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Induction of central venous catheter-associated sepsis (CAS): A novel sheep model simulating the complex pathophysiology observed in humans.

A paper to be submitted to Shock

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ABSTRACT:

Catheter-associated blood stream infections (CABSIs) are serious, yet common, outcomes in both human and animals with indwelling catheters. The increasing rate of these infections is partially due to hindered knowledge on how to stop the spred of these pathogens, this due, in part, to inadequate animal models. Current experimental models fail to mimic various aspects of sepsis pathogenesis, diverse clinical symptomology, and in most cases lack the ability to test novel therapies for use in human medicine. In response to the urgent need for a more clinically relevant animal model of CAS, this large animal model was conceptualized and validated through this prospective study. Eight clinically healthy domestic cross-bred mature female sheep were obtained and double-lumen peripherally inserted central catheters (PICCs) were nonsurgically placed in the left jugular veins. A novel inoculation method was developed using a luminal volume of *Klebsiella pneumoniae subsp. pneumoniae 43816Rif*^{R} isolate and blood mixture (1:3). The mixture was injected into the lumen and was allowed to clot where is remainded for the duration of the study, enabling the development of a chronic catheter infection with the slow prolonged release of bacteria. This continual shedding of bacteria more closely mimicks the natural pathogenesis of catheter-associated sepsis (CAS) in clinical settings. A diverse yet dose-dependent host immune response was observed including; tachypneas, tachycardias, pyrexias, leukopeneas, neutrophilias, thrombocytopenias, hyperlacemias, and in some sheep biochemical signs of organ injury/damage with SOFA scores reaching ≥5. All challenged sheep fulfilled clinical sepsis criteria, as well as acute sepsis-induced organ injury and sepsis-induced coagulopathies to varying degrees. At necropsy all challenged animals showed evidence of recent bacteremia, acute organ injury and positive cultures of the parent *43816Rif*^{R} isolate from several tissues, organs and catheters. In contrast, none of the negative (control sheep) developed any signs of infection or positive bacterial cultures for the

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43816Rif^{R} isolate. Validation of this novel *in vivo* animal model of catheter-associated sepsis demonstrates its potential to serve as a robust, reproducible and reliable platform for future biomedical sepsis research.

Introduction:

Despite the historical significance of sepsis in human medicine, it remains one of the least understood and most enigmatic disease processes to date. It is not only hard to properly diagnose and treat, but until recently was best defined only by its clinical presentation [1-3]. This is due, in part, to highly heterogeneous intrinsic factors including; genetics, age, race, sex and health/comorbidities [4-7]. In addition, there are several extrinsic factors including treatment type, infection type, microbe(s), degree of insult, and time of infection [8-10]. Due to this multifaceted and highly varied host immune response [11-13], an intricate understanding of the complex pathophysiology of sepsis has not been fully described. This gap in knowledge hinders our ability to develop better diagnostics, therapies, and prevention methods.

Animal models are an essential component in biomedical and sepsis research, enabling investigators to study the molecular and cellular pathology of sepsis in experimental designs not feasible in human patients. However, current animal models of sepsis are subject to critique regarding the clinical translation of experimental data to human clinical sepsis [4, 7, 14-23]. While they provide an extremely valuable resource for studying the pathophysiology of several types of sepsis [4, 9, 18, 19, 21-24], current animal models of sepsis are limited in their ability to provide clinically relevant data specific to catheter-associated septicemias (CAS). In fact, several recent publications have argued that the use of animal models, particularly murine models, have resulted in significant misinterpretation and failure of upwards of 150 human clinical trials [7, 15, 16, 24].

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Murine models often fail to properly reproduce clinical symptoms of human sepsis due to significant differences in the host-species tolerance for lipopolysaccharide (LPS), a key mediator of the inflammatory cascade associated with sepsis [25, 26]. In addition, most species used in current models are hindered by an inability to non-surgically place and maintain long-term intravenous catheters, repeatedly sample large volumes of blood, monitor clinical parameters (hemodynamic, cardiopulmonary, biochemical, etc.), or administer IV therapies or fluids to test current and novel treatments. There is a substantial need for the development of robust large animal models that more closely mirror the pathogenesis, immunology and pathophysiology of sepsis.

Similar to human internist, large animal internal medicine specialists routinely deal with sepsis and bacteremia in hospitalized horses, sheep, goats and cattle. Like humans, the source of infection is often associated with long-term IV catheterization. The use of sheep as an animal model of sepsis has been suggested in several articles [17, 21, 27-33], and the similarities in body size, anatomy, immunology and pathophysiology make them an ideal species for studying human disease processes. Sheep can be easily acquired and housed, have a proven track record in human biomedical research, and are amenable to a full array of advanced medical diagnostics and monitoring [27, 31, 34]. They provide marked advantages over swine in that we can easily place and maintain peripherally inserted intravenous central catheters (PICC) and urinary catheters without surgery or sedation, can be administered continuous rate infusions of IV fluids or antibiotics, and they are much more easily handled for evaluation of blood pressure, echocardiography and closed-system urinary catheter collection . In addition, they have LPS sensitivities similar to humans, and have similar pharmacokinetics/pharmacodynamics to many antibiotics used in humans, such as carbapenems [26-28, 33]. There are currently several validated ovine models simulating the critical care situation in humans, and they arguably are much more clinically relevant to study specific disease processes than previous animal models. [29, 34]

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Current literature states that up to 90% of patients admitted into hospitals will require some type of vascular access to deliver intravenous (IV) medication, blood products, and/or fluids [35-42]. Among these catheterized patients, an estimated 40% will require a centrally placed vascular access point commonly referred to as a central venous catheter (CVC) or more commonly in human medicine, a central-line (CL) [43, 44]. CVCs are a necessity for the proper care of critically or chronically ill individuals, as they are required for infusion of certain medications (due to toxicities, volume, etc.), nutritional support, fluid replacement, blood collection, renal replacement therapy, and hemodynamic monitoring [39, 43, 45-49]. However, despite their significant benefits to public health, and the millions of lives they save every year, there are substantial risks involved with their use.

Catheter-associated blood stream infections (CABSIs) have various etiologies, but disease usually is initiated as a monomicrobial infection of the catheter lumen. These infections of the catheter lumen form a biofilm and seed a long-term systemic infection (sepsis). Many CABSIs and CAS are caused by members of the *Enterobacteriaceae* family, including *Klebsiella pneumoniae subsp. pneumoniae* [50, 51].

Our *a priori* hypothesis for this study was that the intraluminal seeding of *Klebsiella pneumonia* in central intravenous catheterized sheep would result in a dose-dependent host immune response that would fulfill the criteria of sepsis in humans (both Sepsis-2 and Sepsis-3 definitions) [1-3]. In addition, confirming the ease of placement and ability to maintain a double lumen peripherally inserted central catheters (PICC) in sheep without the use of general anesthesia of sedation. Successful proof-of-concept induction of sepsis in sheep using this novel induction method combined with the ability to collect frequent samples of large volumes of blood provides a much needed platform on which to further evaluate novel therapies and study the pathobiology of sepsis.

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Materials and Methods:

Animal preparation and general experimental design/setup. Eight adult domestic crossbred clinically healthy female sheep were obtained and housed in the livestock infectious disease isolation facility at Iowa State University. The average body weight for the animals was approximately 70 kg at the start of study. The sheep were housed in individual pens, with control groups physically separated from the challenge groups. The sheep were allowed four days for acclimatization to their new environment prior to the initiation of the experimental procedures. All subjects were fed identical diets of free choice grass hay and a ruminant concentrate pellet twice daily and were given free access to clean water at all times. The eight ewes were randomly placed into three groups; Control Group (N=2), Challenge Group #1 (N=3) and Challenge Group #2 (N=3). During the course of the study, each of the challenge groups received two inoculations, with the second challenge occurring after the return to baseline cardiovascular and physiologic parameters following the first dose. On day 0 of the study, all ewes were administered their first inoculation (all subjects inoculated within 1 hour of each other). Challenge group #1 received (Dose #1 of 10^3 CFU) *K. pneumonia 43816Rif*^{R} isolate while challenge group #2 received (Dose #2 of 10^7 CFU) of the *K. pneumonia 43816Rif*^{R} isolate. The control group received sterile bacterial media (TSB) as a sham inoculation. In order to assure that physiological parameters returned to baseline prior to re-challenge, the second inoculation was administered at different time points for the challenge groups. On day 7 of study, challenge group #1 received (Dose #3 of 10^8 CFU) *K. pneumonia 43816Rif*^{R} isolate, while challenge group #2 received (Dose #4 of 10^9 CFU) *K. pneumonia 43816Rif*^{R} isolate on day 12 of the study. Beginning two days prior (day -2) to the first inoculation and continuing through the study endpoint, physiologic assessments were performed, and samples collected for diagnostics as summarized in Appendix I.

