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RESEARCH ARTICLE

MOLECULAR CHARACTERIZATION OF UNIVERSAL MYCOPLASMAS AND *ACHOLEPLASMA LAIDLAWII* FROM BUFFALOES AT KARACHI, PAKISTAN

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ABSTRACT

The present study was conducted to identify the bovine *Mycoplasmas/Acholeplasmas* on clinical examination and PCR based laboratory confirmation from 1590 buffaloes and 470 lung samples collected from Abattoir Karachi during 2014 to 2016. Clinical field survey along with macroscopic examination revealed that total 467 (13.48%) buffaloes had signs of respiratory distress including sticky nasal discharge, sunken eyes, dyspnea and fever. Out of 470 lung samples collected from slaughter house183 (16.76%) were found culture positive for Mycoplasma species. All the culture positive samples were further evaluated by PCR using universal mycoplasma primers. PCR revealed that 201 (5.80%) nasal discharge from buffaloes and 142 (13.00%) lung tissues collected from abattoir were positive for mycoplamas infection. The PCR for *Acholeplasma laidlawii* was also performed using specie specific primers and the amplified product was sequenced for re-validate the specie. This revealed that 92 (2.66%) nasal discharge samples were positive; and 44 (4.03%) lung samples were positive for *Acholeplasma laidlawii*. It is concluded that the frequently isolation and molecular identification of *Acholeplasma laidlawii* from nasal discharge and lung tissue samples are very significant and this study also indicated alarming months for dairy farmers for precautionary measures.

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INTRODUCTION

Mycoplasmosis has become a serious issue for livestock animals such as buffaloes, cattle and sheep, goats, chickens. This infection is causing great economic losses in Pakistan and also in others countries including USA and Canada (Fox *et al*., 2005; Gonzalez and Wilson, 2003). Mycoplasma is not only causing respiratory symptoms but it is also responsible for mastitis, inflammation of joints, genital disorders etc., (Pfutzner and Sachse, 1996; Wilson *et al*., 2007). Many of other factors, like bacteria, fungi, virus or even climate conditions may also contribute significantly in bovine respiratory symptoms (Poumarat *et al*., 2001). Mycoplasmosis is caused by a number of Mycoplasma species. Amongst various species, Acholeplasma is insignificant in past but now appeared mild pathogen in an experimental infection in lambs (Concha *et al*., 1996). However, two lambs developed swelling of joints and were unable to move or reluctant to move and besides this the rectal temperature was also elevated. On the basis of gross pathological lesions a small area of intermediate lung lobe was observed consolidated (Concha *et al*., 1996). In another study, disgusting lesions of the lungs were recorded as broaden interlobular septa with inflammation that resulting in marbled stone like lining of lung(s). After the outbreak of respiratory disease the infection spreads rapidly in the animal herds (Pfützner *et al*., 1983; Pfutzner and Schimmel 1985; Stipkovits *et al*., 2000). Nevertheless, mycoplasmas/ 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 (Whitford, 1994). Eventually culturing is the Gold standard for diagnosis of infectious diseases, but it is generally laborious and time consuming. Molecular assay (PCR) is more receptive,

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reliable and efficient diagnostic tool for the identification and detection of Mycoplasmas (Hidetoshi *et al*., 2011). An effective and true detection is very imperative to manage the disease with immediate effect (Pfützner and Sachse, 1996). The objective of the study was to isolate the respiratory mycoplasmas/ acholeplasmas from nasal discharge of buffaloes and lung tissue samples.

MATERIALS AND METHODS

Study Area

The study was carried out for clinical and molecular diagnosis of bovine mycoplasmosis/acholeplasmas in live and slaughtered buffaloes. The nasal discharge from live buffaloes and affected lung tissues from slaughtered buffaloes were collected during the year 2014 to 2015 for the isolation of mycoplasmas. All the samples were transported safely to the laboratory for further process.

Clinical Survey & Sample Collection

Livestock farms and herds were visited regarding the clinical assessment. In this respect, large population of livestock animals was surveyed. The animals (*n=1120*) were randomly examined for respiratory signs and symptoms with the coordination of five veterinary experts. 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 transported to lab in cool conditions. Likewise, the affected lungs (*n=470*) tissue samples were collected from slaughtered buffaloes from abattoir, Karachi and examined macroscopically for typical lesions.

