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Molecular Diagnostics of Mycoplasmas: Perspectives from the Microbiology Standpoint

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1. Introduction

Some of the smallest self-replicating bacteria, the wall-less mycoplasmas belonging to Class Mollicutes, are pathogenic for mammals and humans, showing tissue and host-specificity. In humans, the pathogenic species of the *Mycoplasma* or *Ureaplasma genus* cause covert infections that tend to chronic diseases. At present, 7 species of *Mycoplasma*, 2 species of *Ureaplasma* and 1 of *Acholeplasma* have been consistently isolated/detected from several specimens from diseased subjects, specially through the use of molecular detection techniques [Mendoza *et al.,* 2011; Waites & Talkington, 2005; Waites, 2006].

Current laboratory diagnosis of these infections relies on cultural methods, however this is complicated and emission of results may delay up to 5 weeks. Thus development and application of molecular methods, such as polymerase chain reaction (PCR), have allowed direct detection in clinical specimens and shortened the time to get the final results. Nevertheless some pitfalls still hampers the widespread use of these technologies, mainly due to technical difficulties in collecting representative specimens and optimizing sample preparation. There are countless reports on new nucleic acid-based tests (NATs) for mycoplasma detection, however there is a great variation between methods from study to study, including variability of target gene sequences, assay format and technologic platform [Waites *et al.,* 2000; Waites , 2006;].

The processing of the clinical samples is crucial for the improvement of PCR assays as part of routine diagnostic approaches. In general, for the strength of performance of any diagnostic PCR, the overall setting-up of the assay should consider the following four basic steps: 1) sampling, 2) sample preparation, 3) nucleic acid amplification, and 4) detection of PCR products [Rådström *et al.,* 2004].

As occurred with much of the emerging or reemerging pathogens, the molecular detection plays a key role in the discovery, identification and association or such pathogens with human disease [Relman & Persing, 1996]. Nevertheless, routine clinical microbiology

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laboratories still lack of skilled personnel in molecular detection techniques, and consequently in the appropriate sample preparation procedures [Cassell *et al*, 1994a; Talkington & Waites, 2009]. Unlike other fast-growing pathogens, the pathogenic Mollicutes species exhibit unique features that make them the last link in the diagnostic chain, only sought after failure in other diagnostic approaches [Cassell *et al.*, 1994a].

2. Relevant features of mycoplasmas

The term mycoplasmas will be used to refer to any member of the Class Mollicutes. The mycoplasmas are the smallest microorganisms (0.3 - 0.8 μm diameter) capable of self-replication, which lack a rigid cell wall. These bacteria also incorporate exogenous cholesterol into their own plasma membrane and use the UGA codon to encode tryptophan. Due to their reduced cell dimensions, they possess small genome sizes (0.58-2.20 Mb) and exhibit restricted metabolic alternatives for replication and survival. As a result of the above mentioned, the mycoplasmas show a strict dependence to their hosts for acquisition of biosynthetic precursors (aminoacids, nucleotides, lipids and sterols), in a host- and tissue-restricted manner, reflecting their nutritional demands and parasitic lifestyle [Baseman & Tully, 1997; Razin, 1992; Razin *et al.*, 1998].

Mycoplasmas infecting humans mainly colonize the mucosal surfaces of the respiratory and genitourinary tracts [Cassell *et al.*, 1994, Patel & Nyirjesy, 2010; Taylor-Robinson, 1996]. The mycoplasma species commonly isolated from humans and their attributes are listed in Table 1. Of the pathogenic species, *Mycoplasma pneumoniae* is found principally in the respiratory tract, whereas *M. genitalium, Ureaplasma parvum, U. urealyticum., M. hominis, M. fermentans*

Species	*Primary colonization sites*		*Main metabolic substrates*			*Pathogenicity*
	Respiratory tract	*Urogenital tract*	*Glucose*	*Arginine*	*Urea*	
Mycoplasma. salivarium	+	-	-	+	-	-
M. orale	+	-	-	+	-	-
M. buccale	+	-	-	+	-	-
M. faucium	+	-	-	+	-	-
M. lipophilum	+	-	-	+	-	-
M. pneumoniae	+	-	+	-	-	+
M. hominis	+	+	-	+	-	+
M. genitalium	+	+	+	-	-	+
M. fermentans	+	+	+	+	-	+[a]
M. primatum	-	+	-	+	-	-
M. spermatophilum	-	+	-	+	-	-
M. pirum	¿?	¿?	+	+	-	-
M. penetrans	-	+	+	+	-	+[b,c]
Ureaplasma urealyticum	+	+	-	-	+	+
U. parvum	+	+	-	-	+	+
Acholeplasma laidlawii	+	-	+	-	-	-
A. oculi	¿?	-	+	-	-	-

[a]Lo *et al*,1993; [b]Lo *et al*, 1992; [c]Yáñez *et al*., 1999.

Table 1. Mycoplasmas which infect humans.
Adapted from: Taylor-Robinson, 1996.

and *M. penetrans* are primarily urogenital residents, but exceptionally they can be isolated from other unusual tissues and organs, especially in immunocompromised patients or in patients undergoing solid organ transplantation [Cassel *et al*., 1993; Waites & Talkington, 2004; Waites *et al*, 2005; Waites *et al*., 2008].