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Animal Welfare Assessments. The Iowa State University Institutional Animal Care and Use Committee approved all of the experiment protocols in this study under protocol 3-15-7965-O. Based on the recommendations of a recent publication regarding the development and refinement of animal models of sepsis [52, 53] we elected to limit the treatment group sizes in lieu of trying to achieve statistical significance. As argued by that publication, this study was designed as a descriptive proof-of-concept effort [53]. In addition, in order to assure humane endpoints for animal euthanasia we developed and the IACUC approved a monitoring algorithm (Appendix I). This algorithm included a four-level score for each of the following parameters: activity, head position, breathing pattern, rectal temperature, hydration status, heart rate, eating and ability to stand. Scores for each parameter were recorded twice daily beginning two days prior to the first inoculation through the duration of the study, with more frequent evaluations immediately following the highest dose challenge of 10^9 CFU (every 15 minutes for two hours, then every hour for five hours). Sheep receiving a severity score of four in six or more of the assessed parameters would be euthanized immediately.

Preparation of bacteria. *Klebsiella pneumoniae subsp. pneumoniae* (Schroeter) Trevisan (ATCC® 43816™) serotype 2, was the inoculum strain used throughout this study. In order to allow for easier monitoring, the parental ATCC 43816™ strain was sub cultured (10^9 CFU) onto a Trypticase soy agar (TSA) plate containing 100 ug/ml rifampin. An ATCC 43816 Rifampin resistant (43816Rif^R) isolate was selected and isolated, then stored for use throughout study (MIC to rifampin > 256 ug/ml).

Central intravenous (PICC) catheter placements. Intravenous catheters identical in material and placement to catheters used in long-term IV catheterization in humans were selected. These double-lumen (2x18 gauge) wire guided long-term Mila silicone peripherally PICC inserted central catheters (Mila order #3565) were placed in the left jugular vein of each ewe. Prior to insertion of the catheters, the animals were restrained , the fiber was removed using hair clippers and the neck was prepped

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aseptically (Povidone/Iodine) along the jugular vein. Maximal sterile barrier precautions were used as described in [47] to avoid contamination of the catheters. Catheter placement did not require general anesthesia or sedation, however 1.5ml of 2% lidocaine solution was injected subcutaneously at the site of catheter placement to provide local anesthesia. The appropriate catheter length was estimated by measuring the distance from the site of insertion to the approximate site of the distal vena cava or right atrium (as is typical for central catheters in humans) and aseptically cut to length according to manufacturer. Catheters were secured using 2-0 suture and needleless injection ports (Mila order #8095) were placed on each line. Once catheters were placed and secured, both ports were flushed with 10ml heparinized saline solution and tested for patency. Thereafter, 10ml heparinized saline solution was administered after each sample collection and twice daily to ports that were not sacrificed for inoculation.

Sample collection and cultures. Approximately 20ml blood samples were obtained daily from the second catheter lumen following standard aseptic preparation. Samples were aliquoted as necessary for the testing required at the given sample period. Initially blood culture methodology utilized a low volume of blood (1 mL) directly applied to the culture media as a spread plate. However, following inoculation #1, absence of growth in cultures of the isolates was observed in all blood or fecal samples collected. As a result, on day 7 (Dose #3) of the experiment, the decision was made to increase the volume to 5 mls of blood and adding enrichment to cultures to facilitate the growth of the *43816Rif*^{R} isolate in-vitro. The enrichment of blood samples was performed by adding 5 ml of blood samples into 50 ml TSB broth containing 100 ug/ml rifampin. These enriched samples were incubated at 37 ºC overnight and 250 ul of culture was spread out uniformly on TSA + Rifampin plates. Broth and agar plates for cultivation of *K. pneumoniae 43816Rif*^{R} isolate included tryptic soy agar + 100 ug/ml vancomycin + 100 ug/ml rifampin to quantitatively culture the blood and fecal samples. Fecal samples were obtained from rectum using clean gloves for each animal and placed in airtight sealed bags for culture. Culture of feces was

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performed as described above. Select tissue and catheter samples were cultured following necropsy. For samples not receiving the enrichment step, CFU counts were obtained by physical counts of colonies on selective media. When enrichment was performed, samples were recorded as positive or negative without quantification.

Cardiovascular, hematological and coagulation profile monitoring. Heart and respiratory rates were obtained by auscultation and visual observation of thoracic cavity for movement from inhalation. Two methods were used for temperature monitoring. First, rectal temperatures were obtained by inserting a digital thermometer approximately 3 inches into rectum and pressing tip gently against rectal wall. Secondly, rumen temperature data loggers (iButton) were administered via oral bolus for continuous temperature monitoring and were recovered at necropsy. Blood samples were collected from the second catheter lumen in the jugular vein and placed into vacutainers containing EDTA and sodium citrate as anticoagulants or into serum tubes (free of anticoagulant) for biochemical analysis. Hematological and serum samples were placed at 4ºC and analyzed by an accredited veterinary clinical pathology laboratory (Iowa State University College of Veterinary Medicine) within 4 hours of collection. Complete blood counts (CBCs) were monitored daily for the first week of the study and during intensive monitoring of high dose challenge (dose #4), then every other day thereafter. Serum chemistry was analyzed every other day throughout the study and banked for future analysis. Complete coagulation profiles including D-Dimers, prothrombin (PT) and partial thromboplastin (PTT) times were performed intermittently during the study when evidence of sepsis induced coagulopathies (such as; signs of petechia, dehydration, and/or mottling of the skin).

Inoculation #1. Dose 1 (~$1x10^3$ CFU into three ewes in challenge group #2) and Dose 2 of (~$1x10^7$ CFU into three ewes in challenge group #1) were administered by "sacrificing" a single lumen of each PICC catheter. Prior to placement, the catheter residual volume was determined to be just over 400 ul.

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In all ewes, the *K. pneumoniae 43816Rif*^{R} isolate (resuspended in 100 ul of broth) was mixed with 300ul of fresh blood collected from the animal by inversion of the syringe. The 400ul of mixed blood/inoculum was then injected back in the lumen allowing it to fill the lumen without injecting into the bloodstream. The blood was then allowed to clot, resulting in the occlusion of a single catheter lumen contaminated with a monoculture of *K. pneumoniae 43816Rif*^{R} isolate. By utilizing a single lumen of the catheter to serve as a reservoir for microbial growth it enabled the prolonged shedding of a monoculture of bacteria from the contaminated catheter, which is a key element to the overall pathogenesis and pathobiology seen in many types of catheter associated sepsis. Since no previous sheep models have used a similar method of inoculation of this pathogen, the doses were empirically selected. After inoculation, the lumen port that received the inoculations was closed and sealed with red tape labeled "inoculation port" and remained undisturbed throughout the remainder of the study.

Inoculation #2. Dose 3 (~10^{8} CFU to the three ewes previously given Dose #1 in Challenge Group #1) and Dose 4 (~10^{9} CFU to the three ewes previously given Dose #2 in Challenge Group #2) were subsequently administered due to neither of the first two inoculation doses resulting in sufficient changes in clinical signs indicative of severe sepsis. This approach minimized animal usage (consistent with 3R approach to developing animal models) while maximizing our data collection on dose responses. These second dose inoculations of the *K. pneumoniae 43816Rif*^{R} isolate were given to both challenge groups using the same technique as Dose 1, with the exception of using the second lumen of the double lumen catheter. Challenge group #1 was inoculated a second time on day 7 and challenge group #2 was inoculated a second time on day 12.

