

Western Blot Analysis of the IgG-Antibody Response to Acid- Glycine-Extracted Antigens from *Campylobacter fetus* subsp. *fetus* and *C. jejuni* in Naturally Infected Sheep

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Abstract

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IgG-antibody response in aborting sheep and in apparently healthy sheep in a flock against acid-glycine-extracted antigens from three strains for each *C. fetus* subsp. *fetus* and *C. jejuni* were analysed by Western blot. One strain of *C. fetus* subsp. *fetus* was isolated from aborting sheep. Western blot analysis of the sera revealed the presence of IgG antibody binding to the common antigens including proteins with the Mw of 63 kDa and 54 kDa in extracts from both *C. fetus* subsp. *fetus* and *C. jejuni* strains. In addition, IgG antibodies in sera from aborting sheep reacted more strongly with the antigens from *C. fetus* subsp. *fetus* strains with Mw of approximately 100, 95 and 86.5 kDa than those of apparently healthy sheep. The binding profile of the antibodies with these antigens appeared to be unique for each *C. fetus* subsp. *fetus* strain. On the other hand, IgG antibodies only in sera from aborting sheep recognized strongly the antigens of each *C. fetus* subsp. *fetus* strain at the Mw ranged from approximately 26 to 22 kDa. However, the antigenic components between 26 and 22 kDa were not detectable in coomassie blue stained gel and thought to have non-protein nature. These low molecular weight antigens of *C. fetus* subsp. *fetus* may be related to a recent infection in aborting sheep. These observations indicate that such species-specific antigens or conjugated protein antigens could be used for improving the specificity of the serological tests to detect *C. fetus* antibodies in sheep sera, and may be the candidates for subunit vaccines against ovine abortion.

Ovine abortion, Western blot, *C. fetus* subsp. *fetus*, *C. jejuni*

Various campylobacter species are found in the reproductive organs, intestinal tracts and oral cavities of both animals and humans. *C. fetus* subsp. *fetus* is well known as a pathogen causing sporadic or epizootic abortions in sheep and cattle as well as systemic infections in humans. Considerable economical losses in animal production may ensue. *C. jejuni*, a human pathogen, is also recognized as a cause of abortions in sheep (Blöbel and Schliesser 1982). Sheep aborting due to campylobacter infection produce high titres of serum antibodies in the response to these organisms. As for detecting humoral antibody response, several serological tests, such as agglutination, complement fixation, enzyme immunoassay have been reported (Rautelin and Kosunen 1983; Gröhn and Genigeorgies 1985; Melby 1987; Gürtürk et al. 2002). But the use of these tests is limited due to low sensitivity and specificity for the diagnosis of campylobacter infections in sheep. Since effective use of serological tests is mainly related to the specificity of the antigenic or immunogenic components of bacteria used in the tests, the characterization and use of such antigenic components are necessary for enhancing the specificity of the serological tests.

In *C. fetus* infection, a surface layer (S layer) protein plays an important role in the invasion and survival within the host (McCoy et al. 1975; Blaser and Pei 1993). These proteins

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of *C. fetus* represent a family of high molecular weight proteins including proteins of 98 to 100, 127 and 149 kDa that have been demonstrated by SDS-PAGE and Western blot analysis with rabbit immune sera (Pei et al. 1988; Grogono-Thomas et al. 2000, 2003). Immunoblot studies of *C. fetus* and *C. jejuni* with rabbit and human immune sera showed also that *C. fetus* subsp. *fetus*, *C. jejuni* and *C. coli* share antigenically cross-reactive epitopes including flagellin with a molecular size of 50 kDa and 61 - 62 kDa and other major OM proteins (Wenmann et al. 1985). It has been reported that the 31 kDa acid dissociable protein is the antigenic determinant common to the thermophilic Campylobacters but the 92.5 kDa protein of thermophilic campylobacters might be strain-specific (Logan and Trust 1983; Jin and Penner 1988; Dubreuil et al. 1990a).

However, there is little information on the issue, whether these membrane antigens characterized with rabbit immune sera were also recognized with antibodies elicited after natural infection in sheep.

