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1 **Serological and spatio-temporal analysis of anthrax in**
2 **Mongolia**

3 (モンゴルにおける炭疽の血清学的解析と時空間分析)

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2022

Contents

29		
30	Abbreviations	3
31	Unit abbreviations	5
32	Notes	6
33	Preface.....	7
34	CHAPTER I: Development of ELISA based on <i>Bacillus anthracis</i> capsule biosynthesis	
35	protein CapA for naturally acquired antibodies against anthrax	10
36	Summary	10
37	Introduction.....	11
38	Materials and methods	14
39	Horse test sera	14
40	Ethical statement.....	14
41	<i>In silico</i> analyses	15
42	Construction of strains and plasmids	15
43	Protein expression and confirmation of immunogenicity	18
44	Expression and purification of CapA322.....	18
45	CapA322 in-house ELISA	19
46	Statistical analysis	20
47	Results.....	21
48	Screening of CDS-encoding secreted and surface-associated proteins on pXO2 plasmid.	
49	21
50	Expression and immunoreactivity of candidate proteins	29
51	Evaluation of CapA322 as ELISA antigen	33
52	CapA322-ELISA and PAD1-ELISA	39
53	Discussion	41
54	CHAPTER II: Risk Factors and Spatio-temporal Patterns of Livestock Anthrax in Khuvsgul	
55	Province, Mongolia.....	46
56	Summary	46

57	Introduction.....	47
58	Materials and methods	49
59	Data source.....	49
60	The cattle anthrax prevalence per area in Khuvsgul Province by districts (1986–2015)	50
61	The spatial mean and standard deviational ellipse analyses	50
62	Multi-distance spatial cluster analysis	50
63	Kernel density estimation analysis.....	51
64	Statistical analyses	52
65	Results.....	53
66	Old and recent trends of anthrax between 1986 and 2015.....	53
67	The spatio-temporal anthrax pattern and high-risk areas.....	55
68	Positive association between cattle population, temperature, and anthrax case numbers	64
69	Cattle population, mean summer temperature, and outbreak magnitude.....	64
70	Discussion.....	68
71	General conclusion.....	73
72	Acknowledgments.....	76
73	Reference	78
74		
75		

76 **Abbreviations**

77

AMP	Adenosine monophosphate
Bcbva	<i>Bacillus cereus</i> biovar anthracis
CBB	Coomassie brilliant blue
CapA	Capsule biosynthesis protein CapA
CapA322	C-terminal region of CapA
cAMP	Cyclic adenosine monophosphate
CDS	coding sequence
SCVL	State Central Veterinary Laboratory
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EF	Edema factor
ELISA	Enzyme-linked immunosorbent assay
GST	Glutathione S transferase
GIS	Geographic information system
HiConEnv	Upper confidence envelope
IgG	Immunoglobulin G
IPTG	Isopropyl β -D-thiogalactopyranoside
KDE	Kernel density estimation
KPL	3,3',5,5'-tetramethylbenzidine substrate
LB	Lysogeny broth
LF	Lethal factor
LoConEnv	Lower confidence envelope
MES	2-(N-morpholino) ethane sulfonic acid
Mw	Molecular weight
NaCl	Sodium chloride
NC	Negative control
NLPC/60	papain-like cell wall hydrolase domain
NLRP1	NLR family, pyrin domain containing 1
OD	Optical density
OIE	World Organization for Animal Health

PA	Protective antigen
PBS	Phosphate buffer saline
PC	Positive control
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonyl fluoride
PMB	Polychrome methylene blue
PAD1	Recombinant protective antigen domain 1
PAGE	Polyacrylamide gel electrophoresis
rpm	Rotations per minute
RT	Room temperature
SDE	Standard deviational ellipse
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TMB	3,3',5,5' tetramethylbenzidine
TMHMM	transmembrane-hidden Markov model
TB	Terrific broth
Vac_H	Vaccinated horse serum
WB	Western blotting
WHO	World Health Organization
γ-D-PGA	poly- γ -D-glutamic acid

79 Unit abbreviations

%	percent
g	gram
kDa	kilodalton
km²	square kilometer
km	kilometer
L	liter
max	maximum
min	minimum
ml	milliliters
mM	millimolar
ng	nanogram
µg	microgram
µl	microliter
°C	degree Celsius

80

81 **Notes**

82

83 The contents of Chapter I and Chapter II have been published in *PLoS One*.

84

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87 Higashi. **Development of ELISA based on *Bacillus anthracis* capsule biosynthesis protein
88 CapA for naturally acquired antibodies against anthrax.** *PLoS One*. 2021;
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93 Natsagdorj Norov, Baasansuren Lkham, Jargalsaikhan Enkhtuya, Hideaki Higashi. **Risk
94 factors and spatio-temporal patterns of livestock anthrax in Khuvsgul Province,
95 Mongolia.** *PLoS One*. 2021; 16(11):e0260299.

96 Preface

97 Humans have a long history with anthrax, and outbreaks were recorded in the Bible as the
98 sixth plague in Egypt around 5000 BC (Ben-Noun, 2002). German microbiologist Robert Koch
99 first cultured the causative agent *Bacillus anthracis* in 1876; since then, anthrax has been
100 studied for some 140 years (Koch, 1876). Regardless of this, pathogenesis, ecology, and
101 epidemiology of the disease in animals remain surprisingly poorly understood.

102 Anthrax causative agent, *B. anthracis*, is a Gram-positive, spore-forming, non-motile, rod-
103 shaped bacterium (Mock and Fouet, 2001). *B. anthracis* has two different lifestyles, the
104 vegetative bacilli and a dormant spore. Anthrax is not directly transmissible between animals;
105 instead, herbivores such as cattle and horses are infected primarily via ingesting a high dose of
106 spores from grazing pastures. Within the host, *B. anthracis* spores germinate and form
107 vegetative bacteria capable of multiplying and producing virulence factors, which lead to
108 potentially fatal disease (Hugh-Jones and de Vos, 2002). When the infection is fatal to the host,
109 vegetative cells of *B. anthracis* are shed into the soil, forming infectious spores capable of long-
110 term survival. Furthermore, the places where an animal dies and/or their carcass disposal sites
111 most often serve as the source of future infection (Beyer and Turnbull, 2009). In addition,
112 spores are highly resistant to environmental stress, such as UV light, heat, and chemical
113 disinfectants (Stephens, 1998).

114 Cattle exhibit dose-dependent clinical symptoms against orally given *B. anthracis* spores
115 (Schlingman et al., 1956). Oral administration of 10^9 spores culminates in peracute death in
116 cattle, but lower doses cause either subclinical or inapparent infections. In comparison,
117 carnivores and birds are pretty resistant and often have naturally acquired antibodies to anthrax.
118 These antibodies are less common in herbivores, which may have led to the assumption that
119 anthrax is always fatal to these animals (Hugh-Jones and de Vos, 2002). However,
120 susceptibility to anthrax varies widely among species because of inherited genetic factors,
121 immunological state, coinfection, and physiological condition. Even within a susceptible
122 species, there is considerable evidence that individuals survive exposure to anthrax (Turnbull
123 et al., 1992).

124 Inhaling the spores, handling and slaughtering infected livestock, or consuming
125 contaminated meat and meat products can lead to pulmonary, cutaneous, and gastrointestinal
126 anthrax disease in humans (Kaufmann and Dannenberg, 2002). In addition, an unusual route
127 of infection via intravenous injection of heroin contaminated with *B. anthracis* spores has been
128 reported to be associated with several deaths in England, Scotland, and Germany (Price et al.,
129 2012).

130 Anthrax is distributed across the globe, from the tropics to frigid polar regions. Outbreaks
131 behave very differently worldwide, and the seasonality, frequency, and dynamics of anthrax
132 are poorly understood. After an animal vaccine invention, anthrax has been drastically
133 decreased in many parts of the world, and anthrax has become a minor concern of veterinary
134 and public health. However, the adverse consequence was a loss of interest and increasing
135 failure to diagnose and stop animal mass vaccination. Today, anthrax is still regarded as
136 endemic among the livestock in Africa, Asia, and several countries of the former Soviet Union
137 and tends to re-emerge in countries due to climate changes (Fasanella et al., 2010). The re-
138 emergence of anthrax in Siberia showed how an increase in summer air temperature triggered
139 the outbreak through the effect of permafrost thawing, which might release the spores from
140 formerly infected carcasses (Liskova et al., 2021). This phenomenon entails that the process of
141 permafrost degradation and thawing rates of its active layer potentially lead to the re-
142 emergence of pathogens, particularly anthrax in the northern latitude of the globe, where the
143 warming effect is more pronounced. Hence, there are interdisciplinary research is necessary to
144 understand this disease.

145 Anthrax is hyperendemic in the northern region and endemic in areas of Mongolia except
146 the desert region in the south (Odontsetseg et al., 2007). Frequent outbreaks occur in wildlife
147 and livestock, taking their toll on the income of pastoralist communities and threaten public
148 health security in Mongolia. Mongolian traditional livestock includes sheep, goats, cattle,
149 horses, and camels, and the total livestock number was estimated as 67 million in 2020, which
150 is twenty-two times higher than the human population (National Statistics Office of Mongolia).
151 Livestock in Mongolia are reared under the free-ranging system, and nomadic pastoralists
152 move several times a year searching for water and pasture for their herds. Livestock is the main
153 source of income and food in the pastoralist community, and it produces more than 80% of the
154 agricultural product of Mongolia. However, livestock anthrax cases have been annually

155 reported since the 1990s, damaging the income of pastoral communities. Human cases are
156 usually reported after the animal incidence.

157 Despite the current practice involving livestock vaccination, movement control, and
158 disinfecting or burying affected animal carcasses (General Authority for Veterinary Services,
159 2019), the persistence and frequency of anthrax outbreaks in livestock remain an issue of great
160 concern. Thus, to mitigate anthrax infection, there is a need to design a more prudent control
161 strategy, such as interdisciplinary research with a holistic approach. Furthermore, the global
162 distribution of anthrax demands the convergence of diagnostic technologies to aid detection
163 and surveillance worldwide. However, one major limitation towards achieving anthrax control
164 is the lack of a diagnostic tool devoted to detecting the naturally acquired antibodies of anthrax
165 in animals. Altogether, addressing the problem of anthrax and its associated challenges requires
166 a new approach supported by unprecedented innovations.

167 This dissertation aims to provide different entry points pertinent to the effective control of
168 anthrax in Mongolia and beyond. In Chapter I, a new ELISA test capable to specifically
169 diagnose anthrax natural infection caused by virulent *B. anthracis* and non-cross reactive to
170 anthrax vaccine-induced antibodies was established to assist anthrax serosurveillance and
171 improve estimates of burden and at-risk populations in Mongolia. In Chapter II, to gain insight
172 into the anthrax epidemiology in Mongolia, spatio-temporal patterns of anthrax in livestock
173 between 1986 and 2015 were analyzed based on the carcass burial sites of animals that died of
174 anthrax in Khuvsgul Province, which showed the highest anthrax incidence rate in Mongolia.
175 The study determined the historical hotspots of carcass sites, places that need to be prioritized
176 in public health intervention, and the factors that precipitate the recurrence of anthrax outbreaks.
177 Moreover, it provided essential epidemiological data that will inform policy and evaluation of
178 current anthrax control measures in Mongolia.

179 **CHAPTER I: Development of ELISA based on *Bacillus***
180 ***anthracis* capsule biosynthesis protein CapA for naturally**
181 **acquired antibodies against anthrax**

182

183 **Summary**

184 Anthrax is a zoonotic disease caused by the Gram-positive spore-forming bacterium
185 *Bacillus anthracis*. Detecting naturally acquired antibodies against anthrax sublethal exposure
186 in animals is essential for anthrax surveillance and effective control measures. Serological
187 assays based on protective antigen (PA) of *B. anthracis* are mainly used for anthrax
188 surveillance and vaccine evaluation. Although the assay is reliable, it is challenging to
189 distinguish the naturally acquired antibodies from vaccine-induced immunity in animals
190 because PA is cross-reactive to both antibodies. Although additional data on the vaccination
191 history of animals could bypass this problem, such data are not readily accessible in many cases.

192 In this study, a new enzyme-linked immunosorbent assay (ELISA) specific to antibodies
193 against capsule biosynthesis protein CapA antigen of *B. anthracis*, which is non-cross-reactive
194 to vaccine-induced antibodies in horses was established. Using *in silico* analyses, I screened
195 coding sequences encoded on pXO2 plasmid, which is absent in the veterinary vaccine strain
196 Sterne 34F2 but present in virulent strains of *B. anthracis*. Among the 8 selected antigen
197 candidates, capsule biosynthesis protein CapA (GBAA_RS28240) and peptide ABC
198 transporter substrate-binding protein (GBAA_RS28340) were detected by antibodies in
199 infected horse sera. Of these, CapA has not yet been identified as immunoreactive in other
200 studies to the best of my knowledge. Considering the protein solubility and specificity of *B.*
201 *anthracis*, the C-terminus region of CapA was prepared, named CapA322, and developed
202 CapA322-ELISA based on a horse model. Comparative analysis of the CapA322-ELISA and
203 PAD1-ELISA (ELISA uses domain one of the PA) showed that CapA322-ELISA could detect
204 anti-CapA antibodies in sera from infected horses but was non-reactive to sera from vaccinated
205 horses. The CapA322-ELISA could contribute to the anthrax surveillance in endemic areas,
206 and two immunoreactive proteins identified in this study could be additives to the improvement
207 of current or future vaccine development.

208 **Introduction**

209 Anthrax is a widely distributed zoonotic disease that occurs in every populated continent
210 (Carlson et al., 2019). After the invention of an effective animal vaccine, outbreaks declined in
211 many parts of the world; however, anthrax remains endemic in some regions of Africa and
212 Asia (Chen et al., 2016; Driciru et al., 2018; Kanankege et al., 2019; Sitali et al., 2018). Anthrax
213 has a substantial economic and public health impact on countries with limited resources to
214 develop anthrax control measures (Vieira et al., 2017). For example, Mongolia is a resource-
215 limited country where anthrax is endemic, except the semi-desert and desert areas in the south
216 (Okutani et al., 2011).

217 Anthrax is caused by an encapsulated Gram-positive spore-forming bacterium, *Bacillus*
218 *anthracis*. *B. anthracis* infects a wide range of mammals, including humans. Herbivores
219 such as antelopes, buffaloes, cattle, sheep, goats, and horses are susceptible to anthrax, whereas
220 birds and canids are comparatively resistant (Mock and Fouet, 2001). The infection occurs in
221 herbivores through browsing, ingestion, or inhalation of a high dose of spores from grazing
222 lands; besides, carnivores are usually exposed through scavenging an infected animal carcass
223 (Hugh-Jones and de Vos, 2002). In addition, the role of tabanid flies and other blood-feeding
224 insects in anthrax transmission in animals has been demonstrated (Ganeva.D.J., 2004; Turell
225 and Knudson, 1987). Humans often acquire anthrax infections from infected animals or
226 materials contaminated with spores, such as wool, hide, and meat (WHO, 2008).

227 Two plasmids, pXO1 and pXO2, are essential for the virulence of *B. anthracis*. After
228 ingestion or inhalation by the host, spores of *B. anthracis* germinate into vegetative cells that
229 secrete the three pXO1-encoded toxin components: protective antigen (PA), edema factor (EF),
230 and lethal factor (LF). PA is a host cell receptor-binding protein (Mogridge et al., 2002), EF is
231 adenylate cyclase and a potent inhibitor of immune cell function (Leppla, 1982), and LF
232 cleaves mitogen-activated protein kinase and hinders cellular signaling pathways (Duesbery et
233 al., 1998). The pXO2 encodes genes involved in poly- γ -D-glutamic acid (γ -D-PGA), which
234 protects the bacteria from the host phagocytic cells (Drysdale et al., 2005; Jelacic et al., 2014).
235 A lack of either of the plasmids results in a significant loss of virulence of the bacterium
236 (Glinert et al., 2018). The Sterne 34F2 strain, which lacks pXO2 but still secretes the three

237 major toxin components and retains immunogenicity with less virulence; thus, it is commonly
238 used in anthrax veterinary vaccine production (Turnbull, 1991).

239 It was previously understood that anthrax mostly resulted in host death; however, field
240 surveys in anthrax-endemic areas have suggested that herbivores infected with a sublethal dose
241 of *B. anthracis* spores could survive (Turnbull et al., 1992). Furthermore, studies have indicated
242 that exposure to a sublethal dose of spores likely elicits adaptive immune responses to *B.*
243 *anthracis* (Hampson et al., 2011; Lembo et al., 2011). However, the effect of sublethal infection
244 on the adaptive immune response of animals to anthrax is still poorly understood; scaled field
245 studies are needed to detect naturally acquired antibodies in animals.

246 There is currently no serological test dedicated to distinguishing naturally acquired
247 antibodies against *B. anthracis* from vaccine-induced immunity. Most of the available assays
248 for anthrax serological diagnosis have been developed based on the PA of *B. anthracis* (Marcus
249 et al., 2004; Reuveny et al., 2001). However, due to PA secretion from both naturally virulent
250 and vaccine strains of *B. anthracis*, PA-based assays cannot distinguish the antibodies acquired
251 by vaccination from those acquired by natural infection. So far, additional information such as
252 the vaccination history of herds must differentiate the source of antibodies. However, while
253 anthrax vaccination history is easily obtainable in countries with a good farm management
254 system, accessing such data in developing countries is challenging because of poor record-
255 keeping. For instance, herders in Mongolia have nomadic pastoralism where households
256 migrate to various places to seek pastures for their livestock. Such movements often lead to the
257 loss of important animal records, including vaccination history. In addition, animal
258 identification systems in most local communities are largely uncoordinated, thus complicating
259 the discrimination of vaccinated animals from unvaccinated animals.

260 Herein, a new enzyme-linked immunosorbent assay (ELISA) test for detecting antibodies
261 against capsule biosynthesis protein CapA of *B. anthracis*, which is non-cross-reactive to
262 vaccine-induced antibodies was developed. First, I screened genes on the pXO2 for ELISA
263 antigen candidates because of the differences between virulent *B. anthracis* (pXO1⁺, pXO2⁺)
264 and anthrax vaccine strains (pXO1⁺, pXO2⁻). Further, capsule biosynthesis protein CapA
265 (GBAA_RS28240) and peptide ABC transporter substrate-binding protein (GBAA_RS28340)
266 were identified as immunoreactive to hyperimmune horse anti-*B. anthracis* serum. I also found

267 that the C-terminus region of CapA, named CapA322, is soluble and specific to *B. anthracis*.
268 Therefore, the CapA322-ELISA was developed using the antigen.

269 **Materials and methods**

270 **Horse test sera**

271 Hyperimmunized antiserum of horses infected with virulent *B. anthracis* Pasteur No. 1
272 strain (pXO1⁺, pXO2⁺), named PC1 in this study (also known as the Ascoli serum), was
273 obtained from the National Institute of Animal Health, Japan.

274 Two naturally infected horse sera (PC2 and PC3) were provided by the Institute of
275 Veterinary Medicine of Mongolia. According to records, the two horses showed clinical
276 anthrax symptoms, and *B. anthracis* was isolated from the nasal discharge samples and
277 confirmed with polymerase chain reaction (PCR) (Beyer et al., 1995; Stear, 2005).

278 To prepare serum samples of vaccinated horses, four 2-year-old female horses (Vac_H1
279 to Vac_H4) were subcutaneously injected with Sterne vaccine containing 2×10^6 spores/ml, as
280 recommended by the manufacturer (KM Biologics, Japan) at Japan Racing Association. Serum
281 samples were collected before and after vaccination. After vaccination, serum samples were
282 collected every three days starting from day 3 to day 45 postvaccination. Later, samples were
283 obtained every seven days until day 56 postvaccination. The antibody response to PA of *B.*
284 *anthracis* was evaluated by PAD1-ELISA (Simbotwe et al., 2019). Serum samples with high
285 anti-PA-D1 immunoglobulin G (IgG) titers were obtained on day 21 postvaccination (named
286 Vac_H1D21–Vac_H4D21 for the four horses, respectively) and were used to test the cross-
287 reactivity of CapA322-ELISA.

288 Two naive horse sera (NC1 and NC2) were purchased from Invitrogen, USA, and KOHJIN
289 BIO, Japan, to serve as negative controls.

