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Research paper

Clinical and immunological responses in sheep after inoculation with Himar1-transformed *Anaplasma phagocytophilum* and subsequent challenge with a virulent strain of the bacterium

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ABSTRACT

In Norway, the tick-transmitted bacterium *Anaplasma phagocytophilum* is estimated to cause tick-borne fever (TBF) in 300 000 lambs on pastures each year, resulting in economic and animal welfare consequences. Today, prophylactic measures mainly involve the use of acaricides, but a vaccine has been requested by farmers and veterinarians for decades. Several attempts have been made to produce a vaccine against *A. phagocytophilum* including antigenic surface proteins, inactivated whole cell vaccines and challenge followed by treatment. In the current study, a virulent wild type strain of *A. phagocytophilum* named Ap.Norvar1 (16S rRNA sequence partial identical to sequence in GenBank acc.no M73220) was subject to genetic transformation with a Himar1-transposon, which resulted in three bacterial mutants, capable of propagation in a tick cell line (ISE6). In order to test the immunogenicity and pathogenicity of the live, mutated bacteria, these were clinically tested in an inoculation- and challenge study in sheep. One group was inoculated with the Ap.Norvar1 as an infection control. After inoculation, the sheep inoculated with mutated bacteria and the Ap.Norvar1 developed typical clinical signs of infection and humoral immune response. After challenge with Ap.Norvar1, 28 days later all groups inoculated with mutated bacteria showed clinical signs of tick-borne fever and bacteremia while the group initially inoculated with the Ap.Norvar1, showed protection against clinical disease. The current study shows a weak, but partial protection against infection in animals inoculated with mutated bacteria, while animals that received Ap.Norvar1 both for inoculation and challenge, responded with homologues protection.

1. Introduction

The gram-negative bacterium *Anaplasma phagocytophilum* is transmitted mainly by the tick *Ixodes ricinus* in Europe and can cause disease in humans and several other species (Stuen et al. 2013). The bacterium infects host neutrophil granulocytes and cause pasture fever in cattle and tick borne fever in sheep (Hudson, 1950; Macleod and Gordon, 1933). A previous estimate suggests that 300 000 sheep are infected annually in Norway (Stuen et al., 2002a), and up to 80–100 % of the sheep may become infected on certain tick infested pastures (Ogden et al., 1998; Stuen and Bergstrom, 2001b). Clinical signs of TBF are high fever (>40 °C) and apathy, typically commencing 4–14 days after infection (Macleod and Gordon, 1933). Infected sheep are susceptible to secondary infections such as septicemia, arthritis and pneumonia

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(Foggie, 1951; Stuen et al., 2001). The current prophylactic treatments against TBF are the application of acaricides and long acting antibiotics such as tetracyclines (Stuen and Bergstrom, 2001a; Stuen et al., 2012).

A more sustainable approach to preventive treatments is advocated, contributing to lower antibiotic- and acaricide use in order to avoid resistance in ticks and bacteria. The most desirable prophylaxis would be a vaccine, which has been requested by Norwegian farmers and veterinarians for several decades (Stuen et al., 2015). One challenge with vaccines against *A. phagocytophilum* is the presence of several genetic variants and that vaccines may not show cross-protection across variants (Stuen et al., 1998). By identification of single nucleotide polymorphisms, within a variable region near the 5' end of the 16S rRNA gene, several genetic variants have been identified in sheep in Norway (Stuen et al., 2002b). One of these variants is the *A. phagocytophilum* Norway variant 1 (Ap.Norvar1) which is identical to the base pairs 81–126 of the Gene Bank accession number M73220 16S rRNA (Stuen et al., 2002b). Further, this strain is regarded to be the most virulent of those that infect Norwegian sheep (Stuen et al., 2003).

Several attempts have been made to find vaccine candidates against *A. phagocytophilum*. One study used formaldehyde inactivated, whole cell organisms as a vaccine to induce immunity in sheep upon later challenge with viable bacteria. However, the vaccine had no documented effect against the infection in sheep (Stuen et al., 2015). In addition, vaccines based on recombinant outer membrane protein A (OmpA) and *A. phagocytophilum* surface protein 14 (Asp14), were used to induce protection against infection with a Ap.Norvar1 in sheep. The protein vaccines provoked specific serological responses against the outer membrane proteins, however they did not protect against the challenge with live bacteria (Eskeland et al., 2019). This strategy was also previously applied in a study with *Anaplasma marginale* in cattle, which failed to establish protection against the live bacteria (Ducken et al., 2015). In Israel, South Africa and Australia, the former *A. marginale* ssp. *centrale*, now considered to be separate species (*A. centrale*), is currently used as a vaccine to protect cattle against anaplasmosis (Bock and de Vos, 2001; Khumalo et al., 2018; Shkap et al., 2002; Theiler, 1911). The live vaccine is unable to prevent infection with live *A. marginale*, but it results in reduced clinical signs in infected cattle (Shkap et al., 2002). However, it has only been observed that highly virulent strains of *A. phagocytophilum* in sheep may produce cross-protection against less virulent strains of the bacterium (Foggie, 1951; Stuen et al., 2003).

DNA-transposon insertion into eukaryotic and prokaryotic organisms has led to new knowledge on bacterial pathogenesis (Munoz-Lopez and Garcia-Perez, 2010). A mariner element Himar1 (DNA-transposon) can be inserted into the bacterial genome (Lampe et al., 1999) which has been successfully applied with *Anaplasmataceae* (Cheng et al., 2013; Crosby et al., 2015, 2014; Felsheim et al., 2010, 2006; Oliva Chavez et al., 2015). The transposon is randomly inserted between TA-nucleotides in the genome. The insertion may interrupt gene expression if it is incorporated into a coding region (Felsheim et al., 2006). A previous study, Crosby et al. (2015) reported a reduction in the virulence of *A. marginale* mutants infecting cattle, and Cheng et al. (2013) reported reduced replication of transformed *Ehrlichia chaffeensis* in white-tailed deer (*Odocoileus hemionus*). In contrast to bacterial attenuation by repeated passages in cell cultures, bacteria with transposon insertions show a high degree of stability against regaining virulence (Crosby et al., 2015; Pelicic et al., 2000; Rholl et al., 2008). In addition, the incorporation of fluorescence-coding and antibiotic resistance genes, ensures monitoring and selective culturing of the bacteria which is important for the verification of viable mutant colonies (Cheng et al., 2013; Felsheim et al., 2006).

The current study presents a Himar1-transposon mutagenesis of a highly virulent sheep strain, Ap.Norvar1, resulting in three living infectious mutants. These mutants were examined for their potential to cause clinical disease in sheep after inoculation, but also their immune protective potential upon challenge with the virulent strain from which they originated from.

2. Material and methods

2.1. Ethics statement

The use of experimental animals in the current study was based on an assessment of animal welfare taking into consideration the species, breed, sex and the age of the animals. The research protocol was designed in accordance with these parameters, and ethical standards used in the study were approved by the Norwegian Animal Research Authority (protocol approval no. FOTSID12093) upon formal application and in accordance with the EU Directive 2010/63/EU. The import and implementation of genetically modified *A. phagocytophilum* in a sheep model were approved by the Norwegian Environment Agency (protocol approval no. 2017/1332).

2.2. Genomic modifications: Propagation and transposon mutagenesis of Ap.Norvar1

The *Ixodes scapularis* embryo-derived tick cell line ISE6 was used to support the isolation and propagation of Ap.Norvar1 and the derived mutants. Sheep blood, which contained Ap.Norvar1, was collected from an infected sheep (Norwegian University of Life Sciences [NMBU], Sandnes, Norway) and stored in EDTA, prior to inoculation of ISE6-cells. The strain Ap.Norvar1 was maintained in ISE6-cells as described previously (Munderloh et al., 1999) and underwent less than 3 passages before the transformation process. Bacteria released from cells in one 5 mL culture were prepared for electroporation following the procedure outlined by Cheng et al. (2013), with some modifications: Cells were resuspended in 1.5 mL of spent medium and vortexed at the highest setting for 30 s with ~100 µL of silicone carbide rock tumbler grit, 60/90 grade (Loretone, Mukilteo, Washington, USA), in a 2-mL micro-centrifuge tube. The supernatant was passed through a 2 µm pore-size filter to remove cell debris and the bacteria were collected after centrifuging at 11 000 x G for 11 min at 4 °C. The bacteria were incubated for 15 min with 1 µg of plasmid DNA on ice. The plasmid encoded the transposon which comprised of the mCherry fluorescent marker and the aminoglycosideadenyltransferase A (*aadA*) resistance gene for selection, flanked by mismatched LoxP sites. After incubation, the bacteria were electroporated at 1.8 kV (kV), 400 O (Ω) and 25 µF (µF), yielding a pulse time of 6–9 milliseconds (ms). The bacteria were immediately recovered in 0.5 mL of fetal bovine serum (FBS), mixed with ISE6-cells from one 5 mL flask, and centrifuged at 5 000 x G for five minutes at room temperature. Tubes with pelleted cells and bacteria were incubated at 30 °C for 2 h, and then gently resuspended in complete medium for ISE6-cells as described by Munderloh et al. (1999). The suspension was distributed into wells on a 48-well plate and antibiotic selection (spectinomycin) started the next day. The plates were monitored for the presence of fluorescent bacteria starting two weeks after electroporation using a Nikon Diaphot (NY, USA) inverted microscope fitted for epifluorescence. The content of the wells, in which cells with living, fluorescent, mutated bacteria were detected, generated three mutants collectively termed as Himar1-mutants for further use in the study. These mutants were then transferred to fresh 5 mL ISE6-cultures and incubated under antibiotic selection with spectinomycin and went through 3−4 passages until approximately 90 % of the ISE6-cells were infected, before being stored in liquid nitrogen. Prior to inoculation in sheep, the Himar1-mutants were further propagated in ISE6-cells for 4 more passages without spectinomycin or streptomycin, due to the antibiotic selection performed in the initial cell cultivation. Uninfected ISE6-cells were used as negative control.

