Original Article

Polymerase chain reaction with internal amplification control to detect mycoplasmas in cell cultures and raw materials

Reacción en cadena de la polimerasa con control interno de amplificación para detectar micoplasmas en cultivos celulares y materias primas

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ABSTRACT: The presence of polymerase chain reaction **(**PCR) inhibitory substances in cell cultures and raw materials used in the manufacture of monoclonal antibodies could limit the usefulness of this method to detect mycoplasma contamination. The objective of this research was to determine the sensitivity and specificity of a PCR method to detect mycoplasma deoxyribonucleic acid (DNA) in MDCK and HeLa cell lines, hybridoma cells, ascitic fluid and cell supernatant containing monoclonal antibodies. The effect of a thermal shock-based mycoplasma DNA extraction method on PCR sensitivity, as well as the performance of two mycoplasma DNA purification methods: silica/guanidinium thiocyanate and a commercial DNA purification kit, were evaluated. In addition, an internal amplification control (IAC) was optimized to detect inhibitory samples from these matrices after thermal shock treatment. PCR-IAC was specific for the amplification of mycoplasma DNA. The inclusion of IAC plasmid at the concentration selected did not decrease the sensitivity of PCR in mycoplasma-spiked matrices. Seventy-eight samples were analyzed; all represented the matrices under study. As a result, all matrices showed PCR inhibition after thermal shock. The inhibitory effect decreased when silica/guanidinium thiocyanate or commercial DNA purification kit was used. PCR-IAC detected 65 % of inhibitory samples and revealed differential susceptibility to inhibitors among different samples of the same matrix.

Key words: PCR, inhibition, internal amplification control, monoclonal antibodies, mycoplasma.

RESUMEN: La presencia de sustancias inhibitorias de la reacción en cadena de la polimerasa (PCR) en cultivos celulares y materias primas usadas en la fabricación de anticuerpos monoclonales podría limitar la utilidad de esta técnica para detectar la contaminación por micoplasmas. El objetivo de esta investigación fue determinar la sensibilidad y la especificidad de un método de PCR para detectar ácido desorribunucleico (ADN) de micoplasma en las líneas celulares MDCK y HELA, en células de hibridoma, en líquido ascítico y en sobrenadante celular que contenía un anticuerpo monoclonal. Se evaluaron el efecto de un método de extracción de ADN micoplásmico, basado en choque térmico sobre la sensibilidad de la PCR, así como el desempeño de dos métodos de purificación de ADN micoplásmico: sílice / tiocianato de guanidinio y un kit comercial de purificación de ADN. Además, se optimizó un control de amplificación interno (CAI) para detectar muestras inhibitorias de estas matrices después del tratamiento de choque térmico. La PCR-CAI fue específica para la amplificación del ADN de micoplasma; la inclusión del plásmido CAI a la concentración seleccionada no disminuyó la sensibilidad de la PCR en las matrices experimentalmente contaminadas con micoplasma. Se analizaron 78 muestras; todas representaron las matrices en estudio. Como resultado, todas las matrices mostraron inhibición de la PCR después del choque térmico. El efecto inhibitorio disminuyó cuando se utilizó sílice / tiocianato de guanidinio o el kit comercial de purificación de ADN. La PCR-CAI detectó 65 % de muestras inhibitorias y puso en evidencia una susceptibilidad diferencial a los inhibidores entre diferentes muestras de una misma matriz.

Palabras clave: PCR, inhibición, control de amplificación interno, anticuerpos monoclonales, micoplasma.

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INTRODUCTION

The biotechnology industry has grown considerably in recent years, primarily the manufacture of monoclonal antibodies and other products for biomedical applications. These products are widely used in medical therapy and purification processes in the biotechnology and pharmaceutical industry. Regulatory agencies establish procedures to control the quality of such products (1, 2, 3, 4, 5, 6). The control of mycoplasma contamination is included for safety reasons. In addition, this contamination in cell cultures is known to influence the growth, morphology and cell metabolism of infected cells (7,8,9), leading to a failed experiment, a low-quality bioproduct and a waste of time and investment.

The polymerase chain reaction (PCR) methodology has widely been used for the detection of mycoplasma contamination in cell cultures and their products (8, 10,11). This assay has several advantages: it is rapid, highly sensitive, and it does not require living mycoplasmas to evidence contamination. However, the presence of nucleic acid amplification inhibitors in the samples tested can reduce PCR performance, leading to false negative results (12).

