Prevention and Control of Mycoplasma Contamination in Cell Cultures

Guidance on Mycoplasma contamination for everyone using cultured cells for their research or production

Prepared by the IRPCM Mollicute Contamination in Cell Cultures and Bioproducts Team

Introduction

“Mycoplasma” is the trivial name, more familiar to cell biologists, for a group of prokaryotes of limited biosynthetic capacity that formally belong to the taxonomic class Mollicutes, characterised by the absence of a cell wall. Cell cultures may become infected with a variety of species from the genera mycoplasma and acholeplasma and the possibility of infection by other mollicute genera, particularly in plant or insect cell lines cannot be ruled out.

History

The first report of the occurrence of mycoplasma contamination in a cell line was in 1956. It was shown in the late 1950s and early 1960s that mycoplasma infection of cell cultures had become widespread and, as the effects of such infections became apparent it was suggested that many of the results of cell culture investigations during this period should be viewed with scepticism. In 1962 the United States Public Health Service established a mycoplasma test requirement for viral vaccines produced in cell cultures. Testing for mycoplasmas, essentially part of the product sterility testing specifications, continues to be a requirement worldwide for all products produced in, or derived from cell culture.

Thus it is likely that mycoplasmas established themselves in cell culture from the very earliest attempts at in-vitro cultivation and manipulation of cells as, by the time of the first published report, many workers checking their own cell lines as a consequence found they too had mycoplasma contamination.

Cell Cultures- a Perfect Environment for Mycoplasma Growth

Cell cultures provide an ideal environment for mycoplasma growth; over the years they have been and still are, used by mycoplasmologists for the propagation of mollicutes, especially those which are exacting in their growth requirements, or which cannot be grown using conventional cultural methods. A cell culture provides the organisms with favourable incubation conditions, protection from osmotic stress and a good, regularly replenished supply of the complex nutrients most species require. Mollicutes owe their successful colonisation of cell cultures to their ability to remain undetected; they can reach high numbers without causing any overt signs of contamination, such as turbidity, as is usually the case with other adventitious agents.
All types of cell can become contaminated with mollicutes including fish, insect and plant cells although the lower incubation temperatures may alter the range of species which can establish an infection.

**Effects of Mycoplasma Contamination**

Mycoplasma infected cell cultures should not be used for experimental or diagnostic work. Figure 1 shows the results from two cultural tests for mycoplasma detection where a direct inoculation of the cell culture suspended in its normal medium has yielded confluent growth of mycoplasmas. Such gross contamination will inevitably have some effect on the cells.

**Figure 1**

The photographs illustrate the edge of the inoculum on agar plates with confluent growth. The dark (dense) appearance in the picture on the left is characteristic of *M. hyorhinis*.

(Illustration provided courtesy of Mycoplasma Experience, Reigate, UK)

There is an extensive body of publications on the observed effects of mollicute contamination. This is not surprising considering the number of cell types cultured, the variety of media and supplements and the different mycoplasma and acholeplasma species involved. New reports of interactions between cells and their mollicute contaminants continue to appear with the increased use and diversity of cell culture applications as well as new technology. Some of the observations can be readily understood by considering the metabolic activity and nature of the organisms.

**Presence of an infectious agent**

Cells will respond to the presence of an infectious agent, a feature that is exploited in one of the more recently introduced test kits. Alterations in cytokine activity, changes to gene expression detected in microarray systems and changes in the activation of surface receptors can occur.

**Nutrient Competition**

Any actively growing contaminant will compete with the cells for available nutrients, not only will these nutrients be depleted to the detriment of the cells but cytotoxic metabolites may be produced, particularly by fermentative mollicute species. A number of mycoplasma species commonly found in cell cultures hydrolyse arginine as an energy source; these rapidly deplete the available arginine, adversely affecting cell growth and arginine requiring virus replication. Arginine depletion has also been linked with the chromosomal aberrations observed in contaminated cell cultures.
Cytadsorption

Some mycoplasma species attach to the cell surface, this has been associated with cytopathic effects and alteration of specific cell surface antigens.

