Molecular characterization of universal *Mycoplasma* and *Acholeplasma laidlawii* from buffaloes (*Bubalus bubalis*) at Karachi, Pakistan.

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Received: 21-03-2017
Accepted: 15-12-2017
DOI: 10.18805/ijar.B-737

ABSTRACT

The present study was conducted to identify and characterize the bovine *Acholeplasma laidlawii* from 1120 clinically ill buffaloes and 470 lungs collected from buffaloes at abattoir of Karachi. All buffaloes were clinically examined and around 467 (13.48%) buffaloes had signs of respiratory distress including sticky nasal discharge, sunken eyes, dyspnea and fever. All the culture positive samples were further evaluated by PCR using specific primers of *A. laidlawii* and the amplified product to re-validate the sequence of the specie. This revealed that suspected buffaloes, 92 (2.66%) from nasal discharge and 44 (4.03%) from lung tissue samples respectively were positive for *A. laidlawii*. Rests of the samples were still under investigation. However it is first time proclaimed on research basis that *A. laidlawii* was infecting the buffaloes in Pakistan and precautionary measures should be taken to prevent losses from this specie in the months of October and April.

Key words: *Acholeplasma*, Buffaloes, Clinical symptoms *Mycoplasma*, PCR.

Abbreviations: PCR: polymerase chain reaction, PPLO: pleuropnemonia like organism, DNA: deoxyribonucleic acid, NCBI: national center for biotechnology information.

INTRODUCTION

Mycoplasmosis has become a serious issue for livestock animals such as buffaloes, cattle and sheep, goats, chickens in Pakistan (Awan et al. 2010; Mukhtar et al. 2012; Amin et al. 2013; Ahmad et al. 2014). This infection is causing great economic losses in Pakistan and also in others countries including USA and Canada (Gonzalez and Wilson, 2003; Fox et al. 2005; Awan et al. 2010; Mukhtar et al. 2012; Amin et al. 2013; Ahmad et al. 2014). *Mycoplasma* is not only found in respiratory tract but it is also responsible for mastitis, inflammation of joints and genital disorders etc., (Volokhov et al. 2007; Wilson et al. 2007; Fareed et al. 2017).

Mycoplasmosis is caused by a number of *Mycoplasma* species. Amongst various species, *A. laidlawii* that was insignificant in past is now appeared mild pathogen in an experimental infection in lambs (Ahmad et al. 2014). Nevertheless, acholeplasmas are the fastidious organism and rarely detected on media routinely used in diagnostic bacteriology laboratories and they vary in their growth on selective culture media (Ahmad et al. 2014). Molecular assay (PCR) is more receptive, reliable and efficient diagnostic tool for the identification and detection of mycoplasmas (Hidetoshi et al. 2011).

The objective of the study was to isolate the respiratory acholeplasmas from sick animals and lung tissue samples collected from abattoir to identify and evaluate the significance of acholeplasmas.

MATERIALS AND METHODS

Study area: The study was carried out on buffaloes for the clinical and molecular diagnosis of bovine *Acholeplasma*. The nasal discharge from sick dairy buffaloes and affected lung tissues were collected during the year 2015 to 2016 for the isolation of acholeplasmas. All the samples were transported safely to the laboratory for further process.

Clinical sample collection: Large population of livestock animals was screened to select the dairy buffaloes on the basis of clinical assessment. Clinically ill animals (n=1120) were randomly selected for isolation and characterization of acholeplasmas. Rectal temperature of infected animals was also noted. Fresh nasal discharge (from inner nostril) were collected by cotton swab from affected animals and inoculated into sterile PPLO broth then transferred to lab in cool conditions.
Likewise, the affected lung \((n=470)\) samples were collected from buffalo at abattoir and examined macroscopically for typical lesions.

**Isolation of Acholeplasma:** For the isolation of organism, nasal discharge and lung tissue samples were processed. Affected lung tissue was triturated in PPLO medium as described (Allen *et al.* 1991). The inoculated swabbed broth and triturated suspension was filtered with 0.45µm pour size membrane syringe filter and then incubated at 37ºC in sterile broth and agar. The change of color or turbidity in broth and appearance of fried egg colonies were recorded in agar plate. The positive samples were three times streaked on agar plate for the isolation and purification of *Acholeplasma* species.

**Molecular assay:** DNA was extracted from each positive sample by using kit (Miniprep DNA Extraction Kit-Qaigen, USA) as per following instructions given in the kit method literature. The extracted DNA was stored at -20ºC for use of PCR.

