Title:

Fetal *Ureaplasma parvum* bacteraemia as a function of gestation-dependent complement insufficiency: evidence from a sheep model of pregnancy.

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Running title: **Bacteraemia and Complement Insufficiency.**

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Abstract.

Problem Complement is a central defence against sepsis and increasing complement insufficiency in neonates of greater prematurity may predispose to increased sepsis. *Ureaplasma* spp. are the most frequently cultured bacteria from preterm blood samples.

Method of Study A sheep model of intrauterine *Ureaplasma parvum* infection was used to examine *in vivo Ureaplasma* bacteraemia at early and late gestational ages. Complement function and *Ureaplasma* killing assays were used to determine the correlation between complement potency and bactericidal activity of sera *ex vivo*.

Results *Ureaplasma* were cultured from 50% of 95-day gestation lamb cord blood samples compared to 10% of 125-day gestation lambs. Bactericidal activity increased with increased gestational age and a direct correlation between functional complement activity and bactericidal activity ($R^2=0.86$; $p<0.001$) was found for 95-day gestational lambs.

Conclusions *Ureaplasma* bacteraemia *in vivo* was confined to early preterm lambs with low complement function, but *Ureaplasma* infection itself didn't diminish complement levels.

INTRODUCTION

The complement system encompasses a series of >30 serum proteins that interact through an amplification cascade following activation by foreign microbial surfaces, immune complexes, surface-bound antibodies or pathogen-bound pattern recognition molecules. Like most elements of the innate immune system, the complement system does not require previous exposure to be effective. Complement also acts to integrate the innate and humoral immune responses through recruiting innate and adaptive immune cells to the site of activation (via chemotaxin and anaphylotoxin release), improving the humoral response through a natural adjuvant activity, and increasing engulfment of microbes through decorating the surface with opsonins.[1] Complement has direct anti-microbial activity mediated by the formation of lytic pores on activating surfaces, which makes it a pivotal barrier to initial invasion by pathogens. However, as we have previously reviewed, the complement system does not have full potency (for both activation and regulatory factors) at birth in humans due to lower circulating levels of some of the components and this insufficiency is greater with increasing prematurity.[2] The reduced capacity of the complement system at term and birth, which is exacerbated with increasing prematurity, has been proposed to be responsible for the increased susceptibility of neonates to bacterial infection and sepsis.[3]

Intrauterine infection by *Ureaplasma parvum* (UP) is strongly associated with preterm birth, and is among the organisms most commonly isolated from gestational tissues.[4,5] UP is one of the smallest self-replicating microorganisms identified to date with a minimal genome (0.75-0.78 Mbp) that limits it to a parasitic existence.[6] Most notably, the UP genome does not encode any components that make up a cell wall, which is common to most bacteria and therefore inherently resistant to all classes of antibiotics that function by inhibiting cell wall synthesis. The cell wall also confers a protective effect against membrane attack complex-mediated

complement killing (particularly effective for Gram-positive bacteria), which may explain why UP is relatively susceptible to human complement.[7]

Ureaplasma bacteraemia is detected in up to 23% of preterm cord blood samples[4] and was isolated from blood samples in a cohort of 200 preterm neonates[8], showing that this organism represents one of the most prevalent systemic infections in births under 32 weeks gestational age. To determine if sheep could be used as an *in vivo* model to investigate the correlation of complement development and bacteraemia with increasing prematurity observed in humans, we have examined bacteraemia and complement function using a well-established experimental model for *in utero* UP infection.[9-11] Here we investigate the ability of adult and preterm sheep sera to kill *U. parvum in vitro*, and investigate the presence of fetal *U. parvum* bacteraemia following experimental intrauterine infection *in vivo*. We hypothesised that, in a sheep model of pregnancy, the degree of gestation-dependent complement insufficiency in preterm lambs would correlate with fetal UP bacteraemia. To investigate this hypothesis, we examined: **i)** plasma titres of UP from lambs delivered at early- (95 d) and mid- (125 d) term gestations; **ii)** fetal anti-UP antibody responses; **iii)** UP killing activity of serum from adult sheep and preterm lambs; and **iv)** the kinetics and calcium dependence of serum UP killing activity.

