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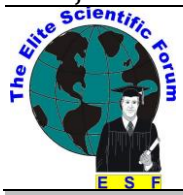

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RESEARCH ARTICLE

A Study on Pathological Effects of *Acholeplasma laidlawii* Isolated from Buffaloes in Mice Model

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ABSTRACT

Respiratory distress has become a hot issue that is causing severe infection in livestock industry of Pakistan. The exact and timely diagnosis is incredible to treat the disease. However, *Acholeplasma* (*A.*) *laidlawii* is found very significant from buffalo lungs but being a ubiquitous organism, its pathogenic description is not completely understood. The study was designed to validate the involvement of *A. laidlawii* in respiratory diseases in buffaloes. For this purpose, experimental trials on mice were conducted to confirm the involvement of the organism in respiratory tract infection. It was re-isolated from experimentally infected mice, showing lesions in respiratory tract (83.3%), proving Koch's postulates. Statistically, the experimental group-A (subcutaneous route) showed significant difference ($P<0.05$) except in case of mortality feature. The group-B (intraperitoneal route) indicated non-significant difference ($P>0.05$) in all cases. Based on current study, it may be concluded that the organism is opportunistic, and can produce either disease or lesions on targeted organs in stressed animals, particularly buffaloes.

INTRODUCTION

Acholeplasma (*A.*) *laidlawii* is a ubiquitous and common contaminant of cell culture (Windsor et al., 2010). It has been isolated from different species of animals and plants (Fareed et al., 2019). This specie belongs to the class *mollicutes*; self-replicating, wall-less bacteria which makes it resistant against antibiotics that affect the cell wall (Taylor and Bebear, 1997).

The numerous strains of *Mycoplasma* (*M.*) and *Acholeplasma* including *A. laidlawii*, *M. dispar*, *M. bovirhinis*, *M. bovigenitalium* and *M. gallisepticum* proved to cause infection in experimentally infected animals (Schnee et al., 2012; Mukhtar et al., 2012; Anderson et al., 1976). The pathogenicity of all species of *Mycoplasma* and *Acholeplasma* was found similar except the pathogenic role of *M. disper*. The appearance of lesions in lab animals were found similar as in infected cattle (Anderson et al., 1976). The *M. canis* and *M. edwardii* were the bacteria recovered from experimentally infected mice organs such as lungs, liver and spleen. *A. laidlawii* has also been reported in the heart muscles as histological examination of mice heart showed focal lymphocytic infiltrations in the myocardium (Vishnyakov et al., 2015; Eberle et al., 1977).

However, little information is available regarding the pathogenic role of *Acholeplasma* in buffaloes. Therefore, the current research was carried out to determine the pathogenic role of *A. laidlawii* in experimental mice. Nevertheless, the proposed hypothesis of this study was that *A. laidlawii* had pathogenic role in experimental mice and had similar lesions like those found in buffaloes.

MATERIALS AND METHODS

The present study was conducted in Department of Microbiology, University of Karachi, Pakistan after the approval of Ethical Committee. The organism *A. laidlawii* was isolated from buffaloes (Fareed et al., 2019) and used to study its pathogenic role in experimental mice. The experiment was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments and National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Experimental plan

The experimental trials were conducted on male mice with average weight of 22-25gm. All mice were purchased from the Institute of Panjwani Center for Molecular Medicine and Drug Research (PCMD), University of Karachi, Karachi, Pakistan. Animals were divided in A & B groups (each containing 03 mice) for induction of *A. laidlawii* infection. The test group A was designated for subcutaneous inoculation, whereas test group B for intraperitoneal route with their respective control groups (Table 1). All the mice were kept in cage (45 x 25 x 15cms) housing system with laboratory grade water and bedding in animal house of Department of Microbiology, University of Karachi under standard environmental conditions (25˚C ± 1˚C relative humidity 52 to 58%). Standard twelve-hour light and dark cycle was maintained.

