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(54) Titre : PROTEINES RECOMBINANTES UTILISABLES DANS UN VACCIN, ANTICORPS DIRIGES CONTRE LESDITES PROTEINES ET METHODES DIAGNOSTIQUES ET THERAPEUTIQUES FAISANT APPEL A ELLES

(54) Title: RECOMBINANT PROTEINS FOR USE IN VACCINE, ANTIBODIES AGAINST SAID PROTEINS, AND DIAGNOSTIC AND THERAPEUTIC METHODS INCLUDING THE SAME

(57) Abrégé/Abstract:
The present invention relates to proteins and/or fragments and derivatives thereof and their use as vaccines and in biotechnological methods. The vaccines particularly include immunogenic proteins in Treponema spp. isolated from digital dermatitis in cattle. The present invention further relates to antibodies raised against said proteins or fragments thereof, and the use of said proteins in diagnostic methods in which antibodies are detected as a sign of digital dermatitis in cattle.

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RECOMBINANT PROTEINS FOR USE IN VACCINE, ANTIBODIES AGAINST SAID PROTEINS, AND DIAGNOSTIC AND THERAPEUTIC METHODS INCLUDING THE SAME.

The present invention relates to proteins and/or fragments and derivatives thereof and their use as vaccines and in biotechnological methods. The vaccines particularly include immunogenic proteins in *Treponema* spp. isolated from digital dermatitis in cattle. The present invention further relates to antibodies raised against said proteins or fragments thereof, and the use of said proteins in diagnostic methods in which antibodies are detected as a sign of digital dermatitis in cattle.

Background

Digital dermatitis (DD) is a contagious claw disease causing lameness in cattle, most commonly seen in intensive dairy production. The disease was first described in 1974 in Italy. In Sweden the first herd with DD was described recently (Hillström and Bergsten, 2005) whereas previously only sporadic, atypical cases have been reported (Manske et al., 2002). There is a strong connection between wet/dirty claw environments and the occurrence of DD (Rodriguez-Lainz et al., 1996), for example in cubicle systems where accumulation of faeces and urine on the alleys is a typical hygienic problem. Besides being an animal welfare problem, economic losses due to reduced milk production and weight loss are associated with DD (Losinger, 2006).

The rapid response to antibiotic treatment of DD lesions strongly supports a bacterial cause. Many bacteria of different genera, such as *Treponema*, *Fusobacterium*, *Dichelobacter*, *Prevotella*, and *Porphyromonas* have been isolated from DD lesions and a polymicrobial cause is often discussed. However, there is strong circumstantial evidence that *Treponema* spp. are central in the aetiology of DD. As early as 1964 spirochetes were observed in smears from different variants of "foot-rot" manifestations in cattle (Gupta et al., 1964). Another early observation of spirochetes was made 1988 when DD was described for the first time in the UK (Blowey and Sharp, 1988). The first spirochete cultures from DD were reported 1995 (Walker et al., 1995). In histological preparations from DD lesions treponemes are found invading the deeper layers of epidermis (Moter et al., 1998). Additionally a humoral immune response against *Treponema* spp. has been demonstrated in infected cattle (Walker et al.,

1997; Trott et al., 2003). Successful experimental transmission of the disease through inoculation with fresh scrapings from DD lesions was described in 1996 (Read and Walker, 1996). It was also confirmed by histopathology that spirochetes invaded the tissue 1-2 weeks after inoculation (Read et al., 1998).

Several phylotypes of *Treponema* can be present in the same lesion. Different phylotypes have been isolated from the same animal (Walker et al., 1995; Evans et al., 2008) and by cloning and sequencing of 16S rRNA genes, five different phylotypes were identified in a pooled sample from four cows (Choi et al., 1997). It has also been demonstrated by fluorescence in situ hybridization on biopsies from DD lesions that the distribution in the dermal layers differs between phylotypes (Moter et al., 1998). The *Treponema phagedenis*-like phylotype was located mainly in the stratum corneum and stratum spinosum. Some phylotypes have not yet been reported as cultured. Recently the *Treponema phagedenis*-like phylotype has been indicated in several studies to be a key agent in the pathogenesis of DD (Klitgaard *et al.* 2008, Nordhoff *et al.* 2008, Yano *et al.* 2009).

In countries where DD is widespread, footbaths containing antibiotics are often used. These footbaths rapidly become contaminated with faeces and dirt and hence function as large selective cultures of antibiotic resistant bacteria. In Sweden tetracyclines are used, but only for topical treatment of individual animals since on herd level footbaths with copper sulphate are recommended.

To date no commercial vaccine or serologic test for DD is available. A humoral response against *Treponema* spp. has been shown in cattle with DD and used for whole cell lysate ELISA investigations in research (Demirkan *et al.* 1999, Trott *et al.* 2003, Vink *et al.* 2009, Walker *et al.* 1997). Novartis produced a whole cell lysate DD vaccine (TrepShield) for the USA market for some years in the early 2000s (Berry *et al.* 2004, Keil *et al.* 2002).

