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Performance of PCR-based and Bioluminescent assays for mycoplasma detection

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ABSTRACT

Contaminated eukaryotic cell cultures are frequently responsible for unreliable results. Regulatory entities request that cell cultures must be mycoplasma-free. Mycoplasma contamination remains a significant problem for cell cultures and may have an impact on biological analysis since they affect many cell parameters. The gold standard microbiological assay for mycoplasma detection involves laborious and time-consuming protocols. PCR-based and Bioluminescent assays have been considered for routine cell culture screening in research laboratories since they are fast, easy and sensitive. Thus, the aim of this work is to compare the performance of two popular commercial assays, PCR-based and Bioluminescent assays, by assessing the level of mycoplasma contamination in cell cultures from Rio de Janeiro Cell Bank (RJCB) and also from customers' laboratories. The results obtained by both performed assays were confirmed by scanning electron microscopy. In addition, we evaluated the limit of detection of the PCR kit under our laboratory conditions and the storage effects on mycoplasma detection in frozen cell culture supernatants. The performance of both assays for mycoplasma detection was not significantly different and they showed very good agreement. The Bioluminescent assay for mycoplasma detection was slightly more dependable than PCR-based due to the lack of inconclusive results produced by the first technique, especially considering the ability to detect mycoplasma contamination in frozen cell culture supernatants. However, cell lines should be precultured for four days or more without antibiotics to obtain safe results. On the other hand, a false negative result was obtained by using this biochemical approach. The implementation of fast and reliable mycoplasma testing methods is an important technical and regulatory issue and PCR-based and Bioluminescent assays may be good candidates. However, validation studies are needed.

1. Introduction

Contamination of cell cultures by mycoplasma remains a significant problem in many laboratories. According to published reports, mycoplasma is present in about 5–35% of all cell cultures (Hay et al., 1989). However, the actual rates are probably higher in a significant number of laboratories that do not test for such phenomena. As reported by Rivera et al. (2009), the level of mycoplasma contamination of the cell cultures evaluated in Mexico was 88.7%.

The massive presence of mycoplasma is unfortunately a big issue, especially because an array of physiological and biochemical parameters are affected by the presence of mycoplasmas in cell culture. It is known that mycoplasma affects cell function, growth, metabolism, morphology, attachment, and membrane properties, contributes to virus propagation in the cell culture, and induces chromosomal abnormalities and DNA damage, as well as cytopathic effects including plaque formation (Lincoln and Gabridge, 1998). The use of contaminated eukaryotic cells may thus cause disastrous effects, since they can alter many cellular parameters, leading to unreliable experimental results and potentially unsafe biological products such as biopharmaceutical products used in cell therapy, tissue engineering and vaccine manufacturing (FDA, 2010). Moreover, mycoplasma contamination is a serious concern for both autologous (Gong et al., 2012) and heterologous (Albon et al., 2013) cell-based therapies and financially, represents considerable economic impact: a study estimated that approximately $350 million

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of NIH-funded research are potentially affected considering the contamination rate of 11% observed via a survey of NCBI RNA-Sequencing data for mycoplasma sequences (Olarerin-George and Hogenesch, 2014).

Mycoplasmas (class *Mollicutes*) are the smallest and simplest self-replicating organisms and are devoid of a cell wall. Despite the fact that over 180 mycoplasma species have been described, the vast majority of cell culture contaminants belong to only six species, primarily of human, bovine or porcine origin: *Mycoplasma hyorhinis*; *Mycoplasma orale*; *Mycoplasma arginini*; *Mycoplasma fermentans*; *Acholeplasma laidlawii* and *Mycoplasma hominis*. Due to their size, mycoplasmas are able to pass through 0.2 μm filters commonly used for the sterile filtration of media and media components (Young et al., 2010). Regarding their concentration, mycoplasmas can reach 10^8 cells per mL of tissue culture medium without causing obvious cloudiness and with no apparent effect on cell growth, unlike ordinary bacterial contamination. The absence of a rigid cell wall makes mycoplasmas unresponsive to antibiotics that target cell wall synthesis, commonly employed for the prevention of bacterial contamination in cell culture.

