Brazilian avian metapneumovirus subtypes A and B: experimental infection of broilers and evaluation of vaccine efficacy
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Avian metapneumovirus (aMPV) is a respiratory pathogen associated with the swollen head syndrome (SHS) in chickens. In Brazil, live aMPV vaccines are currently used, but subtypes A and, mainly subtype B (aMPV/A and aMPV/B) are still circulating. This study was conducted to characterize two Brazilian aMPV isolates (A and B subtypes) of chicken origin. A challenge trial to explore the replication ability of the Brazilian subtypes A and B in chickens was performed. Subsequently, virological protection provided from an aMPV/B vaccine against the same isolates was analyzed. Upon challenge experiment, it was shown by virus isolation and real time PCR that aMPV/B could be detected longer and in higher amounts than aMPV/A. For the protection study, 18 one-day-old chicks were vaccinated and challenged at 21 days of age. Using virus isolation and real time PCR, no aMPV/A was detected in the vaccinated chickens, whereas one vaccinated chicken challenged with the aMPV/B isolate was positive. The results showed that aMPV/B vaccine provided a complete heterologous virological protection, although homologous protection was not complete in one chicken. Although only one aMPV/B positive chicken was detected after homologous vaccination, replication in vaccinated animals might allow the emergence of escape mutants.

INDEX TERMS: Avian metapneumovirus, in vivo replication, vaccine protection.
não tenha sido conferida em uma ave. Apesar de o aMPV/B ter sido detectado em aves vacinadas, a replicação viral em aves vacinadas pode resultar em emergência de mutantes de escape.

TERMOS DE INDEXAÇÃO: Metapneumovirus aviário, replicação in vivo, proteção vacinal.

INTRODUÇÃO

Avian metapneumovirus (aMPV) é classificado como um membro da família Paramyxoviridae (Njenga et al. 2003). O vírus é capaz de replicar na tróca respiratória de turquesas e frangos com significativos custos econômicos, especialmente se a infecção está associada a patógenos secundários (Jirjis et al. 2002, Gough et al. 2008). Na criação, aMPV é um desafio, entre outros agentes, na síndrome do rosto enfedado (SHS) (Cook et al. 1995) e é provável que desempenhe um papel na patogênese de distúrbios testiculares (Villarreal et al. 2007).

Com base em análises moleculares do genoma, aMPV pode ser classificada em quatro subtipos: A, B, C e D (Juhasz et al. 2006). O vírus é capaz de replicar em muitos países. aMPV subtipos A e B (aMPV/A e aMPV/B) são disseminados em todo o mundo, onde o subtipo C está presente nos Estados Unidos e no domínio de aves de criação (Toquin et al. 2006) e o subtipo D foi relato em outros países (Cook et al. 2000, Njenga et al. 2003). No Brasil, onde a alta densidade de criações de galinhas existe, estudos em galinhas e turquesas demonstraram prevalência de aMPV/A e aMPV/B (Chacón et al. 1997) a subtipos A e B, respectivamente. A presença destas duas subtipos pode causar EMD (em emergência de mutantes de escape).

Com base na replicação viral em aves vacinadas pode resultar em emergência de mutantes de escape.
Virus titration
Individual samples from both in vivo experiments were inoculated in CER cells. When the viral cytopathic effect (CPE) was observed, the original samples were titrated in CER cells. Serial 10-fold dilutions were inoculated in CER 96-well plates following standard procedures. Titres were calculated by the Reed and Muench (1938) method and expressed as the log_{10} tissue culture infective dose per mL (TCID_{50}/mL).

Serum neutralization test
The sera were assayed for the presence of antibodies by the serum neutralization (SN) test. All SN titres >3 (log2) were considered as positive.

Statistical analysis
In the phenotypic characterization study, viral titre and Ct values obtained from groups challenged with aMPV/A or aMPV/B were analyzed using the paired and unpaired T test (Graph Pad Prism 5.0 software). Results are given as mean values (± SE) and p values less than 0.05 were considered statistically different.

RESULTS

Virus shedding by chickens inoculated with aMPV/A and aMPV/B
No clinical signs were observed in the control group or in chicks inoculated with aMPV/A. In animals inoculated with the aMPV/B field strain, a clear nasal discharge was seen mainly at 5 and 7 d.p.i. when the nares were squeezed gently.

