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Isolation and identification of pathogenic mycoplasmas in ostrich farms using PCR and culture methods

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ABSTRACT. In ostriches, mycoplasmas are generally associated with respiratory diseases and causes rhino-tracheitis, airsacculitis and inflammation of the upper respiratory tract. The aim of current study was the isolation and identification of pathogenic mycoplasmas in ostrich farms of Iran by the use of PCR and culture methods. In this study, mycoplasmas were isolated from ostrich slaughterhouse; 114 samples were collected from ostriches with respiratory signs and were cultured and PCR methods along with alignment were used to detect the mycoplasmas. For this purpose lung, trachea and airsacs were evaluated. The results indicated that 21.05% of samples were positive in PCR assay and from them 7.89% and 14% was M. gallisepticum and M. synoviae, respectively. The highest rate of M. gallisepticum and M. synoviae was detected in lung, airsacs and trachea. Alignment analysis demonstrated that the M. gallisepticum strains detected in our study have 97% homology to 06/14, 05/14 and 16S strains. In addition, M. synoviae strains have 99% and 98% homology to MSR-812, MSR-795 and MSR-1019 strains. One of the M. synoviae strains has 82% homology to ABSfsdMS2011 strain. The results of our study showed that ostriches in Iran were infected with chicken mycoplasmas but the pathogenesis of them in ostrich respiratory should be further evaluated.

Keywords: Mycoplasma, Ostriches, isolation, PCR, sequencing
INTRODUCTION

Mycoplasmosis is an infectious disease and causes economic losses due to respiratory signs, leg disorders, growth retardation, losses of egg production and reduction of hatch rate (Bradbury and Morrow, 2008; Calnek and Barnes, 1997). Mycoplasmas are widespread in nature and differ from other bacteria, because of their small size and the lack of cell wall (Bradbury and Morrow, 2008). Mycoplasmas are host-specific and completely resistant to antibiotics that affect cell wall synthesis (Al-Ankari and Bradbury, 1996). Pathogenic avian mycoplasmas include the species *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) in chickens and turkeys as well as *Mycoplasma meleagridis* (MM) and *Mycoplasma iowae* (MI) in turkeys (Panangala et al., 1992).

In ostriches, mycoplasmas are generally associated with respiratory diseases and causes rhino-tracheitis, airsacculitis and inflammation of the upper respiratory tract (Huchzermeyer, 1994; Verwoerd, 2000). Rhino-tracheitis, usually appeared in cold, windy weather during winter or in summer following heat stress (Botes et al., 2005; Verwoerd, 2000). MS have been isolated from respiratory infected ostriches, with respiratory symptoms (Huchzermeyer, 1994). Although serological response to poultry mycoplasmas (MG, MS and MM) were reported from northern Italy, infection of ostriches with poultry mycoplasmas was not determined (Peccati et al., 1996). It was reported that the concurrent infections in ostriches cause production loss, carcasses downgrading and thus considerable economic losses in ostrich industry (Botes et al., 2005).

When ostriches were kept near poultry in northern Italy, they had positive immunologic response to poultry mycoplasmas (including MG, MS, and MM), although infection with that mycoplasmas was not recognized (Peccati et al., 1996). In Zimbabwean ostriches slaughteredhouses antibodies against MG and MS were reported (Cadman et al., 1994).

Concurrent infections of mycoplasmas and other pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Pasteurella* species and occasionally *Avibacterium paragallinarum* were reported from clinical syndromes of infected ostriches (Blackall et al., 2005; Verwoerd, 2000).

Experimental infection of MG with clinical signs in ostriches was previously reported (Cline et al., 1997). Researchers indicated that the mycoplasmas occurred in ostriches, were not typical mycoplasmas of poultry which infected respiratory system in poultry (Shivaprasad, 1993). Other investigations suggested that unique ostrich-specific mycoplasmas, causes infections in ostriches (Shane, 1998; Smith, 1993). Previous studies reported isolation of M. synoviae from ostriches in Iran (Tebyanian et al., 2014). Generally, Mycoplasma transmitted within species or between closely related species, with rare exceptions (Kleven, 2008; Nascimento et al., 2005).

The aim of current study was the isolation and identification of pathogenic mycoplasmas in ostrich farms of Iran using molecular and culture methods.