Figure 2

*M. hyorhinis* cells (M) adherent to the cell surface

(Scanning electron micrograph courtesy of J.S. Jensen, Staten Serum Institut, Copenhagen, Denmark)

Enzyme activity

The activity of mollicute scavenging enzymes causes many problems for the cell biologist, the most well known and documented of which are caused by the nucleoside phosphorylases which break down nucleosides into their free bases. The presence of these enzymes from adventitious agents will interfere with any assay of cellular DNA/RNA activity which depends on measuring the uptake of radioactive nucleosides. Over the years numerous assays for mycoplasma detection have been based on this reaction. Depletion of thymidine phosphorylase can lead to a failure to produce hybrid colonies in HAT medium. More recently the inactivation by mycoplasma enzymes, of nucleoside analogues used in cancer chemotherapy has been reported.

The presence of mycoplasma nucleases may account for some of the chromosome damage observed in mycoplasma infected cells and has been suggested as a possible cause for the failure of reverse transcriptase assays in contaminated cells.

Sources of Mollicute Contamination.

All mollicute contaminants originated from a small number of primary sources. The operators were, and potentially still are, a source of the human oral commensal species. Contaminated serum has supplied a wide variety of bovine species as well as the, probably saprophytic, acholeplasma species. The environment is of considerably less importance as a source of mollicutes than it is for other
adventitious agents but it should be noted that *Acholeplasma laidlawii* can be water borne. The original tissues used to develop the cell line can be a source, particularly if the cells are derived from tissue which is colonised by mycoplasmas. Generally, however, primary tissue is considered a relatively rare source although some unusual species specific mycoplasmas that have been detected clearly derived from the original tissues. It is likely that some of the human species, not normally isolated from the upper respiratory tract may have established themselves via original cells.

Secondary infection, cross contamination from another infected cell line has always been the most important and frequent source of contamination. When an infection is detected in a previously clean cell line this should always be the first line of investigation. All material which has been in contact with other cells should be considered suspect including conditioned medium (Mollicutes can readily pass through 0.2µ filters) and virus stocks.

**Mollicute Species Isolated From Cell Cultures**

At the present time all mollicutes isolated from cell cultures have been either mycoplasma or acholeplasma species. The most frequently isolated species, throughout the history of mycoplasma contamination are normally part of the human or bovine flora reflecting the importance of the serum and laboratory staff as the original sources. One porcine species is frequently found but may have originated from serum. Less commonly rodent, canine and avian species have been reported; these have usually been associated with the original tissues used to establish the cell line although *Mycoplasma canis* has been isolated from cattle as well as dogs so could enter via bovine serum.

Two species have been isolated as contaminants without being isolated from their natural host. *Mycoplasma pirum*, when it was first described, had only been isolated from cell cultures and the source had not been determined for any of the isolates. Subsequently the organism was isolated from human peripheral blood lymphocytes during AIDS associated research. It is possible that this organism entered cell cultures via original human tissue and also that other human species became established via the same route. Both *Mycoplasma hominis* and *Mycoplasma fermentans* have been isolated from the respiratory tract but *M.hominis* normally colonises the urogenital tract and *M.fermentans* has been shown to invade B cells. The second species, so far only found as a contaminant is *Acholeplasma vituli*. This organism was isolated from cell cultures from various laboratories in The Netherlands between 1979 and 1992 and also from several lots of foetal calf serum confirming contaminated serum as the source. However *A. vituli* has not yet been directly isolated from cattle or other animals.

There has been a marked change in the prevalence of particular species over time until a small number of species, some which were only found rarely in the early years, dominate. (see figures 2 and 3) This may reflect changes in the types of cell and their popularity with researchers related to the ease with which a particular species can colonise a particular cell type. It is more likely that the major contributory factor is the importance of cross contamination in spreading mycoplasma infection and the dominant species are those which have survived by repeated cross contamination.
### MYCOPLASMAS ISOLATED FROM CELL CULTURES
(Cumulative to 1978)

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<th>Human</th>
<th>Bovine</th>
<th>Swine</th>
<th>Murine</th>
<th>Avian</th>
<th>Canine</th>
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### MYCOPLASMAS ISOLATED FROM CELL CULTURES
FROM 1981 - 2005

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* High isolation rate due to a contaminated serum lot

(Tables provided by courtesy of Helena Windsor, Mycoplasma Experience)

**Survival of The Fittest**

How do mollicutes avoid detection and persist in cell culture

The contaminants circulating in 21st century cell cultures are examples of classic Darwinian biology, they represent the survival of the fittest. In the case of a micro-organism infecting a cell culture
survival means avoiding detection, successful infection of new hosts and resisting attempts to prevent infection. Mollicutes are already well placed as they are able to reach high titres in cell cultures without showing the clear visual evidence of contamination observed with bacterial and fungal contamination; the most successful mollicute species show several other features which have allowed them to survive and persist in this environment.

