MOLECULAR TYPING OF RUMINANT MYCOPLASMAS: A REVIEW

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SUMMARY

Mycoplasmas are aetiologic agents for various diseases that cause tremendous socio-economic losses in the livestock industry such as contagious bovine pleuropneumonia, contagious caprine pleuropneumonia, contagious agalactia, mycoplasmal pneumonia of swine and chronic respiratory disease of chickens. Due to their fastidiousness, mycoplasmas are difficult to propagate *invitro*. Similarly, because of the multiplicity of the syndromes caused by these organisms their diagnosis is difficult. Traditionally, phenotypic methods such as biotyping, serotyping, phage typing, bacteriocin typing and antibiogram typing have been used to characterise and differentiate microbial strains involved in disease outbreaks. However, these methods have some disadvantages such as low discriminatory power and low reproducibility, lack of stability of some of the phenotypes and, they require maintenance of large stocks of reference strains. The deficiencies inherent in the phenotypic methods of microbial typing have led to the evolution of molecular, and particularly nucleic acid-based typing methods which differentiate strains on the basis of their genetic material. Nucleic acid-based typing methods have a high discriminatory power, are highly reproducible and produce stable genotypes. Of the genotypic methods, pulsed-field gel electrophoresis and amplified fragment length polymorphism show a good potential for use in molecular typing and epidemiologic studies of bovine and caprine mycoplasma infections. This review focuses on methods for molecular typing of mycoplasmas with special emphasis on ruminant mycoplasmas.

INTRODUCTION

Mycoplasma infections cause tremendous socio-economic losses in the animal industry. Among the major epizootic diseases of cattle is contagious bovine pleuropneumonia (CBPP), which is caused by *Mycoplasma mycoides* subsp. *mycoides*, small colony biotype (*M. mycoides* SC) (Provost *et al.*, 1987). Historically, this disease has affected cattle in all continents, although nowadays it causes major economic losses in sub-Saharan Africa (Masiga *et al.*, 1996, Windsor and Wood, 1998, Thiaucourt, 1999). Other pathogenic mycoplasmas in cattle include *M. bovis, M. dispar, M. bovigenitalium, M. californicum, M. canadense* and *M. alkalescens* (Jasper, 1987; Kirkbride, 1987). Contagious caprine pleuropneumonia (CCPP), caused by *M. capricolum* subsp. *capripneumoniae* (*M. capripneumoniae*) is one of the most serious epizootic diseases that adversely affect the goat industry in East and Central Africa, the Mediterranean and Middle East (Thiaucourt *et al.*, 1996). *M. agalactiae* causes contagious agalactia, a disease of great importance in the dairy goat and sheep industry (Lambert, 1987). *M. mycoides* susbp. *mycoides*, large colony biotype (*M. mycoides* LC), *M. mycoides* subsp. *capri* (*M. capri*), *M. capricolum* subsp. *capricolum* (*M. capricolum*) and *M. putrefaciens* are involved in the aetiology of the mastitis, arthritis, keratoconjunctivitis, pneumonia and septicaemia syndrome, particularly in goats (Thiaucourt *et al.*, 1996; De Santis *et al.*, 1999). Mycoplasmal pneumonia of swine caused by *M. hyopneumoniae* causes significant socio-economic losses in the swine industry, especially in Europe and America (Kobisch and Friis, 1996). *M. gallisepticum, M. meleagridis, M. synoviae* and *M. iowae* cause production losses in the commercial poultry industry worldwide (Kleven, 1994; Stipkovits and Kempf, 1996).

