

# Isolation, Molecular Identification of *Mycoplasma Putrefaciens* Local Strains and Its Associated Pathological Lesions in Goats

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## Research Article

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# Abstract

*Mycoplasma putrefaciens* (Mp) is a dreadful pathogen that causes a severe, usually fatal disease in domestic goats known as MAKEPS syndrome in small ruminants. The present lack of effective diagnostic and control measures leaves Pakistani farmers at perpetual great risk of financially disastrous MAKEPS outbreaks. Thus far, very limited information is available on the detection and molecular characterization of the unique Mp strains prevalent in Pakistan. This study was proposed to apply modern molecular techniques to characterize endemic Mp isolates and their associated specific lesions in goats to devise an effective control strategies. For the collection of samples Northern areas of Pakistan were focused including Chitral, Gilgit and Swat. Samples were collected from *Mycoplasma putrefaciens* suspected animals (Chitral n=200, Gilgit n=200, Swat n=200). Clinical samples including nasal swab (NS) and synovial fluid (SF) from live animals (n=50), whereas pleural fluid (PF) and lungs samples from dead goats (n=50) were collected from each representative northern area of Pakistan. All samples were subjected to isolation of Mp local strain, while tissue samples were examined for pathological lesions. Culture positive PPLO broth was first confirmed for Mp by specific PCR. From Gilgit Baltistan 38.83% samples were found positive for Mp by PCR, whereas 25% and 19.38% samples from Chitral and Swat, respectively, were detected positive for Mp by generating an amplicon of 540 bp. The PCR positive PPLO broth samples were cultured on PPLO agar for isolation of Mp. A single colony with fried egg like appearance was picked and sub-cultured in PPLO broth for purification and later on confirmed with PCR. The Histopathological study of lungs showed that there was emphysema, rupture of alveoli, in bronchioles ciliated epithelium were sloughed and infiltration of leukocytes. We successfully isolated and purified Mp local strain prevalent in northern area of Pakistan. The Mp was detected prevalent in 27.76% goats of Northern regions by PCR. This study laid down a foundation to develop an effective diagnostic kit and vaccine against Mp infection in Goats.

## Introduction

In Pakistan, livestock is a major subsector of agriculture that contributes 60.54% to the agriculture gross domestic product (GDP) (Economic survey, 2018-2019). Pakistan have the largest population of goats among livestock. Goat is considered as poor man's cow in Indo-Pak sub-continent (Rahman *et al.*, 2003). The population of goat is facing diversified problems in the country because of poor management practices, low feeding and infectious and non-infectious diseases. Among the infectious diseases, *Mycoplasmosis* causes great loss to animals because of high morbidity and mortality (Siddique *et al.*, 2012). The *Mycoplasma* belongs to class Mollicutes having no cell wall, prokaryotes, small size and have unusually small genomes approximately 0.58 to 2.20 Mb. (Khan *et al.*, 2016;2018). . In small ruminants it cause respiratory disease, arthritis, eye lesions, genital lesions and mastitis (Nicholas, 2002; Sharif and Muhammad, 2009).

*Mycoplasma putrefaciens* is responsible for great economic loses in developing countries in term of decrease milk production, meat, hide and hair in most countries (OIE, 2000). *Mycoplasma putrefaciens* adversely affect the goat population throughout the world causing morbidity and an acute illness with

mortality. *Mycoplasma putrefaciens* is considered as an etiological agent of the *contagious agalactiae* syndrome in goats by the world organization (OIE) for animal health (Manual, Paris, France 2000) characterized by Mastitis, Arthritis, Keratoconjunctivitis, Pneumonia, and Septicemia (MAKePS) (Thiaucourt *et al.*, 1996). *Mycoplasma putrefaciens* also found in ear canal as a commensal organisms (Cottew GS *et al.*, 1981). *Mycoplasma putrefaciens* is closely related to *mycoplasma mycoides* cluster that consist of 5 major members, highly pathogenic to ruminants including *M. mycoides* subsp. *capri*, *M. mycoides* subsp. *mycoides*, *M. capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, and *M. leachii* (Manso-Silvan L *et al.*, 2007) but different from these mycoplasmas because the first sign which appear in *Mp* infection is mastitis (Adler *et al.*, 1980). The most impressive biochemical characteristic that differentiate *Mycoplasma putrefaciens* from all other *mycoplasmas* is the production of bed smell that occur in cultured broth (Tully *et al* 1974). The gross pathology macroscopic study revealed arthritis (increase in the size of the carpal joints) and respiratory signs such as serous to mucoid nasal discharge and temperature range from 38.1 to 40C° (Rodriguez *et al.*, 1995).