Detection of mycoplasmas may be difficult for routine cell culture and many cells support low levels of contamination that are only detectable using highly sensitive tests. Therefore, laboratories that do not test for mycoplasma, or use insensitive methods, have a high probability of being infected (UKCCCR, 2000). The source of contamination is usually traced to mycoplasma present in animal serum, or to human oral mycoplasma transferred by droplet infection during cell culture (UKCCCR, 2000); the problem of cross contamination when an infected cell line is obtained from an outside source may also be responsible for mycoplasma contamination.

The gold standard microbiological assay, currently recommended for the mycoplasma testing of biologics by the United States Pharmacopeia (USP), European Pharmacopeia (EP), Japanese Pharmacopeia (JP) and the US FDA, involves the culture of viable mycoplasmas in selective microbiological culture media. Although the procedure enables the specificity of mycoplasma detection in cell culture media ingredients and cell-derived products, the protocols are laborious and are usually very time consuming (up to 28 days for the test completion). The long-term culture required for these conventional assays does not allow their usage for timely "go/no-go" decisions during routine in-process testing (Volokhov et al., 2011).

Recently, PCR-based and other alternative methods for mycoplasma detection, such as Bioluminescent assays, have been proposed as potential approaches for routine cell culture screening. Nucleic acid testing methods employing PCR are widely used and it has been suggested that they have the potential superiority over conventional microbiological methods for mycoplasma testing in terms of analytical sensitivity, simplicity and turnaround time (Louie et al., 2000; Cenciarini-Borde et al., 2009; Molla Kazemiha et al., 2015). Bioluminescent assay, a biochemical method that detects mycoplasma carbamate kinase and/or acetate kinase, is claimed to be very sensitive and fast (30 min) (Mariotti et al., 2008; Molla Kazemiha et al., 2014).

Due to the importance of mycoplasma detection in cell cultures as aforementioned, the aim of this work is to compare the performance of two different commercial assays popularly used for routine cell culture screening, PCR-based and Bioluminescent assays, by assessing the mycoplasma contamination levels in incoming cell cultures at Rio de Janeiro Cell Bank (RJCB) and also from customers' laboratories that wanted to monitor the quality of their cell stocks. The results obtained by both assays were confirmed by scanning electron microscopy. In addition, we evaluated the limit of detection of the PCR kit under our laboratory conditions and the storage effects on mycoplasma detection in frozen cell culture supernatants.

2. Material and methods

2.1. Cell cultures

Cell cultures used in the present study were from Rio de Janeiro Cell Bank (RJCB), a certified repository of cell lines and also from customers' laboratories that agreed to cooperate with this work and/or from customers' laboratories that intended to monitor the quality of their own cell stocks. All of them were located in Brazilian universities or hospitals. Eighty two (82) different cell cultures were analyzed, being 66 samples from RJCB, and 16 samples from other laboratories.

2.2. Sample preparation

Cell lines were cultured for three days in the absence of any antibiotic in order to maximize PCR and Bioluminescent assay responses. Samples were derived from cultures that were at 90–100% confluence.

2.3. Storage effects on mycoplasma detection

To evaluate the storage effects on mycoplasma detection, the supernatants (100 μL) of all samples were frozen at −20 °C. The analysis by PCR and Bioluminescent assays for positive samples was carried out seven (T7) and thirty (T30) days after the sample storage.

2.4. PCR-based assay

The VenorGeM® Mycoplasma Detection Kit (Sigma-Aldrich, USA, Catalog Number MP0025) was employed for the PCR-based assay. The primer set supplied is specific to the highly conserved 16S rRNA coding region (s) in the mycoplasma genome which allows for the detection of the *Mycoplasma* and *Acholeplasma* species, commonly observed as contaminants in cell cultures and also *Ureaplasma* species, least represented in contaminated samples.

