Brazilian avian metapneumovirus subtypes A and B: experimental infection of broilers and evaluation of vaccine efficacy
Brazilian avian metapneumovirus subtypes A and B: experimental infection of broilers and evaluation of vaccine efficacy

Márcia B. dos Santos¹, Matheus C. Martini², Helena L. Ferreira³, Luciana H.A. da Silva², Paulo A. Fellipe², Fernando R. Spilki⁴ and Clarice W. Arns²*

ABSTRACT.- Santos M.B., Martini M.C., Ferreira H.L., Silva L.H.A., Fellipe P.A., Spilki F.R. & Arns C.W. 2012. Brazilian avian metapneumovirus subtypes A and B: experimental infection of broilers and evaluation of vaccine efficacy. Pesquisa Veterinária Brasileira 32(12):1257-1262. Laboratório de Virologia, Instituto de Biologia, Universidade Estadual de Campinas, Rua Monteiro Lobato s/n, Cx. Postal 6109, Campinas, SP 13083-970, Brazil. E-mail: arns@unicamp.br

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.

RESUMO.- [Metapneumovirus aviário subtipos A e B brasileiros: infecção experimental em frangos de corte e eficácia vacinal.] O Metapneumovírus aviário (aMPV) é um patógeno respiratório associado à síndrome da cabeça inchada (SHS) em galinhas. Apesar de vacinas vivas contra o aMPV serem utilizadas no Brasil, os subtipos A e B (aMPV/A e aMPV/B) são ainda encontrados no país, com predominância do subtipo B. Este estudo foi conduzido com o intuito de estudar dois isolados brasileiros de aMPV (subtipos A e B) isolados de frango. Para isto, um desafio experimental em frangos foi conduzido com o intuito de explorar a capacidade de replicação dos subtipos A e B Brasileiros. Posteriormente, a proteção virológica conferida por uma vacina do subtipo B em pintos foi realizada com os mesmos isolados. Após o desafio experimental demonstrou-se, por isolamento viral e PCR em tempo real, que o isolado do subtipo B replicou por maior período de tempo e em quantidades maiores, em comparação com o subtipo A. Para o estudo de proteção, 18 pintos de um dia de idade foram vacinados e desafiados aos 21 dias. Usando isolamento viral e PCR em tempo real, em nenhuma ave vacinada e desafiada com aMPV/A foi detectado o vírus, ao passo que uma ave vacinada e desafiada com a aMPV/B foi positiva. Os resultados mostraram que a vacina do subtipo B forneceu proteção heterológia completa, embora a proteção homológia
isolates as challenge viruses. At first, a challenge trial was performed to explore the behavior of two Brazilian aMPV isolates in chicks. aMPV/A and aMPV/B of chicken origin were selected for this purpose. Both strains were isolated from broiler chicks flocks after serial blind passages in Chicken Embryo Related (CER) cells (Coswig et al. 2010) and a RT-nested PCR was used to confirm virus presence. The strains Chicken/A/BR/775/06 and Chicken/B/BR/877/08 were titrated in CER cells, end points were calculated by the method of Reed and Muench (1938), and expressed in 50% of tissue cell infectious doses per ml (TCID₅₀/mL).

**Vaccine**

The chicks were inoculated with a commercial aMPV/B vaccine, at a titre of 3.8 log₁₀TCID₅₀/mL, by the ocularonasal (o.n.) route at the dose recommended by the manufacturer.

**Experimental design**

In vivo replication study. At two weeks of age, two groups of 25 chicks were divided and inoculated by the o.n. route with 0.2ml of aMPV/A or aMPV/B field strains at 4.3 log₁₀TCID₅₀/mL. A non-challenged control group was included. All groups were maintained in separate isolation units. After inoculation, broilers were observed for clinical signs daily up to 14 days post infection (d.p.i.). At 3, 5, 7, 10, and 14 d.p.i. five birds from each group were euthanized. Sinusal swabs and tissue scraped from trachea were collected individually and suspended in 1 ml of E-MEM containing 1mg/mL enrofloxacin. After shaking, samples were centrifuged for 5 min at 3500g to sediment the cellular debris. The supernatants were harvested and stored at -80°C until used.

**Protection study.** Two groups of 21 birds were either vaccinated (one-day-old) or remained as controls. At the day of vaccination and at weekly intervals during three weeks, blood samples were collected from ten chicks as an indicator of vaccine immunogenicity. At 21 days of age, 18 birds of each group were subdivided in two groups and challenged with 0.2 ml of aMPV/A or aMPV/B field strains at 4.3 log₁₀TCID₅₀/mL by the o.n. route. Samples of the three remaining birds of each group (vaccinated and non-vaccinated), before the virological exposure, were collected to determine whether vaccine virus could be detected 21 days post vaccination (d.p.v.), these birds were then euthanized. A non-vaccinated and non-challenged control group with nine birds was included in the experiment. All groups were maintained in separate isolation units. After inoculation, broilers were observed for clinical signs daily up to 7 d.p.v. At 3, 5, and 7 d.p.c. three birds from each group were euthanized. The sinusal and tissue tracheal samples were individually collected for virological examination and processed as the first experiment.

