only a small proportion of birds in the vaccinated group showed mild signs while in the majority of birds no respiratory noise could be heard, indicating the significant effect of vaccination ($p=0.000$).

Ciliary activity test

Total ciliostasis was seen in the great majority of challenged control chickens at 5 dpch, resulting in 48.3 mean score. Vaccinated and challenged chickens showed mild ciliostasis only (mean score 11.0). Ciliostasis scores in the vaccinated group proved to be significantly lower than in the positive control group at 5 dpch ($p=0.000$).

Statistical comparison of pre- and post-challenge ciliostasis scores (mean score 9.9 versus 11.0, respectively) revealed the lack of a significant effect of challenge in the vaccinated chickens ($p=0.735$).

Evaluation according to the European Pharmacopoeia showed 85% protection in the vaccinated group against the heterologous challenge and full susceptibility of challenged controls. The test was validated by the negative control results.

Histological examination

Moderate histological lesions were seen in the challenged controls (with marked differences among individuals). Mucosal thickening and lymphocyte infiltration was accompanied by epithelial lesions, including loss of cilia, and epithelial degeneration and metaplasia. In 75% of vaccinated chickens only a mild inflammatory reaction without any lesions in the epithelium was seen; a sign of immune response to challenge infection. In a smaller proportion of the vaccinated group mild to moderate epithelial lesions were also found. Vaccination prevented the development of epithelial lesions in the great majority of vaccinated chickens and reduced its severity significantly ($p=0.008$).

Quantification of IBV replication in the trachea by real-time PCR

A high level of challenge virus replication was measured in the challenged controls. Mean Ct value in the positive control group corresponds to the dilution of challenge virus stock having a titer of $5.2 \log_{10} \text{EID}_{50}/\text{ml}$. Vaccination had a strong, significant effect on the suppression of challenge virus replication (approx. $3.7 \log_{10}$ reduction of mean total IBV RNA amount). The qRT-PCR method used does not differentiate between vaccine and challenge strains, therefore the result of the vaccinated and challenged group obtained at 5 dpch was compared to the pre-challenge results of the vaccinated group to estimate the effect of challenge. There was a mild, but statistically significant increase between the two dates (mean Ct decreased from 38.5 to 35.1 indicating an approximately 10-fold increase of IBV load).

Discussion

Epidemiological investigation of IB in Brazil demonstrated that the most prevalent geno-group of IBV is the BR-I group despite the vaccination with Mass-type vaccines. Significant economic losses can be attributed to IBV infection (Assayag (2012a, 2012b), Colvero (2015)), and this calls for an improved IB vaccination program. A homologous live vaccine had been developed, which proved to be efficient against the BR-I-type IBV challenges as demonstrated during the registration process. In the study presented here, the efficacy of a vaccine combination comprising BR-I-type and Mass-type live IB vaccines was tested against an IBV 793B-type challenge, in order to investigate the possible use of this new vaccine protocol against the 793B-type IBV strains. 793B-type IBV strains show a wide geographical distribution and have also been demonstrated in Brazil (Villareal (2010)). Results obtained in SPF chickens indicated that the simultaneous application of IBV BR-I and IBV B48 vaccine strains is able to strongly suppress challenge virus replication in the trachea and, as a consequence, provides significant protection against ciliostasis (85% protection) and histological lesions in the trachea. These data indicate that the combination of B48 and BR-I vaccine strains is beneficial not only due to the improved protection against the most prevalent IBV BR-I field strains, but also due to the additional protection afforded against IBV 793B challenge, which may help reduce the spread of this variant in Brazil.

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Group		Vaccination (D0)	Challenge (D28)	Pre-challenge observations & samplings (D28)	Post-challenge observations & samplings (5 dpch)
		B48 and BR-1 vaccine strains at day-old, eye drop	793B strain (5.0 IgEID ₅₀ /dose; eye drop and intra-tracheal)	Ciliostasis test, trachea for qRT-PCR	Clinical scoring, ciliostasis test, trachea for histology and qRT-PCR
Vaccinated		yes	yes	yes (n=10)	yes (n=20)
Non-vaccinated	Positive control	no		no	yes (n=10)
	Negative control		no	yes (n=5)	yes (n=5)

Group	Clinical score at 5 dpch (mean±STD)	Ciliostasis score (mean±STD)		Protection based on ciliostasis ^a	Histological lesion score at 5 dpch (mean±STD)		IBV load in the trachea (qRT-PCR; Ct mean ± STD)	
		Pre-challenge	5 dpch		All lesions ^b	Epithelial lesions ^b	Pre-challenge	5 dpch
Positive control	1.3 ±0.5	NA	48.3 ±3.4	0 %	6.0 ±3.5	4.3 ±2.9	NA	22.7 ±2.4
Negative control	0.0 ±0.0	0.0 ±0.0	1.2 ±2.2	100%	0.0 ±0.0	0.0 ±0.0	40.0 ±0.0	40.0 ±0.0

^a Evaluation according to the European Pharmacopoeia.

^b „Epithelial lesions” indicate deciliation, epithelial degeneration and epithelial metaplasia; “all lesions” indicate sum score for epithelial lesions, lymphocyte infiltration and thickening of mucosa.

H120 and 793/B type vaccine strains combination protects chickens against challenge with IR-1 like infectious bronchitis virus

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Introduction

Avian infectious bronchitis (IB) is a widely distributed poultry disease that has huge economic impact on poultry industry. The continuous emergence of new IBV genotypes and lack of cross protection among different IBV genotypes have been an important challenge. Methods: The present study was done to evaluate protection caused by two different serotypes' vaccines (Massachusetts & 793/B) in order to evaluate protection against challenge with Ir-1 like-virus. Results: The results showed a protective ability of the used vaccination program. The H120-793 vaccinated group obtained 81% protection.

Conclusions

These results indicate that the H120 + 79 vaccines could be helpful for the reduction of economic losses caused by newly evolving Ir-1 IBV variants.

Broadening the cross-protection of infectious bronchitis provided by a vaccination regime using three different live infectious bronchitis vaccines

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Abstract

To date there are many serotypes or genotypes of infectious bronchitis virus (IBV) which have been identified worldwide. In published cross-protection studies using existing live IB vaccines it has been shown that the use of Nobilis IB Ma5 and Nobilis IB 4/91 vaccines provides excellent protection against many different serotype virus challenges, but for the D1466 serotype the cross protection was limited. We attenuated a Dutch IBV QX isolate for vaccine development as Nobilis® IB Primo QX which is also compatible with Nobilis® IB Ma5 in simultaneous use. This paper focused on whether the regime of administering Nobilis® IB Primo QX and Nobilis® IB Ma5 at day-old followed by Nobilis® IB 4/91 two weeks later could provide a better protection against IBV D1466. Groups of day-old SPF birds were vaccinated with either Nobilis® IB Ma5 and Nobilis® IB 4/91 or Nobilis® IB Primo QX and Nobilis® IB Ma5 or Nobilis® IB Primo QX and Nobilis® IB Ma5 plus Nobilis® IB 4/91 at 14 days of age; in addition, two groups of age-matched birds from the same hatch were included as non-vaccinated-non-challenge controls and non-vaccinated-challenge controls respectively. All birds in all groups were challenged with virulent IBV D1466 strain at 28 days of age and protection was assessed by ciliostasis assay. Excellent protection (100%) was achieved in the group receiving the three different vaccines, and groups receiving two different vaccines were conferred around 30% protection. Outcomes of this study suggest that a suitable vaccination scheme with high immunogenic live IB vaccines can broaden the protection against many serotype or genotype challenges and that it is unnecessary to develop a new IB vaccine to combat each serotype or genotype.

Introduction

The infectious bronchitis virus (IBV) genome consists of a linear, non-segmented, positive-sense, single-stranded RNA of approximately 27.6 kilobases in length, coding for four major structural proteins (the spike (S) glycoprotein, the envelope (E)

protein, the membrane (M) glycoprotein and the nucleocapsid (N) protein). It is known that the S1 part of the spike protein is responsible for determining its serotype or genotype. A few amino acid changes in the S1 spike protein can lead to the appearance of a new variant. To date many serotypes or genotypes of IBV have been identified worldwide. Some variants remain in the field for a long period of time, and others appeared and disappeared very quickly. IBV immunity is generally serotype-specific, and low degrees of cross-protection are observed among serotypes or genome types. In published cross-protection studies IB Ma5 and 4/91 vaccines (belonging to two different serotypes) given 14 days apart improved cross-protection against challenge with many IB viruses of different serotypes or genotypes except IBV D1466 (Cook, *et al.* 1999; Terregino *et al.* 2008). MSD Animal Health recently developed the IB Primo QX vaccine which belongs to the IBV QX genotype and is compatible with IB Ma5 vaccine. The present study investigated whether a regime of administering Nobilis® IB Primo QX and Nobilis® IB Ma5 at day-old followed by Nobilis® IB 4/91 two weeks later could provide a better protection against IBV D1466.

Materials and Methods

Animal experiment

The study design is outlined in Table 1. Briefly, birds were vaccinated with standard commercial vaccines at day-old or both at day-old and 14 days old. The birds were challenged by the ocular-nasal route with virulent IBV D1466 at 28 days of age. Protection was assessed by ciliostasis assay five days post challenge. Briefly, six rings were cut from each trachea, two from the top, two from the middle and two from the bottom. Each ring was examined microscopically. For each ring, ciliary activity was regarded as normal if greater than 50% of cilia showed vigorous beating. The bird was considered protected if greater or equal to 5/6 rings showed normal activity. Ciliary activity for each ring was also recorded as a percentage and the average percentage of ciliostasis per group was then calculated.

IBV RT-PCR and S1 gene sequencing

At 5 days post challenge kidney samples were collected. Viral RNA was extracted from each sample. The first RT-PCR was performed with IBV universal primers located in the non-translated region of the IBV genome. If positive, a second RT-PCR test was run with IBV D1466 S1 gene specific primers (Cavanagh *et al.* 1999), and PCR products were then sequenced to further confirm if the sequences derived from the S1 gene matched with the S1 gene of IBV D1466.

Histopathological examination in kidney samples

At 5 days post challenge kidney samples were taken to evaluate the presence and extent of histological features of interstitial (mononuclear) nephritis consistent with IBV infection. The following scoring system was used:

- 0 = no abnormalities detected
- 1 = minimal/focal
- 2 = mild/focal to multifocal
- 3 = moderate/multifocal
- 4 = severe/diffuse

Results

Evaluation of cross protection

The results are tabulated in Table 1. The group receiving three different vaccines achieved a full protection against IBV D1466 challenge and ciliary activities of tracheas in all birds were not affected. The groups receiving two different vaccines were only conferred approximately 30% protection with an average of approximately 50% ciliostasis. The non-vaccinated challenge group was not protected and ciliary movement of tracheas was completely stopped. The non-vaccinated non-challenge group remained unaffected.

Detection of IBV D1466 viral RNA in kidney samples

No IBV D1466 viral RNA was detected in kidney samples collected from groups 1, 2 and 5 (Table 2). IBV D1466 viral RNA detection was positive in 11 to 33% birds from group 3 and 70% birds from group 4.

Histological examination of kidney samples

All five groups had very low scores with an average between 0.8 and 1.0, indicating no significant nephritis was seen at five days post IBV D1466 challenge (Table 2).

* three samples were positive using primers located in un-translated region, but only one of these three samples was D1466 positive using primers located in the S1 gene.

Discussion

Further improved cross-protection has been achieved by using a combined vaccination schedule incorporating three antigenically different IB vaccines against IBV D1466 serotype challenge in the present study. In addition, several groups have also shown that a vaccination schedule using a live attenuated IB vaccine at one day old, followed

by a heterologous live attenuated IBV vaccine at 14 days, is able to induce considerable cross-protection against challenge with recent new variants (Terregino *et al.*, 2008; Hager *et al.*, 2014). These studies suggest that the concept of protectotypes should be considered in the field in order to develop strategies of controlling IBV infections. Actually, this concept has been used in the field for many years and has proved to be efficacious and safe. To date there is no reported data available regarding S1 reassortant IB viruses becoming epidemiologically significant in the field.

IBVs continuously evolve by developing mutations in antigenic sites. It is both impossible and unnecessary to develop a new vaccine for every variant; therefore, broadening cross-protection will be a highly relevant and practical method in IBV control strategies. In the future it will be worthwhile to investigate the protection spectrum provided by the vaccination schedule against a range of IBV variants which are currently prevalent worldwide.

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Simultaneous vaccination of a live Newcastle disease and two live infectious bronchitis vaccines in commercial broiler chicks

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Abstract

This study examines the interactions between a live Newcastle Disease Virus (NDV; VG/GA-Avinew) vaccine and two different live infectious bronchitis virus (IBV; H120 and CR88) vaccines in day-old IBV-NDV maternal-antibody positive commercial broiler chicks. Chicks were divided into four groups; i) unvaccinated control, ii) live NDV vaccinated, iii) combination of live H120+CR88 vaccinated, iv) combination of VG/GA-Avinew+H120+CR88 vaccinated. Clinical signs were monitored daily and at 0, 3, 7, 10, 14 and 21 days post vaccination (dpv), oropharyngeal swabs were taken for detection of the vaccine viruses by RT-PCR. Blood samples were collected at 21 dpv for IBV and NDV serology. At 21/22 dpv, 10 chicks from each group were removed and challenged with either virulent IBV M41 or QX. After 5 days post challenge (dpc), tracheal ciliary protection assessment was carried out. At 21 dpv, the 793B IBV haemagglutination-inhibition (HI) titre of the VG/GA-Avinew+H120+CR88 group was significantly lower than the H120+CR88 group. In contrast, the HI titre against M41 antigen was significantly higher in the triple-vaccinated birds compared to the H120+CR88 vaccinated group. For NDV, the HI titre in the VG/GA-Avinew+H120+CR88 vaccinated group was significantly lower than the single VG/GA-Avinew group. However, both groups had titres above the NDV HI protective titre. In both IBV vaccinated groups following either a M41 or QX challenge, the ciliary protection remained above 94%. These results show that simultaneous application of live VG/GA-Avinew and live IBV H120+CR88 vaccines on day-old chicks induces protective NDV HI antibody titres, with no adverse effect on the protection conferred against virulent IBVs.

Introduction

Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) cause huge economic losses to producers due to respiratory, reproductive, renal, intestinal and neurological diseases (Alexander, 2000, Jackwood and de Wit, 2013). Previous

studies have shown that IBV interferes with the replication of live Newcastle disease vaccine viruses (Bracewell et al., 1972, Thornton and Muskett, 1973, Thornton and Muskett, 1975, Gelb et al., 2007). In all of these previous studies, single strains of NDV and IBV live vaccine viruses were used. In recent years, for better and broader protection against variant IBVs, two different strains of live infectious bronchitis vaccine viruses are simultaneously administered to young chicks (Awad et al., 2015a). This study examines the interactions between live NDV (VG/GA-Avinew), Massachusetts (H120) and 793B (CR88) vaccine viruses when applied simultaneously to day-old commercial broiler chicks.

Materials and methods

Day-old commercial broiler chicks were allocated into four groups (Table 1). Groups A, B and C received NDV VG/GA-Avinew, IBV H120+CR88 or VG/GA-Avinew+H120+CR88 whereas the control (D) group was sham inoculated with sterile water (SW). Each chick was inoculated by ocular (50 µl) and oral (50 µl) routes (Table 1). Dosages received by each bird were as recommended by the manufacturers. The vaccines mixtures were prepared as previously reported (Awad et al., 2015b). The live vaccines were supplied by Merial S.A.S, Lyon, France. The H120 and CR88 belongs to Massachusetts and 793B serotypes respectively.

Table 1. Vaccination groups and challenge schedule

Group	Vaccination (n)	Group (at 21 days-old)	Challenge Virus (n)
A	VG/GA-Avinew (50)	A1	M41 (10)
		A2	QX (10)
B	H120+CR88 (50)	B1	M41 (10)
		B2	QX (10)
C	VG/GA-Avinew + H120 + CR88 (50)	C1	M41 (10)
		C2	QX (10)
D	Unvaccinated (50)	D1	M41 (10)
		D2	QX (10)

Following either vaccine or sham inoculation, oropharyngeal (OP) swabs were randomly collected from 10 chicks of each group at 3, 7, 10 and 14 days. At 7, 14 and 21 days post vaccination (dpv), 5 chicks from each group were euthanized for pathological assessment, trachea and kidney sampling for histopathology and virus detection. Blood was collected at 21 dpv before 10 birds from each group were removed to separate rooms for challenge by either virulent M41 or QX. Ciliary

protection was assessed as before (Cook et al., 2009). OP and tissue samples were analysed for the presence of viruses by RT-PCR (NDV and IBV) or qRT-PCR (IBV) (Cavanagh et al., 1999, Aldous and Alexander, 2001, Jones et al., 2011).

NDV and IBV antibodies were detected by haemagglutination inhibition (HI) testing (Allan and Gough, 1974; Alexander and Chettle, 1977). The IBV antigens used for the HI assay were M41 and 793B, which were purchased from Animal Health Service, Deventer, Netherlands.

The serological data were analysed statistically using analysis of variance (ANOVA), followed by Tukey's test. Differences were considered to be significant when $P \leq 0.05$. All analyses were conducted using SPSS Statistics 22.

Results

Post-vaccination clinical signs and lesions

No clinical signs or lesions were seen in the unvaccinated group. In the vaccinated groups, mild respiratory clinical signs were observed. No necropsy lesions were found in birds that were euthanized for sample collection. One, two and three chicks died in the A, B and D groups respectively with lesions suggestive of injuries or bacterial challenge.

Post-vaccination RT-PCR OP swabs

Each OP swab was tested for presence of IBV and NDV using RT-PCR. NDV was not detected at any point. IBV was detected at all sampling points in Groups B and C, and was absent from Groups A and D. Interestingly we detected the Mass-type vaccine strain up until 10 dpv in Group B and up to 14 dpv in Group C. After this, the 793B-type vaccine was identified. All Mass-type identified strains had high sequence homology to the original vaccine, ranging from 92.97-99.74%. Similarly, the 793B-type strains had sequence homology ranging from 93.72-99.47%.

Post-vaccination RT-PCR tissues

We did not detect NDV in any samples. IBV vaccine strains were recovered at 7 and 14 dpv from Group B, and at all sampling days in Group C (Table 2). It is worth noting that no trachea samples were IBV-positive at 21 dpv and no Mass-type strains were recovered in the kidney past 7 dpv. Sequence homology to the vaccine strains was high, between 98-100% with a single CR88 exception of 96% at 21 dpv.

Post-vaccination serology - IBV and NDV haemagglutination inhibition (HI):

Table 3 provides the mean NDV HI and IBV (against M41 and 793B antigens) at day-old and at 21 dpv.

Post-challenge clinical signs

Clinical signs were seen in all groups challenged with either the M41 or QX strains. The groups that were not vaccinated against IBV (D: Unvaccinated; A: VG/GA-Avinew-vaccinated) had mild signs from 1-5 dpc. The IBV-vaccinated groups (B: H120+CR88; C: VG/GA-Avinew+H120+CR88) showed mild clinical signs from 2-3 dpc.

Post-challenge ciliary protection

The ciliary protection against M41 in the H120+CR88 and VG/GA-Avinew+H120+CR88 groups were 99% and 94% respectively. Similarly, the protection percentages against the QX challenge were 99% and 97% in the H120+CR88 and VG/GA-Avinew+H120+CR88 groups respectively. There was no ciliary protection against M41 and QX in the unvaccinated and VG/GA-Avinew groups.

Discussion

In this study, we aimed to investigate the protection conferred by separate or combined IBV and NDV vaccination. Broiler chicks received live vaccinations for IBV and NDV at one-day-old and were then separately challenged with two virulent IBV strains (M41 and QX) at 21 dpv. High protection was witnessed against both IBV challenge strains, and no challenge virus strains were recovered in tissue samples from both IBV-vaccinated groups (B: H120+CR88; C: VG/GA-Avinew+H120+CR88) chicks.

Ciliostasis analysis at 5 dpc demonstrated a high level of protection in both IBV vaccinated groups against both challenge strains (M41 and QX). Previous work by Chhabra et al., has shown that the combination of H120+CR88 conferred excellent ciliary protection against Q1, a strain first identified in the same region as QX (Chhabra et al., 2015, Yu et al., 2001, Wang et al., 1998). Furthermore, when combined with the NDV vaccine (VGA/GA-Avinew), protection level still remained high, above 93%, indicating combined vaccination had no detrimental effect on the protection against virulent IBVs.

Table 2. RT-PCR result of kidney and trachea samples (7-21 dpv) tested for both IBV and NDV

Group	Day	Tissue	PCR Result		IBV Genotype	% (Homology)
			IBV	NDV		
Group A: VG/GA	7	Kidney	-	-	-	-
		Trachea	-	-	-	-
	14	Kidney	-	-	-	-
		Trachea	-	-	-	-
	21	Kidney	-	-	-	-
		Trachea	-	-	-	-
Group B: H120+CR88	7	Kidney	-	-	-	-
		Trachea	+	-	H120/CR88	98/99
	14	Kidney	-	-	-	-
		Trachea	+	-	H120/CR88	100
	21	Kidney	-	-	-	-
		Trachea	-	-	-	-
Group C: VG/ GA+H120+CR88	7	Kidney	+	-	H120	99
		Trachea	+	-	H120	99
	14	Kidney	+	-	CR88	99
		Trachea	+	-	CR88	99
	21	Kidney	+	-	CR88	96-99
		Trachea	-	-	-	-
Group D: CONTROL	7	Kidney	-	-	-	-
		Trachea	-	-	-	-
	14	Kidney	-	-	-	-
		Trachea	-	-	-	-
	21	Kidney	-	-	-	-
		Trachea	-	-	-	-

Table 3. Mean HI titres for NDV and IBV (4/91 antigen and M41 antigen) at day-old and 21 dpv

Group	Vaccine Administered	GMT (SD)		
		NDV	IBV (4/91)	IBV (M41)
Day 0	-	5.40 (1.02)a	5.80 (0.75)a	6.40 (0.49)a
A	VG/GA	6.63 (0.99)b	-	-
B	H120+CR88	-	6.75 (0.97)b	9.75 (0.43)b
C	VG/GA+H120+CR88	5.63 (1.41)a	5.88 (0.78)a	6.88 (0.78)a
D	Control	1.67 (0.86)c	1.50 (0.70)c	1.80 (1.05)c

1Standard error margins are shown in brackets. Significant differences between groups ($p < 0.05$) are labelled as either a or b. Groups with no significant differences between them are labelled with the same letter.

Work related to virulent NDVs is not allowed in our laboratory due to local regulations. Serological NDV HI titre of 2 log₂ and above have been reported to correlate with clinical protection of chickens (Ganapathy et al., 2007). In this experiment, the mean NDV HI titres in both NDV-vaccinated (A:VG/GA-Avinew; C:VG/GA-Avinew+H120+CR88) were 5.3 log₂ or higher, demonstrating that the simultaneous vaccination does not compromise the immune responses or protection against NDV.

In conclusion, this study demonstrates that the combined vaccination program of VG/GA-Avinew+H120+CR88 has no detrimental effect on the efficacy of either vaccine when given simultaneously to one-day old NDV-IBV maternal-antibody positive broiler chicks.

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IBV challenge of laying hens

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Abstract

Protection of layers/breeders against IB infections during the laying period is achieved by live IB priming followed by a boost with inactivated IB vaccine. For many IB variants, homologous live vaccines are not available for priming. Very little is known about the efficacy of priming with heterologous live IB vaccines (or combination of live IB vaccines) to induce broad IB protection in long living chickens.

In this study, the value of a broad heterologous priming using vaccines of Mass and 4/91 serotype for a homoogous inactivated vaccine was investigated to protect layers against an IBV D1466 challenge analysing egg production, egg quality, respiratory signs and ciliostasis during 4 weeks post challenge.

The D1466 challenge in layers primed with IB Ma5 and IB 4/91 and boosted with inactivated IB3/G/ND induced a low and short drop in egg production of 2.8% that was comparable to the low and short drop in egg production of 3.3% of the mock-infected group. This was significantly lower ($p=0.003$) than the long-lasting drop in egg production of the non-vaccinated control birds (16.6%). Birds only primed with live IB and birds only vaccinated with inactivated vaccine had intermediate drops of egg production. Only the primed and boosted group had a lower ($p=0.010$) proportion of egg quality disorders post challenge compared to the non-vaccinated control group.

It was concluded that heterologous IB priming of layers can increase the efficacy of IB inactivated vaccines against drops in egg production and egg quality post IB challenge.

Field evaluation of aMPV vaccine (NEMOVAC®) in multicausal infectious respiratory disease in broiler flocks

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Abstract

Multicausal infectious respiratory disease (MIRD) has caused dramatic economic losses in Iranian chicken flocks in recent years. Recent findings based on samples gathered in the field indicated that avian metapneumovirus (aMPV) is one of the most prevalent pathogens involved in MIRD. The aMPV vaccines are commercially available in Iran but to-date vaccination has only been permitted in broiler breeder flocks. In order to evaluate the ability of aMPV vaccine to reduce damage and mortality related to MIRD, NEMOVAC® was administered to 35 broiler flocks with high population density and a history of MIRD in previous production periods. Forty-five broiler flocks located in the vicinity of the vaccinated flocks were left unvaccinated as control groups. Other vaccines for Newcastle disease, Infectious bronchitis, Avian influenza (H9N2) and Infectious bursal disease were administered based on routine local vaccination programs. Blood samples were collected from both groups (vaccinated and unvaccinated flocks) before vaccination and at the end of the production period in order to measure antibody response to aMPV. Mortality rate, feed conversion ratio (FCR) and slaughter weight were recorded and analyzed statistically. Tracheal and choanal cleft swabs were collected from broiler flocks with clinical signs related to MIRD for molecular investigation

The level of antibody before vaccination (maternally derived antibody) is significantly higher than at slaughter age ($P < 0.001$). The serological response in vaccinated flocks was weak and there was no significant difference in level of antibody between vaccinated and unvaccinated flocks. Also, no significant difference was found in slaughter weight. On the other hand, the vaccinated flocks had significantly lower FCR ($P < 0.05$). In addition, a significant difference ($P < 0.001$) was found in the mortality rates and some of the unvaccinated flocks experienced severe MIRD and

higher mortality rates. The results show that in areas where aMPV infection is prevalent, vaccination of broiler flocks may directly diminish the mortality caused by MIRD.

Introduction

Avian metapneumovirus (aMPV) causes an acute contagious upper respiratory tract infection in turkeys and chickens. The aMPV was first isolated in South Africa in the late 1970s and it is now considered a major disease threat to both turkeys and chickens in many parts of the world (Jones and Rautenschlein, 2013 and Pedersen and Gough, 2009). The aMPV has been identified as causative agent of respiratory disease in broilers and egg drop in broiler breeders in Iran (Hosseini H. and Ghalyanchi-Langeroudi, 2012; Ghalyanchi-Langeroudi et al. 2013). The B-subtype is the only subtype that has been detected in Iran so far.

Multicausal infectious respiratory disease (MIRD) has been causing dramatic economic losses in Iranian chicken flocks in recent years. Avian influenza (AI) (H9N2), Infectious bronchitis (IB), Newcastle disease viruses and aMPV along with bacterial infections are considered to be involved in MIRD in broiler flocks. Recent findings indicate that aMPV is one of the most prevalent pathogens involved in MIRD (Akbari et al. 2016; Ghafouri et al. 2016 and unpublished findings). Live vaccines are commercially available for prevention of disease and economic losses caused by aMPV in broiler flocks. However, to date the use of aMPV vaccine in broilers has not been permitted. In order to evaluate the ability of aMPV vaccine to reduce the damage and mortality related to MIRD this study focusses on the use of aMPV vaccine in the field where broiler flocks are more likely to suffer from this disease.

Materials and Methods

Broiler Flocks

Thirty-five broiler farms in nine provinces (Mazandaran, Gilan, Golestan, East Azerbaijan, Kermanshah, Razavi Khorasan, Qom, Tehran and Zanjan) were randomly selected as test (vaccinated) groups. Forty-five neighboring flocks were left unvaccinated as control groups. All farms are located in areas of high poultry population density and have a history of MIRD in previous production periods.

Vaccination. Flocks in the test farms were vaccinated with NEMOVAC® according to the manufacturer's instructions by spray or drinking water routes. The other vaccines (ND, IB, AI (H9N2) and Infectious bursal disease (IBD)) were applied based on routine local vaccination programs.

Sampling

Blood samples were collected from both vaccinated and non-vaccinated flocks before vaccination and at the end of the production period. The response levels of antibodies against aMPV were measured with commercial ELISA kits (Biocheck, the Netherlands) according to the manufacturer's instructions. Tracheal and choanal cleft swabs were collected from broiler flocks showing clinical signs related to MIRD and were analyzed by RT-PCR (Hosseini H. and Ghalyanchi-Langeroudi, 2012).

Statistical analysis

The mean antibody titers, mortality rate, feed conversion ratio (FCR) and slaughter weight were compared using repeated measures where a P -value < 0.05 was considered statistically significant.

Results and Discussion

The majority of the broiler flocks (96%) were seropositive at the first sampling. Seropositivity for aMPV at an early age is related to maternally derived antibodies (MDA), resulting from vaccination against aMPV by live and killed vaccines in broiler breeders. However, some vaccinated (35%) and unvaccinated flocks (32%) became seronegative at the second sampling. The mean antibody titer in all vaccinated and unvaccinated flocks at the end of production period was significantly lower than those at an early age (2889 vs. 6630). Previous studies showed that humoral antibodies do not have an important role in respiratory protection and antibody may not be detected following vaccination (Ganapathy et al. 2010). However, the application of aMPV by drinking water or spray induces sufficient immunity to protect against challenges in the absence of antibodies. The unvaccinated flocks developing MIRD were all seropositive for aMPV and in some cases (18%) aMPV was detected by RT-PCR.

With regards to production performance, no significant difference was found in slaughter weight ($P > 0.05$). This was probably due to differences in age of slaughter. However, there was a significant difference in FCR ($P < 0.05$) and the vaccinated flocks had lower FCR than the unvaccinated ones (2.01 vs 2.14).

The mortality rate was also significantly lower in aMPV vaccinated flocks compared to the unvaccinated ones (7% vs. 18.73%). The mortality rate ranged from 2.3 to 11.5% and 8 to 53% in the vaccinated and unvaccinated flocks, respectively. In fact, some unvaccinated flocks experienced severe MIRD with a high rate of mortality (up to 53%). This difference in the mortality rate of aMPV vaccinated flocks may pertain to the protection achieved by the vaccination against aMPV. In the field chickens are most susceptible to aMPV when they are between 2 and 6 weeks old. This increase

in susceptibility is related to the waning of MDA and although MDA cannot provide full protection, it may reduce virus replication (Aung et al. 2008). Similarly, field observations in Iran demonstrated that most MIRD cases occurred after the second week of the rearing period.

Multiple causative agents may be involved in severe respiratory disease in Iranian commercial broiler farms. Co-circulation and co-occurrence of different respiratory agents may result in variations in clinical outcome. Recent studies revealed that aMPV is one of the most prevalent pathogens in MIRD cases. In more recent reports of MIRD, AIV, ND, aMPV, IBV and *Ornithobacterium Rhinotracheale* (ORT) were the prevalent pathogens (Akbari et al. 2016; Ghafouri et al. 2016; and unpublished findings), showing that the co-occurrence of these four pathogens is likely. It is proposed that vaccinating broiler chicks against aMPV may reduce the severity and duration of MIRD. Regarding the high prevalence of aMPV among MIRD cases, it seems that vaccination not only provides adequate immunity against aMPV, but that it also reduces the severity, duration and mortality rate of the MIRD cases.

This study was carried out in the regions where the majority of Iranian broiler flocks are located and where MIRD is a common reason for economic losses. The results of this study may suggest that in areas where aMPV infection is prevalent, the prevention of aMPV by vaccination may directly affect a reduction in the mortality caused by MIRD.

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Protection conferred by H120 vaccine against IBV Moroccan Italy 02 in commercial broilers and SPF chickens

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Abstract

The ability of H120 to protect against Moroccan-Italy02 IBV was investigated in broilers and SPF chickens. Commercial broilers were vaccinated at day old in the hatchery for IBV (H120) and NDV (Avinew) in a Merial sprayer cabinet, while SPF birds received via oculonasal route a dose of H120 at one day of age. Chickens were challenged by oculonasal with 10^3 EID₅₀ /0.2 ml of IBV Moroccan Italy02 virus at three weeks of age. Control birds (non challenged and unvaccinated but challenged for SPF birds) were housed in the same conditions. Protection was assessed by daily clinical observations, lesions at necropsy 5 days post challenge (pc) and virus shedding in oropharyngeal and cloacal swabs on days 3, 5, and 7.