Sample processing for PCR is critical. Inhibitory substances are not only present in the matrix, but they can also be added to the sample during DNA extraction/purification step. These inhibitory substances can include ethanol, phenol, isopropanol, and ionic detergents such as sodium deoxycholate, sarkosil, and sodium dodecyl sulfate (SDS) (13,14).

Thermal shock extraction of mycoplasma deoxyribonucleic acid (DNA) has been successfully used for molecular detection of *Mollicutes* species (15). It is a fast, efficient, non-laborious, inexpensive, and useful method for processing a large number of samples. However, DNA is not purified with this method, and therefore the exclusion of inhibitory substances is not guaranteed (16). The use of an internal amplification control in PCR assays is very important to detect inhibitory samples and to avoid false negative results due to inhibition. Nevertheless, the concentration of such a control and the reaction parameters for its amplification must be carefully optimized (17,18).

The variety of matrices requiring mycoplasma detection is increasing due to the extension of quality requirements to almost all products derived from the biotechnology industry. Reports on the performance of PCR for the detection of mycoplasma contamination in different matrices of cell cultures are scarce. The purpose of the present study was to evaluate the performance of a PCR assay for the detection of mycoplasma DNA in samples previously processed by thermal shock or subjected to DNA purification. Samples included MDCK and HeLa cell lines, hybridoma cells, ascitic fluid, and cell supernatant with monoclonal antibodies. In addition, an internal amplification control was optimized to detect inhibitory samples from these matrices.

MATERIALS AND METHODS

Cell lines and biological products

Mycoplasma free Madin-Darby Canine Kidney Epithelial Cells (MDCK Line) and HeLa (human cervical carcinoma cells) were obtained from the Mycoplasma laboratory belonging to "Instituto de Ciências Biomédicas-II, Universidade de São Paulo", Brazil. Mycoplasma free hybridoma cells, cell supernatant containing monoclonal antibodies (IgG isotype) and ascitic fluid were kindly supplied by the National Center for the Production of Laboratory Animals (CENPALAB), in Cuba. The absence of mycoplasma in the cell lines and biological products was confirmed by microbiological culture and PCR, following the quality standards of the Reference Laboratory for Mycoplasma Diagnosis (MYCOLAB) at the National Center for Animal and Plant Health, Cuba.

Mycoplasma strain and culture conditions

A *Mycoplasma arginini* culture (NCTC 10129) containing 3,9x10^8CFU/mL was used as positive control for the assays. The strain was grown in modified Hayflick medium (19). Broth culture was placed at 37°C until color change. Afterwards, 100 µL of grown culture were transferred to Hayflick agar plates and placed at 37°C for 48 h. Mycoplasma colonies were visualized and counted to calculate and adjust CFU/mL.

PCR sensitivity for mycoplasma detection in cell cultures and biological products after thermal shock treatment of samples

PCR sensitivity for the detection of mycoplasmas in cell cultures (MDCK and HeLa cell lines) and biological products derived from the manufacture of monoclonal antibodies (hybridoma cells, cell supernatant containing an IgG isotype monoclonal antibody and ascitic fluid) was evaluated after thermal shock treatment of the samples. One milliliter of each matrix and nuclease-free water was spiked with3.9x10^8CFU/mL *Mycoplasma arginini* and ten-fold diluted to 3.9 CFU/mL. Each dilution was processed by thermal shock (20).

Briefly, 1ml of each dilution was centrifuged at 12 000 rpm for 10 minutes. Pellets were homogenized in 1mL of sterile phosphate-buffered saline (PBS), shaken and centrifuged again. Such pellets were then resuspended in 100 µL of nuclease-free water. After boiling for 10 minutes, samples were cooled on ice and stored at -20^{0}C.

