Novel insights into the interaction of human and animal complement regulator factor H with viable Lyme disease spirochetes

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Abstract

Spirochetes belonging to the *Borrelia (B.) burgdorferi* sensu lato (s.l.) complex differ in their ability to establish infection and to survive in diverse vertebrate hosts. Association with and adaption to various hosts most likely correlates with the spirochetes' ability to acquire complement regulator factor H (FH) to overcome the host´s innate immune response. Here we assessed binding of serum FH from human and various animals including bovine, cat, chicken, dog, horse, mouse, rabbit, and rat to viable *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, *B. spielmanii*, *B. valaisiana*, and *B. lusitaniae*. Spirochetes ectopically producing CspA orthologs of *B. burgdorferi* s.s., *B. afzelii*, and *B. spielmanii*, CspZ, ErpC, and ErpP, respectively, were also investigated. Our comparative analysis using viable bacterial cells revealed a striking heterogeneity among Lyme disease spirochetes regarding their FH-binding patterns that almost mirrors the serum susceptibility of the respective borrelial genospecies. Moreover, native CspA from *B. burgdorferi* s.s., *B. afzelii*, and *B. spielmanii* as well as CspZ were identified as key ligands of FH from human, horse, and rat origin while ErpP appears to bind dog and mouse FH and to a lesser extent human FH. By contrast, ErpC did not bind FH from human as well as from animal origin. These findings indicate a strong restriction of distinct borrelial proteins towards binding of polymorphic FH of various vertebrate hosts.

Introduction

Lyme disease (LD) is the most prevalent vector-borne anthropozoonosis in Eurasia and North America. This disease is caused by spirochetes belonging to the *Borrelia (B.) burgdorferi* sensu lato (s.l.) complex (Steere et al., 2004). The *B. burgdorferi* s.l. complex comprises more than 15 species including *B. burgdorferi* sensu stricto (*B. burgdorferi*), *B. garinii*, *B. afzelii*, *B. spielmanii, B. valaisiana*, and *B. bavariensis* (formally designated as *B. garinii* OspA serotype 4). Spirochetes are maintained in multiple vertebrate reservoir hosts (mainly mammals, birds, and reptiles) and transmitted from these hosts to humans and other animals during the blood meal of ixodid ticks (Steere et al., 2016). Upon the tick bite, spirochetes first survive in the blood, migrate from ticks to vertebrate hosts, and establish infection of the skin at the bite site (Hart et al., 2018). *B. burgdorferi* s.l. then disseminate via the bloodstream to multiple tissues and organs (Steere et al., 2004). In humans, the colonization of spirochetes can result in severe chronic infections such as Lyme arthritis, neuroborreliosis or acrodermatitis chronica atrophicans (Koedel et al., 2015; Steere et al., 2016; Ogrinc et al., 2017). Thus, *B. burgdorferi* s.l. requires the ability to survive during the ticks' blood meal and in the hosts' bloodstream to be maintained in the enzootic cycle.

Complement is one of the most powerful innate immune defence mechanisms in vertebrate animals' blood. Complement is composed of a network of more than 50 proteins including inactive precursor molecules, fluid-phase and membrane-bound regulators as well as distinct inhibitors (Walport, 2001b; a; Zipfel, 2009; Ricklin et al., 2010; Trouw and Daha, 2011). This tightly-controlled surveillance system plays an important role for the recognition, discrimination, and elimination of invading pathogens (Walport, 2001b). Activation of complement is initiated through three canonical routes, the classical, the lectin, and the alternative pathways, all of which converge in the generation of the central C3b molecule and subsequently lead to the formation of the C3 and C5 convertases. Cleavage of C5 by the C5 convertases following binding of C5b to the microbial surface initiates the activation of the terminal sequence. Finally, a pore-forming complex known as the terminal complement complex (TCC) or membrane attack complex (MAC) is generated by the unidirectional, sequential binding of components C6, C7, and C8 to deposited C5b. This is followed by binding of numerous C9 molecules to the surface-associated C5b-8 complex. The integration of numerous pores into the cell membrane leads to the bacteriolysis of invading pathogens (Ricklin et al., 2010; Trouw and Daha, 2011). To prevent activated effector molecules from attacking self-cells and -tissues, this system is efficiently controlled at different levels by various soluble and membrane-anchored regulators (Zipfel and Skerka, 2009a). C1 esterase inhibitor (C1-INH) and the C4b-binding protein (C4BP) represent the main soluble regulators of the classical and the Lectin pathway while Factor H (FH) and Factor H-like protein 1 (FHL-1) are the primary regulators of the alternative pathway (Zipfel et al., 2006; Zipfel and Skerka, 2009a). The latter two regulators act as co-factors for factor I mediated inactivation of C3b, and thereby inhibit the formation and accelerate the decay of the C3 convertase of the alternative pathway (Whaley and Ruddy, 1976; Pangburn et al., 1977; Kühn et al., 1995; Zipfel and Skerka, 2009b).

Recruitment of FH and FHL-1 appears to be an efficient and prominent strategy adopted by LD spirochetes to resist complement-mediated killing by termination of alternative pathway activation (Alitalo et al., 2001; Kraiczy et al., 2001b; Stevenson, 2002; McDowell et al., 2003b). *B. burgdorferi* s.l. produce at least five distinct surface-exposed Complement Regulator-Acquiring Surface Proteins (CRASPs), including CspA (CRASP-1), CspZ (CRASP-2), ErpP (CRASP-3), ErpC (CRASP-4), and ErpA (CRASP-5) (for review see (Kraiczy and Stevenson, 2013; Kraiczy, 2016a)). The deficiency of CspA in infectious *B. burgdorferi* results in the inability to bind human FH (Brooks et al., 2005). Conversely, the production of this protein in a spirochete strain leads to greater levels of human FH-

79 binding activity (Brooks et al., 2005; Kenedy et al., 2009). Consistent with the unique expression of 80 *cspA* when spirochetes are within ticks, this gene is essential for *B. burgdorferi* to be transmitted 81 from nymphal ticks to mice by evading complement during ticks' blood meal (Hart et al., 2018). 82 Unlike *cspA*, the other CRASP encoding genes are co-expressed when spirochetes are in vertebrate 83 hosts (Bykowski et al., 2007). Though expressing each of these genes (except *erpC*) in spirochetes 84 enhances human FH binding activity (Kraiczy and Stevenson, 2013), a *cspZ* deletion mutant of *B.* 85 *burgdorferi* strain B31 or Tn-inserted mutant spirochete of *erpP* or *erpA* display little or no defect of 86 human FH-binding activity and/or infectivity, suggesting a potential redundant function of these 87 genes *in vitro* and *in vivo* (Lin et al., 2012). In addition to *B. burgdorferi*, previous studies on other *B.* 88 *burgdorferi* s.l. species revealed that the overall FH binding pattern often resembles the pattern of 89 serum resistance/susceptibility observed among LD spirochetes corroborating the hypothesis of a 90 species-specific, complement-associated host selectivity (Kurtenbach et al., 1998b; Kurtenbach et al., 91 2002a; Kraiczy, 2016b). Taking up this important issue, numerous attempts have been made to 92 determine binding of serum-derived FH from different animals including mouse, rat, cat, dog, sheep, 93 horse, cattle, goat, monkey, mini pig, pig, duck, and chicken by employing Far-Western blot analyses 94 (Stevenson et al., 2002; Alitalo et al., 2004; Hovis et al., 2006; McDowell et al., 2006; Haupt et al., 95 2007; Bhide et al., 2009; van Burgel et al., 2010; Kisova-Vargova et al., 2012) to further substantiate 96 the hypothesis that complement is an important factor for host-specificity and transmissibility of LD 97 spirochetes. However, controversial results have been reported from these studies, possibly due to the 98 usage of misfolded or denatured proteins (borrelial cell lysates or purified proteins) for capturing 99 serum-derived FH (Stevenson et al., 2002; Alitalo et al., 2004; Hovis et al., 2006; McDowell et al., 100 2006; Haupt et al., 2007; Bhide et al., 2009; van Burgel et al., 2010; Kisova-Vargova et al., 2012; 101 Marcinkiewicz et al., 2017). In this study, we re-assessed binding of native FH from different 102 animals' sera to viable *B. burgdorferi* s.l. and gain-of-function borrelial strains producing each of the 103 CRASPs to overcome the obvious limitations of previous approaches. This strategy allowed us to 104 characterize and report the CRASP-mediated FH-binding activity in a fashion that is much closer to 105 physiological conditions.

