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

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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; n=6) or 10^5 infectious units of UP at 70 (**Group 4**; n=8) or 115 d GA (**Group 5**; n=10) for delivery at 125 d GA. Injections were carried out under ultrasound guidance as described previously.[12] Maternal blood was collected into serum separator and EDTA plasma tubes by peripheral venepuncture prior to euthanasia with intravenous pentobarbitone (100mg/kg) at either 95 (Groups 1 and 2), or 125 d GA (Groups 3, 4 and 5). The fetus was surgically delivered under terminal anaesthesia. Fetal viability at time of euthanasia was confirmed by ultrasound cardiac imaging and by fetal arterial cord blood gas analysis (Table 1). Fetal amniotic fluid and arterial cord blood (both serum and plasma) were obtained during surgical delivery by aseptic technique to ensure no cross contamination. Samples for serum (and plasma) separation were immediately dispensed into serum separator tubes (Becton-Dickinson) and placed on ice. Serum was separated by centrifugation at 2500 xg for 20 minutes prior to immediate aliquoting into sterile tubes and transfer to -80°C until analysis. Serum and plasma samples were processed in under 3 hours after collection (kept on ice at all intervals) until separation and freezing at -80°C. UP levels in plasma and amniotic fluid were determined by culture in triplicate immediately upon delivery (on fresh samples, not frozen and thawed), as detailed below. Maternal blood samples for comparative complement function assays were collected from pregnant, UP-naïve ewes into serum separator tubes as above.

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***Ureaplasma parvum* culture and killing assay.**

The serovar 3 strain HPA5 of *Ureaplasma parvum* (UP)[13] was used for all experiments. UP was cultured using commercial Ureaplasma selective medium (USM; Mycoplasma experience ltd., Surrey, UK), using standard techniques as previously outlined.[14] UP titres were quantified by serial 10-fold dilution (or 2-fold dilution for killing assay) in USM, in triplicate

and incubated at 37°C for 48 h prior to recording results. Killing assays were performed as for previous human serum studies,[14] with the exception of experiments to determine the rate of serum killing. For those experiments, 10mM EDTA (final concentration) was added to block further complement activation at defined incubation times and the serum removed by centrifugation and resuspension in USM prior to titration of surviving bacteria. Transient exposure to EDTA was not found to alter growth of UP in separate experiments. Serum killing was calculated as the fold decrease relative to the titration of surviving bacteria following identical exposure to the same sera, except that all complement activity had been removed by heat-inactivation at 56°C for 30 minutes prior to the experiment.

Haemolysis assay

Haemolysis assays were performed as previously published.[15] Guinea pig erythrocytes were purchased from TCS Biosciences (Oxford, UK) and sensitised with rabbit polyclonal anti-guinea pig erythrocyte antibodies purchased from Fitzgerald Industries International (North Acton, MA), as described previously.[15] Sheep serum from pregnant ewes and their singleton lambs were obtained by peripheral venepuncture of Merino- cross ewes and fetal cord blood was obtained from their lambs during surgical delivery at 95 and 125 d GA. Sera from both uninfected control and UP infected sheep were examined. Sera were stored at -80°C prior to use and aliquots only thawed once.

IgG quantitation and anti-UP response

IgG concentrations in all sera were determined by commercial Sheep IgG ELISA (Life Diagnostics Inc., West Chester, PA) as per manufacturer's instructions. Results were performed in duplicate and assays were repeated once. IgG is not transferred across the ovine placenta[16,17] resulting in hypogammaglobulinemia in presuckle lambs;[18] therefore, *in utero*

IgG levels can only have arisen by production by the fetus. The reactivity of fetal antibodies for UP in the fetal sera was determined by immunoblot analysis as previously described for human studies.[14] Total protein equivalent to 30 µg of HPA5 per lane was separated by non-reducing polyacrylamide electrophoresis prior to electrophoretic transfer to nitrocellulose membranes. A non-related UP serovar 6 strain (HPA61) was also included to examine the presence of pan-UP reacting antibodies. Primary antibodies consisted of fetal or maternal sera at a final dilution of 1/100, subsequently detected with peroxidase-conjugated donkey anti-sheep antibodies (minimum species cross-reaction, Jackson ImmunoResearch UK).