In commercial broilers, respiratory signs ranged from mild respiratory distress to severe gasps, as early as 24 hours pc. The maximum severity of clinical signs was observed on days 3 and 4 pc. 90% of the tracheas collected on day 5 showed hyperplasia and deciliation.

In SPF vaccinated birds, respiratory signs started on day one pc in 50% of birds and peaked on day 4 pc. In unvaccinated birds respiratory signs were more pronounced as early as day 3 pc and persisted up to 10 days in all birds. The histopathology investigations on day 5 pc did not show much damage in tracheas of vaccinated birds in contrast to deciliation and hyperplasia observed in unvaccinated chickens. Virus was detected in 45% of the vaccinated birds on days 3 and 5, while 100% of the swabs were positive on days 3 to 7 pc for unvaccinated chickens. IBV Moroccan-It02 is therefore highly pathogenic for chickens and the H120 vaccine alone did not protect birds against clinical signs but contributed to a faster disease recovery.

Production of recombinant N and S1 protein of IBV using the baculovirus expression system and its assessment as a diagnostic antigen

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Abstract

The avian coronavirus-Infectious Bronchitis Virus (AvCoV-IBV) is recognized as an important avian pathogen and new viral variants are a continuous threat to the poultry industry worldwide. Sensitive diagnostic and efficacious prophylactic reagents are necessary to combat IBV infections in chickens. The aim of this study was to produce recombinant N and S1 protein of IBV to use in ELISA diagnostic testing in order to assess the seroprevalence and risk of IBV infections in chickens in Turkey. For this, the gene encoding the N protein of the Beaudette strain of IBV was expressed using a recombinant baculovirus expression system. The recombinant protein was purified using Ni-NTA affinity chromatography. A 49-kDa recombinant protein corresponding to the expected molecular weight of IBV N including the 6xHistag was detected using anti-His monoclonal antibody as well as a 23 kDa protein of partial S1. Specific immunoreactivity of the recombinant protein was confirmed by Western blot using antiserum obtained from chickens naturally infected with a local (Turkish) strain of IBV as well as using a monoclonal antibody raised against the N protein of the IBV Massachusetts strain. These results suggest that the recombinant IBV N protein is broadly cross-reactive with antisera produced against different IBV strains. We conclude that the recombinant baculovirus expressed IBV N protein could serve as a useful diagnostic antigen for detection of IBV infections in chickens.

Introduction

Infectious bronchitis virus (IBV) is an enveloped, single positive-stranded RNA virus in the family Coronavirus, genus Gammacoronavirus. As one of the major contributors to economic losses in the poultry industry globally, the virus poses a persistent threat. IBV is the cause of an acute disease, Infectious Bronchitis, in chickens. The virus is spread mainly by aerosol, consumption of contaminated feed and water, and contact with infected feces or equipment. IBV is characterized by various clinical signs in broilers and layers: coughing, sneezing, and decreased weight gain. Specifically in layers, egg production can drop up to 70% with eggs that have shells that are wrinkled, thin, and soft. In chicks, IBV infection can lead to oviduct cysts and reduced laying potential (Ignjatovic and Sapats 2000; Cavanagh 2007; De Wit et al., 2011).

The genome of IBV encodes nine functional genes, which include the spike protein (S), a viral surface glycoprotein shown to induce the neutralizing antibody response, and the nucleocapsid protein (N) (Cavanagh 2007; De Wit et al., 2011). There is a high rate of emergence of antigenic variants and reassortant strains, making control and vaccine development especially challenging (Cavanagh 2007). The primary objective of this study is to assess the utility of the more conserved N protein as a tool for serodiagnostic development, and the more polymorphic S1 as a potential subunit vaccine candidate.

Materials and methods

Cloning and construction of recombinant bacmid

The N coding sequence (1,230 bp) of the Beaudette IBV strain, and a truncated 600 bp S1 coding sequence of Israel variant 2-4/94 IBV strain were initially amplified by PCR. The PCR products were cloned into pFastBac vector, to create donor plasmids, pFastBac-N and pFastBac-S1, which were transformed into One Shot Mach1 T1 Chemically Competent E. coli strain. The donor plasmids were double digested with restriction enzymes BamH1 and Pst1 to determine the presence of the correct insert in the right orientation (Figure 1). Integrity of the sequences was confirmed by DNA sequencing. The donor plasmid was transformed into MAX Efficiency DH10Bac Competent E. coli strain to construct a recombinant bacmid via site-specific transpositioning.

Expression and purification of recombinant N and S1

The purified recombinant bacmids were used to infect *Spodoptera frugiperda* (Sf9) cells grown in Sf-900 II SFM medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin to rescue the recombinant baculoviruses. The recombinant baculoviruses were used to express the recombinant IBV proteins, and then purified by affinity column chromatography using Ni-NTA superflow resin. Proteins were eluted with 250 mM imidazole and dialyzed overnight against PBS (pH 7.4).

Western Blot Analysis and Immunoreactivity

Western blot analysis was performed to verify specific protein expression. The membranes were probed with anti-His (C-Terminal)- HRP monoclonal antibodies (1:5,000) to determine expression of the correct molecular size proteins. Further confirmation of expression was performed using a mouse monoclonal antibody, B819M, (1:2,000) against the N protein of Massachusetts IBV strain as well as antiserum collected from chickens naturally infected with local Turkish wildtype IBV strains. The membrane was then incubated with anti-mouse IgG-HRP secondary antibody conjugate at 1:5,000 dilution and specific signals were detected using the enhanced chemiluminescent (ECL) detection system.

Results

Expression of the the recombinant proteins, IBV N (52 kDa) and truncated IBV S1 (23 kDa) exhibiting the correct molecular weights was determined using anti-His (C-Terminal)-HRP monoclonal antibody (Figure 2A and B); expression of recombinant IBV N protein was further confirmed by Coomassie blue staining.

Specific reactivity of recombinant N protein was confirmed using monoclonal antibody raised against the N protein of the Massachusetts IBV strain as well as antiserum obtained from chicken naturally infected with Turkish wildtype IBV strains); the recombinant S1 was not reactive with the chicken antiserum.

Discussion

The recombinant N protein derived from sequences of the Beaudette IBV strain was reactive against anti-His (C-Terminal)-HRP monoclonal antibody, indicating expression of the correct molecular size protein. The protein displays specific reactivity against a heterologous monoclonal antibody (B819M) derived from the IBV Massachusetts strain; and against antiserum from chickens naturally infected with Turkish IBV strains. While the expected molecular weight for recombinant S1 protein, based on the Israel variant 2-4/94 IBV strain, was detected using the anti-His (C-Terminal)- HRP monoclonal antibody, it did not show reactivity against the antiserum from naturally infected Turkish chickens. This appears to be due to antigenic variation of S1 among different strains of IBV. Based on these reactive profiles, the recombinant IBV N protein seems to be a strong candidate for development of serodiagnostic tools.

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Epidemiology

Keynote Lecture

Role of wild birds in ecology of avian coronavirus; AvCoVs on the wings

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Abstract

A successful prevention strategy for any animal diseases requires scientific-based monitoring program and up to date epidemiological data. Therefore there is a need for additional or updated epidemiological data regarding currently circulating AvCoVs both in poultry and wild bird population around the globe.

Avian viruses in wild birds has been partly neglected by the veterinary community and are best described in poultry species such as chickens and turkey due to the economic implication of the disease for the poultry industry. There are many contributing factors for the apparent lack of disease studies, including the difficulties of finding sick or dead animals in the field, distinguishing infected from non-infected individuals, availability of molecular tools as well as biosafety considerations for potential zoonotic infection. On top of that, pathogen communities are as complex as host communities, with large genetic variation at species, subtype and strain levels, and display spatial and temporal variation in prevalence rates in their host populations in relation to a large number of environmental, trophic and intrinsic factors. Furthermore, many pathogens tend to infect more than one host species, and most host species tend to be infected by more than one pathogen species, making it hard to study a single pathogen in a single host in isolation. Most surveillance schemes provide information on a single pathogen, in a population of animals and in a snapshot of time.

With exception of isolates from chickens, turkey, guinea fowl and peasant, avian gammacoronavirus has also been detected in variety of avian orders including Anseriformes, Ciconiiformes, Charadriiformes, Columbiformes, Galliformes, Passeriformes, Pelecaniformes, and Psittaciformes. Avian deltacoronaviruses are also composed of viruses detected in wild birds, which suggests that wild birds play an important role in the epidemiology of AvCoVs.

However, our knowledge of the prevalence of AvCoVs in wild bird's species is very limited and few studies have been performed to assess host species range, seasonal prevalence, geographic distribution and genetic variation among these viruses. Therefore the surveillance activities should tailored to meet the global need for knowledge and used to develop and constantly improve the disease prevention strategies for the poultry industry. The Veterinary community has knowledge and areas of expertise, which should undoubtedly be also part of surveillance strategies in wild birds.

Prevalence and genetic diversity of avian coronaviruses in Luxembourg, The Netherlands and Nigeria

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Abstract

Similar to avian influenza and Newcastle disease virus, avian coronaviruses (CoV) seem to use wild birds as reservoirs. The large variety of potential hosts raises the question of what impact avian CoV from wild birds may have on domestic poultry. Here we report surveillance data from three countries, namely Luxembourg, the Netherlands and Nigeria, showing a high genetic diversity of both gamma- and delta-coronaviruses, as well as a high diversity of avian hosts.

Introduction

Increasing evidence suggests that several wild bird species are asymptomatic carriers of a wide variety of avian CoV (Muradrasoli *et al.*, 2010; Wille *et al.*, 2015), but avian CoV prevalence, host range and infectious potential for poultry are currently unknown. In addition, CoV are prone to recombination and as-yet-unknown CoV strains might be the donors of new genes to infectious bronchitis virus (IBV), leading to the generation of new serotypes or new CoV species such as Turkey and Guinea fowl CoV (Brown *et al.*, 2016; Ducatez *et al.*, 2015). In this study, we aimed to detect and characterize the gamma- and delta-CoV strains in wild birds in Luxembourg, the Netherlands and Nigeria and thereby understand the links between viral strains from various species or regions.

Materials and methods

Cloacal and/or tracheal swabs and faeces from wild birds sampled during the surveillance for avian influenza in Luxembourg (2006-2010 and 2014-2015), the Netherlands (2007) and Nigeria (2007-2008 and 2011) were screened for the presence of gamma- and delta-CoV by a modified nested RT-PCR (Chu et al., 2011). Positive samples were sequenced using the same primers. Phylogenetic analyses including partial RNA-dependent RNA polymerase (RdRp) gene sequences available on GenBank and the sequences obtained in this study were performed with MEGA v6 (Tamura et al., 2013).

Results

In total, 5.3% (53/1000) of birds sampled in the framework of continuous surveillance between 2006 and 2010, tested positive for gamma- or delta-CoV in Luxembourg, compared to 7.5% (13/174) of birds sampled during winter 2014-2015. In the Netherlands and Nigeria, 4.4% (34/776) and 1% (16/1576) of birds tested positive, respectively.

Ninety-three partial RdRp sequences were obtained and phylogenetic analyses based on a 366 bp fragment revealed great genetic diversity. Gamma- and delta-CoV were identified in the three countries. In total, 19, 24 and 7 gamma-CoV and 26, 10 and 7 delta-CoV strains were found in Luxembourg, the Netherlands and Nigeria, respectively. Most of the gamma-CoV strains from wild birds grouped within three clusters (A, B and C) distinct from infectious bronchitis viral strains, and interspersed with other strains found in Europe, Asia, Africa or the USA, resulting in no clear geographic or species-specific cluster. In Anseriformes the majority of strains clustered within the gamma-CoV cluster A which includes the largest number of strains, but also within gamma-CoV cluster B and to a lesser extent within delta-CoV. The strains found in pigeons also formed a separate cluster C including mainly strains from pigeons from China.

A greater genetic and host diversity (Anseriformes, Pelaconiformes, Gruiformes, Charadriiformes, Passeriformes) was observed in delta-CoV compared to gamma-CoV. However, most sequences clustering within delta-CoV grouped together with sequences obtained from related bird species. For instance, two strains from great cormorants found in Luxembourg grouped with sequences from great cormorants from Hong Kong. A strain from a Sudan golden sparrow from Nigeria was most closely related to sequences from tree sparrows from Luxembourg.

In addition, inter-species transmission involving poultry was also observed. In Luxembourg, an IBV-like strain was found in a common blackbird. A chicken was harbouring a gamma-CoV strain belonging to the cluster C including mainly pigeon strains, and a delta-CoV strain from a tree sparrow cluster was found in a peafowl.

Discussion

Our data show differences in gamma- and delta- CoV prevalence according to country which is most likely to be due to species sampling differences. However, seasonality and the age of the birds could also play a role in the frequency of virus detection, since the influx of birds at migratory sites and the presence of juveniles could influence virus transmission (Wille *et al.*, 2015). Similarly to avian influenza virus, gamma-CoV seem to be easily exchanged between migratory birds, especially ducks, as shown by the similarity of strains shared by various species. Shared environments such as feeding and resting places, might facilitate inter-species CoV transmission. On the other hand, the surveillance of gamma- and delta-CoV is generally biased by the cohorts used. Indeed, Passeriformes or Columbiformes also host specific delta or gamma-CoV strains. Our results suggest that despite their diversity, delta-CoV tend to be more restricted to certain bird species, compared to gamma-CoV, although exceptions were noted. Three cases of IBV-like strains in wild birds or other gamma- and delta-CoV in poultry were also observed, showing that bridge species with potentially more frequent contacts with poultry could participate in increasing the interspecies transmission of CoV to and from poultry.

In the future, more efforts towards sequencing a larger fragment of the RdRp gene to improve phylogenetic inference, especially of gamma-CoV, is desirable. This would enable a more precise understanding of the species and geographic distribution of viral strains. In addition, sequencing of the spike gene will be needed in order to investigate the relationship between avian CoV strains from wild and domestic birds.

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Multi-strain infection by infectious bronchitis variant viruses in broiler and breeder flocks in Latin America

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Abstract

Infectious bronchitis virus (IBV) causes a highly contagious upper-respiratory tract disease in chickens and some strains are nephropathogenic. The virus can also cause significant egg production losses and mortality in commercial layers and breeders. Three broiler companies in Colombia with several broiler flocks and five breeder flocks presenting overt respiratory signs, nephritis lesions, late mortality, decreased egg production and decreased fertile egg quality were investigated for IBV infection. Results from ELISA serology and a molecular diagnostic survey confirmed that flocks had been infected by either one or concomitantly by more than one of the IBV variant strains (Q1 [China], YEM/L 2865-2005 [Yemen], K46/10 [South Korea] and PRTL 898/04 [Portugal]). Details of the diagnostic and clinical investigation are discussed.

Introduction

Infectious bronchitis virus (IBV) causes a highly contagious respiratory tract disease in chickens, infectious bronchitis (IB), and some strains are nephropathogenic. It is possibly the most economically important viral respiratory disease of chickens in regions where there is no highly pathogenic avian influenza virus or velogenic Newcastle disease (vND) virus and is found everywhere that broilers are commercially produced (Cook *et al.*, 2012; Jackwood and de Wit, 2013). Even in some countries endemic for vND (e.g., Peru and Colombia), IB is regarded as having a similar or even higher economic impact to vND (Sesti *et al.*, 2014a; present report). The virus can also cause significant egg production losses and mortality in commercial layers and breeders (de Wit *et al.*, 2011b).

Coronaviruses, particularly IBV, are RNA viruses that present high genetic mutation rates. When those mutations occur in the spike gene they can result in the emergence of antigenic variant strain viruses or new serotypes (McKinley, 2009;

Lim *et al.*, 2012; Toro *et al.*, 2012). These variant strains are often partially or not controlled at all by live and inactivated IBV Massachusetts strains-based vaccines. During approximately the last eleven years, several countries in Latin America have reported an increasing incidence of clinical and subclinical outbreaks of IBV caused by variant strains of the IBV (de Wit *et al.*, 2011b; Chacón *et al.*, 2011; Jackwood, 2012; Sesti *et al.*, 2014a; Sesti *et al.*, 2014b).

This short article reports the findings in three broiler companies in Colombia (companies A, B and C; Midwest and Northeast regions) which had several broiler breeder (five flocks investigated in company A) and broiler flocks (several flocks in companies B and C) presenting overt respiratory signs, nephritis, late mortality, decreased egg production and decreased fertile egg quality, investigated for IBV infection. Results from ELISA serology and a molecular diagnostic survey confirmed that flocks had been infected by either one or concomitantly by more than one of the following IBV variant strains: a) Q1 originally isolated in China (Yu *et al.*, 2001), b) YE/L 2865/05 original molecular detection in swabs taken from industrial poultry in Yemen (Worthington *et al.*, 2008), c) K46/10 originally isolated in South Korea (Lim *et al.*, 2012) and, d) PRT/L 898/04 original molecular detection in swabs taken from industrial poultry in Portugal (Worthington *et al.*, 2008). Details of the clinical and diagnostic investigation and control attempts will be reported.

Material and methods

IBV molecular detection and genetic characterization

GD Animal Health. All molecular detections and genetic characterizations were carried out at the poultry diseases diagnostic laboratory GD Animal Health (Deventer, Holland). Samples comprised tissue imprints from trachea, lungs, kidneys and cecal tonsils from clinically affected birds on FTA card (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ, USA). The FTA card material was processed for IBV molecular detection (RT-PCR) and sequencing of PCR products as described elsewhere (de Wit *et al.*, 2011a). In short, nine sample discs from the dried spot on each of the four FTA card circles were collected using a 2.0 mm diameter Harris micro punch and placed in a 1.5 mL tube. 270 µL of RNA Rapid Extraction solution (Ambion) was added to the tube and incubated for 5 minutes after mixing. Subsequently 200 µL of solution for RNA extraction (MagMAX system) was added. The RT-PCR was performed as described earlier (de Wit *et al.*, 2011a). A fragment of about 350 base pairs of the S1 gene was amplified with the primers XCE1⁺ and XCE3⁻ (Cavanagh *et al.*, 1992). The S1 amplicons were separated on a

1% agarose gel, and visualized with ethidium bromide staining and an ultraviolet light transilluminator. The purified amplicon was sequenced (BaseClear, Leiden, The Netherlands) using both XC1+ and XCE3 primers. The sequence data were aligned using computer software (Bioedit, Ibis Biosciences, Carlsbad, USA).

Broiler flock sampling and sample submission for molecular diagnostics were carried out on different occasions. Likewise, the detection of strains Q1, YE/L and PT/L in company A was performed in five different breeder flocks sampled at different ages and on different sampling dates and samples were submitted to the laboratory on different occasions.

IBV serology.

When used, IBV serology was carried out locally (laboratories in Colombia) in commercial ELISA kits (Idexx® IBV Ab Test, Idexx® laboratories, Westbrook, Maine, USA).

Field data.

For the flocks suspected of undergoing an Infectious Bronchitis outbreak clinical signs, macroscopic lesions and flock clinical and productivity data were collected when appropriate or possible.

Results

Table 1, presents the variant IBV strains diagnosed in the three poultry companies in Colombia. Four different IBV variant strains were detected (Q1, YE/L, K46/10 and PT/L). The diagnosis of the Q1 and K46/10 strains was carried out in few broiler flocks in companies B and C respectively. Percentage nucleotide homology of the detected partial portion of the S1 spike gene in the variant IBVs on the FTA cards when compared with the originally GenBank-deposited sequences were 96.7-98% for the Q1 (accession numbers: AF286302 = original Q1 and HM446006 = Q1 from Chile), 96.3 % for YE/L (accession number = EF006524), 97% for the K46/L (accession number = JF804679) and 93% for the PRT/L strain (accession number = EF066521). Further genetic comparison of the variant IBVs detected with the only IBV live vaccine strain (Massachusetts H120) available in Colombia indicated a quite small S1 protein gene homology between them (Table 2).

ELISA serology titers at slaughter in broiler flocks affected with the Q1 strain were quite high at company C (maximum titer as high as 6000 and GMTs around 2500; vaccination scheme = 1 dose H120 spray at day one and one dose of Ma5 spray between 2-3 weeks of age) similar to what has been reported elsewhere (Wang *et al*, 2000). For company B, infected with the K46/10 strain, IB titers were at medium

to normal levels (maximum GMT = 2769; vaccination scheme = 1 dose H120 spray at day one; average slaughter age = 36 days). In all investigated flocks, serology for other respiratory diseases (e.g., Newcastle, metapneumovirus infection) was not indicative of field infection.

Clinical signs in broilers were quite diverse in Companies B and C and a more severe incidence of clinical disease was observed in those flocks infected by the K46/10 strain (Table 3). Late mortality (>4 weeks of age), renal and respiratory signs as well as septicemia due to secondary bacterial infection were quite common in affected flocks. In Company B, affected by the K46/10 strain, many infected flocks presented average mortality of up to 15% at slaughter age, mostly occurring during the last two weeks of the grow out period (6th - 5th week).

The five breeder flocks investigated in Company A were all clinically affected in a similar manner (Table 3). The severity of clinical signs varied from flock to flock but they were always present and easily identifiable. Flocks with infection diagnosed by either the Q1 or the YE/L or the PRT/L strain presented clinical disease at the ages of 27, 28, 30, 44 and 65 weeks (Table 1).

Discussion

This is quite an unusual report of clinical infections caused by multistrain variant IBVs occurring simultaneously in a given country in Latin America. The Q1 strain has been causing severe losses to the broiler industry of several South American countries, including Colombia, during the last 7-8 years (Alvarado, 2012; Sesti *et al*, 2014a; de Wit *et al.*, 2011b).

For the first time in Latin America the Q1 strain was not only detected but also correlated with clinical disease in a breeder flock. In addition, three other variant strains never previously reported in Latin America (K46/10, YE/L and PRT/L) were also detected and correlated with clinical disease and macroscopic lesions in broilers and breeders.

Company A, where Q1 and YE/L and PRT/L variant strains were detected, has seen recurrent clinical outbreaks in their breeder flocks, particularly during the first 15-20 weeks of production although, in the present investigation, a 65-week flock was also clinically affected by the YE/L strain. Of particular clinical severity (Table 3 for main clinical signs and lesions) was the infection of two young breeder flocks (27 and 28 weeks of age) by the strain PRT/L. The general perception in the company is that this clinical situation of IBV infection in breeders has been increasing in severity during the last several months despite each flock being vaccinated with four live and four

inactivated doses of IB vaccines during the rearing period (Mass strains only). Live Mass revaccination during production seems to slightly alleviate the clinical condition but never completely prevent or control it.

A very similar situation is occurring in broilers in Companies B and C which have had their broilers flocks challenged by the K46/10 and Q1 strains, respectively. During July 2015, an epidemiologically critical period in Company B, mortality of K46/10 affected broiler flocks averaged 14%. These broilers had been vaccinated via spray at day one with only the H120 strain. Company efforts to improve biosecurity and decrease environmental contamination as well as measures taken to reduce the vertical transmission of mycoplasma to the broiler progeny have considerably improved the IBV epidemiological situation in the company. Accordingly, by the end of 2015 the clinical situation had almost returned to normal on the broiler farms with total mortality decreasing to an average of 5.5%. Clinical IB outbreaks in broilers caused by the Q1 strain in Company C are not new for this production system since the Q1 strain has been diagnosed in the company in the past. Since the first diagnosis the company has been vaccinating broilers against IB at day one and in the field (2-3 wk of age) using two commercial Mass vaccine strains (H120 and Ma5). Nonetheless, the vaccination program has never been totally effective at controlling the Q1 strain. The same situation has been seen in recent years in Chile, Argentina and Peru (Sesti *et al.*, 2014a). Total control of the Q1 strain, at the level of both controlled protectotype trials and in the field, has only been accomplished when broilers were vaccinated with a combination of a Mass vaccine strain with a 793/B-type vaccine strain (Sesti *et al.*, 2014ab; de Wit *et al.*, 2012). Such a vaccination program cannot be used in Colombia at the moment as no 793/B-type live vaccine is registered in the country. As for the K46/10 and YE/L strains, there is no published information from protectotype trials indicating that protection against these strains would be afforded by the Mass H120 strain alone or in combination with a 793/B-type live vaccine. Based on the S1 gene nucleotide homology between the K46/10, YE/L and PRT/L strains with the Mass H120 strain (depicted in Table 2), it seems likely that the protection afforded by the Mass strains alone will be quite low against these new variants circulating in the country as it is against the Q1 strain (Ladman *et al.*, 2006; Wang *et al.*, 2000). Several years ago, Alvarado *et al.* (2005; 2012) were able to carry out the first molecular detection of four unique IBV genotype variants in Colombia although no subsequent work (pathotype and protectotype trials) has been done with those indigenous genotypes which were isolated from commercial layers and broilers.

At the moment, there is no epidemiological evidence or clues as to where from and how these IBV variants were able to arrive in Colombia and establish a new and quite economically damaging clinical picture in broiler and breeder flocks of three different poultry companies. The same question has often occurred in many parts of the world after the appearance of new IBV variants that are genetically very similar to strains originally isolated and detected on another continent several thousands of kilometers away (Jackwood, 2012; de Wit *et al.*, 2011b).

Conclusion

Such an unusual IB epidemiological situation in Colombia should be thoroughly investigated and the extent of the dissemination of the four IBV variants in the Colombian poultry industry should be clearly and quickly determined. In addition, the K46/10, YE/L and PRT/L strains should be isolated, pathotyped, with protectotype trials carried out, and eventually, new and effective vaccination programs made available for the Colombian poultry industry.

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Table 1. Molecular detection of different IBV variants in Colombia in 2015

Poultry Companies	IBV variant strain detected and flocks' age (weeks) at detection				
	Q1 broilers	K46/10 broilers	Q1 breeders	YE/L breeders	PRT/L breeders
A			30 wk	44 and 65 wk	27 and 28 wk
B		4-6 wk			
C	>4 wk				

Table 2. Percentage (%) of nucleotide homology of a portion of the S1 spike gene in the variant IBVs on the FTA cards and the Massachusetts (Mass) H120 live vaccine strain used in all three companies (Sesti *et al.* 2014b; Worthington *et al.*, 2008; Yu *et al.*, 2001; and Dr. Remco Dijkman – GD Animal Health, Deventer, The Netherlands – personal communication)

	Q1	YE/L	K46/10	PRT/L	Mass H120
Q1	100				
YE/L	83.9	100			
K46/10	61.5	62.9	100		
PT/L	81.3	85.4	62.8	100	
Mass H120	74-80	82.4	<58.7-63	82.7	100

Table 3. Relative incidence of clinical signs in broilers and breeder flocks infected with different strains of variant IBVs (incidence score: + = low / ++ = low to medium / +++ = medium / ++++ = high / +++++ = very high)

Company A - breeders (in production)		
Q1 & YE/L & PRT/L strains		
Clinical signs, lesions		
Increased weekly mortality in production with and without overt clinical disease (<i>ranging from 0.11% before to 0.35% during IB challenge in females and from 0.44% before to 2.37% during IB challenge in males [challenge period of 2-4 weeks' duration]</i>)		
Respiratory distress with tracheitis		
Decreased egg production (<i>from 1-4% above down to 2-3% below standard during IB challenge [challenge period of 2-4 weeks' duration]</i>)		
Higher incidence of colorless egg shells		
Salpingitis, congested ovary, nephritis, renal hypertrophy		
Clinical signs, lesions	Company B broilers	Company C broilers
	K46/10 strain	Q1 strain
High late mortality (> 4sem)	++++	++
Severe septicemia	++++	++
Airsacculitis	++++	++
Nephritis / Urolithiasis	+++	++
Swollen head	++++	++
Respiratory distress	++++	++
Tracheitis	++	++

Molecular epidemiology of avian infectious bronchitis virus in Iran

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Abstract

Between 2010 and 2014 more than 2500 specimens of trachea, kidney and cecal tonsil were collected from birds from 250 flocks suspected of being infected with avian infectious bronchitis virus. Partial spike glycoprotein gene sequences revealed seven distinct genotypes including Mass, 793/B, IS720, Variant 2, QX, IR-I and IR-II to be circulating in these chicken farms. The majority of flocks (67.6%) experienced infection with an IBV variant unrelated to the vaccine strains. These variants displayed homologies ranging from 54.1% to 78.5% and from 53% to 86% with H120 and 4/91 respectively. Our findings reveal the existence of IBV variants genetically different from the vaccine strains currently in use and explain the outbreaks of disease observed in the field.

Introduction

IBV Massachusetts (Mass) type was first detected in Iran by Aghakhan *et al.* (1996). In 1998 a virus similar to the European 793/B type was isolated in Iran (Iran/793B/19/08) (Vasfi Marandi & Bozorgmehri Fard, 2001). This IBV genotype is reported to have been highly prevalent in Iran between 1994 and 2004 (Seyfi Abad Shapouri, *et al.*, 2004; Shoushtari, *et al.*, 2008) and consequently vaccines of serotypes Mass and 793/B have been administered to chicken flocks. In spite of different vaccination regimes, IBVs continue to cause considerable economic losses to the poultry industry. IBV outbreaks are often caused by an IBV strain serologically different to the strains (Cavanagh, 2003) in the currently available vaccines which appear to be unable to induce cross-protection. Nucleotide sequencing of a diagnostically relevant fragment of the S1 gene is the most useful technique for the differentiation of IBV strains and has become the genotyping method of choice in many laboratories (Jackwood & De

Wit, 2013). The objective of this survey was the detection, characterization and classification of prevalent IBV genotypes circulating between 2010 and 2014 in chicken flocks in Iran.

Materials and Methods

Sampling

Chickens from IBV-suspected cases from different flock types were submitted to the Veterinary Diagnostic Laboratory (Tehran, Iran). Samples of trachea, kidney and cecal tonsils were collected and used for RNA extraction. Over the period of the survey more than 2500 samples from 250 flocks were tested and analyzed.

Reverse transcription

After viral RNA extraction using RNeasy Mini Kit (Qiagen, Germany), complementary DNA (cDNA) was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, Canada) according to the product manual. The synthesized cDNAs were used as the template for polymerase chain reaction (PCR).

Untranslated Region (UTR)-PCR

Pairs of oligonucleotide primers which are flanking 5'UTR and 3'UTR respectively were used as previously described for the detection of IBVs (Callison, et al., 2006; Hewson, et al., 2009).

Amplification of the spike gene and analysis

Positive samples in UTR-PCR were selected for further analysis of the spike gene. S1 gene partial amplification was performed as described previously (Villarreal, et al., 2010; Worthington, et al., 2008). A portion of 393 base pairs was amplified and sequenced. Nucleotide and predicted amino acid sequence data were aligned with the Clustal W. Phylogenetic trees were constructed based on the amino acid sequences using the MEGA 6 package (Tamura, et al., 2013).

Results

Two hundred and twenty four flocks (89.6%) were found to be positive for infectious bronchitis virus. Further analysis of the positive samples by S1 sequencing revealed the circulation of at least seven genotypes in Iranian chicken flocks. These seven distinct genotypes could be divided into three categories based on their distribution. Three of the IBV genotypes detected in Iran have an extensive distribution as they have been reported in several continents. These viruses include Mass, 793/B and QX

genotypes. Mass-type was detected in 21 flocks (8.4%) and 793/B in 21 flocks (8.4%). In addition, both types of viruses were simultaneously detected in samples of 13 flocks (5.2%). The first evidence for introduction of a virus related to the QX genotype in Iran was found in August 2011 (Iran/QX/179/11) in this survey. During the period of this survey QX genotype was found in 24 flocks (9.6%).

The majority of types of IBVs (35.6%) circulating in Iranian chicken farms fall in the category of regional distribution. The dominant viruses in this survey are closely related to the two types of viruses Variant 2 and IS720 which have been detected in Middle Eastern countries (Jackwood & De Wit, 2013). Both of these viruses have been reported to be nephropathogenic in chickens (Gelb, et al., 2005; Meir, et al., 2004). During this survey, IS720 type was found in 46 flocks (18.4%) and Variant 2 was detected in 43 flocks (17.2%).

A distinct novel IBV genotype was detected for the first time in 2010 and designated Iran-I (IR-I). The closest nucleotide S1 sequence in GenBank was the variant 2 (84.4%). The IR-I genotype was detected in multi-age broiler flocks in the north of Iran showing respiratory signs and increased mortality. Hence, this virus probably emerged solely in Iran and may have a limited geographical distribution.

