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Comparison of different methods for the solubilisation of *Neospora caninum* (Phylum *Apicomplexa*) antigen

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Abstract

Parasite antigens are of interest both for the development of better diagnostic tools and potential subunit vaccines. Particularly, relevant in this regard are membrane proteins as the first point of contact between host and parasite. Here, four different methods for the extraction and solubilisation of *Neospora caninum* proteins were evaluated by comparing protein yield, specific antigenicity and relative protein abundance in the electrophoresis profile. Extraction with SDS and sulphobetaines (SB) gave higher yields than those achieved using the standard sonication method. SDS and SB preparations also gave the best signal to noise ratio when used as capture antigens in an ELISA. Electrophoresis of the three preparations showed an even protein-banding pattern spread out over the 90–10 kDa size range. However, there were a greater proportion of lower molecular weight proteins when the sonication method was used suggesting that this method may have resulted in the degradation/proteolysis of some proteins. Extraction with Triton X-114 resulted in the concentration of three or four proteins but led to a considerable reduction in the overall protein yield. The ELISA indicated that not all of the Triton X-114 extracted proteins were involved in specific antibody binding. The optimal extraction method for parasite proteins depends on the intended application.
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1. Introduction

For years, studies of protozoan parasites have emphasised genomic research rather than proteomics. More recently with the realisation of the limitations of genomics, there has been a renewed interest in proteomics with proteins as the primary targets for diagnostic tools, therapeutic agents and vaccines. Applications range from simple diagnostic tests, such as ELISA, to highly complex analyses of protein expression profiles using 2D-electrophoresis and mass spectroscopy. In protozoan parasites, proteins are usually localised to particular compartments, and frequently restricted to certain stages of the pathogen's

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development. Post-translational modifications are not identified by the genetic code, yet they are vital for the correct functioning and recognition of many proteins. Some of the most important technical challenges of proteomics involve difficulties of protein solubilisation and purification, as proteins differ in their solubility and may become denatured (reversibly or irreversibly) during solubilisation. Moreover, immunologically relevant proteins frequently represent a minute fraction of the pathogen's proteome. Membrane proteins that are of particular interest because they are involved in the first stages of parasite–host interactions typically represent a difficult class of proteins for analysis/separation by SDS-PAGE. Using *Neospora caninum* as a model organism we have compared the yield and protein combination resulting from various parasite protein extraction methods.

N. caninum is an intracellular protozoan pathogen that is an important cause of infectious abortion and stillbirth in cattle world-wide (reviewed by Dubey, 2003). Infection is endemic in many herds and the parasite is frequently passed from mother to calf with no signs of disease. Damage occurs when it multiplies in the developing calf and/or the placenta, resulting in abortion or stillbirth. In Europe, there are no commercially available therapeutic or prophylactic drugs for prevention of transplacental transmission or for the treatment of neosporosis in cattle. Because infected animals may abort in successive years, may abort only once, or may produce normal calves it is difficult to provide rational management advice to farmers on what course of action to take in relation to seropositive animals in their herd.

In the last decade, a large number of *N. caninum* proteins have been described and localised (review by Howe and Sibley, 1999; Howe et al., 1998; Alvarez-Garcia et al., 2002; Schares et al., 2002; Jenkins et al., 2004). Many of these have been put to diagnostic use to detect existing infections and determine the likely time point of infection. Others have been analysed in their structure and function particularly in relation to host cell invasion. Interestingly, most workers have used the same standard technique for parasite solubilisation, consisting of repeated freeze–thawing followed by sonication (Williams et al., 2000; Alvarez-Garcia et al., 2002; Rettigner et al., 2004). In some studies extractions were carried out with the non-ionic detergents Triton X-114 or X-100 in order to enrich parasite antigen preparations with membrane proteins (Bjerkas et al., 1994; Hemphill et al., 1997a,b). However, there is a lack of published information on the effects of different extraction methods on protein quality and abundance.

In this study, we compare the standard sonication method and extraction with Triton X-114. In addition, parasites were solubilised in a combination of detergent and non-detergent sulphobetaines (SB) as described by Blisnick et al. (1998) to enhance the recovery of membrane and/or cytoskeleton-associated proteins. Finally, parasites were extracted in 1% SDS. The four extraction methods were assessed in relation to their usefulness for various applications.

2. Materials and methods

2.1. Parasite in vitro culture and purification

N. caninum tachyzoites (Nc1, kindly provided by E. Innes, Moredun Research Institute) were cultured in VERO-cell monolayers according to Hemphill and Gottstein (1996) except that horse instead of bovine serum was used in order to prevent any non-specific binding by anti-bovine antibodies in later assays. Briefly, VERO cells were maintained in RPMI 1640 (with L-glutamine and 25 mM HEPES) (Biosciences) supplemented with 7.5% heat-inactivated horse serum and incubated at 37 °C at 5% CO_2. Once monolayers had formed the VERO cells were subcultured by trypsinisation in 0.05% trypsin-EDTA (Biosciences). Following washing in fresh culture media, viable cells were counted in a haemocytometer by exclusion of trypan blue stain and used to initiate new VERO cell cultures. For parasite maintenance, VERO cell monolayers were infected with tachyzoites at a 2 tachyzoites:1 VERO cell ratio. After 72 h or once the majority of infected host cells had detached, they were scraped from the culture flask, concentrated by centrifugation ($814 \times g$) and counted. A portion was used to reinfect new monolayers, while the remainder was washed twice in ice-cold PBS, passed repeatedly through a 26G hypodermic needle to disrupt host cells and then purified on a PD-10 column (Amersham). Following purification, tachyzoites were suspended at a concentration of 0.5 to 1×10^8 parasites/ml in PBS containing 10 μl/ml protease inhibitor cocktail

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