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2. **Miller, J. C.**, Cross, A. S., Tennant, S. M., & Baliban, S. M. (2024). *Klebsiella pneumoniae* Lipopolysaccharide as a Vaccine Target and the Role of Antibodies in Protection from Disease. *Vaccines*, 12(10), 1177. <https://doi.org/10.3390/vaccines12101177>
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- clinical correlates of acute and convalescent SARS-CoV-2 infection among patients of a U.S. emergency department. *The American journal of emergency medicine*, 48, 261–268. <https://doi.org/10.1016/j.ajem.2021.04.081>
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3. Oral Presentation: Development of a *Klebsiella pneumoniae* neonatal sepsis model. American Society of Tropical Medicine and Hygiene (ASTMH). 18-22 October 2023. Chicago, IL.

4. Oral Presentation: Development of a *Klebsiella pneumoniae* neonatal sepsis model. Annual Biomedical Research Conference for Minoritized Scientists (ABCRMS) Graduate Symposia. 15 November 2023. Phoenix, AZ.
5. Poster: A *Klebsiella pneumoniae* neonatal sepsis model to evaluate vaccines. Merck: Rising Stars in Measurement Science Symposia. 16-17 November 2023. Rahway, NJ
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Abstract

Title of Dissertation: Development and characterization of a *Klebsiella pneumoniae* neonatal sepsis model to assess passive and maternal immunization efficacy

Jernelle C. Miller, Doctor of Philosophy, 2025

Dissertation Directed By: Sharon M. Tennant, Ph.D., Professor, University of Maryland School of Medicine, Center for Vaccine Development and Global Health

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Klebsiella pneumoniae remains a leading cause of neonatal sepsis in low-to-middle income countries. With the rapid emergence of multidrug resistant strains and the lack of novel antibiotics in the pipeline, there is an urgent need for alternative preventative strategies, such as vaccines, to combat *K. pneumoniae* infections. Several *K. pneumoniae* vaccines are in development, however, the lack of a reliable small animal model of *K. pneumoniae*-associated neonatal sepsis is a significant barrier for preclinical evaluation of vaccine efficacy. In this dissertation, we developed and characterized a *K. pneumoniae* neonatal sepsis mouse model. We demonstrated that 2- to 3-day-old C57BL/6 mice were highly susceptible to *K. pneumoniae* B5055, a serotype O1:K2 hypervirulent strain, following peroral infection. Additionally, neonatal mice exhibited an

age- and dose-dependent effect, where two-day-old mice were bacteremic as early as 2-hours post-infection, with rapid systemic dissemination to tissues including the brain, liver, lungs, and spleen. Interestingly, only the liver and lungs revealed inflammation following histopathological analysis. We then used this model to assess the utility of passive immunization by transferring immune *K. pneumoniae* rabbit antisera to infected neonates pre- and post-lethal challenge. We demonstrated that the passive immunization model is an effective tool, as the transfer of rabbit antisera improved the survival rate of *K. pneumoniae*-infected neonates. Finally, we determined the suitability of a maternal immunization model by vaccinating dams with either heat-killed *K. pneumoniae* or an O polysaccharide (OPS) glycoconjugate vaccine and assessing survival rates and bacterial burden in neonates following lethal challenge. Our maternal immunization model provided significant protection, with neonates immunized with the heat-killed vaccine showing enhanced survival and reduced bacterial burden when compared to nonimmune neonates. Taken together, we successfully generated and characterized a novel and reproducible mouse model for *K. pneumoniae* neonatal sepsis. This work not only provided a much-needed preclinical platform but also identified protective readouts, via passive and maternal immunization, to aid in evaluating vaccine efficacy against *K. pneumoniae* neonatal sepsis.

Development and characterization of a *Klebsiella pneumoniae* neonatal sepsis model to
assess passive and maternal immunization efficacy

By
Jernelle C. Miller

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2025

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Dedication

To my parents, husband, and son.

Acknowledgements

Thank you Dr. Sharon Tennant, my first mentor, for all your support and guidance through this dissertation. I appreciate the opportunity to learn from you, both scientifically and professionally, and am extremely grateful for your wonderful mentorship. To Dr. Scott Baliban, my second mentor, thank you for all the training, knowledge, and answering all of my questions, I would not have made it to this point without you. I am so glad I had the opportunity to grow as a scientist with your support.

To all the Tennant and Baliban lab members, past and present, thank you for making this project possible. Jessica, Khandra, and Shanaliz, thank you for your insight and moral support. Thank you Jasnehta, Garima, Sunil, Joe, Brianna, and Maryam for making this the best work environment to achieve one of my greatest accomplishments.

I am extremely grateful for the friends I have made along the way. To Luisa, Kenny, David, Liz, Jill, Noah, Povilas, Caitlin, and Yaoxian, thank you for being the best cohort! Alex, and Liron I am so glad to have built an ever-evolving friendship with you ladies. We are bonded forever. To my girls, Eris, Damaris, Jasmine, Veronica and Constance, I would not have made it through this journey without yall. The sisters I chose, thank you!

To my family, my parents, parent-in-loves, grandparents, in the physical and watching over me, and brother thank you for always encouraging me and believing in me. To my mother, everything I am, is a reflection of you, I hope I have made you proud.

To my husband and son, Charles III and Charles IV, my reasons why, I love you.

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List of Abbreviations

AMP	Antimicrobial peptides
AMR	Antimicrobial resistance
APC	Antigen-presenting cells
BARNARDS	Burden of Antibiotic Resistance in Neonates from Developing Societies
BCR	B cell receptor
BSL	Biosafety level
CDC	Center for Disease Control and Prevention
CHAMPS	Child Health and Mortality Prevention Surveillance
CRE	Carbapenem-resistant <i>Enterobacteriaceae</i>
CFU	Colony forming units
cKp	Classical <i>K. pneumoniae</i>
COPS	Core O-Polysaccharide
CPS	Capsular polysaccharide
CRKP	Carbapenem-resistant <i>K. pneumoniae</i>
CVD	Center for Vaccine Development and Global Health
DC	Dendritic Cell
d.p.i	Days post infection
ELISA	Enzyme-linked immunosorbent assay
ESBL	Extended-spectrum Beta-lactamase
EOS	Early-onset sepsis
EU	ELISA units

FlaA	Flagellin A
FlaB	Flagellin B
FDA	Food and Drug Administration
FBS	Fetal bovine serum
GBS	Group B Streptococcus
GI	Gastrointestinal
GMT	Geometric mean titer
GSK	GlaxoSmithKline
H-IVIG	Hyperimmune immunoglobulin
HAI	Hospital-associated infections
h.p.i	Hours post infection
HRP	Horseradish peroxidase
HS	Hy-Soy
hvKp	Hypervirulent <i>K. pneumoniae</i>
Ig	Immunoglobulin
IL	Interleukin
K antigen	Capsular polysaccharide
Kleb4V	Quadrivalent bioconjugate
LD ₅₀	Fifty percent lethal dose
LMIC	Low-to-middle income countries
LOS	Late-onset sepsis
LPS	lipopolysaccharide
LPS	Lipopolysaccharide

MALDI-TOF	Mass spectrometry
MDR	Multi-drug resistant
MLST	Multilocus sequence typing
MTD	Mean time to death
NeoOBS	Neonatal sepsis observational cohort study
NET	Neutrophil extracellular traps
O1:rFlaB	O1 polysaccharide conjugated to recombinant FlaB
O antigen	O-polysaccharide
OD	Optical density
OMP	Outer membrane protein
OPS	O-polysaccharide
PBS	Phosphate buffered saline
PTFE	Polytetrafluoroethylene
PRR	Pattern recognition receptors
qKPPA	Quadrivalent <i>K. pneumoniae</i> / <i>P. aeruginosa</i> glycoconjugate vaccine
RPM	Revolutions per minute
Strep ^R	Streptomycin resistance
TCR	T cell receptor
TD	T cell-dependent
T _H	T helper cell
T _{H1}	Type 1 T helper
T _{H2}	Type 2 T helper
T _{H17}	Type 17 T helper

TI	T cell-independent
TLR	Toll-like receptor
UTI	Urinary tract infection
WHO	World Health Organization

Chapter 1 Introduction

Klebsiella spp.

Klebsiella is a genus of bacteria classified within the family *Enterobacteriaceae*. It is a Gram-negative, nonmotile, facultative anaerobe that can metabolize and grow in the presence or absence of oxygen, allowing it to thrive in diverse environments and host niches (1, 2). *Klebsiella* spp. are ubiquitous, with two common habitats, the environment and mucosal surfaces of humans (2, 3). Strains within this genus were first isolated and described in the late 19th century by Carl Friedlander, and thus, they were originally named Friedlander's bacillus (2, 4). The *Klebsiella* genus comprises a wide variety of species, however, *Klebsiella pneumoniae* is the most clinically relevant, accounting for over 80% of infections caused by this genus (3, 5).

There are three subspecies of *K. pneumoniae*, including *ozaenae*, *rhinoscleroma*, and *pneumoniae* (i.e., *K. pneumoniae sensu stricto*), with *K. pneumoniae sensu stricto* being the most commonly recognized, an opportunistic pathogen responsible for the majority of human infections (2, 3, 6). While genetically distinct, species within the *K. pneumoniae* complex lack a clear, formal taxonomic classification below the species level, despite species sharing 95% nucleotide identity with *K. pneumoniae sensu stricto* (5). Species typing of *K. pneumoniae* isolates is reliant on multilocus sequence typing (MLST) and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry for identification. *K. pneumoniae* capsular polysaccharide (K antigen) and lipopolysaccharide (O antigen) are used to differentiate isolates. To date, more than

77 structurally unique K antigens (with over 90 additional K-types proposed based on unique gene sequences) and 10 O antigens have been identified. K1, K2, K20, K54, and K62 are the most common circulating K serotypes, with K1 and K2 considered the most virulent, accounting for approximately 70% of hypervirulent *K. pneumoniae* infections (7–11). Over 80% of *K. pneumoniae* clinical isolates belonging to O1, O2, O3, or O5 serotypes, with O1 and O2 isolates being the most prevalent often associated with hypervirulent or multi-drug resistant (MDR) *K. pneumoniae* infections (7, 12–14).

Clinical presentations

K. pneumoniae is the causative agent of a wide range of infections. From mild colonization to severe, life-threatening infections, *K. pneumoniae* can infect a variety of tissues. The most prominent sites of infection include the lungs, urinary tract, central nervous system, liver, and bloodstream leading to pneumonia, urinary tract infections (UTIs), meningitis, pyogenic liver abscesses, and bacteremia/sepsis, respectively (15). There are two pathotypes, classical and hypervirulent *K. pneumoniae*, which are associated with infections.

Classical K. pneumoniae

Classical *K. pneumoniae* (cKp), an opportunistic pathogen, is primarily associated with hospital-associated infections (HAIs) and has been identified as one of the top three causes of HAIs in Western countries (16–18). cKp predominantly targets immunosuppressed individuals, including diabetics and patients with pre-existing comorbidities (15). HAIs encompass pneumonia, UTIs, bloodstream infections, and surgical site infections (15, 16). cKp is known for its predisposition to accumulate and

disseminate mutations and resistance genes, though they do not enhance virulence of *K. pneumoniae*, leading to the emergence of multiple, extensive or pan-drug resistant clones (5, 15, 19).

Hypervirulent K. pneumoniae

Hypervirulent *K. pneumoniae* (hvKp), unlike cKp, is a highly virulent pathogen capable of establishing infection in both immunocompromised and immunocompetent individuals (5, 17, 20). hvKp is primarily associated with community-acquired infections, infecting individuals of all ethnic and age groups, though infections are most common in the Asian Pacific Rim. (17, 21). The clinical presentation of hvKp is notably more severe and often involves metastatic infections, meaning *K. pneumoniae* spreads from the primary site to distant organs (17, 22–24). Common manifestations include pyogenic liver abscesses, which are frequently complicated by dissemination, such as to the eyes or central nervous system, leading to endophthalmitis and meningitis, respectively (25–31). Other severe presentations include necrotizing fasciitis, severe pneumonia, and bacteremia that can progress rapidly to septic shock (15, 17, 32).

Virulence factors

K. pneumoniae employs a variety of virulence factors to establish infection and overcome host defenses (**Figure 1.1**). Capsular polysaccharide, also known as K antigen, is a hydrophilic matrix coating the bacterial cell surface, essential for *K. pneumoniae* pathogenesis. The capsule primarily evades the host immune response by preventing phagocytosis and opsonophagocytosis of *K. pneumoniae* by professional phagocytes (i.e., macrophages and dendritic cells) (15, 33–35). It achieves this by physically masking

bacterial surface components, thereby hindering recognition and direct interaction by host immune receptors and subsequent engulfment. Furthermore, K antigen can inhibit complement activation and deposition on the bacterial cell surface, preventing complement-mediated lysis and opsonization (36, 37). Beyond direct evasion, K antigen can modulate host immune signaling, influencing dendritic cell maturation and cytokine production, hindering an effective immune response and aiding in bacterial survival and proliferation (15, 37–39).

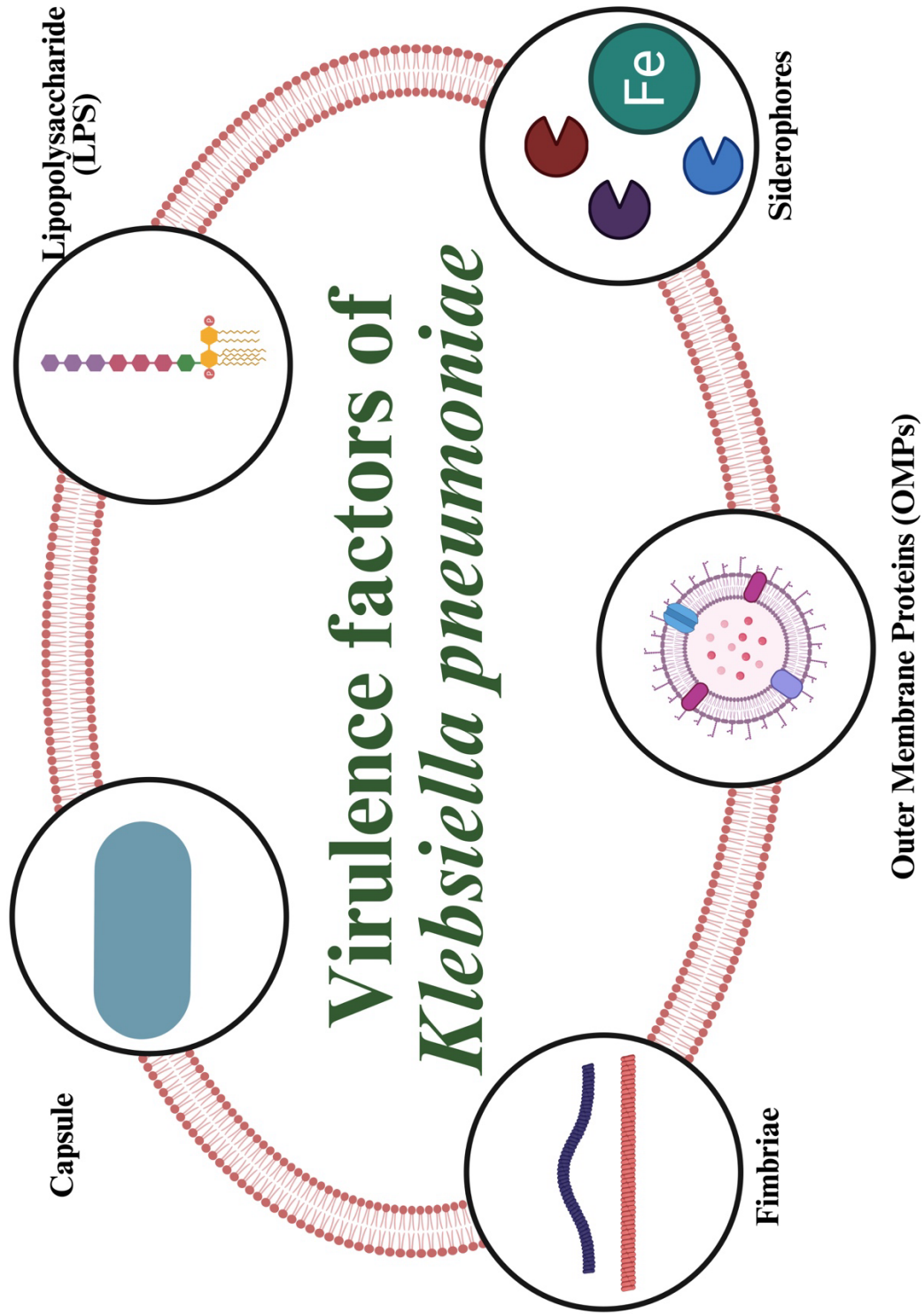


Figure 1. Virulence factors of *Klebsiella pneumoniae*

As a Gram-negative bacterium, *K. pneumoniae* contains lipopolysaccharide (LPS), a known endotoxin, in the outer leaflet of its cell membrane (15). LPS is comprised of an O antigen, a core oligosaccharide, and lipid A, the ligand for toll-like receptor (TLR) 4, which activates an immune response. To evade this, *K. pneumoniae* can utilize its K antigen, particularly K1, K10, and K16 capsules, to shield LPS from detection (15, 40, 41). Additionally, through the use of proteins such as PhoPQ and LpxO, *K. pneumoniae* can modify the structure of LPS to prevent immune recognition (15, 42, 43). Also within its membrane are various outer membrane proteins (OMPs), including peptidoglycan-associated lipoproteins and porins, which aid in its virulence. OMPs aid in cell integrity maintenance and protection against the innate immune response (15).

K. pneumoniae leverages type 1 and 3 fimbriae, adherence factors, to establish colonization and infection (15). Type 1 fimbriae, encoded by the *fim* operon and characterized by the mannose-specific FimH adhesin, enables *K. pneumoniae* to bind to mannosylated glycoproteins present on epithelial cells (15, 44–46). Concurrently, type 3 fimbriae, encoded by the *mrk* operon and featuring the MrkD adhesin, mediates binding to the extracellular matrix (i.e., collagen), contributing to tissue adherence and biofilm formation (15, 46–48). Biofilms provide a protective niche for *K. pneumoniae*, enhancing its resistance to host immune defenses. Within these complex structures, *K. pneumoniae* can significantly enhance antimicrobial resistance, leading to persistent and difficult-to-treat infections.

Finally, *K. pneumoniae* can employ siderophores to overcome the host iron-restricted environment. Iron is an essential micronutrient, vital for bacterial metabolism, enzyme functionality, and DNA synthesis, however, it is tightly bound in the mammalian host to lactoferrin, creating a nutrient deficient state to limit bacterial proliferation (15, 49, 50). *K. pneumoniae* will secrete various siderophores, including enterobactin, salmochelin, yersiniabactin, and aerobactin, to chelate iron from host proteins, facilitating its uptake into the bacterial cell (15, 51). The capacity of *K. pneumoniae* to synthesize multiple distinct siderophores enables it to outcompete the host for nutrients, ensuring its survival, replication, and dissemination.

Antibiotic resistance of *K. pneumoniae*

K. pneumoniae was initially identified as a pathogen of importance in 2008 by its inclusion in ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) pathogens – a designation for bacterial evading multiple antibiotic classes (15). The U.S. Centers for Disease Control and Prevention (CDC) highlighted the *Enterobacteriaceae* family in their 2013 and 2019 Antibiotic Resistance Threats report. Carbapenem-resistant *Enterobacteriaceae* (CRE), which included carbapenem-resistant *K. pneumoniae* (CRKP), were classified as an urgent threat, while extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* were classified as a serious threat. Carbapenems were once considered a drug of “last resort” for MDR *K. pneumoniae*, but the rise of CRKP has limited effectiveness (52). The World Health Organization (WHO) have ranked CRKP highest on its 2024 Bacterial Priority Pathogens List (53–55). Several studies have corroborated the alarming rates of carbapenem resistant *Klebsiella* spp., and

the limited therapeutic options for MDR *K. pneumoniae* directly translates into prolonged hospital stays, increased healthcare costs, and increased patient mortality (55–57). The impact of antimicrobial resistance (AMR) was highlighted in 2019, when approximately 5 million deaths were associated with bacterial AMR, with 1.27 million deaths directly attributed to it (58). Of these, *K. pneumoniae* was a major contributor, accounting for 17.5% of AMR-associated deaths and 19.9% of directly attributable deaths, establishing it as a leading cause of AMR-related morbidity and mortality worldwide.

