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PROTOTHECOSIS: AGENT CHARACTERIZATION AND PATHOGENESIS

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CONTENTS

Units

%	percentage
µg	microgram
µL	microlitre
µm	micrometre
bp	base pair
CFU	Colony forming units
cm	centimetre
g	gram
h	hours
Hz	Hertz
kb	kilobase
kDa	kiloDalton
L	litre
M	Molar
m/z	mass/ charge ratio
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
mol	mole
ºC	temperature – Celsius degrees
rpm	revolutions per minute
s	second
U	Unit
wt/vol	weight/volume
X g	times gravity

Abbreviations

4MU	4-methylumbelliferone
7-AMC	7-amino-4-methylcoumarin
AAR	p-n-p-α-arabinoside
ADP	Adenosine diphosphate
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of variance
ARG	Arginine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BLAST	Basic Local Alignment Search Tool
BPH	p-n-p bis phosphate
C*	Cysteine carbamidomethyl
CI	Confidence interval
CIT	Citrate
CLSI	Clinical Laboratory Standards Institute
CO_2	Carbon dioxide
CW	Cell wall
DNA	Deoxyribonucleic acid
e.g.	*exemplī grātia*
EDTA	Ethylenediamine tretaacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENF	Enteric/Nonfermenter
EST	Expressed sequence tags
FAR	L-arginine-AMC
FGA	4MU-N-acetyl-β-D-glucosaminide
FGN	4MU-β-D-glucuronide
FGS	4MU- β-D-glucoside
FISH	Fluorescent *in situ* hybridization
FNAC	Fine needle aspiration cytology
FPY	L-pyroglutamic acid-AMC
FTIR	Fourier-transformed infrared spectroscopy
FTR	L-tryptophan-AMC
GAL	Galactose
GAPDH	Glyceraldehyde -3-phosphate dehydrogenase
GLY	Glycerol
GMS	Gomori's methenamine silver
GP	Gram-Positive
H_2	Hydrogen gas
HCl	Hydrochloride
Ig	Immunoglobulins
IGS	intergenic spacer
INO	Inositol
IS	Ion score
ITS	Internal Transcribed Spacer
Log	Logarithm
LPS	Lipopolysaccharide
LSU	Large subunit
LYS	Lysine
M*	Methionine oxidation
MAL	D-maltose
MALDI	Matrix-assisted laser desorption/ ionization
MIC	Minimal inhibitory concentration
ML	Maximum likelihood
MNS	Mannose

Mol. Mass	Molecular mass
Mononuc. cells	Mononuclear cells
MP	Maximum parsimony
mtDNA	Mitochondrial DNA
MW	Marker spectra^{TM} multicolor broad range protein ladder
n	number of isolates
N.I.	No information
N_2	Nitrogen gas
NaCl	Sodium chloride
NCBI	National Center for Biotechnology
ND	Not determined
NJ	Neighbor-joining
nr	non-redundant
OM	Organic matter
P (p-value)	probability
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PAS	Periodic-acid-Schiff
PAUP	Phylogenetic Analysis Using Parsimony
Pb	*P. blaschkeae*
PCR	Polymerase chain reaction
PEM	*Prototheca* Enrichment Medium
PHC	p-n-p-phosphorylcholine
PHE	Phenylalanine
PIM	*Prototheca* Isolation Medium
PLN	Proline & Leucine-p-nitroanilide
PMF	Peptide mass fingerprint
PMNs	Polymorphonuclear leukocytes
PRO	Proline nitroanilide
PVDF	Polyvinylidene difluoride
Pz	*P. zopfii*
Pzg1	*P. zopfii* genotype 1
RAF	D-rafinose
RAPD	Random Amplification of Polymorphic DNA
rDNA	ribosomal DNA
RFLP	Restriction fragment length polymorphism
Rib. Prot.	Ribosomal protein
RNA	Ribonucleic acid
RP	Restriction pattern
rRNA	ribosomal RNA
SAS	Statistical Analysis Software
SCC	Somatic cell count
SDS	Sodium dodecyl sulphate
SE	Standard error
spp.	Species
ssp.	Subspecies
SSU	Small subunit
TCA-A	Trichloroacetic acid-acetone
TOF	Time-of-flight
TRE	D-Trehalose
tRNA	transfer RNA
TTC	Tetrazolium
URE	Urea
var.	Variant

Sumário

Membros do género *Prototheca* são algas microscópicas, ubíquas e não fotossintéticas que pertencem à família Chlorellaceae e incluem espécies que podem ser associadas a patologias em humanos e animais. Actualmente, são taxonomicamente reconhecidas cinco espécies de *Prototheca*, sendo a *P. zopfii*, *P. wickerhamii* e *P. blaschkeae* as únicas com potencial patogénico. Todas as espécies patogénicas já foram associadas a protothecose humana, contudo apenas as *P. zopfii* e *P. blaschkeae* foram descritas em associação a casos clínicos e subclínicos de mamites em bovinos. Na protothecose canina foi apenas descrita a *P. zopfii* e *P. wickerhamii*. Nos animais a forma mais relevante de protothecose é a mamite bovina, que é reconhecida como endémica em todo o Mundo apresentando uma incidência gradual crescente e tendo já sido considerada como potencial problema a nível de saúde pública. Estas microalgas são eucariotas unicelulares que se reproduzem assexuadamente por endosporulação com a produção de número variado de endosporos no interior da célula mãe ou esporângio. Os métodos actualmente utilizados no diagnóstico de protothecose são essencialmente fenotípicos e moleculares. Os métodos fenotípicos incluem análise macro e microscópica bem como a determinação do padrão de assimilação de diversos substratos. O padrão de assimilação dos substratos mais comuns está bem determinado para a identificação de *Prototheca*, contudo surgem algumas incertezas quando se utilizam estas metodologias. Por consequência, nesta dissertação, três sistemas de identificação bioquímica foram testados e analisados para a *Prototheca*, que em conjunto com o programa InforBio permitiu determinar caracteres fenotípicos discriminativos. Os substratos, citrato, fosforilcolina e arabinosídeo foram determinados como sendo úteis na diferenciação das espécies patogénicas. No entanto, estes sistemas devem ser usados com prudência no diagnóstico de *Prototheca*. A identificação molecular e análise filogenética destes microrganismos são geralmente alcançadas pela sequenciação e análise de restrição dos produtos de amplificação do ácido desoxirribonucleico do 18S ribossomal (rDNA) e da grande subunidade do ribossoma. Também tem sido amplamente utilizada a reacção em cadeia da polimerase (PCR) específica de genótipos. A caracterização molecular dos isolados de *Prototheca* utilizados nesta dissertação permitiu determinar uma incidência elevada de mamites associadas ao genótipo 2 da *P. zopfii* na região noroeste de Portugal e o primeiro relato de mamite bovina associada a *P. blaschkeae*. O nosso trabalho inclui também um estudo preliminar da amplificação do espaçamento interno transcripto nuclear (ITS) e do operão do rDNA do plastídeo de *Prototheca*. Estes resultados sugerem que estas regiões podem apresentar um elevado interesse na genética de populações e epidemiologia das espécies de *Prototheca*. Esta microalga encontra-se largamente

distribuída em vários ambientes, mas é encontrada com maior frequência em ambientes com elevada humidade e com matéria orgânica, apresentando assim uma elevada capacidade de disseminação e perpetuação ambiental. Como estas algas ubíquas são extremamente resistentes devido à esporopolenina presente nas suas paredes celulares, já foram isoladas de vários valores de pH e de concentrações salinas, tal como de águas tratadas com cloro e de leite pasteurizado. Outro objectivo desta dissertação foi o de avaliar a eficácia de tratamentos físicos geralmente usados no processamento do leite e de outros tratamentos químicos sobre os isolados utilizados neste estudo. As espécies de *Prototheca* mostraram-se capazes de sobreviver entre valores de pHs 1 a 12, apresentando maior crescimento entre os pHs 5 a 9. Foram de igual modo capazes de multiplicarem-se a elevadas concentrações salinas, até aos 9% e 18% para a *P. zopfii* e *P. blaschkeae* respectivamente. Relativamente à sensibilidade à acção de diferentes temperaturas, a *Prototheca* foi capaz de multiplicar-se em pelo menos um dos tratamentos térmicos comummente utilizados na pasteurização, sendo o tratamento de 100ºC/ 1 segundo, o único capaz de a inactivar e impedir a sua multiplicação. Consequentemente, os resultados obtidos sugerem que a ultra-pasteurização é o único procedimento industrial capaz de eficientemente inactivar estas algas no leite. Os mecanismos da patogénese de *Prototheca* e a resposta imune a ela associada ainda estão por determinar. Por conseguinte, torna-se difícil a implementação de estratégias terapêuticas contra as patologias induzidas por esta alga. Estudos prévios identificaram algumas proteínas imunogénicas para cada genótipo de *P. zopfii* e para *P. blaschkeae*, não tendo sido realizados estudos de caracterização dessas proteínas. Propusemo-nos a identificar neste projecto, proteínas de *Prototheca* com capacidades imunogénicas a partir dos isolados obtidos de mamites bovinas. Com a utilização de soro hiperimune produzido, foi possível detectar e identificar as seguintes proteínas em todas as espécies de *Prototheca* testadas: proteína ribossomal, glutamil-tRNA-reductase 1, proteína transportadora do ADP/ATP, ATP sintetase, factor de elongação e gliceraldeído-3-fosfato-desidrogenase. Apesar de estas proteínas serem todas detectadas a nível intracelular, a sua identificação revela-se como um avanço na compreensão de alguns dos mecanismos de patogénese de *Prototheca*. Deve-se contudo prosseguir com estudos que permitam a confirmação destes achados e da sua localização, pois estas proteínas poderão constituir bons candidatos para a aproximação profilática e terapêutica da protothecose. As terapias de rotina actuais utilizadas para as mamites bovinas não são capazes de eliminar esta alga do úbere das vacas infectadas, sendo a única forma de controlo considerada eficiente, a identificação dos animais infectados, a sua separação dos restantes membros do efectivo e a secagem do teto afectado quando apenas um quarto

do úbere está infectado, ou refugo da vaca quando mais quartos do úbere estão afectados.

Durante este programa doutoral foi gerado conhecimento inovador relacionado com as estirpes patogénicas de *Prototheca* que podem estar associadas a mamites bovinas, é fornecida informação sobre a sua caracterização fenotípica (com a determinação de novos substratos discriminativos assimilados por esta alga), molecular e a primeira descrição mundial de mamites bovinas por *P. blaschkeae*. Também, a determinação da susceptibilidade de *P. blaschkeae* a vários pHs, diferentes concentrações salinas, e aos tratamentos térmicos, os últimos correspondentes aos que são geralmente utilizados no processamento do leite, tal como a confirmação da susceptibilidade de *P. zopfii* a estes tratamentos, constitui conhecimento inovador que pode ser útil para as acções de prevenção de infecção e de controlo desta alga. A identificação e caracterização de novos antigénios imunodominantes presentes nas células de *Prototheca* poderão demonstrar-se de utilidade nas acções de controlo de protothecose no futuro.

Summary

Members of the genus *Prototheca* are ubiquitous non-photosynthetic algae that belong to the family Chlorellaceae and include species that can be associated with pathologies in humans and animals. Currently, most of the authors accept five species of *Prototheca* with *P. zopfii*, *P. wickerhamii* and *P. blaschkeae* being the only pathogenic. All pathogenic species have been associated with human protothecosis, but only *P. zopfii* and *P. blaschkeae* were described in association with clinical or subclinical cases of bovine mastitis. Cases of canine protothecosis have been associated involving the species *P. zopfii* and *P. wickerhamii*. The most relevant form of protothecosis in animals is bovine mastitis which is recognized as endemic worldwide with incidence steadily increasing and is considered to be a potential public health issue. These algae are unicellular eukaryotes that reproduce asexually by endosporulation with the production of variable number of endospores inside a mother cell or sporangium. The current methods used for protothecosis diagnosis are essentially phenotypic and molecular. The phenotypic methods include macro and microscopic analyses and determination of the assimilation patterns on several substrates. The assimilation pattern of the most common substrates used for the identification of *Prototheca* are well known, however uncertainties arise when using these methodologies. Therefore, in this thesis, three biochemical identification systems were used and analysed with InforBio software in order to determine discriminative phenotypic characteristics. Citrate, phosphorylcholine and arabinoside substrates were found to be useful in the differentiation of the pathogenic species. However, these systems should be prudently used in *Prototheca* diagnosis. Molecular identification and phylogenetic analyses of these microorganisms are generally performed by the sequencing and restriction analyses after amplification of the 18S and 28S ribosomal deoxyribonucleic acid (rDNA). Also, genotype specific polymerase chain reaction (PCR) analysis has been widely used. The molecular characterization of the *Prototheca* isolates used in this thesis was able to determine a high incidence of *P. zopfii* genotype 2 mastitis in the Northwest region of Portugal and for the first time the involvement of *P. blaschkeae* with bovine mastitis. Our research work included a preliminary study on the amplification of the nuclear internal transcribed spacer (ITS) and the plastid rDNA operon. The results suggested that these regions could be of great value for *Prototheca* spp. population genetics and epidemiology. *Prototheca* are widespread through different environments, but are most frequently found in those with high humidity and organic matter, presenting elevated environmental dissemination and perpetuation. Because these ubiquitous algae are extremely resistant due to the sporopollenin present in their cell wall, they have been isolated from a great variety of pH values and salt

concentrations as well as from water treated with chloride and from pasteurized milk. Another focus of this thesis was the evaluation of the efficacy of physical treatments used in milk processing and chemical treatments on the studied isolates. *Prototheca* species were found to survive between pH 1 to 12, and presented higher growth between pH 5 and 9. The mastitic isolates were also able to grow at high salt concentrations, until 9% and 18% for *P. zopfii* and *P. blaschkeae* respectively. In the temperature susceptibility studies, *Prototheca* could grow in at least one of the common thermal pasteurization treatments, and 100ºC/ 1 second was the only treatment that was able to cause their total growth inhibition. Therefore, the results of our studies suggest that ultra-pasteurization is the only industrial procedure to efficiently inactivate these algae in milk. The pathogenic mechanisms and the associated immune response in protothecosis are still to be clarified. Consequently it is difficult to develop therapeutic strategies against the induced disease. Some studies were able to identify different immunogenic proteins for each *P. zopfii* genotype and *P. blaschkeae*, however no further characterization of these proteins was performed. Thus our project intended to identify these and new immunogenic proteins on *Prototheca* spp. mastitic isolates. Ribosomal protein, glutamyl-tRNA reductase 1, ADP/ATP carrier protein, ATP synthase, elongation factor and glyceraldehyde-3-phosphate dehydrogenase were detected using hyperimmune sera. Nevertheless the identified proteins are intracellular, their identification may reveal an advance in the study and understanding of the mechanisms of pathogenesis by *Prototheca*. Further analyses should be performed in order to confirm and localize these immunogenic proteins that could be used prophylactically or therapeutically in the future. Presently, routine mastitis therapy is not able to eliminate these pathogenic algae from the udder of infected cows. Therefore, the only control measurements which proved to be efficient after the identification of infected animals, were their separation and drying of the teat when only one quarter is affected or culling of the cow when more quarters are affected.

During this research program, further knowledge was generated in relation to the pathogenic strains of these algae. Novel information regarding the determination of new discriminative phenotypic characteristics and molecular characterization of *Prototheca* are provided. *P. blaschkeae* was associated with bovine mastitis for the first time. Also, the determination of *P. blaschkeae* susceptibility to several pH values, different salt concentrations, and to the temperature treatments usually applied in milk processing, as well as the confirmation for *P. zopfii* susceptibility, provide novel knowledge that should be considered for prevention and control issues associated with these algae. The preliminary results on the characterization of new immunogenic antigens in *Prototheca* can be an important contribution in future preventive and therapeutic approaches of protothecosis.

CHAPTER 1

STATE OF THE ART

GENERAL INTRODUCTION

The genus *Prototheca* comprises unicellular, achlorophyllic algae with asexual reproduction characterized by endosporulation with production of a variable number of endospores (1-4). These algae belong to the family Chlorellaceae that together with *Chlorella* are the only plant like organisms that can cause infectious diseases in humans and animals (5-9). In nature *Prototheca* are ubiquitous and saprophytic, however are unusual opportunists that cause pathology when the host immunologic defences are impaired (10-12), or when predisposing factors occur such as deficient animal care and poor milking hygiene in dairy management (13, 14). *Prototheca* is included in the class Trebouxiophyceae (*sensu* Friedl 1995) (15, 16), and is closely related to green algae of the genus *Chlorella* (9, 17), which are among the best studied unicellular green algae (16). Some authors have described *Prototheca* as “colourless Chlorellae” (18, 19), because they lack chlorophyll and therefore are dependent on a heterotrophic source of nutrients (20). But besides the absence of chlorophyll, these algae have other characteristics that clearly distinguish them from members of *Chlorella* spp.: they cannot use nitrate as a sole source of nitrogen (21), they are auxotrophic for thiamine (22), they are acid tolerant (23), and they are able to use a wide variety of hydrocarbons as sole sources of carbon (19, 24, 25). *Prototheca* obtain nutrients from debris and degradation products of other organisms, which in the past led to some controversy about their taxonomic status, because they share important features with algae (reproduction and morphology) but also with fungi (heterotrophic and achlorophylic) (2, 21, 26).

PROTOTHECA CHARACTERIZATION

Taxonomy History

The genus *Prototheca* was first isolated from slime flux of trees and recognized by Wilhelm Krüger in 1894 (27), who described two species, *Prototheca moriformis* Krüger and *P. zopfii* Krüger, and also a new species of *Chlorella*, *Chlorella protothecoides* Krüger (3, 27). The latter species was then lectotypified by Kalina and Puncochárová in 1987 (28), and Krüger’s original strain was elected as the type strain of *Auxenochlorella protothecoides* (Krüger) Kalina et Puncochárová. Negroni and Blaisten (29) and later Cooke (30) designated *P. zopfii* as the lectotype for the genus. Although Krüger

considered *Prototheca* as a colourless green alga, it was also considered to be related to fungi by other researchers (31-34). Nevertheless, the ultrastructural observation of degenerate plastids in their cells clearly identified the genus as an alga (35). With the usage of chemotaxonomic strategies, other investigators were able to infer the closest current representative of the photosynthetic algal progenitor that gave rise to the genus *Prototheca*, *Auxenochlorella* (36, 37). For example, the shared properties detected between *Prototheca* and *A. protothecoides* included thiamine dependency, inability to reduce nitrate, high level of salt tolerance, the presence of glycogen (starch is absent) as the storage carbohydrate, and the distinctive sugar composition of their acid-soluble cell wall fraction, which lacks arabinose but contains galactose, mannose, and sporopollenin. These features were not found in most of the other *Chlorella* species (22, 37-39). Deoxyribonucleic acid (DNA) base composition data (23), and sequence similarities between 18S ribosomal ribonucleic acid (18S rRNA) (40) also confirmed this relationship and separated these two species from the other *Chlorella* spp. (23, 40). Both eukaryotic algae belong to the family Chlorellaceae within the class Trebouxiophyceae (15, 16). *P. wickerhamii* was later described by Phaff *et al*. (22, 31).

Presently, there is no consensus among various authors on the existing number of species in the genus *Prototheca*, but all agree that *P. zopfii*, *P. wickerhamii* and most recently *P. blaschkeae* are the only species that are pathogenic to humans and animals (1, 2, 41-44). However, recently Satoh *et al*. (45) isolated a novel species of *Prototheca* from inflamed skin of a patient with protothecosis, and proposed naming it as *P. cutis*. Although this report was supported by analyses of the nuclear 18S rDNA gene, D1/D2 domain of 26S rDNA gene and by chemotaxonomic studies which determined its close phylogenetic relationship to *P. wickerhamii* and *A. protothecoides* (45), these findings should be further analysed and confirmed to correctly define *P. cutis* as a new species. As mentioned before, the taxonomic status of the genus *Prototheca* has changed during the last decades. Currently, most of the authors consider five species assigned to this genus, which includes the three pathogenic species and also *P. stagnora*, and *P. ulmea* (41, 43, 46, 47). A sixth species, *P. moriformis* is not generally accepted (21, 34, 43). This species is genetically and biochemically very similar to *P. zopfii*, however strains of *P. moriformis* show a marked heterogeneity between each other (48). Phylogenetic studies of *Prototheca* inferred that *P. stagnora* and *P. ulmea* should be regarded as different species and that both species together with *P. moriformis* belong to a cluster represented by *P. zopfii*, while *P. wickerhamii* was not grouped within these species and was more closely related to the autotrophic alga *A. protothecoides* (48). Therefore, this study suggested the transfer of *P. wickerhamii* to *Auxenochlorella* or to a new genus (48).

Previously, *P. zopfii* was differentiated biochemically and serologically into three biotypes (10, 20, 49, 50) with biotype 2 being the only that is considered to be capable of causing pathology in animals, especially in cows as a form of mastitis (10, 49). In this classification the newly defined species, *P. blaschkeae*, was classified as *P. zopfii* biotype 3 (10, 20, 49). This differentiation was supported by Fourier-transformed infrared spectroscopy (FTIR) (50), and also by phylogenetic investigations based on the 18S rDNA, and on cellular fatty acids analyses. Moreover, *P. zopfii* was reclassified as having two genotypes, genotype 1 and 2, with the latter being associated with bovine mastitis (9, 20, 43, 49, 51). One of these studies (9) also suggested the creation of a novel subspecies arising from *P. zopfii* genotype 2, to be designated as *P. zopfii* ssp. *bovimastitogenes* (9, 43). It is also necessary to perform experimental infections in lactating cows to definitely proof the aetiological role of *P. zopfii* genotype 2 on bovine *Prototheca* mastitis. Additionally, pathogenic *P. zopfii* isolates from man and from dogs should also be investigated by genotyping (9). A recent study that analysed the 18S rRNA, suggested that *P. blaschkeae* is more closely related to *P. zopfii* than to other species and that this species can also be associated with bovine mastitis (41) and not only with human onychomycosis as considered previously (43). Until recently, there was no knowledge of different biotypes of *P. wickerhamii*, and studies on this subject only refer to the existence of different strains within this species (3).

Since the pathogenic species have major clinical and economic importance for humans and animals, this state of the art will emphasize these species and only refer relevant information to the non pathogenic species.

Life cycle

Prototheca spp. are unicellular organism, spherical to oval in shape, and reproduce asexually. No sexual form of reproduction has been identified to date in any species of these algae (22). As the cells mature, nuclear division followed by cytoplasmic septation and irregular cleavage occur to form endospores with subsequent rupture and release of the daughter cells (Fig. 1). A sporangium may contain between 2 to 20 or more daughter cells or sporangiospores. The enlarging sporangiospores eventually rupture the mother cell by pressure, and a characteristic split in the cell wall leads to passive release of the spores that continue to develop into mature endosporulating cells (2, 22, 52). The production of endospores inside the mother cell is characteristic for the genus *Prototheca* and other genera like *Chlorella*, which facilitates their differentiation from other organisms. The number and size of spores varies among species (53). When adequate nutrients are present in the environment spore release takes place every 5 to 6 hours (54). A previous

study (55) mentioned that the mean daughter cell number increases linearly with growth rate and this dependency is genetically controlled. However, the lineage analysis showed that daughter cell number variation is not under direct genetic control and it can be altered by changes in the culture media or environmental conditions (55).

P. wickerhamii sporangiospores are spherical with 2 – 11 µm in diameter, and the sporangium size varies between 7 – 13 µm and can include up to 50 sporangiospores inside. These sporangia have a morula like form, with endospores arranged symmetrically like a daisy which are characteristic of this species (14, 22). *P. zopfii* sporangiospores in contrast are about 9 – 11 µm in diameter, and the sporangium size is 14 – 25 µm with about 2 – 20 sporangiospores (2, 14, 22, 56). *P. blaschkeae* forms small, ovoid to globose (5 – 7.5 µm) sporangiospores in sporangia with a mean diameter of 15 µm (43). A resting cell stage, “Dauer cell”, between spores and a new sporangium are formed. This can be recognized by a thick cell wall and no cell divisions, and is characterized as extremely resistant (14, 21, 22). Intracellular granules are often observed and characteristic for these algae, and they increase in number and size with the aging of the cultures (57). *Prototheca* algae are about 16 µm long with a diameter of about 13 µm, the cell wall is about 0.5 µm thick, and they have a prominent nucleus. The cell walls are double layered with a thinner outer and a thicker inner layer (2, 14, 22), although some authors state that they have a trilaminar cell wall (39). This structure is resistant to acid and alkaline hydrolysis, enzymatic degradation and acetolysis, and does not contain glucosamine like in fungi, cellulose like in plants, nor muramic acid like in bacteria (2, 3, 37). Resistance is due to the presence of sporopollenin which is a highly resistant biopolymer known to build up the outer wall of spores and pollens, and originates from the oxidative polymerization of carotenoids (39, 58). Quantitative analyses of amino acid composition of the cell wall of *Prototheca* demonstrated that there are 12 major and 5 minor components, with the most common abundant amino acid detected being arginine followed by alanine and serine (59). Although the existence of a lipopolysaccharide (LPS) like substance (algal LPS) has been shown in *Prototheca* (60, 61), it does not stimulate a defence reaction mechanism in mammals (61) as the LPS of gram-negative bacteria (62, 63). Some non pathogenic *Prototheca* species can develop a mucopolysaccharide capsule that resembles those of *Cryptococcus neoformans* (2, 14).

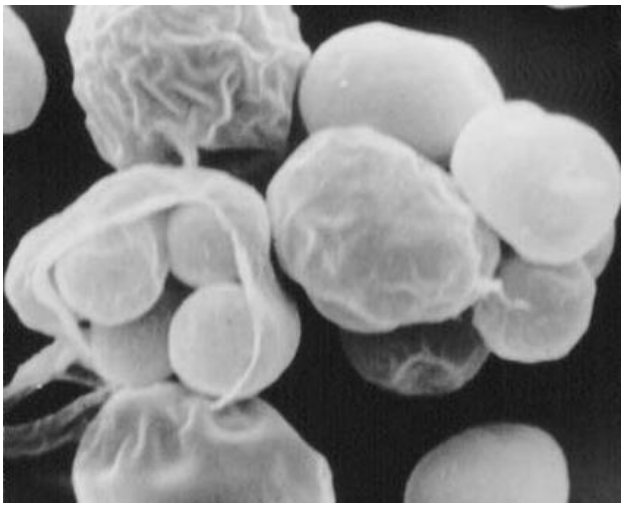

Figure 1 – Photomicrograph obtained by scanning electron microscopy of *Prototheca* cells at different developmental stages. Ruptured mature cell with several endospores inside can be observed. Magnification, X 1,785. Adapted from da Costa *et al.* 2004 (64).

EPIDEMIOLOGY

Prototheca are widespread world-wide throughout different environments, but are found most frequently in those with high humidity and organic matter (2, 14, 17, 65). These algae have been isolated from a great variety of sources, including plants, soil, drinking and marine water, sludge, the faeces of domestic animals (e.g., cattle, dogs, and pigs) or wild animals (e.g., deer, rats, mice, or rabbits), cow's milk, meat products (e.g., beef, pork, crabs, clams), animals beddings, teat cups, milking system, and barn floors (20, 66-72). In cows, *Prototheca* have been isolated from the mammary gland, lymph nodes, supramammary lymph nodes and right horn uterus, gastrointestinal tract and kidneys (73). *Prototheca* spp. are widespread throughout the environments of dairy herds, where outbreaks of bovine mastitis occurred, and can also be found in the environment of dairy farms without history of protothecal mastitis, demonstrating its wide distribution in dairy herds with or without mastitis problems associated with this alga (14, 22, 70, 74). *Prototheca* can also be found to transiently colonize human skin, fingernails, respiratory tract and digestive system (56, 75, 76). These ubiquitous algae are extremely resistant, being also isolated from a great variety of pH values and salt concentrations, from water treated with chloride and from pasteurized milk (5, 22, 77-79). These algae can be found at various pH values (1 to 12), but have optimal grow between 5 and 9. *P. zopfii* (until 9%) and *P. blaschkeae* (until 18%) were also able to grow at high salt concentrations (5). *Prototheca* resistance to standard chlorination contributes to its persistence in domestic and public sewage, from which these algae return to the environment (75). Regarding temperature susceptibility, its total growth inhibition was only achieved at 100 ºC/ 1

second treatment, indicating that ultra-pasteurization is the only industrial procedure to ensure that the milk from endemic regions is free of these agents (80). Also, in this study, *P. blaschkeae* was suggested to be more resistant to heat treatment than *P. zopfii*. Other studies on *P. zopfii* susceptibility to different heat treatments (78-80) found a high variability and resistance in at least one of the thermal pasteurization treatments tested. Faeces of healthy animals can contribute to the environmental dissemination by spreading viable *Prototheca* without showing any signs of infection or of endogenous multiplication (14, 22, 74, 81, 82). These algae are resistant to digestion due to the sporopollenin included in the cell wall, leading to recontamination and promoting the dissemination and perpetuation in the environment (4, 69). In aquatic systems, these algae are transient inhabitants and not indigenous, because they are unable to maintain significant reproduction rates in this type of environments (75). *Prototheca* is distributed globally, and cases of protothecosis were reported from all continents (10, 81, 83).

PATHOGENESIS AND CLINICAL MANIFESTATIONS

The pathogenicity and virulence of *Prototheca* spp. is moderate and these organisms are considered to be rare opportunistic agents (68). In mammals, the clinical presentation depends on the animal species affected. *P. wickerhamii* has been mainly isolated from human infections, and *P. zopfii* predominantly causes infections in animals, particularly in cows and dogs (1, 2, 20, 22, 42, 84). *P. blaschkeae* has been associated with human onychomycosis (43) and recently with bovine mastitis (8, 41, 46). Nevertheless, these species can cause pathology in any animal as long as predisposing factors occur. The mechanisms of the pathogenesis by *Prototheca* spp. are still unclear, especially if we consider that they are ubiquitous and only cause sporadic infections. The incubation period for *Prototheca* infections was never well defined, because it is extremely difficult to determine the exact onset of the infection. Nevertheless it has been speculated to be weeks to months (2, 85). Similarities and differences occur in protothecosis in different species, but the most similar feature found is a cutaneous granulomatous infection (81). As *P. stagnora* does not grow at 37ºC, it does not have pathogenic potential to birds and mammals (14, 21).

Bovine protothecosis (mastitis)

Bovine mastitis is an inflammatory process of the mammary gland that may cause severe losses in milk quality and production. The economic deficit caused by this pathology is primarily due to reduced milk production, followed by premature slaughter of

animals, milk rejection after therapy, drugs and veterinarian costs. Besides the economic features, attention should be paid to public health problems, because several of the pathogenic agents responsible for this disease or their secreted toxins can cause pathology in humans, and some of them can have zoonotic potential. Mastitic agents can be classified into two groups with respect to their origin and means of transmission, such as contagious and environmental agents (4, 86-88). The contagious agents spread from cow to cow primarily during the milking process, and the udder is the first source of infection. Conversely, the environmental microorganisms infect cows from the environment, their growth locations such as contaminated bedding materials and teat dips. These agents can also directly infect cows. Unlike contagious microorganisms, environmental agents can survive during long periods in the herd environment and thus they are not dependent on the mammary gland for their survival, and infection can occur at all stages of the cow's life (87, 89, 90).

The most common environmental pathogens are Gram negative and positive bacteria, primarily coliforms, and *Streptococcus* spp., with the exception of *Streptococcus agalactiae*, which together with *Staphylococcus aureus* are considered to be the most important contagious agents. *Enterococcus* spp. also belong to the environmental group, as well as other pathogens, *Arcanobacterium pyogenes*, *Nocardia* spp., *Bacillus* spp., yeasts, molds and algae (87, 89). From the environmental microorganisms, special attention should be given to the green algae of the genus *Prototheca*.

The prevalent form of protothecosis in animals is bovine mastitis, which generally occurs in a chronic subclinical or a mild clinical inflammatory process in the udder and affects cows that do not respond to routine therapy (4, 10, 78).

The route of infection is by contamination of the teat end with ascension trough the mammary gland duct until the mammary tissue (91, 92). The infectious doses for mastitis by *Prototheca* are higher than the observed for most of the common mastitic agents. However, these algae can cause infection at any stage of the cows' life (84).

P. zopfii was first identified by Lerche in 1952 (93) as a pathogen of bovine mastitis associated with reduced milk yield and characterized by thin watery secretion with white flakes (9, 20, 71). Since then, sporadic cases of this infection were reported across Europe, Asia and American Continents (93, 94) but at the present time cases of acute to chronic mastitis are recognized to be endemic all over the world and gain more economic and public health concerns (10, 41, 95). Despite few reported cases of protothecal mastitis, its real incidence is higher, because this alga can be incorrectly diagnosed as yeast, or cannot be detected in conventional culture media, as they are often overgrown

by other microorganisms (14). In Portugal, some cases of bovine mastitis by *Prototheca* were described in the centre of the country, in Azores islands (96), and a high incidence of cases have been reported in the North of the country (8, 13, 97, 98). These latter findings suggest that bovine protothecosis in the past was under-diagnosed in the country.

Prototheca spp. represent a severe economic significance because they cause mastitis which is difficult to treat, due to their resistance to most common anti-infective mastitic agents. The detection of mastitis by *Prototheca* spp. is an indication of a serious problem which can affect an entire herd (14, 87, 99). Therefore, the correct identification by clinical microbiology laboratories of *Prototheca* infections is important. Presently, its identification is still based on phenotypic characteristics (49), although some laboratories also use molecular genetics as a diagnostic tool (41, 43). This type of mastitis is most often recognized as a chronic, asymptomatic process with high somatic cell count (SCC) and low milk yield; however, acute clinical mastitis may also occur in outbreaks. In subclinical mastitis SCC can be higher than 10^6 /mL (4, 14, 67). Also, seropurulent mastitis can occur in acute cases with large number of algae in milk, in the alveolar epithelial layer, in macrophages, and in the interstitium. Mastitis can progress to a chronic granulomatous infection with regional lymphadenitis leading to atresia of the infected quarter and loss of milk yield (4, 10, 69). High SCC results may not be continuous as an infected cow can also present low milk SCC (100). The milk of infected cows can be of a watery appearance, containing flakes and lumps and the mammary parenchyma is firm on palpation due to the severe inflammatory process (14, 83). There is a higher rate of udder infection in the first weeks of the lactation period, probably because of a limitation of defence mechanisms in this period (101). Although mixed infection with bacteria can occur, synergism between bacteria and algae has not been proven (102).

The histopathological findings are generally characterized by a progressive interstitial mastitis associated with alveolar atrophy. Algae can be found in the alveolar lumen and interstitium, as well as in macrophages (83, 99). Some controversy exists on the role of neutrophils, because only *P. wickerhamii* was observed inside human neutrophils (103). In contrast, bovine neutrophils were never seen to phagocytise *P. zopfii* and consequently their role in algae death is questionable (104). *Prototheca* mastitis induces local and systemic immunity depending on the stage of infection. Cows with acute infection produce high specific antibodies, immunoglobulins G (IgG) and IgG1 in serum and milk samples, respectively. However, specific IgA concentrations in milk of chronically infected cows are higher than in cows with acute mastitis, due to the gradual and increasing answer of these Ig during the course of infection. Cows with chronic infection and microbiologic positive culture have high specific antibody activity in serum and milk, whereas cows with chronic

infection and microbiologic negative culture do not have elevated concentrations of these antibodies (10, 71). In a study by Roesler and Hensel (10) a strong correlation of milk whey IgA and IgG1 antibodies activity with total SCC in milk was observed, but only a weak correlation between these antibodies concentrations and the number of algal cells excreted with milk was observed. This strongly suggests that serological examination for identification of acutely and chronically infected cows and also intermittently shedders should be used concomitantly with SCC of milk, to increase the sensibility of *Prototheca* mastitis identification (10).

As these algae do not respond to routine therapy, the elimination of the infected animals is the best method to control the disease (69, 105, 106). *Prototheca* is considered an environmental pathogen (4, 13), nevertheless the mammary tissue can act as a reservoir for infection (107). Cases of disseminated protothecosis have been described in cows with massive necrotic mastitis, renal oedema, thrombosis of the pulmonary arteries, and colitis (22). Recently, algaemia by *P. blaschkeae* was described in a dairy cow after a chronic episode of mastitis by this pathogen (8). The algae were isolated from milk, joint fluid and blood samples, and the affected cow was culled after molecular confirmation of systemic infection by *Prototheca* (8).

Prototheca is widely distributed in the dairy environment, thus the incidence of bovine mastitis by these algae depends on predisposing factors, such as bad environmental conditions, locals with humidity and organic matter, and insufficient milking hygiene (4, 71). *Prototheca* infections occur when the teats are exposed to elevated concentrations of this agent in the environment, for example during milking breaks, or when the milking system is defective. Therefore, the algae are usually transmitted by direct contact from the environment to the cow and from cow to cow during the milking process, and from the milking units and milkers' hands (2, 14, 69, 71, 84). The inclusion of new cows in the dairy herd can also be responsible for introduction of the agent in the dairy cow environment and of its transmission to other cows (2, 69). Costa *et al.* (87) found a higher occurrence of bovine protothecosis in herds that were not immediately fed after milking. In dairy herds close to wild environments, it was proven that wild animals well adapted to aquatic environments can act as spreaders of the infection trough faecal elimination in the dairy farms (108). Therefore, these animals are considered to be an important link in the epidemiological chain of mastitis by *Prototheca*. In a retrospective study of *Prototheca* mastitis, the age/parity of cows, previous mastitis cases and especially antimicrobial pre-treatment of udder quarters were found to increase the risk of protothecal mastitis (109). High SCC before diagnostic and previous history of mastitis shows that mammary gland lesion is important for *Prototheca* invasion and colonization. As risk factors are more

common in older cows, the correlation between parity and risk of infection can be easily explained. Udder quarters with history of clinical mastitis during lactation and in previous lactations have higher risk of mastitis. Antibiotic therapy increases the risk of infection, because these drugs eradicate the normal flora of the teat skin and thus facilitate *Prototheca* multiplication. However, infusion of algae from the environment through introduction of drugs inside the teat duct cannot be ruled out (14, 22, 107). In another most recent study, pasture systems, pasture and silage feeding, use of milking machine in stalls, cow pen without fresh feed after milking, raising of swine near bovine housing, existence of other domestic animals (dogs, cats) and also rodents, absence of teats hygienization with water, use of pre-immersion devices with return and without changing the antiseptic, calves fed with milk of clinical mastitis cases, and the Holstein breed were determined as risk factors of protothecal bovine mastitis (110). Therefore, *Prototheca* mastitis in well managed herds is often sporadic, but it becomes endemic in herds with poor housing conditions and poor milking hygiene (111).

In conclusion, predisposing factors associated with *Prototheca* bovine mastitis are: bad management and poor maintenance of dairy herds, humid environment, faeces and detritus accumulation, bad milking management, drug infusions with insufficient hygiene and some cow associated factors, like age and history of previous mastitis (14, 83, 107).

Human protothecosis

The first documented human case of protothecosis was described by Davies *et al.* in 1964 (112), and occurred as a chronic localized skin lesion in a rice farmer from Sierra Leone. The first systemic protothecal infection was only described by Cox *et al.* in 1974 (113). Until now, more than 100 cases of protothecosis have been described in humans all over the world (56, 114, 115).

Human protothecosis occurs in healthy and immunocompromised patients. The pathogenic mechanisms of this alga are unclear, but it is believed that they may infect humans by contact with a potential source such as contaminated soil and water, or by traumatic inoculation of the algae. Inoculations have been reported to occur during surgery and orthopaedic procedures, through insect bites and trauma. Infection may also occur by penetration of the agent through any previous skin injury in contact with contaminated water (72, 116-118), but person to person transmission is not known to occur (1, 117). These organisms are of low virulence in patients with an intact immune system. The infection usually spreads indolently in local areas, but may be more widespread in immunocompromised hosts (1). Although rare, the infection caused by these algae in humans has become increasingly important as it can affect

immunocompromised patients (2, 65, 68, 119). In almost every reported patient, systemic or local predisposing factors were detected (42, 119).

Three principal clinical syndromes of human protothecosis have been described: cutaneous infections that mostly affect skin, and subcutaneous tissue of the body exposed sites (head, neck and extremities); olecranon bursitis or infection of other fibrous tissues; and disseminated (systemic) form, which is typically opportunistic and generally occur in patients with various forms of immunosuppression or chronically ill.

Olecranon bursitis and localized cutaneous infections develop in healthy patients and are the most reported forms (1, 42, 82), while disseminated cutaneous infections and visceral involvement are rare and affect primary immunocompromised patients (2, 42, 82, 120), although cutaneous infection can occur as well in immunocompromised patients (117, 121, 122). Also, some cases of human onychoprotothecosis have been described (43, 68, 123, 124). Thus, most of the infections involve skin and subcutaneous tissue and present erythematous nodules, plaques or ulcers, and verrucous or herpetiform lesions. Olecranal bursa may be involved, most likely as a result of antecedent trauma or surgical procedures and subsequent contamination, manifesting a swelling, mild erythema, and occasional drainage in the vicinity of the elbow. Only a few cases (less than ten) of systemic protothecosis have been documented involving patients with cancer, acquired immune deficiency syndrome (AIDS), diabetes mellitus, renal transplantation, steroids or other immunosuppressive therapy. In half of these cases algaemia was present. Only three cases of meningitis due to *Prototheca* spp. have been reported, and although they were not all documented, this pathology is considered rare (2, 82, 116, 119, 125-128). Protothecosis may also be rarely present as urinary tract infections or endocarditis.

Protothecosis can also be a nosocomial infection, complicating endotracheal intubation, peritoneal dialysis, intravenous catheters, corticosteroid injection, hand or wrist surgery, and orthopaedic procedures (119, 129). In a study, *Prototheca* was isolated from faeces of a patient with gastro-intestinal signs after ingestion of these algae from contaminated cheese. However, more studies are needed to prove real zoonotic potential (6).

In cancer patients protothecosis is a rare infection, as only 13 cases have been reported in the literature (125). Another study reported a rare case of disseminated protothecosis due to *P. zopfii* in an immunocompromised patient (130). Moreover, a case of bilateral choroiditis due to algae (*P. wickerhamii*) was also described (131).