Statistical analyses.

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

concentration was found to be logarithmic, therefore, correlation between $\log_{10}$ killing and serum lysis was much clearer and analysis on transformed data was more significant (Pearson $r=0.93$, $R^2=0.86$; $p<0.001$)

RESULTS

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

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

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

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

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

Immunoblot analysis of antibody response in preterm lambs.

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

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

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

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

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

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

116), which 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).

DISCUSSION

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

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

fetuses with bacteraemia were found to have lowest complement function compared to non-bacteraemic fetuses; although~~. Further, in the group with bacteraemia,~~ animal 116 also had the lowest UP plasma titre (Figure 2) and the highest complement function within this this group (Supplementary Figure 1A). The correlation between UP bactericidal activity and complement function was not influenced by experimental infection, suggesting that the natural variation in complement activity at 95 d GA determined whether UP infection in the amniotic fluid became systemic or not.

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

cross-react with guinea pig erythrocytes[15] and this was not detected in any of the sera from the preterm lambs (data not shown).

The HPA5 strain is susceptible to human serum killing in the absence of specific anti-UP antibodies.[14] As with human serum studies with this strain, sheep serum bactericidal activity required the presence of calcium. However, we are unable to determine if the bactericidal activity in sheep is mediated by the classical or lectin pathways (both require calcium), as we lack the reagents to differentially block these pathways as was performed for human sera.[14] The increased presence of *Ureaplasma* spp. in the cord blood of human preterm neonates of increasing prematurity has also been identified in the previously published Alabama preterm study.[4] Furthermore, *Cassell et al*. found that *Ureaplasma* could be cultured from blood samples of 26% of 200 ventilated preterm neonates studied, showing that Ureaplasma bacteraemia is prevalent in this patient group.[8]

Sheep models have recently been developed to investigate hypoxic delivery complications and intrauterine inflammation induced by microbial infection or exposure to lipopolysaccharides.[20-26] None of these studies, however, has investigated the contribution of the complement system to disease pathogenesis or to the role of complement in controlling systemic infection, largely due to the current lack of reagents to measure and manipulate the sheep complement system. The present study thus represents a significant advance in our understanding of the pathophysiology of fetal bacteraemia in the setting of preterm birth.

In conclusion, experimental intrauterine UP infection establishes stable amniotic fluid levels that remain stable for weeks following the infection.[27] Highlighting the importance of gestation/complement function on fetal response to challenge, UP was detected in the cord blood of 50% of fetuses infected at 70 d GA and delivered at 95 d GA (Group 2), but only in

10% of lambs infected at 115 d GA and delivered at 125 d GA (Group 5). No bacteraemia was observed in lambs infected at 80 d GA and delivered at 125 d GA (Group 4), but this was the only group to show a UP-specific antibody response. Interestingly, a range of functional complement activity was observed in uninfected 95 d GA preterm lambs, indicating that development of the complement system is variable in the normal population and is not influenced by intrauterine UP infection. In addition to advancing our understanding of fetal responses to UP infection, our results also suggest that a familial history of complement deficiency may increase the risk of fetal UP infection in pregnancy, concomitant with an increased risk of preterm birth and adverse neonatal outcomes.

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FIGURE LEGENDS.

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

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

Figure 2. UP titres in cord plasma at delivery. GA at delivery and total length of UP infection is indicated for each group. Each point represents the average as determined in

triplicate. Data points for lambs 115 and 116 are separately identified for correlation purposes in other figures. Results were repeated in 2 duplicate experiments.

Figure 3. (A) IgG concentration in 95 d and 125 d GA lambs. ELISA determination of sheep IgG levels in sera from uninfected control lambs (Groups 1 and 3), compared to experimentally UP-infected lambs. Lambs 121 (Group 2) and 235 (Group 5) are identified as they have elevated IgG levels, but do not react with UP by immunoblot in (B). Results were repeated once.