Isolation of Mycoplasma

Isolation of organism from nasal discharge samples and lung tissue samples were processed. Affected lung tissue was triturated in PPLO medium as described by Allen *et al.,* 1991. The inoculated swabbed broth and triturated suspension was filtered (0.45µm pour size membrane filter) in sterile broth and agar respectively then incubated at 37ºC till color change or turbidity of broth and appearance of fried egg colonies in agar plate. The positive samples were streaked on agar plates three times for the isolation and purification of Mycoplasma 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 Mycoplasma universal reported primer MGSO and GPO1 with nucleotide sequence 5'-TGCACCATCTGTCACTCTGTTAACCTC-3' and 5'GGGAGCAAACACGATAG ATACCCT-3' (270 bp) respectively were used to amplify 16s rDNA gene (Van Kuppeveld *et al*. 1992; Roulland-Dussoix *et al*. 1994). The reported nucleotide sequence of specie specific primer, UNI-5'TAATCCTGTTTGCTCCCCAC3', and ACH3 5'AGCCGG ACTGAGAGGTCTAC 3' (505bp) were used to amplify *A. laidlawii* 16s rDNA gene following protocol described by 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

Mycoplasmas/ Acholeplasmas 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 (Figure 1), difficulty in breathing, sounds appeared on chest, off feed, emaciated, depressed, dull coat and elevated temperature was recorded in more than 400 buffaloes examined.

Table 1. Clinical Assessment of Buffalo on the Basis of Various Parameters

Months	Animals Observed	Clinical Assessment Parameters						Cultural Study for general mycoplasmas	Molecular Assay (PCR)		
		Temperature elevated	Respiratory sounds	Dyspnea	Coughing	Sneezing	Nasal Discharge		Universal mycoplasmas	Specie specific (A. *laidlawii*)	Percentage
Oct-Dec	280	191	145	9	98	11	123	57	57	31	28.93%
Jan-Mar	281	146	112	0	114	18	151	82	74	36	29.27%
Apr-June	280	110	12	55	9	0	77	25	25	12	17.47%
Jul-Sep	279	185	97	38	15	0	116	55	45	13	24.33%
G. Total	1120	632	366	109	236	29	467	219	201	92	
Percentage	(32.33%)	(18.24%)	(10.57%)	(2.94%)	(6.81%)	(0.84%)	(13.48%)	(6.32%)	(5.80%)	(2.66%)	100%

The Chi-square 401.76 indicate rows and column variables are significantly associated at P value (P<0.01)

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

Months	Animals Observed	Macroscopic Lesions of Lungs			Cultural Study for general mycoplasmas	Molecular Assay (PCR)		
		Bronchitis	Marbled stone like linings with Pale coloration	Nodular Formation		Universal Mycoplasmas	Specie specific (A. *laidlawii*)	Percentage
Oct-Dec	120	43	26	4	41	36	12	25.82%
Jan-Mar	118	51	44	11	90	65	18	36.36%
Apr-June	116	9	4	2	12	12	2	14.38%
Jul-Sep	116	24	29	6	40	29	12	23.44%
G. Total	*470*	*127*	103	*23*	*183*	*142*	*44*	
Percentage	(43.04%)	*(11.63%)*	(9.43%)	(2.11%)	(16.76%)	(13.00%)	(4.03%)	100%