Corticosteroid therapy can facilitate invasion of opportunistic agents, because these drugs reduce cell mediated immunity, mainly T cells, in a similar way as other

immunosuppressive agents (42). Humans with cellular deficiency are at risk for protothecosis (121), and it has been hypothesized that defects in neutrophil function play an important role in the host defense against *Prototheca* spp. (103, 132). Human polymorphonuclear neutrophils (PMNs) ingest and kill *P. wickerhamii* with the aid of IgG antibodies and heat-stable serum opsonins (103). Therefore, individuals with neutrophils incapable of killing *P. wickerhamii* are predisposed to protothecosis. In addition, other studies demonstrated that protothecosis can not be attenuated by the humoral immune system (22, 42). In contrast, for cancer patients, neutropenia does not appear to be an important risk factor, as only 2 of 13 patients with protothecosis were neutropenic (125). The fact that there are relatively few cases of protothecosis in AIDS patients suggests that a type of immunodeficiency other than that caused by AIDS contributes to susceptibility to this infection (133). Tyring *et al.* (134) also suggested a role for natural killer cell activity in the pathogenesis of protothecal infections.

Canine and feline protothecosis

About 31 cases of canine protothecosis have been described worldwide (135). Although a mucocutaneous pathology has been described, protothecosis is generally detected as disseminated pathology with the involvement of several organs and with an insidious onset, persistent progression and inevitably fatal course. Dogs generally have signs related to the gastro-intestinal tract, particularly the colon, and also ocular and/ or neurologic symptoms (105, 106, 135-137).

Most common clinical signs include large bowel diarrhoea with fresh blood, blindness due to chorioretinitis and retinal detachment, deafness, seizures and ataxia. In several patients, colitis is present during many months prior to the other signs of dissemination. Short periods of remission, or a delay in the progression of the disease were reported in some cases, but the great majority of canine protothecosis cases were euthanized or died soon after presentation of signs (81). In these animals, *Prototheca* infections have been associated with deficient immune system, and dogs from the breed Collie (11, 73, 138) and Boxer (135) are over-represented in the reported cases. An over-representation of cases in females was also determined (135).

In canines the infections were thought to occur principally through the skin. However, pathologic findings of systemic canine protothecosis concluded that the most important means of transmission is ingestion with subsequent haematogenous and lymphatic dissemination. These findings were even detected in cases without gastrointestinal signs and in cases, where the entrance place was not found (81, 138, 139). Canine systemic protothecosis due to *P. zopfii* is frequently accompanied with uveitis and chronic diarrhoea

(81, 138, 140). However, canine protothecosis caused by *P. wickerhamii* is extremely rare. Three canine cases of *P. wickerhamii* infection have been reported, one suffering from subcutaneous lesions (141), another suffering from generalized infection with simultaneous isolation of *P. zopfii* (142), and the most recent suffering from disseminated lesions caused by *P. wickerhamii* (17). Disseminated infection can also cause myocardial infections (22).

Canine protothecosis was classified (135) into three clinical forms: cutaneous infections; systemic infections where dogs show involvement of a single body system (excluding skin); and disseminated infection where clinical cases show evidence of haematogenous and/ or lymphatic dissemination from the primary site of infection to one or more tissues. In most of the cases it was difficult to distinguish from systemic and disseminated forms, because there were not sufficient data. Cases of canine protothecosis generally do not have immunological status assessments, and the few performed show deficiency in both lymphocytes T and neutrophils activities (143).

Feline protothecosis is usually rare and generally causes cutaneous lesions (11, 81, 135, 138, 144). Nevertheless, feline cases are said to be mostly fatal and an effective counter measure is not yet established (145, 146).

The increasing number of protothecosis cases detected in humans and animals substantiate that these algae represent an expanding and prominent risk for public health (9, 56, 65, 78, 97, 98, 135). Therefore, it is important to have a good identification and characterization of this potential zoonotic microorganism.

Experimental infection

An important factor that reflects the low pathogenicity and virulence of *Prototheca* infections is the difficulty to induce an infection in a variety of experimental animal models (114). This has been done successfully only in a very few cases and only via intratesticular injection and parenteral inoculation (75, 147). Some authors (103) were unable to induce *P. wickerhamii* infections in neutropenic guinea pigs or athymic mice. These laboratory animals only showed local reactions at the point of injection despite the high inoculum doses. In contrast, *P. zopfii* was lethal for immunosuppressed mice when used as an inoculum of 10^6 colony forming units (CFU) (103, 114). In a recent study (148) *P. zopfii* genotype 1 was used to experimentally infect normal bovine mammary gland to investigate its pathogenicity. In this study the udder did not present any clinical sign, but histopathology showed granulomatous lesions similar to the lesions induced by *P. zopfii* genotype 2 (148).

DIAGNOSIS

Clinical

Bovine protothecosis (mastitis)

Bovine *Prototheca* mastitis should be correctly diagnosed in order to properly implement the correct control and prevention measures and avoid algae dissemination (69, 149). However, the diagnosis can be difficult because infected cows generally become and remain subclinical (4, 87). The protothecal mastitis diagnosis is generally based on the culture of milk samples in Sabouraud dextrose agar, followed by microscopic analysis of wet mounts with lactophenol cotton blue stain. Furthermore, owing to its slow growth and intermittent shedding, these methods cannot be used for control measures (22, 71, 150).

In the last years, several alternative diagnostic methods for detection of *Prototheca* bovine mastitis have been developed. Fine needle aspiration cytology (FNAC) is one example, which can be used for diagnosis of subclinical mastitis with the algae identification being done by staining or by scanning electron microscopy, together with their isolation from the aspirated material from the mammary parenchyma. FNAC is easy to execute, it has low costs, causes minor aggression to the aspiration local and provides a fast and correct diagnosis together with the usage of different staining procedures (Gram, Giemsa and Shor) (64, 88).

Some attempts to early diagnose this type of infection using serologic tests to detect specific antibodies against *P. zopfii* in milk and blood have been developed (71), although there is little information about the immune reaction to this type of mastitis (10, 151). In the few available immunological studies, the detection of anti-*Prototheca* IgG in serum using counter-immunoelectrophoresis and an enzyme-linked immunosorbent assay (ELISA) showed poor sensitivity and specificity. Additionally, their use for routine diagnosis (99, 152) was limited. Although the presence of specific IgA and IgG1 antibodies against *P. zopfii* in serum and milk from lactating cows is known and could be demonstrated by immunodiffusion, this test system is unsuitable for herd screening because it is too labour-intensive (71). In another study an indirect ELISA for the identification of infected cows and for discriminating among infected cows at various clinical stages was developed. This test demonstrated sensitivity and specificity of 96% and 94% respectively, and was found to be suitable for discrimination between infected and uninfected animals by measuring IgA and IgG1 antibody titers, and might therefore be useful for screening affected herds (10, 71). Despite of the promising benefits of these new diagnostic techniques, they have

a reduced percentage of use by the diagnostic laboratories, because of the amount of time needed to perform and the money dispended on them. In order to overcome these drawbacks and to rapidly identify this pathogen from clinical or environmental sources, several techniques have been developed recently. A real time polymerase chain reaction (PCR) was recently developed by Ricchi *et al.* (44) and allowed a fast molecular identification of these algae. Although this methodology is not available for all laboratories, well known molecular techniques (18S rDNA, and genotype specific PCRs) have similar identification efficiency (9, 41, 43, 153). Also, another study demonstrated that the usage of fluorescent *in situ* hybridization (FISH) could also be used to rapidly identify *Prototheca* spp. (154).

Histopathologically, *Prototheca* cells can be observed in the alveolar lumen, basal membrane of affected alveoli, macrophages, interstitium and alveolar epithelial layer, but not inside epithelial mammary cells. In other organs these algae can also cause destructive and necrotic inflammatory lesions. Histological alterations due to direct or indirect action of the algae and related inflammatory answers in the mammary tissue are significant (86, 155). These algae can cause destruction of blood vessels, acinus and interstitial connective tissue of the udder, leading to atrophy of alveoli. Histologically, severe progressive pyogranulomatous mastitis with irregular distribution in the mammary parenchyma can be observed together with interstitial mononuclear cells (macrophages, lymphocytes and plasma cells) infiltrates. In the alveolar lumen macrophages with algae inside, detached epithelial cells, and also PMNs neutrophils can be observed. The observation of algae inside macrophages suggests that intracellular proliferation is responsible for the failure of the response to infection. *Prototheca* inside macrophages can also have different stages of degeneration when observed by electron microscopy (14, 99, 104, 156). Associated lymph nodes also present chronic progressive pyogranulomatous lesions. With time, glandular tissue is substituted by fibrous tissue leading to reduction of the milk production and alveolar atrophy (4, 155, 156). Milk is extremely important for the diagnosis of bovine mastitis, because the majority of the pathogens are observed there and in the mammary parenchyma (86).

Human protothecosis

Generally, human protothecosis is not suspected clinically, and patients are usually subjected to several treatment modalities for long periods without satisfactory results. The definitive diagnosis of infection depends on morphological identification of the organisms in wet slide preparations of cultures and/or direct identification in tissue specimens. When

suspicious cases are detected, microbiological and histopathological tests should be carried out (1, 42, 117).

As cutaneous protothecosis is uncommon and sometimes its clinical features can be similar to those caused by subcutaneous mycosis or eczema, it can be overlooked or wrongly diagnosed as yeast without precise laboratory diagnosis. Such a diagnosis is sometimes time-consuming and not always possible in hospital laboratories (65, 66). Nevertheless, efforts should be made to obtain an adequate diagnosis of this pathology, because a misdiagnosis causes possible dissemination of the infection, mistreatment, and unnecessary expenses to the patients, and also bad health conditions of the patients (65, 66, 119, 157). So, when protothecosis is suspected, mycological tests should be periodically repeated in order to determine whether the isolated organism is really an alga, and whether it is a nonpathogenic transient colonizer or a cause of protothecosis. The healing process should also be evaluated (65, 68, 119). Also, biopsies and culture of affected tissue should be performed leading to the detection of *Prototheca* (56, 118).

Diagnosis of *Prototheca* infections in humans requires a high index of suspicion. On histological examination, cutaneous protothecosis is characterized by a suppurative or granulomatous infiltration of the dermis and the presence of many solitary spherical spores that measure 6 to 10 μm in diameter, which can be found both within giant cells and extracellularly. Also, great numbers of inflammatory cells, macrophages, lymphocytes, plasma cells and also neutrophils, are present together with epithelial hyperplasia with fibrosis (1, 42, 56, 118). The organisms grow well on Sabouraud dextrose agar and are identified under wet mount preparations (119). Diagnosis is largely made upon detection of characteristic structures observed on histopathologic examination of tissue (56). Although FTIR has been reported to be a suitable and efficient method for distinguishing and characterizing human pathogenic yeasts and animal-pathogenic algae, this technique cannot be used for routine diagnosis, and more conventional methods, such as cultivation and microscopic examination, should be sufficient (50, 56).

Canine and feline protothecosis

The clinical signs of canine protothecosis are so unspecific and its course so insidious that when a definitive diagnosis is made, the agent is already spread. Therefore, an early diagnosis may be accomplished by the introduction of protothecosis in the differential diagnosis of several pathologies, such as ocular (acute blindness), and gastrointestinal (chronic diarrhoea) diseases (11, 81, 138). In a study (135), most of the patients first signs were colitis of various grades of severity that were often present for many months before other symptoms developed. Succeeding dissemination, signs were mostly ocular and/or

neurological. Once dissemination was evident, death or euthanasia quickly emerged (135). Feline protothecosis should also be early diagnosed preventing its local dissemination and allowing better therapeutic results (144).

Therefore, the diagnostic approach for disseminated canine protothecosis should include the collection of clinical data and performance of other tests such as complete blood analysis, urine analysis, thoracic and abdominal X rays and also faecal examination (usually negative). Blood analyses are generally normal, unless the patient has chronic disease and is debilitated. On abdominal X rays, alteration of thickness of the intestinal wall is generally seen. In dogs with neurologic signs, cephalorachidian liquid analysis reflects inflammation and algae can be observed in the culture (81, 135).

Cutaneous lesions are usually diffuse pyogranulomatous dermatitis with various algae inside macrophages and these can be diagnosed on skin biopsies and specific culture of the algae with subsequent microscopical observation (81, 158).

Disseminated canine infection are disperse and white to grayish nodules of 1 to 3 mm of diameter, or a diffuse lesion dispersed through several organs can be observed. Nodules are granulomas with necrotic areas and with inflammatory cells such as macrophages, lymphocytes, plasma cells and neutrophils and also algae (81). Necropsy confirms the disseminated nature of disease, with lesions in a variety of tissues including the colon, eyes, heart, brain, kidneys, skeletal muscle and liver (81, 135).

In ocular protothecosis retinal, iris and ciliary body, and anterior chamber alterations are found. Algae can be observed in vitreous humor, and in histopathology granulomatous separation of retina is observed (81).

Laboratorial

The definitive diagnosis of *Prototheca* infections is usually made through identification of the macroscopic and microscopic morphological characteristics and evaluation of the assimilation pattern of several substrates (4, 74, 96). An unknown isolate must be initially examined by optical microscopy to be identified as belonging to *Prototheca*, and subsequently its assimilation pattern (22, 159) should be determined. Since laboratory differentiation of *P. wickerhamii*, *P. zopfii* and *P. blaschkeae* may be tricky, the separation of species is usually based on macroscopic and microscopic analyses, their growth temperature, and sugar and alcohol assimilation patterns. Most recently the usage of molecular techniques became extremely relevant to allow a fast and efficient species identification (10, 43, 66, 71, 74). Real time PCR and a FISH method were developed to

improve and accelerate the diagnosis of protothecosis. However, these methods are not always accessible to all laboratories because of monetary reasons (153, 154).

Macroscopic analyses

Prototheca can be aerobic or microaerobic, and grows aerobically in several standard laboratory media such as Columbia agar supplemented with 5% sheep blood (blood agar), Sabouraud dextrose agar, and Brain Heart Infusion broth, among others. As contaminant microorganisms, such as bacteria, fungi and yeast, can overgrow these algae, chloramphenicol can be added to the media to suppress their growth. *Prototheca* and *Cryptococcus neoformans* are inhibited by cycloheximide, and media with this inhibitor should be avoided to correctly identify these organisms (14, 26, 66, 135). On blood agar these algae usually show white to greyish, opaque, pasty, nonhemolytic, yeast like colonies with a diameter 0.5 – 1 cm after 24 – 72 hours of incubation at 25 – 37 ºC (4, 64, 160). In this medium *Prototheca* colonies can be misidentified as yeast and coagulase-negative *Staphylococcus*. On Sabouraud dextrose agar, the colonies are yeast-like, cream to white, dry, and show a creamy consistency with granular surface and yeast smell after 24 – 72 hours of incubation at 25 – 37 ºC (2, 4, 14, 116) as shown in Fig. 2. Special media, such as *Prototheca* Isolation Medium (PIM) (38) and *Prototheca* Enrichment Medium (PEM) (161), containing phthalate to inhibit bacteria and 5-fluorocytosine to suppress yeasts, have been used to recover organisms from contaminated specimens.

Incubations at 30ºC during 72 hours are adequate for most *Prototheca* species, while some slow-growing strains require incubation at 25ºC for up to 7 days. Growth is generally optimal between 25 and 37ºC, and organisms usually proliferate within 48 hours. Specifically, *P. wickerhamii* produces smooth, yeast-like colonies, resembling those of *Candida albicans,* which may develop a tan pigment with age. Colonies of *P. zopfii* tend to grow faster, are usually larger, and may be more wrinkled in appearance than those of *P. wickerhamii* after 48 hours of incubation in Sabouraud dextrose agar. They also have unequal borders, granular surface with a central protuberance, and with time tend to develop a yellow-whitish pigment. At last, *P. blaschkeae* colonies are white to cream, smooth, convex and small. Their texture is soft and viscous. Besides the macroscopic differences of the species, exact identification by means of morphological features cannot be used as definitive diagnosis. Under the stereomicroscope, the colonies may also have a ground-glass appearance (2, 4, 14, 22, 43).

Pathogenic *Prototheca* can be distinguished from *P. stagnora*, which grows only at 30ºC and produces mucoid colonies due to the presence of capsules (21, 34, 162).

Selective culture of *P. zopfii* can be obtained in PIM medium at pH 5.1 and with acetate as the only carbon source, or by using its capacity to assimilate 1-propanol (3, 17). The differentiation of some *Prototheca* spp. can also be achieved by a susceptibility test to clotrimazole. In this test, *P. zopfii* is resistant, but *P. wickerhamii* and *P. stagnora* are susceptible, showing an average diameter of inhibition halo of 23 mm and 20 mm respectively (2, 14, 22, 160). No information regarding *P. blaschkeae* susceptibility to clotrimazole was found in the literature.

As *Prototheca* and *Candida* in routine and even in differential chromogenic media have similar appearance, these two genera can be differentiated by the aggregation test, in which the hydrophobic character of *Prototheca* can be observed, and by the susceptibility test to ribostamycin (60 g / disk), in which *Prototheca* isolates demonstrate an inhibition halo of 25 to 28 mm of diameter and yeasts are resistant (14, 66).

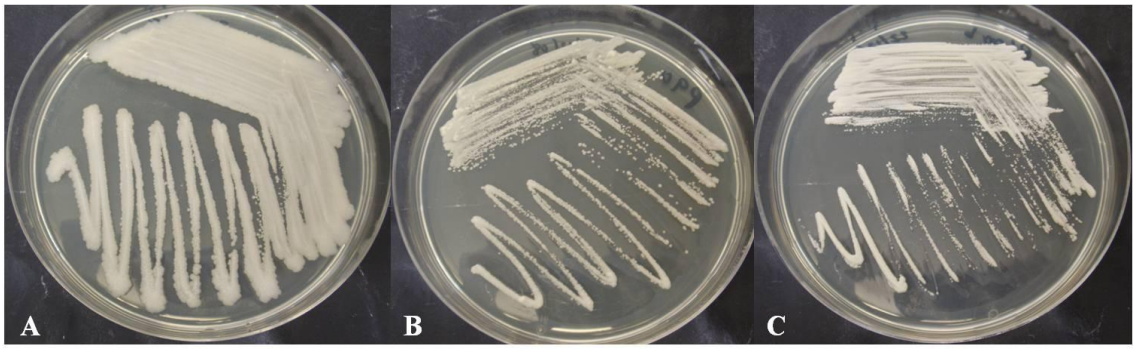

Figure 2 – Macroscopical morphologic characteristics of *Prototheca* on Sabouraud dextrose agar. *P. zopfii* (A), *P. blaschkeae* (B) and *P. wickerhamii* (C) cultures incubated during 48 hours.

Microscopic analyses

Prototheca species can be differentiated from bacteria and fungi by their size, shape, and mode of reproduction (2). *Prototheca* species differ from each other in size and number of produced autospores (74). Round to oval *Prototheca* cells with endospores can be unambiguously identified in microscopic observations (163).

After analysis of the macroscopic morphological characteristics of *Prototheca*, species differentiation of these algae is enhanced by microscopic observations. Smears of *Prototheca* isolates, stained with methylene blue or Gram stains, reveal big (8 – 25 µm), spherical to oval cells, with a coloured central region, and with or without endospores at magnification X 1,000 as shown in Fig. 3. *Prototheca* is an eukaryote and therefore, it has neither a Gram + nor a Gram – cell wall, although the Gram staining lead to a blue colour.

Using this staining procedure *Prototheca* can be confused with some yeast, *Candida* and *Cryptococcus*.

A wet mount preparation of a colony, unstained or stained with lactophenol cotton blue, generally reveals the characteristic morphology of sporangiospores within a sporangium at various development stages surrounded by a hyaline cell wall as shown in Fig. 4 (2, 14, 22, 26, 74). The symmetrical morula or mulberry or daisy-like forms of *P. wickerhamii* are shown in Fig. 4 C1 (adapted from Lass-Florl and Mayr (56)). This organization of the sporangiospores is strongly suggestive for *P. wickerhamii* infections (1, 2, 74). The observation of the algae is also efficient in stained smears using Wright or Giemsa stains (2, 14, 22, 26, 74, 160).

Prototheca can have different appearances, and their size can vary from 1.3 X 13.4 µm to 1.3 X 16.1 µm, depending on the species, development stage, culture medium used for identification, and also environmental conditions (2, 11, 22). But in general, the sporangia of *P. zopfii* are larger and more oval to cylindrical in shape than those of *P. wickerhamii* and *P. blaschkeae* (2, 22, 43). More specifically, *P. zopfii* sporangia are spherical (15 – 30 µm) or ellipsoidal (11 – 20 µm X 14 – 23 µm). The released sporangiospores have a granular cytoplasm and are spherical (4.5 – 15 µm) or ellipsoidal (3 – 7 µm X 5 – 8 µm), and the “Dauer cells” are also spherical (8.5 – 14 µm) or ellipsoidal (6 – 11 µm X 8.5 – 13 µm). All *P. wickerhamii* stages are spherical and the average size is about half of that reported for *P. zopfii*. The sporangia are about 7 – 13 µm, the sporangiospores between 2.5 – 4.5 µm, and the “Dauer cells” 5.5 – 8.5 µm in diameter (14, 22, 74). As for *P. blaschkeae*, small ovoid to globose sporangiospores (5 – 7.5 µm) are formed within a sporangium of a mean diameter of 15 µm (43). The non-pathogenic *Prototheca* spp. produce a capsule similar to the one of *C. neoformans*, and this can be detected by Indian ink staining (14, 21, 22).

Some pathogenic fungi such as *Coccidioides immitis* and *Rhinosporidium seeberi* also form sporangia, but these are much larger and contain smaller endospores compared to those from *Prototheca*. Equally, as *Prototheca* algae do not undergo budding, they can be easily distinguished from yeast-like fungi like *Candida* spp. and *C. neoformans* or dimorphic fungi capable of producing a yeast-like shape such as *Blastomyces dermatitidis*, *Histoplasma capsulatum* var. *duboisii*, *Paracoccidioides brasiliensis* or *Lacazia loboi* (2, 42, 132). *Lacazia loboi* and also *Pneumocystis jiroveci* cannot grow in culture media and the latter show a characteristic cyst formation in histological sections (42, 164). Also, *Prototheca* should be clinically differentiated from other human cutaneous infections such as dermatophytosis and lupus vulgaris, but on culture and microbiological analyses a good differentiation can be achieved (1, 42).

The use of immunofluorescent staining with specific reagents to different species can be performed to confirm *Prototheca* growth on culture plates (2, 43, 52). More recently, the usage of FISH permitted a rapidly identification of *Prototheca* spp. (154).

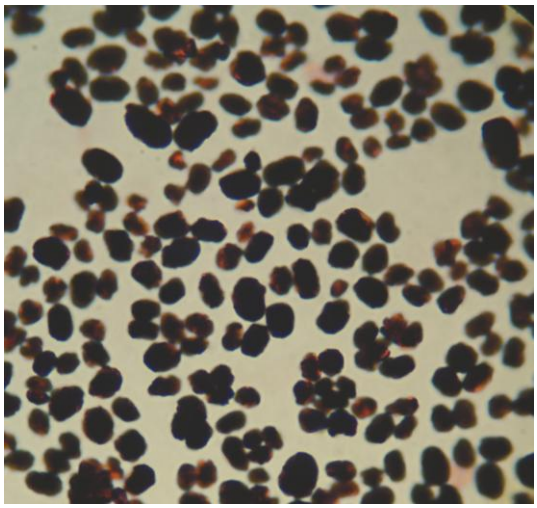

Figure 3 – Photomicrograph obtained by light microscopy of smears of *Prototheca* spp. stained with Gram. Magnification, X 1,000.

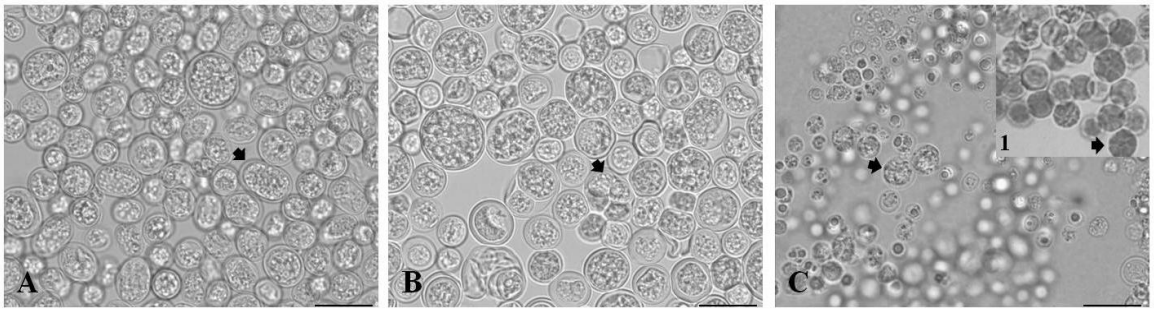

Figure 4 – Photomicrographs obtained by light microscopy of wet mount preparations of *Prototheca* spp. stained with lactophenol cotton blue. Characteristic morphology of *P. zopfii* (A) sporangia (arrow); sporangiospores inside *P. blaschkeae* (B) sporangium (arrow); *P. wickerhamii* (C) sporangia (arrow); characteristic morula like organization of *P. wickerhamii* (C1, arrow – adapted from, Lass-Florl and Mayr (56)). Magnification, X 1,000. Bar, 20 µm.

Prototheca can be differentiated from other algae by electronic microscopy, where the lack of chlorophyll inside the plastids and the presence of cell wall with two layers can be observed (2, 74). The double-layered *Prototheca* cell walls have a thinner outer and a thicker inner layer, in contrast to three layered wall of all *Chlorella* spp. except *A. protothecoides* (2, 165). Also, cytoplasmic vacuoles and numerous mitochondria on the periphery of the cytoplasm can be observed by electronic microscopy (2, 165).

Under histological examination a wide variety of tissue responses from insignificant infiltration to severe granulomatous and suppurative inflammation accompanied by necrotizing foci can be observed (166).

Prototheca cells on histopathological examination are spheroid, ovoid, or elliptical with a prominent wall (21, 35). But, in contrast to wet mounts microscopic analysis, species identification in tissues cannot be based on size, because several algal cells can overlap, host tissues can interfere, no information is available for the age of the algae, and characteristic esporangiospores most of the time are not visible (22, 167, 168). *Prototheca* species stain well with the generally used fungal staining procedures. Thus, these algae stain positively with periodic-acid-Schiff (PAS) with or without diastase, Gomori's methenamine silver (GMS), Grocott's methenamine silver, Gridley and Giemsa stains, but stain lightly with mucicarmine, and can show secondary fluorescence to acridine orange (22, 81, 83, 135). However, hematoxylin or eosin stains only facilitate the observation of *Prototheca*, without a homogeneous staining, and consequently they have a limited use when a detailed study of *Prototheca* is necessary (42, 135). Other green algae can be differentiated from *Prototheca* by PAS, because the latter stain positively, since no starch granules are present in the cytoplasm of this alga (169). *Prototheca* can also be observed with Papanicolaou's, methylene blue, and safranin stains (22).

Biochemical analyses

Morphologic criteria are important for *Prototheca* spp. differentiation but are not enough, because they reflect conditions of an *in vitro* culture and thereby can have considerable fluctuations (21). Hence, identification on the basis of colony and cell morphology should always be followed by more objective methods, such as determination of sugar and alcohol assimilation patterns and also susceptibility to clotrimazole or other drugs (34, 53).

All *Prototheca* species assimilate glucose as carbon source, and nitrogen inorganic salts (ammonium salts) and proteins as nitrogen source, but are unable to hydrolyze urea nor assimilate nitrates, and need oxygen and thiamine for their growth (14, 53). Also, most *Prototheca* species do not assimilate sucrose and xylose, but *P. stagnora* has been found to assimilate sucrose after long periods of incubation (21, 56, 85). The storage carbohydrate is glycogen, it does not have starch, and it can also store fat until 10% of its weight (22, 83).

Commercial identification systems used for fungi can be used to identify isolates as belonging to *Prototheca* (2, 84). From these, API 20C (53), RapID Yeast Plus (170), and Vitek (171) systems are able to identify 96% of yeast and yeast-like microorganisms to the

species level and are easy to use and effective in the majority of cases. The API 20C Aux system can also be used to identify *Prototheca* species, but *P. zopfii* is not considered in the database. *P. zopfii* genotypes differentiation can also be performed by two microbial identification systems, BBL Crystal Enteric/ Nonfermenter®, and BBL Crystal Gram positive®, as described by Roesler *et al.* (20). These three systems were used in a recent study followed by analysis with InforBio software in order to identify discriminative phenotypic characteristics for *Prototheca* (46). Hence, new phenotypic discriminative characteristics were determined to aid on the differentiation of the three pathogenic species of *Prototheca* such as citrate, phosphorylcholine and arabinoside. *P. wickerhamii* was found to assimilate the first two compounds, and demonstrated a weak assimilation of arabinoside. The other two pathogenic species did not assimilate any of these compounds, and only *P. zopfii* showed variable assimilation of citrate (46). However, identification systems have some problems: e. g. their analysis sometimes can be biased, and they require the use of pure cultures. Nevertheless, these systems are still widely used in microbiology diagnosis laboratories (2, 13, 20, 22, 96, 128, 172).

Prototheca are non fermentative and they can be differentiated due to the different sugar assimilation properties. The carbon source assimilation profile can be used to differentiate the three pathogenic species. *P. wickerhamii*, *P. zopfii* and *P. blaschkeae* assimilate glucose and galactose, and the first two also assimilate glycerol, while *P. blaschkeae* does not. Only *P. wickerhamii* assimilates trehalose and this is considered to be the key trait for its differentiation (26, 43, 46, 53, 72, 160). The non-pathogenic species *P. stagnora*, *P. ulmea*, and some strains of *P. zopfii* (*P. zopfii* var. *hydrocarbonea*) and of *P. wickerhamii* are known to possess an anaerobic metabolism similar to that of heterolactic-fermenting bacteria (3). In Table 1 the characteristics generally used to differentiate the three pathogenic *Prototheca* species and also *P. stagnora* can be observed. The doubtful assimilation of galactose by *P. zopfii*, and the assimilation/ non-assimilation of arginine by *P. blaschkeae* (Table 1) are some examples of the intriguing results that could be obtained when using these identification systems. In the case of galactose, some *P. zopfii* strains lately assimilate this carbohydrate and since these are only incubated during 48 hours this cannot be observed. Moreover, even at longer incubation times other strains assimilate or do not assimilate galactose. Arginine is assimilated by *P. blaschkeae* when using BBL Crystal Gram positive®, but is not assimilated when using BBL Crystal Enteric/ Nonfermenter®, and no explanation is obvious for this phenomenon. Generally, *P. blaschkeae* is considered as not assimilating arginine (20, 43, 56).

Table 1 – Phenotypic properties that distinguish four *Prototheca* species (adapted from Janosi *et al*. (14), Roesler *et al.* (43), Möller *et al.* (9) and Marques *et al.* (46)).

Feature	*P. wickerhamii*	*P. zopfii*	*P. blaschkeae*	*P. stagnora*
Colony shape	hemispheric, flat with smooth margin	flat, roughly, with central button and uneven margin	flat, with central button and uneven margin	flat, with smooth margin
Diameter of cells (mm)	4-10	7-30	N.I.	7-14
Assimilation of Glycerol	+	+	–	+
Galactose	+	(+)	+	+
Sucrose	–	–	–	+
Trehalose	+	–	–	–
n-Propanol	–	+	N.I.	–
Arginine	+	+	+ / –	+
Lysine	+	+	–	N.I.
Growth at 37ºC	+	+	+	–

–: no use; (+): doubtful; +: uses; N.I.: no information

The formerly known biotype 3 of *P. zopfii* is clearly a new species, *P. blaschkeae*. Biotypes 1 and 2 are now designated as genotype 1 and 2, respectively (43). In 2003, Roesler *et al.* (20) performed an extensive phenotypic analysis which provided emended standards for *P. zopfii* identification, and proposed the 3 biotypes and the standards for their identification. Thus, this biotype classification was based on glucose, glycerol and galactose assimilation in PIM medium or using API systems, Western Blot and molecular techniques such as PCR and restriction fragment length polymorphism (RFLP) assays (9, 14, 20, 71). The same research group unequivocally identified *P. blaschkeae* and *P. zopfii* genotypes 1 and 2 later by a polyphasic molecular approach, based on sequence analysis of the 18S rRNA gene, and by the pattern of cellular fatty acids (43).

The carbohydrate assimilation is detected within 48 hours, when *P. zopfii* genotype 1 strongly assimilates galactose and glycerol, genotype 2 assimilates glycerol but not galactose or this can only be detected later (after one week of the beginning of the test), and *P. blaschkeae* does not use glycerol. The amino acid assimilation pattern of *P. zopfii* genotype 1 and *P. blaschkeae* show that they do not use lysine, but *P. zopfii* genotype 2 does (14, 20, 49). These and other features are summarized in Table 2. Several studies discuss the presence of heterogeneities in biochemical assimilation and fermentation patterns of *P. zopfii* strains (12, 16, 21, 46). The phenotypic variability demonstrated by

the fermentation pattern was found to be well correlated with the topology of the phylogenetic trees, irrespective of the genetic distances estimated for the long terminal branches (3).

Table 2 – Differentiation of two *P. zopfii* genotypes, and *P. blaschkeae* (formerly *P. zopfii* genotype 3) (adapted from Janosi *et al*. (14); Roesler *et al.* (20, 43), Möller *et al.* (9), Marques *et al.* (5, 46, 80), and Ricchi *et al.* (153))

Feature	*P. zopfii* genotype 1	*P. zopfii* genotype 2	*P. blaschkeae*
Sporangiospores diameter (µm)	11 – 30	5 – 15	N.I.
Shape of cells	spherical/ cylindrical	spherical/ cylindrical	Spherical
Galactose	++	(+)	+
Glycerol	+++	+++	–
Lysine	–	+	–
pH tolerance	2.4 – 9.5	2.1 – 12	3.0 – 12
NaCl tolerance	4 %	9 %	18 %
Growth at 37ºC	+	+	+
Habitats	Cattle stables and pigsties	Predominantly in cattle stables and local waste water	Pigsties and cattle stables
Associated with bovine mastitis	No	Yes	Yes

NaCl: sodium chloride; –: no assimilation; (+): doubtful assimilation; +: weak assimilation; ++: moderate assimilation; +++: strong assimilation; N.I.: no information.

A novel thermotolerant *Prototheca* strain, *P. zopfii* var. *hydrocarbonea*, was isolated from a hot spring (12). Growth of this strain can occur at 25ºC and 40ºC with gas production from glucose and sucrose at 37ºC and 40ºC (12). Throughout fermentation, similar amounts of lactic acid and ethanol, elevated concentrations of carbon dioxide, and little amounts of acetic acid are produced by this strain at 25ºC. At 40ºC in the initial fermentation stages, there is no lactic acid production and ethanol and carbon dioxide concentrations are higher than at 25ºC. In the presence of nitrogen, at both temperatures, ethanol was the only resulting fermentation product. At environmental temperatures, this strain has an anaerobic metabolism similar to that of heterolactic fermenting bacteria, and it can change its metabolism to alcohol fermentation at 40ºC under $N_2/H_2/CO_2$ similarly to thermotolerant yeasts (12).

Some strains of *P. zopfii* were found to have the ability to degrade different types of mainly aliphatic hydrocarbons. This characteristic allows the usage of these strains as biodegradation agents in environments contaminated with these compounds, and show their economic potential (173). *P. zopfii* is able to degrade 10% and 40% of motor oil and petroleum, respectively. In motor oil, this alga degrades a higher percentage of aromatic hydrocarbons, but in petroleum it degrades a higher percentage of saturated hydrocarbons (19). *P. zopfii* var. *hydrocarbonea* can stand large pH and salinity intervals during growth in petroleum hydrocarbons. This thermotolerant strain can grow in n-alkanes at elevated temperatures, and can efficiently degrade 1% of n-alkanes at 35ºC in fresh water, and hence would be a candidate for the removal of paraffinic oil pollutants during summer months (77). *Prototheca* can be used in residual waters of chemical industries treatments, because these contain low concentrations of hydrocarbons and/ or other organic compounds. In this context, the development of a cell system of immobilized *P. zopfii* can offer new perspectives of their use in biotechnology (174). These algae can also be used in the degradation of petroleum in rotating biological contactors used in aerobic treatment processes, with good stability and versatility (175). A most recent study suggested that polyurethane foam harbouring *P. zopfii* var. *hydrocarbonea* cells can be used repeatedly for selective retrieval of polycyclic aromatic hydrocarbons from oil-polluted waters after preferential biodegradation of n-alkanes by these algae (176).

Immunogenic analyses

Great immunogenic differences exist between the two *P. zopfii* genotypes and *P. blaschkeae*. Specific antigens have been identified for each genotype/species, which could be detected by all hyperimmune sera used for its detection (20). Our preliminary results on the characterization of immunogenic factors demonstrated that some cellular factors could be determined and characterized in *P. zopfii* genotypes and *P. blaschkeae*, such as ribosomal protein, glutamyl-tRNA reductase 1, ADP/ATP carrier protein, ATP synthase, elongation factor and glyceraldehyde-3-phosphate dehydrogenase. Although all of these proteins are intracellular, they may represent a progress in the identification of some of the pathogenic mechanisms in these algae.

Phylogenetic analyses

Several phylogenetic studies on the genus *Chlorella* (16, 40, 177-187) are described in the literature. Also, studies on the genus *Prototheca* and on its plastid metabolic functions were performed (3, 15, 44, 48, 51, 188-196). Additionally, a smaller number of studies on *P. zopfii* biotypes/ genotypes classification and new species formation (9, 20, 43, 77, 153) were reported. The increasing number of these studies reveals the

importance and impact of *Prototheca* and the need to understand its evolution and relations with other species/ genus.

The phylogenetic analyses of *Prototheca* spp. have been carried out by sequencing the small subunit (SSU) rDNA and the D1/ D2 domains at the 5' end of the large subunit (LSU) rDNA (3, 12, 40, 48, 51, 77). In 2003, Ueno *et al.* demonstrated that *Prototheca* spp. can be divided into two groups (48). One group included all *Prototheca* spp. except *P. wickerhamii*, and the other one consisted of *P. wickerhamii*, which seemed more closely related to *A. protothecoides* (2, 17) as previously referred. The DNA base composition (mol % GC) values of *P. wickerhamii* strains were similar to those of *A. protothecoides* as shown by Huss *et al.* (23). *A. protothecoides* is the most related taxon that shares a common ancestor with *Prototheca* (16, 40, 48). It is clear however, that further investigation should be performed with more strains to better understand the extent of genetic diversity within and between species (intrageneric and intraspecies levels) of *Prototheca* (3).

Other ribosomal DNA regions such as the non-coding ITS are also used in fungal and yeast phylogenetic studies (12, 197-200). In a preliminary study we intended to amplify this region in several *Prototheca* spp. isolated from bovine mastitic milk with the purpose to differentiate the isolates at the herd level. However, amplification of this region was only successful for three *P. zopfii* isolates. Also, as the plastid 16S rDNA is known to be not as conserved as the nuclear 18S rDNA (15), the study conducted in our laboratory determined the 16S rDNA, intergenic spacer (IGS) and partial 23S rDNA sequences. After plastid rDNA sequence determination more variability between species was observed. This preliminary results suggests that the ITS and plastid rDNA sequences determination could be of great value for the *Prototheca* spp. population genetics and epidemiology.

Mitochondrial DNA (mtDNA) from *P. wickerhamii* contains two mosaic genes as revealed from complete sequencing of the circular extranuclear genome (188). In the mitochondrial genes two types of introns were identified and their classification into group I and group II is generally based on canonical nucleotide sequence motifs and on conserved characteristics of the secondary structure potentially formed by the intron RNA (201). Numerous fungal species contain both groups of mitochondrial introns, and so far only few introns have been detected in mitochondria from angiosperms and gymnosperms all belonging to group II (201). Phylogenetic comparisons of the subunit I of the cytochrome oxidase protein group I intron sequences allowed Wolff *et al.* to conclude that *P. wickerhamii* mtDNA is much closer related to higher plant mtDNAs than to those of the chlorophyte alga *Chlamydomonas* (189). *P. wickerhamii* mitochondrial genome is very characteristic (181), and represents an ancestral type among green algae with, among

other characteristics, a larger size (45–55 kb) and a more complex set of protein coding genes than the mitochondrial genome from the *Chlamydomonas* (181, 195). Recently it was demonstrated that the mitochondrial genome of *Helicosporidium* closely resembles that of *P. wickerhamii*. These two genomes share an almost identical gene complement and display a level of synteny that is higher than any other sequenced chlorophyte mtDNAs. However, the *Helicosporidium* mitochondrial genome contains novel features, particularly with respect to its introns (187).

The presence of plastids in eukaryotic cells is generally associated with their ability to perform photosynthesis. However, colourless plastids with various degrees of functional and structural degeneration have been identified in several eukaryotic lineages including *Prototheca* (202). The loss of plastid-encoded photosynthesis-related genes has been documented in plastids from achlorophyllic lineages among green algae (194, 196) and other organisms, although the functional role of these plastids is largely unknown (193).

Based on phylogenetic analyses inferred from the plastid 16S rRNA genes it can also be stated that *Prototheca* spp. is closely related to the photoautotrophic genus *Chlorella*, as by the analyses of the nuclear 18S rRNA genes (15, 16). The plastid 16S rRNA gene is a chloroplast gene, and its presence has been demonstrated in the non-photosynthetic green algae of the genus *Polytoma* (203), which is closely related to *Chlamydomonas*. There are no records of microscopic observations of a leucoplast in *Prototheca* cells. However, the plastid genome of *P. wickerhamii* has been isolated and partially sequenced (196), and found to be functional (195). The metabolic functions of the *P. wickerhamii* plastid were studied (193), initially searching for expressed sequence tags (ESTs) that corresponded to nucleus-encoded plastid-targeted polypeptides in this alga. From a large number of ESTs, it was found that 71 unique sequences (235 ESTs) corresponded to different nucleus-encoded putatively plastid-targeted polypeptides. These proteins predict that carbohydrate, amino acid, lipid, tetrapyrrole, and isoprenoid metabolism as well as *de novo* purine biosynthesis and oxidoreductive processes take place in the *P. wickerhamii* plastid. Magnesium-protoporphyrin accumulation and, plastid-to-nucleus signalling can also occur in this alga. This can be explained, because a transcript that encodes subunit I of magnesium-chelatase was identified, and this enzyme catalyses the first committed step in chlorophyll synthesis (193).

