

Mycoplasma In-Process and Lot Release Testing: To PCR or Not to PCR

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Mycoplasma are the simplest self-replicating prokaryotes (1, 2). They infect a wide variety of eukaryotic hosts including humans, animals (birds, reptiles, fish, mammals), insects, plants, and bovine rumen. They are the smallest free-living prokaryotes, varying in size from 0.3 to 0.8 μm with a genome size of 580–2220 kb. They are contained by only a cell membrane without a rigid cell wall or peptidoglycan. Therefore, they are resistant to penicillin and can squeeze through 0.2- μm and 0.45- μm membranes routinely used for sterile filtration. They are frequent contaminants of cell cultures. Historically, about 15% of US cultures screened have been contaminated by mycoplasma. The contamination rate can be even higher in products from some other countries.

Biologic products prepared using cell culture substrates are expected to

be free of mycoplasma to assure safety, purity, potency, and consistency. Mycoplasma infection can affect nearly every cell culture parameter and result in decreased quantity or quality of product, inconsistency of manufacture, or possible adverse effects in recipients. Detection of mycoplasma is challenging because of their small size, limited turbidity produced in culture, no readily observable changes in growth or metabolic parameters with infection, requirements for enriched culture media or cell substrates for growth, and the wide diversity of mycoplasma species.

Current testing requirements include US FDA codified regulations (21 CFR 610.30) (3), which apply to virus vaccines produced in cell cultures; however, some “noncultivable” organisms may not be detected with methods described in those requirements. The current industry standard for testing biologic products produced using cell substrates is described in Attachment 2 to the 1993 *Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals* (4). This testing procedure applies to all biologics produced in cell substrates and includes a DNA staining procedure using indicator cell cultures to detect noncultivable strains, in addition to broth and agar culture methods.

The culture methods of mycoplasma detection for cell bank and raw material release, in-process,



JALEEN GROVE (WWW.ISTOCKPHOTO.COM)

and lot release testing, recommended in the documents mentioned above, have a long turnaround time (minimum 28 days). For most biological products, the mycoplasma culture test is the rate-limiting step for lot release. Alternative methods with shorter turnaround times, such as polymerase-chain-reaction (PCR)–based assays, have been developed recently. PCR methods are accepted for use as lot-release tests for biologics with extremely short shelf lives (shorter than the culture method turnaround time). But extending the use of PCR-based mycoplasma assays to other products raises concerns: namely, that PCR primers may not detect all possible mycoplasma species or that the methods may not be sufficiently sensitive.

ALTERNATIVE TESTING METHODS

The fourth Well-Characterized Biotechnology Pharmaceutical (WCBP) Chemistry, Manufacturing and Control (CMC) Strategy Forum was held on 19 September 2003 in

PRODUCT FOCUS: CELL-SUBSTRATE–DERIVED PRODUCTS

PROCESS FOCUS: PRODUCTION

WHO SHOULD READ: MYCOPLASMA EXPERTS, QUALITY CONTROL, ANALYTICAL METHODS DEVELOPMENT, ASSAY DEVELOPMENT

KEYWORDS: MYCOPLASMA, POLYMERASE CHAIN REACTION (PCR), CHARACTERIZATION, RAPID MICROBIAL TESTING

LEVEL: INTERMEDIATE

The fourth Well-Characterized Biotechnology Pharmaceutical (WCBP) Chemistry, Manufacturing and Control (CMC) Strategy Forum was held on 19 September 2003 at the Philadelphia Marriott in West Conshohocken, PA. The event was sponsored by the California Separation Science Society (CaSSS; www.casss.org) as part of an ongoing series of discussions between industry and regulatory participants exploring current practices in analytical and bioprocess technologies for development and communication of consensus concepts. The topic of this forum was "Mycoplasma In-Process and Lot Release Test: To PCR or Not To PCR."

The objectives of this forum were to review a wide variety of rapid mycoplasma detection technologies; discuss the advantages and limitations of each technology as a potential replacement for the culture methods; and summarize and share information regarding mycoplasma species that have been identified during biologics manufacturing. The goal was to facilitate corporative efforts among industry players and jointly develop a plan with regulatory agencies to resolve concerns for potential limitations of alternative rapid mycoplasma testing methods.