290 **Ethical statement**

291 Ethical clearance and research approvals were obtained from the Animal Experiment
292 Committee of Equine Research Institute, Japan Racing Association (Reference: 20-32), and
293 immunization and sampling were conducted according to approved protocols.

294 ***In silico* analyses**

295 All coding sequences (CDSs) on pXO2 of *B. anthracis* Ames ancestor strain (GenBank
296 accession number: AE017335) were obtained from the National Center for Biotechnology
297 Information and analyzed to predict their cellular localization, secretion, and functional
298 domains. PSORT was used to predict cellular protein localization (Nakai and Horton, 1999),
299 and SignalP was used to predict cleavable N-terminus signal peptide regions (Emanuelsson et
300 al., 2007). Lipoprotein signal peptides identified by LipoP (Juncker et al., 2003), and
301 membrane-associated proteins with transmembrane helix were predicted by the
302 transmembrane-hidden Markov model (TMHMM) algorithm (Krogh et al., 2001). The
303 domains and active sites of the proteins were identified by PROSITE (Hulo et al., 2008). The
304 criteria for selecting candidate genes focused on secreted and surface-exposed CDS products.
305 All CDSs were scored based on the presence of the predicted signal peptide, lipoprotein signal,
306 TMHMM domain, putative domain information, and localization on the cell surface or
307 extracellular secretion. Eight CDSs that scored three or more were selected.

308 **Construction of strains and plasmids**

309 Primers for amplification of candidate CDSs were designed and synthesized by Integrated
310 DNA Technologies (Table 1). Candidate CDSs were amplified from genomic DNA of *B.*
311 *anthracis* CZC5 (Ohnishi et al., 2014) using KOD FX Neo (TOYOBO, Japan). The vector F
312 (5'-GGGTCGACTCGAGCGGCCGCA-3') and vector R (5'-
313 GGATCCCAGGGGCCCTGGAACAG-3') were used for the linearization of pGEX-6P-2
314 plasmid, which expresses the glutathione S-transferase (GST) fusion protein. The amplified
315 genes were incorporated into the plasmid using Gibson Assembly (Gibson et al., 2009). The
316 amplified PCR products were analyzed on 1% agarose gel and extracted using the QIAquick
317 Gel Extraction Kit (Qiagen, Germany). The purified fragments with 5' complementary
318 overhangs were combined in a 1:2 molar ratio of the vector, and the fragments were inserted
319 with 20 µl of Gibson Assembly Master Mix (New England Biolabs, MA, USA) and additional
320 nuclease-free water to obtain a reaction volume of 40 µl. The reaction was then performed at
321 50°C for 15 min, and the assembled constructs were desalted for an hour using an MF-Millipore
322 membrane filter 0.025 µm (Merck, Germany) on distilled water. *Escherichia coli* 10β (New
323 England Biolabs, MA, USA) was transformed with each construct through electroporation. The

324 cells and constructs were mixed and transferred to the Bio-Rad 0.1 cm gap Gene Pulser cuvettes.
325 Electroporation was performed using a Gene Pulser Xcell Electroporator (Bio-Rad, CA, USA)
326 set to 1,800 V, 25 μ F, and 200 Ω . After electroporation, the cells were immediately transferred
327 to 1 ml of lysogeny broth (LB) and incubated at 37°C for an hour at 180 rpm. Next, 200 μ l of
328 the culture was plated on LB agar supplemented with 50 μ g/ml ampicillin for selection. The
329 DNA from constructs was purified using a QIAprep Spin Miniprep Kit (Qiagen, Germany),
330 and the sequence of each gene of interest was confirmed by Sanger sequencing using a 3130
331 xl Genetic Analyzer (Applied Biosystems, MA, USA).

332 **Table 1. Primer list**

Primer name	Locus tag	Primer sequence
TZ_F006	GBAA_RS28005	5'-CTGTTCCAGGGGCCCTGGGATCCATGGCAGCTACACAAGAAACAGCC-3'
TZ_R006		3'-TGCGGCCGCTCGAGTCGACCCTCATCTTGGTACTCTTCGAATTCCTG-5'
TZ_F012	GBAA_RS28035	5'-CTGTTCCAGGGGCCCTGGGATCCATGGCTACTATGAAAATAAAAAGAATGG-3'
TZ_R012		3'-TGCGGCCGCTCGAGTCGACCCTTATCTTCTACGCAATTGATCTGTCC-5'
TZ_F029	GBAA_RS28110	5'-CTGTTCCAGGGGCCCTGGGATCCATGTGTAAAAGGTTAAGTTTTATTGGCTG-3'
TZ_R029		3'-TGCGGCCGCTCGAGTCGACCCTTAATTTGTTTTCTTAAATATATTTTGTATTAAACG-5'
TZ_F043	GBAA_RS28165	5'-CTGTTCCAGGGGCCCTGGGATCCATGAACACTAAGGGAATTATAGCAAAAC-3'
TZ_R043		3'-TGCGGCCGCTCGAGTCGACCCTTAGTAATAAGCAGACATGTTATGACCTTTC-5'
TZ_F060	GBAA_RS28240	5'-CTGTTCCAGGGGCCCTGGGATCCATGAGACGAAAATTGACATTTCAAG-3'
TZ_R060		3'-TGCGGCCGCTCGAGTCGACCCTCAAGTTGTTGTCTCCACTGATAC-5'
TZ_F068	GBAA_RS28275	5'-CTGTTCCAGGGGCCCTGGGATCCATGAAAATAATAAAAATTGTTGATTACATATGG-3'
TZ_R068		3'-TGCGGCCGCTCGAGTCGACCCTATTTAGAAATTACTGTAGCTAGAACACGTTTCG-5'
TZ_F083	GBAA_RS28340	5'-CTGTTCCAGGGGCCCTGGGATCCATGTTAAAAAAGTAACGCCTATTGTGG-3'
TZ_R083		3'-TGCGGCCGCTCGAGTCGACCCTTATTTCTTCACTCAGTCCACTTATAG-5'
TZ_F100	GBAA_RS28430	5'-CTGTTCCAGGGGCCCTGGGATCCATGAAGTATAAAACGCATCTTACAACAAG-3'
TZ_R100		3'-TGCGGCCGCTCGAGTCGACCCTTAACTAAATAACGCTTTAAAGGATTCTAAAAT-5'
TZ_F322	capA322	5'-CTGTTCCAGGGGCCCTGGGATCCCCTGATAATGGTACTGCAATTCTTG-3'
TZ_R322		3'-TGCGGCCGCTCGAGTCGACCCTCAAGTTGTTGTCTCCACTGATAC-5'

333

334 **Protein expression and confirmation of immunogenicity**

335 *E. coli* BL21 cells were transformed with each construct for protein expression. The
336 transformed *E. coli* BL21 cells were grown in 3 ml LB supplemented with 50 µg/ml of
337 ampicillin at 37°C for 18 h at 180 rpm. The OD₆₀₀ of the cultures was adjusted to 0.05 in 3 ml
338 LB or terrific broth (TB) with 50 µg/ml of ampicillin, and the cells were grown at 37°C at 180
339 rpm. When the OD₆₀₀ reached 0.8, the expression of proteins was induced by adding isopropyl
340 β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM. The cells were grown
341 at 37°C for 4 h at 180 rpm. In addition, uninduced cell cultures were prepared and used as
342 controls. The cells were harvested at 8,000 rpm for 10 min and washed twice with phosphate-
343 buffered saline (PBS). The cell pellets were resuspended in 250 µl of lysis buffer (PBS
344 containing 0.05% Tween-20, 1 mM PMSF, 0.1 mM benzamidine, pH 7.5) and lysed using a
345 Branson 450 Analog Sonifier (Branson Ultrasonics, CT, USA). The total lysate, supernatant,
346 and pellet fractions of cells were collected. Then, 15 µl of each collected fraction was diluted
347 in 5 µl of sodium dodecyl sulfate (SDS) gel-loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS,
348 10% glycerol, 1% beta-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue), and 5 µl
349 of diluted sample was analyzed using 10% SDS polyacrylamide gel electrophoresis (SDS-
350 PAGE).

351 Detection of expressed GST fusion proteins was achieved by Western blotting using GST
352 Mouse Monoclonal IgG (Santa Cruz, DTX, USA) and anti-mouse IgG-HRP (GE Healthcare,
353 CHI, USA). To identify which proteins were immunoreactive, the hyperimmunized antiserum
354 of horses (PC1) infected with virulent *B. anthracis* strain was used to probe the proteins in the
355 cell pellet fraction, followed by probing with goat anti-horse IgG-HRP (Jackson
356 ImmunoResearch Laboratories Inc, PA, USA) for detection. Upon visualization of the Western
357 blotting, signals of target proteins higher than the background were selected as positive signals.
358

359 **Expression and purification of CapA322**

360 The peptide derived from the 322nd to the 411th amino acid residues of capsule biosynthesis
361 protein CapA was defined as CapA322. Primers for amplifying partial coding sequence of
362 CapA are described in Table 1, and pGEX-6P-2 was used to construct pTZ006. The protein
363 expression in *E. coli* was conducted using the method described above. After the expression

364 process, the cells were harvested and lysed using the Branson 450 Analog Sonifier. The
365 resulting suspension was centrifuged at 4°C for 15 min at 15,000 rpm. From the supernatant,
366 GST-tagged recombinant CapA322 was purified using Glutathione Sepharose 4 beads (GE
367 Healthcare, CHI, USA) according to a batch protocol of the manufacturer. The GST tag was
368 cleaved from the protein using the PreScission Protease (GE Healthcare, CHI, USA) at 4°C for
369 18 h. Then, recombinant CapA322 was eluted with an elution buffer (50 mM Tris, 150 mM
370 NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5). The elution buffer was changed to 25 mM 2-(N-
371 morpholino) ethane sulfonic acid (MES), pH 6.5, using Amicon Ultra centrifugal device
372 (Merck, Germany). After buffer exchange, the collected fraction was loaded onto a Resource-
373 S column (GE Healthcare, CHI, USA) equilibrated with 25 mM MES, pH 6.5. A NaCl gradient
374 from 0 mM to 150 mM was used for elution. The eluted CapA322 protein was stored in aliquots
375 at -80°C. Fractions at all steps of the expression and purification processes were analyzed
376 using 10% SDS-PAGE and Western blotting. For the Western blotting analyses of CapA322,
377 PC1 and NC1 sera were used as primary antibodies, whereas goat anti-horse IgG-HRP was
378 used as a secondary antibody.

379 **CapA322 in-house ELISA**

380 Checkerboard titration was performed to determine the optimal concentration of the
381 reagents (Crowther, 2000). Flat-bottomed 96-well microtiter plates (Corning, NY, USA) were
382 coated with 100 µl per well of serially diluted CapA322 in coating buffer (0.1 M NaHCO₃, pH
383 9.6) and incubated overnight at 4°C. The antigen-coated plates were washed three times with
384 300 µl wash buffer (PBS containing 0.05% Tween-20, pH 7.4), and the wells were blocked
385 with 100 µl blocking buffer (3% skim milk in PBS containing 0.05% Tween-20, pH 7.4) for 1
386 h at room temperature (RT). After being briefly washed three times, the sera of PC1 and NC1
387 were each diluted from 1:100 to 1:6400 in a blocking buffer. Then, 100 µl of the diluted
388 solution was added to wells in triplicate and incubated at RT for 1 h. After being washed again,
389 goat anti-horse IgG-HRP was diluted to 1:15,000 in blocking buffer, and 100 µl of the diluted
390 antibody solution was added to the wells and incubated at RT for 1 h. After washing, an antigen
391 and antibody complex was detected by adding 100 µl 3,3',5,5'-tetramethylbenzidine substrate
392 (KPL, MD, USA) per well. The reaction was stopped by adding 50 µl 1N HCl per well followed
393 by 20 min incubation at RT. Absorbance at 450 nm (OD 450) was measured using a microtiter
394 plate reader (Thermo Scientific, WI, USA).

395 **Statistical analysis**

396 Each horse serum sample was tested in technical triplicate by PAD1-ELISA and CapA322-
397 ELISA. One sample (Vac_H4D21) did not show the expected antibody response to vaccination
398 when analyzed by PAD1-ELISA, suggesting the failure of immunization. Therefore, this
399 sample was excluded from the following statistical analysis. The rest of the samples were
400 categorized as positive group (PC; n = 3), negative group (NC; n = 2), and vaccinated group
401 (Vac; n = 3). Then, the relative OD values of serum samples were calculated by dividing OD
402 values obtained from PAD1-ELISA by those from CapA322-ELISA. Finally, the one-way
403 ANOVA with Tukey's multiple comparison test was conducted using GraphPad Prism v.7.0
404 to test for differences among the groups.

405 **Results**

406 **Screening of CDS-encoding secreted and surface-associated proteins on** 407 **pXO2 plasmid.**

408 To select potential antigen candidates among 105 CDSs encoded on pXO2 of *B. anthracis*
409 Ames ancestor strain, *in silico* screening was conducted. Functional features that could make
410 proteins more antigenic were identified by searching for anchoring domain, secretion signal
411 peptide, and cellular localization (Ariel et al., 2002; María et al., 2017).

412 Five informatics tools were used for the screening. Signal P (Emanuelsson et al., 2007)
413 predicted the product of 12 CDSs to possess the N-terminus signal peptides and secreted
414 through the classical Sec pathway. PSORT (Nakai and Horton, 1999) predicted 2 extracellular
415 proteins, 3 cell wall-associated proteins, 27 cell membrane-related proteins, and 35 cytoplasmic
416 proteins. LipoP (Juncker et al., 2003) predicted 24 proteins: 7 with putative Sec signal peptide
417 SpI sites, 2 with lipoprotein signal peptides SpII sites, and 15 with transmembrane helix domain.
418 TMHMM (Krogh et al., 2001) predicted 33 proteins, including one to six transmembrane helix
419 domains. In addition, PROSITE (Hulo et al., 2008) indicated that several proteins have S-layer
420 homology domains, papain-like cell wall hydrolase domain (NLPC/60), membrane lipoprotein
421 lipid attachment sites, and serine lysine active sites (Table 2).

422 **Table 2. Result of *in silico* analyses on CDSs encoded by pXO2.**

CDS	Locus tag	Protein function	SignaIP ^a	TMHMM ^b	PSORT ^c	PROSITE ^d	LipoP ^e	Ranking					
								SignalP ^a	TMHMM ^b	PSORT ^c	PROSITE ^d	LipoP ^e	Total score
29	GBAA_RS28110	hypothetical protein	+	1	CMSVM	Prokaryotic membrane lipoprotein lipid attachment site	+	1	1	1	1	1	5
68	GBAA_RS28275	signal peptidase	+	1	CMSVM	Signal peptidases I serine active site		1	1	1	1	0	4
83	GBAA_RS28340	peptide ABC transporter substrate binding protein	+		CWSVM	Prokaryotic membrane lipoprotein lipid attachment site	+	1	0	1	1	1	4
100	GBAA_RS28430	metal dependent hydrolase	+	3	CMSVM			1	1	1	0	0	3
12	GBAA_RS28035	hypothetical protein	+	5	CMSVM			1	1	1	0	0	3
43	GBAA_RS28165	amidase	+		CWSVM	S layer homology domain		1	0	1	1	0	3
60	GBAA_RS28240	Capsule biosynthesis protein CapA	+	1	CMSVM			1	1	1	0	0	3
6	GBAA_RS28005	lysozyme	+	1	ECSVM			1	1	1	0	0	3
3	GBAA_RS27990	hypothetical protein	+	1	Unknown			1	1	0	0	0	2
25	GBAA_RS28090	hypothetical protein	+	1	Unknown			1	1	0	0	0	2
33	GBAA_RS28130	hypothetical protein	+	1	Unknown			1	1	0	0	0	2
9	GBAA_RS28020	hypothetical protein		2	CMSVM			0	1	1	0	0	2
10	GBAA_RS28025	hypothetical protein		1	CMSVM			0	1	1	0	0	2
11	GBAA_RS28030	hypothetical protein		2	CMSVM			0	1	1	0	0	2
16	GBAA_RS28050	hypothetical protein		2	CMSVM			0	1	1	0	0	2
19	GBAA_RS28065	hypothetical protein		2	CMSVM			0	1	1	0	0	2
20	GBAA_RS28070	hypothetical protein		4	CMSVM			0	1	1	0	0	2
21	GBAA_RS28075	hypothetical protein			CMSVM/ CWSVM	Sigma 54 interaction domain ATP binding region A signature		0	0	1	1	0	2
28	GBAA_RS28105	hypothetical protein		1	CMSVM			0	1	1	0	0	2
42	GBAA_RS28160	hypothetical protein		3	CMSVM			0	1	1	0	0	2

50	GBAA_RS28195	CPBP family intramembrane metalloprotease	6	CMSVM		0	1	1	0	0	2
52	GBAA_RS28205	TetR family transcriptional regulator		CMSVM	TetE type HTH domain	0	0	1	1	0	2
54	GBAA_RS28215	undecaprenyl-diphosphatase	2	CMSVM		0	1	1	0	0	2
55	GBAA_RS31175	UDP-diphosphatase	1	CMSVM		0	1	1	0	0	2
61	GBAA_RS28245	poly gamma glutamate biosynthesis protein PgsC	3	CMSVM		0	1	1	0	0	2
62	GBAA_RS28250	poly gamma glutamate synthase PgsB	1	CMSVM		0	1	1	0	0	2
74	GBAA_RS28305	amino acid transporter	6	CMSVM		0	1	1	0	0	2
80	GBAA_RS28325	hypothetical protein	3	CMSVM		0	1	1	0	0	2
81	GBAA_RS28330	CPBP family intramembrane metalloprotease	4	CMSVM		0	1	1	0	0	2
91	GBAA_RS28380	hypothetical protein	1	CMSVM		0	1	1	0	0	2
96	GBAA_RS28405	CPBP family intramembrane metalloprotease	3	CMSVM		0	1	1	0	0	2
98	GBAA_RS31215	hypothetical protein	1	CMSVM		0	1	1	0	0	2
5	GBAA_RS28000	hypothetical protein	+	Unknown		1	0	0	0	0	1
13	GBAA_RS31135	hypothetical protein		CytoSVM	EF hand calcium binding domain	0	0	0	1	0	1
14	GBAA_RS28040	conjugal transfer protein	2	Unknown		0	1	0	0	0	1
18	GBAA_RS28060	hypothetical protein	1	Unknown		0	1	0	0	0	1
23	GBAA_RS28085	hypothetical protein	1	Unknown		0	1	0	0	0	1
38	GBAA_RS28145	hypothetical protein		CMSVM		0	0	1	0	0	1
39	GBAA_RS31155	hypothetical protein	1	Unknown		0	1	0	0	0	1
44	GBAA_RS31160	IS3 family transposase		CytoSVM	Integrase catalytic domain	0	0	0	1	0	1
45	GBAA_RS28180	hypothetical protein	1	Unknown		0	1	0	0	0	1
47	GBAA_RS31165	integrase		Unknown	Endoplasmic reticulum targeting site	0	0	0	1	0	1
53	GBAA_RS28210	IS4 family transposase		Unknown	Sugar transport proteins signature	0	0	0	1	0	1

