Archives of Razi Institute, Vol. 64, No. 2, December (2009) 109-114

Short Communication

Using PCR and culture methods for *Mycoplasma* testing in poliomyelitis vaccine

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Received xx Oct 2009; accepted xx Apr 2009

ABSTRACT

Mycoplasma contaminants can be considered important not only for their roles as pathogens but also they may indicate that insufficient care has been taken during vaccine manufacture or quality control. The purpose of this study was to test the poliomyelitis vaccines for *Mycoplasma* by culture and polymerase chain reaction (PCR) methods. During 2008 to 2009, a total of 47 lots of oral poliomyelitis vaccines were produced by Razi Vaccine and Serum Research Institute (RVSRI) in Iran. They were evaluated by culture and PCR for detection of *Mycoplasma*. In culture method, PPLO broth and PPLO agar medium were used and in PCR method, universal primers that were selected from the region 16S ribosomal RNA of *Mycoplasmas* were applied. In culture method, no changes on pH and color in PPLO broth tubes and no colony growth on PPLO agar plates were seen. In PCR method, *Mycoplasma* DNA was not detected in any of the tested vaccines. It was concluded that the current culture method for *Mycoplasmas* is reliable to detect viable *Mycoplasmas* in oral poliomyelitis vaccines, but our results confirmed the use of PCR assay as an efficient supplement to culture method.

***Keywords*:** Poliomyelitis vaccine, *Mycoplasma*, Culture, PCR

INTRODUCTION

Viral vaccines are generally produced in cell lines of diploid cell strains (MRC-5), chick fibroblast, embryonated eggs, continuous cell lines (Vero) and occasionally live animals. Contamination of cell lines by *Mycoplasma* is a well documented phenomenon, large scale surveys revealing contamination rates of 5 to 50% (Thornton 1986, Barile *et al* 1973, McGarrity *et al* 1984). The contamination may arise from the original tissue or the serum used in the culture medium (Barile *et al* 1973, Stipkovits *et al* 1975), or from personnel handling the Cultures. Once established, cross contamination in the laboratory promotes the widespread dissemination of the *Mycoplasma* throughout other cell cultures. Contamination by *Mycoplasma* may not be detected on simple inspection even when present at high

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concentrations, as it may cause no cytopathic effect or turbidity (Thornton 1986). The use of antibiotics is permitted during vaccine manufacture because of the large scale of virus growth involved and the economic consequences that would ensue following casual bacterial contamination. However, the suppression of bacterial growth disguises and even encourages breakdown in aseptic technique, hence allowing opportunities for invasion by *Mycoplasmas* (Thornton 1986). Simple and sensitive tests such as PCR are now available to detect contamination by *Mycoplasma*. However these may not yet be in routine use by quality control laboratories, so there is potential for the presence of *Mycoplasma* in live virus vaccines. Various workers have reported the presence of *Mycoplasma* in vaccines (Jurmanova *et al* 1986, Thornton 1986). In Iran, oral poliomyelitis live viral vaccines are produced. These vaccines are manufactured by using cell strain (MRC-5) cultures. The sterility test for *Mycoplasmas*, which are common contaminants of cell cultures, is required at each single harvest, as well as for the virus pool, and for the bulk suspension (Sasaki *et al* 1996a). Although many new methods to detect *Mycoplasmas* in biological samples have been developed in the past two decades, the culture method plays a key role in the sterility test for *Mycoplasmas*. The culture methods used in Iran and other countries are essentially the same as those recommended by the WHO Expert Committee on Biological Standardization (WHO 1973). Although the direct culture method has been found to be very sensitive, the method is time consuming (requiring at least 24 days), laborious and costly. The PCR has been developed which enables detection of a variety of micro-organisms. Several attempts to detect *Mycoplasma* contamination of cell cultures by PCR have been reported (Sasaki *et al* 1996b). *Mycoplasma* contaminations cause many problems in the area of 'new-technology' products, where cell lines are used for preparing monoclonal antibodies or genetically-engineered pharmaceuticals and vaccines (Thornton 1986). Our purpose was to use PCR and culture methods for *Mycoplasma* testing in poliomyelitis vaccines.