106 **Materials and Methods**

107 **Ethics statement** 108 The study and the respective consent documents were approved by the University Hospital Frankfurt' 109 ethics committee (control number 160/10). All healthy blood donors provided written informed 110 consent. 111 112 **Bacterial strains and culture conditions** 113 Borrelial strains listed in Supplementary table 1 as well as transformed spirochetes listed in 114 Supplementary table 2 were grown until mid-exponential phase (5 x 10^7 cells per ml) at 33 °C in 115 Barbour-Stoenner-Kelly (BSK-H) medium (Bio&SELL, Feucht, Germany) or BSK-H supplemented 116 with streptomycin (50 mg/ml) as described previously (Siegel et al., 2008). The density of 117 spirochetes was determined using dark-field microscopy and a Kova counting chamber (Hycor 118 Biomedical, Garden Grove, CA). 119 120 **Human and animal sera, antibodies, and serum proteins** 121 Normal human serum (NHS) used for serum susceptibility testing and Far-Western blotting or as a 122 source of FH was tested for the presence of anti-*Borrelia* IgM and IgG antibodies by commercially 123 available ELISAs (Enzygnost□ Borreliosis/IgM and Enzygnost□ Lyme link VlsE/IgG, Siemens 124 Healthcare Diagnostics Products GmbH, Marburg, Germany). Only sera proven to be negative for 125 IgM and IgG anti-*Borrelia* antibodies were used to form a serum pool of at least 10 blood donors.

126 Chicken, goat, bovine, mouse, rat, and sheep serum were purchased from Dunn Labortechnik, 127 Asbach, Germany. Cat and dog (beagle) serum were purchased from Sera Laboratories International, 128 West Sussex, UK, and horse and rabbit serum were obtained from Sigma-Aldrich, Taufkirchen, 129 Germany. Purified human FH was purchased from Complement Technology, Tyler, TX, USA, and 130 the polyclonal sheep anti-FH antiserum (dilution 1/1,000) was obtained from Acris, Herford, 131 Germany. 132 133 **Binding of complement proteins to viable spirochetes** 134 Spirochetes (2 x 10^9 cells) grown to mid-log phase were gently washed and resuspended in 750 µl 135 human or animal serum supplemented with 34 mM EDTA (pH 8.0) to avoid complement activation. 136 After 1 h incubation at room temperature and four wash steps with PBSA (0.15 M NaCl, 0.03 M 137 phosphate, 0.02% sodium azide, pH 7.2) containing 0.05% Tween-20, proteins bound to the 138 spirochetal surface were eluted by incubation with 100 mM glycine-HCl (pH 2.0) for 15 min. Cells 139 were then removed by centrifugation at 14.000 x g for 10 min at 4°C, and the supernatant was 140 neutralized by adding 1M Tris (pH 9.0). Both, the last wash and the eluate fraction were separated by 141 12.5 % glycine-SDS-PAGE under non-reducing conditions and binding of human or animal FH was 142 analyzed by Western blotting using a sheep anti-FH antibody (dilution 1:500). Detection of bound 143 proteins was performed using 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. 144 145 **Far-Western blot analysis** 146 Whole cell lysates obtained from each borrelial isolate (15 µg per lane) were subjected to 10% 147 tris/tricine SDS-PAGE under reducing conditions and transferred to nitrocellulose as previously 148 described (Kraiczy et al., 2001a; Siegel et al., 2010; Kraiczy, 2018). Briefly, membranes incubated 149 for 1h with NHS were washed four times with TBS containing 0,2% Tween 20 and thereafter 150 incubated for 1 h with a sheep anti-FH antiserum (dilution 1:1,000). Following four wash steps with 151 TBS containing 0,2% Tween 20, membranes were incubated with an appropriate peroxidase152 conjugated secondary antibody for 1 h. Detection of bound proteins was performed using TMB as 153 substrate. 154 155 **Production of His$_6$-tagged borrelial proteins in *E. coli*** 156 Generation of vectors pQE CspA LW2, pQE CspA PKo, pQE CspA A14S, pQE CspZ, pBLS538, 157 and pBLS528 producing amino-terminally polyhistidine-tagged CspA$_{LW2}$ (CRASP-1 of *B.* 158 *burgdorferi* s.s. strain LW2), CspA$_{PKo}$ (CRASP-1 of *B. afzelii* strain PKo), CspA$_{A14S}$ (CRASP-1 of *B.* 159 *spielmanii* strain A14S), CspZ (CRASP-2), ErpP (CRASP-3), and ErpC (CRASP-4), respectively 160 were described previously (Stevenson et al., 1998; Siegel et al., 2008; Hammerschmidt et al., 2012; 161 Hammerschmidt et al., 2014). Note that CRASP-3/ErpP and CRASP-4/ErpC encoded by the *erpP* 162 and *erpC* genes of *B. burgdorferi* s.s. type strain B31 and the European *B. burgdorferi* s.s. strain 163 LW2 are identical and thus, proteins are hereafter referred to as CspA$_{B31}$, ErpP, and ErpC. Expression 164 of recombinant proteins was induced in DH5α or JM109 at an OD_{600} of 0.6 by the addition of 0.2 165 mM IPTG. Following incubation for 4 h at room temperature, cells were centrifuged (5,000 × g, 20 166 min, 4 °C) and subsequently suspended in lysis buffer (300 mM NaCl, 56 mM NaH_2PO_4 pH 8, 10 167 mM Imidazole) containing 50 mg/ml lysozyme. Cells were lysed by 6 rounds of sonication for 30 sec 168 using a Branson B-12 sonifier (Heinemann, Schwäbisch Gmünd, Germany). After centrifugation 169 (14,000 × g, 20 min, 4 °C), supernatants were filtered through 0.45 µm filters and stored at -20 °C 170 until needed. For affinity purification of His-tagged proteins, cell lysates were incubated with Ni171 NTA agarose and bound proteins were purified as recommended by the manufacturer (QIAGEN, 172 Hilden, Germany). 173 174 **Generation of CRASP producing *B. garinii* cells** 175 The construction of shuttle vectors pCspA, pCspA$_{PKo}$, pCspA$_{A14S}$, pCspZ, pErpP, pErpC allowing 176 ectopical production of surface exposed lipoproteins in the surrogate strain *B. garinii* G1 and the 177 procedure applied to generate transformed spirochete have been previously described in detail (Siegel 178 et al., 2008; Siegel et al., 2010; Hammerschmidt et al., 2012; Hammerschmidt et al., 2014). 179 180 **Detection of interacting serum proteins using magnetic beads** 181 For identification of interacting serum proteins, magnetic beads (Dynabeads TALON, Invitrogen 182 Dynal AS, Oslo, Norway) coated with cobalt ions were used. Briefly, His$_6$-tagged proteins (10 µg 183 each) were allowed to bind to Dynabeads (Life Technologies) for 10 min at room temperature as 184 recommended by the manufacturer. Following several wash steps, recombinant proteins coated to 185 magnetic beads were incubated with 500 µl serum for 1 h at room temperature. After four washes 186 with phosphate buffer (50 mM phosphate, 300 mM NaCl, 0.01% Tween 20), bound proteins were 187 eluted with elution buffer (300 mM imidazol, 50 mM phosphate, 300 mM NaCl, 0,01% Tween 20) 188 for 15 min at room temperature. The eluate and the last wash fraction were separated by 12.5% 189 glycine SDS-PAGE under non-reducing conditions followed by silver staining. 190 191 **Mass spectrometry** 192 The selected proteins were excised from the acrylamide gels and analyzed by mass spectrometry 193 using a commercial service. Data generated were analyzed by Mascot Search Engine, Matrix Science 194 (http://www.matrixscience.com). 195 196 **Results** 197 198 **Detection of polymorphic FH in animal sera** 199 In order to detect native FH in human serum as well as polymorphic FH from rodents (mouse, rat), 200 carnivores (cat, dog),