The first part of the forum consisted of overview comments from FDA senior mycoplasma and NAT

(nucleic acid technology) experts regarding current regulatory guidelines for mycoplasma testing of biological products and considerations for alternative rapid methods, as well as presentations by speakers from ATCC, BioReliance, Cambrex Bio Sciences, the FDA, Genentech, GlaxoSmithKline, and Pall Corporation on alternative technologies for rapid, sensitive, and reliable mycoplasma detection (see the "Participants" box). The attendees then discussed the following topics in detail and developed consensus for the remainder of the forum.

The co-chairs were William Egan (CBER, FDA) and Yuan Xu (GlaxoSmithKline). The members of the permanent CMC advisory committee are Siddharth Advant (Diosynth Biotechnology), John Dougherty (Eli Lilly and Company), Rohin Mhatre (Biogen Idec Inc.), Anthony Mire-Sluis (Amgen, Inc.), Wassim Nashabeh (Genentech, Inc.), Nadine Ritter (Biologics Consulting Group, LLC), Mark Schenerman (MedImmune, Inc.), Heather Simmerman (Amgen, Inc.), and Keith Webber (CDER, FDA).

More than 60 people (mostly mycoplasma experts) attended, representing large and small biopharmaceutical companies, contract service laboratories, government agencies, industry consultants, and academic organizations.

West Conshohocken, PA to develop a plan to address concerns for using PCR-based methods (or other alternate methods) as mycoplasma in-process and lot release tests (see the "Proceedings" box).

When to Use Alternative Methods, and What Sensitivity Is Needed: All biologic products manufactured using cell substrates (e.g., viral vaccines, monoclonal antibodies, immunological modulators, interferon and other cytokines, erythropoietin, and growth factors) must be tested to ensure absence of mycoplasma contamination. Rapid alternative methods are highly desirable wherever culture methods are currently used, which includes but is not limited to the following:

- Raw material release
- Cell-bank release (e.g., master cell bank, working cell bank, and seed bank)
- In-process testing
- Lot-release testing.

The sensitivity for rapid alternative methods should be equivalent to or better than the current culture methods. The detection limit for

current culture methods is 10 cfu/mL and 4 cfu/mL per FDA guideline and *European Pharmacopoeia*, respectively (cfu = colony-forming units).

GENERALITY OF TEST METHODS

Culture methods and all types of rapid alternative methods presented at the forum use different mechanisms for mycoplasma detection and quantification. Table 1 summarizes some advantages and limitations of each method discussed.

What Culture Methods Miss:

Culture methods are currently the gold standards for industry. The tests recommend using a large volume of test samples (e.g., 10 mL), which significantly increases assay sensitivity (with limit of detection, LOD, of 4–10 cfu/mL). However, a culture test requires at least 28 days for completion. Nuclear debris from indicator cells have led to false positive results in the indicator cell culture procedure. Also, culture methods can cultivate most mycoplasma identified as frequent contaminants of cell culture, but there is no assurance

that such methods can support the growth of every unknown mycoplasma species.

What Rapid Alternative Methods

Miss: All alternative methods presented by forum participants aimed at improving the turnaround time for mycoplasma testing. The majority used PCR technology. The 16s and 23s ribosomal RNA (rRNA) coding regions are highly conserved among all known mycoplasma species (1). Those two regions are separated by a short spacer sequence that varies both in length and sequence for different mycoplasma species. Therefore, almost all PCR-based mycoplasma methods use forward and reverse primers in the 16s and 23s rRNA coding regions, respectively.

The nested-PCR method presented by Deborah Polayes of ATCC (www.atcc.org) uses a two-stage PCR procedure. The first-stage reaction amplifies a region spanning the 3' end of the 16s RNA conserved coding region, the spacer sequence, and the 5' end of the 23s RNA conserved coding region. The second-stage PCR

reaction further amplifies the PCR product from the first-stage reaction, therefore significantly increasing assay sensitivity.

Degenerate primers are used for both stages of reactions to ensure coverage of all known mycoplasma sequences. The final PCR products are analyzed by agarose gel electrophoresis. That method has a much shorter turnaround time (one to two days) than do culture methods. The length of those final PCR products, as visualized on the agarose gel, rapidly indicates the identity of contaminating mycoplasma species because each species has its own defined length of spacer sequence.

