Biologics Safety Testing

BioReliance's Approach to Mycoplasma Testing: Introduction of United States Pharmacopoeia 63 Regulation

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BioReliance Corporation is a leading provider of cost-effective contract services to the pharmaceutical and biopharmaceutical industries, offering more than 1,000 tests or services related to biologics safety testing, specialized toxicology and animal health diagnostics. Founded in 1947, BioReliance is headquartered in Rockville, Maryland, with laboratory operations in Rockville and Scotland and offices in Tokyo, Japan, and Mumbai, India. The Company employs more than 650 people globally. For more information, visit www.bioreliance.com.

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- **Virus/TSE Validation Studies** for all biological products
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- **Regulatory and Consulting Services**

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Introduction

Mycoplasma contamination of cell culture (both of primary and continuous eukaryotic cell lines) is common and represents an issue of importance in the basic research, development and production of biologicals. Contamination can alter virtually every physical and chemical property of cells (depending on the contaminating species and the cell type) causing them to yield unreliable results and perhaps unsafe biologicals, biopharmaceutical drugs or viral vaccines. In fact, mycoplasma contamination can be present with no obvious change in the host culture, even when the concentration of mycoplasma exceeds that of the host cells by 10-100 fold. Thus, testing for mycoplasma contamination during development and manufacturing of biologicals is required by the worldwide regulatory authorities in the United States, Europe and Japan. Key regulations are defined under FDA Points to Consider (PTC, 1993[3]; 1997[4] 2010[5]), 21 CFR 610.30 (CFR)[6], European Pharmacopoeia (EP) section 2.6.7[1], Japanese Pharmacopoeia section 14 (JP)[2] and the recently announced United States Pharmacopoeia <63> (USP)[7] monograph which will become effective in October 2010.

All biologics produced for clinical investigation and as licensed therapeutics produced via cell substrates (e.g., viral vaccines, monoclonal antibodies and similar products) must be tested to ensure the absence of mycoplasma contamination. Tests for the presence of mycoplasma contamination in Master Cell Banks (MCB) and Working Cell Banks (WCB) originating from metazoan cells should also be conducted as part of purity testing. Guidance for this testing is detailed in the PTC[3,4], EP[1], and USP[7] publications referenced. Live viral vaccines produced from *in vitro* living cell cultures prior to clarification or filtration and inactivated viral vaccines produced from living cell cultures prior to inactivation must also be tested to ensure the absence of mycoplasma per 21 CFR 610.30[6].

The recently published USP chapter on "Mycoplasma Tests" represents a step forward in bringing requirements in the US closer to those outlined in the EP. BioReliance is updating its mycoplasma detection assay range to include the USP method for our clients who are filing in the US. Our new, cGMP compliant assay will meet or exceed USP, EP, and PTC requirements. In addition to the combined USP/EP/PTC test, a 21 CFR 610.30 compliant assay will be available separately for viral vaccines, as well as a JP-compliant test for those clients who need to meet Japanese regulations.

Comparison of the EP, USP, and PTC methods

While the regulatory documents describe methods that are mostly harmonized, there are still some differences between United States[3–7] and European[1] regulatory guidelines. These differences include:

- The acceptance criteria for quantitative recovery of positive controls in the test for nutritive properties and qualification test for inhibitory substances.
- The incubation conditions and number of positive controls included during testing.
- The number of subcultures performed during broth evaluation.

Qualification Testing: The pharmacopoeias (EP and USP) require testing for inhibitory properties once, while the PTC does not stipulate this testing requirement. This qualification ("mycoplasmastasis") testing entails the recovery of spiked control organisms in the presence of test product. The acceptance criteria for the test for nutritive properties of media and qualification testing in the EP and USP methods are aligned for broth culture **(Table 1)**. The growth of mycoplasma in the presence of test product must be within one subculture of that in the absence of test product. For the direct agar portion of the test, however, the quantitative

evaluation in the USP is more stringent than the EP. The USP states that the test is compliant if the recovery of the spike organisms is within 0.5-log in the presence of the test material as compared to that in absence of test material. By contrast, the EP defines that the plates inoculated with spiked test product must be within one-fifth the number of colonies of those inoculated without the test product. In this instance, the USP criterion is more stringent than the EP.