lagomorphs (rabbit), ungulates (horse, cattle, goat, sheep), and bird (chicken), 201 we first searched for a suitable antibody, exhibiting a broad reactivity to FH molecules of various 202 origin. Of note, serum samples from birds other than chicken as well as from lizards were not 203 commercially available in sufficient quantity at the time of this study. As various monoclonal and 204 polyclonal antibodies tested failed to recognize FH from the selected samples (data not shown), a 205 polyclonal sheep anti-FH antibody raised against the human FH reacted with FH from nearly every 206 animal's serum except with FH from sheep and goat (Fig. 1; FH, approximately 150kDa). As 207 expected, sera from the two latter animals showed a strong reactivity to immunoglobulins and 208 generated a strong background in the Western blot analysis (data not shown). A strong reactivity 209 could be detected for FH from human, mouse, rat, cat, dog as well as rabbit whereby a weaker 210 reactivity was observed for cattle and chicken. In addition, proteins of 25 to 40 kDa could be detected 211 in sera obtained from human, cat, and rat suggesting cross-reactivity of this antibody to factor H-212 related proteins (FHRs). Furthermore, by analyzing cross-reactivity of the secondary anti-sheep 213 antiserum, immunoglobulins from cattle, sheep, and goat showed the strongest signal while 214 immunoglobulins from human displayed a very weak cross-reactivity. None of the other sera tested 215 reacted with the secondary antibody applied (Supplementary figure 1). Owing to the broad reactivity 216 of the sheep anti-FH antibody to almost all FH molecules, we used this particular antibody for all 217 subsequent analyses.

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219 **FH binding to the surface of viable spirochetes** 220 In order to analyze FH binding to spirochetes in a close-to-native context, we first employed whole221 cell ELISA by immobilizing 5×10^7 borrelial cells per well. However, several attempts (e.g.,

222 optimization of the blocking buffer formulation, variation of the antibody and cell concentration) 223 failed to enhance the signal-to-noise ratio for all animal sera tested which yielded very low 224 absorbance values (< 0.2) after subtracting the background values (data not shown). Because of the 225 limitations and failure to increase the sensitivity of the whole-cell ELISA, e.g. by coating higher 226 amounts of spirochetes, we decided to re-assess FH binding by using a fluid phase approach where 227 high concentrations of viable spirochetes (2×10^9 cells) were incubated in inactivated sera including 228 human, mouse, rat, cat, dog, rabbit, cattle, horse, and chicken. Since the sheep anti-human FH 229 antiserum did not reliably detect FH from goat and sheep, these sera were not included in these 230 analyses. Following serum incubation, bound FH was eluted from the surface of the spirochetes and 231 the last wash and the eluate fraction were subjected to glycine-SDS-PAGE under non-reducing 232 conditions. Polymorphic FH molecules were then detected by Western blotting using the sheep anti233 human FH antibody.

234 As expected, *B. burgdorferi* B31, *B. afzelii* FEM1-D15, and *B. spielmanii* A14S bound **human** FH as 235 well as FHL-1 while *B. garinii* G1, *B. lusitaniae* MT-M8, and *B. valaisiana* ZWU3 Ny3 did not bind 236 human FH at all (Figure 2) (Herzberger et al., 2007; Dieterich et al., 2010; Schwab et al., 2013). 237 Concerning *B. burgdorferi* B31, there were four hardly detectable signals below 37 kDa which most 238 likely represent FH-related proteins FHR-1α , FHR-1β, FHR-2, and FHR-2α (Hammerschmidt et al., 239 2012; Hammerschmidt et al., 2016).

240 Employing **mouse**, **rat** or **dog** serum, strong signals corresponding to the molecular weight of human 241 FH could be detected in *B. burgdorferi* B31, *B. afzelii* FEM1-D15, and *B. spielmanii* A14S. (Figure 242 2). In addition, two faint bands of approximately 150 kDa and 75 kDa were present in *B. valaisiana* 243 ZWU3 Ny3 when mouse or dog serum was applied. Notably, *B. garinii* G1 and *B. lusitaniae* MT-M8 244 did not bind FH molecules from any of the animal sera examined. Apparently, none of the borrelial 245 strains bound FH from **cat** with the exception of *B. spielmanii* A14S where a strong signal was 246 visible. **Rabbit** serum showed only very weak signals corresponding to human FH when *B.* 247 *burgdorferi* B31, *B. afzelii* FEM1-D15, and *B. spielmanii* A14S were applied while additional weak 248 signals were observed for *B. valaisiana* ZWU3 Ny3. By incubating spirochetes with serum from 249 **cattle** and **horse**, a stronger signal was only detected for *B. burgdorferi* B31 whereas weak signals 250 were also seen in *B. afzelii* FEM1-D15 and *B. spielmanii* A14S. Moreover, an additional band of 251 approximately 120 kDa and two bands of 90 and 120 kDa were observed when *B. burgdorferi* B31 252 was treated with cattle and horse serum, respectively. Concerning *B. valaisiana* ZWU Ny3, several 253 signals of weak intensity below 100 kDa could be observed. Concerning **chicken** serum, strong 254 reactive bands corresponding to the molecular weight of human FH was observed for *B. afzelii* 255 FEM1-D15, *B. spielmanii* A14S, and *B. valaisiana* ZWU3 Ny3 while a weak signal was visible when 256 employing *B. burgdorferi* B31. In the eluate of the latter strain, a 100 kDa-protein exhibited a strong 257 signal as well as a 75 kDa-protein in *B. valaisiana* ZWU3 Ny3. The data collected from the Western 258 blot analyses are summarized in table 1. 259 260 **Binding of polymorphic FH to CRASP-producing spirochetes** 261 To further explore the role of CRASPs in binding polymorphic FH molecules, *B. garinii* strain G1 262 producing CspA from *B. burgdorferi* B31, CspZ, ErpC or ErpP were incubated with human serum 263 and selected animal sera. ErpA was not included as this protein displayed similar binding patterns as 264 ErpP (Stevenson et al., 2002; Siegel et al., 2010; Hammerschmidt et al., 2012). Transformants 265 producing CspA orthologs from *B. afzelii* PKo ($CspA_{PKo}$) or *B. spielmanii* A14S ($CspA_{A14S}$) were 266 also included as these CspA orthologs were documented to promote binding to human FH 267 (Hammerschmidt et al., 2014). As shown in figure 3, all three CspA orthologs acquired human FH as 268 expected. $CspA_{B31}$ and $CspA_{PKo}$ also bound to horse FH whereas $CspA_{B31}$ also bound to rat FH. None 269 of the CspA orthologs interacted with FH from mouse, dog, and chicken serum. Regarding CspZ, 270 human, rat, and horse serum showed the strongest signals and weak signals were observed for mouse, 271 dog, and chicken serum. Weak signals were visible when human, mouse, and dog serum was applied 272 to the ErpP-producing strain. Notably, the ErpC-producing strain was unable to bind FH from any of 273 the animal sera investigated. The data collected from these analyses are summarized in table 2.

