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Recombinant 35-kDa inclusion membrane protein IncA as a candidate antigen for serodiagnosis of *Chlamydophila pecorum*

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Abstract

Chlamydophila pecorum strains are commonly found in the intestine and vaginal mucus of asymptomatic ruminants and may therefore induce a positive serological response when the animals are tested for *C. abortus*. They have also been associated with different pathological diseases in ruminants, swine and koala. The aim of this study was to identify specific *C. pecorum* immunodominant antigens which could be used in ELISA tests allowing to distinguish between animals infected with *C. pecorum* and those infected with other chlamydial species. A gene encoding 35-kDa inclusion membrane protein *incA* of *C. pecorum* was isolated by immunoscreening of the *C. pecorum* DNA library using ovine anti-*C. pecorum* antibodies. The recombinant IncA protein did not react with a murine serum directed against *C. abortus* but did react with a specific monoclonal antibody of *C. pecorum* and toward several ovine serum samples obtained after experimental infection with different *C. pecorum* strains. This protein could be a good candidate for specific diagnosis of *C. pecorum* infection.

Keywords: *Chlamydophila pecorum*, serodiagnosis, IncA

Introduction

Chlamydophila pecorum strains have been isolated from small and large ruminants, koalas and swine. It is commonly found in the intestine and vaginal mucus of ruminants without any clinical sign. However, some *C. pecorum* strains cause diseases in ruminants and swine, including pneumonia, polyarthritis, conjunctivitis, enteritis, encephalomyelitis and abortion (Kaltenboeck and Storz, 1992; Rodolakis et al., 1998). In koalas, *C. pecorum* is known to cause reproductive disease, infertility and urinary tract disease (Jackson et al., 1999). *C. pecorum* strains can also cause infections in wild animals, for example, conjunctivitis in the western barred bandicoot and abortion in water buffalo (Greco et al., 2008; Warren et al., 2005).

C. pecorum could be responsible for the positive serological response sometimes seen in flocks, tested for *C. abortus*. Serodiagnostic tests, such as the complement fixation test (CFT) and enzyme-linked immunosorbent assays (ELISA), are the simplest methods for detecting chlamydial infections. The CFT was generally used until the development of ELISA tests because of the false positive response obtained with anti-complementary serum samples in the CFT. Thus, neither the CFT nor ELISA tests using whole bacteria or protein extracts are able to differentiate between antibodies directed against *C. abortus* and *C. pecorum* (Brade et al., 1987; Markey et al., 1993). Several experimental (Hoelzle et al., 2004; Kaltenboeck et al., 1997; Longbottom et al., 2001) or commercial ELISAs developed by INRA-Nouzilly (Buendia et al., 2001; Vretou et al., 2007) have been proposed to detect species-specific anti-*C. abortus* antibodies in ovine and bovine sera.

The present study focuses on the search for a specific antigen to identify animals infected with *C. pecorum*. This would allow to estimate the prevalence of *C. pecorum* in epidemiological studies and to improve the diagnosis of *C. abortus* by removing the serological cross-reactivity between *C. pecorum* and *C. abortus*.

Materials and Methods

Chlamydial strains

Five chlamydial strains were used: 4 strains of *C. pecorum* and 1 strain of *C. abortus* (Table 1). All strains were propagated in the yolk sac of chicken embryos and stored at -70 °C as previously described (Rodolakis, 1976).

Serum samples

Serum samples were obtained after experimental infection of sheep and mice with various *C. pecorum* isolates. Maintenance and care of experimental animals were in accordance with National Decree, N° 2001-464, May 2001, concerning animal testing in France. Samples were collected from 10 ewes: 3 inoculated with *C. pecorum* M14, 3 with *C. pecorum* AB10, 2 with *C. pecorum* LW679, and 2 with *C. pecorum* iB5. Approximately 10^6 plaque-forming units (PFU)/Sheep of each strain were inoculated subcutaneously 4 times at 3-week intervals. Two serum samples obtained from mice inoculated with *C. pecorum* M14 or *C. pecorum* AB10 were previously used in our laboratory (Rekiki et al., 2004). One serum was pooled from 10 mice infected experimentally with *C. abortus* AB7. Approximately 10^4 PFU/mouse were inoculated subcutaneously 3 times at 2-week intervals.

