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PREVALENCE OF *MYCOPLASMA CAPRICOLUM SUBSPECIES CAPRICOLUM* AND *MYCOPLASMA PUTREFACIENS* IN GOATS IN PISHIN DISTRICT OF BALOCHISTAN

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ABSTRACT

Several Mycoplasma species cause serious and economically important diseases in goats world-wide. Forty goat flocks in the Pishin district of Balochistan province of Pakistan were examined for the clinical cases of contagious caprine pleuropneumonia (CCPP) during 2008. Thirty goats suspected for CCPP on the basis of respiratory symptoms were euthanized for post mortem examination, microbiological and molecular studies. Two types of Mycoplasma species were isolated and identified by biochemical and growth inhibition (GI) tests and polymerase chain reaction (PCR). *Mycoplasma capricolum* subspecies *capricolum* was isolated from 12(40%) of the nasal swabs and 12(40%) of the respective lung cultures, whereas 2(6.7%) *Mycoplasma putrefaciens* (*Mp*) isolates from nasal swab and lung cultures and 1(3.3%) from liver and intestine cultures were recovered. We report probably for the very first time the isolation and identification of *Mycoplasma capricolum* subspecies *capricolum* (*Mcc*) and *Mp* from the nasal swabs and lungs of goats with respiratory problems in Pishin district of Balochistan. Experimental studies to reproduce pneumonia or pleuropneumonia by *Mcc* and *Mp* organisms in susceptible goats or other laboratory animal models are further needed.

Key words: Mycoplasma, goats, pneumonia, isolation, identification, PCR.

INTRODUCTION

Mycoplasmas are smallest fastidious bacteria which can cause diseases in major species of animals including humans. In small ruminants, they are known for respiratory disease, arthritis, eye lesions, genital disease and mastitis (Nicholas, 2002; Sharif and Muhammad, 2009). Most of the members of *Mycoplasma mycoides* cluster group are the important pathogens for small ruminants. This group comprises six species and subspecies. Some of these Mycolasma species can cause severe and contagious diseases in goats with significant economic impact (Cottew *et al*., 1987). Of the many Mycoplasma diseases, contagious caprine pleuropneumonia (CCPP) is a highly fatal disease that occurs in Eastern Europe, the Middle East, Africa and Asia (Kopcha, 2005).

The majority of the goats in Pakistan are produced on small farms which are distributed almost evenly across the country. The production systems are nomadic, transhumant and sedentary (Ishaque, 1993). Of the 53.8 million goats in Pakistan, 11.8 million are present in Balochistan (Anon, 2006). Of the many diseases, the goat population is prone to many respiratory diseases and cold winter and poor husbandry practices are the important predisposing factors (Tariq, 1980).

Pishin is one of the districts having dense population of goats and has borders with Afghanistan. There is a consistent influx of goats from Afghanistan to this part of Balochistan. A ready to use bacterin, adjuvated with saponin from the *Mycoplasma mycoides* subspecies c*apri* (PG-3 strain) is available and massively used by the goat farmers throughout Balochistan, but caprine pneumonia with or without pleuritis with high morbidity and mortality is still endemic. Moreover, pneumonia along with arthritis, mastitis, keratoconjunctivitis and abortion is noticed in many parts of Balochistan (Tariq, 1980). Seroprevelance of *Mycoplasma mycoides* subspecies *capri* (PG-3 strain) in ruminants and camel was reported in Faisalabad district of Pakisatan, using counter current immunoelectrophoresis (CIE) and indirect haemagglutination (IHA) test (Rahman *et al.*, 2006). Out of 1288 serum samples, highest prevalence of 7.3 (CIE) and 1.6% (IHA) was recorded in goats, followed by sheep, cattle, buffaloes and camels.

Mycoplasma diseases may not be diagnosed solely on the basis of clinical signs, pathological lesions or serological tests because of the close association among the Mycoplasma organisms. Isolation and identification are, therefore, required to confirm diagnosis, but this requires a specialist laboratory with experience of these very fastidious organisms. The classical methods for detecting and identifying mycoplasmas are time consuming and complicated by serological cross-reactions between the closely related organisms.