Technologies and strategies for development of vaccines are described in i.a. Vaccine Design: Innovative Approaches and Novel Strategies (Caister Academic Press, 2011) and Vaccines: From Concept to Clinic: A Guide to the Development and Clinical Testing of Vaccines for Human Use (Informa Healthcare, 1998). The use of a recombinant protein as a vaccine is described in Erdile *et al.* 1997.

Summary

The present invention aims at providing efficient methods for diagnosis of and immuno-protection against dermatitis in animals, particularly digital dermatitis in ruminants, as well as products for said purposes.

The present invention revolves around immunogenic proteins in *Treponema* spp. isolated from digital dermatitis in cattle, and more specifically to recombinant proteins.

In a first aspect, the present invention relates to isolated *Treponema phagedenis*-like proteins, TmpA, Ttm, and PrrA, with amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, respectively, to fragments and derivatives thereof capable of inducing an immune response to *Treponema* spp., and to fragments and derivatives capable of binding to antibodies produced by a subject in an immune response against said protein, as further defined below.

In one embodiment of the invention, the *Treponema* proteins, and fragments and derivatives thereof, are recombinantly produced.

In one aspect, the invention relates to nucleic acid molecules encoding the proteins, fragments and derivatives according to the invention.

The invention also relates to the use of said proteins, fragments and derivatives thereof in veterinary medicine, specifically as a vaccine for prevention of digital dermatitis.

In a further aspect, the present invention provides a veterinary vaccine for protection against digital dermatitis comprising one or more of said recombinant proteins and/or active fragments thereof, and conventional and suitable adjuvants. Such a vaccine may or may not further include other *Treponema* immunogens or whole cell lysates of different *Treponema* spp. in a vaccine for a broader immune response.

According to another aspect, the present invention also relates to a method for prevention of digital dermatitis in animals comprising the step of administering said vaccine to an animal in need thereof.

According to a still further aspect, there is provided a method of detecting presence of antibodies against *Treponema* spp. in a sample in which said recombinant proteins and/or active fragments are used to detect the presence of antibodies against *Treponema* spp. in said sample.

According to a still further aspect, there is provided a method for diagnosis of digital dermatitis in an animal in which said recombinant proteins and/or active fragments are used to detect the presence of antibodies against *Treponema* spp. in an animal.

In one embodiment of said detection method or diagnostic method, said recombinant proteins and/or active fragments are used in an ELISA (Enzyme-Linked ImmunoSorbant Assay) method.

In one aspect, the present invention relates to antibodies raised against said immunogenic proteins, or immunogenic derivatives or fragments thereof. Such antibodies are useful in treatment of disease caused by *Treponema* spp. by way of passive immunization and also in various laboratory methods such as immunomagnetic separation of *Treponema* bacteria.

Brief description of the figure

Figure 1: Enzyme-linked immunosorbant assay with recombinant *Treponema phagedenis*-like strain V1 immunogenic proteins TmpA, Ttm, and PrrA as antigens. The assays were performed with sera from eight dairy cows with acute digital dermatitis (black bars), two cows with no known history of digital dermatitis and five calves 6-7 months of age (gray bars). Horse-radish peroxidase (HRP) conjugated rabbit anti-bovine IgG antibodies (Sigma) (A) or monoclonal 22:26 anti-bovine IgG-HRP antibodies (Svanova Biotech AB) (B) were used as secondary antibodies. Corrected optical density (COD) was measured at 450 nm.

Definitions

An "immunogenic agent", or "immunogen", is capable of inducing an immunological response against itself on administration to a patient, optionally in conjunction with an adjuvant.

An "active fragment" or "active derivative" as used in the present specification is a fragment or derivative of a native immunogenic agent, capable of inducing an immunological response against said native immunogenic agent on administration to a patient, optionally in conjunction with an adjuvant. An active fragment or derivative comprises or mimics at least one "epitope" or "antigenic determinant".

A "binding fragment" or "binding derivative" as used in the present specification is a fragment or derivative of a native immunogenic agent, capable of immunospecific binding to antibodies produced by a subject in an immune response against said native immunogenic agent. A binding fragment or derivative comprises or mimics at least one "epitope" or "antigenic determinant".

A "derivative" of a protein may be a protein showing substantial sequence homology to the original protein. The sequence homology may be 50% identity or more, such as 65%, 80%, 85%, 90%, 95% or 99% identity in amino acid sequence. The substituted amino acids are preferably conservative substitutions. The substituted amino acids may be natural or non-natural amino acids.

The term "epitope" or "antigenic determinant" refers to a site on an antigen to which B and/or T cells respond. B-cell epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e. g., Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66, Glenn E. Morris, Ed. (1996).

Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target immunogen, or fragment or derivative thereof.

The term "antibody" refers to an intact antibody, or a binding fragment thereof. An antibody may comprise a complete antibody molecule (including polyclonal, monoclonal or chimeric), or comprise an antigen binding fragment thereof. Antibody fragments include $F(ab')_2$, Fab, Fab', Fv, Fc, and Fd fragments, and can be incorporated into single domain antibodies, single-chain antibodies, maxibodies, minibodies, intrabodies, diabodies, triabodies, tetrabodies, v-NAR and bis-scFv (See e.g., Hollinger and Hudson, 2005, Nature Biotechnology, 23, 9, 1126-1136).

