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Genotypic Methods for Diagnosis of Mycoplasmal Infections in Humans, Animals, Plants and Cell Cultures

GEORGES RAWADI* AND OLIVIER DUSSURGET[1]

Laboratoire des Mycoplasmes, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France. [1]*Present address: Department of Microbiology, Public Health Research Institute, 455 First Avenue, New York, NY 10016, USA*

Introduction

Mycoplasmas are the smallest self-replicating bacteria, characterized by a wall-less envelop and an unusual small genome size. They are closely related to Gram-positive bacteria with a low G+C content (Maniloff *et al.*, 1992). Mycoplasma genus belongs to the class of Mollicutes which consists of eight genera (Razin, 1992). These organisms are widely distributed in animals and plants (Tully, 1992).

The first isolation of a mycoplasma from humans was reported in 1937. However, there is poor evidence of their pathogenicity in human apart from atypical pneumonia and some genitourinary diseases. Reports of isolation of mycoplasmas in the blood of AIDS patients raised the possibility of the implication of these bacteria as co-agents in this autoimmune disease (Blanchard and Montagnier, 1994). Although the role of these microorganisms as immuno-modulatory agent is well established (e.g. immunoglobulin production, cytokine induction), the pathophysiological significance of this effect remains unclear. The entire genome of two human pathogens, *Mycoplasma genitalium* and *M. pneumoniae*, have been sequenced (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996) and sequence analysis of the genome of *Ureaplasma urealyticum*, another human pathogen, will be completed in a near future (International Organization for Mycoplasmology (IOM) congress, Orlando, Florida, USA, 1996). The wealth of informations provided by the Genome Projects should allow a better understanding of the molecular pathogenesis of mycoplasmas.

Mycoplasmas are also of great importance in veterinary medicine and are responsible of significant economical losses in animal production (Razin and Barile, 1985). Several mycoplasma species have clearly been shown to be pathogenic in caprine, ovine, bovine, porcine and avian (Tully and Whitcomb, 1979).

*To whom correspondence may be addressed.

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The first evidence of the implication of a wall-less prokaryote in plant diseases was reported by Doi *et al.* (1967). These non-culturable organisms, initially called mycoplasma-like organisms (MLOs), are included within the Mollicutes class according to the 16S rRNA genes analysis (Schneider *et al.*, 1993; Seemüller *et al.*, 1994). More recently, based upon the sequence of the 16S and 23S rRNA genes and their spacer region, the name phytoplasmas was recommended by the IOM Subcommittee for the Taxonomy of Mollicutes (IOM Congress, Bordeaux, France, 1994). Phytoplasmas infect a wide variety of agricultural and ornamental plants causing severe damages (McCoy *et al.*, 1989) or desirable traits (Lee *et al.*, 1997). They are also found in insects which are thought to be the transmission vectors. The cultivable and well-characterized spiroplasmas are also plant and insect pathogens, e.g. *Spiroplasma citri* (Calavan and Oldfield, 1979).

Mycoplasmas often contaminate cell cultures. The contamination of primary and continuous eukaryotic cell lines represents a major problem of economic and biological importance in basic research, in diagnosis, and in biotechnological production. This problem is widespread and surveys showed that 5 to 87% of cell lines are contaminated (Bölske, 1988; Uphoff *et al.*, 1992; Pawar *et al.*, 1994).

The isolation and detection of mycoplasmas were, for a long time, hindered by the lack of specific and sensitive detection methods. Unlike most bacteria, these organisms require specific and elaborated culture media and cultivation needs prolonged incubation period (one to more than two weeks). Recent advances in the microbiological diagnosis field have involved the development of a technology based on molecular biology. This technology includes: (i) hybridization of a specific DNA fragment with genus or species-specific probes and (ii) amplification of a specific DNA segment by polymerase chain reaction (PCR). These methods were shown to be rapid, sensitive and specific providing reliable diagnosis tools. Hybridization methods are based on the formation of a duplex between a target single-stranded nucleic acid molecule and a complementary probe. This reaction is widely used in basic research and has been shown to be useful in microbiological diagnosis (Tenover, 1988). *In vitro* DNA amplification by PCR uses oligonucleotidic primers and a thermostable DNA polymerase to synthesize millions of copies of a specific DNA segment (Saiki *et al.*, 1988). PCR-based methods are a fast and highly specific way to detect low level of mycoplasmal contamination that surpass the current methods of diagnosis and overcome the principal limitation of diagnostic probe hybridization, i.e. its relatively low sensitivity.

This review describes the target molecules for hybridization and PCR amplification and the recent advances in the detection of mycoplasmas in clinical and veterinary samples, in plants and in cell cultures by genotypic methods.

Target molecules and strategies for probes and PCR primers design

The choice of genomic target sequence for hybridization and/or for PCR amplification is a critical step that influences the specificity of microbial detection. Two categories of probes are used in hybridization-based techniques: conventional DNA probes and oligonucleotidic probes. The first type of DNA probes is determined either by random cloning of restriction fragments or from known nucleotidic sequences. The oligonucleotidic probes, 20 to 30 bp long, are necessarily deduced from known

sequences and synthesized chemically. The hybridization procedure has been suggested as a complement to classical methods of diagnosis. 16S ribosomal (r) RNA genes have been used to study the microbial diversity (Weisburg *et al.*, 1989). Thus they are considered good candidates for molecular diagnosis. These genes are divided into three regions: highly conserved, semi-conserved and variable regions. This allows the design of species and even subspecies-specific complementary probes. A large number of rRNA sequences are available in databases (e.g. GeneBank), thus sequence analysis by a software package (e.g. Genetics Computer Group) permits one to display alignment and similarity within these genes and to design rDNA probe (Devereux *et al.*, 1984). Molecular diagnosis takes advantage of the fact that rRNA is present in up to 10^4 copies per living bacterium, increasing the sensitivity of detection.

A similar strategy is used to design PCR primers. Specific restriction fragments of cloned DNA can be sequenced and derived primers sets synthesized for PCR amplification. Sequence analysis of rRNA, as described above, is also used to determine genus or species-specific primers for PCR.

Probes and PCR primers design, hybridization and PCR protocols should be tested in order to verify the absence of cross-reactivity species, especially closely related species. If cross-reactivity occurs, new sequence analysis and probe selection have to be performed. Moreover, the sensitivity of the detection should be evaluated for each probe or PCR primers set. Finally, the new protocol must be validated by comparison with conventional methods. The different steps for the design of hybridization probes and PCR primers are summarized in *Figure 1*.

Diagnosis of human major pathogenic mycoplasmas

Mycoplasmas cause severe respiratory, arthritic and urogenital diseases of humans (*Table 1*). Koch's postulates have been fulfilled for two species, *M. pneumoniae* which is the most common pathogen in lung infections among 5 to 35-year-old patients, and *U. urealyticum* which is responsible for nonchlamydial, nongonococcal urethritis of males and meningitis in very-low-birth-weight infants and chorioamnionitis (Taylor-Robinson and McCormack, 1980; Cassel *et al.*, 1991, 1993). *M. hominis* is also considered to be a significant human pathogen associated with pelvic inflammatory disease, postpartum septicemia and endometritis, amnionitis, pyelonephritis, pneumonia and central nervous system infections in newborn infants (Cassel *et al.*, 1991). *M. genitalium* has been associated with respiratory and urogenital diseases. More recently, *M. pirum*, *M. fermentans* and *M. penetrans* have been isolated from patients infected with HIV (Blanchard and Montagnier, 1994). The presence of mycoplasmas in these patients was associated with accentuated pathology, namely AIDS-associated nephropathy, adult respiratory distress syndrome, and enhanced HIV cytopathic effects (Bauer *et al.*, 1991; Lo *et al.*, 1993; Hawkins *et al.*, 1992; Ainworth *et al.*, 1994; Blanchard and Montagnier, 1994). Mycoplasmas may play a role in AIDS by activating the immune system and/or by elucidating the programmed cell death of immune cells (Ruuth and Praz, 1985; Rawadi and Roman-Roman, 1996, Rawadi *et al.*, 1996). This issue should be resolved in the future by additional epidemiological data and therapeutical trials involving anti-mycoplasmal antibiotics.

A number of investigators have used DNA probes to detect mycoplasmas in clinical specimens (Blanchard *et al.*, 1992) as an alternative to conventional microbiological

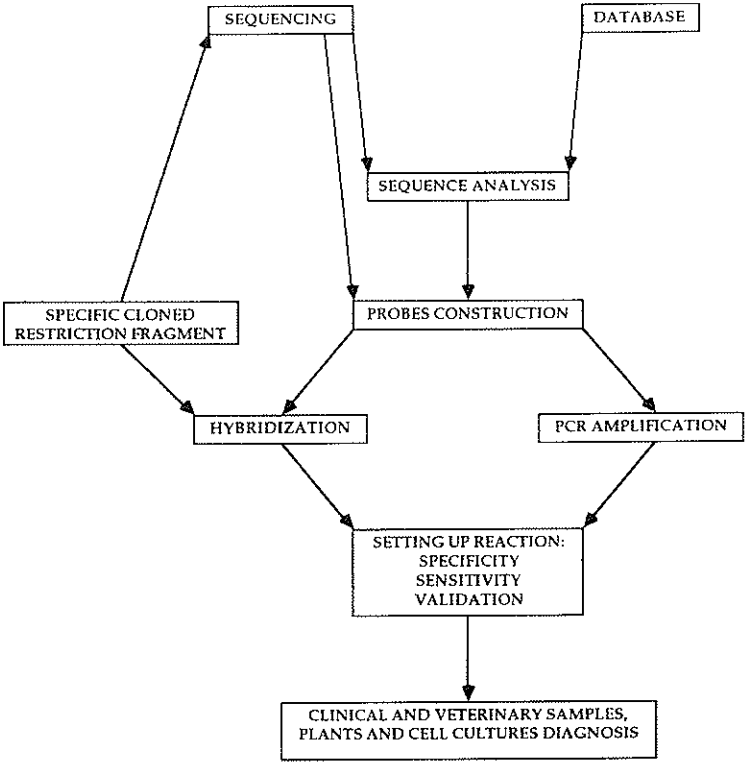

Figure 1. Major procedures for probes and PCR primers design to diagnose mycoplasmal infection in animal samples, in plants and in tissue cultures.

methods. Based up on the 16S rRNA genes, a *M. pneumoniae*-specific probe has been described by Shaw *et al.* (1989) and became commercially available (Gen-Probe Corp., San Diego, CA). Several studies were performed to compare this probe with more classical techniques (i.e. culture and serological methods) for the detection of this microorganism in various clinical specimens (Dular *et al.*, 1988; Tilton *et al.*, 1988; Hata *et al.*, 1990; Kleemola *et al.*, 1990). The numbers of patients examined were, respectively, 116, 82, 163 and 160. Although they all came to the conclusion that this hybridization system is specific and faster, making a same day diagnosis possible, the sensitivity found by some investigators was lower than expected leading to false negative diagnosis.

Göbel *et al.* (1987a, b) developed synthetic oligonucleotides probes, 20 to 30 bp long, complementary to domains of rRNA genes showing group and species specificity. A probe designated MP30 was found to cross-hybridize between *M. pneumoniae* and *M. genitalium* allowing the detection of both. On the other hand, a probe designated MP20 was proved to react only with *M. pneumoniae*, therefore allowing the specific identification of this organism with as sensitivity estimated at 10^3 organisms.

DNA probes specific to *M. pneumoniae* and *M. genitalium* have been developed to overcome the serological cross-reactivity between these species that hinders their identification (Hyman *et al.*, 1987; Risi *et al.*, 1987; Hooton *et al.*, 1988). Two DNA

Table 1. Mycoplasmas which infect humans

Species	Diseases and significance
A. laidlawii	Unknown
M. buccale	None
M. faucium	None
M. fermentans	Possible role in respiratory infections, systemic diseases, AIDS
M. genitalium	Possible role in respiratory and genital tract infections
M. hominis	Direct role in some female pelvic inflammatory diseases, in postpartum infections, in some traumatic wound infections in both males and females
M. lipophilum	None
M. orale	None
M. penetrans	Unknown
M. pirum	Unknown
M. pneumoniae	Direct role in pneumonia and associated extrapulmonary complications involving the central nervous system, cardio-vascular system, skin and joints
M. primatum	Unknown
M. salivarium	None
M. spermatophilum	Unknown
U. urealyticum	Direct role in male non-gonococcal urethritis, pneumonia and meningitis in neonates

fragments from *M. pneumoniae* (pPN4) and *M. genitalium* (pGN3), respectively 8 kb and 20 kb long, were isolated by Razin *et al.* (1987). These probes yielded no or weak non specific signals in dot blots with non-homologous purified DNAs at concentration 1000-fold higher than the minimal concentration of homologous DNA exhibiting a hybridization signal (Hyman *et al.*, 1987).

Cultivation is currently used to diagnose infections due to *U. urealyticum* because it can be isolated easily from clinical specimens. However, this costly procedure is slow. Nucleic acid probes offer an alternative approach for *U. urealyticum* detection in clinical specimens. Conventional DNA probes used by Brogan *et al.* (1992) were determined to be specific but lacked sensitivity.