Most of mycoplasmal diseases are underdiagnosed because the specific laboratory diagnostic strategies are quite different than those for fast-growing bacteria. It is noteworthy that mycoplasmal etiology of diseases in humans is considered only after failure of diagnosis of other common bacterial etiologies. In addition, outside their hosts, the mycoplasmas are highly labile to environmental factors, such as changing osmotic pressure and temperature, desiccation and/or alkaline or acidic conditions [Cassell *et al*., 1994a; Waites *et al*., 2000]. Noteworthy, there are few specialized or reference laboratories for diagnosis of mycoplasmal diseases and therefore, limited skilled laboratory personnel [Cassell *et al*., 1994a].

3. Routine laboratory diagnostic approaches

Several different detection techniques of mycoplasmal infections have been developed, each one of which has its advantages and limitations with respect to cost, time, reliability, specificity, and sensitivity. According to the laboratory's infrastructure, the most common methods include: a) culture-based isolation/detection/identification/antimicrobial susceptibility profile; b) antigen detection, c) mycoplasmal-specific serologic responses; and, d) PCR and other NATs. [Razin *et al*., 1998; Talkington & Waites, 2009; Waites *et al*., 2000; Yoshida *et al*., 2002].

3.1 Culture

Relationship between mycoplasmas as etiologic agents and diseases in humans remains doubtful due to unsuccessful isolation/detection of these microorganisms in specimens from affected persons, as compared with healthy carriers [Taylor-Robinson, 1996]. Demonstration of growth of mycoplasma, by means of *in vitro* culture from clinical specimens, is still required to link the pathogen with the disease; thus culture is considered as the Gold Standard. However, current culture methods for detection of mycoplasmas in clinical specimens are arduous and emission of results may delay up to 5 weeks, which even then may be inconclusive or inaccurate [Cassell *et al*., 1994a; Waites *et al*., 2000].

In this context, detection or isolation of mycoplasmas from clinical specimens requires careful consideration of the type of specimen available and the organism (species) sought [Cassell *et al*., 1994a]. The adequate specimens for culture include: a) normally sterile body fluids (sinovial, amniotic, cerebrospinal, urine, peritoneal, pleural, etc., b) secretions exudates or swabs from sites with associated flora (from nasopharinx, pharinx, cervix, vagina, urethra, surgical wounds, prostate, sputum, etc.), and c) cell-rich fluids (including blood and semen) or tissue biopsies. Overall, specimen collection should reflect the site of infection and/or the disease process. [Atkinson *et al*., 2008; Waites & Talkington, 2004].

Liquid specimens or tissues do not require special transport media if culture can be performed within 1 hour, otherwise specimens should be placed in transport media, such as SP-4, 10B or 2SP broths. When swabbing is required, aluminum- or plastic-shafted calcium alginate or dacron swabs should be used, taking care to obtain as many cells as possible [Atkinson *et al*., 2008; Cassell *et al*., 1994a; Waites, 2006].

There is no ideal formulation of culture media for all pathogenic species, mainly due to their different substrate and pH requirements [Waites *et al*., 2000]. Modified SP-4 media (broth and agar) [Lo *et al*., 1993a], containing both glucose and arginine, can support the growth of all human pathogenic *Mycoplasma* species, including the fastidious *M. pneumoniae* and *M. genitalium*. A set of Shepard´s 10B broth and A8 agar can be used for cultivation of *Ureaplasma* species and *M. hominis*. For cultivation, specimens in transport media should be thoroughly mixed, and then should be 10-fold serially diluted in broth (usually up to 10^{-6}) in order to allow semiquantitative estimation of mycoplasmal load, but subcultures in agar media should also be performed [Cassel *et al*., 1994a]. Inoculated media should be incubated under microaerophilic atmosphere at 37 °C.

Detection of *M. pneumoniae* in broth culture is based on its ability to ferment glucose, causing an acidic shift after 4 or more weeks, readily visualized by the presence of the phenol red pH indicator. Broths with any color change, and subsequent blind broth passages, should be subcultured to SP4 agar, incubated, and examined under the low-power objective of the light microscope in order to look for development of typical "fried egg"-like colonies of up to 100 µm in diameter. Examination of agar plates must be done on a daily basis during the first week, and thereafter every 3 to 4 days until completing 5 weeks or until growth is observed [Waites *et al*., 2000; Waites & Talkington, 2004]. *M. genitalium*, *M. fermentans* and *M. penetrans* are also glucose-fermenting and form colonies morphologically indistinguishable from those of *M. pneumoniae*, thus serologic-based definitive identification can be done by growth inhibition, metabolic inhibition, and mycoplamacidal tests [Atkinson *et al*., 2008].

Hidrolysis of urea by Ureaplasma and hidrolysis of arginine by *M. hominis* cause an alkaline shift, turning the colour of 10B broth from yellow to pink. Tiny brown or black irregular colonies of *Ureaplasma* species develop between 1-5 days on A8 agar plates, due to urease activity in the presence of manganese sulfate. Typical fried egg colonies are produced by *M. hominis* in this medium [Cassell *et al*, 1994a; Waites *et al*., 2000].

3.2 Molecular assays

The nucleic acid-based techniques have several advantages over culture-based methods, including rapid results, low detection limits (theoretically a single copy of target sequence), and specific organism detection. This is critical in a hospital setting, since rapid pathogen detection is important for faster and improved patient treatment and consequently for shortening hospitalization time [Mothershed & Whitney, 2006].