Incubation Conditions and Controls: There are also subtle differences in the incubation conditions between the methods (Tables 1 and 2). The EP defines a temperature range of 35-38 ºC for incubation, while the USP and PTC stipulate it as 36 ± 1ºC. Similarly, the terminology for atmospheres slightly varies between the methods. The PTC refers to it as "anaerobic" incubation while the EP and USP use the term "microaerophilic" (although the definition remains identical across the regulations at 5 – 10% CO_2 in nitrogen).

All regulatory documents specify the number and types of mycoplasma positive controls to be included in the assays; for PTC and USP at least two known mycoplasma species are required, one being a dextrose fermentor and one an arginine hydrolyzer. The EP requires the use of at least one of six mycoplasma species listed in the chapter as a positive control.

Number of subcultures: All methods require sub-culturing from the broth bottle cultures throughout the 28 day period (Table 2 and Figure 1). The number of subcultures ranges from 3 subcultures for PTC (Days 3, 7, and 14) to 4 subcultures for the USP and EP. The additional subculture on day 21 in the USP and EP method is incubated for 7 days. This fourth subculture gives added sensitivity without increasing the duration of the assay.

Indicator cell culture methods. The use of indicator cells to detect non-cultivable mycoplasma is also included

Table 1. Differences between the EP, USP, and PTC methods in the agar culture conditions and criteria.

Method		European Pharmacopoeia	United States Pharmacopoeia	FDA Points to Consider (1993)
Agar Culture	**Inoculum volume**	0.2 ml on each solid media plate	0.2 ml on each solid media plate	0.2ml/2 or more plates
	Positive control recovery	Not more than 100 cfu per inoculum	Not more than 100 cfu per inoculum	Not more than 100 cfu per inoculum
	Temperature /Incubation condition	35-38°C Microaerophilic (nitrogen containing 5-10% CO_2)	36°±1°C Microaerophilic (hydrogen atmosphere containing <0.5% oxygen and/or nitrogen containing 5-10% CO_2 in nitrogen)	36°±1°C Hydrogen atmosphere containing <0.5% oxygen and/or nitrogen containing 5-10% CO_2 in nitrogen
	Inhibition assay	Spike recovery	Spike recovery	No spike recovery required
	Mycoplasmastasis testing	Plates inoculated with TA spiked with control must be within 1/5 of the number of colonies without TA	Plates inoculated with TA are within 0.5log range of number of colonies without TA	N/A
	Positive control selection	Based on type of sample	One dextrose fermentor and one arginine hydrolyzer. Others may be used based on sample type	One dextrose fermentor and one arginine hydrolyzer.
	Test for nutritive properties of media	Essential using all appropriate positive control organisms	Essential using all appropriate positive control organisms	Not required

in all the three regulations **(Table 3)**. Vero cells are most commonly used but another cell substrate may be used if equivalence for detection of mycoplasma is demonstrated. In the PTC, the test article is added to the cells and incubated for 3-5 days before direct staining and examination by epifluorescence microscopy. However, per both the EP and USP methods, the test article is added to indicator cells in a flask and allowed to grow for 3-5 days before passaging onto coverslips for a subsequent 3-5 day growth period. This additional passage step allows for additional amplification of the potential contaminants which enhances the detection of mycoplasma in the test product.

The degree of alignment amongst these monographs (EP, USP, PTC) makes it more practicable to satisfy the regulatory expectations under a single assay system. However, further alignment in the monographs should aid in achieving a more concise assay.

Table 2. Differences between the EP, USP, and PTC methods in broth culture conditions and validity criteria.

