1 **Immunogenicity and efficacy of Heat Inactivated Whole-Cell Vaccine to**
2 **increase murine survival after Extensively drug-resistant *Acinetobacter***
3 ***baumannii* infection.**

4
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11 **Running heads:** HI-WCV increases neutralizing IgG antibody titre

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16 **Key words:** Heat Inactivated Whole Cell Vaccine, *A. Baumannii*, XDR,
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Abstract: Due to the rapid emergence of extensively drug-resistant (XDR) strains worldwide there is a necessity for greater consideration for the role of preventive vaccines to combat these pathogens. The study reported the effectiveness of a heat-inactivated whole-cell vaccine (HI-WCV) against *A. baumannii* to produce protective immunity with evaluation of the bactericidal antibody responses in mice after intramuscular inoculation with different XDR strains of *A. baumannii*. Six XDR *A. baumannii* strains emulsified with complete freund's adjuvant (CFA) were used for intramuscular inoculation in the experimental group of mice (n=4) in three different phases on 14 days interval whereas placebo-controlled mice (n=4) received phosphate-buffered saline emulsified with CFA in same the schedule. Serum was collected from tail blood of each mouse on 10th day after each inoculation and by cardiac puncture on 14th day after lethal dose from the experimental group and after death of the placebo-controlled group. Mice inoculated with heat-inactivated whole-cell vaccine survived and showed a higher neutralizing (IgG) antibody response after 2nd (>7-fold titre) and 3rd inoculation (>12-fold titre) whereas all mice from placebo-controlled group did not survived. Sera from experimental group of mice collected (38th day) after 3rd inoculation resulted in higher serum bactericidal killing index (42.12) after three hours incubation with an XDR *A. baumannii* over sera collected after 1st and 2nd inoculation (50% cutoff value=14.5). These results suggest that intramuscular vaccination with heat-inactivated whole-cell vaccine stimulated IgG antibody responses that can increase murine survival after XDR *A. baumannii* infection.

40 **Introduction:**

41 *Acinetobacter* species are non-fermentative, non-fastidious, catalase-positive,
 42 oxidase negative, non-motile and strictly aerobic gram-negative coccobacilli (1).
 43 Among these species *A. baumannii* causes serious infections that are associated
 44 with high morbidity and mortality rates (2) particularly, in immunocompromised
 45 patients, elderly, premature neonates and patients that had recently undergone
 46 surgery or experienced a major trauma or were previously admitted to contaminated
 47 ICUs (3) causing septicemia, endocarditis, meningitis, skin and soft tissue
 48 infection, wound infection, respiratory tract infections and urinary tract infections
 49 (4). The World Health Organization declared that *A. baumannii* is one of the most
 50 serious ESKAPE organisms (*Enterococcus faecium*, *Staphylococcus aureus*,
 51 *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*
 52 species) that effectively escape the effects of antibacterial drugs (5). Now, MDR
 53 and XDR *A. baumannii* have emerged worldwide as a critical problem and have
 54 been increasingly reported (6) as a result of widespread and indiscriminate use of
 55 antibiotics.

56 *A. baumannii* exhibits an intrinsic resistance to a range of antibiotics such as
 57 amoxicillin, narrow spectrum cephalosporins, ertapenem, trimethoprim and
 58 chloramphenicol (7). In addition, *A. baumannii* has managed to acquire resistance
 59 genes against several classes of antibiotics through the transfer of plasmids,
 60 transposons and integrons from other gram-negative bacteria (8). Currently, there
 61 are a small number of drug candidates like colistin, tigecycline, eravacycline (9),

62 cefiderocol and other therapeutic options such as monoclonal antibody (10), phages
63 (11) that may be used to treat XDR *A. baumannii* infections. Several researchers
64 adopted the idea of solving the *A. baumannii* problem by vaccination (12).
65 However, to date, there are no licensed vaccines against XDR *A. baumannii*.

