

## Detection of *Mycoplasma sp.* in Vaccines Produced by Cell Culture

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*Mycoplasma* is a genus of bacteria that lack a cell wall. Because they lack a cell wall, they are unaffected by some antibiotics such as penicillin or other beta-lactam antibiotics that target cell wall synthesis. *Mycoplasma* is a major contaminant of cell lines and is considered as a serious economic and biologic problem in the basic research, biotechnology products such as vaccines and laboratories diagnosis. 12 types of OPV vaccines in 8 series were randomly collected from the post marketing area. Standard culture method for *Mycoplasma* detection in biological substances, based on BP (A190), EP (2.6.7), VICH GL34 for detection of *Mycoplasma* contamination was performed. None of 96 samples were positive by culture methods while the results of positive and negative controls and performance tests for culture media were acceptable.

**Key words:** *Mycoplasma*, Vaccines, Standard culture method.

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*Mycoplasma* is a major contaminant of cell lines and is considered as a serious economic and biologic problem in the basic research, diagnostic laboratories and biotechnology products such as vaccines. They can be parasitic or saprotrophic agents. Several species are pathogen in humans, including *M. pneumoniae*, which is an important

cause of atypical pneumonia and other respiratory disorders, and *M. genitalium*, which is believed to be involved in pelvic inflammatory diseases. They may cause or contribute to some cancers. The genus *Mycoplasma* is one of several genera within the class *Mollicutes*. *Mollicutes* are bacteria which have small genomes, lack a cell wall and have a low GC-content (18-40 mol%). There are over 100 recognized species of the genus *Mycoplasma*. Their genome size ranges from 0.58 - 1.38 megabase-pairs. *Mollicutes* are parasites or commensals of humans, animals (including insects), and plants; the genus *Mycoplasma* is by definition restricted to vertebrate hosts. Cholesterol is required for the growth of species of the genus *Mycoplasma* as well as certain other genera of

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mollicutes. Their optimum growth temperature is often the temperature of their host if warm bodied (e.g. 37°C in humans) or ambient temperature if the host is unable to regulate its own internal temperature. Analysis of 16S ribosomal RNA sequences as well as gene content strongly suggest that the mollicutes, including the mycoplasmas, are closely related to either the *Lactobacillus* or the *Clostridium* branch of the phylogenetic tree (Firmicutes *sensu stricto*).

Mycoplasmas are often found in research laboratories as contaminants in cell culture. Mycoplasma cell culture contamination occurs due to contamination from individuals or contaminated cell culture medium ingredients. The Mycoplasma cell is usually smaller than 1 µm and they are therefore difficult to detect with a conventional microscope. Mycoplasmas may induce cellular changes, including chromosome aberrations, changes in metabolism and cell growth. Severe mycoplasma infections may destroy a cell line. Detection techniques include culture method using specific broth and agar with the specific procedure, PCR and staining with a DNA stain including DAPI or Hoechst.

## METHOD

12 types of OPV vaccines from 3 sources in 8 series were randomly collected from the post marketing area. Standard culture method for *Mycoplasma* detection in biological substances, based on BP (A190), EP 5.0 (2.6.7), VICH GL34 for detection of *Mycoplasma* contamination was performed. For bulk vaccine or for the final lot (batch), the culture method is used as below:

### Culture method

#### Choice of culture media

The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the chosen incubation conditions of small numbers of mycoplasmas that may be present in the product to be examined. Liquid media must contain phenol red. The range of media chosen is shown to have satisfactory nutritive properties for *Mycoplasma orale* ATCC 23714 and *Mycoplasma pneumoniae* ATCC 15531. The nutritive properties of each new batch of medium were verified for these organisms. (EP 5.0, 2.6.7. 2005, pages 149-152)

### Incubation condition

Divide inoculated media into two equal parts and incubate one in aerobic conditions and the other in microaerophilic conditions; for solid media maintain an atmosphere of adequate humidity to prevent desiccation of the surface. For aerobic conditions, incubate in an atmosphere of air containing, for solid media, 5 to 10 per cent of carbon dioxide. For microaerophilic conditions, incubate in an atmosphere of nitrogen containing, for solid media, 5 to 10 per cent of carbon dioxide.

### Test for Mycoplasmas in the Vaccines to be examined

For solid media, use plates 60 mm in diameter and containing 9 ml of medium. Incubate each of not fewer than two plates of each solid medium with 0.2 ml of the product to be examined and inoculate 10 ml per 100 ml of each liquid medium. Incubate at 35°C to 38°C, aerobically and microaerophilically, for 21 days and at the same time incubate an uninoculated 100 ml portion of each liquid medium for use as a control. If any significant pH change occurs on addition of the product to be examined, restore the liquid medium to its original pH value by the addition of a solution of either sodium hydroxide or hydrochloric acid. On the first, second or third day after inoculation subculture each liquid culture by inoculating each of two plates of each solid medium with 0.2 ml and incubating at 35°C to 38°C, aerobically and microaerophilically, for not less than 21 days. Repeat the procedure on the sixth, seventh or eighth day and again on the thirteenth or fourteenth day of the test. Observe the liquid media every 2 or 3 days and if any colour change occurs subculture immediately. Observe solid media once per week. If the liquid media show bacterial or fungal contamination, repeat the test. If, not earlier than 7 days after inoculation, not more than one plate at each stage of the test is accidentally contaminated with bacteria or fungi, or broken, that plate may be ignored provided that on immediate examination it shows no evidence of mycoplasma growth. If, at any stage of the test, more than one plate is accidentally contaminated with bacteria or fungi, or broken, the test is invalid and must be repeated.

Include in the test positive controls prepared by inoculating not more than 100 CFU of suitable species such as *M. orale* and

*M.pneumoniae*.

At the end of the incubation periods, examine all the inoculated solid media microscopically for the presence of mycoplasmas. The product passes the test if growth of mycoplasmas has not occurred in any of the inoculated media. If growth of mycoplasmas has occurred, the test may be repeated once using twice the amount of inoculum, media and plates; if growth of mycoplasmas does not occur, the product complies with the test. The test is invalid if the positive controls do not show growth of the relevant test organism.

### RESULTS

None of 96 samples were positive by culture methods while the results of positive and negative controls and performance tests for culture media were acceptable and the test was valid.

### DISCUSSION

In 1898 Nocard and Roux reported the cultivation of the causative agent of contagious bovine pleuropneumonia (CBPP). The disease is caused by *M. mycoides* subsp. *mycoides* SC (small-colony type), and the work of Nocard and Roux represented the first isolation of a mycoplasma species. Cultivation was, and still is difficult

because of the complex growth requirements. The name *Mycoplasma*, from the Greek mykes (fungus) and plasma (formed), was proposed in the 1950's, replacing the term pleuropneumonia-like organisms (PPLO) referring to organisms similar to the causative agent of CBPP (Edward and Freundt, 1956). It was later found that the fungus-like growth pattern of *M. mycoides* is unique to that species. Eaton and colleagues cultured the causative agent of human primary atypical pneumonia (PAP) or 'walking pneumonia.' This agent could be grown in chicken embryos and passed through a filter that excluded normal bacteria. However, it could not be observed by high magnification light microscopy, and it caused a pneumonia that could not be treated with the antimicrobials sulphonamides and penicillin (Eaton, et al., 1945a). Control of Vaccines prepared by cell culture and the same biologic products for preventing of mycoplasmas is essential. Detection techniques include culture method using specific broth and agar with the specific procedure as described in this study, ELISA, PCR and staining with a DNA stain including DAPI or Hoechst.

### REFERENCES

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