57	GBAA_RS28225	Capsule synthesis positive regulator AcpB	CytoSVM	PRD domain	0	0	0	1	0	1
59	GBAA_RS28235	Capsule polysaccharide biosynthesis protein	ECSVM		0	0	1	0	0	1
65	GBAA_RS28255	transcriptional regulator	CytoSVM	ArsR type HTH domain type	0	0	0	1	0	1
66	GBAA_RS31190	IS4 family transposase	CMSVM		0	0	1	0	0	1
72	GBAA_RS28295	PRD domain containing protein	CytoSVM	PRD domain	0	0	0	1	0	1
73	GBAA_RS28300	PRD domain containing protein	CytoSVM	PRD domain	0	0	0	1	0	1
76	GBAA_RS28315	Capsule synthesis positive regulator AcpA	CytoSVM	PRD domain	0	0	0	1	0	1
86	GBAA_RS28355	DNA repair protein MucB	CytoSVM	UmuC domain	0	0	0	1	0	1
97	GBAA_RS31210	DNA topoisomerase 3	CytoSVM	Toprim domain and prokaryotic DNA topoisomerase I active site	0	0	0	1	0	1
1	GBAA_RS27980	hypothetical protein	CytoSVM		0	0	0	0	0	0
2	GBAA_RS27985	hypothetical protein	Unknown		0	0	0	0	0	0
4	GBAA_RS27995	hypothetical protein	Unknown		0	0	0	0	0	0
7	GBAA_RS28010	ATP binding protein	CytoSVM		0	0	0	0	0	0
8	GBAA_RS28015	hypothetical protein	CytoSVM		0	0	0	0	0	0
15	GBAA_RS28045	hypothetical protein	CytoSVM		0	0	0	0	0	0
17	GBAA_RS28055	hypothetical protein	Unknown		0	0	0	0	0	0
22	GBAA_RS28080	hypothetical protein	Unknown		0	0	0	0	0	0
24	GBAA_RS31140	hypothetical protein	Unknown		0	0	0	0	0	0
26	GBAA_RS28095	hypothetical protein	CytoSVM		0	0	0	0	0	0
27	GBAA_RS28100	hypothetical protein	CytoSVM		0	0	0	0	0	0
30	GBAA_RS28115	hypothetical protein	CytoSVM		0	0	0	0	0	0
31	GBAA_RS28120	hypothetical protein	CytoSVM		0	0	0	0	0	0
32	GBAA_RS28126	hypothetical protein	Unknown		0	0	0	0	0	0
34	GBAA_RS28135	hypothetical protein	CytoSVM		0	0	0	0	0	0
35	GBAA_RS31145	hypothetical protein	Unknown		0	0	0	0	0	0

36	GBAA_RS31150	hypothetical protein	Unknown	0	0	0	0	0	0
37	GBAA_RS28140	hypothetical protein	CytoSVM	0	0	0	0	0	0
40	GBAA_RS28150	parA family protein	CytoSVM	0	0	0	0	0	0
41	GBAA_RS28155	hypothetical protein	Unknown	0	0	0	0	0	0
46	GBAA_RS28185	hypothetical protein	CytoSVM	0	0	0	0	0	0
48	GBAA_RS31170	hypothetical protein	Unknown	0	0	0	0	0	0
49	GBAA_RS28190	hypothetical protein	CytoSVM	0	0	0	0	0	0
51	GBAA_RS28200	DUF523 containing protein	CytoSVM	0	0	0	0	0	0
56	GBAA_RS28220	hypothetical protein	Unknown	0	0	0	0	0	0
58	GBAA_RS28230	DUF1093 domain containing protein	CytoSVM	0	0	0	0	0	0
63	GBAA_RS31180	hypothetical protein	Unknown	0	0	0	0	0	0
64	GBAA_RS31185	hypothetical protein	Unknown	0	0	0	0	0	0
67	GBAA_RS31195	IS6 family transposase	Unknown	0	0	0	0	0	0
69	GBAA_RS28280	hypothetical protein	Unknown	0	0	0	0	0	0
70	GBAA_RS28285	hypothetical protein	CytoSVM	0	0	0	0	0	0
71	GBAA_RS28290	HTH domain containing protein	CytoSVM	0	0	0	0	0	0
75	GBAA_RS29350	hypothetical protein	Unknown	0	0	0	0	0	0
77	GBAA_RS28320	hypothetical protein	Unknown	0	0	0	0	0	0
78	GBAA_RS31144	hypothetical protein	Unknown	0	0	0	0	0	0
79	GBAA_RS31200	hypothetical protein	Unknown	0	0	0	0	0	0
82	GBAA_RS31205	hypothetical protein	Unknown	0	0	0	0	0	0
84	GBAA_RS28345	hypothetical protein	Unknown	0	0	0	0	0	0
85	GBAA_RS28350	DUF3967 domain containing protein	CytoSVM	0	0	0	0	0	0
87	GBAA_RS28360	Yold like family protein	Unknown	0	0	0	0	0	0
88	GBAA_RS28365	hypothetical protein	Unknown	0	0	0	0	0	0
89	GBAA_RS28370	hypothetical protein	CytoSVM	0	0	0	0	0	0

90	GBAA_RS28375	hypothetical protein	CytoSVM	0	0	0	0	0	0
92	GBAA_RS28385	hypothetical protein	Unknown	0	0	0	0	0	0
93	GBAA_RS28390	integrase	CytoSVM	0	0	0	0	0	0
94	GBAA_RS28395	hypothetical protein	Unknown	0	0	0	0	0	0
95	GBAA_RS28400	hypothetical protein	CytoSVM	0	0	0	0	0	0
99	GBAA_RS28425	DUF3991 domain containing protein	CytoSVM	0	0	0	0	0	0
101	GBAA_RS28435	hypothetical protein	CytoSVM	0	0	0	0	0	0
102	GBAA_RS28440	hypothetical protein	CytoSVM	0	0	0	0	0	0
103	GBAA_RS28445	hypothetical protein	Unknown	0	0	0	0	0	0
104	GBAA_RS28450	hypothetical protein	CytoSVM	0	0	0	0	0	0
105	GBAA_RS28455	hypothetical protein	Unknown	0	0	0	0	0	0

[#]Coding sequence

^bTMHMM: Transmembrane helix domain numbers

^dPROSITE: Protein families, domains, and functional sites

CMSVM: Membrane protein

ECSVM: Extracellular protein

^aSignalP: Signal peptide sequences, if detected; +, If not -,

^cPSORT: Putative cell localization of protein

^eLipoP: Lipoprotein signal, if detected; +, If not -,

CWSVM: Cell wall protein

CytoSVM: Cytoplasmic protein

424 I focused mainly on secreted and surface-exposed CDS products that are considered
425 relevant for identifying targets for eliciting protective immunity. Combining the results of the
426 5 tools, 8 CDSs were selected based on predicted signal sequence, transmembrane helix
427 domain, and cellular localization, considering potential exposure to a host cell and probability
428 to generate host protective response. The candidate proteins were predicted to be secreted, and
429 surface-associated proteins were observed to have 0–5 transmembrane segments (Table 3).

430

431 **Table 3. *In silico* selected CDSs for identification of immunoreactivity**

Locus tag number	Product name	<i>In silico</i> data				
		Cellular localization	Signal peptide ^a	Lipoprotein signal peptide ^a	TMHMM number ^b	Domains and active sites
GBAA_RS28005	Lysozyme	Extra cellular	+	-	1	NlpC/P60
GBAA_RS28035	Hypothetical protein	Membrane	+	+	5	
GBAA_RS28110	Hypothetical protein	Membrane	+	-	1	PROKAR Lipoprotein
GBAA_RS28165	Amidase	Cell wall	+	-	0	SLH
GBAA_RS28240	Capsule biosynthesis protein capA	Membrane	+	-	1	
GBAA_RS28275	Signal peptidase	Membrane	+	-	1	Serine, lysine active sites
GBAA_RS28340	Peptide ABC transport substrate binding protein	Cell wall	+	+	0	PROKAR Lipoprotein
GBAA_RS28430	Metal dependent hydrolase	Membrane	+	-	3	

^aIf secretion or lipoprotein signals were detected; +, If not -,

^bTransmembrane helix domains

432

433 **Expression and immunoreactivity of candidate proteins**

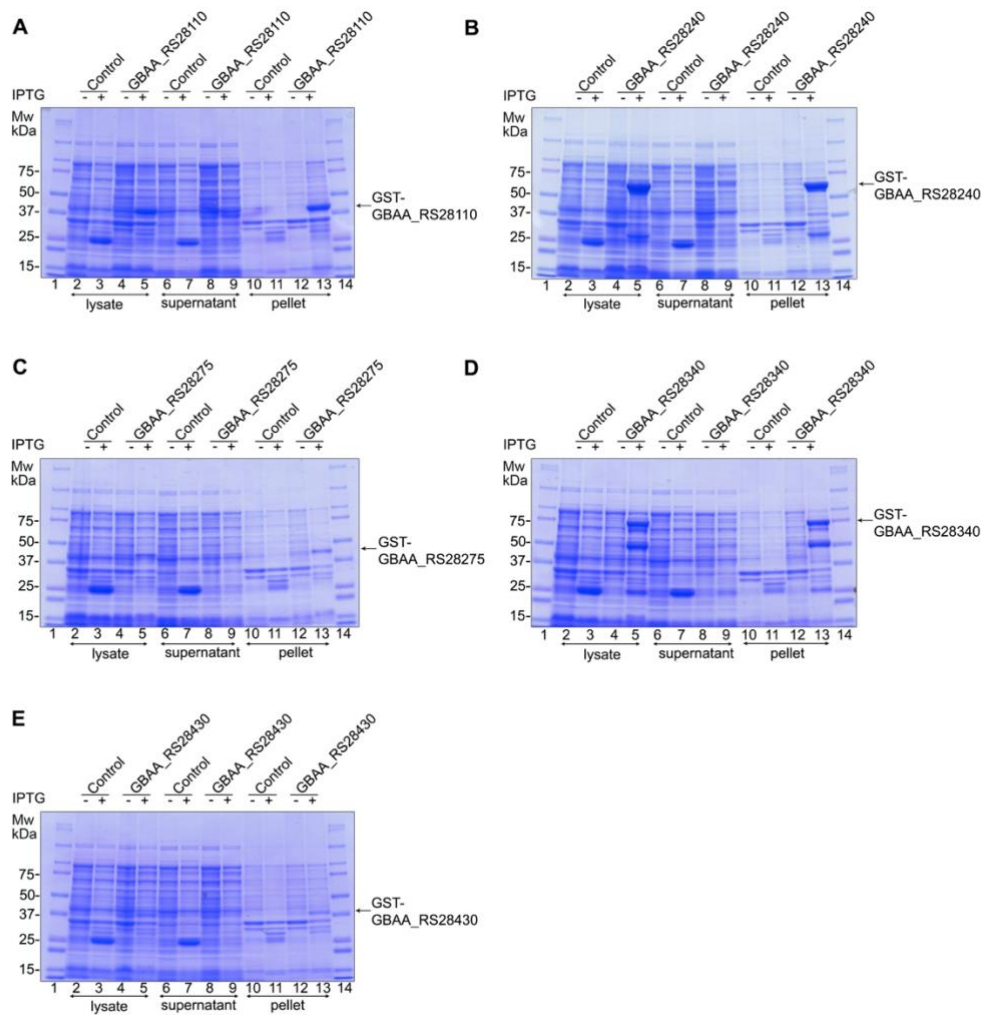
434 To further identify whether candidate proteins were immunoreactive, the proteins were
435 probed with the hyperimmunized antiserum of horses infected with virulent *B. anthracis*
436 (PC1). CDSs were cloned and expressed in *E. coli*. I successfully constructed *E. coli* strains
437 that express our candidate proteins fused with GST tag at the N-terminus for 5 of 8 CDSs
438 selected from *in silico* analyses (Table 4). The target proteins at the expected molecular sizes
439 were visualized in the lysate, supernatant or pellet fraction of the cell lysate, indicating soluble
440 or insoluble expression (Figure 1). The sizes of the target proteins in the cell pellet fractions
441 were verified using SDS-PAGE (Figure 2A) and Western blotting with antibodies against the
442 GST tag (Figure 2B). After expression verification, proteins were probed with hyperimmune
443 antiserum from horses infected with virulent *B. anthracis* (PC1) to identify their
444 immunoreactivity. Of the 5 expressed proteins, only the capsule biosynthesis protein CapA
445 (GBAA_RS28240) and peptide ABC transporter substrate-binding protein (GBAA_RS28340)
446 reacted with PC1 serum (Figure 2C). Serum from the naive horse (NC1) showed only
447 background signal with the screened proteins, indicating that the proteins detected using the
448 PC1 serum resulted from an antibody response induced by fully virulent *B. anthracis* infection
449 (Figure 2D).

450 **Table 4. Constructs and strain list**

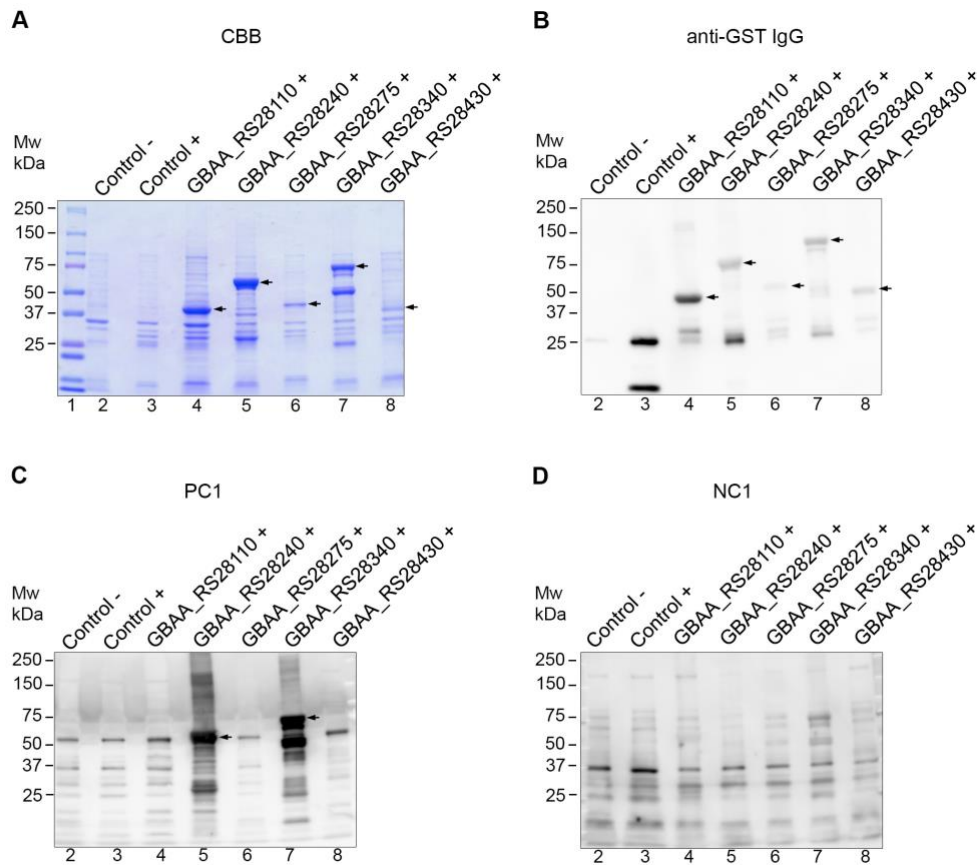
Strain or plasmid	Description	Reference
Plasmids		
pGEX-6P-2	Cloning vector	Reference ¹
pTZ001	pGEX-6P-2 cloned with GBAA_RS28110	This study
pTZ002	pGEX-6P-2 cloned with GBAA_RS28240	This study
pTZ003	pGEX-6P-2 cloned with GBAA_RS28275	This study
pTZ004	pGEX-6P-2 cloned with GBAA_RS28340	This study
pTZ005	pGEX-6P-2 cloned with GBAA_RS28430	This study
pTZ006	pGEX-6P-2 cloned with C-terminus region of GBAA_RS28240	This study
Strains		
<i>E. coli</i>		
BL21	B F ⁻ <i>ompT gal dcm lon hsdSB</i> (τ_B - m_B^-) [malB ⁺] κ -12(λ^S)	Reference ²
BTZ001	BL21 harboring pTZ001	This study
BTZ002	BL21 harboring pTZ002	This study
BTZ003	BL21 harboring pTZ003	This study
BTZ004	BL21 harboring pTZ004	This study
BTZ005	BL21 harboring pTZ005	This study
BTZ006	BL21 harboring pTZ006	This study

¹(Smith and Johnson, 1988)

²(Jeong et al., 2015)



451
 452 **Figure 1. Expression of candidate proteins.** Coomassie brilliant blue (CBB) staining
 453 analyses of proteins in cell lysate (lanes 2–5), supernatant (lanes 6–9), and pellet (lanes 10–13)
 454 fractions of control and candidate protein-expressing *E. coli* strain grown in terrific broth with
 455 or without isopropyl β -D-thiogalactopyranoside (IPTG) at 37°C for 4 h at 180 rpm. Lanes 1
 456 and 14, Mw, molecular weight marker (in kDa). Control, *E. coli* BL21 harboring empty pGEX-
 457 6P-2 plasmid expressing glutathione S-transferase (GST: 26 kDa). (A) *E. coli* BTZ001
 458 expressing recombinant hypothetical protein (GST-GBAA_RS28110: 44 kDa). (B) *E. coli*
 459 BTZ002 expressing recombinant capsule biosynthesis protein CapA (GST-GBAA_RS28240:
 460 72 kDa). (C) *E. coli* BTZ003 expressing recombinant signal peptidase (GST-GBAA_RS28275:
 461 47 kDa). (D) *E. coli* BTZ004 expressing recombinant peptide ABC substrate-binding protein
 462 (GST-GBAA_RS28340: 84 kDa). (E) *E. coli* BTZ005 expressing recombinant metal-
 463 dependent hydrolase (GST-GBAA_RS28430: 46 kDa). +, 0.2 mM IPTG induction; –, without
 464 IPTG induction.



465

466 **Figure 2. Expression and immunoreactivity of candidate proteins.** (A) Coomassie brilliant

467 blue (CBB) staining and (B–D) Western blotting of the proteins in cell pellet fractions of the

468 control and candidate protein-expressing *E. coli* strains grown in terrific broth. In Western

469 blotting, the proteins in the cell pellets were probed with different antibodies: (B) anti-

470 glutathione S-transferase (GST) immunoglobulin G; (C) horse hyperimmunized anti-*B.*

471 *anthracis* serum (PC1); (D) naive horse serum (NC1). Lane 1, Mw, molecular weight marker

472 (in kDa); lane 2, control strain which is *E. coli* BL21 harboring empty pGEX-6P-2 without

473 IPTG induction (-); lane 3, control strain which is *E. coli* BL21 harboring empty pGEX-6P-2

474 with 0.2 mM IPTG induction (+); lane 4, *E. coli* BTZ001 expressing recombinant hypothetical

475 protein (GST-GBAA_RS28110: 44 kDa); lane 5, *E. coli* BTZ002 expressing recombinant

476 capsule biosynthesis protein CapA (GST-GBAA_RS28240: 72 kDa); lane 6, *E. coli* BTZ003

477 expressing recombinant signal peptidase (GST-GBAA_RS28275: 47 kDa); lane 7, *E. coli*

478 BTZ004 expressing recombinant peptide ABC substrate-binding protein (GST-

479 GBAA_RS28340: 84 kDa); lane 8, *E. coli* BTZ005 expressing recombinant metal-dependent

480 hydrolase (GST-GBAA_RS28430: 46 kDa). The arrows indicate target proteins in the expected

481 sizes.