Molecular diagnosis has improved their detection and identification, specifically the polymerase chain reaction (PCR) for *Mycoplasma capricolum* subspecies *capripneumoniae* (*Mccp)* (Woubit *et al.*, 2004), the *Mycoplasma* "*mycoides* cluster" and *Mycoplasma* "*mycoides* sub cluster" coupled with restriction fragment length polymorphisms (RFLP) (Bashiruddin *et al*., 1994) and the recently introduced PCR and denaturing gradient gel electrophoresis (DGGE) method (McAuliffe *et al*., 2005).

Previous work on Mycoplasma diseases in goats in Pishin, Balochistan has been limited (Tariq, 1980; Awan *et al*., 2004). This study recorded for the first time the isolation and identification of *Mycoplasma capricolum* subspecies *capricolum (Mcc)* and *Mycoplasma putrefaciens* (*Mp)* on the basis of biochemical, serological and PCR tests from the nasal swabs and lung cultures of goats in Pishin district of Balochistan, Pakistan.

MATERIALS AND METHODS

Study area

Pishin is one of the larger districts in Balochistan with temperatures ranging from -14^{0}C in winter to 40^{0}C in summer. There is continuous migration of small ruminants from Afghanistan to adjoining parts of Balochistan, having the partial enforcement of quarantine regulations.

Sample size and sampling

During 2008, forty goat herds with a total population of 2010 animals were visited in the Pishin district of Balochistan. The goats were examined for the presence of respiratory signs and symptoms. Thirty sick goats with varying degrees of respiratory manifestations were selected and brought to the animal facility in the Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta.

Ante-mortem examination was carried out on all of the 30 goats and nasal swabs were also taken for immediate processing. The goats were euthanized two days later and post-mortem examination was performed on each of the goat. The lungs and swab cultures from trachea, kidneys, fore-limb joints, liver, intestines and eyes from each of euthanized goats were collected and processed for the isolation of Mycoplasma species.

Isolation and identification of Mycoplasma species

The nasal swabs and samples from lung, trachea, liver, kidneys, intestine, fore-limb joints and eyes were cultured in contagious caprine pleuropneumonia (CCPP) medium such as modified CCPP broth and agar media (OIE, 2008) following methods described by Nicholas and Baker (1998). Briefly, all of the cultured tubes and plates were incubated at 37^{0}C in a humidified CO_2 (5%) incubator. The broth cultures were observed daily for any color change or presence of cloudiness indicating growth of Mycoplasma. The solid agar medium plates were also examined every two days for the presence of "fried egg" micro-colonies of Mycoplasma species using the stereomicroscope (35 x).

Isolates were individually purified and subjected to digitonin sensitivity and a series of biochemical tests such as glucose fermentation, arginine decarboxylation, casein digestion, liquefaction of inspissated serum, phosphatase activity, and reduction of 2, 3, 5, triphenyl tetrazolium hydrochloride for identification of Mycoplasma species (Poveda, 1998). The growth inhibition test (GIT), as described by Poveda and Nicholas (1998), was also performed to further confirm the members of the Mycoplasma "*mycoides* cluster" and other prevailing species of Mycoplasma by using homologous polyclonal specific rabbit antisera.

Polymerase Chain Reaction

DNA was purified from the cultures of triple cloned isolates of Mycoplasma species, using the genomic DNA purification kit (PUREGENE – Gentra System, USA). The purified genomic DNA samples were stored in micro tubes (1.5 ml) at -20^{0}C until tested.

Polymerase chain reaction (PCR) tests for the detection of *Mycoplasma* "*mycoides* cluster" members, *Mycoplasma mycoides* sub cluster (*Mmm*SC, *Mmc, Mmm*LC) members (Bashiruddin *et al*., 1994), *Mccp* (Woubit *et al*., 2004) and *Mp* (Shankster *et al*, 2002) were performed on DNA samples from purified cultures of Mycoplasma isolates. All the primers used in this study were synthesized from Gene-Link, USA.