METHODS

Animal Studies

All experimental sheep procedures were performed in Perth, Australia following approval by the University of Western Australia Animal Ethics Committee (RA/3/100/1289). 37 date-mated merino-cross ewes (*Ovis aries)* carrying singleton pregnancies were separated into 5 groups. Two groups received a received a single intraamniotic (IA) injection of sterile saline (**Group 1**; controls; n=5) or 10^5 infectious units of UP at 80 d GA (**Group 2**; n=8), for delivery at 95 d GA. Three further groups received a single IA injection of sterile saline (**Group 3**; controls;

95 n=6) or 10^5 infectious units of UP at 70 (**Group 4**; n=8) or 115 d GA (**Group 5**; n=10) for
96 delivery at 125 d GA. Injections were carried out under ultrasound guidance as described
97 previously.[12] Maternal blood was collected into serum separator and EDTA plasma tubes by
98 peripheral venepuncture prior to euthanasia with intravenous pentobarbitone (100mg/kg) at
99 either 95 (Groups 1 and 2), or 125 d GA (Groups 3, 4 and 5). The fetus was surgically delivered
100 under terminal anaesthesia. Fetal viability at time of euthanasia was confirmed by ultrasound
101 cardiac imaging and by fetal arterial cord blood gas analysis (Table 1). Fetal amniotic fluid and
102 arterial cord blood (both serum and plasma) were obtained during surgical delivery by aseptic
103 technique to ensure no cross contamination. Samples for serum (and plasma) separation were
104 immediately dispensed into serum separator tubes (Becton-Dickinson) and placed on ice.
105 Serum was separated by centrifugation at 2500 xg for 20 minutes prior to immediate aliquoting
106 into sterile tubes and transfer to -80ºC until analysis. Serum and plasma samples were processed
107 in under 3 hours after collection (kept on ice at all intervals) until separation and freezing at -
108 80ºC. UP levels in plasma and amniotic fluid were determined by culture in triplicate
109 immediately upon delivery (on fresh samples, not frozen and thawed), as detailed below.
110 Maternal blood samples for comparative complement function assays were collected from
111 pregnant, UP-naïve ewes into serum separator tubes as above.
112
113 ***Ureaplasma parvum culture and killing assay.***
114 The serovar 3 strain HPA5 of *Ureaplasma parvum* (UP)[13] was used for all experiments. UP
115 was cultured using commercial Ureaplasma selective medium (USM; Mycoplasma experience
116 ltd., Surrey, UK), using standard techniques as previously outlined.[14] UP titres were quantified
117 by serial 10-fold dilution (or 2-fold dilution for killing assay) in USM, in triplicate and
118 incubated at 37ºC for 48 h prior to recording results. Killing assays were performed as for
119 previous human serum studies,[14] with the exception of experiments to determine the rate of

120 serum killing. For those experiments, 10mM EDTA (final concentration) was added to block
121 further complement activation at defined incubation times and the serum removed by
122 centrifugation and resuspension in USM prior to titration of surviving bacteria. Transient
123 exposure to EDTA was not found to alter growth of UP in separate experiments. Serum killing
124 was calculated as the fold decrease relative to the titration of surviving bacteria following
125 identical exposure to the same sera, except that all complement activity had been removed by
126 heat-inactivation at 56°C for 30 minutes prior to the experiment.
127
128 ***Haemolysis assay***
129 Haemolysis assays were performed as previously published.[15] Guinea pig erythrocytes were
130 purchased from TCS Biosciences (Oxford, UK) and sensitised with rabbit polyclonal anti-
131 guinea pig erythrocyte antibodies purchased from Fitzgerald Industries International (North
132 Acton, MA), as described previously.[15] Sheep serum from pregnant ewes and their singleton
133 lambs were obtained by peripheral venepuncture of Merino- cross ewes and fetal cord blood
134 was obtained from their lambs during surgical delivery at 95 and 125 d GA. Sera from both
135 uninfected control and UP infected sheep were examined. Sera were stored at -80°C prior to
136 use and aliquots only thawed once.
137
138 ***IgG quantitation and anti-UP response***
139 IgG concentrations in all sera were determined by commercial Sheep IgG ELISA (Life
140 Diagnostics Inc., West Chester, PA) as per manufacturer's instructions. Results were performed
141 in duplicate and assays were repeated once. IgG is not transferred across the ovine placenta[16,17]
142 resulting in hypogammaglobulinemia in presuckle lambs;[18] therefore, *in utero* IgG levels can
143 only have arisen by production by the fetus. The reactivity of fetal antibodies for UP in the fetal
144 sera was determined by immunoblot analysis as previously described for human studies.[14] Total