2.3. Verification of transposon insertions

ISE6-cell cultures infected with Himar1-mutants were processed to

release the bacteria from the cells by using centrifugation and silicon carbide rock tumbler grit as described in previous section. The DNA Quick gDNA microprep kit (Zymo, CA, USA) was used for extraction of bacterial genomic DNA, which was sequenced at the University of Florida Genomics center (Interdisiplinary Center for Biotechnology Research, Gainesville, FL 32610).

Three Himar1-mutants were sequenced and named CL2B5, CL1A2 and CL3D3. The CL1A2 mutant was sequenced using the PacBio system (Pacific Biosciences of California, CA, USA), while the CL2B5 and CL3D3 were sequenced on the Illumina platform (Illumina, CA, USA). Confirmation of the transposon insertion was performed by PCR with primers targeting *A. phagocytophilum* genome sequences adjacent to the transposon (tn) (Table 1) as described by Felsheim et al. (2006). The amplicons generated were as following (tn-transposon, uml-unmutated loci); CL2B5 (tn – 2059 bp, uml - 224 bp), CL1A2 (two primer sets; tn1−1973 bp, uml 1–138, tn2−2012 bp, uml 2–177 bp) and CL3D3 (tn-2738 bp, uml -903 bp). Further, the CL1A2 had to be assessed with an additional PCR to assure whether the isolate contained one or two mutants. This was evaluated by comparison of the gene copy numbers of *aadA* and msp5 in a duplex qPCR (Table 1). The quantitative PCR (qPCR) counts were based on a standard curve of a 10-fold serially diluted gBlock dsDNA fragment, which carried both the *aadA* and msp5 sequences. The PCR reactions included 10 ng DNA and the PrimeStar GXL DNA polymerase (Primestar, Denver, USA) was used. Primers, amplicons and PCR-conditions are listed in Table 1. The software Artemis 18.0.3 (Sanger Institute, UK,) was used to present the locations of the Himar1-transposons insertions in the genome in Fig. 1.

2.4. Animals and premises

Twenty-four sheep, six to eight months old, of the breed "Norwegian white sheep" were selected from the research flock at the NMBU, Sandnes, Norway. The selection criteria were sex (only ewe lambs), body conformation and age. The sheep were randomly divided into five groups; each consisted of five sheep except for the negative control group which consisted of four sheep. The sheep were confined indoors, in a tick free environment on plastic slatted floors until the start of the study, when they were placed in a room with standards equal to biosecurity 2 level. Before inoculation, the sheep were confirmed negative for immunoglobulin G (IgG) against *A. phagocytophilum* by immune fluorescent assay test (IFAT) performed by The Swedish Veterinary Institute (SVA, Uppsala, Sweden) and confirmed negative for *A. phagocytophilum* by PCR as described in the subsequent section "Bacterial load of the Himar1-mutants and the Ap.Norvar1 by real time-PCR". The study period was 51 days. On day 0, sheep were inoculated with one of the three Himar1-mutants, Ap.Norvar1 or uninfected ISE6-cells (controls). On day 28 of the study, all groups were challenged with the Ap.Norvar1, except for two sheep in the control group that only received physiological saline solution (Fig. 2). The groups that were inoculated with either CL2B5, CL1A2 or CL3D3 on day 0 are hereafter called Himar1-groups, while Ap.Norvar1 was inoculated in the Ap. Norvar1-group (Fig. 2A).

2.5. Preparation of Himar1-mutants, Ap.Norvar1 and ISE6-cells for sheep inoculation

Heavily infected ISE6-cells (>80 %) were pelleted at 400 x G for 5 min at room temperature. The supernatant was discarded and 1x phosphate buffered saline (PBS) was added for resuspension of the cell pellets into a final volume of 1 mL. The cell suspension was further aspirated using 25 G or 27 G hypodermic needle, to lyse the ISE6-cells and release bacteria. The cell-free bacteria were examined using a fluorescence microscope before the cells were injected into the jugular vein of the sheep (Fig. 2A). Sheep in the Ap.Norvar1-group were

Table 1
Primer and PCR-settings for amplicons in the current study.

Target	Forward primer	Reverse primer	Size (bp)	PCR-setting
CL2B5 tn	5'CCT AAG ACA ACA CCA CAC AAC C'3	5'GGG CTG GGA ACA GTG TAT G'3	tn: 2059 uml: 224	
CL1A2 tn	5'CCC ACT AAG GCT CTA ACA GAT TGT G'3	5'CCA AAT TTC ACG GCG TTT CTC'3	tn: 1973 uml: 138	94°C 2 min, followed by 30 cycles at 98°C 10 sec, 65°C 15 sec, 68°C 2 min and one cycle at 68°C for 7 min
CL1A2 tn	5'GCA CTC TCA CTT CTC CAT CG'3	5'GAG CTG GTA CTC CAT CAA TAG G'3	tn: 2012 uml: 177	
CL3D3 tn	5'ATC ACT GCA ATA CGA TCC TG'3	5'AGA GAT GTA GAT TGC CGT ACA GAC G'3	tn: 2738 uml: 903	
msp5[1]	5'GTGAGGCTGCTGATAGACTTC'3	5' TGCGAATAACTTCCTCATCCC'3	138	30 sec on 95°C, 95°C 5 sec and 30 sec on 60°C
aadA[2]	5'GCGCTGTAGAAGTCACCATT'3	5'TCATTGCGCTGCCATTCT'3	97	
aadA[3]	5'GGT GAC CGT AAG GCT TGA TG'3	5'ACC AAG GCA ACG CTA TGT TC'3	279	45 cycles, included 10 min at 95°C, 10 sec at 95°C, 30 sec at 60°C, 1 sec at 72°C. Cooling: 30 sec at 40°C
gltA[4]	5'TTT TGG GCG CTG AAT ACG AT'3	5'TCT CGA GGG AAT GAT CTA ATA ACG 7'3		Reference Henningson et al. (2015)

Superscript[1] amplicon 138 bp, probe 5'[FAM]-AGGCCTTTGATAGTCGAATTCAGATGCT-[TAM]-3'.
Superscript [2] amplicon 97 bp, probe 5'-[Cy5]-ATCATTCCGTGGCGTTATCCAGCT-[BHQ2]3'.
Superscript[3] amplicon 279 bp, probe 5'[TET]-ACCATTGTTGTGCACGACGACA-[BHQI]-3'.
Superscript [4] amplicon 64 bp, probe 5'-FAM-TGC CTG AAC AAG TTA TG-BHQ 1-3 '.
Ref. (Henningsson et al., 2015).
Abbrevations: tn-transposon, uml-unmutated locus.

