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1 Title: **Caprine humoral response to *Burkholderia pseudomallei* antigens during acute**
2 **melioidosis from aerosol exposure**
3
4 Short Title: **Caprine humoral response to acute melioidosis from aerosol exposure**
5
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24

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26

27 # Abstract

28

29 *Burkholderia pseudomallei* causes melioidosis, a common source of pneumonia and sepsis in
30 Southeast Asia and Northern Australia, that results in high mortality rates. A caprine melioidosis
31 model of aerosol infection that leads to a systemic infection has the potential to characterize the
32 humoral immune response. This could help identify immunogenic proteins for new diagnostics
33 and vaccine candidates. Outbred goats may more accurately mimic human infection, in contrast to
34 the inbred mouse models used to date. *B. pseudomallei* infection was delivered as an intratracheal
35 aerosol. Antigenic protein profiling was generated from the infecting strain MSHR511. Humoral
36 immune responses were analyzed by ELISA and western blot, and the antigenic proteins were
37 identified by mass spectrometry. Throughout the course of the infection the assay results
38 demonstrated a much greater humoral response with IgG antibodies, in both breadth and quantity,
39 compared to IgM antibodies. Pre-infection sera showed multiple immunogenic proteins already
40 reactive for IgG (7-20) and IgM (0-12) in most of the goats despite no previous exposure to *B.*
41 *pseudomallei*. After infection, the number of IgG reactive proteins showed a marked increase as
42 the disease progressed. Early stage infection (day 7) showed immune reaction to chaperone
43 proteins (GroEL, EF-Tu, and DnaK). These three proteins were detected in all serum samples after

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44 infection, with GroEL immunogenically dominant. Seven common reactive antigens were selected
45 for further analysis using ELISA. The heat shock protein GroEL1 elicited the strongest goat
46 antibody immune response compared to the other six antigens. Most of the six antigens showed
47 the peak IgM reactivity at day 14, whereas the IgG reactivity increased further as the disease
48 progressed. An overall MSHR511 proteomic comparison between the goat model and human sera
49 showed that many immune reactive proteins are common between humans and goats with
50 melioidosis.

51

52 **Author Summary**

53 *B. pseudomallei* infection, the causative agent of melioidosis, results in severe
54 disseminated or localized infections. A systemic study of the humoral immune response to *B.*
55 *pseudomallei* infection using the *B. pseudomallei* aerosol caprine model would help understand
56 the detectable antigenic proteins as the infection progresses. To study the immune response, IgG
57 and IgM antibody responses to whole cell lysate proteins were identified and analyzed. Antigenic
58 carbohydrates were also studied. From the results, this study suggests that the caprine humoral
59 immune response to aerosolized *B. pseudomallei* has similarities to human melioidosis and may
60 facilitate the analysis of the temporal antibody responses. In addition, commonly detected
61 immunogenic proteins may be used as biomarkers for the future point of care (POC) diagnostics.

62

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63 # Introduction

64 *Burkholderia pseudomallei* is a Gram-negative, non-spore forming, aerobic, and motile
65 bacillus [1] and the etiological agent of melioidosis. This disease has emerged as a significant
66 public health threat in Southeast Asia and Northern Australia [2]. Both *B. pseudomallei* and its
67 close relative, *B. mallei*, the cause of glanders, are classified by the Centers for Disease Control
68 and Prevention as category B bioterrorism agents [3]. In Thailand, *B. pseudomallei* is widely
69 distributed in water and wet soils, such as rice paddies [4, 5]. In the two highly endemic regions
70 of Northern Australia and Northeast Thailand, *B. pseudomallei* is responsible for a melioidosis
71 fatality rate of around 10% and 40%, respectively [2, 5, 6]. There is also emerging evidence that
72 melioidosis is endemic in Central and South America [7, 8], southern regions of China [9, 10] and
73 India [11, 12]. The global distribution of *B. pseudomallei* endemicity has been linked to
74 anthropogenic dispersal, both ancient and more recent [13]. Furthermore increased travel [14], and
75 soldiers returning from endemic countries have led to many cases in non-endemic regions such as
76 the USA and Europe [14, 15]. Limmathurotsalkul *et al* reported an evidence-based estimate of *B.*
77 *pseudomallei* global distribution across the tropics; 46 countries were identified as suitable for
78 melioidosis and with environmental suitability for the persistence [16]. Detection of cases outside
79 endemic countries is also now helped by an increased awareness of melioidosis by clinicians
80 worldwide [2]. The main routes of *B. pseudomallei* infection are dermal inoculation, inhalation
81 and ingestion [17, 18]. Melioidosis clinical presentation spans from pneumonia (50% of all cases)
82 [18, 19], and sepsis often leading to septic shock often with multiple abscesses in internal organs
83 such as spleen, liver, kidney and prostate [20], to chronic abscesses in the skin without sepsis [18,
84 21].

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85 Melioidosis is successfully treatable if diagnosed early and correctly. However, confirmed
86 evidence of melioidosis infection in a patient currently relies on isolation and identification of *B.*
87 *pseudomallei* from culture, often requiring use of selective medium [22, 23]. The bacteriological
88 method takes a minimum of 24-48 hours, making it too slow to guide early treatment, which is
89 particularly problematic for severe sepsis with its high mortality rate [2, 23-25]. To improve the
90 diagnosis of melioidosis, a number of techniques have been attempted, such as antigen detection
91 in specimens, antibody detection, molecular and rapid culture techniques [2]. A number of
92 serological tests for antibody detection have also been developed for possible early diagnosis of
93 melioidosis, viz. indirect hemagglutination (IHA), complement fixation (CF),
94 immunofluorescence (IFA) and enzyme linked immunosorbent assay (ELISA) [2, 23, 26].
95 However, there are a number of problems associated with the interpretation of the results and
96 sometimes the tests give false positive results [27]. Therefore, a sensitive, specific and rapid test
97 is still needed for diagnosis of melioidosis, especially for those presenting with severe sepsis [6,
98 23]. Recombinant proteins as diagnostic antigens for serology may offer advantages over IHA,
99 which is currently the only serological assay which used. The current IHA tests have low
100 sensitivity early in infection plus poor specificity in endemic regions due to prior background
101 exposure; i.e. false positives are not uncommon [2].

102 In the last 10 years, melioidosis and *B. pseudomallei* have attracted increased global attention
103 and the related research has likewise increased considerably. However, there are large gaps in our
104 knowledge of the pathogenesis of *B. pseudomallei* infection and the host immune response [6].
105 The comparative studies of an animal model and human infection can provide significant insight
106 on the understanding of pathogenesis and immune response. Through the systemic study of the
107 immune response and antigenic protein detection, potential candidates for the diagnostics of the

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108 infection in the early stage of disease process can be found. The purpose of this study was to
109 analyze the humoral immune response of the caprine model that was aerosol-challenged with *B.*
110 *pseudomallei*, mimicking inhalational melioidosis in humans. Melioidosis is common in goats
111 living in melioidosis-endemic locations and the disease in goats has many parallels to human
112 melioidosis [28]. In this present study, we analyzed antibody reactive proteins and determined
113 quantitative humoral antibody response to melioidosis as measured by western blotting and whole
114 cell lysate ELISA. Antibody generation by the humoral response is a key component of
115 understanding the immune response and a foundation of the potential biomarkers and vaccine
116 development. Understanding the progression of immune response through an animal model could
117 give insight into how the host reacts to *B. pseudomallei* infection and what antigens contribute to
118 immune reactivity.

119

120 ## Methods

121 ### Broad characterization of humoral response

122 Whole cell lysate (WCL) generated from the *B. pseudomallei* infection strain (MSHR511) was
123 used as the antigenic material to characterize the humoral response in goat sera for 1, 4, and 5 days
124 before infection (pre-challenge) and for days 7, 14 and 21 after infection (post-challenge).

125

126 ### Design of *B. pseudomallei* aerosol infection (challenge) study and collection of sera

127 *B. pseudomallei* (MSHR511) isolated from an outbreak of melioidosis in goats on a farm
128 outside Darwin, Northern Territory, Australian goat farm was grown in Muller-Hinton (MH) broth

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129 as described in Soffler *et al.* [29]. The bacterium was harvested in mid-log phase and diluted to 1
130 X $10^4$ CFU/ml as final concentration. The bacteria suspension was then delivered as an
131 intratracheal aerosol [29]. Twelve goats (7 males and 5 females) obtained through a private sale
132 were acclimatized for 1 week before being infected with *B. pseudomallei* under anesthesia (Table
133 1). The goats were monitored by rectal temperature and complete blood counts pre- and post-
134 infection [29]. Pre-infection sera were available from 8 goats. At the different time points of day
135 7, 14 and 21 post infection, 2-3 goats were euthanized and sera were collected with the exception
136 of goat no. 16 planned for day 21 but became moribund. The serum from the goat euthanized on
137 day 16 was included with the day 14 for calculations and analysis.

138

139 Table 1. *B. pseudomallei* goat aerosol challenge study design (Soffler *et al*. 2012)

| Goat no. | Sex | Estimated dose (CFU)* | Day after infection each goat was euthanized | Status at euthanasia |
|---|---|---|---|---|
| 14 | M | 1.2 x $10^4$ | 7 | Subclinical disease |
| 19 | F | 1.2 x $10^4$ | 7 | Subclinical disease |
| 27 | M | 2.3 x $10^4$ | 7 | Febrile |
| 15 | M | 1.2 x $10^4$ | 14 | Febrile |
| 26 | F | 1.1 x $10^4$ | 14 | Afebrile, intermittent cough |
| 16 | M | 1.2 x $10^4$ | $^{+}$16 | Afebrile, moribund, thick nasal discharge |
| 21 | F | 1.2 x $10^4$ | 21 | Subclinical disease |
| 22 | F | 1.2 x $10^4$ | 21 | Subclinical disease |

140 Eight goats were infected with *Burkholderia pseudomallei* strain MSHR511 delivered
141 intratracheally via a nebulizer. At each time point, 2-3 goats were euthanized (the day 16
142 goat was originally planned for the day 21 time point, but became moribund and was
143 euthanized at that time).

144 * Estimated dose delivered to lungs calculated as 10% of the total amount of bacteria placed in
145 the nebulizer.
146 $^{+}$ The Day 16 was analyzed with Day 14 goat for calculation of antibodies in sera.
147 Soffler C, Bosco-Lauth AM, Aboellail TA, Marolf AJ, *et al*. (2012) Development and
148 Characterization of a Caprine Aerosol Infection Model of Melioidosis. Plos One 7(8):
149 e43207. doi:10.1371/journal.pone.0043207

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150 http://www.plosone.org/article/info:doi/10.1371/journal.pone.0043207

151

152 **Bacterial Strain and Growth Conditions**

153 *B. pseudomallei* strain MSHR511 was used in this study. The bacterial strain was grown on
154 minimal media (BD) supplemented with casamino acids and glucose agar plates at 37°C for 35–
155 48 hr. After incubation, single colonies of bacteria were scrapped and suspended in phosphate
156 buffered saline (PBS) solution, pH 7.4 to give a turbidity reading of 1.0-1.2 at OD 600 nm.