**Evading Detection**

It was noted in the 1970s that there were cells infected with mycoplasmas which could not be cultured on the mycoplasma growth media available at the time. These were strains of *M. hyorhinis* which were initially dubbed “non–cultivable” although subsequently media formulations which allowed their cultivation were developed. Other mycoplasma species which are more difficult to culture from contaminated cells than their natural hosts have also been noted.

Whilst the typical contamination is high titre it is possible to get a chronic low grade infection with mycoplasma species such as *M. orale* which have a slower growth rate, particularly if they establish infection in a vigorous cell line. Tests which require relatively high titres for a reliable positive result may fail to detect these infections and other low grade infections, for example a cell line which has been treated for mycoplasma removal, where the treatment has suppressed numbers but not successfully eradicated the infection.

Some mycoplasma species cytadsorb and there is evidence that some species can invade the cells and become internalised Where only the supernatant is used for testing these infections may be missed particularly if the numbers are low.

**Figure 4**

**Cytadherent Mycoplasmas**

Antibiotic Resistance

Mollicutes start out with the advantage of no cell wall which not only allows them to pass through sterilising filters under pressure or vacuum but also means they are unaffected by the addition of penicillin to control contamination. Early isolates can be shown to be sensitive to streptomycin but isolates from the later part of the 20th century are typically resistant reflecting its widespread use in cell cultures and reinforcing the evidence that the majority of contaminants are being passed from cell culture to cell culture.

Mollicutes, like bacteria, can develop resistance to antibiotics. Many recent isolates are also resistant to the aminoglycoside antibiotics neomycin, kanamycin and gentamycin although the latter has successfully been used for eradication in the past. Once resistance has developed to one of these three antibiotics the strain will be resistant to all three.
The Most Common Contaminants

Figure 6

Currently the following five species will account for the majority of infections

- *M. arginini*
- *M. fermentans*
- *M. hyorhinis*
- *M. orale*
- *A. laidlawii*

(Illustration provided courtesy of Mycoplasma Experience)

These five species account for the majority of present day cell culture infections. All have fulfilled the requirements of “survival of the fittest” either by their capacity to adapt to the cell culture environment or by their ubiquity in the environment.

*Mycoplasma arginini*

This organism has been isolated from a range of mammalian species including all farm animals. It is one of the two commonest serum contaminants. It is typically very easy to culture, with a rapid growth rate but slower growing, more fragile strains have been isolated from cell cultures. These strains have been associated with antibiotic resistance, particularly to the aminoglycoside antibiotics, neomycin, gentamycin and kanamycin suggesting long term exposure to cell cultures.

*M. arginini* has been reported to be the most likely species to fail to be detected with the Gibco “Mycotect” system due to low enzyme levels.

*Mycoplasma fermentans*

This is another organism that may have become adapted to the cell culture environment and thus more difficult to culture. There can be a marked difference in growth rate between strains isolated from the
host and cell culture isolates. Slower growing species may establish a low grade infection in more rapidly growing cell lines making them more difficult to detect. It is not known whether the increasing prominence of this organism as a cell culture contaminant is due to changes in the types of cell line being cultivated- which are more susceptible to colonisation, or detection failures due to low numbers or adaptation to the cell culture environment.

**Figure 7**

![Growth of Two Strains of *M. fermentans*](image)

(Adapted from data kindly supplied by Mycoplasma Experience, UK)

It has been reported that *M. fermentans* can invade the cell which would also hinder detection in supernatant only tests.