Klebsiella vaccine development

Vaxxon SRP *Klebsiella* is the only U.S. Department of Agriculture (USDA)-licensed vaccine available for the treatment of *K. pneumoniae* infections in cattle (59). However, there are currently no licensed vaccines to prevent *K. pneumoniae* infections and associated morbidity and mortality in humans. K antigen was first targeted by an early *K. pneumoniae* vaccine candidate where 24 capsular polysaccharides were selected, based on seroepidemiology, to cover ~80% of serotypes associated with bacteremic infections (16). This vaccine also contained an 8-valent *Pseudomonas aeruginosa* O-polysaccharide-based conjugate vaccine. Promising preclinical data with this vaccine candidate lead to the production of a human hyperimmune immunoglobulin (H-IVIG), which was administered at a dose of 100 mg/kg intravenously to patients entering the intensive care unit of Veterans Affairs medical centers to determine if it would prevent *K. pneumoniae* and *P. aeruginosa* infections (60, 61). During the follow-up period, there was suggestion of reduced *K. pneumoniae* bacteremia in the H-IVIG group relative to

controls. However, at the conclusion of the observational period there was no significant effect observed.

With the increase emergence of MDR *K. pneumoniae* infections, particularly in low-to-middle income countries (LMICs), there is a renewed effort aimed at preventing these infections. An alternative to targeting the K antigen for vaccine development, is the O-polysaccharide (OPS) of lipopolysaccharide. With approximately 80% of clinically relevant infections stemming from four O-types (O1, O2, O3, and O5), a 4-valent OPS vaccine would allow for broad global coverage (7). Currently there is one OPS-based *K. pneumoniae* vaccine to have entered clinical testing, a quadrivalent bioconjugate (Kleb4V) targeting the O1, O2a, O2afg, and O3b serotypes, that is being developed by LimmaTech Biologics AG in partnership with GlaxoSmithKline Biologics SA (GSK). Phase I and II studies to assess the safety and immunogenicity of Kleb4V were completed in 2022 (NCT04959344), but no data have been published.

There are a plethora of *K. pneumoniae* LPS-targeted vaccine candidates in preclinical development, displaying immunogenicity and efficacy against *K. pneumoniae* (16). For example, a quadrivalent glycoconjugate targeting *K. pneumoniae* O-serotypes O1, O2, O3, and O5 has been developed (62). The four OPS were chemically coupled to recombinant *P. aeruginosa* flagellin A (FlaA) or flagellin B (FlaB), with active immunization in mice demonstrating an increase in serum anti-OPS IgG following a three-dose vaccine regimen (62). Additionally, passive immunization of rabbit anti-sera in naïve mice reduced bacterial organ burden and protected mice following lethal intravenous challenge with an O1:K2 strain of *K. pneumoniae* (16, 62).

Klebsiella as a causative agent in neonatal sepsis

K. pneumoniae stands as a significant causative agent of neonatal sepsis in LMICs. Countries in sub-Saharan Africa, Southeast Asia, and the Caribbean observed an average annual increase of 9% in neonatal sepsis between 1990 and 2019 (63). A meta-analysis of studies between 1980 and 2018 also determined that *K. pneumoniae* was responsible for 21% of culture-positive bacteremia or sepsis cases in neonates in sub-Saharan Africa (64). *K. pneumoniae* was identified as the most common cause of neonatal sepsis in the Burden of Antibiotic Resistance in Neonates from Developing Societies (BARNARDS) study, which was an observational study conducted in 12 sites in sub-Saharan Africa and Southeast Asia (65–67). Additionally, the Child Health and Mortality Prevention Surveillance (CHAMPS) study determined that *K. pneumoniae* was the main Gram-negative bacterium associated with neonatal sepsis, contributing to 31% of mortality cases (68, 69). Intriguingly, over 56% of *K. pneumoniae*-associated neonatal sepsis cases were delivered via caesarean section, suggesting that neonates may acquire *K. pneumoniae* from sources other than the traversing of the birth canal (16).

Neonatal sepsis

Global burden and challenges

Neonatal sepsis is a systemic infection in infants under 28-days-old, resulting in severe organ dysregulation frequently associated with irreversible long-term deficits or death (70–72). Neonatal sepsis is often divided into two groups, based on time of presentation, early-onset sepsis (EOS) and late-onset sepsis (LOS), which can differ in their acquisition and causative risk factors. EOS, occurring within 72 hours of life, is a

result of maternal transmission of invasive pathogens during the perinatal period, often associated with low birth weight (63, 70, 73–76). LOS generally occurs via environmental exposure to pathogens postnatally, where infants requiring invasive procedures that disrupt the mucosa (e.g., catheter insertion) are at increased risk (63, 70, 73–76).

Morbidity and mortality rates

Sepsis was the third leading cause of neonatal morbidity and mortality, accounting for 47% of all child deaths under 5 years of age in 2022 (77). A recent meta-analysis estimates the global incidence of neonatal sepsis to be 2,824 per 100,000 live births with a mortality rate of 18% (63, 71, 78). The impact of neonatal sepsis, in terms of both morbidity and mortality, is most severe in LMICs. This heightened vulnerability stems from the high prevalence of infectious diseases and restricted access to properly equipped and staffed healthcare facilities (71, 78–80). The highest neonatal mortality rate was found in Sub-Saharan Africa at 2.7% (27 deaths per 1000 live births), followed by central and southern Asia at 2.1% (21 deaths per 1000 live births) (77).

Immunobiology of neonatal sepsis

Immaturity of the neonatal immune system

The neonatal immune system, while complex, is inherently immature, rendering neonates vulnerable to infections (81, 82). This immaturity is characterized by insufficiencies in both innate and adaptive immune responses, leading to a distinct immune profile when compared to the mature adult immune system. Innate immunity, the

body's first line of defense, is comprised of reduced phagocytic activity, impaired cytokine production, and limited complement function (81–83). Furthermore, the adaptive immune system, which involves the activation of T and B lymphocytes, is less developed in neonates, leading to suboptimal cellular and humoral responses (81, 82). These immune insufficiencies, coupled with the challenges of transitioning from a sterile intrauterine environment to a microbial-rich external environment, contribute to the increased risk of severe infections in newborns.

Role of innate and adaptive immunity in neonatal sepsis

Neonates rely heavily on their innate immune system as it provides a quick, short-term, nonspecific response to microorganisms. Particularly, neutrophils provide a first line of defense against pathogens, engaging in phagocytosis, the release of antimicrobial peptides (AMPs), and the generation of neutrophil extracellular traps (NETs) in response to an infection (74, 83). Endothelial cells will release cytokines and chemokines recruiting neutrophils to the site of infection. When activated, neutrophils will phagocytose and release reactive oxygen species to kill the pathogen, and they will secrete IL-1 β to recruit additional neutrophils to the site of infection (84–86). Additionally, with neutrophil death, NET formation contributes to the killing of bacteria, as they contain proteases which can degrade bacterial surface proteins leading to their death and clearance (84, 87).

Antigen-presenting cells (APCs), which includes monocytes and dendritic cells (DCs), also play a key role in pathogen recognition and eradication. Monocytes when exposed to cytokines, will differentiate into macrophages, which will engage in the

phagocytosis and the release of AMPs aiding in the degradation of bacteria (84, 88, 89). Through pattern recognition receptors (PRR), specifically TLRs, signaling pathways in APCs are activated leading to cytokine production, antigen presentation to T cells, and the polarization of the T cell response (84, 90). Once activated, the naive T cell will undergo clonal expansion, leading to the rapid proliferation and differentiation of specialized effector cells (91). These effector cells mount a specific, robust, and long-lasting adaptive immune response tailored to eliminate the invading bacterium. However, the contribution of the adaptive immune system in the first days of life are uncertain, as development is ongoing. Since EOS occurs within the first 4 weeks of life, adaptive immunity may play less of a role in protection against severe disease and mortality. Therefore, neonates are believed to rely heavily on their innate immune system to combat sepsis.

Immune dysregulation in neonatal sepsis

The immature neonatal immune system, while capable of responding to pathogens, is often unable to mount an effective and balanced response. This immune dysregulation can manifest as either a hyper- or hypo-inflammatory state (83, 92, 93). In hyper-inflammatory states, excessive production of pro-inflammatory cytokines can lead to systemic inflammation, organ dysfunction, and potential death (83, 92, 93). Conversely, a hypo-inflammatory response may result in inadequate clearance of the pathogen, allowing for persistent infection and sepsis (83, 92, 93). Understanding the mechanisms underlying immune dysregulation in neonatal sepsis is crucial for developing targeted therapeutic interventions to improve patient outcomes.

Neonatal neutrophils exhibit significant deficiencies including decreased baseline reservoirs and neutrophil progenitor cell production when compared to adults, leading to a diminished response to infection and an increased risk of depletion of these reservoirs following sepsis (82, 94). Additionally, neonatal neutrophils minimally express surface receptors L-selectin, Mac-1 (CD11b/CD18), and P-selectin, and when coupled with decreased calcium influx there is an impairment in adhesion to the vascular epithelium and transmigration and chemotaxis to the site of infection (82, 83). Moreover, neonatal neutrophils have reduced phagocytic capabilities, dampened bactericidal activity, and defective NETs production, hindering the degradation and killing of invasive pathogens (82, 83, 94). Neonatal neutrophils are also less responsive to apoptosis, promoting excessive inflammation and tissue damage (83, 94). Overall, neutrophil impairment can make neonates more susceptible to sepsis.

Though surface expression of TLRs on neonatal monocytes and DCs are comparable to adults, function responses (cytokine production) and T-helper (T_H) fates are altered (74, 82, 88). Neonatal cytokine production in response to stimuli are often skewed towards an anti-inflammatory or T_H2/T_H17 polarized response, impairing the production of pro-inflammatory cytokines or a T_H1 response (74, 88, 95, 96). This renders neonates vulnerable to microbial infections, as T_H1 -associated cytokines are necessary to enhance phagocytic activity, induce inflammation, and promotes the differentiation and proliferation of T lymphocytes (83, 88, 95, 96).

The innate immune response provides the necessary signals for the adaptive immune response to develop and function effectively. Therefore, deficiencies in the

innate response will ultimately lead to deficiencies in the adaptive response. T lymphocytes are activated via their T-cell receptor (TCR) binding to epitopes presented by APCs, allowing for the activation, differentiation, and proliferation of T_H cells dependent on pathogen-derived antigens (97). However, in contrast to mature T cells, neonatal T cells have a diminished response following antigen presentation. This is due to both a polarization towards an anti-inflammatory response and reduced TCR signaling, a result of insufficient phospholipase C activation, hindering activation, proliferation, and differentiation (82).

Neonatal antibody production is also impacted when compared to adults due to B cell immaturity, limited B cell repertoire, and/or dampened B-cell receptor (BCR) signaling (82). B cells are differentiated into two subsets: B1 cells where the BCR is activated via T cell-independent (TI) antigens and B2 cells where the BCR is activated via T cell-dependent (TD) antigens, both allowing for sufficient antibody production in response to an invasive pathogen (82, 98). However, B1 cells, mainly found in the marginal zone of the spleen, are reduced in numbers at birth, dampening antibody production upon TI antigen stimulation in neonates (82, 98). B2 cells are dependent on germinal centers, for maturation and class-switch recombination to produce high-affinity antibody responses, however, these structures are absent at time of birth preventing B cell maturation and sufficient antibody responses to neonatal infections and/or sepsis (82, 98). Additionally, insufficient antibody production may also be related to increased expression of inhibitory coreceptors and high-density surface immunoglobulin (Ig) M, hindering BCR signaling and inducing apoptosis (82, 99, 100).

Animal models of neonatal sepsis

Existing neonatal sepsis animal models have primarily focused on polymicrobial infections or specific pathogens like Group B *Streptococcus* (GBS) or *Escherichia coli*, which are common causes of neonatal sepsis in developed regions (101–103). A neonatal murine model is well-established for GBS; pregnant female mice are intravaginally inoculated, exposing neonates to GBS as they traverse the birth canal (104). Multiple rodent (mice and rats) models have been established to study *E. coli* neonatal sepsis, including a humanized neonatal mouse model, antibiotic-treated neonatal mouse model for disseminated infections, and an age-dependent neonatal model using Wistar rats (105–107).

While *K. pneumoniae* infections are recognized as a leading cause of neonatal sepsis globally, particularly in LMICs, and are increasingly associated with AMR, there remains a notable lack of well-established neonatal sepsis animal models for *K. pneumoniae*. This critical gap hinders the comprehensive investigation of *K. pneumoniae* pathogenesis in the vulnerable neonatal population, limits the understanding of age-dependent immune responses to this pathogen, and impedes the development of novel interventions, such as vaccines, against *K. pneumoniae*-induced neonatal sepsis.

Project goals and aims

The ultimate goal of this thesis is to develop a *K. pneumoniae* neonatal sepsis rodent model that can effectively aid in the development and evaluation of novel vaccines aimed at minimizing the global burden of neonatal sepsis. To complete this project, I

developed and characterized a *K. pneumoniae* neonatal sepsis model in mice and identified a suitable immunization paradigm that can be used to evaluate vaccine efficacy. Little is currently known about optimal *in vivo* correlates of protection for *K. pneumoniae* neonatal sepsis, especially in the context of passive and maternal immunization strategies. Thus, in this project, I aimed to establish a robust research methodology and identify primary readouts for evaluating vaccine efficacy against *K. pneumoniae* neonatal sepsis. The specific aims for this thesis were as follows:

Aim 1. Develop a *K. pneumoniae* neonatal sepsis rodent model.

Hypothesis: Neonatal mice will be susceptible to K. pneumoniae infection, with increased mortality and bacterial burden when compared to older animals.

Establish a *K. pneumoniae* neonatal sepsis rodent model by comparing different mouse strains, administration routes, and bacterial strains. Assess survival following lethal challenge with the reference strain *K. pneumoniae* B5055 in neonatal, adolescent, and adult mice. Evaluate bacterial burden following lethal challenge with *K. pneumoniae* in neonatal mice.

Aim 2. Establish the utility of a passive immunization model for assessing protection against *K. pneumoniae* neonatal sepsis.

Hypothesis: Passive immunization of neonatal mice with immune sera will result in increased survival rates, reduced bacterial burden, and attenuated histopathological changes following lethal challenge with K. pneumoniae.

Using the mouse model established in Aim 1, determine the effectiveness of a passive immunization model as a tool for assessing protection in neonates following lethal challenge with *K. pneumoniae* B5055. Determine the ability of hyperimmune *K. pneumoniae* rabbit antisera to improve survival rates and reduce bacterial burden following passive transfer to neonatal mice. Correlate antibody levels induced by hyperimmune *K. pneumoniae* rabbit antisera to degree of protection *in vivo*.

Aim 3. Determine the suitability of a maternal immunization model for evaluating protection against *K. pneumoniae* neonatal sepsis.

Hypothesis: Maternal immunization with Klebsiella vaccines will result in increased survival rates, reduced bacterial burden, and attenuated histopathological changes in neonatal mice following lethal challenge with K. pneumoniae.

Determine if maternal immunization with heat-killed *K. pneumoniae* or an OPS glycoconjugate vaccine results in increased survival and reduced bacterial burden within secondary lymphoid tissues of neonates following lethal challenge with *K. pneumoniae* B5055. Measure the antigen-specific IgG titers post immunization in the dams and their litters. Compare the antigen-specific antibody levels between neonates born to vaccinated or unvaccinated dams.

Chapter 2 Development of a *Klebsiella pneumoniae* neonatal sepsis model

This chapter contains the following published manuscript that has been reformatted accordingly: Miller JC, Choi M, Zhao Z, Mushrush EM, Legesse TB, Cross AS, Baliban SM, Tennant SM. Development of a mouse model for *Klebsiella pneumoniae*-associated neonatal sepsis. *Microbiology Spectrum*. 2025 Aug 1. doi: 10.1128/spectrum.00697-25

Introduction

Klebsiella pneumoniae is an opportunistic healthcare-associated pathogen that is responsible for a substantial burden of bacterial pneumonia and invasive disease worldwide (15, 108). Recently, *K. pneumoniae* has been implicated as a common cause of bloodstream infections and sepsis in neonates (7, 109). Neonatal sepsis is defined as a systemic inflammatory response occurring in infants ≤ 28 days of life and is commonly caused by multiple bacterial pathogens (110–113). A systematic review found that *Klebsiella* spp., *Escherichia coli*, *Enterobacter* spp., and *Pseudomonas* spp. accounted for 38% of blood culture-positive neonatal sepsis cases in Sub-Saharan Africa (64). Other studies, including CHAMPS, BARNARDS, and a global neonatal sepsis observation cohort study (NeoOBS), have implicated *K. pneumoniae* as one of the predominant causative pathogens associated with neonatal sepsis and death in LMICs (66, 68, 114, 115). High rates of antimicrobial resistant *K. pneumoniae* in addition to poor accessibility and affordability of appropriate antibiotics in LMICs have been reported (65, 114, 116). Additionally, the World Health Organization considers multidrug-resistant *K. pneumoniae* to be a critical threat (53). Multiple groups are developing *K. pneumoniae*

vaccines for various clinical indications including prevention of neonatal sepsis (16, 117, 118). However, no vaccines that target *K. pneumoniae* have yet been licensed.

Sepsis in neonates is often divided into early and late onset forms. In general, early onset neonatal sepsis is thought to be the result of maternal transmission of invasive pathogens during the perinatal period, whereas late onset sepsis results from environmental exposure to pathogens postnatally (63, 74–76, 112). Establishing early protection from *K. pneumoniae* might significantly reduce the morbidity and mortality associated with both early and late onset neonatal sepsis. Therefore, maternal immunization affords the optimal opportunity to prevent neonatal sepsis via the transfer of protective maternal antibodies to neonates or blockage of *K. pneumoniae* transmission from mothers to their children. Recent modeling data suggests a maternal *K. pneumoniae* vaccine with ~70% efficacy could avert approximately 80,000 neonatal deaths and 400,000 neonatal sepsis cases yearly worldwide (114).

Adult rodents are the model of choice to investigate *K. pneumoniae*-induced respiratory, gastrointestinal (GI), and urinary tract infections (119). For *K. pneumoniae* respiratory infection rodent models, several methods of inoculation (e.g., intranasal inoculation, intratracheal inoculation with or without surgery) have been described that effectively promote lung disease and show evidence of increased bacterial load in the lung and systemic dissemination (119). *K. pneumoniae* GI infection models primarily utilize oral administration to mimic natural acquisition but these models generally require pre-administration of antibiotics to clear the gut microbiota (119). In terms of eliciting lethal bacteremia in adult mice, we and others have used the intravenous or intraperitoneal routes of infection (62, 120). In an elegant study, Russo *et al* used a

bacteremia mouse model incorporating intraperitoneal or subcutaneous injection to examine the virulence of classical and hypervirulent *K. pneumoniae* isolates (121). Despite the utility of adult rodent models for understanding *K. pneumoniae* disease pathogenesis, they cannot effectively mimic *K. pneumoniae*-associated neonatal sepsis due to age related differences of the host. This is a critical knowledge gap. A reliable neonatal sepsis model would advance our knowledge of *K. pneumoniae* infections and help to develop strategies that mitigate the risk of morbidity and mortality associated with neonatal sepsis.

Animal models are routinely used to evaluate vaccine efficacy. To protect neonates against infection, since there is often not sufficient time to elicit an immune response, vaccines may need to be administered maternally to confer passive protection to their offspring. Rodent neonatal sepsis models for GBS and *E. coli* have been developed; maternally-administered vaccines were shown to confer protection to pups following lethal challenge with GBS (104, 122). Additionally, maternal antibodies elicited by a live attenuated *E. coli* K1 vaccine conferred protection in neonates following lethal challenge with both K1 and non-K1 strains of *E. coli* (107, 123). Collectively, these studies highlight the promise of maternal immunization in eliciting protective immunity against lethal bacterial challenge using a relevant neonatal sepsis animal model.

In this work, we developed a *K. pneumoniae*-associated neonatal sepsis murine model using the well-studied B5055 (O1:K2) hypervirulent strain (124). We evaluated various strains of mice and bacterial isolates, routes of infection, susceptibility to infection by age, and assessed bacterial loads in tissues, as well as histopathological effects early and late in infection. This novel model system provides a useful tool to

examine host-microbial interactions during *K. pneumoniae* bloodstream infection in newborns as well as to develop countermeasures against *K. pneumoniae* neonatal sepsis.

