Veterinary Immunology and Immunopathology 218 (2019) 109936

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Assessing the clinical and bacteriological outcomes of vaccination with recombinant Asp14 and OmpA against *A. phagocytophilum* in sheep

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ARTICLE INFO

Keywords:
Anaplasma phagocytophilum
Recombinant protein
Vaccination
Serological response
Peripheral mononuclear blood cells

ABSTRACT

Anaplasma phagocytophilum is a tick borne bacterium, causing disease in sheep and other mammals, including humans. The bacterium has great economic and animal welfare implications for sheep husbandry in Northern Europe. With the prospect of a warmer and more humid climate, the vector availability will likely increase, resulting in a higher prevalence of *A. phagocytophilum*. The current preventive measures, as pyrethroids acting on ticks or long acting antibiotics controlling bacterial infection, are suboptimal for prevention of the disease in sheep. Recently, the increased awareness on antibiotic- and pyrethorid resistance, is driving the search for a new prophylactic approach in sheep against *A. phagocytophilum*.

Previous studies have used an attenuated vaccine, which gave insufficient protection from challenge with live bacteria. Other studies have focused on bacterial membrane surface proteins like Asp14 and OmpA. An animal study using homologous proteins to Asp14 and OmpA of *A. marginale,* showed no protective effect in heifers. In the current study, recombinant proteins of Asp14 (rAsp14) and OmpA (rOmpA) of *A. phagocytophilum* were produced and prepared as a vaccine for sheep. Ten lambs were vaccinated twice with an adjuvant emulsified with rAsp14 or rOmpA, three weeks apart and challenged with a live strain of *A. phagocytophilum* (GenBank acc.nr M73220) on day 42. The control group consisted of five lambs injected twice with PBS and adjuvant. Hematology, real time qPCR, immunodiagnostics and flow cytometric analyses of peripheral blood mononuclear cells were performed. Vaccinated lambs responded with clinical signs of *A.phagocytophilum* infection after challenge and bacterial load in the vaccinated group was not reduced compared to the control group. rAsp14 vaccinated lambs generated an antibody response against the vaccine, but a clear specificity for rAsp14 could not be established. rOmpA-vaccinated lambs developed a strong specific antibody response on days 28 after vaccination and 14 days post-challenge. Immunofluorescent staining and flow cytometric analysis of peripheral blood mononuclear monocytes revealed no difference between the three groups, but the percentage of $CD4^+$, $CD8^+$, $\gamma\delta$ TcR^+, λ-Light $chain^+$, $CD11b^+$, $CD14^+$ and MHC II^+ cells, within the groups changed during the study, most likely due to the adjuvant or challenge with the bacterium. Although an antigen specific antibody response could be detected against rOmpA and possibly rAsp14, the vaccines seemed to be ineffective in reducing clinical signs and bacterial load caused by *A. phagocytophilum*. This is the first animal study with recombinant Asp14 and OmpA aimed at obtaining clinical protection against *A. phagocytophilum* in sheep.

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https://doi.org/10.1016/j.vetimm.2019.109936
Received 3 April 2019; Received in revised form 22 August 2019; Accepted 30 August 2019

1. Introduction

Vector borne diseases in humans and animals are likely to increase in Europe and elsewhere, due to a warmer and more humid climate (Alkishe et al., 2017; Jaenson and Lindgren, 2011; Wikel, 2018). Thus, climate changes may undermine or overwhelm the effects of control programs, thereby having a severe impact on public health and leaving the current control strategies for vector borne diseases in humans and animals ineffective (Campbell-Lendrum et al., 2015). In sheep, the current preventive treatments against tick borne diseases, mainly involve the use of pyrethroid acaricides (Stuen et al., 2013) and broad spectrum antibiotics in clinically affected animals (Stuen and Bergstrom, 2001a). Pyrethroids reduce the tick infestation on animals, but lambs are still not protected from transmission of tick pathogens (Stuen et al., 2012). There is a perpetual concern for developing resistance against antibiotics (WHO, 2018) and antiparasitic drugs in general (Abbas et al., 2014). Thus, seeking alternatives to current therapies is a priority to meet future challenges. Despite multiple efforts and approaches, there are currently few studies presenting evidence of protection against tick borne infections by the use of vaccines (Contreras et al., 2017; de Vos et al., 2001; Ducken et al., 2015; Shkap et al., 2002; Stuen et al., 2015).

Anaplasma phagocytophilum is the most widespread tick borne pathogen in farm animals in Northern Europe (Stuen et al., 2013), and is known as the agent of tick borne fever (TBF) in sheep (Foggie, 1951). In Norway, it has previously been estimated that 300 000 lambs become infected each year (Stuen et al., 2002). Lambs and naïve sheep are especially susceptible to the infection, and clinical signs normally develop between 4 to 14 days post infection (Macleod, 1933). Clinical signs include high fever (> 41 °C), depression and lethargy (Foggie, 1951; Macleod, 1933). The infection typically causes immune suppression, leaving the sheep vulnerable to secondary infections such as septicemia, pyemia, arthritis and pneumonia (Stuen et al (2013). The infection can be verified by examinations of blood smears for inclusion bodies in neutrophil granulocytes or by PCR (Foggie, 1951; Hart et al., 1992; Henningsson et al., 2015). At necropsy, splenomegaly is commonly observed in infected individuals (Overas et al., 1993).

Previously, a strain of *A. phagocytophilum* causing disease in horses, was inoculated in lambs, but did not generate protection against a strain typically isolated from sheep (Stuen et al., 1998). Stuen et al. (2003) showed that a highly virulent isolate of the bacterium in sheep, provided immunity against a less virulent strain in sheep, but not conversely. In a recent study, *A. phagocytophilum* isolated from sheep neutrophils was formalin inactivated and used as a vaccine in lambs (Stuen et al., 2015). After the vaccination, all lambs became infected and displayed clinical signs of tick borne fever when challenged with live bacteria. The immunological background for the above mentioned outcomes is unknown and supports the need for more knowledge on the host-pathogen interactions and the mechanisms of immunity.

Recent studies on surface proteins of *A. phagocytophilum*, i.e. 14-kDa *A. phagocytophilum* (Asp14) and outer membrane protein A (OmpA) showed that these are potential candidates for vaccine development due to their conserved and immunogenic properties (Kahlon et al., 2013; Ojogun et al., 2012; Seidman et al., 2015). The two proteins have been shown to be upregulated in the early stages of infection in the human leukemia cell line 60 (HL-60 cells), and are possibly involved in the infection of host cells (Kahlon et al., 2013; Ojogun et al., 2012). Pretreatment of HL-60 cells with antibodies against Asp14 or OmpA, significantly reduced the infection which indicated that Asp14 and OmpA should be introduced in animal vaccine studies (Kahlon et al., 2013; Ojogun et al., 2012). In a previous study by Ducken et al. (2015), heifers were vaccinated with the homologous proteins of Asp14 and OmpA from *A. marginale* (Ducken et al., 2015). The vaccination resulted in a serological response, but did not protect the heifers when they were challenged with *A. marginale* (Ducken et al., 2015). This study describes the effects of recombinant Asp14 (rAsp14) and recombinant OmpA (rOmpA) in a sheep model for the first time, emphasizing on the clinical response, bacterial load and the immunological response in sheep after the challenge with *A. phagocytophilum.*

2. Material and methods

2.1. Approval of animal study

The Norwegian Animal Research Authority, approved the ethical standards and the protocol, approval number FOTS ID 8005, used in the study.