Looking at the economic losses, high morbidity and mortality of small ruminants due to *Mycoplasma putrefaciens* a rapid, specific and sensitive detection test was developed called Polymerase chain reaction in order to isolate, identify and characterize the *Mycoplasma putrefaciens* that has never been reported in northern regions of Pakistan. This study will be helpful for the researcher to eradicate this fatal disease.

The Histopathological study *Mp* showed intestinal lesions characterized by a multi focal necrotic-hemorrhagic enteritis and areas of catarrhal enteritis with fusion of villi and infiltration of cells which are composed of mainly neutrophils and eosinophil's. The joints showed acute fibrinopurulent arthritis with hyperplasia of the synovial epithelium (DaMassa and others 1984, 1987, 1992).

## Materials And Methods

### Study Area

The study was approved by the ethical committee of College of Veterinary Sciences, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar. A total of 600 samples were collected from Northern region (Chitral, Gilgit Baltistan, Swat) of kpk, Pakistan during November 2017 to December 2018. All the samples were collected from naturally infected small ruminants having respiratory signs like coughing, muco-purulent nasal discharge, along with decrease milk production in lactating animals.

### Sample size and Sampling

The samples for the bacterial isolation were collected that comprised of nasal cavity through nasal swab, synovial fluid from live animals by aspiration, pleural fluid and lungs tissue were taken from dead animals suspected for *Mycoplasma putrefaciens*. Total 600 samples were collected, 200 from each region. Sterile cotton swab were used for collection of nasal discharge which are inserted gently and touch nasal passage of the goat to get the secretion. Pleural fluid was collected with the help of sterile

5ml syringe and pour into screw capped sterile 15ml falcon tube and placed in icebox immediately. Lung sample were collected aseptically between both normal and morbid part (consolidation, red hepatization) of lung. Lung tissues were also preserved in 10% neutral buffered formalin for Histopathological examination. All the samples were immediately preserved in icebox at -4C° and transported to Pathology laboratory, College of Veterinary Sciences, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar for further processing.

## Cultivation of Samples

Nasal swab, Synovial fluid and Pleural fluid were cultured in PPLO broth media with the composition of PPLO broth (Sigma, USA) 2.1% supplemented with 0.5% Sodium pyruvate (Sigma, USA), 0.09% Yeast extract (Sigma, USA), 0.2% glucose (Sigma, USA), 20% Horse Serum (New Zealand). Lung tissues were first minced and then cultured in PPLO broth media. All the tubes were placed at 37C° in 5% CO<sub>2</sub> incubator (New Brunswick, Galaxy48S, UK) for 3-4 days. The samples were checked every 12 hours for color change, whirling movement and turbidity. One tube containing Sterile PPLO broth having no sample were also kept as a negative control.

### Isolation and Purification of *Mycoplasma putrifaciens*

*Mycoplasma putrifaciens* positive cultured samples were processed further for isolation and purification. Primary positive culture was passed through PVDF syringe filter having pore size 0.45µm for removing large debris and other field bacteria. After filtration positive samples were cultured and sub-cultured in PPLO broth and Agar, incubated for 3-4 days. This process was repeated 4-5 times to get a single pure colony of *Mycoplasma putrifaciens*. The plates were then examined under CCD microscope (Olympus, Japan) at 4X, and 10X on daily basis for 3-4 days.

## DNA Extraction

The culture showing turbidity, whirling movement were transferred to 1.5ml eppendorf tube and centrifuged at 10000rpm for 15 minutes. Supernatant was discarded and genomic DNA was extracted through DNA extraction kit (Thermoscientific, USA). Concentration and purity was checked through Nanodrop (Multiskan, Thermoscientific, USA) which was in the range of 30-50ng/µl.