Medium		European Pharmacopoeia	United States Pharmacopoeia	FDA Points to Consider (1993)
Broth Culture	Inoculum volume	10 ml/100 ml; subculture days: day 2-4, day 6-8, day 13-15, day 19-21	10 ml/100 ml; subculture days: day 2-4, day 6-8, day 13-15, day 19-21	10 ml/50 ml; subculture day 3, 7,14
	Positive control recovery	Not more than 100 cfu per inoculum	Not more than 100 cfu per inoculum	Not more than 100 cfu per inoculum
	Temperature /Incubation condition	35-38°C Aerobic	36°±1°C Aerobic	36°±1°C Aerobic
	Inhibition assay	Spike recovery	Spike recovery	Not required
	Validity criteria of inhibition test	Recovery within 1 subculture in presence of TA compared with positive control	Recovery within 1 subculture in presence of TA compared with positive control	N/A
	Positive control selection	Based on type of sample	One dextrose fermenter and one arginine hydrolyzer. Other may be used based on sample type	One dextrose fermenter and one arginine hydrolyzer.
	Test for nutritive properties of media	Essential using all appropriate positive control organisms	Essential using all appropriate positive control organisms	Not required

Table 3. Comparison of the EP, USP, and PTC cell culture methods.

Method		European Pharmacopoeia	United States Pharmacopoeia	FDA Points to Consider (1993)
Cell Culture	Seeding density	2×10^4 cells to 2×10^5 cells/ml (4×10^3 to 2.5×10^4 cells/cm^2)	2×10^4 cells to 2×10^5 cells/ml (4×10^3 to 2.5×10^4 cells/cm^2)	Not defined
	Inoculum volume	1 ml	1 ml	1 ml
	Positive control recovery	Not more than 100 cfu per inoculum; *M. hyorhinis* and *M. orale*	Not more than 100 cfu per inoculum; *M. hyorhinis* and *M. orale*	100 cfu or less per inoculum; *M. hyorhinis* and *M. orale*
	Temperature condition	35-38°C	36°±1°C	36°±1°C
	Inoculation of test article	Culture 3-5 days then inoculated onto coverslips	Culture 3-5 days then inoculated onto coverslips	TA inoculated directly on cells on coverslips
	Inhibition assay	Spike recovery when using neutralising antiserum	Spike recovery when using neutralising antiserum	No spike recovery

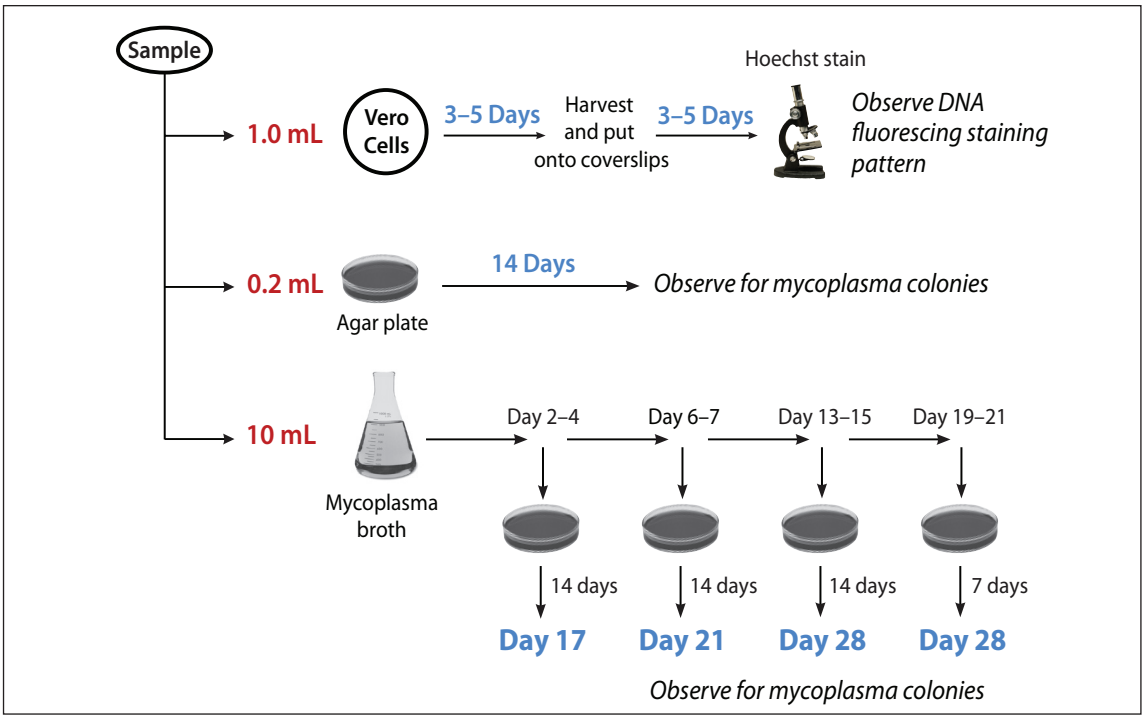

Figure 1 – Schematic depicting basic mycoplasma testing principles.