During this survey another novel IBV genotype was detected in six flocks (2.4%). This IBV, designated Iran-II (IR-II) shows 45.9% and 47% difference in amino acid identity with the H120 and 4/91 genotypes respectively. No similar sequences have been described elsewhere.

Discussion

According to the results of this survey at least five distinct genotypes differing significantly from Mass and 793/B have been circulating in Iran during the last decade. These field strains were found in 169 cases (67.6%) of IBV suspected flocks. The most frequently detected IBV type shows high similarity (98.8%) to IS720 type which was isolated for the first time from a broiler flock with respiratory disease in Israel in 1999 (Gelb, et al., 2005). The second most common field-type of IBV in Iran, belongs to Variant 2-type (IS/223/96) which was described by Callison et al. (2001). Challenge studies revealed that the H120 vaccine provides poor protection (25%) against Variant 2 (Gelb, et al., 2005).

The third genotype of field strain belongs to the new variant Iran-I (IR-I). IR-I and original Variant 2 shared more than 84.4% amino acid identity. IR-I had a higher identity ($\geq 87.7\%$) with Iranian Variant 2.

The genotype called QX was the fourth field-type of virus in this survey. In a matter of almost a year, a QX type virus (PCRlab/06/2012) similar to Iran/QX/179/11 was recognized as the causative agent of "cystic oviduct" (Bozorgmehri-Fard, et al.,

2013). The rate of QX detection in Iran has decreased after its first detection and it never became the dominating type in the period of this survey.

IBV "IR-II" shows close relation to sequences reported from Iran by a research group in 2010 (accession numbers: HQ123349 and HQ123363) but no similar sequences have been described anywhere else. It is believed that the variant may become significant if it spreads, causes disease and is detected frequently over a short period of time (Jackwood, et al., 2005).

Cross-protection between IBV strains depends on the amino acid similarity of S1. Based on the S1 glycoprotein amino acid sequence, Iranian IBV's homology with Mass-type vaccine and 793/B-type vaccine ranges from 54.1% to 78.5% and from 53% to 86%, respectively. These findings explain the poor vaccine performance in the field and show that the disease outbreaks were associated with IBV variants which circumvent vaccination immunity. These findings emphasize the need for new control strategies of IBV in Iran.

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Full-length characterization of the S1 gene of Iranian QX infectious bronchitis viruses isolates, 2014-2015

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Abstract

Avian infectious bronchitis (IB) has been prevalent in most chicken farms during recent years in Iran, despite an IB vaccination program. Methods: To better understand the molecular epidemiology of IBV in Iran, full-length sequences of the S1 gene of Iranian QX IBVs were determined and phylogenetic analysis was performed with some sequences of IBV previously published in GenBank. Results: Iranian QX IBVs belong to the LX4 (Cluster 2) genotype. The nucleotide homologies between these isolates were 99.52% - 100%. Phylogenetic analysis revealed that all the IBV isolates were very similar and probably had a common origin. The hyperactive variable regions of S1 were determined. Conclusions: The results from this study and other results published in GenBank show that, since the first report of QX IBV in Iran in 2011, the predominant genotype of isolates circulating in Iran in recent years is the LX4 (Cluster 2) genotype. This finding provides important information on IBV evolution in Iran.

Keywords: Avian Infectious Bronchitis, QX, Iran, Phylogenetic analysis, Spike

Introduction

Infectious bronchitis (IB) is one of the most contagious diseases affecting poultry worldwide and is responsible for severe economic losses. Infectious bronchitis virus (IBV) is a member of the species avian coronavirus, genus *Gammacoronavirus* (*Nidovirales: Coronaviridae -Coronavirinae*). S antigen, which is known as the main immunogenic antigen of IBV, has two parts called S1 and S2. S1, which is the most variable part, is responsible for viral variations and recombination. There are hundreds of IBV serotypes and the majority of them differ from each other by 20 to 25% of S1 amino acids (Cavanagh, Elus, & Cook, 1997; Jackwood *et al.*, 2001). The precursor S protein is posttranslationally cleaved into S1 and S2 subunits. In the S1 subunit,

three hypervariable regions (HVRs) are located within amino acids 38–67, 91–141, and 274–387. Neutralizing epitopes are associated with the defined HVRs, and variation in these regions is thought to correlate with serotype. Indeed, a recent study typed IBV based on the HVR 1 sequence and demonstrated that genotyping correlated with serotyping using the VN test. Bozorgmehri - Fard *et al.* (2014) have demonstrated the presence of QX viruses in Iranian commercial flocks (2011). Genotypes of IBV strains isolated in Iran were classified into seven distinct phylogenetic groups (Mass, /B like, IS/1494 like, IS/720-like, QX-like, IR-1, and IR-2) based mainly on analysis of HVRs of the S1 gene (Hosseini, Bozorgmehri Fard, Charkhkar, & Morshed, 2015; Najafi *et al.*). In spite of the use of different vaccines (Mass & 793/B type) on poultry farms in Iran, outbreaks of IB with high mortality have been reported. Since outbreaks of IBV still occur in vaccinated flocks and the virus strains isolated frequently differ from the serotypes of the vaccine strains used, continuous identification of the genotype and production of new generations of vaccines are crucial (Mahmood, Sleman, & Uthman, 2011). The aim of this study was to characterize the Iranian QX strain circulating in 2015 by performing full-length sequencing and phylogenetic analysis of the S1 gene.

Material and Methods

Sample collection & Virus Isolations

In this study, samples were collected from Iranian broiler and layer farms in the period 2014 - 2015. The samples (trachea and kidney) were taken from chickens suspected of showing clinical signs of IB. Details of the positive samples are available in Table 1.

RNA extraction and cDNA synthesis

RNA was extracted from tissue samples using Cinna Pure RNA Extraction Kit (Sinaclone, Iran). For cDNA synthesis, 1 μ L (0.2 μ g) of random hexamer primer (SinaClon, Iran) was added to 5 μ L of extracted RNA and the mixture was heated at 65°C for 5 minutes. Fourteen μ L of cDNA master mix containing 7.25 μ L DEPC-treated water (SinaClon, Iran), 2 μ L dNTP mix (SinaClon, Iran), 0.25 μ L RiboLock RNase Inhibitor (Thermo Fisher Scientific, USA), 0.5 μ L Revert Aid Reverse Transcriptase (Thermo Fisher Scientific, USA), and 4 μ L 5X RT Reaction Buffer was added to each tube, resulting in a final volume of 20 μ L. The mixture was incubated consecutively at 25°C for 5 min, 42°C for 60 min, 95°C for 5 min, and 4°C for 1 min.

PCR Reaction for S1 Amplification

RT-PCR was carried out using primer sets New oligo 5'TGAAACTGAACAAAAGACA 3' and New oligo 3' 5' CCATAAGTAACATAAGGRCRA 3' targeting the spike glycoprotein of the S1 subunit of IBV. The products were analyzed on 1.0% agarose gel. The PCR products were cloned into pTZ57R/T vector (Ins TA clone PCR Cloning Kit , Cat No: K1213) for later sequencing.

Phylogenetic analysis

Sequencing was performed with the primers (both directions) used in the PCR (Bioneer Co., Korea). Chromatograms were evaluated with ChromasPro (ChromasPro Version 1.5). The phylogenetic tree was constructed using the MEGA 5.1 software with the neighbor-joining method and each tree was produced using a consensus of 1000 bootstrap replicates (Tamura *et al.*, 2011). The nucleotide sequences of the full length of the S1 gene were compared with several S1 sequences from Genbank.

Results

The full-length sequence of the S1 gene was obtained for the three IBV isolates recovered in this study population. MEGA5 programs were used to determine the sequence homology of the S1 gene from these Iranian QXIBV isolates (QXIRGW4, QXIRGW7, and QXIRGW8). Phylogenetic analysis, based on the full length nucleotide sequences of the S1 gene of the three QXIBV isolates and other results published in GenBank (as well as other Iranian QX IBV data; Partial S1), showed that the Iranian QXIBV isolates belong to the LX4 (Cluster 2) genotype (as shown in Figure 1). The nucleotide homologies between these isolates were 99.52% -100%. The nucleotide homology between QX IBV isolates and CK/CH/LSD (091005), SDIB768 (KJ469743), South Korea (K101903), QXIB (AF193423) were about 99.52% -99.52%, 98.36%-98.84%, 94.60%- 95.37%, and 95.18% -95.95%, respectively.

Discussion

Infectious bronchitis (IB) is one of the most common and difficult-to-control poultry diseases in Iran, causing persistent but infrequent outbreaks of disease in commercial chicken farms. Sun *et al.* (2011) were the first to classify LX4 IB-like viruses into two clusters (I, II) after analysing the genotypes of 78 isolates of IBV obtained from different field outbreaks in China in 2009 and 34 reference strains: Four genotypes of IBV and three new isolates were identified by phylogenetic analysis and BLAST searches of the entire S1 gene. Genotype I included 57 out of the 78 isolates that were grouped with reference strains LX4 and QX. The strains in this group included field viruses isolated between 1997 and 2009. A phylogenetic analysis comparing

the complete S1 gene sequences of the 62 isolates and reference viruses published previously in GenBank revealed that the isolates in this group could be further separated into two genetic clusters, identified as cluster I and cluster II, represented by LX4 and QX, respectively. The minimum amino acid divergence observed between isolates of the two clusters was 96.5%. Interestingly, two of the three South Korean QX-like viruses, three QX-like strains from the Netherlands and two from France fell into the same group as QXIBV by S1 gene phylogenetic analysis (Sun *et al.*, 2011). Furthermore, the viruses isolated in 2009 clustered in the QX cluster closely with strains isolated before 2003. The results of Ma *et al.* (2012) on the genetic diversity of Chinese IBVs complement the previous finding that the LX4 genotype can be grouped into two clusters (Sun *et al.*, 2011). Importantly, we also extended the findings to demonstrate that viruses in clusters I and II have different favored amino acid residues at different positions in the S1 subunit of the spike protein, although some of the positions had a mixture of amino acid residues (Ma *et al.*, 2012). Based on S1 phylogenetic analysis or comparison of the partial S1 genes for amino acid similarity, Chinese QX-like IBVs had a close relationship with strains from the Netherlands, France, Slovakia, Greece, and Hungary. In contrast, partial analysis of the S1 gene revealed that the Russian QX-like IBVs showed high divergence from Chinese QX-like strains. In addition, QX IBV was reported in the south east of Asia. In Japan, four different genetic groups have been identified since 1995: Japan (JP)-I, JP-II, JP-III (LX4), and JP-IV. The JP-III group falls into the China LX4 group (QX IBV) (Masaji Mase *et al.*, 2010; M Mase, Tsukamoto, Imai, & Yamaguchi, 2004). In Korea, the IBV isolates are divided into three genetic groups: Korea (K)-I, K-II (LX4-type), and K-III (LDL-type). QX group located to KII group (Lim *et al.*, 2011). In Iran, Bozorgmehri-Fard *et al.* detected Iran/QX/H179/11 strain in 2011 and Iran/QX/H255/12, Iran/QX/H281/12, and Iran/QX/H284/12 strains in 2012. QX-type isolates of this study had more than 96% homology to these Iranian strains (Bozorgmehri-Fard, Charkhkar, & Hosseini, 2014). Hosseini *et al.* (2015) described QX-type IBVs as the sixth genotype in Iranian poultry farms (6.5%) during 2010-2014 (Hosseini *et al.*, 2015). Najafi *et al.* (2015) reported that QX-type IBVs were the third most common genotype in circulation in Iranian chicken flocks in 2014-2015. In their survey, the IBV QX-type isolates shared more than 96% nucleotide similarity (Partial S1 sequence) with original QX (AF193423), and UTIVO-103 and UTIVO-105 had 99% sequence similarity with Iran/QX/H255/12 (KP310038). UTIVO-14 had 96% sequence similarity with Iran/QX/H179/11 (KP310037) (Najafi, Madadgar, Jamshidi, Langeroudi, & Lemraski, 2014). In 2009, based on the genetic characterization of Iraqi isolates, a putative novel IB variant not related to the QX-like strain was revealed. The phylogenetic analysis showed that the Kurdistan-Iraqivirus was closely related

(98.9%) to QX-strains collected in China between 2009 and 2010. Lower nucleotide similarity (95.4 %) was revealed with the unique QX-type virus sequence from the Middle East deposited in GenBank and isolated in Israel in 2004 (IS/1201 isolate, accession number DQ400359) (Amin *et al.*, 2012). In early 2011, five IBV isolates obtained from respiratory disease outbreaks in Iraq, Jordan, and Saudi Arabia were characterized. The isolates were found to be IBV strain CK/CH/LDL/971 (Ababneh, Dalab, Alsaad, & Al-Zghoul, 2012). QX-type IBVs isolated in this study shared more than 96% nucleotide similarity with the original QX (AF193423). A retrospective analysis of IBV strains isolated in Russia showed some isolates genetically related to the QX-type in 2001 (Terregino *et al.*, 2008).

Due to the irreversible effects of the QX strain on reproduction, further studies should be performed on pullet and layer flocks in Iran. In outbreaks with high prevalence and false layer issues a suitable vaccine and vaccination program are recommended, particularly in the layer and breeder pullet farms. To this end, further detailed investigation of the genome is needed to clarify the evolution of Iranian IBV isolates. In addition, in order to safeguard against the sudden emergence of new predominant strains, continuous monitoring of the IBV strains circulating in the field is of great importance.

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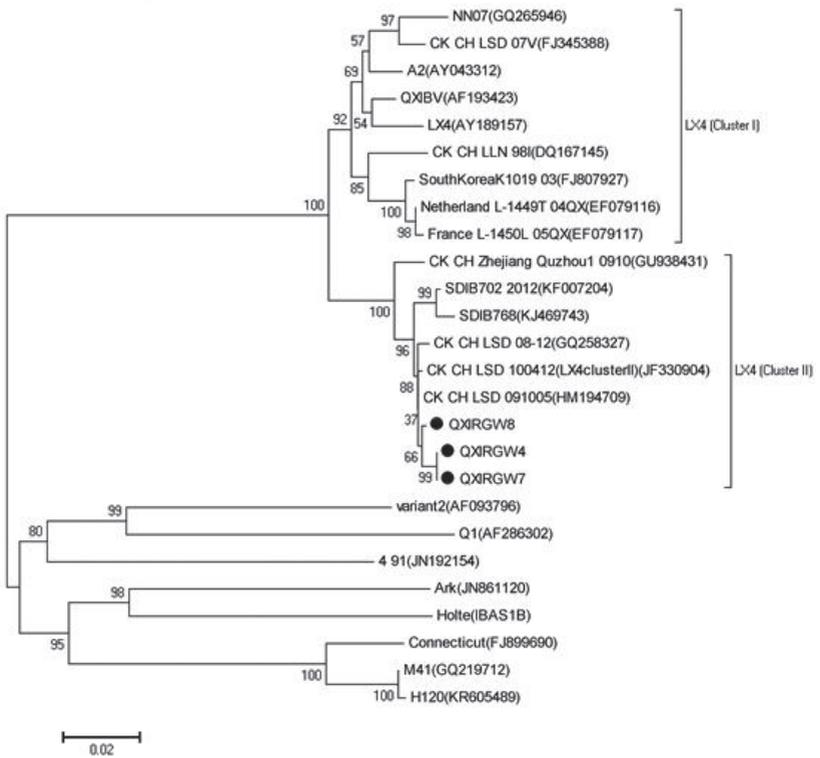


Figure 1. Phylogenetic tree based on a full-length sequence of the S1 gene, showing the relationship between the Iranian and other IBV strains. The neighbor- joining method

Molecular survey of infectious bronchitis virus in commercial flocks in Macedonia reveals presence of different genotypes

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Abstract

Introduction: Preliminary testing of commercial flocks using molecular and phylogenetic analysis revealed presence of genotypes QX and 4/91 in poultry farms with clinical signs and in poultry farms without clinical signs. Poultry farms are performing different vaccination programs without the knowledge of the genotype of the circulating strains. The purpose of this study was to detect presence of IBV in commercial flocks in Macedonia by molecular methods and to genotype the detected strains thus to establish a map of circulating genotypes in the country.

Methods: Molecular survey was performed in 35 poultry farms representative for the whole territory of the country without clinical signs at different production age as part of active surveillance for avian influenza. From each farm 30 cloacal swabs were taken totalling 1050 samples. Samples were pooled by five in the laboratory. Additionally, samples (oropharyngeal swabs, affected organs) were taken from 15 farms with clinically signs (respiratory signs, drop in egg production, altered egg quality) and post-mortem (respiratory, ovary and kidney lesions) findings associated to IBV. A real-time RT-PCR was performed targeting UTR region on all samples. A two-step nested RT-PCR was performed on all positive samples with primers targeting the S1 gene. Genotyping and subsequent phylogenetic analysis was performed by partial sequencing of the S1 gene region.

Results: Results of the present study revealed presence of following genotypes of IBV's circulating in commercial poultry in Macedonia: 4/91, Italy-02, QX, Mass, D274. This is first report of detection of Italy-02 in Macedonia.

Conclusions: Establishing a map of circulating IBV genotypes will help towards better optimisation of vaccination protocol of poultry farms and will contribute towards better understanding of epidemiological situation on a country and regional level.

Economic impact of infectious bronchitis in a poultry complex in Southern Brazil

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Abstract

The aim of this study was to evaluate the economic impact caused by infectious bronchitis virus (IBV) infection in a poultry complex located in Southern Brazil, which divides farms into micro-regions and applies differential biosecurity management as recommended by the OIE. This management includes unidirectional truck transit and mandatory disinfection of all material that goes on and off farms. Over a period of 13 months, flocks were evaluated for the presence of clinical signs of IB infection and subjected to RT-qPCR analysis, ELISA or histopathology. IBV infection was mostly found in flocks near highways with heavy traffic of trucks carrying poultry. The results demonstrated that, compared to flocks in which IBV was not diagnosed, infected flocks showed decreased zootechnical performance and increases in mortality and condemnation at processing resulting in an economic impact of U\$ 3.57 per one thousand chickens housed. Most viruses have been identified as BRs strains, however Massachusetts genotypes were also detected. The study shows that IBV infection led to increased production costs but suggests that these may be minimised by the use of the biosecurity management system which appears to reduce the spread of infection as well as the economic impact.

Introduction

Infectious bronchitis (IB), caused by Infectious Bronchitis Virus (IBV) causes significant economic losses to the poultry industry worldwide. The disease was first identified in North Dakota, USA, when a new respiratory disease in young chickens was reported (Schalk & Hawn, 1931). Since then, IBV has been recognized worldwide, especially in countries with large commercial poultry populations (Cavanagh, 2007).

The first isolation of IBV in Brazil was recorded in 1957 (Hipólito, 1957) and despite the official introduction of IBV vaccination in 1979, outbreaks of infectious bronchitis are ongoing (Ito, 2006; Montassier *et al.*, 2006; Felipe *et al.*, 2010).

The virus is transmitted via the air, directly via chicken to-chicken contact with respiratory secretions and faecal droplets from infected poultry, or indirectly through

contaminated objects. These latter may facilitate the transmission and spread of the virus from one flock to another (OIE, 2013; Ignjatovic & Sapats, 2000).

IB occurs worldwide and assumes a variety of clinical forms. Apart from respiratory infections, IB affects the kidney and reproductive tract, causing renal dysfunction and decreased egg production, respectively. Nephropathogenic IB causes acute nephritis, urolithiasis and mortality (Cavanagh & Gelb, 2008).

Flock management and the strain of virus involved play a major role in the impact of IBV infection. The respiratory disease is debilitating, resulting in poor utilization of feed by young chicks, and hence poor weight gains (Ignjatovic & Sapats, 2000), and it may lead to condemnation of a large number of carcasses in slaughterhouses (Jones, 2010).

Little is known about the loss in value caused by IBV. Thus, our proposal was to demonstrate the economic impact (in terms of increased feed consumption and condemnation at slaughter) of IBV infection on a poultry complex with good biosecurity practices and management. We also hypothesize as to the potential economic impact on one of the largest chicken production states in Brazil.

Material and Methods

A poultry complex in Southern Brazil was evaluated for a period of thirteen months. This complex performs vaccination for IBV (Massachusetts strain) in all chicks at hatchery and poultry are raised on average for 42 days. The sanitary controls carried out in this complex are extremely rigorous, with controlled access of people and materials, disinfection of trucks, origin of progeny, use of heat-treated poultry litter, litter fermentation, a downtime period with disinfection between flocks, heat treatment of the feed, and division of farms into micro-regions.

Veterinarians responsible for poultry health in these areas evaluated the presence of clinical signs, mortality and bird performance. Additionally, the birds were evaluated at the slaughterhouse to identify partial and full condemnation volumes.

For comparative purposes, a second complex, with less developed biosafety standards, that reported outbreaks of bronchitis over a similar period, was evaluated for performance feed conversion. The second poultry complex is at about 156 miles away from the first and uses the same vaccination standards and farm structure.

Initially, veterinarians attended all reports of flock mortality, loss of performance or clinical signs of IB and birds were clinically evaluated according to the company's sanitary procedures and submitted for necropsy. Blood and / or tissues were collected from all flocks. Confirmatory diagnosis of infection with IBV was performed by at least one of the following methods; RT-qPCR as described by Fraga *et al.* (2013), detection of antibodies to IBV in a commercial kit (IDEXX Laboratories, Westbrook,

ME, USA), or observation of histopathological lesions compatible with IBV in the trachea and kidney.

Some samples detected by RT-qPCR were subjected to a new RT-PCR and then partial sequencing of the S1 gene for identification of circulating genotype, according to the methodology described by Fraga *et al.* (2013).

After confirmation of infection, the farms were mapped. The mapping was carried out with the help of applications Google Maps/Google Earth (Google Inc, Mountain View CA). The dollar rate was taken from Central Banking of Brazil (<http://www4.bcb.gov.br/pec/taxas/batch/taxas.asp?id=txdolar>). Values for calculating the cost of feed were obtained from the Brazilian Agricultural Research Corporation (EMBRAPA), affiliated with the Brazilian Ministry of Agriculture (http://www.cnpa.embrapa.br/cias/index.php?option=com_content&view=article&id=61), specific to Santa Catarina State, Brazil.

Results and Discussion

Until August 2014 IBV infection in broiler systems in this region of Southern Brazil, which covers approximately 276 km², was well controlled with only sporadic infections reported. Holdings of various companies are spread across the region but do not typically share the same transport routes. However, at the time of this investigation, the main transport routes of feed trucks and live poultry had been altered due to extensive roadworks and this resulted in heavy traffic of trucks on the roads around the farms in this study. In September 2014, the first episode of IB was identified and diagnosed in a flock located next to a busy road. Subsequently there were similar reports of disease, of varying severity, in other flocks.

These outbreaks were mapped in order to identify the direction of flow of disease spread and to aid control and disinfection programs. In February 2015 there was a fall in the number of cases although this rose again in July 2015 and incidents continued until October 2015.

Over this time a total of 10,06% flocks were diagnosed with and presented mild or acute signs of IB. Signs of infection, characterized by watery diarrhea, hyperthermia and mortality, were consistently most severe between 28 and 32 days of age. In some cases, no symptoms were observed, however IB was diagnosed by one of the following methods: Flocks were considered positive for IBV infection when virus was detected by RT-qPCR or antibodies in samples taken at slaughter (between 40 and 44 days) and / or when the tissues evaluated by histopathology showed characteristic lesions that corresponded with the presence of clinical signs. The sequenced samples were grouped in the group of Brazilian strains or Massachusetts.

Flocks diagnosed for IBV infection lost 67g in feed conversion and 1,89g in daily weight gain when compared to healthy birds. In this period, considering only this poultry complex, the company lost 428,86 tons of feed due to infection by IBV. Feed value was U\$ 433,51 per ton (EMBRAPA, 2015). Considering the dollar rate at that time, R\$ 3,9042 (Central Banking Brazil), the losses incurred by this broiler operation during this outbreak totalled U\$ 185.915,95. The loss per 1000 housed chickens was U\$ 2,92.

The average rate of condemnation at slaughter from flocks with IBV infection was 0,036% greater than in non-infected flocks and total mortality was 1.16% higher. Losses due to condemnations totalled U\$ 0,65 per 1000 chickens. The majority of condemnations were due to airsacculitis and pectoral myopathy.

The combined losses for this broiler operation were U\$ 3,567 per 1000 chickens, totalling U\$ 225.782,14 over the evaluated period.

IB-infected flocks on the second complex showed a 94g increase in feed conversion rate in flocks diagnosed with IBV, amounting to 27g more than was seen on the first complex. These data suggest that biosecurity and management measures are extremely important in the control of viral respiratory diseases, and can contribute to the prevention and control of the spread of IBV. IBV infection in a commercial flock with the best possible management practice reduces income by approximately 3%, in comparison with a hypothetical flock free from IBV (McMartin, 1993). However, in addition to management practices, the strain of virus involved plays a major role in the impact of IBV infection (reviewed by Ignjatovic and Sapats, 2000).

A few studies have genotypically characterized Brazilian samples and evaluated their pathogenicity (Fernando *et al.*, 2013; de Wit *et al.*, 2015). These samples are segregated into a specific group called BR strains, and vary in pathogenicity level. The viruses circulating on the farms in this study, showed pathogenic properties in the poultry but, as shown, the impact in terms of losses can vary.

This state of Southern Brazil accounts for approximately 15% of chicken production in Brazil, and can produce a volume of 0.9 billion chickens per year (IBGE, 2016). The farms in this study contribute 5,1% of this state's production. Based on our findings, the potential loss due to IB infection affecting the entire state over this period would have been U\$ 3,645,410.78. However, it is important to note that the calculation is based on a farm with a high level of biosecurity, so actual statewide losses in feed conversion and condemnation rates are likely to be higher.

Assayag *et al.*, (2012) reports considerable economic loss in feed and slaughterhouse condemnation, with a value of \$ 8.46 US dollars per 1,000 birds housed. We also

evaluated the losses in feed and slaughterhouse condemnation. The lower rate of loss that we report may reflect a difference in currency exchange rates over the periods studied. Additionally, loss may have been minimised by the high levels of biosecurity, including the use of micro-regionalisation and closed systems, on the farms in our study. Assayag *et al.* (2012) based calculations about the loss in feed conversion on standard values published by the Brazilian Agricultural Research Corporation (EMBRAPA, 2012). In this study, we used company data to compare results from flocks with and without a diagnosis of IBV. Our results are, therefore, adjusted to the company norms regarding feed quality, farm structure, progeny and management practices.

It is important that the surveillance, control and biosecurity measures outlined in the law of the Brazilian Department of Agriculture (IN 21) are well understood by all parties involved in the poultry production chain. For companies with multiple sites or complexes the segregation model (micro-regions), referring to animal populations kept in one or more farms under a common biosecurity management system and with a different health status in relation to infection with Avian Influenza and Newcastle disease virus, is particularly important.

Conclusion

Our results showed loss of zotechnical performance in chickens infected with IBV and a consequent increase in production costs. However, these losses are less than those reported in previous studies of other poultry complexes in the region and less than those seen during this study on a second complex with less strict sanitary controls. Favorable results may be related to the improved biosecurity on farms.

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The cost of diversity: Assessing the economic burden of avian coronavirus on poultry

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Abstract

Considering the absence of dependable data on the economic losses caused by avian infectious bronchitis (IB), this survey aimed to estimate such losses in broiler, breeder and layer flocks in which the Brazil type of avian coronavirus (ACoV) is found. To this end, from 2012 to 2014, 49 flocks of Cobb breeders, layers and broilers, all vaccinated with the Massachusetts type were sampled from different regions in Brazil. ACoV screening, S1 typing and economic and production performance indicators were used to assess economic losses. Thirty-two ACoV lineages, all belonging to the Brazil type, were identified. In breeders, the total estimated loss per 1,000 birds was US\$ 3,567.4 and US\$ 4,221.5 for 25-26 and 28-32 week-old birds, respectively, while in layers (29 weeks) and broilers (48 day-old), the estimated loss was US\$ 4,210.8 and US\$ 266.3 /1,000 birds, respectively. These results evidence the significant economic impact of IB in poultry in Brazil when the Brazil type of the virus, against which the Massachusetts type gives poor protection, is involved.

Introduction

Avian coronavirus (IBV in chickens) infection in chickens can manifest in many clinical ways including delayed growth, egg production drop, eggshell malformations, infertility and condemnation in slaughterhouses and might be complicated by co-infection with *Mycoplasma* or *Escherichia coli* (Jones, 2010). Infectious bronchitis (IB) is recognized globally as one of the diseases of most concern due to the economic effects on poultry production and trade (Bagust, 2008).

In Brazil IB occurs with very high frequency, and is caused mainly by field strains of IBV of a divergent type (Di Fabio *et al.*, 2000; Villarreal, *et al.* 2010) named Brazil type, for which the first complete genome has recently been published in GenBank by some of the authors of the present paper (KX258195).

The aim of this survey was to estimate the pre-slaughter economic losses in flocks of broilers, layers and breeders presenting with IB and to examine the diversity of IBV in these cases.

Materials and Methods

Sample collection: From 2012 to 2014 the respiratory tract, kidneys, cecal tonsils, enteric contents, testes and female reproductive organs were collected from 49 flocks of breeders, layers and broilers in poultry farms from different regions in Brazil. IBV screening and typing: Screening was carried out using a nested RT-PCR targeting the 3'UTR of IBV (Cavanagh *et al.*, 2002) after total RNA extraction with TRIzol (Life Technologies), using M-MLV (Life Technologies) and GoTaq (Promega). Positive samples were subjected to a nested-PCR targeted to the partial amplification of the S1 subunit of the spike gene (nucleotides 28 to 477 of the S gene of M41 strain Genbank accession number DQ834384.1) as described by Torres *et al.* (2014).

S1 amplicons (450pb) have been purified using EXOSap It and subjected to bi-directional sequencing with BigDye 3.1 (Life Technologies). An amino acid neighbor-joining phylogenetic tree was built with the Poisson model and 1,000 bootstrap replicates was built with Mega 6 (Tamura *et al.*, 2011).

Estimation of economic losses: The parameters used for the estimation of economic loss were: weight, percent of production, mortality and number of chicks produced. These parameters were compared with standard values for the Cobb 500 line and parameters of the flocks in the field. All the expected data for breeders, layers and broilers are taken from the Cobb Handbook. In this study the costs considered were US\$ 0.4 per chick, US\$ 0.2 per egg and US\$ 0.97 per poultry meat kilogram.

Results

IBV screening and typing: Thirty-two IBV lineages were found amongst the 49 flocks, each flock being positive for at least one lineage, all of which belong to the Brazil type (GI-11 according to Valastro *et al.* (2016). Mean amino acid identity amongst the 32 Brazilian lineages was 91.8% (sd 3.3) and with D274 (closest), 75.6% sd 1.6 and Connecticut (most distant), 61.7% sd 1.1 MSD/30 was the most frequently occurring lineage and was found in 14.3% of the flocks. In breeders and broilers it was present in samples of enteric content/cecal tonsils, respiratory tract, kidney and female reproductive tract, with most of the sequences (n=24) exclusive to a specific flock.

Estimation of economic losses: For older (42 weeks) breeders and layers, in which lineages MSD/006 and MSD/022 were found, the estimated loss per 1,000 birds was US\$ 4,221.5 and US\$ 4,210.8, respectively. In 48-day-old broilers, a total loss of US\$ 266.3 was estimated per 1,000 birds, in which lineage MSD/027 was detected. In breeders from 25 to 26 weeks of age in which lineages MSD/001-004 were found, the total loss per 1,000 birds per flock was estimated as US\$ 3,567.4, while in breeders from 28 to 32 weeks of age with lineages MSD/008, MSD/014, MSD/015, MSD/023 and MSD/32 the estimated loss was of US\$ 4,221.5/1,000 birds. The economic loss could not be estimated for some flocks due to the lack of reliable data from the farms.

Discussion

The per 1,000 birds loss for all ages and bird types described here is a major burden for farmers. It is noteworthy that for breeders there was a 15% increase in the economic losses from 25 to 42 weeks, suggesting a cumulative effect.

The loss amongst older breeders is similar to the one found in layers (US\$ 4,221.5 and US\$ 4,210.8/1,000 birds, respectively) probably due to similar modes of action of IB in these birds, i.e., the involvement of the reproductive tracts and thus decreases in fertility and egg production.

In the 48-day-old broilers the losses are substantially less than those found for layers and breeders, but this is a biased comparison as the reproductive signs of IB are not manifested in broilers and the profit margin in broiler production is smaller.

