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Research Paper

Experimental infection of BHK21 and Vero cell lines with different *Mycoplasma* spp.

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Abstract

Mycoplasma spp, belongs to the class Mollicutes and is capable to produce alterations in cellular cultures causing damages to the biotechnological industry. Bioproducts generally require two essential inputs, bovine serum and cells. The study herein aims to evaluate the mycoplasma concentrations that affect the growing of BHK21 and Vero cells. The species used were: *Mycoplasma orale, M. salivarium, M. arginini* and *M. hyorhinis,* cultivated in a SP4 media. Two contamination tests were performed with BHK21 and Vero cells and one of them applied different concentrations of mycoplasma. In the first one, mycoplasma was applied at the day zero and, in the second one, the contamination was performed after the monolayer establishment. The both cellular cultures presented cytopathic effects with mycoplasma contamination, but the Vero cells suffered more damages than the BHK21 ones. It was also observed that the severity of the cytopathic effect depended on the mycoplasma specie, on the concentration and on the time of contact with the cellular culture, which evidences the importance of controlling the presence of mycoplasma in biotechnological industries.

Key words: growth reduction, PCR, biotechnological products.

Introduction

Cellular cultures contaminated with mycoplasma represent an artificial habitat for such microorganisms. Studies performed in many countries demonstrated that from 10 to 87% of the cellular cultures are infected with mycoplasma (Kasemiha *et al.*, 2009; Netto, 2013). The species *M. orale, M. salivarium, M. hyorhinis* and *M. arginini* are among the ones that are most found as contaminants (Miyaki *et al.*, 1989; Hu *et al.*, 1995; Timenetsky *et al.*, 2006).

Mycoplasma orale and *M. salivarium* inhabit the human oropharynx and, generally, they infect cell cultures through inadequate aseptic techniques. *M. arginini* has bovine origin and it is found in cultures from contaminated bovine serum. *M. hyorhinis* has porcine origin and it can contaminate bovine serum in slaughterhouses when they are also used to slaughter swine (Rottem and Barile, 1993; Smith and Mowles, 1996; Razin *et al.*, 1998).

The contamination through mycoplasma in bovine cells and serum used to produce vaccine antigens or used in diagnosis can generate non-confident results and add potentially noxious effects to biotechnological products. Contaminations through mycoplasma cause alterations in the cellular metabolism, decreasing the cellular division rate through the interference in DNA and RNA synthesis, chromosomal aberrations and death with the monolayer release (Timenetsky *et al.*, 1992). However, there are few studies that quantify which Mycoplasma concentration can affect such growth.

Products, such as vaccines, medicines or monoclonal antibodies, manufactured based on such inputs should be disenabled, which causes prejudices to research laborato-

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ries and to pharmaceutical and biotechnological industries (Uphoff and Drexler, 2002). Thus, the scope of such research was to evaluate the mycoplasma concentrations that affect the growth of BHK21 and Vero cells.

Materials and Methods

Microorganisms cultivation

The strains of *M. orale*, *M. salivarium, M. arginini* and *M. hyorhinis* were cultivated in 10 mL of SP4 broth at 37 °C (Nascimento e*t al*., 2002). The growth of the microorganisms was confirmed due to the acidification of the pH in the broth, absence of turbidity, production of colonies looking like `fried eggs' in SP4 agar plates and through polymerase chain reaction (PCR). Mycoplasma was frozen at -20 °C with 20% glycerol (Sigma-Aldrich, St. Louis, MO and USA) up to its usage.

Polymerase chain reaction

In order to confirm the identity of the mycoplasma strains, a PCR technique standardized by Netto (2013) was used. Briefly, the first step to extract the DNA of the microorganisms, the phenol/chloroform and boiling techniques described by Bashiruddin (1998) were used. The DNA concentration of each sample was measured through the absorbance at 260 nm (NanoDrop2000, Thermo Scientific, Waltham, MA, USA).

