Orchitis in Roosters with Reduced Fertility Associated with Avian Infectious Bronchitis Virus and Avian Metapneumovirus Infections

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Infectious bronchitis virus (IBV) is an epitheliotropic virus that causes acute and severe diseases of the respiratory, renal, and female reproductive tracts of chickens (2,6,10,12,36). Replication in the oviducts results in a decline in the quality and quantity of eggs (2,6,10,12,36). Replication in the reproductive tracts of chickens (2,6,10,12,36). Replication in the genitalia of fowl results in the formation of calcium stones in the epididymus of roosters (2,6,10,12,36). Replication in the genitalia of fowl results in the formation of calcium stones in the epididymus of roosters, a definitive association has not been confirmed. This report describes the detection of IBV and aMPV in the testes of roosters from a Brazilian poultry breeder’s flock with epididymal stones and low fertility. Samples of testis, trachea, and lungs from breeder males aged 57 wk were positive for IBV by reverse transcriptase–polymerase chain reaction (RT-PCR), and virus isolation and testis samples were also positive for aMPV by RT-PCR. The inoculation of testis samples into embryonated chicken eggs via the allantoic cavity resulted in curled, hemorrhagic, and stunted embryo deaths. Based on neutralization patterns using monoclonal antibodies and molecular analysis, the allantoic fluid was positive by RT-PCR aimed to amplify the region coding for the S1 subunit of the IBV S gene, but it was not positive for aMPV. Sequence analysis of the amplified fragment revealed a close relationship with European IBV genotype D274, previously unreported in Brazil. These results indicate that IBV and perhaps aMPV are likely to have played a role in the pathogenesis of the testicular disease described and should be regarded as factors that can influence male fertility disease in chickens.

Key words: avian infectious bronchitis virus, avian metapneumovirus, roosters, epididymus, stones, fertility

Abbreviations: aMPV = avian metapneumovirus; IBV = infectious bronchitis virus; RT-PCR = reverse transcriptase–polymerase chain reaction; S = spike

Infectious bronchitis virus (IBV) is an epitheliotropic virus that causes acute and severe diseases of the respiratory, renal, and female reproductive tracts of chickens (2,6,10,12,36). Replication in the oviducts results in a decline in the quality and quantity of eggs (13,14,21). IBV is a group 3 Coronavirinae in the family Coronaviridae of the Nidovirales (5). The genome is a single-stranded, positive-sense RNA 27.6 Kb (7,35). The surface spike (S) protein and, mainly, its S1 subunit, the ectodomain, are highly variable as a result of mutation and give rise to variation in serotypes/genotypes within the species. This makes this protein and its coding gene an invaluable molecular marker for establishment of interrelationships between IBV strains from different sources and locations (18). Infectious bronchitis (IB) causes considerable economic losses to the poultry industry.

Avian metapneumovirus (aMPV) belongs to the subfamily Paramyxovirinae within the Paramyxoviridae (33). Based on neutralization patterns using monoclonal antibodies and molecular analysis, metapneumoviruses are subdivided in four different groups (A, B, C, and D groups) (3,15,17). In commercial layers, aMPV infection may
affect the quality of eggs (11) and cause a range of reproductive tract abnormalities, including egg peritonitis, folded shell membranes in the oviduct, misshapen eggs, and ovary and oviduct regression (15). aMPV infections are also associated with swollen head syndrome in chickens (22). Furthermore, reduced laying performance has been reported in broiler breeders in association with swollen head syndrome (31).

In previous studies in males, it has been shown that several factors can alter the production of fertile sperm, including the presence of stones in the epididymal region, more specifically in the efferent ductules (20). Others (4) have shown that roosters with stones could have lower testis weight and decreased daily sperm production. Although IBV has been suggested to be one of the possible causes of the formation of calcium stones in the epididymis, a definitive association has not been established. Vaccination with attenuated IBV has been shown to lead to the development of stones and therefore to problems of reduced fertility (4). However, Decheva et al. (28) failed to show a definitive relationship between IBV and the occurrence of epididymal lithiasis.

This article reports the detection of IBV and aMPV in association with epididymal stones in roosters in Brazilian broiler breeder flocks and discusses the possible role of these viruses as a cause of decreased fertility.

**MATERIALS AND METHODS**

**Case history and samples.** A flock of broiler breeder grandparents, aged 57 wk, from São Paulo State, Southern Brazil, with a single-age system of production, presented with respiratory disease, facial edema, nephritis, low hatchability, and decreased fertility. Males in particular were in poor general condition, presenting with broken feathers, cyanotic combs, depression, and mild tachycardia. The fertility rate of the males was 40.7%, as measured by embryo diagnosis using an ovoscope; the expected rate for this age is 86%. At necropsy, testicular atrophy and epididymal stones were found.

The clinical signs and the decreased fertility began when the flock was 33 wk of age. They had received live attenuated H120 vaccine at 5, 28, and 84 days of age and an inactivated H120 vaccine at 20 wk of age. They also received a live aMPV subtype A vaccine at 8 and 14 wk and an inactivated aMPV vaccine at 20 wk.

