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3 ***burnetii* as a potential antigen for immunodiagnostic of Q fever in goats.**

4

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20

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23

24 **Abstract:**

25

26 *Coxiella burnetii* is an intracellular bacterium that causes a worldwide  
27 zoonosis, the Q fever. Currently, to diagnose the infection in ruminants, whole  
28 cell antigens-based ELISAs are used.

29 In this study a heat shock protein, the HspB, was evaluated for its ability to be  
30 recognized by the goat immune system and its capacity to sign a stage of  
31 infection. The *htpB* gene of *C. burnetii* was cloned and sequenced. A high identity  
32 (>90 %) was observed among the *htpB* genes of four ruminant strains tested. A  
33 recombinant protein was expressed in a prokaryotic expression system. The  
34 rHspB protein was used to determine the IgG reactivity by ELISA. Sera from  
35 experimentally and naturally infected goats were tested. The rHspB is recognized  
36 early during the infection course, at 18 days post-infection. Moreover, 80 to 90 %  
37 of the animals tested were positive at 39-60 dpi. In addition, animals presenting a  
38 reactivation of the infection displayed a higher reactivity, statistically significant  
39 ( $p<0.05$ ), than that of the animals in latent infection. These findings suggest that  
40 the rHspB could be a good candidate for the development of an ELISA test  
41 making possible the detection of recent *C. burnetii* infection in goats as well as  
42 reactivation in those with latent infection.

43

44 Keywords : Q fever / goat / rHspB / recent and reactivation of the infection /  
45 immunodiagnostic

46

47 **1. Introduction**

48 *Coxiella burnetii* is an intracellular bacterium that causes a worldwide  
49 zoonosis, the Q fever. Ruminant species represent the main reservoir of *C.*  
50 *burnetii*. In cattle and small ruminants, Q fever can provoke placentitis, abortion  
51 and stillbirth. These species can shed the bacteria into the environment through  
52 birth products, feces and milk (Berri et al., 2002; Guatteo et al., 2006; Berri et al.,  
53 2007).

54 Several immunodiagnostic methods such as ELISA, immuno-fluorescence  
55 assay and complement fixation test may be used to evaluate the serological status  
56 of animals (Rousset et al., 2004). At the moment these tests are poorly  
57 standardized, and discordant results may be observed in the field (Rousset et al.,  
58 2007). These immunodiagnostic tests use whole phase I and phase II *C. burnetii*  
59 antigens, which production is difficult and needs level 3-containment laboratories.  
60 Moreover, these tests cannot distinguish between recent and chronic infections.  
61 Hence, an ELISA test based upon the use of recombinant antigens would be  
62 extremely useful, to improve diagnosis of *C. burnetii* infection in animals.

63 Previous studies have identified several immunodominant antigens of *C.*  
64 *burnetii*, amongst which, HspB, a heat shock protein was characterized (Vodkin  
65 and Williams, 1988). Contrary to humans, the antibody reactivity from ruminant  
66 sera against HspB has not yet been assessed (Williams et al., 1990).

67 In this study, the ability of a recombinant HspB protein (rHspB) to sign  
68 different stages of the infection in goats was studied by ELISA.

69 **2. Materials and methods**

70 *2.1. Bacterial strains used*

71 Caprine (CbC1 and CbC2), ovine (CbO1) and bovine (CbB1) French isolates  
72 obtained from milk or placenta samples have been used in this study (Arricau-  
73 Bouvery et al., 2003; Arricau-Bouvery et al., 2006).

74

75 *2.2. Sera selected for the study*

76 All sera used in this study were tested by a commercial ELISA test (IDEXX,  
77 Eragny-sur-Oise, France or LSI, Lissieu, France) according to the manufacturer's  
78 instructions.

