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Mycoplasma bovis, A Multi Disease Producing Pathogen: An Overview

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INTRODUCTION

Mycoplasma bovis, first isolated in the USA from the milk of a mastitic cow in 1961 (Hale *et al*., 1962) belongs to the class *Mollicutes,* order *Mycoplasmatales,* family Mycoplasmataceae and the genus *Mycoplasma* (Razin *et al*., 1998). Initially it was named as *Mycoplasma bovimastitidis* then it was classified in group 5, *M. agalactiae* var *bovis*, on the basis of its antigenicity and biochemical tests (Leach, 1973) and due to similarity with the clinical signs of contagious agalactia in sheep caused by *M. agalactiae*. Both the species are identical in cell-and colony-form as well as in their metabolic behavior with the sharing of high number of antigens. It is difficult to differentiate them by usual morphological, metabolical and serological methods (Gonzalez *et al*., 1995; Gummelt *et al*., 1996; Kumar, 2000). Later with the advancement of techniques it was ranked as species and named *Mycoplasma bovis* based on 16S ribosomal RNA sequences (Askaa and Erno, 1976).

Among pathogenic mycoplasma species which mainly colonize bovine respiratory mucous membrane (Ter Laak *et al*., 1992) *Mycoplasma bovis* is the most important and most pathogenic bovine mycoplasma causing respiratory, venereal and other diseases of bovines all over the world mainly in Europe and North America. *Mycoplasma bovis* is very versatile pathogen and has been reported from the cases of genital disorders and abortions (Langford, 1975; Ruhnke, 1994; Byrne *et al*., 1999) bovine pneumonia (Srivastava, 1982), reduction of semen fertility (Kissi *et al*., 1985), arthritis and meningeal abcesses (Stipkovits *et al*., 1993), decubital abscesses (Kinde *et al*., 1993), keratoconjonctivitis (Jack *et al*., 1977; Kirby and Nicholas, 1996), otitis (Walz *et al*., 1997), poly arthritis (Henderson and Ball, 1999) and mastitis (Byrne *et al*., 2000; Hirose *et al*., 2001).

Economic losses: There is as such no survey available for the economic losses due to *M. bovis* infections, although it is estimated to cause at least for the quarter or third of total losses approximately a sum of 576 million Euros per year in Europe due to respiratory diseases in cattle (Nicholas and Ayhng, 2003). However, more comprehensive data is available for the losses caused by this organism due to mastitis ($ 108 million) and loss of the weight gain and the diminished carcass value ($ 32 million) in the USA (Rosengarten and Citti, 1999).

According to Campbell (2009) average of a minimum of $100.00 expended per case of *M. bovis* on treatment costs/animal health in Canada with the conservative estimate of approximately $20,000 in expenses due to *Mycoplasma bovis* cases in a 10,000 head feedlot per year.

Source of infection: *Mycoplasma bovis* is supposed to be a natural habitat of respiratory tract in healthy bovines without showing any clinical symptoms and is shed through their nasal discharges for month or years. The genital tract of both male and female animals can also harbor *M. bovis* and can be a source of the infection through coitus and natural service (Kreusel *et al*., 1989) or through artificial insemination with deep frozen bull semen (Jurmanova and Sterbova, 1977) as mycoplasmas can survive in frozen semen for several years. Milk may also be a source of infection which acts as major source of infection for suckling calves (Pfutzner, 1990; Hirose *et al*., 2001). It has also been reported from sheep (Bocklisch *et al*., 1987), goat (Egwu *et al*., 2001), rabbits (Boucher *et al*., 2001), Poultry (Hasan *et al*., 2008) and can be transmitted from these carrier animals and birds. Isolation of *M. bovis* from human respiratory disease (Madoff *et al*., 1979) is suggestive of human carriers. *Mycoplasma bovis* are quite resistant to environmental conditions (Nagatomo *et al*., 2001) therefore, the transmission through fomites and mechanically can also not ignored.