In consideration of the aspect that immune response of sheep against determined antigens after natural infection could be different than those of rabbits, the demonstration of antibodies to the campylobacter antigens with immune sera after natural infection in a compromised animal would give more appropriate information on the membrane antigens implicating in serological tests. This would make it possible not only to improve the specificity of serological tests to be used for the diagnosis of campylobacter infection in sheep, but also to develop strategies for immune protection. Our previous study (Gürtürk et al. 2002) showed also that campylobacter antibodies in sheep sera could be detected with a dot-immunobinding assay and a complement fixation tests by using crude extract of acid dissociable antigens from both *C. fetus* subsp. *fetus* and *C. jejuni* but the tests failed to discriminate antibodies to the antigens from both campylobacter species. In the present study, therefore, ovine-IgG antibody response to acid-glycine-extracted antigens from *C. fetus* subsp. *fetus* and *C. jejuni* strains was analysed to observe possible strain or species-specific antigens reacting with IgG antibodies elicited during ovine abortion.

Materials and Methods

Serum samples

Three sera from different sheep aborting due to *C. fetus* subsp. *fetus* infection in a flock were used. Sera were obtained 3 or 4 weeks after abortion. *C. fetus* subsp. *fetus* was isolated from one of the aborted fetus examined bacteriologically. Additionally three sera were collected from apparently healthy sheep in the same flock which were found to be negative in intestinal culture for Campylobacter. All sera from aborting sheep showed positive antibody titres of $1:20 \leq$ in complement fixation test (CFT). Sera from apparently healthy sheep had antibody titres of $1:5 \geq$ in CFT. In CFT, the adapted micro technique (Kolmer method) using cold fixation was employed as described previously (Gürtürk et al. 2002). All sera were found to be negative for antibodies to Brucellae with Rose bengal plate test and Dot-ELISA and the tests were performed as described previously (Gürtürk et al. 1997).

Bacterial strains

The following *Campylobacter* strains were used in this study: *C. fetus* subsp. *fetus* strain F5 was isolated from aborting sheep (of which homolog serum was also used); *C. fetus* subsp. *fetus* strain F3; *C. fetus* subsp. *fetus* strain F6; *C. jejuni* strain J1 and *C. jejuni* strain J3 were isolated from the contents of intestines or gall-bladders of apparently healthy sheep. *C. jejuni* subsp. *jejuni* DSM 4688 was supplied from DSM (Braunschweig, Germany).

The *C. fetus* subsp. *fetus* strains were resistant to nalidixic acid and grew at 25 °C. The *C. jejuni* strains were sensitive to nalidixic acid, hydrolysed Na-hippurate and grew at 43 °C but not 25 °C. Both strains could be cultured on the Skirrow's selective medium in a microaerobic atmosphere. They were catalase- and oxidase-positive, Gram negative bacteria with a typical S form. Both species were identified by further biochemical characteristics as described previously (Holt et al. 1984).

Extraction of antigen

The antigen was extracted separately from all campylobacter strains and used in Western blot. The bacteria were cultured on Blood agar base (Oxoid No. 2) supplemented with 7% defibrinated sheep blood and Skirrow's selective supplement for 48 - 72 h at 37 °C and 42 °C, respectively.

Acid glycine extraction was performed after the method described by McCoy et al. (1975). The cultures were harvested into distilled water, washed twice and then suspended in 0.2 M glycine-hydrochloride, pH 2.2 (1 g of cell per 25 ml). The suspension was stirred at room temperature (RT) for 30 min and whole cells were removed by

centrifugation at 10,000 *g* for 15 min. The supernatant was neutralized with NaOH and lyophilised. Protein contents of the extracts were determined by using a protein detection kit (Sigma, St. Louis, OM, USA).

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Glycine extracted proteins were analyzed by SDS-PAGE according to the method of Laemmli (1970) with a 4% stacking gel and a 10% running gel. Extracts were mixed with a reducing buffer containing 125 mM Tris, 4% SDS, 20% Glycerol and 10% mercaptoethanol (pH 6.8) and boiled for 90 sec. Approximately 30 - 50 μ g of protein from each glycine extracts was applied per lane and electrophoresis was conducted under a constant current (30 mA per gel) at RT with a Hoefer electrophoresis apparatus (Hoefer Scientific Instruments, USA) and 25 mM Tris-192 mM Glycine buffer (pH 8.3) containing 0.1% SDS. The molecular weight marker (Sigma, St. Louis, OM, USA) contained aprotinin (6.5 kDa), α -lactalbumin (14.2 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa) and albumin (66 kDa). Gels were stained with 0.025% coomassie brilliant blue R-250 dye in 40% methanol, fixed in 50% methanol-10% acetic acid and destained in several changes of 5% methanol-7% acetic acid.

