Vet.Med.J.,Giza. Vol.46, No.4 B (1998):773-783 .

THE SWIFT DETECTION OF MYCOPLASMA CONTAMINATION IN CELL CULTURES BY USING POLYMERASE CHAIN REACTION

BY

A. H. M. HUSSEIN; SEHAM, A. S. EL-ZEEDY; FADIA, S. H. FAHMEY AND A. M. DAOUD

Vet. Serum Vaccine Research Institute, Agriculture Research Center, Cairo, Egypt

SUMMARY

A total of 20 Samples (9 cultures, 7 sera and 4 medium samples) were used in this study. The stained culture samples showed pathological profile resembled that produced by Mycoplasmas. The cell aberrations, clumpings and the increased granularity came out as a result of the Myoplasma impairment influence on the amino acids, RNA and DNA synthesis of the cells which in turn reflected on their metabolic activities.

Using the ordinary culturing technique, only 3 cultures and one serum sample gave the characterestic Mycoplasma colonies 4-6 weeks post culturing. The same samples with two cultures and 2 serum samples more were positive with Mycoplasma Polymerase Chain Reaction (PCR), The test was performed and the results were obtained in the same day. The positive bands on Agarose Gel Electrophoresis were of three sizes. A 600bp band seen in rabbit kidney culture samples which might be contaminated with *M. orale* or *M. arginini.* The second band (620bp) was observed in VERO, CEF and MDBK cultures that most likely infected with *M. hyorhinis* or *M. fermentans.* The third band was 100 bp noticed in the positve serum samples that most probably contaminated with *Acholeplasma laidlauii* .

It could be concluded that PCR technique proved to be accurate, reliable and saving time & can be relied on to detect mycoplasma contamination of cell cultures .

Further investgations should be carried out in order to make use of the set in screening of either conventionally produced or imported TC and egg adapted vaccines .

INTRODUCTION

Mycoplasmas are the smallest and simplest procaryotes capable of self replication.

The genome consists of a circular double stranded DNA molecule having 5x108 doltons.

The unique property that characterizes the organisms from the true bacteria is the absence of cell wall. Mycoplasmas belong to family Mollicutes, they are ubiquitous organisms occurring as parasites or saprophytes (Razin, 1985).

Mycoplasmas are one of the most serious and wide spread problems encountered by workers utilizing cell cultures for virus propagation. They alter various metabolic activities of the cells, reduce cellular growth rate, force diploid cell lines into early senescence, produce chromosomal aberration and induce interferon, Hence they decrease the synthesis of viruses in cell culture (Mc Garrity, 1982 and Del Gwdice and Gardella, 1984).

In some instances mycoplasma contaminants produce an extensive CPE in cell cultures, but in many cases they produce few or no observable changes, so they represent a major intricacy in serologic antigens or immune sera preparations (Barile, 1973). The concentration of Mycoplasma can easily reach 107-108 C. F. U. per milliter with out inducing turbidity (Hog et. al., 1989). The mycoplasma contaminants of tissue culture are not derived from the original tissues, but more likely are introduced in the Laboratory. The majority of mycoplasma strains contaminating cell cultures are human (*M. orale*), bovine (*Acholeplasma laidlauii* and *M. arginini*) or porcine (*M. hyorihinis*) species (Mc Garrity et al., 1984).

The traditional methods for isolation of mycoplasmas in cell cultures are rather fastidious and inaccurate.The sensitivity of culture procedures in detecting small numbers of contaminating organisms (e. g. from bovine sera) could be severely altered unless the so-called large specimen broth culture method is applied (Barile, 1973).

The Mycoplasma PCR primer set manufactured by Stratagene Corporation has furnished rapid and meticulous system for detection of cell culture mycoplasma contaminants even from 1ul of culture supernatant. Cell growth inhibiting or weak mycoplasma infection can also be detected by harvesting and testing the contaminated cells. Moreover, the type of fingerprint seen can be used diagnostically for strain identification.