The reported *Acholeplasma* nucleotide sequence of specie specific primer, UNI- 5’TAATCCCTGTGTCCTCCGAC3’ and ACH3 5’AGCCGACTGAGAGGTCTAC3’ (505bp) were used to amplify *A. laidlawii* 16s rDNA gene (Van Kuppeveld *et al.* 1992). The amplified product was run on 1% agarose (invitrogen, catalog No. 16500-500) containing 1.5µg/ml ethidium bromide and visualized by using Bio Rad gel Doc system and purified using kit (Promega PCR purification kit, Catalog No. A9281) as per manufacturer’s instructions. The PCR fragments were sequenced using dideoxy method and further validated through NCBI blast.

**RESULTS AND DISCUSSION**

*Acholeplasma* are one of the major contributors in respiratory infections with a tremendously damaging economic impact in Livestock industry. The signs with sticky nasal discharge, watery eyes, difficulty in breathing, sounds appeared on chest, off feed, emaciated, depressed, dull coat and elevated temperature were examined in 1120 buffaloes. However, the disease was more prevalent and severe during the months of January to March but lower during the months of April and June (Table 1). The high incidence rate in this quarter may be assumed due to cold effect. While positive samples other than this quarter may be considered as humid climate or also could be due to stress environment. Similar study was closely accorded as carried out in Hungry regarding the clinical study of the disease of calves associated with *M. bovis* infection (Stipkovits *et al.* 2000). Nonetheless, only 219 (6.32%) and 183 (16.76%) nasal discharge and lung tissue samples respectively were found culture positive for general mycoplasmas (Table 1). Lung lesions were observed as marble stone like lining, pale and noted enlarged (affected lobe of lung) as compare to normal lung (Figure 1) which substantiated the lung was pneumonic or affected.

<table>
<thead>
<tr>
<th>Months</th>
<th>Temperature (°C)</th>
<th>Respiratory Dyspnea</th>
<th>Coughing</th>
<th>Sneezing</th>
<th>Nasal Discharge</th>
<th>Cultural Study for general mycoplasmas (%)</th>
<th>Molecular Assay Specie Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-Dec</td>
<td>280</td>
<td>0</td>
<td>145</td>
<td>9</td>
<td>98</td>
<td>181</td>
<td>123</td>
</tr>
<tr>
<td>Jan-Mar</td>
<td>281</td>
<td>9</td>
<td>112</td>
<td>0</td>
<td>11</td>
<td>146</td>
<td>151</td>
</tr>
<tr>
<td>Apr-June</td>
<td>280</td>
<td>9</td>
<td>112</td>
<td>0</td>
<td>11</td>
<td>112</td>
<td>114</td>
</tr>
<tr>
<td>Jul-Sep</td>
<td>279</td>
<td>11</td>
<td>112</td>
<td>5</td>
<td>15</td>
<td>146</td>
<td>151</td>
</tr>
</tbody>
</table>

**Table 1:** Clinical assessment of buffalo on the basis of various parameters

The Chi-square 401.76 indicate rows and column variables are significantly associated at P value (P< 0.01).
Various lung lesions were estimated during the study and 142/470 lungs were found positive for universal mycoplasmas (Table 2). Pathological lesions and bacteriological response were also discussed on the basis of distribution of lung lobes in buffalo (Sayyari and Sharma, 2011).

On the basis of cultural study the obtained colonies of *Acholeplasma* were observed under compound microscope (40x) as fried egg like appearance. The central zone was found deep in the agar while peripheral zone appeared on the surface of the agar as described earlier (Giovanni *et al.* 1980).

This genus was isolated from the large ruminant (buffalo) as documented by many groups (Ayling *et al.* 2004; Zafar *et al.* 2011). *Acholeplasma* was identified on the basis of digitonin sensitivity and morphological characterization but the pathogenicity of *Acholeplasma* was not highlighted due to its non-pathogenic or ubiquitous in nature (Zafar *et al.* 2011). Pathological description described by (Kulp and Kuehn, 2010; Vladislav *et al.* 2014) that the extracellular membrane vesicles which is composed of lipopoly saccharide, lipids, soluble or membrane associated proteins, genetic materials and other factors associated with virulence are boosted with virulent protein which is secreted by *A. laidlawii*.