The PCR assay was performed according to the manufacturer's instructions. Briefly, templates for PCR analysis were prepared by boiling the supernatant of cell cultures. Supernatants (100 μL) from cell cultures were transferred to a sterile microcentrifuge tube; the samples were heated at 95 °C for 5 min and centrifuged for 5 s to remove cell debris before adding to the PCR mixture. The PCR reaction was carried out in a 25 μL mix containing 2 μL of sample supernatant, 2.5 μL of primer mix, 2.5 μL of 10× reaction buffer supplied with $MgCl_2$ (3.0 mM), 0.4 μL of Jumpstart *Taq* polymerase (2.5 U/μL-Sigma-Aldrich, USA), 2.5 μL of internal control DNA (plasmid DNA including mycoplasma-specific primer sequences and an internal sequence of the HTLV-I *tax* gene with a size of ~191 bp) and 15.1 μL of deionized water. A positive control reaction tube was prepared with 2 μL (>10^4 copies) of DNA-fragments of the *M. orale* genome (positive control DNA; yields 270 bp band), supplied by VenorGeM®. A negative control containing 2 μL of deionized water instead of 2 μL of sample supernatant was included in all of the PCR analysis. The cycling conditions comprised of an initial denaturation step for 2 min at 94 °C, followed by 39 cycles: 94 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s (Veriti, Applied Biosystems). The amplified fragments were analyzed by electrophoresis on a 1.5% agarose gel, loading 5.0 μL of each PCR reaction product, mixed with 1.0 μL of bromophenol blue loading buffer and 1.0 μL of Gel Red (Biotium, Inc., USA). The electrophoresis was conducted for 20 min at 100 V and the products were visualized under UV illumination and photographed (Molecular Imager-Gel Doc™ XR + Imaging System, BioRad).

Also, the limit of detection (LOD) of the VenorGeM® Mycoplasma Detection Kit was evaluated under our laboratory conditions. DNA purified from *M. orale* (NCTC 010112, UK) using QIAamp®DNA Blood Mini Kit (Qiagen, Cat. No. 51104) was kindly supplied by Minerva Biolabs GmbH (Berlin, Germany). Three independent 10-fold dilution (from 10^5 copies to 10^{-1} copies) series of mycoplasma DNA were tested

with 4 replicates for each dilution by two operators on different days. 2 μL of each dilution was used for a PCR reaction. The LOD was determined as the lowest copy number of *M. orale* DNA detectable.

All work was performed under the GLP conditions in a laminar flow bench.

2.5. Bioluminescent assay

The Bioluminescent assay was performed using the MycoAlert® Mycoplasma Detection Kit (Lonza, Inc., USA, Catalog Number LT07-318). The MycoAlert® Assay is a selective biochemical test that monitors the activity of selected mycoplasma enzymes. The presence of enzymes provides a rapid screening procedure, allowing the sensitive detection of mycoplasma contamination in a test sample. The enzymes of viable mycoplasmas react with the MycoAlert® Substrate catalyzing the conversion of ADP to ATP. The MycoAlert™ Assay Control Set (Lonza, Inc., USA, Catalog Number LT07-518) that provides a lyophilized positive control and assay buffer for reconstitution that also serves as a negative control was used as control for assay performance before readings of samples to be tested.

The assay was carried out according to the manufacturer's instructions. First, 2 mL of culture supernatant samples was transferred into a centrifuge tube and the cells were pelleted at 1500 rpm (200 ×*g*) for 5 min. Afterwards, 100 μL of cleared supernatants was transferred into a luminometer tube. The luminometer (Lucetta™, Lonza, USA) programmed to take a 1 second integrated reading and 100 μL of reagent supplied by MycoAlert® Mycoplasma Detection Kit was added to each sample. After 5 min, the tube was placed in the luminometer and the program was initiated (Reading A). Subsequently, 100 μL of substrate supplied was added to each sample. After 10 min, the tube was placed in the luminometer and the program was initiated (Reading B). The ratio of Reading B/Reading A was calculated. The ratio was used to determine whether a cell culture was contaminated by mycoplasma or not (ratio >1 = cell culture contaminated).

2.6. Scanning electron microscopy (SEM)

The SEM analyses were performed to evaluate random cell samples and to confirm the positive PCR and Bioluminescent assays results. The cells were grown on sterile glass coverslips for at least 5 days, without antibiotics and with daily medium changes for 3 days. The samples were washed with a phosphate-buffered saline solution (PBS) and fixed with Karnovsky's solution for 2 h at room temperature. The cells were washed again with PBS, post-fixed with 1% osmium tetroxide in distilled water for 1 h and washed twice. The samples were dehydrated in a graded acetone series, critical point dried (Leica EM CPD030, Germany), sputter coated with 5 nm of gold (Leica EM SCD 500, Germany) and imaged in a field emission scanning electron microscope (FEI Magellan 400 XHR-SEM, Netherlands) at an acceleration voltage of 15 kV.

