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# Comparative analysis of the immune responses induced by native versus recombinant versions of the ASP-based vaccine against the bovine intestinal parasite *Cooperia oncophora*

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ABSTRACT

The protective capacities of a native double-domain activation-associated secreted protein (ndd-ASP)-based vaccine against the cattle intestinal nematode *Cooperia oncophora* has previously been demonstrated. However, protection analysis upon vaccination with a recombinantly produced antigen has never been performed. Therefore, the aim of the current study was to test the protective potential of a *Pichia*-produced double-domain ASP (pdd-ASP)-based vaccine against *C. oncophora*. Additionally, we aimed to compare the cellular and humoral mechanisms underlying the vaccine-induced responses by the native (ndd-ASP) and recombinant vaccines. Immunisation of cattle with the native *C. oncophora* vaccine conferred significant levels of protection after an experimental challenge infection, whereas the recombinant vaccine did not. Moreover, vaccination with ndd-ASP resulted in a higher proliferation of CD4-T cells both systemically and in the small intestinal mucosa when compared with animals vaccinated with the recombinant antigen. In terms of humoral response, although both native and recombinant vaccines induced similar levels of antibodies, animals vaccinated with the native vaccine were able to raise antibodies with greater specificity towards ndd-ASP in comparison with antibodies raised by vaccination with the recombinant vaccine, suggesting a differential immune recognition towards the ndd-ASP and pdd-ASP. Finally, the observation that animals displaying antibodies with higher percentages of recognition towards ndd-ASP also exhibited the lowest egg counts suggests a potential relationship between antibody specificity and protection.


## 1. Introduction

The economic impact of gastrointestinal nematode infections in ruminants has been extensively demonstrated over the years (Charlier et al., 2014). To date, control of these infections in livestock relies almost exclusively on the use of anthelminthic drugs, but the increasing spread of anthelmintic resistance worldwide illustrates the need for alternative control strategies (De Graef et al., 2013; Geurden et al., 2015). Successful vaccination against gastrointestinal nematodes with native and recombinant proteins in cattle and sheep (Meyvis et al., 2007; Besier et al., 2012; Nisbet et al., 2013; Vlaminck et al., 2015; Gonzalez-Hernandez et al., 2016), demonstrates that protein-based vaccination is a promising alternative to the current control methods (Matthews et al., 2016). Recently, our group has shown that vaccination with native double-domain activation-associated secreted protein (ndd-ASP) obtained from the excretory-secretory material of the adult stages of the bovine intestinal nematode *Cooperia oncophora* (Borloo et al., 2013) provides protection both under experimental and natural conditions, as indicated by a decrease in the cumulative egg output of 91% and 59%, respectively (Vlaminck et al., 2015). Under natural conditions, vaccination of cattle with the ndd-ASP also resulted in a 65% reduction in pasture larval counts and a decrease in 82% of worm counts when compared with the control group (Vlaminck et al., 2015). Additional studies on the immune response elicited by vaccination with ndd-ASP have shown an increased antigen-specific antibody production, which correlated negatively with the egg output. Moreover, significant T-cell proliferation was induced in ndd-ASP vaccinated animals after in vitro stimulation with the antigen, which was positively

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correlated with a higher proportion of immature larval stages (Van Meulder et al., 2015). Despite these promising results achieved with the native antigen, recombinant production of the antigen would be an absolute requirement for the economic viability of the vaccine (Geldhof et al., 2007; Matthews et al., 2016). Therefore, the objectives of the present study were: (i) to produce and evaluate the protective capacity of a *Pichia*-produced version of the dd-ASP, and (ii) to compare the cellular and humoral responses induced by both native and recombinant versions of the antigen.

## 2. Materials and methods

### 2.1. Native and recombinant antigen production

Preparation of the native *C. oncophora* dd-ASP (ndd-ASP) was carried out as previously described (Borloo et al., 2013a). In short, excretory-secretory material of adult worms was collected and fractionated over a Superdex 200 16/70 size-exclusion chromatography column. The purity of the ndd-ASP fraction, which eluted first from the column, was checked by reducing and non-reducing one-dimensional gel electrophoresis (SDS–PAGE) and visualized by Simply-Blue SafeStain (Invitrogen, USA) staining. Afterwards, the protein band was excised and protein identity was further confirmed by mass spectrometry.