After electrophoresis, proteins were immediately transferred from slab gel to nitrocellulose paper (BA85; Schleicher & Schuell, Germany) by the method of Towbin et al. (1979). Electrophoretic transfer was carried out overnight at 50 - 60 V with a Hoefer Transblot apparatus (Hoefer Scientific Instruments, USA) and 25 mM Tris-192 mM glycine buffer (pH 8.3) containing 20% methanol. For the immunological detection, the nitrocellulose paper was incubated with 10 mM Tris-0.9% NaCl buffer (TBS, pH 7.4) containing 5% skimmed milk powder for 2 h at RT to block non-specific binding. The nitrocellulose paper was then incubated with sera diluted 1 in 100 or more with TBS containing 0.05% Tween 20 (TBS-T) for 2 h at RT. The nitrocellulose paper was washed three times with TBS-T and incubated with horseradish peroxidase-conjugated donkey anti-sheep immunoglobulin G (whole molecule; Sigma, St. Louis, MO, USA) diluted 1 in 1000 with PBS-T for 2h at RT. After washing with PBS-T, the binding was revealed by the treatment of the nitrocellulose paper with 4-chloro-1-naphtol/hydrogen peroxide substrate in TBS.

Results

SDS-PAGE

Protein band profile of acid-glycine extracts from *C. fetus* subsp. *fetus* and *C. jejuni* strains in coomassie blue-stained polyacrylamide gel ranged in molecular weight from 22 kDa to greater than 100 kDa in this gel system (Fig. 1, Plate VIII). Protein bands with the Mw of approximately 63 kDa and 54 kDa were present in each extract from both *C. fetus* subsp. *fetus* and *C. jejuni* strains, whereas several protein bands including those with Mw of approximately 42.4 to 46.5, 36, 30, 26 and 22 kDa could only be observed in the extracts from *C. fetus* subsp. *fetus* strains. The protein bands of *C. fetus* subsp. *fetus* strains at the Mw of approximately 113.4, 100, 95, 86.5 kDa appeared to be unique for each strain, but they were not observed in *C. jejuni* strains.

Western Blotting

Immunoblotting analysis of the sera from aborting sheep and from apparently healthy sheep with the glycine extracted antigens from *C. fetus* subsp. *fetus* and *C. jejuni* strains are shown in Figs 2 and 3 (Plate VIII and IX). IgG antibodies in each serum reacted with the common protein antigens of approximately 63 kDa and 54 kDa from all strains of *C. fetus* subsp. *fetus* and *C. jejuni*. In addition, all sera showed similar binding profile with the antigens from *C. fetus* subsp. *fetus* isolated from aborting sheep which were still distinctly different from those of *C. jejuni* DSM 4688 (Fig. 2). However, the IgG antibodies from aborting sheep sera reacted strongly with the antigens from infecting strain of *C. fetus* subsp. *fetus* in the Mw region from approximately 26 to 22 kDa. On the other hand, any comparable binding profiles of IgG antibodies in sera from apparently healthy sheep with these antigens were not observed (Fig. 2, panel a). Even if the sera were used at a lower dilution (1:100), the same result was obtained (data not shown). Antibodies in sera from aborting sheep recognized also these low molecular weight antigens in each extract from other strains of *C. fetus* subsp. *fetus* as well as from homolog infecting strain of *C. fetus* subsp. *fetus*. Any comparable binding of antibodies to the antigens from *C. jejuni* strains between these molecular weights was not observed (Fig. 3, panel 1). However, antigenic components of *C. fetus* subsp. *fetus* strains between the molecular weights of 26 to 22 kDa were not observed in coomassie blue stained gel (Fig. 1, lanes a, b, c)

As represented in Fig. 3, IgG antibodies in serum from aborting sheep reacted also strongly by binding activity with the antigens from other *C. fetus* subsp. *fetus* strains as well as homolog infecting strain of *C. fetus* subsp. *fetus*, including the proteins with the molecular weights of approximately 100, 95 and 86.5 kDa, but the binding profile of the antibodies with these antigens appeared to be unique for each strain. Any comparable binding of the antibodies with the antigens from all *C. jejuni* strains at the range of these Mw could not be observed (Fig. 3, panel 1). Antibodies in serum from apparently healthy sheep reacted weakly with the antigens of *C. fetus* subsp. *fetus* strains, excepting the 63 kDa and 54 kDa (Fig. 3, panel 2) but no reaction was apparent in Western blots when higher dilutions of serum was used (Plate IX, Fig. 4, panel a, 1 - 2). However, the binding profile of the antibodies in serum from apparently healthy sheep with the antigens from *C. jejuni* strains did not differ from those of aborting sheep (Fig. 3, panel 1 - 2) and remained still undistinguishable, even if a higher dilution of the serum was used (Fig. 4, panel b, 1 - 2).