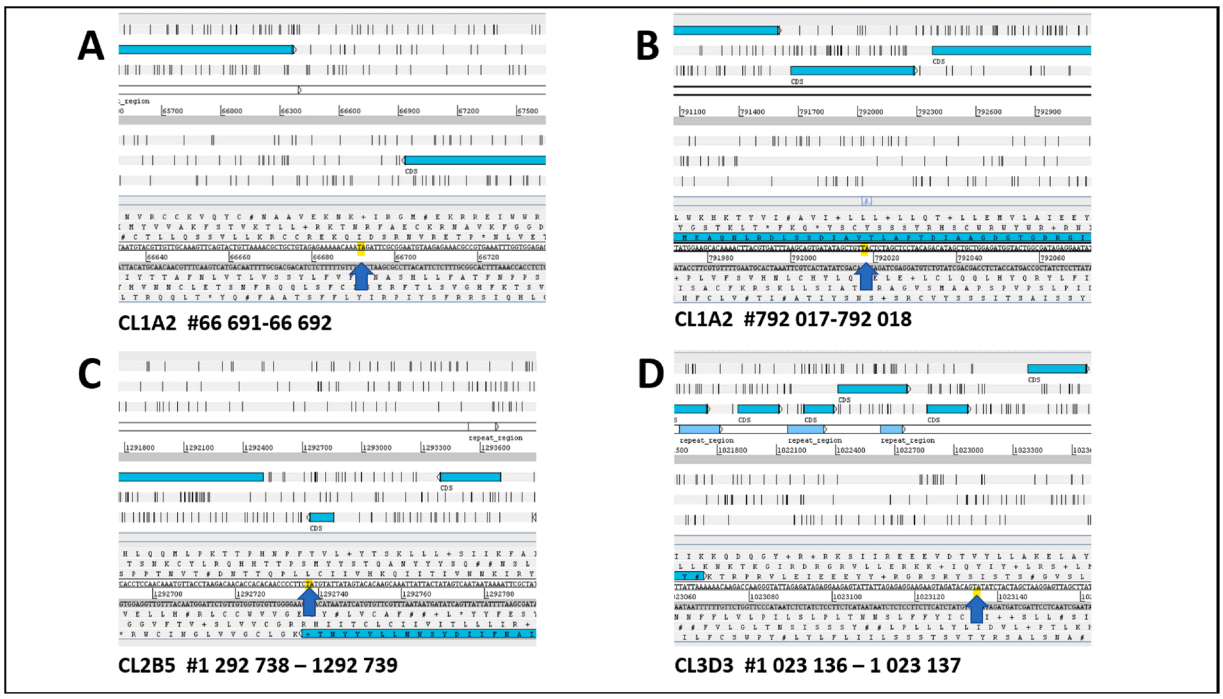

Fig. 1. Insertion sites of transposon in mutants. A. CL1A2, insertion site base pair (bp) 66 691-66 692. **B.** CL1A2, insertion site base pair (bp) 792 017-792 018. **C.** CL2B5, insertion site base pair (bp) 1 292 738-1 292 739. **D.** CL2B5, insertion site base pair (bp) 1 023 136-1 023 136. Reference genome GenBank acc. no CP04663.

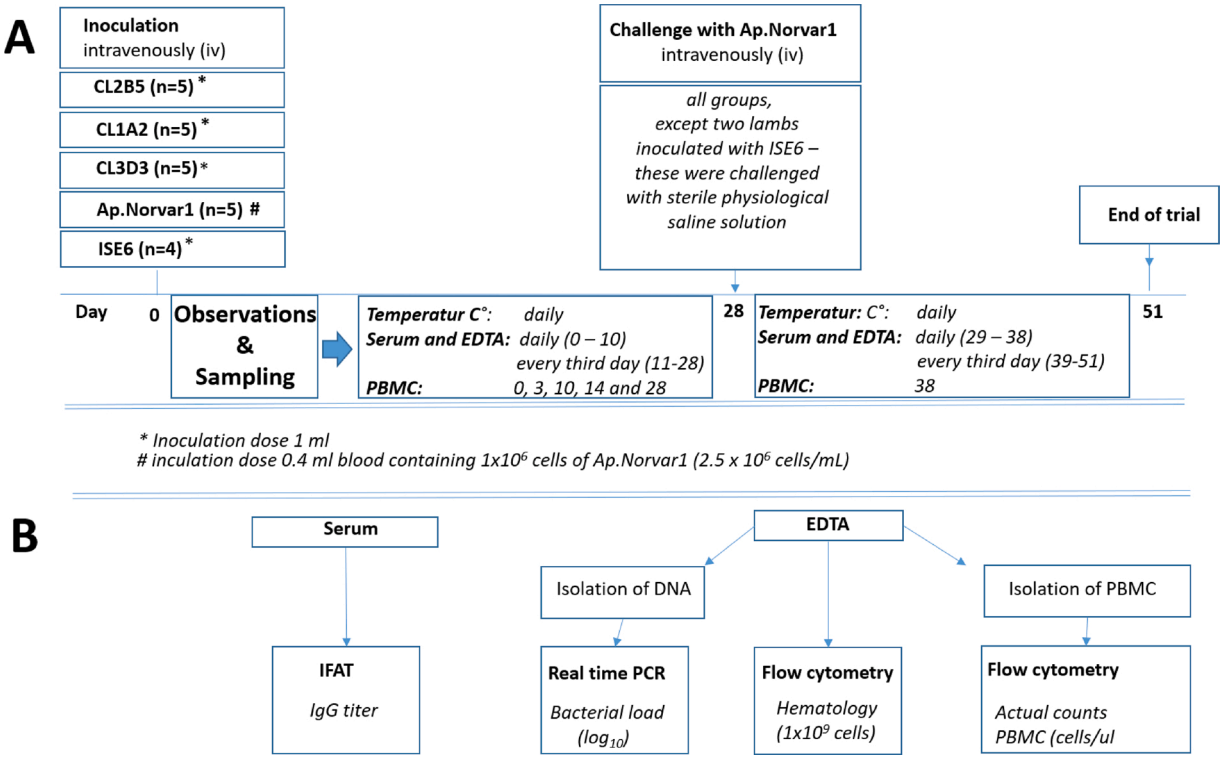

Fig. 2. Flow diagram of experiment and sample outcomes. A. Flow diagram of the animal study. The boxes illustrate the groups of animals and what kind of treatment they received. **B**. Flow diagram for outcomes of blood sampling.

inoculated with the Ap.Norvar1, and the preparation of the inoculation dose was identical to the one described in the subsequent section "Challenge". The four sheep in the control group were inoculated with uninfected ISE6-cells. The uninfected ISE6-cells were prepared in the same way as the ISE6-cells infected with the Himar1-mutants. The rationale for using two different propagation systems; A sheep for Ap. Norvar1 and the ISE6-cell cultures for Himar1-mutants, was to evaluate if cell-cultivated mutant strains, deprived of immunological stimulation may provide protection against a virulent strain derived from an animal host.

2.6. Challenge with Ap.Norvar1 or PBS

The Ap.Norvar1 inoculum consisted of infected sheep blood and 10 % dimethyl sulfoxide (DMSO) as cryopreservative. The Ap.Norvar1 inoculum was stored at −70 °C and thawed at room temperature before injection in the left jugular vein of a naïve sheep (donor), six days prior to inoculation, to ensure that the Ap.Norvar1 inoculum contained live bacteria (Fig. 2A). Blood was collected from the donor sheep during the peak bacteremic phase, six days after inoculation. A challenge dose of 0.4 mL donor sheep blood containing approximately 1×10^6 cells infected (2.5×10^6 cells/mL) was injected into the jugular vein of all experimental sheep except for two sheep in the control group which received PBS. To quantify the inoculation and the challenge doses, 400 neutrophil granulocytes in a May-Grünwald-Giemsa stained blood smear from the donor sheep, were assessed for inclusions of *A. phagocytophilum* (Fig. 2A).

2.7. Clinical and hematological observations

The clinical overall condition and the rectal temperature (C°) were observed and measured daily throughout the study. Threshold level for fever was >40.0 °C. Blood samples were collected from the jugular vein daily during the first ten days after the inoculation or challenge, and then every third day (Fig. 2A). The threshold level for neutropenia was set to $<0.7 \times 10^9$ cells/L. Samples for peripheral blood mononuclear cells (PBMC) analysis were collected and analyzed on the days 0, 3, 10, 14, 28 and 38 (Fig. 2A and B).

2.8. Bacterial load of the Himar1-mutants and the Ap.Norvar1 by real time-PCR

Extraction of DNA from 500 µL blood was performed on the MagNA Pure LC 2.0 instrument (Roche, Basel, Switzerland), using MagNA Pure LC DNA Isolation Kit – Large Volume (Roche, Basel, Switzerland), according to the manufacturer's instructions. Bacterial loads were quantified using a Taqman real-time PCR. Primers and probe targeting the *A. phagocytophilum* citrate synthase gene (*gltA*) (Table 1) were designed to determine the number of Ap.Norvar1 and Himar1-mutants. A pUC57 vector (Genscript USA Inc, NJ, US) carrying a region spanning the nucleotides 304–420 of the *gltA* gene (acc. no AF304137.1) was used for standard curve preparation. The concentration of the plasmid solution was determined using a NanoDrop® ND-1000 instrument (Wilmington, DE, US).

For quantification of the Himar1-mutants, primers and a probe targeting a region of 279 bp within the Streptomycin 3"-adenylyltransferase (*aadA*) gene were used, which is present in the Himar1-transposon. This resulted in a 279 bp long amplicon, which was compared with a standard curve based on a ten-fold serial dilution (10^8 to 1 copies) of the plasmid pHimar cisA7 mCherry-SS, as previously reported (Crosby et al., 2015). After challenge, the bacterial load was determined using both of the previously mentioned primer sets, plasmids and primer conditions due to the possibility of co-infection with Himar1-mutants and Ap. Norvar1. All samples were run in duplicate for each of the primer sets. Primers and PCR-conditions are shown for each primer sets in Table 1.