145 protein equivalent to 30 μg of HPA5 per lane was separated by non-reducing polyacrylamide
146 electrophoresis prior to electrophoretic transfer to nitrocellulose membranes. A non-related UP
147 serovar 6 strain (HPA61) was also included to examine the presence of pan-UP reacting
148 antibodies. Primary antibodies consisted of fetal or maternal sera at a final dilution of 1/100,
149 subsequently detected with peroxidase-conjugated donkey anti-sheep antibodies (minimum
150 species cross-reaction, Jackson ImmunoResearch UK).

151

152 ***Statistical analyses.***

153 All values represent mean ± standard deviation (SD). Statistical analyses were performed using
154 IBM SPSS Statistics for Windows, Version 20.0 (IBM Corporation, Armonk, NY.). Data were
155 assessed for normality using Shapiro-Wilk tests. Normally distributed data, were tested either
156 for significant mean differences by one-way ANOVA (for one factor) or where indicated by two
157 factor ANOVA (e.g. age of serum source versus serum dilution), employing a critical value of
158 0.05. Post-hoc analysis was performed with Bonferroni's post-hoc test comparing only relevant
159 groups to control. Between groups differences in non-parametric data were tested for
160 significance with Kruskal-Wallis one-way ANOVA employing a critical value of 0.05. Multiple
161 post-hoc comparisons for non-parametric data were performed using Rank-Sum tests with a
162 critical value corrected for *n* multiple comparisons. Slope of the growth curve to analyse
163 amniotic fluid titres for UP during experimental infection establishment, as well as Y-intercept
164 and goodness of fit statistics, were performed by GraphPad Prism (La Jolla, CA) using non-
165 linear regression for exponential growth. Correlation between the lysis of guinea pig
166 erythrocytes and UP killing for each serum sample was performed as untransformed data
167 (Pearson r=0.55; R^2=0.30, p<0.05), however, the relationship between UP killing and sera
168 concentration was found to be logarithmic, therefore, correlation between $\log_{10}$ killing and

169 serum lysis was much clearer and analysis on transformed data was more significant (Pearson

170 r=0.93, R^2=0.86; $p<0.001$)

171

172

173 **RESULTS**

174 ***Ureaplasma-cidal activity of serum from adult sheep and preterm lambs.***

175 The capacity for sera collected from UP-naïve adult and preterm lambs from Group 1 (95 d GA)
176 and Group 3 (125 d GA) were examined for its ability to kill the parental UP strain (HPA5) used
177 for experimental intrauterine infection in the present study (Figure 1A). Sera from uninfected
178 adult sheep and preterm lambs was used to assess the innate immune killing and sera were
179 confirmed to be devoid of anti-UP cross-reacting antibodies when tested by immunoblot (data
180 not shown). Adult sera at dilutions as low as 6.25% (v/v) was capable of killing all added UP.
181 Sera from control 125 d GA lambs (Group 3) had reduced capacity to kill *Ureaplasma*, requiring
182 8-fold more sera (50% (v/v) dilution) to achieve bactericidal activity observed for adult sheep
183 (Figure 1A), whereas 50% (v/v) sera from control 95 d GA lambs (Group 1) had 100-fold lower
184 bactericidal activity than 125 d GA lamb sera. The kinetics of bactericidal activity in 50% (v/v)
185 dilutions of sera were investigated by stopping complement function with EDTA at various time
186 points for 95 d GA lamb sera and adult sheep sera (Figure 1B). Despite the reduced bactericidal
187 activity, preterm and adult sera showed identical killing kinetics, achieving most of the killing in
188 10-15 minutes of incubation at 37ºC (Figure 1B) and 95 d GA sera did not show any increase in
189 bactericidal activity even if incubation was extended to 4 h (data not shown). Bactericidal
190 activity was found to be calcium dependent for both preterm and adult sera, as dilution in 10mM
191 EGTA with additional Mg^{2+} showed no killing when complement activity was restricted to the
192 alternative activation pathway (Figure 1B).