Unfortunately, the sample volume for this method, as with all other PCR-based methods, is dramatically smaller (usually 5–10 μL /PCR reaction) than for culture methods (10 mL). As a result, the detection limit is about 100–1000 cfu/mL; this does not meet current FDA and *European Pharmacopoeia* requirements despite the enhancement provided by two rounds of PCR amplification. Another perceived limitation for this method, as with all other PCR-based methods, is a theoretical concern that the degenerate PCR primers may not detect all possible mycoplasma species with unknown sequences.

The quantitative PCR method (qPCR) presented by Shing Mai of GlaxoSmithKline (GSK, www.gsk-us.com) took advantage of real-time quantitative PCR technology (TaqMan) developed by PE Applied BioSystems (Foster City, CA, www.appliedbiosystems.com), which uses the 5' nuclease assay (5–7). Briefly, a specific probe is designed to anneal to the target nucleic acid sequence between the two PCR primers. The probe is labeled with a fluorescent reporter dye at its 5' end and a quencher at its 3' end. When the probe is intact, the proximity of that reporter dye to the quencher

Table 1: Advantages and limitations of various mycoplasma detection and quantification methods

Method	Detection Mechanism	Detection Limit	Advantages	Limitations
Culture (broth or agar)	Mycoplasma replication in growth media	4–10 cfu/mL	<ul style="list-style-type: none"> • Sensitivity • All cultivable species with known and unknown sequences 	<ul style="list-style-type: none"> • Long turn around time (>28 days) • Noncultivable species
Indicator cell culture procedure	Mycoplasma replication in cell cultures	<100 cfu/mL	<ul style="list-style-type: none"> • Detects noncultivable strains • Relatively short turn-around time (three to five days) 	<ul style="list-style-type: none"> • Positive or negative evaluation is subjective • Possible false positives due to indicator cell debris • Possible interference when viral samples induce a cytopathic effect (CPE)
Nested PCR	PCR amplification of conserved sequences	1–5 copies/PCR reaction (~100–1000 cfu/mL)	<ul style="list-style-type: none"> • Short turnaround time (one to two days) • All species with known sequences including the noncultivable species 	<ul style="list-style-type: none"> • Sensitivity not as good as culture methods • Limited to mycoplasma species with known sequences • Possible false positives from contaminating DNA and may detect nonviable/fragmented mycoplasma DNA
Infectivity qPCR	Mycoplasma replication in growth media followed by quantitative PCR amplification of conserved sequences	1–4 cfu/mL	<ul style="list-style-type: none"> • Sensitivity • Short turnaround time (~seven days) 	<ul style="list-style-type: none"> • Limited to mycoplasma species with known sequences • Possible false positives from contaminating DNA and may detect nonviable/fragmented DNA
Hot-start and touchdown-style PCR	PCR amplification of conserved sequences	10 copies/PCR reaction (~1 cfu/mL)	<ul style="list-style-type: none"> • Sensitivity • Short turnaround time (~eight hours) 	<ul style="list-style-type: none"> • Limited to mycoplasma species with known sequences • Possible false positives from contaminating DNA and may detect nonviable/fragmented DNA
PCR-microarray combination	PCR amplification of conserved sequences followed by microarray (hybridization) identification	Not established yet	<ul style="list-style-type: none"> • Short turnaround time (one to two days) • Species identification 	<ul style="list-style-type: none"> • Limited to mycoplasma species with known sequences • Possible false positives from contaminating DNA and may detect nonviable/fragmented DNA
Hybridization protection	Hybridization protection and chemiluminescent labeling	Not established yet	<ul style="list-style-type: none"> • Short turnaround time (hours) • Simple operation 	<ul style="list-style-type: none"> • Limited to mycoplasma species with known sequences • Possible false positives from contaminating DNA and may detect nonviable/fragmented DNA
MycoAlert (Cambrex BioScience)	Enzymic activity found in mycoplasma and other members of the Mollicutes family	~50 cfu/mL	<ul style="list-style-type: none"> • Short turnaround time (hours) • Simple operation • All species with known and unknown sequences, cultivable and noncultivable 	<ul style="list-style-type: none"> • Sensitivity not as good as culture methods • False positive results caused by bacterial contamination