All animal studies were performed under supervision of the bioethics committee of the UNICAMP (protocol number 1716-2), following standard national guidelines.

**Viral shedding by real time RT-PCR and virus titration**

Sinusal swabs and tissue scraped from trachea were collected individually from both in vivo experiments. RNA was extracted from each sample and cDNA prepared, followed by real time RT-PCR based on amplification of the F (fusion) protein gene of aMPV/A (Ferreira et al. 2009) or B (Santos 2010). Threshold cycle values (Ct) were used, as Ct indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold.
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 log10 tissue culture infective dose per mL (TCID50/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 1). The highest virus titre in tracheal samples was observed at 7d.p.i. (2.3 log10 TCID50/mL) and in sinusal samples at 3 and 5 d.p.i. (2.7 log10 TCID50/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 log10 TCID50/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).

Virological 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...
shown in Fig. 3. No virus was detected in chicks which were not vaccinated or challenged. Also, no aMPV/B vaccine could be detected in vaccinated and non-challenged chicks at 21 d.p.v., asserting that aMPV/B detected after 21 d.p.v. were of field origin. In -/A group, viral RNA was detected at 3 and 5 d.p.c., infectious virus was recovered at 5 d.p.c. from trachea and sinus of all birds and at 3 d.p.c. in sinusal samples. aMPV/B strain was detected in -/B group, except for the tracheal samples collected at 7 d.p.c. The highest virus titre and lowest Ct value were observed at 5 d.p.c. from sinusal samples of both -/A group (2.8 log_{10} TCID_{50}/mL, Ct: 31.43) and -/B group (4.0 log_{10} TCID_{50}/mL, Ct: 27.92). In B/B group, viral RNA was detected in 4 out of 9 birds at 3, 5 or 7 d.p.c. Only one bird in B/B group yielded infectious virus: 2.5 and 2.8 log_{10} TCID_{50}/mL in sinusal 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.

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>Titre</th>
<th>Ct</th>
<th>Titre</th>
<th>Ct</th>
<th>Titre</th>
<th>Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>A</td>
<td>3dpc</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>3dpc</td>
<td>-/-2.5a</td>
<td>-/-31.41</td>
<td>-</td>
<td>-</td>
<td>-/-39</td>
<td>-/- &gt;39</td>
</tr>
<tr>
<td>None</td>
<td>A</td>
<td>3dpc</td>
<td>&lt;2.3a</td>
<td>38.64</td>
<td>2.8</td>
<td>31.43</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>None</td>
<td>B</td>
<td>3dpc</td>
<td>2.5</td>
<td>33.07</td>
<td>4.0</td>
<td>27.92</td>
<td>2.3</td>
<td>38.79</td>
</tr>
<tr>
<td>Trachea</td>
<td>A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>-</td>
<td>-/-2.8</td>
<td>-/-39/29.45</td>
<td>-/- &gt;39</td>
<td>-</td>
<td>-/-29.98</td>
<td>-</td>
</tr>
<tr>
<td>None</td>
<td>A</td>
<td>-</td>
<td>-/- &gt;39</td>
<td>2.5</td>
<td>36.77</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>None</td>
<td>B</td>
<td>2.7</td>
<td>31.91</td>
<td>3.0</td>
<td>30.67</td>
<td>32.56</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Titres are expressed in log_{10} TCID_{50}/mL. *Values are means of tree 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 trachea and sinus of all birds and at 3 d.p.c. from sinusal samples of both -/A group (2.8 log_{10} TCID_{50}/mL, Ct: 31.43) and -/B group (4.0 log_{10} TCID_{50}/mL, Ct: 27.92). In B/B group, viral RNA was detected in 4 out of 9 birds at 3, 5 or 7 d.p.c. Only one bird in B/B group yielded infectious virus: 2.5 and 2.8 log_{10} TCID_{50}/mL in sinusal 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 aminoacid 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. 2005). A similar situation is due to amino acid differences between the vaccine and field strains. These studies suggested that the field virus was able to overcome the immunity induced by the vaccine, and circulating in Brazilian vaccinated and non-vaccinated chicken and turkey farms. Avian Dis. 55(1):82-89.


Regarding, 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


Santos MB. 2010. Experimental studies with avian metapneumovirus (aMPV) subtypes A and B isolates in broiler chickens. Dissertação de Doutorado em Genética e Biologia Molecular, Universidade Estadual de Campinas, Campinas, SP, Brazil. 32p.