**Mycoplasma hyorhinis**

*M. hyorhinis* is commonly found in the porcine upper respiratory tract when it is easy to culture, typically giving large colonies on agar after a few days incubation. It is equally common in present day cell cultures where it can be regarded as the most successful “survivor”. Trypsin has often been suggested as the source of *M. hyorhinis* infections as it is sourced from porcine tissue. However this has never been demonstrated and cultures of *M. hyorhinis* inoculated into trypsin solution are rapidly killed although it has been demonstrated experimentally that there is limited survival of the microbial cells if they are aggregated. A more likely original source is bovine serum through abattoir cross contamination. There is evidence to support this proposition as an incidence of a serum lot found to be contaminated with *M. hyorhinis* was reported in the 1970’s. It is likely that this event would occur far less frequently than contamination with the bovine associated species which emphasises how successful this organism has been at evading detection and spreading through cell cultures worldwide.
*M. hyorhinis* was the first species to be recognised as having lost the ability to grow on conventional mycoplasma media after adaptation to the cell culture environment. It was realised in the 1970’s that some cell lines were contaminated with strains of *M. hyorhinis* which could not be cultivated. The Hoechst DNA stain method was specifically developed to allow detection of these strains. A small number of laboratories concentrated on developing medium formulations which would support growth of these “non-cultivable” strains.

**Figure 8**

![M. hyorhinis porcine isolate](image1)

*M. hyorhinis* porcine isolate
3 days growth on Mycoplasma Experience agar

![M. hyorhinis ATCC 29052](image2)

*M. hyorhinis* ATCC 29052
3 days growth on Mycoplasma Experience agar

(Illustration provided courtesy of Helena Windsor, Mycoplasma Experience, UK)
M. hyorhinis strongly cytadsorbs consequently it can still evade detection today if only supernatants are tested.

Mycoplasma orale

This is a slow growing, arginine metabolising species and is a human oral commensal. It does not cytadsorb, which makes it more difficult to detect using the Hoechst stain. In a rapidly dividing cell line it can establish a chronic low grade infection due to its slow growth rate. If only tests requiring relatively high titres for detection are used or a strain has become difficult to culture it can evade detection.

Acholeplasma laidlawii

A special case.

A. laidlawii is easy to culture and, being sterol independent will grow well in simple media such as tryptone soya broth or fluid thioglycollate. It will also grow in serum free cell culture media. It grows over a wide temperature range from, surprisingly, refrigeration temperatures to 37 degrees C, allowing it to thrive in cell lines, such as insect cells incubated at lower temperatures. A. laidlawii was first isolated from London sewage in 1936 and has been isolated from many animals and birds, including all farm animals, as well as plant surfaces.

It is the commonest Acholeplasma species found as a serum contaminant and it has been established that it is part of the bioburden of broth powders although the point of entry is not yet known. It has also been found as a contaminant in serum free cell culture media, as these contain complex plant and animal sourced peptones the source may be the same as for broth powders.

The Problem With Filter Sterilisation

A. laidlawii poses a contamination problem in all three sources due to the need for filter sterilisation as mollicutes can pass through 0.2µ filters under pressure or vacuum. Serum processors now routinely use 0.1µ filters and in addition filters can now be routinely integrity tested to detect and amend filter failure. Whilst this has improved the quality and reduced the contamination risk from serum, it has not completely eliminated risk. Unfortunately some cell culture media formulations cannot be passed through filters with pore sizes below 0.2µ as the growth promoting properties are affected. When mollicutes do succeed in passing through modern filtration systems, it is likely to be in very low numbers, making detection by the manufacturers post production QC largely a matter of chance, even where mycoplasma testing procedures are exemplary. It is also highly likely that a contaminated batch of serum or cell culture medium will consist of contaminated and uncontaminated aliquots making source tracing more difficult.

Prevention, Detection and Control

Operator

There is no excuse for contamination with the human mycoplasma flora from the operator, good aseptic technique will prevent its occurrence. Many cell biologists recommend working with
antibiotic free media wherever possible. This ensures good working practices and in the case of mycoplasma contamination assists prevention as any breakdown is more likely to involve bacterial or fungal contaminants, which are more readily detected.

**Primary Cell Lines**

The use of antibiotics to suppress any microbial flora carried over with the tissue in primary culture is an acceptable practice and thus antimycoplasmal antibiotics may be included. Organisms colonising their natural host should still be sensitive to the commonly used antibiotics. Where work is carried out solely with the primary tissue, cells may remain in antibiotic supplemented medium but if the primary tissue is being used to establish continuous cell lines, or the cells are used for longer periods, they should be passaged into medium without the antibiotics and tested for mycoplasmas according to the established laboratory routine.