PCR reaction was carried out in a 25 µL volume containing 1,25 U of GoTaq® DNA polymerase; 1X of Green GoTaq® Flexi Buffer; 1,5 mM of $MgCL_2$ (GoTaq®Flexi DNA Polymerase kit, Promega); 200 µM of each dNTP (Promega) and 0,5 µM of each primer GPO-3 (5'-GGGAGCAAACAGGA-

TrAGATACCCT-3') and MGSO (5'-TGCACCATCTG TCACTCTGTTAACCTC-3') amplifying a 270 bp region of the mycoplasma 16S rRNA (21). Five microliters of the extracted DNA were added to the reaction. The cycling conditions for PCR were 10 minutes at 94°C, followed by 40 cycles of 30s at 94°C, 30s at 60°C and 35s at 72°C, followed by a final step of 5 min at 72°C. PCR products were visualized under UV light after electrophoresis on a 2 % agarose gel stained with ethidium bromide.

The effect of the matrices on mycoplasma DNA amplification was determined by comparing the PCR detection limit in each matrix with that obtained in nuclease-free water. Matrices with a reduced PCR detection limit or no DNA amplification were considered slightly or totally inhibitory, respectively.

PCR sensitivity for mycoplasma detection in cell cultures and biological products after mycoplasma DNA purification

The usefulness of two different DNA purification methods to reduce the inhibitory effect of the matrices tested on mycoplasma DNA amplification was evaluated. Serial ten-fold dilutions of *Mycoplasma arginini* culture, as described above, were performed in the matrices and in nuclease-free water. Each dilution was processed as follows: first, using a method based on DNA extraction using silica/guanidinium thiocyanate, with modifications (22). Briefly, 500 µL of lysis buffer and 40 µL of silica were added to 1ml of each dilution. The mix was shaken vigorously for 20s and kept at room temperature for 10 min. The pellet was obtained by centrifugation at 14000 rpm for 4 min, subsequently; it was washed twice with 500 µL of wash buffer, homogenized for 30s and centrifuged under the same conditions. It was then diluted in 500 µL of cold 70 % ethanol, homogenized for 30s and centrifuged. The latter was repeated but using acetone instead of 70 % ethanol. It was also homogenized with acetone, oven dried, eluted in 120 µL of nuclease-free water and placed for 10 minutes at 56°C. Finally, tubes were centrifuged and the supernatant was recovered and stored at -20^{0}C until use.

In addition, DNA extraction and purification from the dilutions were performed using Wizard® Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. PCR reactions were performed as described above. The reduction of the inhibitory effect of the matrices was evaluated by comparing the PCR detection limit obtained after using both purification methods and that one obtained in nuclease free water.

Internal amplification control

A PCR with internal amplification control (PCR-IAC) was optimized to detect inhibitory samples from cell cultures and biological products previously processed by thermal shock during the detection of mycoplasma DNA. The plasmid pTtPV was obtained from the Animal Virology Laboratory at the National Center for Animal and Plant Health in Cuba, and it was used as internal amplification control. This plasmid was constructed by inserting a fragment of the L1 gene of the dolphin papillomavirus into the pGEM-T-easy vector (Promega), and it was preserved in recombinant *Escherichia coli* JM 109 recombinant cells. Plasmid DNA was extracted and purified using Wizard ® Plus Midipreps DNA Purification System (Promega), following the manufacturer's instructions. DNA concentration was measured on a NanoDrop 3300 (ThermoScientific).

Optimization of critical parameters

Different annealing temperatures (55ºC, 60ºC and 65ºC), $MgCl_2$ concentrations (from 0,5 mM to 3,5 mM) and IAC primer concentrations (from 0,1 µM to 0,6 µM) were evaluated. All PCR reactions were performed by simultaneously amplifying IAC plasmid and mycoplasma genomic DNA. For the amplification of mycoplasma DNA, 0,5 µM of primers GPO-3 and MGSO was used (21). Five microliters of genomic DNA extracted from a *Mycoplasma arginini* culture containing 39 CFU/mL (the lowest amount of *Mycoplasma arginini* detected by the PCR previously described) were added to the reaction mix. Mycoplasma genomic DNA was extracted by thermal shock. Primers M13 uni (-21) (5`TGTAAAACGACGCCAGT3`) and M13 rev (-29) (5`CAGGAAACAGCTATGACC3`) were used for the amplification of IAC plasmid. Those primers amplified a 654 bp fragment of the pTtPV plasmid. To determine IAC concentration to be added as a template during the optimization reactions, plasmid DNA was tenfold prediluted in nuclease-free water. The lowest IAC concentration that remained amplified by PCR was determined. IAC amplification during the optimization study was performed by adding 56.2 fg/µL of the plasmid pTtPV, being tenfold more concentrated than the lowest IAC concentration still amplified by PCR, as recommended (17). Primer specificity for simultaneous amplification of mycoplasma DNA and IAC was previously evaluated *in silico* and *in vitro* (data not shown).