Sequence listing

SEQ ID NO	Type	Description
1	DNA	TmpA homolog, complete coding sequence
2	Protein	TmpA homolog, protein
3	DNA	putative tail tape measure protein (Ttm), complete coding sequence
4	Protein	putative tail tape measure protein (Ttm), protein
5	DNA	putative proline-rich lipoprotein (PrrA), complete coding sequence
6	Protein	putative proline-rich lipoprotein (PrrA), protein
7	DNA	GSTtmpAF1 (fwd)
8	DNA	GSTtmpAR1 (rev)
9	DNA	GSTkallaF1 (fwd)
10	DNA	GSTkallaR1 (rev)
11	DNA	GSTPGKEEF1 (fwd)
12	DNA	GSTPGKEER1 (rev)
13	DNA	ImpactPGKEEF1 (fwd)
14	DNA	ImpactPGKEER1 (rev)
15	DNA	ImpactkallaF1 (fwd)
16	DNA	ImpactkallaR1 (rev)
17	DNA	primer SAseky

Detailed description

Further outline of aspects and embodiments of the invention

In one aspect the present invention relates to an isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, or fragments or derivatives thereof capable of inducing an immune response to said protein, or a fragment or derivative capable of binding to antibodies produced by a subject in an immune response against said protein. Said protein, fragment or derivate may be used in veterinary medicine, such as in prevention of a disease caused by *Treponema* spp., such as digital dermatitis.

In another aspect, the present invention relates to a method for treatment or prevention of a disease caused by *Treponema* spp. comprising administering to a subject isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, or a fragment or derivative thereof capable of inducing an immune response to said protein. Said method may be used for a disease such as digital dermatitis.

In a further aspect, the present invention relates to a pharmaceutical composition comprising an isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, or a fragment or derivative thereof capable of inducing an immune response to said protein, and optionally pharmaceutically acceptable adjuvants, carriers and/or diluents.

In yet another aspect, the present invention relates to a method for detecting the presence of antibodies against proteins from *Treponema* spp. in a sample, comprising the steps:

- bringing said sample in contact with an isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, or a fragment or derivative thereof capable of binding to antibodies produced by a subject in an immune response against said protein; and
- detecting antibodies binding to said protein, fragment or derivative.

A further aspect of the invention relates to a method for *in vitro* diagnosis of a disease caused by *Treponema* spp. comprising the steps:

- obtaining a sample of body fluid or tissue from a subject;
- bringing said sample in contact with an isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, or a fragment or derivative thereof capable of binding to antibodies produced by a subject in an immune response against said protein; and

- detecting antibodies binding to said protein, fragment or derivative;

wherein the presence of antibodies binding to said protein, fragment or derivative is indicative of a disease caused by *Treponema* spp.

Said method may be used for a disease such as digital dermatitis.

A further aspect of the invention relates to an antibody, or binding fragment thereof, binding specifically to an isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6.

Another aspect of the invention relates to a method for treatment or prevention of a disease caused by *Treponema* spp. comprising administering said antibody to a subject. Digital dermatitis is one example of such a disease.

A further aspect of the invention relates to a method for separation of *Treponema* bacteria from a sample, comprising the steps:

-bringing said sample in contact with said antibody bound to a solid phase;

- allowing said antibody to bind to *Treponema* proteins in said *Treponema* bacteria; and

- separating said solid phase from said sample

thereby separating said *Treponema* bacteria from said sample.

In said method, the separation may be achieved by for example immunomagnetic separation.

Another aspect of the invention relates to a nucleic acid molecule encoding the protein, fragment or derivative according to the present invention. In one embodiment the nucleic acid molecule encoding the protein has a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 and parts thereof.

A further aspect of the invention relates to a vector comprising said nucleic acid molecule and optionally regulatory sequences for expression in a host cell.

Another aspect of the invention relates to a transgenic host cell comprising said vector.

The present invention also relates to a method for producing a protein, fragment or derivative according to the present invention, comprising the steps:

- culturing the host cell according to the present invention in a suitable medium; and
- isolating said protein, fragment or derivative from said medium.

Details

Shotgun phage display was used to identify three immunogenic proteins in an isolate (V1) of the DD *Treponema* phylotype closely related to *T. phagedenis*. This phylotype has been indicated in several studies to be a key agent in the pathogenesis of DD (Klitgaard *et al.* 2008, Nordhoff *et al.* 2008, Yano *et al.* 2009). The phage library was selected against antibodies from a rabbit immunized with live bacteria.

A homolog to the well-characterized immunogenic protein TmpA of *T. pallidum* subsp. *pallidum* was identified as well as two proteins without homology to any known spirochetal protein. The complete amino acid sequence of these proteins was predicted from a genomic sequence of V1 generated by 454 Sequencing™. The three specific immunogenic proteins, and their amino acid sequences, are described in SEQ ID NO: 2, 4 and 6.

The proteins, fragments and derivatives according to the first aspect of the invention may be isolated from a culture of the *Treponema* phylotype closely related to *T. phagedenis* strain V1, or, preferably, recombinantly produced as described below.