If using DNA sequences coding for proteins as probes would *a priori* be a more specific approach than selecting rRNA genes as a target for hybridization, it is also less sensitive because bacterial cell contains up to 10^4 rRNA copies in comparison with a single or a few copies of a particular gene (Waters and Mc Cutchan, 1990). However, the secondary and tertiary structure of rRNA make these molecules more accessible to probes based up on synthetic oligonucleotides than up on entire gene fragments. Therefore the experimental sensitivity in the latest case is lower than expected (personal data). ^{32}P-labelled oligonucleotides complementary to the variable regions of 16S rRNA were found to be 100 times more sensitive than using DNA probe directed against a single copy gene (Göbel *et al.*, 1987; Johansson 1993). Efforts to replace radioelements (^{32}P or ^{125}I) used in hybridization procedures, limiting their use to authorized laboratories, by chemical labels (e.g. sulphonation, biotinylation) accounted for 10-fold losses in sensitivity (Hyman*et al.*, 1987; Rawadi*et al.*, unpublished data).

PCR amplification affords a means to enhance sensitivity with its potential to detect genomic material from one organism (Saiki *et al.*, 1988). PCR-based detection assays have been developed for the vast majority of mycoplasmas known as human pathogens or suspected to be involved in human diseases (*Table 2*). Several target sequences

Table 2. Detection of mycoplasmas which infect humans by *in vitro* DNA amplification

Species	Target	Technique	Reference
M. pneumoniae	Randomly cloned DNA	PCR	Bernet *et al.*, 1989
		PCR	Narita *et al.*, 1992
		PCR	Skakni *et al.*, 1992
		PCR	Vekris *et al.*, 1995
	16S rDNA	PCR	Jensen *et al.*, 1989
		RT-PCR*	van Kuppeveld *et al.*, 1992
		3 Primers-PCR	Kai *et al.*, 1993
		PCR/RT-PCR	Tjhie *et al.*, 1994
		PCR	van Kuppeveld *et al.*, 1994
	P1 adhesin gene	PCR	Jensen *et al.*, 1989
		PCR	Buck *et al.*, 1992
		PCR	De Barbeyrac *et al.*, 1993
		Nested-PCR	Zigangirova *et al.*, 1993
		PCR	Leng *et al.*, 1994
		PCR	Williamson *et al.*, 1994
		PCR	Rezkinov *et al.*, 1995
		Semi-nested PCR	Fink *et al.*, 1995
	P1 adhesin gene/16S rDNA	PCR	Marmion *et al.*, 1993
		PCR	Ieven *et al.*, 1996
	P1+MgPa adhesins genes	Triplex-PCR	Cadieux *et al.*, 1993
	Elongation factor Tu gene	PCR	Lüneberg *et al.*, 1993
	Rep elements of P1	PCR	Ursi *et al.*, 1992
M. genitalium	MgPa adhesin gene	PCR	Palmer *et al.*, 1991
		PCR	Jensen *et al.*, 1991
		PCR	de Barbeyrac *et al.*, 1993
		PCR	Blanchard *et al.*, 1993a
	MgPa+P1 adhesin genes	Triplex-PCR	Cadieux *et al.*, 1993
U. urealyticum	Urease gene	PCR	Willoughby *et al.*, 1991
		PCR	Blanchard *et al.*, 1993b
	Randomly cloned DNA	PCR	Brogan *et al.*, 1992
	16S rDNA	PCR	Roberston *et al.*, 1993
M. fermentans	Insertion sequence-like element	PCR	Wang *et al.*, 1992
		PCR	Blanchard *et al.*, 1993a
		PCR	In Chingbingyong and Hughes, 1996
	16S rDNA	PCR	Blanchard *et al.*, 1993a
	Elongation factor Tu gene	PCR	Berg *et al.*, 1996
M. hominis	16S rDNA	RT-PCR*	van Kuppeveld *et al.*, 1992
		PCR	Blanchard *et al* ., 1993c
		PCR	Grau *et al.*, 1994
	Randomly cloned DNA	PCR	Gallia *et al.*, 1995

*RT-PCR: reverse transcription polymerase chain reaction.

to be amplified have been chosen, randomly cloned DNA from mycoplasmas of interest, repetitive genomic sequence, specific mycoplasmal gene sequence and 16S rRNA gene sequence (*Table 2*). Their use has been previously discussed (Razin, 1994).

The use of PCR to diagnose both *M. pneumoniae* and *M. genitalium* allowed a clear distinction of these two organisms which share many characteristics such as morphology, ultrastructure, antigenic cross-reactivity, and possess adhesin protein homology. PCR primers designed to amplify *M. pneumoniae* 16S rRNA such these described by van Kuppeveld *et al.* (1992) may cross-react with *M. genitalium* when present at high

level (>10^4 cfu/ml). A reliable PCR assay for the detection and differentiation of these mycoplasmas would combine the amplification of rRNA and of species-specific target such as the gene encoding the adhesin P1 (Ieven *et al.*, 1996) from *M. pneumoniae* and MgPa from *M. genitalium*. Although genetic heterogeneity among *M. hominis* strains is well documented, 16S rRNA gene has been successfully used to target specific amplification allowing the detection and the identification of this species (Christiansen *et al.*, 1987; Grau *et al.*, 1994). In addition, Gallia *et al.* (1995) have identified a DNA fragment specific for *M. hominis* that they successfully used as a target for a PCR assay.

Once suitable primers have been designed, the authenticity of the amplified sequence must be assessed by several types of control to avoid false positive reaction due to cross-amplifications of related DNA. One of the most common strategies consists of the use of DNAs from human or non-human mycoplasmas, from human DNA and DNAs from a variety of bacteria as negative controls. The identity of the product is routinely confirmed directly by three-primer PCR (Kai *et al.*, 1993) or semi-nested (Fink *et al.*, 1995) and nested PCR (Zigangirova *et al.*, 1993) or secondarily by hybridization with a specific probe (Jensen *et al.*, 1991; Palmer *et al.*, 1991; Buck *et al.*, 1992; Wang *et al.*, 1992; Blanchard *et al.*, 1993a,b,c; de Barbeyrac *et al.*, 1993; Grau *et al.*, 1993; Vekris *et al.*, 1995; Berg *et al.*, 1996; Ieven *et al.*, 1996; In Chingbingyong and Hughes, 1996) and/or restriction fragment analysis (Jensen *et al.*, 1991; Palmer *et al.*, 1991; de Barbeyrac *et al.*, 1993; Williamson *et al.*, 1994). Experiments conducted in the authors' laboratory lead them to recommend the hybridization assay since nested PCR may yield false positive signal and three-primer PCR is dependent upon amplification conditions which may vary from one laboratory to another.

Exquisite sensitivity has been reached by PCR assays using either purified DNA, bacterial suspension, samples from experimentally infected animals and artificially seeded human samples or clinical specimens. Under optimal conditions, PCR was reported to detect 1 fg of target DNA or between 1 and 10 organisms, even in the presence of background DNA. This sensitivity was achieved for the detection of *M. genitalium* (Palmer *et al.*, 1991), *M. fermentans* (Wang *et al.*, 1992), *M. pneumoniae* (Buck *et al.*, 1992; van Kuppeveld *et al.*, 1992) and *U. urealyticum* (Blanchard *et al.*, 1993b).

Several investigators compared the use of PCR with microbiological culture for the detection of mycoplasmas. *In vitro* amplification was able to detect *M. pneumoniae* (de Barbeyrac *et al.*, 1993; Ieven *et al.*, 1996) and *M. genitalium* (de Barbeyrac *et al.*, 1993) in clinical samples when cultures were negative. Similar observations were reported for the detection of *M. fermentans* in amniotic fluid and for the detection of *M. genitalium* in patients with urethritis and cervicitis (Blanchard *et al.*, 1993a). Buck *et al.* (1992), Skakni *et al.* (1992). Marmion *et al.* (1993) extended the comparison to microplate agglutination assay, gene probe, cell-sheet culture, indirect haemagglutination detection of IgM antibody and antigen capture-enzyme immunoassay. They indicated that PCR was the most reliable method in term of sensitivity, specificity and time. PCR assay for *M. pneumoniae* in clinical specimens was found 100-fold more sensitive than culture technique and 1000-fold more sensitive than gene probe detection (Buck *et al.*, 1992). Blanchard *et al.* (1993b) showed that their PCR assay could detect *U. urealyticum* in less than 24 hours compared within 2–5 days for

culture. The authors emphasized that a fast detection method may be very important in management of very low-birth-weight infants in whom this species accounts for meningitis, respiratory disease and death. PCR was also faster than serological assays and was suitable in immunocompromised patients (van Kuppeveld *et al.*, 1994). A PCR-based method for biotyping *U. urealyticum* was also developed that allows to investigate the relationship between biovars and disease (Roberston *et al.*, 1993).

Before routine use of PCR-based diagnosis of mycoplasmal diseases, some problems remain to be resolved. False negative results, most probably due to polymerase inhibitors, have been reported in clinical samples (Blanchard *et al.*, 1993b; Kai *et al.*, 1993; Marmion *et al.*, 1993; Reznikov *et al.*, 1995; Ieven *et al.*, 1996). The nature of these inhibitors is not clearly identified. However, procedures were described to reduce the number of false negative, for example, reducing the volume of the sample added to PCR reaction and increasing the proteinase K concentration (Blanchard *et al.*, 1993b). Moreover, depending on their origin, DNA polymerases exhibit a different sensitivity to PCR inhibitor in a given specimen (personal data). Therefore, distinct thermostable DNA polymerases should be assayed to overcome this drawback. False positive results may be avoided by including adequate controls. Procedures for this purpose should be applied in all laboratories using *in vitro* amplification either for diagnosis or for basic research.

Veterinary mycoplasmas

Mycoplasmas of veterinary interest are shown in *Table 3*. These mycoplasmas have been proved to be pathogens in their respective hosts. Therefore, particular efforts were focused on developing suitable detection methods to survey outbreaks. Both hybridization- and PCR-based methods were used to detect and identify mycoplasma species in the veterinary field. Herein, we distinguished mycoplasmas included in the *M. mycoides* cluster that are significant bovine, ovine or caprine pathogens (Cottew *et al.*, 1987; Ernø, 1987) from mycoplasmas that are found in small and large ruminants, and from avian mycoplasmas.

THE *MYCOPLASMA MYCOIDES* CLUSTER

The first mycoplasma isolated was the contagious bovine pleuropneumonia (CBPP) agent described by Nocard and Roux (1898). This mycoplasma is now known as *M. mycoides* subsp. *mycoides* small colony (SC) type and is included in the *M. mycoides* cluster. This cluster comprises six groups: *M. mycoides* subsp. *mycoides* with two types, SC and large colony (LC); *M. mycoides* subsp. capri; *M. capricolum*; *M.* sp. strain F38; and *M.* sp. group 7 of Leach (Cottew *et al.*, 1987). *M. capricolum* and *M.* sp. strain F38 were newly classified as *M. capricolum* subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae* (Leach *et al.*, 1993).

Mycoplasmas belonging to this cluster are closely related, show serological cross-reactivity and share several biochemical characteristics and so it is difficult to identify species or subspecies by conventional methods (e.g., serological methods). A DNA probe, CAP-21, described by Taylor *et al.* (1992), allowed the differentiation of strains causing CBPP from those which did not produce the disease. A second DNA probe has been reported by the same group, F38-12, and allowed to distinguish *M. capricolum*

Table 3. Mycoplasmal species of interest in veterinary medicine

Mycoplasma species	Diseases	Hosts
M. mycoides subsp. *mycoides* SC	CBPP	bovine
M. mycoides subsp. *mycoides* LC	septicemia, arthritis and pneumonia	goats
M. capricolum subsp. *capricolum*	septicemia, arthritis and mastitis	goats and sheep
M. cparicolum subsp. *capripneumoniae*	CCPP	caprine
M. mycoides subsp. *capri*	pneumonia and arthritis	goats
M. sp group 7 of Leach	arthritis and mastitis	cattle
M. bovis	mastitis, arthritis and respiratory diseases	cattle
M. agalactiae	contagious agalictia	goats and sheep
M. gallisepticum	respiratory diseases	chickens and turkeys
M. synoviae	respiratory diseases	chickens and turkeys
M. meleagridis		turkeys
M. iowae		turkeys

subsp. *capripneumoniae*, agent of contagious caprine pleuropneumonia (CCPP), from *M. capricolum* subsp. *capricolum* which causes septicemia, arthritis and mastitis in goats and sheep. However, the cross-reactivity of these probes with other mycoplasmas or bacteria and their sensitivity remain to be determined.