The aim of our work is to recognize the mycoplasma contaminated cultures by using concise, precise and reliable method aiming to produce mycoplasma free tissue culture biproducts.

MATERIALS AND METHODS

1- Samples:-

A total of nine culture samples representing chicken embryo fibroplast (CEF) either from commercial eggs or from specfic pathogen free eggs (SPF), Duck embryo fibroplast (DEF), Rabbit kidney (RK) (As primary cultures) as well as African Green Monkey kidney (VERO), Baby hamester kidney (BHK-21) and Madin darby bovine kidney (MDBK), (as cell lines) were histologically examined and kept at 4oC till

screened either with the conventional culturing technique or with Stratagene primer set. Four minimum essential medium samples in addition to seven serum samples (Foetal calf or newly born calf sera either imported or locally produced) were also subjected to the same examinations .

2- Mycoplasma positive strain :

Mycoplasma gallisepticum F. Vax -MG, (Schering Plough Animal Health, USA) after being passaged via the yolk sac of 7 day old specific pathogen free embryonated chicken eggs.

3- Mycoplasma conventional culturing System:

3-1.Mycoplasma broth Medium (Frey,s Medium):

It was prepared in accordance with the recipe given by Frey,s et al., (1968).

3-2. Mycoplasma Agar (Adler medium):

It was processed as the method described by Adler et. al., (1958).

4- Mycoplasma PCR Primer Set

Materials provided by Stratagene imply PCR Primers (Stratagene company U. S. A), internal and positive controls which all were in a lyophilized state, In addition to Strataclean resin and rehydrated buffer [5mM Tris-HCl and 0.1 mM EDTA(pH 7.5)] . The PCR Primres and the controls were be rehydrated in 200 and 100ul of rehydration buffer respectively. The storage temperature of the rehydrated materials is -20°C.

5- Internal and Positive Controls Strategy :

The internal control verifies polymerase activity by serving as an alternative substrate for the PCR primers. In the absence of mycoplasma infection amplification in the internal control will predominate and a 420 bp product will be visualized upon analysis of PCR products.

In contrast, template derived from infected cells will serve as a competitive inhibitor of internal control. So, the PCR signal caused by the internal control will diminish or disappear, coincident with the appearance of PCR products diagnostic of mycoplasma infection. the positive control which is noninfectous genomic DNA from *M. orale* is used to demonstrate the expected size of mycoplasma specific PCR product .

6- Template preparation :

100ul of cell free-supernatant harvested from cell cultures were boiled for 5 minutes then purified with Strataclean resin to eliminate proteins inhibitory to PCR. The extract was centrifuged and diluted 1:10 with UV irradiated water. Template prepared in this manner is stable for several days if stored at 4oC.

7- Polymerase chain Reaction (PCR) :

The master mix per each reaction should include 10mM of tris Hcl (pH8.3) 2.5mM MgCl2, 200uM of each dNTP, 50pM each primer and 2u of Taq DNA polymerase in addition to 2ul of internal control, but if the template was not added, 2ul of H20 were used instead. The mixture was adjusted at 40ul in a thin walled tube (Perkin Elmer Cetus, Norwalk, CT. USA). 10ul of diluted template, 10ul of water, and 10ul of positive template (either from the standard strain or the

Stratagene positive control) each added to the tested Sample, -ve and +ve control respectively. Each tube was overlaid with 60ul of light mineral oil.

8- PCR Program:

The following PCR program (table 1) has been designed to detect all strains of mycoplasma .

9- Analysis of PCR Results :

ThePCR products are analysed using standard agarose gel electrophoresis (3% w/v). Sambrook, et. al., (1989). This ensures good differentiation between PCR products associated with the internal control template and those due to mycoplasmas. Fig.(1) demonstrates typical fingerprint results for five strains of mycoplasma commonly associated with cell culture infection.