157

158 **Purification of Whole Cell Lysate (WCL) Proteins and Antigenic Carbohydrates**

159 WCL proteins were surveyed for immunogenic reactivity using sera from *B. pseudomallei*
160 infected goats by 2-DE western blots. The bacterial cells suspended in PBS buffer, pH 7.4 were
161 washed and centrifuged twice at 16,000 xg for 3 min at 4°C to pellet the cells. The cell pellets were
162 resuspended in lysis buffer (50 mM $KH_2PO_4$, 400 mM NaCl, 100 mM KCl, 0.5% Triton X-100,
163 and 10 mM imidazole), pH 7.4. The bacterial cells were lysed by a freeze and thaw technique using
164 liquid nitrogen and 42°C heat block, respectively, repeated three times. Whole cell lysate proteins
165 were separated by centrifugation at 18,000 xg for 15 min at 4°C. After separation, lysis buffer was
166 exchanged with Tris-HCl buffer, pH 7.8 by centrifugation using microcentrifuge tubes. Protein
167 concentration was determined using the Bradford technique [30] with bovine serum albumen
168 (BSA) as a standard. WCL protein was enzyme treated to remove nucleic acid and precipitated
169 using 15% trichloroacetic acid (TCA) in acetone and centrifuged at 18,000 xg for 18 min at 4°C
170 to purify the proteins. Protein pellets after purification were dissolved in rehydration buffer

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171 containing 7 M urea, 2 M thiourea, 1.3% CHAPS, 30 mM DTT, 0.5% NP40, and 0.25% IPG
172 ZOOM® carrier ampholyte of pH 4-7.

173 For the CPS purification, broth in 2 L baffled Erlenmeyer flasks was inoculated with *B.*
174 *pseudomallei* RR2683 and incubated overnight at 37°C with shaking (200 rpm). Cell pellets were
175 obtained by centrifugation and extracted using a modified hot aqueous-phenol procedure [31].
176 Purified CPS antigens were then obtained essentially as previously described [32].

177 For the OPS extraction and purification, Intron LPS extraction kit reagents were used (Intron
178 biotechnology, South Korea). The cells were collected from agar plates inoculated with B.
179 pseudomallei strain Bp82 and incubated 36 ~ 48 hr. at 37°C. Cells were collected from the plates
180 and transferred to PBS buffer. Cell pellets were obtained by centrifugation 1300 x g. The cells
181 were lysed using lysis buffer, chloroform, and purification as per manufactures instructions. The
182 precipitated LPS was pelleted and suspended in water and sterility was confirmed by plating on
183 5% sheep blood in Tryptic soy agar (Hardy Diagnostics, Santa Maria, CA). The LPS was then
184 further purified using proteinase K and 70% ethanol wash and drying.

185

186 **Two-Dimensional Electrophoresis (2-DE)**

187 The antibody-reacted proteins from matched silver stained gels were identified by mass
188 spectrometry. The strongly reactive immunogenic proteins were cloned, expressed and used in
189 ELISA assays. 2-DE analyses were conducted using isoelectric focusing (IEF) of whole cell lysate
190 proteins on immobilized pH gradient (IPG) gel strips (7 cm, pH 4-7 NL) for the first dimension
191 according to Rabilloud [33] and Gorg *et al.* [34]. IPG strips were passively rehydrated with
192 proteins dissolved in 165µl of rehydration buffer solution (Invitrogen, Carlsbad, CA) containing

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193 100µg protein. IEF was focused on an electrophoresis apparatus (Xcell6™, LifeTech, Carlsbad,
194 CA) for a total of 8000V·hr. Focused proteins in the IPG strips were reduced and alkylated for the
195 second dimensional electrophoresis by reduction and alkylation buffers for two consecutive 20
196 min incubations in 100mM Tris-HCl, pH 8.8 containing 5M urea, 800mM thiourea and 4% SDS,
197 alternately, dithiothreitol (DTT) and iodoacetamide (IAA), each with a concentration of 130mM.
198 The second dimension was separated on 4-20% Tris-Glycine gradient SDS-PAGE gels (Novex
199 gels; Invitrogen, Carlsbad, CA). The gels were run at a constant voltage of 110V for 90 min and
200 visualized using silver staining (Shevchenko *et al*., 1996). The images were captured using the
201 UVP gel documentation system (UVP, Upland, CA). Image analysis was performed using Melanie
202 (GeneBio, Geneva, Switzerland).

203

204 **Western Blot Analysis**

205 Proteins on 2-DE gels were transferred onto nitrocellulose membrane (Immun-Blot™, 0.2 µm)
206 using a dry transfer technique (iBlot®, Dry Blotting System; Invitrogen, Carlsbad, CA) for 10 min
207 at a constant current of 25 mA. The membrane was blocked for 1h in PBS containing 1.5% skim
208 milk. Blotted samples were reacted sequentially with goat serum at a dilution of 1:1000 in PBS
209 containing 1.5% skim milk for 1h, washed three times in PBS, followed by goat anti-human
210 IgG/IgM horse radish peroxidase at a dilution of 1:1000. Protein spots were visualized using a
211 chromogenic substrate, 3, 3′-diaminobenzidine (DAB) solution.

212

213 **In-Gel Trypsin Digestion**

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214 Immunostained protein spots were matched with their corresponding protein spots on silver
215 stained 2-DE gels using Melanie software (Melanie version 7.0.6 software; GeneBio, Geneva,
216 Switzerland). The matched protein spots on silver stained 2-DE gels were excised and destained
217 in 0.02% sodium thiosulfate and 0.5% potassium ferricyanide solution [35]. Gel pieces were
218 washed, dried in 50% acetonitrile, reduced and alkylated in buffer (100 mM Tris-HCl, pH 8.8 with
219 5 M urea, 0.8 M thiourea and 4% SDS) containing 10 mM DTT followed by 100 mM IAA. Proteins
220 were digested overnight in a digestion buffer (50 mM $NH_4HCO_3$, containing 1 mM $CaCl_2$) and
221 12.5 ng/ml trypsin (Promega, Madison, WI) at 37°C. The enzyme treated peptides were extracted
222 using 5% formic acid and 50% acetonitrile. The extraction of the digested peptides was facilitated
223 by vortexing followed by sonication each for 30 minutes.

224

225 **MALDI-TOF Mass Spectrometry**

226 Mass spectrometry analysis was performed using a 4800 Plus Matrix Assisted Laser
227 Desorption Ionization Time of Flight (MALDI-TOF) analyzer (AB Sciex, Toronto, Canada) with
228 a 400Å Anchorchip TM target plate (AB Sciex, Toronto, Canada). Recrystallized α-
229 hydroxycinnamic acid (1 mg/ml) in acetone was diluted 1:2 with ethanol and 1 µl was mixed with
230 0.5 µl of peptides and crystallized on the target. Spectra were analyzed and proteins were identified
231 in a 4000 series Explorer V3.5.3 and Protein Pilot V4.0 software (AB Sciex, Toronto, Canada).
232 Peptide mass fingerprints were searched against RAST annotated protein database. One missed
233 cleavage per peptide was allowed, and the fragment ion mass tolerance window was set to 100ppm.

234

235 **Bioinformatics and Protein Identification**

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236 Using MS peptide sequence results, "immunogenic" proteins were identified with the help of
237 several software programs available online. Protein identity was performed using BLAST
238 (www.ncbi.nlm.nih.gov) against our laboratory created database and NCBI database was used for
239 inferred bioinformatics information of the identified proteins. Theoretical molecular weight and pI
240 values were taken from NCBI database and calculated using Compute pI/Mw tool on ExPASy
241 website (http://web.expasy.org/compute_pi/).

242

243 **Primer Design and PCR**

244 Primers were designed based on the 5' and 3' ends of the gene sequences using online software
245 Integrated DNA Technology. Oligonucleotides were generally between 18-24 bases (S1 Table)
246 with a melting temperature of between $\geq$ 54 – 60°C. PCR was by Pfx DNA polymerase
247 (Invitrogen) [36] to produce blunt-ended PCR products suitable for cloning in pcDNA 3.1
248 (Invitrogen). PCR reactions consisted of 0.3 mM of each dNTP, 1 mM of $MgSO_4$, 3x PCR
249 enhancer solution, 0.3 µM of forward and reverse primers and 0.05 ng/µl template DNA using
250 1.25U/well Pfx DNA polymerase in a final volume of 20 µl. The PCR amplicons were quantified
251 and analyzed by nanodrop spectroscopy and/or gel electrophoresis and working concentrations of
252 DNA made for use in the ligations reactions. The cloned gene inserts were confirmed by Sanger
253 DNA sequencing [37] and transformed into *Escherichia coli* (strain BL21-DE3).

254

255 **Preparation of Recombinant Proteins**

256 **Bacterial cells**

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257 *Escherichia coli* cells were harvested and purified according to the methods previously
258 described [38-40]. *E. coli* cells (strain BL21-DE3) containing his-tagged recombinant proteins
259 were grown at 37°C overnight in 50ml Luria broth containing 100 μg/ml ampicillin. The bacteria
260 were grown in an incubator with an orbital shaker at a speed of ~200 rpm to log phase ($OD_{600}$ =
261 0.5 – 1.0). The cells were then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) of
262 1mM final concentration.

263

264 **Harvesting of *Escherichia coli***

265 The cells were harvested as a pellet by centrifugation at 4,000 rpm for 40 min at 4°C. The
266 supernatant was discarded and the pellets placed in a dry ice/ethanol bath for 5-10 min. The frozen
267 pellets were transferred into 200 ml of neutral (N)-lysis buffer (50 mM Tris-HCl, pH 7.5,
268 containing 0.3 M NaCl and 0.5 mM EDTA) in a 500 ml flask. Lysozyme (0.5 mg/ml) was added
269 to the solution, incubated for 2 hr to digest the bacteria cell wall. The bacteria cells were then lysed
270 by adding 20 ml of 100 mM $MgCl_2$/10 mM $MnCl_2$ salt solution. DNA was removed by adding
271 DNase I (10 μg/ml) to digest DNA for 30 min at RT. The resulting bacteria lysate (~220 ml) was
272 dialyzed overnight with two changes of 4 liter phosphate buffer (20 mM $Na_2HPO4.2H_2O$, 300 mM
273 NaCl), pH 7.4 containing 5 mM imidazole.