2.7. Statistical analysis

Agreements between assays were measured using Kappa analysis (GraphPad Software — Free Web Calculator). McNemar's test was used to compare proportions in 2-by-2 contingency tables. Fisher's exact test was used to compare the contamination level between the RJCB and customers' laboratories. A p value of <0.05 was considered significant.

3. Results

A comparison between the results of the PCR-based and Bioluminescent analysis is presented in Table 1. The cell culture analysis by PCR (Fig. 1) showed a mycoplasma contamination in 20/82 samples (24.4%) and 23/82 samples (28.05%) were shown to be contaminated when the analysis was performed by the Bioluminescent assay. Overall, mycoplasmas were detected in 24/82 of the cell cultures (29.3%) by PCR and/or by Bioluminescent assays. The performance of both assays for detecting mycoplasma was not significantly different ($p = 1.000$; McNemar's test) and they showed very good agreement (98.7%; Kappa = 0.966; 95% confidence interval [CI], 0.899 to 1.032).

Table 1
Mycoplasma Contamination in Cell Cultures: Results Obtained by PCR-based and Bioluminescent assays.

Mycoplasma contamination	PCR-based assay	Bioluminescent assay
Positive samples	20	23
Negative samples	57	59
Inconclusive analysis	5[a]	0
Total of samples	82	82

[a] Absence of amplification in the internal reaction control.

One sample presented contradictory results between the two methods employed: the sample was positive for mycoplasma contamination by PCR and negative (mycoplasma-free) by the Bioluminescent assay. The same discrepant result was observed after retesting the original sample.

The mycoplasma-contaminated sample data stratification showed that 22% (18 samples) of cell cultures received by RJCB from other laboratories were contaminated whereas 7.3% (6 samples) of cultures from customers' laboratories showed positive results. The level of contamination in the incoming cell cultures at the RJCB was 18/66 (27.3%) and it was 6/16 (37.5%) in cultures from customer laboratories. This difference of contamination levels was not statistically significant ($p = 0.5411$, Fisher's exact test), and may represent the mean contamination level of culture cells in Brazil.

Five samples analyzed by PCR showed inconclusive results due to the absence of amplification in the internal reaction control (Table 1); four of them being mycoplasma positive by Bioluminescent assay. None of the samples showed a negative result for mycoplasma contamination by PCR and a positive result by Bioluminescent assay.

Only one disagreement was observed between the techniques: one sample was considered contaminated (positive) by PCR and mycoplasma-free by Bioluminescent assay on first analysis. This cell line was cultured again in the absence of antibiotics for four days, instead of the usual three days without antibiotics. The new analysis presented positive results by both methodologies. This data indicates that the duration of antibiotic-free culture of the studied sample may be critical, and four days may be the lowest limit to obtain safe results.

Additionally, 14 cell cultures were analyzed by a third tool for mycoplasma detection: scanning electron microscopy (SEM) (Fig. 2A and B). The results are presented in Table 2. The data obtained by PCR and Bioluminescent were confirmed by SEM. The two inconclusive results by PCR (FGH and HGE-3 cells) were positive when the cells were analyzed by SEM, being in agreement with the Bioluminescent assay.

The limit of detection (LOD) of the PCR kit was determined using DNA extracted from *M. orale*. The agarose gel electrophoresis results for one representative run are shown in Fig. 3. Therefore, under our laboratory conditions, the validated LOD was 10 copies/PCR with 2 μL sample volume.

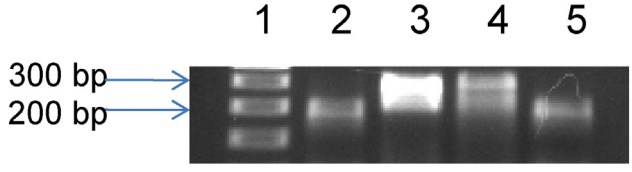

Fig. 1. Detection of mycoplasmas contamination by PCR (VenorGeM® Mycoplasma Detection Kit). Agarose gel electrophoresis for PCR product evaluation. Lane 1—100 bp ladder (Fermentas); lane 2—negative sample; lane 3—positive sample; lane 4—positive control; lane 5—negative control.