Segment	Cycles	Temperature	Time
1	1	94°C 55°C	5 minutes 1 minutes,45 seconds
2	3	72°C 94°C 55°C	3 minutes 45 seconds 1 minutes,45seconds
3	40	72°C 94°C 55°C	3 minutes 45 seconds 45 seconds
4	1	72°C	10 minutes

Table (1): Showing the pest PCR program for mycoplasma detection in tissue Cultures .

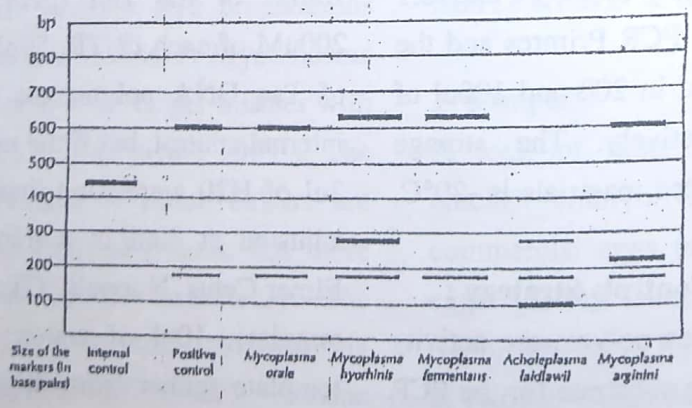

Fig. 1:Typical ferigerprints for five strains of Mycoplasma commonly associated with cell culture infection .

RESULTS

I- Histological examination of cultures suspected to be contaminated with Mycoplasmas:-

After being fixed and stained with HE or crystal violet, several cultures exhibited various aberrations that were in the from of cell necrosis (thread like cells) with multiple black dots along (Fig. 2) while others showed thick and dark cell clumps (Fig. 3) .

II The Conventional Culturing Techniques:-

The above mentioned samples as well as the media and sera incriminated were subjected to Mycoplasma broth for a week followed by Mycoplasma agar for four weeks either in aerobic or anaerobic environments. The positive samples listed in (table 2) revealed microscopic fried egg-colonies of about 0.5mm in diameter (Fig. 4) .

III- Polymerase chain Reaction Results:-

The results of PCR were illustrated in (table 2). The samples that gave two bands (420 and 600 or 620 bp) were considered positive for Mycoplasmas (Fig. 5) while those exhibited merely one band (420bp) were considered negative (Fig. 6) .

samples displayed 100bp band were also considred positive for Acholeplasma laidlauii (Fig.7) .

DISCUSSION

Stratagene has designed a universal pair of primers for exponential amplification of the spacer regions between the 16 S and 23 S r RNA genes of the major mycoplasma species contaminating cell cultures. This two - step PCR method is reported to be very sensitive and it could detect at least 103 c.f.u. / ml of mycoplasmas in cell culture medium (Quinn et al., 1994) .

The deformities seen on culture samples have drown our attention to the possibility of their being contaminated with mycoplasmas . Such deformities might have developed as a result of the direct replication of mycoplasmas that influences the cellular metabolism involving DNA, RNA and protein synthesis (Mc Kenzie, 1992).

Only three out of the nine culture and one out of the seven serum samples gave mycoplasma colonies on M.agar, The specific colonies appeared 4-6 weeks post-cultivation. While four culture and three serum samples were positive with myoplasma PCR primer set. The reaction was performed and the results were obtained in the same day. The fingerprints of the positive samples were of three sizes. A 600bp band observed in rabbit kidney primary culture samples which might be contaminated with *M.orale* or *M.arginini*. The second band was about 620bp noticed in CEF, VERO and MDBK cultures that were most probably contaminated with *M.*

Serial No.	Samples	Samples No.	Conventional culturing technique		PCR technique			
					Internal cont (420bp)		PCR results	
			+	–	+	–	+	–
1	Minimal Essential Medium (MEM).	4	0	4	4	0	0	4
2	Chicken embryo fibroplast. from commercial eggs.	1	1	0	1	0	1	0
3	Chicken embryo fibroplast. from SPF eggs.	1	0	1	1	0	0	1
4	Duck embryo fibroplast	1	0	1	1	0	0	1
5	Rabbit Kidney cells.	2	0	2	2	0	2	2
6	Vero cells.	2	1	1	2	0	1	1
7	BHK	1	0	1	1	0	0	1
8	MDBK	1	1	0	1	0	1	0
9	Serum	7	1	6	7	0	3	4

Table (2): Results of Samples screening either with conventional or PCR technique .