193

194 ***Plasma titres of UP from UP-infected lambs of different gestational ages.***

195 In agreement with the *in vitro* bactericidal assays, the ability to culture UP from the cord blood
196 of experimentally infected 95 d GA lambs was significantly greater than from 125 d GA lambs
197 (Figure 2). Lambs were surgically delivered at either: **Group 2)** 95 ± 2 d (25 d UP infection;

198 n=8); **Group 4)** 125 ± 2 d (42 d UP infection; n=8); or **Group 5)** 125 ± 2 d (10 d UP infection;
199 n=10). Two fetuses in Group 2 died prior to delivery and were excluded from the study.
200 Amniotic fluid UP titres were independent of the length of infection or gestational age (range =
201 $5x10^6$ - $3x10^7$ infectious units/mL; data not shown). However, the identification of fetal UP
202 bacteraemia varied greatly between groups. None of the Group 4 animals had detectable UP
203 bacteraemia; serum UP was detected in only one Group 5 fetus (500 infectious units/mL). In
204 contrast, 50% of Group 2 lambs (delivered at 95 d GA) had UP bacteraemia ranging from 500-
205 500,000 infectious units/mL (Table 1; Figure 2). None of the uninfected control animals (Groups
206 1 and 3) had UP in either AF or fetal serum.

207

208 ***Immunoblot analysis of antibody response in preterm lambs.***

209 Measurement of total serum IgG showed minimal antibody levels in all Groups, apart from
210 Group 4, which were exposed to intraamniotic UP for 42 days prior to delivery at 125 d GA
211 (Figure 3A). Immunoblot analysis was performed on all sera, but only those with elevated IgG
212 levels in group 4 were found to have anti-UP antibody response against the parental infecting
213 strain (HPA5). Development of antibodies reacting to conserved antigens expressed by other
214 non-infected strains was also evaluated by reactivity to an unrelated UP serovar 6 strain by
215 immunoblot (Figure 3B). Despite elevated IgG levels in one of the Group 2 and one Group 5
216 animal, these sera did not react with UP by immunoblot (Figure 3B). However, 66% of Group
217 4 lambs (delivered at 125 d GA following 42 days UP infection) showed variable banding
218 patterns by immunoblot, each recognising between 1 and 3 UP proteins. Three of the lambs
219 raised antibodies only expressed by the infecting strain HPA5 with a mass of 70-72 kDa, and
220 three of the lambs recognised a 50 kDa mass protein only expressed by HPA5. This 50 kDa
221 band represents the major surface protein (multiple banded antigen; MBA) responsible for
222 determining the serovar of the bacteria, and MBA reactivity for these sera was confirmed by

223 probing with a monoclonal antibody raised against the MBA (Figure 3B). Two of the lambs
224 also raised antibodies against a 42 kDa protein that was expressed by both the inoculating
225 serovar 3 strain HPA5 and serovar 6 strain HPA61, suggesting reactivity to a conserved UP
226 protein.

227

228 ***Investigation of serum susceptibility of recovered plasma and amniotic fluid strains.***

229 UP strains recovered from the plasma of Group 2 lambs were examined to determine if they were
230 less susceptible to serum killing than strains recovered from amniotic fluid from bacteraemic and
231 non-bacteraemic lambs. Recovered isolates were separated into 3 groups: **i)** plasma-recovered
232 isolates; **ii)** amniotic fluid recovered isolates from the same animals that had UP recovered from
233 their plasma; and **iii)** amniotic fluid-recovered isolates from animals that did not have
234 bacteraemia. Susceptibility to 50% (v/v) adult sheep sera and 50% (v/v) 95 d GA sera from
235 Group 2 animals are shown in Figure 4. Isolates cultured directly from plasma were equally
236 susceptible to sera as amniotic fluid-recovered isolates from matched or non-bacteraemic
237 animals. All recovered isolates were equally resistant to non-immune sera from control 95 d GA
238 Group 1 animals, except (paradoxically) for one of the plasma-derived isolates (animal 115).