2.9. Separating and phenotyping PMBC

The separation of PBMC, started within eight hours after sampling. A density gradient medium (Lymphoprep; Axis-Shield, Norway) was used for separation of PBMC from the other fractions of EDTA blood, as previously described (Lybeck et al., 2009). Cells were subsequently stored at −80 °C in a freezing solution consisting of FBS and 10 % DMSO (Panreac Applichem ITV, Barcelona, Spain,). The protocol for thawing and analysis of PBMC was in accordance with Eskeland et al. (2019). Further, PBMC 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), followed by incubation with unconjugated primary antibodies targeted selected surface markers and subsequently with the appropriate secondary antibodies. Compensation beads were used following manufacturer's instructions (OneComp eBeads, eBioscience, San Diego, USA). The concentrations of monoclonal antibodies (mAbs) were based on previous studies and are shown in Table 2. A Novocyte flow cytometer (ACEA biosciences, San Diego, USA), and NovoExpress software, version 1.2.4 (ACEA biosciences, San Diego, USA) were used for cellular analyzes. An absolute cell count for the populations of $CD4^+$, $CD4^+CD25^+$, $CD8^+$, $CD8^+CD25^+$, $\gamma\delta TcR^+$, $\gamma\delta TcR^+WC1^+$ and $NKp46^+$ were assessed for the experimental groups during the study period. The gates were set to include mononuclear cells and exclude dead cells and doublets. The positive fluorescence gates were set with reference to negative controls where primary antibodies were omitted.

2.10. IgG response against A. phagocytophilum

An IFAT (Protatek™, MN, USA) was performed on the sera collected before and after inoculation and challenge by SVA in accordance with manufacturer's instructions. The IFAT detected binding of IgG to a known equine strain of *A. phagocytophilum* as previously described (Artursson et al., 1999; Stuen and Bergstrom, 2001b). An antibody titer above the reciprocal 1.6 (dilution 1:40) were considered to be the lower cut off for IgG response against the bacterium (Stuen et al., 2003)

2.11. Statistical analysis

The data were analyzed with Prism Ver. 8.3.0 (GraphPad, San Diego, USA). Further, the groups were compared to each other, and differences within time points in each group were analyzed. Rectal temperature, neutrophilic cell count, bacterial load and IgG were assessed for the two time periods: Between inoculation until challenge (day 0–27) and between challenge until the end of the study (day 28–51). The group comparisons were performed with an unpaired *t*-test for the 'area under the curve' (AUC). Due to only one time point after challenge for the PBMC data, the differences between the groups were analyzed with a repeated two-way ANOVA and Tukey's post hoc test ($p = 0.05$), if the data were normally distributed.

Differences within each group, before and after the challenge, were tested with a paired *t*-test on AUC values for temperature and neutrophil cell counts. The difference in bacterial load within each group was assessed by comparison of maximum bacteria load on days 6 and 34

Table 2
Overview of mAbs used for flowcytometric phenotyping of PBMCs.

Antibodies	Clone	Isotype	Final concentration (µg/mL)
CD4[1]	GC1A	IgG2a	5
CD8[1]	CACT80c	IgG1	5
γδTCR[1]	86D	IgG1	5
CD25[1]	LCTB2A	IgG3	5
WC1[1]	B7A1	IgM	5
NCR1/NKp462	AKS-6	IgG2b	5
Allophycocyanin (APC) conjugated goat anti-mouse IgG1 and IgG2b3			2.5
Fluorescein isothiocyanate (FITC-) conjugated goat anti-mouse IgG2a and IgG33			10
Phycoerythrin (PE)-conjugated goat anti-mouse IgG1, IgG2b, IgG3 and IgM3			2.5

Antibodies with superscript 1 and 2 are primary antibodies, and antibody with superscript 3 are secondary. Producers are listed in regard to superscript; [1]. Monoclonal antibody center, Washington State University, USA. [2]. In-house, Norwegian University of Life Sciences (Olsen et al., 2013). [3]. Southern Biotech, AL, USA.

(equivalent to day 6 after challenge) with a paired *t*-test, while PBMCs and IgG were tested with repeated one-way ANOVA or Friedman test and Dunn's multiple comparison test. In addition, time of incubation, maximum temperature and the duration of fever were calculated for the Himar1-groups with a paired *t*-test or Wilcoxon test depending on the normality of the data.

The Shapiro-Wilk test ($\alpha = 0.05$) and quantile-quantile plot (Q-Q plot) were used to assess if data were normally distributed before the data were analyzed with a paired *t*-test. The non-parametric Wilcoxon test and Friedman test were used on data with non-normal distribution. The level of significance was set to <0.05.

3. Results

In the study, several transformations were conducted of the Ap. Norvar1 strain with Himar1-transposons resulting in mutated bacteria (Himar1-mutants). These Himar1-mutants were sequenced, before being inoculated in sheep to evaluate if they were capable of infecting and causing clinical disease in their hosts. Twenty-eight days after inoculation with Himar1-mutants, the sheep were challenged with the original strain, Ap.Norvar1, to evaluate if the Himar1-mutants provided protection.

3.1. Verification of successful genomic modifications of Ap.Norvar1. strain

Ap.Norvar1 was successfully modified with Himar1-transformation resulting in three mutant populations of the strain, CL2B5, CL1A2 and CL3D3 respectively. The viability of the Himar1-mutants was consistently stable throughout 7–8 cell passages in ISE6-cell culture.

3.2. Verification of the transposon insertions in the Himar1-mutants

CL2B5 had one transposon insertion between TA sites #1,292,738−1,292,739 in an intergenic region (Fig. 1C) while sequence analyses revealed two transposon insertions in the CL1A2. One of the inserts in the CL1A2, was between the TA dinucleotide site #66,691−66,692 in an intergenic region and another at TA site #792,017–792,018, which is within the open reading frame (ORF) of a putative Ankyrin repeat protein (Fig. 1A and B). The CL3D3 had one insertion at TA #1,023,136 and 1,023,137 (Fig. 1D) which is in the 5′ region (intragenic) of a p44 pseudogene. To confirm sequencing results, we performed PCR analysis using primers targeting *A. phagocytophilum* genome sequences adjacent to the transposon (tn) in a PCR (Table 1) as described by Felsheim et al. (2006). Band sizes corresponding to transposon-coding sequences were observed in all the mutants (Supplementary, Fig. 1). However additional bands of smaller sizes corresponding to unmutated-loci or unmutated regions were observed in the CL1A2 and CL3D3 mutants (Supplementary, Fig. 1, arrows). An additional duplex qPCR targeting the *A. phagocytophilum* single copy gene msp5 and the *aadA* gene from the transposon was performed to confirm if the CL1A2 mutant corresponds to a single population carrying two inserts per genome, or two populations each carrying one insert per genome. This analysis revealed close to 1:1 ratio between msp5 and *aadA* (Table 3) indicating two mutant populations carrying one insert per genome. A 1:2 ratio would indicate one population of mutants carrying two inserts per genome. Further, PacBio sequencing of CL1A2 was performed to obtain a complete genome sequence for the Ap.Norvar1 (GenBank accession # CP046639). This sequence does not contain the Himar1-inserts. Given that CL1A2 is a mixed culture of two mutants, each with one Tn insertion at different genomic sites, there were a proportion of reads that matched the mutated locus in one population whereas another proportion of reads matched the same but unmutated locus in the other mutant population. During genome assembly, the software generated a consensus sequence as a result of alignment between subreads, hence during this process, given the presence of the two types of reads, only those containing the unmutated loci were kept in the final assembly. We performed Illumina sequencing in the CL2B5 and CL3D3 mutants to determine Tn insertion sites using the CP046639 and the Himar1 inverted repeats (IR) as references.

Table 3
qPCR ratio of genomic equivalents between *aadA* and msp5 in the CL1A2 mutant.

	aadA	*msp5*
	6.814	6.892
	6.888	6.913
	6.888	6.944
Mean	6.863	6.916
SD	0.043	0.026

3.3. Clinical and hematological observations

After inoculation, all sheep inoculated with Himar1-mutants or Ap. Norvar1 responded with fever (>40 °C) and neutropenia (<0.7 × 10ˆ9 cells/L) (Fig. 3A-D). However, the Himar1-groups showed a significant delay in onset for fever compared with the Ap.Norvar1 group (CL2B5 p = 0.0079, CL1A2 p = 0.0079 and CL3D3 p = 0.0476) (Fig. 3A-D). However, there were no differences between the groups in the duration of fever or maximum temperature in the same time span (days 0–27). The sheep in the control group did not develop fever or neutropenia after inoculation with bacteria-free ISE6 cells (Fig. 3E and J). Three sheep (one in CL3D3- and two in the Ap.Norvar1 group) developed lameness and deteriorated six days after inoculation, and one single dosage of 2 mL Flunixin ™ vet (Biovet, Quebec, Canada) was administered to each of them to improve overall condition. After treatment with Flunixin, their clinical state improved and there was no difference in hematological assays measured at the time of recovery, thus the sheep were retained in the study.