Culture of *A. laidlawii*

The organism was freshly grown in Pleuropneumonia like Organism (PPLO) broth. Growth was observed by taking sample through Color Changing Unit (CCU)/ ml and Optical Density (OD_{560}).

Inoculation

The activated culture was injected at the dose rate of 0.2ml in each mouse. The subcutaneous dose was divided into two halves and injected (0.1ml each) in left and right side of Linea Alba in interior position of mice (Figure 1). The control groups were injected with sterile broth.

Post inoculation examination of mice

The entire groups were examined routinely particularly for the respiratory distress, apparently by body scoring and nasal discharges etc. The control groups were placed separately from the test groups because *Mycoplasmosis* is an infectious and contagious disease. All the groups were examined for 14 days, while on day 15 the mice were slaughtered/ dissected to analyze the necropsy changes. Moreover, all the organs were processed for re-isolation of organisms.

Necropsy examination

All mice were slaughtered for necropsy examination. The organs such as trachea, lungs, heart, kidneys, liver and spleen were macroscopically studied for typical lesions such as hemorrhages, pale coloration and nodule formation etc. All the organ tissues were processed for recovery of causative agent as described by Allen et al. (1991). Recovery of agent was also verified through PCR assay (Fareed et al., 2019). During dissection, the blood was collected and serum was separated for detecting the antibody response against *A. laidlawii* using Agar Gel Precipitation Test (AGPT).

Agar Gel Precipitation Test (AGPT)

1% agarose gel was prepared and poured onto glass slide. Wells were made with 4mm distance between wells. The surrounding wells were filled with testing sera whereas central well containing antigen. The sera of control mice were also evaluated in separate wells. The slide was kept in at refrigerated temperature under moist condition for 1-3 days and then the lines of precipitations were recorded.

RESULTS

Fresh culture of *A. laidlawii* with 10^8 CFU/ ml and Optical Density (OD_{560}) was recorded as 0.535. The dose 0.2ml of CFU/ml (10^8) was established; lesions caused morbidity and mortality in test groups (Table 2).

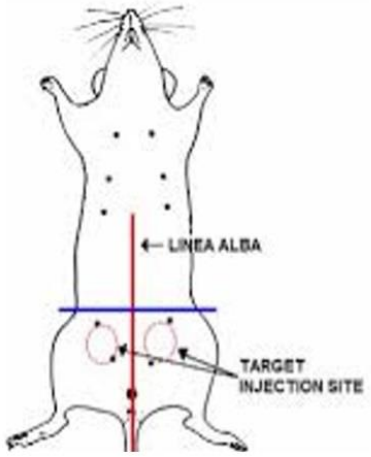

Figure 1: *Subcutaneous inoculation of A. laidlawii in Mice*

Table 1: Experimental groups of mice

Experimental group	Number of mice	Inoculation route
TG-A	3	subcutaneous (S/C) Inoculation
CG-A	3	subcutaneous (S/C) Inoculation of sterile PPLO broth
TG-B	3	Intraperitoneal (I/P) Inoculation
CG-B	3	Intraperitoneal (I/P) Inoculation of sterile PPLO broth

Where; TG- Test group, CG- Control group, PPLO-Pleuropnemonia Like Organism.

Table 2: Clinical, Postmortem and AGPT Response in Experimental Groups

Features	Experimental Mice													Culture quantification		Dose rate (ml)
	Test group A (Subcutaneous)			Control group A			P-Value	Test group B			Control group B (Intraperitoneal)			P-Value	CCU/ ml	OD_{560}	
Mice strength	1	2	3	1	2	3		1	2	3	1	2	3				
Re-Isolation	+	+	+	-	-	-	0.014	+	-	+	-	-	-	0.083			
Morbidity	++	+	+	-	-	-	0.05	-	-	+	-	-	-	0.273			
Mortality	+	-	-	-	-	-	0.237	+	-	+	-	-	-	0.083	10^8	0.535	0.2
Postmortem lesions	++	+	++	-	-	-	0.05	-	+	-	-	-	-	0.273			
AGPT response	+	+	+	-	-	-	0.014	-	+	-	-	-	-	0.273			

+: Positive reaction; ++: Highly reactive; -: Negative reaction; CCU/ ml: Colony changing unit/ milliliter; OD: Optical density; AGPT: Agar gel precipitation test; $p<0.05$ consider as significant.