The primer sense GPO-3 5'GGGAGCAAACAGGA TTAGATACCCT3' and the antisense MGSO 5'TGCACCATCTGTCACTCTGTTAACCTC3' were used according to Van Kuppeveld *et al.* (1994). At the detection through PCR, 2 μL of buffer 10X, 1.5 mM of $MgCl_2$, 0.2 μM of each deoxyribonucleotide triphosphate (dNTP), 12.5 pmol of each primer (GPO3/MGSO*)*, 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 50 ng of DNA (maximum volume of 8 μL) and ultrapure water were added to a final volume of 20 μL. The thermocycler (model Biocycler MG96) was programmed to one cycle at 94 °C during 5 min, 35 cycles at 94 °C during 30 s, 55 °C during 30 s, 72 °C during 30 s and a final step at 72 °C during 5 min.

A negative (ultrapure water) and a positive (DNA of the reference strains) control were added to all amplifications. The expected DNA fragment size after its amplification was of 280 bp.

Experimental contamination of BHK21 and Vero cells with Mycoplasma

Two tests of contamination through mycoplasma in BHK21 and Vero cells were performed. In the test 1, the cellular cultures were contaminated after 24 h of cultivation and, in the test 2, the cellular cultures were contaminated from the zero time of cultivation on. The cellular cultures were incubated with microorganisms' serial dilutions (up to 10^{-8}) from the stock. The total time of the cellular cultivation in the both tests was of 48 h at 37 °C.

T vials of BHK21 cells in MEM supplemented with 10% of fetal bovine serum (FBS) and Vero cells in RPMI 1640 (Gibco®, Carlsbad, CA, USA) with 10% of SFB were peaked and incubated at 37 °C in a stove containing 5% of CO_2 during 48 h. The number of BHK21 cells/mL and of Vero cells/mL was counted in a Neubauer chamber. In order to perform the cellular cultivation, a volume of BHK21 or Vero cells corresponding to 0.3 x 10^5 cells was pipetted and a sufficient quantity of MEM supplemented with 10% of fetal bovine serum or RPMI media supplemented with 10% of fetal bovine serum, respectively, were used, totalizing the volume of 150 μL/pool.

The both tests were performed in flat-bottomed plates with 96 pools in the same distribution. In the pools from A to H of the column 1, 150 μL of MEM supplemented with 10% of fetal bovine serum or RPMI media supplemented with 10% of fetal bovine serum were used to prove the sterility of the media used in the test. In the pools from A to H of the column 2, BHK21 or Vero cells were used as growth controls. In the pools from A to H of the other columns, serial dilutions from 10^{-1} to 10^{-8} from stocks cultivated in SP4 broth were pipetted. In the columns 3 and 4, dilutions of *M. orale* were pipetted; in the columns 5 and 6, *M. salivarium*; in the columns 7 and 8, *M. arginini*; and, in the columns 9 and 10, *M. hyorhinis*.

After 48 h of incubation, the cellular growth was observed and pictured in an inverted microscope with an increase of 100X. Then, the cells were stained with a Crystal Violet solution 0.5% and the absorbance was checked in a microplate reader at 620 nm to quantify the cellular growth.

Results

PCR confirmed the identity of all *Mycoplasma* strains used in this study and all of them presented the expected product of 280 bp (Figure 1). In the first experiment, the contamination through mycoplasma in BHK21 cells, when compared with the non-contaminated control, showed that, in *M. orale*, the dilutions 10^{-1} and 10^{-2} presented inferior results of absorbance and, consequently, the percentage of growth reduction was of 15.60% and of 9.47%, respectively. From the dilution 10^{-3} up to the 10^{-8}, the results were maintained closer to the absorbance values of the negative

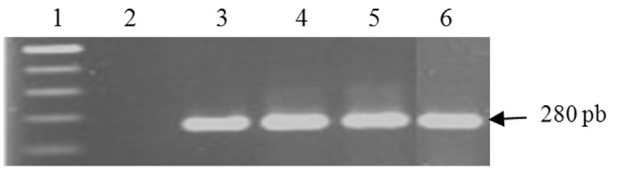

Figure 1 - Agarose gel electrophoresis at 1.5% of PCR products of mycoplasma grown in SP4 culture media. 1: Molecular Weight Marker (100 pb); 2: Negative Control; 3: *M. orale;* 4: *M. salivarium*; 5: *M. arginini*; 6: *M. hyorhinis*.