2.2. Generation of recombinant proteins for animal study

2.2.1. Inserting and propagating the genes asp14 and ompA in a vector

The isolate of *A. phagocytophilum* GenBank acc.no M73220 was used when the open reading frames of *asp14* and *ompa* (devoid of signal peptides) were extracted and PCR amplified using the iProof High fidelity DNA polymerase (Bio-Rad, CA, USA). PCR primers were designed to incorporate specific restriction enzymes vectors at the 5′end of the genes to enable directional cloning into the pET101/D-TOPO expression system (ThermoFisher Scientific, MA, USA) (Table 1). Purified PCR products were then ligated into the pET101/D-TOPO vector, following manufacturer's instructions (ThermoFisher Scientific, MA, USA). The correct orientation of the genes was verified by enzyme restriction and Sanger sequencing, and the amplified and cloned sequences were as expected.

2.2.2. Expression and purification of recombinant proteins

Expression and purification of rAsp14 and rOmpA was performed as previously described (Crosby et al., 2018). Briefly, recombinant *asp14* and *ompa* constructs were introduced into BL21 Star™ (DE3) Chemically Competent *E. coli* cells (ThermoFisher, CA, USA) and incubated in Luria Bertani broth (LB-broth) w/ carbenicillin 50 µg/ml and 1% glucose at 37 °C overnight. After incubation, the cell suspension was centrifuged at 4 000 x G for 20 min at 4 °C and the resulting cell pellets were then incubated in M9 minimal media with carbenicillin 50 µg/ml and 1% glucose. Protein expression was induced with IPTG (1 M isopropyl-β-D-thiogalactopyranoside), (0.5 mM/l final concentration) for 24 h at 4 °C. The low incubation temperature was chosen on the basis of previous

Table 1
Primers for genes *asp14* and *ompa*. Construction of primers were based on sequenced *A. phagocytophilum* (GenBank acc.no M73220) (ApNorLambV1) (Al-Khedery et al., 2012) and analyzed in EMBOSS Explorer. The ABI V2-Veritas machine was used. Life Technologies (ThermoFischer, CA, USA) supplied primers and probe required for *glt*A.

Gene	Forward primer	Reverse primer	PCR setting
asp14	5'-**CAC CAT G**AT ACC ATT AGC TCC TTG GAA GAG-3'	5'-GCT TTC TTT AGG AGT GTT GGT GCC G-3'	72 °C-25 sec, 72 °C-5 min, 4 °C-hold
ompa	5'-**CAC CAT G**AC TCT TCT TCC AGA TAG TAA-3'	5'-CTT AGC GAT TTC GCT AGA GAA TTC AGA AG-3'	98 °C-2 min, 98 °C-10 sec, 60 °C-30 seconds
*glt*A*	5'-TTT TGG GCG CTG AAT ACG AT-3'	5'-TCT CGA GGG AAT GAT CTA ATA ACG T-3'	Ref. (Henningsson et al., 2015)

Underlined and bold nucleotides in forward primers of *asp14* and *ompa* are designed to incorporate vector sequences at the 5′ end for directional cloning in the pET101/D-TOPO directional expression system.

* probe 5'-FAM-TGC CTG AAC AAG TTA TG-BHQ1-3, (acc.no AF3041137.1).

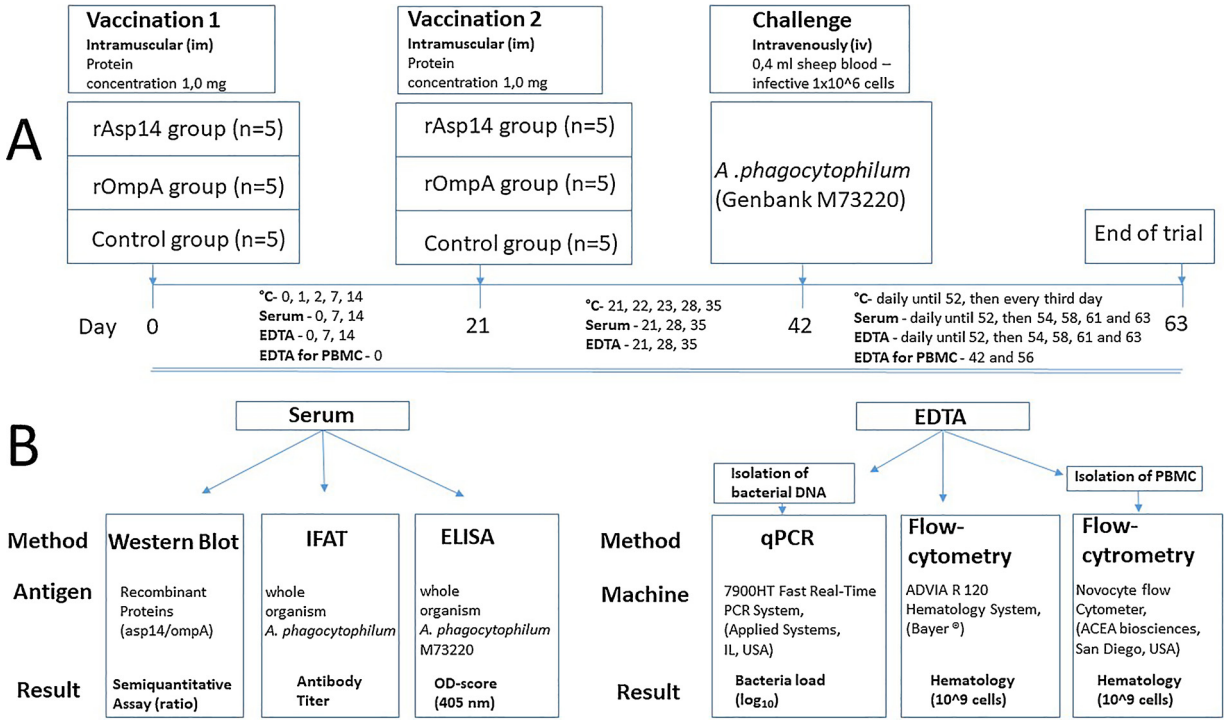


Fig. 1. An overview of the experiment: time line, sampling and the employed techniques for examination of the samples.

studies suggesting increased solubility of the final recombinant protein (Crosby et al., 2018; Ferrer et al., 2004; Sorensen and Mortensen, 2005).

Recombinant Asp14 and rOmpA proteins were purified by using columns (Kimble® Chase Flex®, TN, USA), containing high density nickel beads (Golbio Biotechnology Inc, MO, USA), and eluted with elution buffer (sodium phosphate 50 mM, pH 8, NaCl 300 mM and imidazole 500 mM). Fractions were dialyzed four times or centrifuged in an Amicon Ultra 4 10 K (Merck, Darmstadt, Germany) to remove imidazole. Subsequent, the dialysate was centrifuged at 20 000 x G for 10 min at 4 °C, then frozen at −20 °C.

2.2.3. Verification of the rAsp14 and rOmpA

The molecular mass of rAsp14 and rOmpA plus his tags prepared for the vaccine, were verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. The concentration of the eluted products of rAsp14 and rOmpA were measured on a Quebit (ThermoFischer, CA, USA), then 11.25 µg and 9.75 µg of rAsp14 and OmpA-elution were loaded onto separate lanes on a 4–12 % SDS-PAGE. Further, the SDS-PAGE was stained in Coomassie blue (Bio-Rad, CA, USA), but protein bands were first semi dry transferred to a nitrocellulose membrane for further analysis on a western blot. The western blot was probed with primary antibody; mouse anti-his 1/10,000 (cat.no H99-61), (SignalChem, BC, Canada), secondary antibody was goat anti-mouse-HRP 1/100,000 (cat.no 074-1806) (KPL, MD, USA)and the substrate was SuperSignal West Femto (Thermo Scientific, Waltham, USA).

