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

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## 1 Abstract

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

15  
16 **Keywords:** *Chlamydophila pecorum*, serodiagnosis, IncA

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## 26 **Introduction**

27 *Chlamydomphila pecorum* strains have been isolated from small and large ruminants, koalas  
28 and swine. It is commonly found in the intestine and vaginal mucus of ruminants without any  
29 clinical sign. However, some *C. pecorum* strains cause diseases in ruminants and swine,  
30 including pneumonia, polyarthritits, conjunctivitis, enteritis, encephalomyelitis and abortion  
31 (Kaltenboeck and Storz, 1992; Rodolakis et al., 1998). In koalas, *C. pecorum* is known to  
32 cause reproductive disease, infertility and urinary tract disease (Jackson et al., 1999). *C.*  
33 *pecorum* strains can also cause infections in wild animals, for example, conjunctivitis in the  
34 western barred bandicoot and abortion in water buffalo (Greco et al., 2008; Warren et al.,  
35 2005).

36 *C. pecorum* could be responsible for the positive serological response sometimes seen in  
37 flocks, tested for *C. abortus*. Serodiagnostic tests, such as the complement fixation test (CFT)  
38 and enzyme-linked immunosorbent assays (ELISA), are the simplest methods for detecting  
39 chlamydial infections. The CFT was generally used until the development of ELISA tests  
40 because of the false positive response obtained with anti-complementary serum samples in the  
41 CFT. Thus, neither the CFT nor ELISA tests using whole bacteria or protein extracts are able  
42 to differentiate between antibodies directed against *C. abortus* and *C. pecorum* (Brade et al.,  
43 1987; Markey et al., 1993). Several experimental (Hoelzle et al., 2004; Kaltenboeck et al.,  
44 1997; Longbottom et al., 2001) or commercial ELISAs developed by INRA-Nouzilly  
45 (Buendia et al., 2001; Vretou et al., 2007) have been proposed to detect species-specific anti-  
46 *C. abortus* antibodies in ovine and bovine sera.

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

51

## 52 **Materials and Methods**

### 53 **Chlamydial strains**

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

### 57 **Serum samples**

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

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

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

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

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

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

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

#### 80 **Immunoblotting**

81 Total proteins expressed by cloned *C. pecorum* M14 DNA in *E. coli* XLOR were analyzed  
82 by immunoblotting. *E. coli* XLOR containing the recombinant plasmid was cultured in  
83 Luria-Bertani Media (LB) at 37°C overnight. The cells were pelleted by centrifugation  
84 (5000g/ 10 min), resuspended in Laemmli sample buffer containing 5% of 2-mercaptoethanol  
85 (Biorad, Marnes-la-Coquette, France), and boiled at 95°C for 10 min. The proteins were  
86 separated by SDS-PAGE electrophoresis (Laemmli, 1970) and transferred onto nitrocellulose  
87 membranes in Tris-glycine buffer (containing 0.1 M Tris base, 0.192 M glycine, and 10%  
88 methanol) at 65 mA for 60 min (Towbin et al., 1979). Immunoreactive proteins of *C. pecorum*  
89 were detected by Western blot analysis using an ovine serum sample as the first antibody,  
90 then reacted with anti-sheep alkaline phosphatase conjugate (Sigma-Aldrich, Lyon, France) as  
91 the secondary antibody, and visualized by BCIP/NBT color substrate (Promega,  
92 Charbonnières-Les-Bains, France).