(B) Detection of anti-UP antibodies in fetal sera from infected lambs. Purified whole UP cultures from the infecting serovar 3 strain (HPA5) and separate serovar 6 strain (HPA61) were separated by non-reducing SDS-PAGE and probed with fetal sera to detect immunoreactive bands by immunoblot analysis. Two infected lambs from Group 4 failed to raise specific anti-UP immune response (130 and 132) as well as all of the animals in Group 2 and 4 (only sera with elevated IgG from Groups 2 (121) and Group 4 (235) are shown). Four lambs in Group 4 raised an antibody response to proteins unique to the infecting strain and two of these lambs (136 and 137) also raised cross-reacting antibodies that also recognised strain HPA61 which is a serovar 6 isolate. The MBA isoforms for both strains are identified by mouse monoclonal antibody 6523. Representative results of 3 repeat experiments shown.

Figure 4. Serum susceptibility of UP recovered from plasma or amniotic fluid. Recovered strains were incubated for 30 min with 50% (v/v) adult sheep (A) or 50% (v/v) 95 d GA preterm lamb sera (B). Pooled sera from uninfected animals was used and each point was determined in duplicate. Representative data shown from 3 repeated experiments. Killing calculated as decreased titre relative to UP titre following incubation with matched heat-inactivated sera controls. One way ANOVA analysis found no difference between these groups.

Figure 5. ***Ureaplasma*-cidal activity from bacteraemic, non-bacteraemic, and uninfected 95 d GA lambs.** Serum killing of HPA5 by 50% (v/v) sera from bacteraemic (plasma UP), non-bacteraemic (no plasma UP) and uninfected 95 d GA preterm lambs. Each point represents the average of 3 separate killing assays (each performed in duplicate). Killing calculated as decreased titre relative to UP titre following incubation with matched heat-inactivated sera. Individual points for lambs 115 and 116 are identified.

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

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

Table 1. Fetal delivery data..

Group	UP Infection (d)	Delivery date (d GA)	Delivery Weight (g)	# Male Fetuses	Arterial CB pH	+ve UP AF	+ve UP Bacteraemia
Control (n=8)	N/A	95	778±91	5/8	7.18±0.07	N/A	N/A
Control (n=4)	N/A	125	2558±342	1/4	7.23±0.06	N/A	N/A
1: UP infected (n=8)	70	95	673±77	3/8	7.14±0.06	8/8	4/8
2: UP infected (n=8*)	83	125	2838±297	2/6	7.22±0.2	6/6*	0/6
3: UP infected (n=10)	115	125	2744±212	5/10	7.17±0.06	10/10	1/10

Legend: # = number of male fetuses relative to total number. * = Two of the fetuses were lost prior to delivery. UP, *Ureaplasma parvum* serovar 3 strain HPA5; d, day; GA, gestational age; AF, amniotic fluid. *, two fetuses died in utero after infection but prior to scheduled deliver and were excluded from subsequent analyses