Discussion

Studies on immunogenicity of *C. jejuni* and *C. fetus* cellular components during human infection have been well reported. Sera from rabbits immunized with *C. jejuni* reacted with a number of components in outer-membrane protein preparations and differed from human sera (Nachamkin and Hart 1985). Recently, an investigation has been reported on the role of S layer protein (SLP) during *C. fetus* infection in sheep (Grogono-Thomas et al. 2000). Different isotypes of antibodies directed against SLPs during ovine infection were also demonstrated with enzyme-immunoassay (Grogono-Thomas et al. 2003). A number of specific outer membrane antigens of *C. fetus* and *C. jejuni* are well characterized by immunoblot with rabbit immune sera (Wenmann et al. 1985). However, data on immunoblot analysis of antibody response of naturally infected sheep against these SLPs or other antigenic components of *C. fetus* are not available.

C. fetus is known to be the most common agent of ovine abortion, but the specificity of serological tests for the diagnosis of *C. fetus* infection was limited due to cross-reactivity of the antibodies with the glycine extracted antigens from both *C. fetus* and *C. jejuni*. Glycine extracted antigens from *C. jejuni* were found to be a mixture of different proteins, including flagella antigens and acid dissociable surface antigens. The 62-63 kDa proteins confirmed as the flagellum were antigenically cross reactive with thermophilic campylobacters and *C. fetus* (Logan and Trust 1983; Mills et al. 1986). The 31 kDa acid dissociable protein appeared to be an antigenic determinant common to the thermophilic campylobacters (Dubreuil et al. 1990). A 92.5 kDa protein was shown to be a strain-specific antigen in *C. jejuni* (Jin and Penner 1988).

In the present study, Western blot analysis of the sera from both aborting and apparently healthy sheep revealed similar binding patterns with the antigens from both *C. fetus* subsp. *fetus* (isolated from the aborting sheep) and *C. jejuni*, including approximately 63 and 54 kDa proteins which were one of the major proteins in coomassie blue stained gel. The 63 kDa antigens, that were thought to be components of flagellin, appeared to be major proteins involved in the cross reactivity of the sera with antigens from both *C. fetus* and *C. jejuni* strains. The 54 kDa antigen might be other acid dissociable surface protein or a breakdown product of 63 kDa protein. Similar to our results, Wenmann et al. (1985) reported that *C. fetus* shares only two antigens strongly with *C. jejuni* and *C. coli*, proteins with molecular weights of 62 and 50 kDa reacting with rabbit immune sera to *C. jejuni* in Western blots. Our Western blot studies showed also that the binding profile of antibodies in sheep sera with the glycine extracted antigens from *C. fetus* subsp. *fetus*, except for 63 and 54 kDa antigens, were found to be different from those of *C. jejuni*. *C. fetus* subsp. *fetus* and *C. jejuni* differed also by their protein band profile in coomassie blue stained gel. Therefore,

antibodies in sheep sera did not appear to cross-react with other antigens of both *Campylobacter* species, excepting 63 kDa-54 kDa proteins.

Our Western blot studies demonstrated also antibodies in sheep sera against the approximately 100, 95 and 86.5 kDa proteins of *C. fetus* strains, but their binding profile with the antigen from each *C. fetus* subsp. *fetus* strain were found to be unique in both coomassie blue stained gel and in Western blots. These antigens of *C. fetus* could not be detected in extracts from *C. jejuni* and thought to be S layer proteins (SLP). SLP of *C. fetus* serve as important virulence factors in pathogenesis of *C. fetus* infections (McCoy et al. 1975; Blaser and Pei 1993). These antigens of *C. fetus*, including the high molecular weight proteins of 98 to 100, 127 and 149 kDa are present in glycine extracts from *C. fetus* strains and the diversity of size and structure of the SLP of *C. fetus* have been well reported (Pei et al. 1988; Fujimoto et al. 1991; Brooks et al. 2002). Grogono-Thomas et al. (2000) reported also that most *C. fetus* subsp. *fetus* isolates from natural ovine infections express the 97 kDa protein but the 127 kDa and 149 kDa surface layer proteins are rarely seen.