Methods

Animals and ethics statement

All animal studies were performed in facilities that are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were housed under specific pathogen-free conditions at the University of Maryland School of Medicine, and all procedures were approved by the University of Maryland Baltimore Institutional Animal Care and Use Committee (protocol no. 0820016 and 00000289). To generate neonatal and juvenile mice, 8-10-week-old pregnant CD-1, BALB/c, and C57BL/6 mice were purchased at gestational day 14 (E14), from Charles River Laboratories (Wilmington, MA). Pups were delivered naturally at term gestation and remained with their dams until they reached the desired age except for brief interruptions due to experimental procedures. Pups of both sexes were used for all experimental procedures. For experiments involving adolescent and adult mice, 3-4-week-old and 7-8-week-old female C57BL/6 mice were purchased from Charles River Laboratories and acclimated on site for 7 days prior to bacterial challenge. BSL-2 containment was employed for all experiments involving live bacteria.

Bacterial strains and medium

Bacterial strains used in this study are shown in **Table 2.1**. Streptomycin-resistant *K. pneumoniae* B5055 Strep^R was generated by passaging *K. pneumoniae* B5055 (a hypervirulent O1:K2 strain (124) originally obtained from Frits and Ida Ørskov, Statens Serum Institut, Denmark) on medium containing increasing concentrations of streptomycin and selecting resistant colonies. A mouse passaged version of *K. pneumoniae* 700603 was generated by administering strain 700603 intraperitoneally to a CD-1 mouse and isolating bacteria 24 hours later from the liver. The recovered strain (700603-MP) was verified to be *K. pneumoniae* through analytical profile index testing (bioMérieux, Craaponne, France). All bacterial strains were maintained in animal-product-free Hy-Soy (HS) bacteriological media (10 g/L Soytone [Teknova, CA], 5 g/L Hy-yeast [Kerry Bio-Science, Beloit, WI] and 5 g/L sodium chloride [American Bio, Natick, MA]) at 37 °C. When needed, bacteriological agar (MilliporeSigma, Burlington, MA) was added at 15 g/L. For *K. pneumoniae* B5055 Strep^R bacterial recovery, medium was supplemented with 50 µg/mL of streptomycin sulfate (Research Products International, Mt. Prospect, IL).

Table 2.1: *K. pneumoniae* strains used in this study.

Strain	Characteristics (serotype)	Reference
B5055	Reference strain (O1:K2)	(124)
B5055 Strep ^R	Spontaneous streptomycin resistant B5055 (O1:K2)	This study
TPEVGH-KPN-12	Clinical isolate, Taiwan (O2:K2)	(7)
700603-MP	Mouse-passaged version of ATCC 700603 (O3:K-undetermined)	This study
390	Clinical isolate, Germany (O3:K11)	(125)
15AP507624	Clinical isolate, Sweden (O5:K14)	(7)
12-02000	Clinical isolate, USA (O5:K53)	(7)
4425/51	Clinical isolate, Germany (O5:K57)	(125)

Mouse infections

K. pneumoniae strains were recovered from cryopreservation on HS agar prior to inoculation in HS broth for overnight growth at 37°C, 220 revolutions per minute (rpm) with aeration for 17.5 h. Bacteria were then washed twice by centrifugation at 3000 x g for 10 mins at 4° C using a Sorvall legend XTR centrifuge, XT-1000 rotor (Thermo Fisher Scientific, Waltham, MA) followed by resuspension in sterile phosphate buffered saline (PBS). Bacteria were diluted with sterile PBS to the desired colony-forming units (CFU) based on optical density at 600 nm (OD₆₀₀). All bacterial inocula were confirmed by serial dilution and enumeration on HS agar.

To compare the sensitivity of the mouse strains and routes of administration, two-day-old BALB/c, C57BL/6, and CD-1 mice were infected perorally (p.o.), intraperitoneally (i.p.), or subcutaneously (s.c.) with *K. pneumoniae* B5055 Strep^R suspended in PBS containing 0.5% weight/volume (w/v) Evans Blue Dye (MilliporeSigma, Burlington, MA) at the indicated doses. For p.o. infection, the inoculum was administered to mice using a fixed volume Eppendorf Research plus pipette (MilliporeSigma) attached to a disposable, flexible, polytetrafluoroethylene (PTFE) 20-gauge x 1.5” feeding needle with a 2 mm ball on the tip (Braintree Scientific, Braintree, MA) in a 10 µL volume. For i.p. and s.c. infection, the inoculum was administered to mice in a 50 µL volume either in the lower right quadrant of the abdomen or the dorsal surface, respectively, using a 30-gauge x 5/16” 1 mL insulin syringe (Covidien, Mansfield, MA). Immediately following infection, pups were monitored for signs of stress and marked with a pen for identification and returned to their cage. Mice were then monitored daily for symptoms associated with sepsis for 7 days post infection (d.p.i.). *K.*

pneumoniae-associated sepsis symptoms were scored as per **Table 2.2** and pups with a score ≥ 4 were considered to have met alternative endpoints and were euthanized and recorded as a death.

To assess the virulence of *K. pneumoniae* clinical isolates, two-day-old C57BL/6 mice were infected p.o. with either TPEVGH-KPN-12 (O2:K2), 700603-MP (O3:K-undetermined), 390 (O3:K11), 15AP507624 (O5:K14), 12-02000 (O5:K53), or 4425/51 (O5:K57) suspended in PBS containing 0.5% weight/volume (w/v) Evans Blue Dye (MilliporeSigma) at the indicated doses. The inoculum was administered to mice using a fixed volume Eppendorf Research plus pipette (MilliporeSigma) attached to a disposable, flexible, PTFE 20-gauge x 1.5” feeding needle with a 2 mm ball on the tip (Braintree Scientific) in a 10 μ L volume. Neonatal mice were observed daily for signs of sepsis as per **Table 2.2** and mortality until 7 d.p.i.

For age dependency experiments, C57BL/6 mice at 2, 3, 4, 5, 7, 10, 15, 30, and 60 days of age were infected p.o. with *K. pneumoniae* B5055 Strep^R ($1.4 - 2.0 \times 10^8$ CFU per mouse suspended in PBS containing 0.5% w/v Evans Blue Dye). For mice aged 15 days or younger, the inoculum was administered via a sterile, flexible, PTFE 20-gauge x 1.5” feeding needle with 2 mm ball in a 10 μ L volume. For 30-, and 60-day-old mice, the inoculum was administered via a sterile, straight, stainless steel 20-gauge x 1.5” feeding needle with 2 mm ball (Cadence Science, Cranston, RI) in a 100 μ L volume. Neonatal and infant mice (2-10-day olds) were observed daily for signs of sepsis as per **Table 2.2** and mortality until 7 d.p.i.. Adolescent and adult mice (15-60-day olds) were observed daily for alternative endpoints (weight loss, decrease in activity, labored breathing) until

7 d.p.i.. All experiments were performed at least twice, with representative data from single experiments presented.

Table 2.2: Five-feature scoring system for clinical features of *K. pneumoniae*-associated neonatal sepsis.

Parameters	Scoring for the listed parameters		
	Healthy (0)	Intermediate (0.5)	Unhealthy (1)
Color of the skin	Black color	-	Gray/pale color
Righting Reflex	Immediate reversing upon back placement	Difficulty reversing (> 3 secs) but eventually achieved	Cannot achieved reversing, remaining on backside
Stomach/milk line*	Visible	-	Not visible
Behavior	Active movement, huddling with siblings, feeding	Lethargic moments, slow to feed	Trembling, not within the nest, does not feed
Weight	Gain of 0.5-1g per day	No weight gain	Weight loss

* Milk spot not visible after 5 days post infection. Neonates with score ≥ 4 were euthanized

Quantitative bacteriology

Two-day-old C57BL/6 mice were infected perorally with *K. pneumoniae* B5055 Strep^R suspended in PBS containing 0.5% w/v Evans Blue Dye ($1.3 - 3.3 \times 10^7$ CFU per mouse) or PBS containing 0.5% v/v Evans Blue dye alone (control). Mice were manually restrained, and the inoculum was administered via a sterile, flexible, PTFE 20-gauge-1.5” 2 mm ball feeding needle or with a sterile, straight, stainless steel 24-gauge-1” 1.25mm ball feeding needle (Cadence Science) in a 10 μ L volume. At 2 or 18 hours post infection (h.p.i.), the brain, gastrointestinal (GI) tract, liver, lungs, and spleen were excised aseptically, weighed, and homogenized in PBS using an Omni International TH-01 tissue homogenizer (Swedesboro, NJ). Blood was extracted following euthanasia into EDTA/KE microtubes (Sarstedt, Numbrecht, Germany). Tissue homogenates and blood were serially diluted in PBS and spread plated on HS containing streptomycin to

determine the *K. pneumoniae* B5055 Strep^R load in tissues. Following incubation at 37°C overnight, colonies were counted and data presented as CFU per gram of organ or CFU per 100 µL blood.

Tissue histopathology

Two-day-old C57BL/6 mice were infected with *K. pneumoniae* B5055 Strep^R suspended in PBS containing 0.5% w/v Evans Blue Dye (6.5×10^7 CFU per mouse) or PBS containing 0.5% w/v Evans Blue dye alone (control). Eighteen h.p.i. blood, brain, GI tract, liver, lungs, and spleen were harvested aseptically and instilled in 70% ethanol. Slides were prepared and stained with hematoxylin and eosin (H&E) by the Pathology EM and Histology Laboratory (UMB histology core facility) using standard protocols. Histopathological examination of the whole tissue was performed in a blinded fashion by an experienced pathologist. Histopathology was scored per **Table 2.3** on a scale of 0 to 4, with 4 being the greatest degree of pathology for each parameter assessed.

Table 2.3: Scoring system to assess pathology of neonatal tissues 18 hours post infection.

Tissue	Parameter 1 scoring criteria	Parameter 2 scoring criteria	Parameter 3 scoring criteria	Maximum Score
Liver	Degradation and necrosis of hepatocytes	Infiltration of inflammatory cells, aggregation of inflammatory cells	Not applicable	8
Lung	Reduced alveolar vesicular structure	Airway inflammatory cell infiltration	Not applicable	8
Spleen	Increased number of apoptotic cells, granulocytes, macrophage aggregates and inclusion bodies	Red blood cell depletion	Reduction in white pulp/architectural dysregulation	12

Pathology scored on a scale of 0 to 4. Each tissue was scored on the absence or severity of the listed parameters with brain, liver, and lungs having a maximum score of 8, and the gastrointestinal tract and spleen having a maximum score of 12. 0 = normal/absent, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe.

Statistical analysis

All statistical analyses were performed with GraphPad Prism v10.1.1 (La Jolla, CA). A *p*-value equal to or below 0.05 was considered significant for each test. Survival analyses for Kaplan-Meier curves were accomplished by log-rank test. Fifty percent lethal dose (LD₅₀) values were calculated by linear regression analysis. Statistical significance for recovered CFU after bacterial infection were assessed by two-tailed Mann-Whitney Test. Histopathology significance was determined by Student's *t*-test.

Results

Comparison of the sensitivity of different mouse strains to *K. pneumoniae* B5055 Strep^R.

To compare the susceptibility of different mouse strains to *K. pneumoniae* infection, and the effect of administration route on susceptibility, two-day-old BALB/c, C57BL/6, and CD-1 mice were infected either perorally (p.o.), intraperitoneally (i.p.), or subcutaneously (s.c.) with a streptomycin-resistant strain, *K. pneumoniae* B5055 Strep^R. Use of streptomycin resistance allowed us to selectively enumerate *K. pneumoniae* *in vitro*. Two-day-old mice were selected to model early-onset sepsis. All animals were monitored daily for clinical signs of sepsis (**Table 2.2**), and all remaining surviving animals were euthanized 7 days post-infection (d.p.i.). For p.o. administration, mice were infected with $4.2 - 6.8 \times 10^8$ CFU. C57BL/6 mice exhibited 33% (2/6) survival, while BALB/c and CD-1 mice exhibited 50% (2/4) and 75% (9/12) survival, respectively (**Figure 2.1A**). Perorally infected mice displayed a mean time to death (MTD) of 5.3 ± 2.4 d.p.i. (\pm standard deviation). All animals were susceptible to *K. pneumoniae* B5055 Strep^R following i.p. and s.c. administration of $0.8 - 1.3 \times 10^6$ CFU, succumbing to infection within a MTD of 1.0 ± 0.32 d.p.i. (**Figure 2.1B and C**). Representative data are shown in Figure 1. To examine whether there were any differences in MTD for the peroral route between mouse strains, we combined data from two experiments. We observed a MTD of 3.47 days for C57BL/6 mice (9 mice died/11 mice tested), 4.1 days for BALB/c mice (6 mice died/10 mice tested) and 5.04 days (11 mice died/24 mice tested) for CD-1 mice. There was a statistically significant difference ($p \leq 0.05$) in the

MTD between C57BL/6 and CD-1 mice. Collectively, these data suggest that for the i.p. and s.c. administration routes, all three mouse strains respond similarly to *K. pneumoniae* infection, while neonatal C57BL/6 mice were more susceptible to p.o. infection.

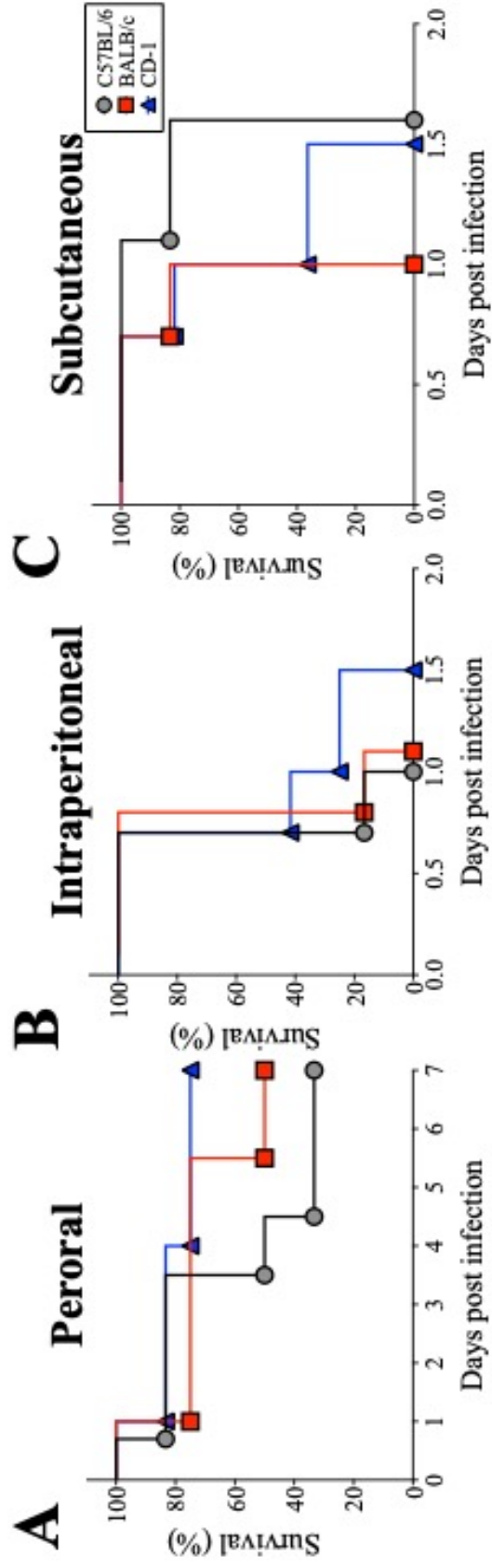


Figure 2.1. Survival comparison of neonatal mouse strains infected with *K. pneumoniae* B5055.

Whole litters of two-day-old C57BL/6 mice (circles, grey lines), BALB/c mice (squares, red lines), or CD-1 mice (triangles, blue lines) were infected with *K. pneumoniae* B5055 Strep^R either (A) perorally with $4.2 - 6.8 \times 10^8$ CFU (n = 4-12 per group), (B) intraperitoneally (i.p.) with $0.8 - 1.3 \times 10^6$ CFU, or (C) subcutaneously with $0.8 - 1.3 \times 10^6$ CFU (n = 4-12 per group). Mice were monitored daily for survival. The p.o. infection experiments were performed at least two times, and the Kaplan-Meier survival curves shown here are from one representative experiment. The Kaplan-Meier survival curves shown for i.p. and s.c. infection experiments are a single experiment for each administration route.

To determine the peroral 50% lethal dose (LD₅₀), two- or three-day-old CD-1 mice (n = 6-15 per group) and C57BL/6 mice (n = 5-9 per group) (BALB/c mice not tested) were infected p.o. with serially diluted *K. pneumoniae* B5055 Strep^R. CD-1 mice exhibited 77-89% survival at infection doses ranging from 6.8×10^5 CFU – 6.8×10^8 CFU, indicating *K. pneumoniae* B5055 Strep^R was marginally virulent following peroral infection (LD₅₀ > 6.8×10^8 CFU) (**Appendix Figure 1**). In contrast, all neonatal C57BL/6 mice infected with a dose of 2.2×10^9 CFU per mouse died within 2.5 d.p.i. (0/9 survived), while 50% (4/8), 50% (3/6), 57.1% (4/7), 100% (5/5) and 87.5% (7/8) survival was observed for mice infected with 2.2×10^8 CFU, 2.2×10^7 CFU, 2.2×10^6 CFU, 2.2×10^5 CFU, and 2.2×10^4 CFU, respectively (**Figure 2.2A**). We calculated the p.o. LD₅₀ of *K. pneumoniae* B5055 Strep^R to be 8.5×10^6 CFU in two-to-three-day-old C57BL/6 mice. We subsequently determined the LD₅₀ for the i.p. and s.c. routes in CD-1 and C57BL/6 mice (BALB/c not tested) to be <100 CFU (**Appendix Figures 2 and 3**). Since the minimum lethal dose for *K. pneumoniae* B5055 Strep^R following i.p. and s.c. administration was low (~10 CFU), we selected the p.o. infection route and C57BL/6 mice for subsequent experiments to enhance the reproducibility of the model.

To ensure that a consistent attack rate was achievable with ~ 10^9 CFU of *K. pneumoniae* B5055 Strep^R, which produced 100% lethality in neonatal C57BL/6 mice,

we evaluated survival in four independent experiments. Two-day-old C57BL/6 mice (n = 5-9 mice/experiment) were infected p.o. with either 1.5×10^9 CFU (experiment 1), 6.0×10^8 CFU (experiment 2), 6.0×10^9 CFU (experiment 3), or 1.4×10^9 CFU (experiment 4). We observed 0% (0/5) survival, 0% (0/8) survival, 11% (1/9) survival, and 12.5% (1/8) survival in experiments 1, 2, 3, and 4, respectively. These data demonstrated that an inoculum of at least 6.0×10^8 CFU could reliably produce 88-100% mortality in neonatal C57BL/6 mice (median survival of $5.55\% \pm 5.92\%$) (**Figure 2.2B**).

To determine if other strains of *K. pneumoniae* were capable of establishing infection in neonatal mice, we assessed several clinical *K. pneumoniae* isolates following p.o. administration. Two-day-old C57BL/6 mice were infected p.o. with $1.0 \times 10^8 - 2.2 \times 10^9$ CFU of TPEVGH-KPN-12 (O2:K2), 700603-MP (O3:K-undetermined), 390 (O3:K11), 15AP507624 (O5:K14), 12-02000 (O5:K53), or 4425/51 (O5:K57). We observed 0% survival in neonatal mice infected with TPEVGH-KPN-12 (0/3), 700603-MP (0/3), and 15AP507624 (0/6) (**Appendix Figure 4**), while 12-02000, 4425/51, and 390 exhibited 50% (3/6), 80% (4/5), and 100% (6/6) survival, respectively (**Appendix Figure 4**). These results highlight the intrinsic variability in virulence amongst *K. pneumoniae* isolates to cause mortality in a neonatal sepsis model following p.o. administration.