239

240 ***Ureaplasma-cidal activity of sera from bacteraemic versus non-bacteraemic lambs.***

241 As no relative serum resistance was observed for isolates recovered from plasma compared to
242 amniotic fluid, the capacity of the sera from bacteraemic and non-bacteraemic 95 d GA preterm
243 lambs was compared for their individual capacity to kill the parental strain inoculated into the
244 pregnant sheep. Endogenous UP in bacteraemic sera was removed by filtration through a 0.22μm
245 filter and removal of endogenous UP was confirmed by titration of filtered sera in Ureaplasma
246 selective medium. The serum killing from the 95 d GA UP-infected animals in Group 2 was
247 higher for non-bacteraemic than bacteraemic with one exception (Figure 5; lamb 116), which
248 was also the lamb with the lowest detectable UP bacteraemia levels (Figure 2). Further, it was

noted that the serum with the lowest UP killing activity was from the animal with the highest plasma titre of UP (lamb 115). These data suggested that killing capacity of the sera was directly linked to UP titres in the serum.

Complement activity of sera from bacteraemic versus non-bacteraemic lambs.

Complement activity (measured by lysis of guinea pig erythrocytes) was examined for the sera from UP-infected and uninfected 95 d GA lambs from Group 1. Complement activity in serum from bacteraemic lambs was significantly lower at 50% and 25% dilutions than those found to have no UP in their plasma (non-bacteraemic) ($p<0.001$; Figure 6A). However, the complement function of 125 dGA lambs was significantly greater than both of these, but still about 4-times less active relative to the complement activity in adult sheep sera (Figure 6A). The individual values for each of these animals can be found in supplementary figure 1, which shows the lowest complement function was observed in the lamb with the highest UP titre (lamb 115; 10^4 culture units/mL in plasma); in contrast, the highest complement activity for the bacteraemic group was found in the lamb with the lowest UP plasma titre (lamb 116; 10 culture units/mL in plasma; Figure 2). Complement function for both bacteraemic and non-bacteraemic groups were representative of the range (30-95% lysis at 50% (v/v) sera dilution) observed from age matched (95 d GA) uninfected lambs from Group 1 (Figure 6B), indicating UP infection (or bacteraemia) did not influence the overall development of the complement system or consume complement through activation. Furthermore, a correlation between *Ureaplasma*-cidal activity and complement activity in 95 d GA sera, irrespective of experimental UP infection, was found (Pearson $r=0.93$, $R^2=0.86$; $p<0.001$; Figure 7).

273 **DISCUSSION**

274 Neonates, particularly those born preterm (<37 weeks' gestation), are frequently
275 immunologically compromised and are more susceptible to morbidity and mortality due to
276 infections.[19] Previously, we developed a haemolysis assay to examine the complement function
277 in sheep, as sheep erythrocytes are the common target for complement function in other species[15]
278 and here we have extended use of our experimental intrauterine UP infection model to examine
279 the role of complement insufficiency as a determining factor for development of in utero sepsis.
280 Sepsis is a life-threatening response to infection leading to tissue and organ damage, often
281 identified through the accompanying fever, tachycardia, tachypnea, and febrile morbidity. Due
282 to the collection of samples in our experimental infection model at Caesarean-section delivery
283 under euthanasia conditions, measurement of temperature, breathing and heart rate could not be
284 obtained in a meaningful manner. Therefore, while we have examined bacteraemia as a surrogate
285 marker of sepsis, it is important to note that sepsis (the body's response to infection) in patients
286 can occur in absence of bacteraemia and still responds to antibiotic treatment.

