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PROPHYLACTIC CONTROL OF MYCOPLASMA CONTAMINATION IN STARTING BIOLOGICAL MATERIALS USED IN VIRAL VACCINE PRODUCTION

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ABSTRACT

Mycoplasma contamination remains a major concern in the biopharmaceutical industry especially in tissue culture based viral vaccine and its presence and/or its endotoxin-like metabolites in the final products can result in pyrogenic responses ranging from fever and chills, to irreversible and fatal septic shock. This study was conducted by in vitro screening of mycoplasma in the different ingredients used in production of Foot and mouth Disease (FMD) vaccine using Polymerase Chain Reaction (PCR) using universal primers that are specific to the 16S rRNA region. Tested items include growth media, cell lines, trypsin, seed virus and working virus. Also the study evaluates the inhibitory effect of different concentrations of neomycin, kanamycin, gentamycin, polymyxin B and ciprofloxacin on mycoplasma contaminated cell lines. Our results showed that the prepared growth media, trypsin, seed virus as well as working virus were mycoplasma free and three tested cell lines were also free while another two lines were mycoplasma positive. The mycoplasma positive cell line are poorly grown in comparison with the free line using the same growth media and the virus yield from the apparently normal contaminated line was very low. Ciprofloxacin can be used for treating valuable cell line after 12 days in 25mg/L and after 18 days in 10mg/L. Ciprofloxacin plus regular antibiotic may keep the line sterile for prolonged time but treatment of contaminated cell line is not advisable. So, prophylactic control by strict personal hygiene and personal protective equipment (PPE) and adopt appropriate aseptic techniques is the core solution.

Key words: FMD, Mycoplasma, PCR. Vaccine, VSVRI .

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INTRODUCTION

Viral vaccines for veterinary use such as foot and mouth disease (FMD) are produced from cell cultures that are used as hosts for the proliferation of virus. Established cell lines such as baby hamster kidney (BHK-21) are commonly used for the large-scale production of vaccines in many countries. In the vaccine production process, the raw materials, including serum-containing cell culture media, need special attention. On top of their substantial costs, animal derived products can contain viruses, mycoplasma bacteria or prions, and therefore require special risk assessments by the supplier and the user **(Genzel *et al.*, 2006).**

Mycoplasmas are the smallest free-living microorganisms, being about 300 nm in diameter.They are bounded by a triple-layered membrane and, unlike conventional bacteria, do not have a rigid cell wall **(DSMZ, 2012).** Mycoplasmas are notorious contaminants of cell culture and can have profound effects on cell biology by depriving cells of nutrients and inducing global changes in gene expression. The main source of contamination is, in many cases, infection by previously-contaminated cell cultures that have been maintained and processed in the same laboratory **(Yueyang *et al.*, 2019)**. Mycoplasmas are spread by using laboratory equipment, media, or reagents that have been contaminated by previous use in processing mycoplasma-infected cell cultures. New cell-culture acquisitions should be quarantined, tested and guaranteed mycoplasma-free before introduction into the tissue-culture laboratory **(Young *et al.*, 2010).**

Although mycoplasma contamination does not create turbidity, factors such as cell line growth rates and viral vaccine production can be adversely affected.

The effects are due to nutrient deprivation, such as the depletion of amino acids, sugars, fatty acids, cholesterol or nucleic-acid precursors **(Barile and Rottem, 1993).** Mycoplasmas have been shown to produce severe cytopathic effects (CPE) characterized by stunted, abnormal growth and rounded, degenerated cells, apparently due to the promotion or inhibition of apoptosis so resulting in erroneous data and misleading publications **(Gerlic *et al.*, 2007)**. In a study by **Timenetsky *et al.*, (2006)**, M. orale, and M. hyorhinis were found to be the most frequent species in cell cultures. While **Uphoff and Drexler, (2002)** observed most of their cell lines were contaminated with M. fermentans.

At least two mechanisms responsible for decreasing viral yields in vitro have been identified. The cytolytic, fermenting mycoplasmas suppress metabolism and growth, resulting in a decrease in viral yields. Arginine-utilizing mycoplasmas decrease the titers of arginine-requiring DNA viruses by depleting arginine from the medium. Mycoplasmas may render cell cultures less sensitive to virus **(Manischewitz et al., 1975)**. Unfortunately, mycoplasmas are immune to common cell-culture antibiotics such as penicillins and streptomycin. Thus, they must be eliminated using specific anti-mycoplasma antibiotics **(Anthony et al., 2015).** Three classes of antibiotics that kill mycoplasma when used at relatively low concentrations, tetracycline, macrolides and quinolones. Tetracyclines and macrolides block protein synthesis by interfering with ribosome translation, whereas quinolones inhibit replication of mycoplasma DNA **(Cord et al., 2012)**.