In particular, for PCR-based detection tests, selection of the appropriate target sequences for amplification appears to be of major concern. Mycoplasmal sequences to be amplified can be chosen from published gene sequences or from a mycoplasma-specific cloned DNA fragments [Kovacic *et al*., 1996; Razin, 2002]. The accelerated rate of genomic sequencing has led to an abundance of completely sequenced genomes. Annotation of the open reading frames (ORFs) (i.e., gene prediction) in these genomes is an important task and is most often performed computationally based on features in the nucleic acid sequence [Jaffe *et al*., 2004; Razin 2002]. Besides complete or almost complete sequences of the 16S rRNA genes for almost all the established mycoplasma species, the published full genome sequences of the human pathogenic mycoplasma species [Fraser *et al*., 1995; Glass *et al*., 2000; Himmelreich *et al*., 1996] will accelerate the process of identification of novel target sequences for PCR

diagnostics. Selection of a variety of target sequences, starting with highly conserved regions of the genes, allowed design of primers of wide specificity ("universal primers") (Table 2) for detection of mycoplasmal infections in anatomic sites where at least 2 or 3 species are frequently found. The use of a single Mollicutes universal primer set in cases of life-threatening infections has the advantage of allowing a rapid positive or negative report to clinicians, and in turn to establish as soon as possible the appropriate treatment [Razin, 1994]. The approach of using *Mollicutes*-specific and *Ureaplasma* spp-specific universal primers allowed better discrimination between organisms of the *Mycoplasma* and *Ureaplasma* genus, and subsequent identification by species-specific primers in urine specimens from HIV-infected patients [Díaz-García *et al*., 2004].

Targets	Applications	References
16S rRNA gene sequence		
Conserved regions of mycoplasmal 16S rRNA genes.	Screening for any mycoplasma species in clinical specimens and cell cultures.	van Kuppeveld *et al*., 1992; van Kuppeveld *et al*., 1994; Yoshida *et al*., 2001.
Variable regions of mycoplasmal 16S rRNA genes:	Species-specific detection.	Blanchard *et al*., 1993a; Grau *et al*., 1994; van Kuppeveld *et al*., 1992.
The 16S-23S intergenic regions	Detection of cell culture contamination.	Harasawa *et al*., 1993.
Mycoplasmal protein genes		
P1 adhesin gene: *M. pneumoniae*	Selective detection and typing	Bernet *et al*., 1989
MgPa adhesin gene: *M. genitalium*	Selective detection	Palmer *et al*., 1991; Jensen *et al*., 1991.
Elongation factor *tuf* gene of *M. pneumoniae*	Selective detection	Lüneberg *et al*., 1993
Ureasa genes: *Ureaplasma spp.*	Genus-specific detection	Blanchard *et al*., 1993b
tet M gene (tetracycline-resistance determinant)	Identification of tetracycline-resistant strains	Blanchard *et al*., 1992
Mba gene	Species-specific detection and typing.	Kong *et al*., 1996
Repetitive genomic sequences		
Is-like elements: *M. fermentans*	Selective detection	Wang *et al*., 1992
Rep elements of P1: *M. pneumoniae*	Selective detection	Ursi *et al*., 1992

Table 2. Nucleic acid sequences suitable for PCR-based mycoplasma testing
Adapted from: Razin, 1994.

When differentiation of the mycoplasmas is required, a multiplex PCR system consisting of a universal set of primers along with primer sets specific for the mycoplasma species commonly involved in a given disease process can be successfully applied [Razin, 2002, Choppa *et al*., 1998]. Moreover, both conserved and variable regions within the mycoplasmal 16S rRNA genes can also be selected for detection at cluster-, genus- species-, subspecies-, biovar- or serovar-specific levels [Kong *et al*., 2000; Razin, 2002].

For diagnostic purposes in mycoplasmology, the nucleic acid tests are more sensitive than culture, and showing a fair to good correlation with serology. PCR testing for species-specific mycoplasmal infection are suitable for both urogenital and respiratory samples [Povlsen *et al*., 2001, 2002]. Interestingly, sample processing prior amplification must be optimized depending of the type of specimen to overcome the presence of undefined inhibitory substances for DNA polymerases, avoiding false negative results. For example, nasopharyngeal samples have a higher rate of PCR inhibition than throat swabs. In general, results obtained by means of NATs will be as good as the quality of the nucleic acid used for the test [Mothersehed & Whitney, 2006; Maeda *et al*., 2004].

Early in the past decade, Loens *et al*., 2003b, stated that the development and application of new nucleic acid tests (NATs) in diagnostic mycoplasmology required proper validation and standardization, and performance of different NATs must be compared with each other in order to define the most sensitive and specific tests. The NATs have demonstrated their potential to produce rapid, sensitive and specific results, and are now considered the methods of choice for direct detection of *M. pneumoniae*, *M. genitalium,* and *M. fermentans* [Cassell *et al*., 1994a; Loens *et al*., 2003b]. There is a great variation in methods used from study to study, including variability of target gene sequences (P1, 16S RNA, ATPase, *tuf*), assay format (single, multiplex) or technologies (end-point PCR, Real-time PCR, NASBA) [Loens *et al*., 2003a, 2003b; 2010]. Also, target DNA has been obtained from different specimens, such as sputum, nasopharyngeal or pharyngeal swabs, brochoalveolar lavages or pleural fluid, and then comparisons of performance between these assays are difficult. For comprehensive understanding of the use of NATs for the detection of *M. pneumoniae*, genital mycoplasmas and other respiratory pathogens in clinical specimens, see the reviews done by Ieven, 2007; ; Lo & Kam, 2006; Loens *et al*., 2003b, 2010.

As with any other diagnostic test, PCR assays designed for mycoplasma detection in the clinical setting offer several advantages over other non-molecular tests, but still have several drawbacks to take into account (Table 3). Notwithstanding, there are several primer sets that have been successfully applied for diagnosis of mycoplasmal diseases in humans (Table 4).