## Polymerase Chain Reaction

Polymerase chain reaction (PCR) tests for the detection of *Mycoplasma Putrifaciens* was performed on purified DNA samples by using specie specific primers *Mp* primers: Forward: (5'-GCGGCATGCCTAATACATGC-3') Reverse: (5' AGCTGCGGCGCTGAGTTCA-3') with the amplicon size is 540bp (Shankster *et al*, 2002). The primer used in this study were synthesized by Invitrogen (USA). About 1.5µl sample of each purified genomic DNA was amplified in 25µl of PCR with a composition of 10µl master mix "1.5 mM Mgcl<sub>2</sub>, 200µM of each dNTP. 1 unit Taq polymerase and 10µM of each forward and reverse primers". All the PCR tubes were subjected to Gradient Thermal cycler machine (Bio-Rad,USA) with a initial denaturation at 95°C for 3 minutes followed by 34X (cycles) consisting of 60 seconds of

denaturation at 94°C, 60 seconds of annealing at 59.5°C. Extension for 60 seconds at 72°C and Final extension for 5 minutes at 72°C. Gel red (Thermo, USA) was added 1% agarose gel for visualization the PCR products by using ultraviolet gel documentation system (Fasgene, Germany).

## Results

### Clinical signs and lesions observed in *Mycoplasma putrifaciens* in suspected goats

Clinically suspected goats for *Mycoplasma putrifaciens* shows Hyperthermia, coughing, mucopurulent nasal discharge, conjunctivitis, arthritis and mastitis were the prominent signs that recorded during this study. The postmortem examination shows unilateral hepatization, consolidation and straw color fluid present in lungs (Fig. 1)

## Cultivation of field samples

Out of total 600 samples 317 samples were positive upon cultivation showing whirling movement by shaking, turbidity and color change (Fig. 2). Separately, of nasal samples (52.66%), synovial fluid (42.66%), pleural fluid (59.33%) and lung tissues (56.66%) showed growth movement for *mycoplasma putrifaciens* when incubated for 2-3 days in 5% CO<sub>2</sub> incubator. Each of the positive culture broth from nasal swab, synovial fluid, pleural fluid and lung tissues gives a circle like colonies having a very small dot in the center of the colony post incubation when cultured on PPLO agar media (Fig. 4).

### Molecular characterization of *Mycoplasma putrifaciens* by PCR

DNA was extracted from all cultured positive samples and subjected to PCR by targeting 16S rRNA gene of *mycoplasma putrifaciens*. PCR results revealed *mycoplasma putrifaciens* isolates in 88 samples by generating an amplicon of 540 bp (Fig. 3). The positive samples for *mycoplasma putrifaciens* through PCR includes 21(26.58%), 5(7.81%), 27(30.33%), 35(41.17%) samples from nasal swab, synovial fluid, pleural fluid and lung tissues.

## Discussion

This work describes the isolation and identification of *Mycoplasma putrifaciens* for the first time in southern regions of Pakistan from goats having respiratory signs along with mastitis, arthritis, keratocconjunctivitis, septicemia, and pneumonia ((MAKePS). *Mp* is also the causative agent of contagious agalactia syndrome (OIE, 2008). *M. agalactiae*, *M. putrefaciens* which are not members of *Mycoplasma mycoides cluster* mostly cause complications when occur along with cluster. (Sadique *et al.*, 2012,) .The isolation and identification of *Mp* is very difficult work because of slow growing rate and require a special media for culturing.

In present study the molecular prevalence of *Mp* was undertaken by using the PCR assay for the first time in southern regions of kpk, Pakistan. Undoubtedly, the previous study on *Mycoplasma* confirm that it is

widely spread in Pakistan. For identification of *Mycoplasma* there are many techniques such as conventional and non-conventional but most of the time gives false negative result. For proper confirmation of *Mycoplasma* it is important to culture the suspected samples but in most cases it is failed because of lack of diagnosis of infected animals and second one is the media which are used for culturing the *Mycoplasma* is expensive and takes time to grow.

*Mp* was isolated successfully when cultured on modified PPLO media from different samples comprised of nasal swab, synovial fluid, Pleural fluids and lungs tissue. The most appropriate sample for the isolation of *Mycoplasma* is pleural fluid. Same findings were also observed by (Sadique *et al.*, 2012, Thiaccourt *et al.*, 1994). Such investigations are confirmed by the fact that *Mycoplasma* cell membrane have lipoglycan that triggers the acute inflammation leads to severe exudation. The *Mycoplasma* livability increases as it is deeply present in host tissue.