274

275 **Purification of Recombinant Proteins by Liquid Chromatography**

276 After overnight dialysis, the supernatant was separated from cell debris by centrifugation at
277 10°C at 10,000 rpm for 30 min. The recombinant proteins were collected as soluble protein. The
278 soluble protein fraction was separated by fast protein liquid chromatography system (BioLogic,

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279 Bio-Rad Laboratories, Hercules, CA) using nickel affinity chromatography. The dialyzed protein
280 solution was applied to a 20 ml nickel nitrilotriacetic acid (Ni-NTA) column and eluted with
281 dialysis buffer containing 500 mM imidazole. The purification of His-tagged recombinant proteins
282 was based on the His-tag protocol[41]. The purification of His-tagged recombinant proteins was
283 monitored by SDS-PAGE and confirmed by western blotting using HisG monoclonal antibody,
284 mouse HRP conjugate (Invitrogen). Protein concentration was estimated by Pierce BCA method
285 with BSA as the standard (ThermoFisher Scientific, Grand Island, NY).

286

287 **Enzyme-Linked Immunosorbent Assay (ELISA)**

288 To understand the humoral response to *B. pseudomallei* in an experimental goat model for
289 melioidosis, we developed the following ELISA assays to quantify the antibody concentration to
290 the whole cell lysate and each individual protein and cell wall antigens. Five of these were protein
291 antigens (dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex, PDHD; thiol
292 peroxidase, TPX; alkyl hydroperoxide reductase subunit C-like protein, AhpC2; enolase, Eno; heat
293 shock protein 60 family chaperone, GroEL1) and two polysaccharides (capsular polysaccharide,
294 CPS and type A O-polysaccharide, OPS A) known to be immunogenic in *B. pseudomallei* (S2
295 Table ) [42]. The polysaccharides were selected based on the fact that *B. pseudomallei* produces
296 both CPS and OPS A which are implicated in *B. pseudomallei* virulence [6, 43, 44].

297 The 96-well immuno plates (Microfluor 2; Fisher Scientific, Pittsburgh, PA) were first
298 coated with individual antigens, namely, recombinant proteins (250ng/well), OPS A (2000
299 ng/well), or CPS (125 ng/well) in PBS coating buffer (137 mM NaCl, 2.7 mM KCl, 10 mM
300 $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.2) (Fisher Scientific, catalog no. BP3991) and left overnight at

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301 4°C. After overnight coating, wells were washed 4 times with 200 µl PBS. The 96 well plates were
302 flicked between the washes to remove all the PBS from the wells and for the fourth wash the plates
303 were smacked onto a stack of paper towels. After washing, the wells were blocked with blocking
304 buffer solution containing 1% (v/v) BSA in PBS for 2 hr at room temperature (RT). After 2 hr
305 incubation the wells were washed 4 times with PBS containing 0.05% (v/v) Tween-20 (PBS/T)
306 and three different goat sera dilutions in blocking solution added to the wells in a volume of 100
307 µl per well. Following the 2 hr incubation with the primary antibody (sera from *B. pseudomallei*
308 infected goats), the wells were again washed 4 times with PBS/T to remove unbound goat serum
309 antibody. A secondary antibody of donkey anti-goat IgG or IgM conjugated with horseradish
310 peroxidase (Santa Cruz Biotechnology, Dallas, TX) was added for immunoglobulin G or M
311 detection. Wells were washed 4 times with PBS/T and the enzyme reaction was detected by adding
312 100 µl substrate (Amplex Red reagent, Life Tech) for a defined time at RT. The fluorescence of
313 the wells was read using a BioTek plate reader at 530/25 excitation and 590/35 emission
314 wavelengths.

315

## 316 Results

317

### 318 1. Initial characterization of total IgG and IgM antibody responses

319 We initially set out to evaluate the general antibody response to *B. pseudomallei* during an
320 aerosol infection of goats. To do this we determined the relative number of the immune reactive
321 antigenic protein spots as an indicator of the diversity of the antigens the immune response
322 generated to and the quantity of the reactive antibodies. This involved ELISA, 2D gel

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323 electrophoresis, and 2D western blot analyses using a whole cell lysate of the infecting strain
324 (MSHR511). Both ELISA and western blot results indicated a much greater humoral response in
325 both breadth and quantity of IgG antibodies compared to that of IgM antibodies throughout the
326 course of infection (Fig 1). Goat IgG antibodies assayed using ELISA on WCL from *B.*
327 *pseudomallei* MSHR511 showed a rapid increase, rising from an average concentration of 11,348
328 µg/ml on day 7 to 26,666 µg/ml on day 14 and reaching a maximum of 35,088 µg/ml by day 21.
329 The concentration of IgM goat antibodies increased over time as well, from a mean of 4,026 µg/ml
330 on day 7 to 8,565 µg/ml on day 14 and reaching 9,590 µg/ml by day 21. The total amount of goat
331 humoral IgM antibody was 2-4-fold less than goat IgG antibodies for all three-time points after
332 challenge with *B. pseudomallei*. In the western blots, the relative number of IgG-reactive protein
333 spots detected after being probed with goat sera mirrored the increase observed using ELISAs for
334 days 7, 14 and 21 (Fig 1). However, the number of antigenic WCL protein spots on IgM western
335 blots remained relatively the same across all time points, with an average of 18 on Day 7, 19 on
336 Day 14, and 12 antigens on day 21. The difference of the IgM immune response between ELISA
337 and 2D western blot might be explained by the particular antigens used by the two methods to
338 detect antibodies. ELISA used whole cell lysate, which included both proteins and carbohydrates,
339 while the western blot used only the whole cell lysate proteins, although this would include
340 glycoproteins. CPS and OPS are carbohydrate antigens known to trigger strong IgM reaction and
341 would only be detected by the WCL ELISA. Overall, the *B. pseudomallei* aerosol challenge
342 induced an increasing and diverse antibody response over the disease progress in the goats.

343

344 Fig 1. Quantitative goat humoral antibody responses to melioidosis as measured by whole cell
345 lysate (WCL) ELISA and western blots.

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346 Goats were challenged by aerosol infection with Burkholderia pseudomallei isolate MSHR511.
347 ELISA and western blot analyses were performed using whole cell lysate (WCL) of cultured
348 MSHR511. Goat sera were drawn prior to challenge and on days 7, 14 and 21 after infection.
349 ELISA results (left y-axis) show the mean antibody titer from all goats per sampling day (7, 14, or
350 21) and are represented by vertical bars for IgG ■, and IgM □. The amount of goat antibody
351 in micrograms (µg) was calculated by subtracting pre-challenge from post-challenge ELISA
352 results (before averaging) relative to the commercially purchased purified standards of goat IgG
353 and IgM. The right y-axis shows the total number of antigenic spots in western blots from each
354 goat, represented by small squares for IgG ■, and IgM □. The total numbers of antigenic spots
355 for IgM and IgG are listed in Table 1.

356

357 **2. Broad survey of goat IgG and IgM responses of pre- and post-infection (challenge)**

358 We characterized the IgG and IgM antibody responses of 8 individual goats' serum samples
359 using 2D electrophoresis and western blots. Our 2DE reference maps prepared from MSHR511
360 WCL allowed us to detect nearly 600 bacterial protein spots by silver stain and to match those
361 spots with antigenic spots detected by IgG and IgM western blots (Sl Fig), thus providing a large
362 population of proteins to investigate. High numbers of immunogenic protein spots were detected
363 (marked in blue), denoting a highly diverse humoral response to bacterial proteins. Immunogenic
364 protein spots detected on the western blots showing strong immunoreactivity against IgG increased
365 in intensity over the 21 days of infection. In contrast, immunogenic protein spots for IgM showed
366 low intensity and remained that way for the entire infection period (Fig 2). Even 2D western blots
367 probed with pre-infection sera showed multiple immunogenic protein spots reactive for IgG (0-20
368 spots) and IgM (0-12) in most goats (Figs 3 and 4). This suggests that the humoral antibody
369 response was previously primed to recognize these or similar antigens from prior bacterial
370 exposure. After infecting the goats with aerosolized *B. pseudomallei,* the number of immunogenic
371 protein spots reacting with goat IgG showed a marked increase over day 7, 14, and 21. The average
372 number of IgG spots shows an increase from 29 protein spots at day 7 to 74 on day 14 and 129
373 protein spots on day 21 (Fig 3).

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374

375 Fig 2. A time series of IgG and IgM responses to infection with *B. pseudomallei*.

376 Goat sera were drawn prior to infection and on the day of euthanasia. Immunoreactive proteins
377 with IgG (A) and IgM (B) were determined by western blot analysis and then mapped onto a silver
378 stained gel. The number of immunogenic protein spots detected (n) is provided at the bottom of
379 each image.

380 Fig 3. Comparison of constant and variable immunogenic proteins among individual goats.

381 All the immunogenic protein spots were identified using Burkholderia pseudomallei MSHR511
382 whole cell lysate protein and eight different goat sera collected during the time course of infection;
383 day 7, 14, and 21 after challenge. All the detected antigenic protein spot count is 282. The number
384 inside each circle denotes the total number of unique detected immunogenic proteins at a particular
385 time point; numbers in parentheses are the total number of pre-challenge immunogenic proteins
386 for that particular goat. The total number of immunogenic proteins detected for each isotype and
387 time point is indicated (N)

388 Fig 4. Comparison of IgG and IgM Immune diversity across eight infected goats against proteins
389 of Burkholderia pseudomallei MSHR511.

390 The average of antigenic spots detected by each individual antibody isotype is shown for each
391 collection time after aerosol challenge and the paired pre-challenge serum sample; 7, 14, and 21
392 days. In addition, spots that were detected by IgG and IgM antibodies were averaged with pre- and
393 post-challenge immune diversity. Vertical Bars represent immunoglobulins M (IgM) ▭ , and
394 immunoglobulin G (IgG) ■ . Circle represents spots detected by both of IgM and IgG ● .

395

396 The number of IgM immunogenic protein spots was much lower compared to IgG. At the pre-
397 infection time point, a mean of about 6 IgM antigenic protein spots were detected but all were very
398 faint (Fig 2). The number of IgM immunogenic protein spots increased 3-fold by day 7 (mean of
399 19 spots), but then remained stable for most goats with a mean of ~25 IgM immunogenic protein
400 spots on day 14 and 21 immunogenic protein spots on day 21 (Fig 4). This result was in agreement
401 with that total IgM response to MSHR511 WCL (above) grew weaker over the 21day time interval.