Euthanasia. No sheep were euthanized due to meeting the criteria outlined in our endpoint's algorithm (Appendix I). Upon termination of the study, each ewe was humanely euthanized by injecting

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10 mls pentobarbital sodium (Fatal Plus© 390 mg/ml) into the jugular vein. The control group and challenge group 1 were euthanized on day 14, while challenge group 2 was euthanized on day 15.

Necropsy. A board-certified veterinary pathologist performed a gross necropsy immediately after death was confirmed for each ewe. Tissue samples collected from various organs were swabbed with a sterile, dry swab and/or placed in a sterile tube for microbiological evaluation or 10% neutral buffered formalin solution for histological analyses. Swabs were taken aseptically from lungs, liver, catheter, spleen, heart valves, feces, duodenal contents, and arterial blood. These swabs were cultured on selective media for *K. pneumoniae 43816Rif*^{R} isolate growth as described above. Tissue samples were collected from lung, liver, spleen, and heart valves for histopathology. The hearts were checked grossly for any evidence of endocarditis. Lastly, the (iButton) rumen temperature data loggers were retrieved from the rumen or reticulum of the subjects.

Results

Dose-Dependent Host Immune Response. The sheep demonstrated a dose dependent host response that displayed consistency with the pathophysiology of sepsis and satisfies the clinical criteria used to define human sepsis, including what was previously considered severe sepsis and septic shock (tables 1-2 and figs. 1-3). Additionally, the severity of sepsis was approximated using human (SIRS and SOFA) criteria and the model successfully induced disease states meeting various levels of these criteria in sheep at specific time intervals of the study (tables 1-2 and figs. 1-3). Clinical signs consistently observed in individual animals following inoculation #1 (Dose #1 & Dose #2) included tachycardia, pyrexia, tachypnea, neutrophilia, leukocytosis, thrombocytopenia, increased total bilirubin and hyperlactemia. A dose-dependent response was observed, with higher doses having a more profound physiologic response in the measured parameters. While the general trends in physiologic response were fairly consistent across all animals for a given dose, there was obvious individual animal variation in the degree of

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response. For example, a much more robust immune response was seen in all subjects within challenge group #2 following Dose #4. In addition to the clinical signs mentioned at the lower doses, these animals demonstrated changes in hematological markers including leukocytosis, lymphocytosis, neutrophilias, neutrophil bands, thrombocytopenia, increased PT, PTT and D-Dimers. The last four findings are consistent with dessiminated intravascular coagulation. Variable changes in biochemical indexes were also seen in these animals with increased serum BUN, lactate, serum creatinine, bilirubin, AST and GGT. Following the highest inoculatioin dose (Dose 4) physiologic changes consistent with human SOFA diagnostic criteria, as well as SIRS, were observed. Lastly, given that each of these animals had a known site of infection (contaminated catheter), all of the animals would meet the human criteria for clinically sepsis, with variable severity correlated to the inoculation dose.

Sham Inoculation (Dose 0): There were no clinical signs of any type of infection observed at any timepoint for either of the two control sheep. There was a notable variability in rumen temperatures during different parts of the day (eating, circadian rhythm) [54], and specifically after drinking cold water (significant changes in temperature at a few timepoints in all sheep were affected by this). Lastly, on average the rumen temperatures were ~.8˚C higher than all of our recorded rectal temperatures. However, this is a well documented phenomenon [54].

Dose #1: $1x10^3$ CFU. Challenge group #1 received their first inoculation dose of ~10^3 CFU on Day 0 of experiment. No significant changes in heart rate, respiratory rate, rumen/rectal temperature, leukocytosis, or neutrophil counts were observed following initial inoculation (fig. 1a-c,f). However, there was an undulant fever present in all three ewes starting 3 days post inoculation of this dose. This is more notable when analyzing the continuously acquired rumen temperature data and was largely missed by twice-daily rectal temperatures (Appendix VI). Concurrent with the periods of the pyrexia,

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we observed intermittent tachycardias, tachypneas, slight increases in serum lactate, mild leukocytosis' at later time points (fig 1a-c, f) and increased total bilirubin (table 2, Appendix VI).

Dose #2: 1x10^7 CFU. Challenge group #2 received their first inoculation dose of ~10^7 CFU on Day 0 of experiment. We observed significant pyrexias (fig. 2a), tachycardias (fig. 2b), tachypneas (fig. 2c), leukocytosis, lymphocytosis, and significant neutrophilias (fig. 2f). The severity of clinical signs slightly varied among individual subjects, but for most parameters lasted for 3-4 days post inoculation (fig. 2a-f). The neutrophilia peaked at one day post-inoculation and increased from a pre-inoculation concentration of ~4 x10^7/ul (+/- 1 x10^7) to ~10 x10^7/ul (+/- 2 x10^7) (fig. 2f, Supplemental VI). Extreme thrombocytopenias were observed post challenge Dose #2 in Ewe #3 on day 1 and day 2 of 60 x10^3/ul and 59 x10^3/ul, respectively (fig 2e).

Dose #3: 1x10^8 CFU. Challenge group #1 received their second inoculation dose of ~10^8 CFU on Day 7 of experiment. Immediately following this inoculation, pyrexias (fig. 1a) and tachycardias (fig. 1c) were observed. There was only a slight increase in the respiratory rates following this dose challenge (fig. 1b). In addition, there were pronounced leukocytosis', hyperlactemias (fig. 1d), neutrophilias, (fig. 1f) and thrombocytopenia (fig. 1e) observed during the few days post inoculation through the termination of the experiment.

Dose #4: High Dose inoculation 1x10^9 CFU. Challenge group #2 received their second inoculation dose of ~10^9 CFU on day 12 of the experiment. Clinical presentations were similar to dose #2 that they received 12 days earlier; however, they were more intense and manifested several additional physiologic changes consistent with more severe host immune response to the systemic infection. Observed were acute pyrexias (fig. 2a), tachycardias (fig. 2b), tachypneas (fig. 2c), leukocytosis, significant neutrophilias (fig. 2f) and extreme thrombocytopenias (fig. 2e). These thrombocytopenias are observed in ewe #4 having values of 20 x10^3/ul, 27 x10^3/ul, 36 x10^3/ul on days 13-15, respectively. In addition,

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the high dose intensive monitoring (fig. 3a-d) displays these physiological changes in more clarity and demonstrates the hyperlactemias observed (fig. 2d, 3d). Band neutrophils were seen increasingly from day 13 through 15 until euthanasia in Ewe #4 (.0726, .2512, .3699 $x10^3$/ul, respectively). Further indication of sepsis, or sepsis induced organ injury/failure were observed including increased BUN from a baseline of ~11mg/dl on day #12 to 16, 31 and 22 mg/dl in ewes 3-5 on day #13, respectively. Serum Creatinine (SCr) increased from an average baseline of ~0.9 mg/dl in all groups (N=8), to ~1.25 mg/dl (N=3). Increased bilirubin in ewe #4 was seen from an average of ~.6mg/dl before dose #4, to 1.17mg/dl, .75mg/dl on days 13 and 14, respectively. Additionally, there were significant changes in MPV, monocytes, AST, fibrinogen, PT, PTT, D-Dimer that were observed with varying intensities and durations (Supplemental I, Appendix VI).

Qualitative/Quantitative Blood and Fecal Cultures. On Days -1 and 0 (pre-inoculation), all cultures for *K. pneumoniae* in blood and fecal samples from sheep were negative. The daily blood and fecal samples from days 0 through day 7 (post-inoculation #1, Doses #1 & 2) were tested for 43816Rif^R isolate in all control and challenge ewes. However, there was no bacteria recovered for any of the samples during this time period. Following the change to a higher inoculation volume and the addition of an enrichment step, all ewes had at least one blood sample positive for the 43816Rif^R isolate following their second challenge dose. Blood and fecal cultures from challenge group #2 on days 13-15 had positive cultures for the 43816Rif^R isolate. Culture of the intravascular tip of the PICC catheter collected at necropsy confirmed that all but one challenge inoculated catheters were culture positive for the inoculated *K. pneumoniae*. Liver, blood, and spleen samples collected during necropsy yielded no growth of *K. pneumoniae* in all eight sheep. Lung swabs collected at the site of gross lung lesions for two ewes (Challenge group #2) were positive for the inoculated *K. pneumonia* strain. Only one ewe had

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a positive culture of intestinal content (~2000 CFU) at necropsy. The control group sheep remained culture negative for the *K. pneumonia* 43816Rif^{R} isolate throughout the study.