#### 93 **DNA sequence analysis**

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

#### 99 **Cloning of *inca* gene**

100 The DNA of the ovine *C. pecorum* AB10 strain was extracted using the Dneasy kit following  
101 the manufacturer's instructions (Qiagen). The *incA* gene was amplified by polymerase chain  
102 reaction (PCR) with two primers, containing *Bam*HI and *Kpn*I restriction sites respectively  
103 (restriction sites are underlined), as follows: In-E58-F (5'-  
104 CTCGGATCCACAGTGAATCCCCTACGAA -3') and In-E58-R (5'-  
105 CTCGGTACCTTTTGAAGCGCTGTTTCAT -3'). PCR was performed using the GoTaq  
106 Flexi DNA Polymerase protocol (Promega) in an automated DNA thermal cycler (Biometra,  
107 Goettingen, Germany). After an initial denaturation period of 5 min at 94 °C, reactions were  
108 subjected to 30 cycles of 30 sec at 94 °C, 45 sec at an annealing temperature of 63 °C, then 72  
109 °C for 1.5 min with a final extension step at 72 °C for 10 min. The PCR product was purified  
110 using the QIAquick PCR kit (Qiagen) and ligated into the pQE30-HisTag expression vector  
111 (Qiagen). The recombinant construct (pQE30/*incA*) was used to transform *E. coli* TG1 cells.  
112 The recombinant IncA protein was purified under native conditions using a Nickel affinity  
113 chromatography column following the manufacturer's instructions (Qiagen).

114

## 115 **Results**

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

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

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

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

133

## 134 **Discussion**

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

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

149 biochemical properties of IncA proteins, including their multimeric structure, were conserved  
150 in all the chlamydial species, the *incA* sequences showed little similarity and antibodies  
151 against IncA in the different chlamydial species did not cross-react (Delevoeye et al., 2004).  
152 This gene has therefore been proposed to detect *C. psittaci* and *C. trachomatis* using  
153 quantitative PCR and immunodiagnostic tests respectively (Menard et al., 2006; Tsai et al.,  
154 2007). Furthermore, a recent *in silico* study proposed IncA as a major antigen for *C.*  
155 *pneumoniae* diagnosis (Park et al., 2009).

156 A coding tandem repeats (CTR) variant was identified along the *incA* gene sequence of *C.*  
157 *pecorum* allowing 19 *C. pecorum* strains isolated from ruminants to be divided into 3 groups  
158 based on the different CTR motifs (Yousef Mohamad et al., 2008a). Seven out of eight  
159 pathogenic strains of different clinical and geographical origins were found in one group in  
160 which the CTR of *incA* contained only alanine-proline (APA) motif. In addition, a multi-  
161 virulence locus sequence typing (MVLST) technique was used to divide *C. pecorum* strains  
162 into 4 groups, one containing only 6 out of 8 pathogenic strains (Yousef Mohamad et al.,  
163 2008b). That study suggested that *ompA*, *incA* and ORF663 genes could be used as molecular  
164 markers in epidemiological studies of *C. pecorum*.

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

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

174 as previously demonstrated (Longbottom, 2004). More than 51% of female calves acquired  
175 natural infection with *C. pecorum* in the first 2 months after birth (Kaltenboeck et al., 2005).  
176 In addition, an investigative study showed that *C. pecorum* was more widespread in cattle  
177 than *C. abortus*, and the bacteria were frequently detected in vaginal swabs and fecal samples  
178 (Jee et al., 2004). It is therefore important to develop an approach able to detect clinically  
179 unapparent intestinal infection caused by *C. pecorum* which has been reported to be prevalent  
180 in both abortion-affected and -unaffected ruminant flocks (Reinhold et al., 2008).  
181 In conclusion, this is the first report about the utilization of a recombinant InCA as a potential  
182 specific antigen for *C. pecorum* diagnosis. Further analysis is needed to evaluate the use of  
183 this antigen in ELISAs.

184

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292 71.
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296 **Tables:**297 **Table 1:** Chlamydial strains used in this study

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

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

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

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329 **Figure captions:**

330 **Fig. 1:** Western Blot analysis of 5 clones using ovine anti-*C. pecorum* serum sample. Lane 1,  
331 clone B3 (33 kDa); lane 2, negative control; lane 3, clone A10 (33 kDa); lane 4, clone B15  
332 (32 kDa); lane 5, clone B11 (30 and 18 kDa).

333

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

337

338 **Fig. 3:** Western Blot analysis of rIncA using MAb PD3. Lane 1, *E. coli* TG1 sample not  
339 induced by IPTG; lane 2, *E. coli* TG1 sample induced by IPTG (35 kDa); lane 3, clone B15  
340 (33 kDa).