79 *2.2.1. Sera from experimentally infected animals*

80 Sera from goats experimentally infected by *C. burnetii* were kindly supplied  
81 by A. Rodolakis (INRA Tours-Nouzilly). Briefly, 25 goats were inoculated with a  
82 range of dilutions of strain CbC1 ( $2.5 \times 10^8$ ,  $2.5 \times 10^6$  or  $2.5 \times 10^4$  bacterium/ml),  
83 obtained from an aborted goat (Arricau-Bouvery et al., 2003). Sera were taken at  
84 different times before and after inoculation and stored at  $-20^\circ\text{C}$  until use.

85 *2.2.2. Sera from naturally infected goats*

86 All sera came from a single herd (42 animals) that suffered an abortion  
87 episode (40 % of the primiparous) due to *C. burnetii* in 2006. Since then, a follow  
88 up was undertaken to ascertain the individual status of the animals in terms of *C.*  
89 *burnetii* excretion and specific antibody response. The excretion level was  
90 measured at the day of delivery or the day of abortion on vaginal swabs (VS) by  
91 using a commercial quantitative PCR kit (Taqvet, LSI). The antibody response

92 was tested by ELISA using sera taken within 1 month after delivery or abortion.  
93 The individual sera were then classified into different categories according to the  
94 status of animals. Latent infection in animals was defined when the ELISA tests  
95 were positive in 2006 and 2007 and the PCR tests were negative in VS in 2007.  
96 On the other hand, the reactivation of the infection was defined when the animals  
97 were seropositive during two consecutive years and the excretion level was  
98  $>1 \times 10^4$  *C. burnetii*/VS.

99 The negative control sera were collected from a goat herd (28 goats) without  
100 known history of *C. burnetii* infection and characterized as follows: Q fever  
101 ELISA negative, *C. burnetii* PCR negative in milk and no abortion in the herd  
102 during the year preceding the sampling.

103

### 104 2.3. Amplifying and cloning of the *htpB* gene fragment

105 The DNA of caprine strains CbC1 and CbC2, ovine strain CbO1 and bovine  
106 strain CbB1 of *C. burnetii* was extracted using the Dneasy kit (Qiagen,  
107 Courtaboeuf, France) following the manufacturer's instructions. The *htpB* gene  
108 was amplified by PCR with two primers, containing *Bam*HI or *Not*I restriction site  
109 (restriction sites are underlined), as follows: HtpB-1 (5'-  
110 CGCGGGATCCGCTGCAAAAGTTTTAAAATTT-3') and HtpB-2 (5'-  
111 ATTTGCGGCCCGCCATCATGCCGCCCATG-3'). Both primers were designed  
112 from the *htpB* gene sequence of the Nine Mile strain (CBU1718; GenBank  
113 GI:29655007) (Seshadri et al., 2003). PCR was performed with the Expand High  
114 Fidelity PCR System (Roche, Meylan, France) in 50  $\mu$ l reaction mixture

115 containing 15 pmol of each primer, 1X buffer with MgCl<sub>2</sub>, 2.6U of enzyme, 0.2  
116 mM of dNTP Mix (Invitrogen, Cergy Pontoise, France) and 100 ng of DNA. The  
117 PCR product was purified using the QIAquick PCR kit (Qiagen), following the  
118 manufacturer's instructions, and ligated into plasmid pGemT Easy (Promega,  
119 Charbonnières-les-Bains, France). Nucleotide sequencing was performed by  
120 MilleGen (Labège, France) and analyzed with the Clustal W Multiple Alignment  
121 feature of the BioEdit software. The *htpB* gene from the CbC1 strain was then  
122 subcloned into the expression vector pET30 (a+) (Novagen, Darmstadt,  
123 Germany). The recombinant construct (pET30/*htpB*) was used to transform *E. coli*  
124 BL21 cells (Lucigen, Middleton, USA).

125

#### 126 2.4. Expression and purification

127 The recombinant *E. coli* BL21, carrying the pET30/*htpB* plasmid, were grown  
128 in Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/ml), at 37°C  
129 up to an O.D.<sub>600nm</sub> of 0.5 – 1. The culture was induced with isopropyl-β-thio-  
130 galactopyranoside (IPTG) at a final concentration of 1mM and incubated at 37°C  
131 in a shaking incubator during 4 hours. Recombinant BL21 cells were then pelleted  
132 by centrifugation.