287

288 The primary findings of this study are that *in vitro* and *in vivo* bactericidal capacity of fetal sheep
289 sera diminished with increasing prematurity. This finding was reflected by the increased
290 incidence of bacteraemia detected in lambs experimentally infected with UP delivered at 95 d
291 GA (50%) relative to 125 d GA (10%) lambs. Within the 95 d GA cohort, the bactericidal serum
292 activity was found directly correlated to the complement function in the serum (R^2=0.30, $p<0.05$).
293 However, the relationship between UP killing and sera concentration was found to be a
294 logarithmic function and analysis of $\log_{10}$-transformed UP killing relative to complement
295 functional assay values (represented by sera lysis of guinea pig erythrocyte targets) had a much
296 stronger correlation (R^2=0.86; $p<0.001$; Figure 7). With one exception (animal 116), fetuses with
297 bacteraemia were found to have lowest complement function compared to non-bacteraemic

298 fetuses; although animal 116 also had the lowest UP plasma titre (Figure 2) and the highest
299 complement function within this this group (Supplementary Figure 1A). The correlation between
300 UP bactericidal activity and complement function was not influenced by experimental infection,
301 suggesting that the natural variation in complement activity at 95 d GA determined whether UP
302 infection in the amniotic fluid became systemic or not.

303

304 Interestingly, measurement of IgG levels in control 95 and 125 d GA sera (Groups 1 & 3; Figure
305 3), 1.6 ± 0.2 μg/ml and 2. 1 ± 0.5 μg/ml respectively, confirmed no transplacental maternal IgG
306 transfer (maternal IgG levels were 14,400 ± 889 μg/ml) which has been speculated to be due to
307 placental structure[16,17] and lack of neonatal Fc expression in the placental tissues and blood
308 vessels. Therefore, measured IgG levels in the fetal sera in experimentally UP-infected animals
309 could only have originated from the fetal immune system. Only 1 animal from Group 2 (95 d
310 GA) had elevated IgG (Figure 3A), but this sera failed to react with UP by immunoblot (Figure
311 3B), and which was also the case for Group 5 (125 d GA, 10 d UP infection). However, variable
312 immune response was observed for Group 4 (125 d GA, 42 d UP infection), but again the highest
313 IgG concentration (137) did not correspond to strongest recognition of multiple UP proteins by
314 immunoblot (Figure 3). On the basis of these observations, it is would appear that complement,
315 rather than adaptive immune response plays a critical role in protecting or resolving fetal UP
316 bacteraemia in pregnancy. Our inability to sample fetal blood prior to delivery prevented us from
317 determining if the seropositive group represent those that were bacteraemic at earlier GA. The
318 anti-UP antibodies can only be of fetal immune origin, as we have previously shown adult, but
319 not fetal, sheep sera immunoglobulins cross-react with guinea pig erythrocytes[15] and this was not
320 detected in any of the sera from the preterm lambs (data not shown).

321

322 The HPA5 strain is susceptible to human serum killing in the absence of specific anti-UP
323 antibodies.[14] As with human serum studies with this strain, sheep serum bactericidal activity
324 required the presence of calcium. However, we are unable to determine if the bactericidal activity
325 in sheep is mediated by the classical or lectin pathways (both require calcium), as we lack the
326 reagents to differentially block these pathways as was performed for human sera.[14] The increased
327 presence of *Ureaplasma* spp. in the cord blood of human preterm neonates of increasing
328 prematurity has also been identified in the previously published Alabama preterm study.[4]
329 Furthermore, *Cassell et al*. found that *Ureaplasma* could be cultured from blood samples of 26%
330 of 200 ventilated preterm neonates studied, showing that Ureaplasma bacteraemia is prevalent in
331 this patient group.[8]
332 Sheep models have recently been developed to investigate hypoxic delivery complications and
333 intrauterine inflammation induced by microbial infection or exposure to lipopolysaccharides.[20-
334 26] None of these studies, however, has investigated the contribution of the complement system
335 to disease pathogenesis or to the role of complement in controlling systemic infection, largely
336 due to the current lack of reagents to measure and manipulate the sheep complement system. The
337 present study thus represents a significant advance in our understanding of the pathophysiology
338 of fetal bacteraemia in the setting of preterm birth.
339
340 In conclusion, experimental intrauterine UP infection establishes stable amniotic fluid levels that
341 remain stable for weeks following the infection.[27] Highlighting the importance of
342 gestation/complement function on fetal response to challenge, UP was detected in the cord blood
343 of 50% of fetuses infected at 70 d GA and delivered at 95 d GA (Group 2), but only in 10% of
344 lambs infected at 115 d GA and delivered at 125 d GA (Group 5). No bacteraemia was observed
345 in lambs infected at 80 d GA and delivered at 125 d GA (Group 4), but this was the only group
346 to show a UP-specific antibody response. Interestingly, a range of functional complement activity