After challenge on day 28, all sheep in the Himar1-groups and two sheep challenged with Ap.Norvar1 in the control group, responded with fever and neutropenia (Fig. 3A-C, F–H and J). This was in contrast to individuals of the Ap.Norvar1 group, which did not display neutropenia or fever. The Himar1-groups had significantly elevated temperature compared to the Ap.Norvar1-group after challenge (Fig. 3A-D), in addition there were significantly reduced neutrophil cell counts for CL2B5 and CL1A2, compared with the Ap.Norvar1-group after challenge (day 28–51) (Fig. 3F and G).

The differences in temperature, within each group before and after challenge, were significant in the Ap.Norvar1-group and the Himar1-groups (Ap.Norvar1 p = 0.0026, CL2B5 p = 0.0065, CL1A2 p = 0.0018 and CL3D3 p = 0.0072) based on comparison of AUC values (Fig. 3A-D). This was also evident when maximum fever and duration of fever was calculated for each of the Himar1-groups (Table 4). None of the groups displayed significant within group differences in neutrophil cell counts before and after challenge.

3.4. Bacterial load

All the groups that were inoculated with a Himar1-mutant or the Ap. Norvar1 developed a mean maximum bacterial load, six days after inoculation, followed by a mean reduction on day ten. After challenge, the bacterial load detected in Himar1-groups increased until day 34 (minimum and maximum. logarithmic scale: 3.96–5.51 genomic equivalents, $\log_{10}$ (GE)), while the Ap.Norvar1-group reached a maximum level on day 38 (minimum and maximum, logarithmic scale: 0.00–4.02 GE). In addition, there was a significantly increased bacterial load in the Himar1-groups compared with the Ap.Norvar1-group after challenge (CL2B5 p = 0.0041, CL1A2 p = 0.0029 and CL3D3 p = 0.0112). However, both CL1A2 and CL3D3 had significantly lower bacterial load than the control group (n = 2) which was inoculated with

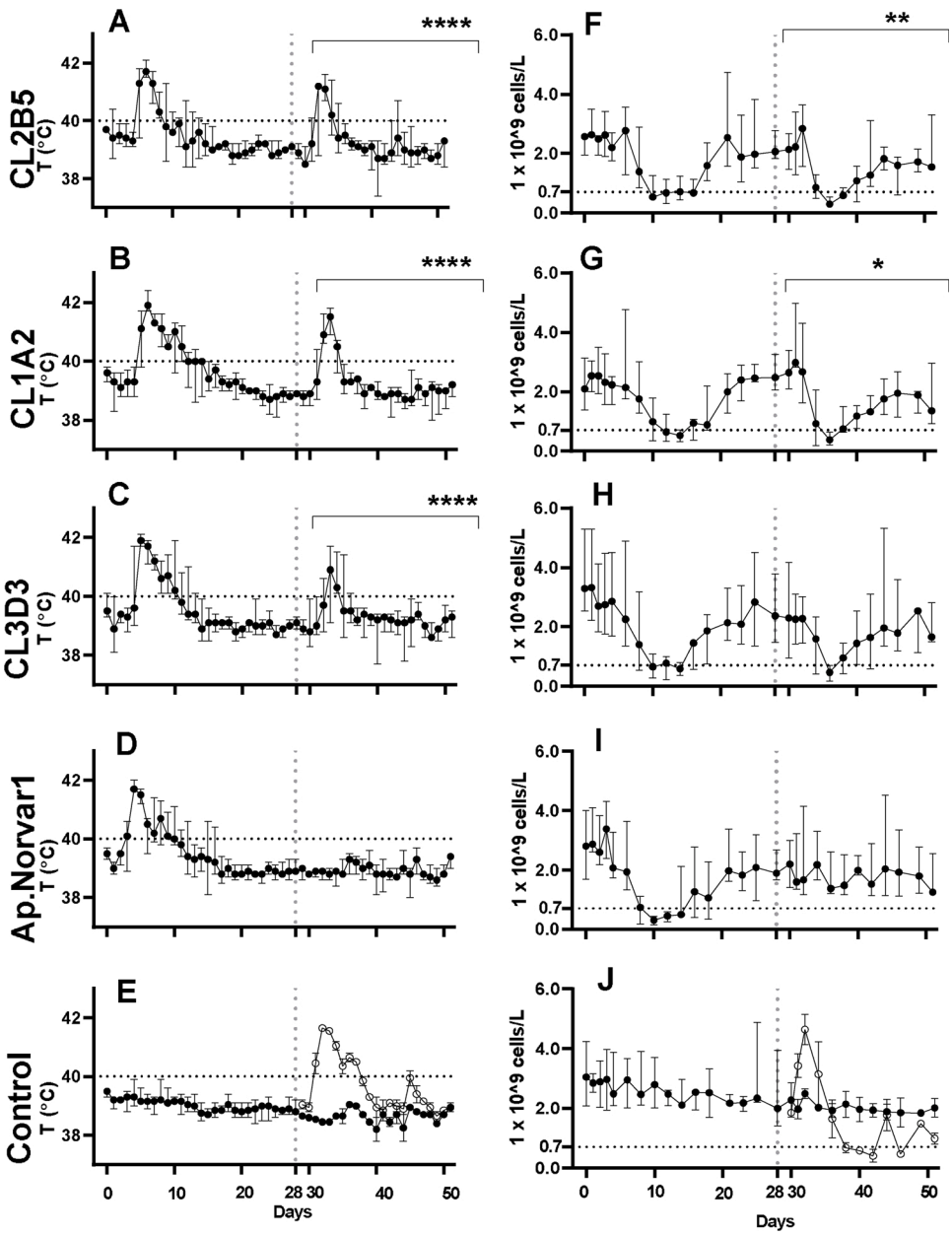

Fig. 3. Clinical and hematological observations. Group median, minimum and maximum of rectal temperature (T °C) and neutrophil granulocytes cell count (1×10^9 cells/L in CL2B5, CL1A2, CL3D3, Ap.Norvar1 and control groups. The Himar1-groups and control groups were compared with the Ap.Norvar1 -group on days 0-27 and 28-51, eg. significant results after challenge in neutrophils in CL2B5 compared with Ap.Norvar1-group for the same time span (28-51 days). The stippled vertical lines mark the time of challenge (day 28) while the stippled horizontal lines mark the threshold value of fever (T > 40.0 °C) and neutropenia ($<0.7 \times 10^9$ cells/L) in the respective graphs. Note that in the graphs of the control group (initially inoculated with ISE6-cells) there are four sheep receiving two different treatments; sheep represented with circles (n = 4) before challenge and further they are differentiated into closed circles (n = 2) and open circles (n = 2) after challenge, the former being challenged with PBS and the latter with Ap.Norvar1. *=p< 0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Table 4
Differences in incubation time, maximum fever and duration of fever within each of the Himar1-groups compared before and after challenge.

	Incubation time (days)	Maximum fever (°C)	Duration of fever (days)
After inoculation			
CL2B5	5 *(5–5)*	41.8 *(41.7–42.1)*	4 *(4–5)*
CL1A2	5 *(5–6)*	41.9 *(41.7–42.4)*	8 *(5–11)*
CL3D3	5 *(4–5)*	41.9 *(41.7–42.1)*	7 *(5–8)*
After challenge			
CL2B5	4 *(3–5)**	41.2 *(41.1–41.6)* *	3 *(2–3)*
CL1A2	4 *(3–4)*	41.6 *(41.2–41.8)* *	3 *(2–4)***
CL3D3	5 *(3–6)*	41.5 *(40.4–41.7)*	3 *(2–4)***

Median, minimum and maximum values are described. *=p<0.05, **=p<0.01, ***=p<0.001.

Ap.Norvar1 for the first time on day 28 (CL1A2 p = 0.0223, CL3D3 p = 0.0262) (Fig. 4B, C and E).

Further, a within group comparison for each of the Himar1-groups, showed a reduction in bacterial load on day 34 when compared with day 6 (CL2B5 p = 0.0005, CL1A2 p = 0.0127, CL3D3 p = 0.0001). In the Ap.Norvar1-group there was a significant within-group reduction in bacterial load (p = 0.019) before and after challenge (Fig. 4D).

There was no detection of the mutants in the Himar1-groups after challenge based on the real time qPCR results targeting the *aadA* gene (specific for the transposon). However, there was one sheep in the CL2B5 group that tested positive for the transposon on day 28 (Fig. 4A). In addition, a total of three sheep from the Himar1-groups, CL2B5 (1 sheep) and CL3D3 (2 sheep) groups tested positive for bacteria with the *gltA* primer set on day 28, but not with the primer set specific for the transposon.