suppresses the reporter fluorescence. During the extension phase of each PCR cycle, the 5' to 3' exonuclease activity of the AmpliTaq Gold DNA polymerase (PE Applied BioSystems) cleaves the probe. That cleavage releases the reporter dye from the probe, increasing the reporter dye fluorescence. The company's PRISM sequence detection system automatically monitors that fluorescence increase during each cycle of PCR throughout the entire amplification procedure.

The exonuclease activity of the AmpliTaq Gold DNA polymerase acts only if a probe hybridizes to the target; it does not cleave free probes in solution. As a result, the increase of reporter dye fluorescence is directly proportional to the amount of PCR product accumulated, which in turn is directly proportional to the amount of target DNA or RNA present in a test sample at the outset. Fluorescence-based detection significantly enhances the assay sensitivity to ~1 copy DNA/PCR reaction. Degenerate primers or probes are used to ensure coverage of all known mycoplasma sequences. But again, due to the much smaller sample size (usually 10 µL/PCR reaction) than that used with culture methods (10 mL), the detection limit for the TaqMan-based qPCR method is ~100 cfu/mL — not meeting current FDA and *European Pharmacopoeia* requirements.

To further enhance assay sensitivity, GSK modified that test procedure by incorporating a culture step for the test samples before qPCR quantification. The modified procedure, which is called the infectivity qPCR method, detected even the relatively slow-growing *M. pneumoniae*, with a detection limit of 1–4 cfu/mL after a five-day culture incubation period before qPCR quantification. This method significantly reduces the turnaround time to about one week, with a sensitivity equivalent to or better than that of culture methods. Therefore, the only limitation for this method is the theoretical concern that degenerate PCR primers/probes may not detect all possible mycoplasma species with unknown sequences.

Touchdown Single-Step PCR: A method presented by Barbara Potts of Genentech, Inc., applies readily available techniques in DNA extraction together with a modified single-step PCR. The method uses a previously characterized primer pair that is homologous to a broad spectrum of mycoplasma species known to infect mammalian cultures (8). Analysis is made easy by the detection of only a single amplification product within a narrow size range 438–470 bp (base pairs). A high sensitivity and specificity for mycoplasma detection is made possible through the combination of three key techniques: 8-methoxypsoralen and UV light treatment to decontaminate PCR reagents; hot-start Taq DNA polymerase to reduce nonspecific priming events; and touchdown PCR to increase sensitivity while reducing nonspecific priming events. The limit of detection for eight mycoplasma species (*M. orale*, *M. hyorhinis*, *A. laidlawii*, *M. salivarium*, *M. agnirini*, *M. fermentans*, *M. hominis*, and *M. pneumoniae*) is 10 genomic copies per PCR reaction. In CHO cell production, the limit of detection for a model organism was 1 cfu/mL. Cell densities were 5×10^6 cell/mL with 0.450 mL used for genomic DNA extraction. This assay was designed to be used as a “hold” step test before cell culture fluid is further processed; thus, it can be run easily within eight hours. However, this method carries a theoretical concern that the PCR primer pair may not detect all possible mycoplasma species with unknown sequences.

A PCR-microarray combination method presented by Konstantin Chumakov of the FDA could enable both detection and identification of contaminating mycoplasma species with a very short turnaround time (one to two days). Degenerate PCR primers could be used to ensure coverage of all known mycoplasma species. PCR conditions may be optimized for most efficient amplification without concern about nonspecific amplification because the PCR products would then be analyzed

by microarray technology. In that step, hybridization stringency would be optimized to help identify contaminating mycoplasma species and screen out the nonspecific amplification products.

However, this method was still in the early development stage at the time of the forum. Therefore, no specific information regarding detection limit and head-to-head comparison with the culture methods was provided.