MATERIAL AND METHODS

Samples. During 2008 to 2009, a total of 47 lots of oral poliomyelitis vaccines were derived from 2 final bulks and 9 harvest produced by Razi Vaccine and Serum Research Institute in Iran. The samples were provided by the manufacture for the sterility tests and were kept at-60°C or lower until subjected to sterility testing for *Mycoplasmas* (Sasaki et al 1996a).

Culture method. The first, Membrane filtration method Poly vinylidene fluoride (PVDF) (Millipore Co.) of 45 µm diameter and 0·1 µm pore size were used. All the samples were cultured at 37°C for 24–48 h in broth medium (pH 7.6-8) which contained 10 ml of basal medium [2.1% (W/V) PPLO broth (Biolife), 0.01% (W/V) glucose, and 0.002% (W/V) phenol red] supplemented with additive [20 ml of equine serum, 1ml of 25% (W/V) yeast extraction, 1 ml of 1% (W/V) b-nicotinamide-adenine dinucleotide, 0.5 ml of 4% (W/V) thallous acetate and 0.5 ml of 50000 units of penicillin G potassium]. After 24-48h, 0.2 ml of all the broth medium were cultured in plate which contained 10 ml of 3.5% (W/V) PPLO agar medium (Biolife) (pH 7.6-8) at 37°C for 21 days. According to European Pharmacopoeia 2005 negative control was PPLO broth medium and also PPLO agar plates. Standard positive controls were *Mycoplasma Pneumonia* (NC10119), *Mycoplasma Orale* (NC10112) and *Mycoplasma galisepticum* (NC10115). All the PPLO broth medium tubes and PPLO agar medium plates were seen every day for pH and color changing in broth media and *Mycoplasma* colony appearing on the agar media.

DNA extraction. DNA extraction on 47 final products (vaccine vials) were carried out. Negative

and positive controls were DDW and standard strain of *Mycoplasma galisepticum* respectively. DNA was extracted by phenol/cholorophorm method.

Phenol/Cholorophorm method. Vaccine samples were cultured on PPLO broth for 24 h in order to primary *Mycoplasma* propagating and enrichment. and then 500 µl of cultured vaccine was placed in a 1×5µl Eppendorf tube and microcentrifuged at 13000 rpm for 15 min. 100 µl of lysis buffer was added to 100 µl of precipitated and tube was placed in a 56°C bath for 4 hours. Then 200 µl saturated phenol was added and tube was centrifuged at 13000 rpm for 20 min. Upper phase was transferred to another tube and equal volume of mixed phenol/cholorophorm (1:1) was added. After centrifuged at 13000 rpm for 20 min the aqueous phase was transferred to another tube and added equal volume of pure cholorophorm and was centrifuged at 13000 rpm for 5 min. Upper phase was transferred to a new tube and Mixed with 1/10 volume of acetate sodium (3M) and were precipitated in -20°C refrigerator with 2 fold volume of cool and pure ethanol (20 min). Then tube was centrifuged at 13000 rpm for 15 min. 200 µl 70% ethanol was added and tube was centrifuged at 13000 rpm for 5 min. The DNA was dried and resuspended in DDW at 4°C and used for PCR (Kojima *et al* 1997).

Primers. In this study two primers: forward 5'-GCTGCGGTGAATACGTTCT-3' (19 bases) and reverse 5'-TCCCCACGTTCTCGTAGGG-3' (19 bases) which have been already designed by Kojima *et al* (1997) were used. They flank and amplify a 163bp regain of 16S rRNA gene of *Mycoplasma* genus.

PCR Parameters and Optimization. DNA amplifications were carried out in a total volume of 35.25 µl containing 17.5 µl DNA, 0.1 µl of each primers, 0.5 µl dNTP mix (10mM) {Cinnagen Inc.}, 4 µl Mgcl2 (25mM) {Cinnagen Inc.}, 2.5 µl PCR buffer (10X) {Cinnagen Inc.}, and 0.25 µl Taq DNA polymerase (5 unit/ µl) {Cinnagen Inc.}. Reaction mixture were thermocycled 30 times beginning with an initial denaturation step of 5 min at 94°C. the temperature and time profile of each cycle was follows: 94°C for 1min (Denaturation), 56°C for 1 min (Annealing) and 72°C for 1 min (Extension), PCRs were finished with a final extension step at 72°C for 5 min. PCR products were stored at 4°C. PCRs were carried out using two programmable thermal cyclers (Primus and Mastercycler gradient). Positive and negative controls were included in all tests. In order to determine the accuracy of the primers, various species *of Mycoplasma* (*M.galisepticum, M. pneumonia* and *M. orale*) and some other bacteria (such as *Salmonella spp*.) were tested by this PCR.