3.5. Peripheral blood mononuclear cells (PBMC)

The absolute cell counts of $CD4^+$, $CD8^+$, $CD4^+CD25^+$, $CD8^+$ $CD25^+$,

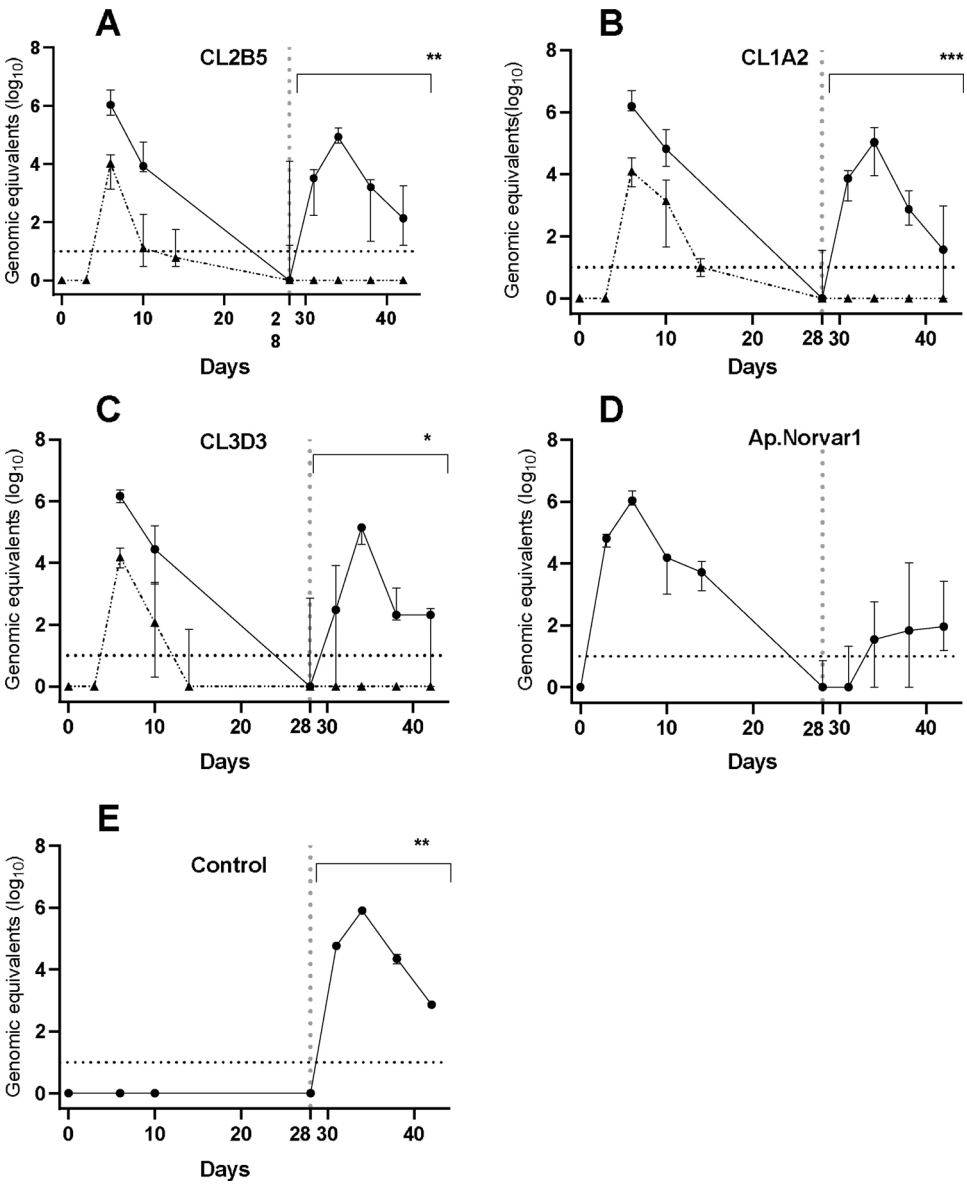

Fig. 4. Bacterial load in CL2B5, CL1A2, CL3D3, Ap.Norvar1 and control groups. Bacterial load was determined solely by the *gltA* (●) gene for the Ap.Norvar1-group and the control-group (n = 2) while both *gltA* and *aadA* genes (▲) were tested for the groups CL2B5, CL1A2, CL3D3 (Himar1-groups). The Himar1-groups were compared with the Ap.Norvar1-group after inoculation until challenge (days 0-27) and challenge until end of study (days 28-51). The stippled vertical lines mark the time of challenge (day 28) while the stippled horizontal lines mark the threshold value of the qPCR (Log units) in the respective graphs. Median, minimum and maximum values are indicated for each time point. *=p<0.05, **=p<0.01, ***=p<0.001.

γδTcR$^+$, γδTcR$^+$WC1$^+$ and NKp46$^+$ in the Himar1-groups were compared with the Ap.Norvar1 group and each other (Figs. 5 and 6). The viability count after resuscitation was 19.48–95.23 % for PBMC, the median of the samples showed a viability count of 61.53 %. The CL3D3 group showed significantly decreased cell counts compared with the CL2B5 and CL1A2 groups on day 14 for γδTcR$^+$ WC1 cells (p = 0.048 and p = 0.024) (Fig. 6B). There were no other significant group differences detected.

The general trends for the total number of CD4$^+$ within each group were small fluctuations, except for the Ap.Norvar1 group which developed a significantly elevated CD4$^+$ count between day 10–38 (p = 0.05) (Fig. 5A). An increase for the same time span was also detected for the Ap.Norvar1. group for CD4$^+$CD25$^+$ (p = 0.04). Further, the CL2B5 group showed an increase in cell counts for CD4$^+$CD25$^+$ between day 14 and 38, while the CL3D3 group showed a significant reduction between day 0 and 10. The CL2B5 group developed a significant increase in CD8$^+$ cells between day 0 and 10 (p = 0.04) (Fig. 5B), and did also develop a significant increase of CD8$^+$CD25$^+$ cells between day 0 and 38 and 14 and 38 (p = 0.002 and p = 0.05) The CD4:CD8 ratio was reduced below 1, 10 days after inoculation in all groups, while only one sheep (from CL3D3 group) developed a ratio below 1, on day 38 (Supplementary Fig. 2).

The general overview of the δTcR$^+$ cells results, suggest that a majority of the cells were WC1 positive. In addition, several significant changes were detected within each of the groups when the γδTcR$^+$ and γδTcR$^+$ WC1 cell counts were analyzed. The CL2B5 group developed significant increase in the number of for γδTcR$^+$ cells between day 0–38, 14–38 and 28–38 (p = 0.0039, p = 0.00426, p = 0.0377) (Fig. 6A and B). The CL3D3 group developed increased cell counts between days 10–28, 10–38 and 14–28 for both cell populations (γδTcR$^+$ p = 0.0080, p = 0.0209, p = 0.0220, γδTcR$^+$WC1 p = 0.0124, p = 0.040, p = 0.0153) (Fig. 6A and B). In addition, the CL3D3 group showed an increase between day 0–28 for γδTcR (p = 0.0150), and day 0–14 and 14–38 for γδTcR$^+$WC1 (p = 0.040 and p = 0.0264) (Fig. 6A and B). In the Ap. Norvar1 group a significant increase was detected between day 10 and 38 for γδTcR$^+$WC1$^+$ (p = 0.0369). The CL3D3 group developed a significant increase of NKp46$^+$ cells between day 3–38 and 10–28 (p = 0.042, p = 0.0193) although total number of cells were low (Fig. 6C).

3.6. IgG response

The IgG levels, measured by IFAT, were not significantly different

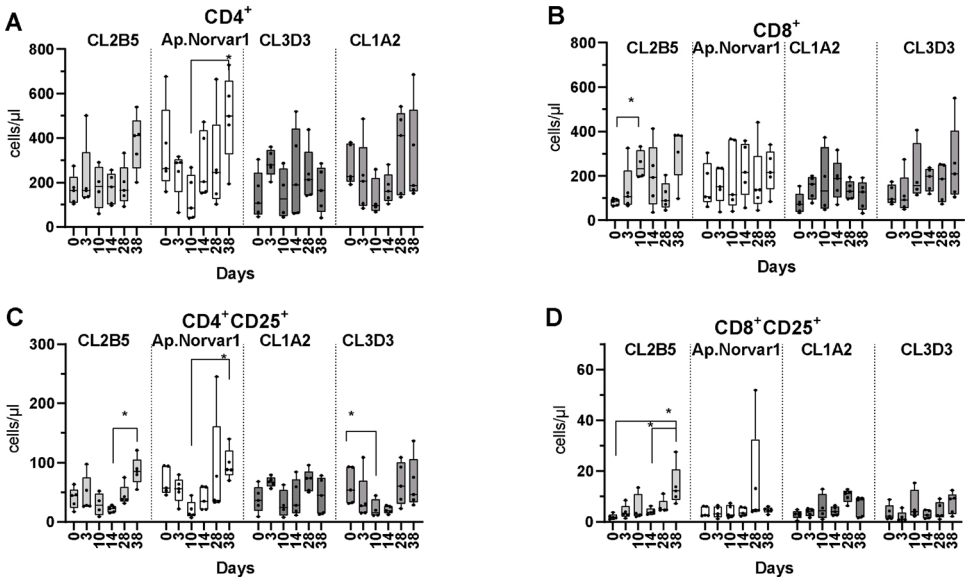

Fig. 5. Flow cytometric phenotyping of PBMCs from sheep during the study on days 0, 3, 10, 14, 28 and 38 after inoculation. Absolute cell counts of cells positive for selected surface markers are shown. A $CD4^+$. B. $CD8^+$. C. $CD4^+CD25^+$. D. $CD8^+CD25^+$. Minimum, maximum values and medians are displayed for each timepoint. Within group differences are marked significant. *=p<0.05, **=p<0.01.