Molecular identification

The phenotypic characterization of the genus *Prototheca* is extremely laborious, time consuming, and is subject to bias. Therefore, it is very important to develop and apply molecular techniques for its correct identification. Nevertheless, the earlier identification

techniques should not be forgotten, and should be used in combination with the new developed molecular techniques.

The molecular identification of *Prototheca* is generally performed by the amplification of the 18S rDNA gene, and of the D1/ D2 domains at the 5' end of LSU rDNA (3, 17, 41, 139). Genotype specific PCR analysis has been widely used as well (9, 43, 153, 204). Most recently a real time PCR was develop and allowed a rapid and high reproducible molecular identification of these algae (44).

The degenerated primers sequences for SSU rDNA that are generally used, are based on sequences reported in previous studies (40, 205), in which the forward primer is, 5'-AAC CTG GTT GAT CCT GCC AGT-3', and the reverse primer is, 5'-TGA TCC TTC TGC AGG TTC ACC-3'. The sequences of the primers for amplification of the D1/D2 domain at the 5' end of LSU rDNA are generally based on the sequences reported previously by O'Donnell in 1993 (206), and Kurtzman and Robnett in 1997 (207), in which primer F63 is 5'-GCA TAT CAA TAA GCG GAG GAA AAG-3' and primer LR3 5'-GGT CCG TGT TTC AAG ACG-3' (17, 71). Other primers can also be used to amplify D1/D2 domain in the 26S rDNA, such as, 26S-F1, 5'-AGC CCA GCG TGT CAA TC-3') and 26S-rDNA-R, 5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') (51).

After amplification of the SSU and LSU rDNA, the PCR products are cloned into a plasmid, and subsequent to transformation, the plasmid DNA is extracted and sequenced by the dideoxy chain termination method (Sanger method) (17, 71).

Direct sequencing can also produce good and reproducible sequences capable to identify these algae (9, 41, 51). Additionally, the usage of endonuclease *Hae*III for RFLP analysis of amplified 18S rDNA aided on the identification of *Prototheca* (41).

For the biotype/ genotype molecular identification of *P. zopfii*, a biotype/ genotype specific PCR was developed by Möller *et al.* (9, 208). In these studies restriction enzymes for RFLP assay were also used on the genotypes identification (208). For the genotype-specific PCR, primers developed from previous results of the 18S rDNA sequences alignments were used: Proto18-4f (5'-GAC ATG GCG AGG ATT GAC AGA-3') and PZ GT 1/r (5'-GCC AAG GCC CCC CGA AG-3') for genotype 1; Proto18-4f (5'-GAC ATG GCG AGG ATT GAC AGA-3') and PZ GT 2/r (5'-GTC GGC GGG GCA AAA GC-3') for genotype 2; and PZGT 3-IK/f (5'-CAG GGT TCG ATT CCG GAG AG-3') and PZ GT 3/r (5'-GTT GGC CCG GCA TCG CT-3') for *P. blaschkeae* (previously biotype 3) (9, 43). All of these primers are specific for a particular *P. zopfii* genotype, with the exception of the *P. blaschkeae* specific primer pair that also recognizes the non-pathogenic species *P. stagnora*. In addition, the genotype-specific endonucleases for RFLP analysis can be:

*Kpn*21 (genotype 1), *Sma*I (genotype 2), and *Bcn*I (*P. blaschkeae*) (9). The primers usually used for RFLP analysis are: Proto18-2f (5'-CGC GCA AAA TTA CCC AAT CC-3') and Proto18-2r (5'-TGA GAC CGG ACA GGG CAA-3') for *Kpn*21/*Sma*I and Proto18-4f (5'-GAC ATG GCG AGG ATT GAC AGA-3') and Proto18-4r (5'-AGG ATG GCT AAC CCA CAC GA-3') for *Bcn*I (9). The PCR- and RFLP-assays are useful tools for the differentiation of pathogenic *P. zopfii* genotype 2 strains from *P. blaschkeae* and non-pathogenic strains of *P. zopfii* genotype 1 (9).

The recently developed real time PCR contains a two-step reaction that is followed by DNA resolution melting analysis. It was developed with the use of reference strains of pathogenic and non-pathogenic species. The method validation was performed by simultaneous amplification of a significant number of isolated *Prototheca* strains with both, real time PCR and the conventional genotype-specific PCR assays, which demonstrated a perfect accordance. Furthermore, the real time PCR was able to identify the other *Prototheca* species (*P. stagnora*, *P. ulmea* and *P. wickerhamii*), which could not be discriminated by conventional PCR assays. Therefore, the newly developed real time PCR is accurate, robust, cost effective and faster than auxonographical, biochemical or conventional molecular biology methods. This new methodology can also be used for the identification of clinical and environmental *Prototheca* species (44).

TREATMENT

The pathogenesis of the infection by *Prototheca* and the associated immune responses are still to be clarified (10), hence it is difficult to develop therapeutic strategies against this infection. Information about effective therapy is limited and the existing is supported in data of isolated cases, limited case series and *in vitro* studies (74, 209). *Prototheca* infections are rare and difficult to eliminate, and consequently limited clinical studies have been published comparing specific treatments for protothecosis. Treatment of protothecal infections remains controversial, and conventional methods for *in vitro* susceptibility tests have not been standardized for algae, and do not have official guidelines for performance, interpretation or quality control (65, 210, 211).

Currently, it is known that minimum inhibitory concentration (MIC) testing is not always reproducible, and that the results not always correlate with clinical success (121). Nevertheless susceptibility tests are not necessary to guide the treatment of *Prototheca* infections, these tests should be recommended if clinical treatment is not successful. Several tests, E-test (158, 212, 213), broth and agar dilution (13, 209, 214, 215), and agar

disk diffusion tests (13, 57, 216) were used for *Prototheca* MIC determination with good correlation between them.

Prototheca algae are extremely resistant to numerous treatment strategies (14, 22), and several susceptibility tests indicated that they are usually resistant to antibiotic agents, and demonstrate different susceptibility to antifungal agents (56, 160). Therefore, in several studies susceptibility to amphotericin B and variable susceptibility to azoles such as ketoconazole, miconazole, clotrimazole, fluconazole, itraconazole and voriconazole, was demonstrated (2, 42, 125, 133, 209, 213, 217). In other studies variable susceptibility to a wide range of antibacterial agents, tetracycline, gentamicin and amikacin, was observed (126, 133, 211, 218, 219). A recent study (213) tested the susceptibility of *P. zopfii* genotype 1 and genotype 2 isolates against amphotericin B, gentamicin, kanamycin and itraconazole. The results showed a higher *in vitro* susceptibility of *P. zopfii* genotype 1 isolates to the first three agents than those of genotype 2. Moreover, all genotype 2 isolates and most of the genotype 1 were not susceptible to itraconazole. Thus, *P. zopfii* genotypes show different drug susceptibilities.

Despite the observed variability, the most effective agents determined in several studies were amphotericin B, nystatin, gentamicin, and polymyxin B among others (4, 13, 22, 56, 216, 220). A possible explanation for *Prototheca* susceptibility to polyene and azole agents could be the presence of small amounts of ergosterol in the neutral lipid fraction of the cell membranes of these algae (220, 221). The susceptibility found to polymyxin B could be related to the organism's phospholipid components. All *Prototheca* spp. in relation to pyrimidine analogues and mitotic inhibitors demonstrated resistant to 5-flucytosine and griseofulvin, respectively (214, 217).

Casal *et al*. (222) demonstrated the existence of a beta-lactamase in *P. zopfii* which is capable of inactivating several compounds, but no further studies were performed in order to identify and characterize this enzyme. In general, *Prototheca* spp. show various susceptibility profiles and, no correlation between *in vitro* activity and clinical response is present with the exception of a few cases (126, 130, 133). Studies on the "*in vitro*" and "*in vivo*" susceptibility to antimicrobials have shown that *Prototheca* spp. is resistant to most of them. Looking for alternative treatments, other studies analysed the susceptibility to antiseptics and other agents. Melville *et al*. (165) analysed the *in vitro P. zopfii* susceptibility to copper sulphate (algaecide effect), silver nitrate (cauterizing agent of mammary glands), and chlorexidine (anti-septic). The minimal microbicidal concentrations for each compound, 0.1%, 0.3% and 0.01% respectively, and also their effect in the ultrastructure of these algae were determined. Silver nitrate induced thickness of the cell wall, while chlorexidine showed changes suggesting degradation of intra-cellular

organelles, and on the other hand, copper sulphate induced fibrillation of the inner layer of the cell wall (74, 165).

It was determined in a recent study, that low concentrations of sodium hypochlorite (0.039% – 0.156%) and iodine (0.156% – 0.625%) were effective against *P. zopfii* isolates (223). Another investigation tested the efficacy of iodine, quaternary ammonium compounds and dodecylbenzenesulphonic acid, and showed that the highest disinfectant concentrations that permitted survival of at least one isolate were dilutions of 1:1000, 1:100 and 1:10, of the respective compounds (79). In this study iodine compounds demonstrated a more pronounced efficacy. These antiseptics could be used for hygiene routines, pre and post-dipping and cauterization of bovine mammary glands infected by *P. zopfii* (223), preventing its dissemination. Also, in another study (219), *Prototheca* spp. strains were tested to determine the susceptibility to different commercial disinfectants, Biocitro® (ascorbic acid, citric bioflavonoids, and other organic acids), eucalyptol, Combicid® (descaling acid), Eco Plus® (chlorinated alkaline solution), and Prodip G® (chlorhexidine digluconate). From these, Biocitro® and Eco Plus® proved to be efficient for all strains, while Combicid® and Prodip G® were not able to inhibit the growth of all *Prototheca* strains. Eucalyptol, however, demonstrated methodological problems during the tests and the efficacy should be reevaluated (219).

The toxic effect of indole-3-acetic acid (a plant growth hormone) combined with horseradish peroxidase was investigated. These agents reduced *P. zopfii* viability and colony formation in a concentration and time dependent manner (224). The algaecide activity of natural essences against *Prototheca* was also tested *in vitro*, and it was concluded that bergamot and tea tree oils can have inhibitory effects on these algae (215). Another study tested the flavonoid activity against *Prototheca* and yeasts isolates, and showed growth inhibition halo diameters of 14.5, 17.8 and 20.7 mm, for only three of the 12 compounds tested against *Prototheca*. No further information regarding *Prototheca* isolates was given (225). Also, the antimicrobial activity of some plant extracts towards yeast and *Prototheca* strains was studied (226). Of those, only extracts of *Camellia sinensis* (obtained from a commercial preparation of green tea) exhibited activity against yeasts and *P. wickerhamii*, with MICs of 300 µg/mL for the algae (226).

The antimicrobial activity of bovine lactoferrin on several microorganisms including *P. zopfii* was also tested (151, 227, 228). Thereby, despite the variability between these studies results, the *Prototheca* isolates were found to be extremely susceptible and showed complete growth inhibition with 1 µg/mL (227) and 7 µg/mL (228) of bovine lactoferrin.

Preliminary results obtained in our laboratory from determination of the *in vitro* susceptibility of some strains of *P. zopfii* and *P. blaschkeae* to borate and phosphate buffers, both at pH 9, showed that all isolates tested grew in the presence of phosphate buffer, but *P. blaschkeae* isolates showed a lower growth. It was found that in the presence of borate buffer, all isolates were inhibited with time, where *P. zopfii* strains were completely inhibited after 1 week of incubation. Thus, borate buffer at pH 9 can possibly have an algaecide effect on *P. zopfii*. Also, when studying *Prototheca* susceptibility to different pH values and saline concentrations, *P. zopfii* strains were able to grow between pH 5 and pH 12 values, and also at pH 1 value, and were completely inhibited at pH 3 values. Whereas *P. blaschkeae* strains had higher susceptibility to all pH values except for pH 3, where a moderate growth was observed compared to *P. zopfii* strains. Therefore, *P. zopfii* had a higher multiplication capacity in all pH buffers except on acetic acid, suggesting that both species, but especially *P. zopfii*, are able to multiply under adverse conditions. With salinity increase, *P. blaschkeae* was more resistant than *P. zopfii*, although a reduction in growth for all strains of *Prototheca* was observed. *P. zopfii* and *P. blaschkeae* are able to grow until 9% and 18% of salt concentrations, respectively (5).

Concerning the temperature susceptibility of *Prototheca*, several studies have demonstrated that these algae have highly variable susceptibilities and that resisted to conventional pasteurization and other thermal treatments such as 62–65ºC/30 min, 72–75ºC/15 s and 72–75ºC/20 s (78-80). The only temperature observed to be effective on the inactivation of *P. zopfii* and *P. blaschkeae* isolates, was 100ºC during 1 second, what demonstrates a high resistance capacity of these algae and its potential risk for public health (80).

Bovine protothecosis (mastitis)

Bovine protothecosis is not susceptible to commonly used antibiotics and antifungal drugs such as amphotericin B and fluconazole have variable effects on *Prototheca*. Although certain antifungals can be effective in bovine protothecosis, treatment is not recommended because of extremely high costs and veterinary public health considerations (79, 219). As the infection causes irreversible damage to udder tissue, granulomatous changes, and since therapeutic failure is certain, the infected cows remain a continuous source of infection and their milk becomes hazardous to public health (14, 71).

Clinical signs of *Prototheca* mastitis sometimes are mild and do not influence the general state of the cows. However, as its course is progressive and infected cows

became intermittent shedders and spontaneous and post therapeutic cure are rare, the most efficient therapeutic measurement is the culling of affected animals. Therefore, immediate removal of the infected animals, separate housing and milking procedures, and early culling are the most recommended measurements (14, 69, 71, 78, 216). Reliable identification of infected individuals would reduce the risk of infection of uninfected cows or contamination of the dairy farm environment (67, 71, 78). The usage of antimicrobial agents can temporarily decrease the severity of clinical signs and its dissemination through the environment. However, as *Prototheca* causes irreversible damage of the mammary gland, even after its elimination milk production will remain low and consequently one of the therapeutic hypotheses is drying the affected udder quarter (10, 22, 150). In a *Prototheca* outbreak in Brazil, thimerosal was clinically used for treatment of mastitis, and demonstrated only microbiological cure without the udder recovering its function (14, 88, 150). Preparations with 4% of tetramisole or levamisole hydrochloride were effective in reducing *Prototheca* in the mammary gland after application of up to 40 ml/quarter for 6 milking times. However, oral administration at elevated doses did not suppress *Prototheca* in milk (229). A different study demonstrated the efficacy of artificial produced bovine lactoferrin against *Prototheca* mastitis. This showed “bacteriostatic” effect, suggesting that this protein could be used together with other agents for therapeutic purposes. However, studies confirming this finding were never published until now (151).

Bovine mastitis prognosis is weak, due to the lack of response to therapy (230).

Human protothecosis

Treatment of protothecal infections remains controversial, and to date the several tested treatments without consistent clinical responses reflect the absence of standard treatment regime for human protothecosis. Usually, treatment involves medical and surgical approaches, and treatment failure is not uncommon. However, for localized lesions the options are surgical excision and topical therapy with amphotericin B, ketoconazole, itraconazole, fluconazole, transfer factor, or the combination of amphotericin B (also liposomal) and tetracycline, and also systemic amphotericin B, with or without excision and oral tetracyclines. The referred antifungal agents have been reported as the most effective, but the therapeutic dose and the therapy duration have not yet been determined. Infection is indolent with no tendency toward self-healing (56, 113, 162).

Although *Prototheca* have low pathogenic potential, they may cause a very difficult treatment problem, especially in immunocompromised patients. If not diagnosed promptly,

even cutaneous protothecosis can produce localized, chronically destructive lesions also in immunecompetent hosts (2, 56, 113, 125, 130, 162).

Synergistic activation of amphotericin B and tetracycline was observed *in vitro*, and some humans with cutaneous protothecosis were successfully treated (57, 231-233) with this combination. However, Takaki *et al*. (234) reported induction of secondary resistance during a 3-year period of therapy and clinical failure. This extent of clinically significant acquired resistance to any drug is generally believed to be uncommon (234).

In a reported case of cutaneous protothecosis, successful treatment was accomplished with long term itraconazole and interferon- γ (210). But in another case of cutaneous protothecosis, itraconazole was not successful in eliminating these algae, amphotericin B together with tetracycline only caused minimal improvements, and total cure was only achieved with surgical excision. However after a few months papules around the scar recurred, and voriconazole treatment was initiated with complete cure (235). In a recent study of cutaneous protothecosis (236), the lesion showed no response to the systemic itraconazole and topical ketoconazole treatments, demonstrating only a complete resolution with the use of thermal therapy as an adjuvant to systemic itraconazole. Excision of infected small localized tissue may be acceptable in superficial infections as was shown by the success of this approach in several previously reported cases (1, 42, 125). Persistent or deep infections may require systemic therapy plus excision (237). The duration of treatment varies from days to weeks or even months (42, 116).

Successful treatment of olecranon bursitis has focused on bursectomy, because repeated drainage has failed (125, 132). However, drainage coupled with local administration of amphotericin B has been curative (132, 238, 239). The role of systemic ketoconazole, fluconazole, and other imidazoles is unclear (121, 237), but itraconazole treatment should be administered for at least 2 months (240).

On the other hand, for disseminated protothecosis a combination of various antifungal agents (ketoconazole, itraconazole, amphotericin B) with various protocols are usually used (2, 56, 113, 125, 130, 162, 241). The use of a liposomal formulation of amphotericin B has been a success in the therapy of protothecosis in patients with cancer (1, 42, 65, 72, 74, 209), but no breakpoints have been established, and no clinical correlation studies have been performed (125). The usage of azoles should be reserved to localized pathologies, because their therapeutic efficacy is not yet determined and is usually associated with failure (125, 130), but in a case of intestinal protothecosis that was not responsive to amphotericin B therapy, the combination of oral itraconazole with

subcutaneous interferon- γ became more effective. Although no further evaluation of the impact of this treatment was accomplished because of its early discontinuation (82, 117, 125, 210). Interferon- γ was also found to improve alveolar and peritoneal macrophages microbicidal activity (210). All catheter-related events were treated by removal of the catheter and debridement, and systemic administration of either, amphotericin B (242, 243), amphotericin B plus oral doxycycline (244, 245), or fluconazole (246), and also voriconazole and interferon- γ (245). In two cases of peritonitis complicating continuous ambulatory peritoneal dialysis, intraperitoneal amphotericin B was used (243, 246).

Administration of amphotericin B appears to be the most effective treatment for systemic protothecosis, although it has not been efficient in six patients. Overall, these patients had a combination of profound immunosuppression and widespread infection (120, 130, 157, 164, 210, 247). In two of these cases, amphotericin B therapy consisted of only 5 days, which is a period probably too short to clear a fungal infection (130, 157). In another case, the treatment failure with amphotericin B was associated with recurrence of olecranon bursitis along with skin lesions and splenomegaly (164).

Nine cases of protothecosis after organ transplantation were described, with 5 presenting localized infection, and 4 disseminated protothecosis. Overall mortality in transplant recipients with *Prototheca* infections was 88%, corresponding to a grave prognosis. All 4 cases of disseminated protothecosis died despite therapy with amphotericin B (241). The optimal dose and duration of therapy are uncertain. So far, amphotericin B therapy is recommended as first-line therapy in cases of dissemination and for patients with severe underlying illness or with immunosuppression (240). The evaluation of new antifungal drug strength, therapy duration and dosage, lipossomic amphotericin B, new azoles and interferon, are required in the treatment of *Prototheca* infections (42, 56, 65).

Canine and feline protothecosis

Canine protothecosis is very difficult to treat. Thus, dogs aggressively treated in the beginning of disseminated protothecosis have greater survival periods than those at the later course of the pathology (81, 138). Agents likely to be most useful against *Prototheca* species include amphotericin B, fluconazole, itraconazole, amikacin, tetracycline and possibly terbinafine (81, 135, 138). The use of oral itraconazole was not effective in the treatment of disseminated protothecosis (135). Colitis cases were treated with local therapy (amphotericin B retention enemas) together with systemic therapy. Combination therapy with more than one agent is likely to be superior to monotherapy with an azole. After retrospective analyses of several studies, it was concluded that usage of

amphotericin B in the therapeutic regimen is mandatory (135). Therefore, newer forms of delivering this antifungal with reduced nephrotoxicity, such as liposomal and lipid complex preparations (81, 135) can be even more effective, but are likely to be cost prohibitive for many animal owners. The usage of amphotericin B in protothecosis is questionable, but in dogs with ophthalmic disease without systemic signs, intravenous administration together with itraconazole demonstrated improvement of the signs in short periods of time (11, 81). Disseminated canine protothecosis have a poor prognosis because response to therapy is weak (11).

In feline protothecosis the major clinical manifestations reported are cutaneous nodules (138, 144), and surgical removal of these lesions should be performed as soon as possible in order to completely remove them (144).

CONTROL AND PREVENTION

The epidemiology and pathogenesis of bovine protothecosis are very important issues to the dairy cow herd level and to public health concerns. Since *Prototheca* can be widely disseminated in the dairy herd environment and are extremely resistant to most antimicrobial agents, the most promising mean of reduction its incidence is to improve the preventive and control measures at the level of dairy herds, alert the clinicians for this emerging pathogen, and include it in the differential diagnostics of bovine mastitis. *Prototheca* mastitis is considered to be a difficult infection to prevent and control and because of that, its prevention and control should involve the implementation of practices that are established for other environmental mastitis pathogens (69, 87, 248). It is extremely important to prevent the contamination of teat ends and to increase cows' resistance to mastitis (89, 90). The reducing of teat-end exposure to *Prototheca* requires attention to all environments in a dairy herd, such as dry cow and close-up heifer housing, calving area, lactating cow's environment, and milking parlour. Clean and dry conditions should be maintained in all areas of the dairy cow's environment. The elimination of moisture, especially in bedding materials, may be one of the more productive ways to reduce the number of environmental pathogens and cases of mastitis in the dairy herd (14, 219, 249). When inorganic materials cannot be used in beddings, every effort should be made to keep organic bedding as clean and dry as possible (89, 111).

Poorly designed facilities can contribute to increased incidence of environmental mastitis (89, 111). The facilities should be designed to maximize cow comfort and minimize stress and physical injuries during all seasons of the year. Ventilation is critical for maintenance of dry conditions and frequently is poor in older facilities in which

sometimes correctable measures become complex or even impossible. In well designed free-stall barns, cows will either be eating or lying down resting (250). In severely infected dairy herds, systematic cleaning with high pressure hot water or spraying with algaecides is essential (111). Pastured cows are generally thought to be at reduced risk for environmental mastitis when compared to cows in confinement housing. However, conditions that can lead to high levels of exposure to environmental pathogens, muddy areas and puddles, also exist in pastures (250).

Improper hygiene at milking time and machine function can contribute as well to the mastitis problem. The solution is to milk clean, dry teats (individual paper towel) and udders with a properly functioning milking machine. Also, favourable hygienic status and regular cleaning of driving ramps are of crucial importance. Additionally, offering freshly served, palatable feed to animals after milking to prevent them from lying down immediately, and giving time for the sphincter of the teat canal to close, are considered good practices (87, 111). Adequate pre-milking hygiene is one of the most important preventive measurements, and has been shown to reduce new environmental mastitis infections during lactation in some herds. Milking time hygiene is also important to prevent the contamination of the milking tools with these algae (90, 251). Post-milking teat dipping with disinfectants is an effective way to reduce potential animal to animal transmission, since *Prototheca* can have contagious behaviour (87, 111, 223).

Diets can influence the resistance of cows to mastitis and several studies refer the importance of vitamin E and the trace mineral selenium (252, 253). However there is no direct correlation of diets on bovine protothecosis, they have influence in other bovine mastitic agents, which can aid in reduction of susceptibility to *Prototheca* infections.

Infected cows have an important role in the maintenance of *Prototheca* contamination of the environment (69, 219). Since the antimicrobial defence mechanisms of the udder cannot overcome and eliminate this pathogen, it can survive for a long time in the udder even during the dry period (9, 14, 254). Consequently, extensive microbiological monitoring should be performed in infected herds, and after identification of infected animals these should be immediately removed from the herd. Early diagnosis, separation of housing and milking, or milking at the end, and early culling of infected cows are considered effective control measurements against protothecosis (64, 67, 91, 111, 150). The culling of cows with more than one quarter infected and chemical cauterization of cases where only one quarter is affected is recommended by some authors (149, 255). Animals recently acquired can introduce *Prototheca* in the new herd. Thus, microbiological analysis of milk of animals that will be purchased or the establishment of strict rules on the buying contracts should be implemented (251). The implementation of control

measurements against *Prototheca* mastitis protects milk quality and decreases its rejection, reduces environmental contamination and new infections in the herd (83, 84, 256).

AIMS OF THE STUDY

The main purpose of this thesis is to contribute to the understanding of *Prototheca* spp. pathogenesis. Specific characteristics of these organisms related to the prevalent species associated with bovine mastitis in Portugal region of the study, their susceptibility to different chemical and physical agents, and the determination of immunogenic factors that can be associated with the immune response and consequently with immune protection are addressed. The knowledge and the understanding of the pathogenic mechanisms by *Prototheca* may contribute to the implementation of more adequate and efficient control measures for this type of opportunistic infections in humans and animals. Therefore the specific aims of this project were:

- The phenotypic and molecular characterization of the *Prototheca* isolates obtained from bovine mastitis
- The evaluation of the phylogenetic relationships between the characterized isolates
- The evaluation of the efficacy of physical treatments used in milk processing and of chemical treatments
- The determination and characterization of immunogenic antigens

Firstly, in Chapter 1, this thesis describes the “State of the Art” on *Prototheca*, followed by the presentation of the main objectives of the research. Subsequently, the work was divided in several experimental studies, which are presented in the next chapters. In Chapter 2, the phenotypic characterization using several commercial available identification systems used for yeasts and bacteria agents is discussed. Also in this chapter, the molecular characterization and evaluation of phylogenetic relationships resulting from the amplification of conserved gene sequences generally used for phylogenetic analyses in eukaryotes are described. As a result of the latter molecular characterization, a case study of *P. blaschkeae* algaemia in a cow is also shown. In Chapter 3, the *in vitro* susceptibility of *Prototheca* spp. to different physical and chemical treatments and the results obtained are discussed. Chapter 4 describes the preliminary studies and results of the identification and characterization of intrinsic and secreted immunogenic factors from *Prototheca*. Finally, a general discussion of the work and significance of the findings is presented in Chapter 5, where conclusions and perspectives for future research are also presented.

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CHAPTER 2

PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF THE *PROTOTHECA* ISOLATES OBTAINED FROM BOVINE MASTITIS

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Phenotypic characterization of mastitic *Prototheca* spp. isolates

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ABSTRACT

Bovine mastitis associated with *Prototheca* is considered a rare pathology, but is increasing in prevalence all over the world and therefore becoming more relevant to the dairy industry. The biochemical characterization of 47 *Prototheca* isolates retrieved from mastitic milk was performed in this study using API 20C Aux and two BBL Crystal Kits, followed by an analysis with InforBio software. The usage of this methodology, allowed the identification of discriminative phenotypic characteristics for the strains tested. The differential-character-finding algorithm used by this software permitted the identification of new phenotypic characteristics to discriminate between *Prototheca zopfii*, *P. blaschkeae* and *P. wickerhamii*, such as, citrate, phosphorycholine and arabinoside. The main objective of this study was to determine new phenotypic characteristics that allowed a better characterization of *Prototheca* spp. Usage of recent bioinformatic tools improved the analyses of several features that are important for a better characterization of *Prototheca* spp.

1. Introduction

Algae of the genus *Prototheca* are one of the few plant-like organisms that cause infections in humans and animals (Matsuda and Matsumoto, 1992; Moller et al., 2007; Marques et al., 2008). These achlorophylic algae are ubiquitous saprophytes that reproduce by multiple splitting with the formation of several endospores within the mother cell (DiPersio, 2001; Malinowski et al., 2002). *Prototheca* are considered rare pathogens that usually cause infections when predisposing factors are present, such as poor dairy cow care and poor milking hygiene, and also impairment of immunological defences (Pore, 1998; Janosi et al., 2001; Roesler and Hensel, 2003; Marques et al., 2006). Sporadic cases were reported in dairy herds of Europe and on the American Continents (Lerche, 1952; Anderson and Walker, 1988; Da Costa et al., 1996), but nowadays, cases of acute to chronic mastitis are recognized increasingly to be endemic worldwide and gaining more economic and public health importance (Roesler and Hensel, 2003; Buzzini et al., 2004; Osumi et al., 2008). This scenario is also detectable in Portugal with several cases of bovine mastitis associated with *Prototheca* spp. being reported mainly on the northern region of the country (Santos and Flor, 2000; Bexiga et al., 2003; Marques et al., 2006, 2008; Thompson et al., 2009). Within the known *Prototheca* spp., only *Prototheca zopfii*, *P. wickerhamii* and *P. blaschkeae* have been associated with pathology in humans and animals (Costa et al., 1998; Roesler et al., 2003, 2006; Zaitz et al., 2006; Thompson et al., 2009). *P. wickerhamii* is generally associated with human pathology presenting essentially cutaneous or subcutaneous lesions and also more rarely generalized infections (Zaitz et al., 2006; Hightower and Messina, 2007; Lass-Florl and Mayr, 2007; Narita et al., 2008). On the other hand, *P. zopfii* has been associated with animal infections and mainly bovine mastitis (Roesler et al., 2003; Buzzini et al., 2004; Moller et al., 2007). *P. blaschkeae* has recently been described as a causative agent of bovine mastitis (Marques et al., 2008), although was first isolated from a human onychomycosis (Roesler et al., 2006). Nevertheless these two species are capable of causing infections in humans as well (Lass-Florl and Mayr, 2007). These algae do not respond to routine mastitis therapy and the only control method to date has been the elimination of the infected animals (Costa et al., 1997; Buzzini et al., 2008). *Prototheca* spp. identification and characterization is usually preformed by macro and microscopic morphological characterization, biochemical profile determination and molecular characterization (Pore, 1998; Roesler et al., 2003; Marques et al., 2006, 2008). Generally the assimilation or non assimilation of some substrates, such as, trehalose, glycerol, galactose, arginine and lysine are well established as being key factors in the characterization of *Prototheca* spp. (Pore, 1998; Roesler et al., 2003). In a previous study (Roesler et al., 2003) several biochemical assimilation systems were able to differentiate various *P. zopfii* strains into biotypes, but no more studies have been preformed

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to detect further biochemical differences to characterize this species or even differentiate the three pathogenic species. Therefore, the main objective of this study was to identify discriminative phenotypic characteristics of 47 pathogenic strains obtained from 47 cows of 20 different dairy herds in the northern region of Portugal, isolated from bovine mastitic milk, by means of three identification systems. The molecular characterization of the isolates in this study was performed and reported in a previous study (Marques et al., 2008).

2. Materials and methods

2.1. Isolation of algae

Prototheca isolates in this study belong to a major collection of several milk pathogens that belong to the Laboratory of Infectious Diseases of Veterinary Medicine from Porto University. These represent a small number of pathogenic organisms that were recovered from about 3500 mastitic milk analyses during a 6 year period (from January 2002 to December 2007). *Prototheca* were retrieved from milk of 47 cows with mastitis originating from 20 different dairy herds from the Northwest of Portugal, which was collected under sterile conditions. The affected cows had a history of persistently high somatic cell counts or with clinical mastitis that was not responsive to antibiotic intramammary treatment. To avoid confusing effects due to the therapy, only milk samples collected after the treatment safety period were analysed. Two *Prototheca* spp. reference strains were kindly provided by Prof. Hasegawa. These strains, *P. zopfii* (accession number AY940456) and *P. wickerhamii* (accession number AB244739), were isolated from a bovine mastitis milk and from a dog with Protothecosis respectively (Roesler et al., 2006; Tsuji et al., 2006). For diagnostic purposes, 40 μl aliquots of milk samples collected from individual quarters of the udder were streaked onto Columbia agar plates supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France). After 42–72 h at 37 °C, plates were examined for *Prototheca* growth, and any colonies resembling this alga were subcultured once on Sabouraud Dextrose Agar medium (Merck Laboratories, Darmstadt, Germany). After macroscopical and microscopical identification, *Prototheca* isolates were spread and grown on Sabouraud Dextrose Agar medium.

2.2. Biochemical characterization and data analyses

Auxonographical and biochemical analyses of all *Prototheca* isolates and reference strains were tested by using API 20C AUX (bioMerieux Portugal Lda) and two BBL systems, Enteric/Nonfermenter and Gram-Positive ID Kits (BBL Crystal, Becton Dickinson, Sparks, Maryland, USA). In total, 50 different carbon and nitrogen sources were checked for each strain with these kits. The system panels were incubated in a humid chamber at 37 °C for 48, 72, and 96 h and for a week period (when possible).

The analysis of the data was performed using the InforBio software (Tanaka et al., 2007) with the aim to identify new discriminative phenotypic characteristics for all the strains tested.

Species identification was performed by amplification of the 18S rDNA and direct sequencing of all isolates as described in a previous study (Marques et al., 2008).

3. Results

3.1. Isolation of algae

The recovery of the 47 *Prototheca* isolates from mastitic milk from 47 different dairy cows was achieved by usual microbiological methods. After streaking milk samples on Columbia agar supplemented with 5% sheep blood or on Sabouraud Dextrose agar and incubating for 48–72 h, grayish and white to cream colored small colonies of 1 mm or 1–2 mm size, respectively, with yeast-like appearance and odor were detected. No haemolysis was observed on the Columbia sheep blood agar plates. Four of the *Prototheca* isolates, obtained from cows of different herds, showed slower growth and paler and smoother colonies. The microscopical observation of *Prototheca* cells stained with lactophenol cotton blue showed a typical appearance with ovoid to globose sporangia with sporangiospores in several developmental stages (Fig. 1A). However, some differences in the internal organization of sporangiospores of the four isolates mentioned above were observed. Their sporangiospores were more consistently and regularly organized compared to those of the other isolates (Fig. 1B), suggesting that they could belong to a different *Prototheca* species. The morphological appearance observed for the *Prototheca* spp. was similar to previous description of other authors (Pore, 1998; DiPersio, 2001; Roesler et al., 2006).

3.2. Species identification; biochemical characterization and data analyses

The species determination of the *Prototheca* associated with bovine mastitis in this study was performed by molecular methods as

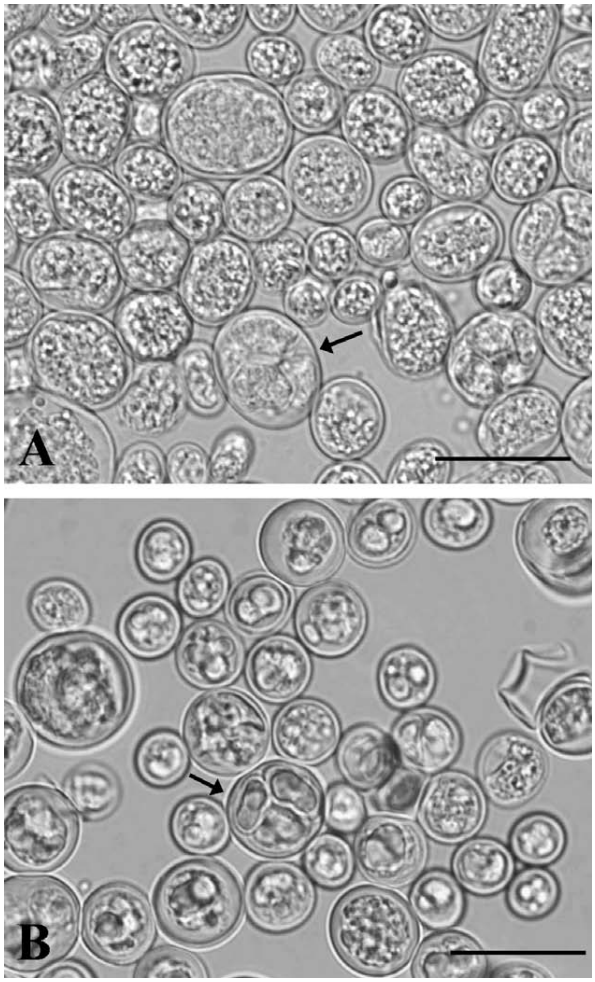

Fig. 1. Photomicrographs obtained by light microscopy of wet mount preparations of *Prototheca* spp. stained with lactophenol cotton blue. (A) Characteristic morphology of *P. zopfii* sporangiospores within sporangia (arrow). (B) Regularly organized sporangiospores of *P. blaschkeae* within the sporangia (arrow). Magnification, ×1000; bar, 20 μm.

previously described (Marques et al., 2008), being the majority of the isolates identified as *P. zopfii* genotype 2 and the four that presented different morphology as *P. blaschkeae*.

The biochemical profile determination for the assimilation of some important substrates, such as glycerol, galactose, trehalose and lysine, presented similar results as previous authors described (Roesler et al., 2003), with all *P. zopfii* assimilating glycerol and lysine and presenting variable assimilation of galactose, *P. blaschkeae* did not assimilate lysine neither glycerol and assimilated galactose. These two species did not assimilate trehalose, but *P. wickerhamii* reference strain was able to assimilate this carbohydrate as it is well known to be a "gold" nutrient for species differentiation (Pore, 1998; Lass-Florl and Mayr, 2007). However some nutrients demonstrated an enormous variability on the assimilations by the isolates. Therefore, the analysis of the data was performed using the InforBio software (Tanaka et al., 2007) to identify discriminative phenotypic characteristics for the strains.

A character dendogram was generated by calculating the distances between the strains with the neighbour-joining method. These distances were obtained from coded data using the Euclidean distance method (Tanaka et al., 2007). According to the differential character algorithm some discriminative characteristics were identified for the strains. It was possible to divide the dendogram into 9 clusters (Fig. 2), which were composed by 8 *P. zopfii* clusters and one cluster containing *P. blaschkeae* and *P. wickerhamii*. Their composition was defined as 7A (13 strains), 7B (4 strains), 7C (6 strains), 7D (8 strains), 7E (5 strains), 7F (5 strains), 7G (5 strains), 7H (2 strains) and 7I (1 strain). While *P. blaschkeae* strains and the reference strain of *P. wickerhamii* (P100) composed the 7E cluster, *P. zopfii* (P79) reference strain, was included in 7H cluster. When analysing the clusters discriminative characteristics, no special character was found to be most discriminative for each cluster, but rather a group of characters was involved. Thus, the *Prototheca* strains were organized into 9 different clusters because they demonstrated consistency of assimilation or not assimilation of some groups of substrates. For example, **7F** cluster presented always usage of Tetrazolium (TTC) substrate after 72 h of incubation, indicating that the cells presented a slower metabolism. However, **7D** cluster did not use TTC at all incubation times, and presented late assimilation of L-tryptophan-AMC (FTR). The **7A** cluster presented no assimilation of L-arginine-AMC (FAR) and FTR at later periods of incubation; the **7B** cluster presented no assimilation of FTR, 4MU-β-D-glucuronide (FGN) and 4MU-β-D-glucoside (FGS) at later periods,

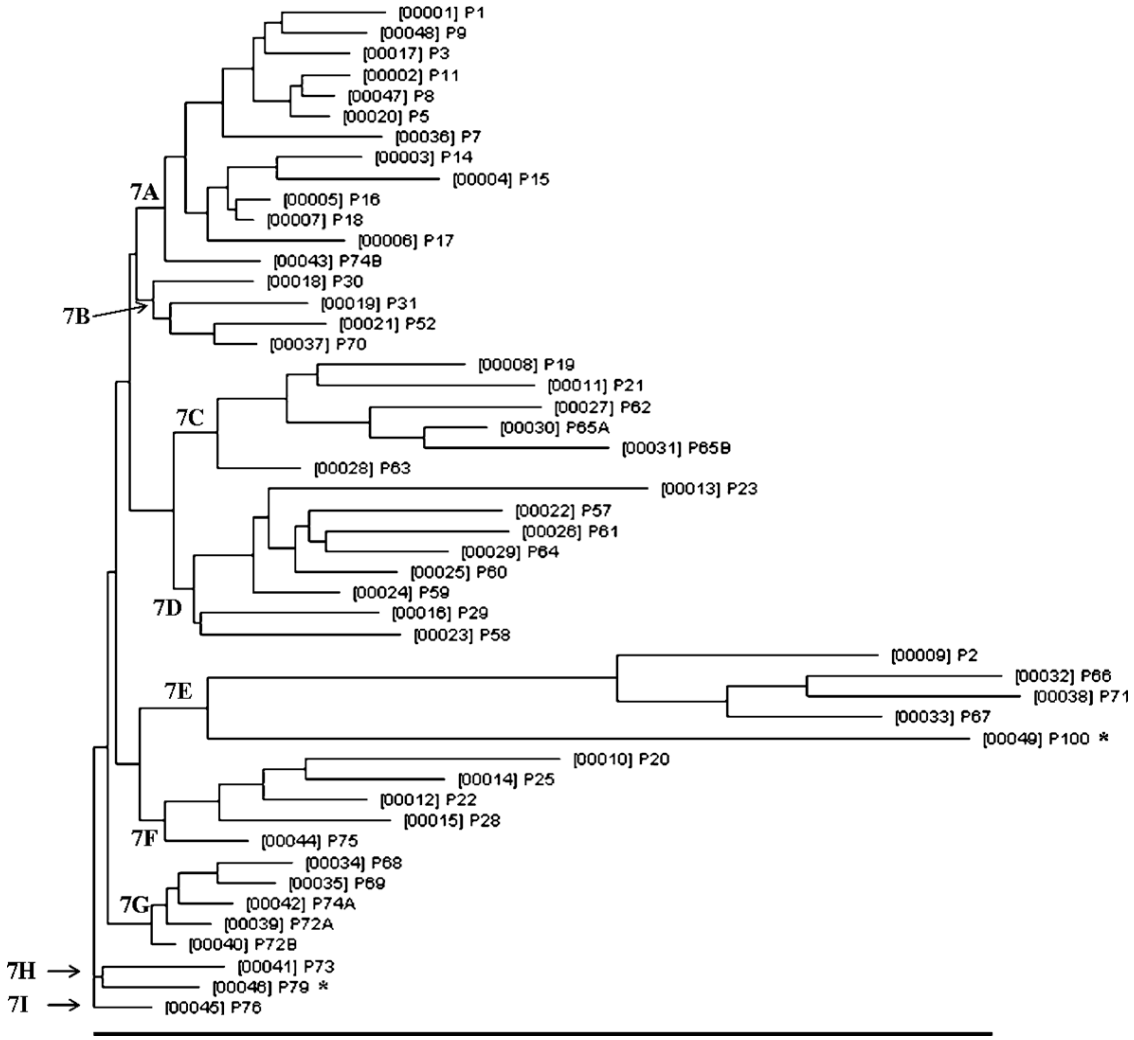

Fig. 2. Character dendogram generated by distances calculations between *Prototheca* strains with the neighbour-joining method. The 9 clusters were divided in 8 *P. zopfii* clusters (7A, 7B, 7C, 7D, 7F, 7G, 7H and 7I) and one cluster (7E) including *P. wickerhamii* and *P. blaschkeae*. Scale bar represents 17 units. Asterisks indicate reference strains used in this study.