4. Importance of the specimen collection and processing

Clinical specimens must be collected with use of strict aseptic techniques from anatomic sites likely to yield pathogenic microorganisms [Taylor, 1998; Wilson, 1996]. In the case of mycoplasmal infections, these are clinically silent or covert, thus it is important to differentiate between asymptomatic carriage and disease. In this context, sampling of representative diseased body sites is critical for successful diagnosis.

The usefulness of a PCR assay for diagnostic purposes is rather limited; this is partially explained by the presence of inhibitory substances in complex biological samples, which then provoke a significant reduction or even blockage of the amplification activity of DNA polymerases in comparison with that obtained with the use of pure solutions of nucleic acids. This in consequence affects the performance and the analytical sensitivity of the PCR assays [Lo & Kam, 2006; Vaneechoute & Van Eldere, 1997].

Advantages	*Disadvantages*
• Overcomes the need for mycoplasma cultivation	• Presence of undefined inhibitors of DNA polymerases may yield false-negative results.
• Emission of results is faster than culture (less than 24 h vs. up to 5 weeks)	• Upon detection, PCR poorly discriminates between disease or carriage
• Allows detection of antibiotic-inhibited or uncultivable species in clinical specimens	• Risk of false-positive results due to carryover contamination with amplicons from previous reactions.
• Selective detection. Presence of nucleic acids from the host or from other microorganisms usually do not affect PCR results	• Setting-up quantitative determination of bacteria in clinical specimens may be a very complicated task.
• Higher sensitivity than other non-molecular diagnostic assays (culture, serology).	• Performance of PCR assays for routine diagnostic purposes in microbiology laboratories is still complex and expensive.
• Use as an epidemiological tool since it allows detection of asymptomatic carriers.	• Skilled personnel are required to carry out tests and analysis of results.
• Allows detection of mycoplasmas at the level of Family, Genus, Species, Subspecies and/or Type.	• Depending on the target sequences used, cross reactivity with closely related bacteria may occur.

Table 3. Considerations for using PCR assays in diagnosis of mycoplasma infections. Adapted from: Razin 1994.

Due to the above mentioned, improvement of PCR assays for routine diagnostic purposes clearly should begin with optimal processing of clinical specimens prior to amplification reaction, thus successful amplification of the target DNA sequence can be obtained in the context of trace amounts of sample-associated inhibitory substances [Horz *et al*, 2010; Lo & Kam, 2006; Rådström *et al*., 2004; Vaneechoute & Van Eldere, 1997].

Group or species	Primer sets.		Sequence (5′→3′)	Target
Mollicutes-specific	Sen: Antisen:	GPO-1 MGSO	ACT CCT ACG GGA GGC AGC AGT A TGC ACC ATC TGT CAC TCT GTT AAC CTC	16S rDNA
	Sen: Antisen:	My-Ins MGSO	GTAATACATAGGTCGCAAGCGTTATC TGC ACC ATC TGT CAC TCT GTT AAC CTC	16S rDNA
M. fermentans	Sen: Antisen: IP:	RW005 RW004 RW006	GGT TAT TCG ATT TCT AAA TCG CCT GGA CTA TTG TCT AAA CAA TTT CCC GCT GTG GCC ATT CTC TTC TAC GTT	Insertion sequence-like element
	Sen: Antisen: IP:	Mf-1 Mf-2 GPO-1	GAA GCC TTT CTT CGC TGG AG ACA AAA TCA TTT CCT ATT CTG TC ACT CCT ACG GGA GGC AGC AGT A	rDNA 16s
M. genitalium	Sen: Antisen: IP:	MGS-1 MGS-2 MGS-I	GAG CCT TTC TAA CCG CTG C GTG GGG TTG AAG GAT GAT TG AAG CAA CGT AGT AGC GTG AGC	MgPa Adhesin gene
	Sen: Antisen: IP:	MGS-1 MGS-4 MGS-I	GAG CCT TTC TAA CCG CTG C GTT GTT ATC ATA CCT TCT GAT AAG CAA CGT AGT AGC GTG AGC	MgPa Adhesin gene
M. hominis	Sen: Antisen: IP:	MYCHOMP MYCHOMN MYCHOMS	ATA CAT GCA TGT CGA GCG AG CAT CTT TTA GTG GCG CCT TAC CGC ATG GAA CCG CAT GGT TCC GTT G	16s rDNA
	Sen: Antisen: IP:	Mh-1 Mh-2 GPO-1	TGA AAG GCG CTG TAA GGC GC GTC TGC AAT CAT TTC CTA TTG CAA A ACT CCT ACG GGA GGC AGC AGT A	16s rDNA
	Sen: Antisen:	RNAH1 RNAH2	CAATGGCTAATGGCCGGATACGC GGTACCGTCAGTCTGCAAT	16S rDNA
M. penetrans	Sen: Antisen: IP:	MYCPENETP MYCPENETN MYCPENETS	CAT GCA AGT CGG ACG AAG CA AGC ATT TCC TCT TCT TAC AA CAT GAG AAA ATG TTT AAA GTC TGT TTG	16s rDNA

Table 4. Primer sets used for end-point PCR detection of mycoplasmas in clinical specimens

bp, Base pairs; Sen, sense or downstream; Antisen, antisense or upstream; IP, internal probe.