MATERIALS AND METHODS

Samples: For this study, 114 samples were collected from ostrich slaughterhouse and investigate the presence of mycoplasma by culture and PCR. From all ostriches with respiratory signs serum samples were collected for antibody evaluation against MS and MG. The samples were collected from lung, trachea and air sacs of ostriches with respiratory signs transported to the laboratory.

Culture: Samples were cultured on pleuropneumonia-like organisms (PPLO) broth and agar media without crystal violet (21 g/l), 20% heat-treated horse serum, 10% fresh yeast extract, 0.2% glucose, 0.4% sodium pyruvate, 0.04% ampicillin and 1% agar noble. For the evaluation of MS by culturing samples 1%, nicotinamide adenine dinucleotide was supplemented to above medium. Firstly, the samples were enriched in PPLO broth at 37°C for 24 hours and then, for PCR investigation, they transported to PPLO agar (Swayne, 1998), in 5% CO2 for 7 to 10 days. The plates were checked daily for the appearance of the characteristic colonies. The mycoplasma specific stain was used for the evaluation of cultured bacteria. A single colony from each isolate and the samples that was collected from various organs was used for DNA extraction and mycoplasma identification.

DNA extraction: For DNA extraction the Genomic DNA extraction Kit (Gene Transfer Pioneers, GTP) was used. Following the instructions to manual 1-4.
ml of bacterial culture centrifuged in 8000g for 1 min and the supernatant was discarded, 250 µl RNase A was added and vortexing for 15 seconds (s). Then 400 µl wash buffer and 400 µl chloroform were added, vortexing for 30s and centrifuged at 12000g for 5 min. 400 µl of supernatant was transferred in new microtube and 400 µl guanidine hydrochloride was added and mixed gently. The solution was transferred to DNA spin column assembled in a clean collection tube and centrifuged at 10000g for 3 min. The extracted DNA was electrophoresed on 1% agarose gel and visualized by staining with ethidium bromide (1.5 µg ml⁻¹).

**Primer for the amplification of 16S rRNA:** To confirm the presence of mycoplasma in samples, general and specific primers of 16S rRNA were used: Primers GPO3F and MGSO to detect the genus (Van Kuppeveld *et al.*, 1992), primers M.syn and M.Syn-R the detect MG (Bagheri *et al.*, 2011) and primers MS-1 and MS-2 to detect MS (Lauerman *et al.*, 1993). Primers are listed in Table 1.

**PCR amplification of 16S rRNA gene:** PCR amplification carried out in 0.5ml tubes in a final reaction volume of 20 µl. The PCR mixture consisted of 5 ng of template DNA, 1 mM MgCl₂, 0.8 µl of each primers, 2 µl of 10X PCR buffer, 100 mM dNTPs, and 1 U Taq DNA polymerase, was amplified according to Botes et al., (2005): 95°C (5min) of initial denaturation and then 35 cycles of denaturation at 95°C (40s), annealing at 52°C (60s) and elongation at 72 °C (60s) and final extension in 72°C (5min). Amplified DNA was electrophoresed on 1% agarose gel and visualized by staining with ethidium bromide (1.5 µg ml⁻¹).

**SEQUENCING OF THE MYCOPLASMA 16S rRNA GENE**

The PCR products of GPO3 and MGSO primer pairs, purified as templates for sequencing reactions. PCR products were electrophoresed on a 0.5% agarose gel. DNA containing bands were excised under a UV light and purified, then samples were concentrated and subsequently analyzed by electrophoresis, as described previously by researchers (Botes *et al.*, 2005). For sequencing, the samples were sent to Macrogen Korea co, (Republic of Korea). Data from sequencing were aligned to and compared on the NCBI nucleotide sequence database.

**RESULTS:**

**Mycoplasmas culture:** After 48-96 hours incubation, mycoplasma colonies were visible. Typical colonies of mycoplasmas, were identified detecting the fried egg morphology in well-developed colonies. From the 114 ostrich samples, 24 were positive for mycoplasmas. Results are listed in Table 2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPO3F (F)</td>
<td>5’-TGGGGAGCAAACAGGATTAGATACC-3’</td>
<td>(Van Kuppeveld <em>et al.</em>, 1992)</td>
</tr>
<tr>
<td>MGSO (R)</td>
<td>5’-TGCAACCATCTGACTCTGTTAACCTC-3’</td>
<td></td>
</tr>
<tr>
<td>M.Syn (F)</td>
<td>5’-GCAGATGACGTGTAGTTATGCTG-3’</td>
<td>(Bagheri <em>et al.</em>, 2011)</td>
</tr>
<tr>
<td>M.Syn-R (R)</td>
<td>5’-CCAATGCAATACTCGTAAAGC-3’</td>
<td>(Lauerman <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>MS-1</td>
<td>5’-GAAGCAAAATAGTGATATCA-3’</td>
<td></td>
</tr>
<tr>
<td>MS-2</td>
<td>5’-GTCGCTCTCCGAAGTTACCA-3’</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Primers used for amplification and detection of mycoplasmas from ostrich samples