Most mycoplasma infections in animals do not produce pathognomonic signs and thus are difficult to diagnose. In the last half of the 20th century considerable efforts were made to improve the isolation procedures for animal mycoplasmas (Bradbury, 1998; Nicholas and Baker, 1998). Media formulations for different species of mycoplasmas have been described (Armstrong, 1994; Kleven, 1994; Rosendal, 1994; Ruhnke and Rosendal, 1994; Sahu and Yedloutschnig, 1994). However, mycoplasmas are very fastidious organisms and are easily overgrown by other bacteria, thus rendering the isolation work very tedious (Simecka *et al.*, 1992; Razin *et al.*, 1998). Moreover, serological diagnosis of mycoplasma infections is hampered by the presence of cross-reactions among closely related mycoplasmas, particularly those belonging to the *M. mycoides* cluster (Cheng *et al.*, 1994; Hotzel *et al.*, 1994).

An important prerequisite for animal disease control is a thorough knowledge of the epidemiology of the diseases. This entails identification of sources of infections and modes of transmission of the diseases (McGowan and Metchock, 1995). In outbreak investigations, microbial typing, that is, differentiation of microbial isolates beyond the species level is helpful in devising appropriate intervention strategies (Olsen *et al.*, 1993). Microbial isolates with identical typing results are allocated in the same group, whereas those with different typing results are allocated to different groups. The different groups of isolates can be traced to their origins.

The traditional phenotypic microbial typing methods include biotyping (Olsen *et al.*, 1993; Quinn *et al.*, 1998), serotyping (Towner and Cockayne, 1993; Rurangirwa *et al.*, 1995), bacteriophage typing (Hickma-Brenner *et al.*, 1991), bacteriocin typing (Towner and Cockayne, 1993) and antimicrobial susceptibility testing (Aarestrup *et al.*, 1995). These methods have several drawbacks. For instance, biotyping is a laborious procedure and some biotypes are unstable. Because of its poor discriminatory power it has limited ability to differentiate strains within a species (Le Minor, 1988). Serological methods are less specific, they require extensive standardization and quality control and their discriminatory power of is poor because many strains may represent the same serotype or may be untypeable. Bacteriophage and bacteriocin typing require maintenance of stock of biologically active substances and control strains and are therefore limited for use in advance laboratories (Holmberg *et al.*, 1984; Towner and Cockayne, 1993). Antibiogram phenotypes vary due to genetic influences such as spontaneous mutations, acquisition of resistance genes via plasmids or transposons from other strains or species, and depend on selection pressure to be stable (Mayer, 1988). Moreover, a comparison of the phenotypic traits may not reflect the true genetic relatedness in a group of clinical isolates and thereby lead to erroneous epidemiological conclusions (Goering, 2001).

Difficulties with the typeability, reproducibility and the low discriminatory power of the phenotypic typing methods led to the evolution of molecular-based typing methods in last half of the 20th century. Molecular methods use macromolecules in the identification and discrimination of microorganisms and, have been very useful in the study of infectious diseases both in humans and animals. The principal macromolecules that have been used include proteins, polysaccharides and nucleic acids (Towner and Cockayne, 1993). Nucleic acid-based diagnosis and typing of bacteria is now being widely used in epidemiological investigations of major diseases of medical and veterinary importance (Belkum, 1994; Maslow *et al.*, 1993a).

Despite the major advances made in the improvement of nucleic acid-based methods for diagnosis and phylogenetic studies of mycoplamas in the past decade (Bashiruddin, 1998; Johansson *et al.*, 1998b), there has been a slow collateral development of the methods for epidemiological typing of field- or outbreak strains of mycoplasmas. As a result, in comparison to other bacteria, there are a few known DNA markers of epidemiological importance in mycoplasmas. The present paper reviews the molecular methods for typing of animal mycoplasmas with emphasis on those affecting domestic ruminants.