66 Among the inactivated vaccine trials there are some single antigen candidates that
67 have been tested includes, the outer membrane protein (OmpA), the K1 capsular
68 polysaccharide, the biofilm-associated protein (Bap) and the membrane associated
69 polysaccharide (poly-N-acetyl- β -1-6-glucosamine) (13). Heat-inactivated whole-
70 cell vaccines are easy to prepare, inexpensive and does not require expensive
71 denaturation of antigens, that may induce conformational changes of the epitopes.
72 previous studies have shown that, emulsification with CFA and IM immunization
73 in mice generate hyper-immune antibodies against its multi-antigenic components,
74 mostly those belonging to the outer membrane (14), providing diverse protection
75 against several *A. baumannii* strains with a complete bacterial clearance from the
76 infected tissues resulting in increased mice survival rates (15). Furthermore, the
77 vaccine proved to induce a protective level of immunoglobulins and reduced the
78 pro-inflammatory cytokines serum levels of IL-1 β , TNF- α , and IL-6 that are
79 normally associated with sepsis and helped to secure high survival rates in
80 vaccinated mice associated with both pneumonia and sepsis (16) that could be life-
81 saving in case of outbreaks or critical conditions (17).

82 We, therefore attempted to develop a HI-WCV derived from multiple strains of
83 XDR *A. baumannii* after detecting several drug resistance genes and investigated
84 potential efficacy of enhancing protective immunity against XDR *A. baumannii*, by
85 evaluating the ability of the vaccine to improve the complement-mediated serum
86 bactericidal activities of the immunized mice sera after intramuscular inoculation.

87

88 **Results:**

89 ***In vivo* assay:**

90 Immunized mice sera were collected before 1st inoculation and on 10th day after
91 each vaccine inoculation from both experimental and placebo-controlled mice and
92 humoral immune response was evaluated by the mean Optical Density (OD) values
93 of duplicates of serum IgG absorbance at 450 nm in 1:160 dilution by indirect
94 ELISA (**Figure 1B**). Among EG mice mean OD values of pre- inoculation and after
95 1st inoculation (Day 10) was 0.29 ± 0.028 and 0.330 ± 0.002 respectively. After 2nd
96 inoculation (Day 24) mean OD value was 2.251 ± 0.262 showing rise of serum
97 antibody titre resulted in a rapid memory response peak ($P < 0.001$). Moreover, the
98 mean OD value was highest (3.487 ± 0.275) after 3rd inoculation where serum was
99 collected on day 38 ($P < 0.001$). However, there was a decrease in mean OD value
100 (2.981 ± 0.119) after lethal dose (Day 56) ($P < 0.001$). The sera from placebo-
101 controlled mice contained very low levels of detectable antibodies at any time-point
102 with an average OD value of 0.0376 ± 0.012 which was very similar to NC mice

103 serum OD value and all the mice from this group died within 24 hours after
104 injection the lethal dose.

105 ***In vitro* assay:**

106 Immunized mice sera collected on days 10, 24, 38 and 56 were tested for the ability
107 to promote *in vitro* complement-mediated serum bactericidal activity of the test
108 XDR *A. baumannii*. **Table 1** showed the organized colony counts per spot from
109 Mueller Hinton agar plates after overnight incubation at 37°C at 8 different
110 dilutions. Bacterial killing is clearly seen for all samples tested in the first three
111 dilutions and as samples are diluted further up the plate, a decrease in bacterial
112 killing is seen where serum is less concentrated. For control A the mean CFU count
113 was 36 in all dilutions. For control B the mean CFU count was 29 in all dilutions.
114 For pre-inoculation the mean CFU count was between 24 to 35 in all dilutions
115 indicating no inhibition of bacterial survival. The calculated non-specific killing
116 (NSK) was 23%. Here, 50% killing index (KI) cut off value was 14.5. The 1st, 2nd,
117 3rd inoculation and after lethal challenge the mean CFU count for 1st three dilution
118 (1:8, 1:24, 1:72) at 25% and 50% guinea pig complement concentration was
119 between 1.5 to 2.5, 0 to 2, 0 to 1.5 and 0 to 1.5 respectively, which indicated
120 bactericidal efficacy of the immunized serums. The highest SBA KI 42.12 was
121 found after 3rd inoculation, whereas the lowest SBA KI 19.1 was found after 1st
122 inoculation. **Figure 2: a), b), c), d).**

123 **Comparison of *in vivo* and *in vitro* assay:** The neutralizing (IgG) antibody titer
124 were significantly increased after 2nd (7- fold) and 3rd vaccination (12-fold). On

125 the other hand, 7-fold rise in SBA KI from 50% SBA cutoff value was only shown
126 after 3rd inoculation (**Table 2**).