402

## 403 3. Comparison of constant and variable immunogenic proteins

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404 Over the time course of this challenge study, we found that a subset of proteins raised a
405 consistently strong immunogenic response at each time point for all individual goats, especially
406 for the IgM response: heat shock 60 family protein GroEL, elongation factor -Tu, ATP synthase
407 beta chain, and heat shock protein DnaK. Many antigenic proteins were observed to remain
408 reactive after the initial response was seen. However, there were also many antigenic proteins
409 observed at only a single time point per goat. Considering all of the antigens detected for IgG and
410 IgM, there were 44 antigenic proteins found at all-time points post challenge, 14 of which were
411 also observed in pre-infection sera (Fig 3). There were 6 protein spots that showed reactivity to
412 both of IgG and IgM. Of all of the antigens for IgG, 38 protein spots were detected at all of the
413 post-infection time points, making proteins reactive to IgG antibody as the most common (38 out
414 of 44 proteins). Eight of these antigens were found in the pre-infection sera (Fig 3 and S Table 4).
415 On the other hand, IgM had the least number of antigenic proteins spots present at all-time points
416 with 19 out of 44 proteins and only 5 antigenic proteins reactive in the pre-infection sera (Fig 3).
417 The total WCL reactive IgM was stable with the majority of antigens (19) present throughout Day
418 7 - 14.

419 To place our results in a broader context, we compared the goat immune response in this study
420 with antigens previously described in human melioidosis patients [42]. *B. pseudomallei* infection
421 leads to strong immune responses in both goats and humans, and many antigens elicited a
422 comparable antibody response. According to these data, there are 98 antigenic proteins detected in
423 both of human and goat immune responses, which is more than half the number of antigenic
424 proteins in the human immune response. Considering the total amount of antigens detected with
425 goat IgG and IgM, 98 out of 282 is still higher number in comparison with 135 total detected
426 human antigenic proteins (Fig 5). The difference in the number of the detected antigenic proteins

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427 might be caused by the various biological facets including anatomy, pathophysiology, and
428 genetics, resulting in the different disease progress due to dissimilar route, dose, and immune state
429 prior to infection in/between human infection and animal model. So, the highly common antigenic
430 proteins detected in both human and animal melioidosis imply that this goat model study could be
431 an appropriate animal model to understand disease progress and humoral immune response with
432 mimicking human disease condition.

433

434 Figure 5. Comparison of immunogenic protein spots common with and specific to goat infection
435 sera and human melioidosis patient sera.

436 Immunogenic whole cell lysate proteins from B. pseudomallei MSHR511 were detected by
437 western blot and sera from human sera (note: human protein list was taken from 4 melioidosis case
438 paper [42]). The number inside each circle denotes the total number of detected immunogenic
439 protein spots from human and goat sera. Each number denotes human specific immunogenic
440 proteins, common proteins detected both of human and goat sera, and goat sera specific
441 immunogenic proteins at all the time points after the infection.

442

443 **4. Characterization of pre-infection immunogenic proteins**

444 Antibodies to a subset of identified *B. pseudomallei* antigens were found in pre-infection goat
445 sera. We compared these 17 antigens from MSHR511 against a subset of common Gram-negative
446 bacteria that could possibly be the source of a pre-challenge infection, including *Pseudomonas*
447 *aeruginosa*, *Escherichia coli*, *Campylobacter jejuni*, and *Mycobacterium tuberculosis* (Table 2).
448 None of these 17 *B. pseudomallei* MSHR511 proteins had an amino acid identity above 83% when
449 compared to their homologues in the selected species of Gram-negative bacteria. Amino acid
450 identity was highest between MSHR511 and *P. aeruginosa*, with 13 antigens in the range of 55-
451 83% and four with much lower similarity (33-40%). Eight proteins from all of the four bacteria
452 evaluated had a high identity of > 50% compared to *B. pseudomallei*: ATP-dependent chaperone

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453 protein (ClpB), elongation factor G2 (EFG2), chaperone protein (DnaK), heat shock protein 60
454 family chaperone (GroEL1), ATP synthase alpha chain (Atp), ATP synthase beta chain (AtpD1),
455 Translation elongation factor Tu (EF-Tu), and enolase (Eno) (Table 3). The remaining 9 proteins
456 out of 17 were the least similar to *B. pseudomallei* proteins across the four Gram-negative bacteria,
457 with one exception, viz. *C. jejuni*, S-adenosylmethionine synthetase having < 50% amino acid
458 sequence identity to the *B. pseudomallei* protein. *C. jejuni* had the lowest number of proteins with
459 similarity to *B. pseudomallei*, with only eight proteins out of 17 having > 50% amino acid sequence
460 identity to *B. pseudomallei* proteins. Whether the < 50% amino acid identity of *B. pseudomallei* to
461 *C. jejuni* means reduced immune reaction to the *C. jejuni* protein antigens and its protein epitopes
462 is unknown. Interestingly, amino acid similarity for GroEL was moderately high across all species
463 (58-73%) and may provide insight into the source of the pre-challenge antibody responses we
464 observed.

465 We compared antigenic spots that were detected using pre-infection sera to the immune
466 reactivity over time. We found that antigenic protein spots detected before the aerosol challenge
467 were also detected at time points after the *B. pseudomallei* aerosol challenge. The chaperone and
468 cell division related proteins in particular were detected pre- and post-infection with high signal
469 intensity (S4 Table).

470 We chose to more fully investigate the antibody response to six proteins that induced an
471 antibody response prior to *B. pseudomallei* infection. Using 2D western analysis and spot area (a
472 relative measure of immune reactive intensity), each antibody response appeared to peak at a
473 specific time point after the aerosol infection. Goat sera probed against the IgG western blots
474 showed that the antigenic spot areas for each protein antigen (GroEL, EF-Tu, AtpD1, DnaK, AtoC,
475 and Eno) generally increased through day 21 (Supplemental Figure S2). This paralleled the pattern

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476 observed for the total IgG response to WCL (above). In addition, the antibody response to these
477 antigens occurred by seven days after infection. In contrast, IgM antibody responses for these six
478 antigens typically showed an early peak in spot area on day 7, which then decreased slowly
479 throughout the remainder of the challenge study. The day 7 peak was particularly strong for GroEL
480 and EF-Tu, and both of GroEL and EF-Tu, and AtoC induced a magnitude of response on par with
481 the IgG antibody at that time point. Interestingly, none of the IgM response for these six antigens
482 matched the overall pattern observed in the total IgM count (i.e., none increased from day 7 to day
483 14). This pattern indicates that the IgM response to other antigenic proteins must generally grow
484 stronger over infection and may represent new antibody responses.

485 Table 2. List of identified antigenic proteins detected in pre-infection sera and their percent identity to four other Gram-negative
486 bacteria.

| No. | Protein names | Symbol | Accession number | Number of pre-infection samples that detected the listed antigenic protein (9 possible samples) | | % identity to *B. pseudomallei* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | IgM | IgG | *Pa* | *Ec* | *Cj* | *Mtb* |
| 1 | ATP-dependent chaperone protein ClpB | ClpB | ABN85130.1 | 0 | 1 | 76 | 72 | 51 | 62 |
| 2 | Elongation factor G2 | EFG2 | YP_109810.1 | 0 | 1 | 81 | 80 | 78 | 75 |
| 3 | Chaperone protein DnaK | DnaK | YP_109422.1 | 2 | 7 | 75 | 73 | 56 | 53 |
| 4 | Dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex | PDHD | YP_108895.1 | 1 | 0 | 55 | 35 | 05 | 34 |
| 5 | 30S ribosomal protein S1 | RPS1 | YP_109111.1 | 2 | 0 | 69 | 65 | 26 | 26 |
| 6 | Heat shock protein 60 family chaperone GroEL | GroEL | YP_109293.1 | 4 | 7 | 73 | 72 | 65 | 58 |
| 7 | Cell division trigger factor | CDTF | YP_108024.1 | 2 | 2 | 40 | 39 | 18 | 14 |
| 8 | Sigma-54 dependent DNA-binding response regulator | GroL | CAH39735.1 | 3 | 6 | 33 | 35 | 28 | 06 |
| 9 | Electron transfer flavoprotein, alpha subunit | ETFα | CAH36505.1 | 1 | 0 | 70 | 22 | 05 | 38 |
| 10 | ATP synthase alpha chain | Atp | YP_109991.1 | 1 | 0 | 76 | 70 | 60 | 54 |
| 11 | ATP synthase beta chain | AtpD1 | YP_109989.1 | 3 | 2 | 80 | 79 | 67 | 60 |
| 12 | Translation elongation factor Tu | EF-Tu | YP_109809.1 | 4 | 6 | 83 | 82 | 78 | 73 |
| 13 | S-adenosylmethionine synthetase | S-AMS | YP_106840.1 | 0 | 1 | 65 | 67 | 34 | 54 |
| 14 | Enolase | Eno | YP_108866.1 | 1 | 1 | 68 | 66 | 54 | 58 |
| 15 | Cell division protein FtsA | FtsA | YP_109617.1 | 2 | 3 | 62 | 48 | 29 | 05 |
| 16 | Flagellin, partial | FliC | AAD24677.1 | 1 | 0 | 33 | 29 | 18 | 05 |
| 17 | Alkyl hydroperoxide reductase protein C | AhpC | YP_108693.1 | 0 | 1 | 26 | 27 | 26 | 45 |

487 Pa = *Pseudomonas aeruginosa*; Ec = *Escherichia coli*; Cj = *Campylobacter jejuni*; Mtb = *Mycobacterium tuberculosis*
488 Detected with pre-infection (= pre-challenge) goat sera; 0 = protein absence.

