CANDIDA PARAPSILOSIS AS VERO CELL CULTURE CONTAMINANT IN *NEOSPORA CANINUM* ANTIGEN PRODUCTION

Raíssa Vasconcelos Cavalcante[1]
Mariana Kikuti[1]
Virginia Bodelão Richini-Pereira[1]
Helio Langoni[1]

ABSTRACT

Neospora caninum has been identified as an important cause of infectious in cattle and dogs. The parasite is successfully established in continuous passage in VERO cell cultures. Cell culture contamination may present itself in two mainly forms: contamination with other cell line or with microorganisms. In this paper we report a VERO cell culture of *Neospora caninum* antigen production contaminated with *Candida parapsilosis.* Sequencing of the ITS amplicons showed 99% similarity with deposited sequences of *Candida parapsilosis* at GenBank. It should be noted through this report the possibility of contamination by *Candida parapsilosis* in cell cultures, thus care in handling cultures should be redoubled.

Keywords: *Neospora,* VERO cells, *Candida,* cell culture contamination

CANDIDA PARAPSILOSIS COMO CONTAMINANTE DE CULTURA DE CÉLULAS VERO NA PRODUÇÃO DE ANTÍGENO DE *NEOSPORA CANINUM*

RESUMO

Neospora caninum tem sido identificado como um importante agente causador de infecções em bovinos e caninos. O parasita é mantido com sucesso em passagens contínuas em culturas de células VERO. A contaminação de culturas células pode ocorrer de duas formas principais: contaminação com outras linhagens celulares ou com micro-organismos. Neste trabalho, relata-se uma cultura de células VERO para produção de antígeno de *Neospora caninum* contaminada com *Candida parapsilosis*. O sequenciamento dos amplicons da região ITS apresentou 99% de similaridade com *Candida parapsilosis* nas sequências depositadas no GenBank. Ressalta-se através deste relato a possibilidade de contaminação por *Candida parapsilosis* em cultivos celulares, devendo-se redobrar os cuidados na manipulação.

Palavras-chave: *Neospora*, células VERO, *Candida*, contaminação de culturas celulares

CANDIDA PARAPSILOSIS COMO CONTAMINANTE DE CULTIVOS CELULARES VERO EN LA PRODUCCIÓN DE *NEOSPORA CANINUM* ANTIGEN

RESUMEN

Neospora caninum ha sido identificado como un importante agente causador de infecciones en bovinos y caninos. Este parásito es mantenido con éxito en cultivos celulares VERO. La contaminación de los cultivos celulares puede ocurrir de dos formas principales: la contaminación con otras líneas celulares o con microorganismos. Este trabajo relata un cultivo de células VERO para la producción de antígeno de *Neospora caninum* contaminada con *Candida parapsilosis*. La secuenciación de los amplicones de la región ITS presentó 99% de similitud con las secuencias de *Candida parapsilosis* depositadas en GenBank. Se destaca en

[1] Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária e Zootecnia, UNESP-Botucatu

esta comunicación la posibilidad de contaminación de los cultivos celulares por Candida *parapsilosis* y, por lo tanto, la importancia de redoblar los cuidados en la manipulación de estos.

Palabras clave: *Neospora*, células VERO, *Candida*, contaminación de cultivos celulares

Neospora caninum is a parasite discovered and described in 1984 and 1988, respectively and affects mainly cattle and dogs. It is traditionally maintained in laboratories in cell cultures for antigen production for serologic diagnosis by indirect immunofluorescence technique (1).

Since cell culture production consists of a aseptic environment, its maintenance depends on physical and chemical controls to prevent contamination. Cell culture contamination may present itself in two mainly forms: contamination with other cell line or with microorganisms. In the second form, contamination may arise from the operator or from laboratory environment, such as media, serum, poor aseptic technique and airborne contamination. Microbial contamination agents are mycoplasma, bacteria, fungi, yeasts and viruses, being mycoplasma the most common agent reported in cell culture contaminations (2,3).

In this paper we report a VERO cell culture of *Neospora caninum* antigen production contaminated with *Candida parapsilosis.* The genus *Candida* is characterized by globose, ellipsoidal, cylindroidal or elongate cells with holoblastic budding reproduction and the ability of form pseudohyphae (4). *Candida* is considered the most important saprophytic opportunistic mycoses reported causing oral, gynecological and dermatological disease (9, 10). It affects mainly patients in intensive care units, with cancer, undergoing surgical procedures and neonates (5).