Foot-and-mouth disease virus (FMDV) is a highly infectious and contagious RNA virus, infecting domestic and wild cloven-hooved animals **(Grubman and Baxt, 2004).** Vaccination campaigns are the way for eradicating FMDV in endemic countries and in case of an outbreak in free country, vaccination is a useful strategy for limiting virus spread **(Parida, 2009)**. FMDV vaccine production is tissue culture based and the most common production cell line is the mammalian baby hamster kidney cell (BHK21, clone 13), Preparation of master and working seed stocks for vaccine production process are the last steps of a successful virus adaption to cell culture systems **(Hassan, 2016)**. The present study aims to provide an effective way dealing with mycoplasma contamination in starting biological material especially cell cultures in order to prepare mycoplasma free vaccines.

MATERIALS AND METHODS

Samples

Fourty five samples were taken during the process of FMDV vaccine production in FMD department VSVRI from 5 separated BHK cell lines, growth media, trypsin, master seed and working virus for mycoplasma detection. Positive cell lines were subjected to treatment regime for 18 days during downstream vaccine production process in addition 48 samples are taken during and after treatment.

Cell culture

BHK cell lines growing in standard disposable plastic roller bottle (Greiner Bio-One, Austria) in basic growth media (MP Biomedical, France) supplemented with 10% mycoplasma-free, heat-inactivated newborn calf serum (Capricon scientific, Germany) supplemented by penicillin (100IU/mL), streptomycin (100µg/mL), gentamycin (80mg/L) under standard cell culture conditions (at 37°C, in 5% CO_2 and 90% humidity). Cultures were passaged according to standard procedures **(DSMZ, 2012).** Antibiotics were directly added to the culture not to the stored medium, antibiotic dilutions were freshly prepared for each treatment cycle. Cell counting were carried out to determine live and dead of cells (Countess® II FL, life technologies USA) by staining with Trypan Blue (0.4% w/v) **(Davis, 2002)**. At the end of the treatment periods, cells were washed twice and resuspended in fresh complete medium without any antimycoplasma antibiotics in order to enrich any residual mycoplasmas up to detectable levels or to get rid of any residual mycoplasmal DNA that may give a false-positive result.

Seed virus

Stock solution of FMDV serotype SAT2 obtained from FMD department, VSVRI was tested for mycoplasma and the effect of viral passage in clean culture on the virus titration were screened during treatment.

Mycoplasma Detection by PCR

Twenty ml trypsin 0.25% (MP, France) used for cell trypsinezation and 5 mL of the detached cell were centrifuged for 6 min at 3,000 rpm. The supernatants were discarded and the pellets were washed twice with phosphate-buffered saline (PBS), the pellets were resuspended in 100 µL PBS. The genomic contents were extracted by GeneJet genomic DNA purification kit according to manufacturer's instructions (Thermo Fisher Scientific, USA). The PCR reactions using Maxime™ PCR PreMix kit according to the manufacturer's instructions (iNtRON, Koria).Oligonucleotide universal primers according to **(Vahid *et al.*, 2009)** and **(Timenetsky *et al.*, 2006)** was forward GTG GGG AGC AAA YAG GAT TAG A and reverse GGC ATG ATG ATT TGA CGT CRT with expected PCR product 425 bp.

Antibiotics

Neomycin (200-500 mg/L, Sigma USA), kanamycin (200-500 mg/L, Sigma USA), gentamycin (80-200 mg/L, EIPICO, Egypt), polymyxin B (50-125 mg/L) and ciprofloxacin (10-25mg/L Amriya Egypt) were tested for their inhibitory effect on mycoplasma. Following the treatment with these reagents, cells were cultured in antibiotic-free medium for 2 passages prior testing for residual mycoplasmal contamination.