Sensitivity and specificity of the PCR-IAC to amplify mycoplasma DNA

To evaluate the analytical sensitivity of the assay to amplify the target DNA (mycoplasma DNA); 56,2 fg/µL of IAC were simultaneously amplified with decreasing concentrations of *Mycoplasma arginini* DNA, (50 ng/µlL, 25 ng/µL, 10 ng/µL, 1 ng/µL, 100 pg/µL, 75 pg/µL, and 50 pg/µL). Analytical

specificity was evaluated using 100 ng of purified DNA from *Lactobacillus spp*, *Streptococcus suis* and *Clostridium sporogenes*, as recommended by the European Pharmacopoeia, 2014 (1). Purified DNA from these bacteria was kindly provided by the Animal Bacteriology Laboratory at the National Center for Animal and Plant Health, Cuba. All PCR reactions were carried out using 1.5 U of GoTaq® DNA polymerase, annealing temperature of 60ºC, 2 mM of $MgCl_2$ (GoTaq®Flexi DNA Polymerase kit, Promega) and 0,4 µM of primers M13 uni (-21) and M 13 rev (-29), as previously optimized.

A DNA extraction method, based on the combination of silica and guanidinium thiocyanate (22) and a commercial DNA purification kit (Wizard®Genomic DNA Purification Kit, Promega), was used to remove inhibitors from the matrices analyzed to increase PCR sensitivity for mycoplasma detection.

Optimal IAC concentration to detect inhibitory samples

PCR sensitivity to amplify mycoplasma DNA or IAC could have varied depending on the type of matrix (18). Therefore, an optimal concentration of plasmid DNA could be used as IAC whatever the matrix was (MDCK and HeLa cell lines, hybridoma cells, cell supernatant containing monoclonal antibodies, or ascitic fluid), without decreasing PCR sensitivity to amplify mycoplasma DNA in the contaminated samples.

Matrices were spiked with the lowest concentration of *Mycoplasma arginini* DNA still amplified in each of them and they were then processed by thermal shock. Five microliters of the DNA extracted in PCR reactions were used with different IAC amounts (5,62 fg/µL, 14,0 fg/µL and 28,0 fg/µL). Those IAC amounts approached the detection limit of the plasmid obtained in nuclease free water (5,62 fg/µL), as determined above. PCR was carried out as described above for IAC simultaneous amplification and mycoplasma DNA on the matrices. The optimal IAC concentration to be added during sample analysis was defined as the plasmid concentration at which two amplification bands (IAC and mycoplasma DNA) were observed in all matrices after visualization in 2 % agarose gel electrophoresis.

Use of the PCR-IAC in biological samples

Seventy-five samples were tested, including three MDCK and three HeLa cell lines, 24 hybridoma cells, 32 cell supernatant samples with monoclonal antibodies (IgG isotype), and 16 ascitic fluid samples. They were previously analyzed by culture and PCR without internal amplification control by the Reference Laboratory for Mycoplasmas Diagnosis (MYCOLAB) at the National Center for Animal and Plant Health, Cuba. All samples were processed by thermal shock (20). Five microliters of each sample and 28.0 fg/µL of IAC (IAC optimal concentration previously determined for matrices) were added to the PCR reaction. The PCR assay for IAC simultaneous amplification and mycoplasma DNA was performed as described above.

RESULTS AND DISCUSION

PCR sensitivity for mycoplasma detection in cell cultures and biological products after thermal shock treatment of the samples

All matrices processed by thermal shock showed an inhibitory effect on mycoplasma DNA amplification. PCR detection limit in the matrices studied was reduced compared to that obtained in nuclease free water. Ascitic fluid was the least inhibitory matrix, whereas hybridoma cells were the most inhibitory, showing a strong reduction in PCR sensitivity for mycoplasma DNA amplification (Figure 1).