347 was observed in uninfected 95 d GA preterm lambs, indicating that development of the
348 complement system is variable in the normal population and is not influenced by intrauterine UP
349 infection. In addition to advancing our understanding of fetal responses to UP infection, our
350 results also suggest that a familial history of complement deficiency may increase the risk of fetal
351 UP infection in pregnancy, concomitant with an increased risk of preterm birth and adverse
352 neonatal outcomes.

353

354

355 **Acknowledgements:** The Authors wish to express their gratitude to Siemens Australia for the
356 donation of Rapidpoint 500 reagents used in this study. MWK is supported by a National Health
357 and Medical Research Council Project Grant (GNT1049148) and the Women and Infants
358 Research Foundation. MSP is supported by a National Health and Medical Research Council
359 Project Grant (GNT1010315). OBS is supported by the Microbiology and Infection
360 Translational Research Group (MITReG) and the Children and Young People's Research
361 Network (CYPRN) as part of the Welsh Government initiative to support research. Bilateral
362 travel between Australian and UK laboratories was funded by an international exchange Royal
363 Society Grant (IE130066). SA was supported by a PhD studentship funded by the Ministry of
364 Higher Education, Iraq Embassy.

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473 **FIGURE LEGENDS.**

474 **Figure 1. (A) Titration of *Ureaplasma*-cidal activity in non-immune sera.** UP killing by
475 diluted sera from adult sheep (closed circle), 125 d GA preterm lambs (open square) and 95 d
476 GA preterm lambs (grey triangle) following 1 h incubation at 37ºC. Killing determined by
477 relative decrease in UP titre compared to incubation with matched heat-inactivated sera controls.
478 Each point represents average and standard deviation of sera from 4 separate animals (performed
479 in duplicate). Results were repeated in 2 duplicate experiments.

480 **(B) UP killing kinetics of non-immune adult sheep and 95 d GA sera.** Sera diluted to 50%
481 (v/v) in calcium and magnesium containing buffer (adult = closed circle, 95 d GA =closed
482 square) were incubated with UP for various times before blocking further complement
483 activation with addition of EDTA prior to titration of surviving UP. No serum killing of UP
484 was observed if sera were diluted in alternative pathway buffer containing EGTA and
485 magnesium (adult = open circle, 95 d GA = open square). Each point represents average and
486 standard error of sera from 4 separate uninfected animals, killing calculated by decreased titre
487 relative to UP titre following incubation with matched heat-inactivated sera. Data from one of
488 three replicated experiments shown. Significant differences were found between all points by
489 two factor ANOVA (dilution and age of serum source/addition of EGTA) and Bonferroni post-
490 hoc analysis found significant reduction relative to adult serum killing for all data points after 5
491 minutes incubation. Significant differences were found by two-factor ANOVA (dilution and age
492 of donor) and Bonferroni post-hoc analysis found significant reduction relative to adult serum
493 killing for all points but 125 d GA sera at 50% dilution. $p<0.01$ = **; $p<0.001$ = ***

494 **Figure 2. UP titres in cord plasma at delivery.** GA at delivery and total length of UP infection
495 is indicated for each group. Each point represents the average as determined in triplicate. Data
496 points for lambs 115 and 116 are separately identified for correlation purposes in other figures.
497 Results were repeated in 2 duplicate experiments.