Western blot has been performed to show that both antibodies from the immunized rabbit as well as naturally infected cattle bind to the recombinantly produced TmpA homolog and the Ttm fragment.

Pilot ELISA runs have been made and a difference in absorbance has been recorded between sera from cattle with and without DD (table 1). There were only a few overlaps between the results in the healthy and the infected group using single antigens (the TmpA homolog or the Ttm fragment) and no overlaps using a combination of the two antigens.

The immunogenic proteins according to the present invention, and active fragments thereof, can be used in vaccines against diseases caused at least in part by the *Treponema* phylotype closely related to *T. phagedenis,* or other *Treponema* spp.

In certain embodiments, the complete proteins are used in vaccines. In certain embodiments only fragments comprising the relevant epitopes are used. In certain embodiments one or more epitopes of one or more proteins are combined in a single molecule and used in a vaccine. The recombinant proteins, derivatives or fragments thereof may be thus used alone or in different combinations or as fusion proteins of the binding epitopes.

Methods

Helper phage, bacterial strains, growth conditions, and DNA-techniques

Phage R408 (Promega) was used as helper phage. *Escherichia coli* TG1 (Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5* (r_K^- m_K^-) *thi-1 supE* [F′*traD36 proAB lacI*^{q}*ZΔM15*]; Stratagene) was used as host in all experiments involving phages or phagemids and grown in Luria-Bertani broth (LB) or on Luria-Bertani agar (LA). When appropriate, 50 µg/ml ampicillin ($C_{16}H_{18}N_3O_4SNa$, Roche) was added. Incubations were at 37 °C. Chromosomal DNA from *Treponema* sp. strain V1 was used for construction of the phage library. The *Treponema* strain was grown in flasks with FABGS (fastidious anaerobe broth, LAB 71, LabM, Lancashire, UK with 2,0 g D-glucose per liter and 25% fetal calf serum, S 0115, Biochrom AG, Germany) incubated at 37°C, in anaerobic jars on a shaker (90 rpm). Broth cultures were washed three times in isotonic saline (pH 6.3), followed by one wash in phosphate buffered saline (PBS, pH 7.3). The *Treponema* DNA was prepared by conventional phenol-chloroform extraction. Restriction and modification enzymes were from MBI Fermentas AB and used according to manufacturer's instructions. Plasmids were prepared using QIAprep™ Miniprep (QIAGEN).

Immunization and purification of polyclonal antibodies

This part of the study was approved by the ethical committee on animal experiments in Uppsala (C 300/8). A New Zealand white rabbit was immunized subcutaneously with a live culture of *Treponema* sp. strain V1. A dose of approximately 10^9 bacteria, washed twice and dispensed in a volume of 0.5 ml isotonic saline, was injected twice with 20 days in between. Serum from the final bleed at day 38 post first immunization was used for purification of antibodies (IgG) for this study. Ten ml serum was sterile filtered through a 0.45 µm syringe filter and applied to a 5 ml HiTrap™ Protein G HP column (GE Healthcare). Rabbit IgG was

purified according to the manufacturer's instructions using the Ab Buffer Kit (GE Healthcare). Eluates of purified antibodies were desalted using Zeba Spin Desalting columns (Pierce) and stored in PBS at minus 20°C.

Construction of the Treponema *sp. phage display library and selection of binding phages (panning)*

The phage library was constructed in the pG8SAET phagemid vector. *Treponema* sp. strain V1 chromosomal DNA was fragmented by sonication until the majority of the fragments were between 0.4–1.5 kb in length. The fragments were made blunt-ended by T4 DNA polymerase and T4 DNA kinase treatment and then ligated into *Sna*B1-digested and dephosphorylated phagemid vector pG8SAET using Ready-To-Go™ T4 DNA ligase tubes (GE Healthcare). The final library was generated by electrotransformation of the ligated material into *E. coli* TG1 cells (2.5 kV, 25 μF, 360 Ω), infection with helper phage, and proliferation of phage particles. This procedure yielded 4 x 10^7 transformants, considered as unique clones, 86% of which carried an insert, as determined by colony PCR on 14 randomly selected clones. The final library had a titer of 1 x 10^{11} colony forming units per ml.

Phage displaying immunogenic polypeptides were isolated by panning against rabbit anti-*Treponema* sp. strain V1 IgG. Three panning experiments were performed. Microwells (MaxiSorp™, Nalge Nunc International) were coated with Zymed recombinant Protein G (Invitrogen) at a concentration of 10 μg in 200 μl 50 mM sodium carbonate, pH 9.5. Thereafter, the wells were blocked with phosphate buffered saline pH 7.4 with 0.05% Tween 20 (PBS-T). Rabbit anti-*Treponema* sp. strain V1 IgG was added at a concentration of 85 or 215 μg in 200 μl PBS or 100 μl PBS + 100 μl crude *E. coli* lysate (for blocking). After washing, 200 μl of the phage library was added. The wells were incubated for 3h at room temperature, or over night at 2 °C, after which they were washed 25 times before phage were eluted by addition of 50 mM Na-citrate/140 mM NaCl pH 2.0. The eluate was immediately neutralised with 2 M Tris-buffer pH 8.0 and used to infect *E. coli* TG1, which were plated on LA-plates with ampicillin (LAamp). In one panning experiment, elution was also carried out by direct infection of TG1 cells added to the well by the bound phages. After incubation overnight, colonies were counted and 100 colonies transferred to an LAamp-plate. These colonies were then transferred to nitrocellulose-filters for screening of E-tag expression using mouse anti-Etag antibodies (GE Healthcare) and secondary horse radish peroxidase-labelled sheep anti-mouse antibodies (GE Healthcare). The remaining colonies were washed off the