127

128 **Discussion:**

129 Over the past few decades, *A. baumannii* has emerged as a major healthcare facility
130 acquired bacterial infection because of resisting many classes of antibiotics by
131 virtue of chromosome-mediated genetic elements (18), leading to rapid emergence
132 of MDR, XDR and PDR strains that causes the consideration for preventive
133 vaccines to combat. Though some single antigen candidates have been tested, these
134 are still under trial. Various studies regarding heat-inactivated whole-cell vaccines
135 showed that these were easy to prepare, inexpensive and did not require expensive
136 denaturation of antigens, that may induce conformational changes of the epitopes
137 and produced protective immunity (IgG Ab) against XDR *A. baumannii*, in murine
138 models after intramuscular administration (19). In the current study, sera collected
139 from each mouse after each inoculation was analyzed for IgG antibody absorbance
140 by indirect ELISA at 450 nm. The OD values of serum IgG absorbance at 1:160
141 dilution showed that there was significant rise ($P<0.001$) in OD value of IgG
142 antibody absorbance within the EG group of mice after 2nd (day 14) and 3rd (day
143 28) inoculation though the level was slightly decreased after intraperitoneal
144 injection (day 42) of lethal dose with 1.5×10^8 CFU/ml live XDR *A. baumannii*
145 which might be due to utilization of the previously formed IgG antibody to

146 neutralize *A. baumannii* antigen. The sera from PC mice contained very low levels
147 of detectable antibodies after all inoculation with an average OD value of
148 0.0455 ± 0.03244 which was very similar to NC group mice. Hence the mice from
149 the EG group survived where all the mice from PC group were dead after lethal
150 challenge as the serum neutralizing antibody was insufficient for them to survive.

151 In a study by Shu *et al.* (2016) in Malaysia showed that, heat inactivated whole-cell
152 vaccine immunized mice sera collected on days 0 to 36 had an increase in IgG
153 antibody. The OD values of IgG absorbance of immunized mice sera collected after
154 second inoculation on day 22 resulted in a rapid memory response peak. However,
155 the OD values on day 36 in immunized mice sera, showed a slight decrease in the
156 antibody response. The sera from placebo-inoculated control mice contained very
157 low levels of detectable antibodies at any time-point with an average OD value of
158 0.30 ± 0.03 which was quite similar with the current study. The findings suggested
159 that inoculation with heat inactivated whole-cell vaccine stimulated high levels of
160 serum IgG antibody recognizing the XDR *A. baumannii* strain as the target antigen.

161 Activation of the complement pathway is an important immune defense mechanism
162 present in serum. Therefore, complement activation could play an important role in
163 minimizing the ability of *A. baumannii* to escape immune clearance. The present
164 study, showed that the sera obtained from mice inoculated with heat-inactivated
165 whole-cell vaccine were able to activate complement-mediated bacteriolysis in the
166 presence of 25% guinea pig complement at 1:216 serum dilution after three hours

167 of incubation with XDR *A. baumannii*. In this study, the calculated 50% SBA cutoff
 168 value was 14.5. It was noted that the bacteriolysis effects in sera of mice from the
 169 1st and 2nd inoculation with HI-WCV collected on 10th day (SBA KI=19.1) and
 170 24th day (SBA KI=20.04) showed only a slight increase from the 50% SBA titre,
 171 suggesting that sera from a two subsequent immunization did not significantly
 172 increase the complement-mediated serum bacteriolysis activity. However, the SBA
 173 KI (42.12) of the EG group (n=4) after 3rd inoculation (Day 38) suggested that they
 174 were able to mount effective responses, resulting in inactivation of the virulent
 175 XDR *A. baumannii* although it was decreased to SBA KI=19.27 after injecting the
 176 lethal dose (1.5×10^8 CFU/ml) which might be due to utilization of the previously
 177 formed antibody to kill the virulent *A. baumannii* injected by intraperitoneal route.

178 In a study by Shu *et al.* (2016) showed that, that the complement-mediated serum
 179 bacteriolysis effects in sera of mice from the first inoculation with inactivated
 180 whole-cell vaccine collected on day 7 and 12 showed only a slight incremental
 181 increase, however, the bacteriolysis effect in the sera collected on day 30 from
 182 immunized mice was much higher in comparison to the sera collected on the same
 183 day from control group mice.