control. For *M. salivarium*, the dilutions from 10^{-1} to 10^{-6} presented growth reducing effects from 47.63% to 13.09%, respectively. The dilutions from 10^{-7} to 10^{-8} presented results closer to the ones of the negative control. For *M. arginini*, the dilutions 10^{-1}, 10^{-2} and 10^{-3} presented cytopathic effect with a reduction in the cellular growth of 44.57%, 10.31% and 5.29%, respectively. From the dilutions 10^{-4} to 10^{-8}, the results were maintained closer to the absorbance value of the negative control. For *M. hyorhinis*, the dilutions from 10^{-1} to 10^{-5} presented growth reducing effects from 49.86% to 5.85%, respectively. The dilutions from 10^{-6} to 10^{-8} presented higher absorbance values (Table 1).

Generally, for BHK cells, in a descending order of cellular growth reduction, the following distribution was observed for the species: *M. salivarium* > *M. hyorhinis* > *M. arginini* > *M. orale.* In the Figure 2, an example of cytophatic effect caused by *M. salivarium* is given.

Experimental contamination with mycoplasma performed in a cultivation of Vero cells, in comparison to the non-contaminated control, showed that, for *M. orale*, the cytopathic effect was higher in the dilutions from 10^{-1} to 10^{-3} with a growth reduction from 30.70% to 9.30%, respectively. From the dilution 10^{-4} up to the 10^{-8}, a lower cytopathic effect was observed. For *M. salivarium*, all dilutions presented growth reducing effects from 59.07% to 7.44%. For *M. arginini,* the dilutions from 10^{-1} to 10^{-4} presented cytopathic effect with cellular growth reduction from 37.67% to 5.12%, respectively. For *M. hyorhinis*, the dilutions from 10^{-1} to 10^{-5} presented growth reducing effects from 39.07% to 6.05%, respectively (Table 2).

Table 1 - Cytopathic effect in BHK21 cell experimentally infected with different mycoplasma spp. after 24 h of incubation. The culture was done in MEM medium supplemented with 10% of Fetal Bovine Serum. The results represent the mean of two replicate and more or less the standard deviation in eight dilutions of *mycoplasma.* The measure was made by absorbance at 620 nm.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
M. orale	0.303 ± 0.007	0.325 ± 0.014	0.352 ± 0.010	0.355 ± 0.008	0.358 ± 0.013	0.359 ± 0.006	0.361 ± 0.004	0.366 ± 0.011
M. salivarium	0.188 ± 0.014	0.269 ± 0.019	0.277 ± 0.014	0.279 ± 0.006	0.29 ± 0.014	0.312 ± 0.010	0.343 ± 0.011	0.353 ± 0.016
M. arginini	0.199 ± 0.011	0.322 ± 0.006	0.340 ± 0.010	0.349 ± 0.011	0.350 ± 0.008	0.355 ± 0.004	0.356 ± 0.014	0.362 ± 0.016
M. hyorhinis	0.180 ± 0.016	0.302 ± 0.016	0.305 ± 0.015	0.330 ± 0.011	0.338 ± 0.011	0.354 ± 0.011	0.359 ± 0.018	0.363 ± 0.016

Negative control: 0.359 ± 0.011.

