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Protections of Cell Culture from Mycoplasma Contamination by an Antibiotic Mixture with Emphasis on Assessment of the Effective and Safe Doses

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Abstract: This study was carried out to validate an antibiotic mixture (Triladyl®) as anti-mycoplasma with the calculation of the effective and safe dilutions for the *in vitro* cell culture (CC). Mycoplasma bovis (*M. bovis*)-inoculated Madin Darby bovine kidney (MDBK) cell culture was treated with that antibiotic understudy as two folds serial dilution. Many giant cells and cells with enlarged and karryorhytic nuclei were observed in the *M. bovis* inoculated MDBK cell culture. By using MTT colorimetric-based assay, the safest dilutions of the antibiotic mixture on MDBK cells were 1/16 and 1/32 while the cytotoxic dilution 50 (CD_{50}) was 1/8. In treated-inoculated MDBK cells, the effective dilution (ED) was 1/32 that inhibits the *M. bovis* multiplication (negative *M. bovis* re-isolation and identification on mycoplasma specific broth medium and by IFA) with no CPEs detection. Specific viral (BHV-1; Colorado strain and BVDV; NADL strain) CPEs were observed in ED treated MDBK cells inoculated with that viruses. In conclusion, the antibiotic mixture (Triladyl®) which commercially used as a concentrate in the egg yolk extender can be safely used as an additive to the CC media at the dilution of 1/32 to protect the CC from mycoplasma as well as other bacterial contamination.

Key words: Mycoplasma · Tissue Culture · Antibiotic mixture

INTRODUCTION

Mycoplasmas are smallest, self-multiplication microorganisms with avid biological and biochemical potentials and high spreading rate in eukaryotes in nature. Infections with mycoplasma species can induce a variety of problems in living organisms and on the genetic aspects of *in vitro* cell culture (CC) [1, 2].

The first isolation of mycoplasma from a CC was reported in 1956 [3]. 15 to 80% of CC has been reported to be contaminated with mollicutes [4]. In most cases, mycoplasmas infection originates from contaminated animal serum. Sharing of the non-certified cells in diagnostic laboratories is the major route of dissemination of mycoplasmas [5, 6].

For the CC laboratory, mycoplasmas contamination has a deleterious event due to their ability to contaminate CC leading to the production of false, unreliable data or, in the worst cases, to the loss of CC itself. Fortunately, mycoplasmas can be eradicated by the use of antibiotics, but early detection of contamination is authoritative. It has been suggested that efforts to eradicate mycoplasmas from contaminated cells should be considered as a last resort and it would be often far better to eliminate the problem completely [7, 8].

Methods of elimination for mycoplasmas contamination should ideally be simple, easy, rapid, efficient, reliable and inexpensive and result in no loss of specialized characteristics of CC. However, there is clearly no a single available method that is both 100% effective and fulfills all the ideal requirements. Chemotherapeutic intervention, antibiotic treatment, appears to be the superior to the other mycoplasma eradication techniques (physical, chemical and immunological procedures) and represents the most practical and efficient option to clean mycoplasma-positive CC [9, 10].

Combination of chemotherapy with antimicrobial agents may be necessary to treat mixed bacterial infections, to achieve synergistic antimicrobial activity and to prevent the emergence of drug resistance [11].

This study was carried out to validate an antibiotic mixture (Triladyl®) as anti-mycoplasma with the calculation of the effective and safe dilutions for the *in vitro* CC.

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MATERIAL AND METHODS

Cell lines: Madin-Darby bovine kidney (MDBK) cells were grown either in 96 well tissue culture (TC) plates (NUNC, Wiesbaden, Germany) for an effective and cytotoxic dilution assays or in 25 cm^2 TC flasks (NUNC, Wiesbaden, Germany) for detection of cytopathic effects (CPEs). Growth and maintenance Eagle's minimal essential medium (EMEM; Gibco, Life Technologies, Scotland, UK) supplemented with 10 and 2 % irradiated and heat inactivated fetal bovine serum (FBS; Biowhittaker, INC, USA), respectively, L-glutamine and sodium bicarbonate were used. Madin-Darby bovine kidney cells were cultured under the standard culture conditions (37 °C, 5% CO_2 and 90% humidity).