These numbers are quite different from those presented in a previous study in Brazilian poultry (Assayag *et al.*, 2012), where the economic losses in breeders was estimated as being US\$ 251.4/ 1,000 birds and in broilers at US\$ 8.46/ 1,000 birds. This discrepancy is probably because those authors based their estimations on clinical signs of IB only, with no definitive diagnostic testing, and also because only birds from a single Brazilian region were sampled while in the present study a diverse geographic area was surveyed.

One cannot accurately associate differences in losses with a given IBV lineage as the intra-geno/serotype diversity was low in the present study. However, as cross-protection decreases with amino acid distance (Jackwood, 2012) and given the distance of IBV lineages described here from the Massachusetts type, a part of the economic losses might be linked to very poor vaccine-induced protection.

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Molecular detection of avian gamma coronavirus in bird parks, Iran

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Abstract

Introduction: Avian coronavirus molecular detection in birds gardens of Tehran Province The infectious bronchitis virus (IBV) is distributed worldwide. Chickens are the most important natural hosts of IB; all ages of chickens can be infected. IBV, or closely related coronaviruses have also been isolated from other species such as turkeys, pheasants, quail and partridges. There has been no molecular surveillance data to describe the epidemiology of coronaviruses in such birds in Iran. **Methods:** The aim of the present study was coronavirus detection in Tehran birds Gardens between 2015-2016. Cloacal swabs were taken and the viral RNA was extracted using Roche extraction kit. The RT-PCR carried out by QIAGEN one step RT-PCR kit using the primers targeting "UTR" and "N" genes. **Results:** Gammacoronavirus was detected in the quails, pheasants, turkeys and partridges. Nucleotide sequences of positive samples were determined and the phylogenetic tree was constructed. **Conclusions:** According to our results, coronaviruses are circulating in different bird types in Iran and further studies are needed to isolate these viruses and evaluate their pathogenesis.

Distribution dynamics of avian infectious bronchitis coronaviruses in poultry population in Asia

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Abstract

The update situation of avian coronavirus (ACoV) new genotypes detected worldwide is necessary regarding profiles of viral distribution and epidemiology especially in commercial poultry population which may result in difficulty of disease control measures. ACoV is commonly referred to as the well-known avian infectious bronchitis virus (IBV) that causes a variety of problems in commercial poultry. To date, not only diseases associated with respiratory symptoms, but a wide range of disease conditions are also recognized. In Asia, the widespread distribution of IBVs is either due to an intra-regional or an inter-regional dynamics. The evolution and dynamic emergence of new IBV variants are found via the recombination and co-circulation between IBV types. This makes it dramatically difficult to prevent and control infections, albeit routine vaccination. Moreover, the existing IBVs continue to evolve in Asian countries where a mixture of housing conditions occurs geographically, including extensive commercial poultry production, medium/small-scale family run business, backyard poultry operation and free-ranging poultry system. Therefore, these sorts of raising systems may also serve as a hub for ACoV transmission within a particular geographic sphere. In addition to commercial and non-commercial poultry, ACoVs have also been identified in other avian species that may confer a threat of cross-transmission to commercial poultry neighborhood. In consequence, all these birds may be capable of disseminating viruses, both to areas of adjacency, and to distant community by the interactions of environmental and social factors, in particular, via migration and trade.

Longitudinal field studies on the pathogenesis, persistence, and molecular biology of avian infectious bronchitis virus in Brazilian broiler flocks

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Abstract

A set of 1640 broiler flocks in a poultry complex located in the Southern region of Brazil was evaluated. Two of the IBV-affected flocks were selected for the longitudinal study. The most frequent clinical signs were watery diarrhea, apathy, fever and mild respiratory signs. At necropsy, enlargement and deposition of urate crystals in renal tissue were observed. Microscopic analysis revealed moderate to severe renal tubular degeneration, presence of urate crystals, necrotic epithelial cells and inflammatory infiltrates. Kidney lesions were more severe in the birds between 29 and 33 days of age, coinciding with the detection of the highest viral loads by RT-qPCR. Tracheal lesions were mild, but the presence of virus was detected from 19 days of age up to 40 days of age. A 5'-region of the S1 gene of one IBV isolate from the beginning (2014) and one from the end (2015) of this study were sequenced and both showed a small but relevant variation between them (2%), and a high nucleotide identity ($\geq 97.1\%$) with the S1 gene of strains classified in BR-I genotype of IBV. Overall, the results indicate that Brazilian IBV strains have a pathogenic profile in different life stages of production of flocks in Southern Brazil, and this emphasizes the necessity of a new control strategy against IBV especially in the periods of intense infection of these broiler flocks.

Introduction

The IBV is the etiologic agent of Avian Infectious Bronchitis (IB), a highly contagious disease that is distinguished in the group of avian infectious diseases as it causes great economic losses to the poultry industry worldwide, despite the routine and widespread use of "live" and/or inactivated vaccines (De Wit *et al.*, 2010, Montassier, 2010). Although this virus primarily causes infection and disease of the respiratory tract there are strains that replicate in epithelial cells of other organs such as the kidney and the gonads (Vilarreal *et al.*, 2007; Fernando, 2013).

The high mutation rate of the S1 gene of IBV generates extensive antigenic variability in the progeny of this virus, which makes the control of IBV infection using vaccines extremely difficult due to the existence of multiple serotypes and variants of the virus, for which full cross-protection may not be afforded by the strains used in IB vaccine formulation. Achieving more effective control of infection IBV through vaccines represents a major challenge (Cavanagh & Gelb, 2008).

The first genetic diversity in the S1 glycoprotein of avian infectious bronchitis virus strains isolated in Brazil was reported by Montassier *et al.*, in 2006. Since then, other Brazilian variant IBV strains have been genotyped (Felippe *et al.*, 2010, Montassier *et al.*, 2012, Fraga *et al.*, 2013) and several of these strains were analyzed for pathogenicity (Fernando *et al.*, 2013; de Wit *et al.*, 2015).

Infections with Brazilian IBV strains constitute the most frequent cases of infectious bronchitis in Brazil and are causing serious problems, such as reduction in production performance, in the Brazilian poultry industry, (Assayag *et al.*, 2012).

Little is known about the course of IB during the rearing cycle of broiler chickens that are naturally infected by the Brazilian strains. Thus, the aim of this study was to determine the pathogenic evolution (including persistence and occurrence of genetic variability) of IBV and the viral load in broilers naturally infected by a Brazilian strain of IBV.

Material and Methods

A longitudinal study carried out in 2014 and 2015 followed flocks of broilers which were housed in a poultry complex in South Brazil. At the beginning (2014) and end (2015, after a maximum interval of seven months) of confirmed bronchitis outbreaks in two flocks, samples of trachea and kidney were collected from birds aged 19, 23, 26, 29, 33, 35 and 40 days.. The samples were placed in a transport medium (Brain Heart Infusion medium plus antibiotic) prior to analysis by RT-qPCR (Okino *et al.*, 2013) and sequencing (Fraga *et al.*, 2013); other samples were collected, fixed in 10% formalin and then routinely processed, embedded in paraffin and stained with hematoxylin and eosin.

The slides were examined by light microscopy and the scores of lesions ranged from 0 to 3 (considered -, +, ++ and +++) according to the severity of the observed lesions. Absence of injury was classified as -, while mild, moderate and severe were classified as +, ++, and +++, respectively (Nakamura *et al.*, 1991; Chen *et al.*, 1996).

Absolute quantification of viral load was performed using oligonucleotides for the S1 gene of IBV (designed by Wang and Tsai, 1996) and the Ct results were used to calculate the log₁₀ of the number of moles using the linear equation from standard curve, optimized previously by Okino et al., 2013 except that the open reading frame of the S1 gene of Massachusetts-41 strain sequence (Genbank accession number AY561711.1) was cloned into the pDTS₂ vector (Integrated DNA Technologies, USA).

The RNA was extracted from tissue homogenates using RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. One-Step-RT-qPCR analyses were run on a 7500 Fast Real-Time PCR System (Applied Biosystems). The reaction was performed with 5 µL of total RNA, 10 pmol each of the forward and reverse primers, 10 µL PowerSYBR® Green RT-PCR Mix (2), 0.16 µl RT Enzyme Mix (125), 4 U RNase inhibitor (Qiagen) and enough nuclease-free water for a final volume of 20 µL/reaction. RT-PCR was performed with subsequent sequencing of the S1 target gene (Fraga et al., 2013).

The PCR products were sequenced with Sequencer ABI 3730 Genetic Analyzer (Applied Biosystems Inc.) using dye terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, USA). The amplified PCR products were sequenced and the nucleotide sequences from both strands were edited, assembled, and analyzed using the ClustalW. The phylogenetic tree of S1 partial gene was constructed using MEGA 6.0 software by the neighbor-joining method with 1000 bootstrap replicates. Evolutionary distances were computed by the pairwise distance method using the Maximum Likelihood model, and amino acid insertion representations were performed using CLC Genomics Workbench 6.5.1.

Serum samples collected from birds were tested for IBV antibodies by a commercial ELISA kit (IDEXX Laboratories, Westbrook, ME, USA). The endpoint titers were calculated according to the manufacturer's instructions.

Results

In total, 165 flocks were diagnosed with IB infection when at least one of the analyses was positive (RT-qPCR, histopathology and/or ELISA). The birds consistently showed more severe signs between days 28-33 of age and these were characterized by watery diarrhea, hyperthermia and increased in the mortality. However, respiratory signs were not common.

At necropsy the most characteristic pathological alterations were enlargement and deposits of pale urate in the kidneys and, in some cases, the presence of airsacculitis.

In the two flocks followed in the longitudinal study samples for antibodies against IBV were collected between 42-44 days old. The geometric mean titer (GMT) was 5395 with a coefficient of variation (CV) of 45.2.

Microscopic examination of the tracheal tissues revealed lesions between 23 and 26 days of age which were more severe on the 23rd day and were characterized by inflammatory infiltrate in the tracheal mucosa, loss of cilia and hyperplasia of epithelial cells. At this time, mild renal disorder was also observed. At 26 days of age, tracheal lesions regressed to a mild inflammatory infiltrate and no relevant lesions were found in the trachea at 40 days of age. Conversely, kidney samples from 29-day-old birds revealed interstitial lymphocytic infiltration, hypercellularity in the glomerulus and necrotic foci of renal tubular cells. Renal changes progressed from 29 to 33 days of age with intense swelling of renal tubular cells, necrosis and moderate presence of inflammatory cells. Between 35 and 40 days of age, the renal lesions were characterized by moderate lymphocytic infiltration in the interstitium (Table 1).

The first viral detection in the tracheal tissue by RT-qPCR, was found at 19 days old. The virus persisted in low levels (1.75 log₁₀) in the tracheal tissues of the infected broilers until the 40th day of age. In addition, IBV was detected from 26 days old in the kidney tissues of these birds, and viral loads remained high until the 35th day before declining over the last interval analyzed when the birds were sampled at 44 days of age. It was notable that the most prominent clinical signs, mortality and gross lesions coincided with the period in which the kidney tissue had the highest viral loads i.e. from 29 to 33 days of age (Fig. 1).

During the trial period, two kidney samples from 33-day-old birds were taken 7-months apart and analyzed by RT-PCR followed by sequencing of PCR products. The samples were named IBV/Brazil/IPX2014 and IBV/Brazil/IPX2015.

The obtained sequences were related to other isolates of Brazilian IBV and grouped in the group of Brazilian strains pertaining to the state of Santa Catarina, Paraná and Rio Grande do Sul, as well as, separate genotypes from other countries (Fig. 2). Sequence analysis of the S1 gene of IBV/BRAZIL/IPX2014 showed high nucleotide similarities (97,1% nt) to IBV/BRAZIL/IPX2015 and recently isolated IBV/BRAZIL/USP CRG/2013/45 (GenBank asses no.KF679874.1). Analyses showed that two amino acid insertions were present at position 120 and 121, and five amino acid insertions at positions 143 - 147 (Fig. 3) in the S1 gene of the IBV/BRAZIL/IPX2014 and IBV/BRAZIL/IPX2015.

Discussion

We phylogenetically characterized the IBV/BRAZIL/IPX2014 and IBV/BRAZIL/IPX2015 strains circulating in broiler flocks in 2014 and 2015 in the south of Brazil as belonging to the Brazilian genotype. These IBV strains were identified in 19- to 40-day-old birds from a vaccinated broiler flock with a history of mild or no respiratory signs, severe renal disease and moderate mortality. The disease in these flocks was assessed by clinical observation, histopathology, viral load and sequencing.

The strains were grouped in the same genotype previously identified in Brazil (Montassier et al., 2006; Villareal et al., 2007; Abreu et al., 2010). Our data show amino acid insertions at positions 120-121 and 143-147 in the S1 gene of the IBV/BRAZIL/IPX2014 and IBV/BRAZIL/IPX2015. The glycoprotein S1 subunit is an important epitope for virus entry into the host cell. Amino acid divergences in the peptide chain were found in strains with different cell tropisms and the modifications in the S1 structure could be affecting the predilection to epithelial cell tropism of IBVs (Kuo et al., 2000; Casais et al., 2003) suggesting that the divergences observed in this study are possibly related to the increased renal tropism observed in these strains.

Most Brazil IBV field isolates belonged to the same genetic cluster (called BR), and show fewer similarities with the vaccine strains currently used in Brazil (H120 and Ma5) (revised by de Wit, 2010). Our results highlight the necessity of an attenuated vaccine that matches with Brazilian strains in order to control IBV infection or an alternative vaccination program. The similarity between isolates from two consecutive years shows that the genetic evolution of the virus circulating in southern Brazil seems to be occurring slowly.

IBV has been shown to replicate in the trachea causing respiratory disease (Cavanagh & Gelb, 2008). However, in the present study, infection was predominantly associated with renal pathology, causing minor or major nephritis and only mild respiratory disease. Recent results from studies investigating the Brazilian genotypes show the high tropism for renal tissue and nephropathogenic activity, such as multifocal nephritis, lymphoplasmacytic infiltration, and necrosis of tubular epithelial cells (Fernando et al., 2013; de Wit et al., 2015). Kidney disease has already been linked to the exacerbation of the inflammatory response due to the high viral load and increased expression of pro-inflammatory cytokines such as IL-6 (Asif et al., 2007; Jang et al., 2013; Fernando et al., 2015).

We conclude that whilst vaccination is considered to be the most cost effective way of controlling IBV infection this approach has been challenged by several factors including the emergence of new IBV serotypes that show little or no cross-protection. Therefore, more detailed analysis of the biologic and antigenic characteristics of the

predominant IBV isolates is warranted, assessments of the efficacy of current vaccines against these isolates are needed, and may highlight the need for new vaccine formulations and / or protocols.

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Detection of meadle east IBV var 2 in broilers in Poland

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Abstract

IBV Var2 strains were firstly detected in 1996 in Israel. It caused intensive losses in poultry industry together with 793B IBV strains in the following years. Later on, incidents of these variants declined, but since 2006 a second wave of IB Var2 has been reported in this country. These new IBV variant was extremely virulent and caused severe damages mainly in broiler flocks. The IBV Var2 has spread in various countries mainly in the Middle-East as Israel, Egypt, Turkey, Iraq, Saudi Arabia but recently its presence was also detected in Lithuania and Armenia.

The IBV Var2 in Poland was detected in 44 day-old broiler flock in December 2015. The flock consisted of 14 200 Ross broilers vaccinated with Mass IBV type in the hatchery and MB IBDV at 18-days of age. The mortality in this flock was observed during the first week of age when more than 800 birds were found dead. However, it was more related to *E. coli* infection. The situation quickly normalized after antibiotics administration and birds started to grow properly. Further health problems appeared at 5 weeks of age. Affected chickens showed decreased feed intake, depression and prolonged, watery diarrhea. The body weight gain over next 8 days was only 350 g and over 600 birds were found dead during this period. The overall mortality reached 10%. The post-mortem examination revealed enlarged livers, crispy kidneys and congestion in both organs. The genome of the Var2 was detected in intestines of affected birds. About 400 base pairs long S1 gene fragment was sequenced. The nucleotide identity between Polish G229/15 and the reference IS/1494/06 strains was 99.2%. As the IBV Var2 has been previously described as displacing all other IBV variants, careful observation of the field situation is needed.

Molecular epidemiology of avian infectious bronchitis and metapneumoviruses in Pakistan (2014-2015)

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Abstract

Viral diseases have been a huge concern for poultry farmers in Pakistan since 1963. Infectious bronchitis virus (IBV) in chicken flocks continues to cause enormous economic losses to the poultry industry in Pakistan. Avian metapneumovirus (aMPV) causes turkey rhinotracheitis and is associated with swollen head syndrome in chickens, which is usually accompanied by secondary infections that increase mortality. In the present study, samples from chickens from 66 flocks with respiratory signs were screened for IBV and aMPV genomes. During surveillance in 2014-2015, oropharyngeal swab samples were collected and spotted on FTA cards. IBV was detected on four chicken farms from two different geographical locations. Partial nucleoprotein gene sequence analysis suggested 793/B genotype circulates in Pakistan. AMPV was detected in chicken farms from the same geographical regions of Pakistan. Sequence analysis of partial G gene was conducted. Only subtype B was detected in two chicken farms during the survey period. These findings demonstrate the circulation of IBV and AMPV in Pakistani commercial chicken farms. The current work constitutes the first comprehensive survey of IBV and aMPV in Pakistan and emphasizes the need for continuous monitoring and rethinking of current preventative measures

Molecular characterization of infectious bronchitis viruses from broiler farms in Iraq (middle and south) during 2014 -2015

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Abstract

Introduction: The infectious bronchitis virus (IBV) is one of the most critical pathogens in the poultry industry. **Methods:** A total of 100 tracheal and kidney tissue specimens from different commercial broiler flocks in the middle and south of Iraq were collected from September 2014 to September 2015. **Results:** 32 IBV-positive samples were selected from among the total and were further characterized by nested PCR. Phylogenetic analysis revealed that isolates belong to four groups (Group I: Variant2 (IS/1494- like), Group II: 793/B- like, Group III: QX- like, and Group IV: DY12-2 like). The present study has discovered that group I (Variant2) is the dominant IBV genotype. **Conclusions:** This is the first comprehensive study on the genotyping of IBV in Iraq with useful information regarding the molecular epidemiology of IBV. The phylogenetic relationship of the strains with respect to different time sequences and geographical regions displayed complexity and diversity.

Molecular surveillance of gamma coronaviruses in pigeon flocks, Tehran province, 2014-2015

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Abstract

coronaviruses have a wide range of host tropism causing respiratory, enteric and central nervous system diseases in pigs, cats, dogs, rodents, cattle, avian species and human. Coronaviruses undergo genetic mutations and recombination at high rates which make them able to infect a wide range of host species from different geographical locations. According to the possible existence of gammacoronavirus in Iranian bird population including pigeons and lack of information about virus prevalence, isolation and molecular characterization of pigeon coronaviruses are needed. The present study was conducted to detect avian coronavirus in pigeons of Tehran province.

Material and Methods

Samples were randomly collected from 25 pigeon flocks of Tehran province (250 tracheal swabs and 250 cloacal swabs, 10 specimens from each flock) between 2014-2015. The viral RNA was extracted from swab samples, the RT-PCR reaction was run using the QIAGEN one-step RT-PCR Kit with primers targeting nucleocapsid (N) gene and 3' untranslated region (3'-UTR) of gammacoronavirus.

Results

Gammacoronavirus was detected in one out of 25 flocks. Conclusions: According to the low prevalence rate of coronavirus in pigeons, samples should be collected from pigeons showing respiratory or enteric signs of disease or from pigeons having contact with other birds or those which are housed near poultry farms.

Host Pathogen & Receptor Interactions

Keynote lecture

Back to the elements: avian coronavirus-host interactions dissected

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Abstract

Interactions between a pathogen and its host determine the outcome of the infection. For avian coronaviruses (AvCoV), like for other viruses, the susceptibility or resistance of a particular species is ultimately determined by the complex interplay between both host and viral factors. Understanding each of these many aspects is elementary in the comprehension of the processes of infection and pathogenesis. More importantly, this knowledge is critical for improvement of the current strategies for disease control and prevention. For many avian coronaviruses, however, the interactions between the virus and the host are hardly understood. While infectious bronchitis virus (IBV) of chickens is the best studied AvCoV, the true differences between IBV serotypes and pathotypes have not been unravelled. Coronaviruses of other fowl are even further from being understood; how they cause disease and what the tropism of each of these viruses is remains to be elucidated.

In this keynote, the essential steps of AvCoV-host interactions will be highlighted. The first fundamental step in the life cycle of a virus includes the binding of the viral attachment protein to the host receptor on a particular cell. The viral spike protein and specific glycan receptors are key elements of this interaction. Subsequent entry and replication of the virus in susceptible cells, and production of new virus particles from those cells, are next steps in the interaction. The success of the virus is now determined by its ability to hijack host proteins for its own benefit, while the host cell starts counteracting the infection to reduce the virus burden. This includes shutting down host protein synthesis and activation of sensors of the innate immune system like for example the interferon system. Once the virus is released from its primary target cell, interactions with the host immune system come into play to ultimately determine the pathogenicity of the virus, as well as persistence of the virus in a particular host and transmission between hosts. For each of the mentioned steps,

examples of known avian coronavirus factors and host elements will be presented to underscore the importance of each of them ultimately determining disease by AvCoVs.

Embryonic kidney cell-adapted infectious bronchitis virus spike protein shows a reduced host cell binding spectrum

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Abstract

Coronavirus spike (S) proteins' ability to bind to host cell receptors is a major determinant of tissue and species specificity and pathogenicity. For infectious bronchitis virus (IBV), for example, recombinant S1 protein representing the attenuated H120 vaccine strain binds to tracheal epithelium with lower affinity than recombinant S1 protein representing the virulent M41 strain (Wickramasinghe et al. 2011). Furthermore, recombinant S1 protein representing an ArkDPI-derived IBV vaccine subpopulation that is selected in chickens binds to tracheal epithelium and other chicken tissues with greater affinity than S1 protein representing the major vaccine population, which is negatively selected in chickens (van Santen et al. 2014). The S1 of this selected vaccine subpopulation differs from that of the negatively-selected major vaccine population in only four amino acid positions (van Santen & Toro 2008). Thus, we hypothesized that the three amino acid changes we observed in S (two in S1 and one in S2) associated with adaptation of the ArkDPI vaccine strain to chicken embryonic kidney (CEK) cell culture (Ghetas et al. 2015) would result in higher-affinity binding of recombinant S protein to CEK cells, thus contributing to adaptation.

Materials and Methods

Expression constructs for soluble trimeric recombinant spike protein (S1 or S ectodomain) representing ArkDPI vaccine or CEK-adapted ArkDPI vaccine (Ghetas et al. 2015) were generated and recombinant spike proteins were produced in transiently-transfected HEK293T cells and purified via affinity chromatography as described (Promkuntod et al. 2013, Wickramasinghe et al. 2011). Recombinant proteins containing only one or two of the three amino acid changes found in CEK-adapted vaccine spike protein were also generated. CEK cultures were prepared as described (Ghetas et al. 2015) in 8-chamber slides. Confluent cultures were acetone-fixed and used for spike histochemistry as described (Wickramasinghe et al. 2011),

with minor modifications: rehydration and antigen retrieval steps were omitted, Tris buffers were substituted for phosphate buffers, and addition of most reagents and washing steps were performed by an IntelliPATH FLX automated slide stainer. Spike proteins and AEC reagent were added manually. Formalin-fixed tissues from a 40-day-old white leghorn chicken were used for spike histochemistry as described (Wickramasinghe et al. 2011), except for substitution of Tris buffers and use of an automated slide stainer

Results

Recombinant S1 protein representing the vaccine strain bound to few CEK cells (Fig. 1a). Contrary to the expected improved binding, recombinant S1 protein representing CEK-adapted vaccine strain exhibited no detectable binding to CEK cells (Fig. 1b). Because inclusion of the S2 ectodomain had been shown to be necessary for binding to chorioallantoic membrane by the spike protein representing the highly embryo-adapted IBV Beaudette laboratory strain (Promkuntod et al. 2013), which is able to replicate in chorioallantoic membrane, we determined whether recombinant spike ectodomain (containing both S1 and S2 ectodomain) representing CEK-adapted virus could bind to CEK cells. Although addition of the S2 ectodomain improved binding of the parental vaccine S1 protein to CEK cells (Fig. 1c), binding of spike ectodomain representing the CEK-adapted vaccine strain was still undetectable (Fig. 1d). Only when spike histochemistry was performed using spike ectodomain at higher concentrations (e.g. 3.5-times the concentration used in Fig. 1) did spike ectodomain representing CEK-adapted vaccine strain bind detectably to CEK cells (not shown).

Effects of each of the three amino acid changes in spike protein associated with adaption to CEK cells on binding to CEK cells were tested individually. Each of the three amino acid changes reduced, but did not abolish binding of spike ectodomain to CEK cells. The combination of the two amino acid changes in S1 reduced binding of the recombinant spike ectodomain to below detectable levels.

Because the CEK-adapted vaccine strain replicates in chickens well enough to stimulate an immune response that protects against virulent IBV challenge (Ghetas et al. 2016), we determined the effect of the changes in the spike protein on the ability of recombinant spike ectodomain to bind to chicken tissues. The spike ectodomain representing the parent vaccine strain bound to epithelial cells of trachea, lung, and kidney, secretory cells and epithelium of choana, epithelial cells of conjunctiva, secretory cells in the nasolachrymal gland, and epithelium and secretory cells in the cloaca. Binding of spike ectodomain representing CEK-adapted vaccine strain to trachea and cloaca was markedly reduced compared to vaccine strain, and

detectable binding to lung, kidney, secretory cells in the choana, and the nasolachrymal gland was abolished. However, binding of spike ectodomain representing CEK-adapted virus to epithelial cells in the choana and conjunctiva was nearly equivalent to that of spike ectodomain representing the parental vaccine virus.

Discussion

Changes in S protein associated with adaption of the vaccine strain to CEK cells abolished rather than improved binding to CEK cells. Thus factors other than improved attachment, associated with other changes in the genome of CEK-adapted vaccine (Ghetas et al. 2015), must be involved in adaptation to CEK cells. This is in contrast to our findings regarding selection of vaccine subpopulations in chickens, where S1 binding assays suggest a role for improved attachment in selection (van Santen et al. 2014). Marked reduction of binding to most chicken tissues by S protein representing CEK-adapted vaccine strain, with the exception of epithelial cells of the choana and conjunctiva, suggests that replication of the CEK-adapted vaccine strain in chickens might be restricted to fewer tissues than the original vaccine strain. From an applied perspective such restriction might be beneficial as it would reduce adverse reactions but still elicit immunity in the upper respiratory tract.

Acknowledgments

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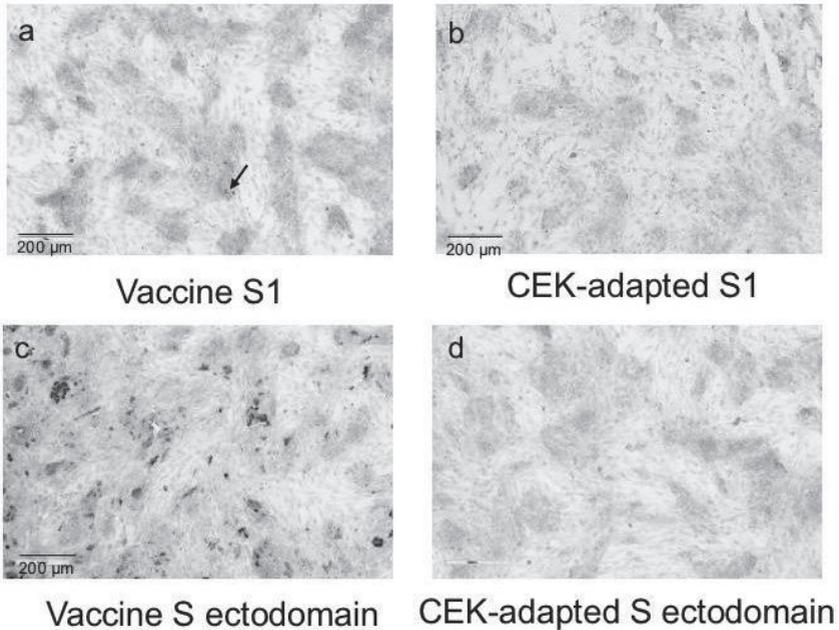


Fig. 1. Neither S1 nor S ectodomain representing CEK-adapted vaccine strain binds detectably to CEK cells.
a and b: Spike histochemistry of recombinant S1 protein representing parent (a) or CEK-adapted (b) vaccine strain on acetone-fixed CEK cells. Arrow in a indicates some S1-bound cells. c and d: Spike histochemistry of recombinant spike ectodomain representing parent (c) or CEK-adapted (d) vaccine strain on acetone-fixed CEK cells. All proteins were used at 100 µg/ml. Thus ectodomain proteins were used at approximately half the molar concentration as S1 proteins. Red color from the chromogenic substrate AEC indicates protein binding.

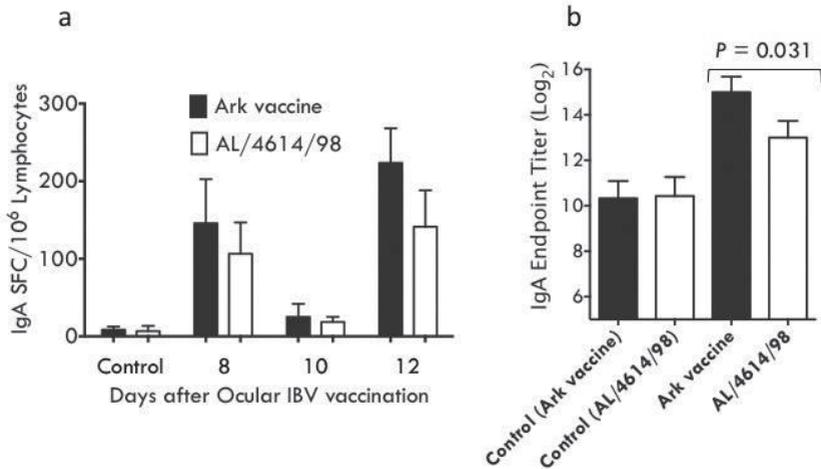


Fig. 2. IgA response to vaccine strain and field strain AL/4614/98 following ocular vaccination with Ark vaccine strain. a) Number of IgA spot-forming cells in HALT (Harderian gland and CALT) against each virus determined by ELISPOT 8, 10 and 12 days post-vaccination. Means +/- SEM for data from 5 chickens for each time point are shown. When results from all three days (N=15) are combined, $P = 0.031$ (2-tailed paired T test). b) IgA endpoint titer in tears against each virus 2 weeks post-vaccination. Means +/- SEM for data from 6 chickens are shown. P value shown is for 2-tailed Wilcoxon matched-pairs signed rank test.

Polymorphisms in the s1 spike glycoprotein of Arkansas-type infectious bronchitis virus show differential binding to various chicken tissues

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Abstract

Arkansas-DelMarVa Poultry Industry (Ark-DPI) infectious bronchitis virus (IBV) has been used as a vaccine for the past 25 years. Concurrently, historical data has shown that Ark-DPI is also the most commonly isolated strain from clinical samples, which is contradictory and unexpected if the vaccine provides protection. In actuality, the high prevalence of Ark-DPI isolations has been attributed to the vaccine continually circulating in flocks. This was confirmed by comparing sequences of the S1 gene of these samples to virus subpopulations in the vaccine. In particular, sequencing of field isolates has shown that subpopulations with specific amino acid changes are selected when the Ark-DPI vaccine replicates in birds as compared to the predominant virus sequence in the vaccine bottle. The most commonly found changes are in the S1 region of the spike glycoprotein, with specific changes in amino acid residues 43 and 344. Position 43 is thought to be important for binding to host tissues while position 344 lies in an antigenically important region of the S1. Based on this data, we hypothesized that the amino acid changes at positions 43 and 344 in the S1 gene are critical for vaccine virus fitness in chicks, as changes at these two positions are always seen in field isolates when compared to the parent vaccine. For this study, we constructed strep-tagged recombinant S1 spike glycoprotein genes with a Tyrosine to Histidine amino acid change at position 43, an Asparagine deletion at position 344, and a gene containing both mutations, and used the expressed proteins as substrate for binding assays. The expressed proteins were bound to fixed chorioallantoic membrane (CAM) or mature chicken trachea tissues and we used the streptavidin detection system to measure binding intensity of each protein to each tissue. The Y43H mutation significantly increased the binding of the Ark S1 protein to trachea when compared to the native sequence. Conversely, the deletion at 344 reduced binding to trachea when compared to the native sequence. When both mutations were present, binding was no different than the native protein. Only the native vaccine sequence bound with any detectability to CAM. These results fit the hypotheses that the Y43H mutation makes a vaccine subpopulation more fit to

replicate in birds by increasing the binding efficiency of the spike protein, which is the first step in infection, while the native vaccine sequence is optimal for growth in embryos which is how vaccines for IBV are produced. The deletion at 344, while reducing overall spike binding efficiency, may play a role in evasion of the chicken immune system as this amino acid falls into a previously reported highly antigenic region of the spike protein. This was further shown by the fact that antisera raised against the pathogenic Ark virus (which contains the Asparagine at 344) did not recognize the proteins with the deletion in western blots. Taken together, it appears that these mutations aid the vaccine subpopulation to outcompete the predominant population in the vaccine when applied to chickens.