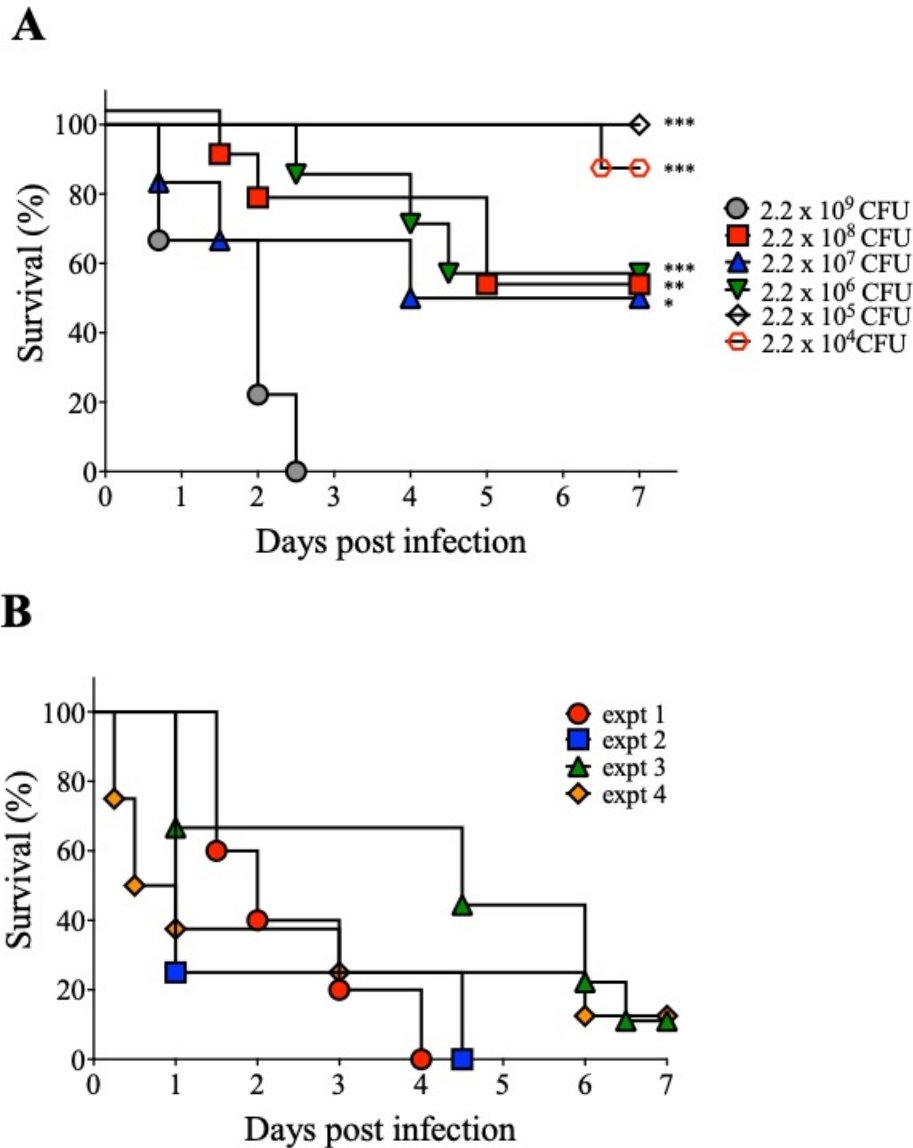


Figure 2.2. The repeatability and stability of the *K. pneumoniae*-associated neonatal mouse infection model.

(A) Whole litters of two- or three-day-old C57BL/6 mice (n = 5-9 per litter) were infected perorally with the indicated inocula of *K. pneumoniae* B5055 Strep^R and monitored daily for survival to determine the 50% lethal dose (LD₅₀). Statistical survival differences were determined by log-rank analysis relative to 2.2 x 10⁹ CFU. *, p ≤ 0.01; **, p ≤ 0.001; ***, p ≤ 0.0001. (B) Whole litters of two-day-old C57BL/6 mice (n = 5-9 per litter) were infected perorally in four independent experiments (expt) with 1.5 x 10⁹ CFU (expt 1), 6.0 x 10⁸ CFU (expt 2), 6.0 x 10⁹ CFU (expt 3), and 1.4 x 10⁹ CFU (expt 4) of *K. pneumoniae* B5055 Strep^R and monitored daily for survival to evaluate the reproducibility of the neonatal mouse model.

Examination of age dependent peroral infection with *K. pneumoniae* B5055

Strep^R.

To assess age susceptibility to *K. pneumoniae* infection, C57BL/6 mice at 2, 4, 5, 7, 10, 15, 30, and 60 days of age were infected p.o. with $1.4 - 2.0 \times 10^8$ CFU *K. pneumoniae* B5055 Strep^R. Two- and 4-day old mice exhibited 0% survival within 5.5 d.p.i. (0/7 and 0/8, respectively) (**Figure 2.3**). Five- and 7-day-old mice exhibited 50% (4/8) and 44% (4/9) survival, respectively. In contrast, 10-, 15-, 30-, and 60-day-old mice were not susceptible to infection with *K. pneumoniae*, with 100% survival at 1.4×10^8 CFU. These results suggest susceptibility to *K. pneumoniae* declines with age and that animals 4 days of age or younger are the most sensitive to *K. pneumoniae* infection following p.o. administration.

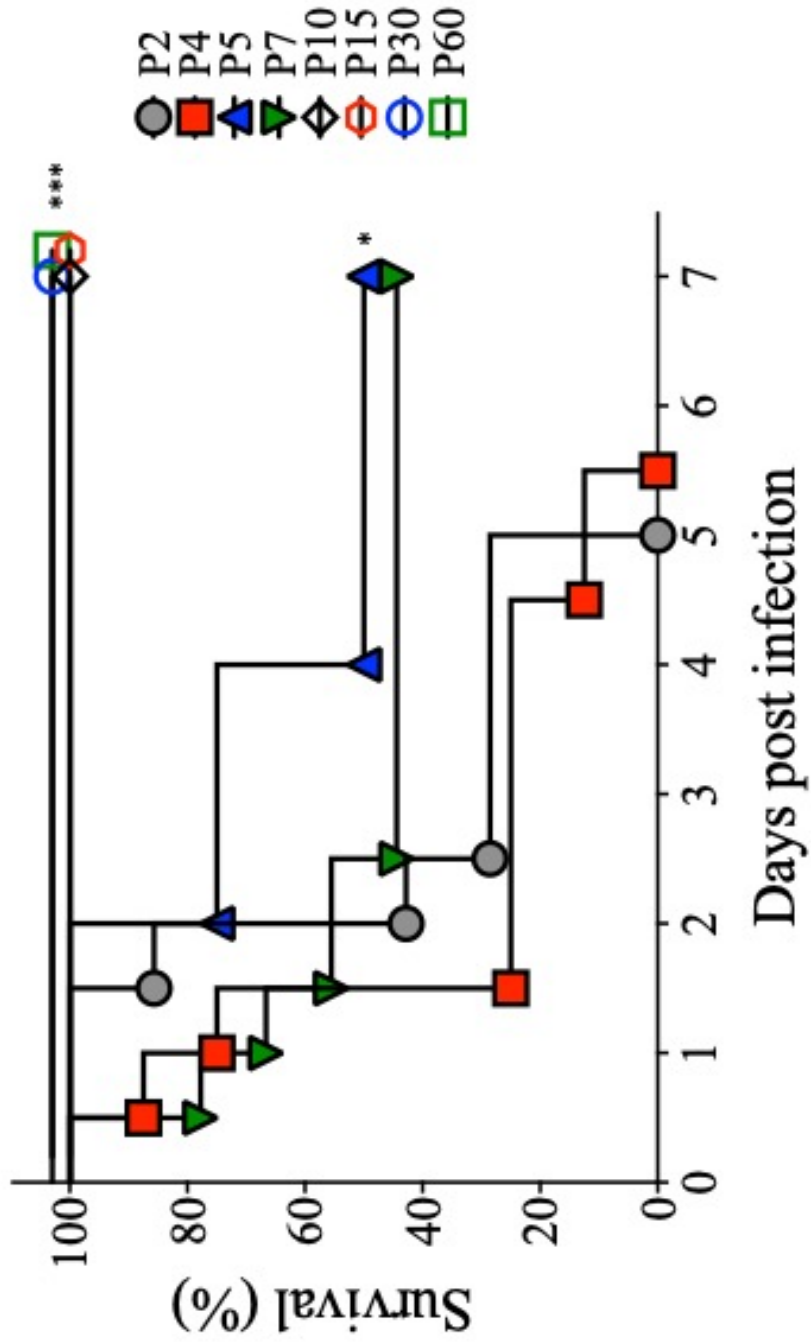


Figure 2.3. *K. pneumoniae* B5055 Strep^R elicits an age dependent effect following peroral infection.

C57BL/6 mice at 2, 4, 5, 7, 10, 15, 30, and 60 days of age (n = 5-9 per age group) were infected perorally with *K. pneumoniae* B5055 Strep^R ($1.4 - 2.0 \times 10^8$ CFU/mouse) and monitored daily for survival. Data are presented as Kaplan-Meier survival curves. Results shown are from one representative experiment performed at least two times. Statistical survival differences were determined using a log-rank analysis relative to P2. *, $p \leq 0.05$; ***, $p \leq 0.0001$.

Evaluation of bacterial burden following peroral infection with *K.*

***pneumoniae* B5055 Strep^R in neonatal mice.**

To understand the temporal dynamics of *K. pneumoniae* infection in neonatal mice, we harvested blood and various tissues (brain, gastrointestinal (GI) tract, liver, lungs, and spleen) and counted viable bacteria at 2- and 18-hours post infection (h.p.i.), which is prior to the expected onset of mortality at 24 h.p.i (Figure 2.2B). Two-day-old C57BL/6 mice were perorally infected with $1.3 - 3.3 \times 10^7$ CFU of *K. pneumoniae* B5055 Strep^R. *K. pneumoniae* was detected in the blood and tissues as early as 2 h.p.i., with no significant difference of *K. pneumoniae* observed in the blood, brain, GI tract, liver, lungs, and spleen between 2 and 18 h.p.i. (Figure 2.4A and B). As expected, the GI tract contained high loads of *K. pneumoniae*. Interestingly, the lungs also contained high loads of *K. pneumoniae*, $\sim 4 \log_{10}$ CFU higher, compared to the bacterial load in the brain, liver, and spleen (Figure 2.4B). To assess whether the gavage technique was contributing to the pattern of *K. pneumoniae* dissemination after local infection of the GI tract, neonatal mice were inoculated p.o. with either a 20-gauge or 24-gauge feeding needle with 1.5×10^7 CFU. The 20-gauge feeding needle allows for delivery into the oral cavity whereas the 24-gauge needle allows for a more precise delivery to the back of the throat at the top of the esophagus. Following inoculation, neonatal mice were euthanized at 2 h.p.i., and bacterial burden was evaluated. We observed no significant differences

between colonization, across tissue type, between 20-gauge and 24-gauge infected neonates (**Appendix Figure 5**). We found that it was easier to infect neonates using the 24-gauge gavage needle and used this method for subsequent experiments.

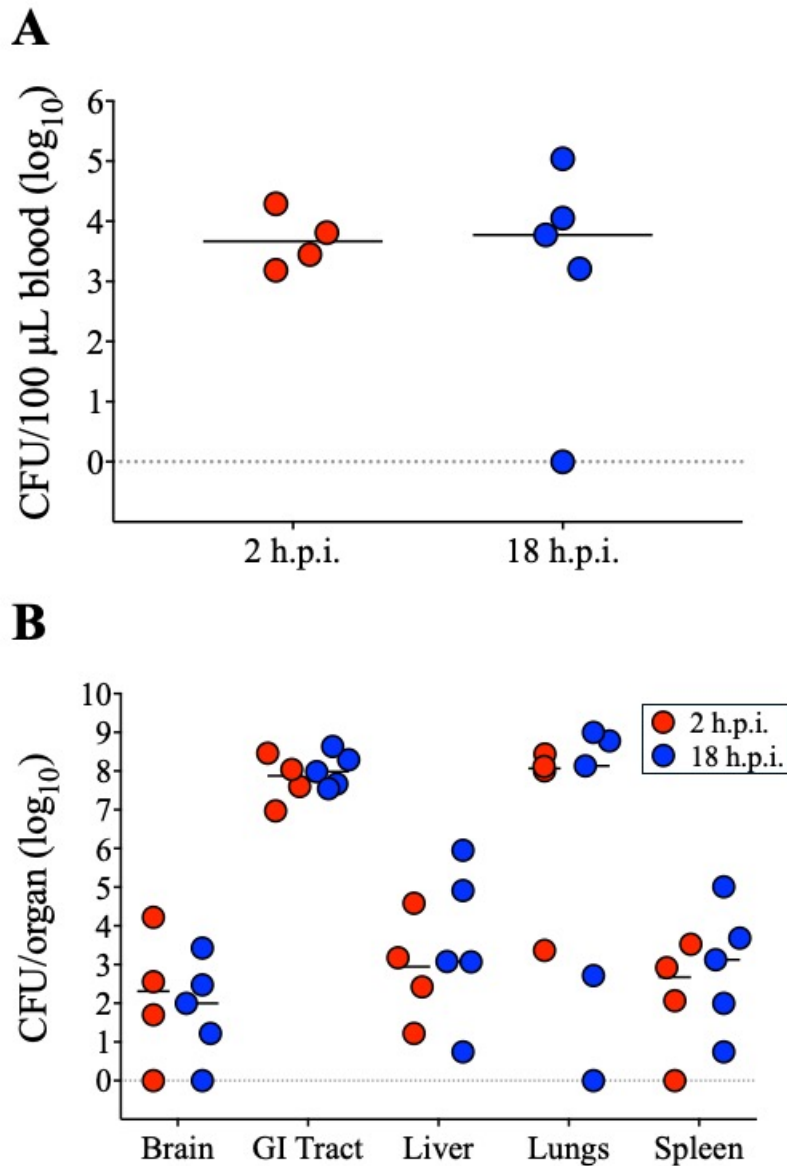


Figure 2.4. Tissue bacterial burden in *K. pneumoniae*-infected neonatal mice.

Two-day-old C57BL/6 mice ($n = 4-5$ per timepoint) were infected perorally with $1.3 - 3.3 \times 10^7$ CFU of *K. pneumoniae* B5055 Strep^R. Bacterial loads in the (A) blood and (B) tissues (brain, intestine, liver, lungs, and spleen) were determined at 2- or 18-hours post infection (h.p.i.). Each point represents an individual mouse. Results shown are one representative experiment performed at least two times. Median is represented by the bar.

Assessment of pathology following peroral infection with *K. pneumoniae*

B5055 Strep^R in neonatal mice.

Having recovered *K. pneumoniae* from the brain, GI tract, liver, lungs, and spleen of infected neonates, we examined whether this dissemination was associated with pathology in these organs. Two-day-old C57BL/6 mice were perorally infected with 6.5×10^7 CFU *K. pneumoniae* B5055 Strep^R and tissues harvested at 18 h.p.i.. We observed no histopathological effects in the brain, GI tract, and spleen (**Appendix Figure 6**), despite bacterial burden in these tissues, while significant inflammation was observed in the liver and lungs of infected neonates at 18 h.p.i. when compared to control Evans blue dye-inoculated mice (**Figure 2.5A**). *K. pneumoniae*-infected neonates displayed inflammatory infiltrate around the portal tract in the liver (**Figure 2.5B**, arrows), and neutrophilic exudate in the alveoli and air spaces, when compared to control Evans blue dye-inoculated mice (**Figure 2.5B**).

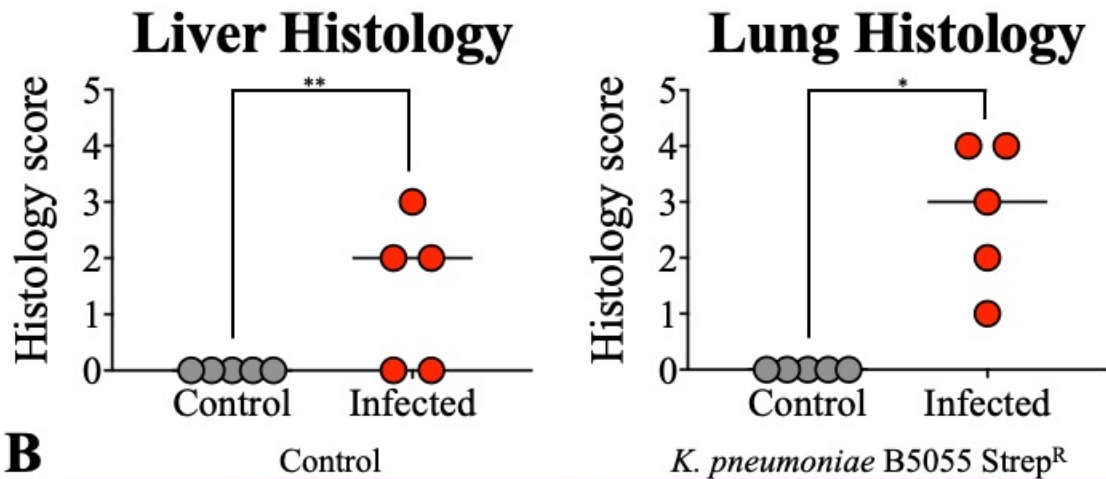
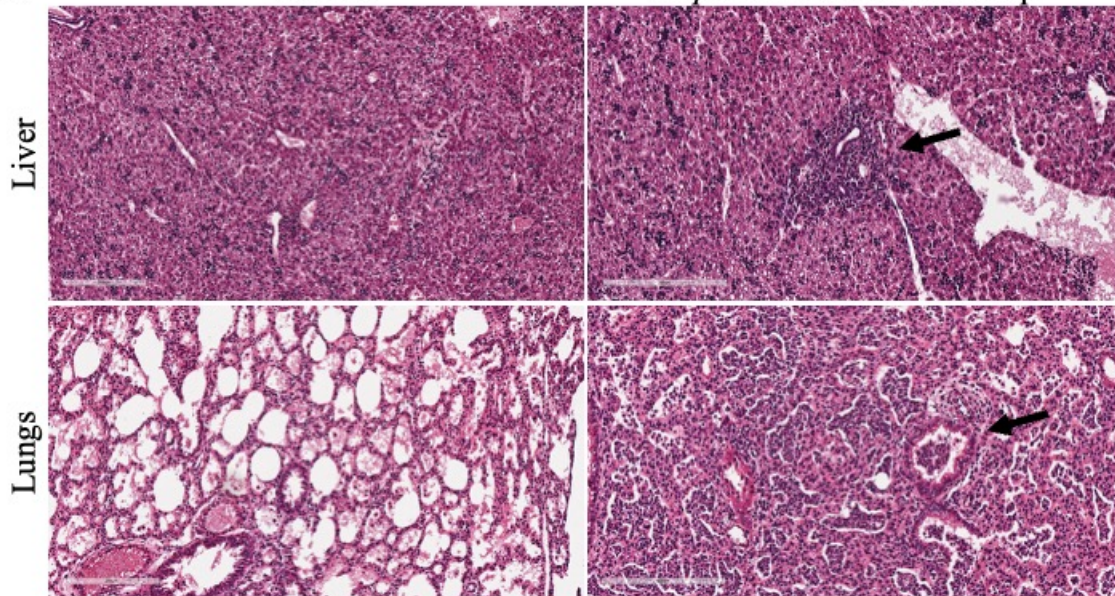
A**B**

Figure 2.5. *K. pneumoniae* B5055 Strep^R induces systemic pathology in neonatal mice.

(A) Liver and lung pathology were scored in *K. pneumoniae* B5055 Strep^R - infected and control two-day-old C57BL/6 mice (n = 5 per group) at 18 h.p.i. Degree of pathology was based on Table 2, with a maximum score of 8. Data points represent an individual mouse from a single experiment (*, $p \leq 0.05$; **, $p \leq 0.001$, by Student's *t* test). (B) Representative images (magnification x 200, scale bar 200 μ m) of H&E-stained liver and lungs sections from neonatal mice at 18 h.p.i. following peroral infection with *K. pneumoniae* B5055 Strep^R (6.5×10^7 CFU/mouse). Arrows indicate the location of liver inflammatory infiltrate around the portal tract and lung neutrophilic exudate in the alveoli and air spaces. Images for *K. pneumoniae* infection are from mice which exhibited pathology, scoring a 3 for the liver and a 4 for the lungs.

Discussion

In this study, we describe a model of *K. pneumoniae*-associated neonatal sepsis using two-to three-day-old C57BL/6 mice, which showed reliable and consistent susceptibility to *K. pneumoniae* following peroral administration. In addition, C57BL/6 mice displayed an age-dependent response to infection and showed an increased inflammatory infiltration into the portal tract of the liver and alveolar spaces of the lungs. This is consistent with observed clinical characteristics of septicemic infants, as studies have demonstrated sepsis-associated liver injury is associated with a higher risk of mortality, and lung injury can be induced by sepsis yielding greater complications including mortality (126–129).