**Serum**

Contamination via serum can be avoided by using serum free formulae or heat inactivated or γ irradiated serum. Where this is not possible operators should be aware that there is a risk, however thorough the suppliers mycoplasma testing protocol and design their mycoplasma testing protocols to take this risk factor into account.

**Media Products**

In commercial and other GMP production operations the use of filter sterilised sterility media may be required for media fill trials or SIP validation. Manufacturers are now offering products designed for filter sterilisation, they offer improved filterability over the standard product and have been γ irradiated to remove the risk of *A. laidlawii* contamination. If sterility media cannot be autoclaved then these products should be used.

The possibility of *A. laidlawii* contamination originating from the culture medium should be borne in mind when organising mycoplasma testing or sourcing an infection.

**Cross Contamination**

Preventing cross contamination is the most important feature of a programme to establish and maintain a mycoplasma free cell culture facility.

**Quarantine and Test**

As infection is most likely to come from another infected cell culture handled in the same facility a strict policy of quarantining all incoming cell lines until they have been mycoplasma tested is essential. All cell lines of unknown status should be handled under quarantine until they have been tested. There should be no exception to this rule, including cell lines obtained from what should be a reliable commercial source such as the ATCC and ECACC or old stocks where data on mycoplasma testing cannot be found. If quarantined cells cannot be handled in a separate area they should be the last cultures handled in the working day, following which the workstation should be thoroughly wiped down. If the incubator has to be shared with tested stock they should be in flasks, not dishes (the filters in the caps which allow gaseous exchange will not allow passage of mollicutes as no force is applied.
Keep it Clean

Good aseptic technique will also prevent cross contamination. There are some cleaning products specifically marketed for prevention of mycoplasma contamination but mollicutes are susceptible to all the normal laboratory disinfectants if they are used correctly. To prevent cross contamination (cell type as well as mollicutes) only one cell culture should be handled in the workstation at a time and media and pipettes should be kept separate. If the same medium is used for several cell lines it should be aliquotted with each cell line having its own bottle of medium. Automatic pipettes should be dedicated to a particular cell culture.

Staff Control

Where several operators use the same facilities it can be helpful to maintain logbooks for hoods and other workstations so that there is a temporal record of the cell lines handled. This can prove invaluable for tracking the source in the event of an outbreak.

Good, ongoing staff training in aseptic handling and mycoplasma awareness together with good recordkeeping for the mycoplasma testing status of all cell lines in the facility will help keep staff motivated to maintain a clean facility.

Choosing a Mycoplasma Testing Regime

The perfect mycoplasma test has yet to be developed! All tests currently available have advantages and disadvantages, The key to instituting an effective testing regime is to be aware of the benefits and drawbacks of the different tests available and make the best match to suit individual testing requirements and any budgetary constraints.

Caveat Emptor (buyer beware)

Extravagant claims for test sensitivity or ease of use from the supplier should be approached with caution. It should also be noted that commercial pressures may lead to test kits being marketed before they have been fully evaluated. A number of systems require an initial investment in expensive instrumentation. However where the equipment is not an integral and dedicated part of the test kit the instrument may already be available in the laboratory or, if purchased can be used for other applications in which case the use of the kit will be more economically viable.

Buy the Best

The critical stages in cell culture handling require the most stringent testing. The chosen test protocol should concentrate on sensitivity and should ensure that a wide range of different mollicute species can be detected. A cultural test procedure is highly recommended, however unless the medium supplier or testing laboratory can prove that the medium used will support growth of the fastidious cell culture adapted strains (validation with a cell culture maintained preparation of a cultivar α strain of M. hyorhinis such as ATCC29052 is a minimum requirement) then at least one additional test method should be included in the protocol. This regime should be applied at the following points

1) Quarantined cells
2) Testing master and working cell banks (Tested cell banks should be established to ensure that, in the event of an infection, the contaminated cells can be destroyed and replaced with clean stock)

3) Before carrying out transfections or any other manipulations which create a unique cell line. (Once the new line has been successfully selected a tested bank should be prepared)

4) Following attempted eradication.

5) For confirmation that any important or unusual findings are genuine, especially if it is expected to progress to publication or product development.