***Gross Histology/Histopathology*.** No remarkable gross or histopathologic lesions were observed in any thoracic or abdominal viscera of the control group. In contrast, all animals in both challenge groups demonstrated varying degrees of multifocal to coalescing gross lung lesions ranging in size from 0.1-4.5 cm in diameter (fig. 4a). Histopathologic evaluation of these lung lesions revealed a consistent fibrinous and seroproteinaceous pneumonia, progressing in more severe cases to a marked multifocal suppurative inflammation with an associated segmental vasculitis and fibrin thrombi (fig 4a). Ewes 3, 7 and 8 had serofibrinous lesions with neutrophils in lung suggestive of acute inflammation. Ewe #8 had a significant vessel with a thrombus and fibrinosuppurative vasculitis. In addition, a single animal demonstrated a moderate lymphocytic periportal hepatitis (Supplemental I). All three ewes in challenge group #1 (Dose #1, and Dose #3) had a subjective splenomegaly. The multifocal pathology in the lung was found predominantly in the middle and caudal ventral lung fields (lesions were not typical of sheep pneumonia and instead were consistent with septic bacterial emboli lung (fig. 4b) and were markedly different from the controls. As expected, animals receiving the highest bacterial doses of 10^7 CFU (Dose #2) and 10^9 CFU (Dose #4) had the most significant lesions at necropsy (figs. 4a-b). These findings suggest a well-established systemic infection in all challenged ewes.

Discussion:

In this study, the observed host immune responses to *Klebsiella pneumoniae* induced catheter associated sepsis in sheep demonstrate clinically relevant and translational similarities to that of humans. The onset of clinical symptoms includes dose-dependent changes in respiratory, cardiovascular, hematological, coagulation and metabolic profiles. The pathophysiologic changes are characteristic of

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the well-documented symptoms of sepsis, septic shock, sepsis-induced acute organ injury and sepsis induced DIC seen in humans [1-3, 55-63]. The novel inoculation method described in this study utilizes monomicrobial bacteria intraluminally seeding of the catheter to better replicate the mechanism of infection, etiology and pathobiology of CAS in humans. As the most common etiology of infection in catheterization is biofilm formation. The results of this study demonstrate that this ovine model of CAS offers a highly reproducible, reliable and clinically relevant research platform to further study sepsis and to test novel treatment approaches of clinically ill patients. Importantly, the model successfully induced the clinical criteria of sepsis in humans, by using both old and new definitions of sepsis (Table 2)[1-3].

Sepsis is currently the most expensive disease process treated in U.S. hospitals and its incidence is steadily increasing each year [64-67]. Additionally, severe sepsis affects more than one million Americans annually [41, 68], an estimated 28 to 57 percent of which die as a result [10, 69-71]. Catheter-Associated bloodstream infections (CABSI) and subsequent Septicemias (CAS) are systemic infections that often involve a pathogen forming a biofilm within the catheter hub where it serves to seed a long-term infection. Additionally, CABSIs are associated with the emergence of several antibiotic resistant pathogens that are nearly impossible to treat and have an additional increase in mortality rates of up to 57% (71-73, 78-85, 99).

Presentation of sepsis, and septic shock using both old Sepsis-1 and Sepsis-2 definitions (including SIRS symptoms) and the newest Sepsis-3 definitions including SOFA criteria (without SIRS symptoms) were observed and evaluated in this ovine model of CAS (table 2) [1-3]. The SIRS cardiovascular criteria include pyrexias, tachypenias, tachycardias, leukocytosis and were observed in a dose dependent manner with varying duration and severity [2]. Using only these cardiovascular symptoms, each challenged ewe met the criteria of SIRS with at least 2/3 criteria being met (pyrexia, tachpnea) [1-2] (table 2, fig 3a-c). These acute temperature spikes were observed in rectal temperatures and were confirmed in the rumen

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temperature data (table 2, Appedix VI). These are especially evident during the high intensity monitoring of high dose (fig. 3a-d). Since there is a documented infection involved (bacterial contamination of the catheter), they are considered to be experiencing clinical sepsis (table 2). SIRS remained evident in each of the challenged ewes throughout the study. The newer SOFA based criteria of sepsis that deemphasizes SIRS was also satisfied by documenting changes of the following clinical symptoms, respiratory (tachypeneas), coagulation (thrombocytosis'), liver dysfunction (increased serum bilirubin), renal dysfunction (increased serum creatinine) and serum lactic acidosis (table 1-2, figs. 1-3) [3]. Consequently, the changes observed fulfilled the requirements for sepsis by several different definitions. Furthermore, since these clinical symptoms developed immedietly following the intentional induction of *K. pneumonia* in the catheter lumens, and none of the control sheep developed similar syptoms, we are confident that these symptoms were not from a secondary source (such as pre-existing ailments). In addition, several sheep displayed early signs of sepsis induced acute organ injury following the highest inoculation dose #4, which manifested as increased hyperlactemia, increased serum creatinine, increased AST, increased BUN, increased bilirubin, thrombocytopenias, tachypneas and tachycardias (tables 1-2, figs. 2-3),.

Signs of disseminating intravascular coagulation (DIC). Further evidence of organ dysfunction includes the observed alterations in coagulation profiles, including disseminated intravascular coagulation (DIC), in a subset of animals with increased PT, aPTT and D-Dimer [72-75] (table 1). A rather diverse onset of clinical coagulopathies characterized by an increased fibrinogen levels, elevated D-Dimers, thrombocytopneas, prolonged activated partial thromboplastin time (PTT) and prothrombin times (PT) (table 1) were observed. These variable, yet dose dependent coagulation profiles observed are expected with these genetically diverse sheep with likely subclinical comorbidities, and previous exposure to LPS or other immunogenic factors. However, a pronounced overt DIC was in fact observed

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in three separate sheep at two different time points, and likely serves as a catalyst in the initial stages of sepsis pathophysiology [49, 58, 73, 76-78]. Two sheep from challenge group #2 displayed extreme thrombocytopenias post challenge (Dose #2, inoculation #1) Ewe #3 (day 1 and day 2 of 60 $x10^3$/ul and 59 $x10^3$/ul, respectively) and post challenge (Dose #4, inoculation #2) Ewe #4 (days 13-15 with 20 $x10^3$/ul, 27 $x10^3$/ul, 36 $x10^3$/ul, respectively) (table 2, Appendix VI).

***Sepsis-induced tissue hypoperfusion*.** Tissues and organs receiving inadequate oxygen and nutrients, often resulting in injury during the early phases of sepsis. Several physiologic responses consistent of this syndrome are seen in challenge group #2 (primarily after high dose #4) including, tachycardia (fig. 3b) and increased lactate levels (fig. 3d). These sepsis-induced tachycardias are likely due, in part to an increased capillary leakage, decreasing the venous return to the heart and cytokines released contribute to myocardial depression. Tachycardias lasting >1-day post dose #4 inoculation increased from an average baseline of ~85 BPM in all samples to ~120 BPM (fig. 2d, 3d). Elevated lactate concentrations were evident post high dose inoculations, with a 4-fold increase from a baseline of ~.6mMol to ~2.4 mMol following dose #4 ($1x10^9$ CFU) on day 13 (fig. 2d, 3d). As suggested in the 2012 surviving sepsis campaign, increased lactate levels are a marker for tissue hypoperfusion ([10, 79-81]. Sepsis induced hyperlactemias are common in early tissue damage due to the aberrant oxygen flow to tissues and organ systems resulting in anaerobic respiration. Evidence of regional hypoperfusion is observed in several organ systems of the body including, renal dysfunction/injury manifest by increased blood urine nitrogen (BUN) and increased levels of serum creatinine (SCr) [79, 80, 82-86]. SCr increased from an average baseline of ~0.9 mg/dl in all groups (N=8), to ~1.25 mg/dl (N=3) following dose #4 ($1x10^9$ CFU) in challenge group #2 (table 2, Appendix VI). The increase in serum creatinine of +0.35 mg/dl in less than 24 hours post high dose challenge is a strong indicator of acute kidney injury (AKI) due to sepsis [84, 87]. According to the RIFLE (Risk, Injury, Failure, Loss and End Stage in relation to kidney function)

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classification system, an increase in serum creatinine levels if ≥.3 mg/dl from baseline levels within the first 24 hours of suspected sepsis is indicative of kidney injury/dysfunction and is a primary indicator in the prognosis of AKI [86, 88]. It is well documented that an elevated level of SCr is a strong indicator of AKI and linked to severe sepsis and septic shock [89]. Furthermore, in a cohort study by Vanmassenhove et al [90], the argument was advanced of using serum creatinine as an independent predictor of mortality, rather than a bystander in cases of sepsis induced AKI. Blood urea nitrogen (BUN), a SOFA criteria also showed a significant increase from an average baseline of ~13mg/dl to ~22mg/dl one day post dose #4 inoculation in group #2 (table #2).