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

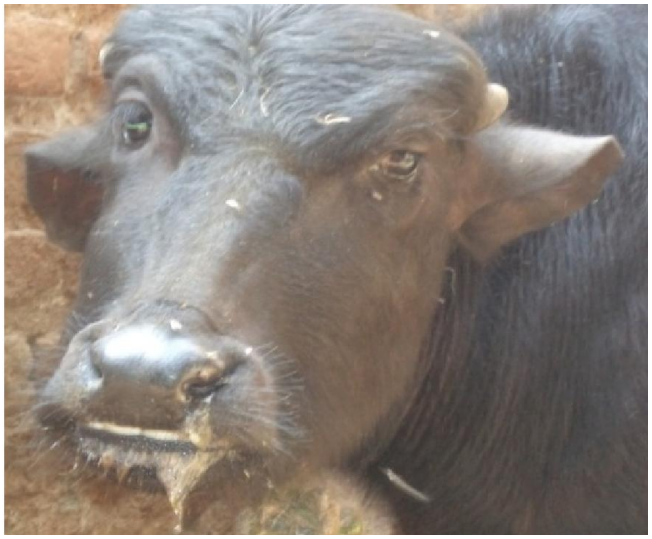
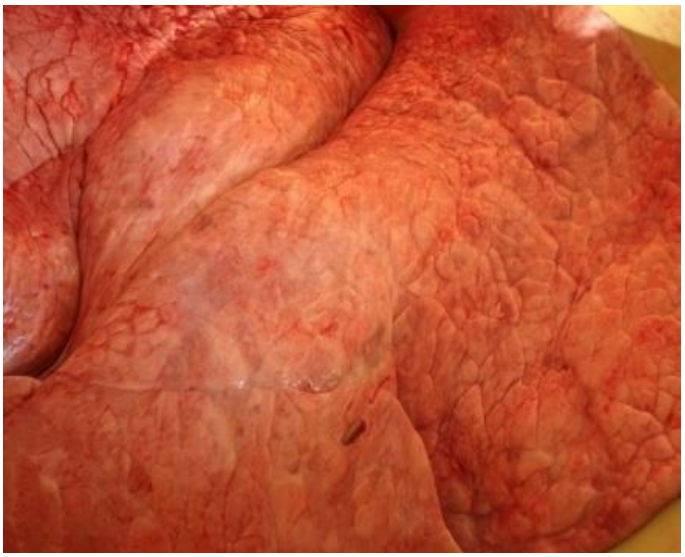
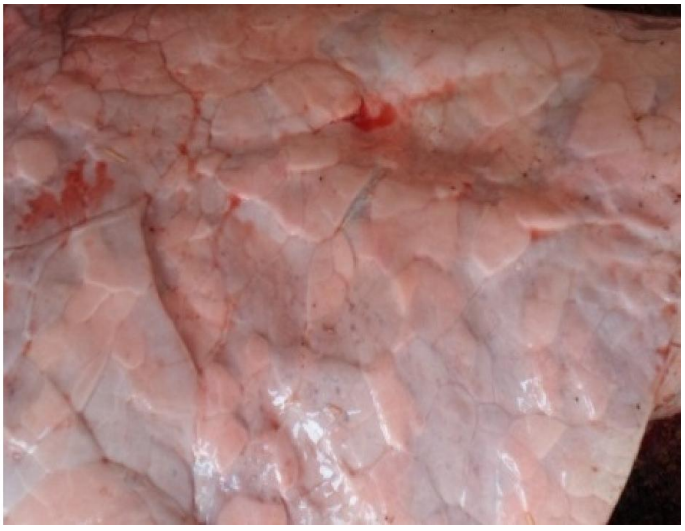


Figure 1. Unilateral sticky nasal discharge with watery and sunken eyes; Head also showing decline position due to emaciation or weakness in diseased condition



(a)



(b)

Figure 2. (a) Asymptomatic buffalo lung without any inflammation and hemorrhages (b) showing enlarged, swelled, pale coloration & marbled stone lining on buffalo lung surface

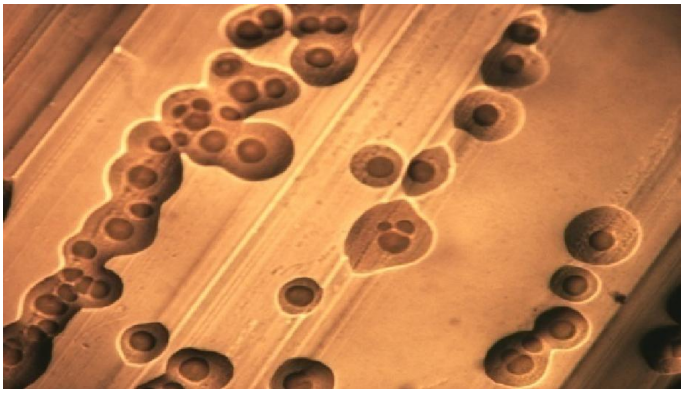


Figure 3. Mycoplasma *fried egg colony* on PPLO agar medium plate under compound microscope at (40x). Central zone of colony deep and peripheral zone appeared on surface of the agar (submerged colonies)