We found that neonatal mice were highly susceptible to *K. pneumoniae* infection, in contrast to previous reports using adult mice. Russo *et al* infected 4 to 6-week-old CD-1, BALB/c or C57BL/6 mice by the intraperitoneal or subcutaneous routes (121). Classical *K. pneumoniae* was only lethal at high doses ($\sim 10^8$ CFU) whereas hypervirulent strains produced an LD₅₀ of 10^3 - 10^5 CFU with mice taking 2-10 days to die. In contrast to these results observed in adult mice, we found that neonatal C57BL/6, BALB/c and CD-1 mice were highly susceptible to intraperitoneal or subcutaneous injection with a hypervirulent *K. pneumoniae* isolate with mice dying within 1.5 days post-infection. Additionally, the LD₅₀ in neonatal CD-1 and C57BL/6 mice was < 100 CFU. Peroral colonization models have also been described for adult mice, but these generally require pre-administration of antibiotics to clear the gut microbiota and circumvent colonization resistance (119). Our results corroborated these previous studies; we observed that C57BL/6 mice aged 10 days or older were resistant to infection with a hypervirulent

strain of *K. pneumoniae*. In contrast, neonatal C57BL/6, BALB/c and CD-1 mice were naturally susceptible to peroral infection with hypervirulent *K. pneumoniae* and did not require antibiotic pre-treatment. The peroral LD₅₀ of *K. pneumoniae* B5055 Strep^R in C57BL/6 neonates was determined to be 8.5×10^6 CFU. Therefore, our neonatal rodent model, unlike current adult models, is capable of establishing robust infection following peroral administration of *K. pneumoniae* which we believe is the natural route of acquisition (119). Our data suggest a heightened susceptibility of newborn mice to *K. pneumoniae* infection that declines rapidly with age. This observation highlights how the normal aging process impacts the susceptibility to *K. pneumoniae*, as has also been observed when comparing pulmonary infection of adult and aged, immunosenescent mice (130).

We found that *K. pneumoniae* B5055 Strep^R infection consistently produced mortality in two-to-three-day old C57BL/6 mice when administered perorally, with *K. pneumoniae* found in tissues as early as 2 h.p.i. (**Figure 2.4A**). *K. pneumoniae* is a leading cause of hospital-acquired infections with colonization and infection driven by environmental contamination, antibiotic selection, and person-to-person transmission (1, 15, 131, 132). Neonatal acquisition of *K. pneumoniae* is believed to occur during the perinatal period, via mother-to-fetus transmission during pregnancy, passage through the birth canal during childbirth, or breast feeding (133–135). Gut colonization with *K. pneumoniae* can be a crucial precursor to bloodstream infection and the development of neonatal sepsis, highlighting the clinical relevance of using peroral administration to study early stages of infection (136, 137). Furthermore, analysis of the CHAMPS data, which identified *K. pneumoniae* as a contributory pathogen in child deaths, determined

21% of child deaths had *K. pneumoniae* in the causal chain of death (68, 69). The most common clinical syndrome among *K. pneumoniae*-related chain deaths in children from this study was sepsis (44%), with *K. pneumoniae* colonizing the blood, heart, lung, liver, and cerebrospinal fluid (69). Our mouse model therefore mimics the early acquisition of *K. pneumoniae*, colonization, and dissemination seen clinically in human neonates.

Human neonates and the elderly are at increased risk for *K. pneumoniae* infection, due to their immature or aging immune responses, respectively; suggesting an age-dependent response to infection (15). Our data corroborate these clinical findings, as adolescent and adult mice were not susceptible to peroral infection with *K. pneumoniae* B5055 Strep^R, in contrast to neonatal mice. This is consistent with other studies showing that adolescent and adult rodents were resistant to *E. coli* and Coxsackievirus infection, whereas neonatal rats or mice were highly susceptible to these pathogens following peroral and intracerebral administration, respectively (107, 138).

We used this optimized neonatal sepsis model to assess bacterial burden in neonatal mice following lethal infection. *K. pneumoniae* B5055 Strep^R colonized the GI tract and lungs and disseminated widely to secondary lymphoid tissues, brain, and liver as early as 2 h.p.i.. In addition, severe pathology was observed in the liver and lungs of infected neonatal mice at 18 h.p.i., compared to uninfected neonatal mice. This pathology is consistent with the hyperinflammatory state that generally develops during sepsis in humans, where neutrophils are activated to produce toxic mediators, damaging the endothelium and alveolar epithelium (139–141). Recent work has also implicated hypervirulent *K. pneumoniae* as a cause of liver damage and abscesses in patients,

particularly in Southeast Asia (142). These findings suggests that neonatal C57BL/6 mice infected with *K. pneumoniae* exhibit important clinical characteristics of disease.

This study has limitations. We optimized the neonatal sepsis model with a reference strain of *K. pneumoniae* belonging to serotype O1:K2 (B5055). Though commonly used in the literature to study *Klebsiella* pathogenesis, this strain may not be representative of neonatal bloodstream isolates. *K. pneumoniae* can be classified into two types, classical and hypervirulent, which primarily cause nosocomial or community-acquired infections, respectively and can be distinguished via genotypic markers (15, 17). B5055 is a hypervirulent strain, and classical *K. pneumoniae* may behave differently in neonatal mice. Additionally, there is significant heterogeneity amongst *K. pneumoniae* isolates in terms of genome composition and virulence factor expression, which could theoretically have an impact on virulence in neonatal mice (143). However, we showed that other clinical *K. pneumoniae* isolates are capable of causing mortality in our model. Future work will be needed to determine if there is a correlation between virulence in neonatal mice and certain characteristics such as genetic loci associated with hypervirulence or other factors. Additionally, we did not evaluate infection of neonates via the respiratory route which is another important route of acquisition of *K. pneumoniae*.

In conclusion, we generated a new animal model for *K. pneumoniae* neonatal sepsis, which is reproducible and provides a strategy for studying microbial pathogenesis and host-microbe interactions. Future studies will expand this model to characterize *K. pneumoniae* isolates collected from neonates with sepsis and will assess protection of

neonates against *K. pneumoniae* infection following maternal immunization with novel vaccines.

Chapter 3 Establish the utility of a passive immunization model to assess protection against *Klebsiella pneumoniae* neonatal sepsis

Introduction

A significant impediment to advancing *K. pneumoniae* neonatal sepsis research has been the lack of a well-characterized and reproducible neonatal sepsis model. This deficiency has limited the ability to investigate pathogenesis, evaluate therapeutic interventions, and assess vaccine candidates in a physiologically relevant manner. Recognizing this critical gap, our work in Chapter 2 successfully characterized and established a *K. pneumoniae* neonatal sepsis mouse model. With this neonatal sepsis model now in place, there is a need to establish an immunization model to assess *Klebsiella* vaccine efficacy. We begin our evaluation focused on establishing a passive immunization model.

Passive immunization represents an attractive approach to provide immediate protection to neonates (144, 145). This strategy bypasses the immature neonatal immune system, arming them with protective humoral immunity. Passive immunization models in the prevention and treatment of bacterial infections are well established (146–150). Specifically, anti-capsular polysaccharide (CPS) antibodies against *K. pneumoniae*, when passively transferred, improved survival, delayed bacteremia, and reduced bacterial burden in a murine sepsis burn model (150).

Building on this evidence of antibody-mediated protection, we pursued an O-polysaccharide (OPS)-based vaccine, as it overcomes the limitation of CPS-based

approaches. *K. pneumoniae* has over 77 known capsular serotypes, thus a vaccine targeting CPS would require the inclusion of >20 K-serotypes to provide broad coverage. In contrast, the limited diversity of O-serotypes makes an OPS-based vaccine a promising strategy for achieving widespread protection (7, 16). Several OPS-based vaccines are currently in preclinical development. The conjugation of OPS to a carrier protein has been shown to elicit robust OPS-specific serum IgG in mice, which were associated with protection against *K. pneumoniae* challenge (16, 62, 151–155). Recently, a novel multiple antigen presenting system (MAPS) vaccine platform was developed (16, 156). This system contains four (O1, O2, O3, and O5) *K. pneumoniae* and eight *P. aeruginosa* OPS antigens biotinylated and chemically linked to fusion proteins. The MAPS vaccine elicited robust antibody responses in mice and rabbits, and the passive transfer of rabbit antisera protected mice against bacterial challenge (156).

Our laboratory previously developed a candidate quadrivalent *K. pneumoniae*/*P. aeruginosa* glycoconjugate vaccine (qKPPA), that includes the chemical linkage of the OPS of *K. pneumoniae* to recombinant flagellin protein FlaA (rFlaA) or FlaB (rFlaB) of *P. aeruginosa*. qKPPA consists of O1:rFlaB, O2:rFlaA, O3:rFlaB, and O5:rFlaB and targets the four major O-serotypes accounting for over 80% of clinically relevant *K. pneumoniae* isolates (7, 62). qKPPA induced robust anti-O1 IgG titers in rabbits which conferred protection in adult CD-1 mice against lethal intravenous challenge with *K. pneumoniae* B5055 following passive transfer (62). The ability of qKPPA antisera to protect against *K. pneumoniae* neonatal sepsis following passive transfer has yet to be assessed.

A critical aspect of evaluating any protective intervention in our neonatal sepsis model relies on relevant outcome measures. Neonatal survival rates following lethal challenge are the most robust indicator of protective efficacy; however bacterial burden can provide insights into control of the infection and the prevention of bacterial dissemination conferred by passively transferred antibodies. Importantly, these two readouts are not always correlated. Tissue damage in sepsis can often precede or occur independently of bacterial clearance, influenced by host inflammatory responses or bacterial toxins, potentially leading to increased mortality (157–160). Therefore, the overarching goal of this study was to provide a comparative evaluation of both survival and bacterial burden in our neonatal sepsis model to establish the utility of a passive immunization model and understand the mechanisms of protection conferred by passive immunization with *K. pneumoniae* antisera.

In this chapter, I determined whether *K. pneumoniae* antisera is protective against neonatal sepsis following passive immunization. Using enzyme-linked immunosorbent assay (ELISA) to measure antibody levels, I confirmed that rabbit antisera generated against heat-killed, capsule-deficient *K. pneumoniae* B5055 (CVD 3001) or the qKPPA conjugate vaccine had elevated levels of anti-O1 and/or anti-K2 serum IgG. Subsequently, utilizing the *K. pneumoniae* neonatal sepsis mouse model established in Chapter 2, I assessed if these antisera, when passively transferred, could enhance survival rates and/or reduce bacterial burden in neonatal mice following lethal challenge with a streptomycin-resistant strain of *K. pneumoniae* B5055 (Strep^R).

Methods

Animals and Ethics Statement

All animal studies were performed in facilities that are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were housed under specific pathogen-free conditions at the University of Maryland School of Medicine, and all procedures were approved by the University of Maryland Baltimore Institutional Animal Care and Use Committee (protocol no. 00000289). To generate neonatal mice for passive immunization, 8-10-week-old pregnant C57BL/6 mice were purchased at gestational day 14 (E14), from Charles River Laboratories. Pups were delivered naturally at term gestation and remained with their dams until they reached the desired age except for brief interruptions due to experimental procedures. Pups of both sexes were used for all experimental procedures. BSL-2 containment was employed for all experiments involving live bacteria.

Bacterial strain and medium

Bacterial strains used in this study are shown in **Table 3.1** and growth conditions are described in Chapter 2.

Table 3.1. *K. pneumoniae* strains used in this study.

Strain	Characteristics (serotype)	Reference
B5055	Reference strain (O1:K2)	(124)
B5055 Strep ^R	Spontaneous streptomycin resistant B5055 (O1:K2)	(161)
CVD 3001	B5055 Δ <i>guaBA</i> Δ <i>wzabc</i> (O1:K-)	(62)

Generation of immune rabbit antisera

K. pneumoniae CVD 3001 and qKPPA were previously generated by Hegerle *et al* (62). We selected CVD 3001 for this study because of its capacity to elicit robust anti-O1 serum IgG without producing anti-K serum IgG. qKPPA was chosen as an experimental vaccine candidate to assess if an OPS-based glycoconjugate vaccine can confer protection against neonatal sepsis.

For CVD 3001, Hegerle *et al* deleted the *guaBA* and *wzabc* genes from wild-type *K. pneumoniae* B5055 by allelic exchange using the lambda-red system (162, 163). Deletion of *guaBA* and *wzabc* were confirmed by PCR sequencing. *K. pneumoniae* OPS (O1, O2, O3, and O5) and recombinant *P. aeruginosa* flagellin FlaA (rFlaA) or FlaB (rFlaB) were produced and purified as previously described (62). Individual OPS were then chemically linked to either rFlaA or FlaB. Conjugation was confirmed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with Coomassie staining.

New Zealand white rabbits were immunized at Cocalico Biologicals (PA) by intramuscular (i.m.) injection at day 0, 14, 28, and 42. Rabbits received either heat-killed CVD 3001 (5 µg/dose) or qKPPA (5 µg of each component/dose; n = 5 rabbits); the first dose was formulated in complete Freund's adjuvant and the remaining doses formulated in incomplete Freund's adjuvant. Sera was collected 28 days following the 4th immunization, qKPPA rabbit antisera was pooled, sterile filtered through a 0.22 µm membrane (MilliporeSigma, Burlington, MA) and stored at -20 °C until use. Rabbit antisera were heat-inactivated prior to immunization.

Immunization and challenge

One-day-old C57BL/6 mice, 24 hours prior to challenge (-24 h), were administered intraperitoneally (i.p.) up to 20 μ L of the following antisera: (i) Heat-killed, CVD 3001 immune rabbit antisera, (ii) qKPPA glycoconjugate vaccine immune rabbit antisera, or no sera (naïve) control. Two hours post challenge (+2 h) mice received a second dose of rabbit antisera i.p. The passive immunization schematic is presented in **Appendix Figure 7**.

Pups were perorally infected at two-days of age ($t = 0$) with *K. pneumoniae* B5055 Strep^R suspended in PBS containing 0.5% weight/volume (w/v) Evans Blue Dye (MilliporeSigma, Burlington, MA) at $5.84 - 6.5 \times 10^5$ CFU in a 10 μ L volume. Mice were then monitored daily for symptoms associated with sepsis for 7 days post infection (d.p.i.). *K. pneumoniae*-associated sepsis symptoms were scored as per **Table 2.2**, and pups with a score ≥ 4 were considered to have met alternative endpoints and were euthanized and recorded as a death.

Quantitative bacteriology

Blood and tissue samples were collected, as described in Chapter 2. Two-day-old C57BL/6 mice were infected perorally with *K. pneumoniae* B5055 Strep^R suspended in PBS containing 0.5% w/v Evans Blue Dye (4.85×10^5 CFU per mouse) at $t = 0$. At 24 hours post infection (h.p.i.), the liver, lungs, and spleen were excised aseptically, weighed, and homogenized in PBS using an Omni International TH-01 tissue homogenizer (Swedesboro, NJ). Blood was extracted following euthanasia into EDTA/KE microtubes (Sarstedt, Numbrecht, Germany). Tissue homogenates and blood

were serially diluted in PBS and spread plated on HS containing streptomycin to determine the *K. pneumoniae* B5055 Strep^R load in tissues. Following incubation at 37°C overnight, colonies were counted and data presented as CFU per gram of organ or CFU per 100 µL blood.

Tissue histopathology

Tissue collection was performed as described in Chapter 2. Two-day-old C57BL/6 mice were infected perorally with *K. pneumoniae* B5055 Strep^R suspended in PBS containing 0.5% w/v Evans Blue Dye (4.85×10^5 CFU per mouse) at $t = 0$. Liver, lungs, and spleen were harvested 24 hours post infection aseptically and instilled in 70% ethanol. Slides were prepared and stained as described in Chapter 2. Histopathological examination of the whole tissue was performed in a blinded fashion by an experienced pathologist. Histopathology was scored per **Table 2.3** on a scale of 0 to 4, with 4 being the greatest degree of pathology for each parameter assessed.

Enzyme-linked immunosorbent assay (ELISA)

Anti-O1 and anti-K2 serum IgG were measured by ELISA. *K. pneumoniae* O1 OPS and K2 CPS were generated as previously described (62, 156) and conjugated to human serum albumin (HSA) and used as coating antigens. 96-well medium binding plates (Greiner Bio-One, Monroe, NC) were coated with either *K. pneumoniae* O1 OPS-HSA or K2 CPS-HSA in PBS at a concentration of 5 µg/mL and incubated overnight at 4 °C. Prior to K2 CPS-HSA coating, plates were incubated with 2 µg/mL of Poly-L-Lysine in PBS for 1 h at 37 °C then washed with PBS-T (PBS containing 0.05% Tween 20). After overnight incubation, plates were washed with PBS-T and blocked with PBS +

10% Omniblok non-fat, dry milk for 2 h at 37 °C. Samples were serially diluted in PBS-T + 10% Omniblok, transferred to blocked ELISA plates, and incubated for 1 h at 37 °C. Plates were washed and incubated for 1 h at 37 °C with horseradish peroxidase (HRP)-labeled anti-mouse IgG (KPL, Gaithersburg, MD). After washing, substrate (3,3',5,5'-tetramethylbenzidine, KPL) was added, and the plates were incubated for 10 min in darkness. The reaction was stopped with the addition of an equal volume of 1 M H₃PO₄, and the absorbance at 450 nm was recorded using a VersaMax microplate reader (Molecular Devices, San Jose, CA). ELISA titers were calculated by interpolation of absorbance values on a standard curve. The endpoint titers reported as ELISA units (EU) /mL represent the inverse of the serum dilution that produced an absorbance value of 0.2 above the blank. Lower limit of quantification (LLOQ) determined by the lowest dilution used in this assay. Seroconversion in immunized adult female mice was defined as a 4-fold increase in the antibody titer compared to the pre-immunization titer.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 7 software (La Jolla, CA). A *p*-value equal to or below 0.05 was considered significant for each test. Survival analyses for Kaplan-Meier curves were accomplished by log-rank test. Statistical significance for recovered CFU after bacterial infection were assessed by two-tailed Mann-Whitney test. Histopathology significance was determined by Student's *t*- test.

Results

Evaluation of anti-O1 and K2 antibody levels in *Klebsiella* rabbit antisera

In preparation for passive transfer experiments, the antigen-specific IgG titers of the immune rabbit antisera were characterized via ELISA. Heat-killed CVD 3001 rabbit antisera exhibited robust anti-O1 (2.12×10^6 EU/mL) and anti-K2 (1.07×10^5 EU/mL) IgG titers (**Figure 3.1**). The production of anti-K2 antibodies was unexpected. This discrepancy suggests pre-existing immunity to K2 in rabbits immunized with heat-killed CVD 3001. Pre-immune rabbit antiserum was assessed to validate these results; we observed anti-K2 (1.3×10^2 EU/mL) IgG titers, confirming pre-existing immunity to K2 in these rabbits. Additionally, pooled qKPPA rabbit antisera had robust anti-O1 (6.13×10^6 EU/mL) IgG titers. As anticipated, given the composition of the qKPPA vaccine, this antiserum did not possess detectable antibodies against the K2 antigen (**Figure 3.1**). These antibody titers allowed for the estimation of antigen-specific IgG titers that would be administered to neonates following passive transfer, as indicated in **Table 3.2**.

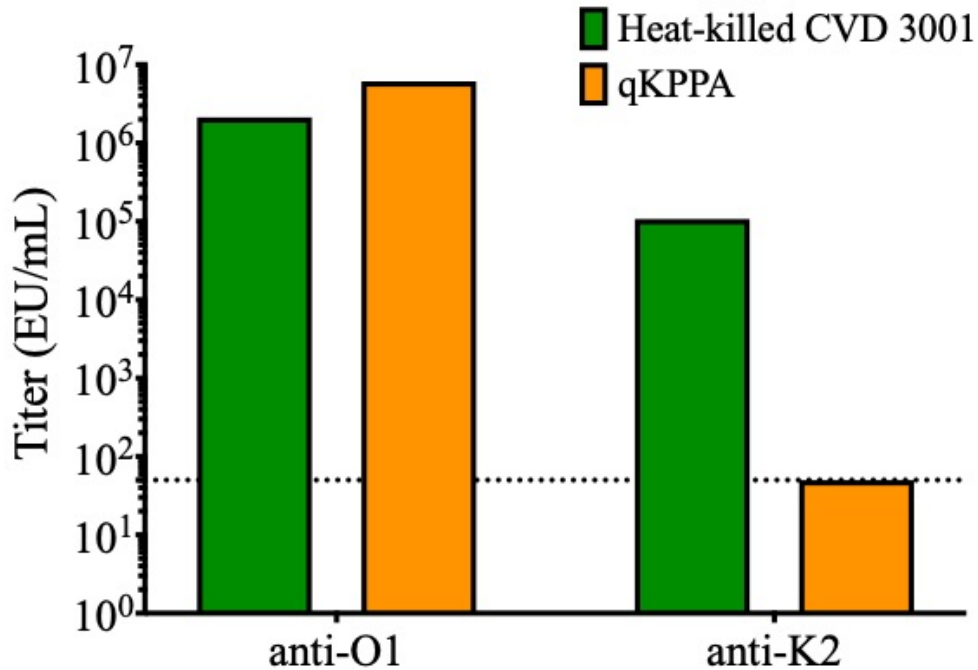


Figure 3.1. Antigen-specific IgG titers for immune rabbit antisera.