341

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

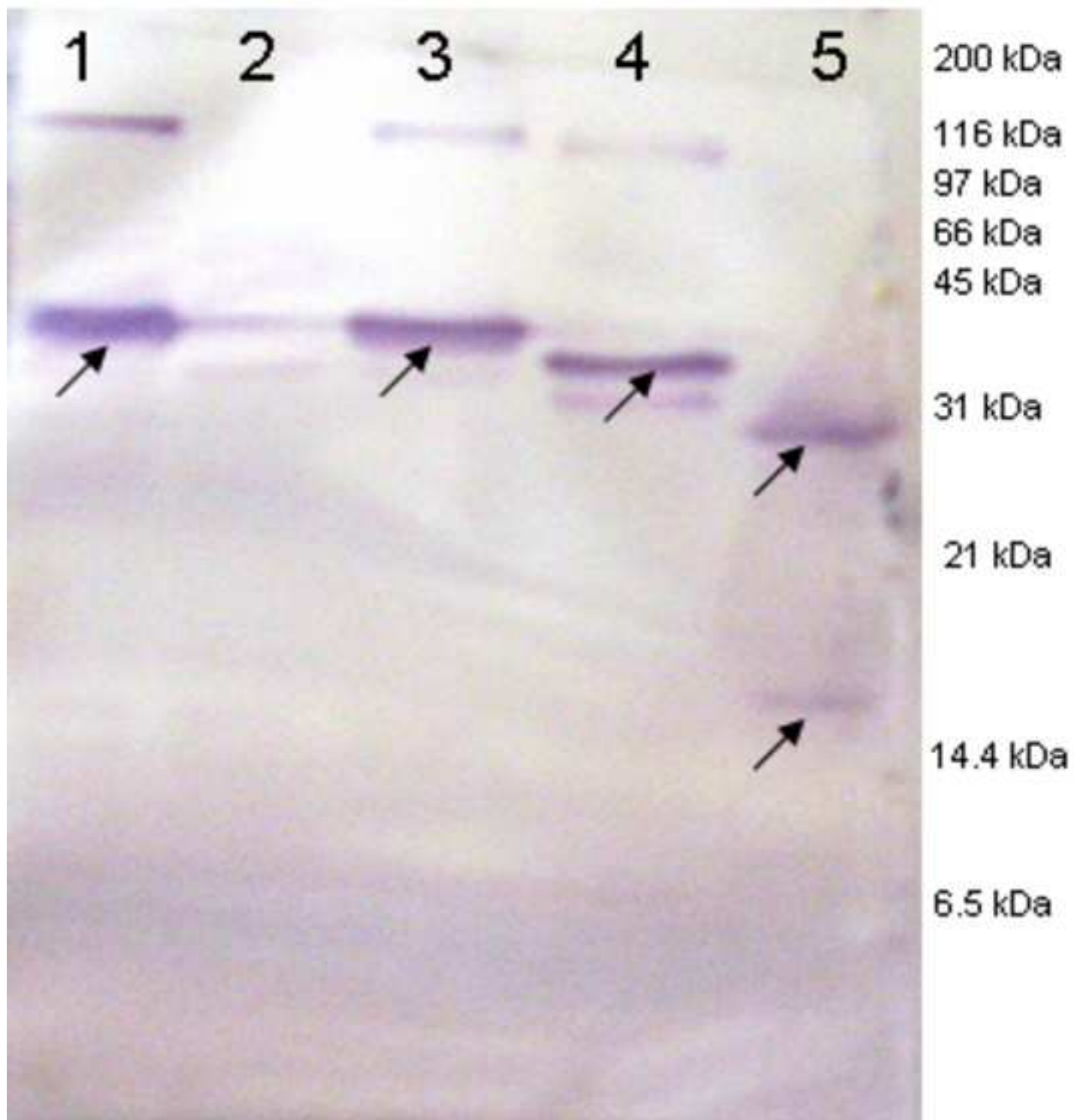


Figure 2