Routine Monitoring

All laboratories handling cell lines should institute a programme of routine monitoring in addition to the critical point testing listed above. The frequency of testing will be related to the risk/expense of a positive finding which would render suspect all the work following the last negative test. Laboratories with many researchers handling a high throughput of cell lines from many different sources would expect to test more frequently than a unit with a small number of established cell lines, particularly if the passage level is restricted where a test at the final passage may suffice.

For reasons of economy, rapidity of results or integration with other tests using the same instrumentation a less stringent test may be selected for all, or some, routine monitoring episodes or for in process monitoring, particularly to obtain a result quickly before harvest and downstream processing in production/pilot production. In some situations, for example with cell therapy products there may be a short period of time available for testing and therefore a rapid test method is needed at the final stage. Provided the limitations of the chosen test are understood mixed test regimes of stringent, critical point testing and a convenient but less sensitive system for monitoring can be effective.

In-House, or Outside Testing Service?

Testing can be carried out by the cell culture user, or a group of researchers teaming up together or samples can be sent to an outside testing laboratory. Large institutions may have central microbiology facilities offering an in-house testing service. Some of these facilities will have many years of experience with mycoplasma testing and can offer a reliable and informed service. Generally, however the potential user should use the same approach for both external and internal testing laboratories to ascertain the suitability of the tests offered. Testing can be split between the user and external testing facilities, for example commercially available kits can be used for monitoring and samples sent out for critical stage testing. Many cell biologists will choose an external service when they require cultural testing and also use these services for identification of contaminants, confirmation of findings using the internal test protocol, or investigation of the source of an infection.
Which Test?

Culture

Initially mycoplasma contamination was detected by the classical microbiological method of amplification of viable mycoplasmas in broth and demonstration of typical colonies on subculture to agar.

This is still the most effective, flexible and sensitive method of detection.

Advantages: A single agar plate can be inoculated for a basic screen but for greater sensitivity larger volumes can also be inoculated into broth at 10% v/v with subcultures at suitable intervals. The method can be used to test realistic volumes of serum or medium and it is the best option for detection of low grade contamination in virus seeds or following elimination treatments. As cells can be inoculated in their suspending fluid with no interference to the test, adherent and internalised contaminants can be detected.

Disadvantages: The most commonly quoted disadvantage is speed yet, in a good growth medium most mollicutes will be detected between one and seven days. Unfortunately, to ensure detection of slow growing or damaged organisms, negative tests need to be incubated for at least 14 days. Culture media are complex and need careful validation, even so many strains of *M. hyorhinis* are unable to grow on the conventional formulations after many years of passage in cell cultures.

Specificity: There should be no false positives; other adventitious agents may grow but are usually readily distinguished from mollicutes. A good medium will allow isolation of a wide range of species.

Sensitivity: Sensitivity is a function of the volume tested and the inherent sensitivity of the test. The sensitivity of culture is not impaired by small sample volumes so the quality of the growth medium is paramount.

Various suitable media formulations, including those developed to support growth of cultivar α strains of *M. hyorhinis* have been published and many testing laboratories have their own tried and tested formulae. Pre-prepared media are also available commercially. Whether media are made in-house, purchased, or an external testing service is used it is important to ensure media lots are validated with low passage strains derived, where feasible, from infected cell cultures, not the natural host. The ability to grow cultivar α strains of *M. hyorhinis* cannot be claimed unless a cell culture maintained preparation of ATCC 29052 (also sometimes listed as DBS 1050) is inoculated in low numbers for validation. Testing laboratories which also offer mycoplasma testing to European Pharmacopoeia or FDA “Points To Consider” protocols should already be using low passage strains for Quality Control. However it should be noted that the strain of low passage *M. hyorhinis* available from the EDQM is sourced from its natural porcine host, not a cell line and is therefore unsuitable.

DNA Binding Stains (Hoechst)

Methods using DNA binding fluorochromes were first developed in the 1970s to detect the infections with *M. hyorhinis* which were being missed by culture. The commonest stain used is Hoechst 33258. All these fluorochromes stain DNA so the cell nuclei will stain together with the DNA of any adventitious agents which will show as small fluorescent spots in the cytoplasm
Infected cell showing contaminant DNA  Control cells, only cell nucleus stained

(Illustrations supplied by courtesy of J. S. Jensen, Staten Serum Institut, Copenhagen, Denmark)

The stain can be used directly on adherent cells but a superior technique is to inoculate a sample of test cells, or other material into an indicator cell line. This test allows for enrichment of low numbers, use of positive controls at specified low titres and a stable cell line not subject to nuclear fragmentation.