184 These results suggest that inoculation of heat inactivated whole-cell vaccine
 185 stimulated an immune response that mediated significantly better clearance or
 186 bacteriolysis activity of XDR *A. baumannii*. Though, A single immunization did
 187 not significantly increase the complement mediated bacteriolysis activity, the

188 results after the booster immunization of the vaccine group were able to mount
189 effective responses, resulting in inactivation of the XDR *A. baumannii*. As a 4-fold
190 or higher increase in antibody titre after vaccination is considered as a positive
191 response, in comparison of the rising fold of IgG antibody titers of the EG group
192 with PC group and analysis of SBA titres in post vaccinated sera showed that the
193 IgG antibody titre were significantly increased after 2nd (7-fold) and 3rd
194 inoculation (12-fold). On the other hand, 7-fold rise in SBA KI from 50% SBA titre
195 was only shown after 3rd inoculation. These variations might be due to several
196 factors including source and quantity of exogenous complements, bacterial strain,
197 test sera, and antigen expression in targeted bacteria.

198 Although there was variation in the rising of titres, both studies showed effective
199 killing of XDR *A. baumannii* *in vivo* and *in vitro* assay. These findings here are
200 consistent with earlier studies, which suggest that a specific antibody response
201 stimulated by a HI-WCV can aid in the clearance of *A. baumannii* and accord
202 protection against *A. baumannii* infections (20). This vaccine development
203 approach may potentially further the development of viable vaccines against XDR
204 *A. baumannii* infections.

205

206

207

208 **Materials and Methods:**

209 **Sample collection:** Clinical samples like endotracheal aspirate, blood, wound swab
210 was collected from patients admitted in different departments, especially intensive
211 care unit and burn unit of Dhaka Medical College Hospital.

212 **Phenotypic identification:** *Acinetobacter spp.* was identified by observing colony
213 morphology on blood agar (non-pigmented, white or cream-colored, smooth to
214 mucoid colonies, 1-2 mm in diameter, non-hemolytic), on MacConkey agar
215 (generally form colorless colonies), Gram staining (gram negative coccobacilli) and
216 biochemical tests- oxidase test (negative), and catalase tests (positive), urease
217 production (variable), indole test (negative), motility (non-motile) and citrate
218 utilization test (positive) (21)

219 **Genotypic identification:** PCR with *blaOXA-51-like* gene was used to identify *A.*
220 *baumannii* (22) from the isolated *Acinetobacter spp.*

221 **Antimicrobial susceptibility test:** Susceptibility was determined by modified
222 Kirby-Bauer disc diffusion technique using Mueller-Hinton agar plates and zones
223 of inhibition were interpreted according to Clinical Laboratory Standard Institute
224 guideline (23). Criteria of the United States Food and Drug Administration (24)
225 was used for the interpretation of zone of inhibition of tigecycline. The
226 antimicrobial discs (Oxoid Ltd, UK) Amoxiclav (amoxicillin 20µg and clavulanic
227 acid 10µg), piperacillin-tazobactam (100µg)/10µg), ceftazidime (30µg),
228 ceftriaxone (30µg), cefepime (30µg), cefoxitin (30µg), aztreonam (30µg),

229 ciprofloxacin (5µg), imipenem (10µg), amikacin (30µg), gentamycin (10µg),
230 doxycycline (30µg), tigecycline (15µg) and colistin (8mg/ml) and *Escherichia coli*
231 ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control
232 strains (25). The isolate that was resistant to all the 14 antibiotics was further
233 isolated for preparation of HI-WCV.

234 **Preparation of HI-WCV:**

235 A loop of six XDR *A. baumannii* was inoculated into TSB and cultured overnight
236 at 37°C and then centrifuged at 3,000g for one minute. The supernatants were
237 discarded, and the cell pellets were washed twice with PBS. Subsequently, the
238 bacterial concentrations were determined by serial dilution with normal saline and
239 plating onto MacConkey agar plates followed by incubation at 37°C for 24 hours.
240 The bacterial cells corresponding to 1×10^7 CFU/ml were heat-inactivated in a water
241 bath by incubating for 30 minutes at 56°C. Complete inactivation of the bacteria
242 was confirmed by plating the supernatant onto Mueller-Hinton agar plates (19) and
243 then stored at -20°C until inoculation.