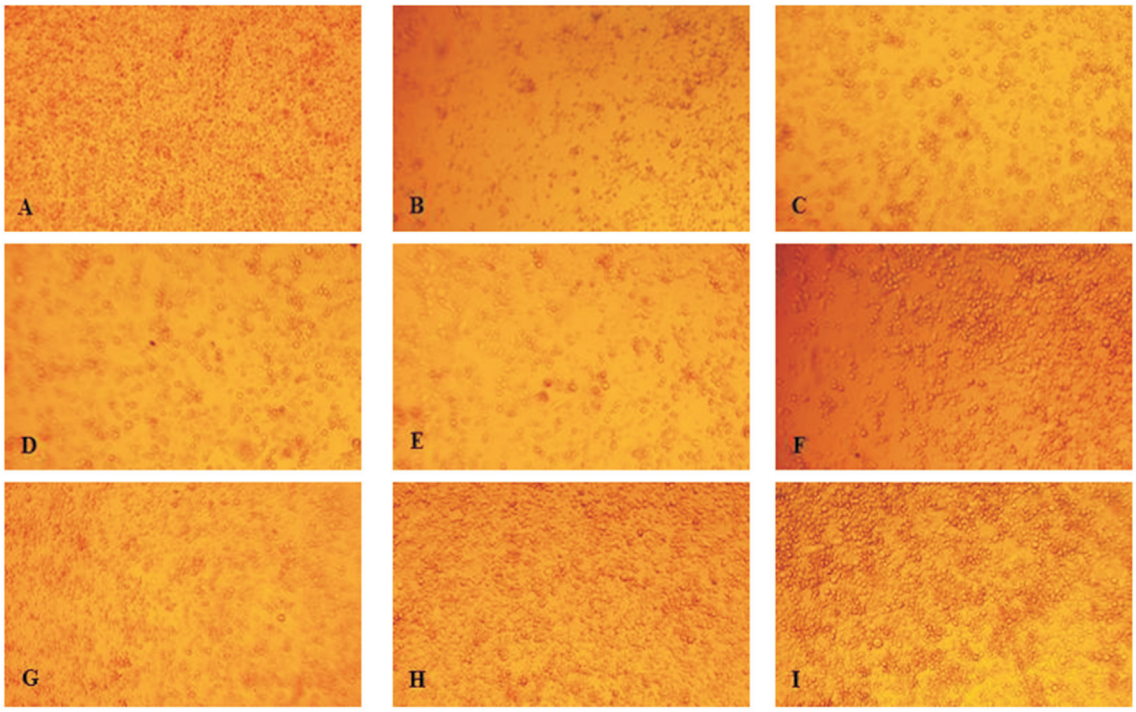

Figure 2 - Culture of BHK21 cells incubated in MEM medium with 10% of fetal bovine serum (FBS) at 37 °C by 24 h, after infected experimentally with serial dilutions of *Mycoplasma salivarium* (X 100). A: Negative Control; the ranges B – I represent BHK21 culture infected by 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}, respectively.

Table 2 - Cytopathic effect in Vero cell experimentally infected with different mycoplasma spp. after 24 h of incubation. The culture was done in RPMI 1640 medium supplemented with 10% of Fetal Bovine Serum. The results represent the mean of two replicate and more or less the standard deviation for eight dilutions of *mycoplasma.* The measure was made by absorbance at 620 nm.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
M. orale	0.149 ± 0.011	0.163 ± 0.013	0.195 ± 0.014	0.210 ± 0.011	0.212 ± 0.010	0.215 ± 0.006	0.217 ± 0.010	0.217 ± 0.006
M. salivarium	0.088 ± 0.014	0.089 ± 0.006	0.104 ± 0.008	0.111 ± 0.003	0.157 ± 0.011	0.187 ± 0.008	0.194 ± 0.006	0.199 ± 0.008
M. arginini	0.134 ± 0.008	0.159 ± 0.013	0.188 ± 0.011	0.204 ± 0.006	0.211 ± 0.014	0.216 ± 0.008	0.216 ± 0.011	0.217 ± 0.008
M. hyorhinis	0.131 ± 0.011	0.145 ± 0.018	0.173 ± 0.017	0.187 ± 0.011	0.202 ± 0.014	0.212 ± 0.011	0.215 ± 0.014	0.216 ± 0.011

Negative control: 0.215 ± 0.005.

For Vero cells, in a descending order of effects over the cellular growth reduction, the following distributions are presented: *M. salivarium* > *M. hyorhinis* > *M. arginini* > *M. orale.* In the Figure 3, the cytopathic effect caused by *M. hyorhinis* in Vero cells is shown.