***In vivo* tracheal α 2,3-linked sialic acid expression changes after infection of chickens with infectious bronchitis virus**

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Abstract

The major receptor and tropism determinant for infectious bronchitis virus (IBV) so far elucidated is the α 2,3-linked sialic acid. Ex vivo analyses on IBV-infected tracheal organ cultures (TOCs) have effectively shown co-localization of α 2,3-linked sialic acid expression and viral antigen presence within ciliated and mucus-producing cells. Furthermore, IBV tropism for those cells has been visualized with spike histochemistry, which uses recombinant produced IBV-spike protein (S1) for specific tissue binding. For highly pathogenic avian influenza virus it has been shown that airway sialic acid expression decreases after infection in mammals. It has been proposed that this might have consequences for viral persistence in the host and may reduce the severity of disease and viral spread. To study sialic acid expression dynamics in chickens after IBV infection, we compared respiratory tract tissue of IBV-infected chickens to uninfected tissue. The presence of mucus-producing cells, α 2,3-linked sialic acid expression, IBV spike-binding and avian influenza hemagglutinin-binding were analyzed five days post infection. Mucus production (visualized using period acid Schiff and alcian blue stains) was significantly decreased in infected tracheas. The location of 2,3-linked sialic acid, shown using the lectin *Maackia amurensis* I (MAAI), shifted from the apical membrane and goblet cell cytoplasm towards the basement membrane region. In contrast, though binding affinity of the IBV spike (M41) was lost at the apical membrane and in goblet cells, no increased spike binding was seen near the basement membrane. Interestingly though, binding of avian influenza hemagglutinin H5 resembled that of MAAI in both the uninfected and IBV-infected tissues. Results were comparable for three IBV strains (M41, 4/91, QX), suggesting that sialic acid binding and the influence of infection on sialic acid expression is similarly induced by various IBV strains. To further elucidate the mechanism of this transition and to study whether spike- and hemagglutinin-binding truly underlie differences between IBV and avian influenza virus regarding their ability to infect the IBV-infected trachea, future studies will focus on the use of

trachea organ cultures (TOCs) of mature chickens. Ultimately, IBV-driven alterations in sialic acid expression in the host might influence the susceptibility of the bird to viruses that use sialic acids as host receptors.

Variation of membrane rearrangements induced by M41 strain of infectious bronchitis virus

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Abstract

Positive-strand RNA (+RNA) viruses rearrange cellular membranes during the virus lifecycle to create replication organelles, thought to allow more efficient viral RNA synthesis. Economically important avian coronavirus, infectious bronchitis virus (IBV), was shown to induce formation of conserved double membrane vesicles induced by all coronaviruses. In addition, IBV caused regions of ER to zipper together and open-necked spherules, strongly resembling spherules induced by other +RNA viruses, were shown to be tethered to the zippered ER. This previous work was performed using an apathogenic, highly laboratory adapted virus strain, Beau-R. In our recent work, we have shown that in cell culture, a pathogenic laboratory strain, M41, induced very low numbers of spherules. In spite of this, overall virus replication was unaffected with comparable accumulation of viral RNA and peak virus titres between Beau-R and M41. A more detailed comparison of a range of apathogenic and pathogenic vaccine and field isolates in *ex vivo* tracheal organ culture demonstrated that M41 was the only viral strain studied to have a low spherule phenotype. Interestingly, a comparison of chimeric recombinant viruses containing gene swaps between Beau-R and M41 indicated that the genetic determinants for spherule formation reside within the region of the genome from the 5' end to nsp14. Therefore, although it appears that for most IBV strains spherules play a role during virus replication, coronavirus replicative apparatus clearly has the plasticity to function in different structural contexts.

Infectious bronchitis virus and the PI3K/AKT signalling pathway

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Abstract

All viruses are required to modulate cellular processes to replicate efficiently within a host cell. Many different cellular pathways can be controlled at various points during viral replication. The PI3K/AKT signalling pathway is a host pathway often modified by viruses because it plays a crucial role in the regulation of many cellular processes including protein synthesis, cell proliferation and survival. We have investigated the role of the PI3K/AKT signalling pathway during avian infectious bronchitis virus (IBV) infection. By studying the phosphorylated form of AKT we have shown that IBV infection induces activation of AKT in a PI3K dependent manner. In avian cells this activation occurs in a biphasic manner at both early and late time points during infection. Furthermore, IBV requires an active PI3K/AKT pathway for efficient replication and over expression of the structural membrane protein M has been found to result in activation of AKT. Finally we have investigated how PI3K/AKT activation allows modulation of downstream cellular pathways. This study highlights the importance of the PI3K/AKT signalling pathway during IBV infection and may be applied to development of novel vaccines or targeted therapeutics.

Generation of a recombinant infectious bronchitis virus suggests that the S protein is a determinant of pathogenicity

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Abstract

The spike (S) protein of IBV is responsible for attachment to host cells and fusion of viral and cellular membranes. Previously it was shown that altering the S protein of IBV can affect the tropism of the virus. It has also been suggested that IBV S plays a role in pathogenicity. In order to investigate this, a recombinant IBV (rIBV) M41K-BeauR(S) has been generated using the reverse genetics system developed at The Pirbright Institute where the S protein from the apathogenic Beau-R strain has been inserted into the pathogenic M41-K background.

The resultant virus (rIBV M41K-BeauR(S)) was used to infect 12 Rhode Island Red chickens and its tropism and pathogenicity was compared to the parent virus M41-K. Clinical signs were observed from day three post-infection and tissues were taken at days four, six and seven post-infection to analyse viral tropism and spread. Viral presence in tissue has been assessed by PCR.

The replacement of the S protein of M41-K with that of the Beaudette strain appears to have vastly reduced the pathogenicity of the recombinant virus, indicating that the S protein is a pathogenicity factor for IBV. Growth kinetics and cellular tropism will also be assessed to ascertain whether the insertion of the Beaudette S protein has affected the *in vitro* characteristics of the virus.

Comparison of respiratory (M41) and nephropathogenic (B1648) infectious bronchitis viruses in *in vitro* and *in vivo* conditions

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Abstract

Infectious bronchitis virus (IBV) is a coronavirus that affects poultry. IBV has a main tropism for the epithelial cells of the respiratory tract. Massachusetts (M41) is a prototype respiratory IBV strain (RIBV). Some IBV-strains are described as nephropathogenic (NIBV) since the respiratory infection is followed by a severe renal infection causing a high mortality. At present, it is not known why some IBV strains have a kidney tropism. This was addressed in the present study. The replication characteristics of respiratory (M41) and nephropathogenic (B1648) IBV strains were compared *in vitro* in respiratory mucosa explants and blood monocytes (KUL01⁺), and *in vivo* in chickens. No major difference in replication characteristics was observed between RIBV and NIBV in the respiratory mucosa explants. IBV penetrated the deeper layers of the respiratory tract by using KUL01⁺ cells as carrier cells. NIBV B1648 was productively replicating in KUL01⁺ monocytic cells in contrast with the respiratory IBV M41. *In vivo*, the virus-shedding pattern in the tracheal mucosa was similar for both strains. In B1648 inoculated animals, 10³ - 10⁵ viral RNA copies were observed in plasma and mononuclear cells, where as in M41 inoculated animals, viral RNA copies were not observed in plasma and mononuclear cells (except at one time point in plasma of one chicken). After two blind passages on embryonated eggs, the plasma and mononuclear cell samples of the B1648 were positive in contrast with M41. Viral RNA copies were detected in kidneys, spleen, lungs and liver of chickens infected with B1648 but not with M41. In conclusion, both M41 and B1648 replicates extensively in the epithelium of tracheal mucosa, after which only B1648 can spread to internal organs via cell associated viremia in mononuclear leukocytes and cell free virus in plasma.

Pathogenicity characteristics of an Iranian IS-1494 like infectious bronchitis virus isolate in experimentally infected SPF chickens

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Abstract

Introduction: Avian infectious bronchitis (IB) is a major cause of economic loss to the poultry industry. IB virus primarily affects the respiratory tract, but strains differ in their tropism for other target organs such as the kidneys and alimentary tract. The objective of this study was to estimate the pathogenicity of an Iranian IBV variant (UTIVO-C, IS-1494 like OR Variant- 2 like) which is one of the most prevalent isolates circulating in Iranian poultry farms. **Methods:** SPF chickens were inoculated intranasally with 104 EID₅₀/0.1 mL of the virus. Sera, fecal swabs, and different tissue samples were collected on different days post infection (p.i.). Clinical signs, gross pathology, and histological changes were recorded. The amount of virus genome was quantified in RNA extracted from different tissue samples using a quantitative real-time-PCR assay. Anti IBV antibody was detected in serum samples. **Results:** The highest IgG antibody titer was found on day 28 p.i. Severe histologic lesions were observed in the trachea, lung, and kidney. Viral RNA was detected in feces and all investigated organs. The highest viral titers were detected in the feces and cecal tonsils. Real-time PCR results demonstrated UTIVO-C tropism for the respiratory tract, digestive system and renal tissue due to its epitheliotropic nature. **Conclusions:** This is the first pathogenicity study of the Iranian Var-2 like IBV isolate. Based on histology and clinical signs this isolate is classified as a nephropathogenic IBV, although the high virus replication in the alimentary tract is worthy of note. Increased knowledge of IBV pathogenesis facilitates more effective prevention practices.

Introduction

Avian infectious bronchitis (IB) is an acute and highly contagious disease of chickens caused by infectious bronchitis virus (IBV). The name of the disease refers to its most frequent clinical manifestation, although it can also infect epithelia of the kidney,

intestinal tract and genital organs (Jackwood & De Wit, 2013). Since 1944, the Massachusetts (Mass) and 793/B were the only IBV types detected in Iranian poultry farms. New IBV genotypes such as IS720, IS-1494 (Variant-2), QX, IR-I, and IR-II have been characterized since 2010 (Hosseini, Fard, Charkhkar, & Morshed, 2015). Little is known about the pathogenic aspects of these new variants. The present study was conducted to evaluate the pathogenicity of one of the most prevalent strains, UTIVO-C, in experimentally infected specific pathogen-free (SPF) chickens.

Material and methods

Strain history and virus titration

The virus was isolated from tissue samples of the trachea and kidney which were collected from suspected broilers. EID₅₀ was calculated by the Reed-Muench method.

Experimental design

Forty-nine one-day-old SPF chicks were randomly divided into two groups (35 chicks in the experimental and fourteen in the control groups). At an age of 14 days, birds in the experimental group were challenged intranasally with allantoic fluid of UTIVO-C virus (IS-1494 like) containing 10⁴ EID₅₀/0.1 ml of the virus. On days 1, 3, 5, 7, 14, 21 and 28 post infection (p.i.), five chickens from the experimental group and two chickens from the control group were randomly selected and sera and tissue samples were collected.

Detection of anti-IBV antibodies. ProFLOK IBV ELISA Kit (Synbiotics-USA) was used for the detection of specific antibody to IBV in the serum samples.

RNA extraction and cDNA synthesis

RNA was extracted from tissue samples using Cinna Pure RNA kit (Sinaclone, Iran). Complimentary DNA (cDNA) was generated using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).

Real-Time PCR assay. Real-time PCR assay was used in order to amplify a conserved sequence within the 5'-UTR of the IBV genome and 28s ribosomal-RNA was used as a reference gene (Rothwell *et al.*, 2004).

Histopathological examination. Specimens from the trachea, lung, and kidney were placed in 10% buffered formalin, sectioned, stained with haematoxylin and eosin and evaluated for histological lesions.

Results

Clinical Signs, Gross Lesion, and Mortality

Chickens inoculated with IBV showed clinical signs of depression, ruffled feathers, nasal discharge, and coughing on day five after exposure and symptoms were more pronounced on day 14 p.i. Half of the infected chicks developed watery diarrhea on day 7 p.i. From day 3 to 7 p.i. small amounts of catarrhal exudate and mucosal hyperemia were observed in the trachea. The kidneys of all infected birds euthanatized between 5 to 14 days p.i. were enlarged and pale. No clinical signs or gross lesions were observed in the control group of chickens. No mortality occurred during the experiment.

Serological results

No antibodies were detected in control chickens. The initial five specimens of the infected group (1, 3, 5, 7 and 14 days p.i.) were negative, while the IgG titers of sera collected at days 21 and 28 p.i. were 437 and 554, respectively.

Real-time PCR results

The highest viral genome copy number was measured on day 3 p.i. in the feces and cecal tonsils. Viral loads in the kidney and proventriculus peaked on day 3 p.i., while those of trachea, lung, and spleen peaked by day 5 p.i. The viral RNA persisted in all tissues and the feces until the end of the investigation period.

Histopathological findings

Focal mononuclear cell infiltration in the renal interstitium was observed from the first day of infection. The main lesions consistent with interstitial nephritis, including vacuolation and necrosis of tubular epithelium, were present on day 5 p.i. Lymphoplasmacytic nodules in the interstitium were observed from day 5 to 14 p.i. after which interstitial hemorrhage and slight lymphocytic infiltrates were recorded. In the tracheal samples, loss of cilia, epithelial degeneration and slight infiltration of inflammatory cells were found at day 5 p.i., progressing to severe mononuclear infiltration on day 7 p.i.. Microscopic examination of the lungs showed hyperemia, mild mononuclear infiltration in the parenchyma and desquamation of bronchial epithelium on day 5 p.i.. Subsequently, lymphoid nodules in the bronchial wall were visible. Hyperemia and mild infiltration of inflammatory cells were the only lesions found on days 14 and 21 p.i..

Discussion: The highest viral genome copy numbers were detected in the faces, cecal tonsils and proventriculus which may reflect a higher viral affinity for the alimentary

tract but is not necessarily indicative of the virus' pathogenicity in these tissues. This discrepancy has also been shown by Dolz *et al.* who studied the pathogenicity of a serotype causing renal disease and found higher levels of viral antigen in the nasal turbinates than in the kidneys (Dolz, Vergara-Alert, Perez, Pujols, & Majo, 2012).

As our results show, the virus was detected in feces and tissue samples collected at different time points. As IBV can replicate in many epithelial surfaces and given that the highest viral load is not always found in the main target organ, real-time results do not accurately demonstrate the pathogenic aspects of a pathogen. Some researchers used histopathological findings coupled with viral genome detection to evaluate the pathogenicity of Mass, SAIBK and Italy02 genotypes (Dolz *et al.*, 2012; Fan *et al.*, 2012).

In the present study, the histological changes were observed in all three investigated organs. As previously reported, severe histologic lesions in respiratory tissues can occur in both nephropathogenic and QX-type IBV infections (Benyeda *et al.*, 2009; Dolz *et al.*, 2012; Purcell, Tham, & Surman, 1976), but lesions indicative of interstitial nephritis are only found in the course of nephropathogenic IBV infections.

Considering the histopathological renal changes, UTIVO-C can be classified as a nephropathogenic IBV. To best determine whether the high viral load observed in the intestinal tract reflects enteropathogenicity further histological examination of different parts of the intestinal tract is required.

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Immunopathogenesis of infectious bronchitis virus Q1 in SPF chicks

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Abstract

We are reporting on an experimental infection of day-old SPF chicks with a Q1-like isolate. Following Q1-like infection at day-old, chicks were observed daily for clinical signs and body weights were taken at weekly intervals. At 1, 3, 7, 14, 21, 28 days post infection (dpi), oropharyngeal (OP) and cloacal (CL) swabs were taken for virus detection, blood was collected from ten birds and five chicks were humanely killed for necropsy. Lesions were scored and trachea, kidney and proventriculus were collected for IBV qRT-PCR and histology. Infected chicks had a significantly reduced body weight compared to the control group at 14, 21 and 28 dpi. Clinical signs of rales and nasal discharge developed in the infected group, which were absent in the control birds. Kidney and tracheal lesions were present in the infected group until 14 dpi. All tissues and swabs were RT-PCR positive and the IBV viral load peaked in the kidneys ($1.4 \log_{10}$ REU) and trachea ($3.8 \log_{10}$ REU) at 7 dpi, whereas the proventriculus ($1.9 \log_{10}$ REU) peaked at 3 dpi. By 28 dpi, 63% of the remaining birds were IBV-antibody positive by ELISA. Virus isolation was achieved from the proventriculus at 3, 7 and 9 dpi.

Introduction

In the period between 1996 and 1998, a new IBV variant (named Q1) was detected in layer flocks in China. Infection was accompanied by respiratory distress, proventriculitis, decreased egg production and diarrhoea (Yu *et al.*, 2001). Upon sequencing, the new variant had a low level of partial S1 nucleotide homology (77-82%) with the M41 vaccine and H120 Massachusetts strain, and this new variant IBV was referred to as Q1 (Ababneh *et al.*, 2012; Wit *et al.*, 2012). A further two isolates (J2 and T3) closely related to Q1 were also identified (Yu *et al.*, 2001). Since the first report of Q1, there has been limited published information on the pathogenesis of this strain, which has since been detected in a number of other countries such as Italy (Toffan *et al.*, 2013), Chile, Peru, Argentina (Sesti *et al.*, 2014),

Colombia (Jackwood, 2012) and in the Middle East (Ganapathy *et al.*, 2015). The aims of this study were to gain further information regarding IBV Q1 pathogenesis after experimental infection in specific pathogen free (SPF) chicks.

Materials and methods

IBV strain

IBV Q1-like $10^{4.5}$ CD50/ml (Chhabra *et al.*, 2015) was propagated in embryonated SPF eggs and titrated in tracheal organ cultures (TOCs).

Gross lesions

On each sampling day (1, 3, 7, 9, 14, 21 and 28 dpi), five chicks were euthanised and assessed for gross tracheal and kidney lesions.

RNA extraction

For tissues, extraction of viral RNA was conducted using the RNA Easy kit (Qiagen) according to the manufacturer's instructions and quantified using a NanoDrop1000 spectrophotometer. For swabs, all 10 swabs were pooled in 3 ml TOC media, then 300 μ l was added to 300 μ l of solution D (stock solution D and mercaptoethanol). RNA extraction was then carried out using the phenol–chloroform extraction as previously described (Ball *et al.*, 2016; Chomczynski and Sacchi, 2006).

RT-PCR. Extracted RNA was subjected to RT-PCR for IBV detection as previously described (Awad *et al.*, 2014; Cavanagh *et al.*, 1999; Worthington *et al.*, 2008).

qRT-PCR

Viral RNA load was quantified using a quantitative real-time RT-PCR (qRT-PCR) assay targeting the IBV 3' untranslated region (UTR) as previously described (Jones *et al.*, 2011).

ELISA

Serum samples were analysed using a commercial IBV ELISA kit (IDEXX) according to the manufacturer's instructions.

Statistical analysis. Data was analysed using Graph Pad Prism 6 and considered significant at $p < 0.05$.

Results

A significant ($p < 0.05$) body weight decrease was seen in the infected group at 14, 21 and 28 dpi (Fig. 1). Clinical signs began after 24 hours pi and continued until 17 dpi. During this time tracheal rales, coughing, sneezing and gasping sometimes

accompanied by diarrhoea were witnessed. Gross lesions consisted of serous, catarrhal, or caseous exudate and/or congestion in the trachea, and kidney swelling, congestion and urate accumulation in the ureter. Histopathological changes in the trachea manifested as loss of cilia, heterophil infiltration, epithelial hyperplasia and lymphoid infiltration. Kidney histopathological findings were limited to interstitial lymphoid infiltration and mild lymphoid follicles. Pooled OP and CL swabs were positive for Q1 on all sampling days by RT-PCR. However, only samples until 21 dpi were qRT-PCR positive.

The trachea, kidney and proventriculus showed differing viral loads. Viral load peaked at 7 dpi in the trachea ($3.8 \log_{10}$ REU) (Fig. 2A), and kidney ($1.4 \log_{10}$ REU) (Fig. 2B), while it peaked at 3 dpi in the proventriculus ($1.9 \log_{10}$ REU) (Fig. 2C). Proventriculus samples (3, 7, 9 dpi) were RT-PCR positive following virus isolation in embryonated SPF eggs. The number of anti-IBV antibody titre positive samples increased from 43% to 63% on days 21 and 28 respectively (Fig. 3).

Discussion

In this study, the pathogenesis of the IBV Q1-like (37089/2013) isolate was determined. Gross lesions, histopathological changes and viral loads in the trachea, kidney and proventriculus were demonstrated. Additionally, re-isolation of the virus was confirmed from proventriculus tissue.

A reduction in body weight following IBV infection has been previously reported for the Australian T-strain (Afanador and Roberts, 1994), M41 (Otsuki *et al.*, 1990) and QX (Ganapathy *et al.*, 2012). Gross and histopathological changes in the current study were similar to those previously reported for Q1 and comparative to other IBVs (Awad *et al.*, 2016). Our results show that the highest histological score for trachea was seen on days 9 and 14 pi, while histological scores in kidney samples remained moderately low, despite being similar to previous reports. Viral load was higher in the trachea compared to the kidney, with both peaking at 7 dpi. This finding was in agreement with recent work (Chhabra *et al.*, 2015) and may be due to replication of the virus directly in the trachea and/or repeat exposure due to the housing and litter environment. Our findings provide further information regarding the pathogenesis and immune responses to this increasingly important variant IBV.

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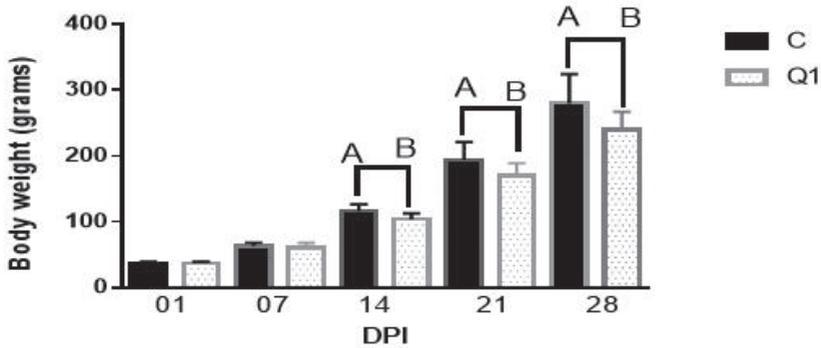


Figure 1. Weekly mean body weight comparison between SPF control and Q1-infected group. Data shows the mean values (n=10) in grams with standard error. Significant differences between the groups indicated with different letters (P<0.05)

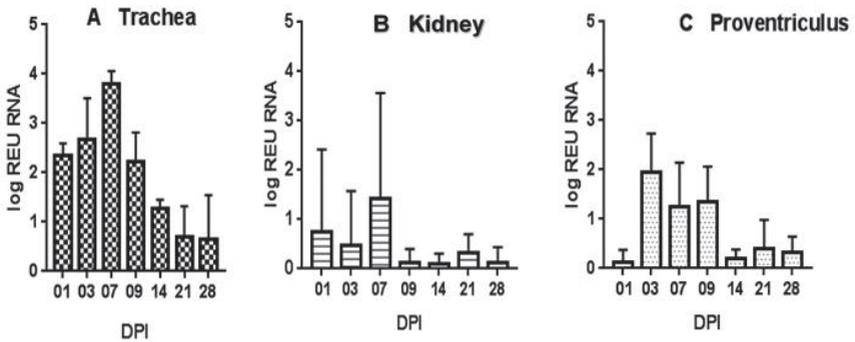


Figure 2. Quantification of viral RNA, expressed as log relative equivalent units (REU) of RNA in (A) Trachea and (B) Kidney and (C) Proventriculus. Infected birds (n=5/group) were inoculated with IBV Q1 ($10^{4.5}$ CD_{50} /bird)

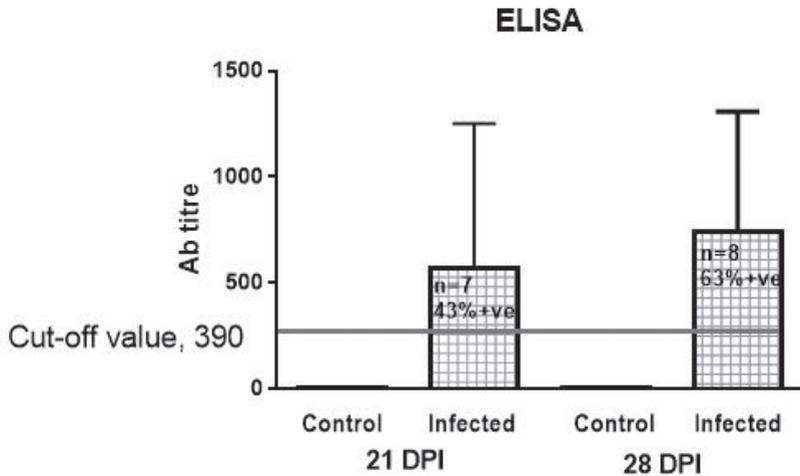


Figure 3. Infectious bronchitis virus (IBV) antibody titres by ELISA. Comparisons shown between the control and infected ($10^{4.5}$ CD_{50} /bird) groups. ELISA cut-off for a positive sample is given as 390. Data is expressed as mean values \pm SEM.

Antiviral activity of mannose-binding lectin against infectious bronchitis virus

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Abstract

The innate host defence system of the chicken respiratory tract is the first to encounter a respiratory pathogen, like infectious bronchitis virus (IBV), when entering the host. Previously, it has been shown that the mannose-binding lectin (MBL), a soluble pattern-recognition receptor and innate immune protein, has role in shaping the responses to IBV *in vivo*. In particular, in high-MBL expressing chicken lower viral loads were detected after infection and MBL haplotypes differed in the production of IBV-specific antibodies (Kjaerup et al. 2014). To further study the interaction between MBL and IBV, we produced recombinant chicken MBL (rcMBL) in a mammalian expression system and purified it using mannan-agarose beads. rcMBL was produced as oligomers and functionally active.

Recombinant cMBL was able to block the infection of IBV-Beaudette on BHK cells in a concentration-dependent manner, as was shown by immunofluorescence assays and qRT-PCR. The specificity of the blocking was confirmed by mannan, the ligand for MBL, which could restore infectivity when present during the infection. The ability of rcMBL to block the infection was due to a direct interaction with IBV as shown by negative staining electron microscopy, IBV-Beaudette agglutinated into clumps in the presence of rcMBL. ELISA assays revealed that in particular the S1 domain of the spike of IBV mediated the binding to MBL in a calcium-dependent way. The relevance of this interaction was finally elucidated by the ability of rcMBL to block the binding of IBV M41 S1 to chicken tissues using our recently developed spike histochemistry assay.

Chickens bred for divergent MBL plasma concentrations differ in innate and adaptive transcriptome immune profiles by IBV challenge

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Abstract

Understanding the molecular mechanisms involved in the interaction between innate and adaptive immune responses to infectious bronchitis virus (IBV) is crucial for further improvement of disease control strategies. Mannose-binding lectin (MBL) is a soluble pattern-recognition receptor important for the innate immune response to bacterial, viral and parasitic infections. Others have earlier shown that recombinant MBL can block IBV-Beaudette infection in cell culture by direct binding of MBL to the IBV S1 protein domain.

Two chicken lines were selectively bred for high (L10H) and low (L10L) plasma concentration of MBL. After 14 generations, offspring were produced for an experimental IBV-M41 infection study. L10H chickens exhibited lower viral loads and less severe damage of tracheal cilia than L10L chickens on days 1-3 after infection. Also, considerable differences between the two lines were observed in the absolute numbers of circulating lymphocytes. In order to study genes of importance for systemic adaptive immunity, spleens were sampled from infected and uninfected

chickens at weeks 1 and 3 pi. Differential gene expression analysis based on RNA-sequencing data as well as functional analysis was performed using Gene Ontology (GO) Immune System Process terms specific for *Gallus gallus*. Comparison of the two chicken lines identified several differentially expressed genes (DE). The two most enriched GO terms were “Lymphocyte activation involved in immune response” and “Somatic recombination of immunoglobulin genes involved in immune response” for uninfected chickens. For infected chickens, the most enriched GO terms were “Alpha-beta T cell activation” and “Positive regulation of leukocyte activation”. In conclusion, significant differences were found in expression profiles of innate as well as adaptive immunity-related genes. The transcriptome differences between chickens with high and low MBL suggest that selection for MBL plasma levels may influence a wide range of immune genes and potentially affect host response to IBV infection and vaccination.

Introduction

The co-evolutionary progress of the host-pathogen relationship enables animals to develop natural immunity towards pathogens. As opposed to disease specific adaptive immunity, genes and molecular pathways related to a broader and general resistance are primarily found among molecules belonging to the innate immune system. Genes important in disease resistance code for receptors such as pattern recognition receptors (PRRs) involved in early recognition of pathogen associated molecular patterns (PAMPs). However, the identification of specific genetic loci responsible for broad resistance against several pathogen types in production animals is yet to be determined. A deeper understanding of this subject matter may lead to the improvement of livestock health and welfare through selective breeding (Glass, 2012).

PRRs exist as membrane-bound, intracellular or even soluble molecules. A particularly interesting family is the collectin family, which is a family of soluble c-type lectin receptors with a collagenous structure enabling them to form higher order oligomers with strong binding potential to carbohydrates present on pathogen surfaces. The soluble CLRs bind to oligosaccharide structures via their carbohydrate recognition domains (CRDs) in a calcium dependent manner, which may lead to complement activation, neutralization or increased opsonophagocytosis. The best studied chicken collectins so far are mannose binding lectin (MBL) and surfactant protein A (SpA). Immunohistochemistry showed increased amounts of MBL and SpA in lung sections from IBV-M41 infected chickens (Figure 1).

In both humans and chickens, large variations in serum levels of MBL between individuals are found. MBL deficiency in humans and the influence on the susceptibility

to different types of infections have been subject to extensive studies, as reviewed in Heitzeneder *et al.* 2012 and Takahashi *et al.*, 2011. Studies have shown that MBL may participate in the protection of the human host against viral infections, such as infections with influenza A virus (Chang *et al.*, 2010) and severe acute respiratory syndrome (SARS) coronavirus (Ip *et al.*, 2005). In chickens, attempts have been made to associate MBL serum concentrations with the severity of a variety of diseases, such as infections caused by IBV (Juul-Madsen *et al.*, 2007), *Escherichia coli* (Norup *et al.*, 2009), *Pasteurella multocida* (Schou *et al.*, 2010), and *Ascaridia galli* (own unpublished data). Interestingly, it was recently shown that recombinant MBL can block IBV-Beaudette infection in cell culture by direct binding of MBL to the IBV S1 protein domain (Verheije, unpublished).

Two genetic chicken lines, selectively bred for high or low MBL serum concentrations (designated L10H and L10L, respectively), were used in the present study. Selective breeding was performed for 14 generations using the combination of two strains (67.5 % UM-B19 chickens and 33.5 % White Cornish) as the starting population, as described by Laursen *et al.*, 1998 (Figure 2). The final result was two divergent lines, with mean MBL serum concentrations of 33.4 µg/ml for the L10H line and 7.6 µg/ml for the L10L line, respectively (Kjaerup *et al.*, 2014). The mean MBL serum concentration for 14 different chicken lines representing both broilers and layers is around 6 µg/ml, but varies from 0.4 to 37.8 µg/ml in normal healthy chickens with protein produced in the liver as the main source of circulating MBL (Laursen *et al.*, 1998).

Understanding the molecular mechanisms involved in the interaction between innate and adaptive immune responses to infectious bronchitis virus (IBV) is crucial for further improvement of disease control strategies. The aim of this study was to characterize the spleen transcriptome of healthy birds from the two chicken lines selected for serum MBL, and to investigate differences in molecular mechanisms behind the development of systemic adaptive immunity between the L10L and L10H lines infected with IBV.