RESULTS

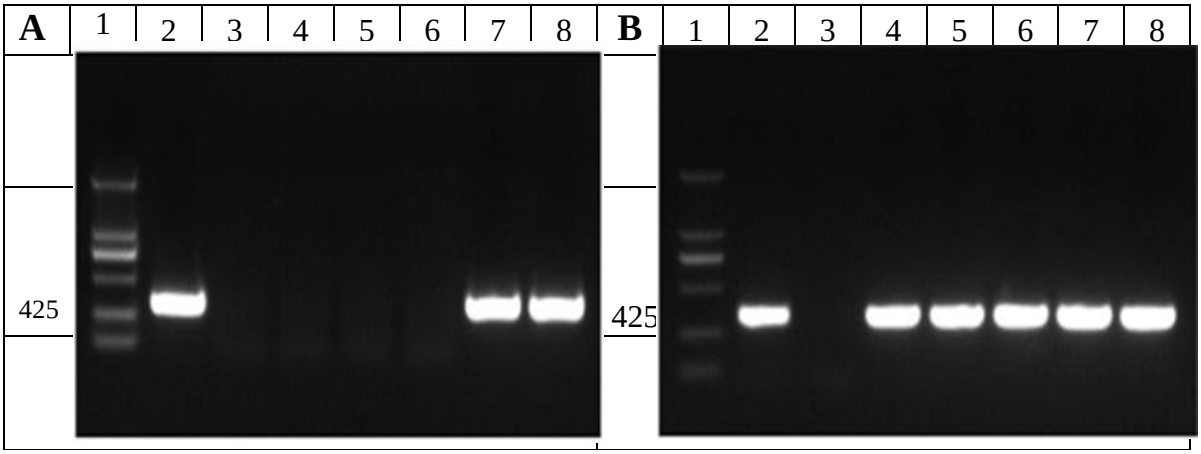
Results obtained from PCR-based detection methods of mycoplasmas showing that all samples taken from growth media, trypsin, master seed virus and working virus (harvested virus) were mycoplasma negative and all samples taken from 1st, 2nd and 3rd cells line were mycoplasma negative while 4th and 5th cell line were mycoplasma positive as shown in table 1.

Table(1):PCR identification of mycoplasma contamination

Samples	Tested material								
	Cell lines No					G.M	Trypsin	M.S	W.V
	1	2	3	4	5				
1	-	-	-	+	-	-	-	-	-
2	-	-	-	+	+	-	-	-	-
3	-	-	-	+	+	-	-	-	-
4	-	-	-	-	+	-	-	-	-
5	-	-	-	+	-	-	-	-	-

G.M Growth Media, M.S Master Seed,W.V Working Virus, - Negative, + Positive

Fig. 1: Agarose gel electrophoresis showing the results of PCR detection of mycoplasma.



Agar gel electrophoresis detecting mycoplasma contamination, positive samples are seen in the level of 425 bp. Photo A, lane 1, DNA ladder. Lane 2, positive control (known mycoplasma contaminated sample). Lane 3, negative control (sterile water). Lane 4, 5&6, media, trypsin and seed virus. Lane 7&8, positive cell lines.
Photo B, lane 1, DNA ladder. Lane 2, positive control. Lane 3, negative control. Lane 4, 5, 6, 7&8 positive cell line not respond to treatment.

Table 2: Effect of mycoplasma contamination on cell growth and virus titration

Mycoplasma free line				Mycoplasma contaminated line			
Tested time	CC		VT	Tested time	CC		VT
	Live	Dead			Live	Dead	
0	$9.5x10^4$	$5x10^4$		0	$9.5x10^4$	$3.5x10^4$	
24h	$0.8x10^6$	$0.2x10^6$		24h	$3.8x10^5$	$5x10^5$	
48h	$2.5x10^6$	$1.2x10^6$	00	48h	$7.2x10^5$	$2x10^6$	
	SC		$1x10^{8.3}$	72h	$0.9x10^6$	$0.5x10^4$	00
0	$9.3x10^4$	$6x10^4$			SC		$1x10^5$
24h	$1.2x10^6$	$0.6x10^6$		0	$7.5x10^4$	$5x10^4$	
48h	$2.6x10^6$	$1.5x10^6$	00	24h	$1x10^5$	$0.5x10^6$	
	SC		$1x10^{8.2}$	48h	$2.3x10^5$	$0.5x10^6$	
				72	$0.8x10^6$	$0.5x10^4$	00
					SC		$1.2x10^5$

CC: Cell count/mL, VT: Virus titration log_{10}/mL, SC: subculture

Each bottle was subcultured in 100ml for cell counting. The initial concentration of cells in sterile line was $9.5x10^4$/mL. After 24 h incubation there was an increase in the live cells population with a mean value of $0.8x10^6$. A complete monolayer was observed on the glass surface 48 h post incubation with $2.5x10^6$ live cell counts and $1.2x10^6$ dead cell and the bottle was subcultured, and the virus titration after 24h was $1x10^{8.3}$ in the 1st passage and $1x10^{8.2}$ in the 2nd passage. While in the mycoplasma contaminated line the initial concentration of cells was $9.5x10^4$/ml and after 24h there is a slow growth rate reached $3.8x10^5$ to $7.2x10^5$ and $0.9x10^6$ after 72h as a mean value with no confluence sheet was observed. When the bottle inoculated with FMDV, the virus titer was low reached $1x10^5$mL in the 1st passage and $1.2x10^5$ in the 2nd passage. The roller is clear and sub cultured after 72h. All harvested virus from contaminated cell line was decontaminated by autoclaving.