PCR inhibitors act through direct interaction with DNA or by affecting the enzymatic activity of the polymerase (14), reducing or even blocking the amplification of target DNA in biological samples with respect to pure nucleic acid solutions (23).

IgG immunoglobulins are among the strongest inhibitors for PCR assays, probably through complex formation with the single-stranded DNA (DNAss). This effect is higher at temperatures above 95°C (24). It is important to notice that IgG is in high concentration in hybridoma cells, ascitic fluid and the cell supernatants analyzed here. Old or high cell density cell cultures may also reduce PCR sensitivity due to inhibitor accumulation in the culture media (HD Biosciences Co. (25).

PCR sensitivity for mycoplasmas detection in cell cultures and biological products after mycoplasma DNA purification

As shown in Figure 1, PCR detection limit increased in MDCK and HeLa cells, in cell supernatant containing monoclonal antibodies and in ascitic fluid after silica/guanidinium thiocyanate treatment. However, not all inhibitors were removed from MDCK and HeLa cells by this method, since the sensitivity of the assay was lower than that obtained with nuclease free water. The treatment with silica/guanidinium thiocyanate failed to reduce PCR inhibition in hybridoma cells. Wizard® Genomic DNA Purification Kit was useful in removing all inhibitors in all matrices, including hybridoma cells, because the detection limit reached the same limit obtained in nuclease free water.

The commercial DNA purification kit employed here can guarantee an efficient nucleic acid extraction, providing high-quality DNA with minimum inhibitors (26). However, it may not be the first choice for laboratories routinely analyzing large numbers of samples

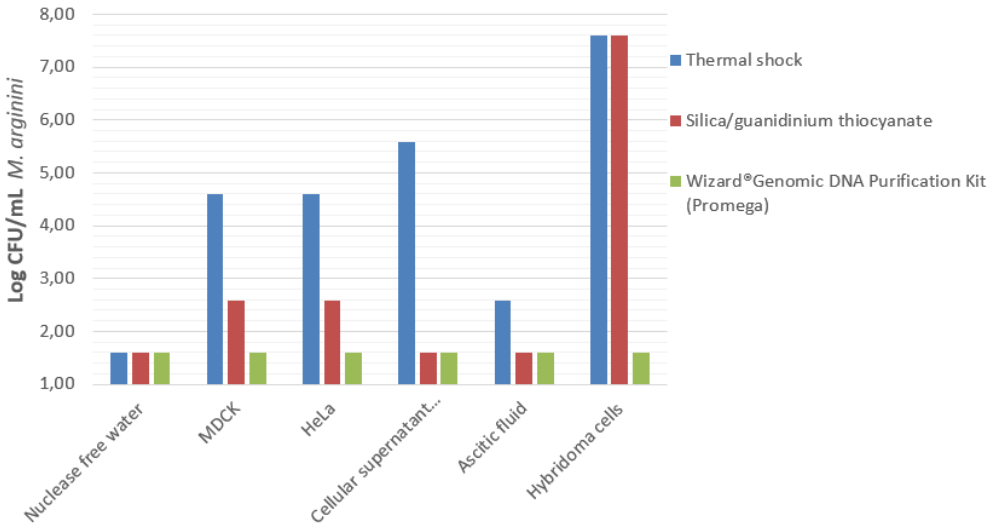

Figure 1. Detection limit of PCR to detect mycoplasma DNA in matrices processed by thermal shock, silica/guanidinium thiocyanate and Wizard®Genomic DNA Purification Kit (Promega). / *Límite de detección de la PCR para detectar ADN de mycoplasma en matrices procesadas por choque térmico, sílice/tiocianato de guanidinio y Wizard ® Genomic DNA Purification Kit (Promega).*

due to increased costs. The extraction of mycoplasma DNA by a thermal shock-based method is fast and inexpensive, but it does not allow DNA purification and nucleic acid remains together with cellular detritus, proteins and other inhibitors after cell membrane rupture (16). Therefore, when using this method, it is important to include an IAC to detect inhibitory samples.

PCR-IAC optimization

IAC primers amplify a 654 bp fragment from the plasmid pTtPV. This fragment size prevents the competence between the target DNA (a 270 bp fragment from mycoplasma genome) and IAC. This is because shorter DNA fragments are preferentially amplified with regard to larger fragments in simultaneous amplification (17).