Chicks in groups infected with aMPV/A or aMPV/B had different viral shedding patterns: aMPV/B could be detected longer and in higher quantities by real time RT-PCR compared to aMPV/A strain. aMPV/A RNA was only detected at 3, 5 and 7 d.p.i. (Fig.1A) whereas aMPV/B was detected at all five time points (Fig.1B). The highest RNA detection was obtained in sinus for both subtypes, at 5 d.p.i. for aMPV/A (Ct: 33.21) and 3 d.p.i. for aMPV/B (Ct: 27.18). When mean Ct value of sinusal and tracheal samples were compared, those in the aMPV/B challenged group at 3, 5, and 7 d.p.i. were significantly higher (P value ≤0.05) than those in the challenged group with aMPV/A. No aMPV RNA was detected in the non-challenged control group.

Virus was recovered from the trachea and sinus at all days p.i. for aMPV/B challenged group (Table I). The highest virus titre in tracheal samples was observed at 7d.p.i. (2.3 log_{10} TCID_{50}/mL) and in sinusal samples at 3 and 5 d.p.i. (2.7 log_{10} TCID_{50}/mL). aMPV/A was detected only in sinusal samples at 3 and 5 d.p.i. with titre mean of 1.7 and 1.9 log_{10} TCID_{50}/mL, respectively. Statistically significant difference (P value ≤ 0.05) between aMPV/A and aMPV/B titres was seen in sinusal samples obtained at 3 d.p.i. As expected, no infectious virus was recovered from the control group.

As described above, aMPV/B replication could be observed longer and in larger amount compared to the aMPV/A in inoculated chickens.

Protection study
Serological response. All pre-vaccination sera were negative for aMPV antibodies by the SN test. At 14 and 21 days post vaccination (d.p.v.) all vaccinated birds were positive for aMPV antibodies. The neutralizing antibodies titres reached their maximal level with titres around 8 log2 at 21 d.p.v. (Fig.2).

Virolological and clinical findings after challenge. No clinical signs were observed in the non-vaccinated and non-challenged (control) or either of the vaccinated-challenged groups (B/A and B/B groups) and in non-vaccinated chicks challenged with aMPV/A strain (-/A group). In the non-vaccinated chicks challenged with aMPV/B (-/B group) at 21 days of age, a clear nasal discharge was seen in individuals mainly at 5 and 7 d.p.c. when the nares were squeezed gently.

The mean viral detection by real-time RT-PCR or virus isolation for the vaccinated and unvaccinated groups is

Table 1. Mean titres in trachea and sinuses of chickens inoculated with aMPV/A or aMPV/B

<table>
<thead>
<tr>
<th>Days post inoculation</th>
<th>Trachea</th>
<th>Sinus</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>1.7*</td>
<td>1.7</td>
</tr>
<tr>
<td>5</td>
<td>1.8</td>
<td>1.9</td>
</tr>
<tr>
<td>7</td>
<td>2.3</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>1.9</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>1.7</td>
<td>-</td>
</tr>
</tbody>
</table>

Titres are expressed in log_{10} TCID_{50}/mL. * Values are mean of five birds.
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Table 2. Mean titres in trachea and sinuses of chickens vaccinated with a commercial aMPV/B vaccine and challenges with field aMPV/A and aMPV/B isolates

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Challenge</th>
<th>Sinus</th>
<th>3dpc</th>
<th>5dpc</th>
<th>7dpc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>-/2.5</td>
<td>-/31.41</td>
<td>-</td>
<td>-/39</td>
</tr>
<tr>
<td>None</td>
<td>A</td>
<td>&lt;2.3*</td>
<td>38.64</td>
<td>2.8</td>
<td>31.43</td>
</tr>
<tr>
<td>None</td>
<td>B</td>
<td>2.5</td>
<td>33.07</td>
<td>4.0</td>
<td>27.92</td>
</tr>
</tbody>
</table>

Titres are expressed in log_{10} TCID_{50}/mL. *Values are means of three birds, except when only some of the samples yielded virus, in which case all the values are shown (*).

The protection study showed that the aMPV/B vaccine provided a complete virological protection against aMPV/A, whereas positive samples were detected in some vaccinated chicks challenged with the aMPV/B isolate.

