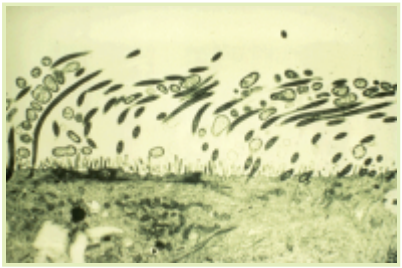
IVT NETWORK
INSTITUTE OF VALIDATION TECHNOLOGY | an informa business
Unlimited Validation Knowledge Awaits...
Published on *IVT Network* (http://www.ivtnetwork.com)

Mycoplasma And Cell Therapy Risks

By **Tim Sandle**

Feb 24, 2022 8:00 am EST



INTRODUCTION

Mycoplasmas are undesirable, common contaminants, and difficult to detect risk factors for human and animal cell lines. Mycoplasmas can alter infected cells at the molecular level and trigger visible changes in cell morphology and growth characteristics. Cell lines are of great importance of biopharmaceutical manufacturing and research, and they are required for many of the new generation of Advanced Therapy Medicinal Products (ATMPs). One reason why ATMPs are attracting such interest is because where no treatment or no cure was available for a disease ATMPs may open new doors to enable the curing of a disease by replacing diseased cells or tissues or correcting diseased genes; by treating disease by adding cells or genetic material; or by removing defective cells by programming the body to attack them.

A recurrent of concern with the maintenance of viable cell cultures is contamination with mycoplasmas (1) and mycoplasma contaminations are difficult to detect and undesirable in ATMP, especially for immunosuppressed patients. Rates of contamination vary, and these are control dependent, although a contaminated eukaryotic cell line can contain 10^6-10^8 organisms/mL (2). The effect of mycoplasma contamination within a cell culture is to alter cell physiology, produce erroneous results and lead to the loss of unique cell lines. This review paper looks at mycoplasmas, the importance of cell lines (with special focus on ATMPs), and methods to eradicate and detect them.

MYCOPLASMAS

Mycoplasma is both a reference to a genus and a generic term used to refer to any of the members of the class *Mollicutes*, which includes the*Mycoplasmataceae*:*Mycoplasma, Acholeplasma, Anaeroplasma,, Asteroleplasma* and*Ureaplasma* genera (the latter can hydrolyses urea for generation of ATP) (3) The ‘Mycoplasma’ is a genus of bacteria defined by a morphological characteristic: The lack a rigid cell wall around their cell membranes (some microbiologists describe mycoplasmas as having the absence of a cell wall. This connects with the word "*Mollicutes*", which is derived from the Latin*mollis* (meaning "soft" or "pliable"), and*cutis* (meaning "skin" (4). Mycoplasmas are distinct from L-form bacteria (which also lack cell walls), on the basis that L-form bacteria (the ‘L’ being named after Lister). These organisms can acquire this characteristic under certain conditions (that is they are specific strains of bacteria that normally have a cell wall) (5); whereas mycoplasma naturally do not have a cell wall. Mycoplasmas have this characteristic because they lack peptidoglycan. Instead, they supplement their membranes with sterols for structural stability.

The organisms were first reported in 1898 (described as “pleuropneumonia-like organisms”) and they were initially considered to be viruses before being identified as the smallest free-living organisms (6). The first isolation from a human was in 1937 (7) (with the role of*Mycoplasma pneumoniae* being associated with pneumonia in humans established in 1962) (8) and the first method of cultivation was developed in 1951 (9). Mycoplasmas are parasites exhibiting strict host and tissue specificities (specific mycoplasmas enter a target host in which they multiply and survive for long periods of time) (10).

Mycoplasma characteristics (11):

- Lack of rigid cell wall.
- Flexible cell membranes.
- Extremely small genome (0.58–2.20 Mb compared with the 4.64 Mb of*Escherichia coli*).
- Generation times of 1–9 hours (extended lag-phase).
- Smallest self-replicating bacteria: 0.3–0.8 µm.
- Filterable through 0.45 µm filter (and potentially through a 0.22 µm rated filter).