Group or species	Primer sets.		Sequence (5′→3′)	Target
M. pneumoniae	Sen: Antisen: IP:	MP5-1 MP5-2 MP5-4	GAA GCT TAT GGT ACA GGT TGG ATT ACC ATC CTT GTT GTA AGG CGT AAG CTA TCA GCT ACA TGG AGG	Unknown gene
	Sen: Antisen: IP:	P1-F P1-R PI-P	GCC ACC CTC GGG GGC AGT CAG- GAG TCG GGA TTC CCC GCG GAG G CTG AAC GGG GGC GGG GTG AAG G-	P1 adhesin gene
	Sen: Antisen: IP:	16S-F 16S-R 16S-P	AAG GAC CTG CAA GGG TTC GT CTC TAG CCA TTA CCT GCT AA ACT CCT ACG GGA GGC AGC AGT A	16S rDNA
	Sen: Antisen: IP:	MP-P11 MP-P12 MP-I	TGC CAT CAA CCC GCG CTT AAC CCT TTG CAA CTG CTC ATA GTA CAA ACC GGG CAG ATC ACC TTT	P1 Adhesin gene
Ureaplasma spp.	Sen: Antisen: IP:	U5 U4 U9	CAA TCT GCT CGT GAA GTA TTA C ACG ACG TCC ATA AGC AAC T GAG ATA ATG ATT ATA TGT CAG GAT CA	Urease locu
	Sen: Antisen: IP:	Uu-1 Uu-2 UUSO	TAA ATG TCG GCT CGA ACG AG GCA GTA TCG CTA GAA AAG CAA C CAT CTA TTG CGA CGC TA	16s rDNA
U. parvum *U. urealyticum*	Sen: Antisen:	UMS-125 UMA-226-	GTA TTT GCA ATC TTT ATA TGT TTT CG CAG CTG ATG TAA GTG CAG CAT TAA ATT C	*mba* gene

Under certain conditions PCR detection/identification/confirmation of mycoplasmas could be attempted from culture broths used for primary isolation, whether or not it have bacterial growth. According to broth turbidity boiling of small aliquots can be sufficient to release the DNA, but presence of precipitated material may inhibit the amplification assay.

In our experience, an alkaline shift around pH 8 frequently results in bacterial lysis, mainly of ureaplasmas, therefore concentration of insoluble material by ultracentifugation prior to DNA extraction is unproductive. This is due to spontaneous release of mycoplasmal DNA that easily dissolves in the aqueous phase and cannot be sedimentated by centrifugation. In such cases, one can take advantage of the alkaline condition to precipitate the dissolved DNA by adding one tenth of 1M NaCl and twice the volume of cold 100% ethanol, and proceed with conventional DNA extraction protocols (unpublished data).

4.1 Exudates and secretions

These types of specimens are fluids closely associated with mucosal surfaces, in low quantities, so collection should be done with the aid of swabs, cytological brush or small syringes. Secretions and exudates can be taken from upper respiratory airways and from lower genital tract, and exceptionally from surgical wounds [Waites, 2006].

4.1.1 Respiratory tract

Respiratory *M. pneumoniae* infection can be assessed by culture and PCR in nasopharyngeal and oropharyngeal secretions, sputa, bronchoalveolar lavage and lung tissue obtained by biopsy. There are reports that nasopharyngeal and oropharyngeal specimens are equally effective for detection of *M. pneumoniae* by PCR, although it is desirable that both sites are screened in parallel for better diagnostic yield [Waites *et al*., 2008].

When neonatal mycoplasmal infections are suspected, endotracheal, nasopharyngeal and throat secretions are appropriate to evaluate respiratory infection., though specimens for culture should be transported quickly to laboratory since they are likely to contain at least a few contaminating microorganisms [Waites *et al*., 2005].

Presence of mucous material in this kind of specimens frequently hampers appropriate processing for culture or PCR. Use of aggressive mucolytic agents (NaOH, n-acetyl-cisteine) can damage as well the mycoplasma cells, thus thorough homogenization by wide-bore pippeting is required prior to culture attempt. For nucleic acid extraction, addition of starch has been of help to enhance recovery of total genomic DNA from sputum samples [Harasawa *et al*., 1993]. In other study, dithiotreitol was used as the mucolytic agent without any apparent detrimental effect on mycoplasmal DNA integrity [Raty *et al*., 2005].

It this worthy to note that differential sample preparation from the same specimen may be necessary when testing separate single-species PCRs on BAL, as described by [de Barbeyrac *et al*., 1993]. In that report, freeze-thawing cycles were applied for sample preparation for *M. genitalium* detection, while standard DNA extraction was needed for *M. pneumoniae* detection. During a study of Finnish patients with radiologically confirmed pneumonia, [Raty *et al*., 2005], evidence further supported the notion that selection of the appropriate specimen is crucial for diagnosis of *M. pneumoniae* infection. By means of a *M.*

pneumoniae-specific 16S rDNA PCR, they obtained positive amplification frequencies of 69%, 50% and 37.5% for sputum, nasopharyngeal aspirate and throat swab specimens, respectively.

4.1.2 Urogenital tract

Since genital tract mycoplasmas are closely associated to live epithelial cells, collection of exudates must be avoided and vigorous scraping of epithelia must be done to obtain as many cells as possible. In this case, a higher associated flora is frequently present in the samples; therefore use of transport liquid media (for culture) of buffered solutions (for DNA extraction) is required immediately after sampling.