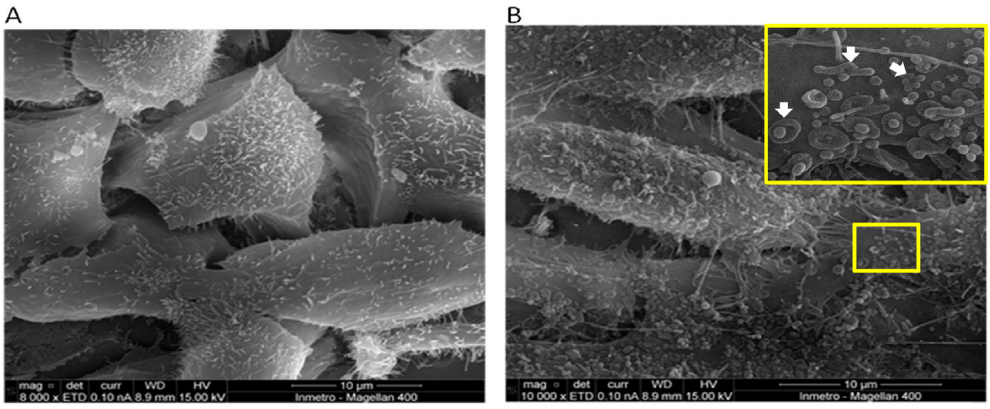

Fig. 2. A and B: Scanning electron micrographs showing C3A cells without mycoplasma (A) and HGE-3 cells with mycoplasma infection (B). Some individual mycoplasma cells are highlighted by arrows (2B).

The frozen supernatant of 12 mycoplasma positive samples by both methodologies, PCR and Bioluminescent assays, were re-evaluated seven (T7) and 30 (T30) days after sample storage (Table 3).

The analysis by PCR showed positive results in 11/12 (91.7%) and 9/12 (75%) samples, seven (T7) and thirty (T30) days respectively, after sampling at −20 °C. Three cell lines, RT4, IT45-R1 and MV3, presented inconclusive results (no amplification). All samples (100%) showed positive results when evaluated by the Bioluminescent assay, regardless of the storage time.

4. Discussion

The purpose of the current study was to compare two different rapid commercial assays, PCR-based and Bioluminescent ones, used widespread for routine mycoplasma detection in laboratories. The present study has shown that these two different assays have a similar performance to assess mycoplasma contamination in cell culture. However, false negative results may be more inconvenient than false positive results, especially when cells or products obtained from cell cultures are used in humans. The mycoplasma assays thus have to be carefully standardized. According to our results, the Bioluminescent assay for mycoplasma detection was slightly more dependable than PCR-based assay due to the lack of inconclusive results produced by the first technique. This was more evident in the analysis of the ability of the assays to detect mycoplasma contamination in cell culture supernatants frozen up to 30 days. However, cell lines should be pre-cultured for four days or more without antibiotics to obtain safe results. On the other hand, a false negative result was obtained by using this biochemical approach.

Table 2
Results obtained by three different assays: PCR, Bioluminescent and SEM.

Cell cultures	Mycoplasma detection assays		
	PCR-based assay	Bioluminescent assay	SEM
A549	Negative	Negative	Negative
H4	Negative	Negative	Negative
RD	Negative	Negative	Negative
FGH	No amplification[a]	Positive	Positive
VERO[b]	Positive	Positive	Positive
9L/LacZ	Negative	Negative	Negative
HGE-3	No amplification[a]	Positive	Positive
MDA-MB-231	Negative	Negative	Negative
VERO[b]	Negative	Negative	Negative
C3A (HEPG2/C3A)	Negative	Negative	Negative
HFF-1	Negative	Negative	Negative
HRT-18	Positive	Positive	Positive
LLC-MK2	Positive	Positive	Positive
IEC-18	Positive	Positive	Positive

[a] Absence of amplification in the internal reaction control.
[b] Vero cell lines from different sources.

The validity of any experimental results, or bioproducts produced using unknown infected cultures, is not reliable. Regulatory bodies and many ISO (International Organization for Standardization) standards require that cell cultures used either for toxicological analysis, the production of reagents for diagnostic kits or therapeutic agents must be mycoplasma-free. The policy of some prestigious scientific journals, such as Nature Biotechnology, and others, requires an author's statement of the use of mycoplasma-free and authenticated cells (UKCCCR, 2000; Nature Biotechnology Editorial, 2013). However, Olarerin-George and Hogenesch (2015) showed that top peer-reviewed journals such as Nature, Cell, PNAS, Genome Research, and Genes and Development present publications having some of the most contaminated series of cells with the highest number of mycoplasma. Moreover, these articles are generally well cited, half of them receiving more than 50 citations since 2009 or later. According to the Second ECVAM Task Force on Good Cell Culture Practice, mycoplasma testing is recommended and should be carried out on all samples received. It is essential that all cell stocks have been tested for the presence of mycoplasma and that a regime of routine testing has been put in place (Coecke et al., 2005).