PROTEIN-BASED METHODS

WESTERN BLOTTING

Whole-cell protein analysis by SDS-PAGE and immunoblotting has been widely used to characterize mycoplasmal isolates (Mew *et al.*, 1985; Khan *et al.*, 1987; Sachse *et al.*, 1993). By using 2-dimensional polyacrylamide gel electrophoresis, Rodwell (1982) demonstrated that *M. mycoides* LC was more closely related to *M. capri* than *M. mycoides* SC and that *M. capricolum*, *Mycoplasma bovine* group 7 and *Mycoplasma* strain F38 were closely related to the *M. mycoides* species. Similarly, Costas *et al.* (1987) used a computerized numerical analysis of 1-dimensional SDS-PAGE protein patterns to differentiate 26 strains belonging to the *M. mycoides* cluster into four distinct phenons comprising of *M. mycoides* SC, *M. mycoides* LC and *M. capri*, *M. capricolum* and *Mycoplasma* F 38-like strains and, *Mycoplasma bovine* serogroup 7. A marked polypeptide and antigenic heterogeneity among 22 strains of *M. ovipneumoniae* when analysed by SDS-PAGE and immunoblotting was demonstrated by Thirkell *et al.* (1990). In a study by Sachse *et al.* (1992), only minor differences in the protein patterns were discernible and which were only confined to quantitative variations of some bands when 34 field isolates of M. bovis were compared by whole-cell SDS-PAGE and immunoblotting, implying a high degree of homogeneity among them. However, Poumarat *et al.* (1994), detected marked differences in the immunoblots of 20 *M. bovis* strains recovered from different lesions and geographical locations within France. Solsona *et al.* (1996), observed uniform protein profiles among *M. agalactiae* originating from different animals species and geographic locations in France. Protein profile and antigenic differences between European, African and Australian strains of *M. mycoides* SC have been demonstrated by using SDS-PAGE and immunoblotting techniques (Gonçalves *et al.*, 1998).

Being a phenotypic trait, protein expression is not stable and variation in profiles within the same strains can be demonstrated after repeated examination. Protein profiles produced by SDS-PAGE are too complex and difficult to compare. Moreover, the discriminatory power of protein profiling techniques is limited to reveal strain specific differences in certain mycoplasma species (Kokotovic, 2001). This deficiency has been overcome by the use of nucleic acid-based typing methods, which utilize microbial genetic materials as a target for analysis (Wolcott, 1992).

NUCLEIC ACID - BASED METHODS

Nucleic acid-based typing methods differentiate strains of microorganisms on the basis of genetic differences. Because the genetic material is not subject to phenotypic variation, genotypes are reproducible and stable. Nucleic acid-based typing methods are specific for target species and can distinguish strains within a species. Some of the nucleic acid-based techniques can allow specific differentiation of organisms from clinical materials without prior cultivation, thus speeding up the diagnostic procedure. Below are the genotypic methods that have been used for characterization of mycoplasma isolates.

RESTRICTION ENZYME ANALYSIS OF CHROMOSOMAL DNA (REA)

Analysis of the profiles of microbial chromosomal DNA digested by restriction enzymes that have frequent recognition sites is one of the simplest genotypic methods for bacteria typing (Razin *et al.*, 1983). Restriction enzyme analysis (REA) generates hundreds of bands in the size range of 0.5-50 kb which can be separated by constant field gel electrophoresis and detected by ethidium bromide staining (Maslow *et al.*, 1993b; Arbeit, 1995; Rodriguez *et al.*, 1997). This method has been used to demonstrate the genomic diversity among strains of *M. bovis* and *M. agalactiae* collected from a variety of specimens originating from different geographical areas in France (Poumarat *et al.*, 1994; Solsona *et al.*, 1996). REA patterns generated by the restriction enzymes *Bam*HI and *Pst*I revealed genomic differences between European and African field strains of *M. mycoides* SC (Poumarat and Solsona, 1995). Using the restriction enzymes *Pst*I and *Hind*III, Thiaucourt *et al.* (1998) also demonstrated that genomic differences exist among strains of *M. mycoides* SC isolated from outbreaks of CBPP in different parts of Africa. In addition, genomic differences between field and vaccine strains were evident. Ionas *et al.* (1991), demonstrated that 60 strains of *M. ovipneumoniae* displayed a marked genomic heterogeneity when analyzed by REA. Restriction enzyme

analysis is easy to perform and yield stable and reproducible profiles. The major demerit of REA is that the procedure generates complex patterns comprising of hundreds of overlapping and unresolved fragments that are difficult to interpret (Versalovic *et al.*, 1993).