Several PCR techniques have been developed to specifically detect and identify the etiological agent of CBPP, *M. mycoides* subsp. *mycoides* SC (Bashiruddin *et al.*, 1994; Dedieu *et al.*, 1994a; Hotzel *et al.*, 1996). Bashiruddin *et al.* (1994) used the CAP-21 probe sequence to design PCR primers that amplify all the members of the *M. mycoides* cluster and identified the biotype SC by restriction analysis. Hotzel *et al.* (1996) used the CAP-21 sequence as well to develop a nested-PCR assay that allowed the identification of the biotype SC. Dedieu *et al.* (1994a) used sequences from a randomly cloned DNA fragment to design primers that detect specifically the biotype SC. However, neither the structure nor the function of these DNA fragments have been described. Recently, a lipase operon was cloned from *M. mycoides* subsp. *mycoides* LC and found to be specifically present in all the species and subspecies within the *M. mycoides* cluster (Rawadi *et al.*, 1995c). Primer sets chosen from this operon allowed specific detection of the biotype SC, the *M.* sp. PG50 and *M. capricolum*. Although the biotype LC and the subspecies *capri* could not be separated, they were easily distinguished from the other members (Rawadi *et al.*, submitted for publication). Although the 16S rRNA genes from the members of the *M. mycoides* cluster are closely related thus preventing the design of species-specific primers, the amplification of this gene and restriction analysis allowed the identification *M. capricolum* subsp. *capripneumoniae* (formerly F38 type; Ros Bascunana *et al.*, 1994; Bölske *et al.*, 1966)

Arbitrarily primed PCR (AP-PCR), a variation of the basic PCR technique that permits typing of bacteria by comparing the polymorphism of genomic fingerprints generated by random amplification (Caetono-Anollés, 1993), has been applied to identify mycoplasmas within the *M. mycoides* cluster (Rawadi *et al.*, 1995b). This method allowed an accurate distinction among the six species or subspecies from this cluster (*Figure 2*). However, this technique, in contrast to basic PCR amplification, requires a highly purified genomic DNA and can be used only after a enrichment step of the culture.

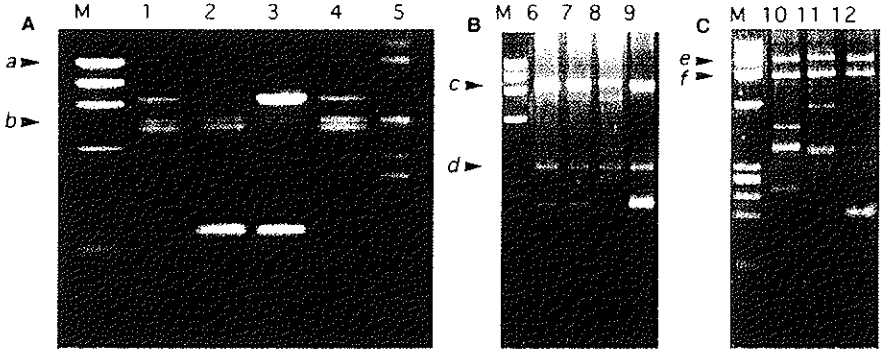

Figure 2. Characterization of *Mycoplasma mycoides* cluster strains by AP-PCR fingerprinting (Rawadi *et al.*, 1995b). A– strains belonging to *Mycoplasma capricolum* subsp. *capricolum*, lanes: 1, California kid; 2, Limoge; 3, Vienne; 4, Goat-189; 5, 7714. B– *Mycoplasma mycoides* subsp. *mycoides* LC, lanes: 6, LC type; 7, 1177-2; 8, Farche; 9, 7730. C– *Mycoplasma mycoides* subsp. *capri*, lanes: 10, PG3; 11, 88-117; 12, L. Arrows indicate polymorphic bands specific for each subspecies. Lane M, molecular weight marker ϕX174-*Hae*III.

OTHER SMALL AND LARGE RUMINANTS MYCOPLASMAS

M. agalactiae is the agent of contagious agalactia in sheep and goats, an infection of economic importance (Cottew, 1979). Different molecular tools were recently developed for the detection of this infection. An oligonucleotide probe complementary to the V8-region of the 16S rRNA was described by Mattsson *et al.* (1991). This probe has been shown to cross-react with some bovine mycoplasma species. However, these are not found in goats and sheep. Therefore, this probe can be useful to detect mycoplasmas in milk from animals. In order to set up a PCR assay for the amplification of *M. agalactiae*, both Dedieu *et al.* (1994a) and Tola *et al.* (1996) have cloned and sequenced a specific but distinct DNA fragments from *M. agalactiae* genomic library and then afterwards designed specific primers sets. The identity of the amplified fragment can be verified by restriction analysis of the amplicon (Dedieu *et al.*, 1994b). Although PCR is well recognized as the most sensitive method for bacterial diagnosis, particular attention should be taken when applying this technology upon veterinary specimens. the sensitivity of PCR-based assay for *M. agalactiae* detection in animal milk was evaluated at 10^3 cfu/ml in experimental situation. Therefore, survey of the mycoplasma milk contamination from animals need to be improved by determining adequate DNA extraction methods in order to increase the detection sensitivity and minimize the presence of thermostable DNA polymerase inhibitors.

M. bovis is, after *M. mycoides* subsp. *mycoides*, probably the most important pathogenic mycoplasma isolated from cattle (Gourlay and Howard, 1979). An oligonucleotide probe complementary to the V6-region of the 16S rRNA gene was described for the detection of this mycoplasma species (Mattsson *et al.*, 1991). No cross-reactivity has been observed using this probe with other bovine mycoplasmas, whereas a positive signal has been obtained with *M. agalactiae*. Recently, the complete 16S rRNA sequence of *M. bovis* was reported by Mattsson *et al.* (1994). This should allow the development of a PCR-based assay.

AVIAN MYCOPLASMAS

M. gallisepticum, *M. synoviae*, *M. meleagridis* and *M. iowae* are the most significant pathogens in domestic fowls. These mycoplasma species are transmitted *in ovo*, and lead to growth rate decrease as well as reduced production and hatchability of eggs (Kleven and Yoder, 1989). Sensitive detection methods for systematic testing are required to guarantee that animals and their progeny are free from these mycoplasmas. DNA probes for *M. gallisepticum* and *M. synoviae* were utilized in hybridization protocols because of their ability to detect new infection (Khan *et al.*, 1987; Santha *et al.*, 1987; Geary *et al.*, 1988; Stipkovits *et al.*, 1988; Hyman *et al.*, 1989; Levisohn *et al.*, 1989). Geary and colleagues (1988) described a detection procedure with a biotinylated DNA probe which could be advantageous for safety reasons. Oligonucleotide probes complementary to the V8-region of 16S rRNA were reported for species-specific detection of both *M. gallisepticum* and *M. synoviae* (Fernandez *et al.*, 1993). The latter technique increased the sensitivity of detection up to 2 to 3×10^4 organisms. In addition, a PCR-based method for the specific detection of *M. gallisepticum* has been described by Nascimento *et al.* (1991).

Detection of phytoplasmas and spiroplasmas

The detection of phytoplasmas (formerly known as mycoplasma-like organisms or MLOs) relies upon electron microscopy observation (Doi *et al.*, 1967). *Figure 3* shows phytoplasmas infecting *Populus alba* roots detected by transmission electron microscopy. However, this technique requires skilled technicians and costly equipment. Although phytoplasmas cannot be cultivated *in vitro*, monoclonal antibodies could be produced that allowed the detection of these organisms by enzyme linked immuno-sorbent assay (ELISA) or immunofluorescence (IF) (Martin-Gros *et al.*, 1987). In addition, phytoplasmas DNA could be extracted from infected plants (Kirkpatrick *et al.*, 1987; Sears *et al.*, 1989; Kollar *et al.*, 1990; Fiaro and Locci, 1993), thus DNA-based technology was applied to detect these organisms. Phytoplasmas, as unculturable mycoplasmas, are typical candidates for diagnosis using genomics methods. Several DNA probes have been described for the diagnosis of phytoplasmas in plants and in insects that are vectors of the diseases. Garnier *et al.* (1991) reported a specific DNA probe for witches' broom disease phytoplasma and Bonnet *et al.* (1990) identified a specific probe for apple proliferation-phytoplasma. The latter group showed that RNA probes synthesized from cloned inserts by a RNA polymerase were about 7-fold more sensitive than the corresponding DNA probes. Similarly, RNA probes transcribed from DNA inserts using adequate cloning vector, allowed to increase the sensitivity and the specificity of dot hybridization-based diagnosis of western X disease-phytoplasma (Lee *et al.*, 1988). Two DNA fragments cloned from the western aster yellow-phytoplasma were used in a DNA hybridization assay to differentiate phytoplasmas that produce symptoms of virescence from those which do not produce these symptoms (Kuske *et al.*, 1991).

Firrao *et al.* (1994) reported oligonucleotidic probes, complementary to the 16S rRNA genes able to specifically differentiate phytoplasmas of a group of related diseases: apple proliferation, plum leptonecrosis, pear decline and peach decline. Moreover, within this group, the use of tetramethyl ammonium chloride in the

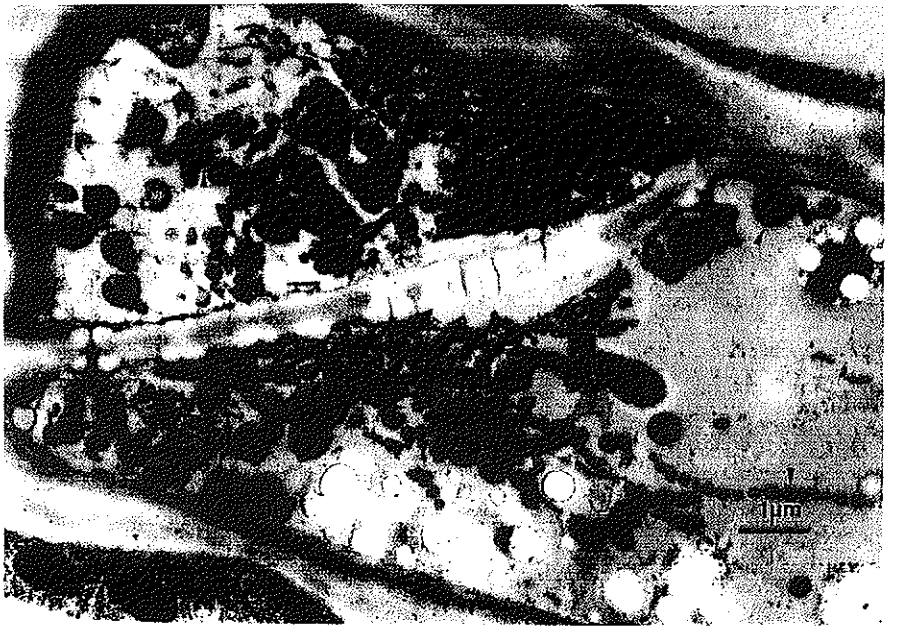

Figure 3. Phytoplasmas detected by transmission electron microscopy. Phytoplasmas are observed in the roots of *Populus alba*. Arrows indicate phytoplasmal bodies (Photograph, courtesy of M.T. Cousin).

hybridization buffer increased the pairing stringency and allowed them to distinguish plum leptonecrosis-phytoplasma from the other phytoplasmas.

Since MLOs 16S rRNA genes have been sequenced, PCR using these targets has been applied to their diagnosis (Ahrens and Seemüller, 1992). G35pm primers have been described for the amplification of the aster yellows-phytoplasma strains (Davis *et al.*, 1992). Later, universal primers sets that prime amplification from all known phytoplasmas, have been designed and found to be suitable for phytoplasmas detection (Lee *et al.*, 1993a; Saeed and Cousin, 1995). Lee *et al.* (1993a) defined group-specific primers for the specific amplification of 16S rDNA by a nested PCR-assay to identify phytoplasmas affiliated with defined groups including aster yellows, peach X diseases and elm yellows-phytoplasmas. However, difficulties to identify strain-specific PCR primers using 16S rRNA sequences were observed for some phytoplasmas. The 16/23S rRNA spacer sequences have been suggested as a complementary approach to design primers for problematic MLO strains (Kirkpatrick *et al.*, 1994; Smart *et al.*, 1996). Sequence analysis showed that the 16S–23S rRNA spacer region (SR) from phytoplasma isolates contains a single and well-conserved Ile-tRNA gene (*Figure 4*). PCR primers deduced from the SR sequences combined with primers from within the 16S rDNA has allowed group-specific amplification. This approach was found to be effective for the detection and identification of phytoplasmas from field samples.