The specific monoclonal anti-*C. pecorum* antibody PD3 was produced at INRA- Nouzilly as previously described (Salinas et al., 1996).

In addition, 8 bovine serum samples were collected from a herd with no clinical signs.

All these serum samples were examined using microimmunofluorescence (MIF) as previously described (Salinas et al., 1996).

Construction and immunoscreening of *C. pecorum* genomic DNA expression library

Genomic DNA was prepared from purified *C. pecorum* M14 strain as previously described (Boumedine and Rodolakis, 1998). The genomic library was constructed using Lambda ZAP

Express vector following the manufacturer's protocol (Stratagene, Lyon, France). The recombinant phages were selected using the ovine anti-*C. pecorum* M14 serum. Several phage clones were plaque-purified and converted to the recombinant pBK-CMV plasmid in *E. coli* XLOR following the manufacturer's instructions (Stratagene).

Immunoblotting

Total proteins expressed by cloned *C. pecorum* M14 DNA in *E. coli* XLOLR were analyzed by immunoblotting. *E. coli* XLOLR containing the recombinant plasmid was cultured in Luria-Bertani Media (LB) at 37°C overnight. The cells were pelleted by centrifugation (5000g/ 10 min), resuspended in Laemmli sample buffer containing 5% of 2-mercaptoethanol (Biorad, Marnes-la-Coquette, France), and boiled at 95°C for 10 min. The proteins were separated by SDS-PAGE electrophoresis (Laemmli, 1970) and transferred onto nitrocellulose membranes in Tris-glycine buffer (containing 0.1 M Tris base, 0.192 M glycine, and 10% methanol) at 65 mA for 60 min (Towbin et al., 1979). Immunoreactive proteins of *C. pecorum* were detected by Western blot analysis using an ovine serum sample as the first antibody, then reacted with anti-sheep alkaline phosphatase conjugate (Sigma-Aldrich, Lyon, France) as the secondary antibody, and visualized by BCIP/NBT color substrate (Promega, Charbonnières-Les-Bains, France).

DNA sequence analysis

Plasmid DNAs were purified by a QIAprep Miniprep kit (Qiagen, Courtaboeuf, France) from cultures of *E. coli* XLOLR clones containing different *C. pecorum* M14 DNA inserts. The cloned DNAs identified by Western blotting were sequenced at Genome Express (Cogenic, Meylan, France) using T7 forward and reverse primers. The cloned genes were identified using BLASTn and BLASTx in NCBI against the complete genome sequence of *Chlamydia*.

Cloning of *incA* gene

The DNA of the ovine *C. pecorum* AB10 strain was extracted using the Dneasy kit following the manufacturer's instructions (Qiagen). The *incA* gene was amplified by polymerase chain reaction (PCR) with two primers, containing *BamH*I and *Kpn*I restriction sites respectively (restriction sites are underlined), as follows: In-E58-F (5′-CTCGGATCCACAGTGAATCCCCTACGAA -3′) and In-E58-R (5′-CTCGGTACCTTTTGAAGCGCTGTTTCAT -3′). PCR was performed using the GoTaq Flexi DNA Polymerase protocol (Promega) in an automated DNA thermal cycler (Biometra, Goettingen, Germany). After an initial denaturation period of 5 min at 94 °C, reactions were subjected to 30 cycles of 30 sec at 94 °C, 45 sec at an annealing temperature of 63 °C, then 72 °C for 1.5 min with a final extension step at 72 °C for 10 min. The PCR product was purified using the QIAquick PCR kit (Qiagen) and ligated into the pQE30-HisTag expression vector (Qiagen). The recombinant construct (pQE30/*incA*) was used to transform *E. coli* TG1 cells. The recombinant IncA protein was purified under native conditions using a Nickel affinity chromatography column following the manufacturer's instructions (Qiagen).

