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Original Research Article

A Molecular Approach to Understand the Involvement of *Mycoplasma* in Suppurative Otitis Cases of Cattle in Chittoor District of Andhra Pradesh

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Abstract: In bovines the economic losses mainly due to the outbreak of diseases, leads to mortality, morbidity, treatment cost and reduced production. Recently in August month of 2024, received 13 bovine suppurativeotitis samples from outbreak in KuppamMandal of Chittoor district. The history having the purulent discharges with foul smell from ear infections of cattle that to crossbreed cattle of all age groups are affected. After 5 days of appearing aural discharges the animals were also shown the nervous signs, head tilting and other clinical findings includes unilateral or bilateral ear droop, epiphora, ptosis, abnormal nystagmus, strabismus, regurgitation, stiff neck, opisthotonus, facial hyperesthesia, purulent aural discharge, nasopharyngeal collapse, recumbency and finally death were noticed in severely affected animals. All the samples were processed for cultural tests by inoculating in PPLO selective broth and selective media PPLO agar then incubated anaerobically at 37^0C and observed the color development in broth pink to yellow color after 3- 5 days of incubation indicative of positive growth and fried egg micro colonies on selective PPLO agar media after 8-14 days of incubation observed under low power and 40X. The DNA was isolated from all the samples and screened for presence of *Mycoplasma* by targeting *16s rRNA* gene and found that out of 13 samples 10 were positive for genus *Mycoplasma* and produced the predicted 280bp size product in all positive samples. The suppurativeotitis in cattle usually is caused by *Actinomyces spp*., *Corynebacterium pseudotuberculosis*, *E. coli*, *Heamophilus*, *P.multocida*, *Pseudomonas spp*, *Streptococcus spp*., and *Mycoplasma bovis*. This study was targeted only the emerging pathogen i.e the cellwall deficient bacteria *Mycoplasma* because of infections is hampered by a lack of effective vaccines and specific treatments, leads to increasing trends in antimicrobial resistance. Concluded that isolation and identification of the pathogenic organisms is helpful for specific treatment to control the infection. The cultural methods are gold standard but time consuming, to overcome the difficulties more recently the molecular approach is an alternative for early detection followed by implementation of control strategies. In keeping in view the objective of this study was a molecular approach to understand the involvement of *Mycoplasma* in suppurative Otitis cases of cattle in Chittoor district of Andhra Pradesh.

Keywords: Suppurative Otitis, Cattle, Mycoplasma, PPLO Media, 16s Rrna Gene, Molecular Detection, PCR.

INTRODUCTION

The principal reported agents of otitis in cattle are *Actinomycesspp*, *Corynebacterium pseudotuberculosis*, *E. coli*, *Heamophilus*, *P. multocida*, *Pseudomonas spp*, *Streptococcus spp*., and *Mycoplasma bovis* (Duarte and Hamdan 2004). *Mycoplasma* infections are responsible for substantial health and welfare problems worldwide but mostly undiagnosed and neglected. *Mycoplasma bovis* is a major, but often overlooked pathogen causing respiratory disease, mastitis, arthritis, otitis media (OM) and otitis media interna (OMI) in cattle (Nicholas *et al*., 2008). The *mycoplasma* bacterium is considered to be one of the major emerging pathogens of cattle threatening livestock production (Nicholas, 2011). *M. bovis* associated otitis media occurs in dairy cattle as enzootic disease or as out breaks, and also occurs sporadically in feedlot cattle.

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Clinical signs are because of ear pain, and cranial nerve VII deficits, especially eardrop and ptosis (Lamm *et al.*, 2004; Francoz *et al*., 2004). Ear pain evidenced by headshaking and scratching or rubbing ears. Epiphora and exposure keratitis can develop secondary to eyelid paresis. Unilateral or bilateral and purulent aural discharge can be present if the tymphonic membrane has ruptured. Otitis externa and vestibulocochlear nerve deficits can occur as sequelae, head tilt, is the most common clinical sign, but severely affected animals can exhibit nystagmus, circling, falling or drifting toward the side of the lesionand vestibular ataxia. In advanced otitis media-interna, meningitis can develop, spontaneous regurgitation, loss of pharyngeal tone, and dysphagia have also been reported, indicative of glossopharyngeal nerve dysfunction with or without vagal nerve dysfunction (Van Biervliet *et al*., 2004).