Materials and methods

Animal experiment

The offspring from L10 parents F14 were reared together in a bio-secure IBV-free environment until they were three weeks of age, n=64 (32 L10L and 32 L10H). The chickens were transferred to biosafety level 2 facilities and randomly allocated to one of four groups and placed into four isolators. Two isolators contained noninfected chickens and two isolators contained infected chickens. The virulent IBV-M41 (a kind

gift from Dr. med. vet. Hans-C. Philipp at the Lohmann Animal Health GmbH, Cuxhaven, Germany) was obtained as freeze-dried vials containing $10^{8.2}$ EID50 /1.5 mL/vial. The virus had been passaged twice in specific pathogen-free embryonated eggs. All IBV inocula were prepared in phosphate-buffered saline (PBS) just before use and contained at least $10^{5.2}$ EID50/100 μ l of IBV-M41 virus. The control chickens were treated with 200 μ l PBS per bird as a mock challenge. Infected chickens were treated with 200 μ l PBS per bird containing IBV-M41. The solutions were given half intranasally and half per os.

Measurement of immunological parameters

As described in Kjaerup *et al.*, 2014.

RNA extraction and sequencing

Tissue samples were homogenized on a TissueLyzer LT (Qiagen, Hilden, Germany). Total RNA was extracted with the Qiagen RNeasy Kit (Catalog ID 74104, Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The quality of the 64 total RNA samples was verified using a 2200 TapeStation RNA Screen Tape device (Agilent, Santa Clara, CA, USA) and the concentration ascertained using an ND-1000 spectrophotometer (Nano-Drop, Wilmington, DE). Libraries were prepared with the Illumina TruseqRNA sample prep kit (Catalog ID FC-122-1001, Illumina, San Diego, USA) following the manufacturer's protocol and evaluated with the Agilent Tape Station 2200. Libraries were quantified by Picogreen and then normalized to 10 nM as recommended by Illumina for cluster generation on the Hiseq2000. Equimolar amounts of each library were mixed before NaOH denaturation. The Illumina Truseq PE cluster kit v3 (Catalog ID PE- 401-3001) was used to generate clusters on the grafted Illumina Flowcell and the hybridized libraries were sequenced on six lines of a Flowcell on the Hiseq2000 with 100 cycles of a paired-end sequencing module using the Truseq SBS kit v3 (Catalog ID FC-401-3001).

Analysis of differentially expressed genes

As described in Hamzic *et al.*, 2016.

Results

MBL concentration and IBV disease parameters

Throughout the experiment, the MBL concentrations in serum stayed constant for the uninfected control chickens showing mean concentrations of ≈ 4 μ g/ml for L10L and ≈ 28 μ g/ml for L10H. In infected chickens MBL concentration increased 1-2 fold

by day 3 post infection (pi.), which was followed by a pronounced decrease at day 7 pi. Serum concentrations in infected chickens returned to the baseline levels three weeks pi.

As detected by RT-PCR the viral load in tracheal swaps was significantly lower in L10H chickens than in L10L chickens at day 3 pi. Furthermore, L10H chickens exhibited less severe damage of tracheal cilia upon infection compared to L10L chickens.

Immune parameters in peripheral blood

The presence of IBV-specific immunoglobulins was detected at week 2 pi.; at this time-point, no difference in titer was observed between L10L and L10H. However, L10H chickens showed higher titers than L10L at week 3 pi, although this observation was not statistically significant.

The leucogram was assessed in peripheral blood and it was evident that the absolute numbers of different leucocyte subsets differed significantly between uninfected chickens from the two L10 sublines. Thus, in the control group, B cell counts were significantly higher in L10L chickens than in L10H chickens and the opposite was true for monocyte counts. Furthermore, significantly higher numbers of CD4+, CD8+ and gamma-delta T cells were found in L10H chickens compared to in L10L chickens. IBV infection induced changes in the leucogram and different changes were observed in L10L vs L10H: IBV infection reduced the number of circulating B cells in L10L chickens but not in L10H chickens. IBV infection reduced the number of circulating CD8+ gamma-delta T cells in L10H but not in L10L. These differences may reflect different kinetics of immune responses in L10L and L10H and we hypothesized that differences would be observed if investigating systemic immune responses by identifying differentially expressed genes in the spleen.

Expression of immunological genes in spleen

Differential gene expression analysis based on RNA-sequencing data as well as functional analysis was performed using Gene Ontology (GO) Immune System Process terms specific for Gallus gallus. Comparison of the two chicken lines identified several differentially expressed immune genes (DE) (Figure 3). The two most enriched GO terms were "Lymphocyte activation involved in immune response" and "Somatic recombination of immunoglobulin genes involved in immune response" for uninfected chickens. For infected chickens, the most enriched GO terms were "Alpha-beta T cell activation" and "Positive regulation of leukocyte activation". In conclusion, significant differences between sublines were found in the expression profiles of innate as well as adaptive immunity-related genes in

control chickens. In IBV infected chickens significant differences between sublines were found in expression profiles of only adaptive immunity-related genes.

Discussion

The results show that the two lines differ greatly in the expression of adaptive immunity-related genes following infection, which may imply the presence of different modes of gene regulation. The sampling times were chosen to access responses both in the effector phase (week 1) and the memory phase (week 3) of the adaptive immune response to IBV. In accordance, at 1 week post infection, subsets of genes actively involved in T cell proliferation show differences between the lines. Also, at week 3, immune-related gene expression profiles in response to IBV infection that differ between the lines, are more related to maintenance of T cell memory. MBL is known to be involved in the regulation of dendritic cell maturation as well as cytokine production (Wang *et al.*, 2011). Dendritic cells, which are the main antigen presenting cells and are actively involved in the regulation of adaptive immune responses, possess the receptors for MBL in mammals (Downing *et al.*, 2003). Therefore, the two lines selected for different MBL serum concentration may display differences in adaptive immune responses and development of adaptive immunity as a result of differences in response to cytokine signaling from dendritic cells. However, further investigation is needed to prove this hypothesis.

Other studies of the two lines, L10L and L10H, have shown that they differ in disease response parameters after being challenged with different pathogens. In the present experiment, significantly lower viral loads ($p < 0.03$) were observed in birds from line L10H in comparison to infected birds from line L10L (Kjaerup *et al.*, 2014). Furthermore, L10H birds in the present study exhibited less severe damage to tracheal cilia following IBV infection in comparison to the L10L line (unpublished data). In the current experiment phenotypic differences in additional traits related to adaptive immunity were observed, including numbers of circulating B cells and cytotoxic T cells (Kjaerup *et al.*, 2014). Based on these observations it seems that selection for high MBL serum concentration allows birds to cope better after being infected. We hypothesize that the transcriptome differences observed here indicate that selection for MBL plasma levels may influence a wide range of immune genes and potentially affect host response to IBV infection and vaccination.

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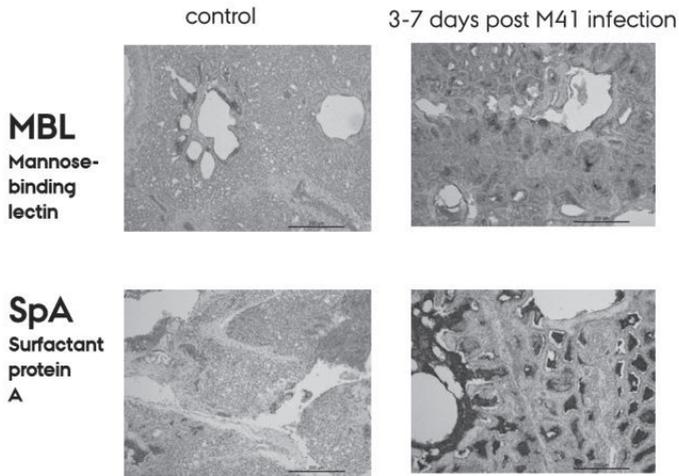


Figure 1. Immunohistochemistry of chicken lung sections. Representative pictures of tissue stained with antibodies against chicken MBL and SpA, respectively.

Uninfected Week 1 L10H versus L10L	<ul style="list-style-type: none"> • Innate immunity: ↓TYRO3, ↓TRAF3, ↓TLR7 <u>in the L10H line</u> • Adaptive immunity: IL7 ↑, FKBP1B ↑, FAS ↑, PTPN22 ↑ and TGFB3 ↑ <u>in the L10H line</u>
Uninfected Week 3 L10H versus L10L	<ul style="list-style-type: none"> • Adaptive immunity: IL7 ↑, FKBP1B ↑ and FAS ↑ <u>in the L10H line</u>
Infected Week 1 L10H versus L10L	<ul style="list-style-type: none"> • Adaptive immunity: CXCR4 ↑, PTPN22 ↑ and FAS ↑ <u>in the L10H line</u>
Infected Week 3 L10H versus L10L	<ul style="list-style-type: none"> • Adaptive immunity: CXCR4 ↑, PTPN22 ↑, FAS ↑ VCAM1 ↑ and JMJD6 ↑ <u>in the L10H line</u>

Figure 2. Differentially expressed immune genes between L10L and L10H. At the top, a comparison between uninfected controls Below, IBV infected chickens.

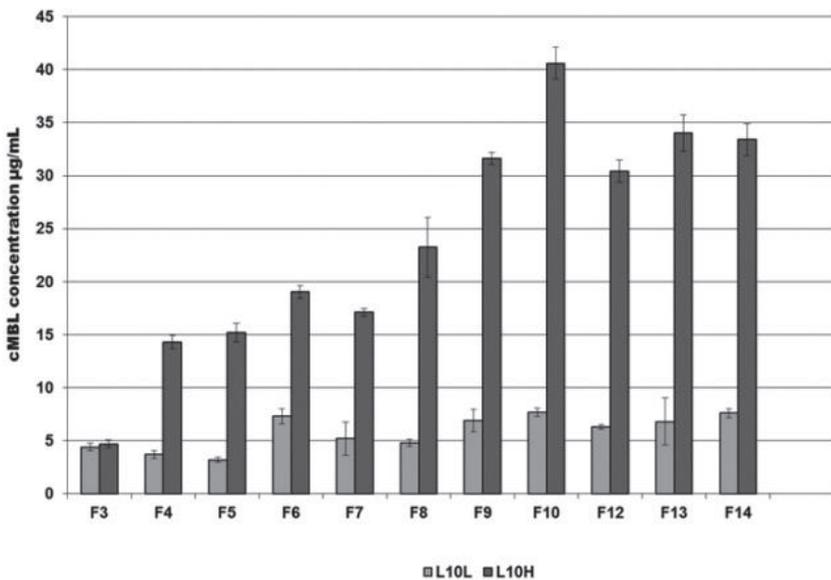


Figure 3. The inbred chicken line selectively bred for MBL levels for 14 generations. Mean values \pm s.e.m of serum MBL conc. are shown (N%20, per subline).

Persistence of Massachusetts- and 793B-type infectious bronchitis vaccine strains in commercial broilers following day-old vaccination

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Abstract

Detection and characterization of Infectious Bronchitis Virus (IBV) from field samples by molecular methods is more and more commonly used. These studies aim to evaluate vaccine-take as an indicator of vaccination efficacy or for use in epidemiological investigations. Proper evaluation of the molecular test results obtained from field samples requires information on the vaccination program and the expected persistence of the vaccine strains used. The aim of our study was to compare the persistence of the two most widely used types of live IB vaccine, representing distinct genetic groups, in commercial broilers. For our study we selected one vaccine of Mass-type (containing IBV B-48 strain) and two vaccines of 793B-type (containing either IBV 1/96 or IBV 4-91 strain). Chicks were assigned to one of four groups at day-old. Three groups of chicks were vaccinated by the oculo-nasal route with one of the IBV vaccine strains described above, the fourth group served as non-treated controls. Oro-nasal swabs and organ samples (trachea, lung, kidney and cecal tonsil) were collected weekly from 10 chickens in each vaccinated group and from five birds in the control group between three to seven weeks of age. Samples were analysed using a one-step real-time RT-PCR method based on the primers and probe described by Callison *et al.* (2006). Mass-type vaccine (B48 strain) was hardly detectable during the tested period, while the two 793B-type vaccine viruses (1/96 and 4-91 strains) persisted during the whole lifetime of commercial broilers. Mass-type vaccine could be detected most frequently in the upper respiratory tract, although in low amounts, while the great majority of lung, cecal tonsil and kidney samples were negative. After 793B-type vaccination the most uniform and highest vaccine virus load was found in the cecal tonsil samples, followed by the oro-nasal swabs. Trachea samples contained significantly lower amounts of IBV while in the kidney and lung samples, the virus was detected for a limited time and at low levels in only a small proportion of the birds. The prolonged persistence of the 793B type vaccines suggests that a long duration of immunity can be expected after a single vaccination at day-old with these vaccines (Kollár *et al.* (2015)). These findings

call for careful analysis of molecular test results obtained from field samples submitted for diagnostic purpose or for epidemiological surveys to allow differentiation of vaccine strains from closely related field strains.

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Clinical signs and pathology associated with Guinea fowl coronavirus field cases in France (2011-2015)

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Abstract

For decades, French guinea fowl have been affected by fulminating enteritis of unclear origin. In a previous study we identified a novel avian gammacoronavirus (gfCoV) associated with this disease that is distantly related to turkey coronaviruses. The present study aimed at understanding the clinics and pathology associated with the disease in field conditions.

Passive surveillance was carried out between February 2014 and November 2015 in guinea fowl farms with severe mortality (>10%). Cases were reported from all poultry producing regions in France: in Brittany and Vendée (Western France), South-western and South-eastern France. Fifteen gfCoV cases confirmed by rt-RT-PCR (Maurel et al, Av Path 2011) were further investigated.

As previously described, birds showed severe prostration, a dramatic decrease in water and feed consumption, and a daily death rate of up to 20% (Liais et al, EID 2014). Upon necropsy, signs of enteritis and dehydration were observed. In particular, whitish enlarged pancreases were consistently reported. Histopathological analyses revealed pancreatic necrosis and lesions of various intensities on the enteritis epithelium.

Transmission kinetics of European turkey coronavirus during experimental infection of SPF turkeys

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Abstract

For several decades, numerous viruses (reo-, adeno-, picorna-, corona-, astroviruses etc), mostly occurring in mixed infections, have been found to be associated with poult enteritis complex (PEC) worldwide. Although known in the USA since the 1970s to be the causative agent of bluecomb, until 2001 coronavirus had not been detected in turkeys in Europe. In 2008 a coronavirus (Fr-TCoV 080385d) was isolated from turkey poults exhibiting clinical signs compatible with PEC. In studies published in 2011 and most recently in 2016, analysis of the full length sequence of this virus has demonstrated a complex recombination history between Fr-TCoV 080385d, US TCoV, European coronaviruses isolated from guinea fowl, and IBVs.

In this paper infections of SPF turkeys with Fr-TCoV 080385 were performed under experimental conditions so as to i) determine the minimum infectious dose (MID) for the virus and ii) determine its basic reproduction ratio (R_0). Results showed that Fr-TCoV 080385 was still infectious beyond the limit of detection by a well characterized AvCoV specific qRT-PCR targeted to the N gene (Maurel *et al.*, 2011). Such a low MID has also been reported for the porcine coronavirus, porcine epidemic diarrhoea virus (PEDV) and this MID was also beyond the limit of detection by qRT-PCR (Goyal *et al.*, 2014). Results obtained relating to the R_0 showed that horizontal transmission was extremely rapid (within approximately 10h) and that detection of virus genome in intestinal secretions extended beyond five weeks post inoculation which was similar to previous reports for turkey coronaviruses in North America (Breslin *et al.*, 2000). However, when the same intestinal secretions were used for inoculation of turkey embryonated eggs, infectious virus was only detected up to two weeks post inoculation. The R_0 will be calculated in respect to the actual infectious period when results obtained from egg inoculation have been confirmed in SPF turkeys.

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Evaluation of pathogenesis and cross-immunity with Massachusetts vaccine strain of a variant isolate of avian infectious bronchitis virus from Brazil

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Abstract

In this study a Brazilian field isolate of infectious bronchitis virus (IBV), previously classified as BR-I variant genotype, was characterized comparatively with the M41 strain of IBV, by evaluating the pathogenesis in different organs (trachea, lung, kidney, gonads and caecal tonsils) and the cross-immunity with Massachusetts (Mass) vaccine strain, including the evaluation of systemic and local humoral immune responses. Experimental groups of specific pathogen free (SPF) chickens were vaccinated or not with attenuated Mass strain of IBV and challenged with this variant isolate, or with M41 virulent strain. The viral replication and histopathology in different tissues and organs, the ability to inhibit ciliar movement of tracheal epithelial cells, and local and systemic humoral immune responses were evaluated in these chickens. The pathogenesis and tissue distribution of these IBV strains were examined by histopathology, immunohistochemistry and RT-qPCR and showed marked differences so that, while the M41 strain damaged more the respiratory tract, especially the trachea, the variant isolate had a wide tissue distribution, showing slightly less replication and lesions in the trachea, but affecting more severely the kidneys and the testicles and replicating without causing tissue lesions in caecal tonsils. The Mass vaccine elicited relevant anti-IBV antibody levels in serum and lachrymal secretion, as well this vaccine induced a partial protection against this variant isolate to the infection and the development of pathological alterations of trachea and kidney and no cross-protection to the infection of testicles. In conclusion, a new pathotype and a partially different protectotype of a Brazilian variant genotype of IBV isolate were characterized in this study with regard to Massachusetts strains of IBV, indicating the importance of future investigations with other Brazilian variant IBV isolates in order to define more effective programs for the control of IBV infection in this country.

Molecular Virology

Keynote lecture

Stability and diversity: the Yin and Yang of gammacoronaviruses genome

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Abstract

Maintaining the balance between genome stability and diversity is strategic for an efficient replication and transmission of viruses. In particular, the fittest virus should possess mechanisms to limit the accumulation of deleterious mutations while concomitantly maintaining the genetic diversity required for adaptation. Differently from other RNA viruses, which possess a limited proofreading-repair ability, it has recently been demonstrated that CoVs possess an enzyme apparatus able to faithfully replicate their large RNA genome without impairing its integrity and stability. On the other side, coronaviruses have the capability to generate genetic diversity through a high mutation rate and recombination. The existing knowledge of molecular evolution and epidemiology shows that, in the end, genetic variability is the main force driving the evolutionary history of gammacoronaviruses and, in particular, of infectious bronchitis virus (IBV). A wide range of genetically distinct IBV types exists and novel variants continue to emerge, which makes it difficult to properly and promptly identify the virus in poultry. Furthermore, the broad diversity of IBV genomes has resulted in heterogeneous genetic group designations and in inconsistent epidemiological investigations. Despite the advances in sequencing techniques and the availability of RGs system, a wide collection of full-length sequences of IBV and a comprehensive understanding of the mutations affecting IBV virulence are still lacking. This has been hampered by the intrinsic difficulties of the subject and the diversity existing between distinct variants. In addition, when exploring IBV intrahost genetic diversity, the majority of deep sequencing studies have once again demonstrated that, even at the subpopulation level, several polymorphisms exist. Understanding the influence of vaccination, poultry density and ecological factors on the rates of nucleotide substitution and strength of natural selection of IBV is urgently needed to improve our ability to intervene on the mechanisms driving virus diversity and establish appropriate control measures.

Phylogenetic network analysis of the infectious bronchitis virus S1 gene, a molecular epidemiological approach

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Abstract

In the U.S.A, a combination of vaccines against different infectious bronchitis virus (IBV) types are often given to commercial poultry to provide protection against currently circulating strains. However, the reemergence of one or more specific IBV types from one year to the next are often unpredictable and can affect decisions on which combination of vaccines are used. If the vaccines selected are not appropriate, outbreaks can occur especially during the winter months. Typically, diagnosis and identification involves reverse transcriptase-polymerase chain reaction amplification and sequencing of the hypervariable region of the spike gene subunit 1 (S1). The sequence of the identified virus is then used to report a percent similarity to previously sequenced isolates, but that data does not provide information on the genetic footprints and spread of the virus. In this study, we use a molecular epidemiological approach to follow the genetic changes and spread of specific strains of IBV. We conducted a phylogenetic network analysis on the full-length S1 sequence to determine the genetic trajectory and spread of specific IBV types currently circulating in the U.S.A. Constructing median-joining networks for individual strains of IBV will aid in our understanding of what appears to be unpredictable reemergence and movement of specific IBV types in commercial poultry.

Using Next Generation Sequencing to investigate the mechanism of IBV attenuation

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Abstract

A rapid replication rate, large population and high error rate are characteristic of RNA viruses such as infectious bronchitis virus (IBV) and allow the viral population to respond to changes in its environment and exploit changes in selection pressure. A viral population exists as a cloud of variants that are genetically linked through mutation and interact on a functional scale as has been shown in polioviruses. Here poliovirus was experimentally manipulated to include a higher-fidelity polymerase and was associated with a reduction in viral diversity and pathogenicity compared to wild-type. This pathogenicity could be restored by application of a mutagen. These findings suggest that a lower mutation rate reduces the adaptability of the quasispecies and that complementation between different variant members enable the virus to colonize different host tissues and systems. It is therefore the combined characteristics of each of the individual variants that collectively contribute to the characteristics of a virus rather the dominant, consensus level sequence being solely responsible.

The proofreading of capability of nsp14 in coronaviruses (Denison, Graham et al. 2011) therefore means diversity within a coronavirus population must have a purpose and as such must provide an adaptive benefit. For coronaviruses, a balance must be struck between fidelity/stability, diversity and pathogenicity. Attenuated viruses for use as vaccine candidates are produced by serial passaging of virulent IBV in embryonated eggs where the viral genetics change to favour growth *in ovo* rather than *in vivo*. The molecular basis for attenuation however remains unclear. Given the evidence, we therefore suggest that attenuation is the result of altering overall diversity within viral population and as such patterns of variation may exist within attenuated populations. There we are studying this using the high-throughput methods of next-generation sequencing to reveal how the viral population changes over the course of this passaging.

Using the industry's standardised procedure for vaccine development as a model, M41-CK a pathogenic strain of IBV adapted for growth in chick kidney cells was passaged in egg 106 times in four independent replicates. By this method, each of the four replicates have the same point of origin so the emergence of single-nucleotide polymorphisms can be traced. The resultant viruses were tested in an animal study and all showed a degree of attenuation. The same five isolates (four resulting isolates and the starting inoculum) were then deep sequenced using Roche 454 pyrosequencing.

Consensus sequences for the starting inoculum (M41-CK EP4) and each of the four independent replicates (M41-SKA, M41-SKA1, M41-SKC and M41-SKD) were generated and variants in SKA, SKA1, SKC and SKD called against the EP4 consensus. Frequency cut offs at all called variants, variants occurring at a frequency >5% and at a frequency of >50% were used. The location of each polymorphism was assigned to its corresponding gene (by position in genome) and substitution per nucleotide was used as a measure to account for the length of each gene. The mutations are widespread across the genome but the structural and accessory proteins of final third of the genome shows the highest number of substitutions per nucleotide (used as a measure relative to the size of the region) consistently across the four isolates.

The effect of each polymorphism at a frequency greater than 5% was assessed as being either protein-changing (non-synonymous) or non-protein changing (synonymous). The majority of mutations (65%) were non-synonymous while 35% were synonymous. Genes showing the highest number of mutations were selected for further analysis and haplotype reconstruction.

High-throughput sequencing generates an enormous number of overlapping reads which are predominately short in length. When sequencing a viral population using such methods it is difficult to identify mutations that occur in association with each other and miss the link between different point mutations. Haplotype reconstruction therefore attempts to model the variant sequences within a population. Using the consensus sequence for each of the five isolates and their corresponding reads, haplotype reconstruction was performed. Multiple haplotypes were predicted for most of the isolates for the regions selected for reconstruction. The percentage of these varied with some variants occurring at minute frequency (<2%) while others represent a more significant proportion of the population with frequencies reaching up to 40%.

Our study shows the importance of using high-throughput techniques for the study of RNA viruses and IBV. Without next-generating sequencing, the heterogeneous

nature of the population remains invisible. The majority of mutations at a frequency >5% lead to a protein-coding changes though a considerable proportion are synonymous suggesting that the attenuation process may not solely be due to structural changes in proteins and as such the effect of synonymous mutations cannot be overlooked. The structural proteins show the highest rate of mutation relative to their size suggesting these are the genes where selection pressure is focussed during the process of attenuation. Haplotype reconstruction for selected genes in this region confirms diversity within the population.

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Full versus partial S1 gene sequencing of classical and variant avian infectious bronchitis viruses

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Abstract

Infectious bronchitis virus (IBV) genotyping is based on the S1 gene. In recent years, different studies have utilised different sequence portions of the gene, making cross-study comparisons and field isolate identification difficult. To provide a uniform approach to IBV genotyping, we aimed to develop an RT-PCR scheme to amplify and sequence the full IBV S1 gene. Seven strains were grown in SPF eggs and RNA extracted from allantoic fluid. Full S1 RT-PCR was achieved using two amplification primers and products were sequenced using a total of four sequencing primers for full S1 coverage. Detection limits for the partial assay were greater than for the full S1 assay, suggesting a higher viral concentration is necessary for full S1 amplification. Extraction from FTA cards provided further evidence for this, with the partial assay detecting IBV at later time points and different temperatures when compared to the full S1 assay.

Introduction

Following the first IBV identification in the 1930s (Schalk and Hawn, 1931), more than 25 serotypes have been recognised. The spike (S) protein consists of 1,145 amino acids with functions including attachment to the host cells, neutralization of antibodies and initiation of protective immunity (Ignjatovic and Galli, 1994; Johnson *et al.*, 2003). The translated protein is cleaved into S1 and S2 subunits (Cavanagh and Davis, 1986; Jackwood and De wit, 2013). With the advancements of molecular and genetic diagnostics, strain identification has been based on genotyping, typically using a partial sequence of the S1 gene (Ganapathy *et al.*, 2015; Worthington *et al.*, 2008). Sequence variability in the S1 gene (approximately 1.76kb) determines the genotype specificity of IBV strains, differing by up to 50% at the amino acid level. Furthermore, it is the most variable of the IBV proteins, with most mutations and recombination events mapping to the S1 region (Adzhar *et al.*, 1997; Kingham *et al.*, 2000). These events can lead to the emergence of new variant strains, or even new genotypes (Zhang *et al.*, 2015).

Recently, there has been growing interest in complete S1 sequencing with reports from Thailand, Sweden and India (amongst others) aiming to further understand the molecular characterisation of IBV (Abro *et al.*, 2012; Kamble *et al.*, 2014; Pohuang *et al.*, 2011). Previous studies have aimed to obtain the full S1 sequence using two primers to amplify and sequence around 1.7kb of the S1 gene, following purification and digestion (Kwon *et al.*, 1993) and conventional RT-PCR (Abdel-Moneim *et al.*, 2006). A separate investigation looked into the differences in sequencing the entire S1 and partial nucleocapsid (N) genes for grouping IBV genotypes (Huang *et al.*, 2004).

As a result of numerous studies using different partial S1 schemes, there is a lack of uniformity in published sequenced data. This restricts the usefulness for future work looking to compare field isolates with those previously investigated. In an attempt to address this issue, we aimed to develop an RT-PCR scheme to amplify and sequence the full S1 gene of classical and variant IBVs.

Materials and Methods

Classical (M41) and variant (D274, 793B, IS/885/00, IS/1494/06, Q1 and QX) IBV strains were grown in specific pathogen free (SPF) eggs. The virus-rich allantoic fluid was collected and titrated (titres ranged from $10^{5.5}$ to $10^{6.75}$ CD_{50} /ml). RNA was extracted from the allantoic fluid and a partial S1 RT-PCR was conducted (Worthington *et al.*, 2008), targeting a hyper-variable region of S1. Positive amplicons were sequenced to confirm strain identity. Following an extensive literature review, and in-house analysis, primer locations were identified to allow for the amplification of a number of IBV genotypes. Full S1 amplification was achieved using two amplification primers; Primer A (Pohuang *et al.*, 2011) and 22.S1. The RT-PCR products were sequenced using a further three primers to obtain a sequencing region which included S1. Sequences were then trimmed to achieve full S1 gene coverage for all seven strains. Pairwise analysis compared the relatedness between full and partial sequence data.

To further compare the full and partial S1 assays, sensitivity testing was then carried out using four genotypes. Ten-fold dilutions of virus rich allantoic fluid were tested by both full and partial RT-PCR assays. Additionally, FTA cards were inoculated with each virus and stored at three temperatures (4°C, RT and 40°C) to mimic field sampling conditions. Cards were sampled at six separate time points and RNA was extracted for both RT-PCR assays.

Results and Discussion

Following serial dilutions of allantoic fluid, it was found that the detection limit of the partial S1 gene (10^{-6} - 10^{-7}) was higher than the full S1 gene (10^{-2} - 10^{-3}). Given these low detection limits, it is apparent that a high viral concentration is required for full S1 amplification. One limitation of the current assay is the inability to amplify QX-like strains, despite being able to amplify all other genotypes during this study. As a result, the QX strain was amplified and sequenced using a previously published assay (Falchieri *et al.*, 2013). However, given that the sensitivity and obtained sequence data was similar for QX compared to the current assay, all amplification and sequence comparisons appear to remain justified.

Partial S1 sequences exhibited a higher average nucleotide homology percentage (79%; 352bp) when compared to full S1 sequences (77%; 1,756bp) (Table 1). Results suggest that this full S1 protocol has greater accuracy for strain identification, as point mutations have a lower effect on pairwise comparisons, given the greater sequence length. Phylogeny comparisons demonstrated that the full S1 scheme is able to correctly genotype a range of strains, with a high level of confidence (Figure 1). Additionally, the genotype clustering further demonstrates that the full S1 sequences were more distantly related compared to the partial data.

Four genotypes were then chosen for inoculation onto FTA cards (Mass, 793B, IS/1494/06 and Q1), which were stored at three temperatures and sampled at set time points. The partial S1 assay was able to detect IBV RNA at all time points and temperatures. However, the full S1 scheme was only able to detect cards stored at 4°C (until 14dpi) and RT (until 3 dpi). It is apparent that for clinical samples, or propagated virus being transported on FTA cards, the cards must have a high viral load and be stored at 4°C to allow for the greatest opportunity of detection by full S1 sequencing.

The greater sensitivity seen with partial S1 amplification may be in part due to the considerable difference in amplicon length (0.35kb *versus* 1.76kb). Previous work has highlighted the difficulties in amplifying and sequencing larger genomic regions (Moscoso *et al.*, 2005; Sakai *et al.*, 2015). This full S1 sequencing scheme appears to be suited to samples enriched in growth medium (e.g. eggs) rather than those obtained directly from the field (e.g. tissues, swabs and impression smears).

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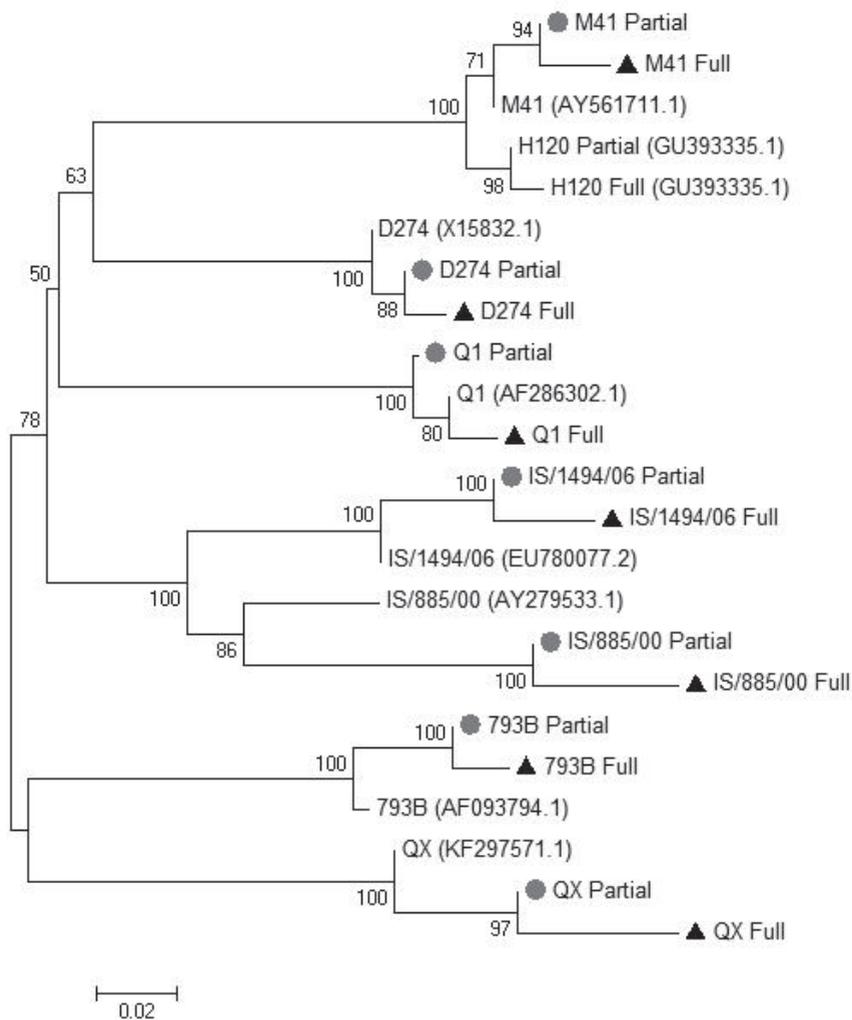


Figure 1. Figure 1. Phylogenetic tree based on both the partial (grey circle) and full (black triangle) S1 nucleotide sequences

Nucleotide identity (%) / Full S1								
	M41	D274	793B	IS/885/00	IS/1494/06	Q1	QX	
M41		77	73	77	79	73	72	M41
D274	80		77	82	84	79	77	D274
793B	80	81		79	77	75	76	793B
IS/885/00	84	79	82		82	79	73	IS/885/00
IS/1494/06	81	79	79	83		82	81	IS/1494/06
Q1	82	80	81	83	80		77	Q1
QX	77	75	78	72	74	79		QX
	M41	D274	793B	IS/885/00	IS/1494/06	Q1	QX	
Nucleotide identity (%) / Partial S1								

Figure 2. Table 1. Nucleotide identity (%) comparison of the seven IBV strains used in this study based on the full S1 and partial S1 sequences.