274 Direct comparison of the pattern obtained from the CRASP-producing transformants with that gather 275 from the wild-type strains revealed that in most instances CRASP proteins are responsible for the 276 acquisition of serum-derived FH (Supplementary figure 3). CspA orthologs from *B. burgdorferi* B31, 277 *B. afzelii* PKo or FEM1-D15, and *B. spielmanii* A14S were primarily responsible for the binding of 278 human FH and FHL-1 as well as horse FH. CspZ like CspA strongly bound human FH and FHL-1 279 (Hartmann et al., 2006; Siegel et al., 2008) as well as horse FH. Hardly visible bands at the size of 280 human FH could also be observed employing mouse, dog and chicken serum. In addition, CspZ 281 bound an additional protein of 120 kDa derived from horse serum. ErpC did not bind FH at all 282 whereby ErpP seemed to bind FH from human, mouse, and dog. However, cross-reactive bands 283 below 37 kDa were only visible when CspZ, ErpC, and ErpP-producing strains were investigated but 284 these signals were absent in the wild-type strains (see Figure 2).

285 **Identification of CRASP-interacting serum proteins** 286 A pull-down assay was conducted to identify further serum proteins interacting with CspA, CspZ, 287 ErpC, and ErpC (Siegel et al., 2010). His-tagged CspA, CspZ, ErpC, and ErpC were coupled to 288 magnetic beads and after incubation with the respective serum samples, bound proteins along with 289 the immobilized borrelial proteins were eluted. Following separation of the eluates by SDS-PAGE, 290 proteins were visualized by silver staining (Figure 4). The protein bands present in the eluate fraction 291 but absent in both wash and control fractions (uncoated beads) were then applied the in-gel digestion. 292 The resulting peptides were analyzed using mass spectrometry. The obtained spectra were analyzed 293 using the NCBI.fasta protein database, and results yielding a score of greater than 80 were considered 294 confident ($p<0.05$). 295 296 For the horse serum incubated with CspA and CspZ-coupled beads, three bands of 150-, 120-, and 297 45-kDa could be detected in the eluate fraction but not in the wash or control fractions (Figure 4A). 298 As expected, the 120-kDa of the band yielded a score as approximately 450, representing horse FH 299 (gi|338722817). The 45-kDa band was identified as a histidine-rich glycoprotein of *Equus cabalus* 300 with a score of 120 (gi|338715996). However, the 150-kDa band did not yield results with scores 301 greater than 80. Compared to the control, two additional proteins of 150-and 37-kDa were found in 302 rat serum bound to CspA and CspZ, but we were unable to identify these proteins using mass 303 spectrometry as scores of these proteins were not greater than 80 (Figure 4B). 304 305 The incubation of CspA$_{PKo}$ with mouse serum yielded two proteins of 150-and 29-kDa whereas the 306 interaction of this protein with horse serum gave three proteins of 150-, 120-, and 29-kDa (Figure 307 4C). Regrettably, all attempts to identify these proteins by mass spectrometry failed because all 308 scores were lower than the threshold (80). We also treated magnetic beads coated with ErpC and 309 ErpP with mouse serum, but no obvious differences in the protein patterns were detected between the 310 ErpC/ErpP-immobilized and the uncoated control beads (Figure 4D). 311 312 **Discussion** 313 314 Vector-transmitted pathogens, especially borreliae have developed a range of subtle strategies to 315 infect, disseminate, and persist in their natural reservoir hosts. Different outer surface proteins act in 316 concert to enable spirochetes to overcome the mammalian innate immune system, in particular the 317 destructive effects of complement. One elegant way used by certain spirochetes is the recruitment of 318 host-derived complement regulators such as FH to inhibit and breakdown complement at central 319 activation points (Hellwage et al., 2001; Kraiczy et al., 2001b; Alitalo et al., 2002; Kraiczy, 2016a). 320 In this study, we re-assess the concept of a species-specific, FH-associated host selectivity of LD 321 spirochetes (Kurtenbach et al., 2002a; Kraiczy, 2016b) by investigating bacterial cells challenged 322 with human and various animal sera in a close-to-native context. In agreement with previous studies, 323 the data presented herein also revealed that the FH binding patterns are highly consistent with the 324 serum resistance phenotypes of the respective genospecies. Moreover, we demonstrated that both 325 CspA and CspZ constitute the primary ligands for polymorphic FH molecules as recently 326 demonstrated for the selective binding pattern of CspA orthologs of *B. burgdorferi*, *B. afzelii*, and *B.* 327 *garinii* to mouse, horse or quail FH (Hart et al., 2018). 328 329 Previous studies on the interaction of FH with different genospecies are largely based on *in vitro* 330 experiments where either whole borrelial cell lysates, purified borrelial proteins or the respective 331 serum samples were subjected to SDS-PAGE following immobilization of the separated proteins to a 332 solid membrane (Stevenson et al., 2002; Hovis et al., 2006; McDowell et al., 2006; Bhide et al., 333 2009; van Burgel et al., 2010). These investigations revealed obvious discrepancies between the FH 334 binding patterns obtained by different experimental approaches. For example, binding of FH from 335 human and mouse but not from rat, cat, dog, cattle, horse, and guinea pig was found if cell lysates of 336 *B. burgdorferi* strain SKT2 were applied (Bhide et al., 2009) while in another study where purified 337 borrelial proteins of *B. burgdorferi* strain B31 were investigated, binding of FH from human, mouse, 338 rat, cat, dog, cattle, rabbit, cattle, horse, and goat could be

detected (Stevenson et al., 2002). In a 339 reverse setting where serum samples were separated and immobilized FH was used for capturing of 340 selected borrelial proteins (see below), strong signals in the range of human FH were visible in the 341 sera from mouse, dog, cow, monkey, mini pig, guinea pig, and pig but not in the sera from rat, cat, 342 rabbit, horse, goat, duck, and chicken (Hovis et al., 2006; McDowell et al., 2006; Rogers and 343 Marconi, 2007). Although, Far-Western blotting has been proven as a valuable methodology for 344 detecting protein-protein interactions (Wu et al., 2007), the utilization of denatured or partially 345 misfolded proteins for capturing may often lead to false-positive results as recently demonstrated for 346 the interaction of human FH with ErpA, ErpC, and ErpP (Siegel et al., 2010; Hammerschmidt et al., 347 2012). To overcome the technical drawbacks of those immunoassays, we incubated viable 348 spirochetes in native but complement-inactive serum. In contrast to a previous study employing 349 ELISA (McDowell et al., 2003b), we were able to utilize considerably greater amounts of spirochetes 350 (2×10^9 cells) and serum concentrations (750 µl) to increase the sensitivity in order to detect even 351 minute amounts of cell-bound FH. Because specific antibodies recognizing FH molecules of different 352 animals are currently not available, we used a sheep anti-human FH antibody that exhibited a broad 353 species-specificity and yielded a low signal-to-noise ratio to the most relevant sera included in this 354 study except sheep, cattle, and goat (figure 1 and supplementary figure 1).