plates and superinfected with helper phage to make an enriched library/phage stock, which was used in the second enrichment cycle (repanning) according to the same protocol. In total, two repannings were performed. More than 200 E-tag positive colonies were chosen for plasmid preparation and sequence determination of the inserts using primer SAsekv (5'-TAT CTG GTG GCG TAA CAC CTG CT-3', SEQ ID NO: 17). Plasmid DNA was sequenced on a 3730xl DNA Analyzer (Applied Biosystems) at Uppsala Genome Centre and analyzed with the CLC Main Workbench software (CLC bio). Analyses of the inserts revealed nine, nine, and eight overlapping partial sequences, respectively, from three different genes.

Genome sequencing and sequence analysis

The chromosomal DNA of *Treponema* sp. strain V1 was sequenced and assembled at the KTH Genome Center at KTH Royal Institute of Technology, Stockholm, Sweden, using the Genome Sequencer FLX System, with long-read GS FLX Titanium chemistry and the 454 de novo assembler, Newbler (454 Life Sciences, Branford, CT, USA). An additional De Novo assembly of the reads was made with CLC Genomics Workbench 3 (CLC bio) and for further sequence editing CLC Main Workbench 5 (CLC bio) was used.

The genome sequence was used to predict the full open reading frames and the corresponding amino acid sequences of the three immunogenic proteins. Homology searches were performed using the BLAST algorithm at the National Center for Biotechnology Information. The SignalP 3.0 Server with Gram-positive data was used for prediction of signal peptides. One protein was predicted as a lipoprotein according to Setubal *et al.* 2006.

Construction of clones for protein expression and purification

Genomic *Treponema* sp. strain V1 DNA for PCR was prepared with the DNeasy Blood & Tissue Kit (QIAGEN) following the protocol for Gram-negative bacteria. A 50 µl reaction mixture of 5 µl 10X *Pfu* Buffer with $MgSO_4$ (Fermentas), 0.2 mM of each deoxynucleotide, 0.2 µM of forward and reverse primers as indicated in Table 2, 1.25 U *Pfu* DNA polymerase (Fermentas) and 50 ng genomic DNA, was prepared. The thermal cycling conditions were 95°C for 1 min, 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 3 min and a final extension at 72°C for 5 min. PCR products were analyzed by agarose gel electrophoresis and purified with the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). Purified amplicons were digested with either BamHI and XhoI or NdeI and SapI according to the manufacturer's instructions (Fast digest, Fermentas) and purified as described earlier. The

digested amplicons were ligated into the respective vector – BamHI and XhoI digested pGEX-6P-1 (bulk GST purification module, GE Healthcare) or NdeI and SapI digested pTXB1 (IMPACT™ Kit, New England BioLabs) – using the ReadyToGo T4DNA Ligase (GE Healthcare). Ligated material were electrotransformed into competent *Escherichia coli* strain BL21(DE3) (GST) or ER2566 (IMPACT) and spread on LA supplemented with ampicillin (final conc. 50µg/ml). The presence of inserts in a number of colonies was analyzed by PCR using the vector sequencing primers. Clones with a correct size insert were further analyzed by DNA sequencing.

Production of recombinant immunogenic Treponema *proteins*

Commercially available protein expression and purification systems such as the bulk GST purification module (GE Healthcare) or the IMPACT™ Kit (New England BioLabs) were used for production of recombinant immunogenic *Treponema* proteins according to manufacturer's instructions. Recombinant clones were grown at 37°C in LB media supplemented with ampicillin (final conc. 50µg/ml). At an optical density ($OD_{600\ nm}$) ~0.6, the growth medium was supplemented with IPTG (final konc. 0.3 mM) and the growth temperature shifted to 20°C. After incubation over night the cells were harvested and resuspended in a buffer [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1 mM EDTA, and 0.05% (v/v) TWEEN20] and lysed by freezing and thawing. After centrifugation, the supernatants were sterile filtrated and applied onto a chitin column. The columns were washed extensively using the same buffer and treated subsequently with cleavage buffer [20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, and 30 mM dithiothreitol (DTT)]. The eluted samples containing the antigens were dialysed against phosphate-buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7.4)].

In the GST-glutathione affinity system, according to the procedure described above, after growth, induction and harvest, the *E. coli* cells were suspended in PBS supplemented with TWEEN20, final conc. 0.1% (v/v) (PBST) whereupon the cells were lysed by freezing and thawing. After centrifugation, the supernatant was sterile filtrated and batch purified with Glutathione-sepharose beads. After extensive washing using PBST the fusion protein was eluted with glutathione elution buffer or treated with scissor protease to release the produced protein. Finally, the amounts of antigens obtained were determined using spectrophotometry and the quality analyzed by SDS-PAGE coomassie staining. The proteins were stored finally at −20°C.