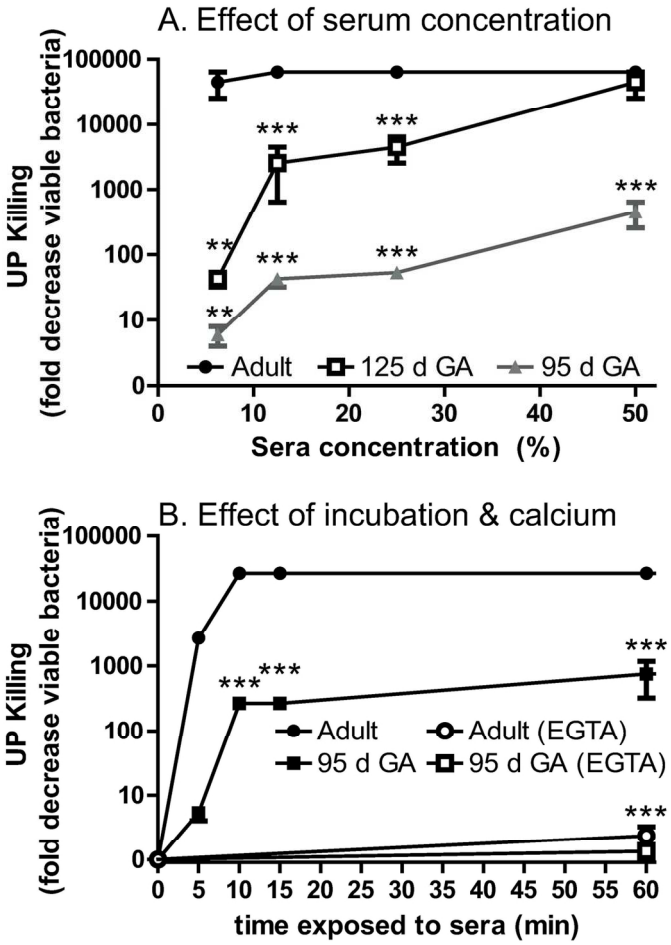

Figure 1. (A) Titration of Ureaplasma-cidal activity in non-immune sera. UP killing by diluted sera from adult sheep (closed circle), 125 d GA preterm lambs (open square) and 95 d GA preterm lambs (grey triangle) following 1 h incubation at 37oC. Killing determined by relative decrease in UP titre compared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

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

Figure 1
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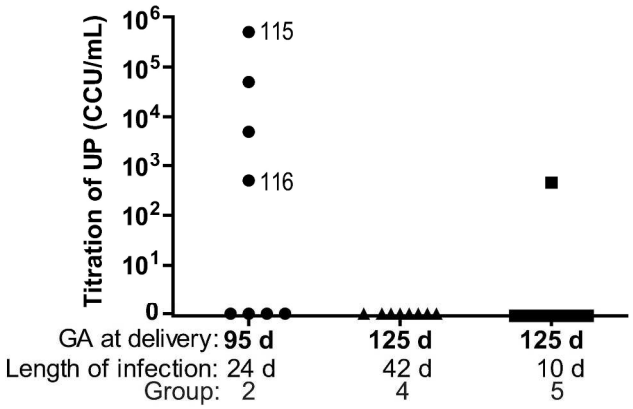

Figure 2. UP titres in cord plasma at delivery. GA at delivery and total length of UP infection is indicated for each group. Each point represents the average as determined in triplicate. Data points for lambs 115 and 116 are separately identified for correlation purposes in other figures. Results were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

Figure 2
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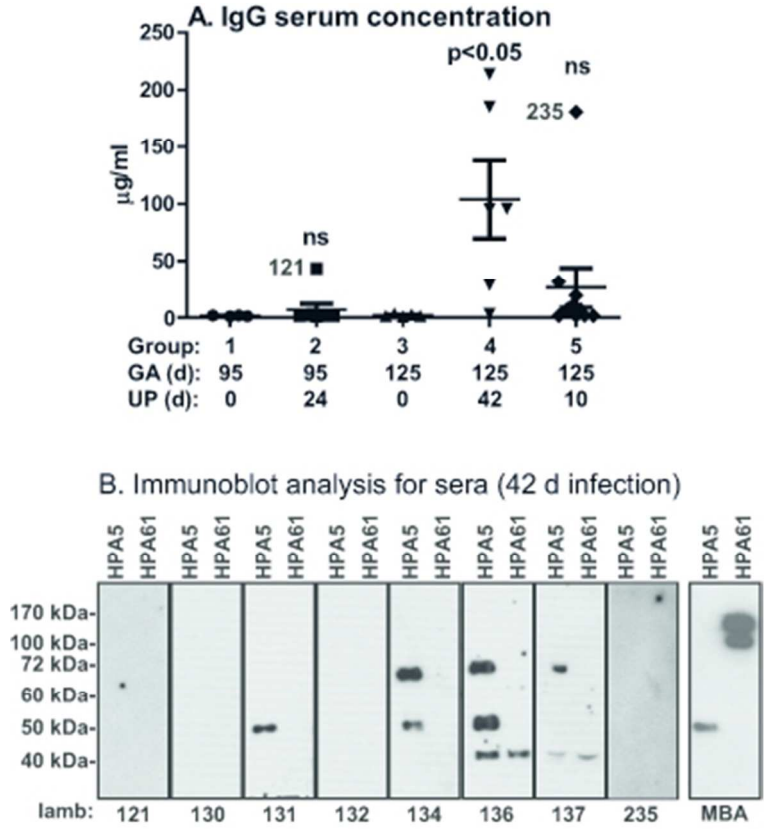