PCR restriction digest in which a specific DNA sequence is amplified by PCR and the resulting amplicon is digested with restriction endonucleases has been employed to detect polymorphism in the P1 cytadhesin gene of *M. pneumoniae* (Sasaki *et al.*, 1996; Dorigo-Zetsma *et al.*, 2000).

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

This method is based on the description by Southern (1975) and it involves digestion of bacterial DNA with restriction enzymes, separation of the DNA fragments by constant field agarose gel electrophoresis and then transferring (or blotting) the fragments onto a nitrocellulose or nylon membrane. The fragments containing specific DNA sequences are then detected by using a labelled piece of homologous DNA (probe). Under appropriate conditions, the probe binds (or hybridises) to complementary base pair matching only to those fragments containing identical nucleotide sequences. The restriction fragment length polymorphism (RFLP) reflects the variation in the number of loci that are homologous to the probe and the location of the recognition sequences of a specific restriction enzyme (Walker and Dougan, 1989; Arbeit, 1995). All strains carrying loci homologous to the probe are typeable and the results are highly reproducible. RFLP analysis was developed in an attempt to reduce the number of fragments detected after restriction enzyme digestion of genomic DNA and thereby reducing the complexity of RFLP profiles. Insertion sequence analysis and ribotyping are examples of genotypic methods of mycoplasma subtyping which are based on Southern blot analysis.

Insertion sequence analysis

Insertion sequences (IS) are transposable short DNA segments of about 800-2500 nucleotide long which are present in multiple copies and positioned at different chromosomal loci in bacterial genomes (Lupski, 1987; Madigan *et al.*, 1997). RFLP analysis in which insertion sequences are used as probes has proven to be a reproducible method for molecular typing and epidemiological studies of bacterial infections (Hermans *et al.*, 1990; Lawrence *et al.*, 1989; Stanley *et al.*, 1993). Insertion sequence IS*1296* has been discovered in *M. mycoides* SC and it has been found that there are high copy numbers (up to 20) of IS*1296* in this mycoplasma and few copies in closely related organisms such as *M. mycoides* LC

and *Mycoplasma* bovine group 7 (Frey *et al.*, 1995). IS*1296* fingerprinting has facilitated the elucidation of the molecular epidemiology of CBPP (Cheng *et al.*, 1995; Frey and Nicolet, 1997; Vilei *et al.*, 2000). The European strains of *M. mycoides* SC that were isolated in the 1980s had a characteristic 3.4 kb *Hind*III fragment detected by the IS*1296* probe. This fragment was absent in the African strains, which instead possessed a 4.4 kb fragment. The Australian strains of *M. mycoides* SC possessed IS*1296* profiles that were closely related to the African ones. Thus, on the basis of IS*1296* profiles, *M. mycoides* SC strains have been grouped into two major clusters, one containing the European strains and another comprising the African and Australian strains. *M. mycoides* LC has been found to possess fewer copies of $IS1296_{varLC}$ that can also be used for fingerprinting isolates within this species (Frey and Nicolet, 1997).

M. mycoides SC also possesses high copy numbers (up to 30) of another insertion sequence, IS*1634* (Vilei *et al.*, 1999). IS*1634* is highly specific for *M. mycoides* SC and is useful for its identification and differentiation from closely related members of the *M. mycoides* cluster, which are devoid of the insertion sequence. The profiles of IS*1634* has fewer differences when compared to IS*1296* but it has been shown that the IS*1634* profiles of European strains possess a 7.0 kb *Hind*III fragment which is absent in the African and Australian strains. The use of IS*1296* and IS*1634* typing has made it possible to refute the notion that outbreaks of CBPP in Europe in the 1980s originated from Africa (Vilei *et al.*, 1999, 2000). IS*1296* and IS*1634* have also been used for subtyping of vaccine strains of *M. mycoides* SC (Cheng *et al.*, 1995; Frey and Nicolet, 1997; Vilei *et al.*, 1999).