Spiroplasmas are well-established infectious agents of plants and insects. Unlike phytoplasmas, these microorganisms can be detected by classical cultivation procedures. *In vitro* cultivation is routinely used in specialized diagnosis laboratories (Lee

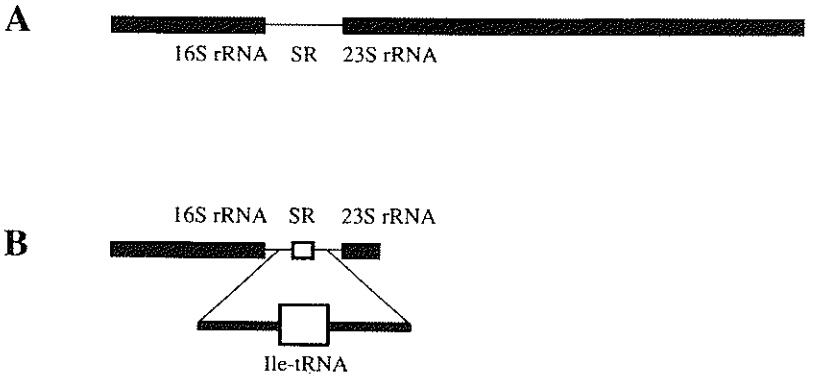

Figure 4. Comparison of the 16S-23S rRNA spacer region (SR) between phytoplasmas (B) and the other Mollicute species (A). In phytoplasmas, the SR includes a well-conserved Ile-tRNA gene, which allows their differentiation from other mycoplasmal species.

and Davis, 1984). In addition, an ELISA-based technique has been developed (Clark *et al.*, 1978). More sensitive techniques such as DNA hybridization were reported for the detection of spiroplasmas (Nur *et al.*, 1986). DNA probes were based on the plasmid pRA1 (Razin *et al.*, 1987b) and on the spiralin gene encoding the major protein from *S. citri* (Mouches *et al.*, 1984). These probes have been shown to be able to detect spiroplasmas in plants without cross-hybridization with phytoplasmas or other microorganisms. The sensitivity of these assays was about 10^3 organisms.

Very few PCR assays for the detection of spiroplasmas appeared in the literature. It may be due to the presence of PCR inhibitors in crude extracts from plants. An immunocapture-PCR (IC-PCR) assay has been developed that allowed to overcome this limitation (Saillard *et al.*, 1994). The single-stranded circular DNA virus, SpV1, found in all *S. citri* strains (Renaudin *et al.*, 1990), was used as a target in IC-PCR. The sensitivity of this assay was estimated to be about 10^2 organisms per 50 µl of plant homogenate. More recently, random amplification of polymorphic DNA (RAPD), has been applied to identify spiroplasmas strains (Ahad *et al.*, 1994). This technique displayed amplification patterns that allowed the distinction of reference strains. Although this technique appears very promising, the results are highly dependent on experimental conditions (PCR mixture, DNA extraction methods, origin of the *Taq* polymerase, etc.) and more information is required to determine its utility as a diagnosis tool.

Detection of mycoplasmas contaminating cell cultures

Unlike bacteria and fungi, mollicutes contaminating cell cultures usually produce no turbid growth and are resistant to antibiotics commonly used in culture media. The most common contaminants found in cell cultures are two bovine mollicutes, *M. arginini* and *Acholeplasma laidlawii*, two human mollicutes, *M. orale* and *M. fermentans* and a porcine mollicute, *M. hyorhinis* (Barile and Rottem, 1993). However, other species such as *M. pirum*, *M. salivarium* and *M. hominis* have also been isolated. Mycoplasmas usually grow on the cell surface (*Figure 5*) and are known to be able to virtually alter every property and parameter measured in cell cultures

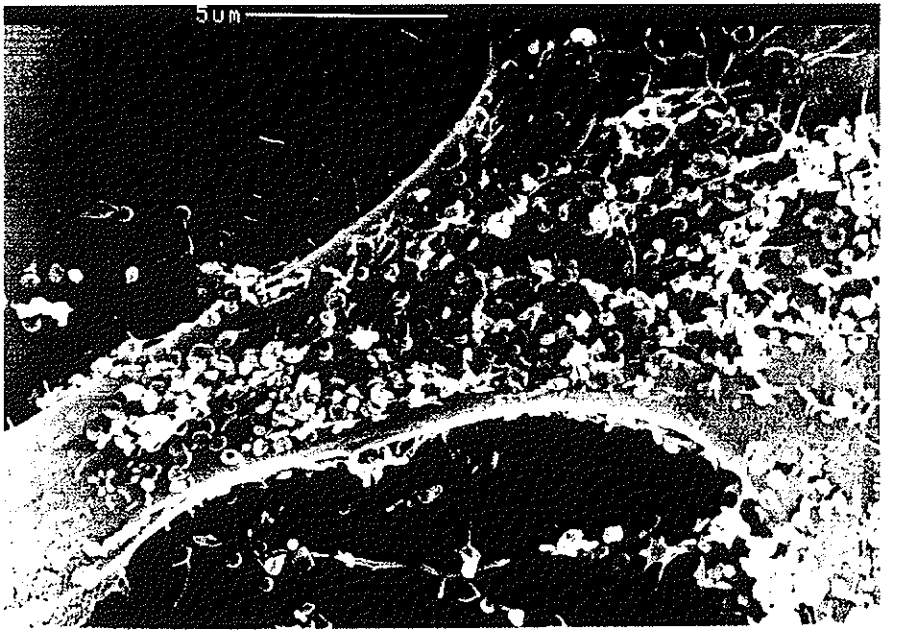

Figure 5. 3T6 cell infected with *Mycoplasma salivarium*. shown by scanning electron microscopy. The photograph shows adherent mycoplasmas growing upon the cell surface (Photograph, G. Rawadi and D. Lecaque).

leading to unreliable experiments and unsafe biopharmaceutical drugs and virus vaccines. Although the origins of contamination are laboratory personnel and commercial animal sera used in culture media, the main way of contamination of clean cultures are mollicute-infected cultures (Hay *et al.*, 1989). Periodic screening is therefore essential to control contamination and to maintain mollicute-free cell lines.

Numerous methods for detecting mollicute infection in cell cultures have been developed. Direct tests are based on microbiological culture using specific media. Although these methods are considered as the most sensitive and specific, 10 to 14 days incubation is necessary to obtain the result. Moreover, some mycoplasma species (e.g. *M. hyorhinis*) are difficult to cultivate and remained very often undetected by these methods (McGarrity *et al.*, 1979). Indirect tests consist on measurement of specific markers or characteristics associated with mollicutes, including DNA fluorochrome staining, ELISA, IF, electron microscopy, autoradiography and biochemical assays (McGarrity and Kotani, 1985; Hay *et al.*, 1989; Barile and Rottem, 1993). Comparative studies of these methods all agreed that there is no 'gold standard' method, since each may yield both false negative or false positive results, even under standardized conditions. At least two techniques should be conducted on the same sample in order to obtain a reliable diagnosis. DNA hybridization and PCR amplification-based methods have been developed and surpass all these conventional methods by their reliability and sensitivity.

DNA–DNA hybridization is based on either the 16S rRNA genes or 23S rRNA

genes hybridization (Johansson *et al.*, 1990; Johansson, 1993). These methods are rapid but have been shown to cross-react with Gram-positive bacteria, especially the H900 probe, yielding unspecific hybridization (Johansson *et al.*, 1990). The detection limit is 10^3 to 10^4 organisms, thus it is not suitable for monitoring eradication of mycoplasma contamination nor for the detection of the onset of contamination.

PCR has been widely and successfully applied to the detection of mycoplasmas contaminating cell cultures (van Kuppeveld *et al.*, 1992, 1994; Spaepen *et al.*, 1992; Hopert *et al.*, 1993; Rawadi *et al.*, 1993; Teyssou *et al.*, 1993; Roulland-Dussoix *et al.*, 1994). Recent advances in this field have been reviewed elsewhere (Rawadi and Dussurget, 1995).

Once more the 16S rRNA genes are used to design primers. One can distinguish two type of primer sets, mollicutes-specific primers that are able to detect all mycoplasmas contaminating cell cultures and species-specific primers that can be used to identify the species. This aspect was discussed elsewhere (Rawadi and Dussurget, 1995). Cross-reactivity of primers with other bacterial species and especially with the closely related clostridial species was reported by some investigators (Spaepen *et al.*, 1992; Teyssou *et al.*, 1993). However, bacterial contamination can usually be spotted visually and clostridia are not common cell culture contaminants.

The 16S–23S spacer region of rRNA operons has been used as an amplification target and a nested-PCR approach was suitable for detection and identification of mycoplasmas in cell cultures (Uemori *et al.*, 1992; Harasawa *et al.*, 1993).

The detection limit of PCR-based methods has been shown to be as little as one mycoplasma contaminating 10^3 cells (Rawadi *et al.*, 1993). Such a sensitivity should allow the detection of onset contamination and the monitoring of mycoplasmas eradication from cell cultures. In a previous study conducted upon more than 300 cell cultures we have shown that PCR-based detection was able to cover all mycoplasma contamination found in cell culture (Roulland-Dussoix *et al.*, 1994). These results were in agreement with other reports (Teyssou *et al.*, 1993; Rawadi *et al.*, 1993; Hoppert *et al.*, 1993; van Kuppeveld *et al.*, 1994). So far, and over a 4-year period, we have extended this study to include more than 1000 cell cultures that have been submitted to our laboratory for mycoplasma screening (unpublished data). Neither false positive nor false negative results have occurred, therefore we consider the PCR to be the most suitable assay for mycoplasma detection and in cell cultures.

Perspective

Diagnostic microbiology laboratories are continuously undertaking efforts to update methods to provide highly accurate, costless, and timely results to clinicians and research and development laboratories. This review highlights advances in molecular detection of mycoplasmas. Traditional detection methods rely mainly on *in vitro* cultivation and serology. Molecular technology overcomes the problems of fastidious cultivation (e.g. phytoplasmas and *M. hyorhinis*), and of anti-serum cross-reactivity (e.g. *M. mycoides* cluster, *M. pneumoniae*).

Genotypic methods have been shown to be the most sensitive and reliable ones. Nevertheless, two major handicaps have been encountered. First, although hybridization procedures using radiolabelled probes have been simplified to be more accessible, many laboratories cannot perform it for reasons of safety and cost. Moreover, non-

radioactive markers (e.g. biotin, digoxigenin) which have been proposed to substitute radioactive ones, are less sensitive. Secondly, the validation of PCR-based results relies upon two criteria: availability of reliable positive and negative controls. PCR yields a million of DNA copies starting from a few ones, therefore negative control is essential to verify the absence of carry-over contamination which may lead to false positive results. It has been reported that samples may contain trace inhibitors of DNA polymerase enzymes producing false negative results. Several techniques are available to set-up positive control. Briefly, positive controls consist on the coamplification of a foreign plasmid with a distinct primers set or on the coamplification of a genetically modified DNA target with the same primers set. Recently, low stringency PCR has been used to detect mycoplasmas contaminating cell cultures and provides a reliable positive control (Rawadi and Dussurget, 1995). Laboratory practices necessary to minimize the hazard of contamination and inhibition prevented fast diffusion of PCR-based diagnosis, especially in clinical laboratories. Indeed, the reliability of the results depending on the controls appears to be crucial in the field of human diseases diagnosis.

Accumulation of informations and standardization efforts are needed in order to facilitate evaluation of the molecular diagnosis techniques. Data bases exist for gene and protein sequences and DNA, RNA and protein motifs (Doolittle, 1996). Now, a PCR primers database is being developed on the Internet (http://www.ebi.ac.uk / primers_home.html). The database is searchable at www-srs.caos.kun.hl/srs.wgetz. Such a database should save time and money and facilitates standardization because it includes only optimally designed sets of primers and their optimal conditions of reaction, resulting in a high level of reproducibility. A similar database for synthetic probes up to 100 nucleotides long, Molecular Probe Data Base (MPDB), is available on-line at http://www.biotech.ist.unige.it/interlab/mpdb.html (Campi *et al.*, 1997). Enhancements of such databases and/or introduction of commercial assays with standard hybridization procedures and quality control of reagents would help molecular diagnosis techniques to fulfil regulation requirements and become routine techniques in diagnosis laboratories.

Quantitative detection would be of great value in every field reviewed above. Several systems have been proposed to quantitatively detect specific DNA. Sidhu *et al.* (1995) used competitor internal standards for quantitative detection of mycoplasmal DNA which also served as positive controls. The TaqMan detection system by Perkin Elmer combines probe-hybridization and PCR, avoiding the time consuming steps of electrophoresis and/or blotting of the PCR products. It employs the 5' to 3' exonuclease activity of *Taq* DNA polymerase (Holland *et al.*, 1991) that cleaves a probe bearing a reporter dye and a quencher dye when it hybridizes to the target DNA during the amplification. The increase in the fluorescence of the reporter dye that has been released is detected on a fluorometer and allows quantitation of the amplicons. In addition, multiplex amplifications can be applied since different fluorescent dyes can be used. Tyagi and Kramer (1996) have developed probes called molecular beacons, that fluoresce only when hybridized to target DNA (*Figure 6*). These highly specific probes allow monitoring of amplification in real-time and their fluorescence can be used to determine the initial template concentration (*Figure 7*). Alternatively, Wittwer *et al.* (1997) reported fluorescence monitoring of rapid cycle DNA amplification by three techniques: (i) the double-strand-specific dye SYBR Green I, (ii) a dual-labeled

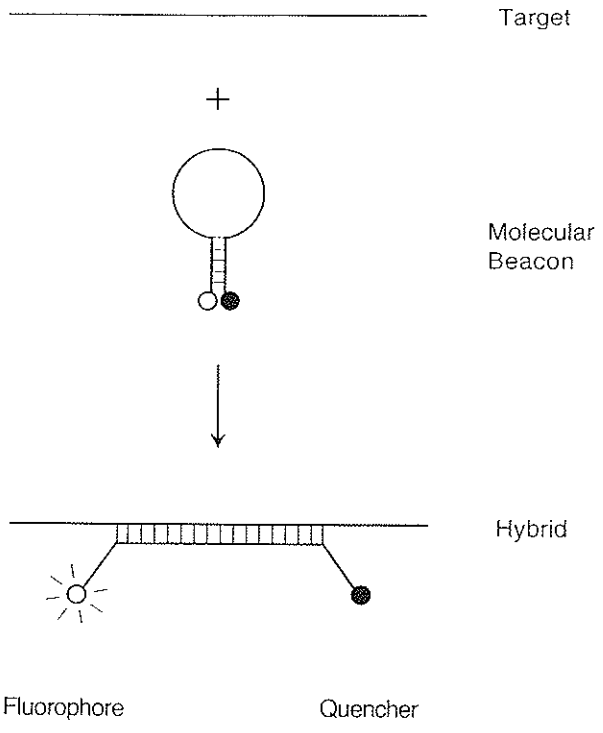

Figure 6. Principle of operation of molecular beacons. The hairpin stem formed by the complementary arm sequences cannot coexist with the rigid double helix that is formed when the probe hybridizes to its target. Consequently, the molecular beacon undergoes a conformational change that forces the arm sequences apart and causes the fluorophore to move away from the quencher (reproduced with permission from Tyagi and Kramer, 1996).