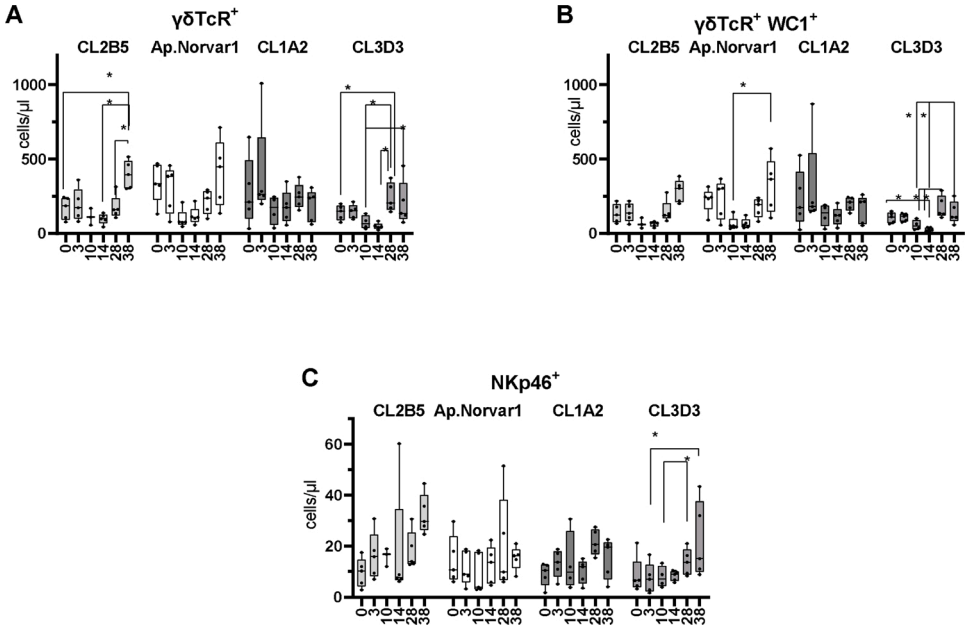

Fig. 6. Flow cytometric phenotyping of PBMCs from sheep during the study on days 0, 3, 10, 14, 28 and 38 after inoculation. Absolute cell counts of cells positive for selected surface markers are shown. A. $\gamma\delta TcR^+$ B. $\gamma\delta TcR^+$ $WC1^+$. C.$NKp46^+$. Within group differences are marked significant. In addition, the CL3D3 group was significant different from CL2B5 (p = 0.0479) and CL1A2 (p = 0.0236). *=p<0.05, **=p<0.01.

Table 5
Reciprocal IgG titer against *A. phagocytophilum* on days 14, 28, 38, 42 and 49 after inoculation for the Himar1-, Ap.Norvar1- and ISE6 groups.

	14	28	36	42	49
CL2B5	**3.41** *(3.41–3.71)*	**3.41** *(3.41–3.71)*	**3.71** *(3.41–4.01)*	**3.71** *(3.71–4.31)*	**3.71** *(3.41–4.31)*
CL1A2	**3.71** *(3.41–3.71)*	**3.71** *(3.41–4.01)*	**3.71** *(3.41–4.01)*	**4.01** *(3.71–4.31)*	**4.01** *(3.71–4.01)**
CL3D3	**3.41** *(3.41–3.71)*	**3.41** *(3.11–3.71)*	**3.41** *(3.41–3.71)*	**3.71** *(3.41–3.71)*	**3.71** *(3.41–4.01)*
Ap.Norvar1	**3.41** *(3.11–3.71)*	**3.41** *(3.11–3.71)*	**3.41** *(3.11–3.71)*	**3.41** *(3.11–3.71)*	**3.11** *(3.11–3.71)*
Control	**0.00**	**0.00**	**1.56** *(0.00–3.11)*	**3.71** *(3.71–3.71)*	**3.56** *(3.41–3.71)*

Median values and 95 % CI are described for the different groups on days 14, 28, 36, 42 and 49 after inoculation. *=p<0.05.

from the Ap.Norvar1 group in any Himar1-group after initial inoculation (Table 5). After challenge on day 28, only group CL1A2 developed significantly elevated IgG titer compared to the Ap.Norvar1-group ($p = 0.034$) (Table 5). IgG responses increased significantly within each group during the study period.

4. Discussion

The overall aim of this study was to develop a vaccine against a virulent sheep strain of *A. phagocytophilum*. Previous vaccine studies have focused on protein-based emulsions and inactivated *Anaplasma* spp. to induce immunological protection against ticks or the pathogen (de Vos et al., 2001; Ducken et al., 2015; Eskeland et al., 2019; Stuen et al., 1998, 2015). These studies did not culminate in an effective vaccine but highlighted the need for a more broad-based strategy. Therefore, the current study used Himar1-transposon technology to generate a multiepitope, attenuated live vaccine against *A. phagocytophilum* in sheep. A possible attenuation is a result from transposon insertion into TA nucleotide sites in a gene that is important for virulence (Lampe et al., 1997). The potential of Himar1-mutants functioning as a vaccine was previously shown by Crosby et al. (2015), who documented the decreased ability of Himar1-transformed *A. marginale* to infect and propagate in cattle, and by McGill et al. (2016) who immunized dogs with an attenuated Himar1 *E. chaffeensis*. Further, the Himar1-mutants are more preserved through passages in cell culture than non-transformed bacteria, and can be monitored for viability (Cheng et al., 2013; Crosby et al., 2015; Felsheim et al., 2006). Himar1-mutants recovered in cell cultures, may demonstrate deficiencies in the ability to infect and replicate in host animals (Crosby et al., 2015; Felsheim et al., 2006). The current study focused on measuring the outcome of the immunization by clinical testing and molecular diagnostics in sheep.

Three Himar1-mutants were obtained after transformation of Ap. Norvar1, of which one (CL2B5) had the transposon insertion in one intergenic region. Mutations in intergenic regions may affect downstream regulation by polar effects and this has been shown for *A. phagocytophilum* in *in vitro* studies (Dumler et al., 2016), although there is no indication for this effect in CL2B5 based on the current observations. Cheng et al. (2013), however showed that Himar1-mutants of *E. chaffeensis* with transposon insertions into intergenic regions were unable to propagate in white-tailed deer (*Odocoileus hemionus*), which corroborated our decision to study CL2B5 in a sheep model. In contrast to the CL2B5, the mutant culture CL1A2 had two insertions. The gel-electrophoresis of CL1A2 showed a profile which corresponded in size with the predicted CL1A2 amplicons, but also had additional bands which likely indicated that unmutated DNA, was still present in the culture. Mixed Himar-1 cultures have been reported previously and may exist under antibiotic selection (Cheng et al., 2013). In addition, the transposon insertion sites of the CL1A2 were in an intergenic region and in a putative ankyrin repeat protein. Additional analyses of the of the genome populations showed that these two insertions most likely belong to two genomes due to a 1:1 ratio of msp5 and the *aadA* gene, suggesting a mixed mutant culture. The ankyrin repeat is a common protein interaction motif in eukaryote organisms (Al-Khodor et al., 2010), that has been adopted by pathogenic bacteria to mediate interactions of effectors with host proteins. This was demonstrated for the ankyrin A protein (AnkA) in *A. phagocytophilum* that binds to the DNA of human granulocytes (Garcia-Garcia et al., 2009; Park et al., 2004) and causes modulation of gene expression (Dumler et al., 2016). In a previous report by Felsheim et al. (2006) a transposon was inserted into ankA of the HGE1 strain of *A. phagocytophilum*, which did not result in a notable change to the phenotype of the bacterium growing in HL-60 cell cultures (Felsheim et al., 2006). However, since the transposon was inserted in another putative ankyrin repeat protein gene in the CL1A2 of the current study, any prediction of the effect on the mutated gene would be presumptive since the function of it has yet not been determined. The mutant CL3D3 had one transposon insertion, but displayed one additional band (unmutated-locus band) on gel-electrophoresis which suggests a mixture of Himar1-mutants and the original Ap.Norvar1 in the culture. The insertion of CL3D3 was located in a p44 pseudogene which was unlikely to alter functional characteristics of the bacterium (Barbet et al., 2006; Dunning Hotopp et al., 2006; Park et al., 2003). A mutation in the expression site of p44 would more likely affect the ability of *A. phagocytophilum* to evade host immunity (Granquist et al., 2010; Thomas et al., 2013).