4.2 Sterile body fluids

Collection of normally sterile body fluids is made through invasive procedures, usually performed by physicians under aseptic conditions [Wilson, 1996]. When specimens are going to be collected through puncture, careful disinfection of the skin spot must be done, this is crucial to both avoid contamination of the specimen with the skin's associated flora and to minimize the risk of introduction of bacteria into patient's body. Clinically, access of mycoplasmas to sterile body sites may be associated with an underlying immune compromise, and probably the bacteria spread from pulmonary or genital infectious foci [Cassell *et al*., 1994b; Waites & Talkington, 2004]. Ureaplasmas and mycoplasmas should always be sought from synovial fluid when hypogammaglobulinemic patients develop acute arthritis [Waites *et al*., 2000].

Since mycoplasma-containing body fluids rarely became turbid; these specimens should be concentrated 10-fold by high-speed centrifugation (aprox. 12,000 x g) and immediately resuspended in one tenth of the original supernatant if culture will be performed. Prior to DNA extraction, the resulting pellet can be washed 1-2 times with Hank's balanced salt solution or PBS, pH 7.4.

4.3 Cell-rich fluids and tissues

Unlike normally sterile body fluids, blood and semen are cell-rich fluid specimens, thus processing for culture or PCR is quite different. It is important to note that mycoplasmas have the ability to invade several cell types, including leukocytes and spermatozoa [Andreev *et al*., 1995; Baseman *et al*., 1995; Díaz-García *et al*., 2006; Girón *et al*., 1996; Jensen *et al*., 1994; Lo *et al*., 1993b, Rottem, 2003; Taylor-Robinson *et al*., 1991; Yavlovich *et al*., 2004], consequently a high input of cells into culture media may result in a higher probability of detection.

In contrast, when DNA extraction must be performed for PCR assays, depuration of the sample must be done, (i.e. erythrocyte lysis and selective enrichment for leukocytes in blood; density gradient-based purification of spermatozoa). Noteworthy, the average content of leukocyte DNA per milliliter of blood ranges from 32 to 76 µg, therefore surpasses considerably the amount of bacterial DNA in a specimen from an infected subject. [Greenfield & White, 1993], so a high amount of sample DNA should be added to the PCR reaction mixture to raise the chances to detect bacterial target sequences.

In the case of solid tissues, mechanical homogenization is required to release single cells, either for culture or DNA extraction. A challenge for DNA extraction is when tissues have been formalin-fixed and/or paraffin-embedded since there is high risk of DNA damage [Shi *et al*., 2004].

A summary of the processing of different specimen types for intended mycoplasma detection is depicted in Figure 1.

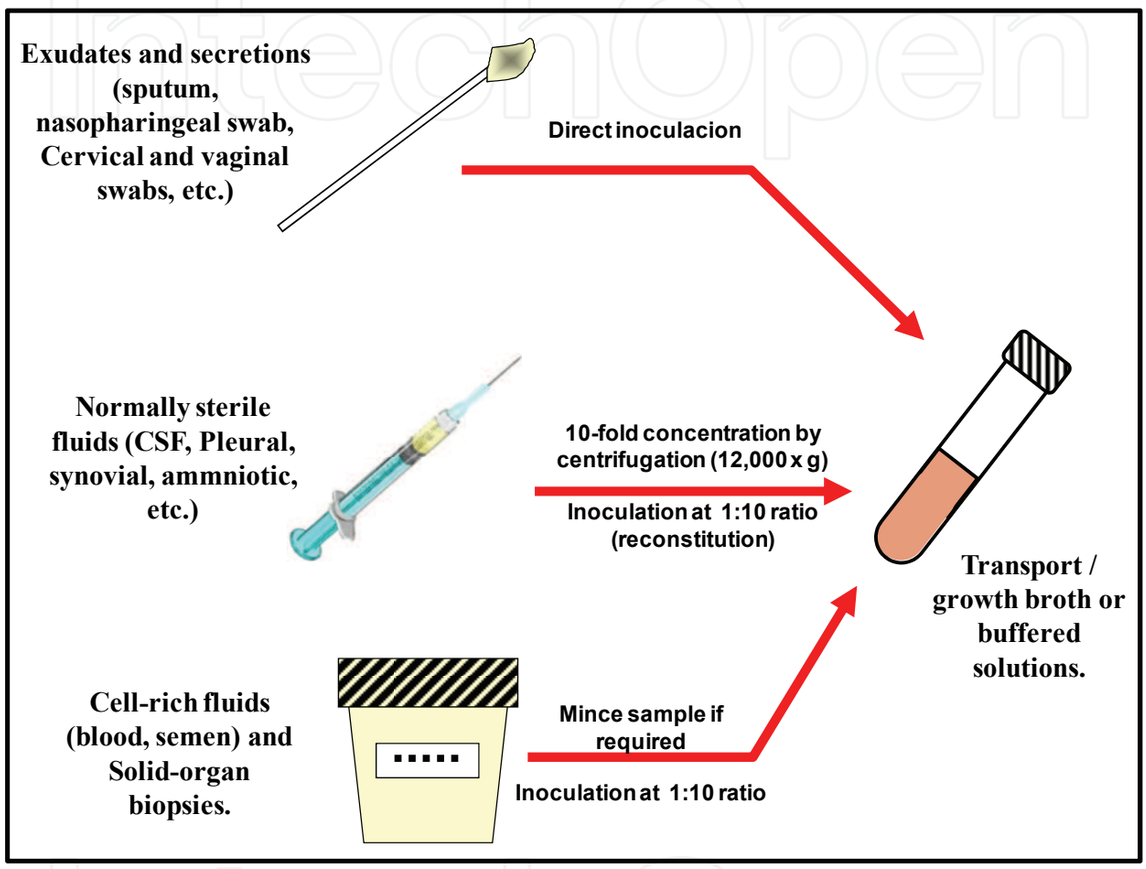

Fig. 1. Differential specimen processing for mycoplasma detection.