482 **Evaluation of CapA322 as ELISA antigen**

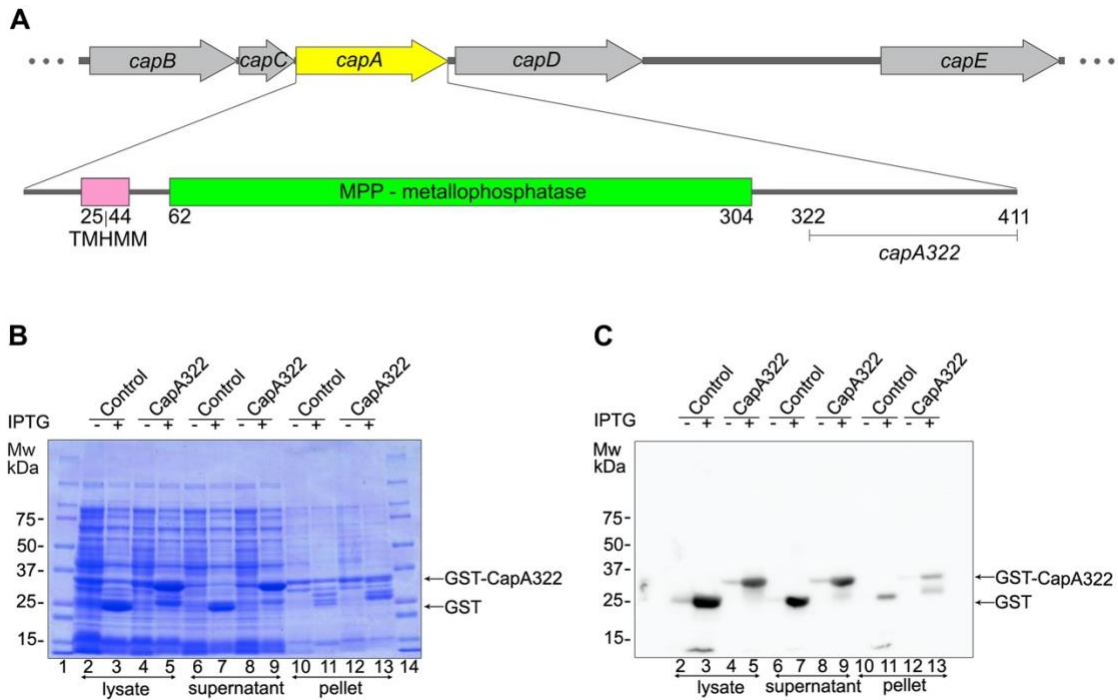
483 The sequences specificity of the two proteins, CapA (GBAA_RS28240) and peptide ABC
484 transporter substrate-binding protein (GBAA_RS28340), that showed immunoreactivity to
485 PC1 was analyzed. When the entire length of the proteins was analyzed with tBLASTn, the
486 peptide ABC transporter substrate-binding protein showed high sequence similarity with a
487 protein encoded in *Bacillus cereus*. Alternatively, the CapA showed low sequence similarity
488 to common *B. cereus* strains (~56%) and other *Bacillus* species (55%–60%) evolutionarily
489 related to *B. anthracis*. A few unusual *B. cereus* strains possess virulent plasmids similar (85%–
490 89%) to those of *B. anthracis*, including the toxin and capsule-coding genes (Table 5).
491 Considering the low solubility of whole length CapA, its soluble region at the C-terminus from
492 322nd to 411th (Figure 3A) was selected. This region also showed even lower sequence
493 similarity (55%) with homologs in common *B. cereus* strains than the N-terminus region. Thus,
494 I tested if the CapA peptide of this region, defined as CapA322, shows better solubility and
495 retains immunoreactivity. The CapA322 was expressed as a GST fusion protein in *E. coli* and
496 solubilized in the supernatant fraction of the cell lysate. The protein size was verified using
497 SDS-PAGE and Western blotting with anti-GST IgG (Figure 3). In GST-tag affinity
498 purification before and after GST-tag cleavage, a GST-CapA322 band of approximately 37
499 kDa and CapA322 of 11 kDa were detected (Figure 4A). The CapA322 reacted with
500 hyperimmune antiserum from horses infected with virulent *B. anthracis* (PC1) but did not
501 react with serum from the naive horse (NC1) (Figures 4B and 4C). After the first purification
502 process, the remaining host cell derivative contaminants were removed using an ion-exchange
503 chromatography column (Figures 4D and 4E). From a 1L culture, approximately 0.6 mg of
504 CapA322 was obtained with a purity of >90%. As expected, the CapA322 did not react to
505 vaccinated horse serum (Vac_H3D21), which had a high anti-PAD1 IgG concentration (Figure
506 4F), indicating that the CapA322 is specific to antibodies resulting from virulent *B. anthracis*
507 infection. In addition, its substantial soluble expression confirms that CapA322 is a potent
508 antigen candidate for developing a diagnostic tool.

509 **Table 5. Sequence similarity of two immunoreactive proteins identified by Western**
 510 **blotting with horse hyperimmune antisera and CapA322**

Whole length CapA						
ID	Species	Similarity	Positive	Coverage	Plasmid	Chromosome
CP020941.1	<i>B. cereus</i> strain BC-AK plasmid pBCXO2	89%	100%	100%	+	
CP001748.1	<i>B. cereus</i> biovar anthracis str. CI plasmid pCI-XO2	89%	100%	100%	+	
CP009317.1	<i>B. cereus</i> 03BB102 plasmid	86%	96%	94.10%	+	
CP001406.1	<i>B. cereus</i> 03BB102 plasmid p03BB102_179	86%	96%	94.10%	+	
CP009636.1	<i>B. cereus</i> 03BB108 plasmid pBFI_2	85%	94%	94.10%	+	
CP017574.1	<i>B. thuringiensis</i> strain SCG04-02 plasmid PSCG...	57%	79%	93.70%	+	
CP015177.1	<i>B. thuringiensis</i> serovar alesti strain BGSC 4...	60%	79%	87.30%	+	
CP023179.1	<i>B. cereus</i> strain CC-1	56%	79%	93.60%		+
CP030926.1	<i>B. butanolivorans</i> strain PHB-7a	55%	76%	93.90%		+
CP017080.1	<i>B. muralis</i> strain G25-68	55%	76%	93.90%		+

CapA322						
ID	Species	Similarity	Positive	Coverage	Plasmid	Chromosome
CP020941.1	<i>B. cereus</i> strain BC-AK plasmid pBCXO2	81%	100%	100%	+	
CP001748.1	<i>B. cereus</i> biovar anthracis str. CI plasmid pCI-XO2	81%	100%	100%	+	
CP009317.1	<i>B. cereus</i> 03BB102 plasmid	91%	95%	73.30%	+	
CP001406.1	<i>B. cereus</i> 03BB102 plasmid p03BB102_179	91%	95%	73.30%	+	
CP009636.1	<i>B. cereus</i> 03BB108 plasmid pBFI_2	86%	89%	73.30%	+	
CP017704.1	<i>B. simplex</i> NBRC 15720 = DSM 1321	59%	86%	70%		+
CP011008.1	<i>B. simplex</i> strain SH-B26	60%	84%	70%		+
CP030063.1	<i>Brevibacterium frigoritolerans</i> strain ZB201705	57%	84%	70%		+
CP030926.1	<i>B. butanolivorans</i> strain PHB-7a	56%	83%	70%		+
CP017080.1	<i>B. muralis</i> strain G25-68	57%	76%	70%		+

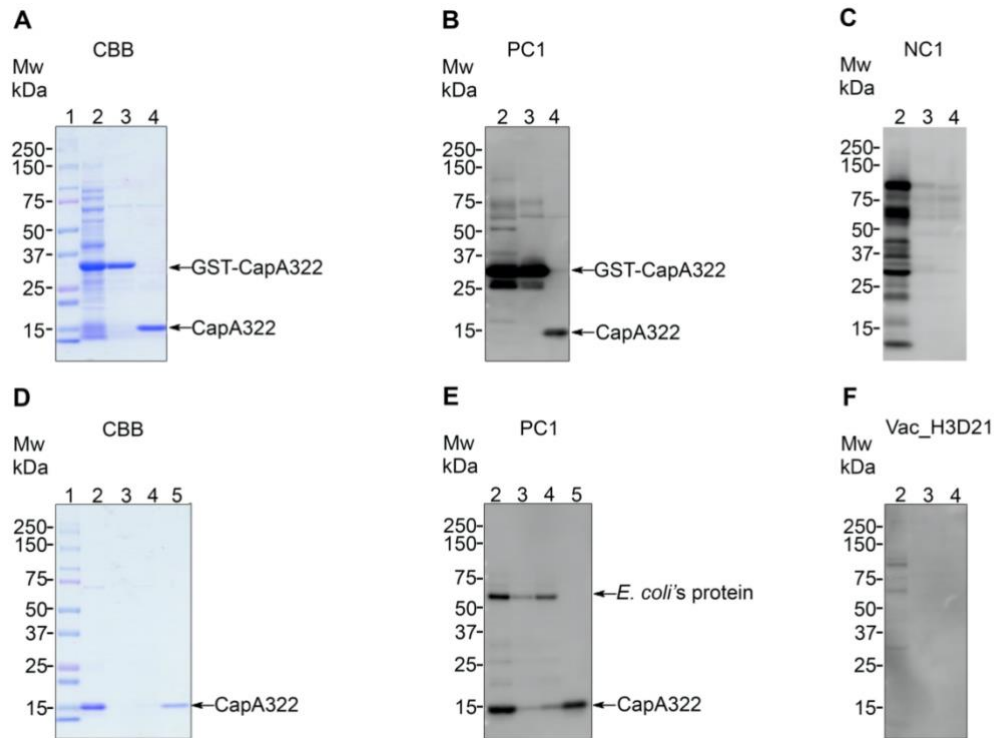
Peptide ABC substrate-binding protein						
ID	Species	Similarity	Positive	Coverage	Plasmid	Chromosome
CP001748.1	<i>B. cereus</i> biovar anthracis str. CI plasmid pCI-XO2	93%	95%	100%	+	
CP020941.1	<i>B. cereus</i> strain BC-AK plasmid pBCXO2 sequence	90%	94%	100%	+	
CP015180.1	<i>B. thuringiensis</i> serovar alesti strain BGSC 4	90%	94%	100%	+	
DQ025752.1	<i>B. thuringiensis</i> serovar kurstaki plasmid pAW	90%	94%	100%	+	
CP018742.1	<i>B. cereus</i> strain FORC_047 plasmid pFORC47_2	90%	94%	100%	+	
CP003691.1	<i>B. thuringiensis</i> MC28 plasmid pMC183	87%	92%	100%	+	
CP024687.1	<i>B. wiedmannii</i> bv. thuringiensis strain FCC41	86%	93%	100%	+	
MG710485.1	<i>B. thuringiensis</i> serovar israelensis strain B	86%	92%	100%	+	
CP015154.1	<i>B. thuringiensis</i> strain Bc601 plasmid pBTBC4	86%	92%	100%	+	
CP013059.1	<i>B. thuringiensis</i> strain YWC2-8 plasmid pYWC2-	86%	92%	100%	+	



511

512

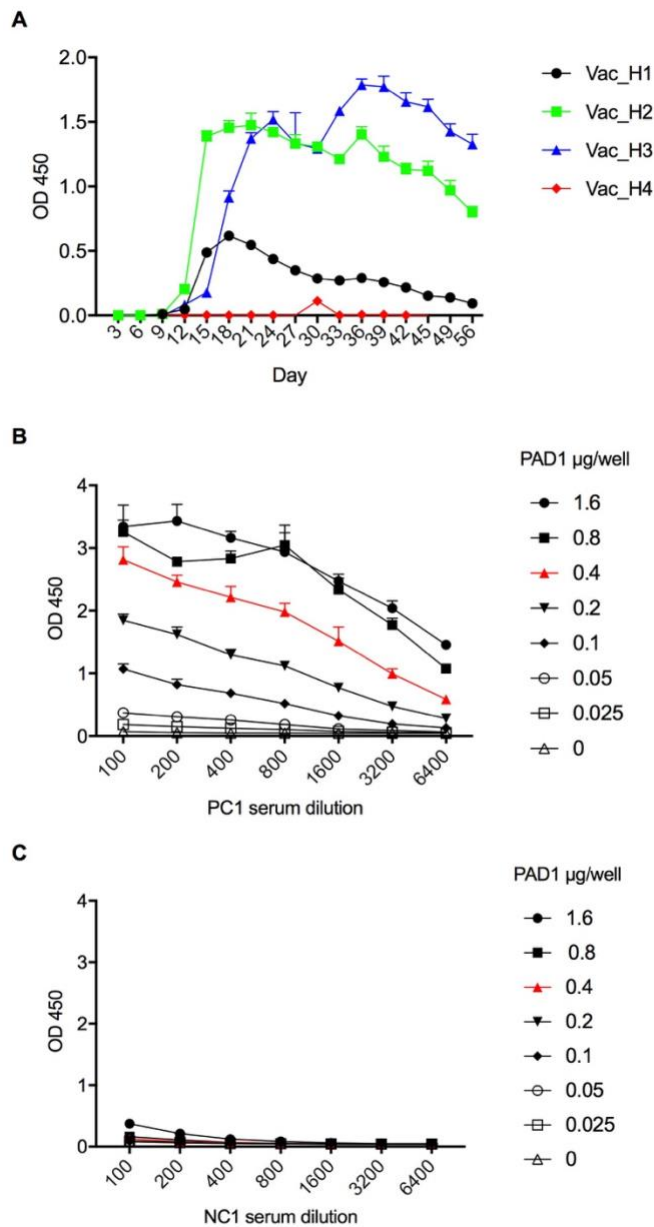
513 **Figure 3. Expression of CapA322.** (A) Genetic map of *B. anthracis* capsule-coding operon
 514 revealing the CapA322, C-terminus region (amino-acid 322–411) of CapA used in antigen
 515 preparation. Coomassie brilliant blue staining (CBB) (B) and Western blotting (C) of proteins
 516 in cell lysate (lanes 2–5), supernatant (lanes 6–9), and pellet (lanes 10–13) fractions of control
 517 and CapA322 expressing *E. coli* BTZ006 grown in terrific broth with or without isopropyl β -
 518 D-thiogalactopyranoside (IPTG) at 37°C for 4 h at 180 rpm. In Western blotting, the proteins
 519 were probed with anti-glutathione S-transferase (GST) immunoglobulin G. Lanes 1 and 14,
 520 Mw, molecular weight marker (in kDa). Control, *E. coli* BL21 harboring empty pGEX-6P-2
 521 plasmid expressing GST (GST: 26 kDa). BTZ006, *E. coli* expressing recombinant CapA322
 522 (GST-CapA322: 37 kDa). +, 0.2 mM IPTG induction; –, without IPTG induction.



523

524 **Figure 4. Purification of CapA322 from cell culture supernatant.** Coomassie brilliant blue
 525 staining (CBB) (A) and (B, C, and F) Western blotting of the fractions collected after affinity
 526 batch purification. In Western blotting, proteins were probed with different horse sera: (B)
 527 horse hyperimmunized anti-*B. anthracis* serum (PC1); (C) naive horse serum (NC1); (F)
 528 vaccinated horse serum (Vac_H3D21). Lane 1, Mw, molecular weight marker (in kDa); lane
 529 2, the supernatant fraction applied to affinity beads (GST-CapA322: 37 kDa); lane 3, beads
 530 bound; lane 4, elute after treatment with PreScission Protease (CapA322: 11 kDa). (D and E)
 531 CBB and Western blotting of the fractions collected during the cation exchange process. Lane
 532 1, Mw, molecular weight marker (in kDa); lane 2, sample loaded onto the cation exchange
 533 chromatography column; lanes 3 and 4 flow-throughs; lane 5, the eluted protein. Host cell-
 534 derived contaminants indicated as *E. coli*'s protein.

535 Further, to elucidate whether CapA322 is cross-reactive to vaccinated horse serum or not,
536 serum samples from four horses vaccinated with the Sterne vaccine were prepared and the
537 immune responses were evaluated using a PAD1-ELISA (Figure 5A). PA-D1, domain one of
538 the PA of *B. anthracis*, was prepared as previously described (Simbotwe et al., 2019). The
539 optimal titration condition of the PAD1-ELISA for a horse was determined as the PA-D1
540 antigen concentration of 0.4 µg/ml, serum dilution of 1:100, and a secondary antibody dilution
541 of 15,000 (Figures 5B and 5C). The Sterne vaccine elicited immune responses in three horses
542 out of four starting at 15 days postvaccination. Despite being inoculated with the same dose of
543 vaccine, horses 2 (Vac_H2) and 3 (Vac_H3) showed higher antibody responses than horses 1
544 (Vac_H1) and 4 (Vac_H4) at all time points. The antibody titers in horses 2 and 3 declined
545 only after the second peak around 5 weeks postvaccination. In contrast, horse 1 showed a weak
546 immune response to the vaccination, with titers decreasing soon after the initial peak. However,
547 horse 4 did not show a detectible immune response despite receiving the same dose of vaccine
548 (Figure 5A).



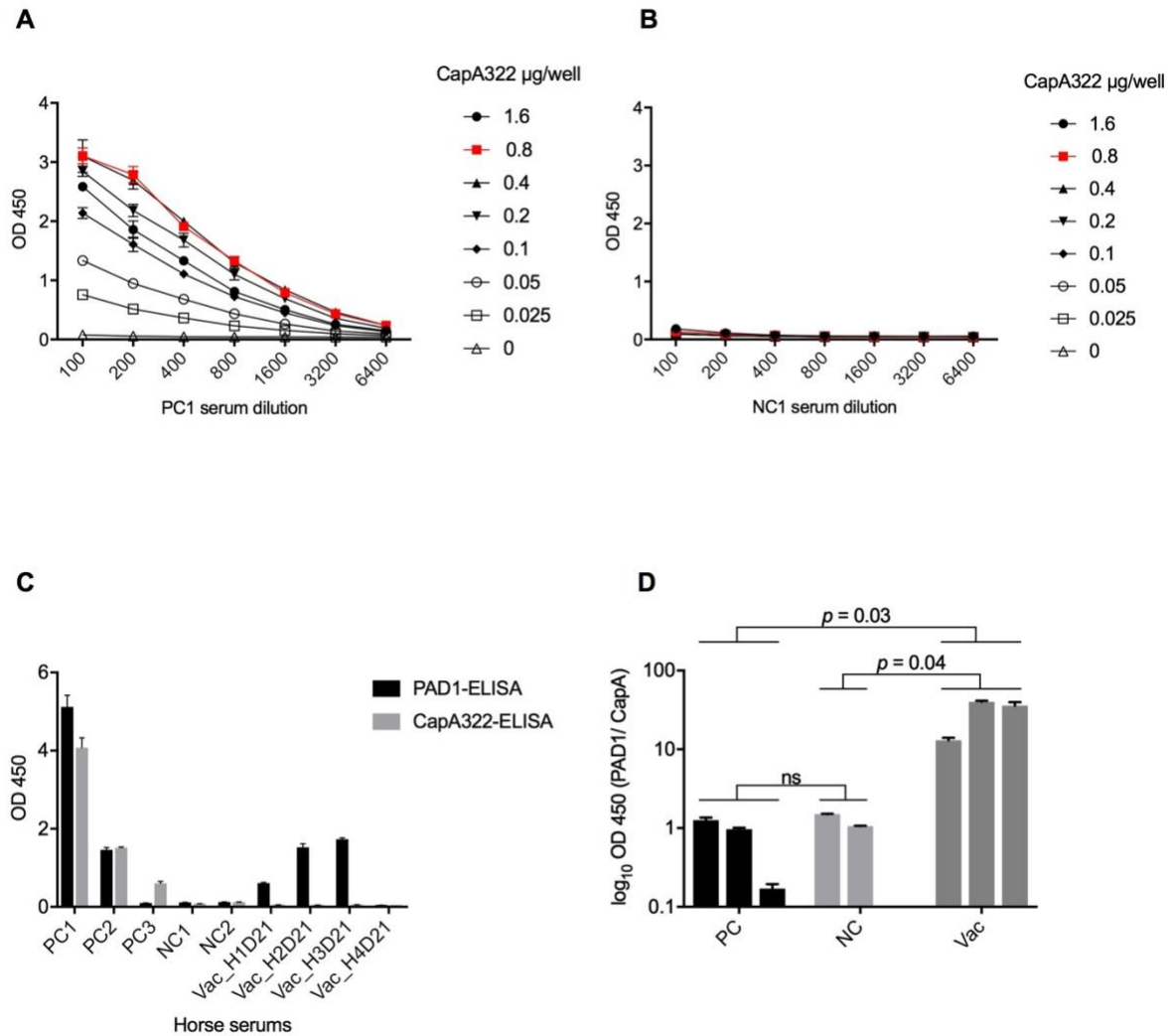
549

550 **Figure 5. Horses anti-PAD1 immunoglobulin G (IgG) responses against subcutaneously**
 551 **injected *Bacillus anthracis* Sterne 34F2 strain spore vaccine.** (A) anti-PAD1 IgG response
 552 of vaccinated horses in serum dilution 1:100. Each serum sample was tested in technical
 553 triplicate by PAD1-ELISA. Checkerboard titration between PAD1 and (B) horse
 554 hyperimmunized anti-*B. anthracis* serum (PC1) or (C) naive horse serum (NC1) in PAD1-
 555 ELISA. Each dilution of serum sample was tested in technical triplicate. The twofold serum
 556 dilution starts with a dilution of 1:100, and PAD1 dilution starts with 1.6 $\mu\text{g}/\text{well}$. From the
 557 result, the optimal concentrations of the antigen, antibody, and serum dilutions were
 558 determined as follows: antigen, 0.4 $\mu\text{g}/\text{well}$; serum dilution, 1:100; second antibody dilution,
 559 1:15,000.