Results

Immunoscreening of the *C. pecorum* genomic DNA library identified 16 immunoreactive clones with varying signal intensities (Table 2). The molecular masse of four (B3, A10, B15 and B11) of the sixteen clones were identified by Western blotting (Figure 1). Three clones (B3, A10 and B15) with a molecular mass of 32-33 kDa reacted with the same MAb PD3 (Fig. 2). Two immunoreactive proteins showing a molecular mass of 18 and 30 kDa were identified in the fourth clone (B11) (Figure 1).

Sequence analysis and BLAST search of the complete chlamydial genome sequence showed that the 18, 30 and 32-33 kDa proteins corresponded to the rRNA methylase family spoU

protein (459 bp), peptidyl-prolyl cis-trans isomerase Mip precursor (744 bp), and inclusion membrane protein IncA (981 bp), respectively.

The full-length *incA* gene of *C. pecorum* was cloned in pQE-30 vector and a recombinant 35 kDa protein was expressed. Immunoblotting analysis showed that the purified rIncA reacted with the MAb PD3 (Figure 3) and with all murine and ovine serum samples obtained after experimental infection with different *C. pecorum* strains. However, no reaction was seen with the murine serum directed against *C. abortus*. Moreover, the ability of the recombinant IncA to detect anti-*C. pecorum* antibodies in healthy ruminant herds was tested using 8 bovine serum samples: 6 of these samples were found positive (Figure 4).

Discussion

To diagnose *C. pecorum*, several DNA-based techniques have been proposed using conventional PCR (Everett et al., 1999; Kaltenboeck et al., 1992) or real-time PCR with primers derived from 23S rRNA or *ompA* genes (DeGraves et al., 2003; Yang et al., 2006). A multiplex PCR has recently been developed to detect *C. abortus*, *C. pecorum* and *Coxiella burnetii* (Berri et al., 2009). However, at present, no serodiagnostic system can specifically detect *C. pecorum* infection.

In this study, we proposed *C. pecorum* inclusion membrane protein *incA* as a diagnostic antigen. Our results showed that the incA was obtained by immunoscreening the *C. pecorum* genomic library with the ovine anti-*C. pecorum* serum sample. Three out of sixteen clones had the *incA* gene, and a strong band reacting with ovine serum directed against different *C. pecorum* strains was detected, suggesting that this protein could be a common and immunodominant antigen for *C. pecorum*. The recombinant IncA protein reacted with the specific anti-*C. pecorum* MAb PD3, but not with the murine serum directed against *C. abortus*, suggesting that IncA could be a specific *C. pecorum* antigen. Although the

biochemical properties of IncA proteins, including their multimeric structure, were conserved in all the chlamydial species, the *incA* sequences showed little similarity and antibodies against IncA in the different chlamydial species did not cross-react (Delevoye et al., 2004). This gene has therefore been proposed to detect *C. psittaci* and *C. trachomatis* using quantitative PCR and immunodiagnostic tests respectively (Menard et al., 2006; Tsai et al., 2007). Furthermore, a recent *in silico* study proposed IncA as a major antigen for *C. pneumoniae* diagnosis (Park et al., 2009).

A coding tandem repeats (CTR) variant was identified along the *incA* gene sequence of *C. pecorum* allowing 19 *C. pecorum* strains isolated from ruminants to be divided into 3 groups based on the different CTR motifs (Yousef Mohamad et al., 2008a). Seven out of eight pathogenic strains of different clinical and geographical origins were found in one group in which the CTR of *incA* contained only alanine-proline (APA) motif. In addition, a multi-virulence locus sequence typing (MVLST) technique was used to divide *C. pecorum* strains into 4 groups, one containing only 6 out of 8 pathogenic strains (Yousef Mohamad et al., 2008b). That study suggested that *ompA, incA* and ORF663 genes could be used as molecular markers in epidemiological studies of *C. pecorum*.

