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FLORESCENT *IN SITU* HYBRIDIZATION (FISH) METHOD OPTIMIZATION FOR RAPID DETECTION OF *PROTOTHECA* IN CLINICAL SAMPLES

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Abstract: Members of the genus *Prototheca* are aerobic; unicellular algae related to the green algae of the genus *Chlorella;* but without chlorophyll. They are resistant and ubiquitous and can be isolated from a great variety of environmental sources.

Many species of *Prototheca* genus have been identified but only two *P. zopfii* and *P. wickerhamii*; are able to produced infection in humans and animals. Frequently *P. zopfii* is most incriminated into the pathologic process in animals; and *P. wickerhamii* in humans; but is still hard to establish a strict delimitation. By different animal species; the most sensitive seems to be the cows; in witch will evolve as enzootic mastitis; and dogs in witch some systemic infections are described. Majority of humans infections are produced by *P. wickerhamii* and were mostly described in patients with immunosuppressant disorders; chronic diseases or after intensive treatments with various antibiotics.

In this study we report a FISH method optimization; for *Prototheca*; which can be used for rapid diagnosis both in humans as well as in animals protothecosis. Reliable and quick identification of *P. zopfii* and *P. wickerhamii* is important for accurate treatment and understanding their role in the pathogenesis of infections. Fluorescent *in situ* hybridization (FISH) of *Prototheca* algae with specific eukaryotic probe EUK 516 ACCA GAC TTG CCC TCC leads to a reduced time to identification. In clinical practice; FISH therefore can be used in situations in which quick identification is necessary for optimal treatment of the patient.

INTRODUCTION

Members of the genus *Prototheca* are unicellular algae related to the green algae of the genus *Chlorella;* but without chlorophyll (Pore; 1998; DiPersio; 2001). They are resistant and ubiquitous and can be isolated from a great variety of environmental sources (Melville *et al.*; 1999; Răpuntean; 2002; Bexiga *et al.*; 2003; Zhao *et al.*; 2004).

The taxonomic status of *Prototheca* has evolved in recent decades; and many species are currently assigned to the genus; among this only two: *Prototheca zopfii and Prototheca wickerhamii* have been reported as etiological agents both for mans as well as for animals protothecosis (Răpuntean Gh. & Răpuntean S.; 1999; Huerre M.; *et al.*; 1993; DiPersio; 2001).

Numerous reports have indicated a pathogenic potential for *P. zopfii* and *P. wickerhamii*. *P. wickerhamii* is predominantly isolated from clinical cases of human infections (Bianchi *et al.*; 2000; Matsuda & Matsumoto; 1992). *P. zopfii* causes infections in animals; particularly of diary cows and dogs (Blogg & Sykes; 1995; Ginel *et al.*; 1997; Schultze *et al.*; 1998). Worldwide; *P. zopfii* has been identified as inducing a therapy-resistant inflammation of the mammary gland in dairy cows that may lead to severe economic losses in an infected. Therefore; rapid and correct identification of *Prototheca* isolates is important in clinical microbiology laboratories.

At present; fluorescent *in situ* hybridization (FISH) is considered in the field of clinical microbiology as a rapid and specific detection method. The FISH technique is based on fluorescently-labeled oligonucleotide probes that specifically target and hybridize to ribosomal RNA (rRNA); so that the whole microbial cell can be visualizied directly by fluorescence microscopy (Moter et al.; 1998).

FISH is a technique with a large number of applications in molecular biology; medical science; clinical research and allowed to detect directly the presence of the suspect on small samples of patient's tissue (Nath et al.; 2000). Therefore; the objective of this study was to report a FISH method optimization; for *Prototheca*; which can be used for diagnosis both in humans as well as in animals protothecosis.

MATERIAL AND METHODS

The investigations took place during March-May 2008 within the Microbiology Laboratory of the Faculty of Veterinary Medicine Lisbon; Portugal. The research was based on 6 mastitis isolates of *Prototheca zopfii* (from Faculty of Veterinary Medicine; Cluj Napoca; Microbiology Discipline Collection) and one reference strain of *Prototheca wickerhamii* (strain RE-4608014 ATCC 16529 from American Type Collection).