Amplicon electrophoresis. Eight µl aliquot of each PCR products was mixed with 2 µl loading buffer (6X). The PCR products and 100bp DNA ladder were then separated by electrophoresis on 1% agarose gel and stained with 0.5 µl /ml ethidium bromide (100 volts for 1 hr) following UV Transillumination.

Specificity and Sensitivity of the test. The sensitivity and specificity of the PCR used by a modified method of Kojima *et al* (1997). The sensitivity of the PCR procedure was carried out by testing the DNA extractions of serial dilutions of bacterial suspension of *Mycoplasma gallisepticum*. The specificity of the PCR was confirmed by testing DNA extracted of *Salmonella spp*. and water.

RESULTS AND DISCUSSION

In tested samples (47 vaccine lots)**,** after 4-7 days and even 24 days no changes in pH or color has occurred in PPLO broth medium tubes and after 24 days no colony growth of *Mycoplasma* on PPLO agar medium plates was observed. In positive controls PPLO broth medium was changed from red to yellow color and acidic pH (Figure 1).

In PPLO agar medium *Mycoplasma Pneumonia* and *Mycoplasma Orale* growth colonies were observed (Figure 2). The *Mycoplasma* PCR exhibited a sensitivity equivalent to 10 cfu/ml and did not react with DNA template of *Salmonella entritidis*.

Figure 1. Color changes of negative and positive control in PPLO broth medium.

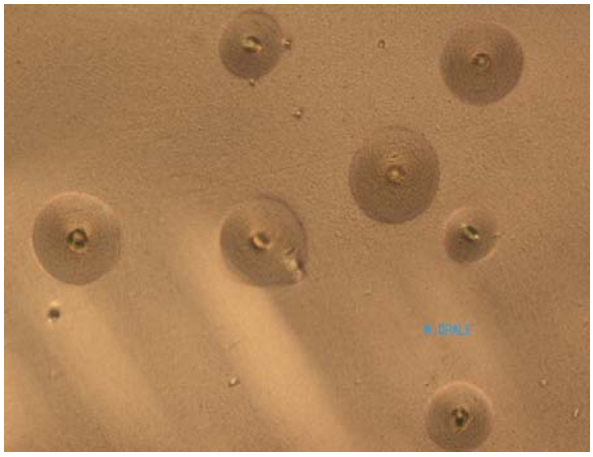

Figure (2). Colonies of *Mycoplasma orale* on the PPLO agar (positive control) (X40).

In positive controls the DNA fragment, approximately 163 bp, was amplified and all of the tested samples (47 vaccine lots) and negative controls turned out to be PCR negative (Figure 3).

Therefore all tested vaccines showed negative results in the PCR. The detection of *Mycoplasma* contamination provides an insight into manufacturer's production and quality control procedures. The *Mycoplasma* may have been presented in the substrates or virus seeds and such materials require careful screening before use. One of the important disadvantages of culture method for *Mycoplasma* detection is some false negative results. Because it must be ensured that the culture media are able to support the growth of a small inoculum of a range of *Mycoplasma* species which have not been adapted to growth in the laboratory. Moreover, because the presence of *Mycoplasma* generally results from a breakdown in aseptic technique or use of unsatisfactory ingredients at some stage of vaccine development or production, it suggests the possibility of other forms of contamination which are associated with the same sources as the *Mycoplasma.* Bovine serum which has been produced or treated unsatisfactorily or tested inadequate, may contain in addition to *Mycoplasma*, viruses, fungi, bacteria, bacteriophages and endotoxin (Boone *et al* 1972, Orr *et al* 1975). However, live viral vaccines are produced; minimal concentrations of antibiotics such as penicillin or other beta-lactam antibiotics are generally added. Most live viral vaccines show growth-inhibiting activity against *Mycoplasma*, and it is difficult to detect *Mycoplasma* by means of the current sterility test (culture method) for *Mycoplasma* (Sasaki *et al* 1996a). Antibiotic treatment is sometimes successful, but resistant strains may emerge (McGarrity *et al* 1984). As judged from the effects of antibiotics on the growth of *Mycoplasma*, even if *Mycoplasma* contamination does occur during vaccine production, the possibility of survival or active growth of the contaminating *Mycoplasma* seems to be very low. Therefore, when the sterility test with membrane filtration for *Mycoplasma* is performed for live viral vaccines containing antibiotics, it may be sufficient