The species *C. parapsilosis* was described by Langeron and Talice in 1932 (12). It is the second leading agent of candidemia in Latin America and it is distributed through all ages, including neonates. *C. parapsilosis* cluster in the northern hemisphere, and associated with lower mortality rates (5).

The NC-1 strain of *Neospora caninum* is maintained in Vero cell culture in the Zoonosis Diagnostic Laboratory (FMVZ-UNESP/Botucatu) for production of antigens for Indiret Immnunofluorescence (IIF), used in routine and surveys diagnostic in the laboratory.

Vero cells are grown in sterile and disposable polystyrene bottles of 25 cm^2. The culture is maintained with 6 ml of RPMI 1640 medium supplemented with 10% of inactivated and sterile fetal bovine serum (FBS) and added 1% of antibiotic-antimycotic solution containing streptomycin, penicillin and amphotericin B (Invitrogen Cat. n. 15240-096). It is incubated at 37ºC, 5% CO_2 and with humidity control, favoring cell growth.

The cell monolayer is generated from growth and confluence of cell being dispersed, in order to obtain cells free to chime in new bottles with the aid of the enzyme trypsin. After the establishment of the monolayer, which occurs after 48 hours of incubation, the bottles are inoculated with 500 µL of suspension of tachyzoites of *N. caninum* NC-1 strain. In four days, formation and disruption of the cysts occurs. For the production of antigen through the infection of cell it is used medium without addition of FBS. The inverted microscope "Leica MS60" and magnification of 400X is used to observe the growth of cell and parasites.

Before and after handling the bottles, a strict aseptic protocol is carried out using 10% sodium hypochlorite solution, 70% ethanol and UV light for 20 minutes around the material to be used such as bottles of medium and trypsin, pipettes, automatic pipettor, disposal container, gloves and gauze.

In an attempt to reduce the risk of contamination between bottles of *N. caninum* tachyzoites inoculated and cells bottles, the bottles inoculated with tachyzoites are handled before the bottles containing only cell culture.

It was first observed in a cell culture bottle uninoculated with tachyzoites, the presence of oval to spherical structures, individually or grouped in clusters, which does not destroy the cell monolayer. The bottle that was contaminated was discarded and reinforced the procedures of antisepsis and asepsis.

Then, two bottles were presented contaminated with the same structure and were selected to perform microbiological examination, along with aliquots of the means used (RPMI enriched with antibiotics-antimycotics with and without FBS, fetal bovine serum and tripsin. These samples were plated on blood agar and incubated in a bacteriological incubator at 37ºC, with evaluation for 72 hours. In 48 hours, there was growth of small colonies round, shiny, smooth and creamy from the contaminated cell culture. Gram staining was performed in which yeast-like organisms were observed, gram-positive cells presenting as varying from globose to elongated, thin-walled features of *Candida* spp.. The samples were sent for molecular detection. To control contamination, culture media, trypsin and FBS were discarded and replaced with new sterile jars, despite the negative result in the blood agar. The cleaning procedures for the room and the sterile laminar flow were reinforced and contaminated bottles were discarded, obtaining good results in controlling contamination.

The DNA extraction from *Candida* spp. was performed according to Van Burik et al. (6) using the Kit Illustra Tissue & Cells genomic Prep Mini Spin® (GE Healthcare, USA). The amplification was carried out by PCR reactions, using as primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAACG-3') that amplified 634 bp as described by White et al. (7). The process was performed in 25 µL containing 1X PCR buffer (10 mM Tris HCl pH 8.0, 50 mM KCl), 1.5 mM MgCl2, 200 µM dNTP, 10 ρmol of each primer, 10 ng of purified DNA and 0.2 U.µL-1 of Platinum Taq DNA polimerase system (Invitrogen, Brazil). Thermal cycling conditions were 94°C for 5 min, followed by 25 cycles at 94°C for 1 min, at 60°C for 2 min and 72ºC for 2min and a final extension at 72°C for 7 min. PCR products were identified by agarose gel electrophoresis. Amplicons were purified by employing ExoSap (USB, USA). Using the sequencer 3500 Genetic Analyser (Life Technologies, USA), reactions was carried out in both strands according to DYEnamicTM ET Dye Terminator Cycle Sequencing Kit (GE Healthcare). The obtained sense and antisense sequences were visualized (Chromas 2.3 software, Technelysium Pty Ltd, Australia), aligned by the MEGA 4 software and compared with the NCBI database using BLASTn (Basic Local Alignment Tool for Nucleotide).