Although, there were consistent trends observed as a function of the dose administered, the severity and duration of symptoms varied slightly between each ewe within a challenge group. This is an expected observation considering these sheep are outbred and genetically diverse. Similar to a diverse population of humans, the sheep displayed individual differences in host immune responses to the infection. This is an important point when considering that many murine models utilize inbred populations of mice, which sometimes fail to replicate the individual variation observed in human disease.

Animal models, which closely reproduce the human condition, serve a vital role in the efficient development of preventative, diagnostic, and therapeutic strategies in various types of disease in humans. Currently, two murine models of sepsis are most commonly used in laboratory settings: the cecal ligation and puncture (CLP) model and the colon ascendens stent peritonitis (CASP) model [16, 53, 91]. They are currently considered the most credible animal models of sepsis, with CLP considered the gold standard for sepsis research, according to Lilley, et al.[53]. In the CLP model, the cecum of the mouse is ligated and then perforated using a needle allowing bacteria in the cecum to migrate into the abdomen and establishing a mixed polymicrobial bacterial peritonitis that progresses to sepsis[53, 91] While the CASP model relies on placement of a stent in the antimesenteric side of the colon which allows for

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continual passage of fecal material into the peritoneum, again resulting in a polymicrobial peritonitis. Although widely used and comparatively inexpensive, these mouse models have several significant shortcomings.

First, they require general anesthesia and surgery, which have documented impacts on the host immune response and subsequent development of sepsis [16]. Second, while these models may replicate post-operative sepsis, they fail to replicate the most clinically relevant routes of exposure for emerging causes of sepsis, associated with the placement of indwelling central venous catheters, urinary catheters and mechanical ventilators. Third, these mouse models do not lend themselves to the standard medical interventions routinely utilized in human sepsis therapy, including IV therapies (drug, fluid replacement) and the frequent large volume blood collection for monitoring clinical parameters or allow testing of novel therapeutics. Fourth, the progression of the septic process in the mice often occurs more rapidly than in humans, with the mouse model often culminating in death after 3 days[16], while in humans a longer period of 2-3 weeks is commonly observed [8]. Fifth, unlike mice, which are several 1000-fold less sensitive to LPS than humans, sheep show similar sensitivities to LPS as humans [33]. Sixth, at a gene expression level, acute systemic inflammatory responses in mice appear to be quite different from that of humans [16, 21, 23, 53, 92]. And finally, even though sepsis in humans is, by and large, a disease that occurs at the extremes of age [4, 18-21] and the short lifespan and rapid maturation of mice limits the ability to study the role of age in the murine models.

Collectively, these issues coupled with the concerns over how well the mouse immune system mimics the human disease process suggests a critical need for the development of new animal models of sepsis. In particular there is significant need for a model that more closely mimics the human development of CAS originating as a monomicrobial infection of nosocomial origins. To address this issue, several models have been developed that directly inject monocultures of bacteria into the vascular system,

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however these models also have significant shortcomings. As outlined in a review by Deitch (36), "The problems inherent with current sepsis models created through intravenous bacteria infusion are as follows: a) they do not correlate with clinical disease; b) they typically produce a hypodynamic circulatory response; c) survival time is generally short and therefore there is limited time for progression of disease; d) the serum cytokine response is transient and much greater in magnitude than that observed in septic patients; and e) antisepsis agents shown to be effective in these types of animal models have not been effective when tested in clinical trials." In this manuscript we provide proof-of-concept evidence that these deficiencies can be overcome by utilizing a novel induction method that relies on seeding of a blood clot in a PICC without direct injection of bacteria into the bloodstream. This approach allows for gradual and continual low level shedding of bacteria into the bloodstream, more closely mimicking CAS and resulting in a more prolonged event.

Despite demonstrating physiologic evidence of sepsis, many of the blood samples collected remained culture negative, especially at the lower doses. Only animals in the higher dose groups (doses #3 and #4) were found to be blood culture positive following inoculation. In our initial efforts with the lower doses, we were only performing low volume (0.1 mL) direct culture of the blood, a technique that has low sensitivity. In human medicine it is routine to preform multiple large volume blood cultures over the course of a 24 hour period in order to demonstrate bacteremias. It is possible that by using the higher volume enrichment protocol adopted following dose 3 and 4 we would likely have been able to demonstrate blood culture positive results. This situation is consistent with that observed in human medicine, where according to current studies more than 50% of severe sepsis cases are culture negative[93, 94].

Several additional benefits of the sheep model for future sepsis research were demonstrated in our results. First, we demonstrated the ability to maintain a double lumen central venous catheter throughout

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the study allowing for the potential for concurrent administration of IV antibiotic infusions, fluid resuscitation, cardiotropic drugs or other therapeutic interventions. These types of interventions are often difficult in the murine and porcine models since these species often require sedation or anesthesia when administering continuous rate IV infusions. Second, the ability to easily collect frequent blood and urine samples allows concurrent pharmacokinetic monitoring during sepsis in order to evaluate the role that sepsis plays in altering pharmacokinetics and pharmacodynamics.

Under recommendations of a recent report focused on balancing science with animal welfare, we elected to use only eight sheep, in order to minimize animal usage for our proof-of-concept model demonstration (3Rs) [53, 91]. The premise of this report advocates that new animal models of sepsis be developed using a minimal animal numbers in a proof of concept manner. The limited number of test subjects made statistical analysis unsuitable, however the consistency of the data provides compelling information regarding the benefit of the model in future studies. The dose-dependent response also provides a good starting point for decision making regarding the inoculation dose necessary to induce varying degrees of disease severity. We considered utilizing four independent groups of animals to evaluate the dose-dependency, however elected to reduce animal usage by using a second higher dose inoculation in each group of the animals. While this does complicate some of the interpretation of the data, it still provides a strong foundation for future research design concerning appropriate dose and the expected variability between individuals. Therefore, the data presented here provides critical information for future power studies in similar studies.

There are several potential drawbacks to the approach we utilized in this study or to the use of sheep for sepsis research in general. First, the limited number of test subjects made statistical analysis unsuitable for interpreting the data, and thus was left out of this report. Future use of the model can easily overcome this issue by enrollement of larger numbers of animals based on power studies using the data

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generated here. Another potential drawback to using sheep is that as a ruminant species the use of oral antibiotics has the potential to be significantly impacted, however oral antibiotics are rarely used as first-line choices in sepsis. In fact, previously published pharmacokinetic studies suggest that the sheep correctly modeled the human pharmacokinetics of many parenteral drugs of interest, included the carbapenems[28]. Finally, the duration of our study was limited, limiting the expected late onset of late phase sepsis and MODS as commonly seen in chronically catheterized human sepsis patients. Although we attempted to utilize oscillometric non-invasive blood pressure measurement as part of this study it was determined to be unreliable and inconsistent when applied to animals that were standing and occasionally moving. However, there are commercially available implantable telemetric invasive blood pressure, blood gas, and temperature monitoring systems that have been validated for use in sheep [34]. Also, perhaps the most important limitation to the use of ovine models, being the lack of validated reagents (including Procalcitonin, CRP, and other biochemical quantitative assays). However, this lack of reagents for use in ovine models will be solved, especially with the continued sequencing of the ovine genome [95], which will allow the rapid PCR of genes necessary for the manufacture of biological tools.