Due to difficulties in achieving highly purified recombinant proteins for vaccination of the lambs, additional batches of rAsp14 and rOmpA was made to enable detection of antigen specific antibody response in sera from vaccinated lambs on western blots. The additional batches were further used for liquid chromatography mass tandem spectrometry (LC/MS/MS). These batches underwent an additional step of ion exchange chromatography (IEX) providing purer rAsp14 and rOmpA, and this step was added after the removal of imidazole as described in "Expression and purification of recombinant proteins". The batches of rAsp14 and rOmpA, which underwent the additional step, were concentrated and diluted with phosphate buffered saline (PBS) to their initial volume of 10 ml. This was repeated five times. Then the samples underwent a buffer exchange, using Amicon® Ultra centrifugal units (Merck, Darmstadt, Germany) from PBS to 50 mM NaCl, 10 mM Tris pH 8.0, 10 mM B—ME buffer. The sample was then added to an equilibrated Resource™ Q-anion exchange column (6 ml) (GE Healthcare Life Sciences, IL, USA) on a Bio-Rad NGC™ Chromatography System (Bio-Rad, CA, USA), before the protein was eluted with high salt buffer (1 M NaCl, 10 mM Tris pH 8.0, 10 mM B—ME) gradient 0–100% to increase the purity of the protein. The eluted proteins from IEX purified rAsp14 or rOmpA, underwent 5 min of boiling (100 °C), before proteins ran on an Amersham ExelGel SDS-PAGE gradient 8–18% (cat.no 80125553)) (GE Healthcare Life Sciences, IL, USA) with ExelGel SDS Buffer strips. Proteins diffused onto a nitrocellulose membrane used in western blot, before the gel was stained with Simply Blue Safe Stain (Novex, Life Technologies, IL, USA). In the western blot, each target protein was probed with 1:4000 Anti-His monoclonal antibodies (mAb) (SignalChem, BC, Canada) followed by a secondary antibody, i.e., Donkey anti-rabbit IgG (cat.no NA934-1 ML)(GE Healthcare Life Sciences, IL, USA) conjugated to horseradish peroxidase with enhanced chemiluminescence (ECL) in PBS (1:1500) and incubated at 4 °C. The bands were detected with diaminobenzidine (DAB) in 0.1 M Na-acetate buffer at pH 4 and 0.01% H_2O_2 (Merck, Darmstadt, Germany). Bands with appropriate size to rAsp14 and rOmpA on the SDS-PAGE and the western blot, were cut out from the SDS-PAGE and confirmed by LC/MS/MS as described in a previously published protocol (Faeste et al., 2010). Peptide data from LC/MS/MS were analyzed in Clustal O 1.2.4 (UCD, Dublin, Ireland) (Sievers et al., 2011).

2.2.4. Study design, animals and sampling routine

Fifteen, 5-6 months old lambs of the "Norwegian white sheep" breed were randomly selected from the experimental sheep flock at the research station of the Norwegian University of Life Sciences in Sandnes, Norway. The lambs were randomly allocated to three groups: rAsp14 and adjuvant, rOmpA and adjuvant and control, the latter injected with PBS and adjuvant. Each group comprised of five lambs.

Lambs were confined indoors and groupwise in pens, on plastic

slatted floor and kept isolated from ticks from birth to the end of the study (days 0–63) (Fig. 1). During the study, blood was regularly sampled from *V.jugularis* in conjunction with the temperature observations (Fig. 1). The lambs were screened for serological responses against *A. phagocytophilum* with an indirect immunofluorescence antibody test (IFAT) before the start of the study (Artursson et al., 1999; Stuen and Bergstrom, 2001b) and all the lambs tested negative.

2.2.5. Vaccination and challenge procedure with A. phagocytophilum

Vaccine doses comprised of 1 mg of rAsp14 or rOmpA, which was emulsified with the adjuvant Montanide™ ISA 61 VG (Seppic, France) in a single-use, two syringe apparatus with I-connectors (Promepla, Monaco). The emulsification procedure was in accordance with the producer's instructions for adjuvant preparation, and resulted in a 2 ml vaccine dose (60/40 vol ratio – of adjuvant/recombinant protein). The control lambs received PBS emulsified with Montanide™ ISA 61 VG (Seppic, France) prepared in the same way as rAsp14 and rOmpA vaccine dosages, resulting in 2 ml of injection dosage for control lambs. The rationale for emulsifying the adjuvant with PBS was to standardize the injection volume for all the groups. Injection of the vaccines and control was intramuscular as previously described (Kalyanasundaram et al., 2015; Rainard et al., 2015) The injection site was thoroughly shaved and disinfected with 70% ethanol before each injection. An oversight of the timeline for the study is provided in Fig. 1.

Prior to challenge, an inoculum (Genbank accnr. M73220) stored at −70 °C was injected into one naïve lamb for propagation of bacteria (not shown). When the lamb presented clinical signs of infection, blood was collected by jugular venipuncture and the amount of circulating bacteria was determined by blood smear microscopy according to Foggie et al (1951). The lambs of the rAsp14, rOmpA and the control groups were then challenged intravenously with 0.4 ml sheep blood containing 1 × 10^6 *A. phagocytophilum* infected cells (2.5 × 10^6 infected cells/ml). Estimation of the infected cells were done by counting 400 neutrophils for morulae in blood smear microscopy. This provided important information on the pathogenicity of the isolate since morulae are formed by live bacteria. Since studies have indicated that a small quantity of bacteria also can cause infection and clinical disease, the numbers of bacteria inoculated was of less importance than standardization (Stuen and Artursson, 2000).

2.2.6. Quantification of A. phagocytophilum by citrate synthase (gltA) gene real-time PCR

Extraction of DNA from 500 µl blood was performed with MagNA Pure LC 2.0 Instrument (Roche, Penzberg, Germany), using MagNA Pure LC DNA Isolation Kit – Large Volume according to the manufacturer's instructions (Roche, Penzberg, Germany). Detection of *A. phagocytophilum* was performed using a TaqMan real-time PCR assay designed to target the *gltA* gene, amplifying a 64 bp long product, primers are listed in Table 1. To quantify the number of *A. phagocytophilum* cells, a serial dilution of plasmid standard was used. The plasmid contained the target sequence of the TaqMan real-time PCR assay, spanning the nucleotides 304–420 of the *gltA* gene (acc. no AF304137.1), synthesized and cloned in a pUC57 vector (Genscript USA Inc, NJ) (Table 1). The concentration of the plasmid solution was determined using NanoDrop® ND-1000 (Wilmington, DE).

2.2.7. Antibody responses against whole-body A. phagocytophilum and the recombinant proteins

Indirect immunofluorescence test (IFAT) (Protatek™, MN, USA) was conducted at SVA (National Veterinary Institute, Sweden). The method was previously described (Artursson et al., 1999; Stuen and Bergstrom, 2001c). Antibody titer 1:40 was determined as the cut off value (Stuen et al., 2003).