Meningitis can occurs as a complication of *mycoplasma* otitis media- interna (OMI), clinical findings includes unilateral or bilateral ear droop, epiphora, head tilt, ptosis, abnormal nystagmus, strabismus, regurgitation, stiff neck, opisthotonus, facial hyperesthesia, purulent aural discharge, nasopharyngeal collapse and recumbency in severely affected (Stipkovits *et al*., 1993). Majority were referred during winter. In complicated cases, the neurological signs are prominent, includes eyelid ptosis, head tilt, paresis of the pinna, ataxia, strabismus, convulsions and death. *M. bovis* is a cause of bronchopneumonia, mastitis and arthritis but may also affect other main organs in cattle such as the eye, ear or brain (Nicholas *et al*., 2008).

Postmortem lesions includes fibrinosuppurative to caseous exudate filled the tympanic airspaces. *M. bovis* spreads to multiple organs and is capable of invading various kinds of host tissue cells. The intracellular localization may be favorable for evading host immune responses (Hussam Askar *et al*., 2021). Epizootic sistuations, *M. bovis* infections and new sources routes of transmission of the infection, caseo-necrotic pneumonia, mastitis, arthritis however, case of infectious keratoconjuctivitis, suppurative otitis media, meningitis, decubital abscesses, endocarditis and reproductive disorders have been associated with *M. bovis* (Hussam Askar *et al*., 2021).

The Varible surface lipoproteins, adhesions, invasion of host cells, modulation of the host immune system, biofilm formation and release of secondary metabolites like hydrogen peroxide (H_2O_2) as well as synergistic infections with other bacterial and viral pathogens are factors contributing to the dissemination and persistence of this pathogen in the bovine host (Song *et al*., 2012; Guo *et al*., 2017; Zhao *et al*., 2017). Highly variable antigenic profiles of *M.bovis*, which are strain independent. The antigenic heterogeneity of *M. bovis* strains is unrelated to the geographical origin, organ of isolation, type of disease induced by single stains but is varaible among different sub clones of the same strain. The antigenic variability mainly due to several prominent amphiphilic, integral, membrane proteins containing cross-reactive epitopes acting as major immunogens. Morbidity rate in *M. bovis* recorded as 8-40% whereas the mortality rate as 100%. *M. bovis* affects all age groups of cattle including preweaned, post weaned, neonate and adult, all cattle sections such as beef, milk or rearing (Nicholas *et al.*, 2008). The organism can persists in a herd for very long period of time, with probability of pathogen shedding by the infected animals for a few weeks to several months (Hazelton *et al*., 2020; Punyapornwithaya *et al*., 2010).

Nasal swabs, lungs, ears (swabs), lymph nodes (cranial and pulmonary), brain and heart are the suitable samples for confirmatory diagnosis of infection. Their fastidious nature made isolation and identification an extremely difficult task, indeed, it had only been in the last two decades with the introduction of DNA amplification techniques that detection and identification have become routine in many parts of the world. *M. bovis* infections is hampered by a lack of effective vaccines and treatments, leads to increasing trends in antimicrobial resistance (Dudek *et al*., 2019; Klein *et al.*, 2019). The evolutionary absence of a cell wall in principle makes *M.bovis* resistant to antimicrobial agents like tetracycline, newer generation macrolides. Epizootic situation of *M. bovis* infections new sources or routes of transmission of the infections, become infected *via* imported cattle (Haapala *et al*., 2018), most important primary cause of mastitis, artritis, keratoconjuctivitis, bovine respiratory complex-BRD. Internalization /spread trade in cattle and cattle products like semen has established its spread to all continents (Vahanikkila et *al*., 2019; Nicholas *et al*., 2011). Control and treatment of bovine otitis is not standardized and there is little evidence based support for the diverse treatments available in the literature. To fill the gap, molecular based PCR detection and conventional isolation and identification of the pathogen is the only method. In this study molecular detection of *mycoplasma* was done by targeting 16S rRNA gene directly from clinical otitis samples from outbreak and simultaneously cultivation of organism in selective media in the laboratory by using conventional cultural methods.