560 **CapA322-ELISA and PAD1-ELISA**

561 Based on the CapA322 antigen, the CapA322-ELISA was developed. An antigen
562 concentration of 0.8 µg/ml, serum dilution of 1:100, and a secondary antibody dilution of
563 15,000 were determined as the optimal titration conditions of CapA322-ELISA for horses
564 (Figures 6A and 6B). Each serum sample from horses experimentally (PC1) or naturally
565 infected with virulent *B. anthracis* (PC2 and PC3) and horses immunized with Sterne vaccine
566 (Vac_H1D21-Vac_H4D21) as well as naive horses (NC1 and NC2) was technically triplicated
567 and comparatively tested by the CapA322-ELISA and PAD1-ELISA (Figure 6C). As expected,
568 PC1 and PC2 gave a significantly higher OD absorbance than the NC1 and NC2 in both
569 CapA322 and PAD1-ELISAs. Vaccinated horse sera Vac_H1D21–Vac_H3D21 showed high
570 OD absorbance in PAD1-ELISA, whereas all were negative in CapA322-ELISA. However,
571 the PC3 serum exhibited a positive reaction only with CapA322-ELISA but was negative in
572 PAD1-ELISA.

573 Furthermore, except one sample (PC3), relative OD values in group PC were around 1,
574 suggesting that the two tests (PAD1-ELISA and CapA322-ELISA) were comparable in
575 detecting virulent *B. anthracis* infection. Similarly, relative OD values in group NC were
576 around 1, indicating that the two tests give the same result. However, relative OD values in
577 group Vac ranged from 13.0 to 39.9, showing the non-cross reactivity of CapA322-ELISA with
578 vaccine-induced antibodies. This result was supported by the observation that the relative OD
579 values in vaccinated group were significantly higher than those in the positive ($p = 0.03$) and
580 negative ($p = 0.04$) groups (Figure 6D).



581

582 **Figure 6. Comparison of PAD1-ELISA and CapA322-ELISA.** Checkerboard titration
 583 between CapA322 and (A) horse hyperimmunized anti-*Bacillus anthracis* serum (PC1) or (B)
 584 naive horse serum (NC1) in CapA322-ELISA. Each dilution of serum sample was tested in
 585 technical triplicate by CapA322-ELISA. The twofold serum dilution starts with a dilution of
 586 1:100, and CapA322 dilution starts with 1.6 $\mu\text{g/well}$. From the result, the optimal
 587 concentrations of antigen, antibody, and serum dilutions were determined as follows: antigen,
 588 0.8 $\mu\text{g/well}$; serum dilution, 1:100; second antibody dilution, 1:15,000. (C) Comparison of
 589 PAD1-ELISA and CapA322-ELISA on horse sera. Each serum samples of horse
 590 hyperimmunized anti-*Bacillus anthracis* (PC1), horse naturally infected with *B. anthracis* (PC2
 591 and PC3), naive horses (NC1 and NC2), and horse vaccinated with Sterne34F2 strain
 592 (Vac_H1D21-Vac_H4D21) was analyzed in technical triplicate by PAD1-ELISA and
 593 CapA322-ELISA. (D) One-way ANOVA with Tukey's multiple comparison test on relative
 594 OD values of positive (PC; n = 3), negative (NC; n = 2), and vaccinated (Vac; n = 3) groups.

595 **Discussion**

596 In this study, potential antigen candidates encoded by pXO2 were screened by *in silico*
597 analyses, and the CapA322-ELISA was developed to detect antibodies against CapA, which is
598 secreted from virulent *B. anthracis* strains that possess pXO2.

599 Identifying the *B. anthracis* natural infection, specifically sublethal infection in animals,
600 is vital in predicting outbreaks in endemic areas. Additionally, identifying sublethal infection
601 can also aid in describing anthrax dynamics in ecosystems and understanding the host-pathogen
602 interaction, which is still poorly understood (Carlson et al., 2018). Current conventional
603 serological assays for anthrax diagnosis and evaluation of immune responses to anthrax
604 vaccines have been developed based on the PA of *B. anthracis*, which is reactive to both
605 naturally acquired and vaccine-generated antibodies. Though animals with natural infections
606 could be distinguished from vaccinated animals based on vaccination history, collecting such
607 data is somewhat cumbersome due to data accessibility and missing records. Besides, although
608 the duration of solid immunity among vaccinated animals is no longer than a year (Stear, 2005),
609 a previous study found that residual antibodies in vaccinated animals were still significantly
610 higher than non-vaccinated animals even after a year had passed (Simbotwe et al., 2019). Given
611 the importance of serodiagnosis for distinguishing *B. anthracis* natural infection from vaccine
612 immunity, the CapA322-ELISA that detects antibodies against CapA encoded by pXO2 of *B.*
613 *anthracis* without cross-reacting with sera from vaccinated animals (Figures 4F and 6C) was
614 developed.

615 Except for a few unusual *B. cereus* strains (Table 5), the C-terminus region of CapA,
616 named CapA322, has lower sequence similarity with other bacterial species and showed
617 immunoreactivity with sera from horses infected with virulent *B. anthracis*. The γ -D-PGA
618 capsule of *B. anthracis*, a critical virulence determinant of *B. anthracis*, is synthesized by CapB
619 and CapC, and transported by CapA and CapE across the cell membrane (Candela and Fouet,
620 2005). Thus, CapA is essential for transporting the γ -D-PGA, but the immunoreactivity of this
621 membrane protein is yet to be determined. To the best of our knowledge, this study is the first
622 to identify CapA as immunoreactive. While the whole length of CapA, as well as its C-terminal
623 region (CapA322), are immunoreactive (Figure 4B), the CapA322 was selected for ELISA

624 development because it was more soluble (Figure 3) compared to the whole length protein
625 (Figures 1 and 2A).

626 CapA is a single-pass transmembrane protein that belongs to the metallophosphatase
627 superfamily (Lu et al., 2020). A single transmembrane helix domain is located at the N-
628 terminus region from 25 to 44 of CapA, translocating the protein in the cell membrane, thereby
629 exposing the C-terminus region to the cell exterior. This exposed cell surface can define the
630 immunoreactive characteristic of the CapA322. Many antigens or vaccine candidate-searching
631 studies have been targeting surface-exposed proteins (Chakravarti et al., 2000; Pizza et al.,
632 2000; Sutcliffe and Harrington, 2002). Cell surface molecules have a greater chance to generate
633 a host cell immune response by its position as they are more exposed to host cells (Chaudhuri
634 et al., 2014). Previously, a few unusual *B. cereus* strains have been reported to cause anthrax-
635 like disease in animals and humans due to the acquisition of virulence plasmids that are highly
636 similar to the *B. anthracis* virulence plasmids pXO1 and pXO2 (Hoffmaster et al., 2006; Klee
637 et al., 2010). Those *B. cereus* strains are divided into two variants; atypical strains such as
638 03BB102 and 03BB108 and *B. cereus* biovar anthracis (Bcbva) strains such as BC-AK and CI
639 (Baldwin, 2020). *B. anthracis* CapA exhibited relatively high sequence homology with the so-
640 called Bcbva and atypical *B. cereus* strains (Table 5); therefore, it is possible for CapA322-
641 ELISA to cross-react with such strains. However, these strains have only been reported in rare
642 cases (Cachat et al., 2008; Han et al., 2006; Sergeev et al., 2006). Also, studies suggest that
643 pXO2 plasmid is not commonly distributed in *B. cereus* and *B. thuringiensis* strains, which are
644 closely related to *B. anthracis* (Kim et al., 2005; Ramiisse et al., 1996). Moreover, screening of
645 pXO2 ORFs among *B. cereus* group strains revealed a restricted distribution of *cap* genes other
646 than in *B. anthracis* (Pannucci et al., 2002). Further, despite the presence of *cap* genes, it is
647 doubtful whether *cap* genes in atypical *B. cereus* strains 03BB102 and 03BB108 are expressed
648 because there was no capsule expression detected in-vitro conditions where it is normally
649 expressed in *B. anthracis* (Cachat et al., 2008; Hoffmaster et al., 2006). A part of the plasmid
650 harbored by strain 03BB102 is highly similar to a portion of the *B. anthracis* pXO2, but the
651 rest of the sequence is different (Baldwin, 2020); therefore, *cap* genes expression might be
652 attenuated in this strain, resulting in the absence of capsule. Although CapA homologs in Bcbva
653 and atypical *B. cereus* strains are not expected to significantly confound the result of CapA322-
654 ELISA because of the rarity of such strains in nature, further validation is required to examine
655 the specificity of CapA322-ELISA by assessing possible antibody cross-reactivity.

656 A comparison of results from CapA322-ELISA and PAD1-ELISA showed that CapA322-
657 ELISA could detect anti-CapA antibodies in the sera of horses experimentally and naturally
658 infected with virulent *B. anthracis* strains. Several ELISAs have been used for serodiagnosis
659 of *B. anthracis* infection, including assays for detecting anti-LF (Ghosh et al., 2013) and PA
660 (Ghosh and Goel, 2012) IgGs; however, detected antibodies themselves are insufficient to
661 distinguish the natural infection of *B. anthracis* from vaccine immunity. Moreover, there is an
662 ELISA for detecting anti- γ -D-PGA IgG of *B. anthracis* (Harrison et al., 1989). However, Chen
663 *Z et al.* found γ -D-PGA antibodies in sera of *B. anthracis* non-infected chimpanzees (n = 9)
664 and humans (n = 6), which were likely the result of exposure to other *Bacillus* species (Chen
665 et al., 2015). In our study, anti-CapA antibodies were not detected in any horse sera (n = 6)
666 other than positive samples (n = 3) when the starting dilution of 1:100 was used (Figure 6C).
667 In addition, unlike PA-based ELISA, CapA322-ELISA showed an advantage of non-cross-
668 reactivity with vaccinated horse sera, suggesting that the CapA322 can be a helpful tool for
669 determining the naturally acquired immune response of animals. For further validation of the
670 CapA322-ELISA, screening a larger sample size for horses, including cattle, is needed. Future
671 work will therefore include follow-up work designed to validate the assay.

672 In preparing vaccinated horse serum samples, significant diversity in the antibody response
673 was observed (Figure 5A). This is in line with data observed by Phaswana *et al.* (Phaswana et
674 al., 2017) where five individual Boer goats vaccinated with Sterne 34F2 *B. anthracis* strain
675 showed variable levels of immune response. The Sterne vaccine elicited an immune response
676 in three out of four vaccinated horses in our study. However, the anti-PA IgG titers
677 progressively declined from around 5 weeks postvaccination (Figure 5A). This could explain
678 the negative result observed when the serum of PC3 collected from *B. anthracis* naturally
679 infected horse was tested by PAD1-ELISA (Figure 6C). Although the time interval from
680 infection to sample collection could not be determined, it can be speculated that the anti-PA
681 IgG titer in PC3 serum had already decreased below detectible levels by that time. Also, as a
682 major limitation of this study, weekly anti-PA IgG titers in PC2 and PC3 could not be evaluated
683 as these were collected at a single point in time and analyzed retrospectively. Nevertheless, the
684 anti-CapA antibodies were detectable by our developed assay, suggesting better stability
685 relative to anti-PA IgG. Although the horses were carefully selected as the same age and sex
686 and subcutaneously vaccinated them with the same lot of vaccines using the same procedure,
687 one horse did not show any immune response (Figure 5A). Therefore, this observation may

688 have been due to differences in the horse's immunological state or genetic background rather
689 than technical vaccination failure. Therefore, more studies with various vaccine doses and
690 challenging tasks will be needed to determine the optimal amount for horse vaccination.

691 In addition to the CapA (GBAA_RS28240), peptide ABC transporter substrate-binding
692 protein (GBAA_RS28340) was identified as immunoreactive. The peptide ABC transporter
693 substrate-binding protein was previously reported to be seroreactive with antisera of rabbit and
694 mice infected with *B. anthracis* Ames spore, and convalescent serum from rhesus macaques
695 vaccinated with Anthrax Vaccine Adsorbed (AVA, BioThrax) (McWilliams et al., 2012).
696 Chitlaru T *et al.* (Chitlaru et al., 2007) highlighted that substrate-binding proteins of ABC
697 transporters are highly immunogenic protein classes. It also noteworthy that Orit Gat *et al.*
698 conducted a similar study in search of potential immunogen proteins from the *B. anthracis*
699 genome, including two virulence plasmids. Three of their eight selected proteins from pXO2,
700 lysozyme (GBAA_RS28005), amidase (GBAA_RS28165), and metal-dependent hydrolase
701 (GBAA_RS28430), coincided with our selection. Although I failed to clone CDSs of lysozyme
702 (GBAA_RS28005) and amidase (GBAA_RS28165), they determined that these two proteins
703 are immunoreactive with guinea pig and rabbit hyperimmunized with Vollum strain of *B.*
704 *anthracis* (pXO1⁺, pXO2⁺) (Gat et al., 2006). In contrast, metal-dependent hydrolase
705 (GBAA_RS28430) was negative, which was the same as our result. The immunoreactive
706 proteins identified here and in previous studies (Ariel et al., 2002; Chitlaru et al., 2007; Gat et
707 al., 2006; Kempell et al., 2015; McWilliams et al., 2012) may be the first candidates for future
708 diagnostic developments. These proteins may provide valuable additives for AVA
709 improvement; the vaccine is currently licensed for humans and consists primarily of PA
710 (Turnbull et al., 1986). Although the vaccine is effective, expanding the vaccine protection by
711 including additional antigens in its formulation is necessary for a less demanding vaccination
712 regimen and as a defense against bioterrorism (Grabenstein, 2008). Further, regardless of the
713 protective immunity provided by PA, previous studies noted that relying on antibodies against
714 PA as the sole protector against anthrax is unconvincing due to the variable protection level
715 conferred by antibodies generated by the PA-based vaccine (Ivins et al., 1995; Ivins et al.,
716 1998; Ivins et al., 1992; Welkos and Friedlander, 1988). Our study showed the possibility of
717 antibodies against PA being short-lived in the sera of vaccinated animals, which declined to
718 range from 3 to 5 weeks after immunization (Figure 5A). Considering these aspects, adding
719 recombinant antigens to the PA might increase the durability and protective efficacy of the

720 AVA vaccine with a less demanding vaccination regimen through a multi-antigen cocktail
721 vaccine, as has been achieved against *Bordetella pertussis* infection (Jefferson et al., 2003).

722 **CHAPTER II: Risk Factors and Spatio-temporal Patterns** 723 **of Livestock Anthrax in Khuvsgul Province, Mongolia**

724 **Summary**

725 Anthrax, a worldwide zoonotic disease, has long been a public health and socio-economic
726 issue in Mongolia. Presently, there is no spatial information on carcass burial sites as a potential
727 hazard of future anthrax outbreaks and possible risk factors associated with anthrax
728 occurrences in Mongolia.

729 Here, retrospective data (1986–2015) on the disposal sites of livestock carcasses was
730 analyzed to describe historical spatio-temporal patterns of livestock anthrax in Khuvsgul
731 Province, which showed the highest anthrax incidence rate in Mongolia. From the results of
732 spatial mean and standard deviational ellipse analyses, the study found that the anthrax spatial
733 distribution in livestock did not change over the study period, indicating a localized source of
734 exposure. The multi-distance spatial cluster analysis showed that carcass sites distributed in
735 the study area are clustered. Using kernel density estimation analysis on carcass sites, two
736 anthrax hotspots were identified in low-lying areas around the south and north regions. Notably,
737 this study disclosed a new hotspot in the northern part that emerged in the last decade of the
738 30-year study period. The highest proportion of cases was recorded in cattle, whose prevalence
739 per area was highest in six districts (i.e., Murun, Chandmani-Undur, Khatgal, Ikh-Uul,
740 Tosontsengel, and Tsagaan-Uul), suggesting that vaccination should prioritize cattle in these
741 districts. Furthermore, size of outbreaks was influenced by the annual summer mean air
742 temperature of Khuvsgul Province, probably by affecting the permafrost freeze-thawing
743 activity.

744 **Introduction**

745 Anthrax is a zoonotic disease caused by the Gram-positive bacterium *Bacillus anthracis*,
746 existing as a spore outside its host animal (Mock and Fouet, 2001). The spores are highly
747 resistant to extreme temperatures, radiation, and chemical substances and can persist in soil for
748 several decades (Stephens, 1998). Anthrax has a wide range of mammalian hosts, including
749 humans, livestock, and wildlife. Domesticated herbivores such as cattle, sheep, and goats are
750 the most susceptible to anthrax; grazing with ingesting or inhaling high doses of spores usually
751 culminates in fatal disease (Hugh-Jones and de Vos, 2002). Anthrax is not contagious as direct
752 transmissions between herbivores are thought to be rare (Beyer and Turnbull, 2009; Fasanella
753 et al., 2010). Although herbivores are the primary hosts for anthrax, humans also get infected
754 with anthrax through contact with infected animals or contaminated animal products, such as
755 meat, hide, and wool (Kaufmann and Dannenberg, 2002).

756 Several environmental factors influence the persistence of *B. anthracis* spores and the
757 onset of anthrax outbreaks. Long-range dispersal of spores is influenced by weather conditions,
758 such as floods and strong winds (Fox et al., 1977; Munang'andu et al., 2012), and by biological
759 vectors, such as birds, scavengers, and biting flies (Cieslak and Eitzen, 1999; Dragon et al.,
760 2001; Turell and Knudson, 1987). Soil conditions, such as high moisture, rich calcium, high
761 organic content, and pH neutral to alkaline, correlate with the onset of outbreaks (Dragon and
762 Rennie, 1995; Hugh-Jones and Blackburn, 2009). Spores can be concentrated in low-lying
763 areas through runaway by rain and water streams, affecting the spatial distribution of outbreaks
764 (Van Ness, 1971). Recent studies highlight the role of permafrost in preserving *B. anthracis*
765 spores for a long time in the frozen ground below 0 °C, and the effect of increasing temperature
766 on permafrost thawing due to the spore spillover to the soil surface (Timofeev et al., 2019).

767 Anthrax is globally distributed, and sporadic cases have been reported on every populated
768 continent (Carlson et al., 2019). In industrialized countries where focused, comprehensive, and
769 sustained livestock vaccination programs have been successfully implemented, the disease has
770 dramatically declined (Hampson et al., 2011). However, the burden of anthrax remains high in
771 low- and middle-income countries in Africa and Asia, where animal vaccination is erratic
772 (Vieira et al., 2017).

773 In Mongolia, anthrax has posed severe challenges to public health and veterinary services
774 for a long time. Approximately 25% of the national population of 3,000,000 residents live a
775 nomadic pastoral lifestyle, raising livestock under a free-ranging system. Livestock products
776 are an essential income source for these people (Shagdar, 2002), thus, making anthrax control
777 a great priority. Mongolia was once a socialist country with close connections to the Soviet
778 Union from around 1920 to the late 1980s. The introduction of routine animal vaccination from
779 1948 onward resulted in a drastic decrease in anthrax incidence (Odontsetseg et al., 2007).
780 However, following the collapse of the Soviet Union, political revolution and economic
781 transition from socialism to a free market began in the early 1990s in Mongolia. Due to the
782 transformation recession, healthcare delivery and mass vaccination in the veterinary sector
783 were ceased (Ebright et al., 2003). Among 19 of 21 provinces in Mongolia, with records of
784 anthrax incidences, frequent outbreaks are restricted to the provinces at the northern and
785 northeastern regions of the country (Odontsetseg et al., 2007). Among them, Khuvsgul has the
786 highest livestock anthrax rate, accounting for 40.35% of all cases (Badmaeva et al., 2014).

787 The spores of *B. anthracis* can persist in soil for several decades, and may even be
788 preserved longer in frozen grounds (Stella et al., 2020). The potential hazard of the historic
789 carcass sites was confirmed by surveys of viable spore detection in previous carcass sites
790 (Dragon et al., 2001). The current disposal practice of animals dying from anthrax in Mongolia
791 mainly involves burying, and those carcass burial grounds are generally identical to outbreak
792 locations (General Authority for Veterinary Services, 2019). Although disease control
793 measures include restriction of access to carcass burial sites, which are most likely to be
794 contaminated with spores, presently, there is a lack of information on the spatial pattern of
795 carcass sites, thus limiting the implementation of interventions. Here, retrospective data (1986–
796 2015) on the burial sites of livestock carcasses was analyzed to describe historical spatial
797 patterns and temporal trends in livestock anthrax across the Khuvsgul Province to answer the
798 following questions; What is the spatial pattern of carcass sites? Where are the areas of high
799 carcass site concentration in previous and recent times? Which districts have more burden of
800 anthrax? Also, The study was interested in exploring the relationship between the number of
801 anthrax cases and animal population, annual mean precipitation, and annual mean summer air
802 temperature, considering the anthrax seasonality in Mongolia (Odontsetseg et al., 2007). This
803 study is the first spatio-temporal study on anthrax in Mongolia and could serve as a baseline
804 for future anthrax studies and public health interventions.