Figure 3. (A) IgG concentration in 95 d and 125 d GA lambs. ELISA determination of sheep IgG levels in sera from uninfected control lambs (Groups 1 and 3), compared to experimentally UP-infected lambs. Lambs 121 (Group 2) and 235 (Group 5) are identified as they have elevated IgG levels, but do not react with UP by immunoblot in (B). Results were repeated once.
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Figure 3
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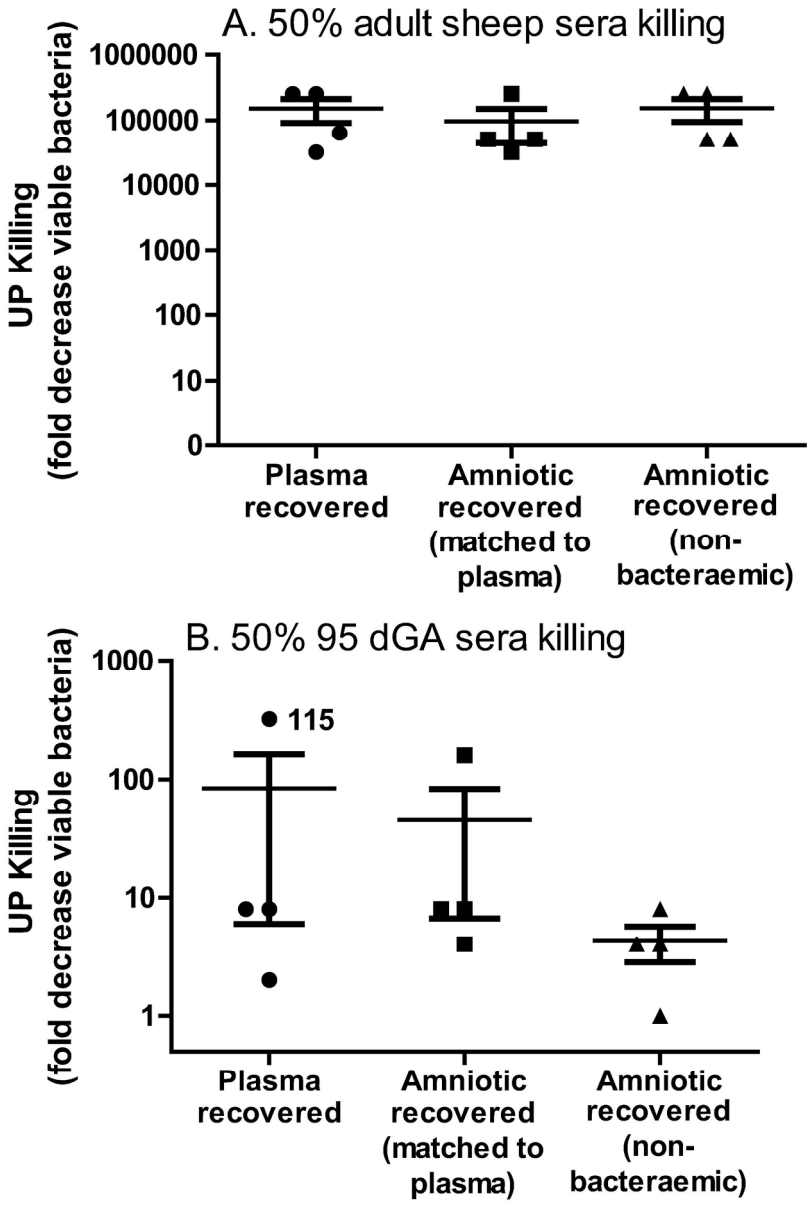