Going forward. Instead of exhausting more billions of more dollars in funding, precious research time and inevitably patient's lives, the use of a more clinically relevant species of sepsis is crucial to our understanding of the disease process. This has been a largely debated topic and remains largely unsolved/unaddressed. This novel ovine model of induced CAS can be used in a variety of studies regarding human disease pathobiology and be used as a platform to increase treatment efficiency. In addition, future studies can be designed to including inoculations of either monomicrobial or polymicrobial infections or different types of pathogens (bacterial, fungal, viral), can utilize this in-vivo animal model of sepsis. However, preliminary small scale studies needs to be conducted to test sheep's susceptibility to these potential pathogens prior to large number studies [91]. Furthermore, as rational

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research design can be utilized to induce specific comorbidities in these sheep to test the different pathophysiologys in; immunocompromised, diabetic, ketonic, malnourished, genetically diverse, age, race and gender to study how they affect the pathophysiology and ways to treat them.

There are many potential applications to warrant the use of this sheep model of sepsis such as; establishing a better understanding of the colonization, adaptation and shedding of carbapenem-resistant *Enterobacteriaceae* (CRE), as well as KPC spread and epidemiology. Additionally, testing new drug therapies or optimal drug dosing regimens, studying in-vivo biofilm formation leading to seeding of infection and ways to treat and prevent them (49). Likewise, a pre-challenge drug intervention can be tested prior to microbial insult to protect certain populations, as well.

Conclusion. It's an undeniable truth that most animal models used in modern research are frequently the most convenient model utilized, instead of the best available. However, in order to make clinical advancements, the most translatable and reliable animal models ought to be used, and these are often non-murine models [4, 18-21, 24]. In sheep, the size, temperament, and ease of catheter (placement and maintenance) coupled with aforementioned intrinsic factors shared with humans make them great platforms for pre-clinical evaluation and optimization of biomedical and sepsis research.

This proof of concept study addresses the urgent need for an improved animal model in sepsis research. In particular, central catheter associated sepsis which has a complex pathobiology that includes biofilm formation and has one of the highest mortality rates in human sepsis. The development of CAS depends on several intrinsic and extrinsic factors in both the host and pathogen, characterized by the high heterogeneity seen in human and sheep sepsis. Further, the nonsurgical (no sedation and no general anesthesia) model of central catheter placement coupled with seeding catheter lumen with a monomicrobial infection and can be housed and tested using both short- and long-term catheter studies. Where sheep and can serve as a model for testing novel therapeutic interventions with IV fluids, specific

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biomarkers, vasopressors and intravenous antibiotics, all of which are not feasible in the most common mouse model. Lastly, the routine hemodynamic, immunological, coagulation, biochemical and microbiological monitoring of these sheep can provide great insight to sepsis pathophysiology and can be analyzed in great detail. Future sheep (or other large animal) research models of CAS is warranted to further understand this medical enigma we call sepsis.

An evolved method of effectively diagnosing and categorizing this multifactorial disease processes is essential to the improved prognosis of sepsis. As the complex pathophysiology of sepsis and its associated syndromes (acute organ injury or dysfunction) become better understood, it is likely that sepsis will no longer be considered generalized syndromes with a broad range of clinical symptoms and treatments. Rather, each syndrome would contain various sets of well-defined disease subtypes that can be characterized by specific cellular changes, clinical symptomologies and/or accompanied biomarkers. Each of which can have therapy regimens developed to optimize their efficacy and improve overall sepsis survivability.

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*Note: varied sample frequency based on Ewe condition, ability to attain viable samples and experimental setup.

Figure 2. **a-f,** *Control Group vs. Challenge Group #2: various measurements (physiological, hematological, and biochemical) taken throughout study.* A. Rumen "Core" (Temperatures, ^{o}C), B. Heart Rates (BPM), C. Respiratory Rates (/Minute), D. Serum Lactate Concentration (mM), E. Platelets-Auto (x10^3/ul), F. Neutrophil Counts (x10^7/ul). *Note: varied sample frequency based on Ewe condition, ability to attain viable samples and experimental setup. See figure 3 for high resolution graphs for intensive monitoring data (shaded area in 3.A- 3.D).

Figure 3. *Intensive monitoring for Challenge Group #2* administered highest inoculation Dose, (Dose #4 of ~1x10^9 CFU). Intensive monitoring of thermoregulation and cardiovascular parameters Control Group vs. Challenge Group #2: High Dose intensity monitoring of A. Respiratory Rates (Top-Left), B. Heart Rates (Top-Right), C. Rumen Temperatures (Bottom-Left), and D. Serum Lactates (Bottom-Right). thermoregulation and cardiovascular recorded every 15 minutes during the first hour post inoculation, then once an hour thereafter for 5 hours. Rumen Temperatures were sampled every 30 minutes and Serum Lactates every 2 hours post high dose administration for 6 hours.

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Table 2. *Severity of sheep sepsis based on both old and new human definitions: (SIRS, SOFA).* 1. Heart rate mean: 75.88, 2σ=7.78, 2. Respiratory rate mean: 27.02, 2σ= 8.06, 3a. Rumen temperature mean: 39.567, 2σ=.413, 3b. Rectal temperature mean: 38.76, 2σ=.33, 4. Lactate concentration mean: .66, 2σ: .33, 5. White blood cell count mean: 5.86, 2σ: .74, 6. Neutrophil Mean: 3.09, 2σ: .79. 7. Creatinine Mean: 0.869, 2σ: 0.1676 (or +30%: 1.13) **Note: Challenge group #1 (Ewe #'s 1 and 2). *Individual Ewe's SOFA score throughout study timepoints.*

APPENDICES

Appendix I. *K. pneumoniae inoculation, Parent Isolate, sample culture results and culture methods and inoculation doses/dates.*

Appendix II. *Histopathology Report. Klebsiella pneumoniae* septicemia in ewes.

Appendix III. *Monitoring and treatment matrix for sheep IV-sepsis model.*

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- *All Gross Histology Photos*

-Sheep Placement and outlining group layouts, experimental setup, dose inoculations and day of inoculations.

-All Analysis &, Related files (.exe, .pzf, .doc, .en

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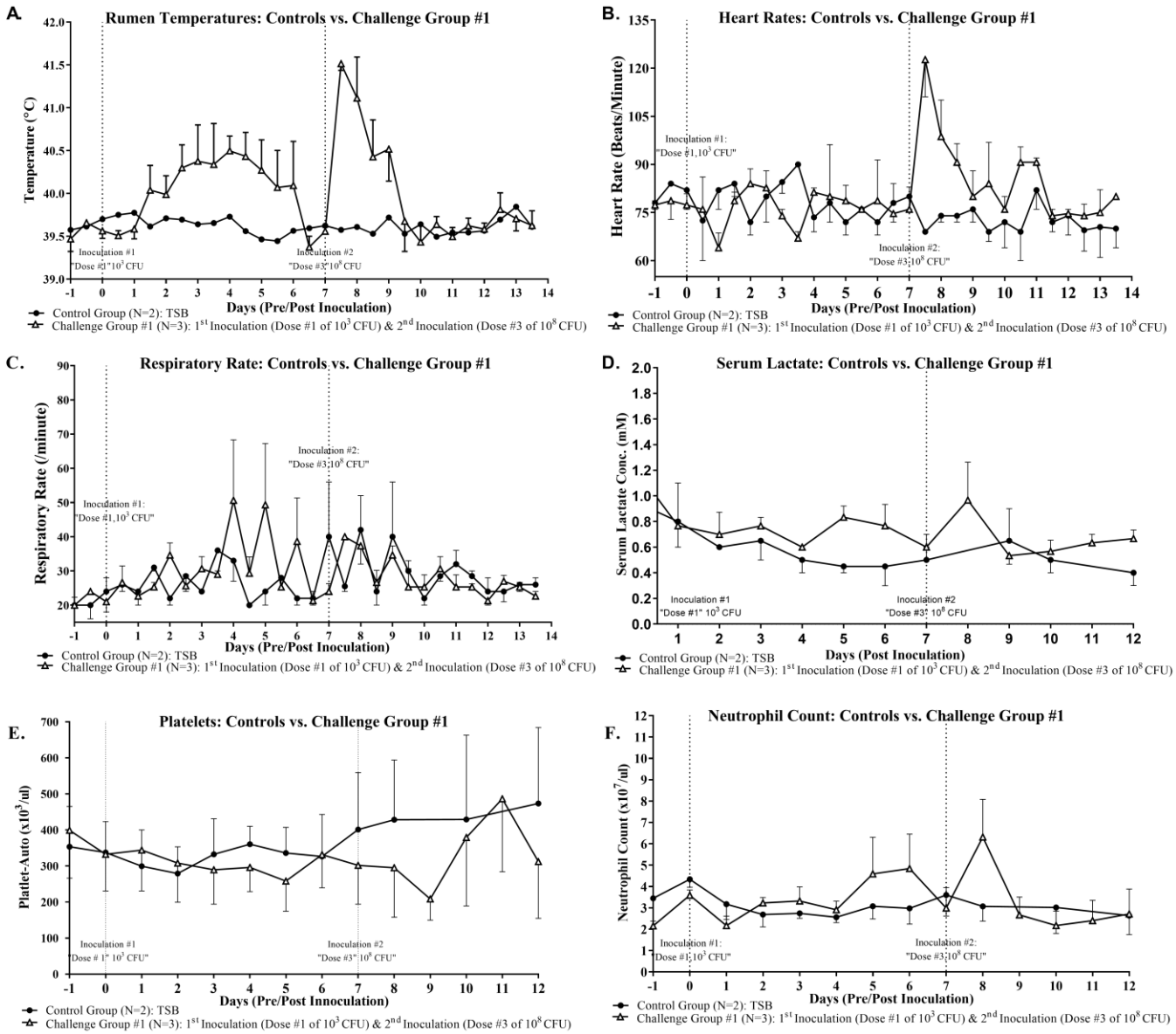