Enzyme-linked immunosorbant assays (ELISAs)

Advantages with using purified proteins recombinantly produced in *E. coli* compared to whole cell lysates in an ELISA are the possibilities of large-scale production and optimization of the test (different combinations of proteins, often less background due to higher specificity). Initial ELISA tests were performed with sera from cattle with and without digital dermatitis (DD) and with sera from the immunized rabbit (above). Microplates (PolySorp™, Nalge Nunc International) were coated with recombinant TmpA homolog and/or recombinant tail tape measure protein fragment at a concentration of 2.5 µg and/or 0.31 µg, respectively, in 100 µl 50 mM sodium carbonate, pH 9.5, overnight at 2°C. Wells were washed twice with 400 µl phosphate buffered saline pH 7.4 with 0.05% Tween 20 (PBS-T). Thereafter, the wells were blocked with PBS-T for one hour at room temperature. One hundred µl serum or PBS-T was added to each well. Four dilutions of each serum were used – 1:25, 1:50, 1:100, and 1:200. The microplates were incubated at 37°C for one hour and then washed three times with 400 µl PBS-T. Horse-radish peroxidase conjugated swine-anti rabbit (Dako) and rabbit-anti cow (Dako) antibodies were added to the relevant wells, diluted 1:4000 and 1:500, respectively, and plates were incubated for one hour at 37°C. The wells were washed three times with 400 µl PBS-T after which 100 µl solution consisting of 20 mM tetramethylbenzidine (TMB) mixed 1:20 with 0.1 M potassium citrate/H_2O_2 (230 µl/l) pH 4.25, was added. The plates were incubated for 10 minutes at room temperature. To stop the reaction 50 µl 10% sulfuric acid was added. Optical density (OD) was measured at 450 nm and the readings were corrected against a sample buffer blank.

Immunomagnetic separation using antibodies

Treponema spp. are fastidious organisms that require complex culture media and anaerobic environments for growth. Additionally, samples from cattle claws have a plethora of other bacteria contaminating the cultures. An option to concentrate and purify *Treponema* spp. for culturing and DNA isolation is immunomagnetic separation (Demirkan *et al.* 1999, Demirkan *et al.* 2001, Choi *et al.* 1996). Immunomagnetic beads covalently coated with for example anti-rabbit IgG coupled with rabbit antibodies raised against said immunogenic proteins, derivatives or active fragments thereof can be used for specific separation of the DD *Treponema* phylotype closely related to *T. phagedenis*.

Vaccines

Bacterial proteins stimulating the immune system to antibody production can also be used for vaccine development. Recombinant proteins can be combined with immune-stimulating complexes (ISCOMs) and/or whole cell lysates to increase the immune response in the animal and hence the protection against the disease-causing agent/s.

5

Further experiments performed

Enzyme-linked immunosorbant assay (ELISA)

Materials and methods: The assays were performed with sera from eight dairy cows with acute DD from the herd from which Tpl strain V1 was isolated, two cows from another herd
10 with no known history of DD, and five calves 6-7 months of age. Digital dermatitis diagnosis was made by visual examination. Microplates (PolySorp™, Nalge Nunc International) were coated with recombinant proteins at concentrations of 1 µg/ml TmpA, 0.8 µg/ml Ttm or 0.02 µg/ml PrrA in 100 µl 50 mM sodium carbonate, pH 9.5, overnight at 2°C. Wells were washed twice with PBS-T and blocked with PBS-T for one hour at room temperature. 171 One
15 hundred µl serum diluted 1:100 in PBS-T was added to each well. The microplates were incubated at 37°C for one hour and then washed with PBS-T. Horse-radish peroxidase (HRP) conjugated rabbit anti-bovine IgG antibodies (Sigma) diluted 1:8000 or monoclonal 22:26 anti-bovine IgG-HRP antibodies (Svanova Biotech AB) diluted 1:4000 were added to the wells and plates were incubated for one hour at 37°C. The wells were washed three times with
20 PBS-T after which 100 µl solution consisting of 1 mM tetramethylbenzidine and 0.006% H2O2 in 0.1 M potassium citrate pH 4.25, was added. The plates were incubated for 10 minutes at room temperature. To stop the reaction 50 µl 10% sulfuric acid was added. Optical density was measured at 450 nm and the readings were subtracted by the optical density of a sample buffer blank giving the corrected optical density (COD).