MATERIALS AND METHODS

Samples: Collected from the otitis cattle includes 13 swabs from outbreak area.

Isolation of *Mycoplasmas*

***Mycoplasma* Enrichment Supplement (Hi-Media)**

A selective supplement for the isolation of *Mycoplasma***,** Each vial sufficient for 70 ml medium, Horse serum 20 ml, Yeast extract (25%w/v solution) 10 ml, Thallus acetate 25 mg and Penicillin G 20,000 IU.

***Mycoplasma* Broth Base /PPLO Broth Base (Hi-Media)**

Beef heart infusion 250 gms/lit, Peptic digest of animal tissue 10 gms/lit, Sodium chloride 5gms/lit and Crystal violet 0.01gms/lit. Final PH (at 25ºC) 7.8 was adjusted with 20 per cent NaOH

PPLO Broth Medium

Mycoplasma broth base 21 gms, Distilled water 700 ml, Sterilized by autoclaving at 15lbs pressure/121ºC/15min. cooled to 45ºC and aseptically and added 10ml of *Mycoplasma* enrichment supplement. Final PH 7.8 was adjusted with 20 per cent NaOH sterilized by filtration with 0.22 mm filters. Mixed well and poured aseptically into sterile Eppendorf tubes.

PPLO Agar Medium

Mycoplasma broth base 21gms, Agar agar powder 14gms, Distilled water 700ml. The PH 7.8 was adjusted with 20 percent NaOH (sterilized by filtration with 0.22mm syringe filters). Sterilized by autoclaving at 15lbs pressure/121ºC/15min. cooled to 45^{O}C and aseptically and added 10ml of *Mycoplasma* enrichment supplement. Mixed well and poured aseptically into sterile petridises (15ml for 100×15mm plate).

Methods for Isolation of *Mycoplasma:* Isolation of *Mycoplasma* from Field Samples

The otitis samples after collection were immediately inoculated into sterile Eppendorf tubes containing sterile PPLO broth. Eppendorf tubes were incubated anaerobically at 37ºC with 90 percent relative humidity in BOD incubator, until the phenol red indicator changed from red to yellow. The cultures were tested for the presence of *Mycoplasma* by Genus specific PCR and simultaneously plated on PPLO agar medium and incubated anaerobically in candle jar to avoid drying of plates, at 37ºC with 90 per cent relative humidity for 8 days in BOD incubator. The colonies were detected by microscopic examination at low power magnification for "Fried egg" appearance (Saritha *et al.*, 2010; Pourbakhsh, 2014; Sumitha *et al.*, 2015 and Logesh *et al.*, 2018, thopireddy *et al.*, 2021, 2022, 2023).

DNA Extraction: DNA was extracted from the samples using TRIsol (TRIsoln of Genei, Banguluru) reagent (Baert *et al.,* 2007).

Preparation for DNA Isolation

The surface of laminar air flow cabinet and work table were wiped clean with 70 per cent ethanol, followed by ultraviolet (UV) lamp was switched on for 30 min. prior to isolation of DNA. All plastic ware used *i.e.,* pipette tips and micro centrifuge tubes were certified by manufacturers as RNase free (Invitrogen, USA). All solutions and buffers used for DNA isolation were prepared using 0.1 per cent DEPC treated water.

Method Used for the Extraction of DNA

Triturated tissue samples (0.25 millilitre) was pipetted into a 2 ml RNase free micro centrifuge tube and 0.75 mL of TRIzol LS reagent was added into it subsequently and homogenised by pipetting the suspension up and down several times. The homogenized sample was incubated at room temperature for five minutes. Chloroform (0.2 mL) was added to the tube followed by vigorous shaking for 15 sec. Incubation was done for 10 min. at room temperature. Centrifuged at 12,000 xg for 15 min. at 4°C. The mixture was separated into a lowered phenol chloroform phase, an interphase and a colourless upper aqueous phase. The inter phase was carefully transferred to a fresh tube. To the inter phase, 0.3 mL of 100 per cent ethanol was added and mixed thoroughly by inverting the sample several times. The mixture was incubated at room temperature for 2 min. Centrifugation was carried out at 2,000xg for 5 min. at 4°C.The supernatant was discarded from the tube leaving only the DNA pellet. The pellet was washed with one millilitre of sodium citrate /ethanol solution. Incubated at room temperature for 30 min (mixed occasionally by gentle inversion). The mixture was centrifuged at 2000xg for five minutes at 4°C and the supernatant was discarded. To the pellet, 1.5 ml of 75 per cent ethanol was added and incubated at room temperature for 20 minutes. The mixture was centrifuged at 2000xg for 5 min at 4°C and the supernatant was discarded. The pellet was air dried for 10min. Air dried pellet was suspended in 0.6 ml of 8mM NaOH. Insoluble material was discarded by centrifuging at 12000 xg for 10 min at 4°C.The supernatant containing DNA was transferred to a new tube and stored at -80°C until further use. The purity of DNA stock was estimated by finding the ratio between the OD readings at 260/280 nm. The samples showing OD value between 1.8 and 2 were chosen for further studies.