Anti-O1 and anti-K2 serum IgG titers from heat-killed CVD 3001 rabbit antisera or qKPPA rabbit antisera measured via ELISA. Lower limit of quantitation (LLOQ) indicated by dash line.

Table 3.2. Antigen-specific IgG titers for immune rabbit antisera.¹

	Immune Rabbit Antisera	
	Heat-killed CVD 3001	qKPPA
Anti-O1 IgG titer	2.12 x 10 ⁶ EU/mL	6.13 x 10 ⁶ EU/mL
Anti-K2 IgG titer	1.07 x 10 ⁵ EU/mL	< LLOQ
Estimated anti-O1 IgG transferred to neonates	~ 4.24 x 10 ⁴ EU	~ 1.23 x 10 ⁵ EU
Estimated anti-K2 IgG transferred to neonates	~ 2.13 x 10 ³ EU	not applicable

¹Lower limit of quantification (LLOQ) = < 50 EU/mL

Examination of survival rates following passive immunization with *Klebsiella* rabbit antisera in neonatal mice and subsequent lethal challenge with *K. pneumoniae* B5055 Strep^R

Having confirmed robust *Klebsiella*-specific IgG titers in the immune rabbit antisera, we proceeded to examine their ability to confer protection in our neonatal sepsis model, established in Chapter 2. One day post-birth which corresponded to 24 h prior to challenge (-24 h), neonatal C57BL/6 mice were passively administered 20 μ L i.p. of either: (i) Heat-killed CVD 3001 rabbit antisera, (ii) qKPPA antisera, or (iii) No sera (naïve control). Twenty-four hours later, two-day-old mice were perorally challenged with $5.84 - 6.50 \times 10^5$ CFU *K. pneumoniae* B5055 Strep^R (t = 0). Two hours post-infection (+2 h), neonatal mice were given a second dose of antisera i.p. and monitored daily for clinical signs of sepsis (**Table 2.2**) and/or mortality for 7 d.p.i.

Survival outcomes revealed a protective effect from heat-killed CVD 3001 rabbit antisera. Neonatal mice administered heat-killed CVD 3001 rabbit antisera exhibited a 57.1% (4/7) survival rate (**Figure 3.2**). In contrast, 0% survival was observed for the mice that received the qKPPA rabbit antisera (0/8) and for the naïve control mice (0/9) (**Figure 3.2**). There was a statistically significant difference ($p = 0.0196$) in survival between the heat-killed CVD 3001 rabbit antisera mice and the naïve control mice. These results highlight the potential of passively transferred antibodies, particularly those elicited by heat-killed *K. pneumoniae*, to confer protection against lethal challenge in our neonatal sepsis model.

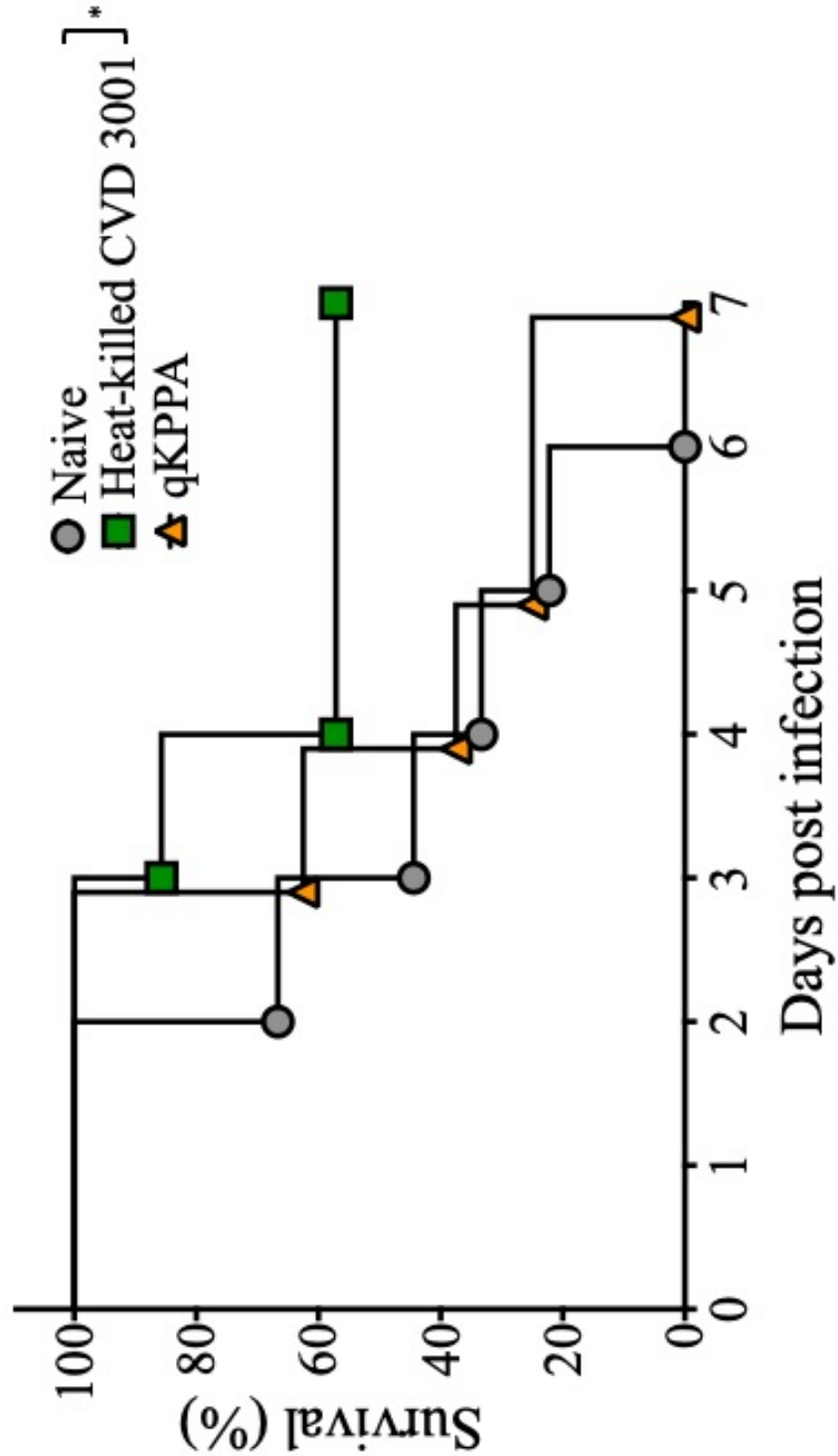


Figure 3.2. Survival curves of neonates passively immunized with *Klebsiella* rabbit antisera and lethally challenged with *K. pneumoniae* B5055 Strep^R.

Survival of neonates passively administered (-24 h and +2 h) either heat-killed CVD 3001 rabbit antisera, qKPPA rabbit antisera, or no sera (naïve) control, and challenged perorally at t=0 with $5.84 - 6.50 \times 10^5$ CFU *K. pneumoniae* B5055 Strep^R (*, $p \leq 0.05$; by log-rank analysis).

Evaluation of bacterial burden following passive immunization with *Klebsiella* rabbit antisera in neonatal mice lethally challenged with *K. pneumoniae* B5055 Strep^R

To evaluate the ability of passively transferred antibodies to prevent bacterial colonization in neonates upon lethal challenge with *K. pneumoniae*, two-day-old mice were passively transferred immune antisera at -24 h and +2 h and perorally challenged with 4.85×10^5 CFU of *K. pneumoniae* B5055 Strep^R at t=0 h. Twenty-four h.p.i., blood, liver, lungs, and spleen were harvested and bacterial loads assessed.

We observed no significant difference in the bacterial load in the blood between heat-killed CVD 3001 rabbit antisera and qKPPA rabbit antisera of neonates when compared to naïve control neonates (**Figure 3.3A**). Similarly, neither heat-killed CVD 3001 rabbit antisera nor qKPPA antisera significantly reduced the bacterial burden in the liver, lungs, and spleen of neonates compared to naïve control neonates (**Figure 3.3B, C, and D**). It is noteworthy, however, that the bacterial burden in the liver of neonates administered heat-killed CVD 3001 rabbit antisera approached significance ($p = 0.0625$) when compared to naïve control neonates, suggesting a trend towards reduced bacterial burden in this specific organ. Collectively, these results suggest the survival advantage conferred by heat-killed CVD 3001 antisera does not correlate with a reduction in bacterial load.

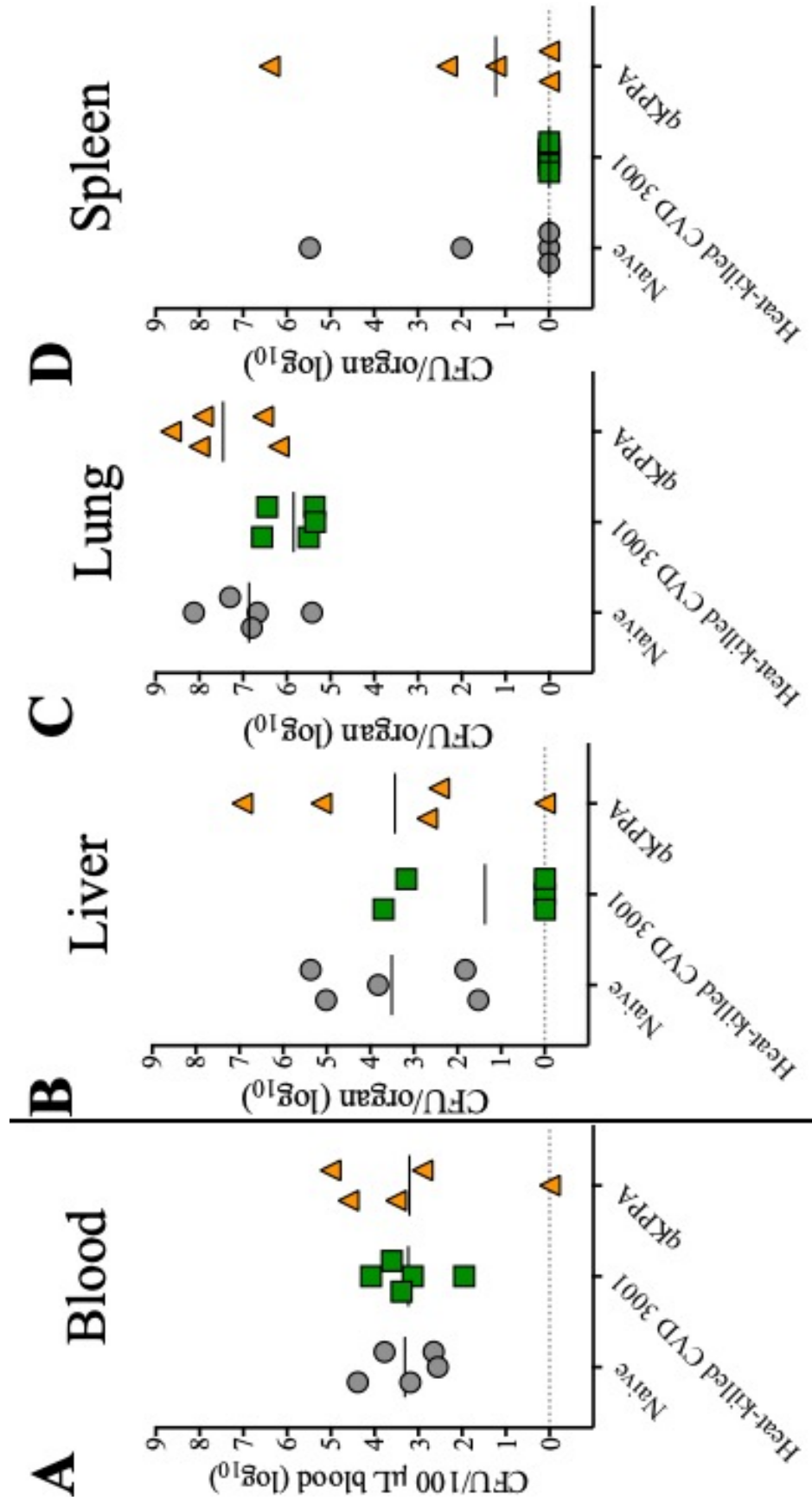


Figure 3.3. Bacterial burden of neonatal mice passively immunized with *Klebsiella* antisera and challenged with *K. pneumoniae* B5055 Strep^R.

Bacterial burden in the A) blood, B) liver, C) lung, and D) spleen of neonatal mice passively immunized with either heat-killed CVD 3001 rabbit antisera, qKPPA rabbit antisera or no sera (naïve control), and subsequently challenged perorally with 4.85×10^5 CFU of *K. pneumoniae* B5055 Strep^R. Median represented by bar. Limit of detection (LOD) indicated by dash line.

Assessment of pathology following passive immunization with *Klebsiella* rabbit antisera in neonatal mice

Organ damage is a hallmark of sepsis, capable of rapidly developing due to a dysregulated host inflammatory response and is often lethal regardless of subsequent bacterial clearance (157–160). Therefore, we aimed to assess whether passively transferred antibodies could mitigate pathology in key organs. Following the established - 24 h/+2 h administration regimen, two-day-old C57BL/6 mice were passively transferred sera and perorally infected with 4.85×10^5 CFU *K. pneumoniae* B5055 Strep^R, and tissues (liver, lungs, and spleen) harvested 24 h.p.i. for histopathological examination.

We observed no histopathological difference in the liver, lungs, or spleen of neonates that received either heat-killed CVD 3001 rabbit antisera or qKPPA rabbit antisera when compared to naïve controls (**Figure 3.4A, B, and C**). This data suggests that while heat-killed CVD 3001 antisera conferred a survival benefit (**Figure 3.2**), antibodies did not prevent tissue damage within these organs at 24 h.p.i. Therefore, antibodies can confer the ability to survive lethal challenge without necessarily correlating with bacterial clearance or reduced tissue pathology.

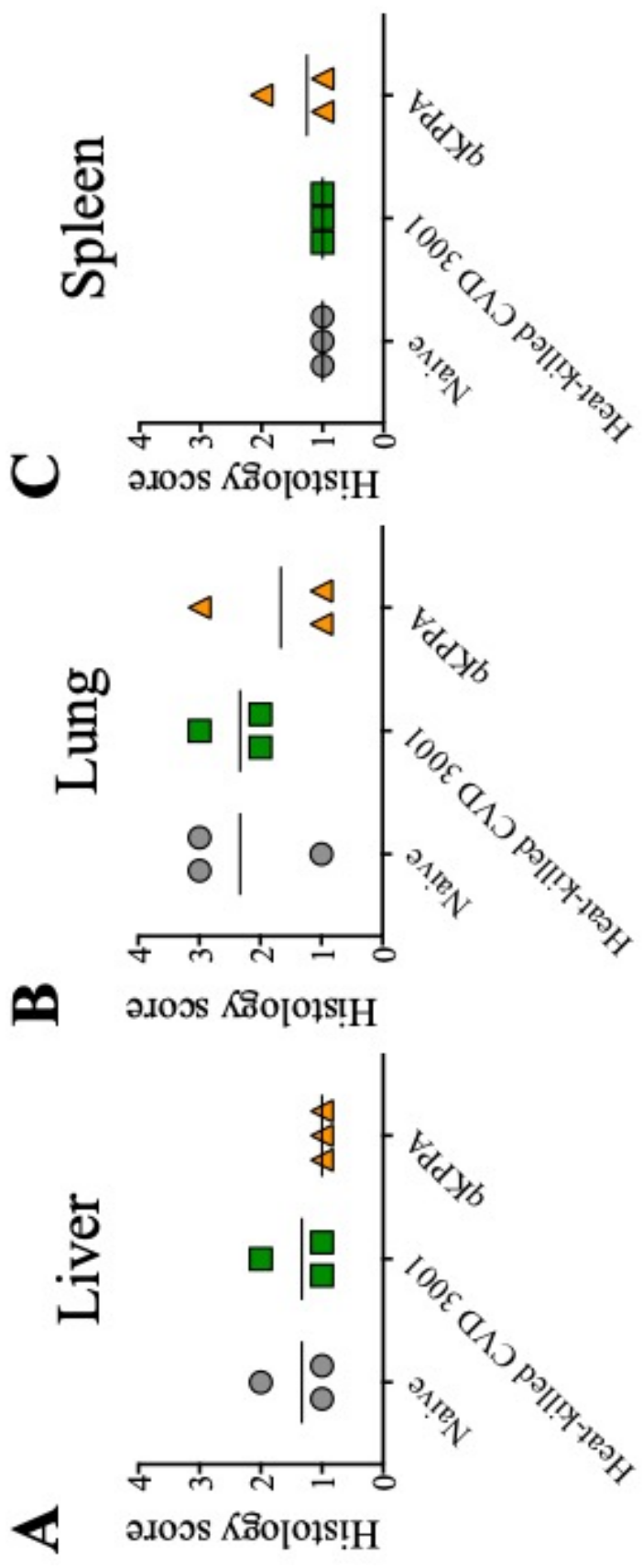


Figure 3.4. Histopathology of neonatal mice passively immunized with *Klebsiella* antisera after challenge with *K. pneumoniae* B5055 Strep^R.

A) Liver, B) lung, and C) spleen pathologies were scored at 24 h.p.i in two-day-old C57BL/6 mice passively immunized (-24 h/+2 h) with either heat-killed CVD 3001 rabbit antisera, qKPPA rabbit antisera, or no sera (naïve control), and subsequently challenged perorally with 4.85×10^5 CFU of *K. pneumoniae* B5055 Strep^R. Degree of pathology was based on Table 2.2, with a maximum score of 4 for each parameter. Data points represent an individual mouse from a single experiment. Bar at GMT.

Discussion

In this study, we investigated the utility of passive immunization to assess protection in a *K. pneumoniae* neonatal sepsis model with two distinct immune rabbit antisera generated against 1) Heat-killed, capsule-deficient *K. pneumoniae* strain CVD 3001 and 2) the qKPPA conjugate vaccine. Our initial characterization of these rabbit antisera revealed robust anti-O1 IgG titers in both the heat-killed CVD 3001 and qKPPA preparations. Interestingly, heat-killed CVD 3001 rabbit antisera contained robust anti-K2 IgG titers, where detectable anti-K2 titers were not observed in the qKPPA antisera. These results were unexpected as heat-killed CVD 3001 is a capsule-deficient strain of *K. pneumoniae*.

Despite robust antibody profiles, a diverse protective outcome was observed. Passive administration of heat-killed CVD 3001 rabbit antisera conferred a significant survival advantage for neonates when compared to naïve controls ($p = 0.0196$). These findings were not applicable for the qKPPA rabbit antisera. Despite having high anti-O1 titers, qKPPA rabbit antisera did not reduce mortality in neonates when compared to naïve controls. Furthermore, bacterial burden in the blood, liver, lungs, and spleen at 24 h.p.i. reveal no significant reduction in bacterial load for neonates receiving either heat-killed CVD 3001 or qKPPA antisera across tissues when compared to naïve controls.

Consistent with the bacterial burden data, histopathological examination of the aforementioned tissues showed no pathological differences between neonates that received either heat-killed CVD 3001 or qKPPA antisera compared to naïve controls.

The dissociation between survival conferred by the heat-killed CVD 3001 rabbit antisera and the lack of reduction in bacterial load or tissue pathology presents a paradox. These findings suggest that protection is conferred via mechanisms other than direct bacterial killing or through tissue damage prevention. Several potential mechanisms could explain this including toxin neutralization, immune modulation, decreased bacterial dissemination, or reduced adhesion (164).

The heat-killed CVD 3001 rabbit antisera is a polyclonal preparation against whole bacteria, thus containing a diverse array of antibodies which are potentially capable of neutralizing various secreted virulence factors/toxins. Prevention of these antigens from triggering an inflammatory cascade, could reduce systemic toxicity and improve survival, even in the presence of high bacterial loads. This aligns with the concept that a dysregulated immune response drives sepsis (157–160). Therefore, antibodies elicited against the heat-killed CVD 3001 preparation could improve survival through neutralization, which in turn can effectively promote the dampening of cytokine release (i.e., immune modulation), reduce overall toxicity, and improve host outcomes.

