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ORIGINAL ARTICLE/ARTICLE ORIGINAL

Molecular characterization of *Prototheca* strains isolated from bovine mastitis

Caractérisation moléculaire de souches de *Prototheca* isolées de cas de mammite bovine

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Summary
Objective. — *Prototheca zopfii* are colourless algae that can cause mastitis in dairy cattle. Based on sequence analysis of the *18S* rRNA gene, three genotypes are described.
Material and method. — To differentiate them, a recent method of diagnostic was proposed based on rDNA amplification by Polymerase Chain Reaction (PCR) and Restriction Fragments Length Polymorphism analysis (RFLP). A total of 30 strains isolated from mastitis cases becoming of various farms were investigated.
Results. — The molecular study allowed the identification of 27 strains belonging to genotype II, three to genotype III and no one to genotype I.
Conclusion. — The simple PCR/RFLP system enabled the rapid, accurate and easily performed identification of genotypes of the species ''*zopfii*''.

MOTS CLÉS
Algue ;
Prototheca ;
Bovin ;
Mastite

Résumé
Objectif. — *Prototheca zopfii* est une algue microscopique incolore responsable de mammite bovine. Sur la base de l'analyse du gène de l'ARN ribosomal *18S*, trois génotypes sont décrits au sein de cette espèce.
Matériel et méthode. — Pour les différencier, une méthode récente permet le diagnostic de ces génotypes au moyen de l'amplification du rDNA par la réaction de polymérisation en chaîne (PCR) et du polymorphisme de longueur des fragments de restriction (RFLP).

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Résultats. — Sur les trente souches de *Prototheca* isolées de mammite bovine provenant d'exploitations différentes, vingt-sept appartenaient au génotype 2 et trois au génotype 3.
Conclusion. — La PCR-RFLP permet une identification rapide et précise des génotypes de l'espèce « *zopfii* ».

Introduction

The *Prototheca* species are aerobic, unicellular, achlorophyllous algae that propagate asexually by endosporulation. They are ubiquitous in nature having been isolated from the soil, water, animal, waste and sewage. They can also be found in many food products. Colourless algae of the genus *Prototheca* of the family *Chlorellaceae* have been identified as causative agents of infections in humans and animals. The only known plants that cause infectious diseases in humans and animals are the colourless algae *Prototheca zopfii* and *Prototheca wickerhamii*. In 1952, *P. zopfii* was first identified as a pathogen of bovine mastitis associated with reduced milk production characterized by thin watery secretions with white flakes [8]. While in the past, only sporadic cases of *Prototheca* mastitis have been observed, this form of mastitis is now endemic in most countries of the world [1,5].

The taxonomic status of *Prototheca* has evolved over several decades and four species are currently assigned to the genus: *P. zopfii*, *P. wickerhamii*, *Prototheca stagnora* and *Prototheca ulmea*. A fifth not generally accepted species was assigned as *Prototheca moriformis* [2,6,9,10]. This species is genetically and biochemically very similar to *P. zopfii*; however, there is a marked heterogeneity between strains of *P. moriformis* [9,14]. Preliminary studies have shown that *P. zopfii* can be differentiated biochemically and serologically into at least three different biotypes [3,11,13].

In contrast to the mastitis caused by other microrganisms, those due to the *Prototheca* are characterized by moderate inflammatory reaction, accompanied by a weak clinical expression of chronic evolution. From milk, the culture of *Prototheca* is easy in a simple Sabouraud medium and the three genotypes of this species edge can be identified by their biochemical characteristics. [12] Roesler and al., 2006 recently proposed a method of diagnosis of *Prototheca* by PCR-RFLP. Most recent phylogenetic investigations based on the *18S* rDNA unequivocally revealed discriminating molecular characteristics between the three different *P. zopfii* biotypes.

The present study involved the comparative investigation of three biotypes by means of sequence analysis of the *18S* rDNA gene. This study is an approach to elucidate the epidemiology of bovine *Prototheca* mastitis by molecular characterization from 30 *P. zopfii* [7] isolated from different exploitations of Belgium and France.

Materials and methods

The reference strains SAG 2063 genotype I (GenBank #AY933040), SAG 2021 genotype II (GenBank #AY 940456) and SAG 2063 genotype III (GenBank #X63519) were investigated with German mastitis isolates and were used by Roesler et al. as controls [11].

A total of 30 *Prototheca* strains were cultured on Sabouraud dextrose agar plates during 48 h (Fig. 1).

Preparation of genomic DNA was carried out with DNeasy® Plant kit (Qiagen). The following genotype II and genotype I specific primer pair was used for genotype specific PCR [12]:

- forward: Proto 18-2f: 5′-CGCGCAAATTACCCAATCC-3′;
- reverse: Proto 18-2r: 5′-AACGGGACGGCCAGAGT-3′.