A hybridization protection method presented by Miguelina Mathews of Pall Corporation (www.pall.com) revolves around hybridization of all-bacterial DNA probes to mycoplasma rRNA in the test samples. The “all-bacterial probes” used ensures detection of *Mycoplasma* and *Acholeplasma* species that commonly infect tissue culture cells. The probes are linked with a chemiluminescent label. Following a hybridization step, the chemiluminescent label is cleaved from free probes that are not hybridized to the target mycoplasma rRNA. The amount of probe that hybridizes to the rRNA is then quantified by chemiluminescent detection that is directly proportional to the rRNA quantity in the test samples.

The easy-to-use Gen-Probe Leader 50 Luminometer (www.gen-probe.com) is designed to perform such a hybridization protection test and record data for use in regulated environments in approximately 75 minutes. However, no data were shown at the time of the forum regarding the detection limit, and no side-by-side comparison to the culture method was offered. In addition, this method still carries the theoretical concern that the “all-bacterial probes” used may not detect all possible mycoplasma species with unknown sequences.

The MycoAlert method presented by Kevin Slater of Cambrex Bio Science (www.cambrex.com) is a bioluminescent assay that detects mycoplasma-specific metabolism. The assay uses enzymatic activity found in mycoplasma and other members of the mollicutes family. The enzymes are

associated with energy generation pathways that result in ATP synthesis and are not expressed in mammalian cells. ATP from mycoplasma-specific metabolism is then measured by the bioluminescent reaction using the luciferase enzyme.

This simple test requires adding two reagents to culture supernatant, and it takes only 15 minutes to complete. The limitation is its detection limit, which at about 50 cfu/mL does not meet current FDA and *European Pharmacopoeia* requirements. Another theoretical concern is that false positive results from ATP could be generated by bacterial contamination or by ATP leakage from host cells being tested.

ASSESSING FALSE RESULTS

Regulatory agencies and the regulated industry are concerned about false negative results for the safety of public health and false positive results for product availability. A false positive result in CGMP testing would require an OOS (out of specification) investigation that could significantly delay lot release and pose a compliance risk during an audit or inspection. Such compliance and business risks have often delayed industry acceptance of using more sensitive methods for adventitious agents as routine lot release tests.

Forum participants were asked whether they had seen alternative methods fail when culture methods had tested positive, or vice versa — when those alternative methods had sensitivity equivalent to or better than the culture methods. Participants identified no such examples from their work in large and small biopharmaceutical companies, contract service laboratories, government agencies, industry consultants, and academic organizations. That was partly because controls were included and validation was performed by the industry to minimize false positive and false negative results when considering alternative methods. Another factor was that none of the alternative methods presented had been officially approved by regulatory agencies as replacements for culture

methods or implemented by the industry for routine testing in parallel with the culture methods. Therefore, the industry simply had not gained enough experience with the alternative methods for CGMP testing.

CONTAMINATION IN PRODUCTION

The participants did not provide specific case information on mycoplasma contamination detected during clinical and commercial production, even though all participants knew that such contamination does happen at different stages during clinical and commercial production.

So far, about 200 mycoplasma species have been identified. A literature search identified the following 10 species as the most common mycoplasma contaminants of cell cultures, counting for >95% of contaminations: *M. arginini*, *M. hominis*, *M. orale*, *M. salivarium*, *M. hyorhinae*, *M. fermentans*, *M. pirum*, *A. laidlawii*, *M. pneumoniae* and *M. arthritidis* (9–12).

VALIDATING ALTERNATIVE METHODS

It was agreed that validating alternative methods should, in general, follow ICH guidelines Q2A (13) and Q2B (14).

Defining the Detection Limit: For PCR- or NAT-based (nucleic acid amplification testing) alternative methods, it was proposed that one copy of mycoplasma DNA detected be equivalent to one cfu by the culture methods. This is a highly conservative proposal because each mycoplasma genome may have multiple copies of the target DNA sequence; and not all detected target DNA sequences can eventually lead to cfu formation. Some may stem from dead and/or noninfectious mycoplasma particles and some may be in the form of fragmented and/or free DNA in test samples.