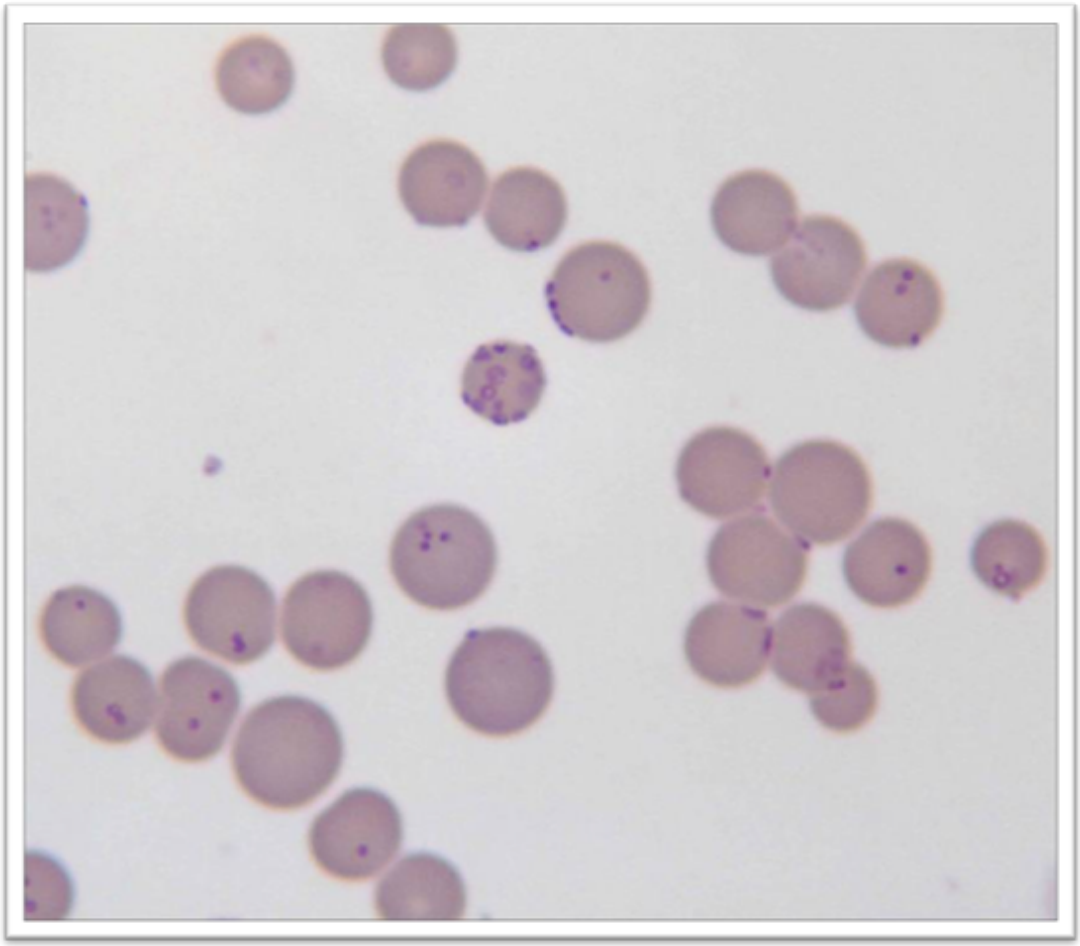


Image: Mycoplasma haemofelis, a causative agent of feline infectious anemia. By Nr387241 (CC BY-SA 3.0: https://commons.wikimedia.org/w/index.php?curid=14945255)

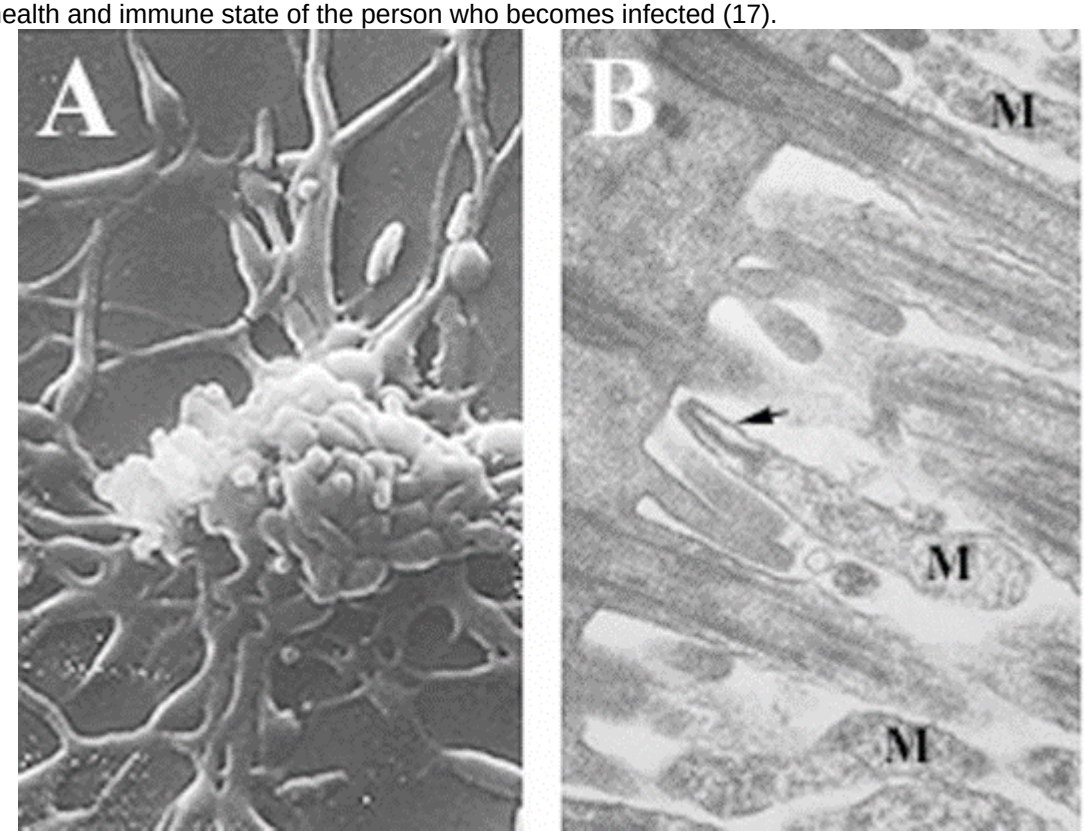
The characteristic of an absence of a cell wall is what makes the organisms resistant to many antibiotics (since a large number of antibiotics target the bacterial target cell, such as beta-lactam antibiotics); it may also help facilitate contact between mycoplasma and their host cell. The characteristic further enables the organisms to change their cell shape (exhibiting pleomorphism), with the typical shape being ‘funnel-like’ (12). It also confers a ‘simplicity’ in that mycoplasmas contain the absolute minimum machinery necessary for survival. The cell is built of a minimum set of organelles including a plasma membrane, ribosomes, and a highly coiled circular chromosome.

With cell cultures, most mycoplasmas present a risk by remaining attached to the surface of epithelial cells, although some mycoplasmas have evolved mechanisms for entering host cells. This is well characterized using ultrastructural studies drawing upon immunochemistry and electron microscopy. Since mycoplasmas are parasitic, this becomes a factor of their limitation is being able to perform many functions and they are invariably dependent upon their host for nutrients. The bacteria are generally found in association with the tissues and cells of humans, plants, animals, and insects (either residing on outer surfaces or as intracellular parasites). Mycoplasmas tend to grow very slowly, with a prolonged generation time and a very long lag phase (this means it typically takes one week for colonies to be visible on agar plates).

In terms of human pathogens, some species of mycoplasma can selectively infect the lungs (respiratory), skin, or urinary tract (urogenital epithelium) (13). While there are over 200 different species of mycoplasma and there are eighteen that have been detected in humans (as of 2021) (14), most are commensal, and few are pathogenic.

The ones of most concern to humans are:*Mycoplasma pneumoniae, Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma urealyticum* and*Ureaplasma parvum* (15).