Clinical samples can be constituted by: cutaneous lesions; tissue samples from biopsy collected; diarrhea feces; blood; milk; internals with granuloma lesions.

The method comprises of three basic steps: fixation of a specimen on a microscope slide; hybridization of a labeled probe with homologous fragments of genomic DNA; and enzymatic detection of targed hybrids.

The isolates (irrespectively of the strain) were cultivated in Sabouraud Dextrose Broth for 24 h; at 37 ºC. After treatment of the 10 well teflon microscopic slides used as hybridization supports; 10 µl algal suspension were placed in each well. Afterwards the slides were keept at laboratory temperature for surface complet drying.

Alga fixation was made with 4% paraformaldehyde (10 µl/well; 2 h at room temperature).

Because *Prototheca* cells wall is formed by cellulose (polisaccharide formed by glucose molecules); for permeabilization we used cellulase β 1.4 with a high concentration (3; 6 mg/ml); during 3 h; at 60ºC. The cellulase we used; (CelA); belongs to a cellulosic enzyme system consisting of three major components: endo-β-1.4 glucanase (CelA); exo-β-glucanase and β-glucosidase [Pétré *et al*.; 1981]. CelA was one of the first cellulase that was purified from the culture supernatant of the thermophilic anaerobe *Chlostridium thermocellum* [Pétré *et al*.; 1981]. This bacterium secretes an extracellular multiprotein complex; the cellulosome that is very efficient in degrading crystalline cellulose [Petro *et al*.; 1996].

10 µl hybridization buffer; pH 7;2 (0;9 M NaCl; 20 mM TRIS; 0;1% SDS) containing 5 ng/ml eukaryotic probe (EUK 516 ACCAGACTTGCCCTCC) were added. Slides were incubated in a humid chamber (Omnislide Thermal Cycling Block; Hybaid Omnislide System; Thermoelectron corporation; USA) at 46ºC during 3 h.

After hybridizatin the slides were washed in a buffer solution at 48ºC during 15 minutes; mounted in DAPI (4';6-diamidine-2'-phenylindole dihydrochloride) Vectashield Mounting Medium (10 µl/well) for 5 min and then visualized by fluorescent microscopy 1000 x; in a Leica DMR microscope; equipped with a mercury lamp of 100W; an I3 filter for excitation between 450-490 nm; and an N2;1 filter for excitation between 515-560 nm.

RESULTS AND DISCUSSIONS

In conditions mentioned above; cellulase β 1.4 at a high concentration (3; 6 mg/ml) allowed nucleic acid denaturation (separated the two strands); and the access of the single strand of eukaryotic probes to their complementary combed single strand.

The application of FISH protocol to alga suspension in Sabouraud Broth allowed the direct observation of *Prototheca* cells in clinical samples. The protocol mentioned above prove to be efficient (fig. 1 and 2) and the method can be used for rapid diagnosis in *Prototheca* infections.

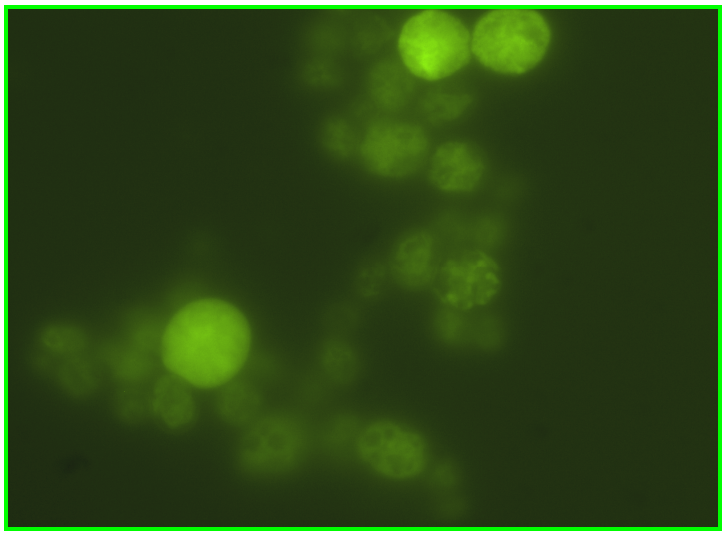

Fig.1; *P. zopfii* detection by FISH; after hybridization with specific eukaryotic probes (EUK 516 ACCA GAC TTG CCC TCC); visualization by fluorescent microscopy 1000 x; in a Leica DMR microscope.