Furthermore, though not statistically significant, the trend towards significance in the bacterial load present in the liver of neonates administered the heat-killed CVD 3001 rabbit antisera ($p = 0.0625$) suggest these antibodies could limit early bacterial dissemination. This may be due to the presence of ant-MrkA antibodies. MrkA, a type 3 fimbriae, is involved in the colonization and adherence of *K. pneumoniae* to host cells,

which is critical for subsequent dissemination and establishment of infection (15, 47). By targeting MrkA, these antibodies would hinder bacterial adhesion and, inadvertently decrease bacterial colonization and dissemination. While a trend in the liver was observed, the quantity of these antibodies may not have been sufficient to reduce the bacterial load across tissues.

The inability of parenterally-administered qKPPA antisera to confer protection, despite having elevated anti-O1 IgG titers suggests at least three possibilities: (i) IgG directed solely against the O-antigen may not be sufficient for protection against *K. pneumoniae* B5055 sepsis in neonatal mice, (ii) protection against *K. pneumoniae* infection requires synergy between anti-O and other antibody specificities, which are not present in this antisera or (iii) a broad and sustained immune response, is necessary to effectively establish protection with a glycoconjugate vaccine, which is not achieved through passive administration. To improve outcomes and further elucidate protective mechanisms, future studies will focus on strategies to elicit a comprehensive and sustained immune response to improve the qKPPA vaccine's protective efficacy.

This study has limitations. Safe i.p. administration of rabbit antisera was limited to a maximum volume of 40 μ L per mouse. This constrained volume required very high antibody titers in the sera to achieve a therapeutic effect. Furthermore, the timing of administration was highly constrained by the window of susceptibility to *K. pneumoniae* in C57BL/6 neonates. As previously described in Chapter 2, susceptibility to *K. pneumoniae* infection declines with age, thereby narrowing our window for both infection and subsequent antisera administration. Additionally, we only quantified anti-O1 and anti-K2 serum IgG titers, which may not fully represent the diverse antibody

repertoire or their functional activity. Future studies will characterize antibodies against other antigens and will also evaluate different antibody classes and subtypes to elucidate their protective mechanisms.

In summary, passive administration of heat-killed CVD 3001 rabbit antisera, effectively conferred protection against *K. pneumoniae* through improved survival rates but failed to significantly reduce bacterial burden or pathology using our *K. pneumoniae* neonatal sepsis model. Additionally, qKPPA rabbit antisera failed to improve survival rates or confer protection against *K. pneumoniae* bacterial burden and pathology at the doses evaluated in this study. Although the qKPPA rabbit antisera were not effective in our neonatal sepsis model, the results of this study are informative in that it suggests that protection against *K. pneumoniae* can be achieved in neonates. Second, our study suggests that a broad, polyclonal antibody response is critical for survival in a neonatal infection, even if substantial reduction in bacterial load or tissue damage is not observed. Likewise, these results raise interesting immunological questions about how maternal immunization, which would establish a sustained immune response, could potentially overcome the observed limitation of the quadrivalent glycoconjugate vaccine.

Chapter 4 Determine the suitability of a maternal immunization model for evaluating protection against *Klebsiella pneumoniae* neonatal sepsis

Introduction

Maternal immunization is a promising strategy to protect neonates from infectious diseases, especially during the critical window before their immune system is fully developed. This approach relies on the transplacental transfer of maternal antibodies and the transfer of antibodies through breast milk, providing immediate and constant passive immunity to their offspring. While maternal immunization animal models exist for other neonatal pathogens, such as GBS, *E. coli*, and *Bordetella pertussis*, there remains a significant gap in model development for *K. pneumoniae*.

K. pneumoniae remains a leading cause of neonatal sepsis in LMICs (66, 68, 114, 115). With the prevalence of MDR and hypervirulent *K. pneumoniae* strains increasing globally, coupled with the lack of novel antibiotics in development, there is an urgent need for alternative preventive strategies. Given the immunologic immaturity of neonates, passive protection via maternally derived antibodies represents an opportunity to prevent neonatal sepsis. Our quadrivalent *K. pneumoniae* vaccine (qKPPA), as previously described, is immunogenic and able to protect adult mice against lethal infection with *K. pneumoniae* ((62) and unpublished data). We hypothesized that the qKPPA vaccine can produce a robust humoral response in pregnant mice, allowing for the transplacental transfer and transfer via breast milk of antibodies to neonates, conferring protection to neonates following lethal challenge with *K. pneumoniae*.

Here, I establish proof of concept for maternal *K. pneumoniae* vaccination with one of the components of the qKPPA vaccine, O1:rFlaB. I describe the immunogenicity of the O1:rFlaB glycoconjugate in neonatal mice following maternal immunization and assess protection utilizing the neonatal sepsis mouse model developed in Chapter 2. I also use heat-killed *K. pneumoniae* B5055 Strep^R as a positive control vaccine. Antigen-specific antibody titers elicited by maternal immunization and protection against tissue colonization and pathology upon *K. pneumoniae* B5055 Strep^R challenge in neonatal mice will be described herein.

Methods

Animals and ethics statement

All animal studies were performed in facilities that are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were housed under specific pathogen-free conditions at the University of Maryland School of Medicine, and all procedures were approved by the University of Maryland Baltimore Institutional Animal Care and Use Committee (protocol no. 00000289). Female C57BL/6 mice were used to examine survival and bacterial burden following maternal immunization in neonatal mice. Five-week-old C57BL/6 (both sexes) mice were purchased from Charles River Laboratories (Wilmington, MA). Pups were delivered naturally at term gestation and remained with their dams until they reached the desired age except for brief interruptions due to experimental procedures. Pups of both sexes were used for all experimental procedures. BSL-2 containment was employed for all experiments involving live bacteria.

Bacterial strain and medium

A streptomycin-resistant strain of *K. pneumoniae* (B5055 Strep^R) strain was used in this study, and growth conditions are described in Chapter 2.

Enzyme-linked immunosorbent assay (ELISA)

Serum IgG was measured by ELISA as described in Chapter 3.

Maternal immunization

K. pneumoniae B5055 Strep^R was recovered from cryopreservation on HS agar prior to inoculation in HS broth for overnight growth at 37°C, 220 revolutions per minute (rpm) with aeration for 24 h. Bacteria were concentrated by centrifugation then diluted with sterile PBS to achieve the correct dosage. Bacteria were heat-killed at 60°C for 30 minutes prior to immunization.

Five-week-old female C57BL/6 mice were injected intraperitoneally (i.p.) in the right abdomen, before pregnancy, on day 0, 14, and 28 or injected i.p. on day 0, 28, and intramuscularly (i.m.) at embryonic day 14 (E14) ,during pregnancy, with either 1×10^8 CFU heat-killed *K. pneumoniae* B5055 Strep^R suspended in 100 μ L of PBS, *K. pneumoniae* O1:rFlaB glycoconjugate vaccine ($\leq 20 \mu$ g) mixed 1:1 with aluminum hydroxide (Alum) adjuvant (Alhydrogel 2%, InvivoGen, San Diego, CA), or Alum alone (**Table 4.1**). Female mice were injected i.m. at E14 to avoid accidental injection or injury to the embryonic sac. Blood was collected retro-orbitally one day prior to each immunization and two weeks after the third immunization to determine serum antibody titers.

Table 4.1. Immunogens/vaccine used in this study.

Immunogen	Dose
<i>K. pneumoniae</i> B5055 Strep ^R	Up to 10 ⁸ CFU
<i>K. pneumoniae</i> glycoconjugate vaccine O1 OPS:rFlaB + aluminum hydroxide 2% (1:1 ratio)	≤ 20 µg
Aluminum hydroxide 2%	Not applicable

Vaccine efficacy in neonates as determined by survival

To assess the protection of pups, following immunization, two female mice from each group were mated with male mice. Two days after birth, half litters from each group, were challenged perorally (p.o.) with *K. pneumoniae* B5055 Strep^R suspended in PBS containing 0.5% weight/volume (w/v) Evans Blue Dye (MilliporeSigma, Burlington, MA) at $6.29 \times 10^5 - 2.59 \times 10^6$ CFU in a 10 µL volume. Remaining pups were euthanized and their blood collected and pooled to detect and quantify *K. pneumoniae*-specific antibodies. Neonatal mice challenged p.o. were observed daily for signs of sepsis as per **Table 2.2** and mortality until 7 d.p.i.

Vaccine efficacy in neonates as determined by bacterial burden of blood and tissues

Blood and tissue samples were collected as described in Chapter 2. Two-day-old C57BL/6 mice were infected perorally with *K. pneumoniae* B5055 Strep^R suspended in PBS containing 0.5% w/v Evans Blue Dye ($2.75 - 3.3 \times 10^5$ CFU per mouse). At 24 hours post infection (h.p.i.), the liver, lungs, and spleen were excised aseptically, weighed, and homogenized in PBS using an Omni International TH-01 tissue homogenizer (Swedesboro, NJ). Blood was extracted following euthanasia into EDTA/KE microtubes (Sarstedt, Numbrecht, Germany). Tissue homogenates and blood

were serially diluted in PBS and spread plated on HS containing streptomycin to determine the *K. pneumoniae* B5055 Strep^R load in tissues. Following incubation at 37°C overnight, colonies were counted and data presented as CFU per gram of organ or CFU per 100 µL blood.

Tissue histopathology

Tissue collection, slide preparation, and pathology analysis were accomplished as described in Chapter 2. Two-day-old C57BL/6 mice were infected perorally with *K. pneumoniae* B5055 Strep^R suspended in PBS containing 0.5% w/v Evans Blue Dye (2.75 – 3.3 x 10⁵ CFU per mouse). Twenty-four hours post infection liver, lungs, and spleen were harvested aseptically and instilled in 70% ethanol. Histopathology was scored per **Table 2.3** on a scale of 0 to 4, with 4 being the greatest degree of pathology for each parameter assessed.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 7 software (La Jolla, CA). A *p*-value equal to or below 0.05 was considered significant for each test. Survival analyses for Kaplan-Meier curves were accomplished by log-rank test. Statistical significance for recovered CFU after bacterial infection were assessed by two-tailed Mann-Whitney test. Histopathology significance was determined by Student's *t*- test.

Results

Optimization of the immunization schedule

We aimed to examine the ability of maternal immunization prior to pregnancy, and during the perinatal period, to prime the immune system. In an initial experimental phase, we administered five-week-old female mice with three doses of the following immunogens or vaccines via the i.p. route: (i) 10^8 CFU of heat-killed *K. pneumoniae* B5055 Strep^R (heat-killed B5055), (ii) 10 μ g of O1:rFlaB formulated 1:1 with alum, or (iii) alum alone two weeks apart (day 0, 14, and 28) (**Appendix Figure 8**). We observed robust anti-O1 IgG titers in mice immunized with heat-killed B5055 (**Appendix Figure 9**), conferring protection in neonates following lethal challenge (**Appendix Figure 10**). However, the O1:rFlaB vaccine at 10 μ g/dose generated low anti-O1 IgG titers and did not confer protection. To address this, we revised the immunization schedule, increasing the O1:rFlaB dose to 20 μ g, with animals receiving two doses i.p., four weeks apart (day 0 and 28), and a third dose i.m. during pregnancy (at E14) to enhance the primary response and potentially establish a more robust antibody pool. Female mice were injected i.m. at E14 to prevent penetration or damage to the embryonic sac. A schematic of this immunization schedule is shown in **Figure 4.1**.

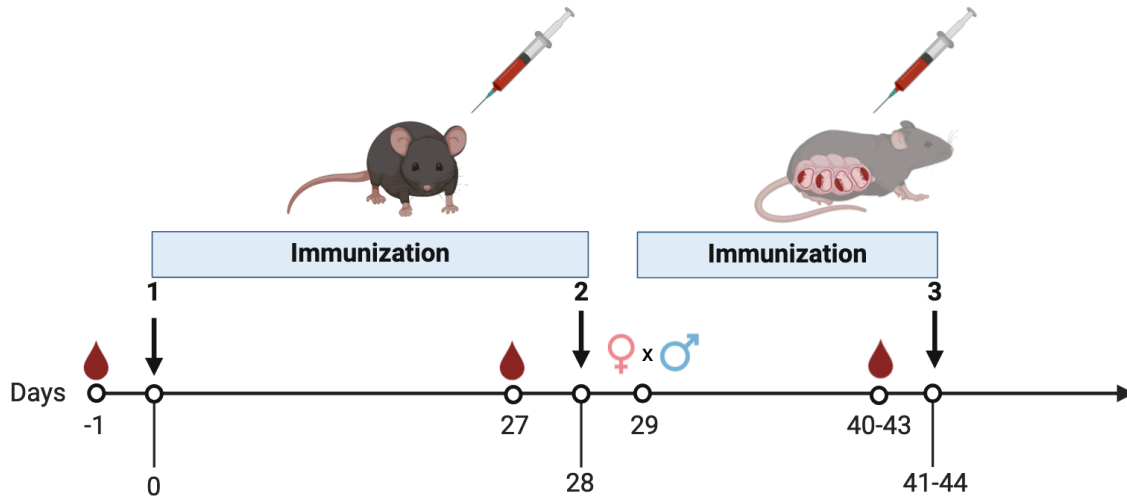


Figure 4.1. Immunization schematic to assess immunogenicity and bacterial burden following maternal immunization.

Evaluation of antibody responses following immunization to assess immunogenicity

Heat-killed B5055 and O1:rFlaB + alum elicited robust anti-O1 and anti-K2 serum IgG titers in adult C57BL/6 mice as compared to alum alone-treated C57BL/6 mice (**Figure 4.2A, and B**). Following three doses, anti-O1 serum antibody titers in heat-killed B5055-immunized dams were elevated compared to adjuvant alone dams for both immunogenicity and bacterial burden studies, with seroconversion (\geq four-fold rise above baseline titers) occurring after one dose. Similarly, anti-K2 serum titers in heat-killed B5055 immunized dams were also elevated when compared to adjuvant alone mice. A more complex pattern emerged with the O1:rFlaB + aluminum hydroxide immunization. Dams designated for immunogenicity studies had elevated anti-O1 and anti-K2 serum IgG titers (post-immunization) when compared to the adjuvant alone group. However, anti-K2 serum IgG titers were not present in O1:rFlaB-immunized dams

designated for the bacterial burden studies, despite receiving the same three-dose regimen. Given that the O1:rFlaB does not contain any capsular polysaccharide antigens, this discrepancy suggests a potential exposure to *K. pneumoniae* or another pathogen with a similar capsule during the study. Overall, this data confirms both immunization strategies successfully stimulated a strong antigen-specific immune response to both O1 and K2 antigens of *K. pneumoniae*.

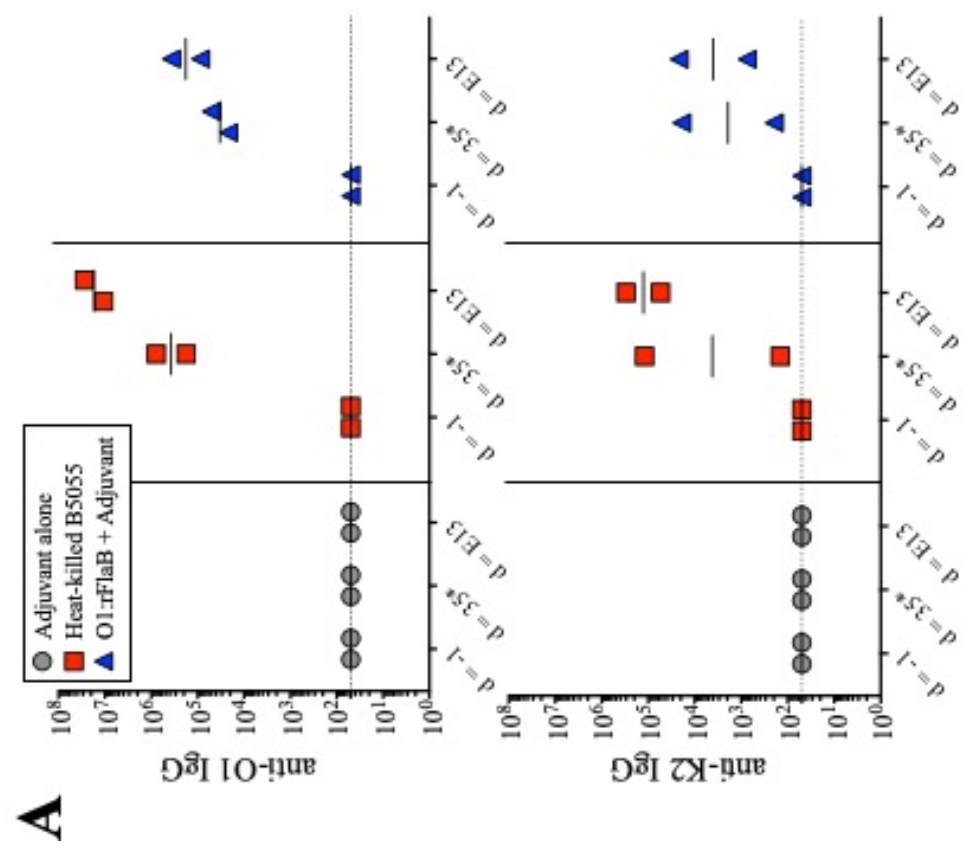
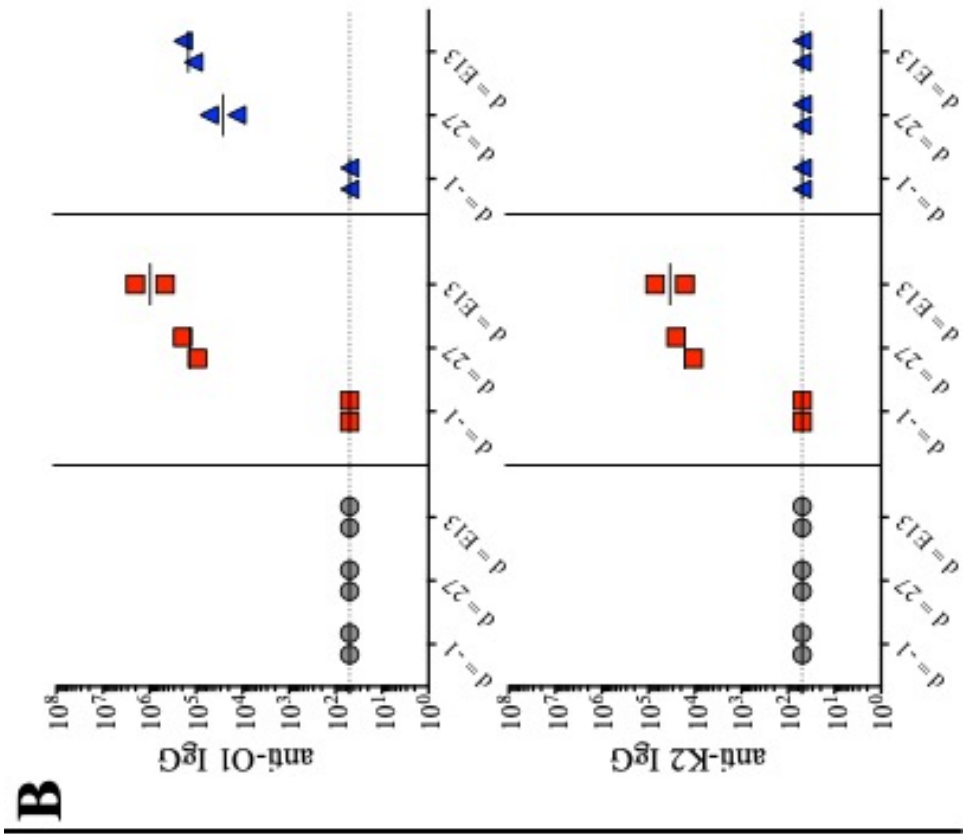


Figure 4.2. Maternal antibody responses following immunization with heat-killed *K. pneumoniae* B5055 Strep^R, O1:rFlaB + adjuvant, or adjuvant alone.

Five-week-old female C57BL/6 mice received two doses of either heat-killed *K. pneumoniae* B5055 Strep^R, O1:rFlaB + adjuvant, or adjuvant alone, spaced 4 weeks apart. A third dose was administered ~2 weeks later during the perinatal period, at embryonic day 14, with serum collected 1 day prior to each immunization (day -1, day 27 (35*), day E13). A) Anti-O1 and anti-K2 serum IgG titers in dams utilized to assess immunogenicity. B) Anti-O1 and anti-K2 serum IgG titers from dams utilized to assess bacterial burden. Bar at GMT.LLOQ (< 50 EU/mL) indicated by dash line.