The molecular identities of these amplicons were confirmed by sequencing showing 99% similarity with *Candida parapsilosis* DNA sequence deposited at Gen Bank (gb|HQ 263346.1).

Although some authors have reported contamination with yeast, the data in the literature are scarce (8). It remains unknow the source of the contamination described. The commercial culture media used, trypsin, bovine sera, the technical staff responsible for the ringing of the cultures, as well as the aerosol formed during tripsinization of bottles of contaminated cell cultures can be sources of contamination, although in this case growth of the agent was not observed in the mediums used.

The medium used for cultures maintenance was increased by the antifungal amphotericin B in order to reduce the risk of fungal contamination. However, it is recommended to control yeast and especially *C. parapsilosis*, the use of ketoconazole and itraconazole (8). In addition, there is controversy about the use of antibiotics, as some authors argue that these may lead to aseptic technique failure in addition to inhibiting some processes of eukaryotic cells and hide the presence of microbial contamination, since, without the use of antibiotics, they shall become apparent as soon as possible, and indicates that culture should be discarded before spreading contamination to other bottles or that one should try to decontamination. The decontamination should be done only as a last resort, since it is not always successful and can lead to the development of antibiotic resistance (9, 10).

Another important source of contamination is the people who handle the cell cultures, so the main contaminants will be the microorganisms present in human skin flora. It is know that *C. parapsilosis* is typically a commensal of human skin and its pathogenicity is limited by intact skin (5).

In a study, it was observed that most opportunistic pathogens microorganisms were human recovered, which are inhabitants of healthy skin, throat, mucous membranes and are also found in the environment. People disperse hundreds to thousands of organisms per minute in the air. So, these microorganisms can travel through the air and be deposited in cell cultures and later produce contamination (10).

The culture media and reagents, such as trypsin and fetal bovine serum were exchanged, and the care of the manipulator with cleaning materials and equipment used and the maintenance of cell cultures have been strengthened.

It should be noted through this report the possibility of contamination by *Candida parapsilosis* in cell cultures, thus care in handling cultures should be redoubled.

REFERENCES

1. Dubey JP. Review of Neospora caninum and neosporosis in animals. Korean J Parasitol. 2003;41:1-16.

2. Lincoln CK, Gabridge MG. Cell culture contamination: sources, consequences, prevention, and elimination. Methods Cell Biol. 1998;57:49-65.

3. Langdon SP. Cell culture contamination: an overview. In: Cell culture: methods and protocols. 2nd ed. New Jersey: Humana Press; 2011. p.309-17.

4. Lodder J. The yeasts: a taxonomic study. 2nd ed. Amsterdam: North Holland; 1970.

5. Nucci M, Queiroz-Telles F, Tobón AM, Restrepo A, Colombo AL. Epidemiology of opportunistic fungal infections in Latin America. Clin Infect Dis. 2010;51:561-70.

6. Van Burik JAH, Schreckise RW, White TC, Bowden RA, Myerson D. Comparison of six extration techniques for isolation of DNA from filamentous fungi. Med Mycol. 1998;36:299-303.

7. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. San Diego: Academic Press; 1990. p.315-22.

8. Behrens UJ, Paronetto F. Elimination of the yeast Candida parapsilosis from lymphoid cells and monolayer cells in culture. In Vitro. 1994;20:391-5.

9. Freshney RI. Culture of animal cells: a manual of basic technique and specialized applications. 6th ed. New York: John Wiley & Sons; 2010.

10. Cobo F, Cortes JL, Cabrera C, Nieto A, Concha A. Microbiological contamination in stem cell cultures. Cell Biol Int. 2007;31:991-5.

Recebido em: 05/06/12
Aceito em: 18/10/12