Genetic characterization of the Belgian nephropathogenic infectious bronchitis virus (NIBV) reference strain B1648

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Abstract

The virulent nephropathogenic infectious bronchitis virus (NIBV) strain B1648 was first isolated in 1984, in Flanders, Belgium. Despite intensive vaccination, B1648 and its variants are still circulating in Europe and North Africa. Here, the full-length genome of this Belgian NIBV reference strain was determined by next generation sequencing (NGS) to understand its evolutionary relationship with other IBV strains, and to identify possible genetic factors that may be associated with the nephropathogenicity. Thirteen open reading frames (ORFs) were predicted in the B1648 strain (5'UTR-1a-1b-S-3a-3b-E-M-4b-4c-5a-5b-N-6b-3'UTR). ORFs 4b, 4c and 6b, which have been rarely reported in literature, were present in B1648 and most of the other IBV complete genomes. According to phylogenetic analysis of the full-length genome, replicase transcriptase complex, spike protein, partial S1 gene and M protein, B1648 strain clustered with the non-Massachusetts type strains NGA/A116E7/2006, UKr 27-11, QX-like ITA/90254/2005, QX-like CK/SWE/0658946/10, TN20/00, RF-27/99, RF/06/2007 and SLO/266/05. Based on the partial S1 fragment, B1648 clustered with the strains TN20/00, RF-27/99, RF/06/2007 and SLO/266/05 and, further designated as B1648 genotype. The full-length genome of B1648 shared the highest sequence homology with UKr 27-11, Gray, JMK, and NGA/A116E7/2006 (91.2 to 91.6%) and was least related with the reference Beaudette and Massachusetts strains (89.7%). Nucleotide and amino acid sequence analyses indicated that B1648 strain may have played an important role in the evolution of IBV in Europe and North Africa. Further, the nephropathogenicity determinants might be located on the 1a, spike, M and accessory proteins (3a, 3b, 4b, 4c, 5a, 5b and 6b). Overall, strain B1648 is distinct from all the strains reported so far in Europe and other parts of the world.

A real-time TaqMan RT-PCR for specific detection of D1466 type of infectious bronchitis virus

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Abstract

The existence of the D1466-like variant, quite different from other European IBV strains, has been known of for more than 40 years. It seems to have low pathogenicity but recently there was some evidence of an increased incidence of infections connected with D1466. Moreover, these new strains may have elevated virulence. Current diagnostic nested RT-PCR assay is labour intensive and prone to cross-contaminations. A specific TaqMan real-time RT-PCR for the detection of D1466 variant strains of IBV was developed and validated. The method was 10 times more sensitive than standard nested RT-PCR with a detection limit of 85 RNA copies per reaction. The specificity of the assay was evaluated with a panel of common poultry pathogens. Repeatability and reproducibility of the method was very high with coefficients of variation lower than 4%. One hundred and two IBV positive samples were tested by this method and D1466-like strains were detected in five of them (4.9%). Partial sequencing of the S gene obtained from positive samples revealed 89.6-89.7% nucleotide identity with the reference D1466 strain.

Introduction

Diagnostic methods for the D1466-like genotype of IBV (which differs significantly from others in the nucleotide sequence of the S1 spike gene) rely on standard nested PCR which is labour intensive and presents an increased risk of cross-contamination due to the manipulation of previously amplified material. Here we describe the development of TaqMan-probe-based real-time RT-PCR which overcomes these problems. The detection of D1466-like IBV strains using this assay is about 10 times more sensitive compared to standard nested RT-PCR assay and similarly sensitive to the universal real-time RT-PCR assay used for detection of all gammacoronaviruses. Moreover, D1466 real-time RT-PCR reduces the costs, the risk of contamination and the necessary hands-on time in comparison to standard nested RT-PCR assay. We also describe the conclusions from the routine use of this method, which was implemented in our laboratory in 2014

Material and methods

Primers and probe design

The S1 gene fragment sequences of D1466-like IBV strains were retrieved from the GenBank database and aligned using the MEGA6 (<http://www.megasoftware.net>, Molecular Evolutionary Genetics Analysis Version 6.0) software package. Primers and probe were designed manually based on a conserved region within the aligned S1 gene fragment sequences.

Standard nested RT-PCR and real-time RT-PCR assays

Standard nested RT-PCR amplification was performed using the primers D1-D4 described by Cavanagh *et al.* The real-time RT-PCR assays were performed using 7500 Fast real-time PCR system (Applied Biosystems) in a 96-well optical plate format. Amplification was carried out in a 25 µl mixture volume containing 12,5 µl 2x QuantiTect Probe RT-PCR Master Mix (QuantiTect Probe RT-PCR Kit, Qiagen, Germany), 0,25 µl QuantiTect RT Mix, primers to a final concentration of 400 nM, probe to a final concentration of 20 nM, 2,5 µl RNA template and nuclease free water. Thermal cycling conditions included one cycle at 50°C for 30 min for reverse transcription, one cycle at 95°C for 15 min for Taq polymerase activation and 40 cycles of 95°C for 10 s, and 60°C for 60 s for cDNA amplification. Fluorescence was acquired during each extension step. Negative controls contained PCR-grade water. Universal real-time RT-PCR assay used for all *gammacoronaviruses* detection was run with the same chemistry and in similar conditions but using the primers and probe according to Callison *et al.*

Limit of detection and specificity assay

To determine the limit of detection of the D1466 real-time RT-PCR, tenfold dilutions of the known RNA amount of D1466 IBV vaccine strain (Nobilis IB D1466, MSD Animal Health, Netherlands) were tested. The concentration of the RNA was quantified using a Nanodrop instrument (Thermo Fisher Scientific Inc, USA) and the value obtained was used to calculate the exact number of RNA molecules (of 87 bp long) using the software convertor (<http://scienceprimer.com/copy-number-calculator-for-realtime-pcr>). The dilution series, ranging from 100 to 10⁻⁹ of viral RNA containing a known number of molecules was tested in triplicate in both D1466 diagnostic assays. To evaluate the specificity of the D1466 real-time RT-PCR, the RNA preparations from other common poultry viral pathogens (793B, Mass, QX and D274 genotypes of IBV, IBDV, NDV, AIV, reovirus, astrovirus and rotavirus) were used.

Sampling and sample preparation

Tissue samples (kidney, oviduct, respiratory and intestinal tracts) and oropharyngeal/ cloacal swabs from various chickens from broiler, commercial layer and broiler breeder farms where clinical or post-mortem observations indicated IBV infection were submitted by veterinarians or farm managers. For molecular assay optimization the live attenuated IB vaccine was used. Tissue samples were homogenized and vaccine and swabs hydrated in 10–20% (w/v) suspension in phosphate-buffered saline (PBS) with addition of antibiotics (100 u penicillin and 100 µg streptomycin/ml), centrifuged at 3000 x g for 15 min. Total RNA was extracted from 250 µl of tissues, vaccine and swabs (5 pooled swabs) supernatants into 50 µl RNase-free water using commercial kits (RNeasy Mini Kit, Qiagen, Germany) according to manufacturer's instructions. Extracted nucleic acids were stored at -70°C for further molecular analysis.

S1 gene of D1466 sequencing and analysis

D1466-positive field samples in specific real-time RT-PCR assay were tested by the standard nested RT-PCR and obtained products were sequenced in both directions by the commercial service Genomed (Poland). The received forward and reverse nucleotide sequences were edited and aligned in final consensus sequence using the SeqMan (DNASTAR, Madison, WI). Multiple alignments of the novel amino acid sequence and several isolates available in the GenBank database (www.ncbi.nlm.nih.gov) were constructed with the use of the Clustal W method using MEGA6 software. A phylogenetic tree was constructed by the neighbor-joining method (figure 1).

Results

TaqMan RT-PCR assay calibration

The standard curve and linear regression analysis were performed using tenfold serial dilutions of D1466 IBV. Cq values ranged from 16.8 to 33.5 cycles with a linear regression (R²) of 0.997. The slope of -3.266 reveals a high RT-PCR efficiency (99.7%).

Limit of detection, specificity and precision of the D1466 real-time RT-PCR assay. The limit of detection of the *gammacoronavirus* universal and D1466 real-time RT-PCR were similar at the level of approximately 85 copies of viral RNA. However, for elimination of any false positive results, the limit of detection of D1466 real-time RT-PCR assay was slightly raised and determined to be about 300 template copies (Cq value = 36). The sensitivity of the standard nested RT-PCR was tenfold lower and detected the presence of about 850 copies of viral RNA in dilution 10⁻⁵ (Table 1). No

fluorescent signals were detected with different IBV genotypes or other chicken RNA viral pathogens.

Detection and characterization of D1466 IBV in field samples

A total of 148 field samples from chicken (broilers, commercial layers and broiler breeders) were tested by *gammacoronavirus* universal real-time RT-PCR assay. One hundred and two out of 148 (68.9%) were positive for IBV. When these samples were tested by D1466 real-time RT-PCR, five out of 102 (4.9%) were positive. Interestingly, no D1466 positive sample was detected in 2014 but three samples in 2015 and two in May of 2016 were identified. All D1466 positive samples were subjected to the standard nested RT-PCR and obtained amplicons were sequenced. Of the 26 tested samples found positive by standard nested RT-PCR the presence of this genotype of IBV was only confirmed using the real-time RT-PCR in 19 of them.

Discussion

In the present work we have successfully developed a highly sensitive TaqMan real-time RT-PCR assay for detection of the D1466-like IBV genotype. So far, standard nested RT-PCR assay was commonly used for this purpose. Both methods target the same fragment of the S1 gene which is crucial for IBV genotype determination. However, the primers and probe designed for real-time RT-PCR met single mismatches in D1466 IBV sequences previously detected in Poland and they include some degenerative nucleotides (one in the forward primer and two in the probe) in order to avoid failure in the detection of this genotype. Real-time RT-PCR proved to be ten times more sensitive than standard nested RT-PCR and equally as sensitive as universal real-time RT-PCR. Moreover, the assay developed in the present study proved to be highly specific. This technique enabled the detection of D1466-like IBV genotype in five samples (4.9%) delivered to the lab in the period between December 2014 and May 2016. We also checked the samples that had previously been identified as D1466 and only in 19 of them was the presence of this genotype confirmed. The most probable explanation for this observation is cross-contamination during sample processing or assay performance which can happen in spite of the most rigorously implemented principles of good laboratory practices. An alternative explanation could be the degradation of nucleic acids up to undetectable levels due to multiple freezing and thawing of tissue samples. Real-time RT-PCR enables the estimation of the amount of viral RNA copies, which could be a useful indicator of the infection phase and correlate with viremia or clinical disease. D1466 real-time RT-PCR assay is able to detect as few as 300 RNA molecules of this IBV genotype. When testing field samples, the C_q values obtained were relatively high (mean C_q

values of 32.4 ± 3.8) which corresponds to a mean amount of virus per milligram of tissue of about 1500 copies. The low number of D1466 IBV RNA copies in most cases did not parallel the Cq values obtained in universal real-time RT-PCR which were much lower. This was due to the presence of other IBV genotypes in these samples. Subsequent assays aimed at identifying these other IBV genotypes detected variants such as 793B, Mass, QX or Var2, in almost all of them. There was only one case when the Cq values in both assays were equal, indicating a low number of RNA copies (Cq about 31). It seems that this variant replicates poorly in the tissues of chickens and its excretion in faeces is also low, raising doubt about the pathogenicity and virulence of the D1466-like strains as poorly replicating viruses are unlikely to cause signs of disease. In our study, five chicken flocks tested positive for the D1466 IBV variant. Partial S1 gene sequence analysis showed 89.6-89.7% and 91.6-92.3% nucleotide identity with reference strains D1466 and V1397, respectively. In conclusion, the TaqMan real-time RT-PCR assay described here is a time-saving, specific, sensitive and reliable method of detection of the D1466 genotype of IBV and could successfully replace standard nested RT-PCR which is extremely prone to cross-contamination. Moreover, this new assay enables the assessment of viral load which might be useful for epidemiology and pathogenesis studies.

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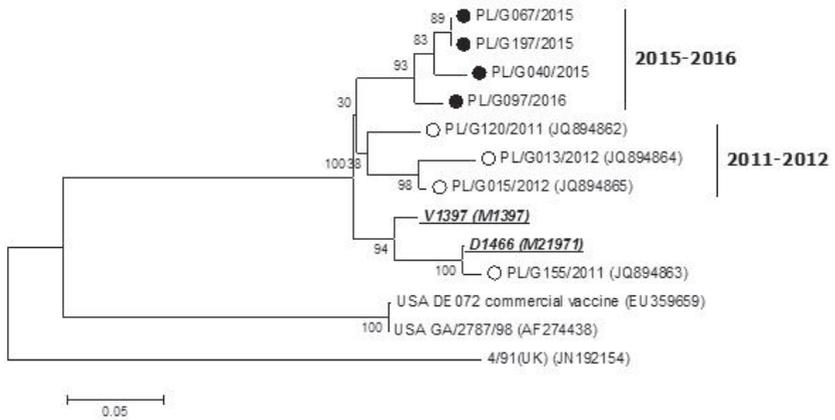


Figure 1. Phylogenetic tree of S1 gene fragment of D1466-like IBV strains detected in Poland. Black dots are strains identified in the period between 2015-2016, empty dots are strains identified between 2011-2012. Reference strains are bold underlined. The strain 4/91 from 793B-like lineage is used as the out-group

Successive occurrence of recombinant infectious bronchitis virus strains in a restricted area of the Middle East

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Abstract

Routine molecular diagnostic testing by our laboratory, performed using a primer pair with conservative binding sites on the spike glycoprotein coding sequence, has indicated the recurrence of a unique phylogenetic cluster of chicken infectious bronchitis viruses (IBV) in the Middle East since 2010. Differences in the nearly full length S1 subunit of the spike gene phylogeny of selected strains, however, split up this grouping, suggesting potential recombination in the S1 gene. In order to clarify this, various bioinformatic analyses of the strains were carried out, and confirmed this supposition. Two patterns of recombination were found among the strains, one of which could also be identified in GenBank-deposited IBV sequences from the region. These findings demonstrate that IBV strains of different recombinant patterns occur simultaneously in the same geographic region and could circulate for an extended period of time, thus contributing to the knowledge on IBV evolution.

Molecular epidemiology of Guinea fowl coronavirus in France (2011-2015)

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Abstract

For decades, French guinea fowl have been affected by fulminating enteritis of unclear origin. By using metagenomics, we identified a novel avian gammacoronavirus (gfCoV) associated with this disease that is distantly related to turkey coronaviruses. Since the first molecular characterization in 2011, we aimed at understanding the molecular evolution of gfCoV in France. Passive surveillance was therefore carried out between February 2014 and November 2015 in French guinea fowl farms. Digestive content, pancreas, and/or cloacal swabs were collected from flocks with severe mortality (>10%). Samples were kept at 4°C between the farm and the laboratory, and then stored at -80°C before further processing. Organs were homogenized, RNA extracted and gfCoV genome detected by real-time RT-PCR as previously described (Maurel et al, Av Path, 2011). A partial S gene fragment of positive samples was then amplified and sequenced (Liais et al, EID 2014). Sequences were analyzed using BioEdit, ClustalW, and Maximum Likelihood phylogenetic trees were drawn using Mega v6.06.

Fifteen gfCoV cases were confirmed by rt-RT-PCR and partial S gene sequence could be obtained from 8 flocks. The recent gfCoV partial S genes clearly clustered with GfCoV/FR/2011 (bootstrap value: 100). 2014-2015 partial sequences shared 85 to 87% identity with GfCoV/FR/2011, while the 2014-2015 sequence diversity ranged between 0.0 and 7.0%. Further genetic characterization is ongoing to better assess gfCoV evolution in France.

Genotyping and classification of recent Iranian infectious bronchitis virus isolates based on S2 protein gene

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Abstract

Infectious bronchitis virus (IBV), the causative agent of infectious bronchitis, is an important disease that produces severe economic losses in the poultry industry worldwide. IBV is classified into different serotypes and genotypes. IBV isolates have been widely genotyped by sequencing of the S1 protein gene. To characterize and classify the IBV strains isolated in Iran, we determined the genotypes of these IBVs based on the S2 protein.

The S2 gene of nine Iranian IBV isolates was amplified by RT-PCR and sequenced. The amino acid sequences were compared with the published sequences of non-Iranian strains. RESULTS: Iranian IBV isolates were classified into three genotypes (I, VII, VIII); Genotype I includes Massachusetts like viruses. Genotype VII consisted of two branches, the first branch VIIa (IS-1494 like viruses), and second branch VIIb that were related to QX- like viruses. Genotype VIII includes 793/B like viruses.

This is the first data for the S2-based genotyping and classification (new method) of Iranian IBV isolates.

Keywords: Infectious Bronchitis Virus, Spike, Genotyping, Iran.

Introduction

Infectious bronchitis virus (IBV), a member of genus *Gammacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae*, order *Nidovirales* (single-stranded positive sense RNA genome, 27.6 kb, surrounded by a lipid envelope) is the causative agent of infectious bronchitis (IB), an acute, highly contagious disease of chickens worldwide. The spike (S) glycoprotein, which is post-translationally cleaved into two subunits, S1 (92 kDa) and S2 (84 kDa) is located on the surface of the viral membrane. It is the major inducer of neutralizing antibodies and is also responsible for virus binding and entry to host cells. It is post-translationally cleaved into the amino-terminal S1 (~535 amino acids) and the carboxyl-terminal S2 (~627 amino acids)

subunits at a multi-basic cleavage site (Jackwood, 2012). The S1 subunit forms the distal, bulbous part of the spike, and the S2 subunit anchors the S1 subunit to the viral membrane. The S2 subunit contains an antigenic region that may play a role in protection (Mo et al., 2013). The S2 subunit contains a fusion peptide-like region and two heptade regions approximately 100 to 130 Å in length (771-879 amino acid in IBV) that are involved in oligomerisation of the protein and entry into susceptible host cells (Abro, 2012). Although the S1 subunit of IBV has been examined extensively, the S2 subunit remains enigmatic. Based on the highly conservative nature of the S2 subunit among different members of the Coronavirus genus and different strains of IBV, it would appear that it plays little or no role in the induction of a host immune response. Most of the molecular studies performed to determine IBV genotypes have been based on the S1 glycoprotein. However, the S2 subunit may also induce serotype-specific neutralizing antibodies and S2 subunits are conserved within a serotype but not between serotypes (Ammayappan & Vakharia, 2009; Scott Andrew Callison, Mark W Jackwood, & Deborah Ann Hilt, 1999). Results of genotyping of IBV strains isolated in Iran were classified into seven distinct phylogenetic groups (Mass, 793/B like, IS/1494 like, IS/720-like, QX-like, IR-1, and IR-2) based mainly on analysis of hypervariable regions of the S1 glycoprotein gene (Hosseini, Bozorgmehri Fard, Charkhkar, & Morshed, 2015; Najafi et al.). At present, despite the use of different vaccines (Massachusetts and 793/B type) in the Iranian poultry industry, IB outbreaks are still reported.

Materials and Methods

Samples:

Nine IBV isolates (different genotypes) in Iran were used in this study. Samples were taken from broiler chicken farms with respiratory manifestations, nephritis and mortalities during surveillance from 2014 -2015. Detailed information about the isolates, including type of flock, specimens and genotypes based on the S1 gene, are available in Table 1);

Table 1. IBV strains employed in this study

Name	Chicken species	Organ	Genotypes based on S1 gene
IBNG-1	Broiler	Trachea	Massachusetts
IBNG-2	Broiler	Trachea	793/B
IBNG-3	Broiler	Kidney	793/B
IBNG-4	Broiler	Trachea	QX
IBNG-5	Broiler	Kidney	QX
IBNG-6	Broiler	Trachea	QX
IBNG-7	Broiler	Kidney	IS-1494 like (Var-2)
IBNG-8	Broiler	Trachea	IS-1494 like (Var-2)
IBNG-9	Broiler	Kidney	IS-1494 like (Var-2)

RNA extraction and cDNA synthesis

Total RNA was extracted from the viruses using CinnaPure RNA (Sinaclon, Iran). The extracted RNA was stored at -70°C until use. The extracted RNA was used in reverse transcription (RT) reactions to generate cDNA using Revert Aid Reverse Transcriptase (Thermo Scientific, Canada), Ribolock Rnase inhibitor (Thermo Scientific, Canada), dNTP mix (Sinaclon, Iran), and DEPC-treated water (Sinaclon, Iran).

PCR for amplification of partial segment of S2 gene

PCR was performed to amplify the S2 gene using the primer pairs comprised of S2F2 and S2R2. The primer sequences used are: Forward S2F2 5' GCTGCGTCRITTAATAAGGCCAT 3' and, reverse 5' CTAGGCTGCCACAACAATAAC 3'. The reaction mix was prepared in a volume of 20µL with 3µL of cDNA, 13µL of master mix (Sinaclon, Iran), 1µL of each primer.

Phylogenetic analysis:

The AccuPrep® PCR Purification Kit (Bioneer Co., Korea) was used for purification of the PCR products. Sequencing was performed with the primers (both directions) used in the PCR (Bioneer Co., Korea). Chromatograms were evaluated with ChromasPro (ChromasPro Version 1.5). The multiple alignment of S2 nucleotide was achieved using Clustal W using MEGA 5.1 software. The phylogenetic tree was constructed using the same software with the neighbor-joining method and each tree was produced using a consensus of 1000 bootstrap replicates (Tamura et al., 2011). The amino acid sequences of the S2 gene were compared with several S2 sequences from GenBank: H120(KF188436), 4/91(JN19215.4), M41(X04722), Beaudette (DQ001335), Iowa97 (GU 393337), Holte (Gu39336), Gray (AF394180), Call99 (AY514485), QX (KJ469737), Delaware (GU393332).

Results

Amino acid sequences of the S2 gene from the nine IBV isolates obtained in the present study were aligned and compared with the reference strains from GenBank and available from the National Center for Biotechnology Information (NCBI). Phylogenetic analysis based on the S2 gene showed that Iranian IBV isolates were grouped into three distinct clusters I, VII & VIII. (Figure 1) Group I (IBNG-1) were closely related to Massachusetts viruses. Group VII was split into two sub-clusters, the first one VIIa containing IBNG-4, IBNG-5 and IBNG-6 (Variant 2 like the virus) and second sub-cluster VIIb including IBNG-7, IBNG- 8 and IBNG-5 that were related to QX IBV isolate. Group VIII (IBNG-2 & IBNG-3) were in the same cluster as 793/B viruses. The amino acid identity within group VIII was 90.21% and within group VII was 93.81%-100%. The amino acid identity between group I and group VIII was 88.19%, and between group VII and group VIII was 85%. The amino acid identities between group I and H120, M41, and Beaudette were 99.35%, 98.58% and 98.36%, respectively. Sequence identity between group VIII and 4/91(JN192154) was 99.39%-99.89% and the amino acid identity between group VII and QX (KJ1469737) was about 99%.

Discussion

Infectious bronchitis virus (IBV) is a major cause of economic losses in the poultry industry. In the S2 subunit of the viral spike protein, regions of sequence variation were found interspaced with highly conserved regions, contributing to the overall diversity of the S gene. Genotyping correlates with serotype, and is often used because it is fast and convenient compared with virus neutralization (VN) testing or other traditional serotyping tests (Lee, Hilt, & Jackwood, 2003). In the past, the genetic diversity of IBV was principally monitored by analysis of the S1 gene because this region is associated with many biological functions such as attachment to cellular receptors and tissue tropism. In addition, this region is a major antigenic site which induces neutralizing antibodies. In contrast, although a major antigenic site has not been identified in the S2 gene, this region is also thought to be associated with antigenicity due to its influence on conformation. Therefore, analysis of the S2 gene is also important for further understanding of the antigenicity of IBV isolates, and antigenic diversity among IBV strains may arise from recombination events between the virus strains classified into different genetic groups (Scott Andrew Callison *et al.*, 1999). The first isolation of IBV in Iranian chicken flocks, identified as Massachusetts serotype, was reported by Aghakhan *et al.* (1994) (Aghakhan, Abshar, Fereidouni, Marunesi, & Khodashenas, 1994). In Iran, Hosseini *et al.* isolated the Iran/QX/H179/11 strain in 2011 and the Iran/QX/H255/12, Iran/QX/H281/12, and Iran/QX/

H284/12 strains in 2012. QX-type isolates in this study had more than 96% homology to these Iranian strains (Bozorgmehri-Fard, Charkhkar, & Hosseini, 2014). The presence of variant 2 viruses (IS/1494/06 like) in Iranian commercial flocks has been demonstrated (Hosseini *et al.*, 2015). Recently, six distinct phylogenetic groups (IS/1494/06 [Var2] like, 4/91-like, IS /720-like, QX-like, IR-1 and Mass-like) based on phylogenetic analysis of the S1 glycoprotein gene were described (Najafi, Madadgar, Jamshidi, Langeroudi, & Lemraski, 2014). Phylogenetic analysis based on the S2 gene revealed the IBV strains of our study could be classified into three genetic groups: genotypes I (Massachusetts like), VII (QX & IS 1494 like) & VIII (793/B like). The present study was partially consistent with Mase *et al.* (2008) who reported a method of grouping IBV based on the S2 glycoprotein gene, IBV genotypes were divided into eight clusters; their strains, belonging to the Massachusetts, Gray and Connecticut types, were classified into group I, II and III, respectively. The strains belonging to the foreign Iowa-609 type were classified into group IV. Group V consisted of only Japanese strains, including the C-78 vaccine strain, and group VI also consisted of mainly Japanese strains, including the comparatively newly established vaccine strains, the Miyazaki, TM86 and GN strains. All viruses belonging to groups VII or VIII were strains isolated after 2000, which were not employed in the previous study (Mase, Inoue, Yamaguchi, & Imada, 2009). Zulperi *et al.* performed phylogenetic analysis and sequencing of the S1 and S2 genes of Malaysian IBV strains. Sequence analysis of the S1 genes indicated that MH5365/95 and V9/04 belong to non-Massachusetts strain. However, the strains share only 77% identity. Phylogenetic tree analysis based on the S2 gene showed that both MH5365/95 and V9/04 isolates were grouped together in a separate sub-cluster (Zulperi, Omar, & Arshad, 2009). Callison *et al.* reported that the S2 gene sequence was more conserved than that of the S1 gene but still showed sequence variability among different strains of IBV. Indeed, serotypes were grouped based on S2 gene sequence. The nucleotide sequences of the S2 genes available to date differ in general by less than 10% (S. A. Callison, M. W. Jackwood, & D. A. Hilt, 1999). In this study, we reveal the diversity of the N-terminal region of the S2 gene of IBV in Iran, and confirm that this region was useful for the grouping of IBV strains. There are different reports about the genotyping of Iranian IBV based on the S1 gene. Our findings demonstrate for the first time the use of IBV genotyping based on the S2 gene in Iran. More studies should include full-length sequencing and genotyping of the S2 gene of IBV isolates.

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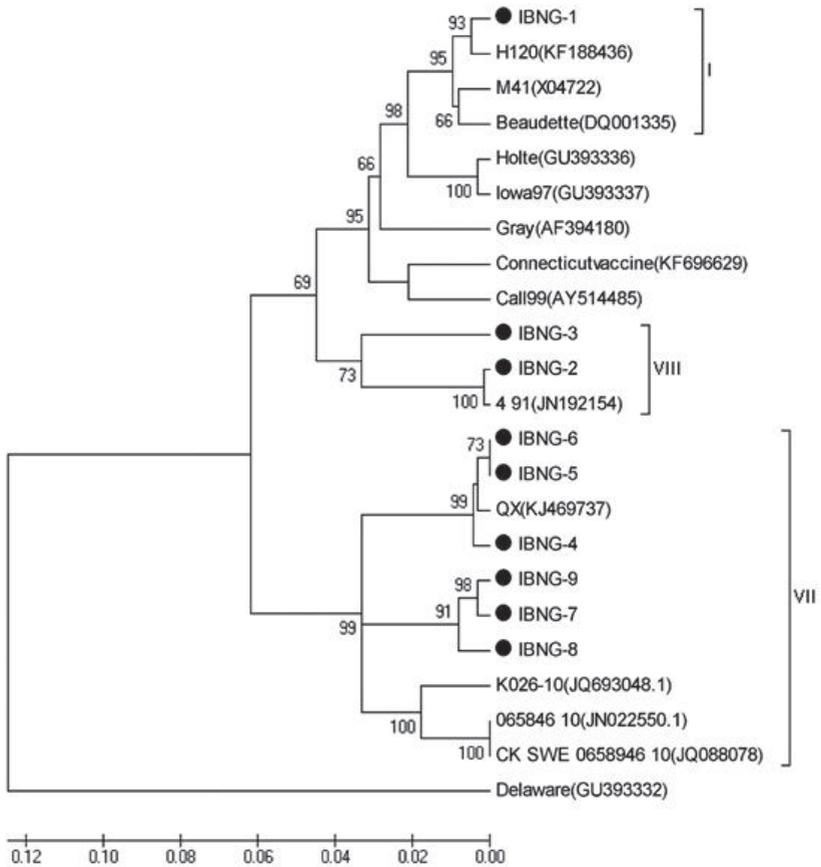


Figure 1. Phylogenetic tree based on the amino acid sequence of the S2 gene of IBVs, where the nine Iranian strains are marked with black circles and the others represent reference samples.

Phylogenetic analysis of avian infectious bronchitis virus isolated in Morocco: a retrospective study, 1983 to 2014

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Abstract

Infectious bronchitis viruses (IBV) are causing great economic losses to the poultry industry worldwide. The emergence of new variant strains complicates disease control. Sixty two field strains of IBV isolated from chicken flocks in Morocco from 1983 to 2014 were characterized by sequence analysis of the N-terminal subunit (S1) of the spike gene. The genetic diversity was addressed using MEGA software. A phylogenetic tree was constructed and Maximum composite Likelihood (ML) distances between and within genetic groups were calculated. The Moroccan isolates were classified into genotypes and subgenotypes based on their S1 protein gene sequence and they were compared with regional and international reference strains available in EMBL/GenBank databases.

Three genotypes have been present in Morocco over the last 31 years: Massachusetts, 793B and Italy 02 genotypes. High genetic diversity was observed among three genotypes with ML distances ranging from 9.4% to 35.6% and 14.5% to 49.8% for nucleotide and amino acid analyses, respectively. The evolutionary distances within the three genotypes Massachusetts, 793B and Italy 02 were respectively 4.6%, 2.9%, and 0.06%. The evolutionary distances comparisons for each genotypes confirmed the sub-genotypes (clusters) identified on the ML tree.

The present study suggests that the distribution of IBV subgenotypes should be carefully studied in order to develop and implement appropriate vaccination programs for given areas.