In the evaluation of the infection and of the cytopathic effect of the already established monolayer in BHK21 cells, in comparison to the control that was not contaminated with mycoplasma, it was possible to observe that, for *M. orale*, the dilutions 10^{-1} and 10^{-2} presented inferior absorbance results and a percentage of growth reduction of 39.40% and 6.25%, respectively. In the dilutions from 10^{-3} up to 10^{-8}, the results were maintained closer to the absorbance value of the negative control. For *M. salivarium*, all dilutions presented growth reducing effects from 55.16% to 13.59%. For *M. arginini*, the dilutions from 10^{-1} to 10^{-5} presented growth reducing effects from 57.34% to 4.89%, respectively. The dilutions from 10^{-6} to 10^{-8} presented results closer to the absorbance value of the control. For *M. hyorhinis*, all dilutions presented growth reducing effects from 57.61% to 15.76% (Table 3). In a descending order of effects over the cellular growth decrease, the following distribution was presented: *M. hyorhinis* > *M. salivarium* > *M. arginini* > *M. orale*. In the Figure 4, an example of cytopathic effect caused by *M. salivarium* in BHK cell culture is shown.

In the second contamination test performed with Vero cells, in comparison to the control that was not contaminated by mycoplasma, it was checked that the cytopathic effect was observed in all dilutions for all species. The growth reduction varied from 51.38% to 14.68% for *M. orale*, from 74.77% to 43.12% for *M. salivarium,* from 50.46% to 29.82% for *M. arginini* and from 64.68% to 28.90% for *M. hyorhinis* (Table 4). In a descending order of

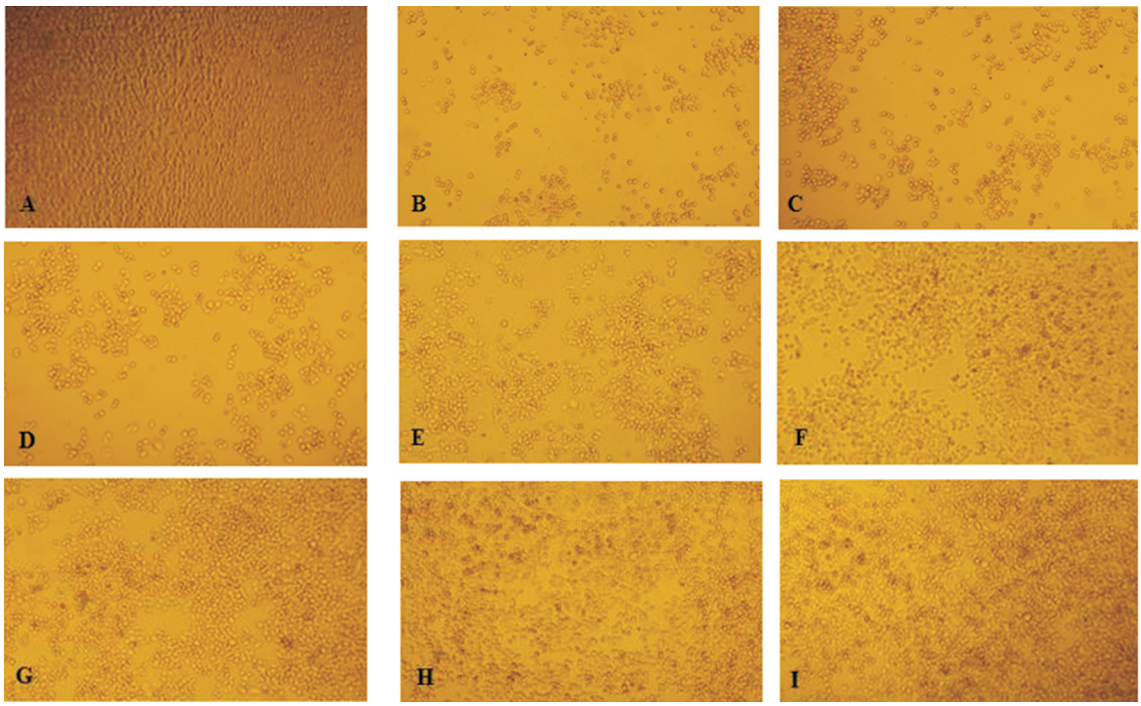

Figure 3 - Culture of Vero cells after 24 h of cultivation in RPMI 1640 medium supplemented with 10% of fetal bovine serum (FBS) at 37 °C and infected experimentally with serial dilutions of *Mycoplasma salivarium* (X 100). A: Negative Control. The ranges B – I represent Vero cells culture infected by 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}, respectively.