805 **Materials and methods**

806 **Data source**

807 Among 21 administrative provinces in Mongolia, Khuvsgul Province is the northmost
808 province, bordering Siberia, Russia, with 24 districts. Anthrax is notifiable under the law on
809 Livestock Health and Gene Protection of Mongolia (General Authority for Veterinary Services,
810 2019). Thus, reporting all suspected cases of anthrax is mandatory. An animal or animal carcass
811 with symptoms suggestive of anthrax (e.g., high fever, breathing difficulty, sudden death, a
812 carcass without rigor mortis, etc.) was considered as a suspicious case, while a positive
813 standard bacteriology and/or molecular laboratory tests was defined as a confirmed case (Stear,
814 2005). Here, basic yearly information on livestock in Khuvsgul Province, including livestock
815 and human population, the number of livestock anthrax cases, and GIS data of carcass burial
816 sites from 1986 to 2015 were collected. The Department of Veterinary Services of Khuvsgul
817 Province, Mongolia, provided the geographic information system (GIS) data for anthrax
818 carcass disposal sites recorded between 1986 and 2015. All these sites represent confirmed
819 cases previously diagnosed by clinical examination and standard bacteriology method at the
820 local veterinarian and the State Central Veterinary Laboratory (SCVL), Ulaanbaatar, Mongolia.
821 The standard bacteriology methods included Giemsa and polychrome methylene blue (PMB)
822 staining for microscopic detection of *B. anthracis* and its poly-D-glutamic acid capsule in blood
823 smears. The methods also involved culture and isolation of the bacterium, as previously
824 described in the OIE manual (Stear, 2005). In addition, samples collected after 2000 were
825 further confirmed by polymerase chain reaction (PCR) for the detection of toxin (*pagA*) and
826 capsule (*capB*) coding genes of *B. anthracis* at the SCVL. This improvement in disease
827 diagnosis would not significantly impact the anthrax surveillance since the disagreement
828 between the standard bacteriology and PCR tests for anthrax confirmation is negligible
829 (Beradze, 2019).

830 The livestock and human population data in the Khuvsgul Province was obtained from the
831 Statistics Office of Khuvsgul Province, Mongolia (National Statistics Office of Mongolia).
832 Data on annual precipitation and air temperature of Khuvsgul Province were provided by the
833 Information and Research Institute of Meteorology, Hydrology, and Environment, Mongolia.
834 Administrative areas, elevation, and inland water maps of Mongolia were downloaded from

835 the database of DIVA-GIS (Hijmans et al., 2004). The project was approved by the review
836 board of the Institute of Veterinary Medicine, Mongolia, Reference number: 20082001.

837 **The cattle anthrax prevalence per area in Khuvsgul Province by districts** 838 **(1986–2015)**

839 The annual average prevalence of anthrax per 1,000,000 cattle population per 1000 km²
840 was calculated at the district level. *B. anthracis* is a non-invasive pathogen regarded as non-
841 contagious; therefore, direct animal-to-animal transmission is not expected to occur except in
842 osteophagia or carnivore activities (Beyer and Turnbull, 2009; WHO, 2008). Indeed, soil is the
843 natural reservoir of the *B. anthracis* spores and becomes the primary source of animal infection
844 (Carlson et al., 2018). Thus, considering the mode of anthrax transmission, the cattle anthrax
845 prevalence was estimated, taking into account the area (km²) of each district as follows:

$$846 \quad \textit{prevalence per area} = \frac{\textit{annual average anthrax cases}}{(\textit{average animal population} \times \textit{area})}$$

847 A cartographic map was used to visualize the distribution of cattle anthrax prevalence
848 using ArcGIS v.10.6.1 (ESRI Inc., Redlands, CA, USA).

849 **The spatial mean and standard deviational ellipse analyses**

850 The analyses were conducted on ArcGIS v.10.6.1 software (ESRI Inc., Redlands, CA,
851 USA) (Environmental Systems Research Institute). The standard deviational ellipse (SDE)
852 analysis was performed to summarize the spatial attributes of geographic features with
853 coordinates (Yuill, 1971). The unweighted spatial mean and SDE analyses were used to
854 determine the directional trend and spatial characteristics of carcass sites in the study area. To
855 reveal the temporal changes of carcass sites resulting from anthrax incidences, the entire study
856 period was divided into three time parts: 1986–1995, 1996–2005, and 2006–2015.

857 **Multi-distance spatial cluster analysis**

858 According to the guide on the manufacturer's website, a multi-distance spatial cluster
859 analysis tool in ArcGIS v.10.6.1 software (ESRI Inc., Redlands, CA, USA) was used to

860 determine the maximum distance relationship between animal carcass sites (Environmental
861 Systems Research Institute). The tool uses Ripley's K function as shown in the equation

$$862 \quad L(d) = \sqrt{\left[A \sum_{i=1}^N \sum_{j=1, i \neq j}^N k(i, j) \right] / [\pi N(N - 1)]}$$

863 where d is the distance, N is the total number of events, A is the area, and the weight k(i, j) is
864 the influence of the elements within the distance. When the distance between i and j is less than
865 or equal to d, k(i, j) is 1, and k(i, j) is 0 when the distance between i and j is greater than d.

866 To analyze the spatial pattern of carcass sites, observed K-values, determined using actual
867 GIS coordinates, were compared with the expected K-values, calculated through the random
868 spatial distribution of carcass sites. The defaults 10 times was used as the number of distance
869 changes with 999 simulations, equal to confidence levels of 99.9%. The minimum enclosing
870 rectangle was utilized as the study area method. Positive value from the difference between the
871 observed K and expected K (Diff K) indicates clustering. When the observed K-value for a
872 specified expected K-value is larger than the upper confidence envelope (HiConEnv) value,
873 the spatial value is statistically significant. In the following kernel density estimation analysis,
874 to avoid underestimating the hotspot areas, the maximum expected K with a statistically
875 significant value was used as the maximum distance for the relationship between carcass sites
876 in the Khuvsgul Province.

877 **Kernel density estimation analysis**

878 The kernel density estimation (KDE) analysis was used to identify hotspots of animal
879 carcass disposal locations. The analysis was performed using ArcGIS v.10.6.1 software (ESRI
880 Inc., Redlands, CA, USA) by employing the quadratic kernel function described by Silverman
881 *et al.* (Läuter, 1988) to estimate carcass densities. The distances 88, 58, 83, and 88 km,
882 calculated from Ripley's K function corresponding to the periods (i.e., 1986–2015, 1986–1995,
883 1996–2005, 2006–2015), were applied as a search radius. The KDE output is classified into
884 five categories, according to the equal interval method.

885 **Statistical analyses**

886 Univariate and multivariate logistic regression models were used to determine potential
887 risk factors associated with a large number of anthrax cases among livestock in the Khuvsgul
888 Province. In the univariate analysis, anthrax outbreaks were categorized into a large or small
889 number of cases. Annual cases that were above the mean of total cases (≥ 51) during the whole
890 study period (1986–2015) were considered a large number of cases and were separately
891 analyzed with different variables, including total livestock population, cattle population,
892 human population, annual mean air temperature in summer months (June to August), and
893 annual mean precipitation. In Mongolia, anthrax is a seasonal disease that mostly occurs in
894 summer when animals graze on pastures (Odontsetseg et al., 2007). In addition, most
895 permafrost in the Khuvsgul region is at temperatures close to 0 °C, and thaws only in summer
896 (Sharkhuu et al., 2007), which possibly leads to spore spillover from buried carcasses to the
897 soil surface. Thus, the effect of temperature variability in summer was investigated. The
898 variables that met the criteria of $p < 0.2$ in the univariate analysis were further evaluated in the
899 multivariate logistic regression model, with a statistical significance level at $p < 0.05$.
900 Afterward, correlation tests were conducted between anthrax cases and cattle population or
901 temperature to determine the direct relationships.

902 Simple linear regression analyses were used to determine livestock anthrax trends
903 corresponding to before and after the Mongolian economic transition (1986–2000 and 2001–
904 2015, respectively). A linear regression model was also applied to identify the annual summer
905 (June to August) temperature changes in the Khuvsgul Province throughout the 30-year study
906 period from 1986–2015. All statistical analyses were conducted using R v.3.5.0.

907 **Results**

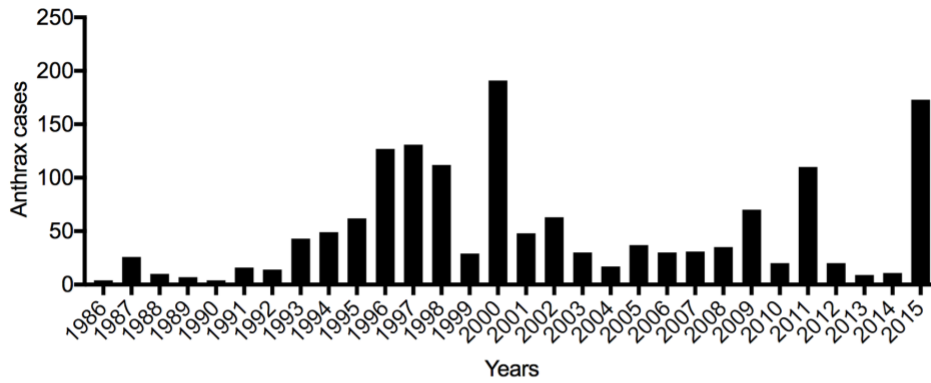
908 **Old and recent trends of anthrax between 1986 and 2015**

909 A total of 1529 livestock cases were reported over the study period (1986–2015), with the
910 majority of cases reported in cattle (76.5%; $n = 1169$), followed by horse (10.3%; $n = 157$),
911 sheep (9.2%; $n = 141$), and goat (4.1%; $n = 62$) (Figures 7A and 7B). Regarding the disease
912 prevalence in livestock by animal species for 30 years, cattle were the highest, followed by
913 horse, sheep, and goat, with proportions of 352, 90, 12, and 4 per 100,000 population,
914 respectively (Figure 7C). There were no anthrax reports in sheep and horses before 2000, and
915 few outbreaks were reported in goats. After 2000, the host range expanded, and reports of
916 anthrax incidents in goats, sheep, and other animal species had increased. Anthrax outbreaks
917 in horses were sporadic and occurred only three times over the entire 30-year period. However,
918 a large outbreak occurred in 2015, affecting more than 150 horses (Figure 8).

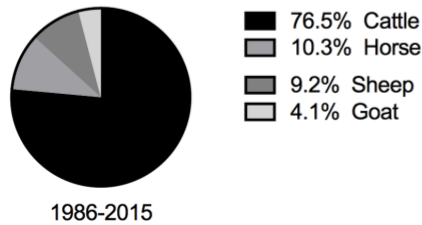
919 Further, simple linear regression analyses were conducted to determine livestock anthrax
920 trends before and after the Mongolian economic transition (1986–2000 and 2001–2015,
921 respectively). A dramatic increase was observed in the annual number of anthrax cases between
922 1986 and 2000, with an average rate of 10.1 ± 2.3 ($p < 0.001$) in a year. Several large outbreaks
923 occurred after 2000, and the average annual case number was 2.5 ± 2.6 without significant
924 increase or decrease in cases ($p = 0.35$) (Table 6). Although neither a significant increasing nor
925 decreasing trend was observed in the total livestock anthrax cases, cattle anthrax cases were
926 significantly reduced (Table 7).

927 A total of 1169 cattle anthrax cases were reported in Khuvsgul Province between 1986 and
928 2015. Annual cattle anthrax prevalence per 1,000,000 population per 1000 km² was high in the
929 districts Murun (ID 11), Chandmani-Uundur (ID 5), Khatgal (ID 24), followed by Ikh-Uul (ID
930 8), Tosontsengel (ID 16), and Tsagaan-Uul (ID 18) (Table 8 and Figure 9). The average human
931 and livestock population density for 30 years was highest in the Murun district (Figure 10).

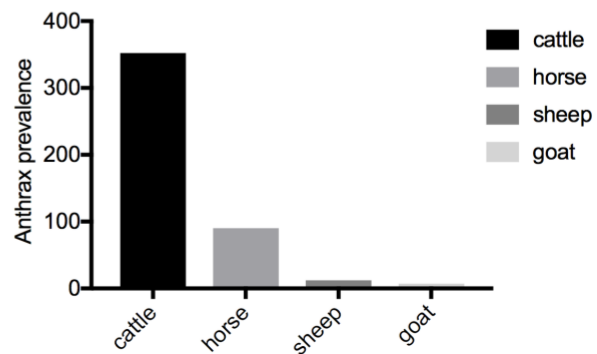
A



B



C



932

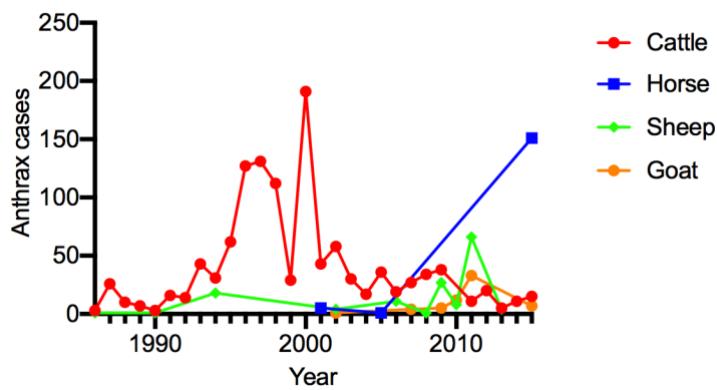
933 **Figure 7. The distribution of anthrax in Khuvsgul Province between 1986 and 2015. (A)**

934 Annual dynamics of registered livestock anthrax cases. (B) Reported anthrax cases in livestock

935 species. (C) Prevalence of anthrax in livestock by species for 30 years.

936

937



938

939 **Figure 8. Anthrax in livestock species. Anthrax dynamic in livestock from 1986 to 2015.**

940 **The spatio-temporal anthrax pattern and high-risk areas**

941 The three identified SDEs with their spatial means of carcass locations are shown in Figure
942 11. There were no significant directional changes in anthrax occurrence between the three
943 periods and the spatial means of carcass sites adjoined in the southern part of the study area.
944 By overlaying the ellipses and spatial means on an elevation map of Khuvsgul Province, it was
945 observed that carcass sites were distributed along the rivers and seasonally dry riverbeds that
946 confluent into wide rivers in the southern region.

947 To determine the distribution pattern of carcass sites and the maximum distance between
948 carcass sites in the three periods, multi-distance spatial cluster analysis was conducted. The
949 result indicated that the maximum distances of the significant spatial association between
950 carcass sites corresponding to the periods (i.e., 1986–2015, 1986–1995, 1996–2005, and 2006–
951 2015) were 88, 58, 83, and 88 km, respectively (Table 9). This result showed that carcass sites
952 distributed in the study area are clustered rather than dispersed in the specified range of
953 distances, and the observed relationships were statistically significant. The identified
954 maximum distances were then used in the KDE analysis to estimate the density of carcasses to
955 identify anthrax hotspots. consecutive anthrax hotspots were found in the southern region of
956 the Khuvsgul Province across the entire study period and an emerging new hotspot in the
957 northern part of the area between 2006 and 2015 (Figure 12).

958 **Table 6. Annual livestock anthrax incidences in Khuvsgul Province during (1986–2000)**
 959 **and after (2001–2015) the economic transition of Mongolia.**

Linear regression						
Period	Anthrax cases in livestock	Ann ¹ min	Ann ² max	Slope	<i>p</i>	R ²
1986-2000	825	4	191	10.10 ± 2.261	0.0006	0.6058
2001-2015	704	9	173	2.529 ± 2.622	0.3524	0.06678

¹Minimum annual number of cases; ²Maximum annual number of cases

960

961 **Table 7. Annual cattle anthrax incidences in Khuvsgul Province during (1986–2000) and**
 962 **after (2001–2015) the economic transition of Mongolia.**

Linear regression						
Period	Anthrax cases in cattle	Ann ¹ min	Ann ² max	Slope	<i>p</i>	R ²
1986-2000	805	3	191	10.08 ± 2.313	0.0008	0.5933
2001-2015	364	0	58	-2.475 ± 0.6898	0.0033	0.4976

¹Minimum annual number of cases; ²Maximum annual number of cases

963

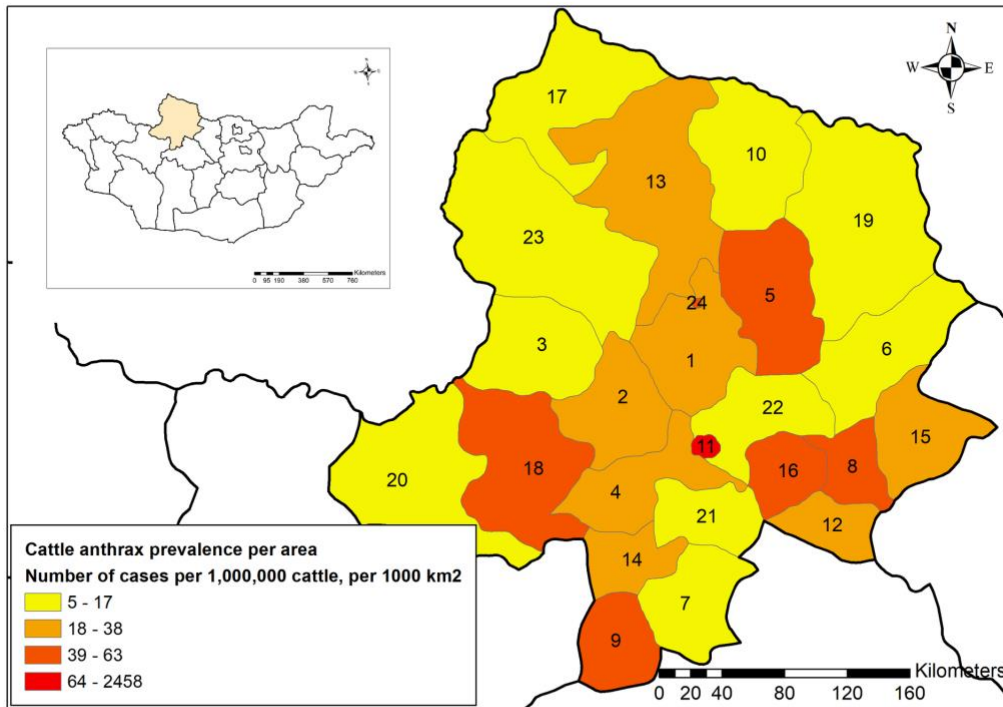
964 **Table 8. Annual cattle anthrax prevalence per 1,000,000 population per 1000 km² in**
 965 **Khuvsgul Province by districts.**