5'-exonuclease hydrolysis probe, and (iii) resonance energy transfer of fluorescein to Cy5 by two adjacent probes.

Other amplification techniques showing an advantage over PCR have been described. Tyagi *et al.* (1996) reported a gene detection assay using binary probes and Qβ replicase. The strategy is based on (i) hybridization of two different RNA probes to adjacent positions on a target nucleic acid, (ii) ligation of the probes in a target-dependent reaction and (iii) isothermal amplification of this newly formed reporter RNA by the Qβ replicase leading to billion copies of the template in 30 minutes (*Figure 8*). They demonstrated that their simple assay avoids all major PCR-related problems. It uses a universal protocol for all infectious agents suspected and all tissues tested and does not require expensive equipment. The process described rules out the possibility of inhibition and false positive reaction. Multiplex assay as well as quantitative measurement can be applied to Qβ replicase detection. Stone *et al.* (1996) described a prototype automated infectious agent detection system using Qβ replicase RNA probe amplification in conjunction to real-time fluorescence detection of amplification. They used the instrument to detect rRNA from four respiratory pathogens including *M. pneumoniae*. The sensitivity was comparable to some PCR assays, since 10 to 100 cfu could be detected. However, the assay showed some cross-

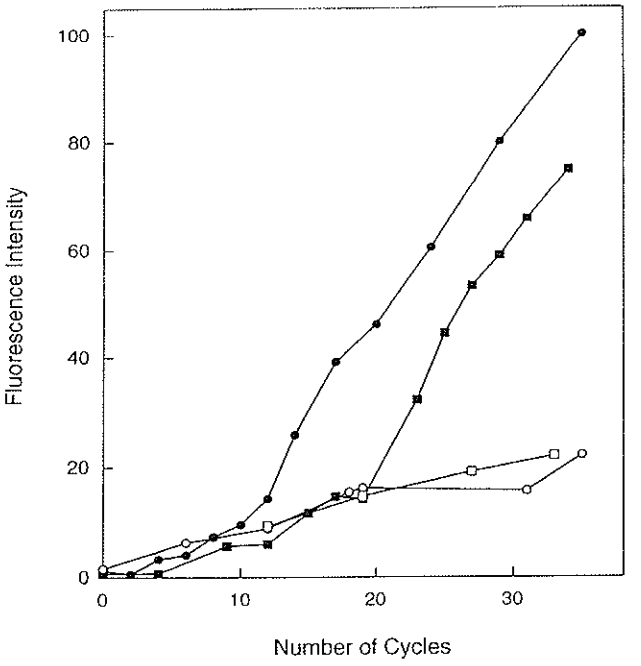

Figure 7. Monitoring polymerase chain reactions in real time. A molecular beacon was included in four different polymerase chain reactions. One reaction was initiated with 10^9 template molecules containing a complementary target sequence (l); a second reaction was initiated with 10^7 molecules of the same template (n); a third reaction was initiated with an unrelated set of template molecules and primers (m); and a fourth reaction was initiated without adding template molecules (o). The reactions were divided into aliquots. Each aliquot was incubated for a predetermined number of amplification cycles, and its fluorescence was measured at 37°C (reproduced with permission from Tyagi and Kramer, 1996).

reactivity when a 10^3-fold excess of *M. genitalium* was used. The automated assay was performed in 7 h for a 10-sample batch with 3 h to first result while reducing hands-on time and risk of cross contamination, two important parameters in clinical laboratories. Nucleic acid sequence-based amplification (NASBA), is another alternative approach to PCR. The technique is based on an isothermal RNA amplification using avian myeloblastosis virus reverse transcriptase, T7 RNA polymerase and RNase H, without the need for specialized equipment (e.g. thermocycler). NASBA has been shown to be a sensitive, specific and rapid method to detect and identify infectious agents in biological samples (Kievits *et al.*, 1991; van der Vliet *et al.*, 1993). Ovyn *et al.* (1996) also used NASBA followed by an hybridization in an enzyme-linked gel assay for typing a collection of *M. pneumoniae* strains. Other isothermal *in vitro* nucleic acid amplification techniques have been tried to detect infectious agents in specimens, e.g. strand displacement amplification (SDA) reported by Down *et al.* (1996).

Recently, microfabricated chip devices have been applied to DNA analyses and diagnosis including microbial identification (Beattie *et al.*, 1995; Yersov *et al.*, 1996). This technology enables the use of smaller samples and faster reactions. Cheng *et al.* (1996) successfully used silicon-glass chips for PCR from extracted genomic DNA and from intact lymphocytes. They showed that HotStart PCR using a mixture of

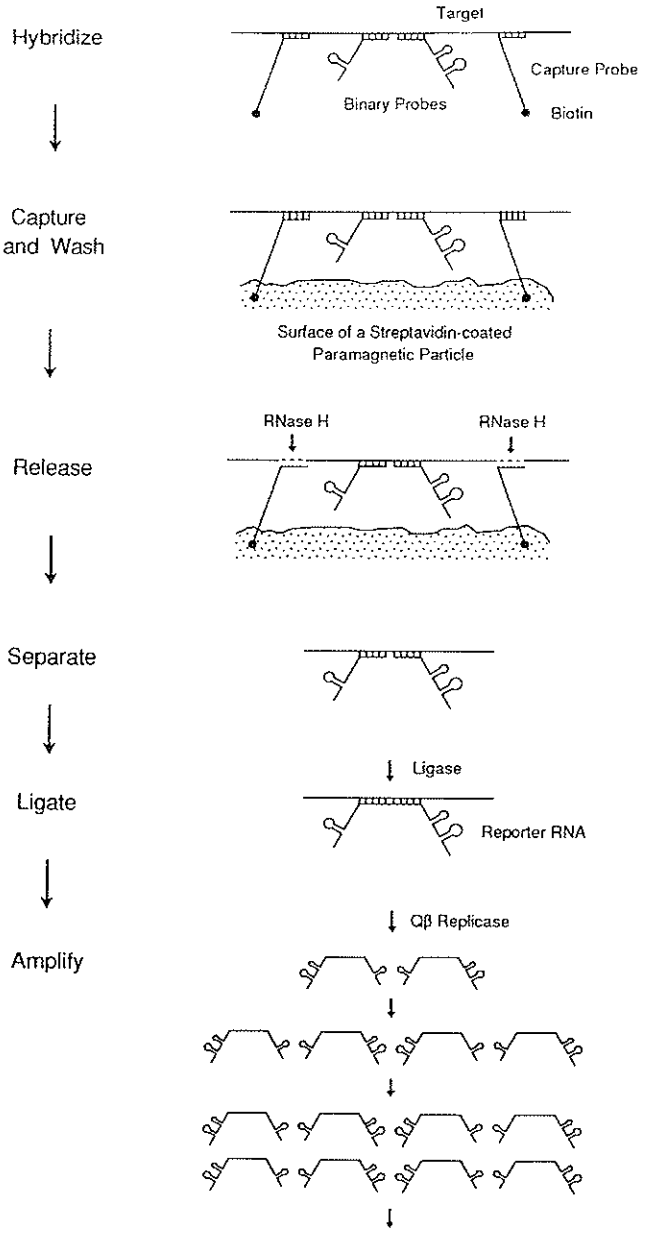

Figure 8. Schematic representation of the key physical and enzymatic steps used in a binary probe assay for gene detection. Both the target and the binary probes are RNA molecules. The capture probes are DNA molecules. Two different capture probes are used to increase the efficiency of capture. They hybridize to the target RNA on opposite sides of the sequence to which the binary probes are bound. Incubation with ribonuclease H digests the target RNA where it is bound to each capture probe, selectively releasing the binary probe-target hybrid from the surface of the paramagnetic particle. After removal of the particle with a magnet, the isolated hybrid is incubated with T4 DNA ligase (which serves here as an RNA-dependent RNA ligase), resulting in the formation of a reporter RNA that is then amplified exponentially by incubation with Qβ replicase (reproduced with permission from Tyagi *et al.*, 1996. Copyright (1996) National Academy of Sciences, USA).

TaqStart antibody and Taq DNA polymerase was more suitable for chip-PCR than the conventional system using Taq DNA polymerase. Eggers and Ehrlich (1995) used a genosensor technology involving microchips on which oligonucleotidic probes complementary to bacterial rRNA are bound at predetermined addresses. Fluorigenic hybridization of the probe to a nucleic acid target and its address are monitored by a charge-coupled device camera. The sensitivity of the assay was 1000 rRNA molecules, i.e. 1–10 bacteria. Current developments are directed toward optimization of DNA chips technology in order to increase its performance, e.g. reducing human intervention, costs and complexity by automation and integration of extraction, reaction and detection in a single system. These are fundamental steps before its clinical application for molecular diagnosis of infectious diseases.

In conjunction to these advances in technology, the development of our knowledge of molecular genetics of mycoplasma, and the information following genome projects make it likely that many more genotypic methods will be applied to laboratory diagnostics in the next decade.

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References

AHAD, P., ALFARO, A., DAVIS, R. AND DALLY, E.L. (1994). Identification of *Spiroplasma* strains by random amplification of polymorphic DNA (RAPD). *IOM Letters* **3**, 193–194.

AHRENS, U. AND SEEMÜLLER, E. (1992). Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytopathology* **82**, 828–832.

AINWORTH, J.G., KATSENI, V., HOURSHID, S., WALDRON, S., BALL, S., CATTELL, V. AND TAYLOR-ROBINSON, D. (1994). *Mycoplasma fermentans* and HIV-associated nephropathy. *Journal of Infection* **29**, 323–326.

BARILE, M.F. AND ROTTEM, S. (1993). Mycoplasmas in cell cultures. In *Rapid Diagnosis of Mycoplasmas*, ed. A. Adoni and I. Kahane, pp. 155–193. New York, Plenum Press.

BASHIRUDDIN, J.B., NICHOLAS, R.A.J., SANTINI, F.G., READY, R.A., WOODWARD, M.J. AND TAYLOR, T.K. (1994). Use of the polymerase chain reaction to detect mycoplasma DNA in cattle with contagious bovine pleuropneumoniae. *Veterinary Research* **5**, 240–241.

BAUER, F.A., WEAR, D.J., ANGRITT, P. AND LO, S.C. (1991). *Mycoplasma fermentans* (incognitus strain) infection in the kidneys of patients with acquired immunodeficiency syndrome and associated nephropathy: a light microscopic, immunohistochemical and ultrastructural study. *Human Pathology* **22**, 63–69.

BERG, S., LÜNEBERG, E. AND FROSCH, M. (1996). Development of a amplification and hybridization assay for the specific and sensitive detection of *Mycoplasma fermentans* DNA. *Molecular Cell Probes* **10**, 7–14.

BERNET, C., GARRET, B., DE BARBEYRAC, B., BÉBÉAR, C. AND BONNET, J. (1989). Detection of *Mycoplasma pneumoniae* by using polymerase chain reaction. *Journal of Clinical Microbiology* **27**, 2492–2496.

BLANCHARD, A. AND MONTAGNIER, L. (1994). AIDS-associated mycoplasmas. *Annual Reviews in Microbiology* **48**, 687–712.

BLANCHARD, A., OLSON, L.D. AND BARILE, M.F. (1992). Sexually transmitted mycoplasmas in humans. In *Molecular and Cellular Biology of Sexually Transmitted Diseases*, ed. L. Archad and D. Wright, pp. 55–83. Chapman and Hall, London.

BLANCHARD, A., HAMRICK, W., DUFFY, L., BALDUS, K. AND CASSEL, G.H. (1993a). Use of PCR for detection of *Mycoplasma fermentans* and *Mycoplasma genitalium* in the urogenital tract and amniotic fluid. *Clinical Infectious Diseases* **17**, S272–279.