Advantages:- This test is very effective for detection of *M. hyorhinis* and other cytadsorbed contaminants. It is not species specific and the equipment required is already available in many facilities. It has traditionally been combined with culture for effective, sensitive testing.

Disadvantages:-Non cytadsorbing mollicutes are more difficult to detect with this method. Test results can sometimes be difficult to interpret and the test is often unsuitable for live virus samples. The indicator cell method offers no increase in speed of detection, compared to culture, with fast growing species.

Specificity:- Detects all adventitious agents.

Sensitivity:- The indicator cell method is validated with 100 colony forming units in Regulatory Tests

**Polymerase Chain Reaction (PCR)**

PCR has increased in popularity as a testing method. There a variety of different kits commercially available, however it should be noted that many testing laboratories have developed their own methods using their own selected primers. For those laboratories that have established reliable protocols PCR has replaced the DNA stain as an adjunct to culture. Many cell biologists would like to rely on PCR alone but it does have some limitations.
Advantages: Speed is the most often cited advantage, however, where it is not economically viable to run the test system until a particular number of samples have accumulated this can be negated. If PCR instrumentation is already available together with personnel experienced in handling samples correctly it can easily be introduced in-house for general monitoring.

Disadvantages: False positives are possible. It should be remembered that this test detects complementary nucleic acid only, not whole or viable organisms; PCR cannot be used to monitor elimination attempts for this reason. Staff must take appropriate precautions to avoid cross contamination with PCR product. False negatives have also occurred, particularly where only supernatants are sampled. (In some cases whole cell preparations interfere with the reaction).

Specificity: There may be some cross reactivity with bacterial species most closely related to mollicutes with generic primers but these are unlikely to present as contaminants. Kits including speciation, for example microarrays should also include a generic primer to ensure detection of unusual mollicutes.

Sensitivity: Correctly performed, with suitable primers, PCR is extremely sensitive but the typical volumes tested compromise this sensitivity, as will use of supernatants only. The absolute sensitivity with typical sample volumes equates to about 100-1000 colony forming units per ml (cfu/ml). Claims of greater sensitivity than culture should be treated with caution, for example, sensitivities of less than 1 cfu/ml are simply not possible, unless much larger sample volumes have been used. Apart from the possibility that the comparison has been made using poor quality culture medium, two other factors need to be taken into account in comparative studies as the test is measuring nucleic acid not viable organisms which are essential for cultivation. Firstly broth cultures used to prepare the test organisms need to be in early log phase to avoid nucleic acid from dead organisms and secondly 1 cfu may consist of more than one microbial cell. This effect is most marked with cytadherent species where a single cell in the sample volume may be covered with many mycoplasmas, on agar this would tend to form one colony. If there is no reaction inhibition the sensitivity can be improved by concentrating a suitable test volume before sampling for PCR either by centrifugation or filtration.

The pressure to develop and validate test protocols which are comparable to compendial tests to allow more rapid release of products and to allow pre-release testing of cell therapy products with short shelf lives has focussed kit manufacturers on designing protocols which meet the sensitivity requirements of the Regulatory Authorities.

Other Nucleic Acid Tests (NATs)

A commercially available test utilises hybridisation. A labelled ssDNA probe hybridises with rRNA of target organisms. Bound probes produce chemiluminescence on addition of the detection reagent.

Advantages: The test is relatively simple and gives a rapid result.

Disadvantages: Sample is prepared from the tissue culture medium. The luminometer supplied by the manufacturer is expensive if this is its only application and there is not a high throughput of tests.

Specificity: All prokaryotes.

Sensitivity: The manufacturers claim $10^5$ cfu/ml (unusually honest). This kit would only be suitable for routine monitoring not critical point testing.
RNA amplification: Tests which amplify mollicute RNA have been in development for a few years and are starting to come to market. RNA is present in multiple copies and, as it is less stable than DNA, it is considered that the detection of RNA when there are no viable organisms present is unlikely. The amplification techniques used do not require high temperatures.