133 The rHspB protein was purified, in denaturant condition (8M urea, 0.1M  
134 NaH<sub>2</sub>PO<sub>4</sub>, 0.01M Tris-HCl, pH8) by affinity chromatography in nickel column  
135 using the QIAexpress<sup>®</sup>Type ATG kit (Qiagen). The purified protein fractions  
136 were pooled and the concentration was determined by using the Bio-Rad Protein  
137 Assay (Bio-Rad, Marnes la Coquette, France).

138

139 *2.5. ELISA*

140 Individual sera were tested by ELISA with the purified rHspB antigen. One  
141 hundred ng of recombinant antigen were mixed with 100  $\mu$ l of coating buffer  
142 (0.05 M carbonate/bicarbonate, pH9.6), deposited into each well of 96-well  
143 microplates (Nunc, Wiesbaden, Germany) and incubated overnight at 4°C. After 3  
144 washes with PBS-0.2% Tween20 (PBST) at room temperature, the plates were  
145 blocked with 2 % BSA in TNE (0.15 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH  
146 7.4) and incubated for 1 hr at 37° C. Then, they were incubated for 1 hr at 37°C  
147 with serial dilutions of sera samples in TNE buffer containing 0.2 % Tween 20  
148 and 0.2 % BSA (100  $\mu$ l). The Peroxidase-conjugate AffiniPure Rabbit Anti-Goat  
149 IgG, F(ab')<sub>2</sub> Fragment Specific (Interchim, Montluçon, France) was added at a  
150 dilution of 1:40000 and the plates were incubated for 1 hr at 37°C. One hundred  
151  $\mu$ l of TMB (Sigma-Aldrich) were used as the substrate. The reaction was stopped  
152 using Stop Reagent for TMB substrate (Sigma-Aldrich), and optical density was  
153 read at 620 nm using an ELISA reader (Multiskan RC, Thermolabsystems).

154

155 *2.6. Statistical analysis*

156 The O.D<sub>620 nm</sub> values obtained by the rHspB ELISA tests for the sera obtained  
157 from the different categories of animals were compared by using the non  
158 parametric Wilcoxon test, as implemented in the JMP software. Differences were  
159 considered significant when  $p < 0.05$ .

160

161 **3. Results**

162 *3.1. Sequence analysis*

163 The *htpB* sequence of CbC1, CbC2, CbO1 and CbB1 was determined  
164 (Accession numbers EU888861, EU888862, EU888863, EU888864). The  
165 sequence analysis of 1623 bp of the ruminant *htpB* sequence indicated 99.5 to  
166 100% of nucleotide identity compared to the Nine Mile sequence and 99.6 to  
167 100% at the protein level (data not shown).

168

169 *3.2. Evolution of anti-rHspB antibodies during the course of infection*

170 The control of the *HtpB* gene expression in *E. Coli* showed the presence of a  
171 60 kDa protein, which is the expected molecular weight (data not shown).

172 Sera of goats experimentally infected by *C. burnetii* were tested by ELISA  
173 using the purified rHspB. A variability of reaction of individual animals was  
174 observed (figure 1). However a general tendency was observed. The sera  
175 reactivity increased with time, reaching a plateau at days 39 to 60, and then  
176 decreased towards a basal level. In order to determine if this protein could be a  
177 marker of recent infection, the O.D<sub>620 nm</sub> values at each time of the infection were  
178 compared with those obtained from sera of animals in latent infection. The O.D<sub>620</sub>  
179 nm values of sera at days 18 to 81 post-infection were significantly higher ( $p < 0.05$ )  
180 than those of the latent group. At day 96 no statistics had been performed due to  
181 the low number of samples.