Further, the antibody responses against native Asp14 and OmpA were tested by an ELISA coated with whole-body *A. phagocytophilum* (GenBank acc. no. M73220). Bacteria were isolated in accordance with previous published protocol by Crosby et al (2018). Two 384-well microtiter plates (Sigma-Aldrich, MO, USA) were coated with *A. phagocytophilum* (Genbank acc.no M73220) organisms, 1×10^8 cells/well, isolated from infected cultures of *Ixodes scapularis* (ISE6) tick cells, in 50 µl of carbonate-bicarbonate buffer (Sigma-Aldrich, MO, USA). Between every step, plates were rinsed in 1 X PBS (pH 7.3), 0.05% Tween 20). The coated wells were blocked with 100 µl of 1% BSA in PBS for 2 h at room temperature. Serum samples from vaccinated lambs from rAsp14, rOmpA and control groups, at the dilutions: 1:150, 1:300, 1:600, 1:1200, 1:2400 and 1:4800, were added to the wells and incubated for 2 h at 700 rpm. Then the plates were incubated for 1 h, rotating at 700 rpm at room temperature, with the 1:5000 recombinant protein A/G-Alkaline phosphatase (Sigma-Aldrich, MO, USA). Subsequent to incubation, 4-Nitrophenyl phosphate (Sigma-Aldrich, MO, USA) was added, and optical densities (Synergy HT Bio-Tek, VT, USA) were measured in a microplate reader at 405 nm. As positive serum controls, serum from *A. phagocytophilum* infected lambs were used at the same dilutions as described above. Negative controls were lambs vaccinated and challenged with PBS during the study (not shown). Cut off was set to 3x standard deviation (SD) of the three group's mean on day zero.

Antibody responses against rAsp14 and rOmpA were tested and semi-quantified on a western blot. The procedure was equal to the description of western blot above, in "Verification of the rAsp14 and rOmpA", however slightly modified as diluted sera (1:100) were used as a probe instead of 1:4000 Anti mAb (SignalChem, BC, Canada), and the secondary antibody was peroxidase-conjugated monoclonal anti-goat/sheep IgG (Sigma-Aldrich, St. Louis, USA)(1: 10 000). The ImageJ 1.51k (NIH, MD, USA) software was used in semi-quantifying the antibody response on the western blot. The program measured the band densities vs. their background, resulting in a ratio used in the interpretation of the groups antibody responses. Cut off was set to three SD above the mean on day zero for the vaccinated and control groups.

2.2.8. Separating and phenotyping peripheral blood mononuclear cells (PBMC)

Sampling of EDTA blood were performed on day 0, 42 and 63, the former two were important in providing a baseline before evaluating PBMCs after challenge on day 56. After sampling, EDTA blood were stored at 4 °C over-night. Peripheral blood mononuclear cells (PBMC) were isolated with a density gradient medium (Lymphoprep; Axis-Shield, Norway) as previously described (Lybeck et al., 2009), and stored at −80 °C in freezing solution consisting of fetal bovine serum (FBS) and 10% DMSO (dimethyl sulfoxide) (Panreac Applichem ITV, Barcelona, Spain).

Prior to analyzes, PBMCs were thawed in a 37 °C water bath before adding prewarmed RPMI 1640 medium with 20% fetal bovine serum (FBS) (Biowest, ID, USA) dropwise. Further, PBMCs were transferred to 96-well plates (3×10^5 cells/well) and stained with LIVE/DEAD fixable Aqua Dead cell stain kit (Invitrogen, CA, USA). This was followed by incubation with unconjugated primary antibodies, catalogue.no in brackets, and producers in superscript; CD4[a] (S-GT2001), CD8[a] (BOV2017), γδ-TCR[a] (S-GT2011), CD25[a] (BOV2076), MHCII[a], CD21[a] (BAQ15A), λ-Light chain[a] (S-BOV2063), WC1[a] (S-BOV2050), CD14[b] (MCA1568), CD11b[b] (MCA1425GA) and NCR1/NKp46[c]. Superscript [a] was Monoclonal antibody center (Washington State University, USA), [b] was Bio-Rad (Hercules, CA, USA) and [c] is described in acknowledgements. The secondary antibodies and respective catalogue no. are shown here; allophycocyanin (APC) conjugated goat anti-mouse IgG1 (1070-115) and IgG2b (1090-11 L), fluorescein isothocyanate (FITC-) conjugated goat anti-mouse IgG2a (1082-02) and IgG3 (1100-02) and phycoerythrin (PE)-conjugated goat anti-mouse IgG1 (1070-09), IgG2b (1090-09), IgG3 (1100-09) and IgM (1020-09) (all Southern Biotech, AL, US). All incubations were 30 min on ice. Between every incubation, plates were washed with PBS or PBS with 1% BSA. Ten percent goat serum was used to block unspecific binding before secondary antibodies

were added. Novocyte flow cytometer (ACEA biosciences, San Diego, USA), using the NovoExpress software, version 1.2.4 (ACEA biosciences, San Diego, USA) was used for cellular analyzes. Cells were gated in forward/side scatter plots to include lymphocytes and monocytes. The positive fluorescence gates were set with reference to negative controls where primary antibodies were omitted. Concentrations of mAbs were based on initial or previous studies (not shown).

2.3. Statistical analysis

Normality tests were performed for all data, both directly and after preliminary regression analyses followed by inspection of residuals and the data were further investigated for normality in a Shapiro-Wilk test test ($\alpha = 0.05$), with non-normality as the overall result for the data. Thus, clinical and hematological data were analyzed with Spearman rank's order correlation and cluster analyzes in Stata 14.2 SE (StataCorp. 2015. *Stata Statistical Software: Release 14*. College Station, TX: StataCorp LP). The PBMC and the serological data were analyzed with a non-parametric Friedman's test, followed by Dunnet's multiple comparison test in GraphPad Prism Software Version 7.04 (CA, USA). The cutoff for statistical significance was set to $p < 0.05$.

3. Results

3.1. Verification of the rAsp14 and rOmpA

The predicted molecular mass for rAsp14 and rOmpA was 17.4 kDa and 23.6 kDa, respectively including V and His-tag. However, the bands measured 19 kDa and 28 kDa on the western blot for rAsp14 and rOmpA respectively (Figs. 2A and B and 3 A and B). The LC/MS/MS score for rAsp14 was 14.29 and peptides were identical to 64.1% of the amino acids in Asp14 (Fig. 3 C and E). In the band derived from rOmpA, LC/MS/MS identified 81.2% of the amino acids present in OmpA, while the score of the protein was 433.8 (Fig. 3D and F). In addition to the recombinant proteins, *E. coli* proteins were detected in both of the bands. Eleven peptides matching with *E. coli* in the rAsp14 band had an increased score compared to rAsp14 detected peptides (18.05–146.96, coverage 36.23–95.11) while in the rOmpA band; all peptides matching with *E. coli* were below 91.16 (coverage 1.56–70.99).

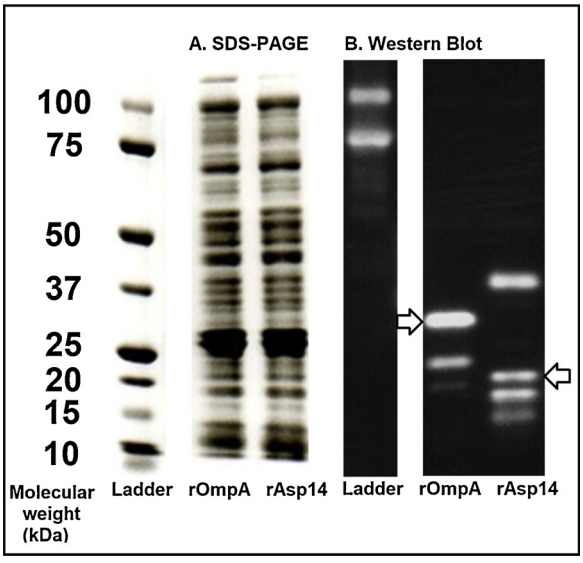


Fig. 2. Purity and quality of rOmpA and rAsp14 used for vaccination of lambs in this study. **A.** SDS-PAGE on rOmpA and rAsp14 **B.**Western blot on rOmpa and rAsp14. Arrows point to the site for where we find the recombinant proteins in the other western blots, and is slightly above predicted molecular weight.