Table 1
Assimilation of new discriminative phenotypic characteristics by *P. wickerhamii*, *P. zopfii* and *P. blaschkeae*.

	P. blaschkeae	*P. wickerhamii*	*P. zopfii*
CIT	–	+	Variable
PHC	–	+	–
AAR	–	Weak	–

Legend: CIT – citrate; PHC – p-n-p-phosphorycholine; AAR – p-n-p-α-arabinoside; (–) no assimilation and (+) assimilation.

and doubtful assimilation of D-rafinose (RAF) and D-maltose (MAL) at short incubation periods; **7C** cluster was characterized to present a doubtful assimilation of L-pyroglutamic acid-AMC (FPY) and 4MU-N-acetyl-β-D-glucosaminide (FGA) at all incubation periods. The **7G** cluster was characterized by the non assimilation of Galactose (GAL), FGN and FGS, and for the doubtful assimilation of MAL. Cluster **7H** presented a non assimilation of GAL, FGN and FGS, but a weak assimilation of Inositol (INO), Proline nitroanilide (PRO) and Proline & Leucine-p-nitroanilide (PLN), and assimilation of urea (URE). Cluster **7I** presented a weak assimilation of PLN, phenylalanine (PHE) and TTC, and did not assimilate RAF, MAL, GAL, FGS and FGN. The **7E** cluster, with *P. wickerhamii* and *P. blaschkeae*, was characterized for assimilation of GAL, non assimilation of RAF, FTR, FGN and FGS, and for a variable assimilation of p-n-p bis phosphate (BPH). This last cluster presented assimilation differences between *P. wickerhamii* reference strain (P100) and the *P. blaschkeae* strains (P2, P66, P67 and P71), such as, Lysine (LYS), Arginine (ARG), Glycerol (GLY), D-Trehalose (TRE), Citrate (CIT), p-n-p-phosphorycholine (PHC) and mannose (MNS) are assimilated by P100 and not by *P. blaschkeae*. Another characteristic difference found was the weak assimilation of p-n-p-α-arabinoside (AAR) and TTC by P100 and non assimilation by *P. blaschkeae*. Within these cluster analyses, it could be recognized that the same substrate, ARG, could present opposite results when using different assimilation kits (E/NF and GP).

InforBio software allowed the determination of new discriminative phenotypic characteristics for the three species analysed in this study, such as, CIT, PHC, AAR, and MNS (Table 1). For *P. wickerhamii* and *P. zopfii* the assimilation of PHC and MNS by the first were found to better discriminate these species, and for *P. wickerhamii* and *P. blaschkeae* the assimilation of CIT, PHC and MNS by the first were discriminative characters for these species. The weak assimilation of AAR by *P. wickerhamii* was also considered a discriminative feature to the other species.

4. Discussion

Prototheca is known to be a ubiquitous saprophyte that rarely causes pathology in humans and animals (Janosi et al., 2001; Lass-Florl and Mayr, 2007). Human Protothecosis has been classified into three clinical forms, namely, cutaneous lesions, olecranon bursitis, and disseminated or systemic infections (rare) (Leimann et al., 2004; Lass-Florl and Mayr, 2007). Among domestic animals, dogs have been affected with severe disseminated forms of Protothecosis involving several organs (Tsuji et al., 2006). In cattle, this pathogen is in general associated with chronic mastitis (Roesler et al., 2006), but has also been associated with systemic disease (Taniyama et al., 1994; Thompson et al., 2009). Therefore, algae characterization is extremely valuable, in dairy cow management health, in order to avoid the spread of the disease condition to other animals and also to improve the milk production and quality of a herd.

The usage of only morphologic characteristics and biochemical assimilation of different substrates for species identification is not appropriate in these times. Therefore, the identification of species should always combine several phenotypic and genotypic features to characterize *Prototheca* taxa. This study reports the determination of new phenotypic characteristics that facilitate a better characterization of *Prototheca* spp. The usage of InforBio (Tanaka et al., 2007) software allowed the identification of new discriminative phenotypic characteristics for the strains tested. The usage of phenotypic and genotypic methods allowed the characterization of 43 *P. zopfii* and four *P. blaschkeae* isolates, with genotyping being the key method for species identification. These facts are in agreement with previous reports on this issue (Roesler et al., 2006; Moller et al., 2007; Marques et al., 2008). The InforBio (Tanaka et al., 2007) software allowed the generation of a character dendogram by using distances calculated with the neighbour-joining method (Tanaka et al., 2007). The *P. zopfii* clusters presented as major discriminators a group of characters instead of a single character (Fig. 2). For example, although clusters 7G and 7H did not assimilate GAL, FGN and FGS they could be distinguished by the weak assimilation of INO, PRO and PLN and for the assimilation of URE of the later cluster. Despite this study accomplishing the purpose of finding discriminative characters for characterization of *Prototheca* spp., the practical results revealed a high variability within *P. zopfii*, which are in concordance with previous reports (Kerfin and Kessler, 1978; Huss et al., 1999; Ueno et al., 2002; Roesler et al., 2006; Marques et al., 2008) referring to the presence of heterogeneities in biochemical assimilation and fermentation patterns of *P. zopfii* strains. Although these findings suggest novelty, and since there is no species consistency on the assimilation of these subtracts some caution should be taken considering the weak or late assimilations as discriminatory, because weak positives can be due to small amounts of impurities and late assimilations can be due to subtract decomposition. The biochemical assimilation pattern for some well known discriminative substrates, glycerol, trehalose, lysine and arginine, allowed the characterization of the three different species analysed in this study, which are in agreement with previous studies (Pore, 1998; Roesler et al., 2006; Tsuji et al., 2006; Lass-Florl and Mayr, 2007). Nevertheless in this study some differences on the assimilation of arginine by the two BBL kits were determined. Although it was not possible to determine new discriminative characters between *P. blaschkeae* and *P. zopfii* the well known substrates utilization of lysine, arginine and glycerol by the last one (Roesler et al., 2006) was confirmed. InforBio software allowed the detection of additional discriminative characteristics for differentiation of *P. wickerhamii* and *P. zopfii* and *P. wickerhamii* and *P. blaschkeae* (Table 1). Therefore, *P. wickerhamii* for the first pair assimilated phosphorycholine, and for the second pair the assimilation of citrate and phosphorycholine by this species, were discriminative characters for them. In this study mannose was also identified as discriminatory between *P. wickerhamii* and the other species, however a previous study by Pore (Pore, 1985) reported that some of *P. wickerhamii* isolates did not use mannose as sole source of carbon for growth, suggesting that it can not be used to discriminate *Prototheca* species. Although *P. wickerhamii* presented a weak assimilation of arabinoside this substrate was considered to have a good discriminative power with the other species. Thus, these new findings represent additional progress into the phenotypic characterization of *Prototheca*. This report shows the improvement provided by the usage of bioinformatic tools for the analysis of several features that are important for a better characterization of *Prototheca* spp. Although the phenotypic characteristics alone are not sufficient for deriving phylogenetic relationships, it is possible to deduce their phylogenetic significance by comparing these data with the molecular derived phylogeny.

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Bovine Mastitis Associated with *Prototheca blaschkeae*▽

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Bovine mastitis is an important and complex disease responsible for economic losses in the dairy industry. Biotype II strains of the green alga *Prototheca zopfii* can be involved, most often resulting in chronic mastitis of difficult treatment associated with reduced milk production. This type of infection is rare, but the number of reported cases is increasing worldwide. In order to determine the kind of species involved in mastitis by *Prototheca* in northwest Portugal, 41 *Prototheca* isolates were genetically characterized. The algae are part of *Prototheca* isolates that were collected during a 6-year period, isolated from the milk of 41 dairy cows in a total of 22 herds with a history of increasing somatic cell counts, mild clinical signs of udder infection, and unsuccessful response to the usual therapy. PCR amplification of the 18S ribosomal DNA (rDNA), amplified rDNA restriction analysis, and phylogenetic analyses of the 18S rDNA sequences were performed. Thirty-seven isolates were identified as *P. zopfii* var. *hydrocarbonea* and four as *Prototheca blaschkeae*. These data suggest a high incidence of *P. zopfii* var. *hydrocarbonea* mastitis in the region and demonstrate for the first time the involvement of *P. blaschkeae* with bovine mammary gland infections.

Green algae of the genus *Prototheca*, and more rarely *Chlorella*, are the only known plant-like organisms that cause infectious diseases in humans and animals (6, 11–13). The genus *Prototheca* consists of microscopical, unicellular, achlorophyllic algae with asexual reproduction by autosporulation with variable numbers of autospores (5, 10). These algae are ubiquitous and saprophytic, but some species may turn into unusual opportunists causing pathology when the host immunological defenses are impaired (16, 18, 21) or when predisposing factors occur, such as, in the case of dairy cows, poor animal care and poor milking hygiene (8, 11, 18). The incidence of bovine mastitis associated with *Prototheca* infections is steadily increasing and gaining more and more economic and public health importance (18). *Prototheca* is included in the class Trebouxiophyceae (14) and is closely related to green algae of the genus *Chlorella* (13, 23), which are among the best-studied unicellular green algae (7). Within the last decade, *Prototheca* taxonomy has developed and five species are currently assigned to the genus, namely *Prototheca zopfii*, *P. wickerhamii*, *P. stagnora*, *P. ulmea*, and most recently, *P. blaschkeae* (19). The validation of another species, *Prototheca moriformis* (1, 17), has been questioned by molecular studies (19, 25). Until recently, the only species known to cause infectious diseases in humans and animals were *P. wickerhamii* and *P. zopfii*, respectively (4, 20, 28). The new species *P. blaschkeae* was identified in 2006 from a previous case of human onychomycosis (19). This species was previously defined as biotype III of *P. zopfii*, which, together with biotype I, was reported to be nonpathogenic, while biotype II has been associated with bovine mastitis (20). Biotype classification was based on phenotypic characteristics, as well as auxonographical and biochemical analyses (3, 20). Serological analyses revealed differences in the pattern of immunogenic structures between the biotypes (20). Further phylogenetic studies of 18S ribosomal DNA (rDNA) sequences were able to determine discriminatory molecular characteristics to define the new species *P. blaschkeae* and reclassify the *P. zopfii* biotypes into two genotypes (19).

The present study aimed to elucidate the epidemiology of bovine mastitis by molecular characterization of *Prototheca* isolates obtained from 41 dairy cows of 22 herds in Portugal. We report the predominant occurrence of *Prototheca zopfii* var. *hydrocarbonea* in milk of cows with mastitis associated with *Prototheca* and for the first time the association of *P. blaschkeae* with bovine mastitis.

MATERIALS AND METHODS

Isolation of algae. *Prototheca* isolates in this study belong to a major collection compiling several milk pathogens that belong to the Laboratory of Infectious Diseases of the Veterinary Medicine School of Porto University. These represent a small number of pathogenic organisms that were recovered from about 3,500 mastitic milk analyses during a 6-year period (from January 2002 to December 2007). *Prototheca* cells were retrieved from milk of 41 cows with mastitis originating from 22 different dairy herds from northwest Portugal, which was collected under sterile conditions. The affected cows had a history of persistently high somatic cell counts or clinical mastitis that was not responsive to intramammary antibiotic treatment. To avoid confounding effects due to the therapy, only milk samples collected after the treatment safety period were analyzed. For diagnostic purposes, 40-μl aliquots of milk samples collected from individual quarters of the udder were streaked onto Columbia agar plates supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France). After 42 to 72 h at

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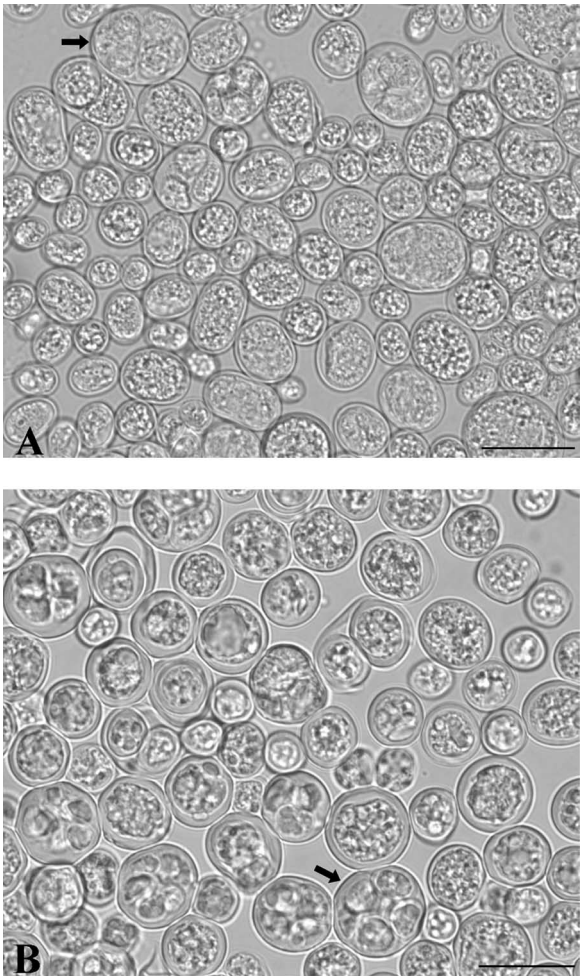

FIG. 1. Photomicrographs obtained by light microscopy of wet mount preparations of *Prototheca* spp. stained with lactophenol cotton blue. (A) Characteristic morphology of *P. zopfii* sporangiospores within sporangia (arrow). (B) Regularly organized sporangiospores of *P. blaschkeae* within the sporangia (arrow). Magnification, ×1,000. Bar, 20 μm.

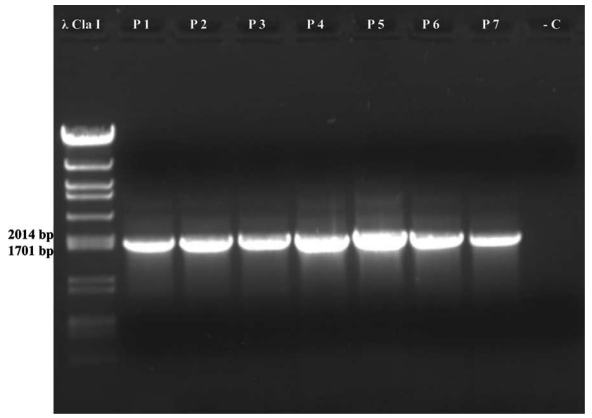

FIG. 2. 18S rDNA amplification of some *Prototheca* isolates (P1 to P7) with eukaryote-specific PCR amplification primers. All PCR fragments have a size of approximately 1,800 bp. Lane λ ClaI, molecular weight standard; lane −C, negative control without DNA.

37°C, plates were examined for *Prototheca* growth and any colonies resembling this alga were subcultured once on Sabouraud dextrose agar medium (Merck Laboratories, Darmstadt, Germany). After macroscopical and microscopical identification, *Prototheca* isolates were spread and grown on Sabouraud dextrose agar medium.

Preparation of genomic DNA. Algal cultures were grown on Sabouraud dextrose agar for 48 h at 37°C. Cells were harvested by centrifugation (1,600 × *g*, 10 min) and mechanically ground with glass beads and extraction buffer (200 mM Tris-HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate) in 1.5-ml Eppendorf tubes in a cell mill (Qiagen tissue lyser) for 10 min at 30 Hz. The efficiency of cell disruption was controlled by microscopical observation. Subsequently, 200 μl of chloroform was added and the suspension was shaken for 1 min. After centrifugation (21,000 × *g*, 5 min), the supernatant was transferred to a new tube and mixed with 200 μl of isopropanol for DNA precipitation. The DNA was pelleted by centrifugation (21,000 × *g*, 5 min) and washed with 70% ethanol. The pellet was air dried and resuspended in a maximum of 50 μl of Milli-Q sterilized water for further use.

rDNA amplification and amplified rDNA restriction analysis. For amplification of the 18S rDNA, the conserved eukaryote-specific primers previously designed by Huss et al. (7) (forward primer, 5′ WACCTGGTTGATCCTGCCAGT 3′; reverse primer, 5′ GATCCTTCYGCAGGTTCACCTAC 3′) were used. Thirty-five cycles were run in a Biometra T3000 thermal cycler with 60 s of denaturation at 95°C, 60 s of annealing at 54°C, and 150 s of extension at 72°C. The amplification products were analyzed on a 0.8% (wt/vol) agarose gel after staining with ethidium bromide. The amplified rDNA was submitted to restriction analysis with HaeIII (NEB, Inc., Frankfurt, Germany) and analyzed on a 1.8% (wt/vol) agarose gel after staining with ethidium bromide. Several of the amplified rDNAs were purified with a PCR purification kit (Qiagen, Inc., Hilden, Germany) and directly sequenced using the following primers: 300 F (5′ AGG GTTCGATTCCGGAG 3′), 1055 F (5′ GGTGGTGCATGGCCG 3′), 536 R (5′ GWATTACCGCGGCKGCTG 3′), and 1200 R (5′ GGGCATCACAGACCTG 3′) (7). Nucleotide sequence determination was carried out by Macrogen, Ltd. (Korea).

Phylogenetic analyses. The 18S rDNA sequences determined in this study were manually aligned on a MicroVAX computer with the sequence editor program distributed by G. Olsen (15). For the phylogenetic analyses, the sequences were aligned with sequences of representative trebouxiophycean green algae extracted from a larger alignment and with two chlorophycean algae that were used as an outgroup. To improve the alignment of the data set, secondary structure models were constructed according to the model provided by Wuyts et al. (27). Highly variable regions that could not be aligned unambiguously for all sequences were excluded from the analyses together with PCR primer binding regions, resulting in a total of 1,782 positions. Phylogenetic trees were inferred from the aligned sequence data by the neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) method using PAUP* 4.0 (22). One

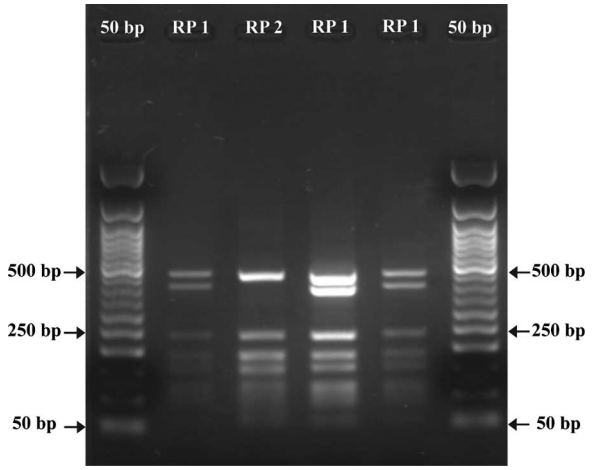

FIG. 3. Restriction patterns of amplified 18S rDNA fragments of four *Prototheca* isolates. PCR fragments were digested with HaeIII, yielding two different patterns, RP 1 and RP 2. RP 2 lacks the 400-bp fragment observed in RP 1. Lanes 50 bp, molecular weight standard (50-bp ladder).

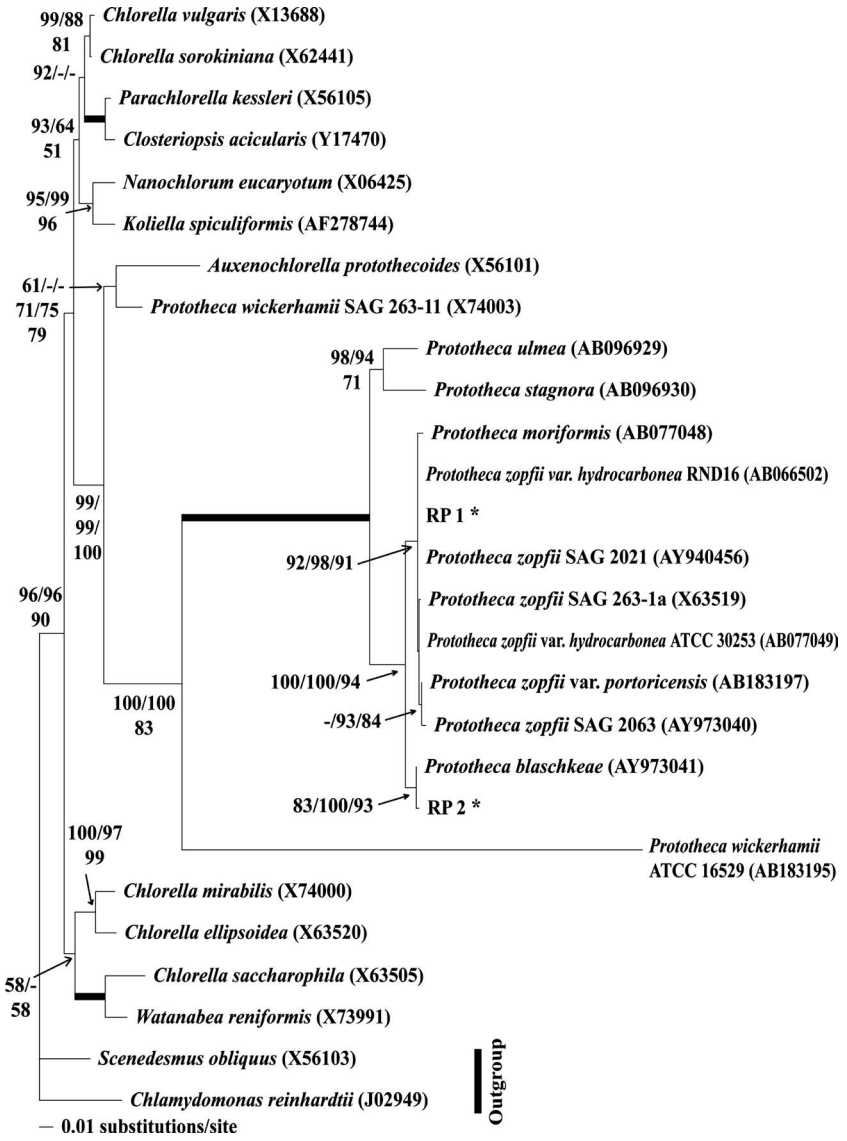

FIG. 4. Phylogenetic tree inferred from complete or almost complete 18S rRNA gene sequences of trebouxiophycean green algae showing species affiliation of *Prototheca* isolates representing RP 1 and RP 2 (see Fig. 3). The tree topology is derived from an ML analysis. Bootstrap values are shown at the internal nodes for each 1,000 resamplings of NJ and MP and 100 resamplings for ML, if values are higher than 50%. Thick lines indicate 100% bootstrap support for all three methods. Asterisks indicate sequences determined in this study. Branch lengths correspond to evolutionary distances. Bar, 0.01 nucleotide substitution per site.

thousand bootstrap replicates each were carried out for NJ and MP, as well as 100 replicates for ML.

Nucleotide sequence accession numbers. The 18S rRNA gene sequences determined in this study have been deposited in GenBank under accession no. EU439262 and EU439263.

RESULTS

The recovery of the 41 *Prototheca* isolates from mastitic milk from 41 different dairy cows was achieved by usual microbiological methods. After milk samples were streaked on Columbia agar supplemented with 5% sheep blood or on Sabouraud dextrose agar and incubated for 48 to 72 h, grayish and white to cream-colored small colonies of 1 mm or 1 to 2 mm, respectively, with yeast-like appearance and odor were detected. No hemolysis was observed on the Columbia sheep blood agar plates. Four of the *Prototheca* isolates, obtained from cows of different herds, showed slower growth and paler and smoother colonies. The microscopical observation of *Prototheca* cells stained with lactophenol cotton blue showed a typical appearance with ovoid to globose sporangia with sporangiospores in several developmental stages (Fig. 1A). However, some differences in the internal organizations of sporangiospores of the four isolates mentioned above were observed. Their sporangiospores were more consistently and regularly organized than those of the other isolates (Fig. 1B), suggesting that they could belong to a different *Prototheca* species. The morphological appearance observed for the *Prototheca* spp. was in concordance with the previous description of Pore (16) and DiPersio (5) for *P. zopfii* and of Roesler et al. (19) for *P. blaschkeae*.

To determine the species of *Prototheca* associated with bovine mastitis in this study, the 18S rRNA genes of all isolates were amplified (Fig. 2). For each isolate analyzed, a PCR fragment of about 1,800 bp was detected as expected, since the sequence length of *P. zopfii* 18S rDNA is about this size (24). Each amplified rDNA was submitted to restriction analysis with HaeIII, resulting in two different restriction patterns (Fig. 3). For 37 isolates, a restriction pattern defined as restriction pattern 1 (RP 1) was identified. The four morphologically distinct isolates showed a different restriction pattern referred to as RP 2, which could be clearly distinguished from RP 1 by the lack of a 400-bp fragment (Fig. 3). The 18S rRNA genes from eight isolates representing restriction pattern RP 1 and four with RP 2 were completely sequenced. All sequences with RP 1 had a length of 1,807 bp and were identical to each other and also to the published sequence of *Prototheca zopfii* var. *hydrocarbonea* strain RND16 (GenBank accession no. AB066502). The four isolates displaying RP 2 were also identical among themselves, had a length of 1,815 bp, and showed 99.8% identity (with three differences) within the 1,449 bp available for the 18S rDNA sequence of *P. blaschkeae* SAG 2064 (AY973041). The three differences were found in variable regions of the 18S rRNA. Similarity between the two types of sequences that displayed different restriction patterns was only 98.0%, corresponding to 40 differences including indels (insertions/deletions). Phylogenetic analyses with the NJ, MP, and ML method confirmed that most of our isolates belong to the *P. zopfii* clade, specifically to *P. zopfii* var. *hydrocarbonea* and *P. zopfii* SAG 2021, while there is high bootstrap support that four isolates are more closely related to *P. blaschkeae* (Fig. 4).

DISCUSSION

Prototheca species are known to be widely dispersed within dairy farmlands. The occurrence of mastitis due to these algae usually takes place during periods of warm weather with high rainfall (8, 12, 16), reflecting poor management and hygiene combined with insufficient premilking cleaning and disinfection of the teats. Generally, infections are maintained in a herd by clinically healthy shedders (8, 18). The objective of this work was to determine the species of *Prototheca* that are associated with bovine mastitis in the northwest of Portugal. The results of morphological characterization and of the molecular analyses of the 18S rRNA genes demonstrated that most of our isolates, with RP 1, belong to biotype II of *P. zopfii* represented by strain SAG 2021 in Fig. 4, as suggested by Roesler et al. (20). This was not unexpected, as all algae associated with bovine mastitis have been identified as *P. zopfii* biotype II (2, 13, 18) and are generally considered to be its causative agent (13). Surprisingly, these isolates share complete 18S rDNA sequence identity with *P. zopfii* var. *hydrocarbonea* RND16, a variety of *P. zopfii* which was first described in 2002 by Ueno et al. (26). This thermotolerant strain was isolated from a hot spring and was never associated with bovine mastitis. It would be interesting to know if this strain is potentially infectious or not. Differences in infectivity combined with the knowledge of characteristic physiological differences could be used to specify prerequisites for the pathogenicity of biotype II strains. In this context, determination of fermentation patterns of our isolates might reveal essential differences, as heterogeneities for strains of a single species of *P. zopfii* are well known (9).

In a recent study (19), biotype I and II strains were reclassified as two genotypes of *P. zopfii* by 18S rRNA gene sequence analysis and determination of cellular fatty acids, and biotype III strains were defined as a new species, *P. blaschkeae*. Interestingly, four of our isolates, those with RP 2, share much more sequence similarity with *P. blaschkeae* than with *P. zopfii* (99.8% compared to 98%). As *P. blaschkeae* so far had been isolated only from a case of human onychomycosis (19), the results of our study demonstrate for the first time the involvement of *P. blaschkeae* in the etiology of bovine mastitis. To quickly discern between the two species, we also want to point out the important additional information provided by amplified rDNA restriction analysis, which allows fast screening of large numbers of isolates for the detection of different infectious species.

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Preliminary studies on the nuclear internal transcribed spacer and the plastid ribosomal RNA operon for phylogenetic analyses of pathogenic *Prototheca* strains

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Abstract

The increasing incidence of rare mastitic pathogens has urged the implementation of fast and efficient diagnostic and control measurements. *Prototheca* algae are known to be associated with diseases in humans and animals. These algae are resistant and are widely distributed on variable environments. We report a preliminary study on the amplification and sequencing of the nuclear internal transcribed spacer (ITS) and almost complete plastid ribosomal RNA operons (16S rDNA, intergenic spacer [IGS] including two tRNAs, and partial 23S rDNA) of *Prototheca* strains recovered from bovine mastitis.

Keywords

Prototheca spp.; internal transcribed spacer (ITS); 16S rDNA; intergenic spacer (IGS)

Introduction

Members of the genus *Prototheca* are ubiquitous non-photosynthetic algae that are associated with pathologies in humans and animals (1-4). The only pathogenic species of this genus are *P. zopfii*, *P. wickerhamii* and *P. blaschkeae* (5-9). *P. zopfii* and *P. blaschkeae* have been associated with clinical or subclinical cases of bovine mastitis (1, 4, 10, 11). At the present time this pathology is recognized as endemic worldwide and is considered a public health problem (12, 13). These algae are unicellular eukaryotes that reproduce asexually by endosporulation with the production of several endospores inside a mother cell or sporangium (3, 12). Generally, *Prototheca* identification is performed by phenotypic and genotypic methods. The genotypic methodologies, 18S ribosomal deoxyribonucleic acid (rDNA) and 28S rDNA amplification, genotype specific polymerase chain reaction (PCR) and also real time PCR, can easily identify *Prototheca* spp., with the two latter being able to additionally identify the different genotypes of *P. zopfii* (1, 4, 14-18). Phylogenetic relationships of these algae are inferred from the small subunit (SSU) and large subunit (LSU) rDNA sequence comparisons (14, 19), and further strains should be investigated in order to understand the genetic diversity within and between *Prototheca* species. A previous study conducted in our laboratory determined that 18S rDNA amplification of *P. zopfii* and *P. blaschkeae* strains showed identical sequences within each species and that restriction analyses with *Hae*III were able to quickly discern between them (1). When comparing 18S rDNA sequences the similarity between the two species was 98.0%, corresponding to 40 differences including insertions and deletions. As it is well know that internal transcribed spacer (ITS) regions are characterized by a lower

degree of evolutionary conservation than 18S and 28S rRNA genes, exhibiting greater interspecific differences that are useful for the differentiation of strains, the present study intended to determine genotypic variability within *P. zopfii* and *P. blaschkeae* strains by amplification and sequencing of ITS. This region is widely used, together with other ribosomal genes, in fungal and yeast identification and phylogenetic studies (20-23). Therefore, the application of these techniques could be of great value for pathogenic algae as well. In the course of the experimental work, *P. zopfii* and *P. blaschkeae* sequences of plastid 16S rDNA, intergenic spacer (IGS) and partial 23S rDNA were also amplified and analysed. These amplifications were done because of general difficulties to amplify the nuclear ITS region of *Prototheca* strains and because the plastid 16S rDNA has been sequenced for numerous photosynthetic and non-photosynthetic algal species and found to be not so conserved as the nuclear 18S rDNA (24).

Materials and methods

Prototheca isolates used in this preliminary study belong to a major collection of several milk pathogens retrieved from milk of cows with mastitis originating from different dairy herds from the Northwest of Portugal. Forty two *Prototheca* isolates were identified by phenotypic analysis and molecular characterization of the 18S rDNA as previously described (1). Thirty seven isolates were identified as *P. zopfii* genotype 2 (GenBank accession number EU439263) and five as *P. blaschkeae* (EU439262). *Prototheca* isolates were spread and grown on Sabouraud Dextrose Agar medium (Merck Laboratories, Darmstadt, Germany). The related alga *Chlorella vulgaris* (AB001684) was used as a control and reference.

Genomic DNA preparation was performed exactly as described in a previous study (1). For amplification of the ITS region several sets of primers were used. First, the conserved eukaryote-specific primers 1400 forward (F) – 5' TGY ACA CAC CGC CCG TC 3' – (25, 26); and ITS-3´reverse (R) – 5' CCT CAC GGT ACT TGT TC 3' – (27) were used. Amplification cycles were similar to those used for 18S rDNA (1), differing only on the extension time, 120 seconds. The amplification products were analysed on a 0.8% (wt/vol) agarose gel after staining with ethidium bromide. Since some of the amplifications produced two bands, these were purified with the QIAquick® PCR purification kit (Qiagen, Crawley, UK) and cloned into Topo® XL PCR Cloning Kit (Invitrogen, Carlsbad, USA). The inserts from individual colonies were amplified using the ITS region primers. After colony PCR the products were sequenced with T7 and M13R universal primers. As some sequence displayed similarities to the *Prototheca* plastid intergenic spacer region between

16S and 23S rDNA, several other universal primers for the ITS region and also newly designed primers were tested.

Primer sequences that produced amplification products are the following: ITSF – 5' GCT GGT GAA GCG CTC GGA TTG G 3' –; ITSR – 5' GCT GCA TTC CCA AGC AAC CCG A 3' –; 1400Fm – 5' CCT TTG TAC ACA CCG CCC GTC 3' –; NLR 204/21 – 5' ATA TGC TTA ART TCA GCG GGT 3' –. These 4 primers were used in different combinations also with the 1400F and ITS-3'R primers and tested at several annealing temperatures. If more than one band was obtained after amplification, the bands were separately cloned. Cloning and sequencing of the amplified products were performed as previously. Direct sequencing of single amplification products from some strains was possible after use of the following primers: 1400Fm; ITSR; P1-ITS 2mR – 5' GCT GCG TTC TTC ATC GAT 3' – (27); P1-28 SR – 5' TTC GCT ATC GGT CTC CCG 3' – (28); P1-ITS 3mF – 5' CGA TGA AGA ACG CAG CGA 3' – (27). Nucleotide sequence determination was carried out by GATC-Biotech (Germany).

Since most of the primer pairs amplified the plastid IGS region instead of the nuclear ITS region, we decided to amplify the 16S rDNA, IGS and part of the 23S rDNA of some isolates. 16S rDNA was amplified with the primer pair 16S 5' – 5' GAG TTY GAT CCT GGC TCA GGA 3' – and 16S 3' – 5' TCC AGC CGC ACC TTC CAG TA 3' –. Direct sequencing was performed by using the following primers: 16S 21F – 5' CTG GCT CAG GAT GAA C 3' –; 16S 1304R – 5' GCG ATT ACT AGC GAT TC 3' –; and 16S 495R – 5' ACC GCG GCT GCT GGC AC 3' –. The intergenic spacer and part of the 23S rDNA were accidentally amplified with the presumed ITS-specific primers 1400Fm and ITS-3´R, because we found out later, that the 1400Fm primer binds also with only three mismatches to the 3' end of the 16S rDNA, and the ITS-3'R primer is completely homologous to the 23S rDNA of *Prototheca*. Thus, the sequences of these regions were obtained instead the desired ITS sequences.

Results

ITS amplification of *Prototheca* isolates with the primer pair 1400F and ITS-3´R retrieved bands of the expected size only in few cases, but bands of approximately 900 bp or two bands (1,300 and 900 or 800 bp) in other cases (Fig. 1). ITSF, ITSR, 1400Fm and NLR 204/21 primers together or combined with the former primer pair also produced two or even more bands. Direct and indirect sequencing of the different amplification products retrieved nuclear ITS of only three *P. zopfii* isolates with some differences between them. No ITS sequences were obtained for *P. blaschkeae*.

Specific amplification of the nuclear ITS region for other *Prototheca* isolates constantly failed. Instead, the amplified products showed similarities to the intergenic spacer of the plastid rRNA operon. In fact, the obtained sequences covered the region from the 3'end of the plastid 16S rDNA, IGS including tRNAs for isoleucine and alanine, and part of the 23S rDNA. In addition, we used plastid-specific 16S rDNA primers to get the complete 16S rDNA as well (Fig. 2). By this, sequences for the almost complete plastid rRNA operon were successfully obtained for eight *Prototheca* isolates. Three *P. zopfii* isolates had identical sequences, and the remaining five sequences from *P. blaschkeae* isolates differed at only one position.

Discussion

The retrieved sequences of the nuclear ITS region and of the complete plastid 16S rDNA, intergenic spacer and partial 23S rDNA plastids were not sufficient to increase the resolution of intraspecific relationships in *Prototheca*, mainly due to the small number of determined sequences. Nevertheless, the sequences looked promising, because they displayed much more variability than observed for the 18S rRNA.

Within the ITS sequences of the three *P. zopfii* isolates, about 39 differences were found with similarities ranging from 82 to 93%. This demonstrates variability between different strains of *P. zopfii* as expected for this region. However, more sequences of *P. zopfii* and *P. blaschkeae* strains are needed for detailed intra- and interspecific comparisons of both species.

The plastid sequences obtained for *P. zopfii* and *P. blaschkeae* isolates showed only 82.8% similarity between each other. Similarity of the complete 16S rDNA alone was 86.8%, which was considerably lower than that of the respective 18S rDNA (98.0%). This indicates that the plastid 16S rDNA is more variable than the nuclear 18S rDNA and may be used for phylogenetic analyses of these species. The plastid intergenic spacer of both species was found to be much shorter than those of *P. wickerhamii* and especially of the related alga *C. vulgaris* used in the alignments, whereas the order of tRNAs was the same (please refer to addendum). As expected, the IGS region was even more diverse than the 16S rDNA.

The preferred amplification of plastid sequences from the rRNA operon instead of the nuclear ITS region may at least in part be explained by the high similarity of the respective binding sites in 18S/16S and 28S/23S rDNAs. Nevertheless, the homologous primer binding sites on the nuclear genome should have been preferred over the partly heterologous sites on the plastid genome. Perhaps the 3' end of the *Prototheca* 18S rDNA

represents a conformational barrier preventing correct primer annealing and subsequent amplification.

In general, plastid 16S sequences are not as conserved as the 18S rRNA sequences, and both IGS and ITS are more variable than these (24, 28-30). Therefore, if we were able to amplify more isolates for these regions and also for the 23S rDNA, the higher variability could be used for a better phylogenetic resolution of our strains.

This preliminary study was the first to demonstrate the variability of nuclear ITS and plastid rDNA sequences between and within *P. zopfii* and *P. blaschkeae* strains. Solving the ITS amplification problem and subsequent sequencing of this region together with the plastid rRNA operon of all our isolates should be of great value for *Prototheca* spp. population genetics and epidemiology.

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Figures

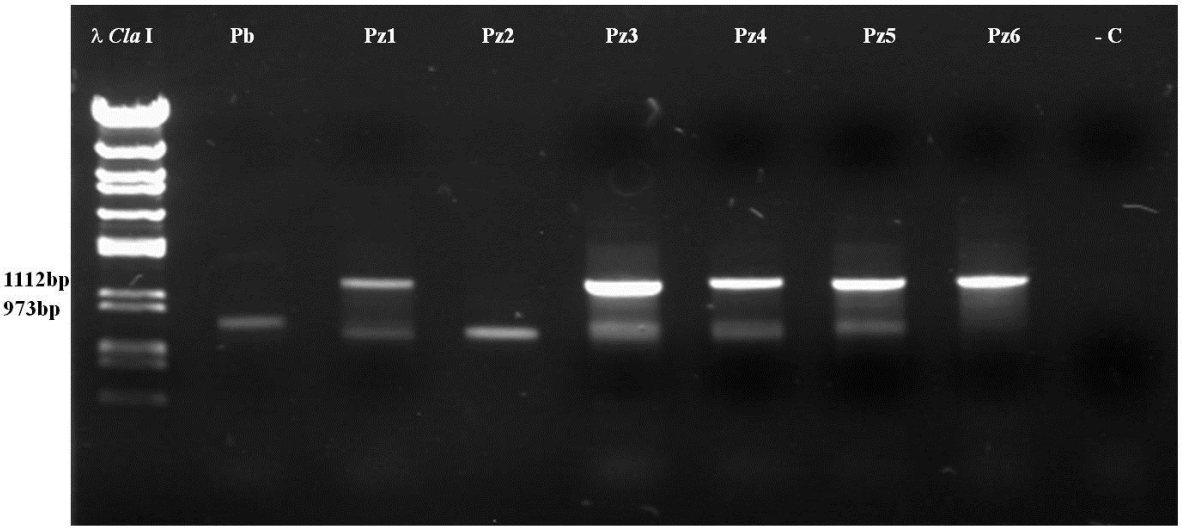

Figure 1. ITS amplification of some *P. zopfii* (Pz1-6) and *P. blaschkeae* (Pb) isolates with ITS primers (1400F and ITS-3´R). All PCR fragments should have a size of approximately 1,300bp, however different sizes were found and in some isolates two bands were amplified. Lane λ *Cla*I – molecular weight standard, -C, negative control without DNA.

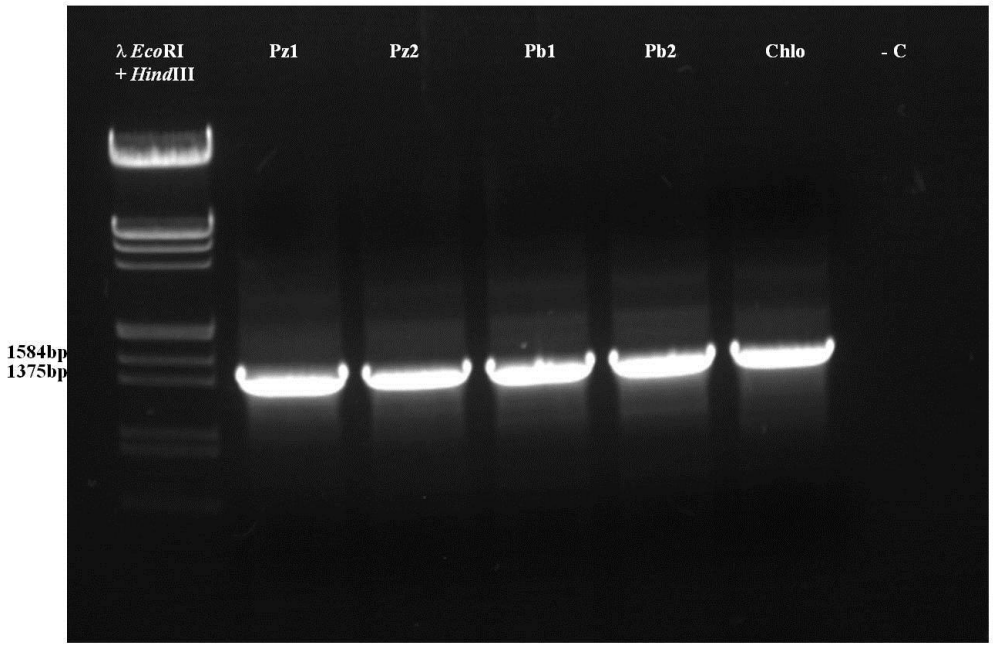

Figure 2. 16S rDNA amplification of some *P. zopfii* (Pz1-2), *P. blaschkeae* (Pb1-2) isolates and *Chlorella vulgaris* (Chlo) with 16S universal primers. All PCR fragments have a size close to the expected size of about 1,500 bp. Lane λ *Eco*RI + *Hind*III – molecular weight standard, -C, negative control without DNA.