Molecular Detection of Genus *Mycoplasma:* The molecular detection of genus *Mycoplasma* from suspected samples was done by using *16S rRNA* gene primers.

Primers Used for Detection of 16s*r*RNA Gene of Genus *Mycoplasma*

The oligonucleotide primers were obtained from Sigma Aldrich India Pvt. Ltd., Bangalore. Details of the primer sequence are enlisted in (Table 1). Working solutions of both the primers were prepared from original stock (100pmol/μl) in nuclease free water to 10pmol/μl.

Table 1: Primers used for detection of genus *Mycoplasma* (thopireddy *et al.*, 2021)

Primers	Primer Name	Nucleotide Sequence	Amplicon Size
16S rRNA gene	GPO3F	5'-TGGGGAGCAAACAGGATTAGATACC-3'	280bp
	MGSO	5'-TGCACCATCTGTCACTCTGTTAACCTC-3'	

Preparation of PCR Reaction Mixture

Taq buffer A (10X) 2.5 μl
MgCl2 (25mM) 1.5 μl
dNTP mix (10mM) 0.5 μl
Taq DNA Polymerase (1U/μl) 0.3 μl
Forward primer (10pmol/μl). 1.0 μl
Reverse primer (10pmol/μl) 1.0 μl
Template DNA 5.0 μl
Nuclease free water 12.5 μl

The total volume of the reaction mixture was made up to 25μl with nuclease free water. The tubes were then spun for 10 sec and PCR was carried out in Thermal cycler (Eppendorf Pvt. Ltd., Hamburg, Germany). Cyclic conditions used for amplification of 16S*r* RNA gene are given in detail in (Table 2).

Table 2: Cyclic conditions used for amplification of 16S*r*RNA gene of genus *Mycoplasma*

S. No.	Step	Temperature (°C)	Time	No of cycles
1.	Initial Denaturation	94	4 min	1
2.	Denaturation	94	30 sec	35
3.	Annealing	56	30 sec	
4.	Extension	72	30 sec	
5.	Final extension	72	10 min	1