For genotype III the specific primer pair was:

- forward: Proto 18-4f: 5′-GACATGGCGAGGATTGACAGA-3′;
- reverse: Proto 18-4r: 5′-AGCACACCCAATCGGTAGGA-3′.

PCR amplification (25 μl/reaction) was carried out with the Master mix (4 μl (5 U/μl) TAQ Polymerase, 1.5 mM $MgCl_2$, 5 to 10 μl (≈50 ng) genomic DNA, 1.25 μl (10 μM) each primer and 1.25 μl (4 mM) (dNTP). PCR conditions were 40 cycles with 1 mn denaturation at 95 °C, 1 mn annealing at 53 °C (58 °C for genotype III) and 1 mn extension at 72 °C.

In addition, the following genotype specific endonucleases were chosen for RFLP analysis: *Kpn 21* (biotype I), *Smal* (biotype II) and *Bcn I* (biotype III) (Fermentas). The RFLP analysis was performed using non-specific amplicons of the *P. zopfii 18S* rDNA. The target sequences of the specific restriction enzymes differed from the target sequences of the above described genotype specific oligonucleotides.

The RFLP analysis was performed on products using the oligonucleotides Proto 18-2f and Proto 18-2r for amplified *Kpn 21*/*Smal* and Proto 18-4f and Proto 18—4r for *Bcn I*.

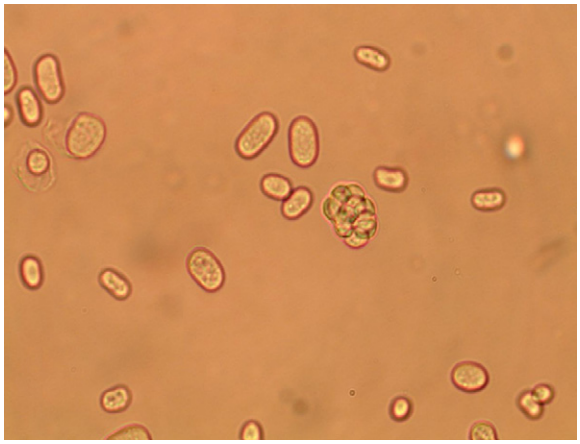

Figure 1 Micrograph of *Prototheca zopfii*.
Microphotographie de Prototheca zopfii.

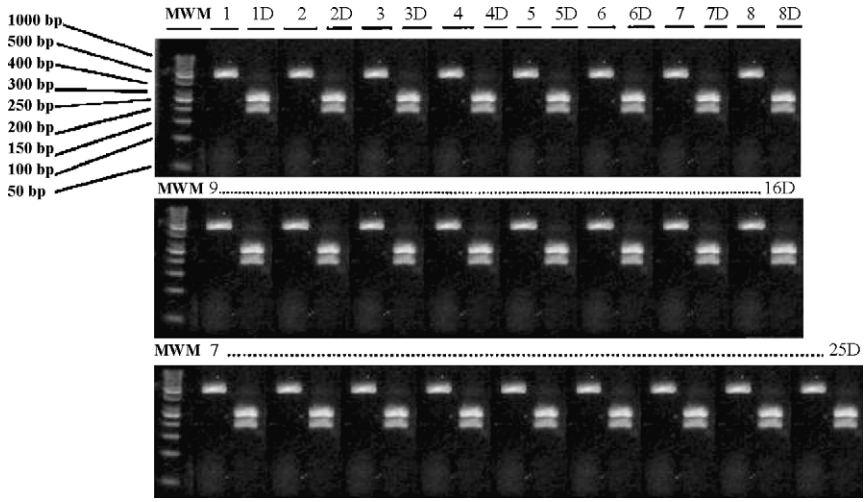

Figure 2 Analysis by an agarose gel electrophoresis of the products amplified by PCR with the primer pair proto18-2f/2r (lanes 1 to 25) then digested by *SmaI (lanes1D to 25D)*. The 450 bp fragment is cut in two fragments of 315 and 135 bp by *SmaI*. MWM = molecular weight markers.
Analyse par électrophorèse sur gel d'agarose des produits amplifiés par PCR par les amorces proto18-2f/2r (lignes 1 à 25) puis digérés par SmaI (lignes1D à 25D).

The amplified PCR products as well as the RFLP fragments were analyzed by electrophoresis on a 4% agarose and detected under UV light at 250 nm after staining gel with ethidium bromide.

Results

We first aligned the sequences of the primer pairs proposed by Roesler and al., 2006 to distinguish the three genotypes on the rDNA sequences. The alignment 18S of the reference strains (see Section Materials and methods) showed a difference of 24.2% between genotype III (#X63519) and the genotype I (#AY973040), 23.7% between genotype III and II (#AY940456) and 0.9% between genotype I and II. Primers proto18-2f/2r aligned as expected. However, primers 18-4f/4r could only be aligned on sequence upon inversion of the 5' and 3'ends given the 18S rDNA of genotype III in Roesler et al. [12].