5. Culture vs. nucleic acid amplification methods

5.1 The gold standard for mycoplasmal infections

Some genital mycoplasmas, *Ureaplasma spp*. and *M. hominis*, are the fastest growing species among the *Mollicutes*, and due to this, culture-based detection is still the first-line diagnostic approach. However, extrapolating this particular feature to all pathogenic human mycoplasmas is inaccurate. PCR amplification has become essential if fastidious, slow-growing, mycoplasma species are sought in certain clinical conditions, especially in patients with high risk of invasive infections (neonates) or when invasive methods of sampling are required [Waites *et al*., 2005]. It is well recognized that culture techniques are of poor or null value for detection of some mycoplasma species (i.e. *M. genitalium*) [Razin *et al*., 1998].

Since culture rely on viability of mycoplasmas to give a positive result, analytical comparisons between culture and PCR invariably will regard the second as less sensitive and less specific, which by any means is wrong. The PCR assay ideally detect target DNA sequences present in the sample, whether it comes from live, dead or uncultivable bacteria [Persing, 1993].

Performance of non culture-based detection tests is frequently evaluated against culture, as the gold standard. Indirect assays measuring serologic responses as correlates of mycoplasma infections, have shown poor sensitivity and required at least 2 serum samples taken several days apart, to be informative [Waites, 2000].

5.2 Culture-enhanced PCR approach

When very few mycoplasma cells are present in a given specimen there is a high probability of obtaining false-negative results, even when the sensitivity of the specific PCR assay is high. To overcome this, several authors have developed culture-based pre-enrichment protocols for mycoplasmas, mycobacteria and *Actinobacillus* [Abele-Horn *et al.,* 1998; Díaz-García *et al.,* 2004; Flemmig *et al.,* 1995; Noussair *et al.,* 2009]. The effects of this procedure are, on one hand the dilution of potential undefined inhibitors, and on the other hand the promotion of short-term bacterial multiplication. This experimental approach has been termed as Culture-enhanced PCR (CE-PCR) [Abele-Horn *et al.*, 1998]. The genomic DNA content in overnight enriched mycoplasma cultures are extracted by standard or commercial techniques, and then subjected to broad-range or species-specific PCR assays. Under this approach, improved detection of *M. pneumoniae* has been achieved in respiratory specimens [Abele-Horn *et al.,* 1998], and of genital mycoplasmas in urine specimens [Díaz-García *et al.,* 2004].

Another culture-based enrichment approach for improvement of PCR detection of mycoplasmas is the cocultivation of these bacteria with permissive immortalized mammalian and/or insect cell lines [Kong *et al*., 2007; Volokhov *et al*., 2008]. Although this approach has been design for intentional screening of cell-derived biological and pharmaceutical products, including vaccines and cell culture substrates, it is a potential tool for biological enrichment of normally-sterile clinical specimens such as CFS, sera, synovial fluid, etc.

Interestingly, strains of mycoplasma-free *Trichomonas vaginalis* are readily infected *in vitro* by *M. hominis* isolates, but not by other urogenital mycoplasmas. The infection can be detected by a *M. hominis*-specific PCR assay after long-term incubation, since the mycoplasma can be transmitted between the protozoan cells [Dessi *et al*., 2006; Rapelli *et al*., 2001]. The symbiotic interplay between *M. hominis* and *T. vaginalis* has been well established, as well a significant correlation between detection of both microorganisms in vaginal specimens from infected women [Dessi *et al*., 2006]. Thus it is likely to take advantage of such symbiosis and employ mycoplasma-free *T. vaginalis* cultures for specific enrichment of *M. hominis*-containing clinical specimens prior to PCR detection tests.

6. Commercial molecular diagnostic kits

Unlike the in-house PCR assays for diagnostic purposes, developed by several researchers, the commercial PCR kits are well standardized in terms of sensitivity and specificity,

allowing their global use in clinical microbiology laboratories. Thus inter-laboratory performance comparisons of such kits are suitable, including testing of several specimen types. Indeed, according to the *In Vitro Diagnostic Medical Devices Directive* 98/79/EC, all commercial diagnostic kits used in European countries must have the CE (*Conformité Européene*) label [Dosà *et al*., 1999].

Among commercially available real-time PCR kits are intended for *M. pneumoniae* detection, mainly targeting the P1 cytadhesin gene, including Nanogen Mycoplasma pn Q-PCR Alert kit (Nanogen Advanced Diagnostics); the Simplexa *Mycoplasma pneumoniae* kit (Focus Diagnostics, California); the Diagenode detection kit for *Mycoplasma pneumoniae/Chlamydophila pneumoniae* (Diagenode SA, Liège, Belgium); the Cepheid *Mycoplasma pneumoniae* ASR kit (Cepheid, Paris, France), and the Venor Mp-Qp PCR detection kit (Minerva Biolabs GmbH). It has been shown that these commercial kits had acceptable analytical sensitivity and performance with clinical specimens [Touati *et al*., 2009].

Interestingly, many commercially available extraction kits incorporate a buffer to lyses the bacteria and a silica matrix membrane (typically in column format) to trap the DNA or RNA. Several wash steps are required to remove protein and other macromolecules, and the purified DNA and RNA is then eluted from the membrane. Many of the manual extraction methods require several centrifugation steps. To reduce hands-on time, operator error, and sample contamination, semi-automated DNA or RNA extraction kits and equipment have been designed and are commercially available.