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Figure 4
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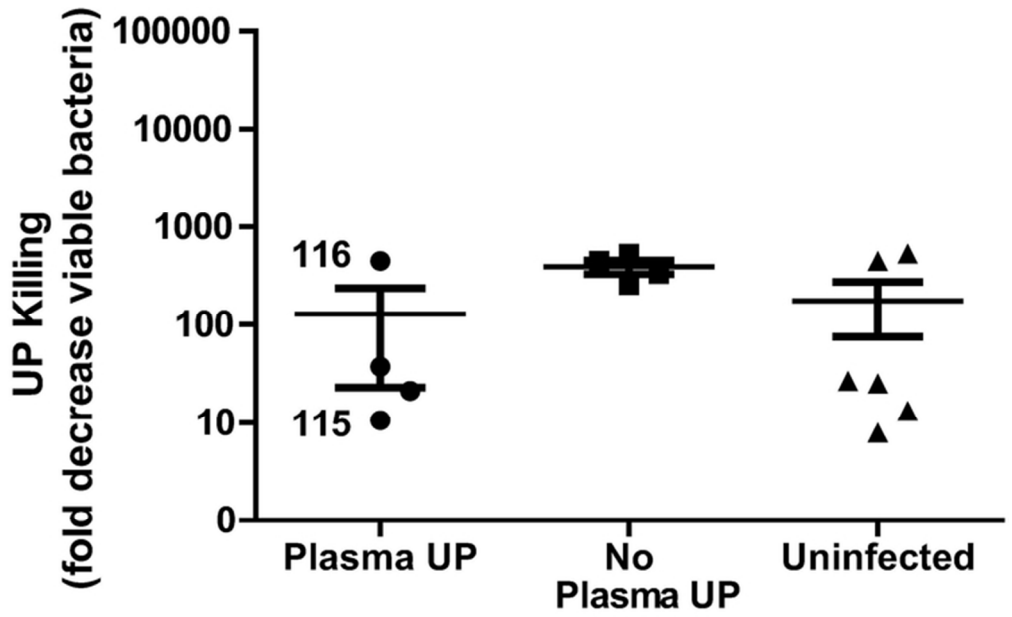

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Figure 5
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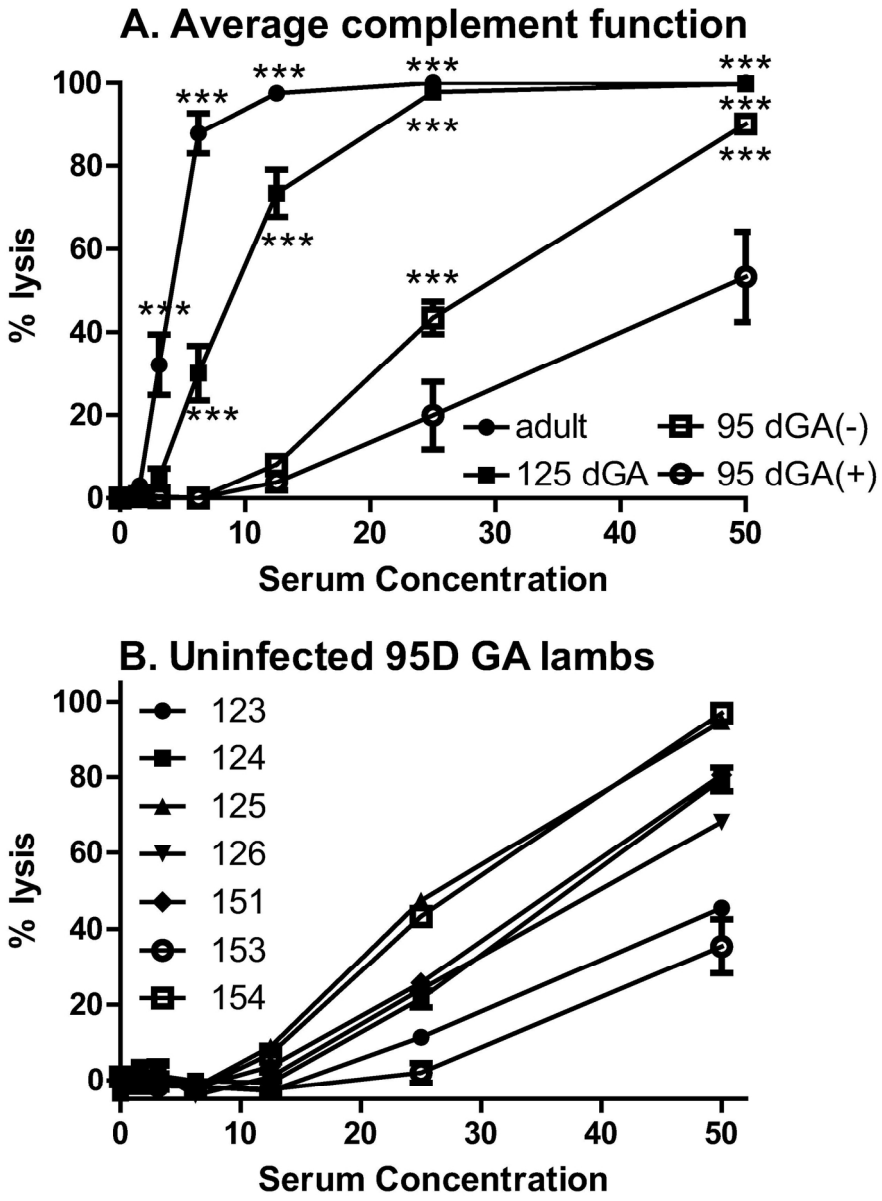