355 In addition, our comparative analysis revealed binding of proteins at the size of human FH and 356 additional serum proteins greater than 150 kDa (Figure 2). Assuming that the bands at the size of 150 357 kDa corresponded to the polymorphic FH molecules, *B. burgdorferi*, *B. afzelii*, and *B. spielmanii* 358 bound FH derived from human, mouse, rat, and dog origin. Both, *B. afzelii* and *B. spielmanii* 359 interacted with chicken FH while *B. spielmanii* was the only genospecies that acquired FH derived 360 from cat serum. Binding of horse FH was detected for *B. burgdorferi* and weak signals were also 361 found when assessing *B. afzelii* (Table 1). Of note, the 120-kDa band was confirmed by mass 362 spectrometry to represent horse FH but not the 150-kDa band. *B. burgdorferi*, *B. afzelii*, and *B.* 363 *spielmanii* appeared to bind rabbit FH to a lesser extent as the signals obtained were faint. A different 364 binding pattern was observed for *B. valaisiana* ZWU3 Ny3 as only chicken serum gave a strong 365 signal whereas weak signals were obtained for mouse, and dog. Finally, *B. garinii* G1 (OspA 366 serotype 6) and *B. lusitaniae* MT-M8 did not bind FH at all. Initial studies unveiled that host 367 complement is an important factor for tick-to-host transmission, host specificity, and niche368 adaptation, and therefore for the global ecology of LD spirochetes (Kurtenbach et al., 1998a; 369 Kurtenbach et al., 1998b; Kurtenbach, 2002; Kurtenbach et al., 2002a; Kurtenbach et al., 2002b). Our 370 results, thus strongly support the previous findings of a species-specific host adaption by which the 371 main complement regulator of the alternative pathway FH is a key tie-in.

372 The second objective of our study was the identification of the surface-exposed determinants 373 capturing serum-derived polymorphic FH molecules. In contrast to former investigations employing 374 purified proteins or whole cell lysates as ligands (McDowell et al., 2003a; McDowell et al., 2003b; 375 Hovis et al., 2006; McDowell et al., 2006; Bhide et al., 2009), we aimed at analyzing spirochetes 376 producing either native CspA (CRASP-1, BBA68), CspA orthologs from *B. afzelii* PKo and *B.* 377 *spielmanii* A14S, CspZ (CRASP-2, BBH06), ErpP (CRASP-3, BBN38) or ErpC (CRASP-4) 378 (Supplementary figure 2) on the cell surface. As wild-type *B. garinii* G1 did not bind FH molecules 379 at all (Figure 1), the signals obtained are attributed to the CRASP produced in the transformed strains 380 (see Supplementary figure 3 for direct comparison of the FH binding pattern of wild-type and 381 CRASP-producing strains). In this study, we observed that spirochetes producing $CspA_{B31}$ and 382 $CspA_{Pko}$ acquired FH and FHL-1 from human serum and FH from mouse serum, which is consistent 383 with previous studies (Bhide et al., 2012). Similarly, we found that $CspA_{Pko}$ was able to interact with 384 FH from horse serum (Figure 2 and Table 2). Note that recombinant $CspA_{Pko}$ or this protein produced 385 on the surface of a *cspA*-deficient *B. burgdorferi* strain is incapable of binding to purified horse FH 386 (Hart et al., 2018). These results imply that the horse FH may have to be in the serum to bind to 387 $CspA_{Pko}$. Additionally, $CspA_{B31}$ was previously shown to be incapable of binding to mouse and horse 388 FH using Far-western blotting (McDowell et al., 2006). As the structure of immobilized FH may be 389 altered or even destroyed, this finding suggests the essentiality of FH's native structure for CspA to 390 display binding activity. Furthermore, consistent with previous studies showing broad spectrum of 391 interaction with various hosts' FH (Rogers and Marconi, 2007), we found that CspZ binds to FH 392 from human, rat, dog, and horse as well as mouse, dog, and to a lesser extent chicken (Figure 2 and 393 Table 2). These findings suggest that both CspA and CspZ play a role in evading different hosts' 394 complement. Binding of FH by CspA and CspZ, which are produced while spirochetes are within the 395 ticks and within the vertebrate hosts, respectively, raises the possibility that these determinants 396 facilitate survival and persistence of LD spirochetes in these distinct and hostile environments (Hart 397 et al., 2018).

398 In contrast to ErpC, which failed to exhibit any binding capability for human and animal FH, ErpP 399 appeared to strongly bind FH derived from dog serum but also displayed weak binding to human and 400 mouse FH (Figure 3 and Supplementary figure 3). Although, binding of murine FH has been shown 401 previously using solid-phase immunoassays with purified proteins (Stevenson, 2002; Alitalo et al., 402 2004; Haupt et al., 2007; Bhide et al., 2009) others investigators did not detect binding of Erp 403 orthologs to mouse FH (Hovis et al., 2006). In addition, potential FH-binding proteins belonging to 404 the Erp protein family have been detected in *B. afzelii*, *B. garinii*, *B. bavariensis*, *B. valaisiana*, and 405 *B. andersonii* which seems to bind FH derived from mice, rats, dogs or cats (Bhide et al., 2009). 406 These proteins might also contribute to the pathogenesis of LD spirochetes.

407 **Conclusion**

408 In closing, the overall FH binding patterns as demonstrated both in previous and the present study 409 largely resembles the pattern of serum susceptibility observed among LD spirochetes corroborating 410 the concept of species-specific, complement-associated host selectivity.

411 **Conflict of Interest**

412 The authors declare that the research was conducted in the absence of any commercial or financial 413 relationships that could be construed as a potential conflict of interest.

414 **Author Contributions**

415 JJM: study design, experimental work, data interpretation, figure preparation, and final approval, 416 YPL: data interpretation, wrote the manuscript and final approval 417 PK: study design, data interpretation, figures and table preparation, drafting the article, wrote the 418 manuscript, and final approval.

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602

603 **Figure legends**

604 **Figure 1. Detection of FH in animal sera.** Sera (2 µl each) of different origins diluted in PBS were 605 subjected to 12.5% glycine-SDS-PAGE and transferred to nitrocellulose. Human and animal FH 606 were detected using a sheep anti-FH serum (dilution 1:500). Purified human FH (500 ng) was used as 607 control. The mobility of the molecular mass standard is indicated on the left.

608 609 **Figure 2. Binding of polymorphic FH to viable LD spirochetes.** Indicated *Borrelia* strains were 610 incubated in human serum as well as in mouse, rat, dog, cat rabbit, cattle, horse or chicken serum. 611 Following incubation, spirochetes were washed extensively, and surface-bound proteins were eluted 612 using 0.1 M glycine (pH 2.0). Both, the last wash (w) and the eluate (e) fractions obtained from each 613 reaction were separated by glycine-SDS-PAGE under non-reducing conditions and transferred to 614 nitrocellulose. FH molecules were detected by using a sheep anti-FH antibody (1:500). Purified 615 human FH (500 ng) served as a control. The mobility of the molecular mass standard is indicated on 616 the left of each panel.