(Van Kuppeveld *et al.*, 1992), primers M.syn and M.Syn-R the detect MG (Bagheri et al., 2011) and primers MS-1 and MS02 to detect MS (Lauerman et al., 1993). Primers are listed in Table 1.

**PCR amplification of 16S rRNA gene:** PCR amplification carried out in 0.5ml tubes in a final reaction volume of 20 µl. The PCR mixture consisted of 5 ng of template DNA, 1 mM MgCl₂, 0.8 µl of each primers, 2 µl of 10X PCR buffer, 100 mM dNTPs, and 1 U Taq DNA polymerase, was amplified according to Botes et al., (2005): 95°C (5min) of initial denaturation and then 35 cycles of denaturation at 95°C (40s), annealing at 52°C (60s) and elongation at 72 °C (60s) and final extension in 72°C (5min). Amplified DNA was electrophoresed on 1% agarose gel and visualized by staining with ethidium bromide (1.5 µg ml⁻¹).

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**RESULTS:**

**Mycoplasmas culture:** After 48-96 hours incubation, mycoplasma colonies were visible. Typical colonies of mycoplasmas, were identified detecting the fried egg morphology in well-developed colonies. From the 114 ostrich samples, 24 were positive for mycoplasmas. Results are listed in Table 2.
Polymerase Chain Reaction: The growth of *Mycoplasmas* in PPLO broth media causes changes in color due to the biochemical activity of the bacteria. One hundred and fourteen (114) suspected samples were analyzed by PCR test. In 24 samples *Mycoplasma* isolates were detected (Figures 103). All positive samples were evaluated by PCR test for the detection of MG and MS. From the 24 positive *Mycoplasma* samples, 14 (58.33%) were identified as M.S and 9 (37.5%) as MG. Microbiological methods indicated 7 MG and 8 MS positive samples. Results are listed in Table 3.

**Table 2: The Clinical origin of mycoplasma positive samples**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mycoplasma</th>
<th>MG</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>14</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Trachea</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Air sacs</td>
<td>6</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>9</td>
<td>14</td>
</tr>
</tbody>
</table>

**Table 3: Results of microbiological and PCR methods in detection of Mycoplasma from ostriches samples**

<table>
<thead>
<tr>
<th>Mycoplasma, PCR</th>
<th>MG PCR, No (%)</th>
<th>MG culture method, No (%)</th>
<th>MS PCR, No (%)</th>
<th>MS culture method, No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>24 (21.05)</td>
<td>7 (6.14)</td>
<td>14 (12.28)</td>
<td>8 (7.01)</td>
</tr>
<tr>
<td>Negative</td>
<td>90 (78.94)</td>
<td>107 (93.85)</td>
<td>100 (87.71)</td>
<td>106 (92.98)</td>
</tr>
</tbody>
</table>

M, marker 100 bp; positive control (+); negative control (-); 15 to 33 samples; a 207 bp band characteristic for MS.

M, marker 100 bp; positive control (+); negative control (-); 1 to 14 samples; a 366 bp band characteristic for MG.
Sequencing: Alignment data showed that the MG strains detected in our study had 97% homology to 06/14, 05/14 and 16S strains. MS strains had 98.99% homology to MSR-812, MSR-795 and MSR-1019 strains. One of the MS strains had 82% homology to ABSfSDMS2011 strain.

Serological results: Serological survey with rapid serum agglutination test demonstrated 22.80% and 30.70% of serum samples have antibodies against M. gallisepticum and M. synoviae, respectively.

DISCUSSION

In ostriches, mycoplasmas cause respiratory diseases, rhino-tracheitis, airsacculitis and upper respiratory tract inflammation (Huchzermeyer, 1994; Verwoerd, 2000). In current study pathogenic mycoplasmas from lung, trachea and air sacs of ostrich farms in Iran were isolated and identified using PCR and culture methods. The results of our study indicated that the M. gallisepticum and M. synoviae were isolated and identified in ostrich from Iran.

