DETECTION OF MYCOPLASMA IN BIOPHARMACEUTICALS VACCINES AND GENE CELL THERAPIES

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Mycoplasma are a form of bacteria that are characterized by the absence of a cell wall surrounding the cell membrane. This renders them inert to many familiar antibiotics, including the beta-lactams, whose activity relies on cell wall disruption. Many different mycoplasma are pathogenic in humans, and they are also commonly found as contaminants in cell cultures. Although the contamination does not create turbidity, factors such as cell line growth rates and viral vaccine production can be adversely affected. This clearly poses a problem for processes that involve cell culture, and thus, being able to detect them is important.

Mycoplasma are too small to be seen under a standard lab microscope, yet the contamination problem is significant—and colonization by mycoplasma is notoriously laborious and is a lengthy process to detect by conventional culture methods. A 2009 study indicated that half of all lab scientists fail to check for the presence of Mycoplasma in their cell cultures 1,which is problematic, as this contamination can disrupt patterns of human gene expression2.

Despite the challenges, it is vital that cell cultures are tested for Mycoplasma contamination if biopharmaceuticals made in this way are to meet regulatory guidelines. The U.S. Code of Federal Regulations, FDA's Points to Consider, the International Conference on Harmonization and both European and U.S. Pharmacopeia all lay out technical documentation for the detection of Mycoplasma.

The two pharmacopeia techniques rely on the agar/broth culture test to detect cultivable mycoplasma, along with the indicator cell culture assay, which detects fastidious mycoplasma that might only grow within mammalian cell cultures. The main drawback with these protocols is that the tests take 28 days to complete in the case of agar/broth culture method, and about one week for the indicator cell culture test. While they are applicable for the manufacture of biologics such as monoclonal antibodies, this timescale is problematic for products whose shelf-life is short, such as cell therapies and cytotoxic viral suspensions, or those that require rapid testing, such as in-process samples. For this reason, more rapid alternatives are routinely accepted by the regulators, as long as they are well validated.

Polymerase chain reaction (PCR) based tests can provide the necessary speed and accuracy. Commercial tests include MycoTOOL from Roche, and MycoSEQ from Life Technologies. The Roche product employs conventional PCR strategies, in combination with gel electrophoresis end-point analysis. The Life Technologies technique, meanwhile, is a rapid, highly controlled, closed PCR well real-time system 3.In both cases, the limit of detection is the lowest number of genomic DNA copies of Mycoplasma that can be found within the sample. In contrast, the detection limit of those slow culture-based methods is typically the number of viable cells that generate colony forming units (CFU) on the surface of the solid medium.

PHARMACOPEIA APPROVED TESTS

The pharmacopeia-prescribed tests can be used to detect the presence of Mycoplasma in cell lines, both purified and unpurified bulk harvests, substrates, biologicals, biopharmaceuticals and final products. In all cases, the culture and indicator method should be used. Virus seeds, harvest and bulk vaccines can also be screened by the indicator method, depending on the virus suitability in this system, and the availability of a qualified neutralizing anti-sera. PCR may be an acceptable alternative for the indicator method if the neutralization studies with the virus preparation are problematic.

The culture method is designed to detect low levels of mycoplasma contamination that other tests might miss. Phenol red is present in the broth medium, and it is subcultured onto agar plates to detect any pH change—a color change in the indicator denotes mycoplasma contamination. Subcultures are performed to confirm the results in the broth. In parallel, the sample is also directly inoculated onto agar plates and incubated at 35–37°C microaerophilically under 5–10% CO2 in nitrogen for at least 14 days—7 days for the broth—and the plates are then studied under a microscope to enumerate the mycoplasma colonies. The pharmacopeia define acceptance criteria for the positive and inhibitory controls regarding the number of colonies, and if colonies are observed then it is possible to identify them by sequencing rather than a microscope.

The volume of broth or agar used is important to ensure that even low levels of mycoplasma can be detected, and the exact volumes to be used are defined within the pharmacopeia. It should also be noted that the test requires frequent observations, making it laborious to undertake, even after the sampling and preparation is complete.

The second test laid out in the pharmacopeia is the indicator cell culture method. In this test, samples are cultured on Vero cells for 6-8 days, then stained with the fluorescent dye Hoechst 33258, which binds specifically to DNA. The dye facilitates the detection of mycoplasma, as there is a characteristic particulate or filamentous pattern of fluorescence on the cell surface, and in surrounding areas too, if the contamination is particularly heavy. While mitochondria within the cytoplasm may also be stained, it is easy to tell these apart from mycoplasma. The dye will also stain the nuclear material of any bacterial or cellular contaminants, which, under a microscope, will be visible and will be larger than mycoplasma, allowing them to be differentiated. These can be reported as an atypical mycoplasma stain result with no need for further analysis. The test is standardized using a Vero indicator cell line.

qPCR TESTING PROCEDURES

The European pharmacopeia Section 2.6.7 includes nucleic acid amplification test (NAT)-based assays as an alternative (if properly validated) to the standard culture detection tests and gives guidelines for the validation.

Such tests, based on quantitative PCR to enumerate mycoplasma, have been developed and validated recently, and have significant advantages in terms of time taken, and comparability studies show greater sensitivity with PCR than the cell culture tests. qPCR techniques can be validated as an alternative to both of the prescribed methods for batch release tests as well as for process control and raw material validation purposes. Against the cell culture method, any technique must be sensitive enough to detect at a level of 10 CFU/ml, as opposed to against the indicator cell culture method, where the system must detect 100 CFU/ml. Notable applications include situations where the pharmacopeia requires two techniques to be used, including PCR before and after an enrichment period on indicator cells and Hoechst staining. PCR can also be used for samples where only a low volume is available, the therapy has a short shelf life and for confirmation if an initial culture based result is dubious. Comparing qPCR results before and after enrichment also allows dead and live Mycoplasma to be differentiated.

In summary, the detection of mycoplasma contamination is of utmost concern in bio-therapeutic and vaccine manufacturing. Now that streamlined assay validation procedures and GMPvalidated NAT tests are readily available, these methods can provide manufacturers with confidence in results required in production and batch release of products. The high reproducibility and fast throughput of cutting-edge NAT-based methods reduces the amount of time needed for development, safety testing, and final marketing of novel biotechnology products.

REFERENCES

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