Transmission: The main route of transmission is aerosol route by the small, inhaled droplets from infected animals and contaminated dust particles (Pfutzner *et al*., 1983; Stipkovits *et al*., 2000). The hematogenous transmission is essential for joint affections (Romvary *et al*., 1977). Calves are affected in both the ways via horizontal transmission through aerosol infection of respiratory tract and vertical transmission as milk is as one of major source of infection particularly from the mastitic udder. Moreover, newborn calves can become infected vertically from the uterus (Pfutzner and Schimmel, 1985).

As small number of living organisms is enough to cause the infection of the teat canal (Bennett and Jasper, 1980); the udder may be infected through teat canal and the milking process (Thomas *et al*., 1981) due to contaminated milking pens, milking machines, wiping cloths, milkers and fomites.

Both male and female genital tracts are affected through ascending infections from the contaminated environment or by direct contact of other animals shedding *M. bovis*. In females artificial insemination of cows with infected semen are major source of infection (Eaglesome and Garcia, 1990). Uterine discharges and aborted fetuses may be source of infection (Bocklisch *et al*., 1987; Pfutzner and Schimmel, 1985).

Diagnosis of the *M. bovis* infection: The *M. bovis* organism remains infective in the respiratory tract and can be transmitted to the next generation. Moreover, frequent way of vertical infection for suckling calves is the milk of asymptomatic shedder or mastitic cows. Thus, the diagnosis of infection is an essential part to reduce the economic losses caused by *M. bovis* infection.

SAMPLING

The nasal mucus of other affected calves or cows is an important factor in horizontal spreading of *M. bovis*. The type and site of sample should be selected depending upon the clinical signs. Nasal, genital, conjunctival, tracheal swabs and samples of milk, synovia, semen and other liquid samples are collected into Medium-B broth containing phenol red and glucose (Erno and Stipkovits, 1973a)

and kept at 4°C and rushed to the diagnostic laboratory at the earliest. However, from respiratory and uterine affections *M. bovis* can be better recovered from broncho-alveolar lavages and deep intra uterine sampling. Tissues are collected aseptically in small pieces and after homogenization with Phosphate Buffer Saline (PBS) of pH 7.2 to 7.6 are submitted as previously mentioned method of culturing.

CULTURE AND IDENTIFICATION

Mycoplasma bovis grow well in suitable culture media and produces typical fried-egg shape colonies after 72 to 96 h of incubation. Medium B and Hayflick's medium are two commonly used media although certain variants are also available with certain modification and are used to identify the *M. bovis* on the basis of cultural characteristics eg. detection of *M. bovis* colonies based on the lipase reaction (Shimizu, 1983) and red color reaction of *M. bovis* colonies (Windsor and Bashiruddin, 1999). Then biochemical properties are assessed as per the method of Erno and Stipkovits (1973a, b). Lauerman (1994) recommended growth inhibition test, metabolic inhibition test and indirect immno fluorescence test with anti *M. bovis* hyper immune sera. Specific Mab-based sandwich or capture ELISA (Ball *et al.*, 1994) or dot immunobinding test using polyclonal sera (Poumarat *et al.*,1991; Kumar, 2000) are also used to confirm *M. bovis* from culture.

IMMUNOLOGICAL TESTS

Antibodies to *M. bovis* can be detected within two week of infection and persist for several months therefore, can be detected with various immunological methods (Kumar *et al.*, 2004) viz., counter current electrophoresis, ELISA, immunoblotting, Immunobinding and immunohistochemistry methods. The use of these tests is particularly useful, when the isolation of the agent is difficult due to chronic infection or regular treatment with antibiotics at a high dosage (Nicholas and Ayling, 2003). The presence of *M. bovis* in nasal cavity may not be immunogenic so these tests are effective whenever there is invasion of *M. bovis*.

ELISA: The ELISA tests are useful diagnostic methods but only for screening purposes (Pfutzner and Sachse, 1996). There are commercially available tests with mixed antigens in order to minimize the false negative reactions due to the antigenic variability of *M. bovis* (Le Grand *et al.*, 2001). There are also non-commercial ELISA tests developed to detect *M. bovis* infection (Boothby *et al.*, 1981; Uhaa *et al.*, 1990; Byrne *et al.*, 2000). The ELISA tests performed from milk can also be applied in the examination of mastitis outbreaks (Byrne *et al.*, 2000).