Table 3 - Cytopathic effect in BHK21 cell experimentally infected with different mycoplasma spp. after 48 h of incubation. The culture was done in MEM medium supplemented with 10% of Fetal Bovine Serum. The results represent the mean of two replicate and more or less the standard deviation for eight dilutions of *mycoplasma.* The measure was made by absorbance at 620 nm.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
M. orale	0.223 ± 0.008	0.345 ± 0.007	0.355 ± 0.011	0.359 ± 0.011	0.361 ± 0.014	0.362 ± 0.008	0.363 ± 0.014	0.365 ± 0.007
M. salivarium	0.165 ± 0.010	0.233 ± 0.008	0.289 ± 0.010	0.308 ± 0.011	0.315 ± 0.011	0.315 ± 0.007	0.316 ± 0.006	0.318 ± 0.015
M. arginini	0.157 ± 0.014	0.325 ± 0.006	0.331 ± 0.009	0.340 ± 0.011	0.350 ± 0.007	0.359 ± 0.011	0.360 ± 0.011	0.369 ± 0.008
M. hyorhinis	0.156 ± 0.006	0.257 ± 0.015	0.268 ± 0.013	0.278 ± 0.011	0.280 ± 0.015	0.283 ± 0.008	0.289 ± 0.011	0.310 ± 0.013

Negative control: 0.368 ± 0.013.