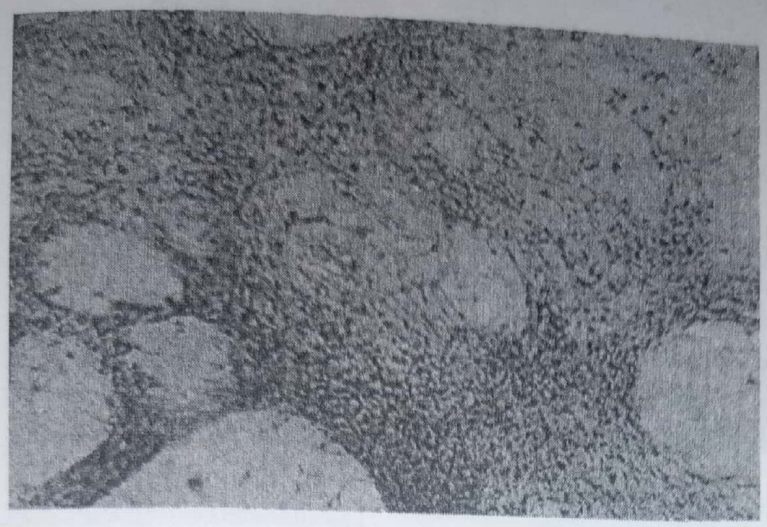

Fig. (2): Mycoplasma infected CEF cells exhibiting cell necrosis and increase in Their granularity H & E (x 40) .

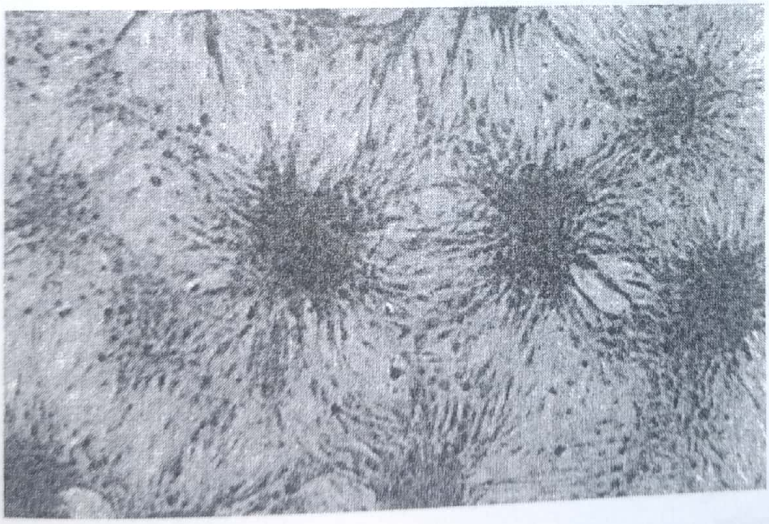

Fig. (3): VERO cells contaminated with Mycoplasma showing cell clumpings. H & E (x 100).