Titters to IBV and aMPV antibodies, as identified by enzyme-linked immunosorbent assay (Idexx™, Westbrook, ME) at weeks 20, 30, 40, 50, and 60, are shown in Table 1.

<table>
<thead>
<tr>
<th>Weeks of age</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBV</td>
<td>876 (56.3)</td>
<td>6567 (30.2)</td>
<td>5317 (43.5)</td>
<td>7030 (53.8)</td>
<td>17,390 (11.6)</td>
</tr>
<tr>
<td>aMPV</td>
<td>ND</td>
<td>ND</td>
<td>3980 (32.6)</td>
<td>ND</td>
<td>8731 (22.0)</td>
</tr>
</tbody>
</table>

^ND = not done.

**RESULTS**

**Histopathologic examination of testses.** The histopathologic lesions found in all of the 12 tests samples comprised infiltration with plasma cells, lymphocytes, and cellular debris surrounding the affected ductules. The columnar epithelium of the efferent ductules was shortened. There were epithidymal stones in the efferent ductules, the epithelium of which consisted of a thin envelope of squamous cells, continuous to the efferent ductile epithelium. Furthermore, the seminiferous ductules were atrophic and showed a low concentration of viable spermatozoa.

**IBV screening.** All samples (separate pools of tests, kidneys, trachea, and lungs) were positive for IBV by RT-PCR. Negative controls (ultrapure water) produced no bands.

**Virus detection.** Virus detection was accomplished using by reverse transcriptase–polymerase chain reaction (RT-PCR) and virus isolation.

**RT-PCR—RNA extraction.** Total RNA was extracted with TRIsol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions, directly from tissue suspensions (for IBV and aMPV screening), from the allantoic fluid of embryonated eggs for the confirmation of IBV and aMPV isolation (see below) and IBV sequence analysis, negative (ultrapure water) and positive (IBV H120 and aMPV subtypes A and B) controls.

**IBV screening.** Primers UTR 41+, UTR 31-, and UTR 11- and reaction conditions described by Cavanagh et al. (9) were used to amplify a 179-base pair fragment of the 3′ untranslated region of avian coronaviruses. aMPV detection. All samples were screened for aMPV with primers G6–, G1+ , G5–, G8+A, and G9–B and reaction conditions described by Cavanagh et al. (8) to amplify 268-bp or 361-bp fragments, corresponding to the subtypes A and B, respectively, of the G gene of the virus.

**Virus isolation and propagation.** The pool of testses was used for attempted virus isolation in embryonated chicken eggs. A suspension of the tests pool in ultrapure water was filtered through 0.45-μm- and 0.22-μm membranes; 10 mg/ml of gentamicin (Sigma-Aldrich, St. Louis, MO) was added; and it was inoculated into 9-to-11-day-old specific-pathogen-free, embryonated chicken eggs (Laboratório Biovet, Vargem Grande Paulista, Brazil) by the allantoic cavity route. Four blind serial passages were performed and screened for IBV and aMPV by RT-PCR, as described above.

**PCR to the IBV S1 coding region.** The set of primers S1OLIGO 5’ and C2K (23,26,27) and the reaction conditions described by Ziegler et al. (38), with some modifications, were used to amplify a product of approximately 706 bp of the region coding for the S1 subunit of the S gene of IBV from the tests pool passed in embryonated eggs, now on named strain IBV/Brazil/2005/USP-01.

**DNA sequencing and phylogenetic analysis.** The PCR product corresponding to the S1 region from strain IBV/Brazil/2005/USP-01 was purified with the QIAquick Spin Miniprep kit (Qiagen, Hilden, Germany) and submitted to bidirectional DNA sequencing with DYEEnamic ET Dye Terminator (Amersham Biosciences, Piscataway, NJ), according to the manufacturers’ instructions, the sequences resolved in a MegaBACETM 1000 (Amersham Biosciences).

**RESULTS**

**Histopathologic examination of testses.** The histopathologic lesions found in all of the 12 tests samples comprised infiltration with plasma cells, lymphocytes, and cellular debris surrounding the affected ductules. The columnar epithelium of the efferent ductules was shortened. There were epithelial stones in the efferent ductules, the epithelium of which consisted of a thin envelope of squamous cells, continuous to the efferent ductile epithelium. Furthermore, the seminiferous ductules were atrophic and showed a low concentration of viable spermatozoa.

**IBV screening.** All samples (separate pools of tests, kidneys, trachea, and lungs) were positive for IBV by RT-PCR. Negative controls (ultrapure water) produced no bands.
AMPV detection. The RT-PCR aimed to amplify the G gene resulted in a positive only with regard to the pool of testis, with a fragment of 268 bp, which corresponds to subtype A of aMPV. Negative controls (ultrapure water) produced no bands.

Virus isolation and propagation. Four serial passages of testis samples in fertile eggs resulted in curled, hemorrhagic, and stunted embryos 3 days postinoculation. IBV, but not aMPV isolation, was confirmed by the respective RT-PCRs.