182 The cut-off value of 0.4 was determined as the mean of O.D<sub>620 nm</sub> taken before  
183 inoculation plus 3 standard deviations ( $0.16 + 3 \times 0.08$ ). At this cut-off value, only

184 one animal out of ten was positive at day 0. Between day 39 and day 67, 80 to  
185 90% of the tested animals were positive while 50% of them still had a positive  
186 response at 96 dpi (figure 2). When these sera were tested with a commercial  
187 ELISA, using a whole cell extract as antigen, the percentage of positive sera  
188 increased with time and reached a plateau at 80 – 100 % until the end of the  
189 experiment (figure 2).

190

### 191 *3.3. Relationship of the anti-rHspB response with different status in the field.*

192 Twenty eight negative sera from a herd with unknown history of Q fever have  
193 been tested with the developed rHspB ELISA. The O.D<sub>620 nm</sub> values of all sera  
194 were below the cut-off of 0.4 (figure 3). The combination of the results (O.D<sub>620 nm</sub>  
195 values) obtained from sera of the negative herd and from the experimental  
196 infection at day 0, delineates a specificity rate of 97%.

197 To determine if the rHspB could be a marker of reactivation, 11 sera of  
198 animals naturally infected by *C. burnetii* were tested. Five of them were obtained  
199 from animals in the reactivation phase and 6 were from animals with a latent  
200 infection (figure 3). All sera from the latent group were negative at the cut-off of  
201 0.4 whereas 3 sera from the reactivated group were positive. The O.D<sub>620 nm</sub> values  
202 of sera from the reactivated group were significantly ( $p < 0.05$ ) higher than those  
203 from the latent group.

204

## 205 **4. Discussion**

206 In epidemiological studies, the development of tests that can distinguish recent  
207 and former infections is important in order to limit the contamination of the  
208 environment and to prevent abortions in herds. This study was performed to  
209 characterize the immunoreactivity of the purified recombinant HspB protein of *C.*  
210 *burnetii* in ruminants. Moreover, the possible role of HspB to sign a recent  
211 infection and reactivation in goats infected by *C. burnetii* was evaluated for the  
212 first time.

213 HspB is a heat shock protein (HSP), member of the Hsp60 family. HSPs are  
214 present in both eukaryotic and prokaryotic cells and achieve important functions  
215 such as the folding and unfolding or translocation of proteins. A basal level of the  
216 expression of this protein can be detected in cells but an increase of this level is  
217 found in response to a variety of stress stimuli, such as infection or inflammation,  
218 in order to protect the cells (Zugel and Kaufmann, 1999). In *C. burnetii*, HspB  
219 was demonstrated to be more abundant in the metabolic and replicative LCV form  
220 than in the SCV form (Coleman et al., 2007), reflecting a stress response to the  
221 acidic lysosomal environment. Due to their universal function, HSPs are relatively  
222 well conserved. The amplification and the sequencing of the *hspB* gene, deleted of  
223 30 nt, of different ruminants isolates of *C. burnetii* was undertaken. As expected,  
224 a high homology in the nucleotide and protein sequences of HspB was observed  
225 among the four ruminant strains. The comparison of these sequences with the  
226 Hsp60/65 of different bacteria that can infect ruminants (*Brucella melitensis*,  
227 *Mycobacterium ovis*) and of *Chlamydiae*, showed 59 to 65% of homology, in  
228 agreement with other studies (Cerrone et al., 1991).

229 HSPs are demonstrated as immunodominant antigens of humoral and cellular  
230 immune responses in a wide range of infections (Kaufmann, 1990). In Q fever, an  
231 immunological response against HspB was demonstrated in different laboratory  
232 animals and in humans, indicating its possible role in protection (Li et al., 2005).  
233 It was also observed that sera of convalescent patients in acute Q fever recognized  
234 HspB in the SCV and LCV forms of the bacteria (Coleman et al., 2007). Our work  
235 is the first study on the immunological evaluation of this protein in goats. As  
236 HSPs have large common parts, the specificity of the rHspB ELISA assay was  
237 first tested. A specificity rate of 97% was found by using sera from goats without  
238 history of Q fever and from the negative group of the experimental infection.  
239 Moreover, no cross reaction was observed with sera from goats experimentally  
240 infected with *B. melitensis* (not shown). In addition, the level of reactivity, of a  
241 pool of negative sera, against the native form of the protein was higher than that  
242 obtained with the denatured form of the protein (not shown). This might be  
243 explained by the presence of non specific conformational epitopes. Thus, the use  
244 of the denatured form of HspB increases the specificity of the test which rate,  
245 obtained in our experiments, was still high.