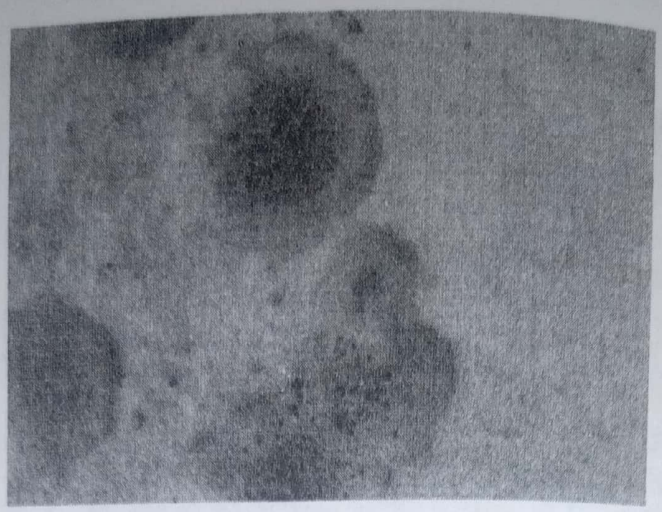

Fig. (4): Mycoplasma colonies as developed on M. agar (Adler meduim) x 40 .

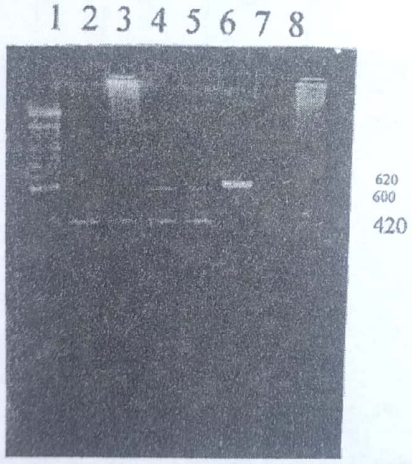

Fig. (5): PCR fingerprints (reaction 1):

Lane 1: 100bp DNA Marker
Lane 2: CEF + Internal control
Lane 3: VERO + Internal cont.
Lane 4: RK + internal cont.
Lane 5: RK + internal cont.
Lane 6: + ve Mycoplasma Sample (F. strain).
Lane 7: +ve control .
Lane 8: _ve control .

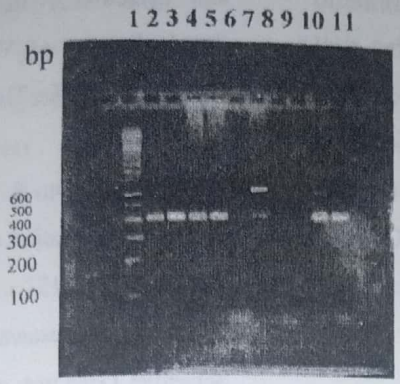

Fig. (6): PCR fingerprints (reaction-2) :

Lane 1: 100 bp DNA Marker (Gibco) BRL.
Lane 2: MEM Without Serum+int. cont.
Lane 3: MEM with Newly born calf serum+int. cont.
Lane 4: CEF (SPF)+int. cont. (Media with Serum).
Lane 5: CEF (SPF)+int. cont. (Media withont serum).
Lane 6: CEF+ .
Lane 7: MDBK+int. cont.
Lane 8 & 9: -ve cont.
Lane 10 & 11: + ve control +int. cont.

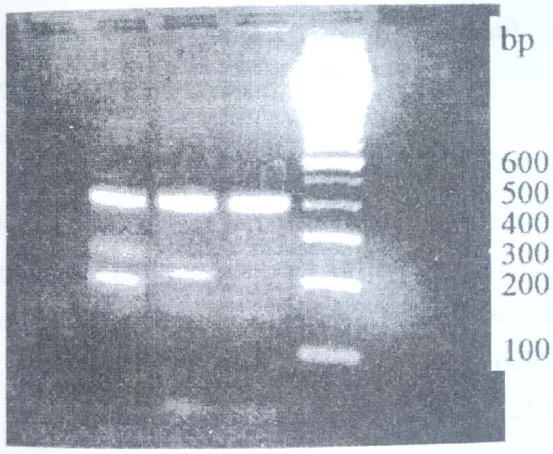

Fig. (7): PCR fingerprints (reaction-2) :

Lane 1: serum sample 5 + int. cont.
Lane 2: serum sample 6 + int. cont.
Lane 3: serum sample 7 + int. cont.
Lane 4: 100 bp DNA Marker .

hyorhinis or M. fermentans while the third band was 100 bp seen in positive serum samples that were more likely infected with *Acholeplasma laidlauii* (Stratagene catalog, 1997). Coincidently, Haraswa et. al. (1993), as well as Mengdong and Charles (1993), used successfully the PCR for detection of mycoplasma either in cell cultures or in viral stocks .