In the clinical setting, concern with mycoplasmas is increasing due to increased accounts of antimicrobial resistance in tandem with reports indicating rising numbers of infections. An example is with*Mycoplasma pneumoniae* infections.*M. pneumoniae* is an important cause of respiratory tract infections in children and adults (16). During epidemics, based on U.S. data, the organism causes about 20 to 40 percent of community-acquired pneumonias for the general population, and up to 70 percent within relatively closed communities such as colleges, military barracks, and nursing homes. While infection is generally mild, the clinical outcome depends upon the health and immune state of the person who becomes infected (17).



Images: A) Filamentous Mycoplasma pneumoniae cells B) M. pneumoniae cells (M) attached to ciliated mucosal cells by the attachment organelle (indicated by arrow). By Rottem, S., S., N., and D., J. (2012) in Biomedical Tissue Culture (Ceccherini-Nelli, L., ed.), InTech [online] http://www.intechopen.com/books/biomedical-tissue-culture/contamination-of-tissue-cultures-by-mycoplasmas CC BY 3.0, https://commons.wikimedia.org/w/index.php?curid=29989585

An additional concern in relation to these infections is where *M. pneumoniae* pathogens have shown resistance to the macrolide antibiotics, such as erythromycin and azithromycin (18). These antimicrobials are typically used to treat patients with respiratory infections (19). While patterns of resistance vary, within Europe a recommendation has been made in 2021 (in the journal *Infectious Disease* Reports). This is to establish a coordinated and structured pan-European surveillance program adequate for public health surveillance, in order to tract geographical patterns of antimicrobial resistance. The main methods of acquired resistance are via chromosomal mutations from mobile genetic elements carrying a gene called tet(M) and through efflux pumps, which provide a means to drive the antimicrobial out of the cell. Other mycoplasmas also present patterns of concern. For example, as per a *British Medical Journal* report infection and antimicrobial resistance patterns of *Mycoplasma genitalium* among pregnant women in the southwestern U.S. are increasing. The rates of the resistant organisms among young black women are the highest.

An additional challenge presented by many mycoplasmata based on the mechanism by which the pathogens make DNA, which potentially provides a means to may protect some species from the human body's immune response. Each time bacteria divide they copy their entire DNA, with the nucleic material providing the blueprint for the resultant cell. For this function, large amounts of the four different DNA building blocks need to be produced. The enzyme that makes the building blocks is RNR (ribonucleotide reductase). For most bacteria, RNR requires metal ions for function. This has led clinical microbiologists to determine that the body's immune system uses a mechanism to attempt to starve bacteria of the required metals. However, researchers at Stockholm University discovered a family of RNR proteins in some species of*Mycoplasma* that does not need metals (20).
These recent trends suggest that research into mycoplasma infections and resistant rates should be elevated higher in many national medical community research programs. While these developments are of medical importance, the central focus of this review paper is with contamination of cells and the impact upon cell therapy products.

CELL THERAPY PRODUCTS

Human cell-based medicinal products are heterogeneous with regard to the origin and type of the cells and to the complexity of the product. Cells may be self-renewing stem cells, more committed progenitor cells or terminally differentiated cells exerting a specific defined physiological function. Cells may be of autologous (originated from the same patient) or allogeneic (from a person other than the patient) origin. In addition, the cells may also be genetically modified (21). While cells may be used alone, associated with biomolecules or other chemical substances what is of relevance to this chapter are where cells are combined with structural materials that alone might be classified as medical devices (that is combined advanced therapy medicinal products).
The products produced fall under the general cover-all of Advanced Therapy Medicinal Products (ATMPs) (22). These are gene therapy medicinal products, somatic cell therapy medicinal products and tissue engineered products. These therapies offer ground-breaking new opportunities for the treatment of diseases and injuries. They are particularly important for severe, untreatable, or chronic diseases for which conventional approaches have proven to be inadequate. There is, in addition, a specific category for those products that consist in an ATMP combined with a medical device (combination products) (23). In terms of pharmaceuticals, they are classed as biologics. ATMPs connect with innovations in personalized medicine, precision medicine, and point of care (POC) medicines and devices manufacture.
An example includes ATMPs designed to address a condition where a single gene mutation in the IL2RG gene, results in no functioning immune system leading to the so-termed "Boy in bubble syndrome" (or Severe Combined Immunodeficiency). Children with this condition lack the ability to produce an immune system and gene therapy approaches are being developed to help to extend the life expectancy of those affected. A further example is with the approval of Zolgensma (onasemnogene abeparvovec), which is an adeno-associated virus gene mediated therapy for spinal muscular atrophy. Zolgensma is a medicine intended for patients with inherited mutations affecting genes known as SMN1, who have either been diagnosed with SMA type 1 (the most severe type) or have up to three copies of another gene known as SMN2. A third example is with a novel gene therapy for hemophilia, a therapy that leads to sustained expression of clotting factor and reduced bleeding events. As of November 2021, there are more than 3000 ongoing clinical trials related to gene therapy (24).

GENERAL APPROACH TO CONTAMINATION AND QUALITY CONTROL

It is important that cells used in the process have been assessed for viability and the integrity of the cellular component is most critical for the product and this must be assessed by the ability of cells to survive, and maintain the genotype or phenotype needed for the intended functions.
It should be noted that the detection of possible changes in cellular nature that may influence the intended function. A viable cell is a cell that has a functional cytoplasmic membrane. Viability is assessed by laboratory assay in order to demonstrate cytoplasmic membrane integrity and activity. This can be undertaken by cell staining using viability dyes or through manual or automated analysis, under a light microscope or by flow cytometry, of a cell suspension in order to determine the percentage of viable cells.
To minimize contamination in relation to the cell bank, no other living or infectious material (such as virus, cell lines or cell strains) should be handled simultaneously in the same area. Other requirements include:

- The number of generations (doublings, passages) should be consistent with specifications in the product license.
- For stages prior to the master seed or cell bank generation, documentation should be available to support traceability including issues related to components used during development with potential impact on product safety (such as reagents of biological origin) from initial sourcing and genetic development.
- Cell bank safety testing and characterization are important for batch-to-batch consistency and to prevent contamination with adventitious agents.
- Seed lots and cell banks should be stored and used in such a way as to minimize the risks of contamination (such as stored in the vapor phase of liquid nitrogen in sealed containers) or alteration.
- Control measures for the storage of different seeds/cells in the same area or equipment should prevent mix-up and take account the infectious nature of the materials to prevent cross contamination.