246 The immunoreactivity of sera from experimentally infected goats to rHspB  
247 was investigated. Inter individual variation was observed, as already found for  
248 bovine tuberculosis, where secretory antigens induced a variable antibody  
249 response in infected cattle (Lyashchenko et al., 1998). Findings from the present  
250 study show that the anti-rHspB antibody level of sera from goats taken within 18  
251 and 81 days post-infection is significantly ( $p < 0.05$ ) higher than that of sera of

252 animals in latent infection. Therefore, the data suggest that HspB is preferentially  
253 detected early post-infection and could be a marker of a recent infection. These  
254 results are in accordance with the presence of HspB on the surface of the bacteria  
255 phase II (Williams et al., 1990) and the development of a primary antibody  
256 response against phase II antigens (Tissot-Dupont et al., 1994).

257 Previous studies, in other pathogens, have shown a correlation between  
258 antibody reactivity to Hsp60 and the pathogenic form of infection. For example,  
259 during an infection with *Chlamydia trachomatis*, the anti-chlamydial Hsp60 IgG  
260 level was higher during recurrent infection than in primary infection (Agrawal et  
261 al., 2007). Therefore as in chlamydial infection, *C. burnetii* HspB can also sign a  
262 reactivation of infection in goats, since the O.D<sub>620 nm</sub> values of sera from the  
263 reactivated group were significantly ( $p < 0.05$ ) higher than those from the negative  
264 or latent groups.

265 To our knowledge, this is the first report about the use of a recombinant HspB  
266 as a potential antigen to diagnose caprine Q fever. In conclusion, this protein  
267 seems to be able to indicate a recent infection, as well as a reactivation of  
268 infection. Further analysis of this recombinant protein in ovine and bovine  
269 infection might allow its use in an ELISA diagnosis test.

270

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273 experimentally infected by *C. burnetii* and the *C. burnetii* strains. The  
274 experimental infection on goats was performed at INRA Nouzilly according to the

275 French law and were approved by the Institutional Ethical Committee. We also  
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277

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352 **Figure legends**

353 Figure 1. Boxplot representation of the kinetic of appearance of anti-rHspB  
354 antibody during the experimental infection and in animals from the latent group.  
355 At each time and group, median, quartile and standard deviation are represented.  
356 The dotted line indicates the cut-off value (means + 3 S.D), the dots represent the  
357 individual optical value ( $O.D_{620nm}$ ). Groups significantly different ( $p < 0.05$ ) from  
358 the latent group are represented by an asterisk (\*).

359

360 Figure 2. Percentage of positive sera at different times of experimental infection.  
361 rHspB ELISA was performed as described in Materials and Methods. Positive  
362 sera are defined when  $O.D_{620nm}$  values are superior to the cut-off value (0.4 O.D).  
363 The same panel of sera was tested with a commercial ELISA (IDDEX). A positive  
364 result was defined as the manufacturer's instructions.

365

366 Figure 3. Boxplot representation of the negative, latent and reactivated groups.  
367 For each group, median, quartile and standard deviation are represented. The dots  
368 represent the individual optical value ( $O.D_{620nm}$ ). A significant difference was  
369 observed between the reactivated and the latent groups ( $p < 0.05$ ) as represented by  
370 an asterisk (\*).