Figure 1. A-F, *Control Group vs. Challenge Group #1: various measurements (physiological, hematological, and biochemical) taken throughout study. A. Rumen "Core" Temperatures oC, B.Heart Rates (BPM), C. Respiratory Rates (/Minute), D. Serum Lactate Concentration (mM), E. Platelets-Auto (x103/ul), F. Neutrophil Counts (x107/ul).*

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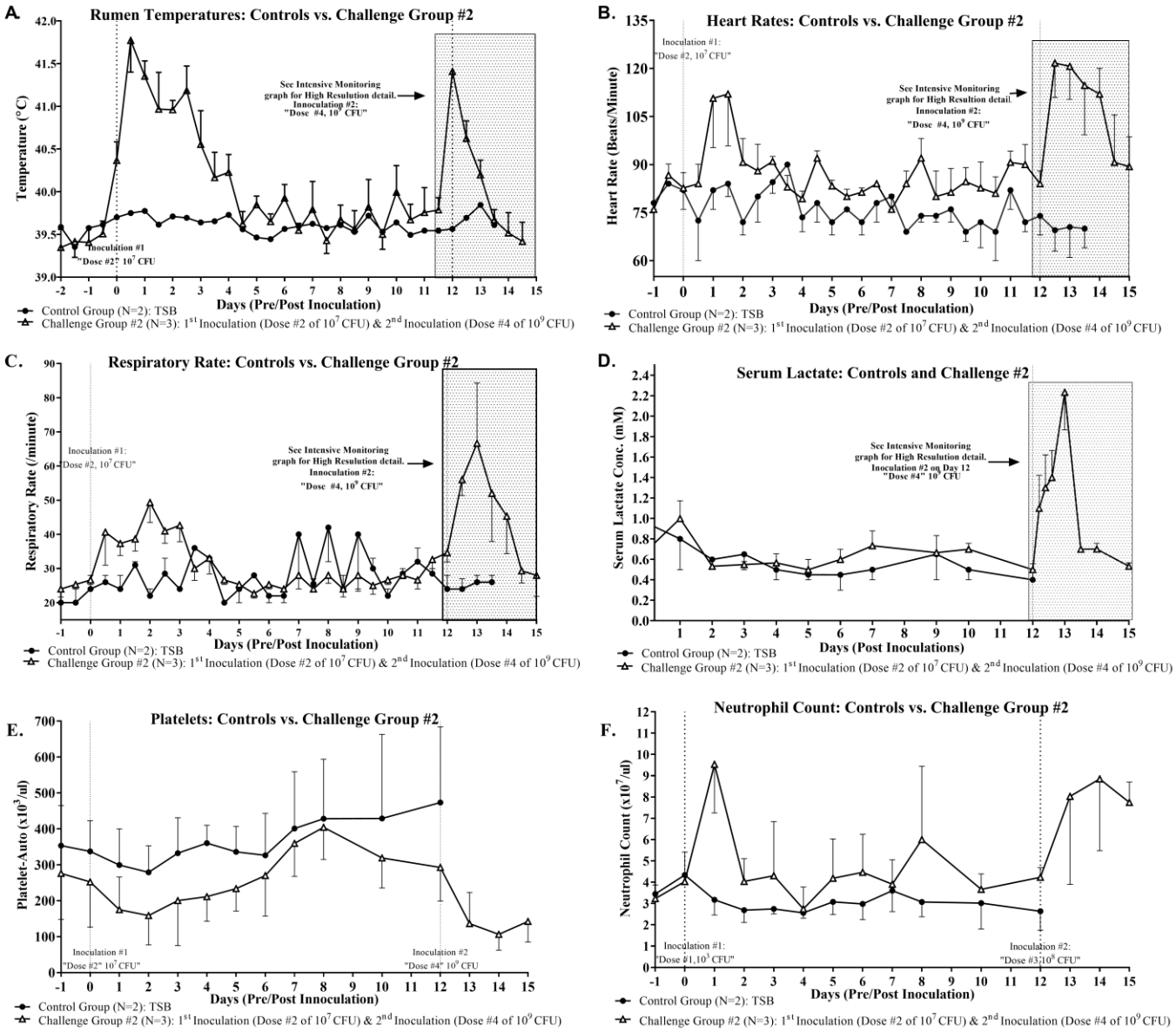

Figure 2. A-F, *Control Group vs. Challenge Group #2: various measurements (physiological, hematological, and biochemical) taken throughout study. A. Rumen "Core" Temperatures* ^{o}*C, B.Heart Rates (BPM), C. Respiratory Rates (/Minute), D. Serum Lactate Concentration (mM), E. Platelets-Auto (x10*^{3}*/ul), F. Neutrophil Counts (x10*^{7}*/ul).*

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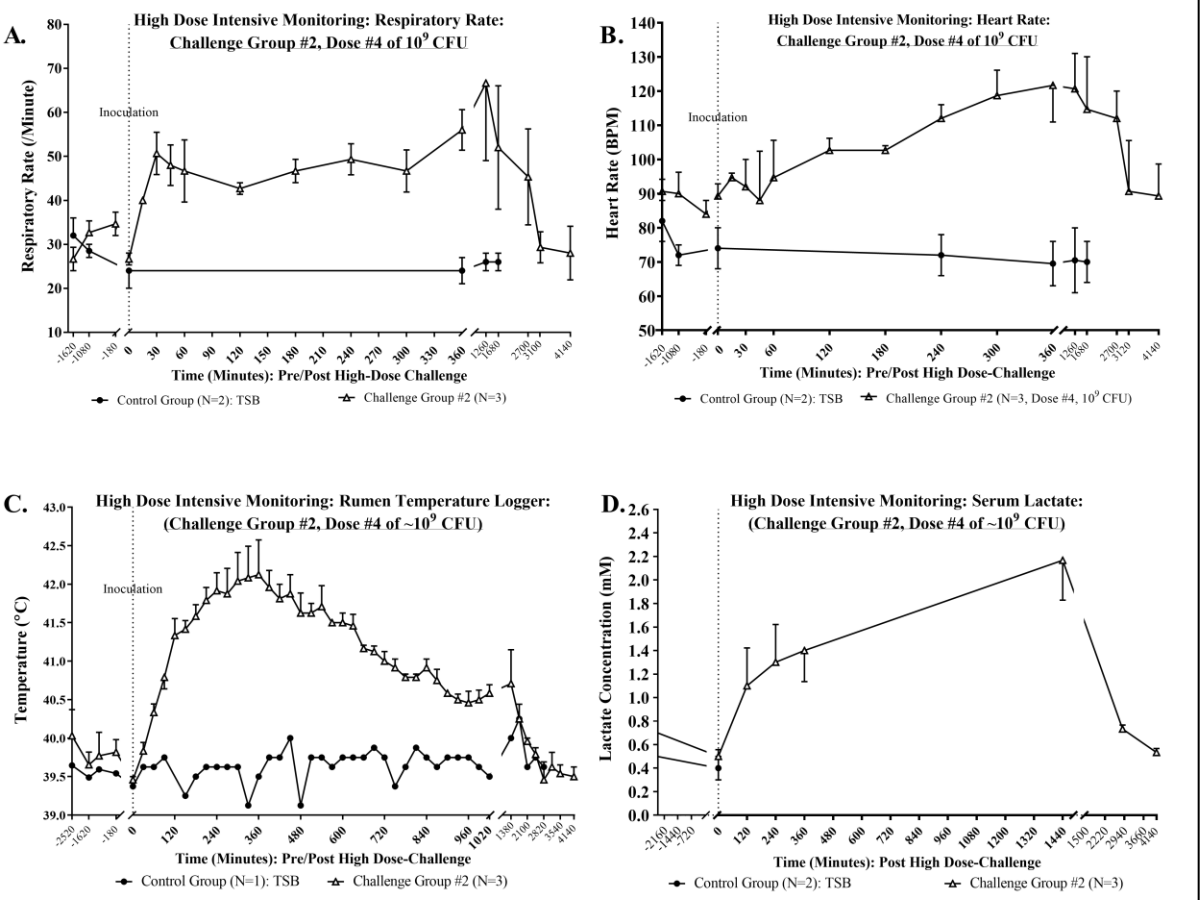