SDS-PAGE and western blot: This technique can be used to compare the antigenic structure of the strains or for the examination of the humoral immune response patterns of the host animal. However, the use of monoclonal antibodies specific to *M. bovis* can be used for the diagnostic purpose. Rosengarten *et al.* (1994) observed 12 major strain variable antigens whereas Sachse *et al.* (1992) identified 34 isolates of *M. bovis* with a high degree of similarity in most of the isolates. Boothby *et al.* (1983) used SDS-PAGE and GED ELISA for the characterization of antigens from mycoplasmas of animal origin and observed certain areas of homology without any common protein band in different species along with high reactivity of proteins with homologous and heterologous antisera. Poly acrylamide gel electrophoresis (PAGE) used for separation of native proteins of *M. bovis* revealed protein profiles almost similar to *M. agalactiae* (Kumar *et al.*, 2001b). However, Kumar *et al.* (2000) characterized Sonicated Supernatant Antigen (SSA) and

Whole Cell Antigens (WCA) of an Indian isolate of *M. bovis* (NC317) by SDS-PAGE and immunoblotting. SDS-PAGE profile revealed 21 and 22 polypeptides in the region of 181.97 to 20.89 kDa in WCA and SSA, respectively. On immunoblotting, with polyclonal hyper immune serum raised against *M.* bovis NC317, only 12 and 14 polypeptides were found immunogenic in WCA and SSA, respectively. Alberti *et al.* (2006) reported that Western blotting might represent a useful tool for discriminating *M. bovis* from *M. agalactiae* after the PCR RFLP test. Rifatbegovic *et al.* (2009) studied the isolates of Bosnia and Herzegovina by SDS PAGE and Immuoblotting to find out immunogenic and protein similarity.

Immunobinding assay (IBA): Immunobinding tests were also used for the diagnosis of *M. bovis*. Kumar *et al.* (2002) used immuno binding assay of *M. bovis* but it showed cross reactivity with *M. agalactiae*. Recently an immuno binding test was developed on nitrocellulose paper with monoclonal antibody to diagnose *Mycoplasma bovis* in cultural isolates from the genital tract of artificially-infected heifers polymerase chain reaction was used as the gold standard (Flores-Gutiérrez *et al.*, 2009).

Immunohistochemistry: The use of specific antibodies either by Immuno Fluroscence (Knudtson *et al.*, 1986) or Immuno Histochemical (Adegboye *et al.*, 1995) can be used for the *in situ* detection of *M. bovis*. However, IH enables the visualization of the antigen together with the specific lesions (Rodriguez *et al.*, 1996).

Monoclonal antibodies: Monoclonal antibodies can differentiate between strains belonging to the same species (Poumarat *et al.*, 1994) but the monoclonal antibodies produced against the *M. bovis* antigen react with antigens prepared from other *Mycoplasma* species (Berthold *et al.*, 1992; Rasberry and Rosenbusch, 1995) due to genetic similarity between the so-called PvpA membrane protein of *M. gallisepticum* and the variable surface lipoproteins (Vsps) of *M. bovis* (Yogev *et al.*, 1994). Mab against the 27 kDa antigen determinant of *M. bovis* is responsible for adherence to cells (Sachse *et al.*, 1993). This surface glycoprotein enables *M. bovis* bacteria to adhere to alveolar phagocytes (macrophages) (Howard *et al.*, 1976) and to neutrophilic granulocytes (Thomas *et al.*, 1991).

Variable surface lipoproteins (Vsps)-is closely related to the polymorphism of *M. bovis*. The family of the Vsp antigens comprises three members: VspA, VspB and VspC (Behrens *et al.*, 1994; Rosengarten *et al.*, 1994; Beier *et al.*, 1998). The produced Vsps-specific antibodies allow us to study the host cell-*M. bovis* relationship, the differences between the isolated strains and their association with virulence.