BLANCHARD, A., HENTSCHEL, J., DUFFY, L., BALDUS, K. AND CASSEL, G.H. (1993b). Detection of *Ureaplasma urealyticum* by PCR in the urogenital tract of adults, in amniotic fluid, and in the respiratory tract of new borns. *Clinical of Infectious Diseases* **17**, S148–153.

BLANCHARD, A., YANEZ, A., DYBVIG, K., WATSON, H.L. AND CASSEL, G.H. (1993c). Evaluation of intraspecies genetic variation within the 16S rRNA gene of *Mycoplasma hominis* and detection by PCR. *Journal of Clinical Microbiology* **31**, 1358–1361.

BÖLSKE, G. (1988). Survey of mycoplasma infections in cell cultures and a comparison of detection methods. *Zentralblatt für Bakteriologie Mikrobiologie und Hygiene* **269**, 331–340.

BÖLSKE, G., MATTSSON, J.G., BASCUNANA, C.R., BERGSTROM, K., WESONGA, H. AND JOHANSSON, K.E. (1996). Diagnosis of contagious caprine pleuropneumonia by detection and identification of *Mycoplasma capricolum* subsp. *capripneumoniae* by PCR and restriction enzyme analysis. *Journal of Clinical Microbiology* **34**, 785–791.

BONNET, F., SAILLARD, C., KOLLAR, A., SEEMÜLLER, E. AND BOVÉ, J.M. (1990). Detection and differentiation of the mycoplasma-like organism associated with apple proliferation disease using cloned DNA probes. *Molecular Plant–Microbe Interactions* **3**, 438–444.

BROGAN, J.M., ACCIAI, J., GALLIA, G.L., MCCLESKEY, F.K. AND DELVECCHIO, V.G. (1992). Development of a DNA probe for *Ureaplasma urealyticum*. *Molecular Cell Probes* **6**, 411–416.

BUCK, G.E., O'HARA, L.C. AND SUMMERSGILL, J.T. (1992). Rapid, sensitive detection of *Mycoplasma pneumoniae* in simulated clinical specimens by DNA amplification. *Journal of Clinical Microbiology* **30**, 3280–3283.

CADIEUX, N., LEBEL, P. AND BROUSSEAU, R. (1993). Use of a triplex polymerase chain reaction for the detection and differentiation of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* in the presence of human DNA. *Journal of General Microbiology* **139**, 2431–2437.

CAETONO-ANOLLÉS, G. (1993). Amplifying DNA with arbitrary oligonucleotide primers. *PCR Methods Applications* **3**, 85–94.

CALAVAN, E.C. AND OLDFIELD, G.N. (1979). Symptomatology of *Siproplasma* plant diseases. In *The Mycoplasmas*, ed. J.G. Tully and R.F. Withcomb, vol. III. pp. 37–64. Academic Press, New York.

CAMPI, M.G., CASTOLDI, M., ROMANO, P., THÜROFF, E., MANNIELLO, M.A., IANNOTTA, B., RONDANINA, G., RUZZON, T. AND SANTI, L. (1997). Molecular probe data base (MPDB). *Nucleic Acids Research* **25**, 92–95.

CASSEL, G.H., WAITES, K.B. AND CROUSE, D.T. (1991). Perinatal mycoplasmal infections. *Clinical Perinatology* **18**, 241–262.

CASSEL, G.H., WAITES, K.B., WATSON, H.L., CROUSE, D.T. AND HARASAWA, R. (1993). *Ureaplasma urealyticum* intrauterine infection: role in prematurity and disease in newborns. *Clinical Microbiology Review* **6**, 69–87.

CHRISTIANSEN, G., ANDERSEN, H., BIRKELUND, S. AND FREUNDT, E.A. (1987). Genomic and gene variation in *Mycoplasma hominis* strains. *Israel Journal of Medical Sciences* **23**, 595–602.

CLARK, M.F., FLEG, C.L., BAR-JOSEPH, M. AND ROTTEM, S. (1978). ELISA assay for *Spiroplasma* detection. *Phytopathology* **92**, 332–337.

COTTEW, G.S., BREARD, A., DAMASSA, A.J., ERNØ, H., LEACH, R.H., LEFEVRE, P.C., RODWELL, A.W. AND SMITH, G.R. (1987) Taxonomy of the *Mycoplasma mycoides* cluster. *Israel Journal of Medical Science* **23**, 632–635.

DAVIS, R.E. AND PRINCE, J.P. (1994). Molecular diagnosis of mycoplasma-like organisms (MLOs) in plants – a review. *Applied Biochemistry and Biotechnology* **48**, 23–26.

DE BARBEYRAC, B., BERNET-POGI, C., RENAUDIN, H., DUPON, M. AND BÉBÉAR, C. (1993). Detection of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* in clinical samples by PCR. *Clinical Infectious Diseases* **17**, S83–89.

DEDIEU, L., MADY, V. AND LEFEVRE, P.C. (1994a). Development of a selective polymerase chain reaction assay for the detection of *Mycoplasma mycoides* subsp. *mycoides* SC (contagious bovine pleuropneumonia agent). *Veterinary Microbiology* **42**, 327–339.

DEDIEU, L., MADY, V. AND LEFEVRE, P.C. (1994a). Development of two polymerase chain reaction assays for the identification of contagious agalactia related mycoplasmas. *IOM Letters* **3**, 31–32.

DENG, S.J., FORSTER, R.J., HIRUKI, C. AND TEATHER, R.M. (1993). Simultaneous amplification and sequencing of genomic DNA (SAS) – sequencing of 16S rRNA genes using total genomic DNA from *Butyrivibrio fibrisolvens*, and detection and genotyping of nonculturable mycoplasma-like organisms directly from total DNA isolated from infected plants. *Journal of Microbiological Methods* **17**, 103–113.

DEVEREUX, J., HAEBERLI, P. AND SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the Vax. *Nucleic Acids Research* **12**, 387–395.

DOI, Y., TERENAKA, M., YORA, K. AND ASUYANA, H. (1967). Mycoplasma or PLT group-like organisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows or paulownia witches' broom. *Annals of the Phytopathology Society Japan* **33**, 359–266.

DOOLITTLE, R.F. (1996). *Computer Methods for Macromolecular Sequence Analysis. Methods in Enzymology*, vol. 266, Academic Press, San Diego.

DULAR, R., KAJIOKA, S. AND KASATIYA, S. (1988). Comparison of Gen-Probe commercial kit and culture technique for the diagnosis of *Mycoplasma pneumoniae* infection. *Journal of Clinical Microbiology* **26**, 1068–1069.

EGGERS, M. AND ERLICH, D. (1995). A review of microfabricated devices for gene-based diagnostics. *Hematological Pathology* **9**, 1–15.

ERNØ, H. (1987). Mycoplasmosis of ruminants: a general introduction. *Revue Scientifique et Technique de l'Office International des Epizooties* **174**, 8152–8158.

FERNANDEZ, C., MATTSSON, J.G., BÖLSKE, G., LEVISOHN, S. AND JOHANSSON, K.E. (1993). Species-specific oligonuleotide probes complementary to 16S rRNA of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. *Research in Veterinary Science* **55**, 130–136.

FINK, C.G., READ, S.J. AND SILLIS, M. (1995). Direct sample polymerase chain reaction for the detection of *Mycoplasma pneumoniae*: a simple system for clinical application. *British Journal of Biomedical Science* **52**, 9–13.

FIRRAO, G. AND LOCCI, R. (1993). Rapid preparation of DNA from phytopathogenic mycoplasma-like organisms for polymerase chain reaction analysis. *Letters in Applied Microbiology* **17**, 280–281.

FIRRAO, G., MALISANO, G., GOBBI, E. AND LOCCI, R. (1994). Detection and differentiation of MLOs affecting fruit trees in Italy using oligonucleotides as probes and PCR primers. *IOM Letters* **3**, 96.

FRASER, C.M., GOCAYNE, J.D., WHITE, O., ADAMS, M.D., CLAYTON, R.A., FLEISCHMANN, R.D., BULT, C.J., KERLAVAGE, A.R., SUTTON, G., KELLEY, J.M., FRITCHMAN, J.L., WEIDMAN, J.F., SMALL, K.V., SARDUSKY, M., FUHRMANN, J., NGUYEN, D., UTTERBACK, T.R., SAUDEK, D.M., PHILLIPS, C.A., MERRICK, J.M., TOMB, J.F., DOUGHERTY, B.A., BOTT, K.F., HU, P.C., LUCIER, T.S., PETERSON, S.N., SMITH, H.O., HUTCHISON, III C.A. AND VENTER, J.C. (1995). The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**, 397–403.

FUTO, S., SOTO, Y., MITSUSE, S. AND YORI, Y. (1992). Detection of *Mycoplasma hyopneumoniae* by using rRNA-oligonucleotide hybridization. *Journal of Clinical Microbiology* **30**, 1509–1513.

GALLIO, G.L., PETROZIELLO, J.M., BROGAN, J.M., MCCLESKEY, F.K. AND DEL VECCHIO, V.G. (1995). Development of a diagnostic PCR assay for the detection of *Mycoplasma hominis*. *Molecular Cellular Probes* **9**, 415–421.

GARNIER, M., ZREIK, L. AND BOVÉ, J.M. (1991). Witches' broom, a lethal mycoplasmal disease of lime trees in the Sultanate of Oman and the United Arab Emirates. *Plant Disease* **75**, 546–551.

GEARY, S.J., INTRES, R. AND GABRIDGE, M.G. (1988). Species-specific biotinylated DNA probe for the detection of *Mycoplasma gallisepticum*. *Molecular Cell Probes* **2**, 237–244.

GÖBEL, U.B., MAAS, R., HAUN, G., VINGA MARTINO, C. AND STANBRIDGE, E. (1987a). Synthetic oligonucleotide probes complementary to rRNA for group- and species-specific detection of mycoplasmas. *Israel Journal of Medical Science* **23**, 742–746.

GÖBEL, U.B., GEISER, A. AND STANBRIDGE, E.J. (1987b). Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between mycoplasma species. *Journal of General Microbiology* **133**, 4969–1974.

GOURLAY, R.N. AND HOWARD, C.J. (1979). Bovine mycoplasmas. In *The Mycoplasmas II*, ed. R.F. Whitcomb and J.G. Tully, pp. 50–102. Academic Press, New York.

GRAU, O., KOVACIC, R., GRIFFAIS, R. AND MONTAGNIER, L. (1994). Development of PCR-based assays for the detection of two human mollicute species, *Mycoplasma penetrans* and *Mycoplasma hominis*. *Molecular Cell Probes* **8**, 139–148.

HARASAWA, R., MIZUSAWA, H., NOZAWA, K., NAKAGAWA, T., ASADA, K. AND KATO, I. (1993). Detection and tentative identification of dominant mycoplasma species in cell cultures by restriction analysis of the 16S-23S rRNA intergenic spacer regions. *Research in Microbiology* **144**, 489–493.

HATA, D., KUZE, F., MOCHIZUKI, Y., OHKUBO, H., KANASASHI, H., MAEDA, S., HIWA, N. AND HIKAWA, H. (1990). Evaluation of DNA probes test for rapid diagnosis of *Mycoplasma pneumoniae* infections. *Journal of Pediatrics* **116**, 273–276.

HAWHINS, R.E., RICKMAN, L.S., VERMUND, S.H. AND CARL, M. (1992). Association of mycoplasma and human immunodeficiency virus infection: detection of amplified *Mycoplasma fermentans* DNA in blood. *Journal of Infectious Diseases* **165**, 581–585.

HAY, R.J., MACY, M.L. AND CHEN, T.R. (1989). Mycoplasma infection of cultured cells. *Nature* **339**, 487–488.

HIMMELREICH, R., HILBERT, H., PLAGENS, H., PIRKL, E., LI, B.C. AND HERRMANN, R. (1996). Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. *Nucleic Acids Research* **24**, 4420–4449.

HOLLAND, P.M., ABRAMSON, R.D., WATSON, R. AND GELFAND, D.H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proceedings of the National Academy of Sciences* **88**, 7276–7280.

HOOTON, T.M., ROBERTS, M.C., ROBERTS, P.L., HOLMES, K.K., STAMM, W.E. AND KENNY, G.E. (1988). Prevalence of *Mycoplasma genitalium* determined by DNA probe in men with urethritis. *Lancet* **i**, 266–268.

HOPERT, A., UPHOFF, C.C., WIRTH, M., HAUSER, H. AND DREXLER, H.G. (1993). Specificity and sensitivity of polymerase chain reaction (PCR) in comparison with other methods for the detection of mycoplasmas contamination in cell lines. *Journal of Immunological Methods* **164**, 91–100.

HOTZEL, H., SACHSE, K. AND PFUTZNER, H. (1996). A PCR scheme for differentiation of organisms belonging to the *Mycoplasma mycoides* cluster. *Veterinary Microbiology* **49**, 31–43.

HYMAN, H.C., YOGEV, D. AND RAZIN, S. (1987). DNA probes for the detection and identification of *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. *Journal of Clinical Microbiology* **25**, 726–728.