Ribotyping

Analysis of the restriction fragment length polymorphism of ribosomal DNA (ribotyping) is a variation of Southern blot analysis in which microbial strains are characterized for RFLPs associated with the ribosomal operons (Yogev *et al.*, 1988a, 1988b; Johansson *et al.*, 1998a). The ribosomal operons comprise nucleotide sequences coding for 16S rRNA, 23S rRNA and one or more tRNAs. Ribosomal sequences are highly conserved and probes prepared from rRNA of one bacterial species or a cloned ribosomal operon (*rrn*) can hybridise to the chromosomal ribosomal operons of a wide range of bacterial species (Stull *et al.*, 1988). All bacteria carry these operons and are therefore typeable. Ribotypes are stable and reproducible, with isolates from an outbreak having the same ribotype (Tenover *et al.*, 1994). Although ribotyping has a good discriminatory power, epidemiological unrelated isolates may exhibit the same ribotype patterns thus reducing the discriminatory power or specificity

(Poh *et al.*, 1992). The discriminatory power of ribotyping can be increased by using multiple restriction enzymes (Yogev *et al.*, 1988a).

Probes made from 16S rRNA genes have been extensively utilized to elucidate the phylogenetic and evolutionary relationship between mycoplasma species (Heltander *et al.*, 1998; Petterson *et al.*, 1996a, 1996b; Petterson *et al.*, 1998). This method has also been employed to elucidate the molecular evolutionary and epidemiological trends for *M. capripneumoniae* (Kokotovic *et al.*, 2000). Nevertheless, ribotyping is not widely applied for epidemiological typing of mycoplasmas because mycoplasmas have low numbers of ribosomal operons (Amikan *et al.*, 1984; Swaminathan and Matar 1993; Cheng *et al.*, 1995).

Although analysis of RFLPs by hybridization is a sensitive, specific and universally applied technique for epidemiological typing of bacteria, the discriminatory power of the method decreases as number of detected restriction fragments decreases. Moreover, in Southern blotting information is provided only for specific regions within the total genome that can hybridise with the particular probe that is used (Towner and Cockayne, 1993). In addition, the procedure is time consuming although the generation of DNA probes by PCR and the development of more efficient non-radioactive labelling systems have simplified it.

RANDOM AMPLIFIED POLYMORPHIC DNA ASSAY (RAPD)

Random amplified polymorphic DNA (RAPD) assay, also known as arbitrary-primed PCR (AP-PCR) involves the amplification of random segments of genomic DNA using oligonucleotide primer sets constructed in the absence of specific nucleotide sequence information about the target (Williams *et al.*, 1990). Because the number and locations of these random sites vary among different strains so will the number and sizes of the fragments detected by electrophoresis of the amplicon. RAPD has been used to differentiate strains of *M. hyopneumoniae* and *M. gallisepticum* (Geary *et al.*, 1994; Artiushin and Minion, 1996). Rawadi *et al.* (1995), described a RAPD assay that demonstrated genomic heterogeneity among strains within the *M. mycoides* LC, *M. capri* and *M. capricolum* subspecies while strains within *M. mycoides* SC, *Mycoplasma* F38 and *Mycoplasma* bovine group 7 subspecies were homogenous.

RAPD is cheap and quick typing method but because less stringent PCR conditions are used, polymerization is initiated with variable efficiency at different sites having sequence mismatches. This result in variable amounts of DNA produced among the different sites amplified from a given isolate and, hence a variable number and intensity of individual bands produced from a single isolate (Arbeit, 1995;

Vaneechoutte, 1996). The reproducibility of the results can be improved by purification of the DNA and optimisation of the PCR conditions (Rawadi, 1998).

PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

Pulsed-field gel electrophoresis, developed by Schwartz and Cantor (1984) is a technique of separating chromosomal-sized DNA molecules by agarose gel electrophoresis using alternating electric field across the gel. PFGE enables separation of large DNA fragments (>50 kb) that are poorly or not separable by conventional agarose gel electrophoresis and about 5-30 large restriction fragments of chromosomal DNA can be separated (Maslow *et al.*, 1993b; Swaminathan and Matar, 1993). The principle of PFGE has been well-described (Birren and Lai, 1993; Maslow *et al.*, 1993b; Swaminathan and Matar, 1993; Tenover *et al.*, 1995). Different PFGE systems have been described with variations in the electrode geometry and method of reorientation of the electric field (Carle and Olson, 1984; Carle *et al.*, 1986; Gardiner *et al.*, 1986; Southern *et al.*, 1987; Clark *et al.*, 1988; Poh, 1998). The contour-clamped homogenous field (CHEF) system is presently the most widely one because it produces stable, highly reproducible and discriminatory profiles and, it is cheaper than the programmable autonomously controlled (PACE) system (Chu *et al.*, 1986; Birren and Lai, 1993).

PFGE has been applied to compare the sizes and genomic fingerprints of isolates of *M. hyopneumoniae, M. agalactiae* and *M. bovis* (Frey *et al.*, 1992; Tola *et al.*, 1996; 1999). More recently, PFGE protocols for molecular typing of bovine and caprine mycoplasmas have been described (Kusiluka, 2000). PFGE analysis using the restriction enzyme *Sma*I demonstrated a genomic heterogeneity among 11 strains of *M. bovis* isolated from nine Danish dairy cattle herds (Kusiluka *et al.*, 2000b). However, isolates from animals in the same herd had identical PFGE profiles. The profiles of the field strains were clearly different from the type strain of *M. bovis* (PG45^T).

PFGE typing has also been used to elucidate the epidemiology of CBPP in Tanzania (Kusiluka *et al.*, 2001a). Analysis of 56 strains of *M. mycoides* SC isolated from six distantly located CBPP-affected regions using *Bam*HI revealed indistinguishable PFGE profiles, leading to a supposition that the spread of the disease in the country was caused by clonal strains. The PFGE profiles of *M. mycoides* SC strains from Kenya, Botswana and Portugal were different from each other and from the Tanzania strains, reflecting possible differences in their epidemiological origins. Notably, the profiles of the two strains from Botswana were indistinguishable from each other, which was suggestive of their common origin. Moreover, the

PFGE profiles of the type- ($PG1^T$) and vaccine- (T_1-SR49) were different from those of the field strains. Although several restriction enzymes; *Apa*I, *Bam*HI, *Bg*lII, *Eco*RI, *Hind*III, *Nae*I, *Pst*I, *Sal*I, *Sma*I, *Sau*3AI, *Xba*I and *Xho*I were used, *Bam*HI produced well-resolved DNA fragments in the range of 9-388 kb when electrophoresed for 17 h at 0.5 to 20s pulse ramp time and hence the enzyme was considered suitable for generation of reliable PFGE profiles for *M. mycoides* SC.

PFGE has also been applied for genomic fingerprinting *M. capripneumoniae* from recent CCPP outbreaks in Tanzania (Kusiluka *et al.*, 2001b). Analysis of 11 strains of *M. capripneumoniae* from CCPP-affected goats in four regions of Tanzania using *Bam*HI revealed indistinguishable PFGE profiles. The profiles were also identical to those of six strains (including the type strain, $F38^T$) collected from Kenya between 1976 and 1996. The observation suggested a possible epidemiological link between outbreaks of the disease in the two countries in which cross-border movements of semi-nomadic tribes particularly the Maasai are rampant. For PFGE typing of *M. capripneumoniae*, the restriction enzymes *Bam*HI (Kusiluka *et al.*, 2001b) and *Kpn*I (Kusiluka, unpublished observation) produced easily interpretable profiles.