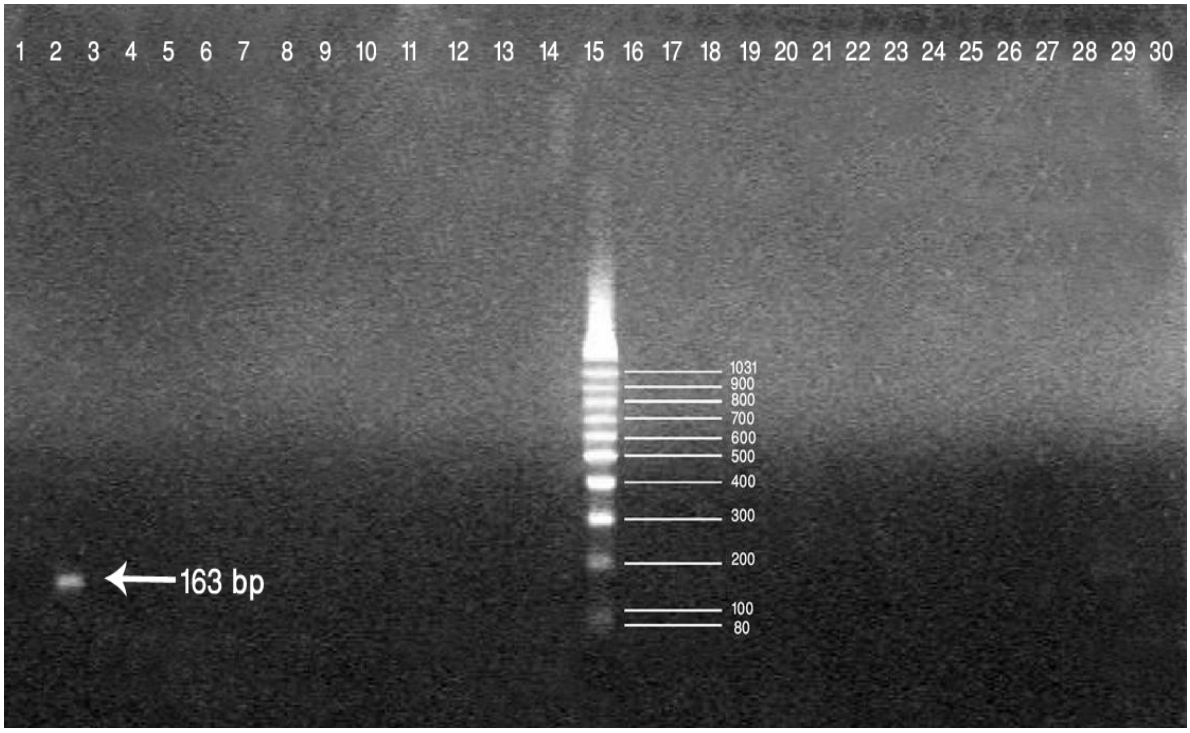

Figure 3. PCR electrophoresis analysis in %1 gel agarose. *Lane 1*: Negative control (PPLO broth). *Lane 2:* Positive control (163bp band) (*Mycoplasma galisepticum*). *Lane 15:* Marker (100bp DNA ladder) and *Lane 3 to 14* and *16 to 30* are negative samples.

to apply this test to a single processing step, such as the single harvest stage (Sasaki *et al* 1996a). Other methods (Stanbridge *et al* 1977) include enzyme studies, nucleic acid analysis, electron microscopic observation, fluorescent antibody staining, and measurement of uridine to uracil uptake ratio or staining with fluorescent DNA-binding compounds. These techniques can be useful supporting methods for detecting that *Mycoplasma* which cannot be readily grown in culture media (Thornton 1986).