- Storage containers should be sealed, clearly labelled, and kept at an appropriate temperature.
- A stock inventory must be kept.
- The storage temperature should be continuously monitored, and records retained.
- Deviation from set limits and corrective and preventive action taken should be recorded.
- Evidence of the stability and recovery of seeds and banks should be documented and records should be kept in a manner permitting trend evaluation. Stability data should be available before the product is used in a clinical trial; after this, the stability data should be built-up with real-life data as the clinical trial progresses.
- Cell-based products should be generated from a cell stock obtained from a limited number of passages, in order to avoid phenotypic changes. It is recommended to split stocks and to store the split stocks at different locations so as to minimize the risks of total loss.

Some specific manufacturing considerations include:

- The procedure to obtain the cells from the organ/tissue needs to account for the type of enzyme, media, etc. and validated. Consideration should be given to the degree of disruption applied to the tissue in order to preserve the intended functional integrity of the cellular preparation and to minimize cell-derived impurities in the product (cell debris, cross contamination with other cell types).
- Procedures used to isolate and / or purify the cell population of interest should be described. The effectiveness should be addressed in relation to the intended use and the method(s) must be validated.
- With cell culture, during in vitro cell culture, consideration should be given to ensure acceptable growth and manipulation of the isolated cells. The processing steps should be properly designed to preserve the integrity and control the function of the cells. The procedures for any manipulation should be documented in detail and closely monitored according to specific process controls. The duration of cell culture and maximum number of cell passages should be clearly specified and validated. The relevant genotypic and phenotypic characteristics of the primary cell cultures, of the established cell lines and the derived cell clones should be defined and their stability with respect to culture longevity determined. When addressing the phenotype of the cells, relevant markers can be used (for example, with cellular components of allogeneic origin, identity should include histocompatibility markers). These markers may be based on gene expression, antigen presentation, biochemical activity, response to exogenous stimuli, capability to produce biologically active or otherwise measurable molecules, and so on. For adherent cells, morphological analysis may be a useful tool in conjunction with other tests. Where applicable, a description of the procedures which could lead to a modification of the characteristic of the product, including adhesion, absorption, degradation, presentation of components of the culture media, should be provided. With genotypic aspects, this may need to include the dentification of genetic polymorphisms.
- The consistency and repeatability of the cell culture process should be demonstrated and the culture conditions including the media and the duration should be optimized with respect to the intended clinical function of the cells.
- Various treatments (physical, chemical, or genetic) can be applied to cells. The method used to modify the cells needs to be fully described and scientifically justified.
- Where cells are grown directly inside or on a matrix/device/scaffold, the quality of the combined advanced therapy medicinal product relies predominantly on the properly controlled manufacturing process. To achieve this, the cell culture process has to be thoroughly validated and the effect of the device on the cell growth, function and integrity has to be considered. The effect that the cells may exert on the device (such as with understanding the rate of degradation) should also be considered.

In terms of batch specific issue:

- The number of cell passages should be defined.
- The number of cell duplications should be defined.
- Pooling strategies must be defined.
- The batch numbering system should be defined.

Special consideration should be given to open processing steps and to sampling. For instance, the addition of materials or cultures to fermenters and other vessels and sampling should be carried out under carefully controlled conditions to prevent contamination. Moreover, care should be taken to ensure that vessels are correctly connected when addition or sampling takes place. These activities should focus on the prevention of the release of viral or infected material.
In support of manufacturing, the water used in the manufacturing of ATMPs should be of appropriate quality and regular checks should be carried out to verify the absence of contamination (chemical and biological).

In-process controls are important during manufacturing, particularly in relation to in-process controls at the level of critical steps or intermediate products. Intermediate cell products are products that can be isolated during the process; specifications of these products should be established in order to assure the reproducibility of the process and the consistency of the final product. If storage occurs during manufacturing, it is important to validate the storage conditions (especially time and temperature).

With manufacturing, the use of antimicrobials may be necessary to reduce bioburden associated with the procurement of living tissues and cells. The application of antimicrobials is not permitted as a replacement the requirement for aseptic manufacturing. When antimicrobials are used, they should be removed as soon as possible, unless agreement has been reached with the appropriate regulator that the antimicrobial is permitted in the end-product. Where sterility testing is undertaken, the antibiotics or antimicrobials must be verified as not interfering with the sterility test (25).

While ATMPs are generally filled as aseptic products, ideally within isolators and verified by aseptic process simulations, there are some exceptional circumstances when the final formulation and filling is undertaken under different conditions. This is when the manufacturing of the ATMP takes place in the operating theatre and it is not possible to move the production to an outside cleanroom because the time since the donation and administration of the product is very short and the patient is also in the operating theatre waiting for administration of the ATMP), closed systems may be placed in a controlled but non-classified environment. This is on the basis of a medical assessment where the expected clinical benefit for the patient outweighs the risks linked to the absence of a classified background.

For quality control, due to the life-expectancy of the product (in that the product may need to be administered immediately after manufacture to the patient), it may not be possible to perform the release tests on the active substance or the finished product. Other reasons for different quality control release strategies include the point that it may not be possible to perform the release tests on the combined components of certain combined products or when the amount of available product is limited to the clinical dose. Where this is the case, ensuring an adequate control strategy for the testing of key intermediates (instead of the finished product) or in-process controls (instead of batch release testing) where the relevance of the results from these tests to the critical quality attributes of the finished product can be demonstrated. Important quality control examinations include identity, purity, potency, viability, and suitability, and for the end product, sterility. Covering some of these aspects is an examination of mycoplasmas.

MYCOPLASMA CONTAMINATION OF CELL CULTURES

Mycoplasma contamination of cell culture is the foremost concern, in terms of cell lines used for both research and biopharmaceutical processing (26, 27). This is not to infer that contamination with other bacteria and fungi cannot occur, but more so that these other microbial contaminants are more readily detectable (such as through visible turbidity) (28). It is of further concern that mycoplasmas can spread vastly among the cell cultures. Some, although not all, species of mycoplasma are cytopathic; however, many will cause the alteration of cellular metabolism or cellular morphology and ruination of a cell line. An additional concern arises in relation to viruses. Viruses contaminated with mycoplasma may show no observable effects or their growth may be altered (29).