*A. axanthum* specie was isolated from ewes vulvar in United Kingdom, on the other hand, it was also recovered from pneumonic lesions of small animals including sheep and goats in India (Banerjee *et al.* 1979). *A. laidlawii* was also reported from Japanese homebred cattle and cows (Uemura *et al.* 2010).

Furthermore, *A. oculi* was recovered from sheep eyes in case of conjunctivitis or keratoconjunctivitis (Arbuckle and Bonson, 1979). The variable percentages of *A. laidlawii* from different animals were determined (Banerjee *et al.* 1979; Taoudi *et al.* 1985) which are closed agreement with our study.

In present study all the isolates were confirmed through PCR assay as described by Van *et al.* (1992); Roulland *et al.* (1994) and on PCR basis *Acholeplasma* was detected from field nasal discharge and lung tissue were 201 (5.80%) and 142 (13.0%) respectively (Fig 2).

All the general *Mycoplasma* / *Acholeplasma* positive samples were also analyzed using specie specific primer of *A. laidlawii*. Clinical nasal swabs were found positive 92 (2.66%) followed by lung samples 44 (4.03%) (Fig 3).

Tenk *et al.* (2005) illustrated that 34% buffalo lungs had pneumatic lesions; while mycoplasmas were isolated from 60% of the lung samples tested. In 25% of the cases *M. bovis* was isolated from lungs with no macroscopic lesions. But contrary, samples collected in our study showed massive strength of clinical symptoms and macroscopic lesions, however,

Table 2: Macroscopic Assessment of Buffalo Lungs on the Basis of Various Lesions

<table>
<thead>
<tr>
<th>Months</th>
<th>Animals Observed</th>
<th>Bronchitis</th>
<th>Marbled stone like linings with Pale coloration</th>
<th>Nodular Formation</th>
<th>Cultural Study for general mycoplasmas</th>
<th>Specie specific Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-Dec</td>
<td>120</td>
<td>43</td>
<td>26</td>
<td>4</td>
<td>41</td>
<td>12 (25.82%)</td>
</tr>
<tr>
<td>Jan-Mar</td>
<td>118</td>
<td>51</td>
<td>44</td>
<td>11</td>
<td>90</td>
<td>18 (36.36%)</td>
</tr>
<tr>
<td>Apr-June</td>
<td>116</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td>12</td>
<td>2 (14.38%)</td>
</tr>
<tr>
<td>Jul-Sep</td>
<td>116</td>
<td>24</td>
<td>29</td>
<td>6</td>
<td>40</td>
<td>12 (23.44%)</td>
</tr>
<tr>
<td>G. Total</td>
<td>470</td>
<td>127</td>
<td>103</td>
<td>23</td>
<td>183</td>
<td>44 (4.03%)</td>
</tr>
<tr>
<td>Percentage</td>
<td>(43.04%)</td>
<td>(11.63%)</td>
<td>(9.43%)</td>
<td>(2.11%)</td>
<td>(16.76%)</td>
<td>(4.03%)</td>
</tr>
</tbody>
</table>

The Chi-square 101.6 indicate rows and column variables are significantly associated at P value (P<0.01).

Fig 1 (a): Asymptomatic buffalo lung without any inflammation and hemorrhages

(b): Showing enlarged, swollen, pale coloration & marbled stone lining on buffalo lung surface
Fig 2: Amplified products of Universal Mycoplasma/ Acholeplasma with a product size of 270 bp.
Where L: Ladder (100bp of Promega); C: Positive Control for universal mycoplasmas; and 1 to 6 (field nasal samples) and 7 to 12 (lung tissue samples) showed positive samples with reported Universal Mycoplasmas/ Acholeplasmas primers.

Fig 3: Amplified product of specie specific (A. laidlawii) primer with product size of 505 bp.
Where L: Ladder (100bp of Promega); C: Positive Control; 1 to 3 (field nasal samples) and 4 to 5 (lung tissue samples) showed positive samples all the samples were not found positive for general mycoplasmas. It could be suggested that the symptoms and lesions might be due to many other predisposing factors or pathogens.

The specie specific amplified products were sequenced and compared with data present in GeneBank by using the blast program. The sequence was found highly similar to A. laidlawii with 99% similar index.

A. laidlawii is an agent commonly isolated from lungs or respiratory tract of buffaloes, usually during January to March. Further, the isolation and PCR based identification of A. laidlawii is very significant from buffaloes lungs but its pathogenic description is very rear. More study would be required to investigate the virulence by performing experimental trials.

REFERENCES