Therefore, to investigate whether *Mycoplasma* contamination has occurred in the production of live viral vaccines, the authors employed the PCR technique. Based on *Sasaki* results of the PCR and the effects of antibiotics on the growth of *Mycoplasma*, even if *Mycoplasma* contamination had occurred in the virus propagation stage in the cell cultures, the possibility of active growth and survival of *Mycoplasma* in the vaccine may be very low (Sasaki *et al* 1996b). The reactivity of the PCR primers was confirmed by Kojima *et al* (1997). This technique can be useful supporting methods for detecting that *Mycoplasma* which cannot readily be grown in culture media (Kojima *et al* 1997, Rolfs *et al* 1992, Ehlen *et al* 1989). In conclusion, as both culture and PCR methods are accurate identification of *Mycoplasma* contamination but the culture method not only can be costly and time consuming but also may show some false negative results. In other hand, this report demonstrates that the PCR method is rapid, reliable and simple method for the detection of *Mycoplasma* contamination in several oral poliomyelitis live virus vaccines.

References

Barile, M.F., Hopps, H.E., Grabowski, M.W., Riggs, D.B. and DelGiudice, R.A. (1973). The identification

and sources of *Mycoplasmas* isolated from contaminated cell cultures. *Science* 225: 251-254.

Boone, C.W., Mantel, N. and Caruso, T.D. (1972). Quality control studies on fetal bovine serum used in tissue culture. *In Vitro* 7: 174-189.

Ehlen, T. and Dubeau, L. (1989). Detection of ras point mutations by polymerase chain reaction using mutation-specific, inosine-containing oligonucleotide primers.*Biochemistry Biophysics Research Community* 160: 441-447.

European Pharmacopoeia (2005). *Test for Mycoplasmas*. Pp: 1: 2.6.7.

Jurmanova, K. and Mackatkova, M. (1986). Detection of *Mycoplasma* in cell cultures and biologicals. *Archive Experimental Veterinary Medicine* 40: 136-140.

Kojima, A., Takahashi, T., Kijima, M., Ogikubo, Y., Nishimura, M., Nishimura, S., Arasawa, R. and Tamura, Y. (1997). Detection of *Mycoplasma* in avian live virus vaccines by polymerase chain reaction. *Biologicals* 25: 365-371.

McGarrity, G.J., Vanaman, V. and Sarama, J. (1984). Cytogenic effects of *Mycoplasma*l infection of cell cultures. *In Vitro* 20(1):1-18.

Orr, H.C., Sibinovic, K.H., Probst, P.G., Hochstein, H.D. and Littlejohn, D.C. (1975). Bacteriological activity in unfiltered calf sera collected for tissue culture use. *In Vitro* 11: 230-233.

Rolfs, A, Schuller, I. and Frickh, U. (1992). PCR: Principles and reaction components. In*: PCR: Clinical diagnostics and research*. Springer-Verlag, Berlin, Heidelberg. Pp: 1-18.

Sasaki, T., Harasawa, R., Shintani, M., Fujiwara,H., Sasaki, Y.,Horino, A., Kenri, T., Ishii, K. and Chino, F. (1996a). Evaluation of current sterility tests for human live viral vaccines. *Biologicals* 24: 51-55.

Sasaki, T., Harasawa, R., Shintani, M., Fujiwara, H., Sasaki, Y., Horino, A., Kenri,T., Asada, K., Kato, I. and Chino, F. (1996b). Application of PCR for detection of *Mycoplasma* DNA and pestivirus RNA in human live viral vaccines.*Biologicals* 24: 371-375.

Stipkovits, L., Bodon, L., Romvary, J. and Varge, L. (1975). Direct isolation of *Mycoplasma*s and *acholeplasmas* from sera and kidneys of calves. *Acta microbiologica Academiae Scientiarum Hungaricae* 22: 45-48.

Stanbridge, E.J. and Schneider, E.L. (1977). The need for non-cultural methods for the detection of *Mycoplasma* contaminants. *Developmentof Biological Standard* 37: 191-194.

Thornton, D.H. (1986). A survey of *Mycoplasma* detection in veterinary vaccines. *Vaccine* 4: 237-239.

WHO (1973). General requirements for the sterility of biological substances (Requirements for biological substances No. 6). In: *WHO Expert Committee on Biological Standardization. twenty-fifth report.* Geneva, World Health Organization. 1973. Annex 4 (WHO technical report series. No. 530).