244 **Mice and inoculation schedule:** Eleven 6-8 weeks old male swiss albino mice
245 were collected and kept under specific pathogen-free conditions with non-
246 medicated diet. The mice were randomly divided into 3 groups where EG and PC
247 group contained 4 mice and negative control (NC) group contained 3 mice.

248 **Inoculation schedule of mice:** Three IM inoculations were performed on day 0,
249 14, 28 (**Figure 1A**) in the alternate thigh of the EG group of mice with 20µl of heat-

250 inactivated whole-cell *A. baumannii* emulsified with 20µl CFA (1:1), and with 20µl
251 of PBS emulsified with 20µl CFA (1:1) for the PC group of mice on the same day.
252 The IM inoculation was done with an insulin syringe BD Ultra-Fine TM (31G).
253 CFA (Sigma-Aldrich, Missouri, 50 United States) was used for the first inoculation.
254 The prepared mixtures were inoculated after intraperitoneal injection of ketamine
255 (ketamine 100 mg/kg) and chloroform by inhalation.

256 **Serum collection:** Tail blood was collected after sterilization with 70% alcohol
257 before inoculation and on 10th day after each inoculation and about one ml blood
258 was collected in a sterile test-tube by cardiac puncture from euthanized NC group,
259 immediately after death from PC group and 14 days after the lethal challenge from
260 EG group (**Figure 1A**).

261 **Detection of IgG antibody:**

262 Bacterial cells used in preparation of HI-WCV of *A. baumannii* (1×10^7 CFU/ml),
263 was used as antigen after sonication. Protein concentration was detected by
264 nanodrop spectrophotometer (ThermoFisher Scientific). Immunoplates (Maxisorp,
265 Nun) were coated with 100 µl/well of 10µg/ml antigen overnight at 4°C in the
266 carbonate buffer. Subsequently, wells were blocked with 200µl blocking buffer
267 (5% w/v non-fat dry milk in PBS-0.05% tween 20) and incubated at 37°C for 1-2
268 hour(s) after covering with adhesive plastic and washed 3 times. 100µl of diluted
269 serum sample 1:100 in 1% bovine serum albumin in 1×PBS containing 0.1%
270 Tween (PBST) were added to duplicate wells and incubated at 37°C for one hour.

271 To each well, 100µl of a 1:5000 dilution of goat anti-mouse IgG peroxidase
272 conjugated antibodies (Thermo Fisher Scientific, USA) was added and incubated
273 at 37°C for one hour. The plates were washed three times with a wash buffer
274 (1×PBST) post all incubations. The plate was developed using TMB, following 1M
275 sulfuric acid addition to stop the reaction, and read at 450/630nm by ELISA plate
276 reader (Biotek Inc., USA). Cut off value of OD was calculated by following
277 formula:

$$278 \quad \text{OD} = M (\text{mean}) + 3 \times \text{SD} (\text{Standard deviation})$$

279 **Serum Bactericidal Antibody Assay:**

280 An XDR *A. baumannii* isolated stock was made by using PBS to determine the
281 dilution (1:1,250) necessary to yield ~100 CFU/spot on Mueller-Hinton agar plates.
282 The serum samples from EG of mice were heat-inactivated in a water bath by
283 incubating at 56°C for 30 minutes. 3-4 weeks old two guinea pigs' sera were used
284 as a source of complement and to heat-inactivate, serum was incubated at 56°C for
285 30 min. SBA was performed with some modifications (26). After diluting of the
286 bacteria in 20ml of PBS according to the pre-determined optimal dilution factor
287 (1:1250), 10µl of diluted bacteria was added to each well of the assay plate. 50µl
288 of 20% solution heat-inactivated guinea pig complement was added to all wells in
289 column 1 (control A wells) and 50µl of 20% solution native guinea pig complement
290 was added to all wells in columns 2 through 12 (Control B and test sample wells).
291 Plate was incubated at 37°C for 3 hours after brief mixing. 10µl of the reaction
292 mixture was taken from each well onto a Mueller-Hinton agar plate, tilted and

293 allowed the spots to run for ~1cm and incubated in the incubator upside-down for
294 16-18 hours.