IAC plasmid concentration in PCR reaction is very important. To optimize critical PCR-IAC parameters, the plasmid was added at a concentration of 56.2 fg/µL, as the PCR detection limit for IAC amplification was 5.62 fg/µL (data not shown). This agrees with the recommendation that IAC be added in the reaction at a concentration close to its own PCR detection limit (27), not only to limit competence with the target DNA but also to demonstrate PCR sensitivity (8).

IAC primer concentration is also a critical point. In PCR-IAC assays based on a non-competitive amplification system, the plasmid and target DNA are amplified using a different primer pair. IAC amplification should be limited by keeping the concentration of its primers at a suboptimal level to reduce competition between IAC and target DNA for reaction components (17). In this research, better results were obtained in the simultaneous amplification of IAC and mycoplasma DNA with 0.4 µM IAC primers at annealing temperature of 55°C - 60°C with 2.0 mM MgCl2, (data not shown).

PCR-IAC sensitivity and specificity

PCR-IAC sensitivity for mycoplasma DNA amplification in nuclease free water was 39 CFU/ml, the same detection limit of PCR without IAC. This demonstrates that the inclusion of IAC at the selected concentration (56.2 fg/µL) did not affect the efficiency of mycoplasma DNA amplification in nuclease free water. The assay was specific for *Mycoplasma arginini* DNA, as no cross-reactivity was observed with DNA from phylogenetically closed bacteria (*Lactobacillus* spp., *Streptococcus suis* and *Clostridium sporogenes*).

Use of PCR-IAC in biological samples after thermal shock treatment

Inhibitory substances can reduce the analytical sensitivity of PCR-IAC in biological samples (18,28). Therefore, the optimal quantity of IAC in the PCR reaction was carefully determined for each matrix, before testing the samples.

Although in this experiment, IAC detection limit in nuclease free water was 5.62 fg/µL, a higher concentration of the plasmid was needed to amplify IAC in hybridoma, MDCK and HeLa cells. At 28.0 fg/µL of IAC plasmid, mycoplasma DNA and IAC were simultaneously amplified in almost all the matrices tested. This strategy agrees with that of Moalic *et al.* (28), who increased IAC concentration to guarantee its amplification during the detection of *Mycoplasma meleagridis* in clinical samples from chickens. In the

Table 1. Simultaneous amplification of mycoplasma DNA and different IAC concentrations in the matrices. / *Amplificación simultánea del ADN micoplásmico y de diferentes concentraciones del CAI en las matrices.*

Matrix	Processing method	Detection limit (CFU/mL of *M. arginini*)	Positive amplification of target DNA and IAC		
			IAC concentrations (fg/µL)		
			5,62	14,0	28,0
Nuclease free water	-	39	IAC +Target	IAC +Target	IAC +Target
MDCK	Thermal shock	$3.9x10^4$	Target	IAC +Target	IAC +Target
HeLa	Thermal shock	$3.9x*10^4$	Target	Target	IAC +Target
Cell supernatant containing monoclonal antibodies	Thermal shock	$3.9x10^5$	IAC +Target	IAC +Target	IAC +Target
Ascitic fluid	Thermal shock	$3.9x10^2$	IAC +Target	IAC +Target	IAC +Target
Hybridoma cells	Thermal shock	$3.9*10^7$	Target	Target	Target
Hybridoma cells	Wizard® Genomic DNA Purification Kit	3.9x10	Target	Target	IAC +Target