The presence of disease in different selected areas confirms that Gilgit Baltistan have high prevalence of *Mp* by comparing to swat and chitral. This above statement is proved by the physical features and location of Gilgit Baltistan that are mostly hilly areas and harsh climatic change due to rain on daily basis. The local people of Gilgit Baltistan migrated from their areas because of small ruminants looking for pasture. Due to migration of the local peoples the small ruminants comes in stress that leads to predisposed of the animals to *Mp*. Due to lack of awareness, education about proper vaccination, diagnosis and treatment *Mp* is spreading from neighbour's countries due to freely movement of nomads from one place to another. The same observations also reported by (Mondal *et al.*, 2004).

For isolation of *Mp* a special media was used called PPLO (Pleuropneumonia like organism). *Mycoplasma* is completely dependent on host because to get fatty, amino and nucleic acids, precures of lipid and vitamins. The medium must contain cholesterol which may replace by other sterols like cholesterol or ergosterol (Rodwell., 1979). Antifungal (Fluconazole) was also added to the medium because of chances of fungal growth in medium is a common problem. Typical fried egg colonies were appeared when streaked on PPLO agar.

Thermo scientific gene jet genomic DNA kit was used for the extraction of DNA from cultured broth showing color change, turbidity and whirling and then perform PCR to confirm the presence of *Mp*. By DNA extraction or culturing the *Mp* positive broth on PPLO agar there was a putrid smell of culture. The findings were also noticed by (Tully *et al* 1974). PCR is the most sensitive method as compared to biochemical tests as most of the time biochemical tests gives false negative results.

Typical fried egg like colonies were appeared from PCR positive culture broth when streaked on PPLO agar. Same findings were also observed by (shah *et al.*, 2017). The colonies were appeared after 2 to 3 days post culturing. Every single colony was picked up with sterile needle of syringe and pour in PPLO broth in order to obtain pure culture broth and then place in Incubator having 5% CO<sub>2</sub> at 37C°. Out of 200 samples, the total prevalence of *MP* in Gilgit Baltistan through PCR was 38.85%; in which the percentage of nasal swab, synovial fluid, pleural fluid and lung tissues were 9, 2, 13 and 16 respectively. In Chitral, 25% samples were positive having nasal swab 5%, synovial fluid 2%, pleural fluid 9% and lung tissues

13%. The total positive samples in Swat were 19.38%, comprised of 7% positive samples of nasal swab, 1% synovial fluid, 5% pleural fluid and 6% lung tissues. Same primers were used as used by (Shankster *et al.*, 2002) having amplicon size of 540bp.

Gross pathology during postmortem examination there was polyarthritis, various degree of pneumonia, red hepatization of one lung, pleural fluid was seen that was also reported by (Rodriguez *et al.*, 1994, Peyraud *et al.*, 2003, Awan *et al.*, 2009). The Histopathological study showed arthritis having acute fibrinopurulent hyperplasia of the synovial epithelium. There is congestion in lungs of septal vessels and also alveolar edema along with cell debris and alveolar macrophages.

The *Mp* is highly prevalent throughout Pakistan and cause high economic loss to goat population in northern and southern regions of the country (Fauzia *et al.*, 2016; Shahzad *et al.*, 2012; Saddique *et al.*, 2012).

## Declarations

### 1. Acknowledgment:

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### 2. Statement of Animal Rights:

This study was approved by the ethical committee of The University of Agriculture Peshawar. All experimental procedures was performed under the institutional guidelines and animal ethics.

### 3. Conflict of interest:

The authors have no conflict of interest to disclose.

4. **Author's contribution:** MS, FAK, and FR designed and conceived the study. MS, HK, FA, FR, MA, AK and QU carried out the research. MS, FAK, HK, FA, and FR analyzed the data. MS, FAK, HK, and FA wrote the manuscript. FAK, and HR critically reviewed and revised the manuscript.

5. **Consent for publication:** All the authors showed agreement on the publication of this manuscript in this esteemed journal.

6. **Data Availability:** Data sharing not applicable to this article.