Culture of *M. bovis*: Mycoplasma bovis (*M. bovis*) from the mycoplasma laboratory at Animal Reproduction Research Institute (ARRI) was grown in heart infusion agar and heart infusion broth medium (Difico, USA) as described by Ruhnke and Rosendal [12]. At 10^7 colony forming unit (CFU)/ml, the growing *M. bovis* was collected and divided into aliquots and stored at -80 °C until used.

Cytopathic Effects: Half ml of growing *M. bovis* was diluted in 4 ml of 2% EMEM and inoculated into 80% confluent MDBK cell lines in 25 cm^2 TC flask and incubated under the standard culture conditions with daily examination for the CPEs detection. After 48 hrs post-inoculation, the inoculum was removed and the cells were stained with crystal violet stain (0.75% crystal violet in a solution of 50% ethanol, 0.25% NaCl and 1.75% formaldehyde) as outlined by Adler *et al.* [13], examined and photographed.

The Antibiotic Mixture (Triladyl®): The antibiotic mixture (Triladyl®; MiniTÛb, GmbH, Germany) that is used as a sterile concentrate in the egg yolk extender to eliminate mycoplasma and other bacterial pathogens in frozen bovine semen was assayed in this study as an additive to CC media *in vitro*. The antibiotic mixture was consisted of 25 mg Tylosin, 125 mg Gentamycin, 150 mg Spectinomycin and 75 mg Lincomycin per 100 ml of the solution (Tris, Citric acid, Sugar, Buffers, Glycerol and ultra pure water).

Effect of the Antibiotic Mixture on *M. Bovis* Inoculated in Cell Culture: According to Hamasuna *et al.* [14], MDBK cells grown in 75 cm^2 TC flasks were trypsinized and resuspended in EMEM with 10% FBS and adjusted as 1.7 x 10^4 cells/ml. A 200 µl portion of the cellular suspension was dispensed in each well of the 96 well TC plate and incubated for the next day under the standard culture conditions. The antibiotic was twofold serial dilutions in 2% EMEM to 1/128 and each dilution was assayed in one column of the plate. To each well, 10 µl of 10^7 CFU/ml of growing *M. bovis* was added. Cellular column without mycoplasma inoculation and cellular column with mycoplasma inoculation but without antibiotic treatment were included as negative and positive controls, respectively. The plate was incubated under the standard culture conditions and microscopically examined daily for the *M. bovis* CPEs detection. After that, the supernatant was removed and the inoculated MDBK cells were stained with crystal violet stain and photographed. The effective dilution (ED) of the treated antibiotic was calculated as the lowest dilution of the antibiotic causing complete inhibition of *M. bovis* CPEs in MDBK cells in the tested wells as well as can't re-isolated *M. bovis*.

Re-Isolation of *M. bovis* from the Treated-Inoculated MDBK Cells: From the cellular suspension of the treated-inoculated MDBK cells, re-isolation of *M. bovis* on heart infusion agar and heart infusion broth medium was done as described by Ruhnke and Rosendal [12].

Immunofluorescence antibody technique: Immunofluorescence antibody (IFA) technique was carried out on the ED treated-inoculated MDBK cells as well as on the *M. bovis* strain using anti-mycoplasma polyclonal antibodies and fluorescence isothiocyanate (FITC) conjugated anti-bovine antibodies according to the standard method as cited by Angulo *et al.* [15].