**Immunological Test Kits**

A wide variety of test kits have been marketed over the years based on immunological reactions. Test kits have included ELISA tests, “combs” and fluorescent antibodies. While any of these tests, particularly the use of fluorochrome labelled antibodies are useful for identification or monitoring of situations where a known species is present most, perhaps all, are unsuitable for general testing. Claims that the kit is based on a “universal” monoclonal antibody should be checked by asking for a complete list of mollicute species checked.

**Cellular Reaction to Infection**

A test kit has been introduced which exploits the ability of Toll-like receptor 2 to recognise adventitious agents including mollicutes and induce a signalling cascade. Special indicator cells, supplied by the manufacturer have been engineered to secrete alkaline phosphatase when activated which is detected by a colour change in the detection medium. This is clearly a simple system for cell biologists to use requiring no special equipment, no independent information is available on its efficacy.

**Enzyme Based Detection**

A number of tests have been developed based on the observed effects of mycoplasmal nucleoside phosphorylases such as the uridine/uracil uptake ratio method. A commercial test was available for many years which utilised an indicator cell line and detected the presence of mollicutes by conversion of 6-methylpurine deoxyribose to the cytotoxic 6-methylpurine. This is no longer available, probably because sales have dropped due to the popularity of other methods not available when it was introduced.

There is one kit currently available based on the generation of ATP by either acetate kinase or carbamate kinase activity by the contaminating mollicutes. The ATP is detected by the luciferin/luciferase reaction.

Advantages: The test is easy to use and extremely rapid.

Disadvantages: A luminometer is required but the kit manufacturer has validated a wide range of instruments for suitability. There is one significant failing, the cells must be removed from the sample to prevent spurious ATP detection from cellular activity.

Specificity: bacteria are not lysed by the reagent so a positive reaction should be due to mollicute activity.

Sensitivity: The manufacturers claims have not been met in studies by IRPCM members. Titres required for a reliable response are in the order of $10^4$-$10^5$ cfu/ml depending on species. As most infections are high titre this is a useful system for routine monitoring but not suitable for critical point testing.
Hybrid Testing

The demand for more rapid results without loss of sensitivity, particularly in the biotech industry has led to the development of combined test methods employing enrichment followed by a rapid method, usually PCR. Where the limit of detection is known any indirect test method can be used to replace the DNA stain in the indicator cell test or liquid to solid subculture in cultural testing.

Elimination

The best strategy is prevention. The importance of banking tested cells has been referred to earlier. If clean banks are available then contaminated cells can be discarded and replaced.

A number of products are available for elimination when the cell line cannot be discarded. Most are antibiotics or combinations of antibiotics but some membrane disrupting compounds are also used. Testing laboratories offer a variety of services from taking the cell line in to run the complete process to running preliminary tests with the contaminant to allow an effective regime to be chosen.

The most important stage is the follow up testing. The cell line should not be banked or considered free of infection until it has been re-tested after at least five passages following the completion of the treatment protocol. Invasive mycoplasmas may be particularly difficult to eradicate. Studies with a cell invasive model evaluating four commercially available kits showed that the organism was suppressed but not permanently eliminated. Failed eradication attempts are regularly reported and some Team members attribute these failures to successful cell invasion, however antibiotic resistance is also widespread which is why some testing laboratories pre-test the actual contaminant to check antibiotic susceptibility before treating the cells.

Further Reading

Some of the best reviews were written in the last three decades of the twentieth century. There has been considerable progress in detection methods but the advice given in these articles regarding infection control has stood the test of time.

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There is a special section on Mycoplasma – P181-248 –in this issue, organised by the PDA Mycoplasma task Force.


Section F has a several good chapters on Mycoplasma contamination in cell cultures.
Steiner, T. and McGarrity, G., 1983 Mycoplasmal Infection of Insect cell Cultures, In Vitro Vol.19 No.9 P 672-682.

For all those working with insect cells and other cell types incubated at lower temperatures


A good general review

Rapid Diagnosis of Mycoplasma, ed. I Kahane, Plenum Press. Barile, M.F. and Rottem,S. Mycoplasmas in Cell Cultures, P 155-193

Cellular Senescence and Somatic Cell Genetics Vol. 3, McGarrity, G. J., Methods of Prevention, Control and Elimination of Mycoplasma Infection, P 213-241

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