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Case Report

Algaemia in a dairy cow by *Prototheca blaschkeae*

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We describe the first known case of an algaemia by *Prototheca blaschkeae* in a dairy cow, which occurred after a chronic episode of mastitis caused by this pathogen. The organism was isolated from milk, joint fluid and blood samples, and microbiologic and molecular methods were performed to obtain a definitive identification of the algae. The affected cow was culled only after confirmation of a systemic infection by *Prototheca*.

Keywords algaemia, bovine mastitis, *Prototheca blaschkeae*

Introduction

The only known plant-like organisms that cause infectious diseases in humans and animals are green algae of the genus *Prototheca* and *Chlorella* [1–4]. Members of the genus *Prototheca* are ubiquitous saprophytes, and can be isolated from a variety of environmental sources such as plants, soil, sludge, drinking and marine water, swimming pools, feces of domestic and wild animals, barn floors and meat products [5]. Some species may be opportunistic pathogens, causing pathology in immunocompromised hosts [6–8]. Similarly, they act as pathogens when there are predisposing factors, such as, in the case of dairy cows, poor animal care and poor milking hygiene are present [3,7,9]. Protothecal mastitis has been reported sporadically in dairy herds of Europe, Brazil and the United States [10–13]. These algae do not respond to routine mastitis therapy and the only control method to date has been the elimination of the infected animals [14]. Bovine mastitis caused by members of this genus has been almost exclusively associated with *Prototheca zopfii* genotype 2 [2]. Until recently the only species known to cause infectious diseases in humans and animals were *P. wickerhamii* and *P. zopfii*, respectively [15–17]. The new species *P. blaschkeae*, was identified from a human case of onychomycosis [18], was defined as biotype III of *P. zopfii* that together with biotype I was reported to be non-pathogenic [15]. Biotype classification was based on phenotypic characteristics, as well as auxonographical and biochemical analyses [15,19]. Recently, mastitis caused by *P. blaschkeae* has been reported in cattle [20] but never as an agent of systemic infections. We report the first known case of algaemia by *P. blaschkeae* in a dairy cow, after a chronic episode of mastitis.

Case report

A 4½-year-old Holstein cow was diagnosed with chronic mastitis in the udder's front right quarter. The culturing of milk samples from all udder quarters resulted in the isolation of *Prototheca* spp. in pure culture and was identified based on its growth and morphological features. Because mastitis by *Prototheca* is a chronic infection with no successful treatment, the owner was recommended to remove the cow from the herd. However, the owner decided to keep it in the farm because the cow was 3 months pregnant. Two months

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after the first milk analysis, the infection had extended to the right back quarter as determined by testing milk from all quarters. The cow was dried off and delivered a viable calf six months after our first diagnosis of mastitis. The animal was lethargic and anorectic for about 8 weeks after parturition.

A generalized mastitis by *Prototheca* spp. was later diagnosed (6 months after calving) in all quarters of the mammary gland. Somatic cell counts showed a steady increase since parturition up to the time the animal was culled (248×10^3, 577×10^3, 446×10^3, 778×10^3, 1.843×10^3 and 1.248×10^3 measured on a monthly basis). Meanwhile, the cow developed abscesses, first in both back leg joints (Fig. 1) then in the perinea region and some of these abscesses ruptured over time, and the animal was subsequently treated for the piodermatitis. At the time of our last visit to the farm, the cow presented an accelerated respiratory frequency rate but with no fever and fully recovered from her previous lethargic and anorectic condition. The following samples were collected; milk, whole blood (with and without EDTA) and joint fluids which appeared bloody and purulent.

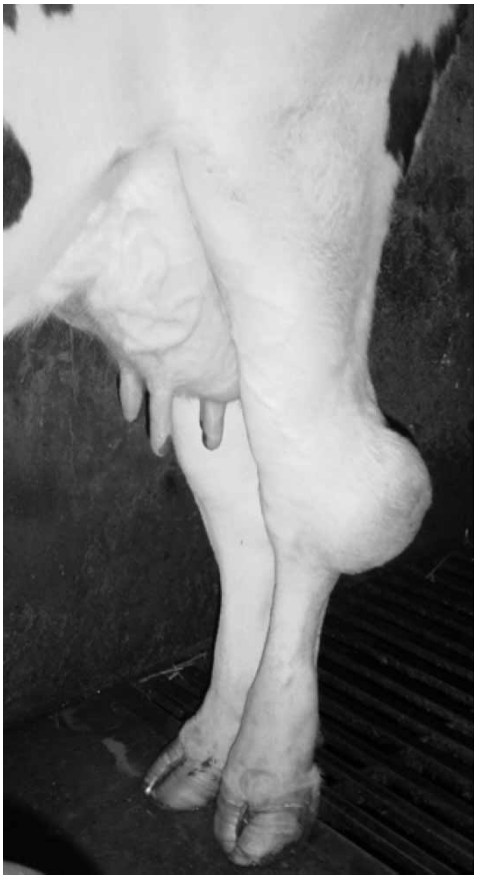

Fig. 1 Rear part of the cow with algaemia caused by *Prototheca blaschkeae*. An abscess in the left back leg joint can be observed. Atrophy of the udder is also noteworthy.

The laboratory hematology findings revealed a monochromic and monocytic anemia represented by values below normal in red blood cells, hemoglobin and hematocrit. Cytology of all samples, stained by Gram and Diff-Quick, showed yeast like organisms in variable amounts under light microscopy. In order to increase the white blood cell fraction from the blood samples, mononuclear cells were separated by Lymphoprep (Axis-Shield PoC AS, Norway) gradient and washed. Stained smears of this preparation permitted an easier visualization of extracellular and intracellular algae bodies, with the evidence of protothecal bodies in wet-mount preparations (Fig. 2). The laboratory analysis consisted of inoculating samples on Columbia agar plates supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) and Sabouraud dextrose agar plates (Merck Laboratories, Darmstadt, Germany) and incubation at 37°C for up to 96 h. After 96 h, the plates were kept at room temperature for up to 15 days. Following the incubation period, pinpoint colonies were observed in both Columbia and Sabouraud dextrose agar cultures, first from milk samples and later from specimens of other fluids. *Prototheca* spp. colonies recovered from milk were visualized in pure culture after 72 h of incubation and their abundance was related to the larger number of algae cells seen in the milk cytology. Colonies of *Prototheca* spp. isolated from the joint fluids presented a much slower growth and were only visualized 5 to 6 days post-inoculation. A mixed flora was retrieved from cultures of the

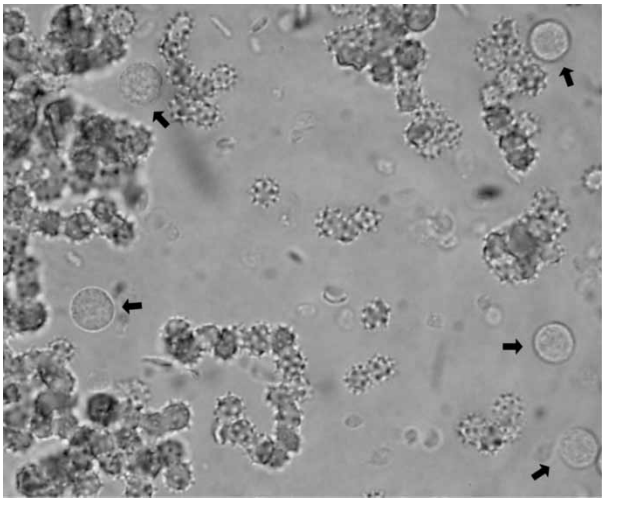

Fig. 2 Photomicrograph obtained by light microscopy of wet mount preparations of *Prototheca* spp. from the white blood cell fraction separated by Lymphoprep. Extracellular *Prototheca* spp. sporangia (arrow). Magnification ×1000.

collected joint fluids on the Columbia agar plates, consisting of *Corynebacterium* spp. and *Streptococcus* spp. that were visualized after an incubation period of 24 h at 37°C. Recovery of *Prototheca* spp. from blood samples was the slowest, with visible colonies detected only after 10 days post-inoculation. Pregnancy and parturition may have contributed to lower the cow's immunocompetence and allow the dissemination of the algae from the mammary gland to the blood stream. Following the evidence of systemic involvement of *Prototheca* spp. in this cow, the owner agreed to remove the animal from the herd.

The identification of *Prototheca* spp. in this study was attempted using the API 20C system (BioMerieux, Portugal Lda). However, the database of the API 20C system only includes *Prototheca wickerhamii*, and the biochemical reaction of trehalose was inconclusive. Therefore, the species identification of the isolates recovered from all indicated samples was accomplished through the use of PCR amplification of the 18S rDNA, amplified rDNA restriction analysis, and by direct sequencing of the 18S rDNA sequences. The results indicated that the isolates were all *P. blaschkeae* (GenBank accession no. EU439262). This isolate is also deposited in the Sammlung von Algenkulturen Göttingen (SAG) culture collection with the strain number SAG2291. PCR amplification of the 18S rDNA region was carried out as previously described [20], presenting a fragment of about 1800 bp. Amplified rDNA restriction analysis was performed using *Hae*III (NEB, Inc., Frankfurt, Germany), which presented a restriction pattern similar to the one determined for *P. blaschkeae* in a recent study [20] (Fig. 3). The amplified rDNA restriction analysis of the isolate (SAG 2291), presented differences when compared to the restriction patterns of the type strains of *P. zopfii* genotype 1 (SAG 2063) and 2 (SAG 2021). However, its rDNA restriction pattern (SAG 2291) was similar to that of *P. blaschkeae* type strain (SAG 2064) which lack the 400 bp fragment (Fig. 3). The sequence had a length of 1815 bp and showed 99.8% identity within the 18S rDNA sequence available for *P. blaschkeae* SAG2064 (AY973041).

Discussion

Prototheca are unicellular organisms closely related to the green alga of the genus *Chlorella* but lacking chlorophyll [5]. Members of the genus *Prototheca* are ubiquitous saprophytes, and can be isolated from a variety of environmental sources [5]. The genus comprises several species, but until recently the only species known to cause infectious diseases in humans and animals were *P. wickerhamii* and *P. zopfii*, respectively [15–17]. Among domestic animals, dogs have been affected with severe disseminated forms of the disease involving several organs. In cattle, this pathogen is in

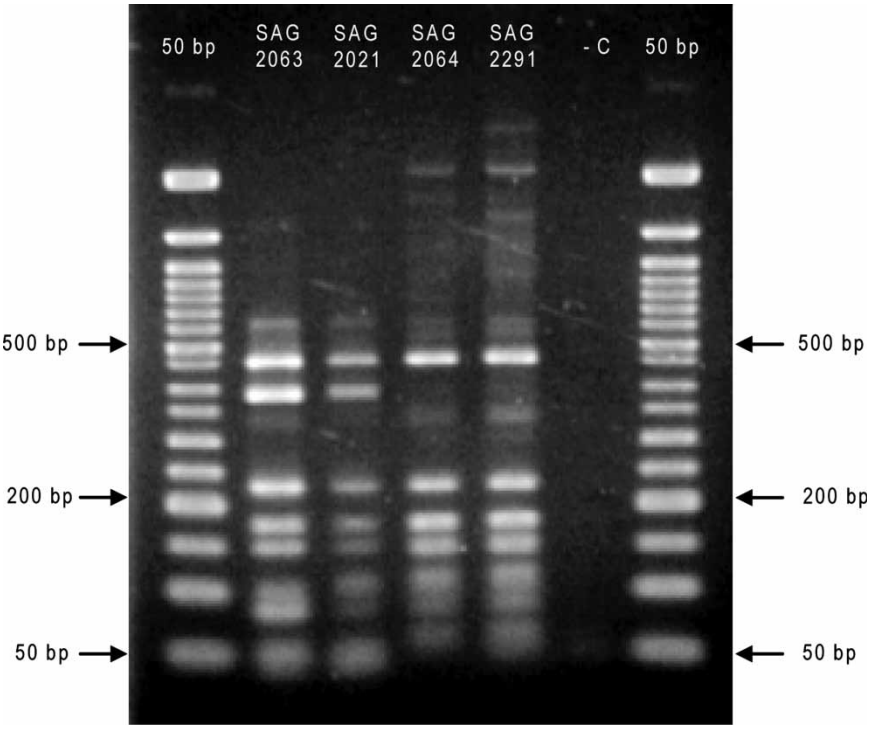

Fig. 3 Restriction patterns of amplified 18S rDNA fragments of the *Prototheca* isolate (SAG 2291) and three *Prototheca* type strains kindly provided by Dr Roesler. PCR fragments were digested with *Hae*III, yielding different patterns, presenting *P. zopfii* genotype 1 and *P. zopfii* genotype 2 similar restriction patterns, and *P. blaschkeae* and our isolate the lack of the 400 bp fragment. Lane 50 bp, molecular weight standard (50 bp ladder); lane SAG2063, *P. zopfii* genotype 1; lane SAG2021, *P. zopfii* genotype 2; lane SAG2064, *P. blaschkeae*; lane SAG 2291, our isolate; and lane – C, negative control.

general associated with chronic mastitis. The first report associating *P. zopfii* with mastitis in cattle was published in 1952 [12]. Taniyama *et al*. [21], described the first case of disseminated protothecosis caused by *P. zopfii* in several organs of a cow through the use of immunohistochemical and histopathological techniques. While *P. wickerhamii* is in general associated with systemic infections in humans [22–24], *P. blaschkeae* was first identified from a human case of onychomycosis [18]. There have been reports of algaemia, with the recovery of *P. wickerhamii* in culture after its visualization in peripheral blood smears but this has only been demonstrated in a few human cases [22,24]. In addition, *Prototheca* in this case study could be seen in cytology of all collected samples, but its recovery in culture from blood specimens was somehow difficult. To our knowledge this is the first report of an algaemia in a cow involving this infectious pathogen. The stress of pregnancy, aggravated by the lethargic and anorectic condition that followed the parturition, may have induced a debilitating status reducing the cow's defenses and resistance mechanisms. Although generally saprophytic, some *Prototheca* species may cause pathology when the host immunological defenses are impaired [5–7], or when predisposing factors such as deficient animal care and poor milking hygiene occur in dairy management [3,7,9]. The literature [13,20,25,26] suggests that the incidence of bovine mastitis by *Prototheca* spp. is increasing all over the world. This fact probably reflects the inclusion by laboratories of explicit tests in their diagnostic routines.

Recently, Roesler *et al*. [15] suggested that isolates belonging to biotype II of *P. zopfii* represented by strain SAG 2021, are the causative agents of bovine mastitis [2]. Moreover, *P. zopfii* was divided into three biotypes on the basis of phenotypic characteristics and auxonographical and biochemical analyses. In this scheme, *P. blaschkeae* belongs to biotype III [15, 19,25]. More recently, mastitis by *P. blaschkeae* has been reported in cattle [20] but never involved in a systemic type of condition. With the emergence of infectious diseases in the world, the potential consequences of bovine protothecosis on public health remains to be determined through studies of the resistance of the new taxonomic groups to milk pasteurization. This study reports the first known case of algaemia by *P. blaschkeae* in a dairy cow, after a chronic episode of mastitis.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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CHAPTER 3

PROTOTHECA SPP. SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL TREATMENTS

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In Vitro Susceptibility of *Prototheca* to pH and Salt Concentration

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Abstract *Prototheca* sp. can assume high economic significance in the dairy industry and pose a potential risk for the public health. We investigated the in vitro susceptibility of *Prototheca* isolates retrieved from mastitic milk (*P. zopfii* and *P. blaschkeae*) to different pH buffers and salt concentrations using a microbroth assay adapted from the Clinical Laboratory Standards Institute guidelines. Different pH buffer solutions ranging from pH 1 to pH 12 and different sodium chloride concentrations, 4.5, 9 and 18%, were tested. *P. zopfii* strains presented an optimal growth between pH 5 and 9, a complete growth inhibition at pH 3, and limited growth at pH 1 and 12, whereas *P. blaschkeae* strains showed higher susceptibility to all pH values except for pH 3 where it demonstrated a moderate growth when compared to *P. zopfii* strains. When salinity was incremented, *P. blaschkeae* was more resistant than *P. zopfii*, although a reduction in growth for all strains of *Prototheca* was observed. This study demonstrated differences in the in vitro susceptibilities of *P. zopfii* and *P. blaschkeae* to different pH and salt concentrations and intend to be a contribution on the understanding of some of the physiologic features that can be associated with the survival of these microalgae in the environment.

Keywords *Prototheca* sp. · pH sensibility · Salinity sensibility

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Introduction

The genus *Prototheca* includes unicellular achlorophyllous microalgae that belong to the family *Chlorellaceae*. These reproduce asexually by formation of variable numbers of sporangiospores within a sporangium [1–3]. Members of this genus are ubiquitous saprophytes and can be isolated from a variety of environmental sources such as plants, soil, drinking water, sludge, marine water, swimming pools, feces of domestic or wild animals, barn floors and meat products [4–6]. Currently five species, *Prototheca zopfii*, *P. wickerhamii*, *P. stagnora*, *P. ulmea* and *P. blaschkeae* have been recognized [2, 7]. From

these, *P. zopfii*, *P. wickerhamii* and *P. blaschkeae* are known to be associated with diseases in animals and humans, specially when predisposing factors occur or when the host immunologic defences are impaired [1, 2, 7, 8]. The most prevalent form of Protothecosis in animals is bovine mastitis, which generally occurs in a chronic subclinical or a mild clinical inflammatory process in the udder and affects cows that do not respond to routine therapy [9–11]. In humans, this disease presents three clinical forms, olecranon bursitis, cutaneous lesions and disseminated or systemic infections [3, 5, 12]. *Prototheca* is largely distributed globally, and cases of this infection have been reported in the five continents [11, 13, 14]. Several reports refer that these extremely resistant algae have been isolated from a great variety of conditions, from water treated with chloride to pasteurized milk [7, 9, 13, 15–17]. These studies refer only to *P. zopfii*, and no further investigations have been performed to analyze the susceptibility of the more recently described pathogenic species, *P. blaschkeae* recovered from animal sources. The objective of this study was to determine the in vitro effects of different pH buffers and salt concentrations on *Prototheca* sp. to better understand some of the physiologic characters that can be involved in its survival and propagation in the environment.

Materials and Methods

Prototheca Isolates

The field isolates of *Prototheca* used in this study belong to a major collection of several milk pathogens of the Laboratory of Infectious Diseases of Veterinary Medicine from Porto University. *Prototheca* isolates were retrieved from milk of cows with mastitis originating from different dairy herds from the northwest of Portugal, representing a prevalence of 1.31% of all isolated microorganisms. All samples were collected under sterile conditions. For diagnostic purposes, 40 µl aliquots of milk samples collected from individual quarters of the udder were streaked onto Columbia agar plates supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France). After 42–72 h at 37°C, plates were examined for *Prototheca* growth, and any colonies resembling this alga were subcultured once on Sabouraud dextrose agar medium (Merck Laboratories, Darmstadt, Germany). After macro and microscopical identification, *Prototheca* isolates speciation was performed by amplification of the 18S rDNA and direct sequencing of all isolates as previously described [1].

Reagents and Solutions

To test the pH sensitivity of the isolates, hydrochloric acid (Merck Laboratories, Darmstadt, Germany), acetic acid (Merck Laboratories, Darmstadt, Germany), sodium acetate trihydrate (Sigma, Steinheim, Germany), disodium hydrogen phosphate (Merck, Darmstadt, Germany) and sodium hydroxide (Sigma, Steinheim, Germany) were used to prepare the buffer solutions. For salinity sensibility, sodium chloride (Merck, Darmstadt, Germany) solutions were used.

Effects of Different pH Values and Sodium Chloride Concentrations on *Prototheca* Viability

Although there are no guidelines and interpretative criteria for *Prototheca* sp., the Clinical Laboratory Standards Institute (CLSI) M27-A2 guidelines were followed [18]. The susceptibility tests for the nine *Prototheca* isolates to different pH values and sodium chloride concentrations were adapted according to these guidelines for a microbroth assay. The experimental design included three replicates in all treatments.

Six different pH values were tested and buffer solutions were prepared according to European Pharmacopeia [19] and United States Pharmacopeia [20]. Therefore, for all buffer solutions, a concentration of 0.1 M was prepared. All solutions and buffers were prepared in the following concentrations: pH 1—hydrochloric acid buffer, 4.14 ml hydrochloric acid 37% and 495.86 ml distilled water; pH 3—acetic acid buffer, 2.86 ml acetic acid 100% and 497.14 ml distilled water; pH 5—acetate buffer, 178.5 ml acetic acid and 321.5 ml sodium acetate (0.1 M); pH 7—phosphate buffer, 378 ml disodium hydrogen phosphate and 122 ml hydrochloric acid; pH 9—phosphate buffer, 477.5 ml disodium hydrogen phosphate and 22.5 ml hydrochloric acid; pH 12—sodium hydroxide buffer, 2 g sodium hydroxide and 500 ml distilled water.

To test for salinity sensibility, three different solutions were prepared in the following concentrations: 4.5, 9 and 18% of sodium chloride corresponding

to 5-, 10- and 20-fold sodium chloride physiologic concentration, respectively.

Prototheca suspensions were prepared in sodium chloride at physiologic concentrations, with the concentration of 1×10^6 to 5×10^6 cells per ml, after which, several other dilutions were performed until reaching the working solutions (5×10^3 to 2.5×10^4). For both tests, the following methodology was applied: 270 µl of buffer or salt solutions were introduced in the test wells in triplicate, and 30 µl of *Prototheca* working solution was added into the wells, with several positive and negative controls wells also included. The positive controls were *Prototheca* suspensions in RPMI 1640 (Gibco, Invitrogen, Paisley, UK) with 0.01% Tween 20 (Merck, Darmstadt, Germany) and the negative controls were solutions of sodium chloride, RPMI with 0.01% Tween 20, buffer solutions 5, 10 and 20 times of sodium chloride and water. Following incubation periods of 5 min, 24 and 48 h, and 1 week at 37°C in a humid chamber, 100 µl of the suspension was spread on Sabouraud dextrose agar plates and further incubated during 48 h at 37°C. *Prototheca* viability was determined by means of growth inhibition, counting the number of colony forming units (CFUs) observed in each plate. For the pH susceptibility testings, pH values were determined before and after the incubation periods.

Statistical Analyses

To evaluate the effects on the growth inhibition of *Prototheca* using the different pH buffers and the three sodium chloride solutions, the Student's *t*-test was used in all comparisons with $P < 0.05$ as the threshold to determine significance of the differences.

Results

Algae Identification

The species determination of the *Prototheca* associated with bovine mastitis in this study and in the region was performed by molecular methods as previously described [1]. Four of the used strains were identified as *P. zopfii* genotype 2 and 5 as *P. blaschkeae*.

Effect of Different pH Buffers

Prototheca growth was variable at different pH buffers and incubation periods, as can be observed in Fig. 1. Generally, the isolates of *P. zopfii* presented an optimal growth between pH 5 and 9, presenting inhibition of growth at pH 3, and limited at pH 1 and pH 12. Despite, the *P. blaschkeae* strains presented limited and uniform growth between pH 3 and pH 12, at pH 1 their growth was completely inhibited. After 5 min of incubation both *Prototheca* sp. presented a similar growth at all pH values, although a significant difference ($P = 0.018$) was detected at pH 9, at which *P. zopffii* strains presented a higher growth than *P. blaschkeae*. After 24 and 48 h of incubation all *P. zopfii* presented a significant higher growth than *P. blaschkeae* strains at pH 5 ($P = 0.006$ and $P < 0.001$, respectively), 7 ($P < 0.001$ and $P < 0.001$, respectively) and 9 ($P = 0.036$ and $P = 0.043$, respectively). However, *P. blaschkeae* strains presented a significant higher ($P = 0.009$ and $P = 0.022$, respectively) growth than *P. zopfii* at pH 3. At pH 1, after 24 h, despite limited, the growth of *P. zopfii* was significantly higher ($P = 0.043$) than of *P. blaschkeae*, and the same occurred at pH 12 ($P = 0.035$) but only after 48 h of incubation. Strains of both species were further incubated during 1 week, and all strains did not grow at pH 1 and 3, and could grow at pH 5 without significant differences between them. Moreover, between pH 7 and 12 *P. zopfii* strains presented a significant higher ($P = 0.001$, $P = 0.007$ and $P = 0.012$, respectively) growth than *P. blaschkeae*. All the *P. blaschkeae* strains were more susceptible than *P. zopfii* strains to all pH values, except for pH 3. The buffer effect of all solutions was maintained during all incubation periods, being only detected small variations on the pH values (data not shown).

Effect of Different Sodium Chloride Concentrations

The results on the different salt concentrations treatments showed that the increment in salinity inhibited the growth of all *Prototheca* strains used in this study, with *P. blaschkeae* isolates presenting a slightly higher resistance than *P. zopfii* strains (Fig. 2) to the same treatment. All *Prototheca* strains showed higher growth at lower concentrations of

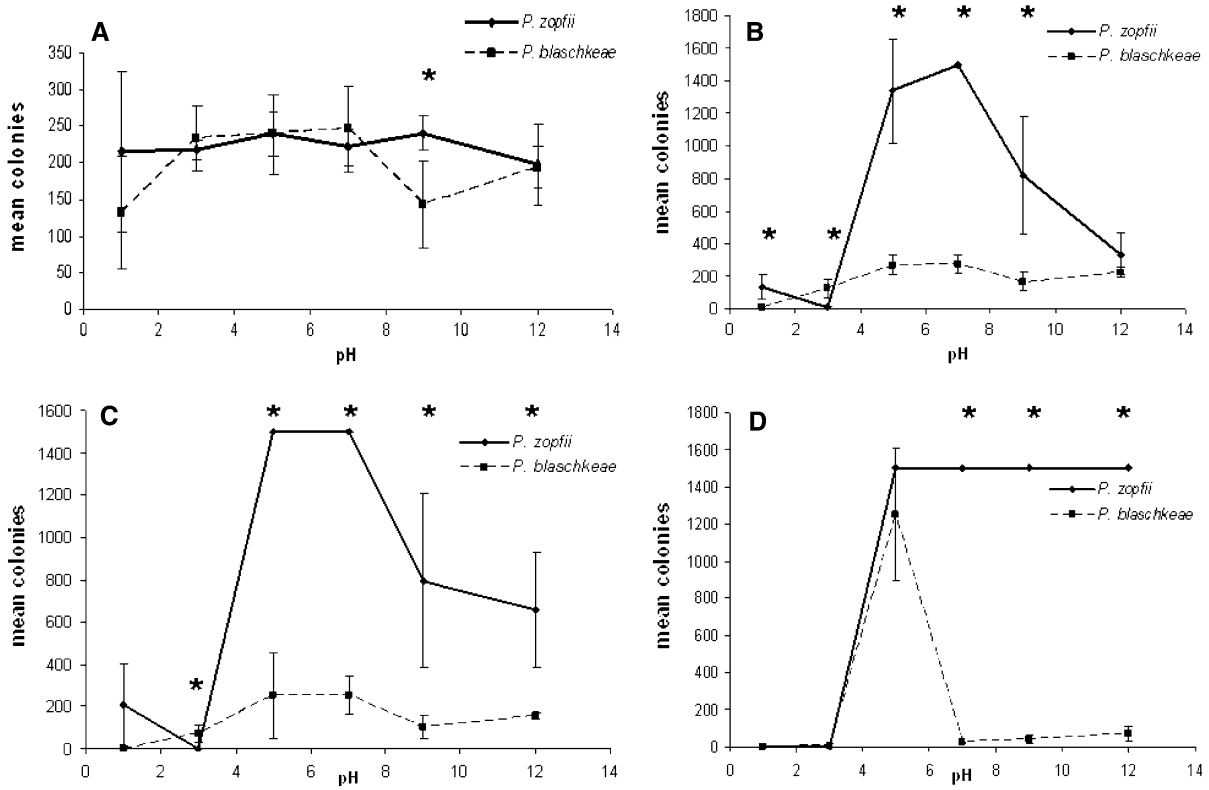

Fig. 1 *Prototheca* growth at different pH values during different incubation periods. **a** Growth after 5 min, *P. zopfii* ($n = 4$), *P. blaschkeae* ($n = 5$). **b** Growth after 24 h, *P. zopfii* ($n = 4$), *P. blaschkeae* ($n = 5$). **c** Growth after 48 h, *P. zopfii* ($n = 4$), *P. blaschkeae* ($n = 5$). **d** Growth after 1 week, *P. zopfii* ($n = 2$), *P. blaschkeae* ($n = 2$). * Significant different values, $P < 0.05$

sodium chloride (5 and 10 times), presenting after 5 min of incubation a uniform growth at all sodium chloride concentrations. However, after 24 h of incubation, growth inhibition was detected for all strains for increased salt concentrations. Interestingly, *P. blaschkeae* was more resistant to this treatment, presenting a significantly higher ($P = 0.001$) growth at 20 times the salt concentration. After 48 h of incubation, a significant higher ($P = 0.023$, $P < 0.001$, 10 and 20 times the salt concentration, respectively) growth of *P. blaschkeae* was observed at higher sodium chloride concentrations, although this growth was moderate. After 1 week of incubation the growth of *P. zopfii* strains was completely inhibited at higher salt concentrations, but in contrast, the *P. blaschkeae* still presented some growth, although at a lower level. For all susceptibility tests in this study, all positive controls showed growth of *Prototheca* and the negative controls presented no growth (data not shown).

Discussion

Prototheca sp. are widespread worldwide throughout different environments, but are found most frequently in those with high humidity and organic matter, being its environment dissemination and perpetuation elevated [21, 22]. These ubiquitous algae are extremely resistant due to the sporopollenin included in the cell wall that allow recontamination of the environment and promote its propagation and maintenance on the environment [10, 23]. Several authors [7, 9, 13, 15–17] state that *Prototheca* have been isolated from a great variety of pH values, from water treated with chloride and from pasteurized milk, but no studies have been conducted to support these findings. Also, no characterization of the most recent specie, *P. blaschkeae*, isolated from cases of bovine mastitis, has been reported regarding their susceptibility to physical and chemical factors. Therefore, the main objective of this work was to evaluate the in vitro

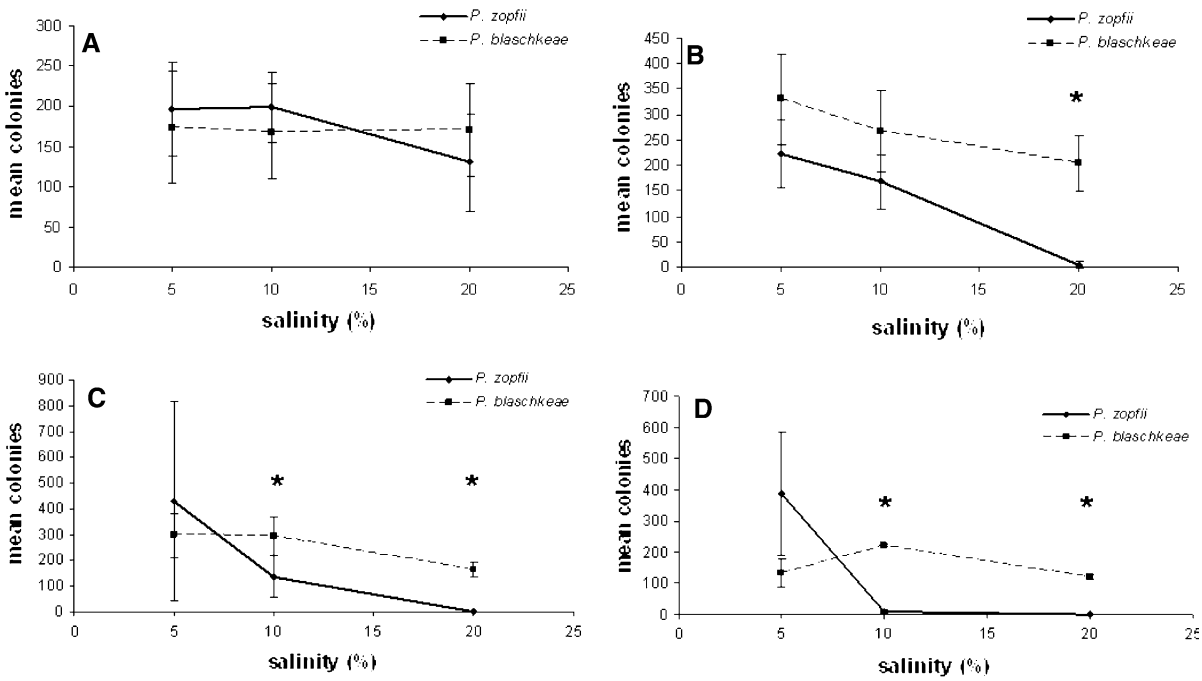

Fig. 2 *Prototheca* growth at different salt concentrations during different incubation periods. **a** Growth after 5 min, *P. zopfii* ($n = 4$), *P. blaschkeae* ($n = 5$). **b** Growth after 24 h, *P. zopfii* ($n = 4$), *P. blaschkeae* ($n = 5$). **c** Growth after 48 h, *P. zopfii* ($n = 4$), *P. blaschkeae* ($n = 5$). **d** Growth after 1 week, *P. zopfii* ($n = 2$), *P. blaschkeae* ($n = 2$). * Significant different values, $P < 0.05$

susceptibility of *P. zopfii* and *P. blaschkeae* isolates recovered from bovine mastitis to the effects of different pH buffers and salinity concentrations.

Results of our study determined that *Prototheca* sp. presented variable sensibility to the pH buffers tested, which may suggest different environmental survival capability between species, nevertheless the well-known biologic differences. *P. zopfii* presented a significantly higher ($P < 0.04$) resistance between pH 5 and 9 than *P. blaschkeae*, demonstrating that it was not only able to survive under these pH values, but also presented a high multiplicity rate under those conditions, even after 1 week of incubation when a slower growth could be expected due to lack of nutrients. Moreover, at pH 12, *P. zopfii* was able to grow, however, at a lower rate when compared to lower pH values and could maintain cell division at least for 1 week. At pH values lower than 5, *P. zopfii* showed a lower multiplication rate, presenting at pH 1 the capacity to survive for 48 h, but was completely inactivated over this period of time. At pH 3, *P. zopfii* growth was completely inhibited after 24 h of incubation. On the other hand, *P. blaschkeae* could grow between pH 3 and 12 for at least 48 h, presenting the optimum growth conditions between pH 5 and 7. Around pH 3 and between pH 9 and 12, *P. blaschkeae* could grow demonstrating moderate resistance. Although its growth rate was near zero, the reasons for this could be that the strains could not rapidly multiply or may have entered in a quiescence condition. At pH 1, *P. blaschkeae* growth was inhibited after 24 h, and it was able to survive between pH 3 and 12 for 1 week, although its multiplication capacity was absent for all pH conditions except to pH 5 where a higher multiplication rate could be observed. The results of this study show that *P. zopfii* presented a higher multiplicity capacity in all pH buffers except to acetic acid. These data suggest that both species, but especially *P. zopfii*, are able to multiply under very adverse conditions such as pH 12.

The increment of salinity concentration in solutions was directly proportional to growth inhibition, however, with a major impact on *P. zopfii* than on *P. blaschkeae* which can lead to speculate that the sporopollenin content of the cell wall in *P. blaschkeae*

is higher than that of *P. zopfii*. Although the growth inhibition of *P. zopfii* was detected only after 1 week of incubation at 9% of sodium chloride, its total growth inhibition was observed after 24 h of incubation at a higher concentration (18%). Therefore, *P. zopfii* can only multiple at lower sodium chloride concentrations, 4.5%. On the other hand, *P. blaschkeae* presented only a reduction on the multiplication rate which can be speculated that this alga can be found in environments with high salt concentrations when compared to *P. zopfii*.

In conclusion, *P. zopfii* can survive and propagate in environments with pH values between 5 and 12, and also at 4.5% of sodium chloride concentrations. On the other hand, *P. blaschkeae* could survive and multiply at high salinity concentrations, up to 18% of sodium chloride, but showed more susceptibility to pH buffers, multiplying at pH 5 and at a lower capacity at pH 9 and 12.

This is the first study that compares the susceptibility of *P. zopfii* and *P. blaschkeae* strains to different pH values and sodium chloride concentrations. Due to the limited number of strains used in the study (which are representative of the sub-regions of the Norwest of Portugal, the larger milk production area in the country), the results herein reported imply the need for further investigations using isolates from different regions. The generated knowledge is a contribution on the understanding of some physiologic characters of these algae that may explain its capacity to survive and perpetuate in different environment conditions.

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In vitro algaecide effect of borate on *Prototheca* strains isolated from bovine mastitic milk

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Abstract

The incidence of mastitis by *Prototheca* spp. is increasing all over the world. Known to be extremely resistant, this alga has been isolated from a great variety of environments. We report a preliminary study regarding the *in vitro* algaecide effect of borate buffer on *Prototheca* spp. recovered from bovine mastitis.

Keywords

Prototheca spp.; borate susceptibility

Algae of the genus *Prototheca* are one of the few plant-like organisms that cause infections in humans and animals (1-3). These microscopic unicellular organisms are achlorophyllic and reproduce asexually by endosporulation producing a variable number of endospores (4, 5). Members of the genus are ubiquitous saprophytes and can be isolated from a variety of environmental sources such as plants, soil, drinking and marine water, sludge, faeces of domestic or wild animals, barn floors, and meat products (6-8). However, some species, *P. zopfii*, *P. wickerhamii* and *P. blaschkeae*, may turn into unusual opportunists and cause pathology when host immunologic defences are impaired or when predisposing factors occur (7, 9-12). *Prototheca* spp. was classified as an environmental agent associated with bovine mastitis (13, 14) and, in the past, only sporadic cases were observed. Nowadays it is recognized as endemic worldwide (15) and may be considered a public health issue. These algae do not respond to routine mastitis therapy, and until recently the elimination of the infected animals is the only control method (15-17). Several studies also show that these extremely resistant algae have been isolated from a great variety of pH values, from water treated with chloride to pasteurized milk (6, 7, 17-21). Susceptibility studies on *Prototheca* using conventional mastitis antimicrobial agents, have demonstrated their resistance in the majority of the cases (10, 22-24). As no official guidelines for performance, interpretation and quality control of *in vitro* susceptibility studies are available for these algae, yeast guidelines are generally adapted in these studies (8). *Prototheca* susceptibility to unconventional drugs has been also performed (23), and the interesting findings obtained in this and other studies led us to further investigate the usage of new products. Borax compound can be used in several ways. It is a component of many detergents, and can also be used on the preparation of buffer solutions (25-27). In this study, we determined the *in vitro* effect of

borate and phosphate buffers at pH 9 on *Prototheca* isolates retrieved from bovine mastitic milk.

Prototheca isolates used in this study were retrieved from milk of cows with mastitis originating from different dairy herds from the Northwest of Portugal. Species identification was performed as previously described (2). *Prototheca* isolates were spread and grown on Sabouraud Dextrose Agar medium (Merck Laboratories, Darmstadt, Germany). For the susceptibility testing, sodium tretraborate (Merck, Darmstadt, Germany) and disodium hydrogen phosphate (Merck, Darmstadt, Germany) were used on the preparation of the pH 9 buffer solutions at 0.1 M. The buffers were prepared according to European Pharmacopeia (27) and United States Pharmacopeia (25).The Clinical Laboratory Standards Institute (CLSI) M27-A2 guidelines were followed, although there are no guidelines and interpretative criteria for *Prototheca* spp. (28). Briefly, the preparation of borate buffer solution consists in adding: 316 mL borax solution to 184 mL hydrochloric acid. The phosphate buffer was prepared as previously described (19). The *in vitro* susceptibility tests were performed as described before (19). Suspensions of *Prototheca* in sodium chloride at physiologic concentrations were subjected to several dilutions, and the used working solution contained between 5 X 10^3 to 2.5 X 10^4 cells per mL. All *Prototheca* suspensions were diluted 10 fold in each buffer solution and tested in duplicate. Several positive (*Prototheca* suspensions) and negative (sodium chloride, RPMI with 0.01% Tween 20, buffer solution and water) controls were included in each treatment. Following incubation periods of 5 minutes, 24 and 48 hours, and one week at 37 ºC in a humid chamber, 100 µL of each well were spread on Sabouraud Dextrose Agar and incubated for 48 hours at 37ºC in order to confirm growth characteristics. To evaluate the effects of two different buffers on the growth inhibition of *P. zopfii* and *P. blaschkeae* strains and to compare them, T "Student" test was used with the logarithmic transformation of colony forming units (CFU) to normalize the data. The threshold for significance was defined at $P < 0.05$.