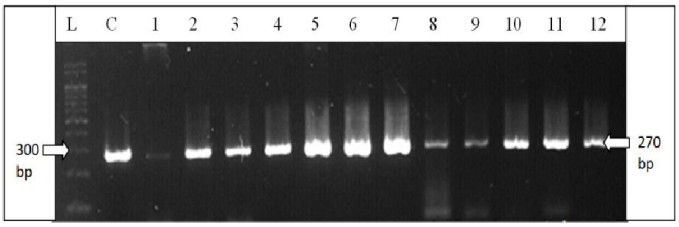


Figure 4. L: Ladder (*100bp of Promega*); C: Positive Control for universal mycoplasmas; and 1 to 6 (field nasal samples) and 7 to 12 (lung tissue samples) showed positive samples with reported Universal Mycoplasmas/ Acholeplasmas primers and the product size is 270bp

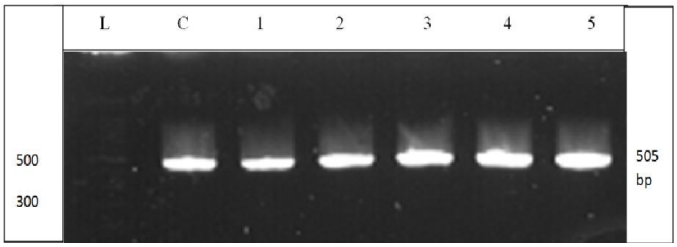


Figure 5. L: Ladder (*100bp of Promega*); C: Positive Control; 1 to 3 (nasal samples) and 4 to 5 (lung tissue samples) showed positive samples with reported Specie specific primer (Acholeplasma laidlawii) with product size 505bp

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 effect of cold weather. 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 *Mycoplasma 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 2) which substantiated the lung was pneumonic or affected. Various lung lesions were estimated during the study and 142out of 470 lung tissues were found positive for universal mycoplasmas (Table 2). Pathological lesions and bacteriological response was 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 found deep in the agar while peripheral zone appeared on the surface of the agar (Figure 3). This appearance was same as described earlier by (Giovanni *et al*. 1980). This genus was isolated from the large ruminant (water buffalo) as documented by many groups (Zafar *et al*., 2011; Ayling *et al*. 2004 and Tully 1984). Another research was carried out to identify the Mycoplasma/ Acholeplasma on the basis of digitonin sensitivity and morphological characterization but the pathogenecity of Acholeplasma was not highlighted due to its non-pathogenic or opportunistic nature (Zafar *et al*., 2011).

Acholeplasma axanthum specie was isolated from ewes vulva from United Kingdom, on the other hand, it was also recovered from pneumonic lesions of small animals including sheep and goats in India (Jones *et al*., 1983; Banerjee *et al*., 1979). Acholeplasma granularum was also reported from sheep and goats, but the researchers did not focus on the significance of this specie (Cottew, 1983). Furthermore, A. oculi was recovered from sheep eyes in case of conjunctivitis or keratoconjunctivitis (Arbuckle and Bonson 1979); moreover it was also isolated from goats (Al-Aubaidi 1975), cows, calves, horses, camels (Rae *et al*., 1987), and pigs (Cottew, 1979). The variable percentages of A. laidlawii from different animals were determined (Taoudi *et al*. 1985 and Banerjee *et al*., 1979) 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) and Roulland *et al*. (1994). Universal Mycoplasmas was detected through PCR from nasal discharge of 201 (5.80%) infected buffaloes and 142 (13.0%) lung tissue samples collected from slaughtered buffaloes (Figure 4). All the general mycoplasmas positive samples were also analyzed using specie specific primer of *Acholeplasma laidlawii.* Clinical nasal swabs were found positive 92 (2.66%) followed by lung samples 44 (4.03%) as shown in (Figure 5). Tenk *et al*. (2005) illustrated that 34% buffalo lungs had pneumonic 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, all the samples were not found positive for general mycoplasmas. It could be suggested that the symptoms and lesions may 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 100% similar index. *Acholeplasma 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's lungs but its pathogenic description is very rear. More study would be required to investigate the virulence by performing experimental trials.

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