295 Photograph of the plates was taken using a digital camera and transferred images
296 to a computer to count the number of colonies in each spot. Calculation of Non -
297 specific killing and 50% Killing Index was done by the following formula:

298 Non -specific killing (NSK) = 1-Control B/Control A,

299 50% Killing Index (KI) = Control B/2

300 Because a serum dilution would rarely yield exactly this 50% KI value, it was
301 interpolated from two sequential serum dilutions, one that kills less than 50% and
302 one that kills more than 50%, (**Figure 2. a, b, c, d**)

303 The formula for calculating the interpolated SBA KI is shown below:

$$304 \quad \text{SBA KI} = 10^{\left\{ \log X_1 \frac{(Y_{50} - Y_1) \times (\log X_2 - \log X_1)}{(Y_2 - Y_1)} \right\}}$$

305

306 **Statistical analysis.**

307 The data from the antibody response, complement-mediated serum bacteriolysis
308 were expressed as the mean± standard deviation from repeated assays. The data
309 were subjected to two-way analysis of variance (ANOVA). Statistical significance
310 was considered as $P < 0.05$. All the statistical analyses were performed using Stata
311 software version 14. (StataCorp LLC, USA).

312 **Ethical clearance:**

313 The animal experiments were performed in accordance with the protocols and
314 guidelines approved by Dhaka Medical College Ethical Review Committee (MEU-
315 DMC/ECC/2019/256). Informed written consent was obtained from all patients or
316 legal guardians following a detailed explanation of the study.

317

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Tables:

Table 1: The organization of CFU counts/spot from Mueller Hinton agar plates

	1	2	3	4	5	6	7	8	9	10	11	12	Dilution
A	41	35	36	34	43	45	32	30	23	21	15	35	Dil 8 1:17496
B	38	33	31	37	39	35	24	21	12	15	19	8	Dil 7 1:5832
C	39	31	37	33	25	29	17	18	8	10	10	9	Dil 6 1:1944
D	35	34	32	36	14	19	5	6	4	8	7	6	Dil 5 1:648
E	32	26	21	27	9	7	0	1	1	1	1	1	Dil 4 1:216
F	33	28	23	25	2	3	3	1	1	0	2	1	Dil 3 1:72
G	38	26	24	28	2	3	1	0	1	2	0	1	Dil 2 1:24
H	34	22	29	27	1	2	0	0	0	0	0	0	Dil 1 1:8
	control A	control B	Pre- inoculation		1 st inoculation		2 nd inoculation		3 rd inoculation		After lethal challenge		

Table 2: Comparison of the differences of rising in IgG antibody and SBA titers after vaccination between PC and EG group mice.

Serum collection	Day	PC group (mean)	± SD	EG group (mean)	± SD	Titre fold rise	50% SBA titre	SBA KI	Titre fold rise
After 1st inoculation	10	0.0127	0.0011	0.029	0.0284	2	15	19.1	1.27
After 2nd inoculation	24	0.0376	0.0104	0.330	0.0026	8	10	20.04	2
After 3rd inoculation	38	0.080	0.0077	2.252	0.2629	27	6	42.12	7
After lethal dose	56	0.078	0.0121	3.488	0.2759	42	4	19.27	4.8

Figure Legends:

Figure 1: A) Both EG and PC group (n=4) was inoculated with HI-WCV on Day 0, 14, 28 intramuscularly and lethal challenge was given intraperitoneally on Day 42. Serum was collected from tail blood before inoculation and on Day 10, 24, 38. Blood was collected by cardiac puncture from euthanized NC group, immediately after death from PC group and on Day 56 after the lethal challenge from EG group.

B) Mean Optical Density of serum samples before and after each inoculation (Day 0, 14, 28) and after lethal challenge (Day 42) of both EC Group and PC Group of mice (n=4). Here, $P < 0.001$ except in pre-inoculation ($P = 2.77$).

Figure 2: a), b), c), d), shows a schematic of linear interpolation after each inoculation. The number of surviving bacteria (y-axis) at each dilution of serum tested (x-axis) is plotted (balls) and individual points were connected by a thin line. The solid and dashed horizontal lines indicated 0% and 50% killing, respectively. The serum dilutions above (e.g., Dilution 5) and below (e.g., Dilution 4) the 50% killing line were connected by a red line and bactericidal titer (KI) was indicated.

A)

B)

a)

b)

c)

d)