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489 **5. Using seven highly immunogenic antigens to quantify immune responses**

490 We used ELISA assays to more thoroughly investigate seven antigens (PDHD, TPX, AhpC2,
491 Eno, GroEL, CPS and OPS A) that were immunogenic in this goat study and a previous human
492 melioidosis study including 4 patients (Fig 6). The overall reactivity against IgG increased over
493 the time. The amount of IgG antibody detected on day 14 for 4 antigens (PDHD, AhpC2, Eno and
494 CPS) ranged from 110.8 to 133.2 μg/ml, with the remainder antigens having < 48.9 μg/ml (Fig 6).
495 The exception was GroEL. IgG response specific to GroEL were at a concentration of 329.8 μg/ml
496 on day 7 and reached up to 5579.1 μg/ml by day 21 (Fig 6). The concentration of IgM on the other
497 hand, increased mostly from day 14 for CPS with 28.4 μg/ml to 65.2 μg/ml for GroEL antigen.
498 The 3 antigens showing high levels of antigen-specific IgG antibodies compared to IgM by day 21
499 were AhpC2, Eno and GroEL. In contrast, the antigens; PDHD, TPX, CPS and OPS A on day 21
500 had elicited fairly high concentrations of IgM-specific antibodies compared to that of IgG
501 antibodies (Fig 6). The percentage of individual antigens for the goat antibody immune response
502 was calculated relative to the total immune response of the 7 selected antigens (Supplemental
503 Figure S3). IgM antibody responses constituted the highest percent of the immune response with
504 the exception of GroEL antibody immune response, which had very similar percentages for both
505 IgG and IgM antibodies for days 7 and 14 and a significantly high level of IgG goat antibody
506 response for day 21 (Supplemental Figure S3). Because GroEL showed the highest immune
507 response for both IgG and IgM antibodies, we compared the individual antigens antibodies
508 immune response to GroEL (Supplemental Figure S3). The heat shock protein GroEL elicited the
509 strongest goat antibody immune response compared to the other six antigens (PDHD, TPX,
510 AhpC2, Eno, CPS and OPS A) measured in this study, specifically for IgG. This response may be
511 due to prior exposure of the goats to GroEL protein found in other Gram-negative bacteria which

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512 may possess conserved amino acid sequences similar to *B. pseudomallei* GroEL. For goat IgM
513 antibody immune response, three antigens, CPS, GroEL and OPS A showed the strongest immune
514 responses for day 7. The other antigens (PDHD, TPX, AhpC2 and Eno) produced similar goat IgM
515 antibody responses for day 7. However, for day 14 the proportion of goat IgM antibody immune
516 response was very similar for all the 7 antigens (Supplemental Figure S3). But by day 21, CPS
517 showed the highest goat IgM antibody response compared to the other antigens; GroEL1, PDHD,
518 TPX, AhpC2, Eno and OPS A. There was not much change in IgM antibody response for both
519 GroEL and OPS A from day 14 to day 21; while the antibodies to the other antigens remained at
520 similar levels for day 14 and 21.

521 Figure 6. Goat humoral antibody responses to individual protein and polysaccharide antigens.
522 Goat sera were drawn prior to challenge and on days 7, 14 and 21 after infection (goat serum
523 from day 16 was averaged with the day 14 calculations). Goat antibody quantities (μg/ml) was
524 calculated by subtracting pre-challenge from post-challenge ELISA results relative to the
525 commercially purchased purified standards of goat IgG and IgM. (A) PDHD, Dihydrolipoamide
526 dehydrogenase of pyruvate dehydrogenase complex; (B) TPX, Thiol peroxidase; (C) AhpC2,
527 Alkyl hydroperoxide reductase subunit C-like protein; (D) Eno, Enolase; (E) GroEL, Heat shock
528 protein 60 family chaperone; (F) CPS, Capsular polysaccharide; and (G) OPS A, Type A O-
529 polysaccharide.

530

## 531 Discussion

532

### 533 Overall immune response to the challenge

534 The overall humoral immune response of goats showed an increase of antibody intensiy
535 and immune diversity during the infection progression, especially for IgG. In contrast, IgM
536 reaction showed only a slight increase in antibody intensity and the immune diversity decreased

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537 after 14 days post challenge. The overall immune response intensity increase of IgG from the
538 earliest stages of infection implies that there are pre-existing immune conditions for many of the
539 antigens including individual proteins tested in this research. The IgG immune response was
540 greater than the intensity for IgM reactivity. IgM is the primary adaptive immune response to
541 infection while IgG usually develops in the later stage of the infection course after class switching
542 from IgM. However, our investigation showed that the overall IgG humoral response was stronger
543 than IgM at day 7 (initial assay date after goat aerosol infection). This stronger intensity of the IgG
544 response may be explained by protein epitope ability to induce a strong IgG antibody response to
545 protein epitopes of pre-existing conditions such as memory B-cells or pre-primed antibodies which
546 could be produced by cross-reactive epitopes [45].

547 The immune diversity results as determined by western blot analysis showed a slightly
548 different pattern from the overall humoral response determined by ELISA. The count of IgM
549 immunoreactive proteins showed a similarity to IgG reactive protein spots on day 7 and decreased
550 with the decreased intensity of IgM reactive protein spots over the infection progress (Figure 2 and
551 Figure 2S). At day 14 onward, we detected more IgG reactive proteins than IgM reactive ones
552 (Figure 1 and 2). The IgG antibody demonstrated more unique immunoblot protein spots from day
553 14 to 21, whereas, IgM reacted with more protein spots on day 7 compared to IgG (Figure 3).

554 The antibody isotype switch happens between day 7 and day 14 when an antigen(s) persists
555 [46, 47]. Even though there are more detected IgM reactive protein spots on day 7, the intensity of
556 humoral immune response was stronger to IgG. The strong IgG response at the early infection
557 stage like day 7 may arise from the memory cells and prior existing health conditions, which
558 produce faster and stronger humoral immune response with the current infection. The
559 immunogenic proteins sequence comparisons of the selected bacteria showed low similarity to the

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560 selected immunogenic proteins of the infecting strains, which could produce memory cells for the
561 fast and strong immune response. However, there is the potential that conserved sequence or
562 structure epitopes of the selected immunogenic proteins between the selected bacteria and the
563 infecting strain or other antigens cause fast and strong IgG response among the unexamined
564 antigens. The T helper cells contribute the class switch and affinity maturation for stronger
565 immunoreactivity. Other researchers have reported similar results of elevated IgG and IgM post
566 infection with *B. mallei*, a closely related species to *B. pseudomallei* [48].

567

568 **Immunity of commonly detected protein**

569 We found 44 antigenic proteins commonly detected in all of the individual goats, and 8
570 immunogenic proteins detected in pre-infection sera for IgG and IgM using western blot. Those
571 immunogenic proteins are a small portion out of the total 282 immunogenic proteins identified in
572 this research (S4 Table). Each or several goat specific immunogenic proteins were detected many
573 more than the commonly detected immunogenic proteins. These results demonstrate how variable
574 individual immune responses are to even infection of the same infecting strain with the same
575 conditions. The individual animals may vary in their prior exposures to other bacterial infections.
576 In addition, these are outbred animals with different genetics, perhaps creating different immune
577 responses because of the polymorphic differences of MHC alleles. However, commonly detected
578 immunogenic proteins with the different timelines for antibody characterization generate the
579 antibody response to the same or similar epitopes despite the host variability to infection. These
580 common immunogenic proteins are mainly immunodominant proteins (S4 Table) [49]. These
581 common immunogenic proteins give the insight of how the host generally reacts to the infection

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582 and it also demonstrates those proteins' potential as general diagnostic antigens for bacterial
583 infection.

584

585 **Highly immunoreactive antigens**

586 By investigating and identifying highly immunogenic proteins, we found that five proteins
587 (PDHD, TPX, GroEL, AhpC2, and Eno) and two polysaccharides (CPS, OPS A) that showed high
588 immunoreactivity to melioidosis patient sera against four *B. pseudomallei* strains in our previous
589 study (Yi *et al.*, 2016). Those highly immunogenic proteins were also commonly detected proteins
590 using sera from the eight different goat individuals. The western blot and ELISA results for the
591 selected antigens showed a strong ELISA signal for IgG and IgM antibodies from day 14 onwards.
592 Specifically, as for the proteins, GroEL, AhpC2, and Eno induced a stronger IgG response, while
593 the IgM response was strong for all five antigenic proteins (Figure 6). This is probably due to
594 antigenic protein epitopes inducing a stronger IgG antibody response with class switching after an
595 initial immune response [50].

596 Polysaccharides, namely CPS and OPS A were assessed in this study. CPS showed a strong
597 ELISA signal for IgG and IgM antibodies from day 14 onwards. While OPS A antigen
598 demonstrated a good IgM antibody response from day 7. CPS and OPS A are thymus-independent
599 antigens known to activate B-cells to elicit low-affinity IgM antibodies [45]. Therefore the
600 presence of IgM up to 21 days in goat sera can likely be explained by the persistence of
601 polysaccharide antigens for an extended period in the lymphoid tissues, continually stimulating
602 newly maturing B-cells to produce IgM antibodies [46].

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603 As is evident from the results, there were differences in the levels of goat antibody titers
604 expressed against the seven highly immunoractive antigens, which may suggest possible
605 differences in the amounts of each protein and polysaccharide antigen produced by *B.*
606 *pseudomallei* or differences in antigen immunogenicity. Vasu *et al*. [50] has shown that the
607 primary antibody response to *B. pseudomallei* protein and polysaccharide antigens in melioidosis
608 patients was IgG, subclasses IgG1 and IgG2 antibodies, suggesting a Th1 antibody response in
609 both septicemic and non-septicemic melioidosis cases.

610 As the most immunoreactive antigen, GroEL is one of the immunodominant antigenic proteins,
611 which gave a strong immune intensity even at the day 7 (Figure 6). This result could be explained
612 by the presence of memory B cells from a prior non-melioidosis infection with a related organism
613 and may cause a strong immune response rapidly right after the host encountered the antigenic
614 proteins. Amemiya *et al*. [51] using ELISA assays reported a 10-fold increase of IgG antibodies
615 against the heat shock protein (hsp), GroEL. This is in agreement with our results, where GroEL
616 specific goat antibody titer was ~12.8-fold higher than it was to AhpC2 protein; the second highest
617 goat IgG antibody titer to GroEL (Figure 6).

618 Because of this strong IgG and IgM antibody immune response to GroEL, it was decided to
619 examine this result further. First, GroEL family of proteins is reported to contain epitopes that are
620 highly conserved from prokaryotes to humans [52]. GroEL-like proteins are reported to be
621 immunodominant antigens from infectious pathogens, such as *Mycobacterium leprae*, *M.*
622 *tuberculosis*, *Coxiella burnettii* and *Legionella pneumophila* [52]. During infection, a pathogen
623 undergoes selective pressure which increase microbial heat shock protein synthesis to withstand
624 the harsh environment inside the host [53]. This reason is why hsps are major antigens in infectious
625 agents that induce a strong innate and cellular immune response [54].

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626

627 **Prior immune condition and potential cross-reactivity of selected antigens.**

628 Most of the pre-infection sera showed some IgM and IgG immune reactivity to *B. pseudomallei*
629 proteins. Most of the antigenic proteins identified using pre-infection sera were immunodominant
630 proteins [42], and were mostly faint spots of low intensity. Strongly reactive proteins to IgG from
631 the pre-infection sera set were detected, which were mostly immunodominant proteins and also
632 detected at post-infection sera (S4 Table). The goats showing pre-primed immune reactivity
633 showed fewer detected antigenic proteins, thus exhibiting less immune diversity (Figure 4). The
634 results imply that the pre-primed response and immunodominant antigens impede developing a
635 broader antibody response against multiple epitopes of the strain. Those antigens may be cross-
636 reactive with proteins from prior bacteria that the goat came in contact with.