ID	District name	Number of cattle cases	Average cases (min ^a -max ^b)	Average cattle population (min ^c -max ^d)	Area of the districts (km ²)	Ann ¹ prevalence
1	Alag-Erdene	59	1.97 (3-29)	12106 (8315-20094)	4503	36
2	Arbulag	45	1.5 (1-28)	16115 (7579-27823)	3529.2	26
3	Bayanzurkh	36	1.2 (2-20)	16275 (11593-23319)	4299.1	17
4	Burentogtokh	66	2.2 (1-26)	15174 (5755-24978)	3768.6	38
5	Chandmani-Undur	120	4 (1-42)	14054 (11159-16975)	4487.5	63
6	Erdenebulgan	34	1.13 (1-18)	13845 (10447-16497)	4694.4	17
7	Galt	32	1.07 (1-12)	17300 (9654-27537)	3596.8	17
8	Ikh-Uul	51	1.7 (1-20)	13709 (9873-18942)	2023.8	61
9	Jargalant	44	1.47 (1-15)	12624 (6100-20375)	2549.2	46
10	Khankh	16	0.53 (1-13)	8589 (903-14919)	5498.7	11
11	Murun	85	2.83 (1-24)	11200 (6207-20150)	102.9	2458
12	Rashaant	19	0.63 (1-3)	12230 (8874-17462)	1982.5	26
13	Renchinlkhumbe	121	4.03 (1-18)	22632 (16469-29020)	8448.3	21
14	Shine-Ider	25	0.83 (1-7)	13897 (7140-24723)	2053.6	29
15	Tarialan	50	1.67 (1-25)	17058 (12000-22176)	3430.7	28
16	Tosontsengel	43	1.43 (1-9)	13592 (8823-21358)	2042.2	52
17	Tsagaan-Nuur	4	0.13 (2)	2602 (835-3991)	5408.3	9
18	Tsagaan-Uul	170	5.67 (2-113)	18345 (4913-37194)	5866.3	47
19	Tsagaan-Uur	21	0.7 (2-12)	12855 (10419-15131)	8735.3	6
20	Tsetserleg	59	1.97 (1-24)	16990 (5967-33153)	7451.6	16
21	Tumurbulag	16	0.53 (1-8)	12603 (6557-19964)	2521.7	17
22	Tunel	16	0.53 (1-4)	12646 (8828-17166)	3577.3	12
23	Ulaan-Uul	27	0.9 (1-18)	18739 (13615-23438)	10057.5	5
24	Katgal	10	0.33 (4-6)	6990 (3915-9657)	911.4	52

¹Annual anthrax prevalence per 1,000,000 cattle per 1000 km²

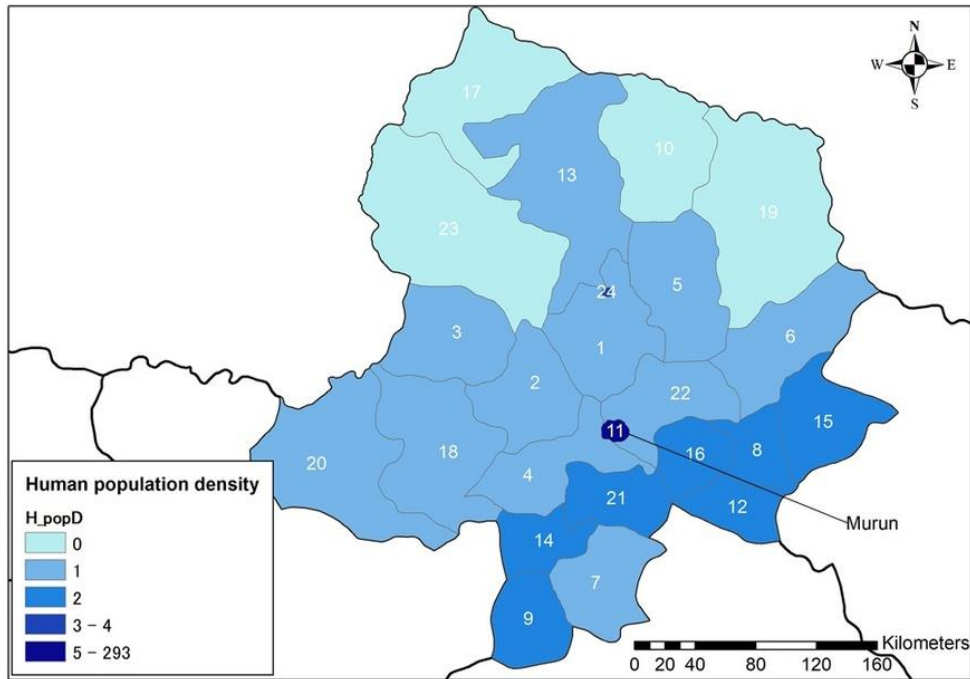
^a Minimum annual number of cattle anthrax cases; ^b Maximum annual number of cattle anthrax cases

^c The smallest annual cattle population number; ^d The largest annual cattle population number



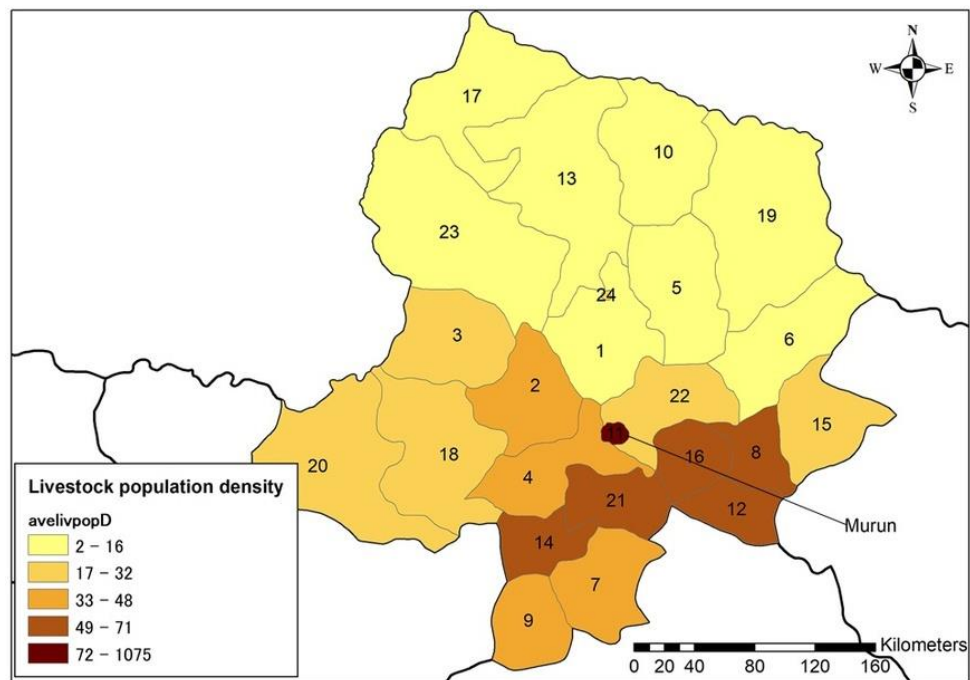
967
 968 **Figure 9. Annual cattle anthrax prevalence per 1,000,000 population per 1000 km² in**
 969 **Khuvs gul Province by districts.** Numbers correspond to the ID number in Table 8. The Murun
 970 (district ID 11), the central administrative district of Khuvs gul Province, had the highest cattle
 971 anthrax prevalence per area. The maps are reprinted from (Hijmans et al., 2004) under a CC
 972 BY license, with permission from DIVA-GIS and Dr. Robert Hijmans.

973



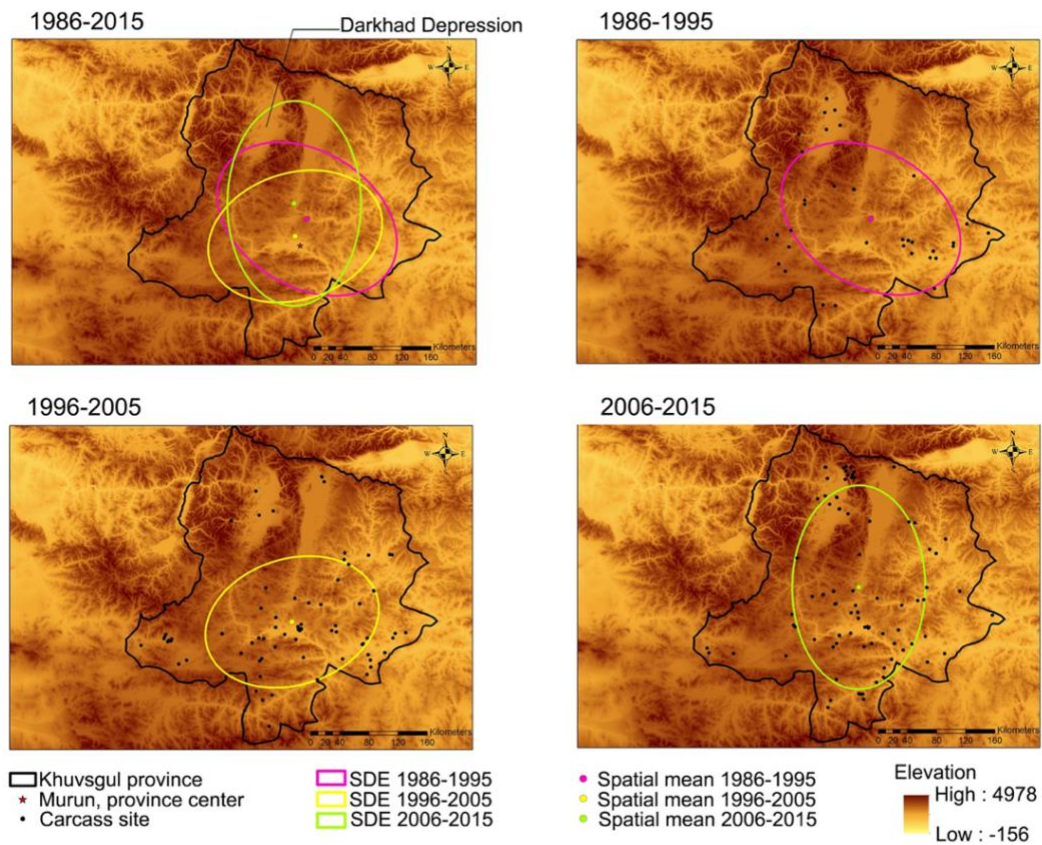
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976

977 **Figure 10. Average human and livestock population densities by districts of Khuvsgul**
978 **Province (1986–2015).** Murun district is the administrative center of the province and is
979 estimated with the highest human and livestock population densities. The maps are reprinted
980 from (Hijmans et al., 2004) under a CC BY license, with permission from DIVA-GIS and Dr.
981 Robert Hijmans.



982

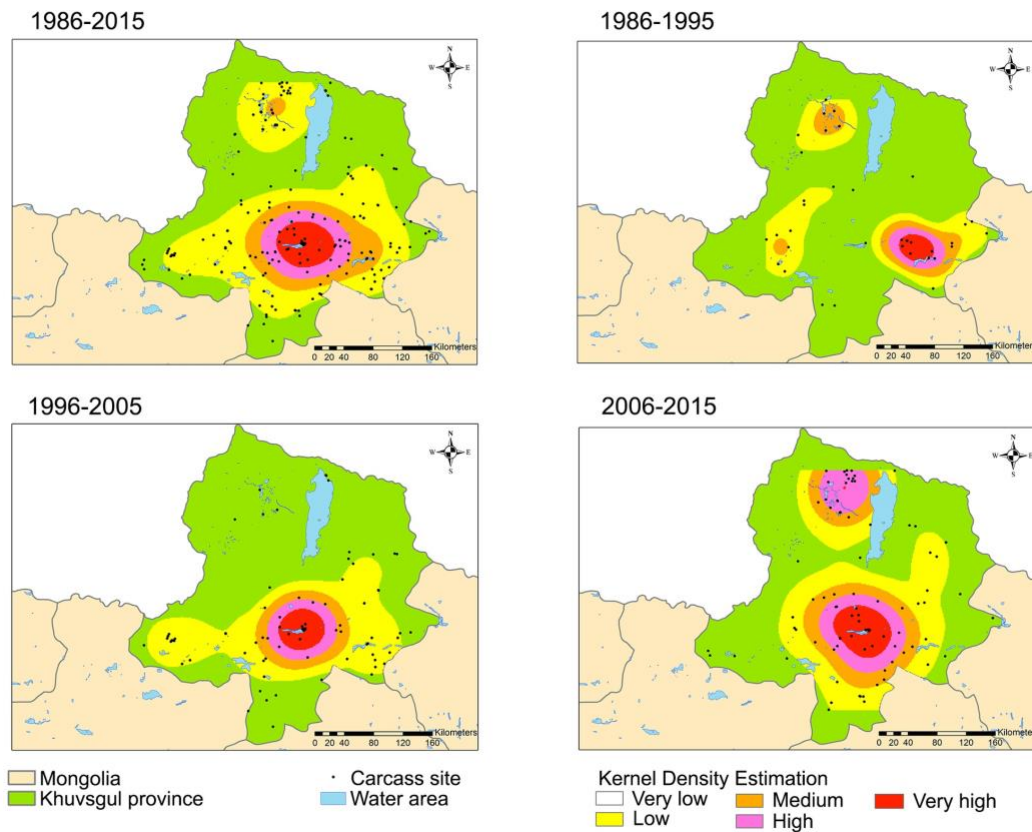
983 **Figure 11. The directional distribution of animal carcass sites registered between 1986**
 984 **and 2015.** Unweighted spatial means and standard deviational ellipse (SDE) were determined
 985 in three periods (1986–1995, 1996–2005, and 2006–2015). The unweighted spatial mean
 986 centers indicate the average value of carcass locations in the given time phases. Ellipses with
 987 mean centers were combined to denote the directional distribution of carcass sites. The base
 988 map is the elevation in meters, with lighter areas being lower in elevation. The maps are
 989 reprinted from (Hijmans et al., 2004) under a CC BY license, with permission from DIVA-GIS
 990 and Dr. Robert Hijmans.

991 **Table 9. Result of multi-distance spatial cluster analyses.**

	expectedK (m)	observedK (m)	diffK (m)	LwConfEnv	HiConfEnv
1986–1995	7212.506103	17579.10497	10366.598	0	14916.3645
	14425.01221	31446.4574	17021.4452	7861.61435	24103.2532
	21637.51831	37373.68796	15736.1697	17223.9341	31837.1116
	28850.02441	44053.1086	15203.0842	22785.1231	39308.0717
	36062.53051	50092.73595	14030.2054	30244.2343	46376.8617
	43275.03662	57770.83113	14495.7945	38353.0772	54125.3735
	50487.54272	64253.97011	13766.428	45298.1825	60997.2115
	57700.04882	69698.43213	11998.3833	52029.3968	69520.8562
	64912.55492	74747.37592	9834.821	59457.9252	76464.0512
	72125.06103	8018.72247	7893.66144	66056.276	84012.6701
1996–2005	8324.025878	50481.65039	42157.6245	5820.17166	13505.5768
	16648.05176	53586.4411	36938.3893	14617.4307	22015.0963
	24972.07763	56819.80914	31847.7315	22367.3602	29456.8453
	33296.10351	60247.58951	26951.486	30712.7286	38131.2653
	41620.12939	68884.11614	27263.9867	39408.2589	45970.0043
	49944.15527	76807.26993	26863.1147	47228.1572	54954.8258
	58268.18115	83379.06679	25110.8856	55450.4559	63264.4483
	66592.20703	88679.60196	22087.3949	63715.9032	71810.3452
	74916.2329	96178.56519	21262.3323	71574.0964	79212.2517
	83240.25878	99951.69691	16711.4381	79212.2517	87719.4381
2006–2015	8813.435842	25903.4777	17090.0419	4729.30635	13652.3315
	17626.87168	35757.64284	18130.7712	13788.1789	23881.8264
	26440.30753	44364.82609	17924.5186	23408.8721	32822.431
	35253.74337	52592.49506	17338.7517	31725.1515	40911.4613
	44067.17921	62324.05206	18256.8728	41047.9091	50752.898
	52880.61505	70913.31648	18032.7014	49901.0924	59383.7187
	61694.0509	82864.14146	21170.0906	58530.142	68425.2892
	70507.48674	90538.73804	20031.2513	66182.1323	77156.8129
	79320.92258	98713.05977	19392.1372	75149.8535	85629.6171
	88134.35842	104989.8909	16855.5325	83379.8751	94487.5484
1986–2015	8821.76704	34127.46953	25305.7025	7653.6782	10929.5374
	17643.53408	39099.76416	21456.2301	16324.0946	19805.2562
	26465.30112	46413.42786	19948.1267	25122.8531	29104.9007
	35287.06816	52656.71269	17369.6445	34085.3732	37663.319
	44108.8352	61914.98147	17806.1463	42647.5556	46444.3516

52930.60224	70904.43218	17973.8299	51067.6488	55533.7825
61752.36928	80358.26118	18605.8919	59906.6969	64401.9258
70574.13632	88048.28697	17474.1507	68322.2185	72936.6722
79395.90336	95759.47909	16363.5757	76914.7807	81190.1273
88217.6704	102592.5447	14374.8743	85190.684	89747.3321

The maximum expected K distances with statistically significant values are highlighted with gray background in the table. The spatial values corresponding to the time phases were then used for kernel density estimation analysis on anthrax carcass sites.



993

994 **Figure 12. Hotspot analysis of carcass sites using kernel density estimation.** The heat maps
 995 show the estimated density of anthrax carcass sites per square kilometer from very low
 996 (transparent) to very high (red): very low <20%, low 40%, medium 60%, high 80%, and very
 997 high >80% of the estimated highest values in each period. The maps are reprinted from
 998 (Hijmans et al., 2004) under a CC BY license, with permission from DIVA-GIS and Dr. Robert
 999 Hijmans.

1000 **Positive association between cattle population, temperature, and anthrax**
1001 **case numbers**

1002 Two factors, cattle population number and annual mean temperature of summer months,
1003 have met the criteria in the initial univariate analyses. In multivariate logistic regression
1004 analysis, the odds of having a large number of anthrax cases (≥ 51) were multiplied by 7.63 for
1005 every 100,000 increase in cattle population size and by 2.86 for every 1 °C increase in mean
1006 temperature of summer (Table 10).

1007 **Cattle population, mean summer temperature, and outbreak magnitude**

1008 After identifying that cattle population and temperature are positively correlated with the
1009 anthrax case number, I investigated the extent to which the two factors affected the magnitude
1010 of an anthrax outbreak. A simple linear regression was used to model the dependence of anthrax
1011 cases on the cattle population over the entire 30-year period. A steady increase was observed
1012 in livestock population in the first half of the study period (1986–1999), followed by a decrease
1013 in 2000 before rising again in the second half (2001–2015) (Figure 13A). A drastic reduction
1014 in cattle number in 2000 was attributed to a disaster associated with severe climatic conditions,
1015 which is called zud in Mongolia. Mongolia was hit by three consecutive zuds between 1999
1016 and 2002. Drought up to 60% of the national territory resulted in reduced pasture growth in
1017 summer and limited forage preparation by herders for the winter. Weakened by inadequate
1018 summer feeding and insufficient supplementary forage, several millions of animals died in
1019 extremely harsh winter with temperatures up to -50°C in some areas. Overall, the national
1020 livestock population decreased by about 12 million because of the three-year sequent zud
1021 (Batima et al., 2008). A significant relationship between anthrax cases and cattle population
1022 ($r = 0.52$, $p = 0.003$) (Figure 13B) was found. The regression line's slope was 3.95 ± 1.2 ,
1023 suggesting that for every 10,000 increase in cattle population size, the number of anthrax cases
1024 in cattle increased by an average of about 4.

1025 A steady rise in annual mean summer temperature of 0.08449 ± 0.02077 ($p < 0.001$)
1026 (Figure 14A) was observed, which exhibited a positive correlation with the total anthrax cases
1027 in livestock over the entire study period (1986–2015) ($r = 0.46$, $p = 0.009$) (Figure 14B). The
1028 slope of the simple linear regression line was 19.24, suggesting that for every 1 °C increase in

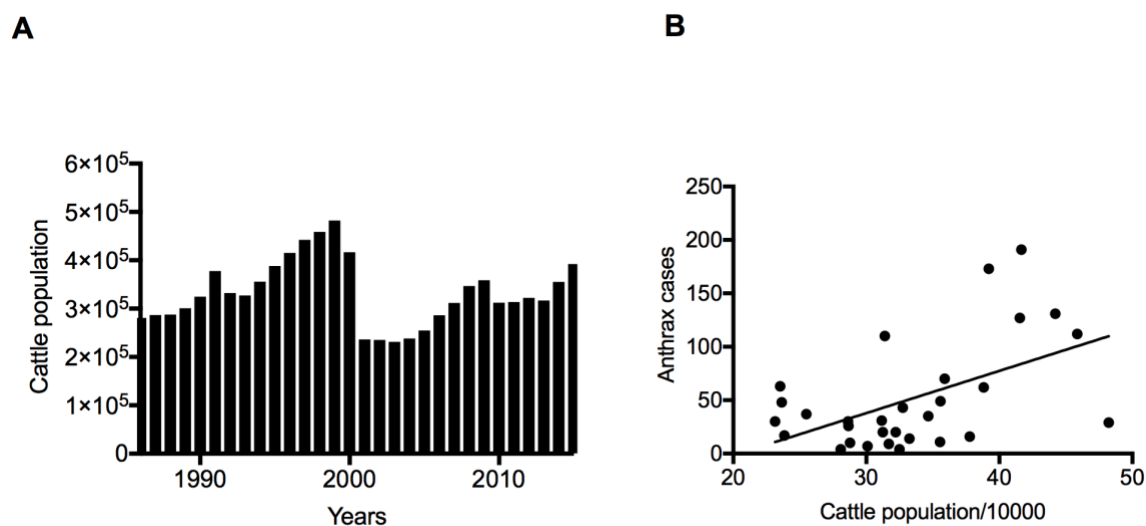
1029 air temperature, the number of livestock cases increased by 19. No positive correlation was
1030 observed between the cattle population and temperature changes (Figure 15).