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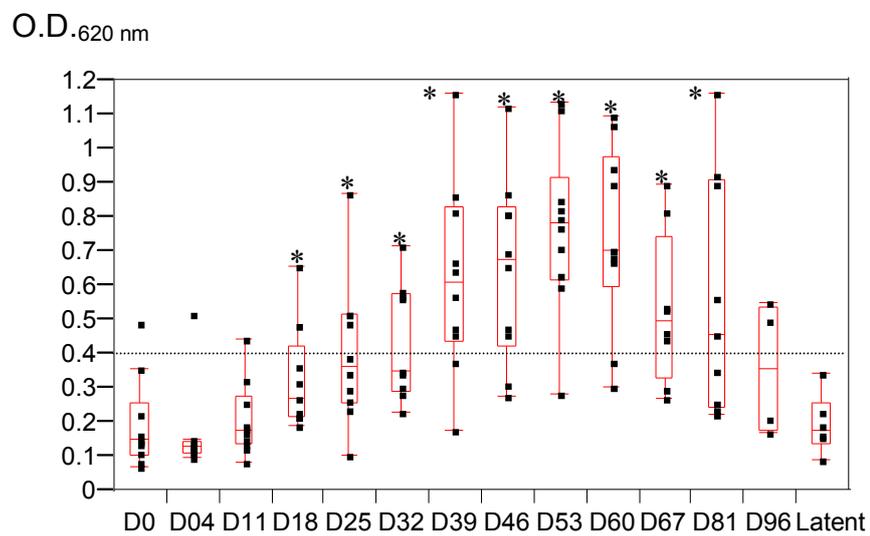
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2 Figure 1



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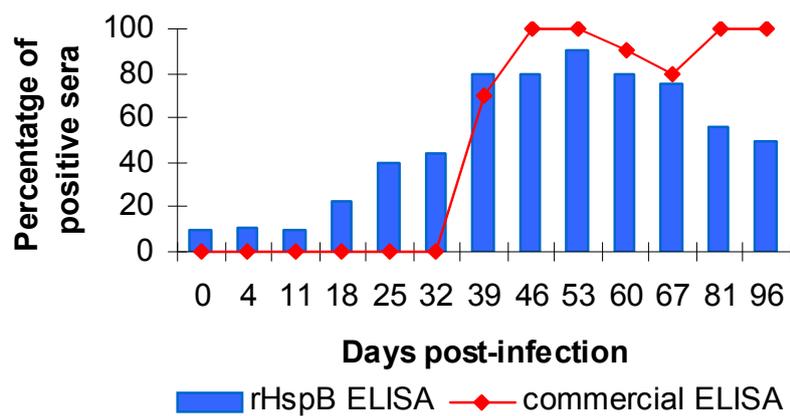
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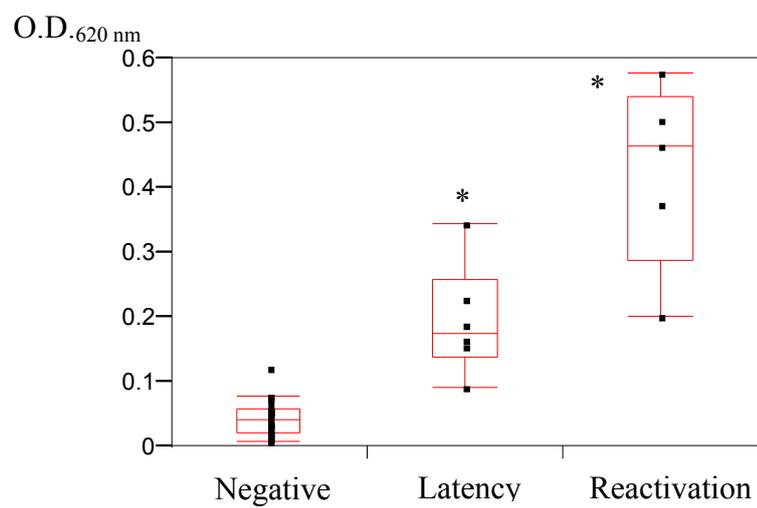
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2 Figure 3

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