PFGE is one of the most reproducible and highly discriminatory typing techniques so far available and it is currently the method of choice for many species of microorganisms (Arbeit, 1995; Lee *et al.*, 1996; Sahm, 1996; Poh, 1998). All bacterial species are typeable by PFGE and the results are highly reproducible. The interpretation of PFGE results is relatively easy and consensus guidelines for interpretations have been published (Tenover *et al.*, 1995, 1997; Struelens *et al.*, 1996). Despite its merits, PFGE requires expensive equipment and is time consuming, requiring at least 2-4 days to produce results (Arbeit, 1995; Struelens *et al.*, 1996; Tenover *et al.*, 1997).

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

Amplified fragment length polymorphism (AFLP) is a genomic DNA fingerprinting technique that is based on selective amplification of restriction fragments from a total digest of genomic DNA (Zabeau and Vos, 1993; Vos *et al.*, 1995; Janssen *et al.*, 1996). Because stringent PCR conditions are used, specific amplification of a high number of restriction fragments (» 50-100) in DNA of any origin and complexity renders AFLP a powerful tool for molecular epidemiological typing. Although AFLP is a relatively new technique when compared to other genomic fingerprinting methods, it is increasingly being employed for

phylogenetic and molecular epidemiological studies of bacteria (Janssen *et al.*, 1996, 1997; Keim *et al.*, 1997; Koeleman *et al.*, 1998; Kokotovic and On 1999; Savelkoul *et al.*, 1999). Kokotovic *et al.* (1999), developed AFLP procedure which demonstrated a very high discriminatory power for strains within *M. dispar, M. hyopneumoniae, M. flocculare, M. hyosynoviae, M. genitalium, M. pneumoniae* and *M. hominis* species. Kokotovic *et al.* (2000), described an AFLP procedure for molecular typing of *M. capripneumoniae* whose results demonstrated that there were two evolutionary lines of descent for the species as it had also been established by 16S rRNA analysis (Petterson *et al.*, 1998). Kusiluka *et al.* (2000a), described an AFLP protocol for elucidation of the genetic relatedness of *M. bovis* strains isolated from Danish cattle over a 17 years' period (1981-1998). A remarkable genomic homogeneity was demonstrated among the 42 field strains analysed. Through AFLP, it was possible to establish an epidemiological link between two outbreaks of *M. bovis*-induced mastitis in two districts located 300 km apart.

A clonal spread of CBPP in Tanzania in the 1990-1999 period was demonstrated through genetic fingerprinting of *M. mycoides SC* isolated from CBPP cases using AFLP (Kusiluka *et al.*, 2001a). Analysis of the *Eco*RI/*Csp*6 patterns of 56 strains of *M. mycoides* SC from six regions revealed indistinguishable profiles except for one strain which was polymorphic at only one position. Strains from Kenya, Botswana and Portugal were different from each other and from the Tanzanian ones, as were the type- ($PG1^T$) and vaccine- (T_1SR49) strains. A close link between *M. mycoides* SC isolated from CBPP-affected cattle and sick goats kept in the same geographical location was demonstrated using AFLP (Kusiluka *et al.*, 2001b), suggesting that cross transmission of the organism between the two animal species was possible. This may be an epidemiologically important factor in the perpetuation of CBPP in areas where cattle and goats are kept together. Based on AFLP analysis, Kusiluka *et al.* (2001b) demonstrated an epidemiological relationship of 11 strains of *M. capripneumoniae* collected from CCPP outbreaks in four regions of Tanzania in the 1998/99 period. Of the 11 strains analyzed by *Eco*RI/*Csp*6 enzyme combination, 10 of them showed indistinguishable profiles, which were also identical to two strains from Kenya and one ovine strain from Uganda. The identical *M. capripneumoniae* strains from Tanzania also showed a 16S rRNA gene sequence polymorphism that was similar to those predominant in Uganda and western Kenya (Msami *et al.*, 2001), probably reflecting a cross-border transmission of the disease between these countries.