21 CFR 610.30

Clients who are producing viral vaccines (either live or inactivated) are required to perform testing on the unprocessed bulk according to the guidance in 21 CFR 610.30 **(Table 4)**. For live viral vaccines or inactivated virus vaccines, the virus harvest pool and control fluid pool must be tested for the presence of mycoplasma in broth and agar culture. Unlike the EP, USP or PTC methods, the 21 CFR does not require detection of non-cultivable mycoplasma on indicator cells. Instead, the method provides guidance on more extensive agar cultivation methods including the use of at least two different solid media in addition to semisolid broth incubated under both aerobic and anaerobic conditions. Both the aerobic and anaerobic broth cultures are further subcultured at days 3 and 14 onto agar plates for an additional 14 day incubation under the same atmospheric conditions.

Due to the more extensive agar and incubation conditions required by the 21 CFR method, it is not feasible to offer a combined assay with the USP/EP/PTC compliant test. For this reason, BioReliance will offer a separate 21 CFR compliant test to clients who require it. The combined USP/EP/PTC method allows comparable detection of mycoplasma to the 21 CFR 610.30 method despite the latter method including 2 incubations conditions and 2 media types.

Japanese Pharmacopoeia, Section 14

Although the regulatory documents describe methods that are mostly harmonized, there are still minor differences between the USP, EP and JP.

The JP regulation, in the recent update[8], provides reference to testing for inhibitory substances similar to the EP and USP methods. As with other regulatory documents

Table 4. Key aspects of the 21 CFR 610.30 Test for Mycoplasma.

Method		21 CFR 610.30
Agar Culture	Inoculum volume and media types	2 ml over 10 plates of two different media
	Positive control recovery	Not more than 100 cfu per inoculum
	Temperature /Incubation condition	36°±1°C Aerobic and anaerobic conditions
	Inhibition assay	No spike recovery
	Acceptance criteria of inhibition test	N/A
Broth Culture	Inoculum volume	1ml across 4 x 10 ml; subculture day 3,14
	Positive control recovery	Not more than 100 cfu per inoculum
	Temperature /Incubation condition	36°±1°C Aerobic and anaerobic conditions
	Inhibition assay	No spike recovery
	Acceptance criteria of inhibition test	N/A
Cell Culture	Test for non-cultivable mycoplasma	Not specified

the number and type of mycoplasma controls to be included in the assays are defined as at least two known mycoplasma species or strains, including one dextrose fermentor and one arginine hydrolyzer.

The exacting observation requirements during agar culture testing and the validity criteria for the indicator culture test in this method have directed BioReliance to provide a separate protocol for detection of Mycoplasma in accordance with JP regulations.

Summary

BioReliance is updating its mycoplasma detection assay range to include a family of cGMP assays that will meet or exceed the USP, EP, and PTC requirements. This new assay range will offer optimal growth conditions for mycoplasma as covered in the new USP as well as existing EP and PTC documents. (This "combined" assay range will include appropriate growth media, incubation conditions, subculture schedule, and the use of indicator cells for noncultivable mycoplasma to effectively satisfy these regulations.) Better harmonization of these testing methods will allow for manufacturers of biologically based therapeutics to more easily assess the safety of their products with respect to regional requirements and will significantly streamline the process of mycoplasma testing.

References

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5. Center for Biologics Evaluation and Research. Food and Drug Administration. Guidance for Industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications, Feb 2010.
6. *Code of Federal Regulations*, Title 21: Food and Drugs, Part 610. General Biological Product Standards, Section 610.30. Test for Mycoplasma.
7. General Chapter 63, "Mycoplasma Tests," USP 33–NF 28 Reissue. (US Pharmacopoeial Convention, Rockville, MD, 2010), pp. 88-91.
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