617

618 **Figure 3. Identification of CRASP involved in binding of polymorphic FH molecules.** *Borrelia* 619 strains ectopically producing CspA$_{B31}$, CspA$_{PKo}$, CspA$_{A14S}$, CspZ, ErpC or ErpP were incubated in 620 human serum and various animal sera. Following incubation, spirochetes were washed extensively 621 and surface-bound proteins were eluted using 0.1 M glycine (pH 2.0). Both, the last wash (w) and the 622 eluate (e) fractions obtained from each reaction were separated by glycine-SDS-PAGE under non623 reducing conditions and transferred to nitrocellulose. FH molecules were detected by using a sheep 624 anti-FH antibody (1:500). Purified human FH (500 ng) served as a control. The mobility of the 625 molecular mass standard is indicated on the left of each panel.

626

627 **Figure 4. Identification of FH molecules bound to CRASP proteins.** Purified, polyhistidine628 tagged proteins (10 µg each) coupled onto magnetic beads and incubated with selected animal sera. 629 Immobilized CspA$_{B31}$ and CspZ were incubated with horse (A) or rat (B) serum, CspA$_{PKo}$ with mouse 630 or horse serum (C) and ErpP and ErpC with mouse serum (D). Uncoated beads were also incubated 631 under the same conditions and used as a control to identify nonspecific binding of serum proteins. 632 After extensive washing, bound proteins were eluted with 100 mM glycine and the eluate fractions 633 were separated by glycine-SDS-PAGE, following silver staining. Protein bands indicated were cored 634 from stained gels and proteins were identified by mass spectrometry. The mobility of the molecular 635 mass standard is indicated on the left of each panel and recombinant proteins eluted were indicated 636 on the right.

637 **Tables**

638 **Table 1. Binding of polymorphic FH to diverse borrelial species.** 639

Species	human	mouse	rat	cat	dog	rabbit	cattle	horse	sheep	goat	chicken
Bb* B31	+	+	+	-	+	±	±	+	-	-	±
Ba FEM1-D15	+	+	+	-	+	±	(±)	±	-	+	+
Bg G1 Bs A14S	±	+	+	+	+	±	±	±	+	+	+
Bl MT-M8 Bv ZWU3 Ny3	-	±	-	-	±	-	-	-	-	±	

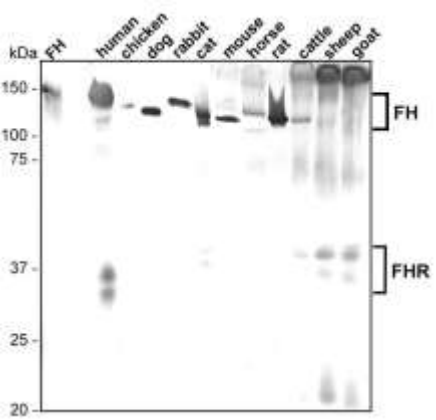
640 641 Bb, *B. burgdorferi*; Ba, *B. afzelii*; Bg, *B. garinii*; Bs, *B. spielmanii*; Bl, *B. lusitaniae*; Bv, *B.* 642 *valaisiana* 643

+, strong signal; ±, weak signal; -, no signal detectable

644 **Table 2. Binding of polymorphic FH to diverse CRASP.** 645

Species	CspA$_{B31}$	human	+	mouse		rat	-nd	dog		horse	+ +	goat		chicken
CspA$_{PKo}$	+ + + -±			---± -±		nd	+ --	---± -+		-+ --		-nd	nd	---± --
CspA$_{A14S}$	CspZ											---		
ErpC	ErpP													

64 +, strong signal; ±, weak signal; -, no signal nd;
6 not determined
64
7
64
8


kDa
FH
human
chicken
dog
rabbit
cat
mouse
horse
rat
cattle
sheep
goat
150
100
75
37
25
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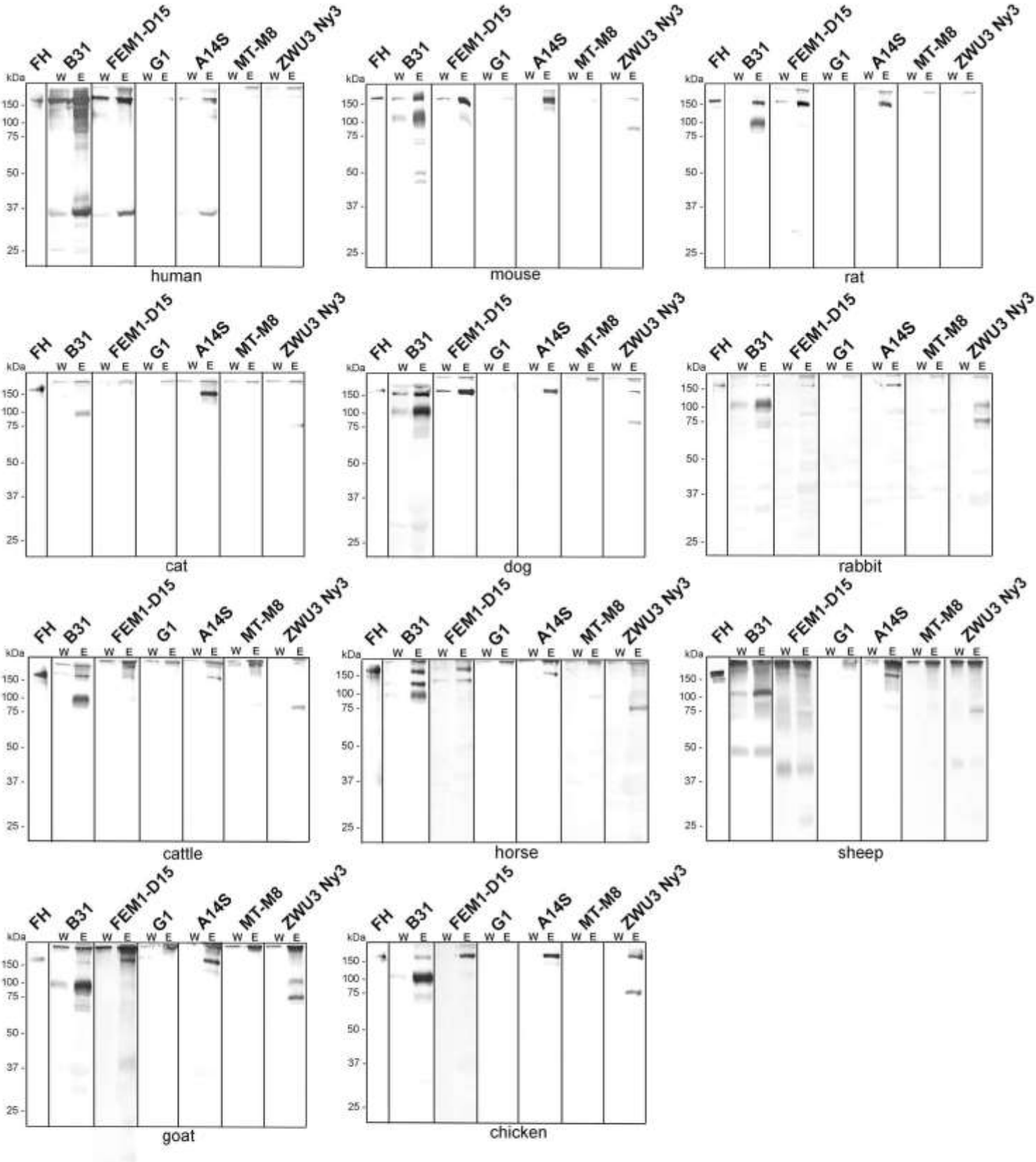
FH
FHR