Mycoplasma "*mycoides* cluster and *Mycoplasma* "*mycoides* sub cluster" members produced PCR products (Amplicon) of 1500 and 574 bp, respectively. Moreover, *Mycoplasma capricolum* subspecies *capripneumoniae* (*Mccp*) and *Mycoplasma putrefaciens* produced PCR products of 316 and 800 bp, respectively.

RESULTS

Ante-mortem examination

Of the 30 goats, 24(80%) showed marked respiratory symptoms such as watery to thick nasal discharge, difficult breathing and temperatures ranging from 38.2 to 40^{0}C. Two goats showed signs of pain on touching the chest. None of the goats showed lameness or any other clinical manifestation.

Post-mortem examination

Gross pathology during post mortem examination revealed various degrees of pneumonia in 27 goats. Fibrinous pleuropneumonia was observed in four goats.

Thirteen goats had lungs adhered to the inner chest cavity. None of the goats showed hydrothorax. Lobar (n=15) and broncho-pneumonia (n=8) were the consistent finding in the investigated goats.

Isolation and identification

Mycoplasma capricolum subspecies *capricolum* isolates grew within 3-5 days, whereas *Mp* grew within 2-3 days, with a characteristic putrefying smell. The colonies produced by the *Mcc* isolates were comparatively larger than the *Mp* isolates.

All the isolates were confirmed as members of Mycoplasma genus on the basis of digitonin sensitivity test prior to the biochemical, GIT and different PCR tests (Table 1). *Mycoplasma capricolum* subspecies *capricolum and Mp* produced a growth inhibition zone greater than 4 mm with the specific polyclonal rabbit antiserum.

Of the six members of *Mycoplasma* "*mycoides* cluster" (M. m cluster), only *Mycoplasma capricolum* subspecies *capricolum* and a non M. m. cluster member such as *Mp* were isolated. *Mycoplasma capricolum* subspecies *capricolum* was isolated from 12(40%) of the nasal swab and 12(40%) of lung cultures, whereas 2(6.7%) isolates of *Mp* from each of the nasal swab and lung cultures and 1(3.3%) from each of liver and intestine cultures were isolated (Table 2). None of Mycoplasma species was isolated from the trachea, kidneys, fore limb joints, and eyes of the goats at post-mortem.

Polymerase Chain Reaction

The results of PCR (Table 3) for M. m cluster (Fig. 1), sub cluster members (Fig. 2), and *Mccp* on the Mycoplasma isolates from nasal swabs and lung cultures excluded all members of Mm cluster except 24 isolates of *Mcc* (nasal swab culture n=12, and lung culture n=12) which were also characterized by the biochemical and GI tests. The *Mp* isolated from nasal swab (n=2), lung samples (n=2), and one from each of liver and intestine cultures were positive in specific PCR for *Mp* (Fig. 3).

DISCUSSION

In the present study, the isolation and identification of caprine respiratory Mycoplasma species were carried out in Pishin district of Balochistan. The isolation of *Mmc* from the lung tissues of goats in few areas of Balochistan (Tariq, 1980) has previously been reported on the basis of classical biochemical tests.

Overall, the high prevalence rate of *Mcc* in nasal swabs and lung samples of goats is alarming in the present investigation. In addition, the isolation of *Mp* from the lungs, nasal swabs, liver and intestine of goats was also unexpected, and stressing to further explore their pathogenic role in caprine respiratory problems. *Mycoplasma mycoides* subspecies *mycoides,* large colony *(Mmm*LC), *Mmc and Mccp* could not be recovered from any of the processed tissue samples of goats.