The problem presented by mycoplasmas is with their ability to adhere to cells (hence adherence is the major virulence factor). Mycoplasmas have developed various genetic systems enabling their attachment to host tissues as well as a highly plastic set of variable surface proteins. It is thought that the versatility of their surface coat and size variation provides mycoplasmas with mechanisms for immune system avoidance (30).

Image: Mycoplasma hyopneumoniae attached to swine cilia. From the International Organization for Mycoplasmology (CC license, via:https://microbewiki.kenyon.edu/index.php/File:Mhyo_cilia.gif)

For bioprocessing, manufacturing steps should be taken to prevent or control contamination by viruses, bacteria, fungi, mycoplasma, and transmissible spongiform encephalopathy (TSE). The production techniques deployed need to demonstrate that they can effectively minimize any impurities inherent from the production processes as well as being able to prevent the introduction of contaminants external to the manufacturing process. Potential impurities include mycoplasmas. Suitable emphasis should be placed on minimizing the risk of contamination from the environment or cross-contamination from other products and consider the operational and design features of the purification suite, Heating Ventilation and Air Conditioning (HVAC) and other support systems, equipment, transfers of any intermediates or substances, and movement of personnel.
This leads on to a consideration of the sources of contamination. These are varied and include personnel, materials, as well as these consumables, equipment, media, and reagents used in cell culture. For example, as a result of the ease of droplet generation during handling of cell cultures and the fact that some commercial cell cultures are received contaminated. The issues are compounded by mycoplasmas being quite hardy with the ability to survive for periods in dry conditions. These sources help to drive the appropriate practices for minimizing the potential for contamination, such as good aseptic technique and controlling the sources that can generate aerosols.

Where cell cultures have been contaminated, attempts can be made to remove mycoplasmas without damaging human or animal cells. However, such processes are complex, and they are not always effective. Among the methods that can be selected are filtration and the application of ultraviolet light (31). In addition, selective antibiotics can be used (complicated by the fact that mycoplasmas are resistant to commonly used antibiotics) (32) Where the continuation cannot be addressed then a reliable decontamination step should be undertaken prior to disposing of the culture (and hence to avoid an opportunity for further contamination). Given the low-level of heat resistance, decontamination using moist heat to 121oC for 15 minutes or greater is effective for this purpose.

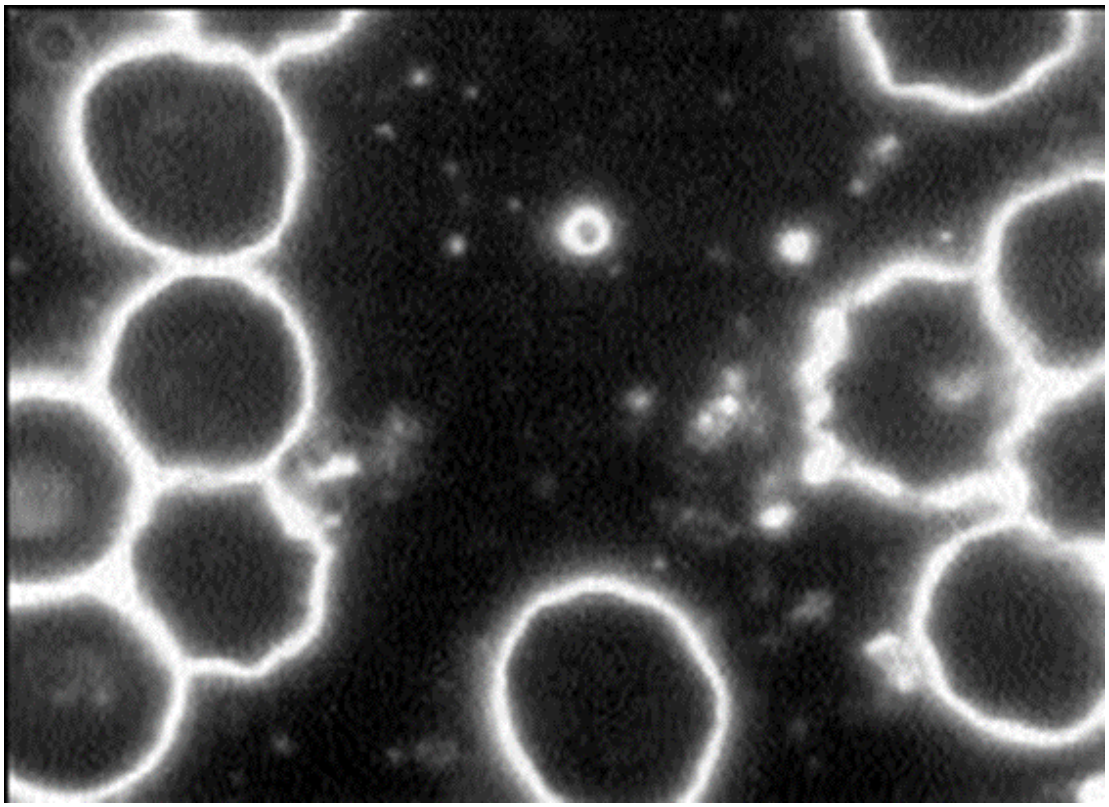


Image: Mycoplasma seen using darkfield microscopy (1000x). By Michael Coyle and Christine Baillie. (CC license via:https://microbewiki.kenyon.edu/index.php/File:Sporoidcopy.jpg)

Sterile Filtration Risks

Mycoplasmas, based on their cell size and flexibility, can penetrate 'sterilizing grade' filters, which are generally accepted to be filters that are 0.22 ?m porosity rated. This presents concern for the filtration of mammalian and bacterial cell culture media and sera and for such purposes 0.1 ?m rated filters are required for mycoplasma clearance. This requires additional testing by filter manufactures using mycoplasma cells with test conditions designed to represent the actual conditions of use (33). There are other applications where the ability of mycoplasmas to pass through sterilizing grade filters is a concern. For example, where culture media is used of media simulation trials (such as tryptone soya broth), some lots of culture media have been found to be contaminated with*Acholeplasma laidlawii* (34). Under ideal conditions, the presence of this bacterium can lead to media turbidity. The optimal approach is to ensure that the culture medium has been examined for the absence of mycoplasmas or that the medium has been subjected to irradiation.

