Identification of immunodominant proteins of the microalga *Prototheca* by proteomic analysis

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**Abstract**

*Prototheca zopfii* associated with bovine mastitis and human protothecosis exists as two genotypes, of which genotype 1 is considered as non-infectious and genotype 2 as infectious. The mechanism of infection has not yet been described. The present study was aimed to identify genotype 2-specific immunodominant proteins. *Prototheca* proteins were separated using two-dimensional gel electrophoresis. Subsequent western blotting with rabbit hyperimmune serum revealed 28 protein spots. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis resulted in the identification of 15 proteins including malate dehydrogenase, elongation factor 1-alpha, heat shock protein 70, and 14-3-3 protein, which were previously described as immunogenic proteins of other eukaryotic pathogens.

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**Keywords:** Bovine mastitis, immunodominant proteins, MALDI-TOF MS, proteomics, *Prototheca*, western blotting

**Original Submission:** 17 July 2014; **Revised Submission:** 18 September 2014; **Accepted:** 16 October 2014

**Available online 8 November 2014**

Protothecosis refers to a rare infection caused by chlorophyllous unicellular algae of the genus *Prototheca*, which are reported to be saprophytic, ubiquitously distributed in the environment and closely related to *Chlorella* species [1,2]. Currently, among the six generally accepted species of *Prototheca*, *Prototheca zopfii*, *Prototheca wickerhamii*, *Prototheca blaschkeae* and *Prototheca cutis* have been associated with infections in cows, dogs, cats and, rarely, in humans. *P. zopfii* and *P. blaschkeae* are associated with antimicrobial-resistant bovine mastitis, which leads to heavy economic loss while *P. zopfii* appears to be frequently reported [3,4]. On the basis of biochemical, serological and phylogenetic analysis, *P. zopfii* has been divided into two genotypes, of which genotype 1 is considered to be non-pathogenic and genotype 2 is associated with bovine mastitis and human protothecosis [5–8]. Recently, differences at the proteomic level between these two genotypes of *P. zopfii* have been described [9–11]; however, the mechanism of infection has still not been described. Hence, the present study was carried out to identify the immunogenic proteins of *P. zopfii* genotype 2 using two-dimensional (2D) gel electrophoresis—western blotting with hyperimmune serum from experimentally immunized rabbits.

The type strain *P. zopfii* genotype 2 (SAG 2021), isolated from bovine mastitis [7], was utilized for extraction of protein [9]. In brief, cells cultured overnight were harvested by centrifugation at 1000 g for 5 minutes, reconstituted in lysis buffer (20 mM HEPES, pH 7.4, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 0.5% benzonase and 0.5% protease inhibitor cocktail tablet), sonicated and the supernatant was collected for further study. The specific rabbit primary polyclonal antibodies were raised in New Zealand White rabbits as described previously [2]. In brief, rabbits of body weight 2.5–3.5 kg were intradermally injected with 1.0 mL of emulsion containing equal volume of phosphate-buffered saline containing 10⁷ cells/mL and Freund incomplete adjuvant (Sigma-Aldrich, Steinheim, Germany). Following 3 weeks of priming, the rabbits were boosted intravenously, biweekly three times with 10⁷ viable cells of the homologous strain. Seven days after the last booster
application and 10 weeks after priming, the rabbits were bled, serum was collected and stored at −80°C until further use. The immunization experiments with the rabbits were performed according to the German law on animal welfare and as approved by the German authorities (Regierungspräsidium Leipzig, Permission No. V 4/04).

Western blotting was carried out following the 2D separation of *Prototheca* proteins on 12% polyacrylamide gel after rehydration and isoelectrofocusing of 250 μg of whole cell lysate on an immobilized pH gradient strip of 7 cm and pl 3–10 (non-linear) (Immobiline drystrip; GE Healthcare, Munich, Germany). The separated proteins were then transferred to a nitrocellulose membrane (Trans Blot trans medium pure nitrocellulose membrane; Bio-Rad, Munich, Germany) using semi-dry transfer unit (80 mA per gel for 90 min) (GE Healthcare). Electrophoresis and blotting quality were checked by staining the membrane for 30 s in Ponceau S (1% Ponceau S, 0.1% acetic acid) followed by short destaining with Tris-buffered saline with Tween-20 (TBST). The membrane was incubated overnight in 1% skimmed milk powder (Carl Roth, Karlsruhe, Germany) in TBST, washed and incubated for 90 min with 1 : 100 diluted rabbit hyperimmune serum. The membrane was briefly rinsed with TBST and incubated for another 90 min with 1 : 2500 diluted goat anti-rabbit horseradish-peroxidase conjugated IgG-h-I (Biomol, Hamburg, Germany). Detection was carried out using 3,30,5,50-tetramethylbenzidine kit (Sigma Aldrich Chemie, Steinheim, Germany). The respective protein spots on a 2D gel performed in parallel and stained with Coomassie Brilliant Blue were identified after overlaying of the western blot image using Delta2D software version 4.0 (Decodon, Greifswald, Germany) [12].