7. PCR, sequencing, phylogeny and molecular epidemiology

The mycoplasmas may have evolved through regressive evolution from closely related Gram positive bacteria with low content of guanine plus cytosine (G+C), probably the Clostridia or Erysipelothrix [Bove, 1993; Brown *et al*., 2007; Razin *et al*., 1998]. The massive gene losses (i.e. genes involved in cell wall and aminoacid biosynthesis) had left mycoplasmas with a coding repertoire of 500 to 2000 genes [Sirand-Pugnet *et al*., 2007]. The G+C content in DNA of mycoplasmas varies from 23 to 40 mol%, while genome size range is 580–2200 Kbp, much smaller than those of most walled bacteria [Razin *et al*., 1998].

After PCR amplification and sequencing of the conserved 16S rDNA gene sequences from representative members of the Mollicutes, the resulting phylogenetic tree was shown to be monophyletic, arising from a single branch of the Clostridium ramosum branch [International Committee on Systematics of Prokaryotes- Subcommittee on the taxonomy of Mollicutes (ICSP-STM), 2010]. The Mollicutes split into two major branches: the AAP branch, containing the *Acholeplasma*, *Anaeroplasma* and *Asteroleplasma* genera, and the Candidatus *Phytoplasma* phyla; the other is the SEM branch that includes the *Spiroplasma*, *Entomoplasma*, *Mesoplasma*, *Ureaplasma* and *Mycoplasma* genera [Johansson *et al*., 1998; Maniloff, 1992; Razin *et al*., 1998]. Interestingly, the genus Mycoplasma is polyphyletic, with species clustering within the Spiroplasma, Pneumoniae and Hominis phylogenetic groups [Behbahani *et al*., 1993; Johansson *et al*., 1998; Maniloff, 1992]. Nevertheless, additional phylogenetic markers such as the elongation factor EF-Tu (tuf) gene, ribosomal protein

genes, the 16S-23S rRNA intergenic sequences, etc, have been already used as complementary comparative data, thus there is no unique phylogenetic tree for Mollicutes [Razin *et al*., 1998].

There are several in-house species-specific end-point or real-time PCR assays developed to detect mycoplasmas in diverse respiratory and urogenital tract infections [Blanchard *et al*., 1993b; Loens *et al*., 2003b; Sung *et al*., 2006; van Kuppeveld *et al*., 1992; Wang *et al*, 1992]. Of those clinical entities, more than one mycoplasma species are commonly associated as etiologic agents, i.e. urethritis, infertility, pelvic inflammatory disease, etc. [Cassell *et al*, 1994b; Taylor-Robinson, 1996]. Thus, simultaneous testing of several species-specific or multiplex PCRs to determine all possible pathogenic mycoplasmas associated with a particular clinical entity would be very complicated. Combination of PCR amplification of a given highly conserved target genome sequence with determination of its nucleotide sequences and phylogenetic analysis has been successfully applied for diagnosis and identification of mycoplasmal etiologies in male urethritis cases [Hashimoto *et al*., 2006; Yoshida *et al*., 2002].

Due to their fastidious growth conditions and frequent cross-reactive antigenic profile, identification and typing of human mycoplasmas is a very difficult task. Other approaches termed "Random Amplified Polymorphic DNA" (RAPD) or "Arbitrarily Primed PCR" (AP-PCR), and "Amplified-Fragment Length Polimorphism" (AFLP), are PCR-based typing methods used for intra- and inter-species differentiation of mycoplasma isolates. The RAPD / AP-PCR method involves PCR amplification with a single arbitrary primer at low stringency, while AFLP method selectively amplifies restriction fragments from whole genome. These PCR-based genotyping techniques have allowed faster and reproducible typing of mycoplasmas for epidemiologic studies [Cousin-Allery *et al*., 2000; Geary & Forsyth, 1996; Grattard *et al*., 1995; Iverson-Cabral, *et al*., 2006; Kokotovic *et al*., 1999; Rawadi, 1998; Schwartz *et al*., 2009].

8. Conclusion

In today's clinical microbiology laboratory, introduction of PCR and other NATs has the potential to increase the speed and accuracy of bacterial detection/identification, especially of those fastidious microorganisms such as mycoplasmas. However, those molecular assays still have serious drawbacks that arise from inadequate acquisition, handling and processing of representative clinical specimens. False negative results ultimately can have a significant impact on patient management.

It is widely accepted that molecular methods are more sensitive and specific than culture- and serology-based diagnostic approaches but, what does a ''positive'' test result mean clinically?. This issue is a matter of controversy for genital mycoplasmas since the duality of their relationship with their host: Is it a commensal or is it a pathogen?. The answer depends of an integral clinical evaluation of patients, where a "signs and symptoms"-focused sampling will improve laboratory diagnosis.

In the clinical setting, when negative results after mycoplasma-specific PCR assays are reported, the type and quality of the specimen, history of antibiotic treatment of the patient, and how representative was the specimen used for the assay, should be taken into account.

Therefore, any set of diagnostic results must be reviewed and critically interpreted before diagnosis and intervention measures are made.

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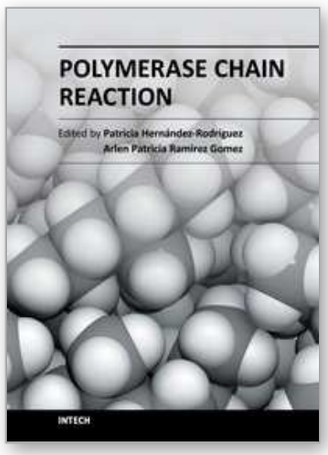

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