Although the CTR in the *incA* sequence varied among *C. pecorum* strains, they did not hamper the reaction of recombinant IncA protein with serum samples from sheep immunized with *C. pecorum*, including pathogenic or intestinal strains. This suggests that the IncA protein has common epitopes recognized by different *C. pecorum* strains. It would be interesting to examine the antigenicity of these motifs and to produce synthetic peptide antigens based on them which could be useful for distinguishing between animals infected by pathogenic and non-pathogenic *C. pecorum* strains.

The rIncA also reacted with 6 of the 8 serum samples collected from healthy bovine herds. This suggests that *C. pecorum* persists in the intestine and vaginal mucus of healthy ruminants

as previously demonstrated (Longbottom, 2004). More than 51% of female calves acquired natural infection with *C. pecorum* in the first 2 months after birth (Kaltenboeck et al., 2005). In addition, an investigative study showed that *C. pecorum* was more widespread in cattle than *C. abortus*, and the bacteria were frequently detected in vaginal swabs and fecal samples (Jee et al., 2004). It is therefore important to develop an approach able to detect clinically unapparent intestinal infection caused by *C. pecorum* which has been reported to be prevalent in both abortion-affected and -unaffected ruminant flocks (Reinhold et al., 2008).

In conclusion, this is the first report about the utilization of a recombinant IncA as a potential specific antigen for *C. pecorum* diagnosis. Further analysis is needed to evaluate the use of this antigen in ELISAs.

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296 **Tables:**

297 **Table 1**: Chlamydial strains used in this study

Strain	Geographic origin	Host	Pathology	Reference
C. pecorum M14	Morocco	Goat	Abortion	(Rekiki et al., 2004)
C. pecorum AB10	France	Sheep	Abortion	(Rodolakis et al., 1989)
C. pecorum LW679	USA	Sheep	Arthritis	(Norton and Storz, 1967)
C. pecorum iB5	France	Sheep	No clinical sign (Fecal carriage)	(Rodolakis et al., 1989)
C. abortus AB7	France	Sheep	Abortion	(Faye et al., 1972)

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Table 2: Signal intensity of the antigenic reaction of the 16 recombinants with the ovine anti-*C. pecorum* M14 serum sample.

Signal intensity	Clone
Strong (+++)	B1, B3, A10
Medium (++)	B2, A7, B14, B15, B17, A4
Weak (+)	A2, A1, B5, B7, B18, B19, B11

Figure captions:

Fig. 1: Western Blot analysis of 5 clones using ovine anti-*C. pecorum* serum sample. Lane 1, clone B3 (33 kDa); lane 2, negative control; lane 3, clone A10 (33 kDa); lane 4, clone B15 (32 kDa); lane 5, clone B11 (30 and 18 kDa).

Fig. 2: Western Blot analysis of 6 clones using MAb PD3. Lane 1, clone B3; lane 2, clone B7; lane 3, clone B15; lane 4, clone B17; lane 5, clone B2; lane 6, clone A10. Three clones, B3, B15 and A10, reacted with the Mab PD3.

Fig. 3: Western Blot analysis of rIncA using MAb PD3. Lane 1, *E. coli* TG1 sample not induced by IPTG; lane 2, *E. coli* TG1 sample induced by IPTG (35 kDa); lane 3, clone B15 (33 kDa).

Fig. 4: Western Blot analysis of rIncA (35 kDa). Lane 1, ovine anti-*C. pecorum* M14 serum sample (positive control); lane 2, 3, 4, 5 and 6, bovine serum samples collected from a healthy herd.