Avian Metapneumovirus

Keynote lecture

Avian metapneumovirus in poultry: update and challenges

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Abstract

In recent years molecular techniques have moved forward in all fields of research on avian metapneumovirus (aMPV). From its first appearance in the late 70s aMPV has spread almost worldwide, causing significant economic losses and welfare issues in poultry due to respiratory disease and reproductive disorders. Four subtypes of aMPV have been identified so far (A, B, C & D) based on antigenic and genetic differences. Reports of aMPV detections in countries previously considered to be free of this disease prove that the geographical distribution of the virus is expanding. Comparison of full-length sequences of known aMPV subtypes with hMPV confirmed that subtype C viruses are more closely related to hMPV than to other aMPV subtypes. A new sub classification of metapneumoviruses, grouping aMPV A, B and D as type I, and aMPV C and hMPV as type II has been suggested. Moreover, studies on the fusion (F) protein functions support the existence of differences between aMPV subtypes in terms of pathogenicity and host-tropism spectrum. The species natural hosts of the virus are turkeys, chickens, pheasants, guinea fowl and Muscovy ducks. In the latter species only subtype C infection has been reported.

Studies recently performed gave new insights in to the pathogenesis and immunopathogenesis of infection, and support the idea of turkeys showing a higher susceptibility to the virus, and the role of aMPV as a primary pathogen in chickens. More sensitive molecular diagnostic protocols have been developed which have allowed the widening of the time window of viral detection from the respiratory tract and permitted more prompt and effective diagnosis. Sequencing and comparison of aMPV field detections with vaccine and vaccine progenitor sequences have also allowed differentiation between field and vaccine strains. While in general disease has been effectively controlled by vaccination, the reversion to virulence of live vaccines of subtypes A and B, and general vaccine failure have been demonstrated

on several occasions. Field and experimental studies, coupled with genome sequencing of field and vaccine strains have allowed the investigation of the molecular basis of reversion to virulence and demonstrate the evolution of field strains in avoiding vaccine-induced immunity. Moreover, although aMPV sequences available in web databases are scarce and limited to attachment and, to a lesser extent, F protein genes, phylogenetic analysis seems to suggest that the virus is evolving worldwide. Given the limits of current vaccines and the evolving molecular epidemiology of the virus, the vaccination regimes need to be improved to facilitate better aMPV control strategies both in turkeys and chickens. In the last 10 years reverse genetics systems for A, B and C subtypes have been developed and used in basic and applied research, and in particular to develop rationally modified aMPV vaccines or vectored vaccines

Avian Metapneumovirus circulation in Italian broiler farms

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Abstract

Avian Metapneumovirus (aMPV) is the causal agent of Turkey Rhinotracheitis (TRT), and also causes a respiratory infection in chickens, which can result in Swollen Head Syndrome (SHS). The current information on the prevalence of aMPV in broilers in Italy is fragmentary and the impact on production remains unknown. Starting in 2014 a survey was carried out in order to draw an epidemiological picture of aMPV in broiler flocks in the parts of Northern Italy with a high poultry density.

Swabs collected during one-off and longitudinal studies were tested using A and B subtype specific real-time RT-PCR. Samples were also tested for Infectious Bronchitis Virus (IBV) using PCRs followed by sequencing. When possible, production data and respiratory disease history were recorded.

Results confirmed the high prevalence of aMPV in broilers older than 30 days in the absence of field IBV. All viruses detected were of the B subtype.

To evaluate the genetic variability in Italian aMPV strains, the fusion (F) and attachment (G) gene nucleotide sequences of selected isolates were determined. These were compared with sequences obtained from turkeys sampled in the same area and in the same period and with previously published aMPV sequences.

Introduction

Avian Metapneumovirus (aMPV) is the type species of the genus *Metapneumovirus* in the subfamily *Pneumovirinae* within the *Paramyxoviridae* family and is grouped in to four subtypes (A, B, C and D) based on the genetic differences (Jones & Rautenschlein, 2013). It causes an upper respiratory tract infection in turkeys leading to turkey rhinotracheitis (TRT), and in some other avian species, including chickens. In laying or breeding birds the virus can cause a drop in egg production and poor eggshell quality. These conditions are associated with serious economic losses in unprotected birds especially when secondary pathogens become involved (Jones & Rautenschlein, 2013).

aMPV has been reported worldwide and in Italy aMPV infection is caused mainly by subtype B and especially affects turkey flocks (Listorti *et al.*, 2014).

The current information on the prevalence of aMPV in broilers in Italy is fragmentary and the impact on production remains unknown. Starting in 2014 a survey was carried out in order to sketch an epidemiological picture of aMPV in broiler flocks in the parts of Northern Italy with a high poultry density.

Material and methods

Farms

Since 2014 a total of 309 samples (mainly rhino-pharyngeal swabs) were collected from 43 broiler flocks located in areas of Northern Italy with high poultry densities, for diagnostic and/or research purposes during one-off and longitudinal studies. Related information including collection data, farm location, age of the birds and observed respiratory signs were recorded.

aMPV detection and characterization

RNA was extracted using the commercial kit (High Pure RNA Isolation Kit, Roche®, Mannheim, Germany) from pools of ten dry swabs and real-time RT-PCR was performed using the method described by Cecchinato et al. (2013) to detect and differentiate A and B subtypes.

Sequencing of fusion and attachment protein genes

F (fusion) and G (attachment) protein genes of three selected strains isolated in 2014 were sequenced. RNA was extracted and RT-PCRs were performed following the method described by Cecchinato et al. (2010). Sequencing was performed at Macrogen Europe (Amsterdam, The Netherlands). Nucleotide sequences were edited and assembled using Bioedit software and then aligned using Clustal W against other Italian and foreign subtype B complete sequences (Cecchinato et al., 2010).

Phylogenetic analysis was carried out with the neighbour-joining algorithm implemented in MEGA4 software. Bootstrap values were obtained with 1,000 replicates. Branches with bootstrapping values >70 were considered significant.

IBV detection and strain genotyping

All samples were also tested for IBV using a real-time RT-PCR commercial kit (Quantification of Avian Infectious BronchitisVirus-IBV-kit; Genesig, Southampton, UK) following manufacturer's instructions. RT-PCRs covering a hypervariable region of the S1 gene were performed on all real-time RT-PCR positive samples. Amplicons were sequenced and the sequences obtained analysed in order to genotype the IBV positive samples as reported by Franzo et al. (2014).

Results and Discussion

Seventy-eight samples collected from 30 broiler farms were found to be positive for aMPV. All detected isolates belonged to aMPV subtype B. These findings confirm the reported high prevalence of this subtype in Italy and that aMPV is endemic in Italian broiler flocks, particularly in areas of Northern Italy with a high poultry density where turkeys and broilers are closely reared. This was further supported by the occurrence of aMPV infection on the same farms for three consecutive production cycles.

aMPV was detected in birds older than 32 days (Fig. 1), and was frequently associated with respiratory signs. Interestingly, during respiratory disease outbreaks (55 excluding samples from longitudinal studies), aMPV was detected more frequently (42%) than IBV (13%) and in the absence of other pathogens, possibly indicating a primary role in the observed clinical signs. aMPV was also found in association with IBV (11%). The majority of detected IBV isolates belonged to QX genotype (26 out of 29) while the other field genotype identified was Q1 (3 out of 29).

Phylogenetic analysis of the F and G genes of three selected strains identified in broilers in 2014 revealed a progressive evolution of this virus in Italy (Fig. 2-3). Furthermore, when these viruses were compared with two strains isolated in turkeys in the same period, all viruses clustered together in the same subcluster suggesting, at least in the considered regions, the absence of any determinant of host-specific adaptation. These findings could imply that in this area, where both species are closely reared, chickens could play a role in the maintenance and circulation of aMPV. In order to control aMPV infection in broilers, a vaccination trial was performed. Two broiler farms (six flocks), located in the area where aMPV was endemic, were selected because of recurrent episodes of aMPV induced respiratory signs. Birds were vaccinated by coarse spray at 11 days of age with an aMPV-B vaccine (strain VCO3) and oro-pharyngeal swabs were collected every week after vaccination for aMPV identification and characterisation. Despite the detection of field aMPV strains, respiratory signs were not observed in any flocks, indicating the possible benefits of vaccination in the control of aMPV infection in broilers, especially in areas with a high infection pressure.

Finally, no aMPV of subtype A was found, probably indicating that the previous detection of this subtype was due to the vaccine spread (Catelli *et al.*, 2006; Lupini *et al.*, 2011) and that no field viruses of this subtype have been circulating in Italy.

The current study gives a comprehensive picture of field aMPV circulation in broiler farms in Italy and provides some suggestions pertaining to the control of this virus.

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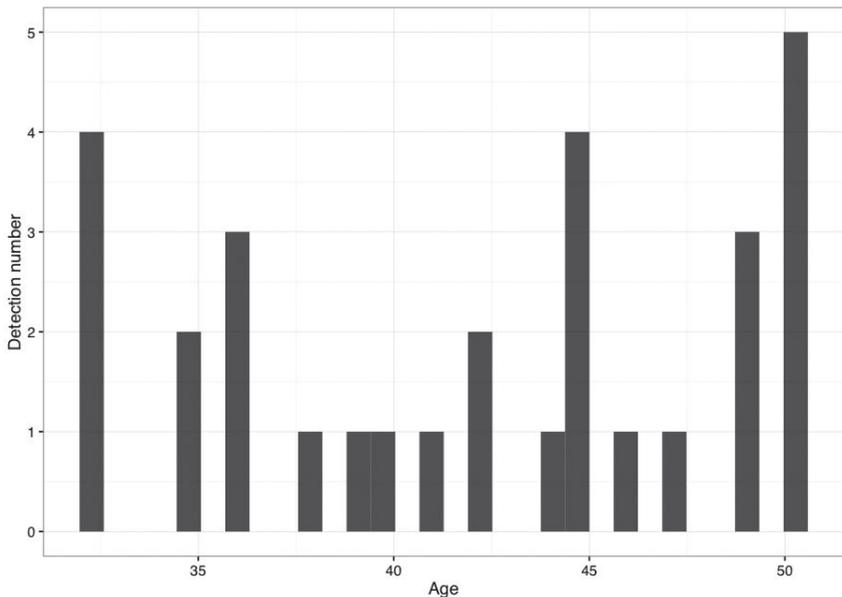


Figure 1. Number of aMPV subtype B detections in chickens at different ages. Data from longitudinal studies have been excluded

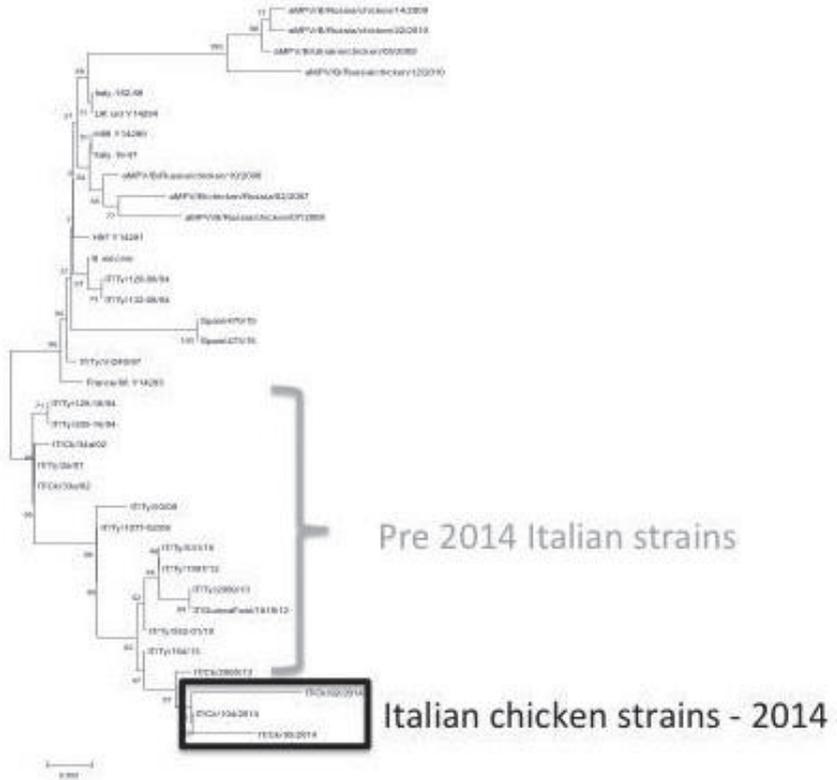


Figure 3. Phylogenetic tree based on F gene sequences of Italian and database logged aMPV strains

Co-infection of the avian respiratory epithelium with aMPV and *Mycoplasma gallisepticum*: comparison of host-pathogen interactions in the tracheal organ model

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Abstract

Avian Metapneumovirus (aMPV) and *Mycoplasma gallisepticum* (MG) are two primary pathogens that share high tropism for the upper respiratory tract of chickens and turkeys. Co-infection with these two pathogens may prolong clinical disease and increase the severity of lesions in affected birds. *In vivo* co-infection studies demonstrated that MG tends to be more invasive in co-infected groups while no effect on virus pathogenesis was observed.

Our objective was to characterize *in vitro* co-infection of chicken and turkey tracheal organ cultures (TOC) with MG S6 strain and aMPV subtype B. TOC were infected with the two pathogens at the same time or 24 h apart in alternating order. Different parameters were investigated including loss of ciliary activity (ciliostasis), pathogen replication, and interferon (IFN) mRNA expression. Significant induction of ciliostasis was only observed in co-infected compared to mono-infected TOC until 72 h post infection (pi, $p < 0.05$) suggesting a synergistic effect between invading pathogens. MG-pre-infection of TOC followed by subsequent aMPV-infection significantly reduced viral titers in co-infected chicken and turkey TOC at 24 h and 96 h pi, respectively, compared to mono-infected groups ($p < 0.05$). While aMPV mono-infection induced significant upregulation in IFN- λ mRNA expression ($p < 0.05$), no major changes were observed in IFN- α mRNA expression up to 72 h pi. Co-infection with MG reduced the increase in IFN- λ mRNA expression levels compared to aMPV-mono-infected TOC. We speculate that this downregulation may either be due to a direct effect of MG on IFN- λ expression or an indirect effect based on interference with aMPV-replication at the epithelial surface, which has to be elucidated further. Overall, our results contribute to the understanding of pathogen-pathogen-host interactions at epithelial surfaces. TOC are a suitable model to investigate possible mechanisms of interference and synergism leading to the exacerbation of clinical disease often observed in the face of multiple infections.

Introduction

Co-infections with different respiratory pathogens can be frequently observed in the field (Sid *et al.* 2015), and exacerbation of clinical disease and lesion development has been experimentally reproduced under *in vivo* conditions in different poultry species (Al-Ankari *et al.* 2001, Rubbenstroth *et al.* 2009, Stipkovits *et al.* 2012a, Stipkovits *et al.* 2012b, Awad *et al.* 2015). *Mycoplasma gallisepticum* (MG) infections show worldwide distribution and it is often detected as a co-infecting pathogen in chickens and turkeys (Levisohn & Kleven 2000, Bradbury 2005). It may be speculated that primary virus infections may aid bacterial invasion and possibly vice versa (Sid *et al.* 2016). Avian Metapneumovirus (aMPV) is also a widespread pathogen in poultry production, which often causes only minor clinical disease known as turkey rhinotracheitis in turkeys but may be exacerbated by secondary pathogens (Jirjis *et al.* 2004, Rubbenstroth *et al.* 2009, Verminnen & Vanrompay 2009, Jones & Rautenschlein 2013). The host-pathogen-interactions in the face of co-infecting pathogens have been only poorly investigated in poultry (Sid *et al.* 2016). Tracheal organ cultures have been shown to be a suitable model to investigate the interaction between host epithelial surface and pathogen not only for MG (Markham *et al.* 2003) but also for mono-infection with aMPV (Hartmann *et al.* 2015). Our objectives were to investigate the impact of co-infection with aMPV and MG on tracheal epithelium by looking at ciliary activity as an indicator of lesion development as well as the antiviral gene response in tracheal organ cultures of turkeys.

Materials and methods

Virus

A virulent aMPV subtype B strain (Italian strain, kindly provided by Dr R.C. Jones, Liverpool, UK), which had been isolated from turkeys and propagated in chicken tracheal organ cultures (Cook *et al.* 1976, Coswig *et al.* 2010) as well as the MG S6 laboratory strain (Sid *et al.* 2016) were used in this study. The MG strain was stored in Frey's broth without thallium acetate. It was thawed and serially diluted in TOC medium to conduct the co-infection studies (Abdul-Wahab *et al.* 1996). Based on preliminary experiments, 10^2 ciliostatic doses (CD)₅₀ of aMPV and 10^3 colony forming units (CFU) of MG were used to infect the tracheal organ cultures.

Tracheal organ culture

TOC were prepared as previously described (Petersen *et al.* 2012). Briefly, tracheae were isolated from humanely sacrificed 25-day-old turkey embryos or 20-day-old chicken embryos and cut into 0.8 mm rings. Chicken TOC and turkey TOC were transferred into

5ml tubes with 800µl of 199 medium with Hanks' salts supplemented with 1% L-glutamine (200mM, Biochrom, Berlin, Germany) and 1% of Penicillin 10.000 U/ml / Streptomycin 10.000µg/ml (Biochrom) and incubated at 37°C. Five days after preparation, TOC were assessed for ciliary activity using an inverted microscope (Zeiss, Germany). Only TOC with 100% ciliary activity were used for the infection studies.

Experimental design

TOC were assigned to one of five different groups. They were infected with MG and aMPV at the same time or 24h apart in variable order. Mono-infected TOC received only one pathogen. Negative control TOC received pathogen-free medium at the same time points. After 30 min incubation time with the primary pathogen, inocula were aspirated and replaced by 1ml medium supplemented with 1% L-glutamine (Biochrom, Berlin, Germany), Penicillin (1650U/mg) (Sigma Aldrich, Steinheim, Germany) and 0.2% bovine serum albumin (BSA) (Carl Roth®, Karlsruhe, Germany). Ciliary activity was assessed at different time points after infection with the second pathogen. Medium was changed daily. In addition, TOC or supernatants were collected (n=5/time point) and investigated for mRNA expression of antiviral cytokines such as IFN- α (Petersen et al. 2012), and the mucosa associated IFN-I. In addition, aMPV genome was detected by qRT-PCR and viral and bacterial antigens were stained by immunofluorescence and analysed using a Confocal Leica TCS SP5 (Leica Microsystems). Methods had been described earlier (Sid et al. 2016). The experiment was repeated at least twice for the different parameters.

Statistical analysis

The Shapiro-Wilk Normality Test was used to test for normal distribution of the data. For multiple comparisons between co-infected, mono-infected and negative control groups, we used Kruskal- Wallis one-way ANOVA. All statistical tests were conducted with Statistix, version 10.0 (Analytical Software, Tallahassee, FL). Differences are considered significant with a p value of < 0.05.

Results and discussion

Tracheal organ cultures of chicken and turkey embryos were inoculated with MG or aMPV, as well as with MG + aMPV in alternating order or at the same time. At 24, 48, 72 and/or 96 h after mono or second infection (psi; 48 h after inoculation of the first pathogen) five rings/treatment group were harvested and investigated. At 48 h psi, chicken (TOC-ch) as well as turkey TOC (TOC-tu) were positive for aMPV and MG antigen. Both pathogens co-localized in the epithelial layer as indicated by confocal microscopy in the dually infected TOC of either species (data not shown).

Co-infection led to earlier induction of ciliostasis, starting at 48 h psi ($P < 0.05$, Figure 1). Differences were observed in ciliostasis development between TOC-tu and TOC-ch after mono-MG and mono-aMPV infection: while TOC-ch developed ciliostasis after 96 h post infection, in TOC-tu, a reduction in ciliary activity was less clear but instead aMPV-mono-infected TOCs had developed some ciliostasis. This confirms previous investigations (Hartmann et al. 2015; Sid et al. 2016) and suggests different susceptibility of TOC-ch and TOC-tu for MG and aMPV, respectively. Subsequent investigations were only conducted with TOC-tu.

In addition to aMPV-antigen detection by immunofluorescence, viral load was quantified by qRT-PCR. Our results indicate that co-infection with aMPV and MG influenced virus replicating rates leading to lower qRT-PCR detection rates at 72 and 96 h psi in comparison to mono-infected TOC. This was significant for TOC-tu, which had received first MG and then 24 h later aMPV ($P < 0.05$).

Previous studies have suggested that aMPV and also human (h) metapneumoviruses may suppress IFN- α expression (Guerrero-Plata et al. 2005). In addition, MG has also been shown to interfere with interferon expression (Sid et al. 2016). IFN- expression was determined by qRT-PCR between 24 and 96 h psi. As expected, no IFN-upregulation was observed during this time period neither in mono- nor in dually-infected TOC. IFN-I (type III IFN) was shown to play a significant role in the resistance of epithelial cells against viral infections. Nothing is known about the involvement of IFN- in aMPV-infections. This study shows for the first time that aMPV induces high upregulation of IFN-I mRNA expression starting at 48 h psi with over a 500-fold increase in some of the aMPV-infected TOC-tu compared to non-infected controls. IFN-I-mRNA-expression was dominated by aMPV also in the dually infected groups. At 72 and 96 h psi, some of the dually-infected groups showed slightly lower expression levels compared to the aMPV-mono-infected group, but this difference was not significant ($P > 0.05$). We speculate that viral load and IFN-I mRNA expression may be correlated and that this interferon may play an important role in the defense against aMPV as shown previously for hMPV (Mordstein et al. 2010).

This study demonstrates that dual infection with aMPV and MG may modify aMPV replication and subsequent innate immune reactions leading to more severe lesion development. Our preliminary data suggest that the order and timing of the co-infecting pathogens may play a significant role in the outcome of the infection for the host. These in vitro observations support previous in vivo studies, and may provide a further step in the understanding of pathogen-pathogen-host interactions.

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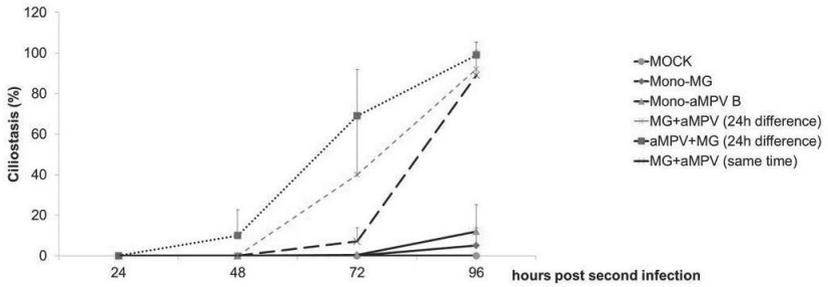


Figure 1. Development of ciliostasis in turkey TOC after mono- or co-infection with *Mycoplasma gallisepticum* and avian Metapneumovirus (aMPV). Co-infection pathogens were applied at the same time or in changing order with 24 h time difference between inoculations. Errors bars indicate standard deviation (n=5/group/time point), presented is the result of one representative experiment.

Evidence of subpopulations in aMPV vaccines using NGS

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Abstract

Avian Metapneumovirus (aMPV) causes an upper respiratory tract infection in turkeys and in some other avian species. Based on genome sequence differences, four subtypes of aMPV (A, B, C and D) have been identified but subtypes A and B have an almost worldwide distribution. Nowadays control strategies are mainly based on vaccination and different commercial live vaccines, originating from both subtype A and B strains, are available. The not absolute coverage of animal population achieved by ordinary mass vaccination in poultry industry allows a prolonged circulation of vaccine viruses in bird populations. Diseases outbreaks have been reported due to reversion to virulence of vaccines; and multiple nucleotide mutations in the aMPV genome associated to this phenomenon have been identified. In the current study, the presence of subpopulations in a subtype B vaccine was investigated by deep sequencing. Of the 19 positions where vaccine (strain VCO3/50) and progenitor (strain VCO3/60616) consensus sequences differed, subpopulations were found to have sequence matching progenitor sequence in 4 positions. However none of these mutations occurred in a virulent revertant of that vaccine, thereby demonstrating that the majority progenitor virus population had not survived the attenuation process, hence were not obviously involved in any return to virulence. However within the vaccine, a single nucleotide variation was found which agreed with consensus sequence of a derived virulent revertant virus, hence this and other undetected, potentially virulent subpopulations, can be involved in reversion. Much deeper sequencing of vaccine derived strains obtained during experimental infections or in the field may clarify if reversion to virulence was due to selective pressure acting on a homogeneous but rapidly evolving vaccine population or to the selection of virulent subpopulations already present in the vaccine batches.

Single reaction, real time PCR, for the detection of all known sub-groups of avian metapneumoviruses

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Abstract

To Date, four sub-groups (A, B, C and D) of avian metapneumovirus (AMPV), which are responsible for respiratory and genital disorders in poultry, have been identified based on genetic and antigenic variation. Molecular PCR diagnostic tools that allow differentiation of sub-groups most often use oligonucleotides corresponding to the surface glycoprotein genes G or SH whereas those with a more broad range of detection most often use oligonucleotides corresponding to the nucleocapsid protein gene N. This is due, in part, to the variation and conservation of nucleotide sequences across subgroups in these genes respectively. Currently no real time molecular diagnostic tool is available which utilizes regions of the AMPV genome that are sufficiently conserved across the four known sub-groups to allow identification of AMPV infection no matter the sub-group concerned. Such a diagnostic test would not only be beneficial financially, but also provide a more accurate analysis of the current or historical distribution of the four known sub-groups.

In this paper a Sybr GREEN single reaction, real time PCR capable of detecting sub-groups A, B, C and D was developed using oligonucleotides targeting a highly conserved region of sequence in the N gene of AMPVs. Results will be presented supporting the assay validation and its implementation to follow AMPV infection in experimental settings.

Efficacy of bivalent avian metapneumovirus subtypes A and B live vaccine in turkeys

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Abstract

Avian metapneumovirus (aMPV) is the causative agent of turkey rhinotracheitis. Because of the high prevalence of aMPV subtypes A and B in Israel and the economic losses caused, live vaccine containing both subtypes was prepared. The purpose of this study was to examine the efficacy of the bivalent vaccine in turkeys. Vaccination of turkeys with bivalent aMPV live vaccine was accompanied by an increased aMPV antibody level. Vaccinated turkeys were protected against clinical signs of TRT after challenge with virulent aMPV viruses.

Introduction

Avian metapneumovirus (aMPV) is the causative agent of turkey rhinotracheitis (TRT) which results primarily in respiratory disease. The virus is known to exist as four subtypes, A, B, C and D based on the attachment glycoprotein (G) gene sequences [Cook & Cavanagh (2002), D'Arce et al. (2005)].

TRT has been described in Israel for more than 20 years and Israeli isolates were characterized as aMPV subtypes A and B [Benet –Noach et al. (2005), Benet –Noach et al. (2009)]. The economic losses associated with TRT necessitate the development of a suitable vaccine for its control.

This study examined bivalent aMPV vaccine in turkeys in terms of its ability to induce seroconversion and provide protection after challenge against clinical signs, and pathological changes in tracheal ciliated cells.

Materials and methods

One-day old turkey poults were obtained from a commercial hatchery. The birds were reared in isolation cabins. Turkey poults were divided into four groups – two vaccinated and two control groups. Each poult was clinically examined and marked with a wing tag for individual identification. Turkey starter feed and water were provided ad libitum.

Experimental design

Vaccination was performed at 14 days of age by the oculonasal route. aMPV antibody levels were tested before the vaccination and before the challenge with a commercial ELISA kit (X-OvO, Flockscreen). Challenges of vaccinated and control groups were performed (separately for each aMPV subtype) with virulent Israeli isolates by the oculonasal route 21 days post-vaccination. Challenged turkeys were monitored daily for clinical signs. At 10 days post challenge the turkey poulters were humanely euthanized and ciliary activity of tracheal explants (ciliostasis score) was determined.

Vaccine

Bivalent experimental live vaccine was prepared from aMPV subtype A strain STG 761/8 and subtype B strain SHS1439. The vaccine was titrated in Vero cells and differentiation between subtypes was done by RT-PCR. Viral RNA was extracted using QIAmp Viral RNA Mini Kit (Qiagen) according to the manufacturer's protocol. A duplex RT-PCR protocol with the three primers Ga, G2 and G12 in a one-tube reaction, designed by Bayon-Auboyer *et al.* (1999), was done for differentiation between subtypes. Ga – G12 gave a 312 bp product specific for subtype B, and Ga – G2 gave a 504 bp product specific for subtype A. RT-PCR was performed using the Verso 1-Step RT-PCR ReddyMix Kit (ABgene, UK) according to the manufacturer's protocol with 5 µl RNA and each one of three primers (10 mM). The whole mixture was incubated at 50°C for 15 min and 94°C for 2 min, followed by 34 cycles of 95°C for 20 sec, 51°C for 30 sec and 72°C for 60 sec, all followed by a final extension step of 5 min. RT-PCR products were visualized after electrophoresis in a 1.5% agarose gel, containing ethidium bromide (0.15 µg/ml). Titer of each subtype was calculated according to the method of Reed & Muench [Villegas & Purchase (1989)] and found to contain $10^{2.5}$ median tissue culture infectious doses (TCID₅₀) of both in one vaccine dose.

Challenge viruses

Subtype A challenge was performed with field Israeli isolate SM04. Subtype B challenge was performed with field Israeli isolate NG02. The challenge viruses were kindly provided by the Division of Poultry Diseases, Kimron Veterinary Institute (Bet Dagan, Israel), propagated and titrated in to the tracheal organ cultures. Challenge dose of subtype A was $10^{2.1}$ TCID₅₀ and challenge dose of subtype B was $10^{2.4}$ TCID₅₀.

Statistics

Data sets were subjected to the student t-test to compare ELISA titers and results of ciliostasis score. P-values ≤ 0.05 were considered statistically significant. The analysis was performed using Excel (Microsoft, 2010).

Results

Antibody levels

aMPV antibodies were not found in the turkeys before the vaccination. Increased antibody level was observed in vaccinated groups 21 days post vaccination. Control groups remained antibody-negative (table 1).

Table 1. aMPV antibodies (ELISA)

Before vaccination		Before challenge (21 days post-vaccination)			
		Vaccinated		Unvaccinated	
GMT*	CV(%)	GMT	CV(%)	GMT	CV(%)
296.6	72.6	1296.1	14.4	342.5	48.3

*GMT- Geometric mean titer, cut-off 877

Note: Significant difference at 0.05 level between vaccinated and control groups.

Clinical observations

Following challenge, vaccinated turkeys were free of clinical signs. In control groups a clear to turbid nasal discharge was observed at different time. Ten days after the challenge in control turkeys clear nasal discharge was found in two turkeys challenged with subtype A group and in three turkeys challenged with subtype B group.

Ciliary activity

No lesions were found in respiratory tract of vaccinated turkeys 10 days post challenge with both subtypes of aMPV.

Difference in ciliary activity of tracheal explants is shown in table 2.

Table 2. Ciliostasis score 10 days after challenge.

Group	Challenged with subtype A		Challenged with subtype B	
	Vaccinated	Control	Vaccinated	Control
1	4	29	5	30
2	6	33	9	32
3	10	33	7	31
4	8	31	8	31
5	7	28	5	30
6	8	30	7	28
7	3	29	8	31
8	6	33	7	33
Average	6.5	30.8	7.0	30.8
STdev.	2.3	2.1	1.4	1.5

Note: Significant difference at 0.05 level between vaccinated and control groups.

Discussion

The aim of the present study was to investigate the response of turkeys to bivalent aMPV live vaccine (containing A and B subtypes) and the protection induced against virulent aMPV viruses. Vaccination is widely used for the control of aMPV in turkey flocks.

Bennet-Noach *et al.* (2009) reported that in Israel, two aMPV subtypes, A and B, are cocirculated. Van de Zande *et al.* (1999, 2000) found that both subtypes are antigenically related and induce development of cross-neutralizing antibodies and cross-protection against infection and disease in turkeys.

Vaccination of turkeys with bivalent aMPV live vaccine in this experiment was accompanied by an increased aMPV antibody level 21 days after the vaccination. After the challenges, in addition to clinical observation, ciliary activity of tracheal explants was examined and revealed that by 10 days after each challenge, ciliostasis had developed (to different extents) in all unvaccinated-challenged birds. In contrast, ciliary activity of the vaccinated groups did not change significantly.

Unvaccinated-challenged birds, inoculated with virulent A or B virus, showed typical clinical signs from three to seven days after the challenge. At the end of the observation period clear nasal discharge remained only in two birds challenged with subtype A and in three birds challenged with subtype B. In the vaccinated groups none of the birds showed clinical signs following either challenge. In terms of protection against clinical signs, the bivalent vaccine was 100% effective (although the conclusion is based on studying a small number of birds).

The present study shows that vaccination of turkeys with aMPV bivalent live vaccine provided protection against the development of clinical signs of TRT after challenge with virulent aMPV viruses.

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