637 As observed with other diseases, potential serological cross-reactivity to proteins of closely
638 related pathogens can produce the immunological memory of the B-cells and cause cross-reactivity
639 in immunoassay, presenting a diagnostic challenge. This cross-reactivity was investigated by
640 comparing the amino acid sequences of seventeen antigenic proteins with the same proteins found
641 in a selected number of Gram-negative bacteria, viz. *P. aeruginosa, E. coli, C. jejuni* and *M.*
642 *tuberculosis* (Table 2). The percent identity of *B. pseudomallei* proteins to the above four Gram-
643 negative bacteria ranged from 5% for PDHD and ETFα of *C. jejuni,* FtsA and FliC of *M.*
644 *tuberculosis* to 83% for EF-Tu of *P. aeruginosa.* The genus, *Pseudomonas* was where *B.*
645 *pseudomallei* was classified before *Burkholderia* was proposed as a new genus [55, 56]. The
646 overall sequence comparison results showed the low similarity of the selected proteins of the
647 chosen bacteria even though the investigated proteins are commonly detected proteins among the
648 bacteria. The low sequence similarity might indicate that the studied proteins do not contribute to

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649 the early stage of immediate antibody response with high humoral response. However, there is
650 potential that there are still cross-reactive epitopes or unstudied antigens causing cross reactive
651 humoral response at the early challenge stage. The faint IgG and IgM immunoreactive protein
652 spots detected in this study support the postulate. Overall, the selected proteins even showed cross
653 reactivity in this research but the intensity and the sequence similarity were very low. Thus, the
654 selected proteins could be the potential biomarkers of *B. pseudomallei* infection (Table 2).

655 In conclusion, this study characterized the overall antibody response of IgG and IgM antibody
656 response, delineating the diversity of immunogenic proteins generated within the host after *B.*
657 *pseudomallei* aerosol challenge. There was a detectable immune response from the early stage of
658 the infection and there are antigens eliciting strong signal intensity for either/both of IgG and IgM.
659 Of the antigens detected, there were 44 commonly detected antigens among the eight individual
660 goats (S4 Table). Many of the detected antigens demonstrated the variation of the immune
661 responses among the goats and during the infection progression. This study also involved
662 expression and purification of five recombinant proteins and 2 polysaccharides detected to be
663 immunogenic using sera from *B. pseudomallei* infected goats and also from human patient sera.
664 Of the seven antigens assayed, AhpC2, Eno and GroEL had a stronger IgG response, while CPS,
665 OPS and TPX showed the stronger the IgM response. Even though we tested the seven potential
666 antigens for their immunoreactivity and the potential as diagnostics biomarkers, we detected
667 additional antigenic proteins with potential as diagnostics targets showing high detection
668 frequency among the goats. Further ELISA assay evaluation of these antigens is needed to
669 determine if they are an improvement over the IHA assay sera and in clinical settings.

670

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# Supporting Information Legends

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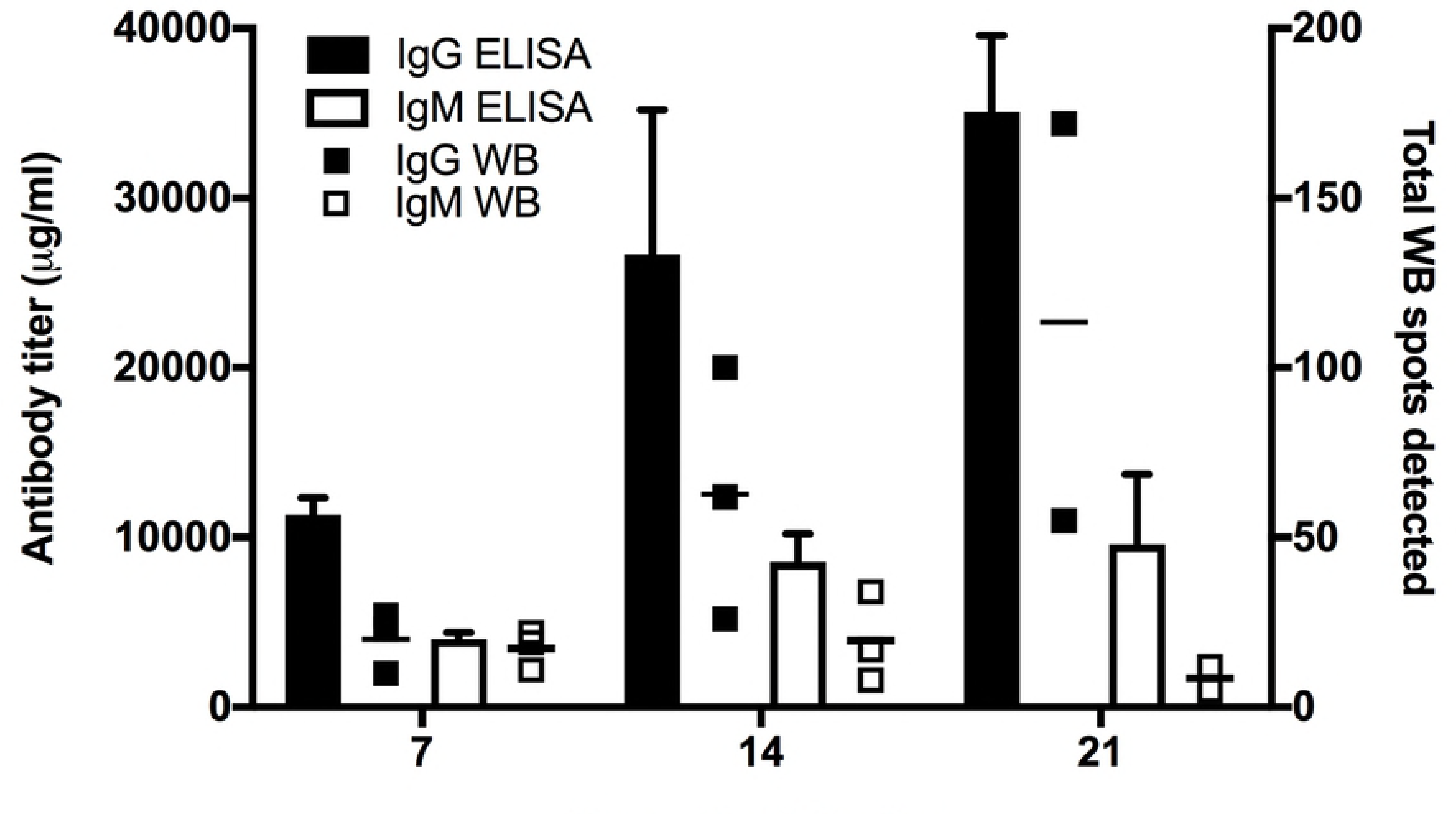
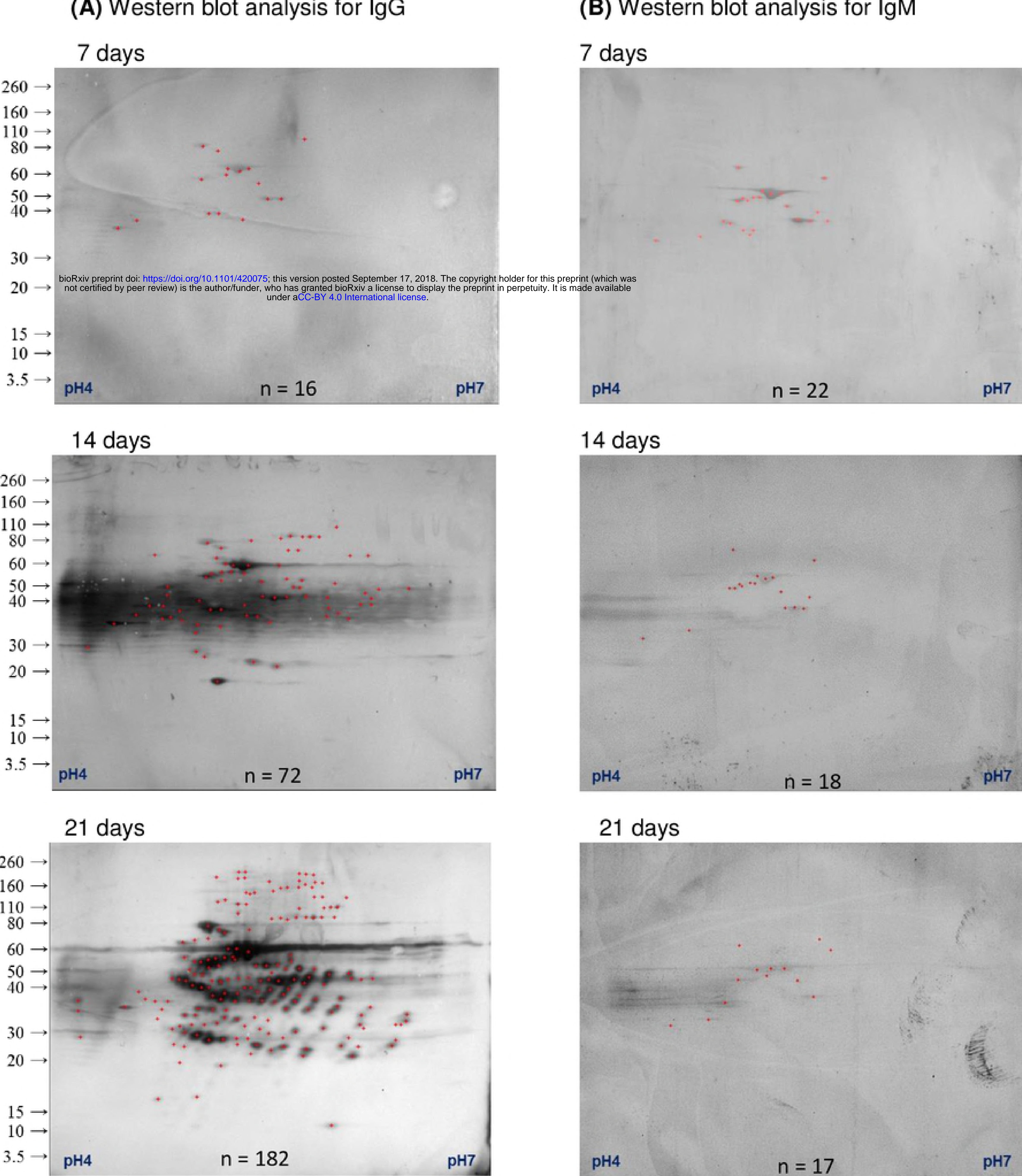
808 S1 Fig. Electrophoretic reference maps of proteins cross reacting to IgG and IgM.
809 The proteins were separated by their isoelectric points followed by size separation by SDS
810 PAGE. Cross reacting spots from western Blots stained for IgG (A) and IgM (B) are mapped
811 onto a gel stained with silver nitrate for protein. Blue marks indicate antigenic proteins that were
812 detected for each. A total of 224 IgG reactive spots and 55 IgM reactive spots detected.
813
814 S2 Fig. Characterization of goat humoral antibody responses to six proteins that were antigenic
815 prior to *Burkholderia pseudomallei* strain MSHR511 infection.
816 Goat humoral antibody responses of individual proteins of B. pseudomallei isolate (MSHR511).
817 Goat sera were drawn prior to challenge or on days 7, 14 and 21 after infection (note: goat sera
818 from day 16 were included with day 14 for calculations). The intensity of goat antibody response
819 was calculated by western blot antigenic protein spot area. (A) Heat shock protein 60 family
820 chaperone, GroL (B) Elongation factor Tu, EF-Tu (C) ATP synthase beta chain (D) Chaperone
821 Protein DnaK (E) Sigmal-54 dependent DNA-binding response regulator, AtoC (F) Enolase, Eno.
822 IgG (Black bars), IgM (White bars). Immunoglobulin G (IgG) ■, and Immunoglobulin M (IgM)
823 □.
824
825 S3 Fig. The relative contribution of seven individual antigens to the humoral antibody response.
826 (A) Immunoglobulin IgG (IgG) antibody response was greatest for GroEL antigen for days 7, 14
827 and 21 followed by Eno on day 14. The humoral IgG response to the rest of the antigens (OPS A,
828 CPS, AhpC2, TPX and PDHD) was typically <5% for each antigen. The high antibody response
829 to GroEL antigen is thought may be due to memory B-cells being present in the immune
830 circulation before challenge with *Burkholderia pseudomallei* strain MSHR511. (B) IgM antibody
831 response was highest to GroEL antigen followed by OPS A for day 7 and these two antigens
832 declined, respectively, for days 14 and 21. IgM response to CPS was relatively the same for days
833 7 and 14 but was most elevated compared to other antigens (Eno, AhpC2, TPX and PDHD) on
834 day 21. The antibody response to the rest of the antigens (Eno, AhpC2, TPX and PDHD) was
835 very similar.
836
837 S1 Table. Primers used to amplify the genes in this study
838
839 S2 Table. List of purified antigens used in immunoassays
840
841 S3 Table. Antigenic proteins identified from an extract of *Burkholderia pseudomallei* using a
842 non-redundant sequence database and MALDI-ToF mass spectrometry data.
843
844 S4 Table. Immunity (immune frequency) of antigenic proteins over days after infection.