Table 3: Antibiotic treatment of the contaminated cell lines

Cell line	Tested antibiotics	Concentration	Day/Passage No			
			3/1	6/2	12/4	18/6
Contaminated	Neomycin	200mg/L	+	+	+	+
		500mg/L	+	+	+	+
	Kanamycin	200mg/L	+	+	+	+
		500mg/L	+	+	+	+
	Gentamycin	80mg/L	+	+	+	+
		200mg/L	+	+	+	+
	Polymyxin B	50mg/L	+	+	+	+
		125mg/L	+	+	+	+
	Ciprofloxacin	10mg/L	+	+	+	-
		25mg/L	+	+	-	-
Free	Day/Passage No		4/2	8/4	12/6	18/9
	Penic/strept	100iu/ 100mg	-	-	-	+
	Penic/strep /Cipro	10mg/L	-	-	-	-

All tested antibiotics shows no value in treating mycoplasma contamination in cell lines even after prolonged passages and in the higher concentration except ciprofloxacin reveal mycoplasma elimination in concentration of 10mg/L after continues treatment until 6 passages (18 day treatment) and also its mycoplasma inhibitory effect appear earlier after passage 4 (12 day) in concentration 25mg/L with no cell cytotoxicity detected in association with the higher concentration. Unfortunately the sterile line using penicillin streptomycin antibiotic as regular cell culture antibiotics get contaminated in the 9 passage in contrast the other line using regular antibiotics with ciprofloxacin still sterile till the end of this study.

DISCUSSION

Mycoplasma-infected cell lines are themselves the single most important source for further spreading of the contamination. This is due to the high concentration of mycoplasmas in infected cultures, and the prolonged survival of dried mycoplasmas. Operator-induced contamination is also a potential issue. Mycoplasmas spread by using laboratory equipment, media or reagents that have been contaminated **(Drexler and Uphoff, 2002)**. Mycoplasma is the most important and common source of cell culture infection, worldwide. In the current study PCR based detection was the ideal and rabid method for mycoplasma detection this finding agreed with **Volokhov *et al.*, (2011)** who mentioned that the molecular-based method is ideal as it is easy and quick to set-up and analyze. Further, it is highly sensitive, specific, reliable, and fairly cost-effective.

Also in this study mycoplasma contamination down regulate cell growth and virus yield as well as time and money consuming, this finding agreed with **Barile and Rottem, (1993)** who indicated that cell line growth rates and viral vaccine production can be adversely affected, the effects are due to nutrient deprivation, such as the depletion of amino acids, sugars, fatty acids, cholesterol or nucleic-acid precursors. Also this study indicate that the aminoglycoside group including neomycin, kanamycin and gentamicin shows no antimycoplasma effect even in higher concentration and for prolonged time (18days) treatment.

Also polymyxin B shows no antimycoplasma effect in a range of 50-125unit/L, these obtained results agreed with **Nikfarjam and Farzaneh, (2012)** who stated that, mycoplasma has 86% resistance to neomycin, 73% resistance to kanamycin and 80% resistance to gentamicin. Also the current study reveal that ciprofloxacin has antimycoplasma activity in concentration of 10mg/L after 18 day treatment and earlier in 12 day treatment in a concentration of 25mg/L without any cell cytotoxicity this finding disagree with **Vahid *et al.*, 2010)** who stated that ciprofloxacin cure 42.5% of infected cell line after 14 day treatment and agreed

with **Nikfarjam and Farzaneh, (2012)** who stated that mycoplasma has only 15% resistance to ciprofloxacin and using ciprofloxacin routinely barrel to regular antibiotics may prolong keeping the line mycoplasma free for longer time, but continues manipulation of the contaminated lines may act as source for contamination to the sterile lines.

CONCLUSION

If a valuable cell line or a primary cell culture is not replaceable our data provide solution for eradication and rendering cell lines mycoplasma free for extended periods of experimental work. Ciprofloxacin is an ideal and available antimycoplasma agent in high concentration without alteration of the growth rate and it is recommended to apply anti-mycoplasma antibiotics in a routine culture medium in order to prevent mycoplasmal infection but it should not be used for treating infected lines because infected cell lines are themselves the most important source for further spreading of the contamination. To both avoid and control the spread of mycoplasma during cell culture, itis recommended the following; discard all infected cultures, because the risk of infection transmission to other clean cultures is high. Only work with one cell line at a time. Aerosols and operator error are two of the most common means of transferring mycoplasma contamination. Wear clean personal protective equipment (PPE) and adopt appropriate aseptic techniques.

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