3.2. Clinical effects of vaccination and challenge

A local skin nodule (5–30 mm) appeared at the injection site in two and three of the lambs in the rAsp14 and rOmpA groups, respectively. Skin reactions were not detected in the lambs of the control group.

All three groups had mean rectal temperatures < 40.0 °C and normal neutrophil counts ($> 0.7 \times 10^9$ cells/L) after the first and second vaccinations (Table 2). The progression of clinical signs and neutropenia was the same for the three groups subsequent to challenge (Table 2). Most evident was the wide range of outcomes in each of the different groups, characterized by wide standard deviations (SD) (Table 2). There were no statistical differences in the clinical or hematological responses between the three groups prior to or after the challenge.

3.3. Bacterial load

The bacteremia was detected from day four post challenge (pc.) in both vaccinated and control groups, and reached maximum levels on day six, which gradually declined by the end of the study (Fig. 4). There were large individual variations within each group, but there were no observed statistical differences in bacterial load between the three groups (Fig. 4).

3.4. Antibody responses

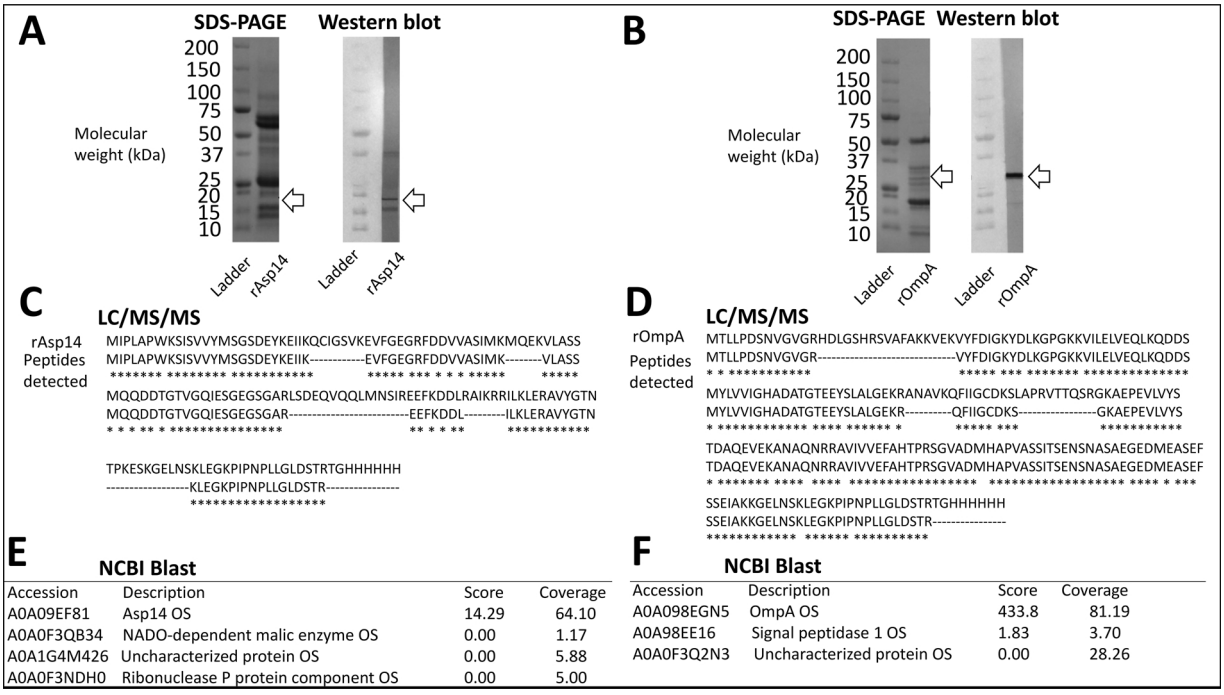
Antibodies against *A. phagocytophilum* were not detected in the rAsp14-group on the IFAT or the ELISA, prior to challenge (Fig. 5A and B). Five and four lambs showed antibody responses on IFAT and ELISA after challenge, respectively. The antibody responses observed against the rAsp14 on the semi-quantitative analysis of the western blot was weak, with one observation above cut off for each of the days 28 and 56 (Fig. 5C).

Likewise, in the rOmpA-vaccinated group, the IFAT and ELISA did not detect antibody responses prior to challenge (Fig. 5A and B), however after challenge, all rOmpA vaccinated lambs displayed antibody responses on the IFAT and ELISA (Fig. 5A and 3B). In addition, there were significant group differences between rOmpA and the control group both in the ELISA and in the semiquantitative assay (Fig. 5B and C). The rOmpA group was significantly increased compared with rAsp14 group in semiquantitative assay (Fig. 4C)

In the control group, no antibody responses were visible on IFAT, ELISA or on the semi-quantitative analysis of the western blot on rAsp14 or rOmpA before challenge (Fig. 5A, B and C). After challenge, antibody responses were detected against the whole-bacteria on both IFAT and ELISA (days 56 and 63) (Fig. 5A and B), although they were absent on the semi-quantitative analysis on the western blot targeting the recombinant proteins (Fig. 3C).

3.5. Phenotyping of peripheral blood mononuclear cells

The percentage of detected surface markers on PBMCs did not differ significantly between the three groups. However, there were statistical differences within each of the three groups when comparing observations on different days during the study (Fig. 6A-H). These differences comprised of a decrease in the concentration of $CD4^+$, $CD4^+CD25^+$cells (Fig. 6A and B) and $\gamma\delta$ TcR^+ T-cells (Fig. 6D) including $\gamma\delta$ T-cell population expressing work-shop cluster ($WC1^+$) (supplementary, Fig. 3A and B). Furthermore, there was an increase in the percentage of λ-Light-chain$^+$, MHC II^+ and $CD14^+$ cells between day zero and 56 (Fig. 6E, G and H). The concentration of $CD8^+$ T cells was stable in the rAsp14 and the control group, but a decrease was observed within the rOmpA group between day 42 and 56 (Fig. 6C). Mononuclear leucocytes expressing $CD11b^+$, increased significantly within the rAsp14 and rOmpA groups between day 0 and 56 (Fig. 6F). Further gating, showed that 88–99% of the $CD11b^+$ cells in all three



Accession	Description	Score	Coverage
A0A09EF81	Asp14 OS	14.29	64.10
A0A0F3QB34	NADO-dependent malic enzyme OS	0.00	1.17
A0A1G4M426	Uncharacterized protein OS	0.00	5.88
A0A0F3NDH0	Ribonuclease P protein component OS	0.00	5.00

Accession	Description	Score	Coverage
A0A098EGN5	OmpA OS	433.8	81.19
A0A98EE16	Signal peptidase 1 OS	1.83	3.70
A0A0F3Q2N3	Uncharacterized protein OS	0.00	28.26

Fig. 3. Verification of recombinant proteins. **A.** SDS-PAGE on rAsp14 and its western Blot detecting His-tag. The arrowhead on the SDS-PAGE, located approximately at 19 kDa, was cut out for LC/MS/MS. **B.** SDS-PAGE on sample containing rOmpA and its western blot detecting His-tag. The arrowhead on the SDS-PAGE, located approximately at 28 kDa, was cut out for LC/MS/MS**. C.** LC/MS/MS results on detected peptides identical to rAsp14 from cut band in fig. A. **D**.LC/MS/MS results on detected peptides identical to rOmpA from cut band in fig. B. **E.** Blast results on peptides matching *A. phagocytophilum* proteins in GenBank. **F.** Blast results on peptides matching *A. phagocytophilum* proteins in GenBank. The batch of rAsp14 and rOmpA in Figs. A and B was used for detecting serological response, and these results are shown in Fig. 5C and corresponding western blots in supplementary Figs. 1D and 2D.