DETECTION METHODS

The impossibility of visual detection with the naked eye and the primary methods of detection are: Culture-based, DNA staining, and PCR (35).

Culture Based Methods

For many years, the standard method was culture on supplemented agar or broth media under appropriate atmospheric conditions. The culture-based approach is, however, limited by the long growth times required and the presence of uncultivatable species (36) (such as*Mycoplasma hyorhinis*). Typical culture media consists of heart infusion, peptone, yeast extract, salts, glucose or arginine, and horse or calf serum (5 to 20 percent). To prevent the overgrowth of the other bacteria (which often accompany mycoplasmas) penicillin, thallium acetate or both are added as selective agents to inhibit the growth of other organisms (37).

As established above, mycoplasmas are reliant upon their hosts for most of their nutrients; hence, to culture mycoplasmas successfully culture media needs to be able to replicate the required nutrients and thus complex media are required to cultivate the organisms. According to Windsor and Windsor (38), the essential requirements for culture media for mycoplasmas needs to comprise of: a broth base, yeast extract, serum source, and solutions of pure chemicals (such as glucose and arginine). The authors caution that the complexity of the medium can lead to variability in manufacture and therefore a rigorous quality control release scheme needs to be in place.

A typical approach is to attempt to grow mycoplasmas in broth and then to transfer a proportion of the broth onto agar. Plates are incubated the plates under microaerophilic conditions (nitrogen containing 5-10% carbon dioxide), at 35-38oC for a period of up to 14 days. Broth media is incubated for a period of up to 21 days. Mycoplasma growth, as colony formation, is microscopic (unlike the macroscopic cultures typical of other bacteria following a suitable incubation time). The typical colony sizes are 100–400 µm, with colonies resembling the shape of a fried egg. The colonies are embedded beneath the surface of the agar. A typical mycoplasma agar formulation is:

- Heart Infusion Agar 25 g
- Heart Infusion Broth 10 g
- Proteose Peptone #3 10 g
- 1% Thallium Acetate 25 ml
- H2O 995 ml
- Heat Inactivated Horse Serum 126 ml
- Yeast Extract 5 ml
- Penicillin (100,000 units/ml) 5.2 ml
- DPN/L-Cysteine 21 ml

Mycoplasmastasis methods are used to demonstrate that a test sample does not contain inhibitory substances. This means the culture method requires validating, both for the culture medium and it the presence of product. The process challenges the detection limit of a laboratory's mycoplasma detection method. The following five strains of mycoplasmas are commonly used, sourced from a recognized collection like International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products:

- *Acholeplasma laidlawii.*
- *Mycoplasma hyorhinis.*
- *Mycoplasma orale.*
- *Mycoplasma synoviae.*
- *Mycoplasma fermentans.*

If growth of the above strains occurs more than one sub-culture sooner without the test material than with the test material, or if plates directly inoculated with the test material have less than one-fifth of the colonies of those directly inoculated without the test material, the test material is determined to contain inhibitory substances. These substances need to be neutralized or their effect otherwise countered. For example, through passage in substrates not containing inhibitors or dilution in a larger volume of medium, before the test for mycoplasma contamination is carried out. For the dilution technique, larger medium volumes may be used, or the inoculum volume may be divided among multiple 100 ml flasks. The effectiveness of the neutralization or other process is confirmed by repeating the test for inhibitory substances after neutralization.
The long-growth periods of culture-based methods led to the introduction of serological based methods for some clinical applications (39).
With non-culture-based methods, immunofluorescence can serve as a diagnostic tool for identification of mycoplasmas*in situ* in infected hosts and for detection of contaminated cell cultures (40). This involves DNA binding fluorochromes and fluoresceinated antibodies (41) The technique has further been used in research investigations to locate mycoplasmas in pathogenicity studies in both animals and in organ cultures (42) While culture-based methods remain important, faster diagnosis can be obtained through PCR (43).

Staining

Prior to the adoption of PCR, staining was a common adjunct to the culture-based methods. This was by DNA fluorochrome staining (such as the Hoechst stain). The stain Hoechst 33258 bisbenzimide, a blue, fluorescent dye, is used in histology, and it was designed for specifically staining the nuclei of living or fixed cells and tissues (44). The stain is excited by ultraviolet light at around 350 nm and visualization is through the application of fluorescence microscopy. Samples infected with mycoplasma are seen as fluorescing nuclei plus extra-nuclear fluorescence of mycoplasma DNA (small cocci or filaments). Fr optimal results, an enrichments step is required so that a sensitivity of 100 cfu/mL of culture is provided. This creates a sufficient cell density to enable sufficient staining. The method suffers from the low signal provided by these dyes compared to the nuclear DNA and hence PCR is the detection method of choice. Dark-field microscopy can be useful when examining plates for mycoplasma colonies. This method excludes the unscattered beam from the image. As a result, the field around the specimen (that is, where there is no specimen to scatter the beam) is generally dark.
Transmission electron microscopy and scanning electron microscopy can also be used for examination, although few facilities will have easy access to such technology.
In relation to the above tests, the U.S. FDA recommends the combination of the two cultural approaches (21 CFR 610.30):

- Direct culture testing, which uses both broth and agar.
- Indirect culture testing, which detects nonculturable mycoplasma strains using the Hoechst DNA stain.

PCR Methods

Advances with mycoplasma detection were advanced during the 1990s with the development of PCR assays (45). Prior to PCR, the common indirect method was the use of species-specific antibodies in immunostaining or ELISA, or with biochemical methods (such as gradient/electrophoresis). The PCR test is based on the detection of 16S and, sometimes 23S, rRNA molecules of the most common species of mycoplasma contaminating cell cultures (both species-/genus-specific PCR primers and universal PCR primers can be used). When test kits use an extracted RNA/DNA mixture for testing, although some live culture variants are also available. These techniques provide sensitive, specific, and rapid diagnosis of mycoplasmal infection.