The use of sequencing technology in AFLP reveals more polymorphisms than the other methods so far used for genomic fingerprinting of ruminant mycoplasmas such as restriction enzyme analysis (Poumarat *et al.*, 1994), insertion sequence analysis (Cheng *et al.*, 1995; Frey and Nicolet, 1997; Vilei *et al.*, 2000) and PFGE (Kusiluka, 2000). The main limitation of AFLP fingerprinting is that the detection system for the profiles largely depends on the use of DNA sequencers, which are very expensive and this limits its use to advanced laboratories.

NUCLEOTIDE SEQUENCE ANALYSIS

The determination of the precise nucleotide sequence of microbial genome is the most specific method for typing microorganisms (Felsenstein, 1988; Swaminathan and Matar, 1993). Nucleotide sequence analysis involves cloning and sequencing an isolate at a particular locus and comparing multiple isolates by sequencing each one at the same locus. DNA sequence analysis has been simplified by PCR amplification of known DNA segments and automated sequencing of the PCR products (Towner and Cockayne, 1993). PCR-based sequencing of ribosomal DNA is now universally used for the detection and identification of a wide range of bacterial pathogens because certain ribosomal sequences are highly conserved across the bacterial kingdom, and thus primers that amplify ribosomal sequences from essentially any bacteria can be designed (Johansson *et al.*, 1998a). The family, genus and species of infectious organisms can be identified by sequencing the amplified product and analyzing the nucleotide sequences at relatively variable areas within the ribosomal operon.

Nucleotide sequence analysis yields precise data about microbial genomes and extensive databases can be shared with relative ease, thus facilitating comparative analyses between laboratories (Towner and Cockayne, 1993; Johansson *et al.*, 1998a). Analysis of the 16S rRNA nucleotide sequence has facilitated the genetic typing and phylogenetic studies of mycoplasmas (Woese *et al.*, 1980; Weisburg *et al.*, 1989; Heldtander *et al.*, 1998). Polymorphisms in the 16S rRNA gene sequences of *M. capripneumoniae* strains from different countries have been demonsrated (Petterson *et al.*, 1998). Furthermore, the sequences of the entire genomes of *M. genitalium* and *M. pneumoniae* have been determined (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996) which makes it easy to type the strains within these species.

One limitation for the application of nucleotide sequencing in strain typing is that, loci with sufficient variability to permit epidemiologically useful strain differentiation must be available for each bacterial species (Arbeit, 1995).

Moreover, the method is technically complex for practical purposes to type a large number of isolates (Swaminathan and Matar, 1993). Furthermore, automated DNA sequencers are expensive.

CONCLUSION

Nucleic acid-based subtyping systems offer several advantages over conventional methods of microbial typing because the genetic material is not affected by phenotypic variations and, genotypes are stable and highly reproducible. Subtle variations between isolates that cannot be detected by phenotypic typing methods can be revealed by genotypic typing techniques. PCR-based systems are rapid, can be applied to a large number of isolates and are suitable for microorganisms which are difficult to propagate *in vitro*. Although universal agreement on methodology, interpretation or quality control in microbial typing has not yet been fully achieved, DNA-based methods show a very promising potential for epidemiological investigations and are increasingly being utilized in clinical laboratories.

PFGE and AFLP show good prospects as additional tools in the genomic fingerprinting and molecular epidemiological studies of bovine and caprine mycoplasmas. These methods produce results that are similar to, but easy to interpret than restriction enzyme analysis. The automation of AFLP analysis permits creation of databases for long-term storage of data and inter-laboratory usage or comparison. However, before these methods can be adopted as standard protocols for genetic fingerprinting of bovine and caprine mycoplasmas, it is recommended to refine the protocols using other restriction enzymes and or primer combinations in order to enhance their discriminatory power. It should also be emphasized that the usefulness of the results obtained by any typing system will depend on the availability of the correct clinical or epidemiological data concerning the microbial strains under investigation.

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