A total of 30 *Prototheca* isolates were grown on Sabouraud medium and their genomic DNA was extracted. The 18S rDNA fragments were then submitted to amplification by PCR. We amplified the 450 bp and 409 bp fragment with the proto 18-2f/2r and proto 18-4f/4r primer pair, respectively.

We then performed further characterization by RFLP. The 450 bp fragment of all investigated mastitis isolates was cut by the *P. zopfii* genotype II specific restriction enzyme *SmaI*. The *P. zopfii* genotype I and III fragments were respectively cut by *Kpn21* and *Bcn1*.

Twenty five *Prototheca* PCR fragments were cut by *SmaI* and thus corresponded to genotype II (315, 135 bp) (Fig. 2). No fragment was cut by *Kpn21*.

Three other *Prototheca* fragments were cut by *BcnI* corresponding to genotype III (235, 179 pb).

Two samples were however not cut (Fig. 3).

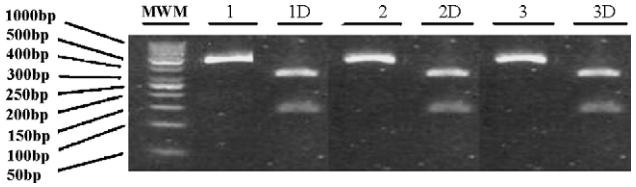

Figure 3 Analysis by an agarose gel electrophoresis of the products amplified by PCR with the primer pair proto18-4f/4r (lanes 1,2,3) then digested by *BcnI (lanes1D, 2D,3D)* .The 409 bp fragment is cut in two fragments of 230 and 179 bp by *BcnI*. MWM = molecular weight Markers.
Analyse par électrophorèse sur gel d'agarose des produits amplifiés par les amorces proto18-4f/4r (lignes 1,2,3) puis digérés par BcnI (lignes 1D, 2D,3D).

Discussion

In order to clarify the taxonomic position of *Prototheca*, a recent study on the phylogeny based on a molecular analysis of *18S* rDNA shows that *P. zopfii* has three different genotypes. The purpose of our work was to study the pathogenic diversity of the species *P. zopfii* and to apply diagnostics test for the routine work of the ARSIA laboratory.

Based on the results of this study, it has to be assumed that bovine algal mastitis is really caused by the pathogenic genotype II of *P. zopfii* as presumed by some other authors [3,4]. Whereas *P. zopfii* genotype I and III do not seem to be involved in the aetiology of bovine *Prototheca* mastitis.

The results of this study show that 25 samples of 30 different exploitations in Belgium and France correspond to the *P. zopfii* genotype II. In spite of the small number of samples, the results obtained are interesting preliminaries for studies that should include also a large number of mastitis strains from other countries with a high prevalence of bovine *Prototheca* mastitis (e.g different European countries). In fact, the comparison between these sequences of different *Prototheca* allowed the classification of *P. zopfii* into three different genotypes. Thus, we showed that the

characterization of the three genotypes by PCR-RFLP compared to the bacteriological examination, provides a technical advantage for the correct identification of *Prototheca* in the milk samples. It provides fast and more precise results on the infection by *P. zopfii*. It reduced the time of diagnosis of an infected animal to 12 h instead of four to five days using a bacteriological analysis. The objective of this study was to identify the diversity of *Prototheca* and to characterize the pathogenic genotype by a molecular approach. This objective was achieved and led to the identification of the pathogenic genotype and the description of the test analysis of *P. zopfii* genotype II.

According to the literature, bovine *P. zopfii* genotype I and III are not involved in the pathology of mastitis and probably are pollutants of milk. Nevertheless, three strains of genotype III were isolated from milk samples submitted for udder infection in the present study. It would be useful to conduct further studies to clarify its isolation. At the preventive level, the eradication of *Prototheca* in cattle and the environment of farms is difficult. The treatments of infected human must associate the surgery operation and injection of antifungal. One the other hand, the treatment of the bovine mastitis is more difficult. The only means to eradicate the infection starts with the reinforcement of hygiene measures in the dairy exploitation, by the setting up with the removal of sick animals reached, even demolition inhalation and by strict control at the least sign of udder infection. The research of *Prototheca* may be improved by the following approaches: It would be interesting to make a new study which includes a great number of samples of mastitis isolated from various exploitations coming from different European countries.

Additionally, pathogenic *P. zofii* isolates from man and from dogs should also be investigated by molecular characterization. It is possible to make extraction of the genomic DNA of *Prototheca* from milk without passing by cultures in Sabouraud medium. The PCR-RFLP technique could be used within the framework of epidemiology studies.

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