40000
30000
20000
10000
0
Antibody titer (μg/ml)
IgG ELISA
IgM ELISA
IgG WB
IgM WB
200
150
100
50
0
Total WB spots detected
7
14
21
Days after infection

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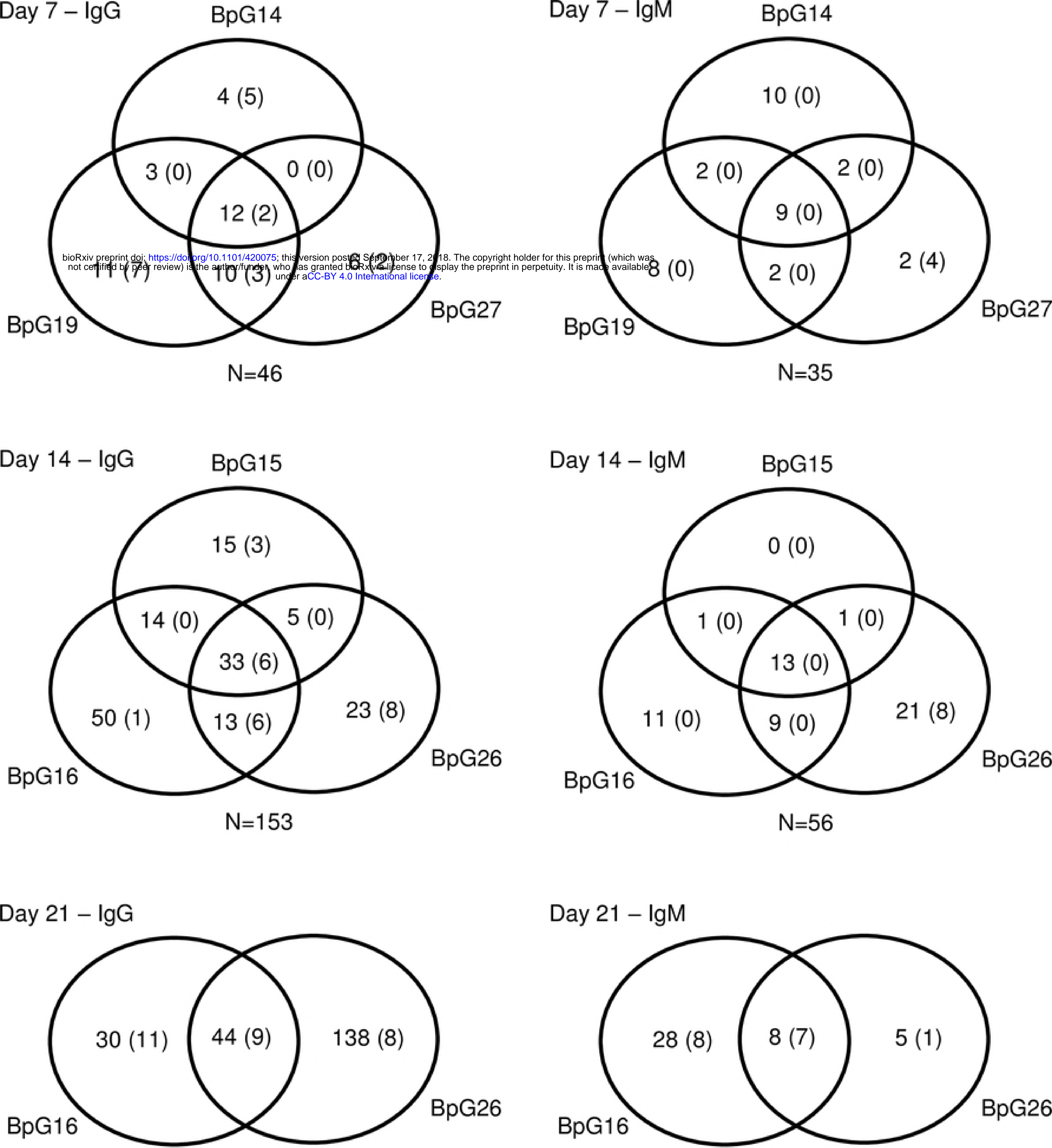


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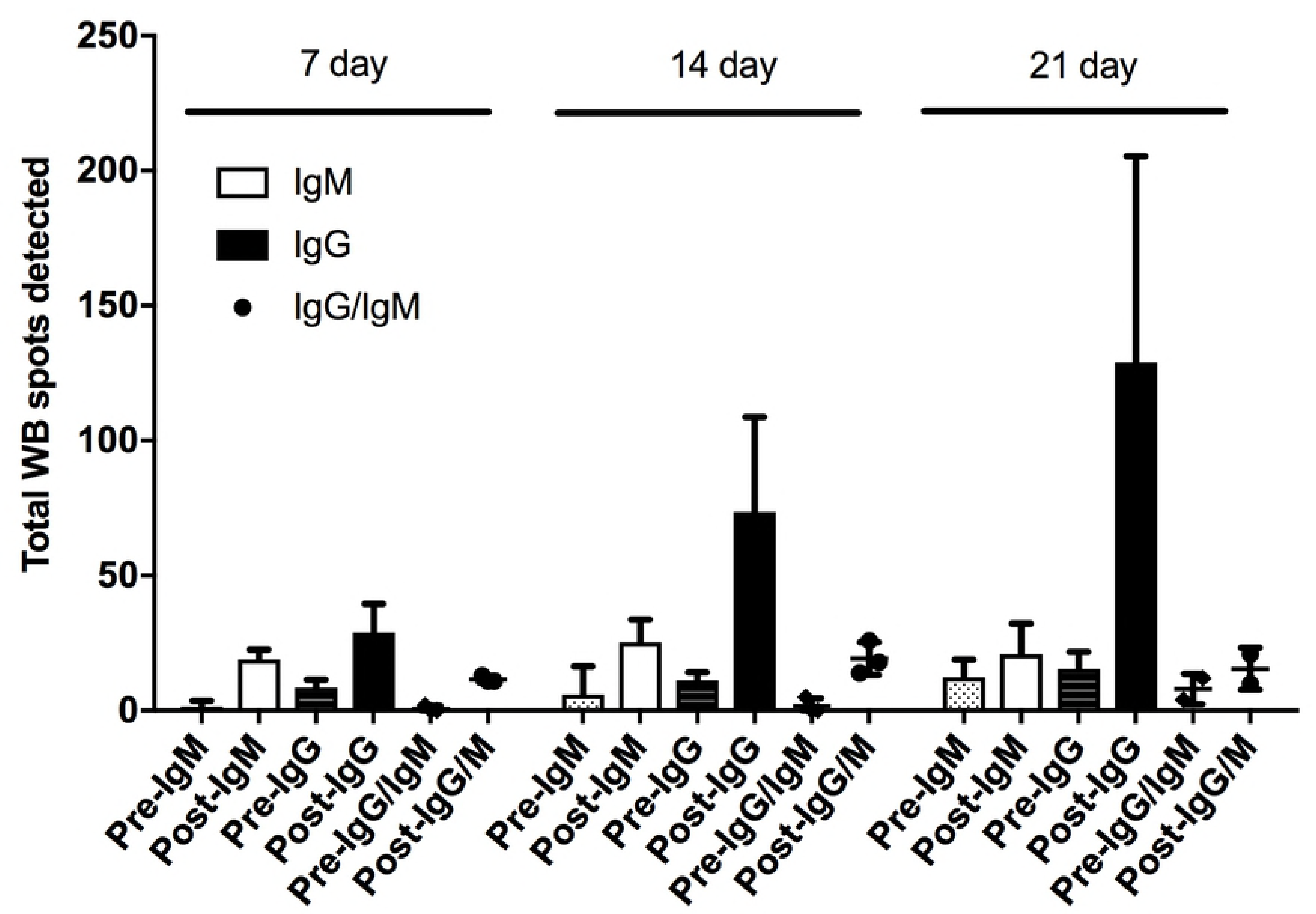