Mycoplasma contamination can cause an impact on biological analysis involving cell cultures, since it affects many cell functions. Nearly 30% of all cell cultures evaluated in this study were contaminated by mycoplasma. In studies carried out in the USA for the Food and Drug Administration (FDA), over 20,000 cell cultures were examined, 15% of which were found to be contaminated (Barile, 1979). In Europe, mycoplasma contamination levels were found to be even higher: over 25% of 1949 cell cultures from the Netherlands and 37% of 327 cultures from former Czechoslovakia were positive (McGarrity, 1988). Employing different approaches, the incidence of mycoplasma contamination was reported to be 57.5% in Iran (Molla Kazemiha et al., 2014), 80% in Japan (Koshimizu and Kotani, 1981) and 88.7% in Mexico (Rivera et al., 2009).

Many methods are available for the detection of mycoplasma, including a microbiological assay, direct or indirect fluorescent staining, ELISA, immunostaining, autoradiography, PCR, and other alternative methods, such as biochemical assays and molecular hybridization

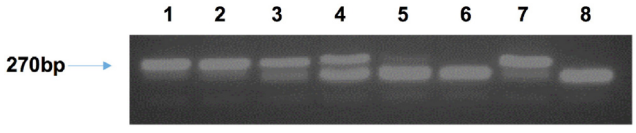

Fig. 3. LOD determination of the VenorGeM® Mycoplasma Detection Kit under laboratory conditions. Agarose gel electrophoresis for one representative run of *M. orale*. Lane 1—100,000 copies of *M. orale* DNA; lane 2—10,000 copies of *M. orale* DNA; lane 3—1000 copies of *M. orale* DNA; lane 4—100 copies of *M. orale* DNA; lane 5—10 copies of *M. orale* DNA; lane 6—1 copy of *M. orale* DNA; lane 7—positive control; lane 8—negative control.

Table 3
Mycoplasma detection in frozen supernatants from positive samples, seven and thirty days after storing.

Cell cultures	T7 days		T30 days	
	PCR-based assay	Bioluminescent assay	PCR-based assay	Bioluminescent assay
B16F1O	Positive	Positive	Positive	Positive
RT4	No amplification[a]	Positive	No amplification[a]	Positive
VERO	Positive	Positive	Positive	Positive
H9C2	Positive	Positive	Positive	Positive
K562 LUCENA	Positive	Positive	Positive	Positive
TS7	Positive	Positive	Positive	Positive
IT45-R1	Positive	Positive	No amplification[a]	Positive
ECV304	Positive	Positive	Positive	Positive
MV3	Positive	Positive	No amplification[a]	Positive
IEC6	Positive	Positive	Positive	Positive
3T3	Positive	Positive	Positive	Positive
3T6	Positive	Positive	Positive	Positive

[a] Absence of amplification in the internal reaction control.

(Volokhov et al., 2011). The PCR-based assay for mycoplasma detection presents many advantages: it is fast, easy, relatively low-cost, and sensitive. A VenorGeM® Mycoplasma Detection Kit provided by Sigma-Aldrich-USA was used in this work and provides primers designed to target 24 different mycoplasma species, including the six mycoplasma species that account for at least 95% of all infections (see Table 1), based on 16S rRNA highly conserved between all species of mycoplasmas. According to the manufacturer, the primers used do not detect eukaryotic DNA or bacterial genera with a close phylogenetic relation to mycoplasmas, and the detection requires as little as 1–5 fg of mycoplasma DNA (corresponding to 2–5 mycoplasma per sample volume) giving a range of specificity and sensitivity. The LOD of the VenorGeM® PCR kit was assessed under our laboratory conditions. The sensitivity detected was 10 copies of mycoplasma DNA/PCR, the same reported by Zhi et al. (2010) in a validation study of a PCR method for mycoplasma detection according to EP. The high sensitivity of this PCR-based assay could explain the result observed for one sample classified as being mycoplasma positive by PCR and mycoplasma-negative by Bioluminescent assay on first analysis, and after a new pre-culture for a longer time (four days without antibiotics), the second analysis showed positive results by utilizing both methodologies. The data sheet from the Bioluminescent kit suggests that cell lines should be pre-cultured for "several days without antibiotics", an unclear and imprecise requirement of the protocol that may contribute to false negative results. This could explain the single discordant result observed in our study, after sample retesting, being the culture classified as contaminated by PCR, and not contaminated by bioluminescence.