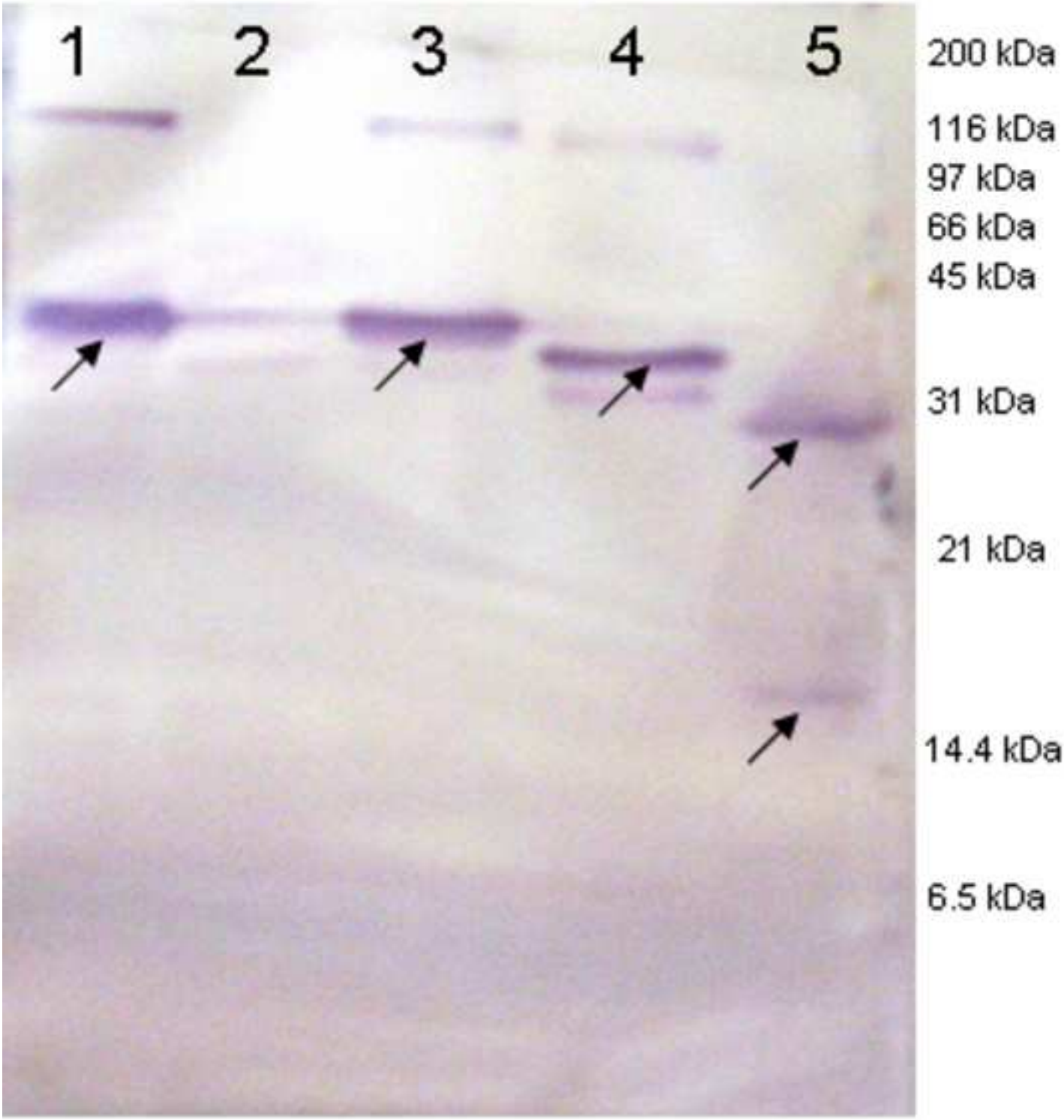

Figure 2