Figure 6. Functional complement activity as determined by guinea pig erythrocyte lysis assay after 30 min by diluted sera. A. Complement activity for uninfected adult sheep (closed circles) and late preterm (125 dGA; closed squares) lamb sera are shown relative to complement function in sera from UP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

Figure 6
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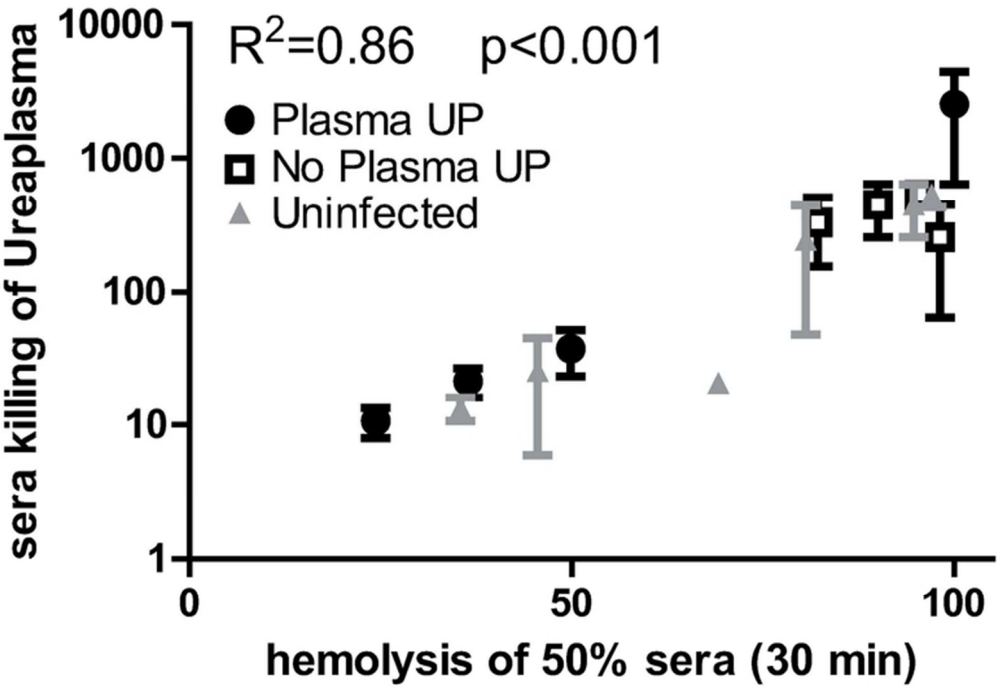