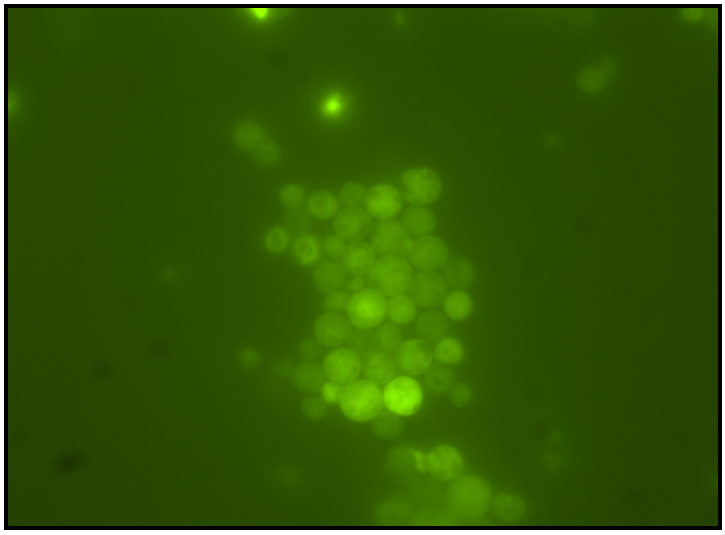

Fig. 2; *P. wickerhamii* detection by FISH; after hybridization with specific eukaryotic probes (EUK 516 ACCA GAC TTG CCC TCC); visualization by fluorescent microscopy 1000 x; in a Leica DMR microscope.

From the pathology point of view the different species of *Prototheca* are regarded as exogenous etiologic agents; with minimum pathogenic activity. Any modifications within the host resistance; especially the immunosupression; are needed first; before these microorganisms could invade the host and act as pathogen agents. Frequently *P. zopfii* is most incriminated into the pathologic process in animals; and *P. wickerhamii* in humans; but is still hard to establish a strict delimitation. The infections have; generally speaking; a chronic aspect; with granulomatous evolutions that are very hard to treat (Răpuntean G et Răpuntean S.; 1999; Răpuntean S.; 2002).

Conventional bacteriological methods require at least 48 h for definite diagnosis of *Prototheca species.* Thus in severe infections and exacerbations empirical; antimicrobial therapy frequently starts without knowledge of the causative microorganism. Specific and rapid detection of infectious agents is essential for administration of appropriate antibacterials and prevention of rapid deterioration of internals functions.

In this study we aimed to report a FISH method optimization for *Prototheca* isolates from bovine mastitis; that would allowed direct observation of alga cells in clinical samples. FISH has been proven to be a rapid; precise and costeffective technique for the identification of *Prototheca* algae within the clinical samples without need for culture.

Since chronic *Prototheca* infection are very difficult to eradicate with antimicrobial therapy; a premature culling of animals and/or an early treatment regime should be implemented for efficient control strategies (Răpuntean Gh. & S. Răpuntean; 1999). Therefore; the development of rapid methods to detect alga cells is essential. In this work; a FISH protocol was developed for a faster detection of *Prototheca*.

The FISH method could be considered an important rapid screening tool for *Prototheca* identification in clinical samples from infected patients and the rapid detection of *Prototheca* would allow the early application of corrective measures.

CONCLUSIONS

- *Prototheca* species are regarded as exogenous etiologic agents; with minimum pathogenic activity. Spite in some specific conditions especially in immunosupression patients; these microorganisms could invade the host and act as pathogen agents.
- Both for mans as well as for animals; protothecosis is very hard to treat; therefore; rapid and correct identification of *Prototheca* isolates is important in clinical microbiology laboratories.
- In this study we report a FISH method optimization for *Prototheca* that would allowed direct observation of alga cells in clinical samples.
- The protocol described here prove to be efficient and and the method can be used for rapid diagnosis in *Prototheca* infections both for mans as well as for animals.

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