M. gallisepticum and M. synoviae isolated from South Africa with respiratory symptoms, regularly in winter from ostriches (Verwoerd, 2000). In northern Italy a serological survey, using the rapid plate test with specific antigen for M. gallisepticum, M. synoviae and M. meleagrisis, demonstrated positive sera to all above mycoplasmas (Peccati et al., 1996). Also, it was reported that the experimental infection of young ostriches with M. gallisepticum causes colonization in trachea (Cline et al., 1997).

It was indicated in ostrich farms of South Africa the new mycoplasma named as Mycoplasma struthiolus had infected ostriches and further studies on them showed three various M. struthiolus with 88.4, 88.7 and 93.1% sequence homology (Botes et al., 2005).

Shivaprasad (1993) reported that there were not clinical signs for the majority of mycoplasmas isolated from lung and trachea and that none of the 32 reported isolates were M. gallisepticum or M. synoviae. M. synoviae was isolated from respiratory tract infection of ostriches (Verwoerd, 2000), and immune response to M. gallisepticum and M. synoviae was documented previously (Cadman et al., 1994; Peccati et al., 1996). Experimental infection with M. gallisepticum with clinical sign also was reported (Cline et al., 1997).

It was indicated that the identification of mycoplasma species in ostriches and the evaluation of their infectivity and pathogenicity is important (Peccati et al., 1996). Due to the susceptibility of ostriches to poultry mycoplasma, it was suggested that ostrich farms should be free of poultry species (Verwoerd, 2000).

The results of our study have been demonstrated that the ostrich farms from Iran were infected with M. gallisepticum and M. synoviae. According to our results, 58.33% and 37.5% of the positive samples in PCR were identified as M. synoviae and M. gallisepticum, respectively. Studies on ostrich farms in Kerman province of Iran indicate that 52% of mycoplasma positive samples was M. synoviae and 48% of other mycoplasmas that was unidentified (Tebyanian et al., 2014). Generally, M. synoviae is considered as sub-clinical respiratory infection in poultry, but economic losses were reported (Elhamnia et al., 2016). The results of alignment analysis indicated that the majority of the MS strains, which was isolated from Iranian ostriches, had 81% and 97-99% homology to poultry origin strains of MS.

M. gallisepticum was isolated and detected from chicken farms by PCR and RFLP in the Fars province of Iran, while M. synoviae was not detected from samples (Ghaleh Golab Behbahani et al., 2005). In addition, M. gallisepticum was identified by RAPD test from different geographical areas of Iran, while M. synoviae was not identified (Peighambari et al., 2006).

The results of our study indicate only M. gallisepticum, and M. synoviae were isolated, the frequency of M. synoviae being higher than this of M. gallisepticum. In addition, our results demonstrated that the mycoplasmas isolated from ostriches have a great homology to poultry origin mycoplasmas, thus keeping poultry away from ostrich farms is necessary. Although studies in South Africa indicated that ostriches kept close to poultry were not infected with poultry mycoplasmas and they were only infected with M. struthiolus some researchers documented infections with poultry mycoplasmas in ostriches. Please add the references (Botes et al., 2005).

From the samples collected from ostriches in slaughterhouse was found high rate of infections with M. gallisepticum and M. synoviae in respirato-
ry system of ostriches. Lung was highly infected with *M. gallisepticum* and *M. synoviae*. It was indicated that mycoplasmas have infected upper respiratory tract of ostriches (Botes et al., 2005) but infections with poultry mycoplasmas and their clinical signs in ostriches was not documented clearly. It should be investigated if poultry origin mycoplasmas cause clinical signs in poultry as the samples analyzed in current study could not demonstrate the clinical signs of the disease, clearly. In addition, other organisms should be evaluated to demonstrate the pathogenesis of mycoplasma in ostriches.

PCR is a precise and ideal method for the diagnosis of mycoplasmas (Kiss et al., 1997), and the detection of infectious agent before onset of signs (Moalic et al., 1997). PCR assays could be used to early detection of infections and evaluation of ostriche-farms. To determine the prevalence of the ostriches mycoplasma infection in Iran, more study is necessary.

**REFERENCES**