In this study, antibodies in aborting sheep reacted strongly with the high molecular weight antigens of *C. fetus*, even if higher dilutions of serum were used. Although antibodies against *C. fetus* antigens with high molecular weights were also present in sera from apparently healthy sheep, they were not detectable in higher dilutions of sera. These results indicate that the antibodies in sera from apparently healthy sheep could be acquired during a past *C. fetus* infection and sheep aborting recently due to *C. fetus* infection could have developed a substantial systemic antibody response directed against antigens with high molecular weight.

Dubreuil et al. (1990b) reported that the acid dissociable protein with low molecular weight, e.g. 31 kDa protein, appeared to be an antigenic determinant that is common to the thermophilic campylobacter strains, but not in *C. fetus* subsp. *fetus*. In our study, Western blots of the sera from aborting sheep, but not from apparently healthy sheep, demonstrated a distinctly different reaction with the antigenic components of *C. fetus* subsp. *fetus* strains between the molecular weights of approximately 26 to 22 kDa. However, except for the proteins with the molecular weight of approximately 26 to 22 kDa, other antigenic components reacting in this region of Western blots could not be observed in coomassie blue stained gel and appeared to be of a non-protein nature. These antigens of *C. fetus* appeared not to be present in *C. jejuni* strains and may be species-specific. The role of these antigenic components of *C. fetus* during ovine abortion are not known but may be related to a recent infection in aborting sheep. Further studies are necessary for the purification and characterization of such antigenic components of *C. fetus* subsp. *fetus*.

In conclusion, the results of this study showed that the acid glycine extractable proteins of both *C. fetus* subsp. *fetus* and *C. jejuni* with the Mw of approximately 63 kDa and 54 kDa were dominant antigens involved in cross-reaction with the IgG antibodies in sheep sera. IgG antibody response in aborting sheep were mainly directed against the high molecular weight antigens of *C. fetus* subsp. *fetus* of which binding profile were unique for each strain and were stronger than those in apparently healthy sheep. We have also demonstrated antibodies only in aborting sheep to a group of acid-glycine extractable antigens of *C. fetus* subsp. *fetus* of Mw ranging from approximately 26 to 22 kDa which may be related to a recent infection in aborting sheep. Such species-specific antigens could be used for improving the specificity of serological tests to detect anti-*C. fetus* antibodies in sheep, and may be candidates for subunit vaccines against ovine abortion due to *C. fetus* infection.

Analyza IgG protilátkové odpovědi na antigeny *Campylobacter fetus* subsp. *fetus* a *C. jejuni* extrahované acid-glycinem pomocí metody Western blot u přirozeně infikovaných ovcí

Pomocí Western blotu byla analyzována IgG protilátková odpověď u abortujících a zdra-

vých ovcí ve stádě na acid-glycinem extrahované antigeny ze tří kmenů pro každý *C. fetus* subsp. *fetus* a *C. jejuni*. Jeden kmen *C. fetus* subsp. *fetus* byl izolován z abortující ovce. Western blot séra odhalil přítomnost IgG protilátek vázajících se na běžné antigeny včetně proteinů s Mr 63 kDa a 54 kDa v pasážích z kmenů *C. fetus* subsp. *fetus* a *C. jejuni*. Navíc protilátky IgG ze séra abortující ovce, oproti těm ze zdravých ovcí, reagovaly výrazně více s antigeny kmenů *C. fetus* subsp. *fetus* o Mr přibližně 100, 95 a 86,5 kDa. Profil vázajících se protilátek s těmito antigeny se zdá být unikátní pro každý kmen *C. fetus* subsp. *fetus*. Na druhou stranu jen protilátky ze séra abortující ovce dobře rozpoznaly antigeny každého kmene *C. fetus* subsp. *fetus* o Mr od asi 26 do 22 kDa. Antigenní komponenty s Mr 26 až 22 kDa nebylo možné detekovat v gelu barveném koloidní coomassie blue. Má se za to, že jsou neproteinové povahy. Tyto antigeny *C. fetus* subsp. *fetus* s nízkou molekulovou hmotností mohou souviset s nedávnou infekcí abortující ovce. Tato zjištění naznačují, že takto dříve specifické antigeny nebo antigeny konjugovaných proteinů mohou být využívány ke zlepšení specificity sérologických testů při detekci protilátek *C. fetus* v séru ovcí, a mohly by být i kandidáty pro subjednotkové vakcíny proti abortům ovcí.