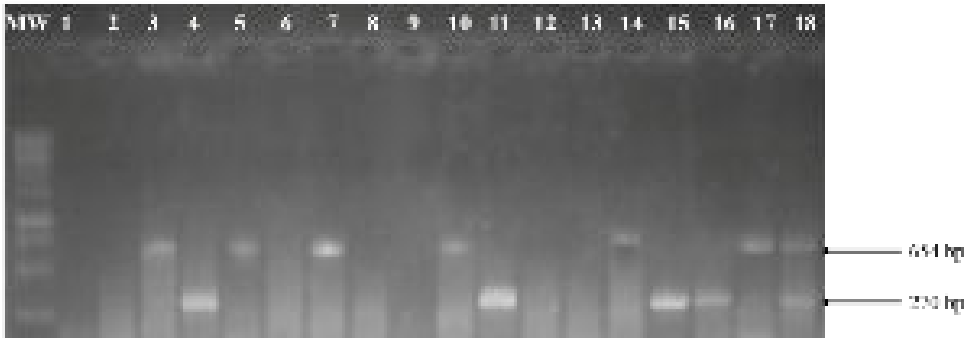

Figure 2. Use of PCR-IAC in samples processed by thermal shock. MW: Molecular weight 1000 pb (Promega). Line 1: Nuclease free water (negative control). Lines 3, 5, 7, 10 and 14: *Mycoplasma* negative samples; Lines 4, 11 and 15: *Mycoplasma* positive sample; Lines 2, 6, 8, 9, 12 and 13: inhibitory samples; Line 16: *Mycoplasma arginini* DNA (*Mycoplasma* positive control); Line 17: IAC; Line 18: *Mycoplasma arginini* DNA and IAC (control reaction of the simultaneous amplification). / *Uso de la PCR-IAC en muestras procesadas por choque térmico. MW: Peso molecular 1000 pb (Promega). Línea 1: Agua libre de nucleasas (control negativo). Líneas 3, 5, 7, 10 y 14: Muestras negativas de Mycoplasma; Líneas 4, 11 y 15: Muestra positiva de Mycoplasma; Líneas 2, 6, 8, 9, 12 y 13: Muestras inhibidoras; Línea 16: ADN de Mycoplasma arginini (control positivo de Mycoplasma); Línea 17: CAI; Línea 18: ADN de Mycoplasma arginini y CAI (reacción de control de la amplificación simultánea).*

case of hybridoma cells, the matrix was also processed using Wizard® Plus Midipreps DNA Purification System (Promega), due to the strong inhibition for DNA amplification observed in latter after thermal shock treatment (Table 1).

IAC should be added to the reaction at a concentration that does not inhibit the amplification of the target DNA (29). At very high concentrations, IAC competes with the target DNA, reducing the analytical sensitivity of the assay and leading to false negative results (17). It is also important that IAC concentration is not too low, as this would limit its own amplification (30).

Using 28.0 fg/µL of the IAC, 51 inhibitory samples were detected (65 % of all samples of the matrices analyzed in this study). Some samples from the same matrix were inhibitory, while others were not. The 75 % (18/24) of hybridoma cell samples, 94 % (15/16) of ascitic fluid samples and 56 % (18/32) of cell supernatant samples containing monoclonal antibodies showed inhibition of DNA amplification in this assay. In the case of MDCK and HeLa cells, no sample (0/3) was inhibited, as mycoplasma DNA and IAC were always amplified. However, in previous experiments, those matrices showed inhibition for mycoplasma DNA amplification. These results suggest that different samples from the same matrix are not homogeneous, and may affect PCR sensitivity differently. Other authors have reported PCR inhibition in cell cultures (8, 24).

The inhibitory samples identified in this study showed no mycoplasma DNA or IAC plasmid amplification band; whereas the non-inhibitory samples showed a mycoplasma DNA and/or IAC plasmid amplification band (Figure 2).

Despite the absence of IAC signal, some samples were considered non-inhibitory in the present experiment, since mycoplasma DNA amplification band was visible (Figure 2). In this case, a high amount of mycoplasma DNA in the sample could suppress IAC, which was found in the reaction mix at a much lower concentration (8). Some authors state other causes of failure regarding IAC amplification. IAC DNA may be degraded or attached to the plastic tube, reducing the plasmid concentration in the PCR. IAC concentration in the reaction should be guaranteed by adequate plasmid conservation or by preparing and using new plasmid dilutions if necessary (7, 8, 10).

CONCLUSIONS

Thermal shock treatment for mycoplasma DNA extraction prior to PCR assay is advantageous. However, the presence of inhibitors in the matrices may affect the sensitivity of the assay and thus the interpretation of the results. In this research, it is shown the inhibitory effect of hybridoma cells, cell supernatant containing monoclonal antibodies (IgG isotype) and ascitic fluid on PCR. It is highly advisable to purify DNA extracted from these matrices prior to PCR, but if thermal shock is the unique method of choice, an internal amplification control should be included in the PCR to detect inhibitory samples. Inhibitory samples identified during routine analyses should be purified and reanalyzed to obtain reliable results.

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