HYMAN, H.C., LEVISHON, S., YOGEV, D. AND RAZIN, S. (1989). DNA probes for *Mycoplasma gallisepticum* and *Mycoplasma synoviae*: application in experimental infected chickens. *Veterinary Microbiology* **20**, 323–337.

IEVEN, M., URSI, D., VAN BEVER, H., QUINT, W., NIESTERS, H.G.M. AND GOOSSENS, H. (1996). Detection of *Mycoplasma pneumoniae* by 2 polymerase chain reactions and role of *M. pneumoniae* in acute respiratory tract infections in pediatric patients. *Journal of Infectious Diseases* **173**, 1445–1452.

IN CHINGBINGYONG, M. AND HUGHES, C.V. (1996). Detection of *Mycoplasma fermentans* in human saliva with a polymerase chain reaction-based assay. *Archives in Oral Biology* **41**, 311–314.

JENSEN, J.S., SØNDEGÅRD-ANDERSEN, J., ULDUM, S.A. AND LIND, L. (1989). Detection of *Mycoplasma pneumoniae* in simulated clinical samples by polymerase chain reaction. *APMIS* **97**, 1046–1048.

JENSEN, J.S., ULDUM, S.A., SØNDEGÅRD-ANDERSEN, J., VUUST, J. AND LIND, L. (1991). Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *Journal of Clinical Microbiology* **29**, 46–50.

JOHANSSON, K.E. (1993). Detection and identification of mycoplasmas with diagnostic DNA probes complementary to ribosomal RNA. In *Rapid Diagnosis of Mycoplasmas*, ed. A. Adoni and I. Kahane, pp. 139–154. Plenum Press, New York.

JOHANSSON, K.E., JOHANSSON, I. AND GOBEL, U.B. (1990). Evaluation of different hybridization procedures for the detection of mycoplasma contamination in cell cultures. *Molecular Cell Probes* **4**, 33–42.

KAI, M., KAMIYA, S., YABE, H., TAKAKURA, I., SHIOZAWA, K. AND OZAWA, A. (1993). Rapid detection of *Mycoplasma pneumoniae* in clinical samples by the polymerase chain reaction. *Journal of Medical Microbiology* **38**, 166–170.

KHAN, M.I., KIRKPATRICK, B.C. AND YAMAMOTO, R. (1987). A *Mycoplasma gallisepticum* strain-specific DNA probe. *Avian Diseases* **31**, 907–909.

KIRKPATRICK, B.C., STENGER, D.C., MORRIS, T. AND PURCELL, A.H. (1987). Cloning and detection of DNA from a non-culturable plant pathogenic mycoplasma-like organism. *Science* **238**, 197–200.

KIRKPATRICK, B.C., SMART, C., BLOMQUIST, C., GUERRA, L., HARRISON, N., AHRENS, U., LORENZ, K.H., SCHEIDER, B. AND SEEMÜLLER, E. (1994). Identification of MLO strain-specific PCR primers obtained from 16/23S spacer sequences. *IOM Letters* **3**, 261–262.

KLEEMOLA, S.R.M., KAIJALAINENE, J.E. AND RÄTY, R.K.H. (1990). Rapid diagnosis of *Mycoplasma pneumoniae* infection : clinical evaluation of a commercial probe test. *Journal of Infectious Diseases* **162**, 70–75.

KLEVEN, S.H. AND YODER, H.W. (1989). Mycoplasmosis. In *A Laboratory Manual for the Isolation and Identification of Avian Pathogen*, ed. L.H. Arp, H.G. Purchasse, C.H. Domermuth and J.E. Pearson, pp. 57–62. Kendal/Hunt Publishing. Dubuque.

KOLLAR, A., SEEMÜLLER, E., BONNET, F., SAILLARD, C. AND BOVÉ, J.M. (1990). Isolation of the DNA of various plant pathogenic mycoplasma-like organisms from infected plants. *Phytopathology* **80**, 233–237.

KUSKE, C.R., KIRKPATRICK, B.C. AND SEEMÜLLER, E. (1991). Differentiation of virescence MLOs using western aster yellow mycoplasma-like organism chromosomal DNA probes and restriction length polymorphism analysis. *Journal of General Microbiology* **137**, 153–159.

LEACH, R.H., ERNØ, H. AND MACOWAN, K.J. (1993). Proposal for designation of F38-type caprine mycoplasmas as *Mycoplasma capricolum* subsp. *capripneumoniae* subsp. nov. and consequent obligatory relegation of strains currently classified as *M. capricolum* (Tully, Barile, Edward, Theodore and Erno, 1974) to an additional new subspecies, *M. capricolum* subsp. *capricolum* subsp. nov. *International Journal of Systems in Bacteriology* **43**, 603–605.

LEE, I.M. AND DAVIS, R.E. (1984). New media for rapid growth of *Spiroplasma citri* and corn stunt Spiroplasma. *Phytopathology* **74**, 84–89.

LEE, I.M., DAVIS, R.E., HAMMOND, R. AND KIRKPATRICK, B. (1988). Cloned riboprobe for the detection of a mycoplasma-like organism. *Biochemical and Biophysical Research Communications* **155**, 443–448.

LEE, I.M., HAMMOND, R.W., DAVIS, R.E. AND GUNDERSEN, D.E. (1993a). Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. *Phytopathology* **83**, 834–842.

LEE, I.M., GUNDERSEN, D.E., HAMMOND, R.W. AND DAVIS, R.E. (1993b). Mycoplasma-like organisms (MLO) 16S rDNA group-specific primer pairs for PCR based classification and identification of MLOs associated with plants and insects. *Phytopathology* **83**, 1400.

LENG, Z., KENNY, G.E. AND ROBERTS, M.C. (1994). Evaluation of the detection limits of PCR for identification of *Mycoplasma pneumoniae* in clinical samples. *Molecular Cell Probes* **8**, 125–130.

LEE, I.M., KLOPMEYER, M., BARTOSZYK, I.M., GUNDERSEN-RINDAL, D.E., CHOU, T.S., THOMSON, K.L. AND EISENREICH, R. (1997). Phytoplasmas induced free-branching in commercial poinsettia cultivars. *National Biotechnology* **15**, 178–182.

LEVISOHN, S., HYMAN, H.C., PERLEMAN, D. AND RAZIN, S. (1989). The use of a specific DNA probe for the detection of *Mycoplasma gallisepticum* in field outbreaks. *Avian Pathology* **18**, 535–541.

LO, S.C., WEAR, D.J., GREEN, S.L., JONES, P.G. AND LEGIER, J.F. (1993). Adult respiratory distress syndrome with or without systemic disease associated with infections due to *Mycoplasma fermentans*. *Clinical Infectious Diseases* **17**, S259–S263.

LÜNEBERG, E., JENSEN, J.S. AND FROSCH, M. (1993). Detection of *Mycoplasma pneumoniae* by PCR and non radioactive hybridization in microtiter plates. *Journal of Clinical Microbiology* **31**, 1088–1094.

MCCOY, R.E., CAUDWELL, A., CHANG, C.J., CHEN, T.A., CHIYKOWSKI, L.N., COUSIN, M.T., DALE, DE LEEUW, G.T.N., GOLINO, D.A., HACKETT, K.J., KIRKPATRICK, B.C., MARWITZ, R., PETZOLD, H., SINHA, R.C., SUGIURA, M., WHITCOMB, R.F., YANG, I.L., ZHU, B.M. AND SEEMÜLLER, E. (1989). Plant diseases associated with mycoplasma-like organisms. In *The Mycoplasmas*, ed. J.G. Tully and R.F. Whitcomb, pp. 545–560. Academic Press, New York.

MCGARRITY, G.J., SARAMA, J. AND VANAMAN, V. (1979). Factors influencing microbiological detection of mycoplasmas in cell culture. *In vitro* **15**, 73–81.

MCGARRITY, G.J. AND KOTANI, H. (1985). Cell culture mycoplasmas. In *The Mycoplasmas IV*, ed. M. Barile and S. Razin, pp. 353–390. Academic Press, New York.

MANILOFF, J., MCELHANEY, R.N., FINCH, L.R. AND BASEMAN, J.B. (1992). *Mycoplasmas: Molecular Biology and Pathogenesis*. ASM Press, Washington DC.

MARMION, B.P., WILLIAMSON, J., WORSWICK, D.A., KOK, T.W. AND HARRIS, R.J. (1993). Experience with newer techniques for the laboratory detection of *Mycoplasma pneumoniae* infection : Adelaide, 1978–1992. *Clinical Infectious Diseases* **17**, S90–99.

MARTIN-GROS, G., GARNIER, M., ISKRA, M.L., GANDAR, J. AND BOVÉ, J.M. (1987). Production of monoclonal antibodies against phloem limited prokaryotes of plants: a general procedure using extracts from infected periwinkles as immunogen. *Annals in Microbiology* **138**, 625–637.

MATTSSON, J.G., GERSDORF, H., GOSBEL, U.B. AND JOHANSSON, K.E. (1991). Detection of *Mycoplasma bovis* and *Mycoplasma agalactiae* by oligonucleotides probes complementary to 16S rRNA. *Molecular Cell Probes* **5**, 27–35.

MATTSSON, J.G., GUSS, B. AND JOHANSSON, K.E. (1994). The phylogeny of *Mycoplasma bovis* as determined by sequence analysis of the 16S rRNA gene. *FEMS Microbiology Letters* **115**, 325–328.

MOUCHES, C., CANDRESSE, T., GADEAU, A., BARROSO, G., SAILLARD, C., WROBLEWSKI, H. AND BOVÉ, J.M. (1984). Expression of *Spiroplasma citri* spiralin gene in *Escherichia coli*, use of the recombinant plasmid carrying this gene as molecular probe. *Israel Journal of Medical Science* **20**, 713–777.

NARITA, M., MATSUZONO, Y., TOGASHI, T. AND KAJII, N. (1992). DNA diagnosis of central nervous system infection by *Mycoplasma pneumoniae*. *Pediatrics* **90**, 250–253.

NASCIMENTO, E.R., YAMAMOTO, R., HERRICK, K.R. AND TAIT, R.C. (1991). Polymerase chain reaction for detection of *Mycoplasma gallisepticum*. *Avian Diseases* **35**, 62–69.

NOCARD, E. AND ROUX, E. (1898). Le microbe de la péripneumonie. *Annals of the Institut Pasteur* **12**, 240–262.

NUR, I., BOVÉ, J.M., SAILLARD, C., ROTTEM, S., WHITCOMB, R.M. AND RAZIN, S. (1986). DNA probes in detection of *Spiroplasma* and mycoplasma-like organisms in plants and insects. *FEMS Microbiological Letters* **35**, 157–162.

PALMER, H.M., GILROY, C.B., FURR, P.M. AND TAYLOR-ROBINSON, D. (1991). Development and evaluation of the polymerase chain reaction to detect *Mycoplasma genitalium*. *FEMS Microbiological Letters* **77**, 199–204.

PAWAR, V., LUCZAK, J., COX, M.S., DUBOSE, J. AND HARBELLE, J.W. (1994). Trends in the incidence and distribution of mycoplasma contamination detected in cell culture. *IOM Letters* **3**, 77.

RAWADI, G. AND DUSSURGET, O. (1995). Advances in PCR-based detection of mycoplasmas contaminating cell cultures. *PCR Methods and Applications* **4**, 199–208.

RAWADI, G. AND ROMAN-ROMAN, S. (1996). Mycoplasmas membrane lipoproteins induce proinflammatory cytokines by a mechanism distinct from that of lipopolysaccharide. *Infectious Immunology* **64**, 637–643.

RAWADI, G., LECAQUE, D., PIROT, D. AND ROMAN-ROMAN, S. (1993). Detection and identifi-

cation of mycoplasma contamination in cell cultures by polymerase chain reaction. *Methods in Molecular Cell Biology* **4**, 147–156.

RAWADI, G., ROMAN-ROMAN, S., CASTEDO, M., DUTILLEUL, V., SANTOS, S., MARCHETTI, P. AND KROEMER, G. (1996). Effects of *Mycoplasma fermentans* on the myelomonocytary lineage. Different molecular entities with cytokine-inducing and cytocidal potential. *Journal of Immunology* **156**, 670–678.

RAWADI, G., LALANNE, J.L. AND ROULLAND-DUSSOIX, D. (1995a). Cloning and characterization of the lipase operon from *Mycoplasma mycoides* subspecies *mycoides* LC. *Gene* **158**, 107–111.

RAWADI, G., LEMERCIER, B. AND ROULLAND-DUSSOIX, D. (1995b). Application of an arbitrarily-primed polymerase chain reaction to mycoplasma identification and typing within the *Mycoplasma mycoides* cluster. *Journal of Applied Bacteriology* **78**, 586–592.

RAZIN, S. (1992). Mycoplasma taxonomy and ecology. In *Mycoplasmas: Molecular Biology and Pathogenesis*, ed. R.N. McElhaney, J. Maniloff, L.R. Finch and J.B. Baseman, pp. 3–22. American Society for Microbiology, Washington DC.