The isolates of *Prototheca* used in this study were identified as *P. zopfii* (four) and as *P. blaschkeae* (five). The results showed that *Prototheca* survival was generally inhibited with time. Nevertheless, *P. zopfii* demonstrated higher significant multiplication rate in phosphate buffer when compared to *P. blaschkeae* (Fig. 1 A and B, $P<0.05$). Previous studies (19) demonstrated that *P. zopfii* has optimal growth between pH 5 and 9, whereas *P. blaschkeae* has a slower and uniform growth between pH 3 and 12. Using the phosphate buffer at pH 9, similar results were obtained in the present study for all tested isolates. However, when borate buffer was used at pH 9, a decrease on *P. zopfii* growth could be observed (Fig.1A and C). *P. blaschkeae* growth (fig.1B and C) was generally inhibited and showed significant differences when compared to *P. zopfii* strains after 1

week of incubation (Fig. 1C). With time, significant differences in growth were observed for *P. zopfii* strains in both buffers. However, in the case of *P. blaschkeae*, their growth was equally and slightly inhibited by both buffers. The results in this study demonstrated that borate buffer at pH 9 had an algaecide effect on *P. zopfii* at 24 and 48 hours of incubation, with total growth inhibition after one week of incubation (Fig.1A). The behaviour of *P. zopfii* to borate buffer in this study was significantly different from treating the same isolates with phosphate buffer (Fig.1A). However, the susceptibility of *P. blaschkeae* to both buffers did not show any significant differences between them (Fig. 1B). The inhibition effect of borate could be due to intrinsic characteristics of this compound and not to its pH value. It is possible that borate can penetrate better the *P. zopfii* than *P. blaschkeae* cell wall. Several studies regarding cell wall composition of *P. wickerhamii* and *P. zopfii* were performed (4, 6, 29-31), detecting that sporopollenin, a highly resistant and chemically stable biopolymer, is the most important component of their cell wall. Thus, *P. blaschkeae* could have a cell wall with higher amount of sporopollenin than *P. zopfii*, because of its longer survival in borate buffer. Moreover, borate could modify the cell wall thickness or structure, or even degrade intra-cellular organelles as demonstrated for other chemical agents (22). This preliminary study indicates that borate buffer compared to phosphate buffer has a more pronounced inhibitory effect on the growth of *Prototheca* strains, especially for *P. zopfii*. Nevertheless, similar studies using higher number of isolates should be conducted to confirm these findings.

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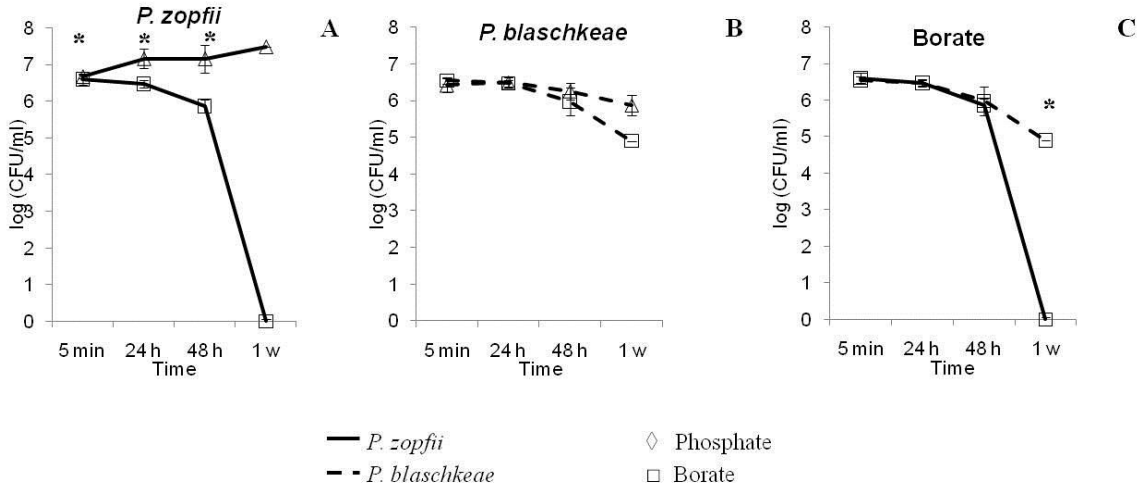
Figures

Figure 1. *Prototheca* growth in phosphate- and borate buffers (pH 9) at different incubation times. (A) Growth of *P. zopfii* (n=4); (B) Growth of *P. blaschkeae* (n=5); (C) Growth of *P. zopfii* (n=4) and *P. blaschkeae* (n=5) in borate buffer. * Significant different values, P ≤ 0.05.



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Short communication: Temperature sensibility of *Prototheca blaschkeae* strains isolated from bovine mastitic milk

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ABSTRACT

Dairy cow mastitis associated with microalgae of the genus *Prototheca* has been reported worldwide. This alga is extremely resistant to most antimicrobials commonly used in mastitis therapy. In milk processing, different thermal treatments are generally efficient at inactivating and eliminating microorganisms. Until recently, no reports on *Prototheca blaschkeae* susceptibility to heat treatment have been described. Thus, considering the potential zoonotic risk that *Prototheca* may represent, the objective of this study was to test the susceptibility of *P. blaschkeae* field isolates retrieved from bovine mastitis to different temperature/time ratios that are generally used in the milk processing industry: 62°C/15 min and 30 min; 70°C/20 s, 15 min, and 30 min; 75°C/20 s; 90°C/1 s; and 100°C/1 s. The results showed a growth reduction of all isolates after the heat treatments, but only at 100°C was a total growth inhibition observed.
Key words: *Prototheca blaschkeae*, *Prototheca zopfii*, bovine mastitis, temperature susceptibility

Green algae of the genus *Prototheca* are one of the few plant-like organisms that cause infections in humans and animals (Matsuda and Matsumoto, 1992; Pore, 1998; Möller et al., 2007). This genus consists of microscopic, unicellular, achlorophyllic algae with asexual reproduction by formation of variable numbers of sporangiospores within a sporangium (DiPersio, 2001; Malinowski et al., 2002). *Prototheca* are ubiquitous and generally saprophytic, being isolated from a variety of environmental sources such as plants, soil, drinking water, sludge, marine water, swimming pools, feces of domestic or wild animals, barn floors, and meat products (Pore et al., 1983; Anderson and Walker, 1988; Lass-Flörl and Mayr, 2007). However, some species in the genus, *Prototheca zopfii*, *Prototheca wickerhamii*, and *Prototheca blaschkeae*, may turn into unusual opportunists and cause pathology in hosts with impaired immunological defenses or when other predisposing factors occur (Pore, 1998; Schultze et al., 1998; Jánosi et al., 2001; Roesler and Hensel, 2003; Marques et al., 2006; Roesler et al., 2006; Marques et al., 2008; Thompson et al., 2009). In humans, *P. wickerhamii* is usually associated with pathology that is expressed essentially by cutaneous or subcutaneous lesions as well as in generalized infections (Zaitz et al., 2006; Hightower and Messina, 2007; Lass-Flörl and Mayr, 2007; Narita et al., 2008). On the other hand, animal infections, mainly bovine mastitis, have been associated with *P. zopfii* (Roesler et al., 2003; Buzzini et al., 2004; Möller et al., 2007). Recently *P. blaschkeae* was also associated with bovine mastitis (Marques et al., 2008), although it was first isolated and described from a human onychomycosis (Roesler et al., 2006). Nevertheless, these 2 species are capable of causing infections in humans as well (Roesler et al., 2006; Lass-Flörl and Mayr, 2007), suggesting a potential zoonotic involvement. In the past, only sporadic reports of cases of *Prototheca* associated with bovine mastitis were described in dairy herds in Europe and in the American continents (Lerche, 1952; Anderson and Walker, 1988; Da Costa et al., 1996). Nowadays, cases of acute to chronic mastitis are increasingly recognized to be endemic worldwide and are gaining economic and public health importance (Santos and Flor, 2000; Bexiga et al., 2003; Roesler and Hensel, 2003; Buzzini et al., 2004; Marques et al., 2006; Marques et al., 2008; Osumi et al., 2008; Thompson et al., 2009). The most prevalent form of protothecosis in animals is bovine mastitis, which generally occurs in a chronic subclinical or a mild clinical inflammatory process in the udder and affects cows that do not respond to routine therapy (Melville et al., 1999; Malinowski et al., 2002; Roesler and Hensel, 2003). Therefore, the implementation of control measures should be the main objective for all dairy herds in order to avoid the spreading of this organism in the parlor environment and to reduce animal culling and, consequently, decrease all the associated economic losses. As previously reported by others (Costa et al., 1998; Melville et al., 1999), milk and dairy products contaminated with *Prototheca* spp. may represent one of the means of transmission of this pathogen to humans.

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Generally, different thermal treatments of milk are efficient for inactivating and eliminating microorganisms. Milk pasteurization and ultrapasteurization are the procedures most used nowadays and consist of the appropriate use of heat to destroy pathogenic microorganisms without changing the organoleptic characteristics and their physical and chemical constitution. Several studies reported that *Prototheca* have been isolated from a great variety of pH values, from water treated with chloride, and from pasteurized milk (Pore, 1998; Melville et al., 1999; Jánosi et al., 2001; Marques et al., 2009). However, the reports evaluating the sensitivity of these algae to pasteurization refer only to a single study with *P. zopfii*, and no other published studies analyze the susceptibility of other species in the genus, in particular *P. blaschkeae*, which has recently been described as being associated with bovine mastitis. Considering the increasing importance of *Prototheca* spp. on bovine mastitis (Melville et al., 1999; Roesler and Hensel, 2003; Roesler et al., 2006; Marques et al., 2008, 2009) and the potential to be transmitted to humans, the purpose of this study was to test the susceptibility of 14 *P. blaschkeae* strains isolated from mastitic milk obtained from 11 dairy herds in the northern region of Portugal to different temperature/time ratios. Seventeen strains of *P. zopfii* genotype 2, also retrieved from mastitic milk (16 dairy herds from the same region), were evaluated as well.

The 31 field isolates of *Prototheca* used in this study belonged to the milk pathogens collection of the Laboratory of Infectious Diseases of Veterinary Medicine from Porto University (Portugal). The molecular characterization for the 18S rDNA region (Marques et al., 2008) demonstrated that the isolates were all genetically similar within each species. Two *Prototheca* spp. reference strains were kindly provided by Uwe Roesler (University of Leipzig, Germany). These strains, *P. zopfii* genotype 2 SAG 2021 (accession number AY940456) and *P. blaschkeae* SAG 2064 (accession number AY973041), were isolated from a bovine mastitis milk and from a human onychomicosis, respectively. Cultures were maintained on Sabouraud dextrose agar medium (Merck Laboratories, Darmstadt, Germany) during the study.

The susceptibility tests to different temperature/time ratios for the 31 *Prototheca* isolates were adapted according to the methodology described previously by Melville et al. (1999). For all evaluations, *Prototheca* cultures grown in Sabouraud dextrose agar with 48 h of incubation were used. Briefly, *Prototheca* suspensions were prepared in sterile sodium chloride solutions at physiologic concentrations (0.85%) at approximately 1×10^6 cells/mL, corresponding to tube 3 of the McFarland scale. From this suspension, one dilution step was performed in sterile milk to achieve the working suspension of 1×10^5 cells/mL, which is in agreement with that defined in the European directives (Hillerton and Berry, 2004) for the acceptable concentration of microorganism present in raw milk for production. Positive and negative controls were always included for all the treatments testing. The positive controls consisted of *Prototheca* suspensions in sterile milk, and the negative controls consisted of sodium chloride solutions and milk. All samples were tested in duplicate and subjected to different temperature/time ratios (62°C/15 min and 30 min; 70°C/20 s, 15 min, and 30 min; 75°C/20 s; 90°C/1 s; and 100°C/1 s) and were immediately placed in ice. The controls were incubated at 37°C during all tested times. Following the heat treatments, 100 μL of each sample, reference strains and controls, was spread on Sabouraud dextrose agar plates and incubated for 48 h at 37°C. The treatment effect was determined by means of the inhibition of growth by counting the number of colony-forming units observed in each plate.

To evaluate the effects on the growth inhibition of *Prototheca* using the different temperature/time ratios, 1-way ANOVA using the logarithmic transformation of colony-forming units (to normalize the data) as the dependent variable was performed using SAS software (SAS Institute, 1989). The linear model included species and time within temperature as main effects. Estimable linear contrasts of least squares means were computed to make inferences and evaluate differences between levels of main effects.

The effect of 8 temperature/time ratios on the counts of *P. zopfii* and *P. blaschkeae* measured in log colony-forming units per milliliter are shown in Table 1. *Prototheca* spp. grew without restrictions in all positive controls (average of 6.3 log cfu/mL) and were significantly different ($P < 0.001$) from all treated samples. On the other hand, no growth was present on the negative controls for each susceptibility test. Both *Prototheca* spp. were affected by the increment of the temperature/time ratios, as shown by the progressive inhibition (% kill) in Table 1. Total growth inhibition was achieved only at 100°C/1 s treatment, indicating that ultrapasteurization is the only industrial procedure that ensures that the milk from endemic regions is free of these agents. A significant difference was found between the 2 species ($P < 0.01$) after adjusting for the time within temperatures effect in the model. This study suggests that *P. blaschkeae* may be more resistant to the heat treatment than *P. zopfii*, with an adjusted log colony-forming units per milliliter mean 1.3 times bigger than the latter (1.33 and 0.97 log cfu/mL, respectively).

No significant differences were found between the field and reference *Prototheca* strains for all temperature/time ratio treatments (Figure 1). Only at 70°C/20

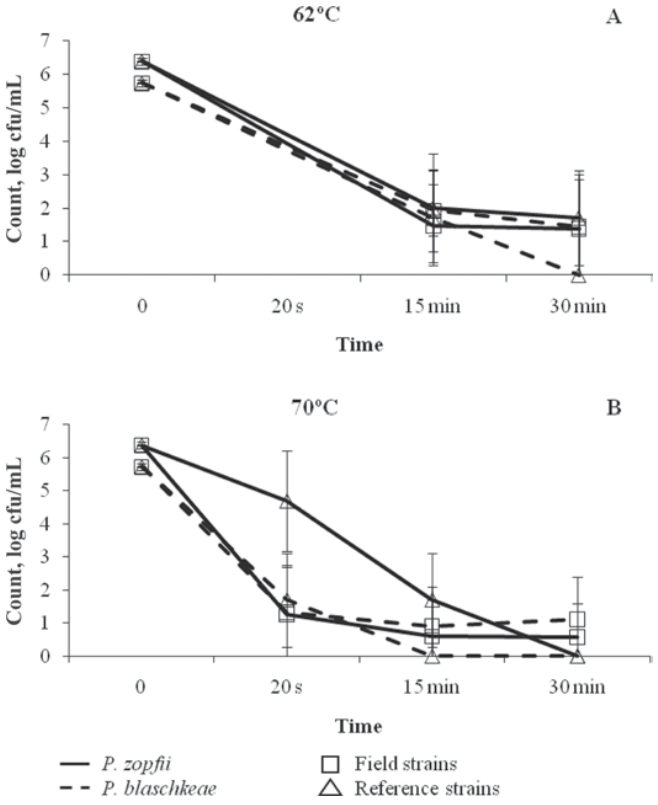

Figure 1. *Prototheca* counts (log cfu/mL) after heat treatment (A: 62°C; B: 70°C) at different time periods. Comparisons are between *Prototheca zopfii* and *Prototheca blaschkeae* originated from field strains and reference strains. Reference strains were *P. zopfii* genotype 2 SAG 2021 and *P. blaschkeae* SAG 2064.

s (Figure 1B) did *P. zopfii* SAG 2021 show an apparent greater resistance than the other strains, but without statistical significance.

Members of the genus *Prototheca* are widespread in many different environments worldwide but are most frequently found in environments with high humidity and content of OM. In these conditions, its dissemination and perpetuation in the environment are enhanced (Jánosi et al., 2001; Tsuji et al., 2006; Zaitz et al., 2006). Mastitic infections by *Prototheca* in dairy herds are generally maintained by clinically healthy shedders (Jánosi et al., 2001; Roesler and Hensel, 2003) and reflect poor dairy cow management and hygiene, especially by defective premilking cleansing and disinfection of the teats (Da Costa et al., 1996; Baumgärtner, 1997; Pore, 1998). Also, these ubiquitous algae are extremely resistant to chemical and physical agents because of the sporopollenin present in the cell wall, which allows recontamination of the environment and promotes its dissemination and perpetuation in the environment and also potentiates the pathogenecity (Costa et al., 1997; Malinowski et al., 2002; Marques et al., 2009). A previous study described the occurrence of human enteritis associated with the consumption of cheese contaminated with *P. zopfii* (Costa et al., 1998). Although direct human infection through dairy products was never confirmed, this finding urges the necessity to implement effective control methods in the dairy farm and active action at the level of milk products quality control. That can be achieved using effective thermal treatments of milk (ultrapasteurization) for human consumption. Melville et al. (1999) also found that *P. zopfii* strains isolated from mastitic milk were resistant in at least 1 of the thermal treatment tests (62–65°C/30 min, 72–75°C/15 s, and 72–75°C/20 s). The temperature/time ratios selected for this study consisted of those recommended for milk pasteurization (Sun, 2005; FDA, 2007). Additional tests were used to expand the temperature/time ratios to further understand the possible effects of time, or temperature, or both, on the growth inhibition of these 2 species. As in the present study, Melville et al. (1999) found a high variability in the susceptibility to temperature/time ratios from all isolates. At pasteurization temperatures (62 and 70°C), the increased exposure time from 15 to 30 min did not significantly increase the growth inhibition in both species (Table 1). One possible explanation is the tendency of these microalgae to form cell clumps, as

Table 1. Effects of temperature/time treatments on counts of *Prototheca zopfii* and *Prototheca blaschkeae*

		Prototheca zopfii genotype 2				*Prototheca blaschkeae*			
			Count, log cfu/mL				Count, log cfu/mL		
Temperature, °C	Time	Isolates, n	Mean	SE	Kill, %	Isolates, n	Mean	SE	Kill, %
62	15 min	34	1.476	0.286	75.94	28	1.933	0.145	78.94
	30 min	34	1.387	0.275	77.40	28	1.435	0.270	69.39
70	20 s	34	1.258	0.245	79.49	28	1.330	0.270	77.28
	15 min	34	0.598	0.191	90.26	28	0.920	0.223	85.44
	30 min	34	0.585	0.171	90.47	28	1.123	0.239	82.23
75	20 s	34	0.745	0.227	87.85	28	1.449	0.230	77.06
90	1 s	34	0.912	0.242	85.14	28	0.374	0.179	94.08
100	1 s	34	0.000	0.000	100.00	28	0.000	0.000	100.00

described for other pathogens by Grant et al. (2005), preventing a complete exposure to temperature of those in the center. Homogenization of the milk during the treatment (not tested) may contribute to a better inhibition process. Only recently *P. blaschkeae* was found to also be associated with mastitis (Marques et al., 2008) and study of its susceptibility to different temperature/time ratios treatments was warranted. These results stress the need for the implementation of more efficient quality control measures at both milk production and milk processing in order to reduce mastitis and milk contamination by this potentially zoonotic alga.

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CHAPTER 4

DETERMINATION AND CHARACTERIZATION OF IMMUNODOMINANT ANTIGENS

Identification of immunogenic proteins associated with *Prototheca*

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Abstract

Infectious bovine mastitis develops in reaction to infection of intra-mammary microorganisms and is considered the most common and costly disease that influences production of dairy herds worldwide. *Prototheca* mastitis is increasing over the years and can be characterized as clinical to subclinical with progress to a granulomatous infection. Since these algae are highly resistant to known chemotherapeutic agents, infected cows should be removed from the herds. Current knowledge of local and systemic humoral and cellular immune responses in *Prototheca* mastitis is limited. Thus, the identification of virulence factors is of great interest in understanding the pathogenic mechanisms of these microorganisms. Therefore the objective of this study was to determine and characterize both secreted and cellular immunogenic factors produced by these microscopic algae. *Prototheca* were submitted to different treatments in order to obtain secreted and cell associated factors that can stimulate the immune system. Using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), western blot and mass spectrometry analyses, antigenic proteins were detected in the cellular fraction, while no secreted immunogenic factors were found. The cellular proteins identified were cytosolic and, our findings constitute a first step towards the understanding of some of the pathogenic mechanisms of these algae.

Keywords

Prototheca, bovine mastitis, immunogenic factors

Introduction

Prototheca is a yeast-like genus composed of achlorophyllous algal species that has been generally accepted as the apochlorotic equivalent of *Chlorella* (1). This alga is ubiquitous and although it was rarely associated with infectious diseases in humans and animals, the number of reported cases nowadays have been increasing worldwide (2, 3). The three pathogenic species of this genus, *Prototheca wickerhamii*, *P. zopfii* and *P. blaschkeae* are associated with pathology in humans, dogs, cattle and also more rarely in cats (4-7). In humans, all species have been associated with protothecosis, being generally divided in three forms, cutaneous or subcutaneous infection, olecranon bursitis and rarely as systemic disease (8, 9). In animals, the most prevalent form of protothecosis is bovine mastitis that is associated with *P. zopfii* and also with *P. blaschkeae*. It generally occurs in a chronic subclinical or a mild clinical inflammatory process in the udder, and

affected cows do not respond to routine therapy (4, 10, 11). *Prototheca* histological lesions are characterized by interstitial infiltrates of macrophages, plasma cells and lymphocytes. Algae have been also observed in the alveolar lumen and interstitium (2).

The increasing number of bovine protothecosis all over the world threatens to be a serious economic and public health problem due to the inherent resistance of *Prototheca* (3, 12). Its survival and multiplication in the environment is due to the presence of sporopollenin in the cell wall, which is considered an extremely resistant biopolymer (10, 13). The mechanisms of the pathogenesis of this infection remain unknown, but its small pathogenicity and low incidence demonstrate that existing diseases or altered host immune response may play an important role in the onset and dissemination of the infection (14, 15).

The vertebrates innate immune system identifies invading pathogens by responding to the pathogen-associated molecular patterns (PAMPs) (16), which are groups of components (*e.g.* lipopolysaccharide (LPS) of Gram negative bacteria) at the surfaces of the pathogens (17), and also to the virulence factors released by them to the environment, such as in the case of *Fusobacterium necrophorum* that produces a leukotoxin (18). The PAMPs and secreted virulence factors are characteristic indicators of the different classes of microbes that signal the presence of microbial intruders to the immune systems of the animals. These molecules are well described for gram-negative and gram-positive bacteria and also for fungi (16, 18-20). However, for *Prototheca* no PAMPs or secreted virulence factors were yet described, mainly because of the rare occurrence of the pathogen. Some authors (15, 21) were able to detect some immunogenic whole cell antigens in *P. zopfii* and one antigen in *P. blaschkeae* (at that time designated as *P. zopfii* biotype 3), when polyvinylidene difluoride (PVDF) membranes were treated with serum and whey antibodies raised against both species. However, the authors did not further analyse or characterize these proteins, and until recently, no additional studies have been published regarding the potential virulence factors in *Prototheca*. Other studies have demonstrated *Prototheca* in their viable forms inside macrophages and neutrophils in a less degree (22, own observation), although a more recent study demonstrates that the latter cells are not effective in the phagocytic capacity of *Prototheca* (23). In spite of this controversy, if viable *Prototheca* cells can be found inside macrophages, we could speculate that this phenomenon would promote the secretion of virulence factors by the algae into the environment and promote its dissemination. Thus, due to the absence of information on virulence factors, to the increasing incidence of new cases of bovine mastitis associated with *Prototheca* and because of its high potential of dissemination and perpetuation in the environment, this preliminary study attempts to identify and characterize potential antigenic factors from mastitic algae in the genus. The detection of

immunogenic factors was attempted by different procedures using different media, incubation times, and also “cell inducers” namely macrophages/ mononuclear cells. To unravel the cellular immunogenic factors, the identification of cellular and secreted proteins of *Prototheca* was pursued.

Material and methods

Algae, growth conditions and sample preparation

The *Prototheca* isolates used in this study are from a collection of milk pathogens that belong to the Laboratory of Infectious Diseases of Veterinary Medicine from Porto University. *Prototheca zopfii* (genotype 2) and *P. blaschkeae* were retrieved under sterile conditions from milk of cows with mastitis. A *Prototheca* spp. reference strain was kindly provided by Doctor Uwe Roesler. This strain, *P. zopfii* (accession number AY973040) genotype 1 (SAG2063) was isolated from cattle liquid manure from cow barn (24). *Prototheca* strains were maintained on Sabouraud Dextrose Agar medium (Merck Laboratories, Darmstadt, Germany) and incubated at 37ºC for 42-72 hours.

Secreted immunogenic factors

Prototheca spp. strains were subjected to different procedures in order to produce sufficient extracellular associated factors. Different conditions (different containers with media at different volumes and various incubation times) were established. These were followed by using Tri Reagent LS (Sigma-Aldrich, Steinheim, Germany) for extraction of proteins, and trichloroacetic acid-acetone (TCA-A) (Merck®, Darmstadt, Germany) for precipitation. The use of mononuclear cells to stimulate the secretion of potential immunogenic factors was also tested (Table 1), as of different filtration procedures. Briefly, duplicates of each *Prototheca* strain were grown in liquid media with slow constant shaking at 37ºC during the different incubation times referred in Table 1. Some *Prototheca* cultures were co-cultured with mononuclear cells, from blood collected from healthy cows, which were earlier separated using Lymphoprep™ (Axis-Shield, Oslo, Norway) following the manufactures instructions. Cultures were maintained at 37ºC during different incubation times. After the incubation period, cells were pelleted by centrifugation (4000 rpm, during 10 minutes at 4ºC) and stored at -20ºC until further analyses. Some of the supernatants were filtered through a 0.45 µm pore-size nitrocellulose filter (Sarstedt, Nümbrecht, Germany). The supernatants were treated by Tri Reagent LS following the manufacture instructions for protein extraction with some modifications. Proteins were concentrated by precipitation with 20% TCA-A (20). The protein quantification was performed by using the Protein Assay Kit II (Bio-Rad, Munich, Germany).

Cell associated immunogenic factors

The *Prototheca* spp. pellets obtained in the previous task were subjected to different treatments in order to obtain cellular and cell wall proteins. For this objective, cell pellets were lysed in a cell disruptor Disruptor Genie® (Scientific Industries, Inc., NY, USA) with glass beads of 0.25 to 0.5 mm at 50 Hz, during 5 minutes cycles with cooling periods on ice between the cycles. This procedure was repeated until 80% of the cells had been lysed as determined by optical microscopic examination. For cytosolic proteins extraction, the cellular homogenates were centrifuged for 15 minutes at 13200 rpm and the supernatants were treated with Tri Reagent LS following the manufacture instructions for protein extraction with some modifications. For the preparation and separation of cell wall proteins the procedure previously described by Takeda (25) was followed with some modifications. Protein quantification was performed by using the Protein Assay Kit II (Bio-Rad, Munich, Germany).

Polyclonal antibodies

Hyperimmune sera directed against *P. zopfii* (genotype 2) and *P. blaschkeae* were developed in rabbits following the same procedure previously described by Roesler *et al.* (15). Rabbits were maintained in an animal house and experiments were conducted in accordance with the National (DL 129/92 6 July and the norm number 1005/92) and European Union guidelines (number 86/609/CE) for the care and handling of laboratory animals.

Sera and milk of cows with mastitis by *P. zopfii* genotype 2 and *P. blaschkeae* were also used in the immunoblotting procedure. Milk of a healthy cow was also used as primary antibody to determine the specificity of the polyclonal antibodies used.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

Up to thirty micrograms of protein were boiled in Laemmli loading buffer containing SDS and were subsequently separated on 4-15% MiniProtean® TGX™ Precast gels (Bio-Rad, California, USA) in Mini-Protean® 3 Cell (Bio-Rad, California, USA) following the manufacturer instructions. After electrophoresis, gels were stained with Coomassie Blue (Imperial Protein Stain, Thermo Scientific, Rockford, USA) for visualization of protein bands. Duplicate gels were transferred onto a Immun-Blot® PVDF membrane (Bio-Rad, California, USA) in a Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad, California, USA). After blocking during 2 hours in Tris-buffered saline-Tween (Merck®, Darmstadt, Germany) 0.05% with skimmed milk at 5%, membranes were washed and incubated

overnight at room temperature with the polyclonal antibodies against *P. zopfii* and *P. blaschkeae* produced in rabbits and also from naturally infected cows as described in the previous section. After washing, the membranes were incubated with anti-rabbit immunoglobulin G (IgG) peroxidase conjugate (Sigma, Saint Louis, USA) and with anti-bovine IgG peroxidase conjugate (Sigma, Saint Louis, USA) respectively, and proteins were visualized by colorimetric detection.

Mass Spectrometry

After the analysis of the stained SDS-PAGE gels and the PVDF membranes, several protein bands that could be of interest from the supernatants, cellular lysates and cell walls were selected. The bands were cut and stored in sterile ultra pure water until further analyses in the Proteomics Unit of the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP). For the identification and characterization of the proteins as well as the peptide MS/MS sequencing, a 4700 Proteomics Analyser Matrix-assisted laser desorption/ ionization time-of-flight/ time-of-flight (MALDI TOF/TOF) mass spectrometer (Applied Biosystems, USA) was used as previously described by Pinho *et al*. (26). Secreted proteins were identified by peptide mass fingerprint (PMF). However for cell proteins, especially cell wall protein PAMPs, in addition to PMF, proteins were identified by MS/MS peptide sequencing, in which several of the highest intensity non-trypsin peptide peaks were selected for MS/MS analysis. The mass spectra were analysed using the GPS Explorer software (Version 3.6; Applied Biosystems), which acts as an interface between the Oracle database containing raw mass spectra and a local copy of the Mascot (Matrix Science, UK) search engine software (Version 2.1.04). The obtained spectra were compared against the non-redundant National Center for Biotechnology (NCBInr) and UniProtKB/Swiss-Prot protein sequence databases and expressed sequence tags (EST) databases with the following modifications: carbamidomethyl cysteine (fixed) and oxidation methionine (variable). To be considered a match, a confidence interval (CI) calculated by Applied Biosystems GPS Explorer of at least 95% was required. Despite the high quality of most of MS and MS/MS spectra obtained, non significant CI values were considered due to the non existing information on *Prototheca* proteins that have been described or annotated in the databases. Several analyses were conducted, including Mascot searches in which Fungi taxonomy was selected, *de novo* sequencing and comparison of the experimental mass spectra data of the tryptic peaks derived from the samples with different proteins that could be possible candidates of pathogenicity by using Protein Prospector software (University of California, USA).

Results

Secreted immunogenic factors

Although several approaches were tested in order to produce and identify secreted immunogenic factors, only the most meaningful results are discussed in this paper. The containers selected for studies on the production of secreted immunogenic factors were 1L Erlenmeyers and glass bottles, and phosphate buffer was selected as the growth medium, as this did not show interferences in the subsequent analyses. The total volume of medium used, in which consistent results were obtained, was 1000 mL, and the incubation periods selected were 72 and 96 hours. From all protein extraction methods applied, low protein concentration yield was obtained only with TCA-A. Filtration with a 0.45 µm pore size filter was selected in order to remove cell contaminants from secreted factors. Jensen *et al*. (22) suggested that macrophages could contribute to *Prototheca* proliferation since viable algae were detected in these cells. Therefore, we tested the concomitant growth of *Prototheca* spp. with macrophages and this demonstrated to be the only procedure in which some detectable proteins were seen in Coomassie Blue stained SDS-PAGE gels. Proteins with 72, 55, 50 and 26 kDa could be observed in the stained gels, however, the last two were very faint (data not shown). From the PDVF membrane of the macrophage stimulated *Prototheca* immunogenic factors, only the 72 kDa protein was detected in the *P. zopfii* strains when both hyperimmune sera were applied. This protein showed, however, a more intense specific staining when hyperimmune sera against *P. zopfii* were used. The 55 and 50 kDa proteins could only be visualized in *P. zopfii* genotype 1 strain when treated with hyperimmune sera against *P. zopfii*. On the contrary, no immunogenic proteins could be demonstrated in the *P. blaschkeae* supernatants when they were treated with any of the hyperimmune sera (data not shown).

As a negative control, PVDF membranes were treated with milk of healthy cows and, as expected, did not show any band.

Cell associated immunogenic factors

In an attempt to identify and characterize new immunogenic antigens and taking into consideration previous studies by others (15, 21), *Prototheca* cytosolic proteins in combination with cell wall proteins were also analysed in this work. Cell wall proteins were considered the most important immunogenic molecules since this structure is the first contact with the host antibodies and other defence mechanisms. Several immunogenic proteins were detected in the cell wall extracts, and the selection of candidate bands was by the criteria of molecular weight bellow 135 kDa, because of a better resolution. A total of 13 common immunogenic proteins were detected in two of the *Prototheca* strains using

hyperimmune antibodies against *P. zopfii* and *P. blaschkeae* (Fig. 1). From these, only 7 proteins were equally detected using both hyperimmune antibodies when combining the three strains, and of these only one band (10 kDa) was common to both *P. zopfii* and *P. blaschkeae* (Fig. 1C). The hyperimmune sera against *P. zopfii* and *P. blaschkeae* respectively recognised proteins of 30 and of 17 kDa for both pathogenic *Prototheca* (Fig. 1 A – C, arrow). Another protein near 60 kDa was observed in *P. zopfii* strains when both hyperimmune sera were used. However, this same protein could only be detected when specific hyperimmune sera (against *P. blaschkeae*) were used in *P. blaschkeae* strains (Fig. 1 A – C, arrows). Hyperimmune sera for both *P. zopfii* and *P. blaschkeae* could specifically stain species specific proteins as shown in Table 2 and Fig. 1.

Several immunogenic bands were observed when whole cell proteins were analysed. The majority of these protein bands were common to those detected in the cell wall. When compared with the cell wall proteins, the whole cell proteins presented different antigens upon treatment with both hyperimmune sera. Thus, *P. zopfii* genotype 1 presented 77 and 46 kDa proteins when hyperimmune sera against *P. zopfii* were used. Also, a protein with 75 kDa common to both *P. zopfii* strains, a specific 80 kDa protein of pathogenic *P. zopfii*, and a 65 kDa protein for non-pathogenic *P. zopfii* (genotype 1) were detected after reaction with *P. blaschkeae* hyperimmune antibodies.

In this study it was not possible to detect all of the previously referred immunogenic whole cell proteins of *P. zopfii* 100, 90, 45, 32 and 30 kDa (15, 21) using different hyperimmune sera. Only the 100, 32 and 30 kDa proteins could be marked, but in the case of the previously described specific proteins, 51 and 37 kDa (21) for *P. zopfii* and *P. blaschkeae*, respectively, difficulties were found in detecting them with both hyperimmune sera.

The discrimination of all immunogenic proteins in the cellular fraction was difficult due to the high density of bands in all SDS-PAGE gels (data not shown). Likewise, the detection of cell wall immunogenic proteins was difficult, although fewer proteins were stained comparing with the whole cell lysates. Thus, it was almost impossible to identify the specific bands previously detected in the PVDF membrane. For the purpose of the study, some of the common antigenic bands in the cell wall with 50, 34, 32, 30 and 10 kDa, were selected for each *Prototheca* strain for mass spectrometry analyses.

For a negative control, the PVDF membranes were treated with milk of healthy cows and, as expected, were not reactive.

Mass Spectrometry

Secreted immunogenic factors. Two bands of approximately 72 and 55 kDa were identified by PMF as serum albumin and immunoglobulin of *Bos taurus* with CI of 100% and 97%, respectively. The protein bands were also detected in the negative control (macrophages alone) of the SDS-PAGE gels. No secreted immunogenic factors could be detected on the SDS-PAGE gels with the usage of described technique and we assume that the observed proteins derive from the blood of the healthy cow.

Cell associated immunogenic factors. The protein identification results from the 50, 34, 32, 30 and 10 kDa gel protein bands for each *Prototheca* strain are shown in Table 3. When MS/ MS analysis was performed for the identification of proteins, only one peptide, with some exceptions, could be determined with statistical significance (highlighted in bold, Table 4 and Fig. 2) for the immunogenic proteins. As it was not possible to obtain a good confidence value for all, in some cases the identification was performed combining PMF and MS/MS peptide sequencing. This poor identification is due to the inexistent information on *Prototheca* and closely related organisms proteins described or annotated in the databases.

The proteins that were not identified with statistical significance or that presented a low CI value were further analysed by comparison of the tryptic digestion peaks derived from the MALDI-TOF/TOF analysis with several proteins that could be possible candidates by using the Protein Prospector software. These proteins were selected by localization, coverage percentage and organism (should be green plant or yeast). From these, the 10 kDa proteins did not retrieve any possible identification for all tested strains, but the 30 kDa protein of *P. blaschkeae* and *P. zopfii* genotype 1 presented some similarity with a multicopper oxidase integral membrane protein present in the cell surfaces of *Saccharomyces cerevisiae* with 27 % identity and “e value” of 6.7 X 10^{-31} and 31 % identity and “e value” of 1.3 X 10^{-36} respectively. In the case of the 32 kDa protein of *P. zopfii* and *P. blaschkeae*, was observed some similarity with a phospholipid synthesis protein (present in membranes) from *Zea mays*, with a confidence interval greater than 56 % for the ion score. Even with this analysis, no further proteins could be properly identified. Thus, of all not determined proteins and of the ones that presented CI < 60 %, *de novo* sequencing was performed, using the DeNovo Explorer software (Applied Biosystems, USA), in order to have sequences that could help in further identification. The sequences retrieved from *de novo* sequencing analysis are present in Table 5. The basic local alignment search tool (Blast) of these sequences in the protein databases did not present additional information in the identification and characterization of proteins. The

main reasons for this were the size of the sequences (very small) and the inexistent information of *Prototheca* or other closely related organism in the databases.

Discussion

Bacteria and fungi are well known to secrete and to have at the cell wall several virulence factors (16, 19, 20). Therefore, this study intended to determine the immunogenic factors present in *Prototheca*, one of the few known pathogenic algae. *Prototheca* spp. does not secrete immunogenic factors at detectable concentrations with the techniques applied. Although no proteins were identified by SDS-PAGE, it was possible to observe some immunogenic proteins that reacted with polyclonal antibodies against *P. zopfii* and *P. blaschkeae* in the PVDF membranes. Thus, other techniques, such as massive growth conditions in liquid media, fraction separation in fast protein liquid chromatography and several concentration procedures by ultrafiltration, should be carried out to allow the production of sufficient concentrations of detected immunogenic proteins in the supernatants.

It can be speculated that *Prototheca* immunogenic factors are mainly present in the cell, rather than in the extracellular environment, and can promote phagocytosis by macrophages. Studies have been performed in order to understand the role of cellular immunity in the defence mechanism against *Prototheca* infection, but some controversies persist. Some authors refer that human neutrophils ingest and kill *P. wickerhamii* (27). Others showed that susceptibility to protothecosis could involve an inability of polymorphonuclear leukocytes (PMNs) to effectively eradicate phagocytised organisms (23, 28). Therefore, more studies are needed to correctly identify the relationship between *Prototheca* and leukocytes. It is known that antibodies can opsonize *Prototheca* cells, because during protothecosis, IgG and IgA could be detected in serum and whey of infected cows (3). However, the mechanisms by which this process occurs were never studied, likely because of the lack of information on cell surface antigens.

Based on these facts, this study intended to identify the presence of cellular associated immunogenic factors with a main focus on the cell wall. On *Prototheca* this is a rigid structure with high tensile strength and considerable forces are required to produce an efficient breakage. This structure is resistant to acid and alkaline hydrolysis, enzymatic degradation and acetolysis, and does not contain glucosamine, cellulose, chitin, nor muramic acid and arabinose, but contains galactose and mannose (6, 29, 30). This resistance is due to the presence of sporopollenin that is a highly resistant biopolymer known to build up the outer wall of spores and pollens, and is originated from the oxidative polymerization of carotenoids (31, 32). Quantitative analyses of amino acid composition of

the cell wall of *Prototheca* demonstrated that there are 12 major and 5 minor components, with the most common abundant amino acids detected being arginine followed by alanine and serine (33). Little is known about its cell wall immunogenic potential. Although the existence of a LPS like molecule (algal LPS) in *Prototheca* has been shown (34, 35), these molecules do not stimulate the defence reaction mechanism of mammals (34) as the LPS of gram-negative bacteria are known to do (36, 37). This study also intended to determine immunogenic molecules present at the cell wall of *Prototheca* and, as for cellular proteins, a great amount of immunogenic proteins could be observed. Thus, immunogenic proteins present at the cell wall were an easier way of selection for MALDI-TOF/TOF analysis. All of the identified immunogenic proteins were cytosolic proteins (38-44), demonstrating that the huge force applied during large periods to break the rigid *Prototheca* cell wall, could have destroyed or even degraded the proteins present in this fraction. Nevertheless, when performing a Blast search in the *Candida albicans* database, some of these proteins, elongation factor (45), glyceraldehyde-3-phosphate dehydrogenase (46) and also ATP synthase (19) were found at the cell surface of this pathogen and are defined as being antigenic during infection. Although *Prototheca* is more closely related to green plants than to yeast, the pathogenic behaviour of these algae could be more close to yeast. However, localization studies should be performed in order to correctly identify these proteins within *Prototheca* cells, because they were marked as being immunogenic to hyperimmune sera against these algae.

The 30 and 32 kDa proteins that did not retrieve identifiable results or that presented low CI identification were similar to proteins present at the cell surface of *S. cerevisiae* (multicopper oxidase) (47) and of *Z. mays* (phospholipid synthesis) (48), respectively. To confirm these results further localization analyses of these proteins should also be performed.

Further experiments are currently ongoing in our laboratory in order to determined cell surface antigens. This preliminary study was able to open the perspectives on the discovery of novel immunogenic proteins for *Prototheca* that could be used for future therapeutic models.

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Figures

Figure 1. Western blot of cell wall extracts of *Prototheca* strains treated with hyperimmune antibodies against *P. zopfii* genotype 2 (A) and *P. blaschkeae* (B, C). A and B were treated with milk of naturally infected cows, and C with serum produced in rabbits. MW – marker spectra^{TM} multicolour broad range protein ladder, (Fermentas, St. Leon-Rot, Germany); CWPzg1 – cell wall *P. zopfii* genotype 1; CWPb – cell wall *P. blaschkeae*; CWPz – cell wall *P. zopfii* (genotype 2). Arrows – immunogenic proteins of 17 (C), 30 (A) and 60 (B, C) kDa.