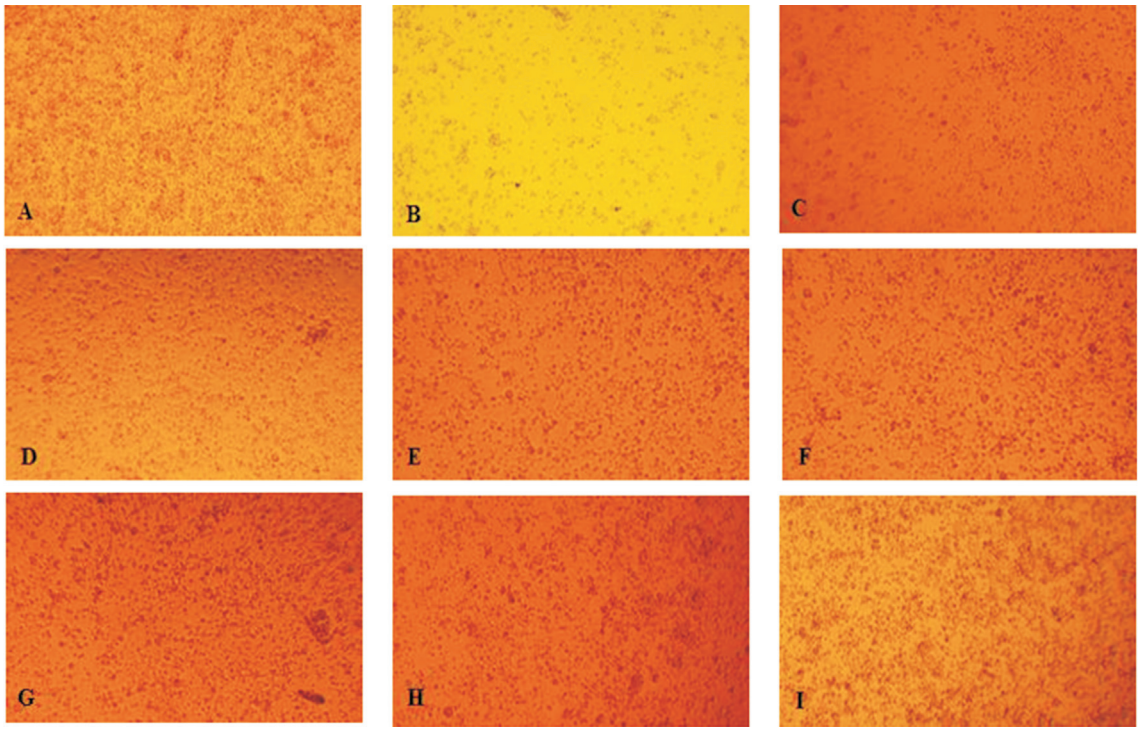

Figure 4 - Cytopathic effect in BHK21 cells after 48 h cultivation in MEM medium supplemented with 10% of fetal bovine serum (FBS) at 37 °C and infected experimentally with serial dilutions of *Mycoplasma hyorhinis* (X 100). A: Negative control; B - I: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, respectively.

effects over the cellular growth decrease, the distribution of the species is the following: *M. salivarium* > *M. hyorhinis* > *M. arginini* > *M. orale*. In the Figure 5, the cytopathic effect of *M. salivarium* is shown.

Discussion

The experimental contamination by mycoplasma in two kinds of cells used to produce immunobiological products showed that, for BHK21 cells, the cytopathic effect was severe for all evaluated species as in the test contaminating the inputs at the beginning of the cultivation as after the monolayer establishment. The contamination after the monolayer produced a higher percentage of cellular growth reduction and/or also a higher number of dilutions that caused such effect. Comparing the results for the BHK21 cells, it was checked, in the test 2, in which mycoplasma contacted the cellular culture during 24 h to, then, infect it, that the reduction in the cellular growth was higher. At the test 1, the specie that caused a more severe effect in the growth of BHK21 cells was *M. salivarium.* However, in the test 2, *M. hyorhinis* presented a more severe effect. Thus, it evidences that, in BHK21 cells, *M. hyorhinis* is capable to cause a higher reduction in the cellular growth with a higher contact time in comparison to other studied species of mycoplasma. In 2003, Rottem reported that *M. hyorhinis* adhered to each cell and that it was capable to completely destroy the membrane of the host cell, causing a generalized cytopathic effect.

Table 4 - Cytopathic effect in Vero cell experimentally infected with different *Mycoplasma* spp. after 48 h of incubation. The culture was done in RPMI 1640 medium supplemented with 10% of Fetal Bovine Serum. The results represent the mean of two replicate and more or less the standard deviation for eight dilutions of *mycoplasma.* The measure was made by absorbance at 620 nm.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
M. orale	0.106 ± 0.010	0.120 ± 0.013	0.152 ± 0.008	0.154 ± 0.014	0.154 ± 0.011	0.159 ± 0.016	0.173 ± 0.016	0.186 ± 0.011
M. salivarium	0.055 ± 0.014	0.065 ± 0.010	0.089 ± 0.008	0.092 ± 0.011	0.110 ± 0.011	0.121 ± 0.017	0.122 ± 0.010	0.124 ± 0.008
M. arginini	0.108 ± 0.008	0.116 ± 0.017	0.146 ± 0.017	0.148 ± 0.008	0.148 ± 0.014	0.150 ± 0.017	0.151 ± 0.013	0.153 ± 0.008
M. hyorhinis	0.077 ± 0.014	0.096 ± 0.008	0.118 ± 0.011	0.127 ± 0.011	0.132 ± 0.014	0.134 ± 0.014	0.140 ± 0.017	0.155 ± 0.014

Control: 0.218 ± 0.012.