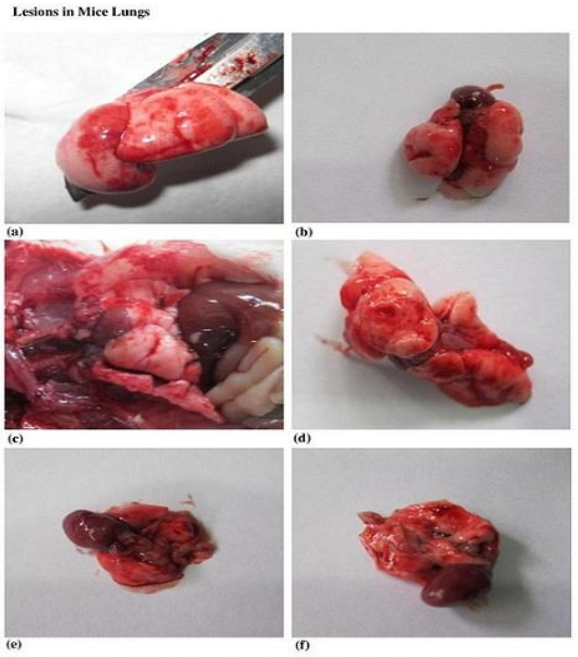

Figure 2. (a), (b) and (c) with intraperitoneal route (I/P) showing inflamed or enlarged lungs. Likewise (d) with subcutaneous route (S/C) also indicated pale, inflamed and enlarge while (e) and (f) are positive control of mice lungs which are in normal state (without inflamed and enlarged)

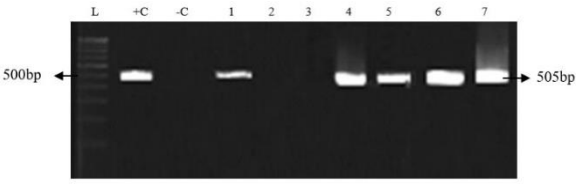

Figure 3: *The Amplified product of 505bp of re-isolated A. laidlawii from lungs and trachea of test group mice. The +C and –C showing the positive and negative controls respectively*

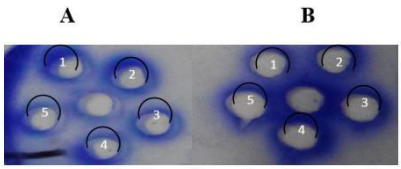

Figure. 4: *A (1-5 wells) indicated samples while central well filled with antigen. The surrounding wells are showing the line of precipitation between the central well. It is specific reaction between the antigen-antibody. B (1-5) as a control that was not showed any positive response or line of precipitation.*

Observation/examination of mice on post inoculation

Morbidity was observed in all mice of test group-A, while in the test Group-B only one mouse showed the symptoms. The morbidity was characterized by laziness, depressed and lethargic condition. However, one mouse of test Group-A was found dead within 35 hours after inoculation. Two mortalities were found in test Group-B, one within 20-24 hours, and second within 50 hours post inoculation. The live mice were bleeding on 15th day (post inoculation) including the control groups. The produced lesions on lungs were found same as occurred in buffalo lungs. The lungs of Group-A showed enlarged and pale coloration but contrary in test Group-B only one mouse represented same lesions (Figure 2).

Recovery of agent

The agent was recovered from five mice, 03 from test Group-A and 02 from test Group-B. On the other hand, it was also re-isolated from mucous present in trachea. However, the entire recovery revealed from test groups was 83.3%. Extracted DNA of re-isolated agent was also positive for *A. laidlawii* confirmed through PCR (Figure 3).