FH
B31
FEM1-D15
G1
A14S
MT-M8
ZWU3 Ny3
W E
kDa
150
100
75
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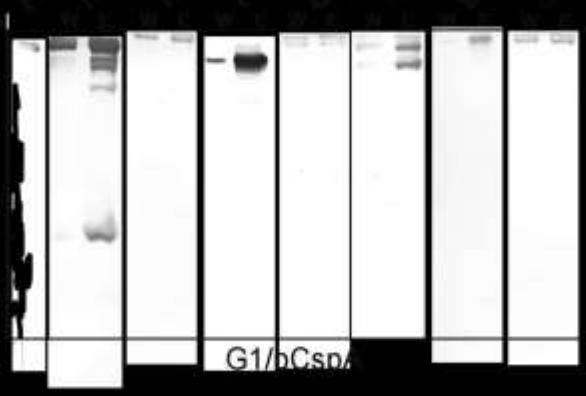
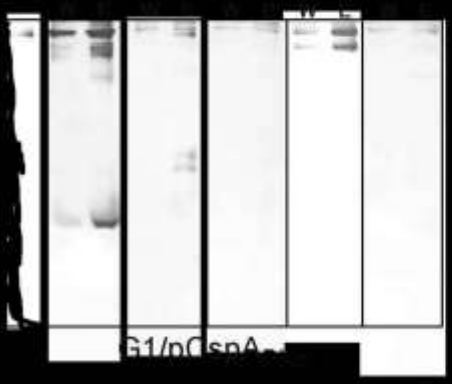
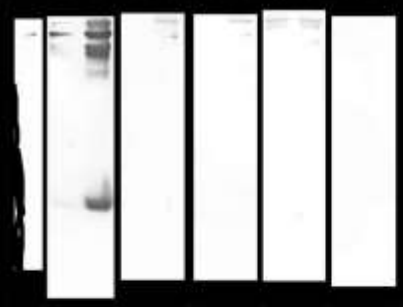
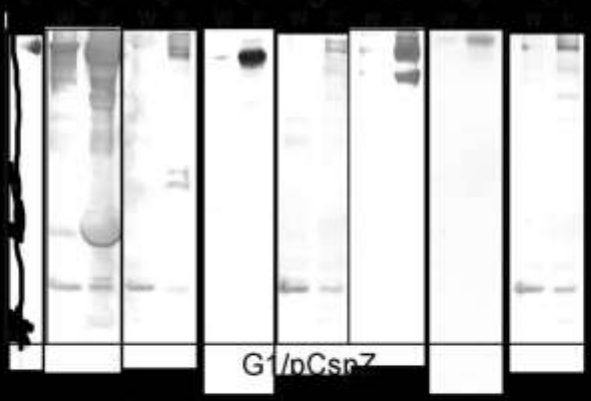
25
human
mouse
rat
cat
dog
rabbit
cattle
horse
sheep
goat
chicken





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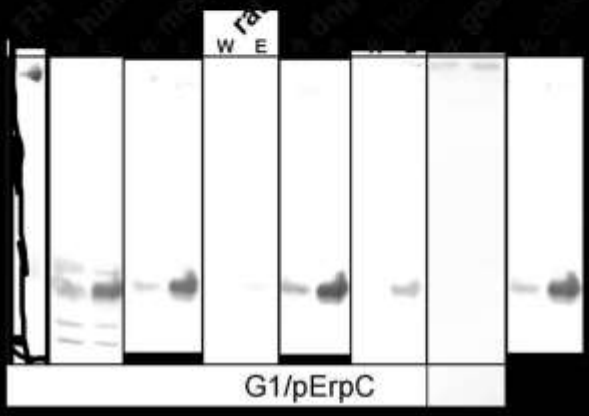
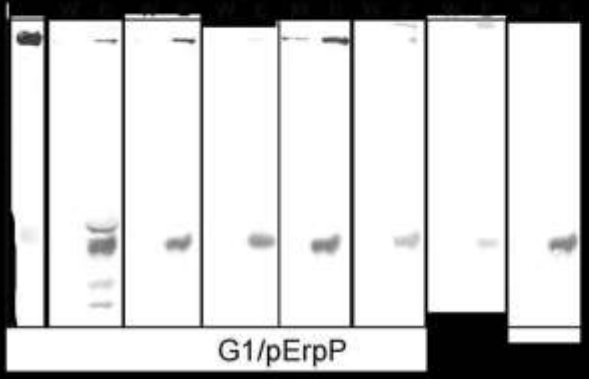