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## Tables

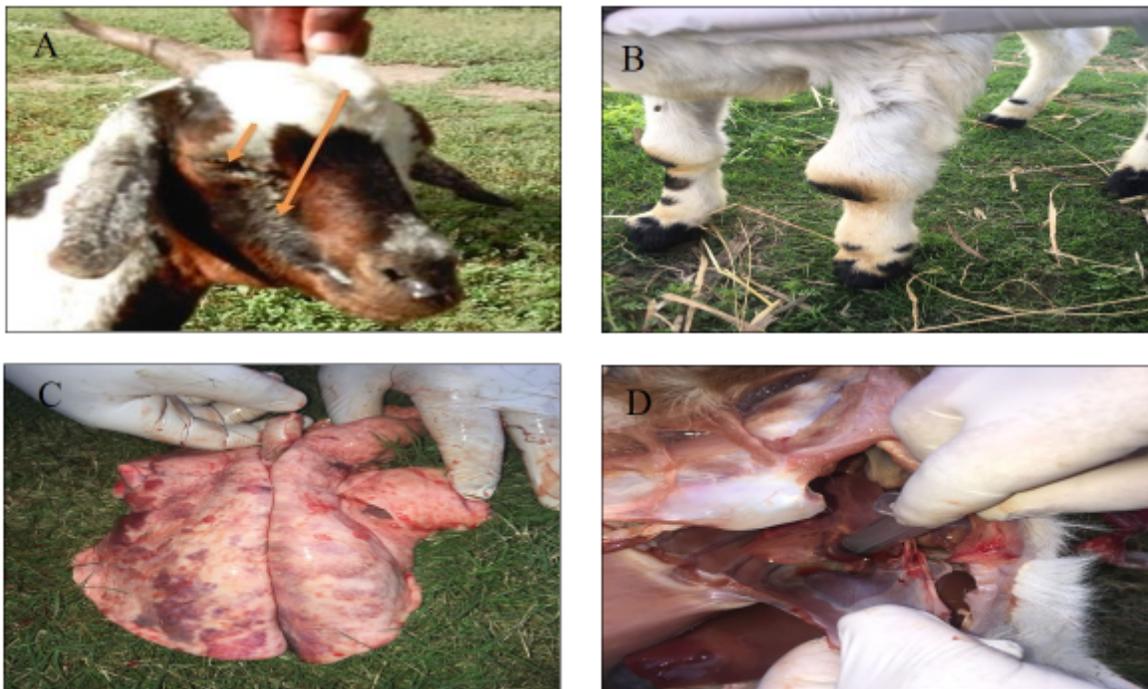
**Table 1** Showing different positive samples for cultivation collected from goats.

Types of sample	No of samples	<i>Mycoplasma</i> +ve samples by cultivation	Percentage (%)
Nasal swab	150	79	52.66
Synovial fluid	150	64	42.66
Pleural fluid	150	89	59.33
Lung tissue	150	85	56.66
Total	600	317	52.83

**Table 2** *Mp* detected through PCR and isolated from goats samples which were positive by cultivation.

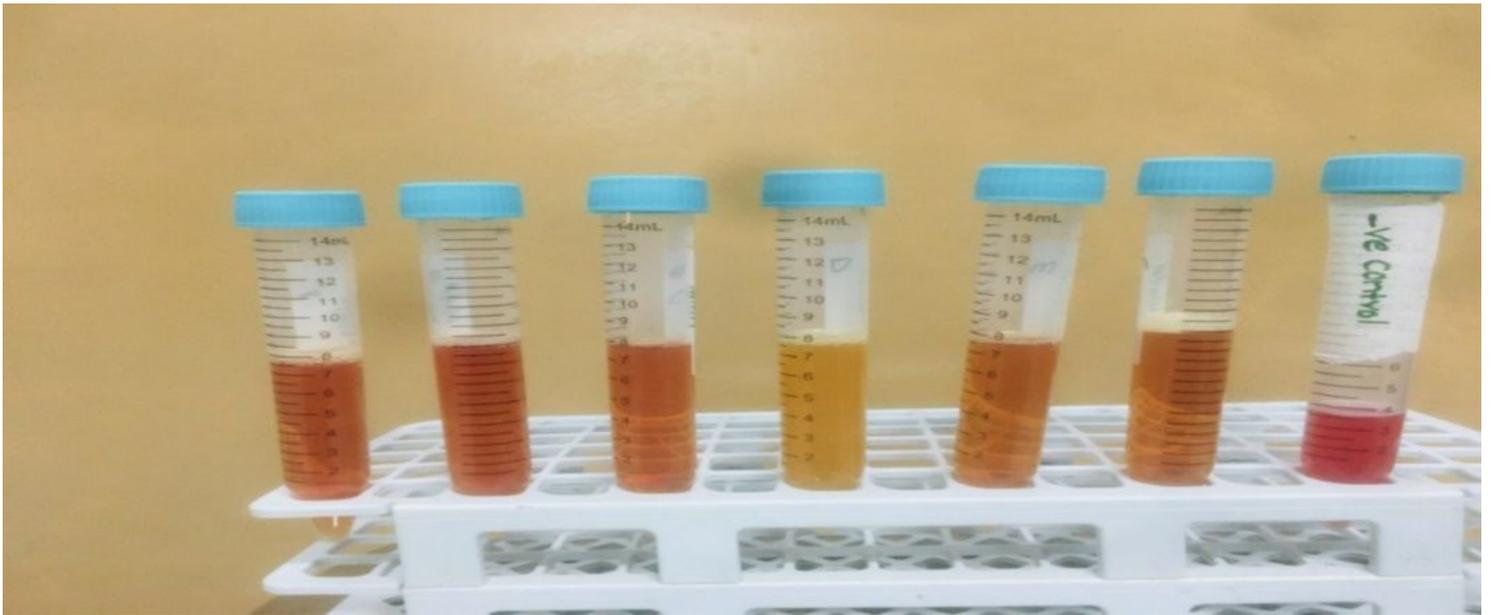
Area	Nasal swab +ve for <i>Mp</i> /No of samples	Synovial fluid +ve for <i>Mp</i> /No of samples	Pleural fluid +ve for <i>Mp</i> /No of samples	Lung tissue +ve for <i>Mp</i> /No of samples	Total +ve/600 samples	Percentage (%)
Chitral	5/29	2/21	9/32	13/34	29	25
Swat	7/24	1/19	5/30	6/25	19	19.38
Gilgit Baltistan	9/26	2/24	13/27	16/26	40	38.83
Total	21/79	5/64	27/89	35/85	317	27.7