In addition to the Vsp lipoproteins occurring in the membrane of *M. bovis,* the membrane protein of 67 kDa molecular weight (pMB67) also has an important role: it induces antibody response during natural infection or disease and may be suitable for the development of vaccines or diagnostic preparations (Behrens *et al.*, 1996).

MOLECULAR TESTS

Isolation of *M. bovis* is difficult, time consuming and cumbersome to perform. Moreover, the cross reactivity of *M. bovis* with many other mycoplasma species dragged the attention on the DNA-based techniques (Kumar *et al.*, 2001a). DNA-based techniques, especially PCR can yield rapid and specific diagnosis of infections caused by *M. bovis* (Hirose *et al.*, 2001).

Plasmid probes containing random genomic fragments used in dot blot hybridization tests produced cross reaction with *M. agalactiae* or *M. arginini* (McCully and Brock, 1992). Synthetic oligonucleotide probes from the 16 S RNA gene were applied by Mattsson *et al.* (1994) are not specific and sensitive enough for the routine diagnosis. The use of PCR in the diagnosis of *M. bovis* reduced the time of diagnosis from organs or the broth cultures even contaminated with bacteria.

The use of 16 S ribosomal RNA gene in PCR (Chavez Gonzalez *et al.*, 1995) also produced cross reactivity with *M. agalactiae*. Thereafter, Ghadersohi *et al.* (1997) designed PCR primers from sequences obtained from a *M. bovis* specific dot blot hybridization probe which was further modified by Hayman (2003) into a seminested setup. The PCR system designed for UvrC gene sequences provide high specificity and clear distinction of *M. bovis* thus UvrC gene could distinguish between *M. bovis* and *M. agalactiae* (Subramaniam *et al.*, 1998).

The Vsp gene based system described by Ghadersohi *et al.* (1997) modified by Hayman (2003) by adding a new forward primer (MbF) to the system-provided rapid detection of this organism. The semi-nested system has been developed by Hayman and Hirst (2003) whereas, Pinnow *et al.* (2001) developed a specific nested PCR test, with which the preservative-treated milk samples can also be examined. Alberti *et al.* (2006) used 16S rDNA sequence to establish the relatedness of strains. A Restriction Fragment Length Polymorphism (RFLP) strategy directed to the identification of phylogenetic clusters was designed to restrict the diagnostic investigation to a few bovine mycoplasma species (Alberti *et al.*, 2006). Reverse-transcription PCR and primer extension analysis indicated that both p68 and p48 are transcribed in *M. bovis* under *in vitro* growth conditions. *Mycoplasma bovis* is the first mycoplasma species in which two malp-related genes have been identified (Lysnyansky *et al.*, 2008). Rossetti *et al.* (2010) described a new specific real-time PCR assay targeting the UvrC gene that was developed to directly detect *M. bovis* from milk and tissue samples without laborious DNA purification.

CONCLUSIONS

Mycoplasma bovis infection can be diagnosed in several ways. Out of them isolation and identification not only requires specially equipped laboratories but also difficult due to secondary bacterial infections or inhibitory effects of antibiotics. Normal presence of *M. bovis* in nasal mucosa cannot be detected due to lack of antibodies. Moreover, serological tests particularly ELISA are useful tools for the herd diagnosis but cross-reactivity of *M. bovis* with other mycoplasmas and individual variation of immune response may sometime produce doubtful results.

Molecular tests particularly Polymerase Chain Reaction (PCR) with specific primers have better chance for the detection of the organism both early and chronic infections. The PCR systems targeting the 16S RNA gene *M. bovis* specific dot blot hybridization probe improve the specificity and sensitivity. The assay targeting the UvrC gene is also very sensitive and specific. Moreover, a conservative prokaryotic lipoprotein signal sequence of N-terminal part of variable cell surface lipoproteins (Vsp) can be an ideal region to be targeted with PCR assays to detect *M. bovis*. Polymerase chain reaction (PCR) and sequencing-based methods are very specific but require a massive amount of work if a wide range of species has to be investigated with dedicated assays.

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