Table 2
Clinical and hematology parameters (mean, SD ±), observed in rAsp14, rOmpA and the control group after challenge with *A. phagocytophilum*.

		rAsp14 (n = 5)	rOmpA (n = 5)	Control (n = 5)
First vaccination until challenge (days 0-41)	Temperature (mean, SD)	39.6 ± 0.40	39.6 ± 0.30	39.6 ± 0.31
	Neutrophilic cell count (1×10^9 cells/L), (mean, SD)	2.6 ± 1.14	2.5 ± 1.00	3.6 ± 2.10
Challenge until end of study (days 42-63)	Temperature (mean, SD)	39.8 ± 0.9	40.2 ± 0.94	40.1 ± 0.92
	Neutrophilic cell count (1×10^9 cells/L), (mean, SD)	1.6 ± 1.15	1.9 ± 0.94	2.3 ± 1.15
	Incubation (days)	3.4 ± 0.49	3.0 ± 0.00	3.4 ± 0.49
	Fever (> 40 °C), (days)	6.2 ± 2.48	10.2 ± 3.60	9.4 ± 2.65
	Neutropenia ($< 1 \times 0.7 \times 10^9$ cells/L), (days)	6.4 ± 2.93	4.8 ± 3.81	3.8 ± 2.31
	Neutropenia ($< 1 \times 10^9$cells/L), nadir	0.29 ± 0.18	0.56 ± 0.31	0.41 ± 0.17

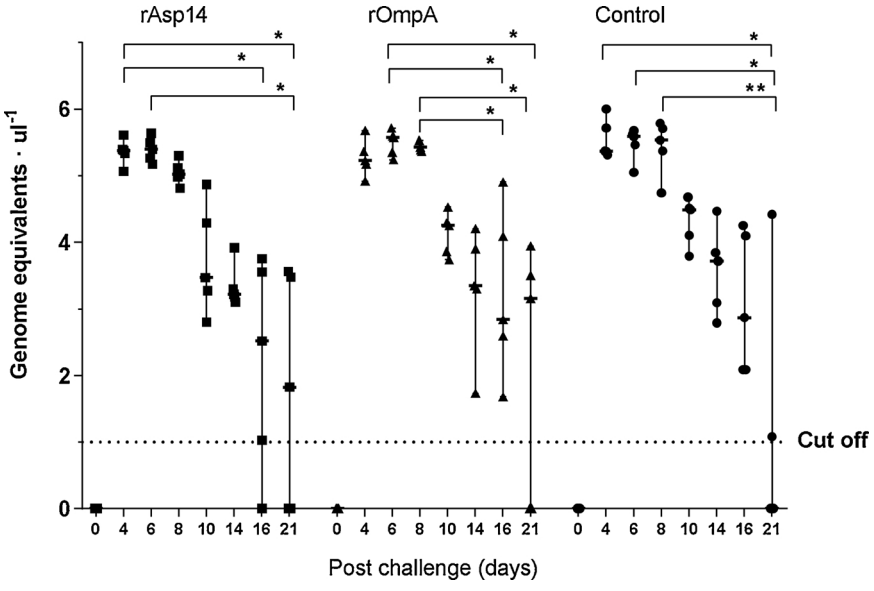


Fig. 4. Bacterial load (real time-PCR) after challenge with *A. phagocytophilum.* rAsp14 (■) and rOmpA (▲), Control group (●), denotes single observations. Median value and range were shown for each group on the different time points, * $p < 0.05$, ** $p < 0.001$. Cut off was 1×10 logarithmic units, the threshold for the detection in real time qPCR.

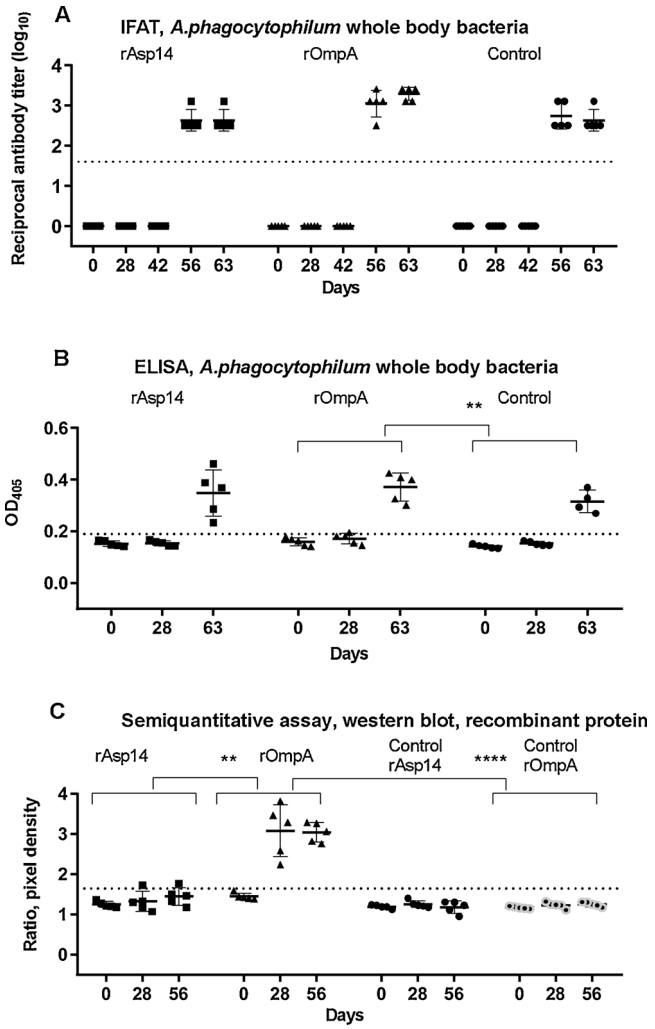


Fig. 5. Serological responses. Figs. A. and B. The serological responses during the study, respectively on days 0, 28, 42, 56 and 63 for IFAT and on days 0, 28 and 63 (21 days pc.) on ELISA, coated with whole bacteria of *A. phagocytophilum*. Means and standard deviations (SD ±) are presented. **C.** A semiquantitative analyzes of serological responses against IEX-fractions of rAsp14 and rOmpA in groups vaccinated with rAsp14, rOmpA or control group detected on western blot on days 0, 28 and 56 (14 days pc.). Each dot denotes a single observation, and mean values are marked for each time point. Standard deviations (SD ±) of the mean are showed for each group and time point. Quality and purity of rAsp14 and rOmpA used for this analyses is shown in Fig. 2A and B. Examples of western blots for this semi quantitation are in fig. D and fig D in the supplementary. * $p < 0.05$, **$p < 0.01$.

groups expressed both $CD14^+$ and MHC II on days 42 and 56 (supplementary, Fig. 3C). The concentrations of NK-cells, $CD21^+$, $CD8^+CD25^+$ and $\gamma\delta$ TcR^+ $CD25^+$ cells were low and are not included in the results and is here denoted with median values for all observations ($n = 45$) in the three groups; $NKp46^+$cells (0.68%), NK^+ $p46CD25^+$ (0.06%), $NKp46^+CD8^+$ (0.03%) and $CD21^+$ (0.42%), $CD8^+CD25^+$ (0 .21%) and $\gamma\delta$ $CD25^+$ (0.11%).