Two-dimensional gel electrophoresis western blots from hyperimmune serum from two rabbits revealed 28 signals following analysis with Decodon software (Fig. 1). The corresponding protein spots were excised from the 2D gel and digested with trypsin. Protein identification was performed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Ultraflex II TOF/TOF; Bruker Daltonics, Bremen, Germany), as described previously [9]. Protein identification was considered to be valid if more than two peptides matched and the MASCOT score was greater than or equal to the significance threshold (p <0.05). As a result, 15 proteins were successfully identified (Table 1). The identified proteins were found to be enzymes of energy metabolism and proteins involved in cellular transport and cell signalling including stress response.

Among the identified proteins, malate dehydrogenase, elongation factor 1-alpha (EF-1α), heat shock protein 70 (Hsp70) and 14-3-3 protein have been described as immunoreactive proteins in serological studies with other eukaryotic pathogens [13–15]. The negative control serum appeared to possess antibodies against S-adenosyl-L-homocysteine hydrolase and EF-1α; however, the latter was earlier identified as an immunoreactive protein of *Prototheca* [16]. On the other hand, four proteins (malate dehydrogenase, S-adenosyl-L-homocysteine hydrolase, EF-1α, and Hsp70) were previously reported to be differentially expressed in *P. zopfii* genotype 2 (SAG 2021). Hence *P. zopfii* genotype 2 hyperimmune serum appeared to possess specific antibodies against two of the proteins, malate...
TABLE 1. List of identified proteins

| Spot ID | UniProt no. | Protein | Organism | Molecular mass (Da) | No. of peptides | pI | MOWSE score | Function |
|---------|-------------|---------|----------|-------------------|----------------|----|-------------|----------|
| 1       | Q1Q17       | Malate dehydrogenase | Aspergillus niger | 105               | 105            | 7.71 | 5          | Energy metabolism |
| 2       | Q3IXY7      | Serine recombinase | Rhodobacter sphaeroides | 87       | 57             | 7.12 | 14         | Site-specific recombination |
| 3       | Q1Q17       | Radical SAM protein | Caenorhabditis remanei | 92       | 38             | 6.28 | 3          | |
| 4       | E1ZTB0      | Hypothetical protein | Chlorella variabilis | 92       | 62             | 6.68 | 10         | |
| 5       | Q1Q17       | Beta chain succinyl-CoA synthetase | Phaeodactylum tricornutum | 94       | 62             | 6.32 | 7          | |
| 6       | Q1Q17       | -adenosyl-L-homocysteine hydrolase, partial | Chondrus crispus | 113      | 54             | 5.31 | 7          | |
| 7       | A4RWG3      | Heat shock protein 70 | Ostreococcus lucimarinus | 93       | 71             | 5.37 | 4          | Stress response, cell signalling |
| 8       | C0LL61      | Translation elongation factor-like protein (EF-1α) | Eubacterium sp. | 83       | 34             | 5.97 | 10         | Ribosome biogenesis |
| 9       | A0LN73      | Porphobilinogen deaminase | Syntrophobacter fumaroxidans | 94       | 71             | 5.97 | 8          | For synthase of porphyrin metabolism |
| 10      | A1F5P3      | ATP synthase subunit | Plasmodium yoelii yoelii | 192      | 58             | 5.93 | 7          | Energy metabolism |

The presence of Prototheca-specific information in the public repositories resulted in the identification of proteins that are best described in other organisms [9]. De novo sequencing of the proteins might provide additional clues on the immunogenicity and virulence nature of Prototheca. The present study utilized serum from experimentally challenged rabbits; however, Prototheca infection in rabbit has not been reported yet. Hence, for a comprehensive description of genotype-specific antigens, which might serve as virulence factors, western blotting using serum of naturally infected animals should be carried out.

**Transparency declaration**

The authors state that they have no conflicts of interest.

**Acknowledgements**

We would like to acknowledge the assistance of the Bio-MS unit of the Core Facility BioSupraMol supported by the DFG. We would like to thank Michael Kühl for technical assistance.

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Hsp70 comprises a highly conserved protein family with housekeeping functions and stress-inducible chaperone functions when located intracellularly [17]. Extracellular Hsp70 in contrast, acts as a cytokine and activates immune cells. We can only speculate about the role of Hsp70 during infection. It should be noted that most of the identified proteins appear to be intracellular in nature, which supports the earlier speculations that the dead Prototheca cells induce a local immune response [18].
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