The Himar1-mutants and the Ap.Norvar1 produced clinical infection in sheep after inoculation and the sheep developed serological responses and changes in the phenotypic expression of PBMC. There were no significant differences between the groups in magnitude and duration of clinical signs, neutropenia and serological responses after the initial inoculation. However, the incubation time was significantly shorter for the Ap.Norvar1group than the Himar1-groups. Incubation time may depend on the inoculum dosage, the rate of bacterial growth in a host or a vector, the transformation process, passage history and possible co-infections (Crosby et al., 2015; Stuen and Artursson, 2000; Wikel, 2018). The inoculum dosage for the Himar1-groups was estimated in cell culture by fluorescence microscopy and was based on infection ratio, while the Ap.Norvar1 dosage was estimated by counting inclusions in 400 neutrophils using light-microscopy (Stuen and Artursson, 2000). A difference in the inoculation dosage was not considered to have influenced the clinical expression or bacteremia during the infection, however it may have affected the incubation time previously shown by Stuen and Artursson (2000). It is also a possibility that different propagation systems might have affected the initial replication of the bacteria in the hosts, however based on our results we cannot find any difference between the groups after the initial inoculation. Further, the Himar1-mutants underwent few passages (7–8) in the tick cell culture after transformation which most likely would not affect the initial replication in the sheep (Woldehiwet et al., 2002). This implies that the Ap.Norvar1 and Himar1 mutants possess the same ability to infect and propagate in the host.

After challenge, the Himar1-groups and the Ap.Norvar1 group developed significant differences in several clinical- and immunological outcomes. The most distinct results were the absence of fever and absence of neutropenia in the Ap.Norvar1 group, which strongly suggest protection against the homologous variant in this group. The absence of clinical signs, combined with the presence of bacteremia, has previously been reported in sheep (Stuen et al., 2009). The Himar1-groups had increased bacterial loads compared with the Ap.Norvar1 group after challenge. However, both CL1A2 and CL3D3 showed reduced levels of bacteremia compared with the control group after challenge. This was accompanied by a reduced temperature in the Himar1-groups compared to pre-challenge levels, which suggest a partial cross-protective immunity. On day 28, several sheep tested positive for the Himar1-mutants, although none were positive for mutants by qPCR after challenge. The detection level between the *aadA* and the *gltA* genes were different, most likely due to different technical properties and PCR conditions (He et al., 1994; Josefsen et al., 2009). Additional analysis could have been undertaken using gBlocks or syntetic dsDNA fragments to standardize an equimolar amounts of amplicon targets. However, our assessment is that both primer sets and probes that were used on day 6, 10 and 28, show the same trend and this suggests that the Himar1-mutants were cleared from the blood after challenge or suppressed by the Ap.Norvar1 as seen in superinfections (Ladbury et al., 2008; Stuen et al., 2009).

In the evaluation of the PBMCs by flow cytometry we did not include negative control animals. This approach was chosen since the Ap.Norvar1 group would function as a control to the Himar1-groups, and thereby we would be able to calculate any difference in the lymphocyte subsets caused by the Himar1-mutants. Further, the sheep were kept in the same facility at all time, which minimized the risk of an environmental factor only affecting one group. There were significant differences between the groups for some of the lymphocyte subsets, although

the results did not provide a cohesive picture of whether these cellular changes were correlated with protection from infection. On the other side, there were several differences between time points within each of the groups, which suggest how levels of lymphocyte populations can change during infection with the Ap.Norvar1 or the Himar1-mutants. In the current study, we observed a trend of low levels of $CD4^+CD25^+$ cells in all groups on day 14, which might suggest limited activation of $CD4^+$ cells after the initial inoculation. This was also discussed by Whist et al. (2003) who reported that $CD4^+$ T cells from sheep, had reduced $CD25^+$ expression up to four weeks after infection with *A. phagocytophilum* (Whist et al., 2003). After challenge, however elevated levels of $CD4^+CD25^+$ was seen in the Ap.Norvar1 and CL2B5 groups. Previous studies have described an initial drop in $CD8^+$ cells followed by an increase in the number of $CD8^+$ cells after infection with *A. phagocytophilum* (Whist et al., 2003). Such an initial CD8 lymphopenia was not observed in the present study, but the CL2B5 group developed a significant increase of $CD8^+$ and $CD8^+CD25^+$ cells during the study. A lower $CD4^+$: $CD8^+$ ratio was seen after the initial inoculation compared to the ratio after challenge. This difference between the two time points appears mostly related to a higher number of $CD4^+$ T cells after challenge compared to after inoculation. A reduction in the $CD4^+$: $CD8^+$ ratio, has been described in canine ehrlichiosis, mainly due to a CD8 lymphocytosis, and was suggested to predispose the dogs to disease complications (Frank and Breitschwerdt, 1999). Woldehiwet (1991) reported also a decrease in CD4:CD8 ratio, but this was observed 13 days after inoculation with *A. phagocytophilum* in sheep (Woldehiwet, 1991) A higher $CD4^+$:$CD8^+$ ratio might be beneficial, as a previous study highlighted the importance of $CD4^+$ T cells in the eradication of *A. phagocytophilum* infection in mice (Birkner et al., 2008). There are small fluctuations of NK-cells, and only the CL3D3 group develop significant increased cell counts throughout the study, but due to the low levels of cells we are cautious in the interpretation of the data.

In the study, we found that a majority of the $\gamma\delta TcR^+$ cells were WC1 positive, and this has also been reported previously (Rogers et al., 2005). Although not significant, there is an observed trend that $\gamma\delta TcR^+$ and $\gamma\delta TcR^+WC1^+$ are reduced 14 days after inoculation in CL2B5, Ap.Norvar1 and CL3D3 groups (CL3D3; only $\gamma\delta TcR^+WC1^+$) could contribute to the lymphopenia after the infection. A reduction in $\gamma\delta TcR^+$ or $\gamma\delta TcR^+WC1^+$ has been reported previously with *A. phagocytophilum* infection in sheep (Eskeland et al., 2019; Whist et al., 2003). The increase in absolute cell counts $\gamma\delta TcR^+$ and $\gamma\delta TcR^+WC1$ after day 14, except for CL1A2, is interesting and might be a result of the cell population levelling at its equilibrium. The $\gamma\delta TcR^+$ cell counts of the CL2B5 group are significant elevated on day 38 compared with day 0, and the Ap.Norvar1 group also display the same trend. The reason for this increase is unknown.

All groups generated antibodies against *A. phagocytophilum* after inoculation and the Himar1-groups displayed elevated levels of antibody titers after challenge. The Ap.Norvar1 group showed a reduction in antibody titers after challenge. A reduction in antibody response has been observed previously for prolonged infections with *A. phagocytophilum* in sheep (Granquist et al., 2010). On the other hand, sheep challenged with heterologous strains of the bacterium developed increased titer (Stuen et al., 2003). Antibodies against *A. phagocytophilum* in mice were only partially protective against the bacterium (Sun et al., 1997) and an increased antibody titer was insufficient for the eradication of *A. phagocytophilum* in sheep (Stuen et al., 2001). However, especially the $CD4^+$ cellular response is believed to be highly important in controlling the infection and propagation of the bacterium (Birkner et al., 2008). The ideal study would need a homologous culture of *A. phagocytophilum* that is genetically identical, to be able to isolate single mutants after transformation. However, since the Ap.Norvar1 has been characterized based on the 16S rRNA gene alone and the genome is currently evaluated based on the CL1A2 genome without transposons (GenBank acc.no CP046639), genetic diversity may occur at different genetic levels of the organism (Bown et al., 2007; Ladbury et al., 2008). The transformation of the Ap.Norvar1 strain will only select for a small fraction of the bacteria present in the ISE6-culture, which can potentially culminate in different clinical- and immunological responses to inoculation and challenge that may explain the partial immunity observed in the Himar1-groups.

In conclusion, Himar1-mutants of Ap.Norvar1 caused infection in experimentally infected sheep with typical clinical manifestations, associated with *A. phagocytophilum*. The sheep inoculated with Himar1-mutants responded by cellular and serological immune responses that were comparable to those caused by Ap.Norvar1. Before challenge, the bacteremia was reduced to pre-inoculation levels in the majority of the sheep. Upon challenge with Ap.Norvar1; bacteremia, clinical signs and neutropenia reoccurred in all the sheep previously inoculated with Himar1-mutants. The Ap.Norvar1 group showed bacteremia, but no clinical signs or neutropenia upon challenge. These results indicate that clinical immunity was achieved against Ap.Norvar1 only in the Ap. Norvar1 group. In order to obtain live attenuated vaccines against *A. phagocytophilum* in future studies, targeted mutagenesis of the bacterium may provide protection and should be priority in further studies.

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Declaration of Competing Interest

The authors report no declarations of interest.

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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.2020.110165.

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