Safe and Toxic Dilution of Antibiotic Mixture on MDBK Cell Culture: The effect of the antibiotic mixture on the viability of MDBK cells was measured using tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, Bornem, Belgium] colorimetric-based assay as outlined by Hansen *et al.* [16] and Al-Jabri *et al.* [17]. For each dilution of the antibiotic mixture and for cellular controls, 8 wells were tested. Briefly, MDBK cells were trypsinized and resuspended in EMEM with 10% FBS and adjusted as 1.7 x 10^4 cells/ml. A 200 µl portion of the cellular suspension was dispensed in each well of the 96 well TC plate and incubated under the standard culture conditions. After 24 hrs, the growth medium was replaced by twofold serial dilutions of the antibiotic mixture in 2% EMEM. Untreated cells served as control. Cells fixed with 4% paraformaldehyde were

included as background condition. After 72 hrs post-incubation, the cells were incubated with 10 µl MTT/well (5 mg MTT powder/ml phosphate buffered saline; PBS) and were incubated for further 3 hrs at 37 °C. Then, the culture medium was removed and 100 µl of detergent reagent (10% sodium dodecyl sulphate; SDS; in 0.01 N HCl) was added and incubated at 4°C overnight. Absorbance was measured on an ELISA reader (Thermo Lab systems, Brussel, Belgium) at wavelength 550 nanometer (nm).

Viral Inoculation of the ED Treated-MDBK Cells: According to Stringfellow *et al.* [18], bovine viral diarrhea virus (BVDV), NADL strain and bovine herpesvirus-1 (BHV-1), Colorado strain, as a reference strains were used and proliferated into ED treated-inoculated MDBK cells with 2% EMEM at titers of $10^{6.3}$ and $10^{7.4}$, respectively. The CPEs in BHV-1 and BVDV inoculated MDBK cells were developed after 24 and 96 hrs post-inoculation respectively.

Data Analysis: Data are presented as mean±SD and evaluated by a one-way analysis of variance (ANOVA) using the SPSS® 6.1.3 software package (SAS, Cary, NC, USA) and the difference between the means were assessed using the test of least significant difference (LSD) at statistical significance $P < 0.05$.

RESULTS

The Cytopathic Effects of *M. Bovis* in Inoculated MDBK Cells: Figure 1. showing *M. bovis* inoculated MDBK cells (B, C and D), cells appeared with many giant cells and karryorhytic chromatin material. In negative control (A), normal cells were observed.

Effect of the Antibiotic Mixture on *M. Bovis* Inoculated in Cell Culture: The antibiotic treated *M. bovis*-inoculated MDBK cells showed that the ED was 1/32 that inhibits the *M. bovis* multiplication with no CPEs detection as shown in Figure 2.

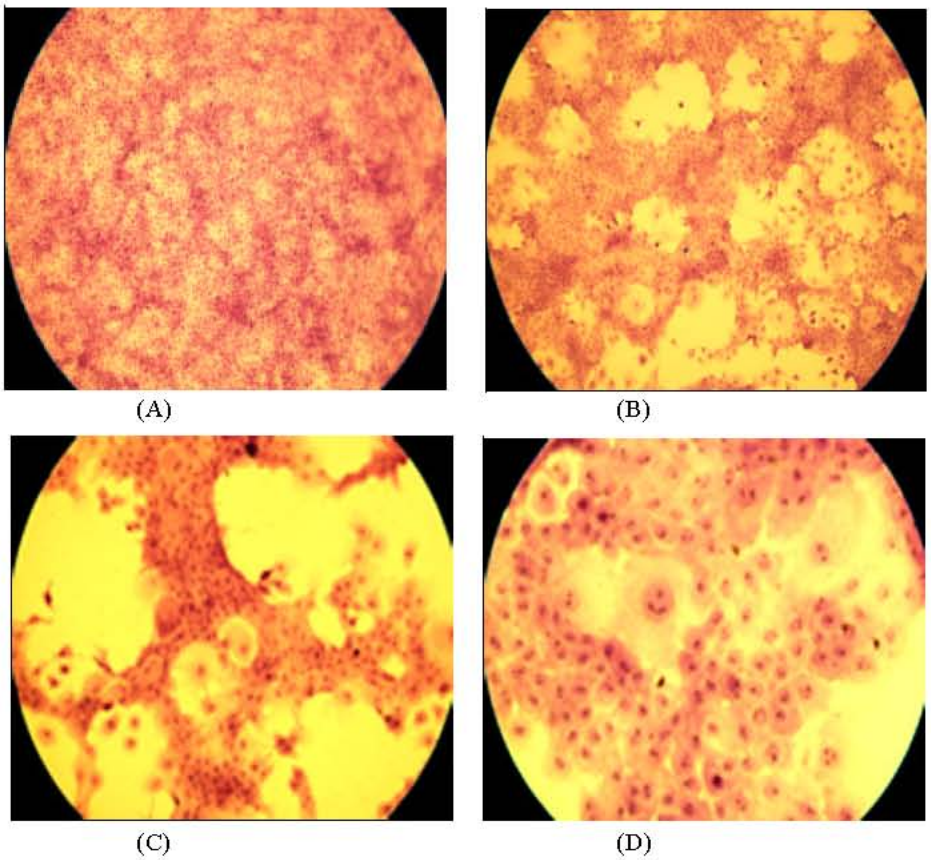
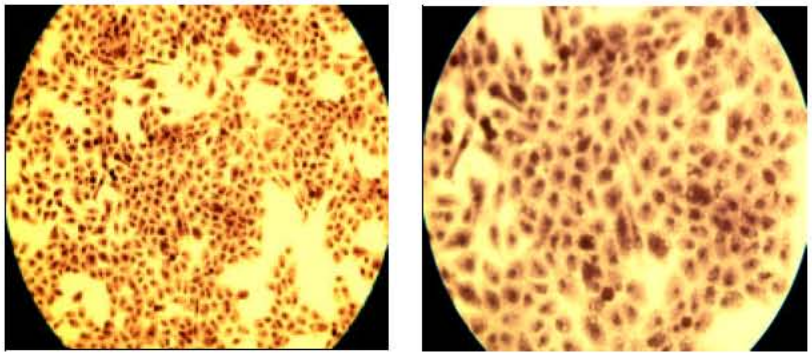