25

Results: The three immunogenic proteins identified in Tpl strain V1 were produced recombinantly; PrrA as a full-length mature protein from aa +1 relative the cysteine residue of the predicted lipoprotein signal peptide to the last aa before the stop codon (aa:s 22-251), TmpA from aa +7 relative the cystein residue of the predicted lipoprotein signal peptide to the
30 last aa before the stop codon (aa:s 29-344), and Ttm as a partial polypeptide covering aa:s 689-970, which are the aa:s constituting the consensus sequence of the overlapping Ttm sequences from the panning experiments. These proteins/polypeptides were used as antigens in indirect ELISAs where serum samples from cattle with and without DD were analyzed for presence of antibodies against the antigens. Different concentrations of antigen, sera, and

secondary antibody were tested in pilot experiments (data not shown). The conditions under which the best discrimination between cattle with and without DD was achieved were used in the final experiment. For the TmpA antigen, the optical density for three samples from cattle with DD was lower than the highest value for the samples from clinically healthy 269 cattle, while the tests with Ttm and PrrA were discriminatory in all cases but one (Figure 1A and B).

Protein/protein fragment	Cattle without DD (OD, 450 nm)							Cattle with DD (OD, 450 nm)								
	1670	1680	242	243	245	246	251	5510	RK	SK	413	571	1004	2326	5542	6437
TmpA homolog	1.23	1.06	1.28	0.91	1.31	0.75	0.73	2.55	1.25	1.7	2.18	2.26	2.44	1.82	2.96	3.15
Putative tail tape measure protein fragment	1.30	1.47	1.08	1.20	1.59	0.75	0.70	2.14	1.66	1.91	1.61	1.86	2.06	1.76	1.74	1.56
TmpA homolog + putative tail tape measure protein fragment	1.36	1.23	0.63	0.90	0.91	0.83	0.60	2.47	1.83	1.84	1.40	1.73	2.17	1.87	2.65	2.03

Table 1. Initial ELISA tests of sera from cattle with and without digital dermatitis (DD). Optical density (OD) was measured at 450 nm and the readings were corrected against a sample buffer blank. Sera were diluted 1:100, the concentration of TmpA was 2.5 µg/well and of the putative tail tape measure protein fragment 0.31 µg/well.

SEQ ID NO	Name of forward (fwd) and reverse (rev) primer pair	Sequence, 5' to 3' of each primer. Restriction enzyme cleavage sites are indicated in bold.
7	GSTtmpAF1 (fwd)	GGT GGT **GGA TCC** AAA GCG GAA CAA GAA GCT CA
8	GSTtmpAR1 (rev)	GGT GGT **CTC GAG** TCA TTG TAC ACC TCC CTC TA
9	GSTkallaF1 (fwd)	GGT GGT **GGA TCC** AAG AAA GAG CTG TTA GAT TT
10	GSTkallaR1 (rev)	GGT GGT **CTC GAG** TTA TTT ATC AAT TTC TGC CAA
11	GSTPGKEEF1 (fwd)	GGT GGT **GGA TCC** CAA GGT CCA GCT AAC CCC ACA
12	GSTPGKEER1 (rev)	GGT GGT **CTC GAG** TTA GAG CTT CTC TAG CAC AAA
13	ImpactPGKEEF1 (fwd)	GGT GGT **CAT ATG** CAA GGT CCA GCT AAC CCC ACA
14	ImpactPGKEER1 (rev)	GGT GGT **TGC TCT TCC** GCA GAG CTT CTC TAG CAC AAA
15	ImpactkallaF1 (fwd)	GGT GGT **CAT ATG** AAG AAA GAG CTG TTA GAT TT
16	ImpactkallaR1 (rev)	GGT GGT **TGC TCT TCC** GCA TTT ATC AAT TTC TGC CAA

Table 2: Deoxyoligoribonucleotides

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CLAIMS

1. An isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, or fragments or derivatives thereof capable of inducing an immune response to said protein, or a fragment or derivative capable of binding to antibodies produced by a subject in an immune response against said protein.

2. Protein, fragment or derivate according to claim 1 for use in veterinary medicine.

3. Protein, fragment or derivate according to claim 1 for use in prevention of a disease caused by *Treponema* spp., such as digital dermatitis.

4. Method for treatment or prevention of a disease caused by *Treponema* spp. comprising administering an to a subject isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, or a fragment or derivative thereof capable of inducing an immune response to said protein.

5. Method according to claim 4, wherein the disease caused by *Treponema* spp. is digital dermatitis.

6. Pharmaceutical composition comprising an isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, or a fragment or derivative thereof capable of inducing an immune response to said protein, and optionally pharmaceutically acceptable adjuvants, carriers and/or diluents.

7. Method for detecting the presence of antibodies against proteins from *Treponema* spp. in a sample, comprising the steps:

- bringing said sample in contact with an isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, or a fragment or derivative thereof capable of binding to antibodies produced by a subject in an immune response against said protein; and
- detecting antibodies binding to said protein, fragment or derivative.

8. Method for *in vitro* diagnosis of a disease caused by *Treponema* spp. comprising the steps:

- obtaining a sample of body fluid or tissue from a subject;

- bringing said sample in contact with an isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6, or a fragment or derivative thereof capable of binding to antibodies produced by a subject in an immune response against said protein; and

- detecting antibodies binding to said protein, fragment or derivative;

wherein the presence of antibodies binding to said protein, fragment or derivative is indicative of a disease caused by *Treponema* spp.

9. Method according to claim 8, wherein said disease is digital dermatitis.

10. An antibody, or binding fragment thereof, binding specifically to an isolated protein having the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6.