Undoubtedly, previous studies have demonstrated that Mycoplasma infections in goats are widespread within Pakistan, particularly in Balochistan (Tariq, 1980). Besides the conventional biochemical and serological (GIT) tests, we have now introduced molecular method (PCR) into our laboratory to detect and identify these organisms that cause such economically important diseases. However traditional test methods are still required to confirm the identification of some Mycoplasma isolates, specifically *Mcc.*

The presence of arthritis especially of the fore limbs in young goats is quite common in this part of Balochistan. In this study, none of the *Mcc* organisms was recovered from any of fore limbs joints of goats, indicating chronic infection by these organisms. *Mycoplasma putrefaciens* is also one of the causative agents of CA syndrome (OIE, 2008). Besides mastitis, it is also reported to inflict septicaemia in kids and arthritis in adults (Peyraud *et al*., 2003) and polyarthritis in kids in Spain (Rodriguez *et al.*, 1994). It was also reported that *Mp* can be isolated from animals with and without clinical signs (Mercier *et al.*, 2001), suggesting that a carrier status could occur. In the present study, the isolation and characterization of *Mp* especially from the nasal swabs and lung cultures of goats from Pishin district can not be ignored and its prevalence and pathogenic role should be investigated in goats in other parts of Balochistan.

The causative agent of CCPP, *Mccp* was not isolated during this study. This could be due to the extremely fastidious nature of the organism, the widespread and indiscriminate use of antimicrobials, especially the macrolides, tetracyclines and fluoroquinolones. The confirmation of purified isolates of *Mcc* and *Mp* from nasal swabs and lung cultures through PCR in this study has also been carried out.

None of the *Mmm*LC and *Mmc* organisms have been isolated from the investigated goats in Pishin district. On the other hand, the high prevalence of *Mcc* and low prevalence of *Mp* in goats is also alarming. These results are in agreement with the study carried out by Waleed *et al.* (2006) in which significantly high number of *Mcc* and *Mp* bacteria were isolated from nasal and milk samples of goats. We report probably for the very first time the isolation and characterization of *Mcc* and *Mp* on the basis of biochemical, GI and PCR tests for the diagnosis of caprine pneumonia in Balochistan, Pakistan.

In conclusion, the use of classical biochemical and serological tests along with the PCR may be used for the identification of Mycoplasma species such as *Mcc* and *Mp* from nasal swabs and lung cultures of goats.

Table 1: Identification of Mycoplasma isolates by biochemical and growth inhibition tests from goats

Source of samples- goat tag #	Standard Biochemical Tests [a, f]								GIT [e] + ve for	*Mycoplasma* species identified as
	b	1	2	3	4	5	6	7		
Liver-233	+	+	-	+	+	-	+[W]	+	*Mp*	*Mp*
Intestine-237	+	+	-	+	+	-	+[W]	+	*Mp*	*Mp*
Nose/Lung-239[c]	+	+	-	+	+	-	+[W]	+	*Mp*	*Mp*
Nose/Lung-240[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-244[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-245[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-246[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-247[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-248[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-249[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-250[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-251[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-252[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-253[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-254[d]	+	+	+	+	-	+	+	+	*Mcc*	*Mcc*
Nose/Lung-326[c]	+	+	-	+	+	-	+[W]	+	*Mp*	*Mp*

[a] Biochemical tests performed as described by Poveda (1998); [b] Digitonin sensitivity; [c] *Mycoplasma putrefaciens (Mp)* isolates from nose and lung +ive or –ve; [d] *Mycoplasma capricolum* subsp. *capricolum (Mcc)* isolates from nose and lung +ive or –ve; [e] Poveda and Nicholas, (1998); [f] Results of standard biochemical tests as described by Nicholas (2002); [1] Glucose fermentation; [2] Arginine hydrolysis; [3] Phosohatase activity; [4] Film & spot; [5] Casein digestion; [6] Tetrazolium HCL reduction (aerobic); [7] Tetrazolium HCL reduction (anaerobic); [+] Positive; [-] Negative; +[W] weak positive.

Table 2: *Mycoplasma* species isolated from goat samples[a] in Pishin district of Balochistan

Nasal swab	Lung	Liver	Intestine
Mcc 12 (40%)	*Mcc* 12 (40%)	*Mcc* 0 (0%)	*Mcc* 0 (0%)
Mp 2 (6.7%)	*Mp* 2 (6.7%)	*Mp* 1 (3.3%)	*Mp* 1 (3.3%)

Mcc: Mycoplasma capricolum subspecies *capricolum; Mp: Mycoplasma putrefaciens*

[a] None of the Mycoplasma species were isolated from samples of trachea, kidneys, fore-limb joints and eyes.