The forementioned data gave a clue for the time saving, the ultimate senstivity and the outstanding accuracy of PCR primer set in detection of mycoplasmas over the ordinary methods. The results also emphasized the capability of the test to distinguish the mycoplasma species from their fingerprints. Rather than being able to detect mycoplasmas as cell culture contaminants, it presumably has the potentiality to survey tissue culture and egg passaged vaccines for mycoplasmosis. This in turn needs further investigations .

REFERENCES

Adler, H. E; Fabricant, J.; yamamato, R. and Besg, J. (1958): Symposium on chronic respiratory disease of poultry isolation and Identification of P. P. L. O. of avian origin . Am. J. Vet Res., 19:440-446 .

Barile, M. F. (1973): Mycoplasma contamination of cell cultures : Mycoplasma culture interactions. In: Fogh. J. ed. " Contamination in tissue culture". New york : Academic Press. 131-172 .

Del, Giudice, R. A. and Gardella, R. S. (1984) : Mycoplasma infection of cell culture: effects, incidence and detection . In Lovine, E. M. ed. Uses and Standardization of Vertebrate cell cultures in Vitro . Gaithersburg MD: Tissue Culture Association, Monogr. 5, 104-124 .

Frey, M. C.; Hanson, R. P. and Anderson D. P. (1968): A medium for isolation of avian Mycoplasma . Am. J. Vet. Res., 29:2164-2171 .

Hara sawa, R.; Mizusawa, H. and Koshimizu, K. A. (1993): Sensetive Detection of Mycoplasma in cell Cultures by using two step Polymerase chain Reaction. In Kahane I. : Aoni, A. eds Rapid Diagvosis of Mycoplasmas New York : plenum Press (1993).

Hay, R. D.; Macy, M. L. and Chen, T. R. (1989) : Mycoplasma infection of Cultured cells Nature. Vol. 339 : 487 - 488 .

Joklik, k. Walfgang; Willett, P. Hilda; Amos, B. and Wilfert, M. Catherine, (1988): " Zinsser Microbiology ". Nineteenth Edition - Prentice - Hall International . Inc. 617-623 .

Mangalog Hu and Charles Buck (1993) : " Detetion of Mycoplasma Contamination in Viral Stocks by polymerase chain Reaction techique " . J. Tiss. Cult. Meth., 15 : 153 - 160 .

Mc Garrity, G. J.; Steiner, T. and Gamon, L. (1984): Prevention, Detection and control of Mycoplasmal infections of cell cultures, In :Kilbey, B. J.; Legator, M.; Nichols, W. and Ramel, C. eds." Handbook of mutagenicity test procedures " 2nd ed. New york Elsevier, 823-839.

Mc Garrity, G. J. (1982): Detection of Mycoplasmal infection of cell cultures. Inc. Maramoresch K. ed. "Advances in cell culture". 2nd ed. New York : Academic Press, 99 - 131 .

Mc Kenzie, D. (1992) : strategies, 5 : 65 .

Quinn, P. J.; Carter, M. E.; Markey, B. K. and Carter, G. R.

(1994): " Clinical Veterinary Microbiology " .

Razin, S. and Tully, J. G. (eds) (1983): "Methods in Mycoplasmology".Vol. I.Mycoplasma Characterrization . New York, Acaclemic Press .

Sambrook, J.; Fristch, E. F. and Maniatis, T. (1989): "Molecular Cloning : A loboratory Manual" 2nd ed. Cold Spring Harbor, Harbor laboratory, NY. USA.

Stratagene Catalog, (1997 - 1998) : " Stratagene Cloning Systems ". 11011 North Torrey Pines Road, LaJolla, CA, 92037 : U. S. A.