Culture in agar is usually considered as the 'gold standard' assay for mycoplasma detection in cell culture. Studies comparing microbiological assays and PCR demonstrate that the nucleic acid-based techniques are robust methods for the detection of mycoplasma (Rivera et al., 2009; Lawrence et al., 2010; Ingebritson et al., 2015). Rivera et al. (2009) reported a significant difference ($p < 0.05$) between microbiological cultures and PCR for mycoplasma detection: ten samples (12.8%) tested were positive by PCR and negative by mycoplasma culture. It is worth to mention that our results do not intend to propose PCR (or biochemical assay) as an alternative method to replace the microbiological assay, therefore no comparison between PCR (or Bioluminescent assay) and culture-based gold standard is presented here.

The Bioluminescent assay evaluated here by the MycoAlert® Mycoplasma Detection Kit (Lonza, Inc., USA) uses the substrate for two mycoplasma enzymes. This is a very fast assay (30 min), with a lower cost when compared to a PCR kit; it is sensitive, and the enzymes detected are found in all six of the main mycoplasma cell culture contaminants and in most of the other 180 mycoplasma species that have been determined. The primary advantage of the MycoAlert® test is that—unlike PCR-based methods—it can distinguish between dead and alive mycoplasmas, and also does not need to rely on universal PCR primer sets. A validation study of the nested PCR and the biochemical method performed by MycoAlert®, as alternatives for mycoplasma detection, showed similar ruggedness, repeatability, and robustness, as the direct culture method being 10^3 and 1 CFU/mL the upper and lower thresholds of the detection limit, respectively (Cheong et al., 2011). However, according to the manufacturer, it is intended as a presumptive screening tool, and any positive samples should be retested by a second confirmatory method, since the enzymes carbamate kinase and/or acetate kinase detected by this assay are also found in other bacteria. In this work, we did not observe any sample presenting a negative result for mycoplasma contamination by PCR, and a positive result by Bioluminescent assay.

Regarding the difference between the number of positive samples obtained by the performed assays, four positive cultures by Bioluminescent assay presented inconclusive results by PCR. The inconclusive results were characterized by the absence of amplification in the internal reaction control, and that could be explained by the presence of inhibitory substances in the supernatant culture (Young et al., 2010; Uphoff and Drexler, 2013, 2014). However, the presence of inhibitors does not seem to be involved in the lack of amplification observed in frozen IT45-R1 and MV3 samples thirty days after sampling since the amplification was obtained when samples were tested seven days after storing (Table 3). The third methodology for mycoplasma detection, SEM, was applied to assess 14 samples, including positive, negative and inconclusive samples, in order to confirm the previous results. The analysis by SEM matched 100% the results obtained by Bioluminescent assay and helped us to classify the samples that showed an absence of amplification by PCR (2/14).

Since mycoplasmas are organisms devoid of a cell wall, they are sensitive to the freezing/thawing conditions necessary for long-term storage at low temperatures (Volokhov et al., 2011; Raccach et al., 1975). For longer storage after sampling, lower temperatures from −20 °C to −80 °C are recommended by the JP, Code of Federal Regulations (CFR), and FDA. In the present work, the mycoplasma evaluation of 12 frozen supernatants from mycoplasma positive cell cultures stored at −20 °C was performed seven and thirty days after sampling. The analysis showed that it is possible to detect mycoplasma by both methodologies applied, but that the Bioluminescent assay was more robust. This result is in accordance with the work by Cheng et al. (2007) who found viable mycoplasmas with little or no reduction in titers after storage for 8 weeks at −30 °C and has a direct impact on how to transport the material to be analyzed. However, freezing/thawing and the storage of mycoplasma samples, prior to their testing, should be carefully controlled, since these factors may affect the viability of the mycoplasma cells. Improper care of the test samples may consequently lead to false-negative results during mycoplasma testing for cell culture screening (Cheng et al., 2007).

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