Table 3: PCR based identification of *Mycoplasma* species isolated (cultures) from goats

Source of samples- goat tag #	PCR [a] *M. mycoides* cluster	PCR [a] *M. mycoides* sub-cluster	PCR [b] *Mccp*	PCR [c] *Mp*
Liver- 233	-ve	ND[*]	ND[*]	+ve
Intestine-237	-ve	ND[*]	ND[*]	+ve
Nose/ Lung-239[c]	-ve	ND[*]	ND[*]	+ve
Nose/ Lung-240[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-244[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-245[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-246[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-247[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-248[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-249[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-250[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-251[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-252[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-253[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-254[c]	+ve	-ve	-ve	ND[!]
Nose/ Lung-326[c]	-ve	ND[*]	ND[*]	+ve

[a] Bashiruddin *et al*., (1994); [b] Woubit *et al*., (2004); [c] PCR + ve or –ve for Mycoplasma culture isolated from both nose and lung samples; [*]ND: Not done because sample was -ve in PCR for *M. mycoides* cluster members; [!] ND: Not done because sample was +ve in PCR for *M. mycoides* cluster members.

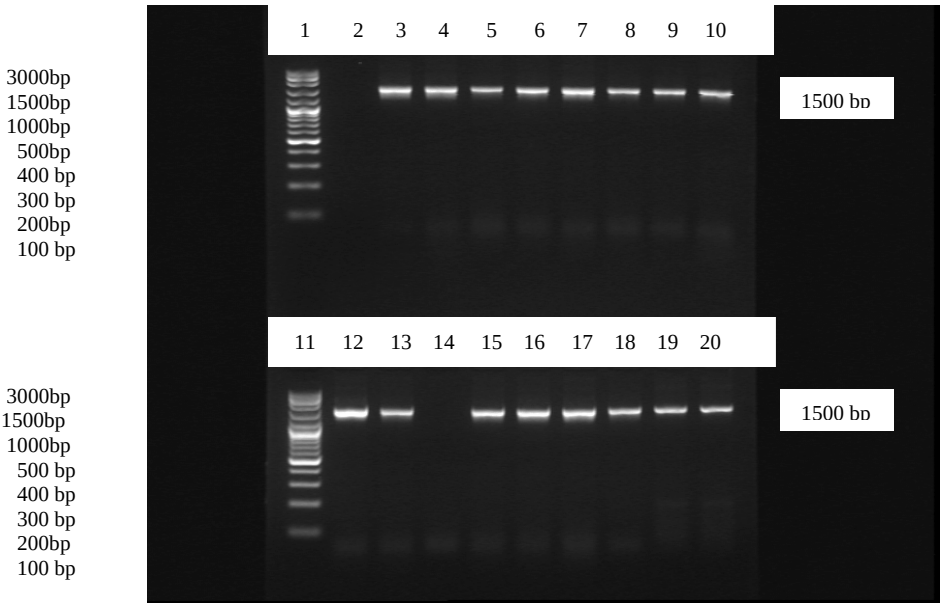

Fig. 1: PCR for the members of *M. mycoides* cluster showing an amplicon size of 1.5Kbp. Lane 1 & 11 Molecular Ladder; lane 2 –ve control; lanes 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 15, 16, 17and 18 test samples +ve for Mycoplasma cluster members; lane 14 –ve sample; lanes 19 & 20 *Mmc* and *Mcc* +ve controls respectively.