Total WB spots detected
250
200
150
100
50
0
7 day
14 day
21 day
IgM
IgG
IgG/IgM
Pre-IgM
Post-IgM
Pre-IgG
Post-IgG
Pre-IgG/IgM
Post-IgG/M
Pre-IgM
Post-IgM
Pre-IgG
Post-IgG
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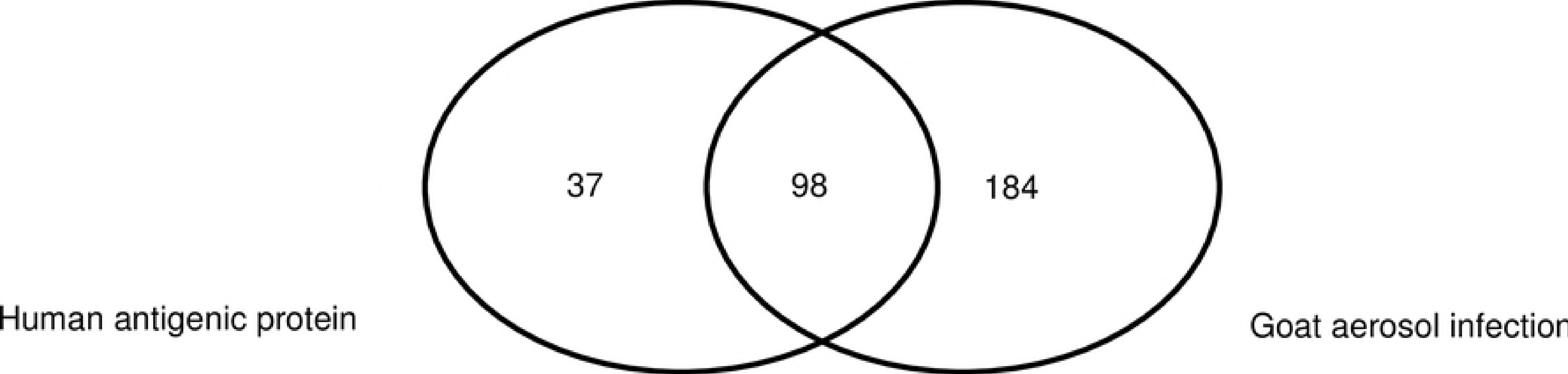
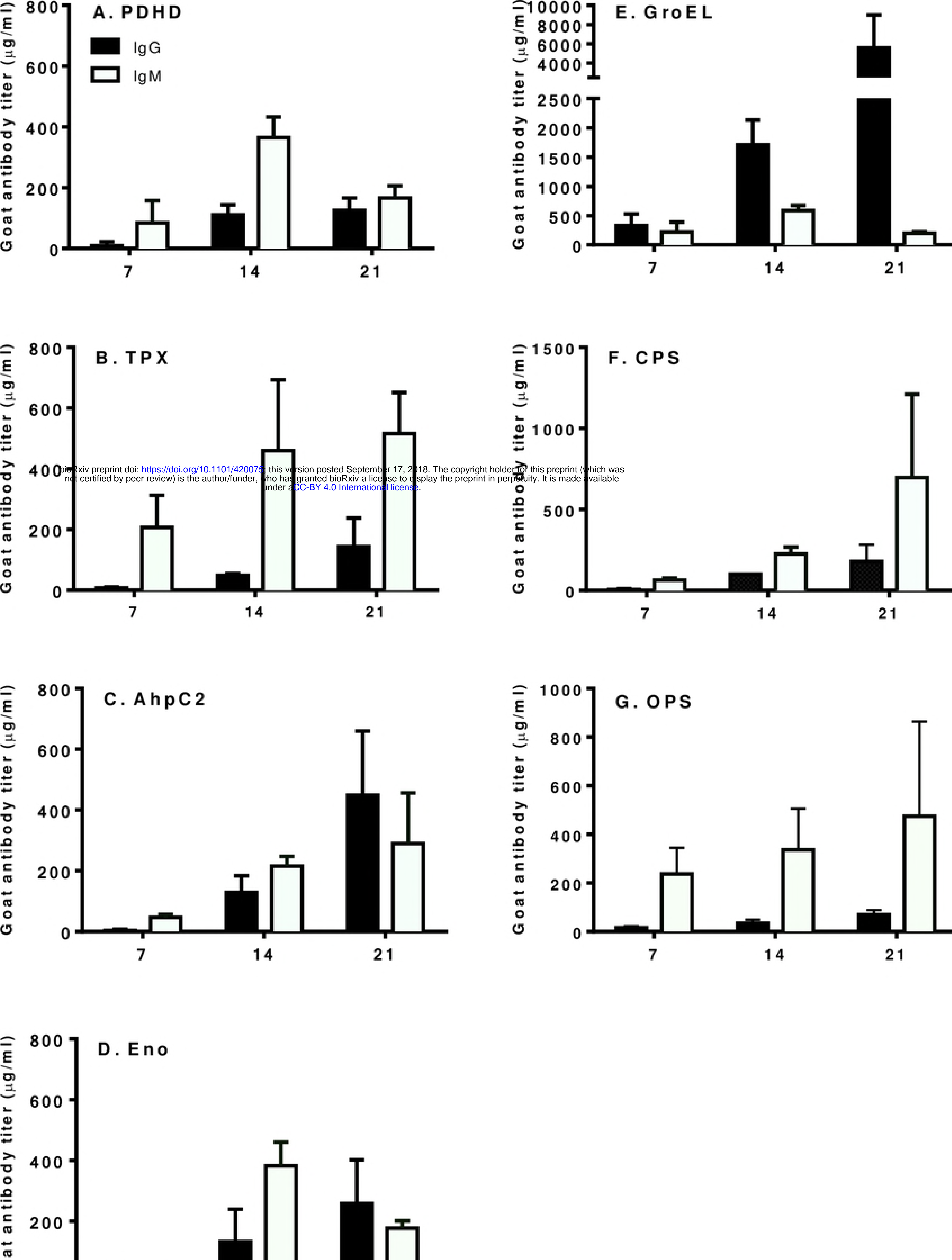
Pre-IgG/IgM
Post-IgG/M


37
98
184
Human antigenic protein
Goat aerosol infection



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(A) IgG

pH4

pH7

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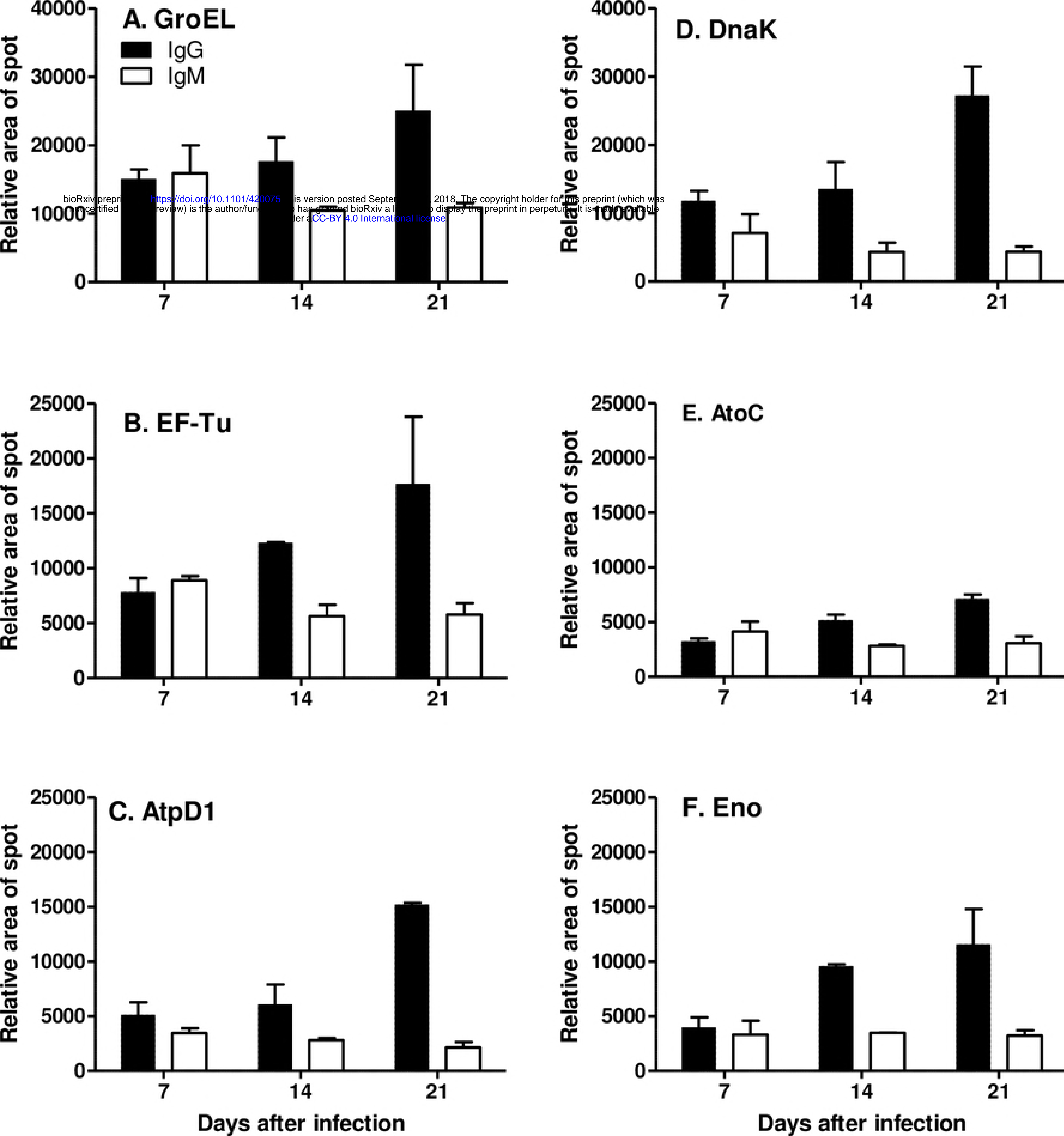
(B) IgM

pH4

pH7

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(A)

100%
80%
60%
40%
20%
0%

7
14
21

OPSA
CPS
Eno
AhpC2
TPX
PDHD
GroEL

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(B)

100%
80%
60%
40%
20%
0%

7
14
21

OPSA
CPS
Eno
AhpC2
TPX
PDHD
GroEL