Recombinant double-domain ASP (pdd-ASP) was expressed in *Pichia pastoris* as follows: its coding sequence (Borloo et al., 2013b) was PCR-amplified and subsequently cloned into the pGEMt-Easy subcloning vector (Promega, USA) according to the manufacturer's instructions. Following transformation into *Escherichia coli* DH5α competent cells (Invitrogen), clone selection on X-gal plates and sequence verification, the pGEMt-Easy-nDD-ASP construct was linearized by *Eco*RI-*Xba*I digestion and inserted in the *Pichia* expression vector pPICZα B (Thermo Fischer Scientific, USA). The resulting expression plasmid was used to transform *P. pastoris* strain KM071H (Invitrogen) by electroporation. Afterwards, individual clones growing on minimal plates were isolated and tested for secretion of pdd-ASP by SDS–PAGE followed by Coomassie Brilliant Blue staining and immunoblotting with bovine-anti-ndd-ASP antibodies. A clone expressing pdd-ASP was freshly grown on minimal plates and then used to inoculate a shake flask culture with buffer minimal glycerol complex medium (BMGY). After 48 h of growth at 29 °C, the cells were pelleted by centrifugation for 15 min at 1,000*g* followed by resuspension of the cells in buffered methanol complex medium (BMMY) and further growth at 29 °C. Every 24 h, extra methanol (0.5%) was added to the culture and after 96 h of induction the cells were finally pelleted by centrifugation for 15 min at 2,000*g*. The cell medium was harvested and filtered over a 0.22 µm membrane, after which the supernatant was concentrated on a Centriprep YM10 (Millipore, USA), dialyzed to 50 mM sodium acetate pH 5.0 and desalted on a HiPrep 26/10 Desalting column (GE Healthcare Bio-Sciences, USA). This fraction was then applied to a Resource-S cation exchange column (GE Healthcare Bio-Sciences AB) equilibrated in 50 mM sodium acetate buffer (pH 5.0) and the bound pdd-ASP was eluted by employing a gradient from 0 up to 1 M sodium chloride (NaCl) in the same buffer. Fractions containing pdd-ASP were pooled and buffer-exchanged to PBS by gel filtration on a Sephadex G25-column (GE Healthcare Bio-Sciences). The protein profile of the obtained material was checked on a 12% reducing SDS–PAGE. Additionally, ndd- and pdd-ASP proteins were blotted onto a poly-vinylidene fluoride (PVDF) membrane for 1 h at 1 mA/cm$^2$ of gel surface followed by a 1 h blocking step in PBS +0.5% Tween80 (PBST). The membrane was afterwards incubated with ndd-ASP-specific antibodies (dilution 1/500) for 1 h, followed by extensive washing with PBST and incubation for 1 h with horseradish peroxidase (HRP)-conjugated rabbit anti-bovine IgG1 antibody. 3,3′-diaminobenzidine (DAB) (Sigma–Aldrich, USA) served as a substrate. All steps were performed at room temperature.

### 2.2. Immunization experiments in cattle

All animal experiments were conducted in accordance with the European Union Animal Welfare Directives and VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products) guidelines for Good Clinical Practice, and ethical approvals to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2014/70, EC2015/40). Two vaccination studies were carried out in cattle as previously described (Van Meulder et al., 2015; Vlaminck et al., 2015).

The aim of study 1 was to investigate the protective capacity of the recombinant antigen. Twenty-one helminth-naïve male crossbreed Holstein calves (6–8 months of age) were randomly divided over three groups of seven animals (QuilA control, ndd-ASP+QuilA and pdd-ASP+QuilA). Subsequently, a second study was performed to analyse and compare the cellular responses induced by the native and recombinant antigens, where 12 helminth-naïve female crossbreed Holstein calves (6–8 months of age) were randomly divided over three groups of four animals (QuilA control, ndd-ASP+QuilA and pdd-ASP+QuilA). For both studies, all animals were immunized three times i.m. in the neck at 3 week intervals. Control animals received 750 µg of QuilA (Superfos Biosector, USA), while the animals in the ndd-ASP+QuilA and pdd-ASP+QuilA groups received 30 µg of antigen in combination with 750 µg of QuilA per immunization. All animals were challenged with a trickle infection of 25,000 L3s (1000 L3s/day; 5 days/week for 5 weeks), which started on the day of the third immunization, and were then euthanized 3 weeks after the last infection. Parasitological parameters (i.e. faecal egg and worm counts) were monitored as previously described (Van Meulder et al., 2015; Vlaminck et al., 2015).

### 2.3. Isolation of peripheral blood mononuclear cells (PBMCs)

In Study 2, blood samples were collected weekly from the jugular vein using vacutainer tubes. PBMCs were isolated by Lymphoprep (Nycomed Pharma, Norway) gradient centrifugation. Additionally, mesenteric lymph nodes (LNs) of the small intestine were isolated at the time of necropsy, and LN mononuclear cells (MNCs) were isolated by homogenization through mechanical disruption of the tissue followed by Lymphoprep gradient centrifugation. After centrifugation, all mononuclear cell fractions were isolated, washed and counted prior to cell culture and flow cytometric analysis.

### 2.4. Flow cytometry

Cells were labelled in Flow Cytometry Staining Buffer (eBioscience, USA) and all antibodies were used at the concentration recommended by the supplier. After incubation for 20 min with primary antibodies, the cells were washed twice prior to staining with fluorescently-labelled secondary antibodies. The cells were then incubated for an additional 20 min, washed and resuspended in PBS to be immediately analysed using a FACS Aria III flow cytometer (BD Biosciences, USA). Non-viable cells were excluded from the analysis based on their propidium iodide (Life Technologies, USA) uptake.

Primary antibodies used were: non-labeled CD3 (MM1A, IgG1), TCRγδ (GB21A, IgG2b), CD21 (BAQ15A, IgM), CD8 (BAQ111A, IgM) (all from Monoclonal Antibody Center, Washington University, USA), CD4 (CC8, IgG2a) and CD335-IgG2b (AKS6, IgG1, kindly

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