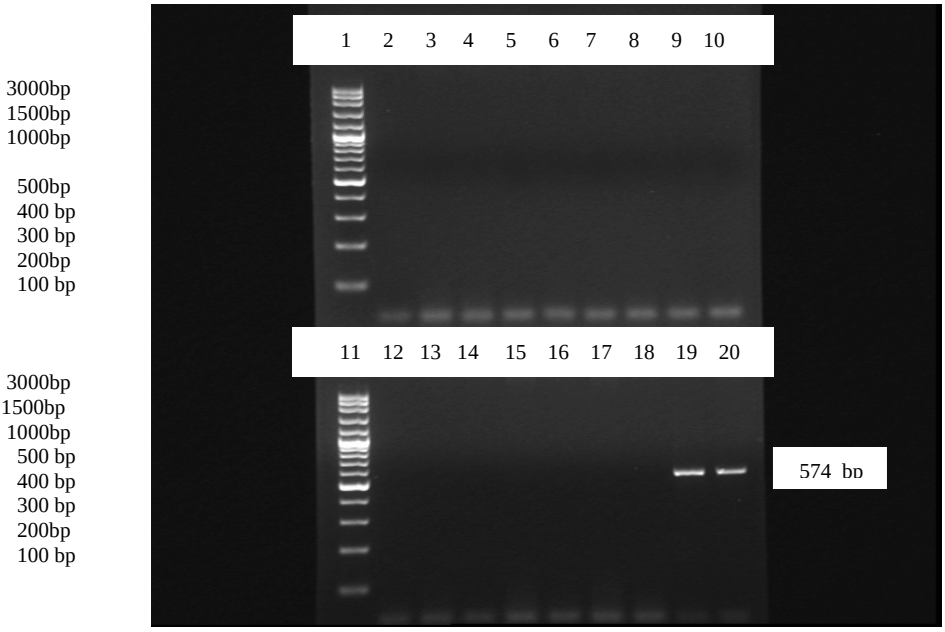

Fig. 2: PCR for the members of *M. mycoides* sub cluster showing an amplicon size of 574bp. Lane 1 & 11: Molecular Ladder; lane 2 –ve control; lanes 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17 and 18 test samples –ve for *M. mycoides* sub cluster members; lanes 19 & 20 *Mmm*LC and *Mmc* +ve controls, respectively.

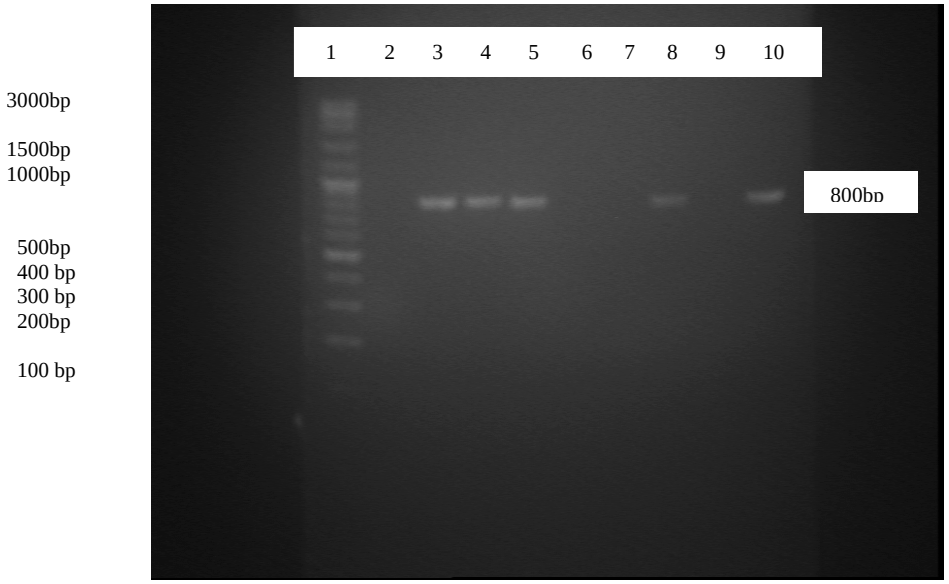

Fig. 3: PCR for the *Mycoplasma putrifaciens* (*Mp*) showing an amplicon size of 800bp. Lane 1 Molecular Ladder; lane 2 –ve control; lanes 3, 4, 5 and 8 test samples +ve for *Mp*; lanes 6, 7 and 9 test samples ve for *Mp*, and lane 10 *Mp* +ve control.

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