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Figure 7
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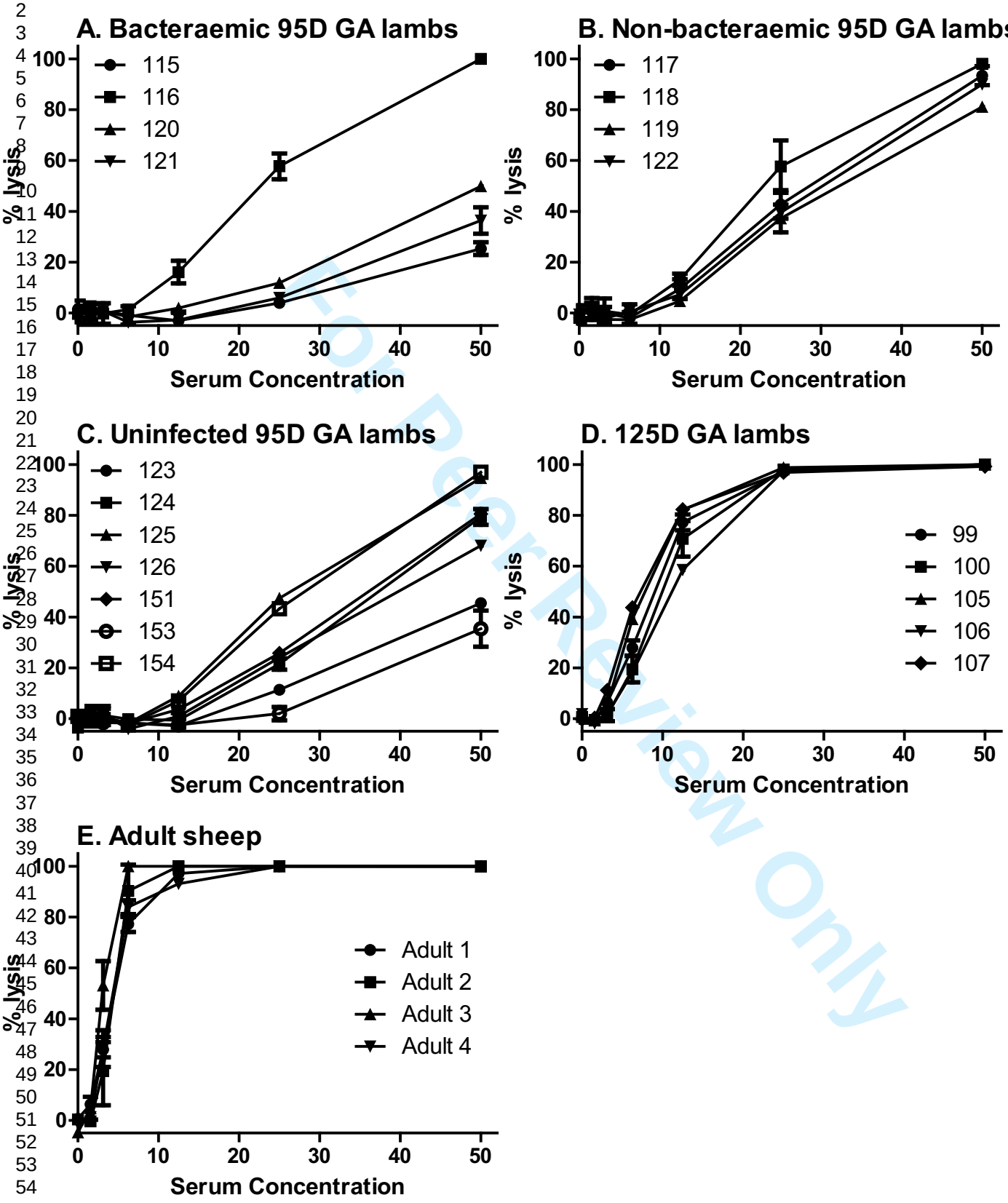

Supplementary figure 1. Complement activity in bacteraemic (A), non-bacteraemic (B) and uninfected (C) 95 d GA preterm lamb serum. Complement activity for uninfected 125 d GA preterm lambs (D) and adult sheep (E) are also included for comparison and show increased function with later gestational age and maturity. Complement activity measured as haemolysis of rabbit antibody-sensitised guinea pig erythrocytes by diluted sera after 30 min incubation at 37ºC. Each point determined in triplicate, unique identifiers for each lamb are also given. Results were repeated in 2 duplicate experiments.