498 **Figure 3. (A) IgG concentration in 95 d and 125 d GA lambs.** ELISA determination of
499 sheep IgG levels in sera from uninfected control lambs (Groups 1 and 3), compared to
500 experimentally UP-infected lambs. Lambs 121 (Group 2) and 235 (Group 5) are identified as
501 they have elevated IgG levels, but do not react with UP by immunoblot in (B). Results were
502 repeated once.
503 **(B) Detection of anti-UP antibodies in fetal sera from infected lambs.** Purified whole UP
504 cultures from the infecting serovar 3 strain (HPA5) and separate serovar 6 strain (HPA61) were
505 separated by non-reducing SDS-PAGE and probed with fetal sera to detect immunoreactive
506 bands by immunoblot analysis. Two infected lambs from Group 4 failed to raise specific anti-
507 UP immune response (130 and 132) as well as all of the animals in Group 2 and 4 (only sera with
508 elevated IgG from Groups 2 (121) and Group 4 (235) are shown). Four lambs in Group 4 raised
509 an antibody response to proteins unique to the infecting strain and two of these lambs (136 and
510 137) also raised cross-reacting antibodies that also recognised strain HPA61 which is a serovar
511 6 isolate. The MBA isoforms for both strains are identified by mouse monoclonal antibody 6523.
512 Representative results of 3 repeat experiments shown.
513 **Figure 4. Serum susceptibility of UP recovered from plasma or amniotic fluid.** Recovered
514 strains were incubated for 30 min with 50% (v/v) adult sheep (A) or 50% (v/v) 95 d GA preterm
515 lamb sera (B). Pooled sera from uninfected animals was used and each point was determined in
516 duplicate. Representative data shown from 3 repeated experiments. Killing calculated as
517 decreased titre relative to UP titre following incubation with matched heat-inactivated sera
518 controls. One way ANOVA analysis found no difference between these groups.
519 **Figure 5**. ***Ureaplasma*-cidal activity from bacteraemic, non-bacteraemic, and uninfected 95**
520 **d GA lambs.** Serum killing of HPA5 by 50% (v/v) sera from bacteraemic (plasma UP), non-
521 bacteraemic (no plasma UP) and uninfected 95 d GA preterm lambs. Each point represents the
522 average of 3 separate killing assays (each performed in duplicate). Killing calculated as decreased

523 titre relative to UP titre following incubation with matched heat-inactivated sera. Individual
524 points for lambs 115 and 116 are identified.

525 **Figure 6. Functional complement activity as determined by guinea pig erythrocyte lysis**
526 **assay after 30 min by diluted sera. A.** Complement activity for uninfected adult sheep (closed
527 circles) and late preterm (125 dGA; closed squares) lamb sera are shown relative to complement
528 function in sera from UP infected 95 dGA lambs with bacteraemia (+; open circles) and without
529 bacteraemia (-; open squares). Two factor ANOVA (age of sera source and dilution) with
530 Bonferroni post-hoc analysis found significantly increased complement function relative to
531 infected 95 dGA lambs with bacteraemia for all points shown ($p<0.001$ = ***). Each point
532 represents mean of triplicate values for N=4 animals per group. **B.** For comparison a wide range
533 of complement function was observed in control sera from uninfected lambs at 95 dGA,
534 indicating UP bacteraemia doesn't reduce complement activity. Each point determined in
535 triplicate, unique identifiers for each lamb are given. Results were repeated in 2 duplicate
536 experiments.

537 **Figure 7. Correlation between complement activity and *Ureaplasma*-cidal activity.**
538 Complement activity shown as haemolysis of 50% (v/v) sera at 30 min relative to fold-killing of
539 50% (v/v) sera at 30 min at 37°C. Data for infected lambs with bacteraemia (plasma UP) or
540 without bacteraemia (no plasma UP) and uninfected preterm lambs are separately identified.
541 Each point determined in triplicate. Correlation for log_{10} transformed killing data relative to lysis
542 (Pearson $r=0.93$; $R^2=0.86$, $p<0.001$) shown for untransformed data.