4. Discussion

In the search for vaccine candidates, effective against tick borne pathogens, proteomic methods have been used to retrieve information about proteins that are important for the infectious agents (Troese et al., 2011). This information has led to the production of recombinant proteins, which can be tested as vaccine candidates (Crosby et al., 2018; Ducken et al., 2015; Seidman et al., 2014).

In the current study, lambs were vaccinated with recombinant Asp14 and OmpA proteins to investigate the clinical protective and immunological response upon challenge with *A. phagocytophilum*. Previous cell culture experiments demonstrated a reduced infectivity of *A. phagocytophilum* when cell cultures were incubated with recombinant proteins, or antisera against these (Kahlon et al., 2013; Ojogun et al., 2012). As a result, Asp14 and OmpA were deemed important in the pathogenesis of *A. phagocytophilum*.

The rationale for using Montanide ISA61 VG is previous experience with this adjuvant in sheep, but also because it is reported that the adjuvant stimulate both a Th1 and Th2 response (Coffman et al., 2010; Stuen et al., 2015). This is beneficial for combating *A. phagocytophilum* in terms of limiting the bacterial load, but also in limiting the immunological responses caused by *A. phagocytophilum* infection resulting in the clinical signs of TBF (Davies et al., 2011). The groups receiving rAsp14 and rOmpA developed a local reaction at the injection site, which is commonly seen in vaccines using water in oil adjuvants (Petermann et al., 2017; Petrovsky, 2015; Stuen et al., 2015). This response was not observed in the control group, which received only adjuvant and PBS. However, intramuscular injection of vaccines may have reduced the reactions in the more superficial tissue layers (Leenaars and Hendriksen, 1998).

All lambs developed a clinical response with fever and neutropenia after challenge. These results are consistent with previous infection experiments using the same variant of *A. phagocytophilum* and a study with formalin inactivated *A. phagocytophilum* (Stuen et al., 1998, 2015). The results show that rAsp14 and rOmpA were inefficient in preventing or reducing clinical signs upon challenge with *A. phagocytophilum*.

The observed course of bacteremia in this study is in accordance with previous studies (Granquist et al., 2008; Stuen et al., 2015; Thomas et al., 2012). Thus, the present results indicate that vaccinations with rAsp14 or rOmpA, using this schedule, were inefficient in reducing infection and bacterial propagation in lambs.

rAsp14 vaccinated lambs developed antibodies against rAsp14, however due to difficulties associated with purifying rAsp14, we were unable to prove that the antibody response was solely against rAsp14 due to remnants of *E. coli* protein, which was also detected in the same band based on MS/LC/LC data. On the other hand, rOmpA vaccinated lambs seemed to have developed a specific antibody response by day 28.

This being evident on the semiquantitative results from western blots and persisted throughout the period after challenge (days 42–56). Lambs vaccinated with rOmpA displayed a strong response after the second vaccination, this being evident on the semiquantitative western blot. The antibody response persisted throughout the period after the challenge (days 42–63). The specific antibody response was demonstrated in several western blots (supplementary Fig. 3C and D) and with LC/MS/MS. The location of the cut band on the SDS-PAGE indicates an antibody response against rOmpA. The immunogenicity of both Asp14 and OmpA has previously been reported in humans and mice (Kahlon et al., 2013; Ojogun et al., 2012). In a previous study by Ducken et al. (2015), it was shown that the homologous protein of *A. marginale* to OmpA (AM854), created a stronger immune response than the protein homologous to Asp14 (AM936) in heifers, which is consistent with the current study. The immune response in heifers was however, insufficient in providing protection against infection with *A. marginale*.

The present study further shows that sera from vaccinated lambs were unable to detect native membrane proteins of *A. phagocytophilum* from equine neutrophils (IFAT), and cultured ISE6-cells (ELISA) on day 42. After challenge on day 42, sera from all lambs reacted against whole bacteria in IFAT or ELISA on days 56 and 63. Two immediate explanations for the negative results on day 42 are either low expression of bacterial Asp14 or OmpA in cell cultures, as shown in OmpA by Ojogun et al. (2012), or alterations in the recombinant protein structure resulting in a changed bioactivity compared with the native Asp14 or OmpA (Fox et al., 2013). The positive results on days 56 and 63 are

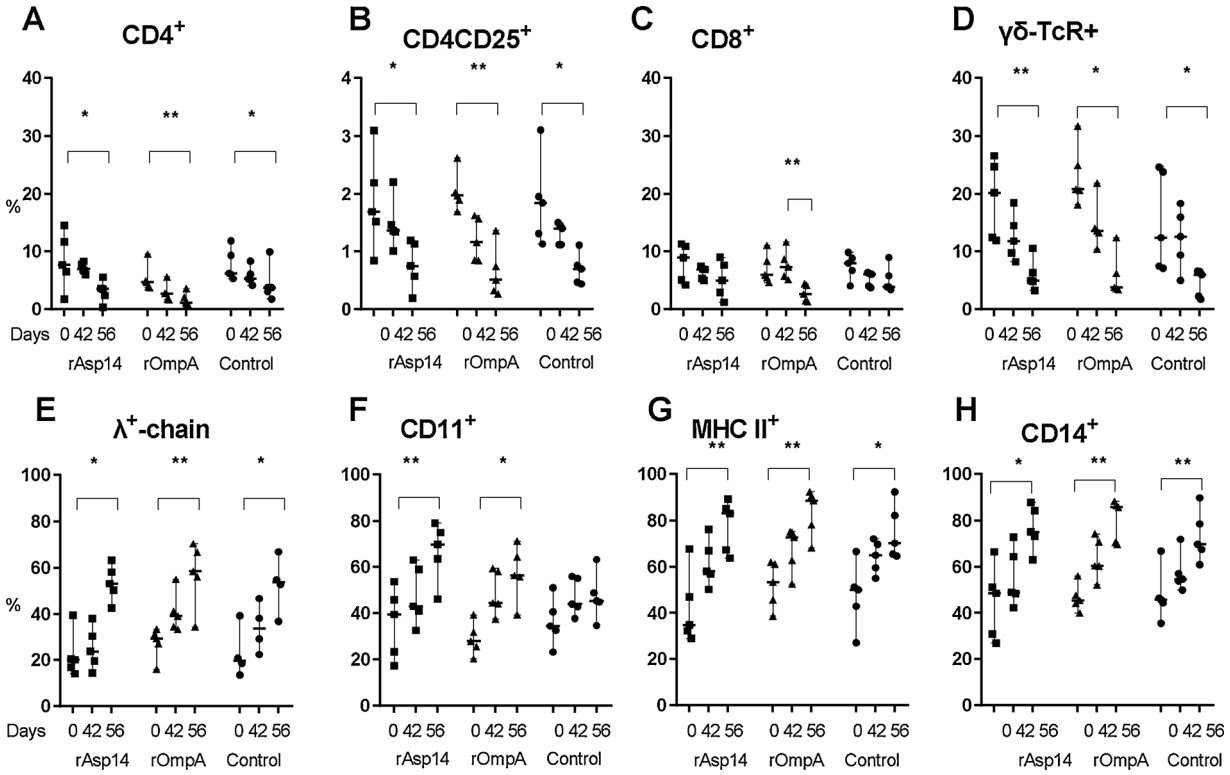


Fig. 6. Percentage of gated PBMCs positive for selected surface markers in rAsp14, rOmpA, and control group on days 0, 42 and 56. rAsp14 n = 5 (■) and rOmpA n = 5 (▲), Control group n = 5 (●) (only four observations in control group on λ-Light chain, Fig. 4E.). **A.** $CD4^+$ cells. **B.** $CD4CD25^+$ cells. **C.** $CD8^+$ cells **D.** γδ-T cells (TcR) **E.** λ^+-Light chain cells, **F.** $CD11b^+$ cells **G.** $MHCII^+$ cells, **H.** $CD14^+$ cells. Observations, median and range are marked for each group for the different time points. P-value are: *$p < 0.05$, **$p < 0.01$.

most likely due to immune response against surface proteins that are prominent on the cell surface eg. MSP2. (Asanovich et al., 1997; Park et al., 2003). Moreover, Ducken et al. (2015) showed that antibodies against recombinant AM936 (the homologue to Asp14 in *A.marginale*) were unable to detect native protein on *A.marginale*. This agrees with the current study's results for the rAsp14 group in IFAT and ELISA with whole body *A. phagocytophilum*.