Phylogenetic analysis. The RT-PCR targeted to the S1 coding region showed a fragment of 706 bp for the IBV-designated strain IBV/Brazil/2005/USP-01. The virus segregated in the same cluster as the IBV European genotype D274, American Cal99 and Ark 99, and field strains detected in China, Korea, and Spain. They were separate from the clusters formed by the vaccine strains H120, Ma5, M41, and Connecticut and were also distant from strains DE072 and 793B (Fig. 1).

DISCUSSION

Data from previous studies support the assertion that the epididymal stones found in the testis samples examined here were the cause of markedly reduced fertility in the male birds studied. It is well established that one of the abnormalities that can lead to low fertility in roosters is the formation of calcium stones in efferent ducts (20). Once stones are formed in the efferent ductules of the epididymal region, the production and viability of the sperm is seriously compromised, as the efferent ductules are responsible for reabsorption of fluid from the testis, and any damage of this function leads to fluid accumulation, fluid back-pressure, testicular atrophy, and infertility (20,28).

The pathogenesis of both IBV and aMPV in reproductive disease in hens derives from viral replication in the ciliated epithelium of the female reproductive tract, resulting in loss of cilia and degeneration and necrosis of the epithelial and glandular cells (4,29,32,37). The epithelium of the efferent duct of the testis also consists of ciliated and nonciliated epithelial cells (1). It seems likely that these cells are susceptible to IBV or aMPV replication.

The epididymal region of roosters consists of a rete testis, efferent ductules, connecting ducts, and the epididymal duct. One important structure of the rooster’s testis is the efferent ductules, and their primary functions include fluid reabsorption, transport and concentration of sperm, phagocytosis of the sperm, and protein secretion (19,25).

Evidence of IBV and aMPV infection has been demonstrated here in the testis of roosters, with epididymal microscopic lesions associated with low fertility and respiratory problems in a breeder flock with poor reproductive performance. Data from antibody titters to both IBV and aMPV (Table 1) reinforce the evidence of a field challenge, as these showed a remarkable increase from week 50 to week 60 in the case of IBV and from week 40 to week 60 in the case of aMPV.

The IBV strain detected by RT-PCR in the testis tissues was also isolated in embryonated chicken eggs, inducing characteristic signs of IBV infection in the embryos. Thus, not only was the viral genome detected, but infectious virus was also found in the testis cells, indicating that IBV was replicating in this organ. However, this would need to be confirmed by a technique showing virus in the

Fig. 1. Phylogenetic tree based on partial S1 coding region of IBV strains from different countries and IBV reference strains showing strain IBV/Brazil/2005/USP-01. Numbers at each node are bootstrap values; the bar represents the number of nucleotide substitutions per site.
cells, such as immunostaining. If there is true IBV replication, this is likely to be the cause of the microscopic lesions found here, namely destruction of the efferent ductules, destruction of the epididymus, seminiferous tubules, and the presence of stones in the epididymus, similar to those symptoms previously reported by Bolton et al. (4).

In view of these results, it seems possible that IBV replication in testis cells of the roosters sampled in the present study caused the severe microscopic lesions that, at the chronic stage, resulted in the formation of stones in the epididymus and were responsible for the reduced fertility.

Phylogenetic analysis of the IBV isolated from the tissues (IBV/Brazil/2005/USP-01) revealed that it is not closely related to the Massachusetts-derived H120 strain, the only IBV that, in an attenuated form, is authorized by the Brazilian Government for vaccination, but closely related to a cluster containing D274, a European virus used as a vaccine.

Considering this clustering pattern, an H120-vaccine–derived pathogenesis of the lesions here described could be promptly discarded, but a larger number of sequences with similar history must be obtained in order to track back the origin of IBV/Brazil/2005/USP-01 and its relationship with other IBV strains that are prevalent in Brazil.

Apart from this, once the prevalence of serotypes different from the H120 is confirmed in a broader way in Brazil, it will be necessary to modify existing vaccination programs to ensure that they control the disease caused by such variants; this effort will perhaps include the development of new vaccines.

Although aMPV was detected in the same testis samples but in no other tissues, it was identified only by RT-PCR, and not by isolation. This virus was not isolated in eggs, and since it was subtype A, it is likely that tracheal organ cultures would have been more successful. Thus, since identification showed the presence of viral RNA only, it is difficult to conclude what role it was playing in the disease processes. Nonetheless, as a result of the tropism of aMPV for ciliated epithelial cells of the reproductive tract of hens, it is tempting to suggest that aMPV could also play a pathogenic role.

Other agents such as Salmonella Gallinarum and Salmonella Pullorum, Chlamydia phila, and aflatoxins have been reported to cause orchitis and to suppress spermatogenesis, leading to abnormalities in spermatozoa and atrophy of the testes (28,30,34). These in turn have been associated with calcification, urolithiasis, and gallstones, and these should not be overlooked.

Experimental studies aimed at the reproduction of the disease in roosters, using the IBV and aMPV isolates and immunostaining, are needed in order to establish a definitive association between these viruses and decreased fertility in roosters.

REFERENCES