Fig. 1: Showing *M. bovis* inoculated MDBK cells (B, C, and D), cells appeared with many giant cells and *karryorhytic* chromatin material. In negative control (A), normal cells were observed

Table 1: Statistical analysis based on analysis of variance (ANOVA)

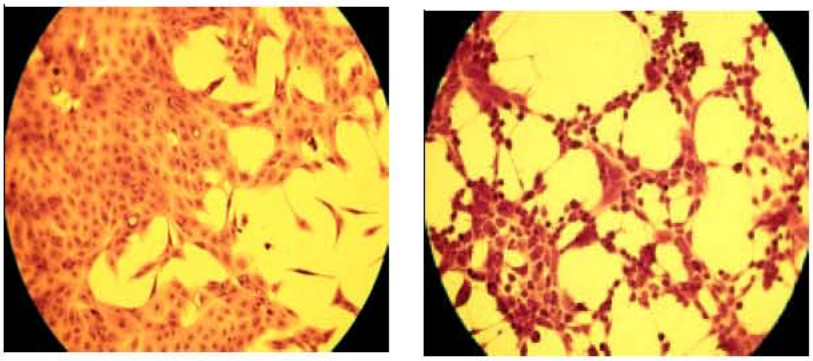
Dilution	Control	Blank	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Mean±SD	1.299±0.192 ^{a}	0.152±0.061 ^{de}	0.055±0.004 ^{f}	0.073±0.008 ^{ef}	0.174±0.014 ^{cd}	0.286± 0.017 ^{b}	0.280± 0.014 ^{b}	0.255± 0.018 ^{bc}	0.243±0.018 ^{bcd}

Means with different superscript letters are different at least at P< 0.05



(A) (B)

Fig. 2: Triladyl® treated *M. bovis* inoculated MDBK cells at dilution 1/32, no CPEs were observed



(A) (B)

Fig. 3: Showing ED treated-MDBK cells; (A) the NADL strain-infected cells with degradation of the monolayer sheet with spider web-like appearance. (B) The BHV-1 infected cells became rounded, dispersed grapes-like appearance in the fluid phase and intranuclear inclusion bodies were observed.

Safe and Toxic Dilution of Antibiotic Mixture on MDBK Cell Culture: The safest dilutions of the antibiotic understudy as presented in the table 1 were 1/16 and 1/32. The cytotoxic dilution 50 (CD_{50}) of the antibiotic on MDBK cells as in the same table was 1/8 that half of the treated cells was degenerated.