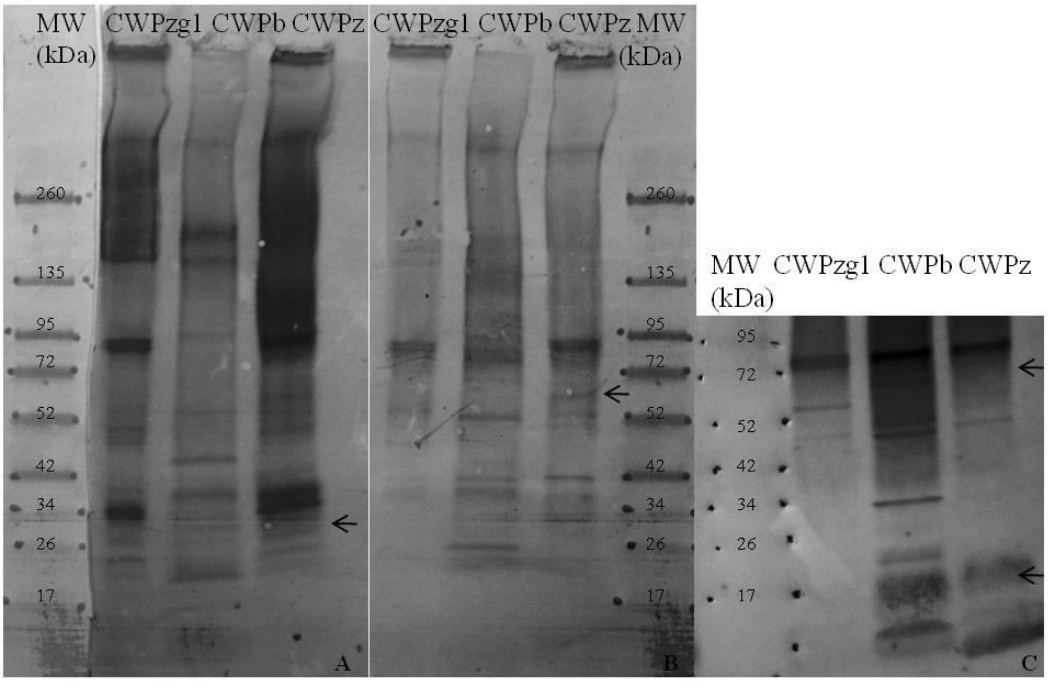
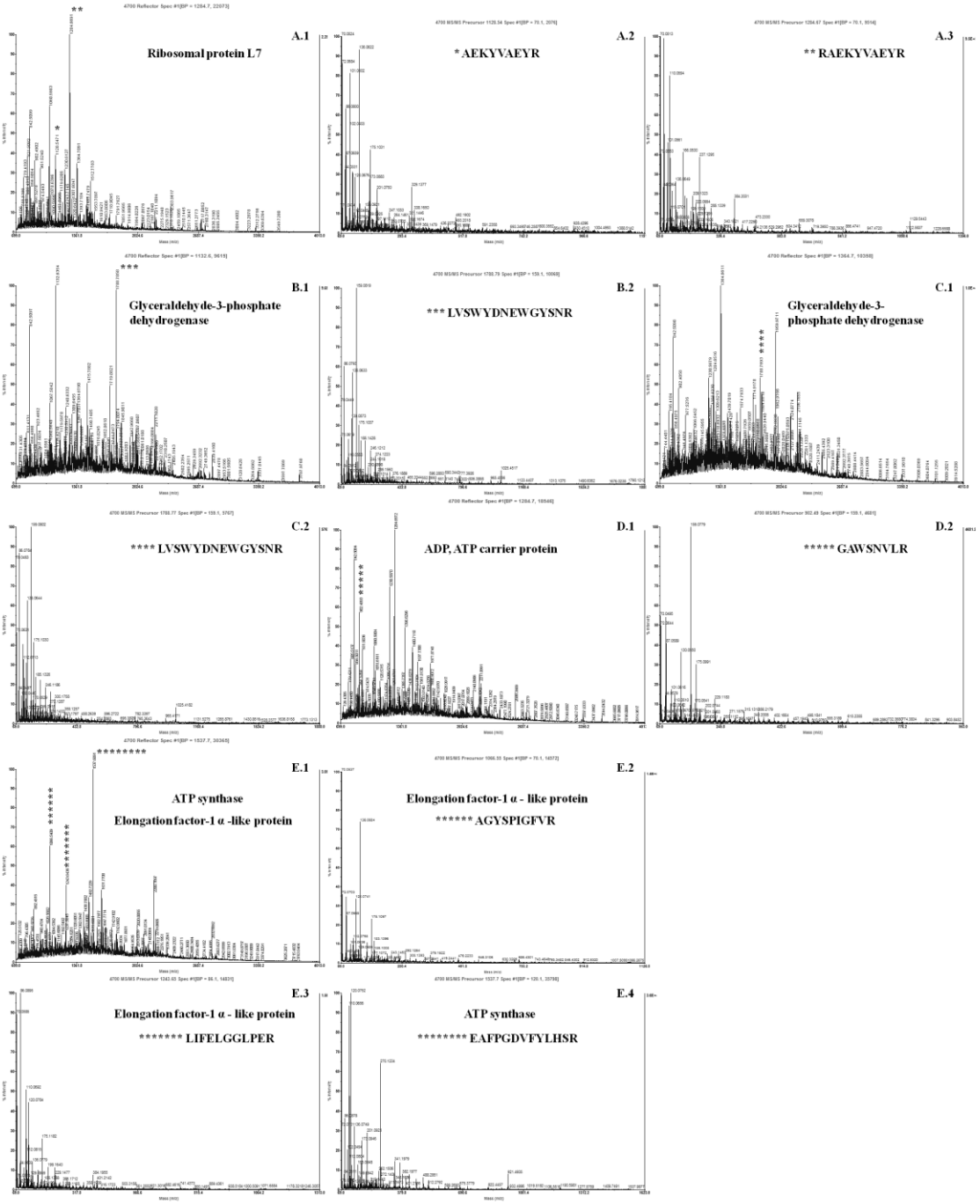


Figure 2. MS and MS/MS spectra of the identified proteins derived from the cell wall preparations of *Prototheca*: A – Ribosomal protein L7, *P. zopfii* genotype 2; B – Glyceraldehyde-3-phosphate dehydrogenase, *P. zopfii* genotype 2; C – Glyceraldehyde-3-phosphate dehydrogenase, *P. blaschkeae*; D – ADP, ATP carrier protein, *P. zopfii* genotype 1; E – ATP synthase and Elongation factor-1 α-like protein, *P. zopfii* genotype 1. MS MALDI-TOF spectra – A.1, B.1, C.1, D.1, E.1. MS/MS MALDI-TOF/TOF spectra of tryptic fragments of several precursor ions: 1128.5 Da (A.2), 1284.6 Da (A.3), 1788.7 Da (B.2), 1788.7 Da (C.2), 902.4 Da (D.2), 1066.5 Da (E.2), 1243.6 Da (E.3), 1537.7 Da (E.4) of the identified proteins. ***** – ************ represent selected peak for MS/MS fragmentation. Note: MS and MS/MS spectra of ATPase F1 α subunit and elongation factor-1 α-like protein from *P. zopfii* genotype 2 were similar to the corresponding proteins from the *P. zopfii* genotype 1.


A.1
Ribosomal protein L7
A.2
* AEKYVAEYR
A.3
** RAEKYVAEYR
B.1
Glyceraldehyde-3-phosphate dehydrogenase
B.2
*** LVSWYDNEWGYSNR
C.1
Glyceraldehyde-3-phosphate dehydrogenase
C.2
**** LVSWYDNEWGYSNR
D.1
ADP, ATP carrier protein
D.2
***** GAWSNVLR
E.1
ATP synthase
Elongation factor-1 α -like protein
E.2
Elongation factor-1 α - like protein
****** AGYSPIGFVR
E.3
Elongation factor-1 α - like protein
******* LIFELGGLPER
E.4
ATP synthase
******** EAFPGDVFYLHSR

TABLES

Table 1: Procedures used to generate *Prototheca* secreted immunogenic factors.

Parameters tested							
Container	**Media**	**Volume (mL)**	**Incubation time (hours)**	**Filtration (µm)**	**Protein extraction**	**Precipitation**	**"Inducers"**
Falcon 50mL	Water	50	24	No	No	No	No
T25 culture flasks	Phosphate buffer	100	48	0.45 filter	Tri Reagent LS	TCA-A	Macrophages / Mononuc. cells
Erlenmeyer 1L	RPMI	500	72				
Glass Bottle 1L	Sabouraud broth	1000	96				
			168				

Mononuc. cells – mononuclear cells

Table 2. Specific immunogenic proteins determined for the *Prototheca* strains when hyperimmune antibodies against *P. zopfii* genotype 2 and *P. blaschkeae* were used.

Prototheca	**Hyperimmune antibodies against *P. zopfii* genotype 2**	**Hyperimmune antibodies against *P. blaschkeae***
P. zopfii genotype 2	100, 70 and 42 kDa	
P. blaschkeae	95, 44 and 28 kDa	95, 70, 44, 26 and 24 kDa
P. zopfii genotype 1 (non-pathogenic)	90 kDa	

Table 3. Identification of proteins of interest for each *Prototheca* strain.

Bands	***Prototheca* strains**		
(kDa)	***P. zopfii* genotype 2**	***P. blaschkeae***	***P. zopfii* genotype 1**
10	ND	ND	ND
30	Rib. Prot. L7 (CI > 97%)	ND	Some peptides related with: • Glutamyl-tRNA reductase 1 (CI > 58%) • ADP, ATP carrier protein (CI IS > 52%)
32	ND	Some peptides related with: • ATP synthase subunit b (CI > 45%)	• ADP, ATP carrier protein (CI > 99%) • Glutamyl-tRNA reductase 1 (CI > 82%)
34	GAPDH (CI > 85%)	GAPDH (CI > 99%)	ND
50	• ATPase F1 α subunit (CI > 97%) • Eukaryotic translation elongation factor 1 α (CI > 40%, with IS > 96%)	ND	• ATP synthase/ F1-ATPase α subunit (CI > 99%) • Elongation factor-1 α-like protein (CI > 99%)

Rib. Prot. – Ribosomal protein; GAPDH – Glyceraldehyde -3-phosphate dehydrogenase; ND – not determined; IS – ion score; CI – confidence interval

Table 4. Immunogenic proteins identified from the cell wall preparations of *Prototheca* spp. Highlighted in bold – peptides with a significant ion score.

Prototheca	**Bands (kDa)**	**Sequence**	**Peptide molecular mass (Da)**	**Protein name**
***P. zopfii* genotype 2**	30	**AEKYVAEYR**	**1128.5**	Ribosomal protein L7
		RAEKYVAEYR	1284.6	
	34	**LVSWYDNEWGYSNR**	**1788.7**	Glyceraldehyde -3-phosphate dehydrogenase
	50	**EAFPGDVFYLHSR**	**1537.7**	ATPase F1 α subunit
		AGYSPIGFVR	**1066.5**	Eukaryotic translation elongation factor-1α
P. blaschkeae	34	**LVSWYDNEWGYSNR**	**1788.7**	Glyceraldehyde -3-phosphate dehydrogenase
***P. zopfii* genotype 1**	32	**GAWSNVLR**	**902.4**	ADP, ATP carrier protein
		IGEDNLNK	902.4	Glutamyl-tRNA reductase 1

Table 5. *De novo* sequencing analyses of the *Prototheca* strains proteins that were not identified with statistical significance. a) *P. zopfii* genotype 2; b) *P. blaschkeae*; c) *P. zopfii* genotype 1.

a) *P. zopfii* genotype 2

Prototheca	**Mol. mass (kDa)**	***de novo* sequencing**		
		Peptides mol. mass (Da)	**Sequence**	**Score (%)**
***P. zopfii* genotype 2**	10	921.5	AGVPYTTLT	50.25
		953.4	KSASAM*GSPT	59.33
		1060.5	ALNTM*GNAPR	63.75
		1397.6	APNHSSEYHKGAT	62.83
	32	1060.5	VLDAAAVM*VR	56.84
		1230.5	VGGSAAAM*GPLGAR	66.68
		1284.5	VHM*SGGEKLGPR	67.86
		1364.6	VTAEKGLASM*WR	75.36
		2018.0	AGDSGLPLYHGGTVENM*ALT	67.94

Mol. mass – molecular mass; M* – methionine oxidation

b) *P. blaschkeae*

Prototheca	Mol. mass (kDa)	*de novo* sequencing		
		Peptides mol. mass (Da)	Sequence	Score (%)
P. blaschkeae	10	765.4	PHWGLR	57.54
		921.5	KLPHGGVLT	66.77
		936.5	KNASYRPT	57.81
		1060.5	ALSAM*GGNAPR	73.69
		1250.6	APSHGMASLAGPR	60.92
	30	1055.5	ALSAVAPSHTT	57.22
		1284.6	VVTGATDAGLNAVP	51.98
		1364.7	AEAAYC*LGVTGPR	66.89
		1960.0	ASLPRSTTWHC*NYPAVT	73.36
		2124.1	ALEPSHTSEC*TLKNAYM*R	81.94
	32	1284.6	LLAAVTGSSALKPG	63.47
		1364.7	TTGALM*MGLNSVGP	65.11
		1714.9	LAGDLADKM*KTVVAGNP	62.42
		1959.9	LM*ANKHVGNEAAEMTGGLT	63.51
		2196.2	AGSAALKVGPAVALSLKAASEER	76.43
	50	1165.5	KALNHGPGKAAT	64.48
		1537.7	KALNHGPGKAAT	64.48
		1631.8	VTLVGRPAAGVNNHAR	78.80
		2199.7	AGGAVLLHPKALATDNTMHEGP	54.84

Mol. mass – molecular mass; C* – cysteine carbamidomethyl; M* – methionine oxidation

c) *P. zopfii* genotype 1

Prototheca	Mol. mass (kDa)	*de novo* sequencing		
		Peptides mol. mass (Da)	Sequence	Score (%)
***P. zopfii* genotype 1**	10	838.4	KAVLGPPR	67.97
		921.5	KAHPVRLT	61.34
		953.5	KTGNHGAAPT	60.68
		1267.7	KGPANLAVKTLR	82.60
	30	902.5	KASVNNLR	65.95
		968.5	KLTPVYFT	54.62
		1060.6	KLGGNAVM*PGT	62.48
		1284.6	VHM*LAGSKDGPR	66.33
		1630.8	ASVLAANVFDAPSVLR	64.96
		2140.1	AGGGALSKRTVLHKPTDLYR	80.71
	34	1132.6	VSLMPGGLLSGT	61.53
		1365.7	VTPTSYLTTVVGK	62.06
		1407.6	KAVNGGTLAKVPPR	78.05
		1561.7	KSSRPYKAVAADLR	77.21
		1788.7	KGGDKYDFLVSGYSPR	84.56

Mol. mass – molecular mass; M* – methionine oxidation

CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

GENERAL DISCUSSION

This dissertation contains five published papers, two manuscripts submitted for publication and one preliminary study in preparation for publication in international Journals. Therefore, the detailed discussion of the experimental work is presented in each publication. In this chapter a general discussion with the overall observations and conclusions obtained is presented.

Prototheca algae are resistant and widely distributed on variable environments (1-3). These algae are known to be associated with diseases in humans and animals (4-7). The only pathogenic species of this genus are *P. zopfii*, *P. wickerhamii* and *P. blaschkeae* (1, 8-11). Although *P. wickerhamii* is mainly isolated from human infections and *P. zopfii* causes predominantly infections in animals, both species are known to infect humans and animals (1-3, 12). The same can also be referred to *P. blaschkeae*, the most recently described pathogenic species, related to a case of human onychomycosis (13) and to bovine mastitis as shown in our investigation (4, 14).

The most prevalent form of protothecosis in animals is bovine mastitis and although *Prototheca* are considered environmental agents, they also have contagious behaviour, since cow to cow transmission can occur (3, 4, 7, 15-17). Presently, this pathology is recognized as endemic worldwide and as a public health problem (2, 18). In Portugal and all over the world, *Prototheca* bovine mastitis incidence is steadily increasing and is gaining more economic importance, because its clinical and subclinical forms result in substantial loss and reduced quality of milk production (3, 4, 7, 10, 14, 19-21). Although protothecosis has low infectivity, the number of studies on its identification, characterization, and susceptibility to different physical and chemical agents reflect the importance that *Prototheca* have been achieving during the years all over the world (14, 19, 20, 22-27). The increasing significance of this pathogen and its global distribution urge the implementation of fast and efficient diagnostic and control measures.

Therefore, the first chapter of this thesis includes the state of the art on *Prototheca* spp. and associated pathologies focusing on the epidemiologic, diagnostic and therapeutic aspects of the infections by these algae.

The second chapter describes the phenotypic and molecular characterization of *Prototheca* isolates from bovine mastitis. Within the phenotypic characterization the most important assimilation patterns were similar to previous studies (6, 10, 28, 29). However, in addition to biochemical characterization of the isolates, another main objective of this

research was to identify additional discriminative phenotypic characteristics of the *Prototheca* strains isolated in the northern region of Portugal by means of three identification systems (API 20C Aux, BBL Crystal Enteric/ Nonfermenter and BBL Crystal Gram positive) and analyses using InforBio software (19). These analyses allowed the determination of three additional discriminative phenotypic characters, citrate, phosphorylcholine and α-arabinoside. Additionally we observed that caution should be taken when analysing biochemical assimilations, because these techniques, when used alone, can misidentify some of the *Prototheca* species. The most relevant problems are due to difficulties of interpreting the results, contamination, and also false assimilation patterns when prolonged incubation times are needed, as required for these algae. Thus, phenotypic identification should always be used simultaneously with other more straightforward identification techniques such as molecular analysis. The results of this research clearly demonstrated that the phenotypic characterization alone was not enough for the identification of some isolates. The molecular identification of the mastitic *Prototheca* isolates intended to elucidate the epidemiology of bovine mastitis associated with these algae (4).

As it is well known, ribosome structure is strictly conserved. Sequences of the deoxyribonucleic acid (DNA) of the ribosome small subunit (SSU) are important and useful molecular clocks for quantifying evolutionary relationships between organisms (30). Generally, the rate of base substitutions, insertions or deletions in various regions of the ribosomal ribonucleic acid (rRNA) gene is not uniform. Within the ribosome some areas are highly conserved and unchanged through millions of years, some are highly variable and others are semi-conserved (31). Thus, these regions are interesting for species identification and phylogenetic analyses, because they develop with a low intraspecific polymorphism and a high interspecific variability (32). *Prototheca* molecular characterization and phylogenetic analysis are usually performed by genotype specific PCR and amplification of the SSU and large subunit of rDNA (7, 13, 24, 33-36). Therefore, the molecular characterization of *Prototheca* field isolates was performed by amplification of the SSU of rDNA through polymerase chain reaction and subsequent restriction analysis with *Hae*III endonuclease (4). *P. zopfii* genotype 2 was found to be highly incident in Northern Portugal, and for the first time *P. blaschkeae* was shown to be associated with bovine mastitis (4). Secondary structure models of the 18S rRNA genes for each species were constructed to improve the alignment of the data set (please refer to addendum). These structures are important for the formation and function of the ribosomes (37), and depend on Watson-Crick and wobble base pairing interactions between rRNA bases. Their use in phylogenetic analyses is considered to be of great importance. Selection for

maintenance of complementary bases in base-pairing regions may interfere with the pattern of independent mutations that provide useful systematic characters. Secondary structure models for the two species identified in this work are shown in the addendum. The phylogenetic analysis of the isolates grouped them in the *P. zopfii* and *P. blaschkeae* clades. Our isolates showed elevated similarity with the representative species sequences of these clades. A high similarity of the *P. zopfii* isolates to *P. zopfii* var. *hydrocarbonea* was also observed. Thus, further studies on the fermentation pattern of the mastitic isolates should be performed, because high variability of fermentation pattern of *P. zopfii* strains has already been described (38). Nevertheless, our isolates were considered to belong to *P. zopfii* genotype 2, because as confirmed in this study, this is the strain generally associated with bovine mastitis (7). Since our first description of *P. blaschkeae* associated bovine mastitis, more cases of bovine mastitis by this species were reported (14, 24), suggesting that our work (4) alerted the community to consider this species as potential pathogen of cattle. Also in our study, the use of restriction analyses of the 18S rDNA proved to be a fast and reliable method to efficiently discern between infectious *Prototheca* species (4).

Since other ribosomal DNA regions are generally used in yeast and fungal identification (39-42), we also amplified the internal transcribed spacer (ITS) region of our *Prototheca* isolates in a preliminary work to obtain a fine resolution of the strains. This region was only successfully amplified for three *P. zopfii* isolates, while the sequences of other isolates showed similarity to plastid rDNA. The plastid 16S rDNA, intergenic spacer (IGS) and partial 23S rDNA of the isolates were also amplified. These regions showed variability within and between species, since they are known to be less conserved than the 18S rDNA. The resolution of the ITS amplification problem and subsequent sequencing of this region together with the plastid rRNA operon of all isolates should be of great interest for *Prototheca* spp. population genetics and epidemiology (please refer to addendum for secondary structures of the plastid small subunit rRNA and alignments of the plastid ribosomal RNA operon).

At the moment, the differentiation of *Prototheca* species in clinical practice is not important. In the future their identification and characterization may help to understand the infection, pathogenicity, and possibly the implementation of species directed therapeutics. Unfortunately, the use of some common molecular techniques for the genotyping of *Prototheca*, such as universal primers or Random Amplification of Polymorphic DNA (RAPD), is still rare (7), maybe because these techniques have poor discriminatory power and reproducibility. However a new real time PCR technique, was recently developed for a fast and reliable identification of these algae (25), revealing their increasing importance

in the scientific community. Therefore, the use of new molecular techniques (RAPD, microsatellites, karyotyping, real time PCR), the improvement of existing ones (RFLP, genotypic-specific PCR), in combination with phenotypic characterization, should be simultaneously performed in the future to improve the typing procedure of this potential zoonotic agent.

Prototheca algae are extremely resistant, and are widely distributed on different environments (1-3), due to the sporopollenin present in the cell wall. They have elevated dissemination and perpetuation on the environments and have been isolated from variable pH values and salt concentrations as well as from water treated with chloride and from pasteurized milk (20, 43-46).

Previous studies on *P. zopfii* var. *hydrocarbonea* demonstrated that this species tolerates pH values of 3 to 9 and salinities from 8 to 24% (43, 46). No studies were performed for pathogenic *Prototheca* associated with bovine mastitis, *P. zopfii* genotype 2 and *P. blaschkeae*. Bearing this in mind, we have for the first time tested the resistance to pH and salt concentration of our isolates, as described in the third chapter of the thesis (20). Generally, algae grew at all pH values (1 to 12), but more significantly between 5 and 9. The only pH that completely inhibited *P. zopfii* growth was pH 3 (acetic acid). However, there is no explanation for this phenomenon, because *Prototheca* cell walls are known to be extremely resistant to acid hydrolysis (6, 34, 47). The only reasonable explanation seems to be that the susceptibility is due to the agent (acetic acid) and not the pH value. *P. blaschkeae* strains show higher susceptibility to all pH values except for pH 3 when compared to *P. zopfii*.

P. blaschkeae (until 18%) was more resistant than *P. zopfii* (until 9%) to increasing salt concentrations. Although, growth reduction was observed for all isolates analysed (20). We can only speculate that *P. blaschkeae* have a higher amount of sporopollenin in its cell wall compared to *P. zopfii*. Future studies should address algae cell wall composition. Also, it can be stated that the pathogenic *P. zopfii* are more susceptible to high salt concentrations, justifying their temporary presence in aquatic systems (48). This study was the first to demonstrate pH and salt susceptibility of *Prototheca* spp. isolated from bovine mastitis. Further studies using more isolates from different regions should be performed in order to establish *Prototheca* susceptibility to these agents. A preliminary study showed that borate buffer at pH 9 is able to inhibit *Prototheca* growth with time. Complete growth inhibition was observed for *P. zopfii* strains after one week. Therefore, future studies should be performed using more strains, higher concentrations of this agent and different pH values to determine if its real efficacy is due to the agent itself or the pH value or both.

A previous study (48) about chlorination showed, that *Prototheca* were resistant to standard chlorination, and this contributed to its persistence in domestic and municipal sewage, from which the algae could return to the environment. However, a more recent study (49) demonstrated that a commercial solution that contains chlorine is efficient in eliminating *P. zopfii* growth and this can be effectively used in the environment. These studies demonstrated that the testing and application of new disinfectants can aid in the elimination of *Prototheca* algae in the environment and therefore reduction of their ubiquity.

Melville *et al*. (44) analysed *P. zopfii* susceptibility to different pasteurization temperature/ time ratios. The *P. blaschkeae* susceptibility to different temperature/ time ratios compared to the demonstrated for *P. zopfii* (45) are also described on chapter 3 of this thesis. The results suggested that *P. blaschkeae* may be more resistant to heat treatment than *P. zopfii*. Cell wall resistance could be the explanation as previously suggested. Therefore, a cell wall analysis is warranted to completely determine its composition, especially in the sporopollenin amount of *P. blaschkeae*. Both temperature susceptibility studies found that *P. zopfii* strains isolated from mastitic milk were resistant in at least one of the thermal treatment tests (62–65 ºC/30 min, 72–75 ºC/15 s and 72–75 ºC/20 s). Furthermore, our study showed that at pasteurization temperatures (62 ºC and 70 ºC), the increase of exposure time from 15 minutes to 30 minutes did not increase significantly growth inhibition in both species. One possible explanation is the tendency of these microalgae to form cell clumps, as described for other pathogens by Grant *et al*. (50), preventing a complete exposure to temperature of those in the centre. Therefore, homogenization of the milk during the treatments may contribute to a better inhibition process and should be tested in further studies. Our study also determined that both species were affected by temperature increase and that 100ºC/ 1 second was the only treatment capable to cause complete growth inhibition. This indicates that ultra-pasteurization is the only industrial procedure to ensure that the milk from endemic regions is free of these potential zoonotic agents.

Prototheca as a cause of mastitis in dairy cows depends on predisposing factors, such as bad management and poor maintenance of dairy herds, humid and dirty environment, bad milking management and hygiene, drug infusions with insufficient hygiene, and cow associated factor like age and previous mastitis (2, 27, 51, 52). In well managed herds this infection is often sporadic (15). Infection causes acute to chronic granulomatous mastitis, leading to reduction of milk production and atresia of the udder. Intermittent shedding and persistent infection generally occur due to its invasive capacity and survival in mononuclear cells (9, 53). Thus, routine mastitis therapy is not able to eliminate

Prototheca from the udder of infected cows. Therefore, the only control measurements that proved to have efficacy after identification of infected animals were their separation and drying of the teat when only one quarter is affected or culling of the cow when more quarters are affected (9, 21, 28, 44, 54). Nevertheless, the search for the discovery of new fast and efficient therapeutic agents can delay the transmission and dissemination of the algae between cows and within the udder and environment. The understanding of the pathogenic and immunologic mechanisms of the infection is of great importance to develop new therapeutic strategies, since great flaws in the investigation of *Prototheca* are found (9, 12). No standard treatment for protothecosis exists in the medical literature and the antifungal agents are not always efficient. Nevertheless, some of them demonstrated efficacy, such as amphotericin B, ketoconazole, or the combination of amphotericin B and tetracycline, and surgical excision (5, 12, 55, 56).

The work presented in chapter 4 describes the identification of antigenic factors associated with these algae, in an attempt to understand their pathogenic mechanisms and to propose therapeutic models that could be used in animals and eventually adopted for humans. These attempts were performed for *P. zopfii* and *P. blaschkeae* by concentration of proteins with trichloroacetic acid-acetone (TCA-A), electrophoresis, western blot and mass spectrometry analyses.

No detectable secreted proteins by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were present even when concentrated thousand times. However, this does not mean that secreted immunogenic factors are absent, because in the polyvinylidene difluoride (PVDF) membranes some immunogenic proteins were observed after their treatment with polyclonal antibodies against *P. zopfii* and *P. blaschkeae*. They can be present at extremely small concentrations or in insoluble mixtures, and therefore not detected by the techniques used. Thus, massive growth in liquid media, fraction separation in fast protein liquid or gel filtration or even ion exchange chromatographies and several concentration procedures by ultrafiltration, should be tried to improve the production and detection of secreted immunogenic proteins.

The results of this study suggest that *Prototheca* immunogenic factors are mainly cellular rather than secreted. Previous studies (2, 29) have determined several immunogenic whole cell proteins with 100, 90, 45, 32 and 30 kDa for *P. zopfii*. These were marked when membranes were treated with polyclonal antisera against *P. zopfii* genotype 1 and 2 and *P. blaschkeae*. Specific antigens, 51 and 37 kDa for *P. zopfii* genotype 2 and *P. blaschkeae*, respectively, were also detected by all hyperimmune sera. A common antigen of 30 kDa was present in all of *P. zopfii* genotypes and in *P. blaschkeae* (2, 29). In Western Blot analyses of our study several immunogenic whole cell and cell wall extract

proteins were detected with polyclonal antibodies against *P. zopfii* genotype 2 and *P. blaschkeae*. Determination of cell wall immunogenic proteins was one of the goals of this study, because this structure can directly interact with immunological cells and inflammatory factors. Although the detection of all previously referred proteins was not possible in this study, selection of some cell wall proteins with approximated molecular weights was performed. Ribosomal protein, glutamyl-tRNA reductase 1, ADP/ATP carrier protein, ATP synthase, elongation factor and glyceraldehyde-3-phosphate dehydrogenase were detected by MALDI-TOF/TOF analyses. All these proteins are known to be localized in the interior of algae and plants, but some of them, elongation factor, glyceraldehyde-3-phosphate dehydrogenase and also ATP synthase in the yeast, could be observed on the cell wall and have immunogenic properties (57-59). Localization studies should be conducted in order to correctly identify immunogenic proteins within *Prototheca* cells, because they were marked as being immunogenic to hyperimmune sera against these algae.

CONCLUSIONS

In order to understand infectious diseases we need to study the microorganism involved, learn about the disease pathogenesis and investigate associated cellular and molecular immune responses and host-pathogen relationships.

Since *Prototheca* has an elevated resistance to several therapeutic drugs, the most promising approach to reduce its incidence, is to improve the prevention and control measures on dairy herds, alert the clinicians (medical and veterinary) for the significance of this pathogen, and include it on the differential diagnosis of several diseases, especially when the diagnosis is difficult and when immunocompromised patients are involved. The diagnostic laboratories should also be alerted for this potential zoonotic agent, because it is simple to misdiagnose or under-diagnose some cases of protothecosis. Studies should be implemented that correlate the *in vitro* with the *in vivo* data for the improvement of diagnosis and disease treatment. The macroscopic and microscopic identification and assimilation patterns should be done simultaneously with the molecular techniques to achieve an adequate identification of *Prototheca*.

In the present thesis, studies on the phenotypic and molecular characterization of *Prototheca* species isolated from bovine mastitic milk, as well as determination of the susceptibility of isolates to physical and chemical agents and the intention to determine virulence factors associated with these infections were presented and discussed in the light of current knowledge, allowing the following conclusions to be made:

- The exclusive use of morphologic characteristics and biochemical assimilation patterns of different substrates for *Prototheca* spp. identification is not appropriate at present. The identification of species should always combine several phenotypic and genotypic features to characterize *Prototheca* taxa. The determination of new phenotypic characteristics facilitates the characterization of these algae. The use of new bioinformatics software can help on the identification of new characteristics to improve the characterization of *Prototheca*.

- The molecular analyses of the 18S rRNA genes demonstrated that some of our isolates were identified as *P. blaschkeae*. This was the first time that *P. blaschkeae* was shown to be associated with bovine mastitis. Most of our isolates belong to genotype II of *P. zopfii*, which was not unexpected, as all algae associated with bovine mastitis have been identified as *P. zopfii*

genotype 2 and are considered to be its causative agent. The restriction analysis of amplified rDNA allowed a fast screening of large numbers of isolates for the detection of different infectious species. The sequence analysis of other rDNA regions such as ITS and plastid rDNA operon suggest that the usage of these regions can help on *Prototheca* molecular characterization.

- Generally, *Prototheca* spp. can survive at all pH values and in several salt concentrations. *P. zopfii* was found to survive and propagate in environments with pH values between 5 and 12, and also until 9% of salt concentrations. However, *P. blaschkeae* showed more susceptibility to pH buffers, multiplying at pH values between 3 and 12. Higher resistance to salt concentrations with survival until 18% was found for this species. Borate buffer at pH 9 generally inhibited *Prototheca* growth. This is a contribution to the understanding of some physiologic characteristics of these algae that may explain its capacity to survive and perpetuate in different environmental conditions.
- *P. blaschkeae* susceptibility to different temperature/ time ratios was analysed for the first time and compared to the determined for *P. zopfii*. The former appears to be more resistant to the temperature treatments tested than the latter. The only treatment capable of completely inhibit both species was 100ºC/ 1 second.
- With the applicable techniques, *Prototheca* immunogenic factors appear to be mainly present in the cell rather than in extracellular environment. MALDI-TOF/TOF analyses allowed the identification of several immunogenic proteins: ribosomal protein, glutamyl-tRNA reductase 1, ADP/ATP carrier protein, ATP synthase, elongation factor and glyceraldehyde-3-phosphate dehydrogenase.

FUTURE PERSPECTIVES

The work presented in this thesis allowed the identification of several issues that may be investigated in the future:

1. The use of new bioinformatics tools can improve the characterization of *Prototheca* spp. by integrating the phenotypic and molecular characterization, and improving the determination of phylogenetic relationships.
2. The identification and characterization of *Prototheca* spp. will promote the understanding of their infection, pathogenicity and the implementation of new therapeutics directed to species. The use of new molecular techniques (RAPD, microsatellites, karyotyping, real time PCR), the improvement of existing ones (RFLP, genotypic-specific PCR), together with phenotypic typing will improve the typing procedure of this potential zoonotic agent.
3. Further investigations using isolates from different regions should be performed to determine *Prototheca* spp. susceptibility to different physical and chemical agents. Our results stress the need for the implementation of more efficient quality control measures at both milk production and milk processing, in order to reduce mastitis and milk contamination by this potentially zoonotic alga. Nowadays, ultra-pasteurization is the only industrial procedure to ensure that the milk from endemic regions is free of these agents.
4. The determination of immunogenic proteins from *Prototheca* can allow the development of control and therapeutic models with low secondary effects to humans and animals. Further experiments are currently ongoing in our laboratory to determine cell surface antigens. Our preliminary study on the determination of immunogenic factors was able to open the perspectives on the discovery of novel immunogenic proteins for *Prototheca* that could be used as therapeutic models.
5. Since host defence mechanisms against *Prototheca* and the predisposing immunologic defects are unknown, the activity of the detected immunogenic factors and immunological response will be further evaluated in healthy, immunocompromised and previously infected animals.

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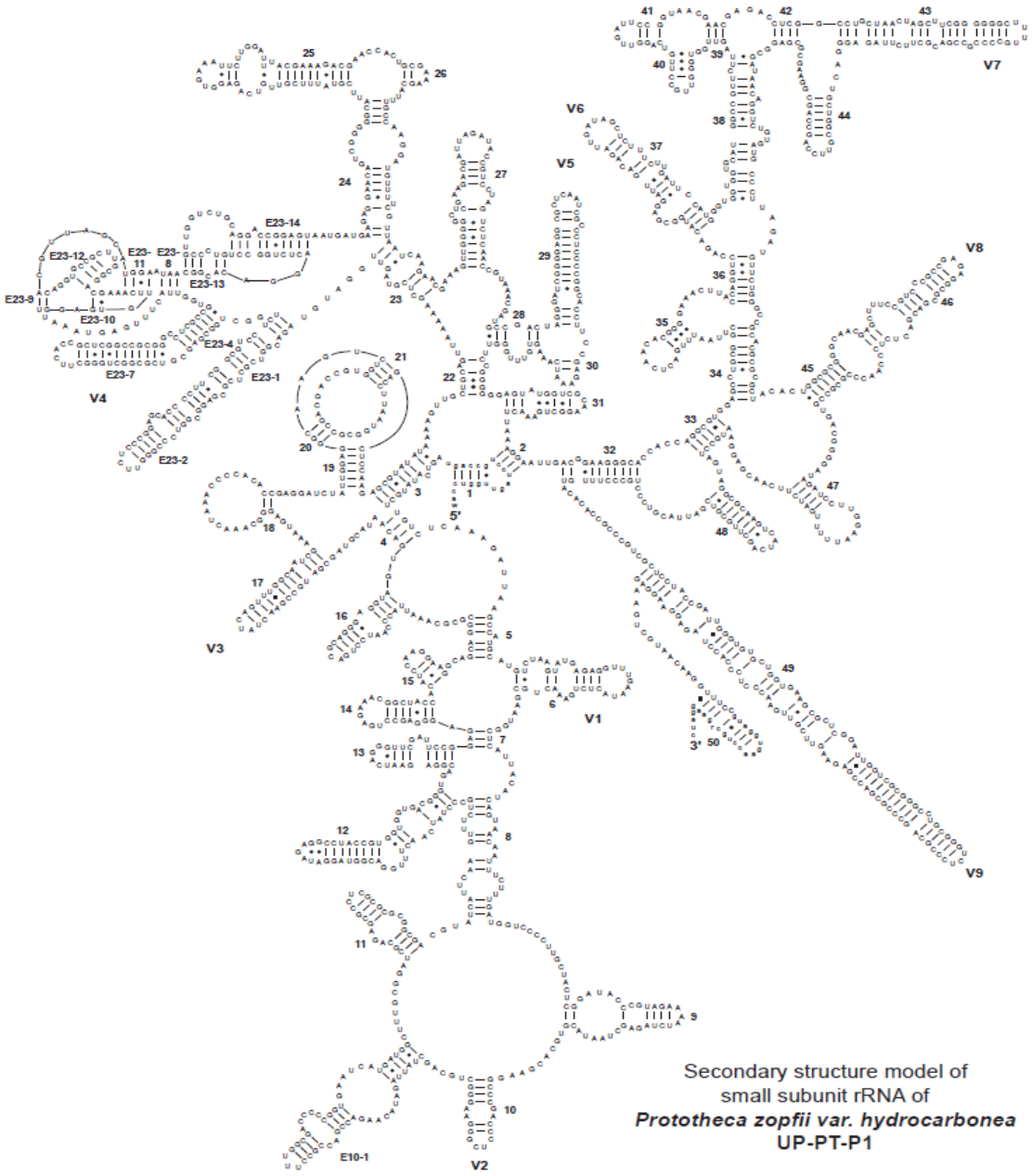
ADDENDUM

CONTENTS

SECONDARY STRUCTURES MODELS OF SMALL SUBUNIT rRNA

Prototheca zopfii var. *hydrocarbonea* UP-PT-P1



Secondary structure model of
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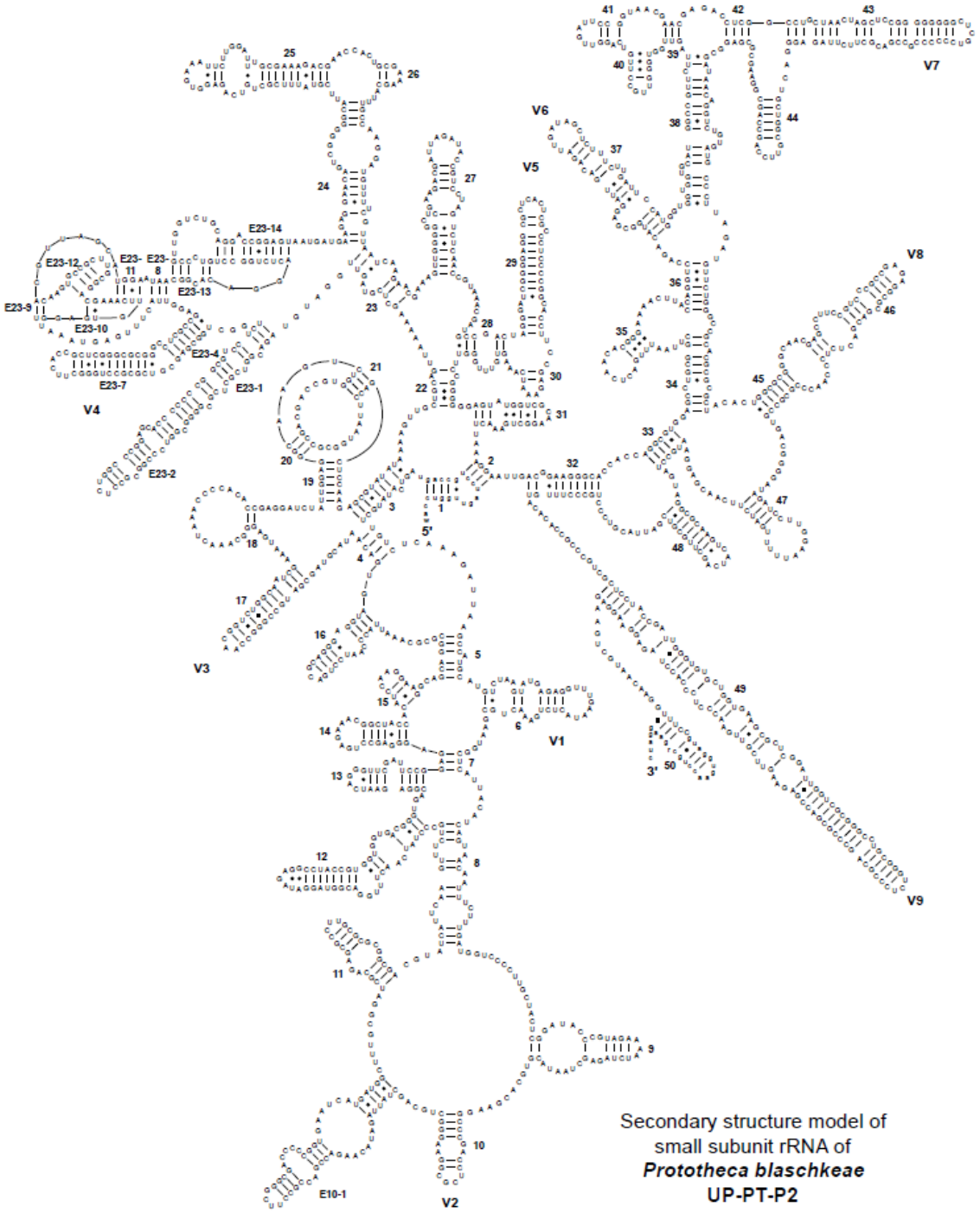
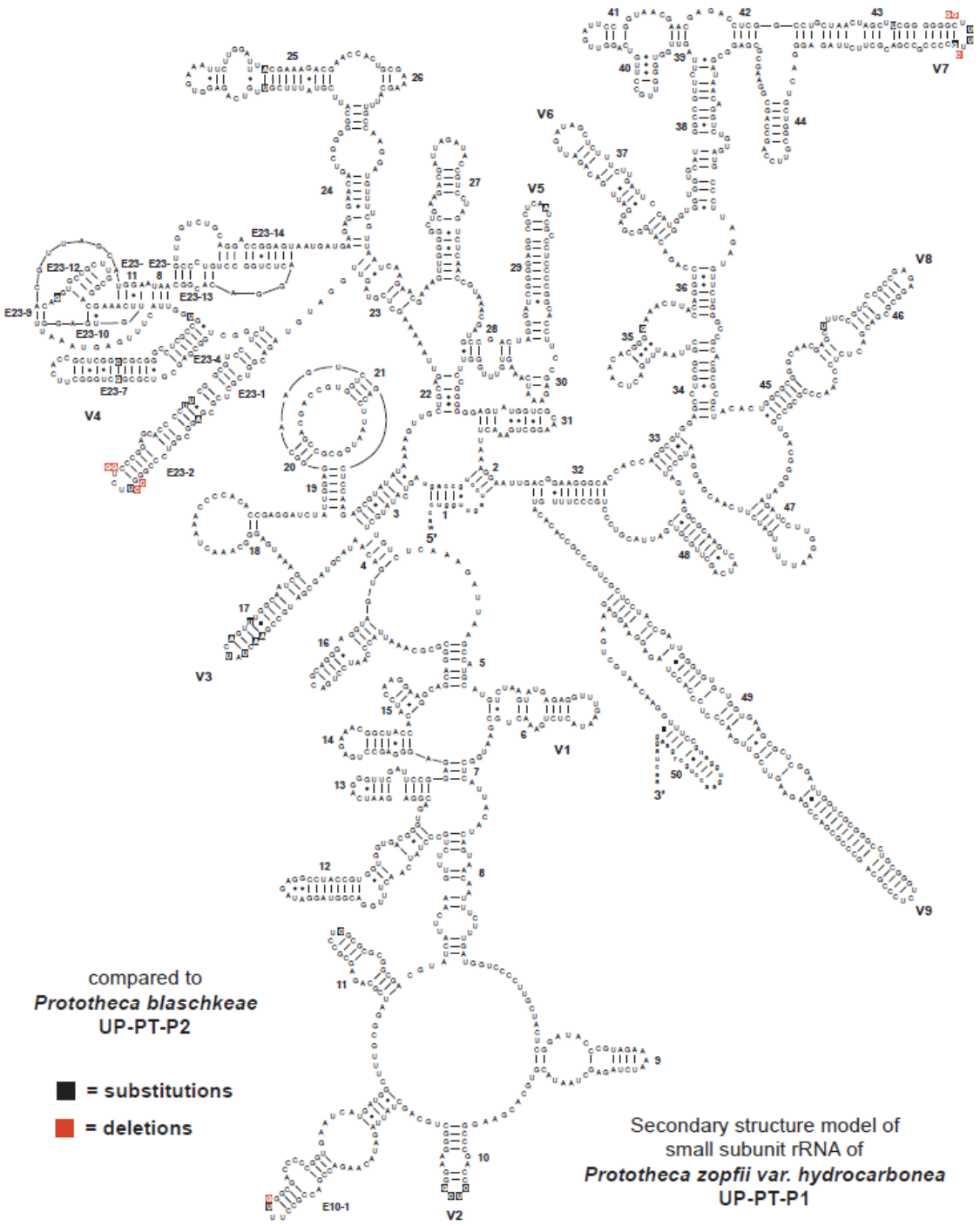
UP-PT-P1