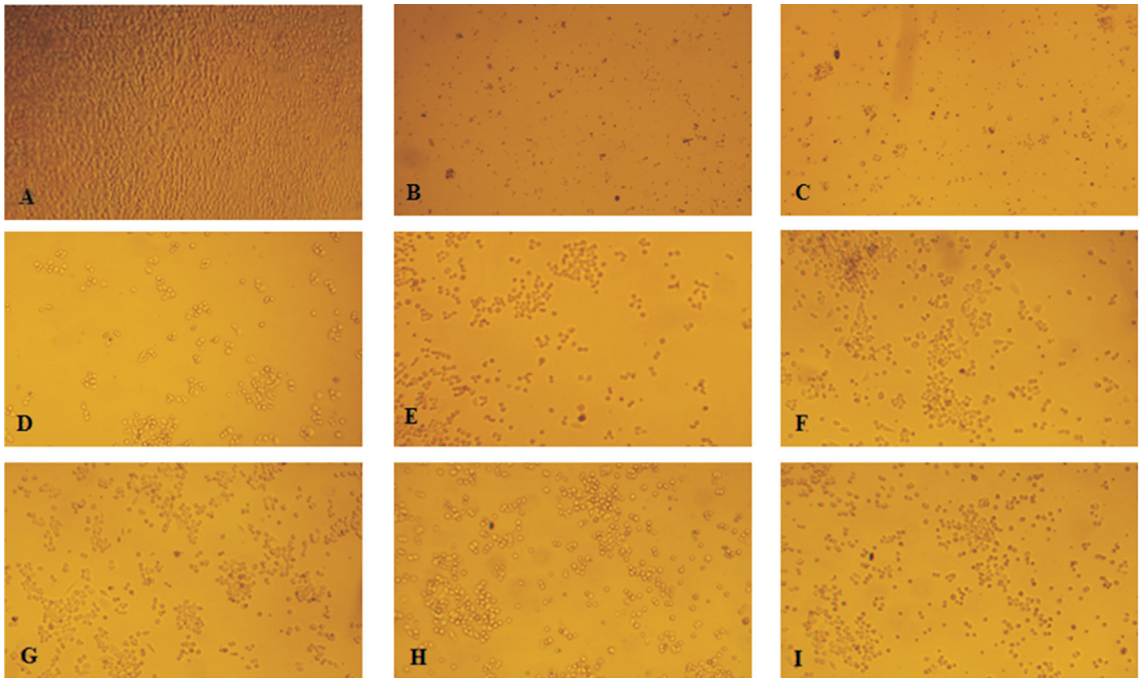

Figure 5 - Vero cells cultures after 48 h of cultivation in RPMI 1640 medium supplemented with 10% of Fetal Bovine Serum and infected with serial dilutions of *Mycoplasma salivarium* (X 100). A: Negative Control; B - I: Vero culture with dilution 10^{-1} to 10^{-8} respectively.

For Vero cells, it was observed that the cytopathic effect was more severe for all species when there was a higher contact with the microorganism. A higher percentage of cellular growth reduction in which the lower concentrations also caused cytopathic effect was checked. For Vero cells, when mycoplasma contacted them during 24 h, a higher reduction in the cellular growth was observed

Through such information, it is possible to evidence that Vero cells had higher cytopathic effects than BHK21 ones in the both tests performed with the same concentrations with all species of mycoplasma. However, the both strains of cellular cultivation had their growth affected in comparison to the control one, especially in the greatest concentrations of the parasite. It was also checked that such effect depends on the mycoplasma specie.

In 2002, Nascimento e*t al.* experimentally contaminated fibrochondrocytes of a rabbit meniscus with many concentrations of mycoplasma during 24 h and they observed that the severity of the cytopathic effect depended on the concentration of the inoculum and on the mycoplasma specie, which are data that corroborate with our research.

Given the harmful effects to cell cultures caused by mycoplasma contamination and, consequently, the financial losses in an attempt to eliminate the contamination or to destruct the infected cell culture, it is essential to emphasize the importance of preventing contamination by these microorganisms. The implementation of quality control of the production process, such as the detection of mycoplasma by PCR from the input to the final product and the use of PPEs, such as masks and gloves, are measured by handlers, which are simpler and cheaper than the damage caused by mycoplasma contamination that is undetected prematurely.

Then, the contamination through mycoplasma causes a reduction in the growth of BHK21 and Vero cells. Vero cells suffered higher cytopathic effects than BHK21 ones in

the tests and the growth of both of them were affected, especially in higher concentrations of Mycoplasma. It was also observed that the severity of the cytopathic effects depended on the mycoplasma specie, on the concentration and on the duration of the contact with the cellular culture.

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