W E
G1/pErpC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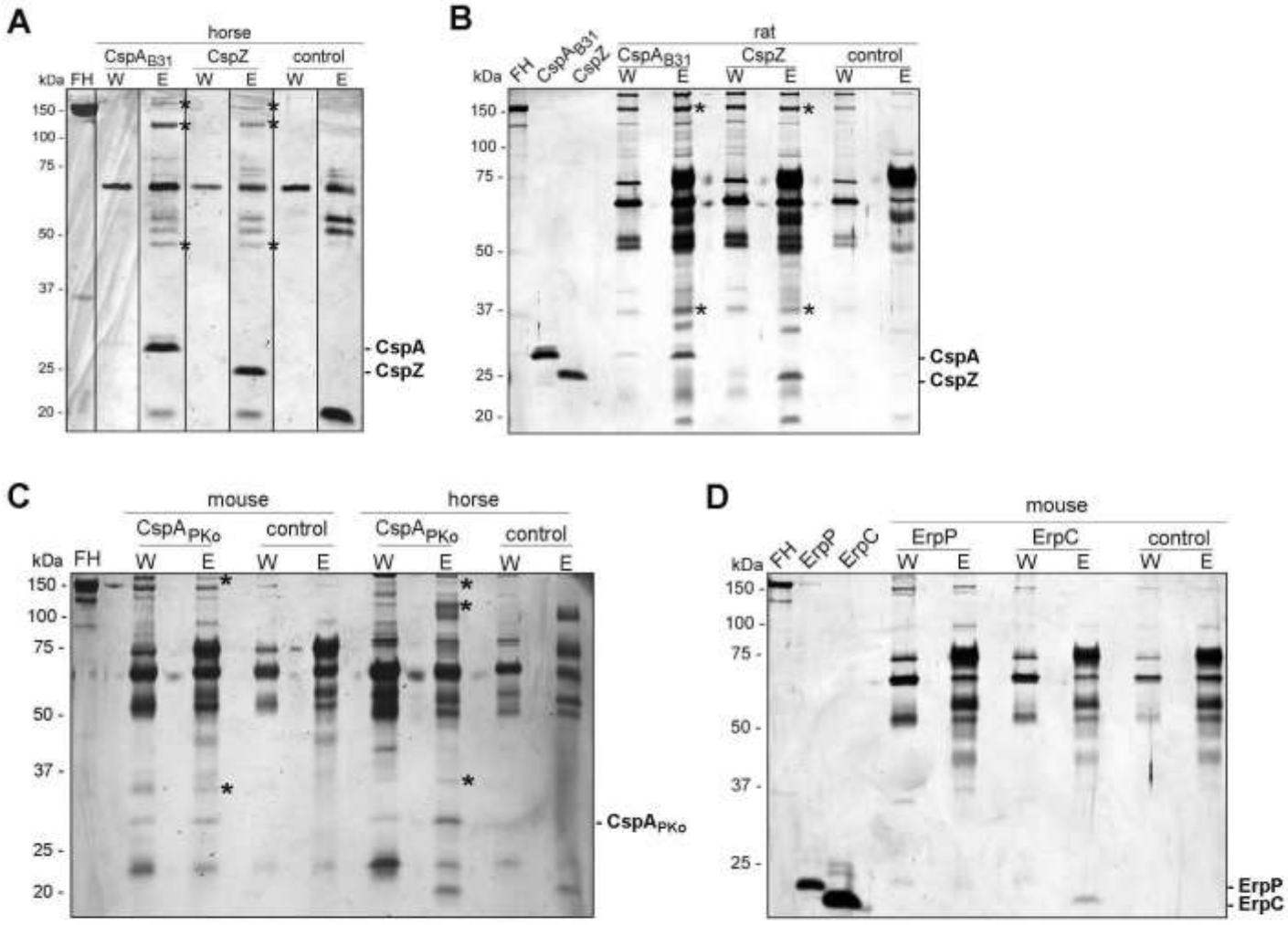
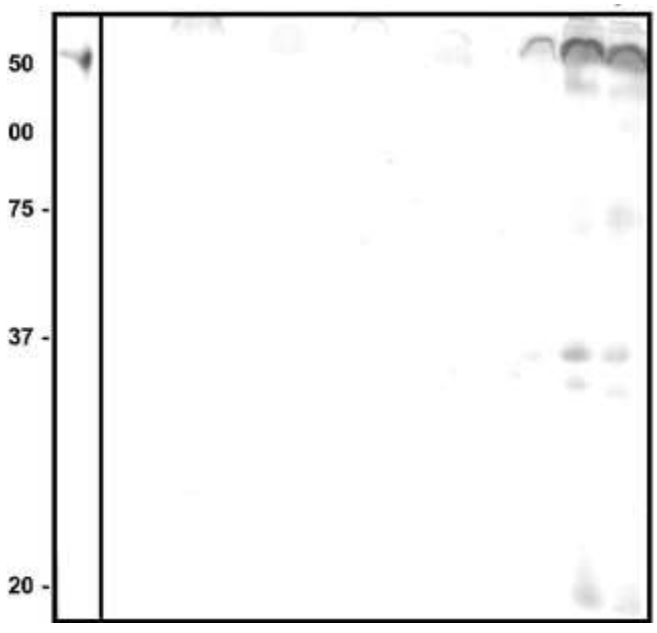
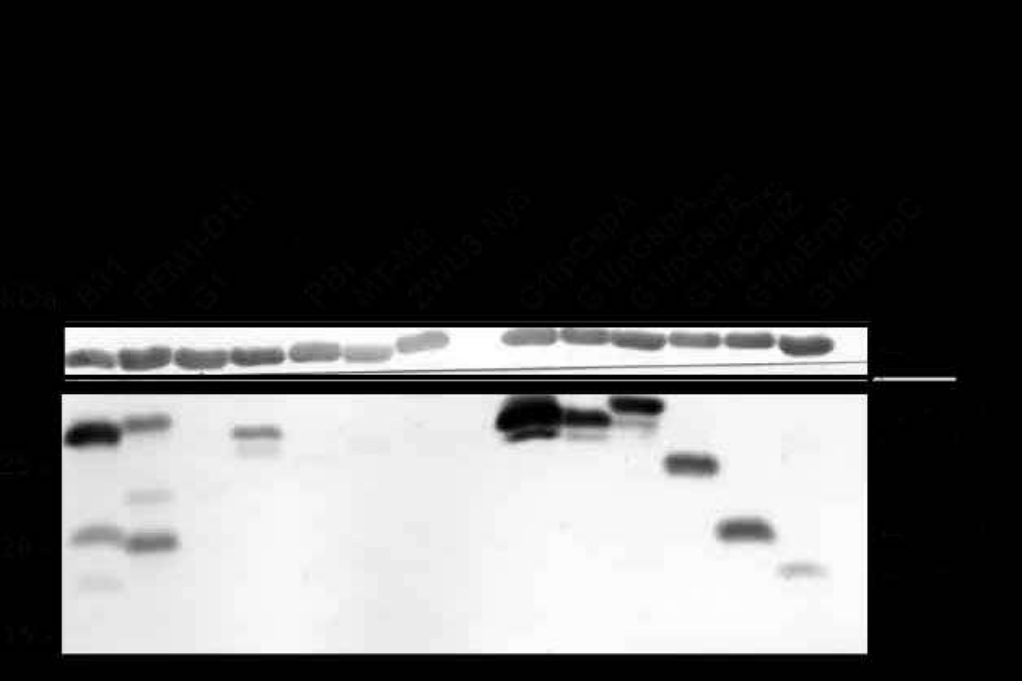

A
horse
CspA$_{B31}$
CspZ
control
kDa FH W E W E W E
150
100
75
50
37
25
20
CspA
CspZ
B
rat
FH
CspA$_{B31}$
CspZ
CspA$_{B31}$
CspZ
control
W E W E W E
CspA
CspZ
C
mouse
horse
CspA$_{PKo}$
control
CspA$_{PKo}$
control
CspA$_{PKo}$
D
mouse
FH
ErpP
ErpC
ErpP
ErpC
control
ErpP
ErpC

Supplementary figure 1

kDa

150 100






W E W E
human
mouse
dog
chicken

Genospecies	Strain	Biological	Origin Geographical	Serum susceptibility[a]
B. burgdorferi	B31	Tick, *I. scapularis*	USA	Resistant
B. afzelii	FEM1-D15	Human, skin	Germany	Resistant
B. garinii	G1	Human, CSF[b]	Germany	Sensitive
B. spielmanii	A14S	Human, skin	The Netherlands	Resistant
B. bavariensis	PBi	Human, CSF	Germany	Resistant
B. lusitaniae	MT-M8	Tick, *I. ricinus*	Portugal	Sensitive
B. valaisiana	ZWU3 Ny3	Tick, *I. ricinus*	Germany	Resistant

Supplementary table 1. Borrelial strains used in the study

[a] Serum resistance was determined by incubation of spirochetes in the presence of 50% NHS by a colorimetric growth inhibition assay and by immunofluorescence staining of deposited complement components C3, C6, and C5b-9 (REF)

[b] CSF, cerebrospinal fluid

Supplementary table 2. Borrelial transformants used in the study

Genospecies	Strain	Ectopically expressed protein	Strain description
B. garinii	G1	CspA (CRASP-1) from *B. burgdorferi* LW2	G1/ pCspA
B. garinii	G1	CspA (CRASP-1) from *B. spielmanii* A14S	G1/pCspA A14S
B. garinii	G1	CspA (CRASP-1) from *B. afzelii* PKo	G1/pCspA PKo
B. garinii	G1	CspZ (CRASP-2) from *B. burgdorferi* LW2	G1/pCspZ
B. garinii	G1	ErpP (CRASP-3) from *B. burgdorferi* LW2	G1/pErpP
B. garinii	G1	ErpC (CRASP-4) from *B. burgdorferi* LW2	G1/pErpC