All control group mice were negative for *A. laidlawii*. In test Group-B, *A. laidlawii* was not recovered from mouse 2 but this mouse produced lesions which were suspected due to other pathogens or factors (Table 2).

Agar Gel Precipitation Test (AGPT)

Antibody response was observed in the form of line of precipitation (Figure 4A), no line of precipitation was observed in control negative groups (Figure 4B).

DISCUSSION

The fried egg like appearance of *A. laidlawii* was isolated from the large ruminant (water buffalo) lungs (Fareed et al., 2019; Ahmad et al., 2011). The variable percentages of *A. laidlawii* from various animals were reported which are similar with our study (Schnee et al., 2012; Taoudi et al., 1985; Banerjee et al., 1979). The first isolation of *A. axanthum* was reported in United Kingdom from vulvar scabs (Vishnyakov et al., 2015;

Jones et al., 1983). It was also recovered from sheep and goats. The various species of *Acholeplasma* were isolated, beside mycoplasmas, from typical pneumonic lesions (Sara et al., 2016; Taoudi et al., 1985). The pathogenic role of acholeplasmas is not clear or even it has become a controversial debate among the researchers. Furthermore, different species of *Acholeplasma* were reported from milk but its significance in such samples is not clear (Gioia et al., 2016). In present study the recovered isolates were re-confirmed through PCR assay (Fareed et al., 2019; Ahmad et al., 2011). Another study was carried out to identify the *Mycoplasma*/ *Acholeplasma* on the basis of digitonin sensitivity and morphological characterization but the pathogenicity of *A. laidlawii* was not highlighted due to considered as non-pathogenic or ubiquitous pathogen (Ahmad et al., 2011). This study validated the pathological role of *A. laidlawii* in mice. As the proteomic approach against *A. laidlawii* documented that extracellular membrane vesicle, secreted by this organism, is highly virulent and pathogenic (Chernov et al., 2014).

Immune response against *A. laidlawii* in mice on day 15 was determined by AGPT. All experimental mice showed humoral response. However, one animal did not show antibody in its serum sample. But as a whole, the antibody titer was high in test Group-A as compared to Group-B. In the subcutaneous inoculation, antigen is slowly released from injected site and antigen persists in the body for long duration that induces strong immune response. In other studies, high antibody titer against *A. laidlawii* was reported in mare measured through ELISA (Khurana et al., 2015). On the other hand, antibody titer through indirect Hemagglutination (IHA) was also recorded against *Mycoplasma bovis* (from buffalo) as described by Vladislav et al. (2014). Unfortunately, we could not determine antibody titer through IHA test due to poor yield of mice serum.

Moreover, the pathological significance was established by inoculating the *A. laidlawii* in mice through subcutaneous (Group-A) and intraperitoneal (Group-B) route. The organism was recovered from almost all infected mice. Some previous studies also reported the similar findings that *A. laidlawii* resulted in changes in lungs of experimentally infected mice (Marantidi et al., 1978; Audrey et al., 2017). Intraperitoneally, *A. laidlawii* inoculation in mice showed focal lymphatic infiltration in the myocardium. Similarly, *M. canis* and *M. edwardii* (PG 24) were re-isolated from lung, liver and spleen (Eberle et al., 1977).

This study fulfills the Koch's postulate and confirms that *A. laidlawii* is opportunistic agent and causes pathological changes under suitable conditions. Experimental infection in mice produces lesions, especially on lungs, which were similar to the lesions seen in buffalo lungs. Nonetheless, this study demonstrated that the *A. laidlawii* is pathogenic or opportunistic agent for buffaloes, therefore, precautionary measures should be taken to prevent damages from this bacterial specie.

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Authors' contribution

All the authors have equally contributed in this study and manuscript preparation.

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