DISCUSSION

After the first detection of aMPV in Brazil (Arns & Hafez 1995), massive vaccination was established in commercial flocks. However, recent studies showed that aMPV/A and aMPV/B are still circulating in Brazil, affecting broilers, breeders, laying hens and turkeys (D’Arce et al. 2005, Chacón et al. 2007, 2011), with a majority of aMPV/B detected. In addition, Villarreal (2009) showed that subpopulations of aMPV/B are present in Brazil and suggested that escape mutants could emerge from the vaccines due to an insufficient protection. Finally, both studies revealed that the aMPV/B vaccine strain used in the country and the Brazilian aMPV/B field strains are grouped in separated clusters (Villarreal et al. 2009, Chacón et al. 2011). Subtype B also predominates in Israel and in six countries of Western Europe (Banet-Noach et al. 2005, Jones, 2010) and, according to the authors, this situation perhaps reflects the efficacy and wider use of subtype B vaccines (as a result of acquiring mutations by the vaccine strain). In order to investigate the protective response induced by an aMPV/B vaccine against Brazilian isolates, this study was conducted into two parts.

In the first part, two aMPV isolates (A and B subtypes) of chicken origin were characterized and were shown to be able to replicate efficiently in vivo, although they had different patterns of replication and clinico-pathological effects. Both viruses could be detected mainly in sinuses, whereas aMPV/B also replicated in trachea at low Ct values in five of six birds. In B/B group, viral RNA was detected in 4 out of 9 birds at 3 d.p.v. Only one bird in B/B group yielded infectious virus: 2.5 and 2.8 log_{10} TCID_{50}/mL in sinus and tracheal samples, respectively. In B/A group, no virus was detected by real time RT-PCR or virus titration. Table 2 shows the recovery of virus from the challenged birds which were initially vaccinated or not.
concerning respiratory signs (Van de Zande et al. 1999), similar observations of differences in virulence between these subtypes were found in ducklings, turkeys, and broilers (Toquin et al. 2006, Aung et al. 2008). Thus, our data indicates differences in replication ability and virulence of type A and B aMPV in chicks.

We also investigated the protection conferred by a commercial aMPV/B vaccine against Brazilian aMPV field strains of A and B subtypes. Our results demonstrate that a complete heterologous protection was conferred by the aMPV/B vaccine. This was expected as the aMPV/A replicated less efficiently than aMPV/B isolate. Interestingly, a partial protection was observed in some birds after vaccination with the aMPV/B vaccine and challenge with Brazilian aMPV/B isolate, despite the high SN titres developed after vaccination. Although the presence of antibodies to aMPV has been shown to have little effect on the fate of virus in the respiratory tract (Jones et al. 1992), antibodies are reliable indicators of vaccine immunogenicity (Jones 1996).

Two recent field studies, carried out in Israel and Italy, reported a lack of complete homologous protection due to genetic divergence between aMPV/B field and vaccine strains. These studies suggested that the field virus was able to overcome the immunity induced by the vaccine, due to amincid differences between the vaccine and field strains in the SH and G gene products, respectively (Banet-Noach et al. 2009, Catelli et al. 2010). A similar situation is found in Brazil, where aMPV/B vaccine strain used in the country forms a monophyletic group different from the Brazilian aMPV/B isolates (Chacón, et al. 2011, Villarreal et al. 2009), which could be an explanation for the partial homologous protection observed in some birds of our study.

Regardless, it should be considered that aMPV/B was not detected in all vaccinated/challenged birds and infectious virus was detected only in one bird. Similarly, Ganapathy (2007) showed that an aMPV/B vaccine was effective against clinical signs in chicks, although one bird was positive by RT-PCR or virus isolation.

CONCLUSIONS

In summary, the present study showed that the Brazilian aMPV/B isolate displays a higher replication ability in chickens than the isolate aMPV/A. Furthermore, the aMPV/B vaccine provided a complete heterologous virological protection, although homologous protection was not complete.

Despite the low number of aMPV/B positive birds detected after homologous challenge, it should be considered that field viruses can replicate in vaccinated birds and contaminate the environment allowing the emergence of escape mutants.

Therefore, a continuous surveillance is required to detect changes in the circulation of aMPV/A and aMPV/B or introduction of new viral subtypes.

REFERENCES