RESULTS AND DISCUSSION

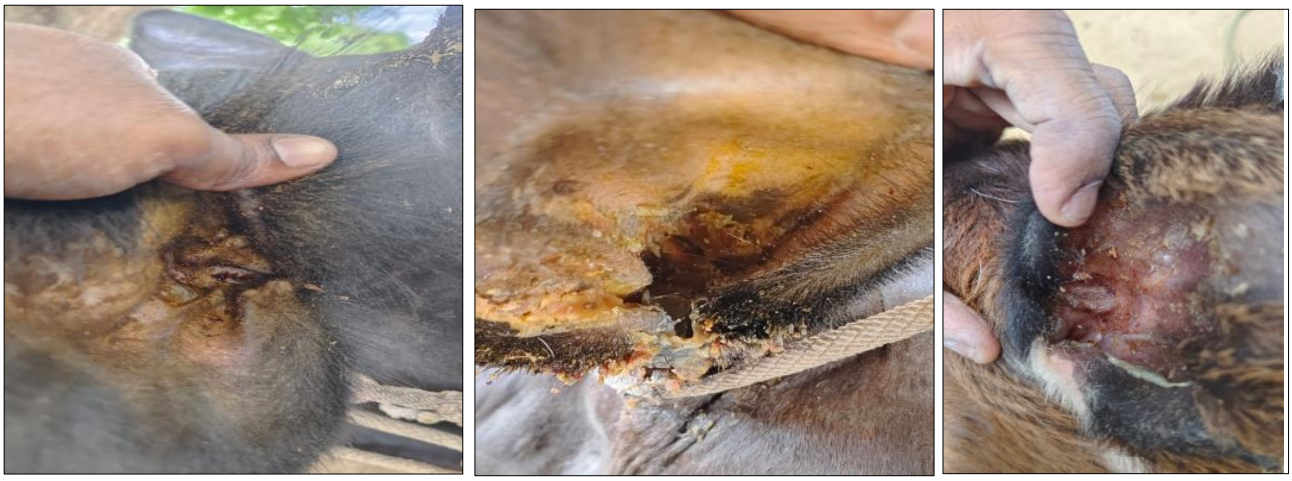

Fig.1: Cattleshowing Suppurative Otitis

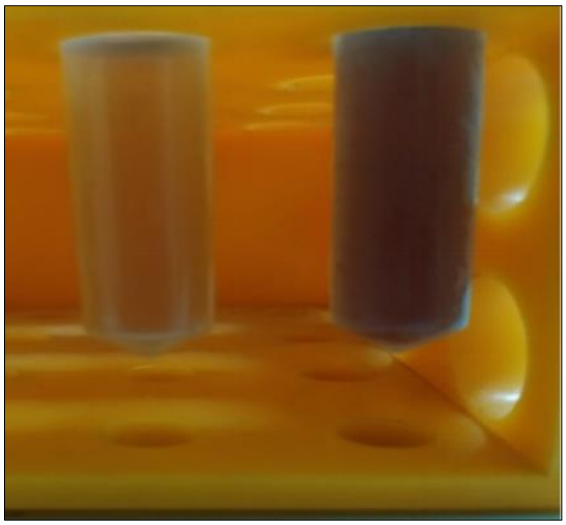

Fig. 2: Showing the Positive culture

Growth of mycoplasma in PPLO broth
Left tube positive and Right tube Control

Fig. 3: Showing the Positive culture

Growth of mycoplasma in PPLO agar
Media- Fried egg microcolonies

Fig. 4: Gel image showing predicted size 280bp product of genus mycoplasma

Mycoplasma species are causing significant diseases worldwide, among them mastitis, arthritis, pneumonia, otitis media (OM), and reproductive disorders are the common disease conditions. They are highly contagious, capable of causing severe diseases, and are difficult infections requiring rapid and accurate diagnosis to prevent and control disease outbreaks. Traditionally identification and diagnosis of *mycoplasma* has been performed *via* microbial culture. More recently the use of PCR to detect various bovine clinical samples has increased PCR has a high efficiency, specificity, and sensitivity for laboratory diagnosis, when compared with conventional culture based methods. Several tools are now available for typing *mycoplasma* species isolates, allowing for genetic characterization in disease outbreak investigations. These methods can contribute to increase knowledge of *mycoplasma* infections in cattle. Within the *mycoplasma* genus *M. bovis* is currently recognized and considered as one of the most important and frequently isolated *mycoplasma* species associated with disease in cattle worldwide (Francoz *et al*., 2004; Duate *et al*., 2004; Maunsell, 2011; Fox, 2012). The lack of the cell wall so that certain widely used antibiotics are not effective. An ability to change the surface proteins so it can evade the cows immune response and has power to produce a biofilms so that it can temporarily hide from both the immune system and antibiotics. It might be the one of the reason most of the commonly used antibiotics may not work.

Recently received samples from the outbreak in kuppam mandal of Chittoor district having the clinical signs, unilateral or bilateral ear droop, epiphora, head tilt, ptosis, abnormal nystagmus, strabismus, regurgitation, stiff neck, opisthotonus, facial hyperesthesia, purulent aural discharge, nasopharyngeal collapse and recumbency in severely affected. In complicated cases, the neurological signs are prominent includes eyelid ptosis, head tilt, paresis of the pinna, ataxia, strabismus, convulsions and death. These nervous signs might be due to invasion of organism in the brain and causes changes. The presenting clinical signs, neurological signs and the frequency of unilateral or bilateral ear involvement were consistent with previous observations (Maeda *et al.*, 2003; Lamm *et al.*, 2004; Francoz *et al*., 2004; VanBiervliet *et al*., 2004; Benier Gosselin *et al*., 2012).