Viral Inoculation of the ED Treated-MDBK Cells: Figure 3. showing ED treated-MDBK cells; (A) the NADL strain-infected cells with degradation of the monolayer sheet with spider web-like appearance. (B) The BHV-1 infected cells became rounded, dispersed grapes-like appearance in the fluid phase and intranuclear inclusion bodies were observed.

DISCUSSION

The infection of CC with mycoplasma has different cytogenetic effects [19]. The biological, biochemical and enzymatic activities of the mycoplasma determine its effect on the cells and the degree of CPEs [20]. All mycoplasmas require nucleic acid precursors (free bases, nucleosides and oligonucleotides), amino acids, energy sources, an absolute requirement for sterols (such as cholesterol) and fatty acids for their growth [21]. The identification of CPEs in accidental or experimental infection of CC may contribute to the understanding of the relationship between mycoplasmas and the host cell [22]. In the present study, the inoculated *M. bovis* on

MDBK cells was able to produce CPEs within 48 hrs post-infection in normal cellular structures. The inoculated *M. bovis* MDBK cells appeared with many giant cells and karryorhytic chromatin material.

Antibiotic intervention appeared to be superior to the other mycoplasma eradication techniques and it is the method of choice [10, 8]. As this antibiotic mixture under study (Triladyl®), several antibiotics act as inhibitors of various biological RNA-catalyzed key processes. The binding of these antibiotics to different functional RNAs can lead to impairment or disturb the decoding process in prokaryotic translation and inhibit the function of various natural ribosomes [23, 24].

Tylosin as one member of macrolide has been widely used to treat mycoplasmosis in animals, as an animal feed supplement and in the preservation of food [25]. It interferes with the protein synthesis by reversibly binding to the 50 S subunit of the ribosome that effect on the peptidyl transferase reaction and prevention the positioning of the peptidyl-tRNA (transfer RNA) [26, 27].

Lincomycin as one member of lincosaminides is active *in vitro* and *in vivo* against clinical isolates of Gram positive organisms. Lincomycin exerts its effect on protein synthesis inhibition by acting on 70 S complexes. It inhibits the binding of phenylalanine-tRNA to the ribosome-messenger complex causing premature detachment of the ribosomes from mRNA or that it inhibits the initiation of polypeptide synthesis [28, 29].

Gentamycin as one member of aminoglycosides-aminocyclitol antibiotics is among the most potent antibiotics known. The spectrum of activity covers most of the common Gram negative bacteria. The codon-anticodon interaction on the ribosome occurs in the A site of the 30 S ribosomal subunit. Aminoglycoside bind to ribosomal RNA in the A site causing misreading of the genetic code and inhibit its translocation [30, 31].

Unlike other aminocyclitol antibiotics, spectinomycin is bacteriostatic rather than bactericidal. The mechanism of action involves the binding to the bacteria 30 S ribosomal subunit and inhibition of protein synthesis that inhibits the catalytic activity of self-splicing group I introns [32].

Herein, the synergistic activity of theses antibiotics would minimize the effective concentration for each antibiotic so prevent the emergence of microbial drug resistance and toxicity to CC. The safest dilutions of this antibiotic mixture were 1/16 and 1/32 while the cytotoxic dilution (CD_{50}) was 1/8. The antibiotic treated *M. bovis*-inoculated MDBK cells showed that the ED was 1/32 that inhibits the *M. bovis* multiplication (negative *M. bovis* re-isolation and identification on mycoplasma specific broth medium and by IFA) with no CPEs detection. Also, there is no contamination with other bacteria species allover the study. Specific viral (BHV-1; Colorado strain and BVDV; NADL strain) CPEs were observed in ED treated MDBK cells inoculated with that viruses.

In conclusion, the antibiotic mixture (Triladyl®) which commercially used as a concentrate in the egg yolk extender can be safely used as an additive to the CC media at the dilution of 1/32 to protect the CC from mycoplasma as well as other bacterial contamination.

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