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Fig. 1. SDS-PAGE (10% polyacrylamide) of the glycine extracted components from different *C. fetus* subsp. *fetus* (lanes a, b, c) and *C. jejuni* (lanes d, e, f) strains. Lane b; infected with strain of *C. fetus* subsp. *fetus* F5; Lane d; *C. jejuni* subsp. *jejuni* DSM 4688. Polyacrylamide gels were stained with Coomassie brilliant blue. Lane M; Molecular weight marker and Molecular weights (MW) are indicated in kDa.

Fig. 2. (A); SDS-PAGE of the glycine extracted proteins of *C. fetus* subsp. *fetus* strain F5 (a) and *C. jejuni* subsp. *jejuni* DSM 4688 (b); Lane M; Molecular weight marker (B); Western blot analysis of sera from aborting sheep (1, 2, 3) and from apparently healthy sheep (4, 5, 6) with the glycine extracted antigens of *C. fetus* subsp. *fetus* strain F5 (panel a) and *C. jejuni* subsp. *jejuni* DSM 4688 (panel b). Homolog serum taken from the aborting sheep from which *C. fetus* subsp. *fetus* F5 was isolated, were shown in lane 1. Sera were diluted 1 in 1000. Molecular weights (MW) are indicated in kDa.

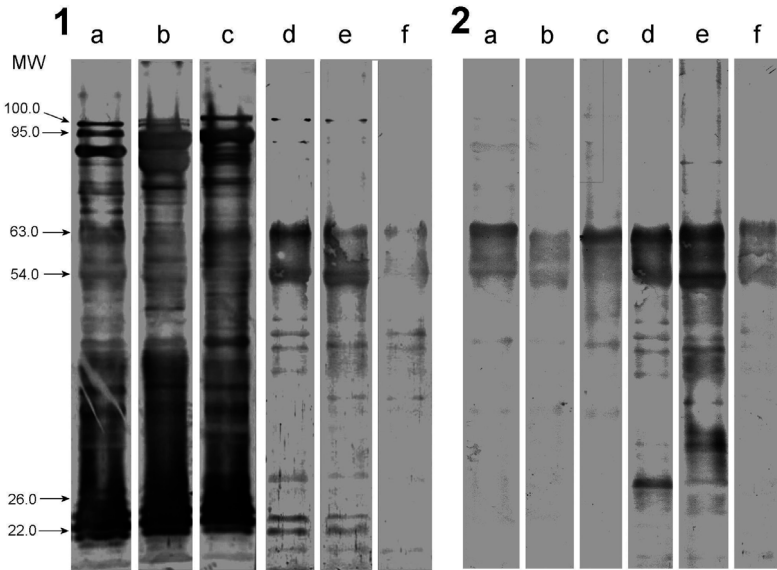


Fig. 3. Western blot analysis of serum from aborting sheep (panel 1) and from apparently healthy sheep (panel 2) with the glycine extracted antigens from different campylobacter strains. Lanes a, b, c; *C. fetus* subsp. *fetus* strain F3, F5 (homolog infecting strain), and F6, respectively; Lanes d, e, f: *C. jejuni* subsp. *jejuni* DSM 4688, *C. jejuni* strain J1 and J3 respectively. Molecular weights (MW) are indicated in kDa.

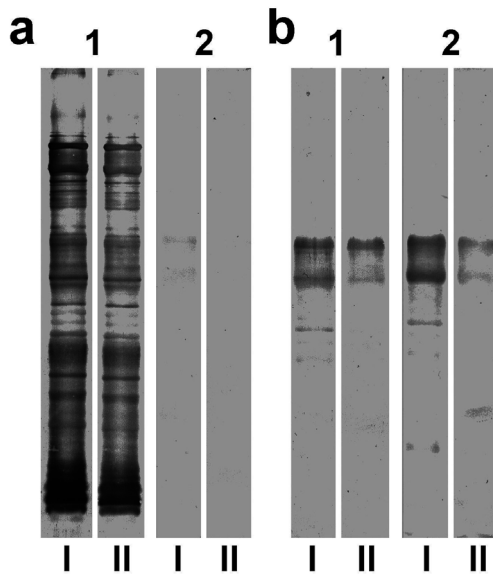


Fig. 4. Western blot analysis of glycine extracted antigens from homolog infecting strain of *C. fetus* subsp. *fetus* strain F5 (panel a) and *C. jejuni* subsp. *jejuni* DSM 4688 (panel b) with the serum from aborting sheep (1) and from apparently healthy sheep (2) diluted 1:2000 (I) and 1:4000 (II).