With the PCR method, positive hybridization signals as dots on filters or scintillation counts and visual demonstration of the PCR amplicon in gels. Many PCR kits are now rapid, with the ability to detect one or two hundred mycoplasma species, within times of around 2 hours (46). PCR can be strengthened through enrichment, where a growth enrichment step is included prior to the nucleic acid test. This is undertaken to delineate viable organisms from non-viable organisms and residual environmental sources, otherwise the methods do not allow for accurate discrimination between viable and non-viable mycoplasma contaminants, which might lead to false-positive results (such as from inactivated raw materials, etc.). Controls are also important in PCR assays, where one aliquot of broth is inoculated with a mycoplasma strain as a positive control and the use of uninoculated media serves as a negative control. These controls also function as controls in the DNA extraction and PCR assay.

While PCR testing is used more often for the detection of mycoplasmas, there is no general approval for PCR-based mycoplasma testing by the regulating agencies. Many laboratories use PCR as a complementary method for in-process controls and product-specific validation. In developing PCR, a comparability should be undertaken with the culture method to demonstrate the methods are equivalent.

CONCLUSION

The controls to avoid mycoplasma contamination and the testing to verify that control is being achieved are important steps for the maintenance and processing of cells. Before being fused or immortalized, cell cultures should be tested for their sterility. All cells should be found negative for bacterial, fungal, viral and mycoplasma contamination. The examination for mycoplasmas is especially important of mammalian cell lines, and quality assurance principles should ensure that quality control testing is regularly performed.

As this review paper has discussed, appropriate tests for detecting mycoplasma should be used and these include direct and indirect culture methods and PCR assays. Regular testing not only provides an assessment of control measures, but it also represents a less expensive option than attempting to remediate an infected cell culture.

REFERENCES

1. Drexler, H. G. and Uphoff, C.C. (2002) Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention.*Cytotechnology*, 39: 75-90
2. Ligasová A, Vydržalová M, Buriánová R, Br??ková L, Ve?e?ová R, Janoš?áková A, Koberna K (2019) A New Sensitive Method for the Detection of Mycoplasmas Using Fluorescence Microscopy.*Cells* 8:1510
3. Maniloff, J., McElhaney, R. N., Finch, L. R., and Baseman, J. B. (Eds.) (1992)*Mycoplasmas: Molecular Biology and Pathogenesis*. American Society for Microbiology, Washington, DC
4. Edward, D.G. and Freundt, F.A. (1967). Proposal for Mollicutes as name of the class established for the order Mycoplasmatales. *Int J Syst Bacteriol*. 17 (3): 267–268
5. Leaver M, Domínguez-Cuevas P, Coxhead JM, Daniel RA, Errington J (2009). Life without a wall or division machine in*Bacillus subtilis*.*Nature*. 457 (7231): 849–53
6. Razin, S. and Hayflick, L. (2010) Highlights of mycoplasma research—An historical perspective,*Biologicals* , 38 (2): 183-190,
7. Diens, L. and Edsall, G. (1937) Observations on the L-organism of Klienberger.*Proc. Soc. Exp. Biol. Med* . 36, 740–744
8. Chanock, R. M., Hayflick, L., and Barile, M. F. (1962) Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO.*Proc. Nat. Acad. Sci*. 48, 41–49
9. Smith, P. F. and Morton, H. E. (1951) Isolation of pleuropneumonia-like organisms from the throat of humans.*Science* 113, 623,624
10. Razin S, Yogev D, Naot Y (1998) Molecular biology and pathogenicity of mycoplasmas.*Microbiol Rev* , 63: 10941156
11. Rottem S. (2003) Interaction of mycoplasmas with host cells.*Physiol Rev*. 83: 417-432
12. Toyonaga, T., Kato, T., Kawamoto, A., Kodera, N., Hamaguchi, T., Tahara, Y. (2021) Chained Structure of Dimeric F1-like ATPase in Mycoplasma mobile Gliding Machinery.*mBio*, DOI: 10.1128/mBio.01414-21