1031 **Table 10. Factors potentially associated with a large anthrax outbreak occurrence by**
 1032 **univariate and multivariate logistic regression: cases (large > 51 ≥ small) vs. factors**

Factors Univariate	OR ¹	95% CI ² for OR ¹	<i>p</i> < 0.2
Total livestock population	1.00	1.00–1.00	0.5062
Cattle population	5.99	1.53–36.46	0.0224
Human population	1.00	1.00–1.00	0.273
Annual mean summer temperature (Jun-Aug)	2.07	1.02–5.11	0.0661
Annual mean precipitation	0.56	0.09–2.40	0.485
Year	1.04	0.95–1.15	0.3988
Factors Multivariate	OR ¹	95% CI ² for OR ¹	<i>p</i> < 0.05
Cattle population	7.63	1.79–59.45	0.0172
Annual mean summer temperature (Jun-Aug)	2.86	1.16–9.71	0.0437

¹Odds ratio; ²Confidence interval

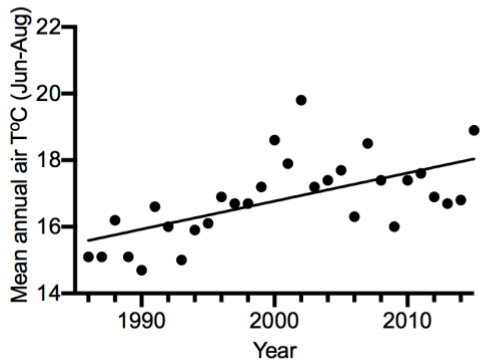
1033



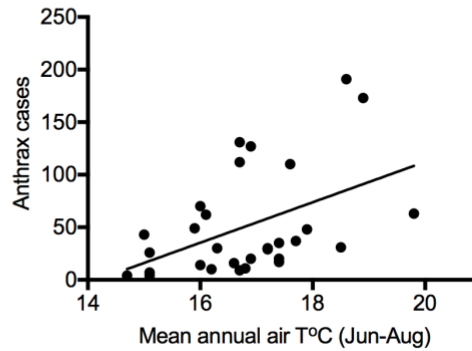
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1035 **Figure 13. The magnitude of anthrax cases in relation to cattle population. (A) Cattle**
 1036 **population by year. (B) Correlation between anthrax cases and cattle population between 1986**
 1037 **and 2015. $r = 0.52$, $p = 0.003$, 95% CI 0.2–0.743.**

A



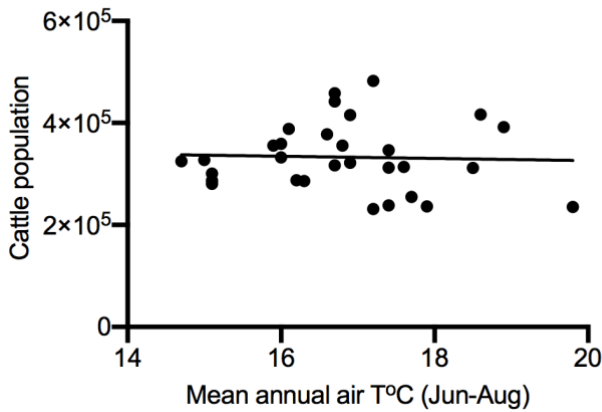
B



1038

1039 **Figure 14. The magnitude of anthrax cases in relation to air temperature.** (A) Linear
 1040 regression of annual summer air temperature 1986–2015, slope = 0.08449 ± 0.02077 , $R^2 = 0.4$,
 1041 $p < 0.001$, 95% CI 0.4195–0.127. (B) Correlation between anthrax cases and annual summer
 1042 air temperature from 1986 to 2015 $r = 0.46$, $p = 0.0099$, 95% CI 0.1238–0.7058.

1043



1044

1045 **Figure 15. Interaction between temperature and cattle population.** There was no
 1046 correlation observed between the two risk factors with $r = -0.0389$, $p = 0.83$, 95% CI -0.3936 –
 1047 0.3259 .

1048 **Discussion**

1049 Here, I report spatial and temporal patterns of anthrax in livestock between 1986 and 2015
1050 based on the carcass burial sites of animals that died of anthrax in Khuvsgul Province, showing
1051 the highest anthrax incidence rate in Mongolia. Our primary objective was to examine the
1052 spatio-temporal dynamics of the disease based on the carcass burial sites and identify where *B.*
1053 *anthracis* spores may persist in the present day. First, the spatial distribution of carcass sites in
1054 three historical periods (1986–1995, 1996–2005, 2006–2015) was determined. It was found
1055 that the spatial distribution of carcass sites had not changed over the 30 years, indicating the
1056 recurrence of anthrax. Using KDE analysis on carcass sites, two hotspots were identified in
1057 low-lying areas around the south and north regions. There was a recently emerged hotspot
1058 identified in the northern part of the province in the last decade of the 30-year study period.
1059 Also, the highest proportion of anthrax cases was recorded in cattle, and cattle anthrax
1060 prevalence was high in several districts. Moreover, the disease trend during and after the
1061 transition period of the country’s political change was shown. Furthermore, a positive
1062 association between outbreak size and cattle population number and the mean annual air
1063 temperature of the summer months (June to August) was observed, highlighting the impact of
1064 these factors on anthrax occurrence in Khuvsgul region.

1065 Results of spatial mean and SDE analyses revealed a localized source of exposure of
1066 anthrax. No significant changes were observed in the distribution of anthrax carcass sites for
1067 the entire 30 years. Unweighted spatial means and spatial overlapping of three SDEs
1068 corresponding to every decade overlaid in the same geographical area. It appears that animals
1069 are repeatedly exposed to spores in the same geographical areas. This suggests that historic
1070 carcass sites are a potential hazard that serves as a source of spore exposure, resulting in
1071 recurrent outbreaks. The localized source of exposure, defined as independent outbreaks
1072 recurring in the same geographical areas, was also identified in several other spatio-temporal
1073 studies on anthrax that characterized outbreaks following a point-source pattern with limited
1074 geographical spread (Blackburn et al., 2014; Dragon et al., 2005; Driciru et al., 2018; Muturi
1075 et al., 2018). Strong, consistent spatio-temporal patterns were observed in two anthrax
1076 outbreaks in hippopotamus (*Hippopotamus amphibious*) with 5-year intervals in the same
1077 location in the Queen Elizabeth Protected Area, Uganda (Driciru et al., 2018). In addition,
1078 consecutive episodes occurred in the same area with a high density of white-tailed deer

1079 (*Odocoileus virginianus*) carcasses in West Texas (Blackburn et al., 2014). Furthermore,
1080 concurrent and spatially localized outbreaks were observed in the Serengeti ecosystem,
1081 Tanzania (Hampson et al., 2011). Known carcass sites could likely have been the source of
1082 anthrax infection over a certain distance range (Dragon and Rennie, 1995; Dragon et al., 2001;
1083 Mongoh et al., 2008). Therefore, proper disposal of carcasses is crucial to prevent further spore
1084 exposure from affected sites.

1085 KDE analysis identified two anthrax hotspots based on the density of animal carcasses that
1086 died of anthrax (Figure 12). One hotspot has been detected around the southern region for the
1087 entire 30 years, whereas the second hotspot emerged in the northern region in the last decade
1088 of the study period. Interestingly, both hotspots were identified in low-lying areas around the
1089 Murun district in the south and the Darkhad Depression in the north. Regarding this particular
1090 geographical feature, Van Ness previously postulated that “anthrax incubator areas” retain high
1091 spore concentrations through the water cycle in a low-lying area or depression, resulting in
1092 spore doses that are lethal to a susceptible host and enough to trigger new outbreaks (Van Ness,
1093 1971). Supporting this postulate, carcass hotspots have been found only in low-lying areas that
1094 have wide rivers and lakes in our study (Figures 11 and 12). Notably, the Darkhad Depression
1095 encompasses many rivers, lakes, small potholes, and wetlands surrounded by mountains (Call,
1096 2018). In agreement with our findings, Relebohile *et al.* (Lepheana et al., 2018), Bengis (Bengis,
1097 2012), and Dragon *et al.* (Dragon and Rennie, 1995) reported a high incidence of anthrax
1098 outbreaks in depressed low-lying areas. Although the effect of water area on the number of
1099 anthrax cases was not explored in this study, I suspect that it might increase the risk of many
1100 animals being infected from one spot. Thus, the disposal of affected animal carcasses near
1101 rivers and lakes should be avoided to restrict the spore dissemination and concentration through
1102 the hydrological cycle in a low-lying area. Besides, 30-year average human and livestock
1103 population densities were highest in Murun, the administrative center of Khuvsgul Province
1104 (Figure 10). The pasture in this area could be overgrazed because of the high animal population
1105 density, thereby increasing the chance of animals ingesting or inhaling the spores. Also,
1106 anthropogenic pressure such as construction and agriculture may contribute to anthrax
1107 occurrence by exposing the spores to the ground surface, resulting in many carcass disposal
1108 sites. Therefore, human activities such as construction, mining, or agricultural development
1109 around carcass sites must be avoided, and those areas must be secured by fencing to minimize
1110 future outbreaks.

1111 Furthermore, univariate and multivariate logistic regression analyses were conducted to
1112 determine the potential association between the number of anthrax cases in livestock and
1113 possible risk factors. I found that the population size of susceptible animals and temperature
1114 increase are the factors that impact outbreak size. Other environmental and climatic drivers,
1115 including drought, rainfall, soil alkalinity, and density of insects or scavengers, have long been
1116 recognized as important factors influencing anthrax ecology (Hugh-Jones and Blackburn,
1117 2009). In this study, however, precipitation including rainfall, did not seem to impact the size
1118 of the outbreaks.

1119 Cattle population number was determined as one of the risk factors for a large outbreak.
1120 From the univariate and multivariate logistic regression analyses, a significant positive
1121 correlation was found between the population number of cattle and annual anthrax cases from
1122 1986 to 2015 (Table 10 and Figure 13B). A similar positive correlation between the number of
1123 anthrax cases and the hippopotamus population was detected in the Queen Elizabeth National
1124 Park, Uganda (Driciru et al., 2018). Hence, the susceptible animal number can be one of the
1125 determinants for outbreak size. Among the livestock species, cattle are most likely to contract
1126 anthrax in many cases (Epp et al., 2010; Mongoh et al., 2007). From the studies conducted in
1127 Kazakhstan (Aikembayev et al., 2010), China (Chen et al., 2016), and Ukraine (Bezymennyi
1128 et al., 2014), anthrax predominantly occurred in cattle compared with other animal species. In
1129 agreement with these reports, cattle anthrax occupied 76.5% of the total anthrax cases in
1130 Khuvsgul Province, and anthrax prevalence for 30 years was highest among cattle (Figures 7B
1131 and 7C), implying that cattle are the most susceptible to anthrax. Thus, in terms of resource-
1132 limited settings, cattle should be prioritized for vaccination. Moreover, cattle anthrax
1133 prevalence per 1 million population per 1000 km² was high in the districts Murun, Chandmani-
1134 Undur, Khatgal, followed by Ikh-Uul, Tosontsengel, and Tsagaan-Uul (Table 8 and Figure 9).
1135 Therefore, more effort in vaccination and disease surveillance should be focused on these
1136 districts with a high burden of anthrax.

1137 Also, increased temperature was detected as another risk factor. Recent studies
1138 emphasized the impact of global warming associated with permafrost melting on anthrax
1139 occurrence in northern latitudes of the globe (Stella et al., 2020). After the reemergence of
1140 anthrax in reindeer over 70 years later in Yamal, Siberia, in 2016 (Liskova et al., 2021) and
1141 Sweden (Ågren et al., 2014), experts believed that infected animal carcasses previously buried

1142 in these regions were long preserved under the freezing effect of permafrost. However,
1143 permafrost melting resulting from global temperature increases the spore spillovers from the
1144 carcass into the ground surface, likely through moving sediments and soil cracking related to
1145 permafrost freeze-thaw activity (Elvander et al., 2017). This hypothesis is further supported by
1146 our findings, where an increasing trend of mean annual summer temperature showed a positive
1147 correlation with the number of anthrax cases in livestock in Khuvsgul (Figures 14A and 14B).
1148 The territory of Khuvsgul comprises a wide area of mountain permafrost, which is a
1149 continuation of the southern fringe of the Siberian permafrost zone, representing the highest
1150 permafrost prevalence in Mongolia (Munkhjargal et al., 2020). From permafrost monitoring
1151 studies, the Khuvsgul region increased in mean annual permafrost temperature, coupled with
1152 intensive degradation of permafrost. These observations have been attributed to climatic factors
1153 such as global warming and anthropogenic elements like changing soil content, vegetation
1154 cover, and hydrologic cycle in the last several decades (Sharkhuu et al., 2007; Sharkhuu and
1155 Sharkhuu, 2012). To this end, the long-term anthrax persistence and the highest incidence rate
1156 in the Khuvsgul Province could be explained by the ecosystem changes, the prevalence of
1157 permafrost, and its freeze-thaw dynamics. These observations suggest that permafrost may
1158 have a role in spore persistence in soil and *B. anthracis* infection cycle, making it a potentially
1159 useful spatial and temporal predictor of infection risk and anthrax outbreaks.

1160 I could not obtain data on the animal anthrax vaccination trends, which is a major
1161 limitation of this study. But, a previous study showed that anthrax vaccination coverage
1162 decreased between 1990 and 2000 because of the country's political revolution and economic
1163 transition phase (Odontsetseg et al., 2007). This phase involved the privatizing of animal
1164 husbandry sectors and the suspension of veterinary services, resulting in a drastic drop in the
1165 anthrax vaccination coverage and a steady increase in anthrax cases. In agreement with the
1166 study, a dramatic increase was observed in yearly livestock anthrax cases in Khuvsgul Province
1167 from 1986 to 2000 (Table 6). Similar increasing anthrax trends were seen in other former Soviet
1168 countries, which was likely a result of socio-political instability (Bezymennyi et al., 2014;
1169 Kracalik et al., 2014). Taken together, these findings highlight that Mongolia's political change
1170 and economic transition affected anthrax occurrence, more likely through livestock vaccination.
1171 Since 2000, although neither a significant increasing nor decreasing trend was observed in the
1172 total livestock anthrax cases, cattle anthrax cases were significantly reduced, which had
1173 progressively increased during the transition period (Table 7). It can be speculated that the

1174 downslope in cattle anthrax cases was likely associated with improving disease control
1175 measures, particularly cattle vaccination (Odontsetseg et al., 2007). Further, extending
1176 vaccination to livestock species excluded in routine vaccination programs is on demand for
1177 successful disease control in Mongolia, especially in areas with high-risk factors.

1178 **General conclusion**

1179 The long-time persistence of highly resistant anthrax spores in the environment and its
1180 detrimental risk to animal and human health have been a great concern. Anthrax continues to
1181 pose a serious public health and socioeconomic threat in several developing countries, and it
1182 tends to re-emerge in some places after a long period of absence. Based on various recently
1183 published literatures, the geographic distribution of anthrax is predicted to expand with
1184 continued warming, suggesting that countries need to strengthen outbreak preparedness and
1185 develop practical control strategies. However, the lack of detailed serological and
1186 epidemiological data in many developing countries limits the successful implementation of
1187 effective anthrax control policies. Presently, there is a lack of serological assays and
1188 epidemiological information to develop an anthrax management and control strategy in many
1189 countries, including Mongolia. Therefore, this work provides a serological tool that can be
1190 utilized to improve anthrax serosurveillance worldwide. Furthermore, using Mongolia's
1191 Khuvsgul Province as an example, the project demonstrates how GIS data on carcass sites can
1192 be valuable for predicting anthrax hotspots that may serve as infection sources for future
1193 outbreaks.

1194 In chapter I, a new ELISA test to detecting naturally acquired antibodies against anthrax
1195 infection was developed. This ELISA was established based on *B. anthracis* pXO2 plasmid,
1196 which lacks in animal vaccine strains, thus distinguishing them from virulent strains. This
1197 research has demonstrated two immunoreactive antigens encoded on the pXO2 plasmid; the
1198 capsule biosynthesis protein CapA and peptide ABC transporter substrate-binding protein.
1199 Concerning the antigen specificity, immunoreactivity, and solubility of protein expression, the
1200 C-terminus end of capsule biosynthesis protein CapA, named CapA322, was further selected
1201 and used for CapA322-ELISA development. The CapA322-ELISA was shown to be specific
1202 and non-cross-reactive to sera from horses vaccinated with *B. anthracis* Sterne 34F2 strain live
1203 spore vaccine. Hence, the CapA322-ELISA can be used to detect naturally acquired antibodies
1204 and ascertain the immunological state of animals. While the results presented here are
1205 satisfactory, further research is essential to optimize the CapA322-ELISA. Such future work
1206 will involve field studies of livestock in endemic and non-endemic areas to validate the assay.

1207 In chapter II, spatial and temporal patterns of anthrax carcass sites in Khuvsgul Province,
1208 Mongolia were analyzed to inform the geographical distribution and hotspot areas of carcass
1209 sites as they are potential infection sources. Moreover, 30-year retrospective data (1986–2015)
1210 of anthrax cases among livestock in Khuvsgul Province was analyzed to reveal past and recent
1211 trends of the disease, anthrax prevalence in districts, and its burden in animals. Furthermore,
1212 the study investigated the risk factors that possibly influence anthrax occurrence to understand
1213 hyperendemicity of anthrax in this province.

1214 The results showed that the spatial distribution of carcass sites had not changed over the
1215 30 years, indicating the recurrence of anthrax. There was one stable hotspot of anthrax carcass
1216 sites around the south and an emerging new one in the north region of the province. These
1217 hotspots exist in low-lying areas with abundant rivers, lakes, and ponds. Further, the burden of
1218 anthrax was higher in cattle than in other livestock species, and the cattle anthrax prevalence
1219 was high in the six districts. Finally, the size of outbreaks was influenced by the annual summer
1220 mean air temperature (June to August) of Khuvsgul Province, probably by affecting the
1221 permafrost freeze-thawing activity.

1222 The study suggests that historical carcass burial sites may serve as a persistent source of
1223 anthrax infection. Thus, regarding the primary action for carcass disposal management, it can
1224 be recommended that fencing the old carcass sites to prevent possible animal spore exposure.
1225 Further, burying animal carcasses that died of anthrax should be banned and replaced by
1226 incineration, considering long-time preservation of *B. anthracis* spores in the frozen ground
1227 (permafrost) and its seasonal thawing effect on spore spillover to the soil surface. At least
1228 carcass disposal near rivers and lakes should be avoided to minimize spore dissemination and
1229 concentration through the hydrological cycle. As additional anthrax control measures, this
1230 study recommends the strategic vaccination of susceptible animals, especially prioritizing
1231 cattle in the six districts mentioned above. Another suggestion would be monitoring the
1232 permafrost condition in endemic areas, which could be helpful to predict future outbreaks and
1233 design epidemic preparedness plans. Future work should involve detailed field surveys on
1234 spore viability around the carcass burial sites and molecular epidemiology of the *B. anthracis*
1235 strains.

1236 Overall, the work presented here highlights a new approach for investigating anthrax.
1237 Firstly, it introduces a novel diagnostic tool that can be used to collect surveillance data

1238 prospectively. Secondly, using data from Mongolia, the study emphasizes the importance of
1239 retrospective data in identifying high risk areas to prevent future outbreaks.

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