M. bovis can be detected directly in clinical specimens by PCR (Cremonesi *et al*., 2007). PCR can be especially useful for stored samples; PCR had a similar sensitivity to culture for detection of *M. bovis* in fresh milk but was much more sensitive than culture in milk frozen for 2 years. Real-time PCR systems with high sensitivity and specificity have been described for the detection of *M. bovis* in clinical samples (Cai *et al*., 2005). Other techniques, including denaturing gradient gel electrophoresis PCR and melting-curve analysis of PCR products, appear promising for the simultaneous detection and differentiation of multiple mycoplasma species (Sachse *et al*., 2010). Significant reductions in mycoplasma recovery rates occur with increased time to processing, regardless of whether samples are refrigerated or frozen, and best recovery rates are achieved when samples are processed fresh within a few hours of collection (Biddle *et al*., 2004).

All the samples were tested initially for presence of *mycoplasma* by conventional cultural tests and molecular test like PCR and found that out of 13 samples 12 were by PCR, 10 were found positive by cultural test. 92% positivity in PCR and 76.9% by cultural test, indicated that the cultural tests are less sensitive than the molecular methods (Thopireddy *et al*., 2021; 2022; 2023). It is also telling that *Mycoplasma* is the pathogenic agent most commonly involved in otitis media. 20% by Foster *et al*., 2009; 17.14%by Mahmood *et al*., 2017 and 89% by Bartone *et al*., 2015; recorded on patho-bacterialogical investigation of an outbreak of mycoplasma bovis in otitis cases of cattle and also observed the histopathological changes like fibrinosuppurative to caseous exudates filled the tympanic air spaces. The partially ulcerated tympanic mucosa was markedly thickened with mononuclear cell infiltration and proliferation of fibrous connective tissue. This outbreak was observed during August month of this year, similarly Francoz *et al*., 2004 recorded the otitis in the inclement weather seems to be a predisposing factor for development of otitis media in cattle.

From total 13 samples were processed and inoculated in selective PPLO broth and observed the yellow color development in 10tubes indicated that the positive growth of mycoplasma may cause the PH changes and indicator at this PH may turn into yellow color. Similar type of changes was documented by Marouf *et al., (*2020) and Thopireddy *et al.,* (2022) observed the colour change in *Mycoplasma* suspected samples after incubation of inoculated Frey's broth and found colour differences in positive sample as developed yellow compare to negative sample indicated as active growth of *Mycoplasma.* All the positive culture inoculated on PPLO agar media after 8-14 days incubation the fried egg micro colonies were observed similar type of findings also noticed by Raza *et al.,* (2022) observed *Mycoplasma* species obtained in Brain Heart Infusion broth had a morphology that was tiny, smooth, circular, and had a fried egg look with a central opaque and outer translucent area and Thopireddy *et al.,* (2022) observed the colonies of *Mycoplasma* isolated on PPLO agar revealed characteristic fried egg appearance upon microscopic examination. Typical micro colonies were appeared as small (0.1-1.0mm), smooth, circular and somewhat flat with a denser central elevation (Kumar *et al*., 2011; Bibak *et al*., 2013; Pourbakhsh., 2014; Senthilnathan *et al*., 2015; Tomar *et al*., 2017; Ammar *et al*., 2016; OIE, 2018; Marouf *et al*., 2020; Basit *et al*., 2021; Thopireddy *et al.,* 2022).

Molecular based PCR assay was standardized for detection of mycoplasma in clinical samples and cultures by targeting the16s r RNA gene and found that out of 13 samples 12 were found positive and produced 280 bp size product in all positive samples. In this study found that the PCR based methods are more sensitive and rapid for detection of mycoplasma in clinical samples when compared to conventional cultural methods (Reddy *et al*., 2022; Haapala *et al*.,2018; Timonen *et al*., 2020; Gille *et al*., 2020; Parker *et al*., 2017; Pansri *et al*., 2020; Thopireddy *et al*., 2022; Thopireddy *et al*., 2023).