## Figures



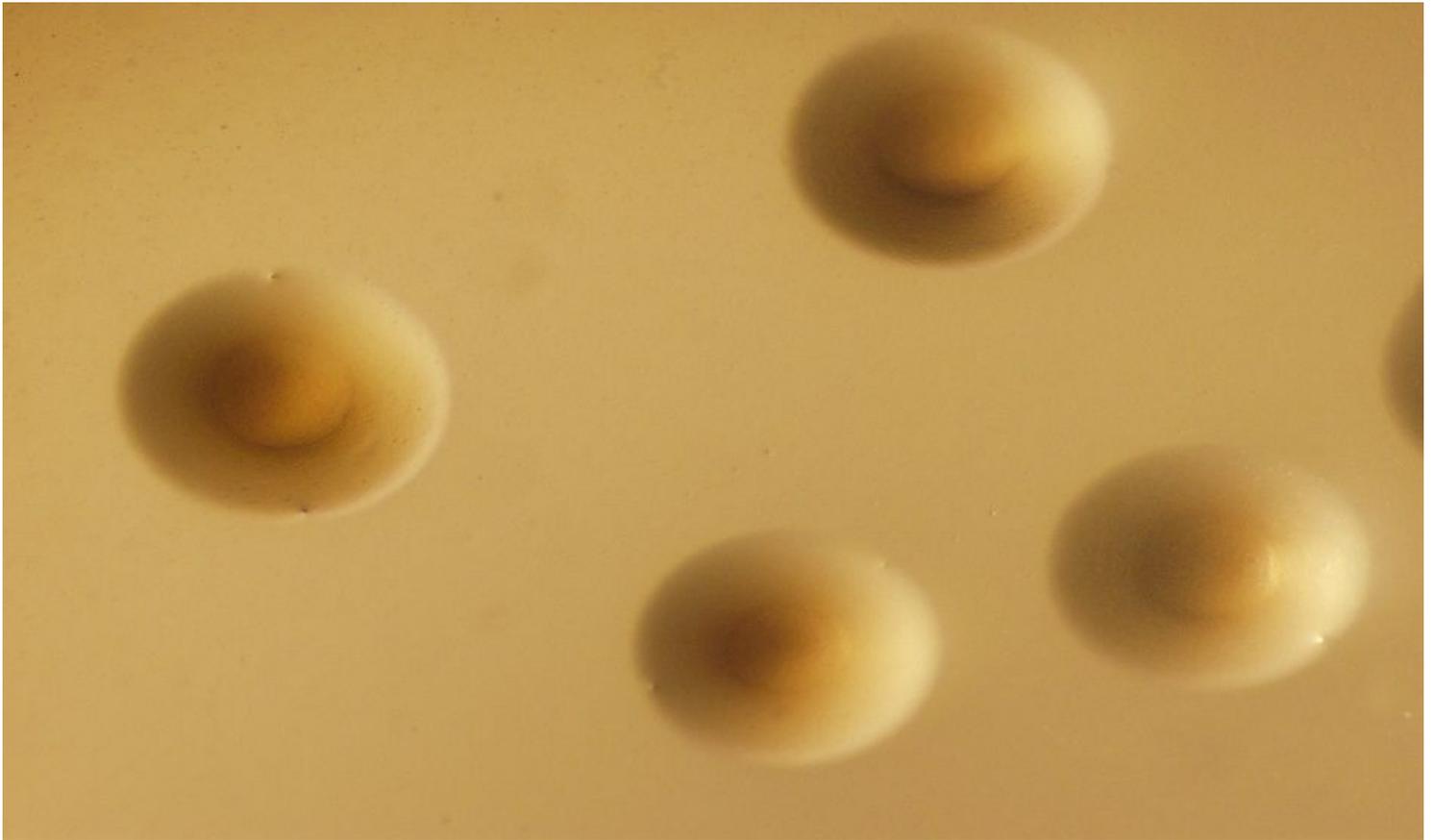
**Figure 1**

(A) Shows conjunctivitis and mucopurulent nasal discharge, (B) shows swelling of joints in kid, (C) postmortem examination shows unilateral hepatization (D) shows pleural fluid aspirated through sterile syringe.



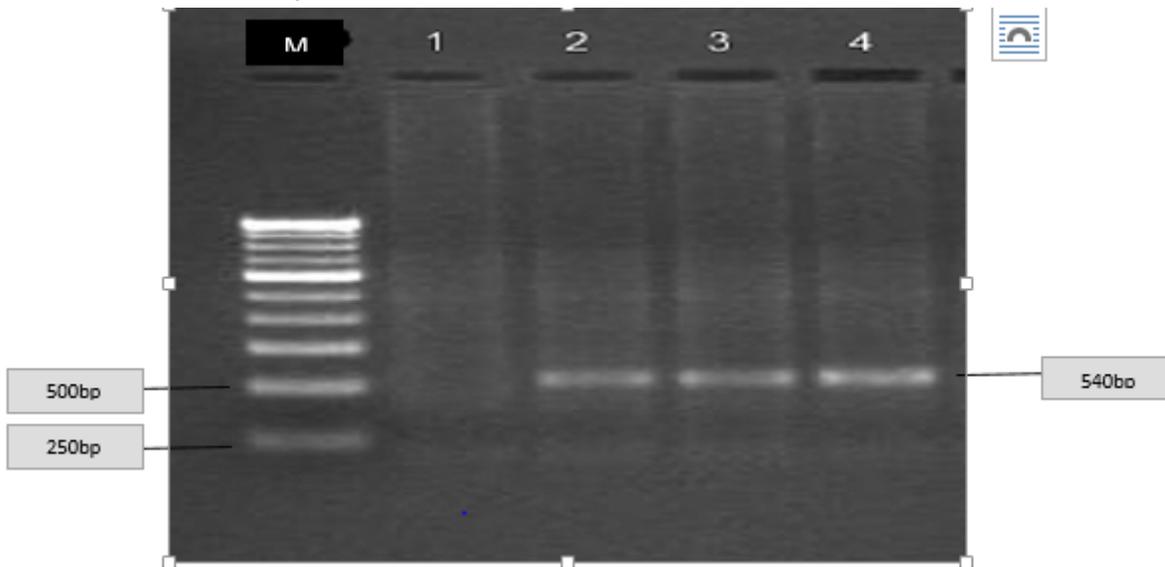
**Figure 2**

Cultivation of field samples collected from naturally infected goats in PPLO broth: from left to right first 6 samples shows *Mycoplasma putrifaciens* growth on 2nd day post incubation while last tube placed as a negative control.



**Figure 3**

*Mycoplasma putrefaciens* (Mp) validation by PCR: DNA Marker (M). Lane 1 indicates Negative control. Lane 2 and 3 indicates field samples revealed Mp local isolates by generating an amplicon of 540bp. Lane 4 indicates as a positive control.



**Figure 4**

Purified colonies of *Mycoplasma putrefaciens* having a very small center on PPLO agar post 2-3 days incubation.