Prototheca blaschkeae UP-PT-P2



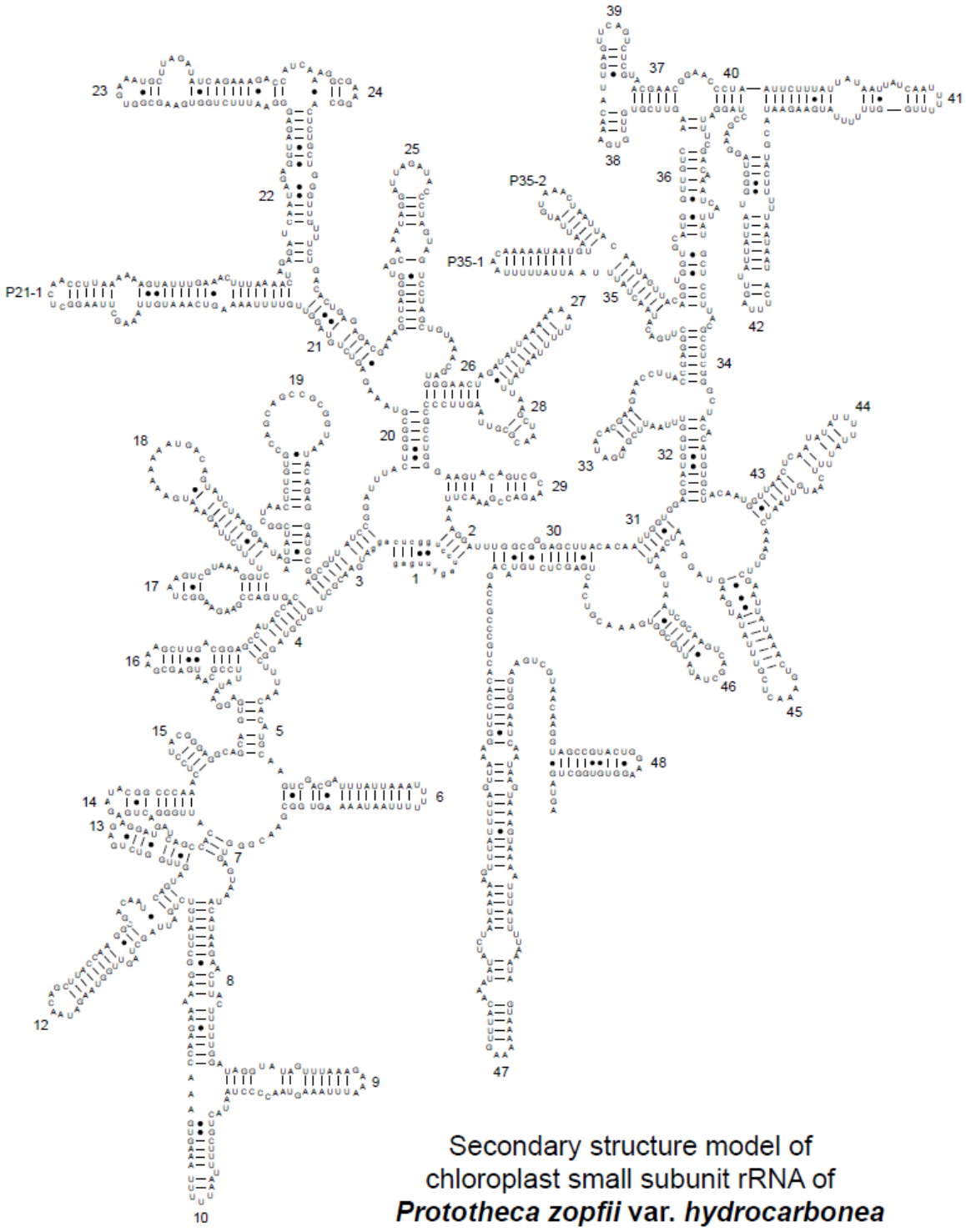
Secondary structure model of
small subunit rRNA of
Prototheca blaschkeae
UP-PT-P2

Prototheca zopfii var. *hydrocarbonea* UP-PT-P1 compared to *Prototheca blaschkeae* UP-PT-P2


compared to
Prototheca blaschkeae
UP-PT-P2
= substitutions
= deletions
Secondary structure model of
small subunit rRNA of
Prototheca zopfii var. hydrocarbonea
UP-PT-P1

SECONDARY STRUCTURES MODELS OF CHLOROPLAST SMALL SUBUNIT rRNA

Prototheca zopfii var. *hydrocarbonea* UP-PT-P1



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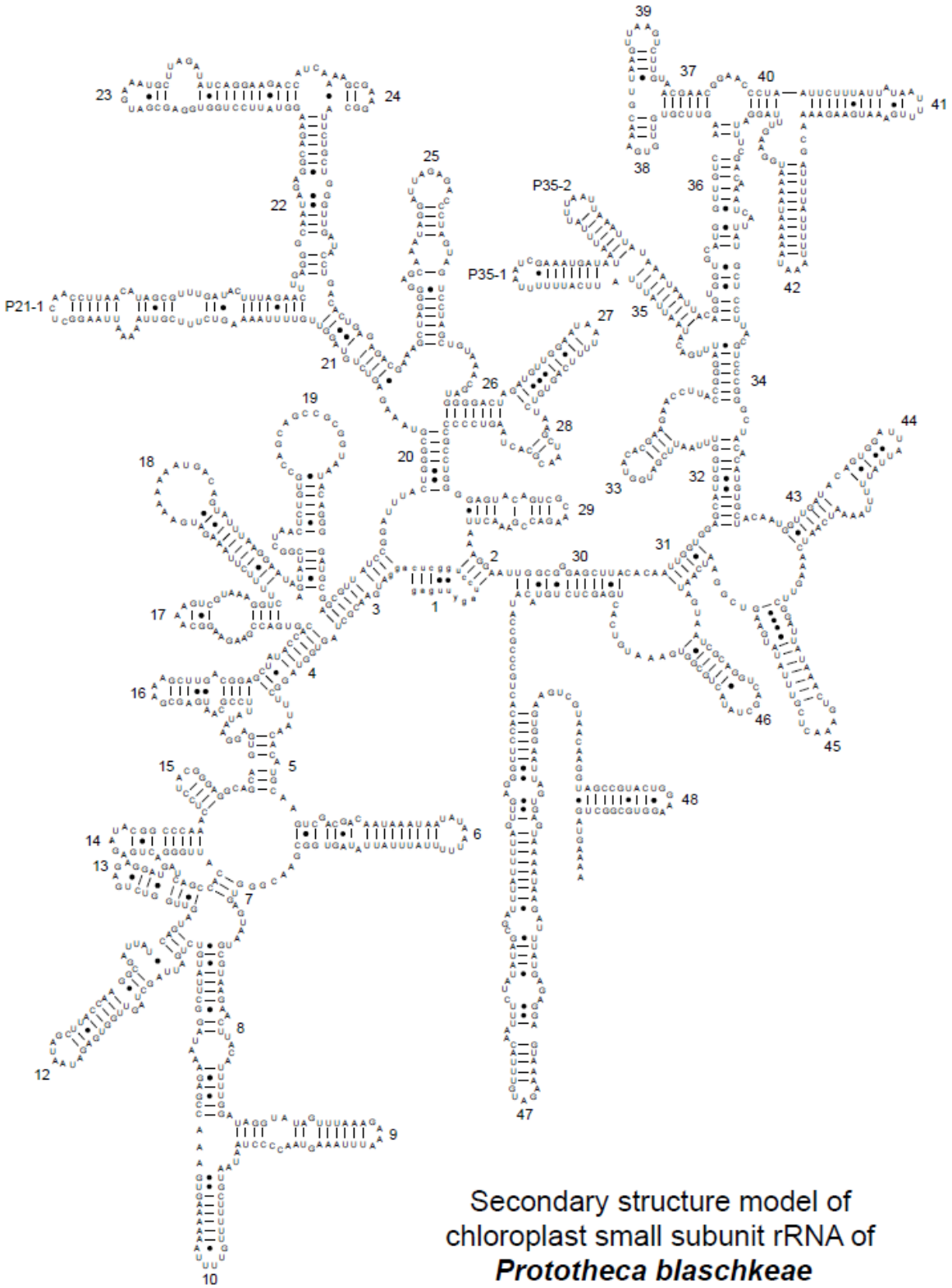
Prototheca blaschkeae UP-PT-P2



Secondary structure model of
chloroplast small subunit rRNA of
Prototheca blaschkeae
UP-PT-P2

ALIGNMENT OF THE PLASTID rRNA OPERON OF *PROTOTHECA*

Legend:

Lower case letters represent helix.

1 (Gene   ): Gene name
2 (helices): Helices in Prokaryotes
3 (numbers): Helix numbers according to Dams *et al.*[1] (for 16S)
4 (E.coli): *E. coli* rRNA operon; J01695 (accession number)
5 (Cv C-27): *Chlorella vulgaris* C-27 chloroplast DNA; AB001684 (accession number)
6 (P.wic pt): *Prototheca wickerhamii* 263-11; pt 16S rDNA; AJ245645 (accession number)
7 (P1 pt+): P1 – *P. zopfii*, 16S-23S; SM – Sara Marques; 2.2.09
8 (P2 pt+): P2 – *P. blaschkeae*, 16S-23S; SM – Sara Marques; 2.2.09

```
Posi-   Sequence
tion:   identity:   Data:

    1   1 Gene                                                                                                                Gene
    1   2 helices                  ---->   ---><---- ----_-----> -------->     ---_-->  ---__--------------->    <------  helices
    1   3 numbers                    1      2    1       3           4            5                   6                   numbers
    1   4 E.coli                                                                                                              E.coli
    1   5 Cv C-27   AAAAAUUUUCAUGGAgaguuUGAuccuggcucAggauGaacgcuGgcggcaugcUU-AAcacAugcAAgucGUacgcaugcaauuugg-CUUG-ccagau  Cv C-27
    1   6 P.wic pt     UGUGAAAACGAAgaguuUGAuccuggcucAggauAaacgcuGgcggcaugcUU-AAcacAugcAAguuGUacgaagguauuuaUcuUUCGagCuaaa  P.wic pt
    1   7 P1 pt+                   GAGUUYGAUCCUGGCUCAGGAUGAACGCUUGUGGUAGGCUUUAACACAUGCAAgucG-acg-Auuuauuaaa--UUUU-----uu  P1 pt+
    1   8 P2 pt+                   GAGUUYGAUCCUGGCUCAGGAUGAACGCUAGUGGUAGGCUUUAACACAUGCAAgucG-acgaCaauaaauaa-UAUAAUUUuuau  P2 pt+

  101   1 Gene                                                                                                                Gene
  101   2 helices   -------------      -->        -------_---__------> ----_____----->    <-----______----   --------->     helices
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  101   4 E.coli                                                                                                              E.coli
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  101   8 P2 pt+    uuauuAuaguggcGAACGGGUGAGUAAUGCGUAAGAACUUACAUUUUGGAUAGGUAUAGUUUAAAGAAAUUUAAAGUAACCCCUAAUAAugcuuuuugUU  P2 pt+

  201   1 Gene                                                                                                                Gene
  201   2 helices     <---------  -->    <-- <------_---------- ---__-_---__------->       <----------_____----  -------  helices
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  201   7 P1 pt+    UU-uuaaagugAAA-----------CCAAGAAAAAGGCUUAUGUCUGAUUAGCUAGUUGGUAAGAUAACAGCUUACCAAGGCGACAAUCAGUAGUUGGUC  P1 pt+
  201   8 P2 pt+    UUaaaaaagugAAA-----------CCGAGAAAUAGGCUUAUGUCUGAUUAGCUAGUUGGUGAGAUAAUAGCUUACCAAGGCGAUUAUCAGUAGUUGGUC  P2 pt+
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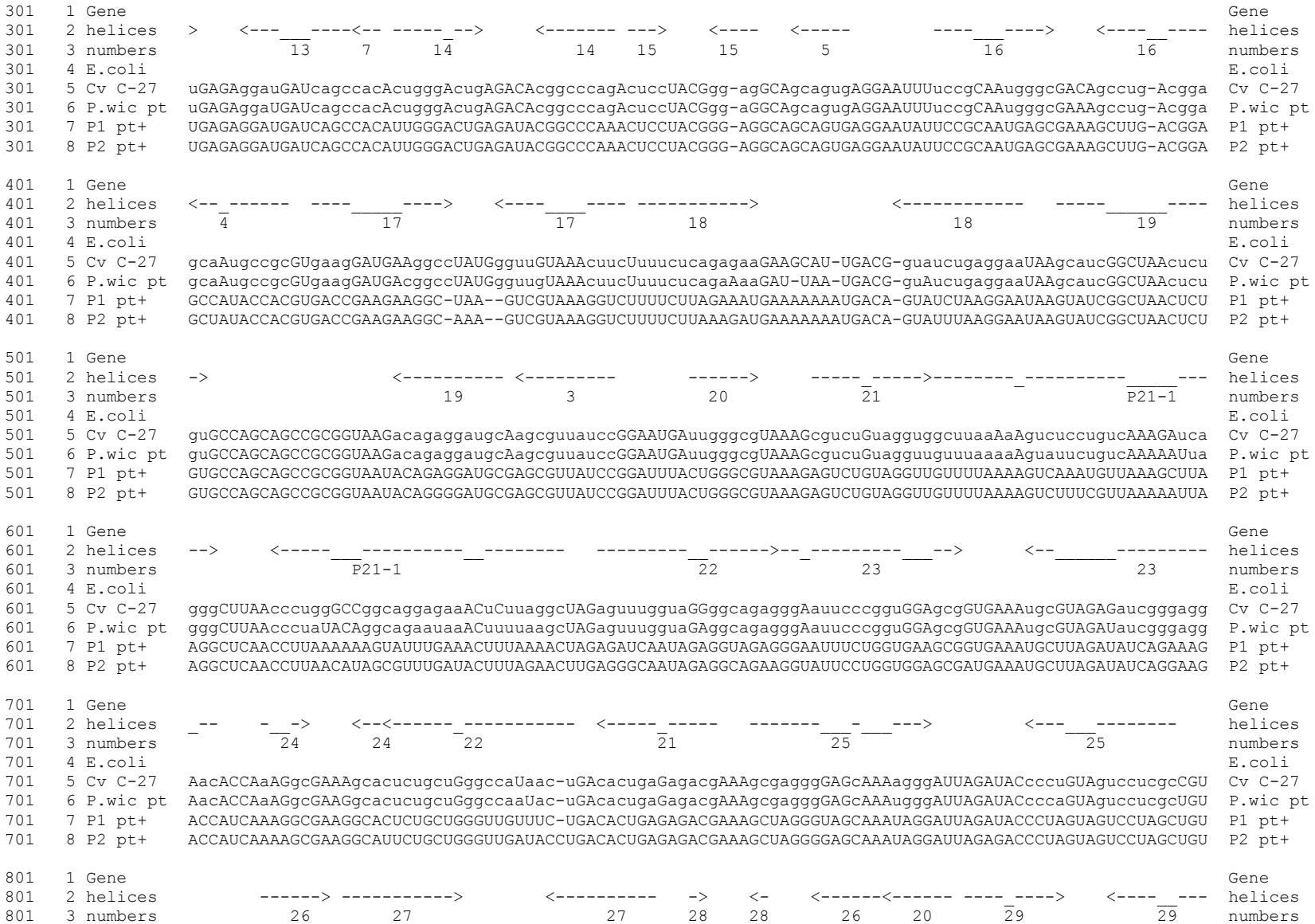
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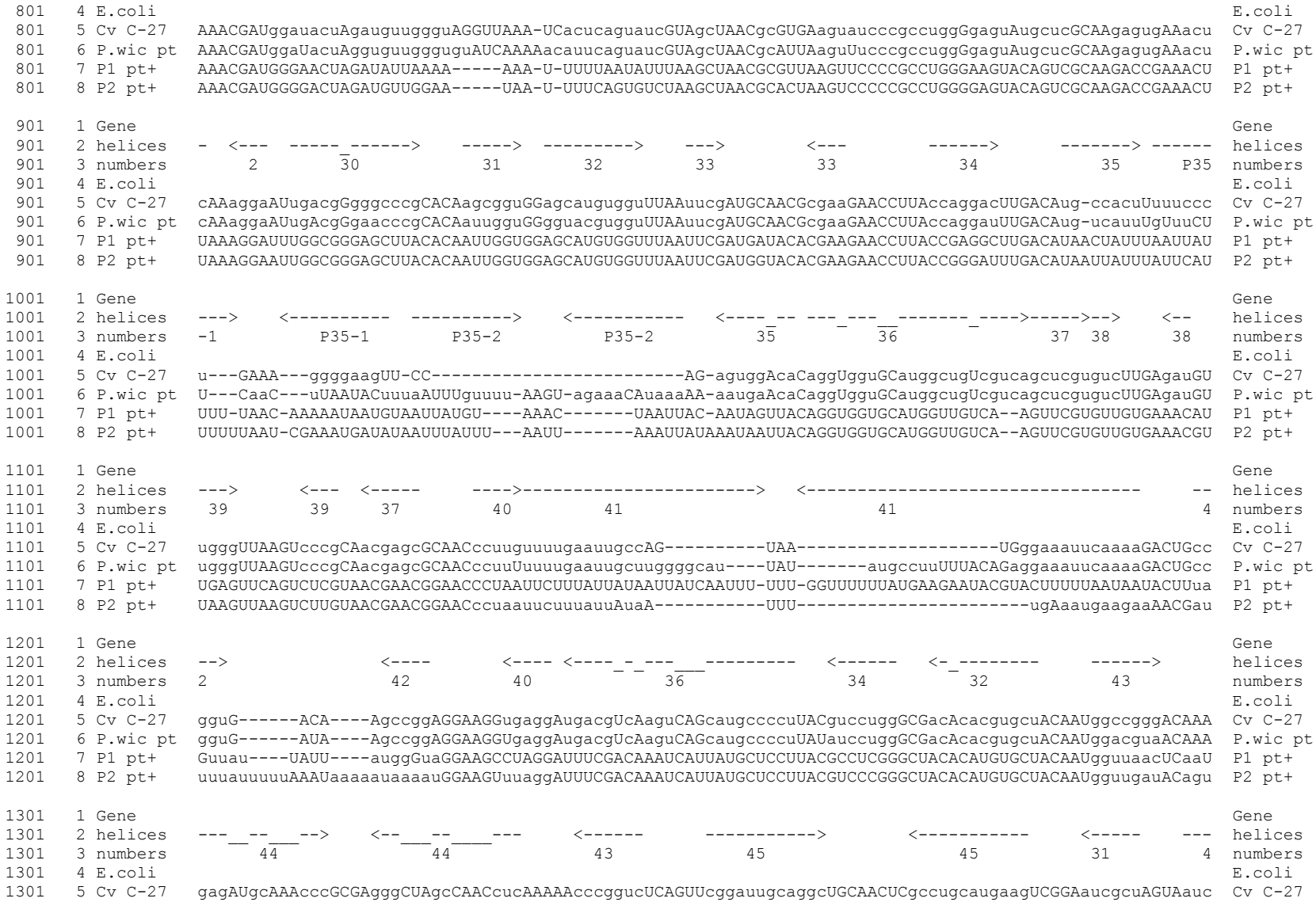
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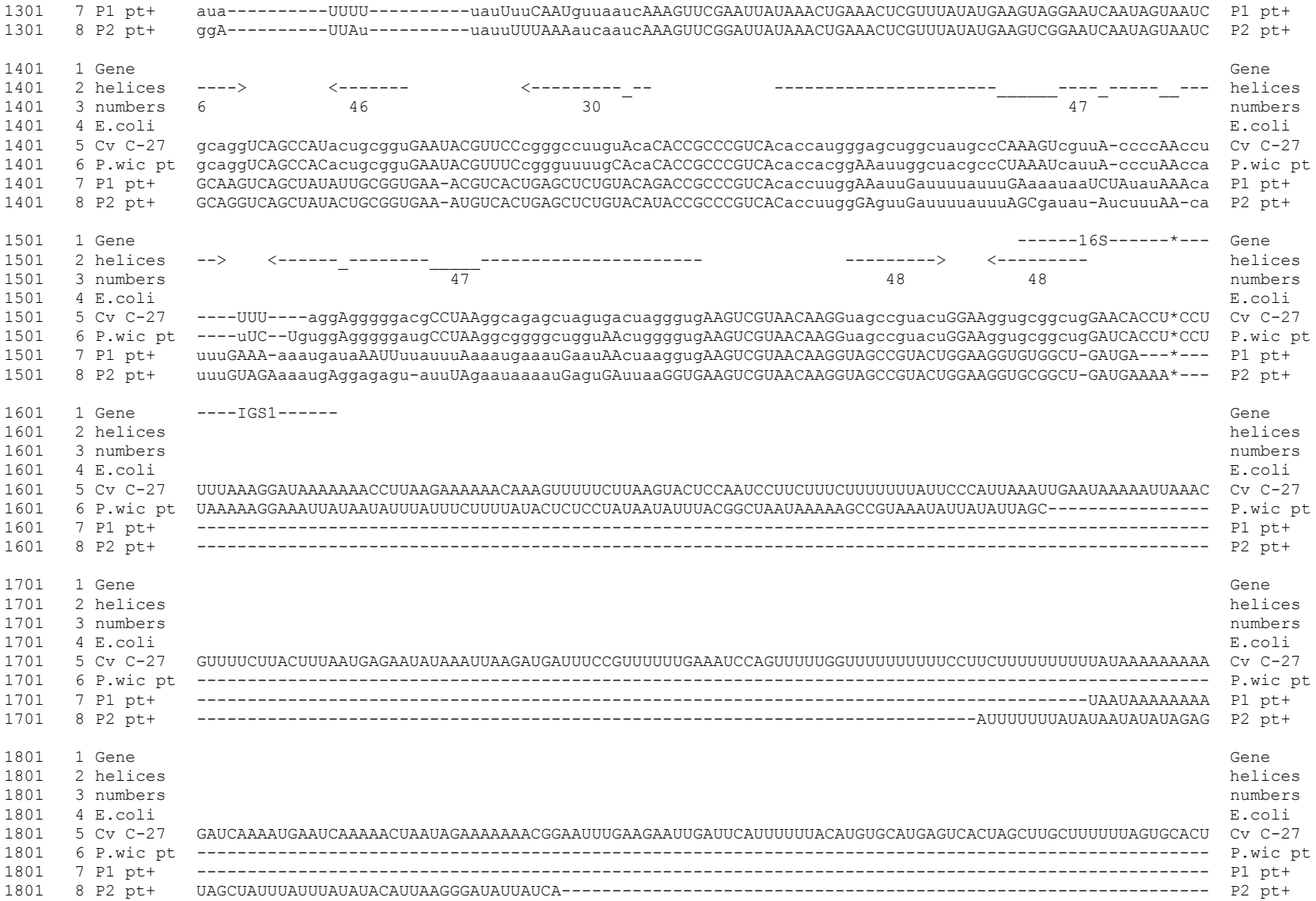
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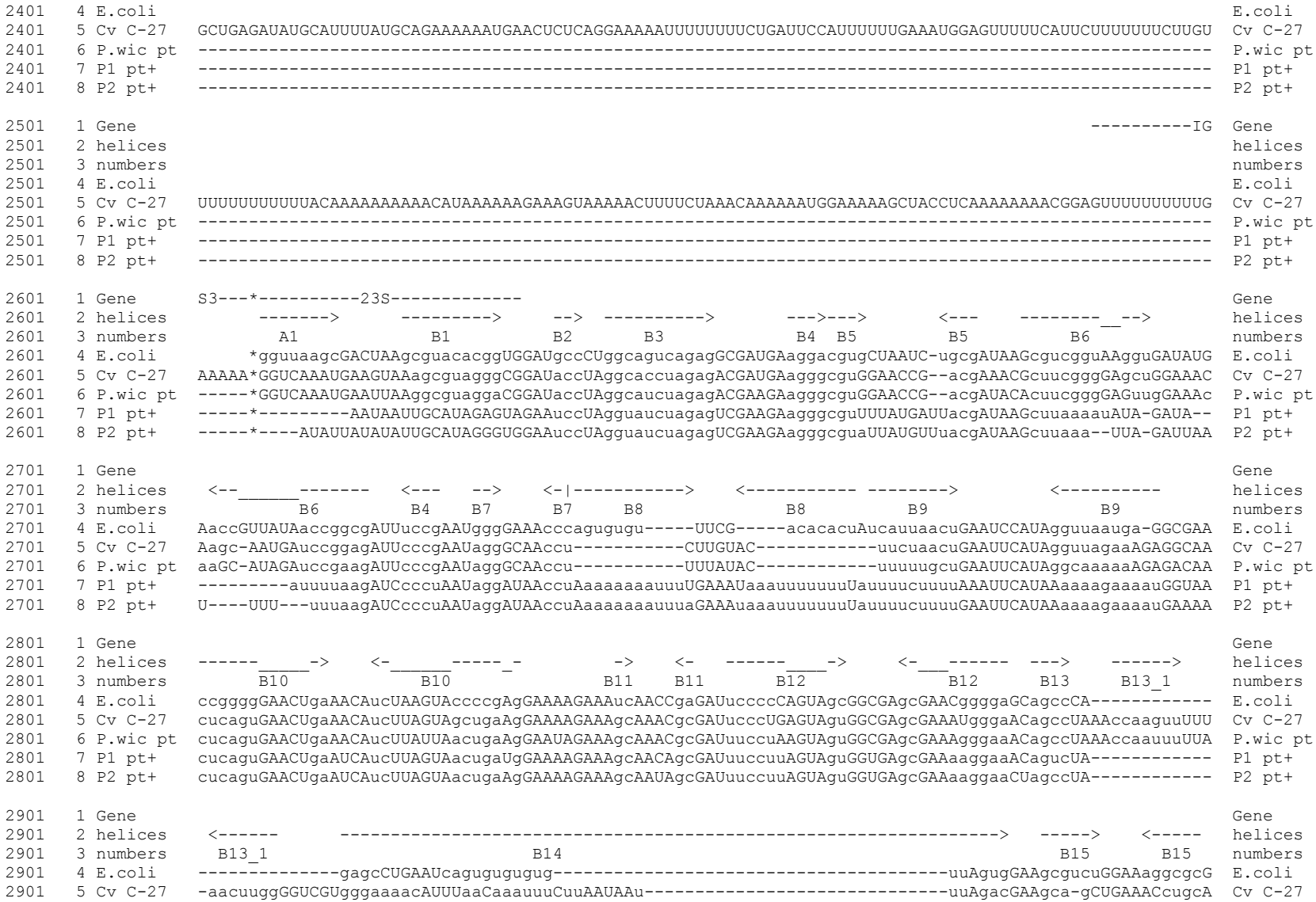
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[1] Dams E, Hendriks L, Van de Peer Y, Neefs JM, Smits G, Vandenbempt I, De Wachter R. 1988. Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Research. 16 Suppl:r 87-173.

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PROTOTHECOSE BOVINA: A MAMITE

As mamites causam perdas severas na qualidade e produção de leite. As perdas económicas provocadas são, principalmente, devido à redução da produção de leite, seguidas do refugo prematuro, rejeição do leite após terapia, fármacos e despesas com veterinários. Para além dos aspectos económicos, deve ter-se em atenção o potencial zoonótico que alguns dos microorganismos envolvidos podem representar. Dos agentes mamíticos de origem ambiental, destaca-se a microalga Prototheca cuja ocorrência tem vindo a aumentar ao longo dos anos.

Estas algas são microscópicas, unicelulares, sem clorofila e são as únicas plantas causadoras de patologias nos humanos e em animais. As Prototheca são ubíquas, encontram-se em fontes ricas em matéria orgânica e em ambientes húmidos, sendo a sua disseminação e perpetuação no ambiente muito elevadas. São, no entanto, agentes oportunistas raros causando patologia, normalmente, quando as defesas imunológicas dos hospedeiros estão comprometidas.

No entanto, a Prototheca foi identificada como detentora de potencial zoonótico. Estas microalgas são agentes extremamente resistentes, pelo que já foram isoladas a partir de uma variada gama de valores de pH, de água tratada com cloro e a partir de leite pasteurizado, desconhecendo-se o efeito destes tratamentos na inactivação da mesma.

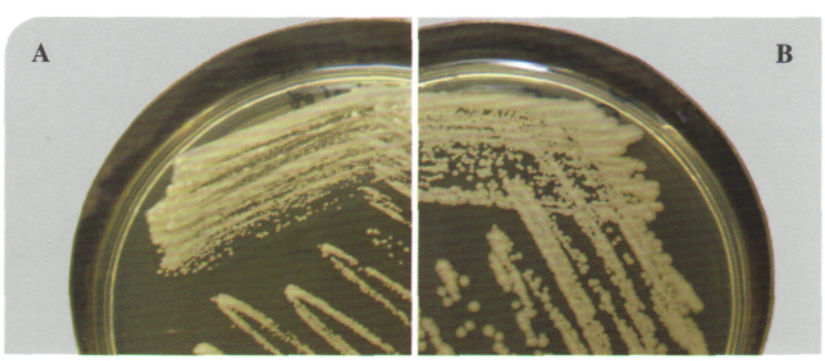

Figura 1. P.blaschkeae (A) e P.zopfii (B) em meio de cultura selectivo para fungos – Agar Sabouraud, culturas com 96 horas de incubação.

O género que engloba esta microalga apresenta três espécies patogénicas, nomeadamente, P. zopfii, P. wickerhamii e P. blaschkeae, em que a P. wickerhamii é primariamente associada a casos clínicos de infecções em humanos, a P. zopfii causa infecções predominatemente nos animais, em particular em vacas e cães, e a P. blaschkeae, causa infecções em humanos e em animais, nomeadamente mamites bovinas.

O diagnóstico de mamites associadas a Prototheca é feito pela inoculação do leite mamítico em meios de cultura, geralmente, utilizados em Microbiologia e após crescimento de colónias características (Figura 1), estas algas são identificadas pela sua morfologia macro e microscópicas e por comparação do padrão de assimilação de diversos substratos.

Um isolado desconhecido deve ser inicialmente examinado por microscópio óptico (exame a fresco e esfregaços corados, (Figura 2) para ser identificado como pertencente ao género Prototheca, e posteriormente determinado o seu padrão de assimilação através das galerias API. Porque a identificação das espécies deste agente é laboriosa e morosa, a utilização de técnicas moleculares apresenta-se extremamente relevante para permitir uma rápida e eficaz identificação das mesmas.

Tem-se registado o aperfeiçoamento das técnicas diagnósticas aplicadas a estas espécies, o que permite a sua melhor caracterização, e pode num futuro, apoiar o desenvolvimento de estratégias terapêuticas inovadoras.

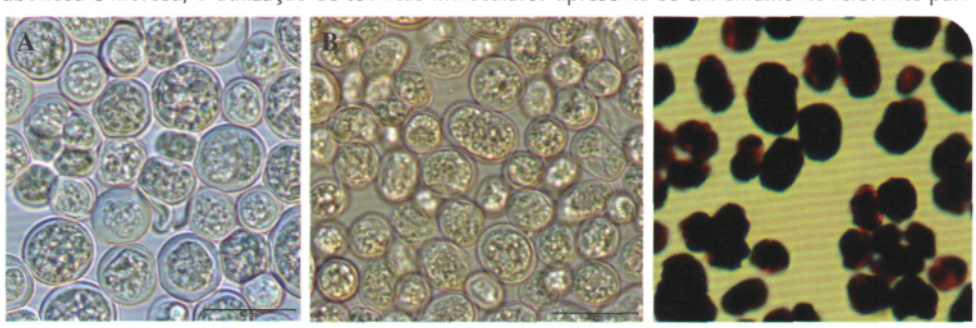

Figura 2. Fotomicrografias de Prototheca. Preparações a fresco: A – P. blaschkeae B – P. zopfii. Coloração de Gram (C) de colónias suspeitas de Prototheca (x1000).

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▶ Continuação da página 24

A **Prototheca** assume elevada importância na produção leiteira por causar mamites (Figura 3) de difícil tratamento, estando a verificar-se um aumento do número de casos em todo o Mundo. Em Portugal, foram descritos alguns casos de mamites bovinas por **Prototheca**, um na zona Centro, outro na ilha dos Açores e, recentemente, uma incidência elevada desta patologia na zona Norte do País, o que pode levar a propor que a mesma se encontra sub-diagnosticada no nosso território. Têm estado a ser reportados vários casos de protothecose humana e, devido à crescente detecção de casos de infecções também nos animais, considerase que estes microorganismos poderão representar um crescente risco para a Saúde Pública.

O tipo de mamites provocada pela **Prototheca** pode ser clínica ou subclínica com evolução para um quadro granulomatoso que pode determinar a perda de função leiteira. Em explorações com bom maneio

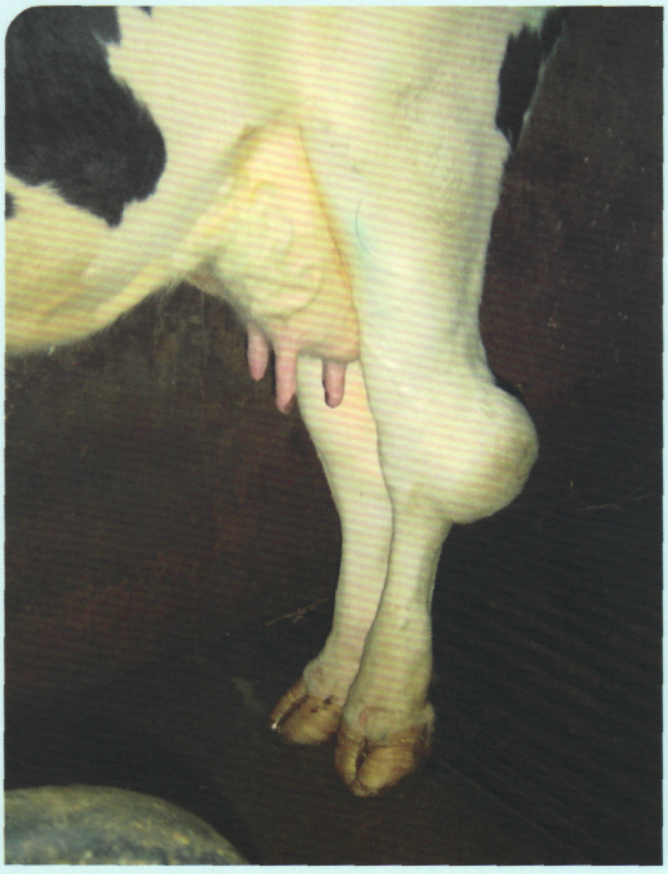

Figura 3. Além de mamites as Protothecas também podem provocar lesões nas articulações.

as mamites por este agente podem ocorrer de forma esporádica, contudo em explorações com más condições de higiene e maneio a patologia torna-se endémica. Apesar de ser um agente considerado como causador de mamite ambiental, ele pode contudo, apresentar um comportamento de agente contagioso.

Como a **Prototheca** está amplamente distribuída no ambiente da exploração, a incidência de mamites bovinas depende de factores predisponentes, tais como, más condições ambientais, locais húmidos em aerobiose e com matéria orgânica, ou insuficiente higiene da ordenha.

A sua natureza ubíqua e lesões da ponta e do canal do teto favorecem a infecção ascendente para a glândula mamária. As infecções podem ocorrer quando os tetos das vacas são expostos a elevadas concentrações desta alga a partir do ambiente, como por exemplo, durante os intervalos entre as ordenhas, ou quando as técnicas de ordenha são deficientes.

Deste modo, a alga pode ser transmitida do ambiente para a vaca e de vaca a vaca durante a ordenha pelas unidades de ordenha e mãos do ordenhador, podendo infectar em qualquer período da lactação e até no período seco.

Bezerros alimentados com leite contaminado por esta alga podem também actuar como disseminadores do agente no ambiente. A sua transmissão geralmente ocorre por contacto directo, podendo esta, ser transmitida pelo leite e fezes, sugerindo que pode ser introduzida na exploração por compra de vacas infectadas.

Nas mamites por **Prototheca**, geralmente, não há sinais sistémicos e em muitos casos os sinais locais são leves. O sinal clínico mais comum pode traduzir-se na aparência aquosa do leite com flocos, mas na maioria dos casos apenas se detecta redução da produção de leite, e aumento prolongado das contagens de células somáticas (CCS).

Nos casos subclínicos, as CCS ultrapassam o milhão de células (106/ml). Nos casos clínicos para além da secreção aquosa com grumos, pode detectar-se com a palpação aumento da densidade do quarto infectado e redução do tamanho, sendo geralmente afectados mais do que um quarto. Em casos raros, pode provocar disseminação aguda com mamite necrótica massiva. Geralmente, o método de controlo desta infecção tem de ser drástico, com a eliminação dos animais infectados das explorações leiteiras afectadas.

O aumento da idade e paridade, história prévia de mamites, especialmente com pré tratamento com antibióticos são factores de risco importantes para o desenvolvimento de mamites bovinas por **Prototheca**.

A elevada CCS, antes do diagnóstico e história passada de mamite, indicam que a lesão na glândula mamária é importante para a **Prototheca** invadi-la e colonizá-la. Como os factores de risco são mais comuns nas vacas mais velhas, a correlação entre paridade e risco de infecção pode ser facilmente explicada. Os quartos com história de mamite clínica durante a mesma ou lactações anteriores têm maior risco do que os outros. A terapia com antibióticos aumenta o risco de infecção, pois a erradicação da flora normal da pele do teto por estes facilita a multiplicação de **Prototheca**, contudo, algas provenientes do meio também podem aceder à glândula mamária através da infusão de fármacos pelo canal do teto. É provável, que a primeira fonte para um surto seja o ambiente e que as glândulas infectadas suportem a libertação da alga para o resto da exploração. Em resumo, são factores predisponentes de mamites por **Prototheca**, o mau maneio, má manutenção da exploração, ambiente húmido, acumulação de fezes e detritos, práticas de ordenha impróprias, infusão intramamária de fármacos com higiene deficiente.

A informação sobre terapia eficaz de mamites associadas a **Prototheca** é escassa, e a existente apoia-se em dados de casos isolados e de estudos in vitro. Terapia contra **Prototheca** é um assunto problemático devido a esta apresentar elevada resistência aos antibióticos geralmente utilizados na clínica e aos antifúngicos. Adicionalmente, não foi detectada cura espontânea de mamites associadas a **Prototheca**, como já foi descrito para leveduras. As alterações patológicas provocadas por esta alga e as suas características intrínsecas, nomeadamente a sua parede celular, contribuem para o insucesso terapêutico deste tipo de mamite. O uso de vários agentes antimicrobianos pode diminuir temporariamente a severidade dos sinais clínicos e a disseminação da alga. Como a **Prototheca** provoca lesão irreversível da glândula mamária, mesmo após a sua eliminação a produção de leite permanece baixa, sendo uma das hipóteses terapêuticas a secagem do quarto afectado.

Devido ao elevado custo e a considerações de saúde pública, o uso de antifúngicos, tais como, anfotericina B ou fluconazole é limitado em vacas.

Em conclusão, devido à crescente incidência de mamites por **Prototheca** e ao seu comportamento insidioso é de extrema importância que produtores, clínicos e investigadores estejam alerta para este agente que, pelas características e potencial zoonótico, deve ser controlado através da aplicação de medidas de maneio e de melhoramento das condições de higiene das explorações.

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Nota: Os leitores que pretendam consultar as Referências Bibliográficas agradecemos que nos contactem para o efeito.