Walz *et al*., (1997) suggested that the outbreak of otitis media due to *M. bovis* in a dairy herd in Michigan might be associated with the increased incidence of mastitis, broncho pneumonia caused by *M. bovis*. The clinical signs of otitis media, or interna, in this study are similar to those previously reported; 10 of 13 cases for which results of aural examination were available had purulent aural discharge. Purulent aural discharge was present in all cases of otitis media in previous reports.Purulent discharge is reported to appear 2 to 3 days after apparent clinical signs and is associated with rupture of the tympanic membrane(Campbell *et al*., 2009). These infections affecting the cerebrum or forebrain (cerebral hemispheres, thalamus, and hypothalamus), such as in bacterial meningitis, are considered infrequent in adult ruminants but are more commonly seen in young animals. The introduction of asymptomatically infected animals is thought to be the primary means by which *M. bovis*-free herds become infected. Transmission is delayed until, and if, shedding occurs; this delay can make it difficult to identify the source of infection and mycoplasma disease outbreaks occur in seemingly closed herds. *M. bovis* has characteristics that enable it to colonize and persist on mucosal surfaces, to invade tissues, and to persist at sites of disease despite an aggressive immune response. Molecules involved in adherence, antigenic variation, invasion, immunomodulation, biofilm formation, and production of toxic metabolites are likely to be important in pathogenesis, but exactly how *M. bovis* interacts with the host is poorly understood. Mycoplasmas lack a cell wall, and exposed membrane proteins form the primary interface with the host. These membrane proteins facilitate adherence to mucosal surfaces, although *M. bovis* adhesions are not yet well characterized. *M. bovis* has a large family of immunodominant variable surface lipoproteins (Vsps), which undergo high

frequency phase and size variation in vitro and in vivo, and exhibit extensive strain variation in their coding sequences. Particular Vsp variants can be selected by exposure to antibodies. These characteristics impart a vast capacity for antigenic variation in *M. bovis* populations that likely contributes to immune evasion and persistence and provides a challenge for vaccine development. After adherence, many mycoplasmas, including *M. bovis*-generate products such as phospholipases, hydrogen peroxide, and superoxide radicals which damage host cells. *M. bovis* can also form biofilms in vitro that impart increased resistance to desiccation and heat stress. Excessive neutrophil recruitment with the subsequent release of large amounts of inflammatory mediators can occur, and the extent of neutrophil recruitment is directly correlated with the severity of mycoplasma disease. Therefore, DNA-based techniques have become the favored diagnostic approach. DNA-based techniques, particularly the polymerase chain reaction (PCR) allows for the rapid and specific detection of *M. bovis* (Hirose *et al*., 2001). The PCR technique offers faster diagnosis compared to culture and serological methods (Thopireddy, 2023), with results available in just a few hours Sachse *et al*.,1993, Further, PCR methods can specifically amplify *M. bovis* DNA, enhancing the identification of Mycoplasma species [Tang *et al*., 2000]. *Genes used for detection mycoplasma bovis buvrC, 16S rRNA, gyrB, polC, 16S-23S rRNA, ITS, oppD, vspB,* and gltX, Moreover, more than one species of *Mycoplasma* as well as non-cultivable or unknown species can be detected when conventional PCR products are run through denaturing gradient gel electrophoresis [McAuliffe *et al*., 2005], identification of virulence factors, the nature of protective and harmful immune responses in *M. bovis* infections, the importance of coinfection with other pathogens in the progression of mycoplasma disease, and the potential of new vaccine technologies to protect from *M. bovis*-associated disease. In applied research, a critical need is the development of cost-effective, sensitive, and specific diagnostic tests to allow accurate identification of *M. bovis*-infected animals. Epidemiological research is required to clarify risk factors for infection and disease, particularly those factors associated with severe outbreaks of clinical disease.

Furthermore, bovine mycoplasmosis has been identified as a major emerging infectious disease by the European Community 7th Framework Programme under the EMIDA ERA-NET, which is funding a three-year study into the development of improved diagnosis and control of bovine mycoplasmosis involving the UK, Germany, Italy and Israel.

These developments coincide with the publication of the complete sequence of the *Mycoplasma bovis* genome, which offers fresh insight into the properties of this tiny wall-less bacterium. With this in mind, it is an appropriate time to review our knowledge of cattle diseases caused by mycoplasmas.

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