Limit Test Versus Quantitative Test:

It was proposed that when rapid alternative methods are used, the outcomes or results of mycoplasma tests should be either positive or negative; that is, the acceptance criterion should be absence of

mycoplasma. Therefore, for quality control purposes, detection of mycoplasma would trigger lot rejection. A mycoplasma lot-release test should be performed at the stage where contamination is most likely to occur and/or be detected (e.g., the bulk fermentation harvest). Quantitative tests may be valuable for method validation. The participants proposed to follow validation requirements for limit tests and quantitative tests as outlined in ICH guidelines Q2A and Q2B.

Interference with Alternative

Methods: Validation studies for alternative methods and controls for the tests should include evaluation for potential interference by the following factors:

- Host substances (such as host cell DNA or host-cell proteins)
- Process-related small molecules (including buffers, reagents, leachables, and antibiotics)
- Product.

Identity and Number of

Mycoplasma Species Used for

Validation: Because each alternative method presented at the forum uses a different detection mechanism, it was proposed that the mycoplasma species for validation be chosen case by case. At a minimum, a species used for validation should include the ten or so species that comprise 95% of contaminants in cell culture. Species identified at a company's production facility also should be included in that company's validation studies.

The general guiding principles for several PCR-based methods were discussed in more detail. For PCR-based alternative methods that use degenerate primers and/or probes, a minimum of one mycoplasma species should be used for each specific set of primers and/or probes for which that mycoplasma species contains the target sequence to be amplified by that set of primers and/or probes. For example, the TaqMan qPCR method presented by GSK uses two sets of PCR primers, each coupled with three different probes to ensure detection of all known mycoplasma species. As a result, the primers-and-probes combination represents six different

target sequences. In that case, it was recommended to include a minimum of six mycoplasma species for validation, each containing one of those six different target sequences. That is mainly to ensure that the PCR conditions used can meet the claimed detection and/or quantification limit for all target sequences.

For the infectivity qPCR developed by GSK, it was recommended that the culture procedure follow the validation and control outlined in Attachment 2 to the 1993 Points to Consider document (4). To validate the length of the culture incubation period needed for the claimed detection and/or quantification limit, at least one of the known slower growing mycoplasma species should be used, and it should be applicable to the product type and production process (e.g., using *M. pneumoniae* for MABs produced using mammalian cell cultures).

Comparison to Current Culture Methods: Participants agreed that validation packages for alternative methods should include head-to-head comparisons with current culture methods. Because each alternative method presented at the forum uses a different detection mechanism, it was proposed that such side-by-side comparison validation studies be designed case by case based on the scientific principle of each method and its specific application. In general, the sensitivity of rapid alternative methods should be equivalent to or better than that of current culture methods. Some trade-offs should be acceptable for some products, including cell-therapy products and radioisotope-labeled products with very short shelf lives; and viral seeds and production harvests for which the indicator cell procedure cannot be performed due to the cytopathic effect (CPE) of the virus. This should be handled case-by-case and should be evaluated for each specific product using a risk-assessment-based approach.

IMPLEMENTING ALTERNATIVE METHODS

As was emphasized repeatedly at the forum, each method has advantages

and limitations. No methods are perfect, including the current gold-standard culture methods. For example, although PCR- and NAT-based alternative methods face the theoretical concern that degenerate primers and/or probes may not detect all possible mycoplasma species with unknown sequences, there is no assurance that the conditions used by current culture methods can support growth of every possible unknown mycoplasma species. Therefore, it was proposed to implement alternative methods as replacements for culture methods whenever an alternative method demonstrates comparable or superior mycoplasma detection and/or quantification capability. Sponsors are encouraged to discuss with regulatory agencies before such implementation to ensure that completeness of the validation package and head-to-head comparison studies with current culture methods are considered acceptable and soundly justified scientifically.

To ease the transition, it was also proposed that an alternative method and a culture method be performed in parallel for a short time in a CGMP environment for clinical and commercial production. That would help mitigate theoretical concerns over perceived limitations of an alternative method with real manufacturing performance data, especially for those companies with relatively high mycoplasma contamination rates.

The PCR-based and most NAT-based alternative methods detect the presence of mycoplasma DNA/RNA in test samples. Because detection of mycoplasma DNA does not always indicate the presence of viable mycoplasma, participants proposed verifying positive results from alternative testing by culture methods to distinguish free, noninfectious, and/or fragmented mycoplasma DNA or RNA from real mycoplasma contamination.

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