11. Method for treatment or prevention of a disease caused by *Treponema* spp. comprising administering an to a subject an antibody according to claim 10.

12. Method according to claim 11, wherein the disease caused by *Treponema* spp. is digital dermatitis.

13. Method for separation of *Treponema* bacteria from a sample, comprising the steps:

-bringing said sample in contact with an antibody according to claim 10 bound to a solid phase;

- allowing said antibody to bind to *Treponema* proteins in said *Treponema* bacteria; and

- separating said solid phase from said sample

thereby separating said *Treponema* bacteria from said sample.

14. Method according to claim 13, wherein said separation is immunomagnetic separation.

15. A nucleic acid molecule encoding a protein, fragment or derivative according to claim 1.

16. A nucleic acid molecule according to claim 15 having the nucleotide sequence according to SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5

17. A vector comprising a nucleic acid molecule according to claim 15 or 16 and optionally regulatory sequences for expression in a host cell.

18. Transgenic host cell comprising a vector according to claim 17.

19. Method for producing a protein, fragment or derivative according to claim 1, comprising the steps:

- culturing a host cell according to claim 18 in a suitable medium; and
- isolating said protein, fragment or derivative from said medium.

Fig. 1A

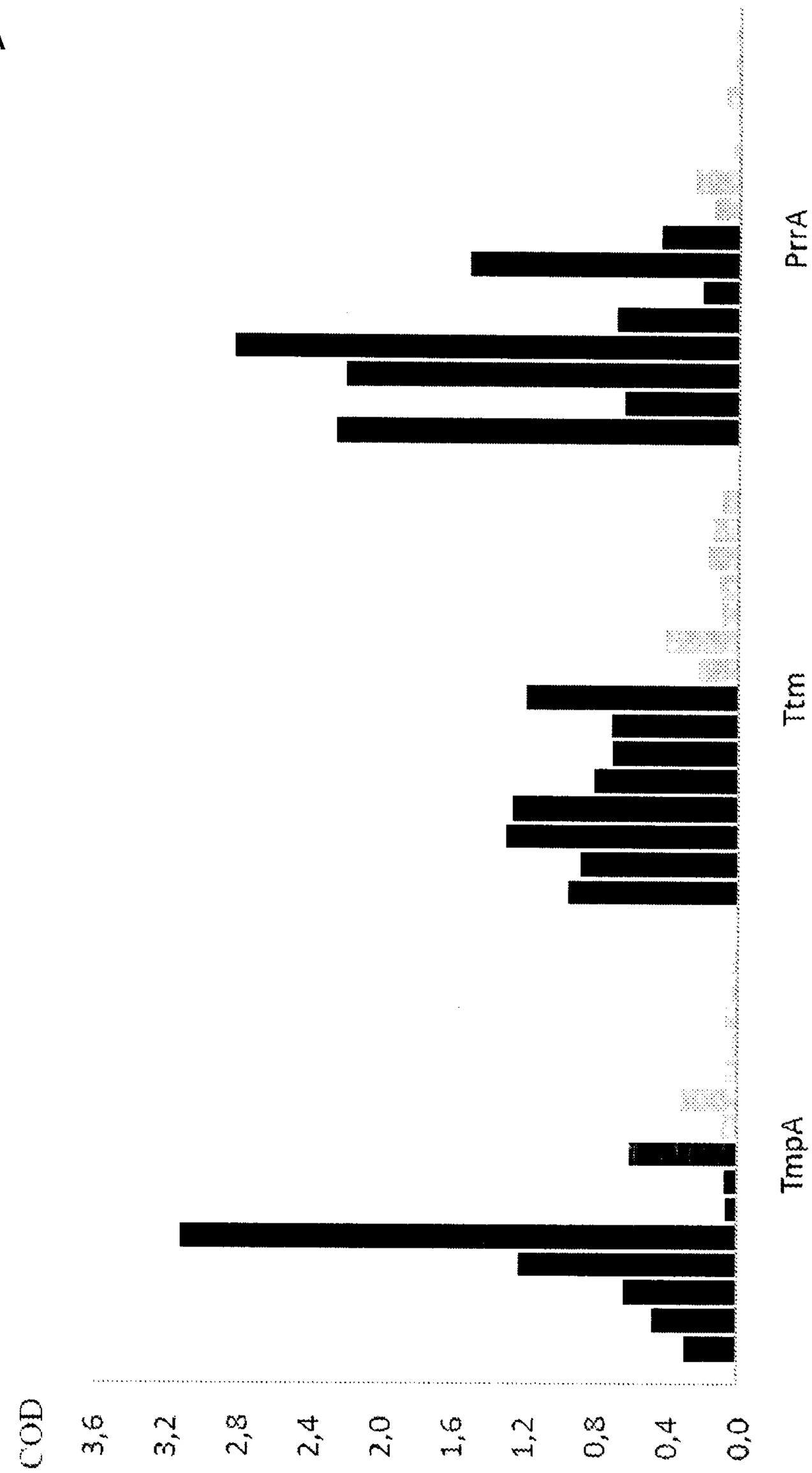

Fig. 1B