13. Barile MF, Rottem S (1993) Mycoplasmas in cell cultures.*Rapid Diagnosis of Mycoplasmas*, Plenum, New York
14. Waites, K.B.; Xiao, L.; Liu, Y.; Balish, M.F.; Atkinson, T.P. (2017) Mycoplasma pneumoniae from the Respiratory Tract and Beyond.*Clin. Microbiol. Rev*. 30: 747–809
15. Pereyre, S.; Bébéar, C.; Bébéar, C. (2015)*Mycoplasma hominis, M. genitalium, Ureaplasma* spp. In *Antimicrobial Therapy and Vaccines.* Volume I: Microbes; Yu, V.L., (Ed).*Esun Technologies*: Pittsburgh, USA,
16. Tully, J. G., Rose, D. L., Whitcome, R. F., and Wanzel, R. P. (1979) Enhanced isolation of M. pneumoniae from throat washings with a newly modified culture medium.*J. Infect. Dis*. 139, 478–482
17. Wincell, J. (2013)*Mycoplasma pneumoniae* – A National Public Health Perspective,*Current Pediatric Reviews*, 9 (4): DOI : 10.2174/15733963113099990009
18. Niitu, Y., Hasegawa, S., and Kubota, H. (1974) In vitro development of resistance to erythromycin, other macrolide antibiotics and lincomycin in*M. pneumoniae*.*Antimicrob. Agents Chemother*. 5, 513–518
19. Pereyre, S. and Tardy, F. (2021) Integrating the Human and Animal Sides of Mycoplasmas Resistance to Antimicrobials,*Antibiotics*, 10 (10): 10.3390/antibiotics10101216
20. Vivek Srinivas, Hugo Lebrette, Daniel Lundin, Yuri Kutin, Margareta Sahlin, Michael Lerche, Jürgen Eirich, Rui M. M. Branca, Nicholas Cox, Britt-Marie Sjöberg, Martin Högbom (2018) Metal-free ribonucleotide reduction powered by a DOPA radical in Mycoplasma pathogens.*Nature*, DOI: 10.1038/s41586-018-0653-6
21. Satake Y, Higa K, Tsubota K et al. (2011) Long-term outcome of cultivated oral mucosal epithelial sheet transplantation in treatment of total limbal stem cell deficiency.*Ophthalmology* 118(8), 1524–1530
22. Rehmann W and Morgan G. (2009). The regulation of advanced therapies: Perspectives from the EU, *RAJ Pharma*:
    http://united-kingdom.taylorwessing.com/uploads/tx_siruplawyermanagement/The_Regulation_of_Advanced_Therapies.pdf
23. High KA and Roncarolo MG. (2019) Gene therapy. *N Engl J Med*. 381(5):455?464
24. Kuick Research (2021) Global Gene Therapy Market Clinical Trials Report 2028: https://www.prnewswire.com/news-releases/global-gene-therapy-market-clin...
25. Lotfi R, Rojewski M, T, Zeplin P, H, Funk W, Pullig O, Nöth U, Schrezenmeier H. (2020) Validation of Microbiological Testing of Cellular Medicinal Products Containing Antibiotics.*Transfus Med Hemother* 47:144-151. doi: 10.1159/000501284
26. Armstrong SE, Mariano JA, Lundin DJ. The scope of mycoplasma contamination within the biopharmaceutical industry.*Biologicals*. 2010;38(2):211–213
27. Uphoff CC, Drexler HG. (2002) Comparative antibiotic eradication of mycoplasma infections from continuous cell lines.*In Vitro Cell Dev Biol Anim*. 38(2):86–89
28. Harlin H, Gajewski TF. (2008) Diagnosis and treatment of mycoplasma- contaminated cell cultures,*Curr Protoc Cytom*. Appendix 3: Appendix 3C
29. V.M. Chernov, O.A. Chernova, J.T. Sanchez-Vega, A.I. Kolpakov, O.N. Ilinskaya (2014) Mycoplasma contamination of cell cultures: vesicular traffic in bacteria and control over infectious agents,*Acta Naturae* , 6 (22): 41-51
30. Razin S. (1999) Adherence of pathogenic mycoplasmas to host cells.*Biosci Rep*. 19: 367-372. 10.1023/A:1020204020545
31. Fleckenstein E, Drexler HG. (1996) Elimination of mycoplasma contamination in cell cultures.*Biochemica* . 1(1):48–51
32. Uphoff CC, Drexler HG. (2011) Elimination of mycoplasmas from infected cell lines using antibiotics. *Methods Mol Biol*. 731:105–114
33. Folmsbee, M., Lentine, K., Wright, C., Haake, G., Mcburnie, L., Ashtekar, D., Beck, B., Hutchison, N., Okhio-Seaman, L., Potts, B., Pawar, V. and Windsor, H. (2014) The Development of a Microbial Challenge Test with*Acholeplasma laidlawii* To Rate Mycoplasma-Retentive Filters by Filter Manufacturers, *PDA Journal of Pharmaceutical Science and Technology* 68 (3) 281-296; DOI: https://doi.org/10.5731/pdajpst.2014.00976
34. Windsor, H., Windsor, G. and Noordergraaf, J. (2010) The growth and long term survival of*Acholeplasma laidlawii* in media products used in biopharmaceutical manufacturing,*Biologicals*, 38 (2): 204-210,
35. Drexler HG, Uphoff CC. (2002) Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention.*Cytotechnology*. 39(2):75–90
36. Hopps, H. E., Meyer, B. C., Barile, M. F., and Del Giudice, R. A. (1973) Problems concerning “noncultivable” mycoplasma contaminants in tissue cultures.*Ann. NY Acad. Sci.* 225, 265–276
37. Razin S. (1985) Molecular biology and genetics of mycoplasmas (Mollicutes).*Microbiol Rev*. 49:419
38. Windsor D., Windsor H. (1998) Quality Control Testing of Mycoplasma Medium. In: Miles R., Nicholas R. (Eds)*Mycoplasma Protocols. Methods in Molecular Biology*, vol 104. Humana Press. https://doi.org/10.1385/0-89603-525-5:61

39. Kenny, C. E., Kaiser, G. G, Cooney, M. K, and Foy, H. M. (1990) Diagnosis of Mycoplasma pneumoniae pneumonia: sensitivities and specificities of serology with lipid antigen and isolation of the organism on soy peptone medium for identification of infections*J. Clin. Micro*. 25, 2087–2093
40. Tully, J. G. (1983)*Introductory remarks, in Methods in Mycoplasmology*, vol. I, Mycoplasma Characterization, (Razin, S. and Tully, J. G., eds.), Academic, New York, pp. 399, 400
41. Del Giudice, R. A. and Hopps, H. E. (1978) Microbiological methods and fluorescent microscopy for the direct demonstration of mycoplasma infection of cell cultures, in*Mycoplasma Infection in Cell Cultures* (McGarrity, G. J., Murphy, D. G., and Nichols, W. W., eds.), Plenum, New York, pp. 57–69
42. Bradbury J.M. (1998) Identification of Mycoplasmas by Immunofluorescence. In: Miles R., Nicholas R. (eds)*Mycoplasma Protocols. Methods in Molecular Biolog*, vol 104. Humana Press. https://doi.org/10.1385/0-89603-525-5:119
43. Tjhie, J. H. T., van Kuppeveld, F. J., Rosendaal, R., Melchers, W. J. G., Gordijn, R., Walbodmers, J. M. M., Meijher, C. J. L. M., and van der Brule, A. J. C. (1994) Direct PCR enables detection of Mycoplasma pneumoniae in patients with respiratory disease.*J. Clin. Micro*. 32, 11–16
44. Latt, SA; Stetten, G; Juergens, LA; Willard, HF; Scher, CD (1975). Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechst fluorescence.*Journal of Histochemistry and Cytochemistry*. 23 (7): 493–505
45. Schaeverbeke, T., Gilroy, C. B., Bebear C., Dehais, J., and Taylor-Robinson, D. (1996) Detection of Mycoplasma fermentans by a PCR assay in synovial fluids from patients with rheumatoid arthritis and other rheumatic disorders.*IOM Letters* 4, 363
46. Russell, B. J., Horiuchi, K., Velez, J. O., Goodman, C. H., & Johnson, B. W. (2020). Mycoplasma detection in a historical arbovirus repository: Commercial kit comparison and implications for improved repository management.*Journal of virological methods*, 276, 113769

Source URL: http://www.ivtnetwork.com/article/mycoplasma-and-cell-therapy-risks