The flow cytometric phenotyping of PBMCs showed similar cell profiles for rAsp14, rOmpA and the control group, which is congruent with the analogous clinical, hematological and bacteriological observations of the groups. This indicate that the protein vaccines used in this study provoked insufficient immunity against the bacterium.

Although there were no significant differences between the groups during the experiment, there were differences between day zero (first vaccination) and the day 56 (14 days post-challenge) for each of the three groups. However, these changes which include decreased levels of $CD4^+$, $CD4^+CD25^+$, $\gamma\delta$-TcR^+ cells and elevated levels of λ^+-chain, $CD11b^+$, $MHCII^+$ and $CD14^+$ cells, are likely to be a result of interaction between the immune system and the adjuvant or the challenge with *A. phagocytophilum* since no group differences were evident. The reduction of $CD4^+$ T-cells subsequent to *A. phagocytophilum* challenge is known from previous studies by Woldehiwet et al. (1991) and Whist et al. (2003). Additionally, the importance of $CD4^+$ cells during *Anaplasma* spp. infection has been discussed in terms of the exhaustion of the $CD4^+$ cell population, possibly resulting in prolongation of bacteremia (Birkner et al., 2008; Turse et al., 2014).

In the present study, the percentage of $\gamma\delta$-TcR^+ cells and the subpopulation $WC1^+$cells, decreased from day 0 to day 56. Whist et al. (2003) reported a similar reduction in γδ-T cells expressing WC1. Furthermore, Woldehiwet et al. (1991) observed a drop in $CD4^-$, $CD8^-$ and $CD5^+$ six days after infection with *A. phagocytophilum* in sheep. Although the cellular markers in that study differs from the current study; they are interpreted as $WC1^+$ (Morrison and Davis, 1991; Wijngaard et al., 1992). There are only a few studies on γδ-T cells in *A. phagocytophilum* infection in ruminants, making it difficult to interpret the impact of these cells over the course of the bacterium's infection cycle.

The increase in λ-Light $chain^+$ cells between day zero and day 56 in the three groups, suggests an elevated level of B-cells responding to the infection, however there is no significant difference between day zero and day 42 or day 42 and day 56. Previous studies have reported that the ovine B-cell level, 14 days post-challenge, was equal to the pre-challenge levels (Whist et al., 2003; Woldehiwet, 1991). This suggests that the adjuvant may have led to an elevated level of B-cells, which is further substantiated by the observed increase in the MHC II+, $CD14^+$ and $CD11b^+$ cells only between days zero and 56. There is limited knowledge on how adjuvants like Montanide™ISA 61 VG stimulate the immune system, but it is believed to induce a local inflammation with subsequent recruitment and activation of antigen presenting cell (Leroux-Roels, 2010). Further, the majority of $CD11b^+$ cells expressed both $CD14^+$ and MHC II^+ after infection, and these cells are likely to represent monocytes. An increase in MHC II^+, $CD14^+$ and $CD11b^+$ cells has been observed in sheep after challenge with *A. phagocytophilum* (Whist et al. 2003), however, the timing of the increase in that study was different for the current experiment, which again could be related to the initial injection of adjuvant in the present study. A previous study in mice reported an increase in $CD11b^+$ cells after challenge with *A. phagocytophilum*, but that study only examined neutrophil granulocytes (Borjesson et al., 2002). However, the latter and the current study indicate an importance of $CD11b^+$ in both mononuclear and peripheral nuclear cells during infection with *A. phagocytophilum.*

Between day 42 and 56, a reduction in $CD8^+$ cells was evident within the rOmpA group. Previous studies have reported a drop in $CD8^+$ concentration after *A.phagocytophilum* infection in sheep, followed by an increase to pre-challenge levels by day 14 post challenge (Whist et al., 2003; Woldehiwet, 1991). In the present study, the

percentage of $CD8^+$ T-cells in the rOmpA group did not reach the pre-challenge level by 14 days post-challenge. Whether this is related to the vaccine, remains unknown.

Overall, this study indicates that rAsp14 and rOmpA, given as vaccines to lambs, stimulate the production of antibodies that are at least detectable from day 28 after vaccination. However, upon challenge there was no enhanced antibody response in vaccinated animals compared with the controls with the methods used. This may indicate that the vaccines used in the current study, did not stimulate an anamnestic response upon exposure to live *A. phagocytophilum*. Additionally, expression of common leukocyte markers were not different in vaccinated compared to control animals.

Considering the current results, the relevance of Asp14 and OmpA as a vaccine against *A. phagocytophilum* in sheep, may be questioned. However, there are several pending factors to the study, e.g. vaccine preparation, protein structure, vaccine dose and route of administration (Fox et al., 2013; Koh et al., 2006; Leenaars and Hendriksen, 1998). In this study, the challenge was performed by intravenous injection which may affect the outcome of the infection and the disease, since the tick is known to modulate the host's immune response (Wikel, 2018). However, in a previous study, mice were infected with *A. phagocytophilum* by injection and by ticks without showing any difference between the two methods (Sun et al., 1997). In addition, outbred sheep are likely to have more diverse immune responses compared to inbred mice used for laboratory experiments (Casellas, 2011; Entrican et al., 2015; Stuen et al., 1998; Stuen and Bergstrom, 2001b; Stuen et al., 2003).

5. Conclusion

This study did not provide evidence that the rAsp14 and rOmpA prevented infection or propagation with *A. phagocytophilum* in sheep, nor that it reduced the clinical outcome of the infection.

Funding

This study was funded by the Norwegian research funding for agriculture and the food industry (Matfondavtalen) grant number: 244173/E50, Norwegian Association of Sheep and Goat Farmers (NSG), Animalia – the Norwegian Meat and Poultry Research Center and ScandTick Innovation (INTERREG ÖKS IVA).

Declaration of Competing Interest

Co-author Tore S. Tollersrud is employed by Animalia - the Norwegian Meat and Poultry Research Center, which is a funding partner to the study.

Acknowledgements

The authors thank Inger Austrheim Heffernan, Wenche Okstad, Solfrid Nevland, Siri Bjerkreim Hamre, Tom Honnemyr, Asbjørn Haga, Ane Odden and Cecilie Ersdal for providing help with sampling and analysis of the samples during the experiment. Siri Kulberg Sjurseth and Lisbeth Hektoen provided valuable help in planning the study. Thanks to Bjørn Dalhus, Pernille Blicher, Bjørn Spilsberg and Christiane R. Fæste at Rikshospitalet, Oslo and Veterinærinstituttet for contributing with ione chromatography purification of proteins and analyzing LC/MS/MS data. Thanks to Anders Moen, who performed the LC/MS/MS at the University of Oslo, Department of Biosciences, The faculty of Mathematics and Natural Sciences. The Anti-NCR1/NKp46 used in flow cytometry was kindly provided by Anne Storset and Preben Boysen at NMBU. Eystein Skjerve has contributed in the analyzing of the data. Thanks to Anna Lundgren at the University of Florida for assistance with the recombinant proteins.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.109936.

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