Figure 3. *Control Group vs. Challenge Group #2: High Dose intensity monitoring of A. Respiratory Rates (Top-Left), B. Heart Rates (Top-Right), C. Rumen Temperatures (Bottom-Left), and D. Serum Lactates (Bottom-Right).*

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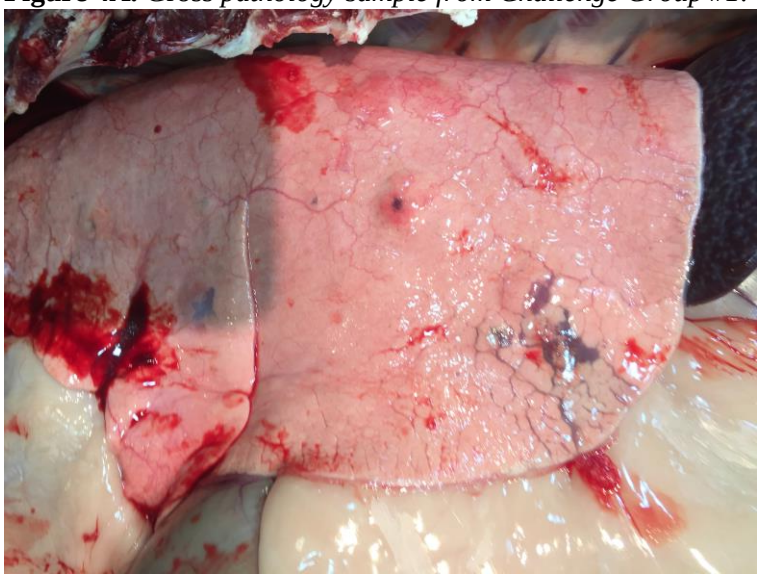
Figure 4A. Gross *pathology sample from Challenge Group #1:*



4B. *Gross pathology samples of two separate lung samples from Challenge Group #2 at necropsy.*

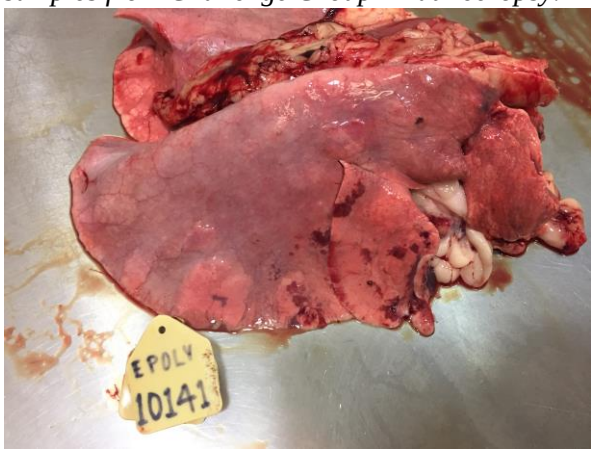


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Table #1. *Coagulopathies of Challenge group #2; displaying possible overt DIC [73-75].*

Coagulopathy of Overt DIC:	**Pre-inoculation (N=8)**	**Day #2 Average (N=3)**	**Day #13 Average (N=3)**	**Day #14 Average (N=3)**
D-Dimer[1]	$\bar{x} = 868$	1002 (↑133.18)	1956 (↑1088)	1584 (↑716)
PT[2]	$\bar{x} = 10.15$ $\sigma = 0.778$	12.03 (↑1.88)	16.73 (↑6.58)	14.27 (↑4.12)
PTT[3]	$\bar{x} = 43.26$ $\sigma = 5.995$	40.1 (↓3.16)	60.7 (↑17.44)	56.27 (↑13.01)
Platelet[4]	$\bar{x} = 367$ $\sigma = 165$	↓306	↓133	↓121
Fibrinogen[5]	$\bar{x} = 350$ $\sigma = 141$	633 (↑283)	500 (↑150)	567 (↑217)

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Table 2. *Severity of sheep sepsis based on both old and new human definitions (SIRS, SOFA).*

Groups	**SIRS[a]** (≥2 of these symptoms concurrently):						**Sepsis[a]:**		**Severe Sepsis[a]:**			**SOFA[b] Scores:**
	HR [*1]: >100	Core Temp. Rumen[*3]: >40ºC	RR[*2] >45	WBC count [*5]: >8.5x10^3/ul	Neutrophil Count [*6]: >7.5x10^3/ul	**Total # with SIRS:**	Known bacterial infection:	**Total # with Sepsis:**	Serum[*7] Creatinine: >1.13(mg/dl)	Lactate[*4] >2(mM/L)	**Total # with Severe Sepsis:**	**Bilirubin ≥1.17 mg/dl Platelets ≤100,000/ml**
Control Group (N=2): Sterile TSB	0/2	0/2	1/2	0/2	0/2	**0/2**	0/2	**0/2**	0/2	0/2	**0/2**	**0, 0, 0**
Challenge Group #1 Dose # 1: ~10^3 CFU	1/3	3/3	2/3	2/3	2/3	**3/3**	3/3	**3/3**	0/3	0/3	**0/3**	**1, 0, 0**
Challenge Group #2, Dose # 2: ~10^7 CFU	2/3	3/3	2/3	3/3	2/3	**3/3**	3/3	**3/3**	0/3	0/3	**0/3**	**0, 0-1, 0-1**
Challenge Group #1 Dose # 3: ~10^8 CFU	2/3	3/3	1/3	1/3	1/3	**3/3**	3/3	**3/3**	0/3	0/3	**0/3**	**1-2, 1-2, 1**
Challenge Group #2 Dose #4: ~10^9 CFU	3/3	3/3	3/3	3/3	2/3	**3/3**	3/3	**3/3**	1/3	2/3	**2/3**	**2-4, ≥5, 1-3**

Heart rate mean: 75.88, 2σ=7.78, 2. Respiratory rate mean: 27.02, 2σ= 8.06, 3a. Rumen temperature mean: 39.567, 2σ=.413, 3b. Rectal temperature mean: 38.76, 2σ=.33, 4. Lactate concentration mean: .66, 2σ: .33, 5. White blood cell count mean: 5.86, 2σ: .74, 6. Neutrophil Mean: 3.09, 2σ: .79. 7. Creatinine Mean: 0.869, 2σ: 0.1676 (or +30%: 1.13)
*Note: control group #1 (Ewe #'s 1 and 2) displayed neither SIRS symptoms nor SOFA criteria of sepsis throughout the duration of the study.

See Appendix VI for individual values, averages and inter/intra group comparisons.

[a]SIRS definitions derived from:[1] [b]SOFA criteria derived from [3, 99]: and modified for use in Sheep.

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