RAZIN, S. (1994). DNA probes and PCR in diagnosis of mycoplasma infections. *Molecular Cell Probes* **8**, 497–511.

RAZIN, S. AND BARILE, M.F. (1985). *The Mycoplasmas: Mycoplasma Pathogenicity IV*. Academic Press. New York.

RAZIN, S., HYMAN, H.C., NUR, I. AND YOGEV, D. (1987a). DNA probes for detection and identification of mycoplasmas. *Israel Journal of Medical Science* **23**, 735–741.

RAZIN, S., NUR, I. AND GLASSER, G. (1987b). *Spiroplasma* plasmids. *Israel Journal of Medical Science* **23**, 678–682.

RENAUDIN, J., AULLO, P., VIGNAULT, J.C. AND BOVÉ, J.M. (1990). Complete nucleotide sequence of the genome of *Spiroplasma citri* virus SpV1-RBA2 B. *Nucleic Acids Research* **18**, 1293–1294.

REZNIKOV, H., BLACKMORE, T.K., FINLAY-JONES, J.J. AND GORDON, D.L. (1995). Comparison of naso-pharyngeal aspirates and throat swab specimens in a polymerase chain reaction-based test for *Mycoplasma pneumoniae*. *European Journal of Clinical Microbiology Infectious Diseases* **14**, 58–61

RISI, G.F., MARTIN, D.H., SILBERMAN, J.A. AND COHEN, J.C. (1987). A DNA probe detecting *Mycoplasma genitalium* in clinical specimens. *Molecular Cell Probes* **1**, 327–335.

ROBERTSON, J.A., VEKRIS, A., BEBEAR, C. AND STEMKE, G.W. (1993). Polymerase chain reaction using 16S rRNA genes sequences distinguishes the two biovars of *Ureaplasma urealyticum*. *Journal of Clinical Microbiology* **31**, 824–830.

ROS BASCUNANA, C., MATTSON, J.G., BÖLSKE, G. AND JOHANSSON, K.E. (1994). Characterization of the 16S rRNA genes from *Mycoplasma* sp. strain F38 and development of an identification system based on PCR. *Journal of Bacteriology* **176**, 2577–2586.

ROULLAND-DUSSOIX, D., HENRY, A. AND LEMERCIER, B. (1994). Detection of mycoplasmas in cell cultures by PCR: a one year study. *Journal of Microbiology Methods* **19**, 127–134.

RUUTH, E. AND PRAZ, F. (1989). Interactions between mycoplasmas and the immune system. *Immunology Review* **112**, 133–160.

SAEED, E. AND COUSIN, M.T. (1995). The genetic relationship between MLOs causing diseases in the Sudan and different continents revealed by polymerase chain reaction (PCR) amplification of the 16S rRNA gene followed by RFLP analysis. *Journal of Phytopathology* **143**, 17–20.

SAIKI, R.K., GELFAND, D.H., STOFFEL, S., SCHARF, S.J., HIGUCHI, R., HORN, G.T., MULLIS, K.B. AND ERLICH, H.A. (1988). Primed-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.

SAILLARD, C., BARTHE, C., MORENO, P. AND BOVÉ, J.M. (1994). Detection of *Spiroplasma citri* in plants by immunocapture PCR. *IOM Letters* **3**, 531–532.

SANTHA, M., BURG, K., RASKO, I. AND STIPKOVITIS, L. (1987). A species-specific DNA probe for the detection of *Mycoplasma gallisepticum*. *Infections and Immunology* **55**, 2857–2859.

SCHNEIDER, B., AHRENS, U., KIRKPATRICK, B.C. AND SEEMÜLLER, E. (1993). Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. *Journal of General Microbiology* **139**, 519–527.

SEARS, B.B., LIM, P.O., HOLLAND, N., KIRKPATRICK, B.C. AND KLOMPARENS, K.L. (1989). Isolation and characterization of DNA from a mycoplasma-like organism. *Molecular Plant–Microbe Interactions* **2**, 175–180.

SEEMÜLLER, E., SCHNEIDER, B., MAURER, R., AHRENS, U., DAIRE, X., KISON, H., LORENZ, K.H., FIRRAO, G., AVINENT, L., SEARS, B.B. AND STACKEBRANDT, E. (1994). Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. Int J Syst Bact 44: 440–446.

SHAW, S.B. (1989). DNA probes for the detection of *Legionella* species, *Mycoplasma pneumoniae*, the members of the *Mycobacterium tuberculosis* complex. In *DNA Probes for Infection Diseases*, ed. F.C. Tenover, pp. 101–117. CRC Press. Boca Raton.

SHAW, S.B., BOAS, L., CABANAS, D.K., FULTZ, T.J., LAWRENCE, T., MCDONOUGH, S.H., PAGE, C., PUTNAM, J.G., KACIAN, D. AND KHONE, D.E. (1986). *Abstracts of the Annual Meeting of the American Society of Microbiology* **G24**, 117.

SKAKNI, L., SARDET, A., JUST, J., LANDMAN-PARKER, J., MONIOT-VILLE, N., BRICOUT, F. AND GARBARG-CHENON, A. (1992). Detection of *Mycoplasma pneumoniae* in clinical samples from pediatric patients by PCR. *Journal of Clinical Microbiology* **30**, 2638–2643.

SMART, C.D., SCHNEIDER, B., BLOMQUIST, C.L., GUERRA, L.J., HARRISON, N.A., AHRENS, U., LORENZ, K.H., SEEMÜLLER, E. AND KIRKPATRICK, B.C. (1996). *Phytoplasma* specific PCR primers based on sequences of the 16S-23S rRNA spacer region. *Applied Environmental Microbiology* **62**, 2988–2993.

SPAEPEN, M., ANGULO, A.F., MARYNEN, P. AND CASSIMAN, J.P. (1992). Detection of bacterial and mycoplasmal contamination in cell cultures by polymerase chain reaction. *FEMS Microbiology Letters* **99**, 89–94.

STIPKOVITS, L., BELAK, S., MCGWIRE, B.S., BALLAGI-PORDANY, A. AND SANTHA, M. (1988). Rapid identification of *Mycoplasma gallisepticum* using a simple method of nucleic acid hybridization. *Molecular Cell Probes* **2**, 339–344.

TAYLOR, T.K., BASHIRUDDIN, J.B. AND GOULD, A.R. (1992). Relationships between members of the *Mycoplasma mycoides* cluster as shown by DNA probes and sequence analysis. *International Journal of Systems in Bacteriology* **42**, 593–601.

TAYLOR-ROBINSON, D. AND MCCORMACK, W.M. (1980). The genital mycoplasmas. *New England Journal of Medicine* **302**, 1003–1010.

TENOVER, F.C. (1988). Diagnosis deoxyribonucleic acid probes for infectious diseases. *Clinical Microbiology Review* **1**, 82–101.

TEYSSOU, R., POUTIERS, F., SAILLARD, C., GRAU, O., LAIGRET, F., BOVÉ, J.M. AND BÉBÉAR, C. (1993). Detection of mollicute contamination in cell cultures by 16S rDNA amplification. *Molecular Cell Probes* **7**, 209–216.

TILTON, R.C., DIAS, F., KIDD, H. AND RYAN, R.W. (1988). DNA probe versus culture for detection of *Mycoplasma pneumoniae* in clinical specimens. *Diagnosis and Microbiology of Infectious Diseases* **10**, 109.

TJHIE, J.H.T., VAN KUPPEVELD, F.J.M., ROOSENDAAL, R., MELCHERS, W.J.G., GORDIJN, R., MCLAREN, D.M., WALBOOMERS, J.M.M., MEIJER, C.J.L.M. AND VAN DEN BRULE, A.J.C. (1994). Direct PCR enables detection of *Mycoplasma pneumoniae* in patients with respiratory tract infections. *Journal of Clinical Microbiology* **32**, 11–16.

TOLA, S., IDINI, G., MANUNTA, D., GALLERI, G., ANGIOI, A., ROCCHIGIANI, A.M. AND LEORI, G. (1996). Rapid and specific detection of *Mycoplasma agalactiae* by polymerase chain reaction. *Veterinary Microbiology* **51**, 77–84.

Tully, J.G. (1992). Mollicutes. In *Encyclopedia of Microbiology III*, ed. J. Lederberg, pp. 181–191. Academic Press, New York.

TULLY, J.G. AND WHITCOMB, R.F. (1979). *The mycoplasmas: Human and Animal Mycoplasmas II.* Academic Press, New York.

TYAGI, S., LANDERGREN, U., TAZI, M., LIZARDI, P.M. AND KRAMER, F.R. (1996). Extremely sensitive background-free gene detection using binary probes and Qb replicase. *Proceedings of the National Academy of Sciences USA* **93**, 5395–5400.

UEMORI, T., ASADA, K., KATO, I. AND HARASAWA, R. (1992). Amplification of the 16S-23S spacer region in rRNA operons of mycoplasmas by the polymerase chain reaction. *Systems in Applied Microbiology* **15**, 181–186.

UPHOFF, C.C., BRAUER, S., GRUNICK, D., GIGNAC, S.M., MACLEOD, R.A.F., QUENTMEIER, H., STEUBE, K., TUMMLER, M., VOGES, M., WAGNER, B. AND DREXLER, H.G. (1992). Sensitivity and specificity of five different mycoplasma detection assays. *Leukemia* **6**, 335–341.

URSI, J.P., URSI, D., LEVEN, M. AND PATTYN, S.R. (1992). Utility of an internal control for the polymerase chain reaction. Application to detection of *Mycoplasma pneumoniae* in clinical specimens. *APMIS* **100**, 635–639.

VAN KUPPEVELD, F.J.M., VAN DER LOGT, J.T.M., ANGULA, A.F., ZOEST, M.J., QUINT, W.G.V., NIESTERS, H.D.G., GALAMA, J.M.D. AND MELCHERS, W.J.G. (1992). Genus and species-specific identification of mycoplasmas by 16S rRNA amplification. *Applied Environmental Microbiology* **58**, 2606–2615.

VAN KUPPEVELD, F.J.M., JOHANSSON, K.E., GALAMA, J.M.D., KISSING, J., BÖLSKE, G., HJELM, E., VAN DER LOGT, J.T.M., NIESTERS, H.D.G. AND MELCHERS, W.J.G. (1994). 16S rRNA based PCR compared with culture and serological methods for diagnosis of *Mycoplasma pneumoniae* infection. *Journal of Clinical Microbiological Infectious Disease* **13**, 401–405.

VEKRIS, A., BAUDUER, F., MAILLET, S., BÉBÉAR, C. AND BONNET, J. (1995). Improved microplate immunoenzymatic assay for PCR products for rapid detection of *Mycoplasma pneumoniae*. *Molecular Cell Probes* **9**, 25–32.

WANG, R.Y.H., HU, W.S., DAWSON, M.S., SHIH, J.W.K. AND LO, S.C. (1992). Selective detection of *Mycoplasma fermentans* by PCR and by using a nucleotide sequence within the insertion sequence like element. *Journal of Clinical Microbiology* **30**, 245–248.

WATERS, A.P. AND MCCUTCHAN, T.F. (1990). Ribosomal RNA : nature's own polymerase amplified target for diagnosis. *Parasitology Today* **6**, 56–59.

WEISBURG, W.G., TULLY, J.G., ROSE, D.L., PETZEL, J.P., OYAIZU, H., YANG, D., MANDELCO, L., SECHREST, J., LAWRENCE, T.G., VAN ETTEN, J., MANILOFF, J. AND WOESE, C.R. (1989). A phylogenetic analysis of the mycoplasma: basis for their classification. *Journal of Bacteriology* **171**, 6455–6467.

WILLIAMSON, J., MARMION, B.P. AND WORSWICK, D.A. (1992). Laboratory diagnosis of *Mycoplasma pneumoniae* infection. *Epidemiology of Infection* **109**, 519–537.

WILLIAMSON, J., MARMION, B.P., KOK, T., ANTIC, R. AND HARRIS, R.J. (1994). Confirmation of fatal *Mycoplasma pneumoniae* infection by PCR detection of the adhesin gene in fixed lung tissue. *Journal of Infectious Diseases* **170**, 1052–1053.

WILLOUGHBY, J.J., RUSSELL, W.C., THIRKEL, D. AND BURDON, M.G. (1991). Isolation and detection of urease genes in *Ureaplasma urealyticum*. *Infections and Immunology* **59**, 2436–2469.

YERSHOV, G., BARSKY, V., BELGOVSKIY, A., KIRILLOV, E., KREINDLIN, E., IVANOV, I., PARINOV, S., GUSCHIN, D., DROBISHEV, A., DUBILEY, S. AND MIRZABEKOV, A. (1996). DNA analysis and diagnostics on oligonucleotide microchips. *Proceedings of the National Academy of Sciences USA* **93**, 4913–4918.

ZINGAGIROVA, N.A., POPOVA, O.V., SOLOJEVA, C.V., GINTZBURG, A.L. AND PROZOROVSKY, S.V. (1993). Development of a PCR-based method for diagnosing of *Mycoplasma pneumoniae* infections. *Letters in Applied Microbiology* **16**, 106–106.