*Female mice utilized to assess immunogenicity in neonates did not receive their second dose until day 35 due to death in male breeders.

Evaluation of maternal antibody transfer to neonates prior to lethal challenge with *K. pneumoniae* B5055 Strep^R

To assess *K. pneumoniae*-specific maternal antibodies transferred to neonates, two-day-old C57BL/6 mice born to immunized dams were euthanized and blood harvested via decapitation, with mice pooled within each group. Serum was then isolated from these pooled samples and used to determine circulating maternal antibody levels.

Robust anti-O1 and anti-K2 serum IgG titers were detected in neonates born to female dams immunized with either heat-killed B5055 or O1:rFlaB + adjuvant (**Figure 4.3, Table 4.2, and Table 4.3**). Neonates from heat-killed B5055 immunized dams exhibited notably higher anti-O1 and anti-K2 IgG titers compared to adjuvant alone control dams. Similarly, neonates born to O1:rFlaB + adjuvant immunized dams showed elevated anti-O1, and anti-K2 IgG serum titers compared to adjuvant alone neonates, though not as robust. As previously mentioned, anti-K2 serum IgG titers were unexpected in our O1:rFlaB + alum group, highlighting a potential *K. pneumoniae* exposure during the course of this study. Furthermore, we examined the ratio of maternal antibodies transferred to neonates. Blood was collected from dams at embryonic day 13 (E13), prior to their third immunization, and sera isolated to evaluate antibody levels. E13 dam sera

was then compared to two-day-old neonatal sera prior to lethal challenge *with K. pneumoniae* B5055 Strep^R. We observed a modest transfer ratio (< 1) of both anti-O1 and anti-K2 IgG in our heat-killed B5055 and O1:rFlaB immune pups (**Table 4.2 and 4.3**). data confirms that maternal immunization with both vaccines successfully elicited and facilitated the transplacental transfer of *K. pneumoniae*-specific IgG antibodies from dams to their offspring.

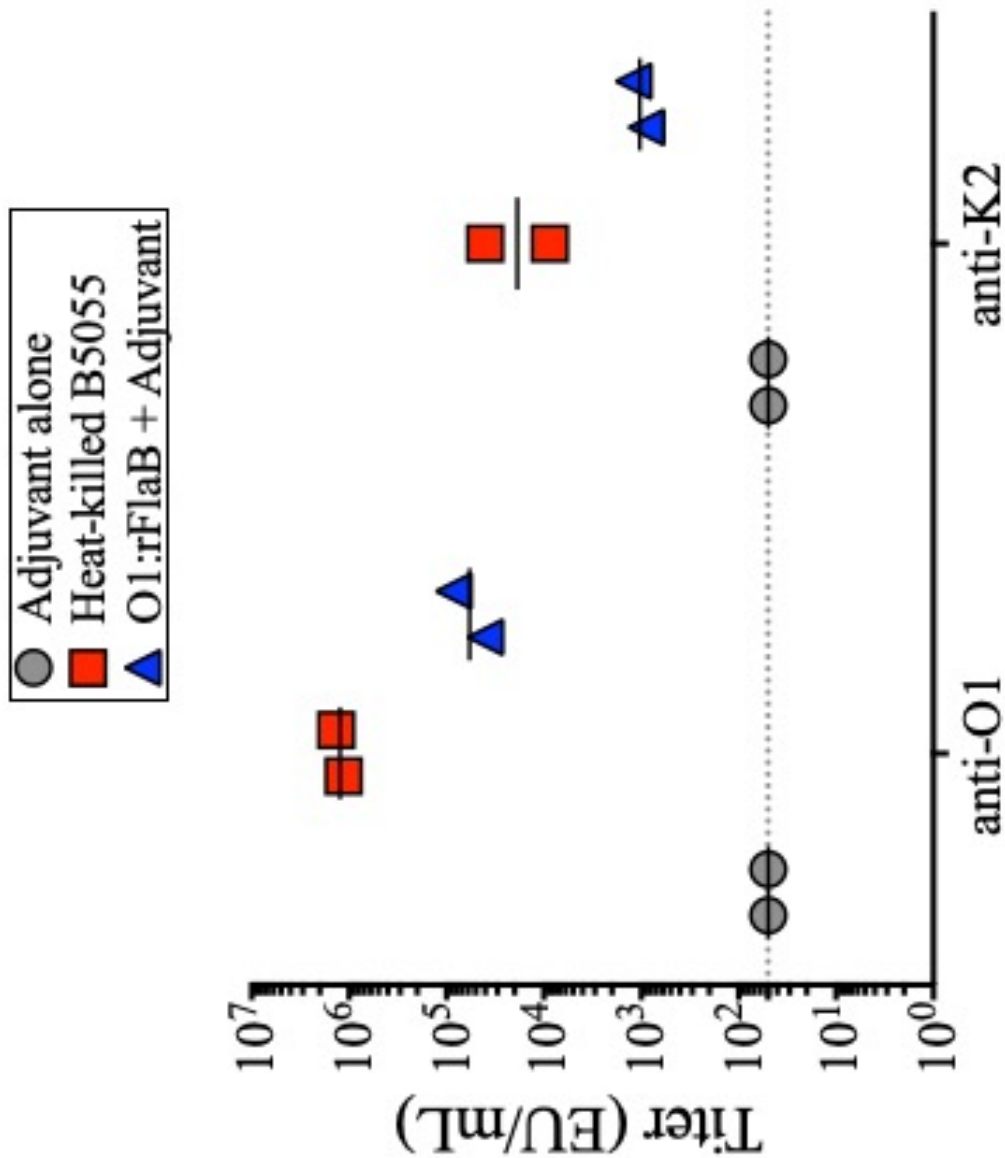


Figure 4.3. Maternal antibody transfer to neonates following immunization with heat-killed *K. pneumoniae* B5055 Strep^R, O1:rFlaB glycoconjugate vaccine + adjuvant, or adjuvant alone.

Anti-O1 and anti-K2 pooled serum IgG titers from two-day-old C57BL/6 neonates born to dams immunized with either heat-killed *K. pneumoniae* B5055 Strep^R, O1:rFlaB + adjuvant, or adjuvant alone. Bar at GMT. LLOQ (< 50 EU/mL) indicated by dash line.

Table 4.2. Maternal transfer of anti-O1 IgG to neonates¹

Dam	Heat-killed		O1:rFlaB		Adjuvant alone	
	Dam 1	Dam 2	Dam 1	Dam 2		
E13 (EU/mL)	2.78 x 10 ⁷	1.08 x 10 ⁷	3.73 x 10 ⁵	8.76 x 10 ⁴	< LLOQ	< LLOQ
Neonatal titer (EU/mL)	1.14 x 10 ⁶	1.36 x 10 ⁶	8.47 x 10 ⁴	4.02 x 10 ⁴	< LLOQ	< LLOQ
Ratio	0.041	0.126	0.227	0.459	Not applicable	Not applicable

¹ Lower limit of quantification (LLOQ) < 50 EU/mL**Table 4.3. Maternal transfer of anti-K2 IgG to neonates¹**

Dam	Heat-killed		O1:rFlaB		Adjuvant alone	
	Dam 1	Dam 2	Dam 1	Dam 2		
E13 (EU/mL)	1.21 x 10 ⁵	5.48 x 10 ⁴	8.92 x 10 ²	2.19 x 10 ⁴	< LLOQ	< LLOQ
Neonatal titer (EU/mL)	4.03 x 10 ⁴	8.73 x 10 ³	7.47 x 10 ²	1.21 x 10 ³	< LLOQ	< LLOQ
Ratio	0.333	0.159	0.837	0.055	Not applicable	Not applicable

¹ Lower limit of quantification (LLOQ) < 50 EU/mL

Examination of survival rates following maternal immunization in neonatal mice lethally challenged with *K. pneumoniae* B5055 Strep^R

Having demonstrated robust anti-O1 and anti-K2 in neonates born to dams immunized with *Klebsiella* vaccines, we proceeded to examine the protective efficacy of these maternal antibodies against lethal *K. pneumoniae* challenge. Two-day-old C57BL/6 mice were perorally infected with 6.29 x 10⁵ – 2.59 x 10⁶ CFU *K. pneumoniae* B5055 Strep^R. As demonstrated in **Figure 4.4**, we observed 0% survival in neonatal mice born to heat-killed B5055 Dam 1 (0/6), O1:rFlaB + adjuvant Dam 1 (0/6), O1:rFlaB + adjuvant Dam 2 (0/4) and adjuvant alone (0/11). Neonatal mice born from heat-killed B5055 Dam 2 exhibited 80% survival (4/5). There was a statistically significant difference ($p \leq 0.05$) in survival curves between heat-killed B5055 Dam 2 neonates and adjuvant alone neonates. There was also a statistically significant difference ($p \leq 0.01$) in survival curves

between O1:rFlaB + adjuvant Dam 1 neonates and adjuvant alone neonates where O1:rFlaB + adjuvant Dam 1 neonates died more quickly than control animals. These results not only highlight the intrinsic variability in protection following maternal immunization but also underscore the potential for whole-cell vaccine preparations (heat-killed B5055) to confer robust protection. The consistent 0% survival across the O1:rFlaB + adjuvant groups suggest this specific glycoconjugate vaccine, under these experimental conditions, does not elicit sufficient protective immunity to prevent mortality following lethal challenge with *K. pneumoniae* B5055 Strep^R.

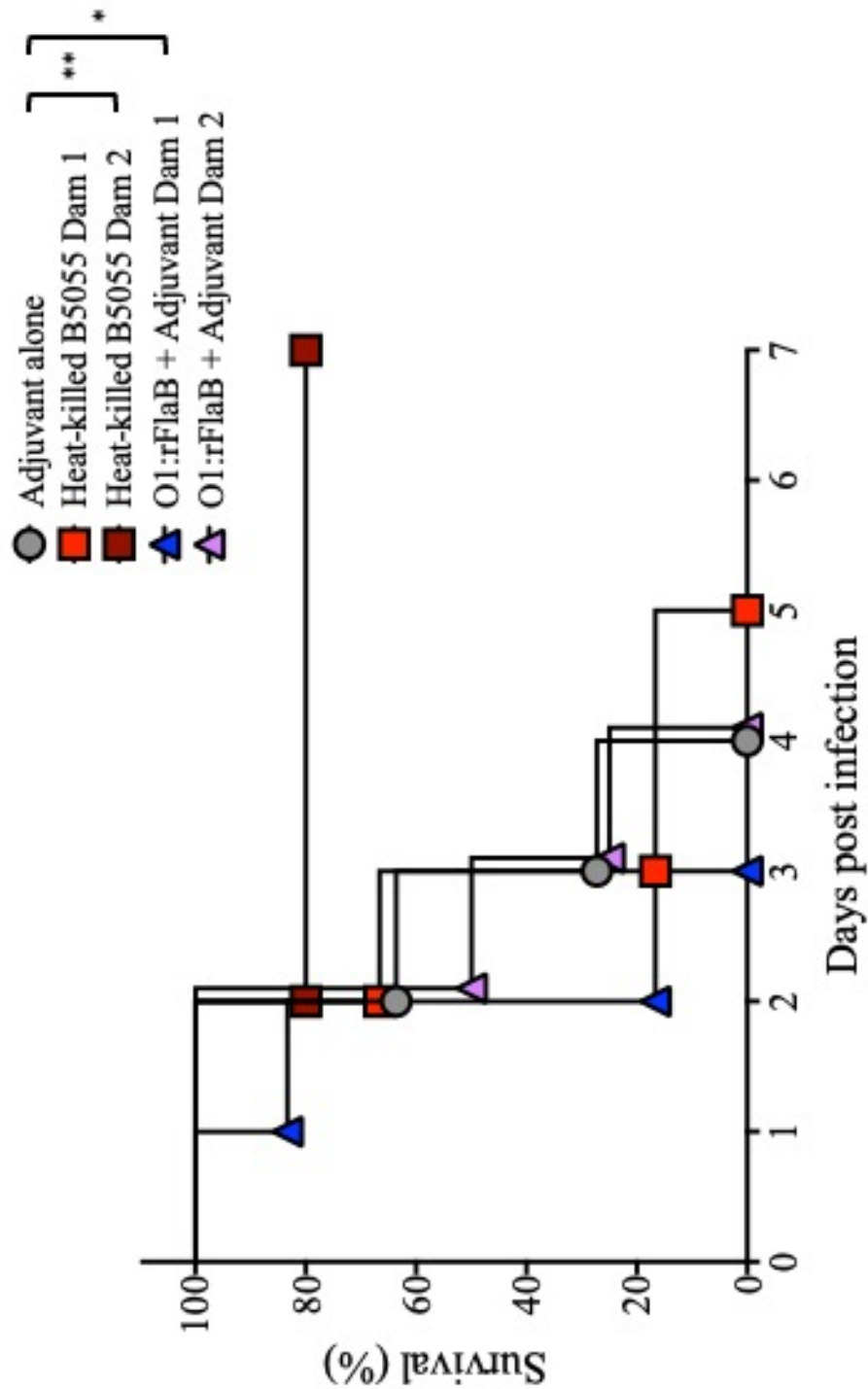


Figure 4.4. Survival curves of neonates born to immunized dams following lethal challenge with *K. pneumoniae* B5055 Strep^R.

Survival of two-day-old C57BL/6 neonates born to dams immunized with either heat-killed *K. pneumoniae* B5055 Strep^R, O1:rFlaB + adjuvant, or adjuvant alone, and challenged perorally at two days post-birth with *K. pneumoniae* B5055 Strep^R (*, $p \leq 0.05$; **, $p \leq 0.01$, by log-rank analysis).

Examination of bacterial burden following maternal immunization in neonatal mice following lethal challenge with *K. pneumoniae* B5055 Strep^R

To evaluate the ability of maternal immunization to reduce bacterial colonization in neonates upon *K. pneumoniae* challenge, two-day-old C57BL/6 mice were perorally challenged with $2.75 - 3.3 \times 10^5$ CFU of *K. pneumoniae* B5055 Strep^R at 24 h.p.i. and then blood, liver, lungs, and spleen were harvested and viable counts were assessed. We observed a significant difference in bacterial burden in the blood between heat-killed B5055 neonates ($p = 0.023$) when compared to adjuvant alone neonates (**Figure 4.5A**). No significant difference was observed in the blood burden between O1:rFlaB + adjuvant neonates and adjuvant alone neonates. Immunization with heat-killed B5055 or O1:rFlaB + adjuvant led to significantly less bacteria present within the liver ($p = 0.002$ and $p = 0.015$) of neonates when compared to adjuvant alone neonates (**Figure 4.5B**). Additionally, heat-killed B5055 significantly reduced the bacterial burden in the lungs ($p = 0.008$) and spleen of neonates ($p = 0.023$) when compared to adjuvant alone neonates (**Figure 4.5C and D**). Notably, there was no statistical difference in bacterial tissue burden of the lungs ($p = 0.061$) and spleen ($p = 0.132$) for O1:rFlaB + adjuvant versus adjuvant alone.

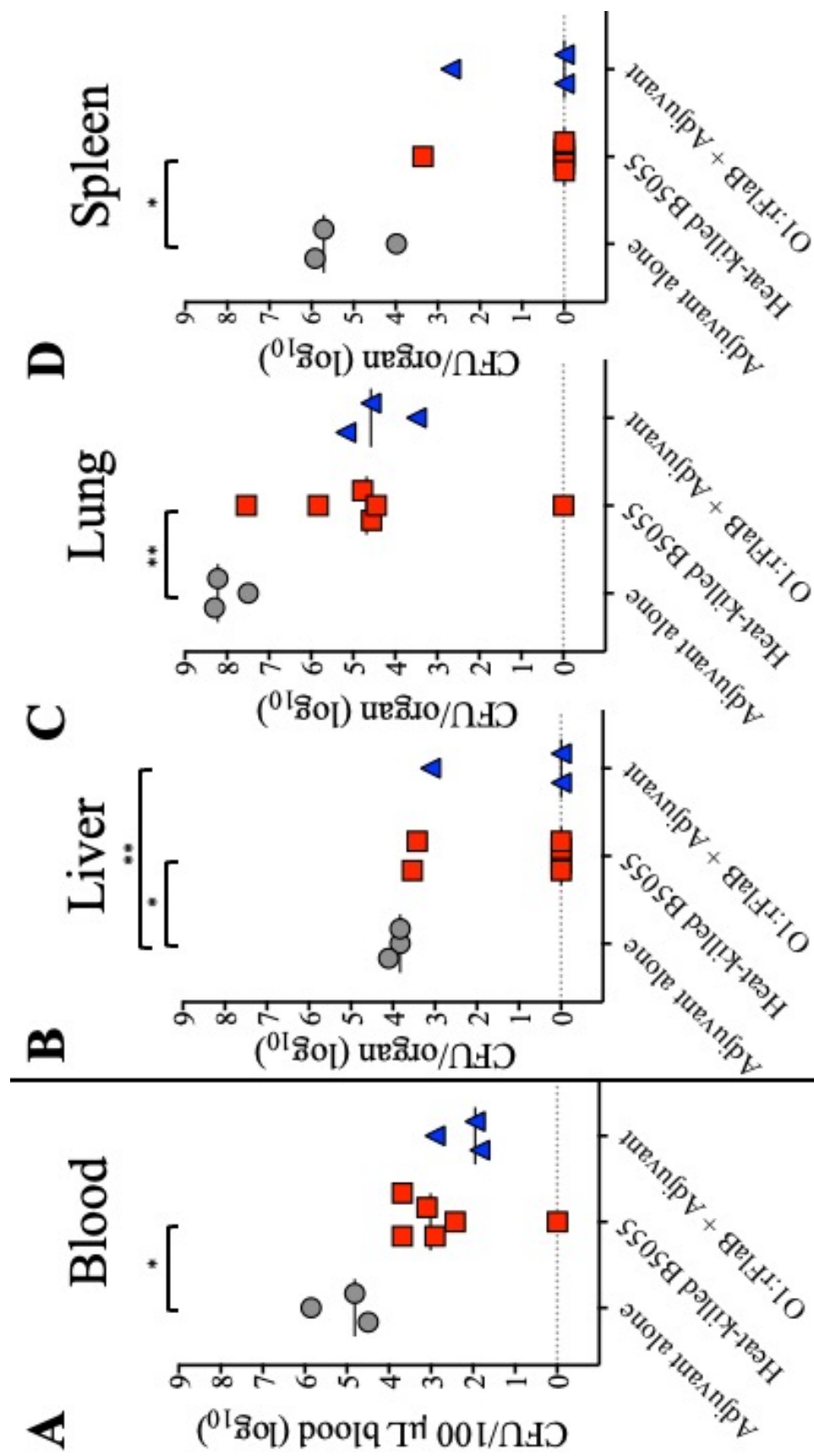


Figure 4.5. Bacterial burden of neonatal mice born to immunized dams after challenge with *K. pneumoniae* B5055 Strep^R.

Bacterial burden in the A) blood, B) liver, C) lung, and D) spleen of two-day-old C57BL/6 neonatal mice born to dams immunized with either heat-killed *K. pneumoniae* B5055 Strep^R, O1:rFlaB + adjuvant, or adjuvant alone, and subsequently challenged perorally with 2.75 - 3.3 x 10⁵ CFU of *K. pneumoniae* B5055 Strep^R. Median represented by bar (*, $p \leq 0.05$; **, $p \leq 0.01$, by Mann-Whitney). Limit of detection (LOD) indicated by dash line.

Assessment of pathology following maternal immunization in neonatal mice

Having observed reduced bacterial burden in the liver, lung and spleen of neonates born to dams immunized with a *Klebsiella* vaccine, we subsequently investigated whether this decreased bacterial dissemination was associated with a reduction in pathology in these organs. Two-day-old C57BL/6 mice were perorally infected with 2.75 – 3.3 x 10⁵ CFU *K. pneumoniae* B5055 Strep^R and tissues harvested at 24 h.p.i. for histopathological examination.

Despite the significant reduction in bacterial burden observed in the liver, lung, and spleen of neonates born to both heat-killed B5055 and O1:rFlaB + adjuvant immunized dams, we observed no histopathological differences in these organs compared to neonates from adjuvant alone immunized dams (**Figure 4.6A, B, and C**). These findings suggest that while maternal anti-O1 and anti-K2 IgG can effectively aid in bacterial clearance, tissue damage might precede the effects of bacterial clearance.

Figure 4.6. Histopathology of neonatal mice born to immunized dams after challenge with *K. pneumoniae* B5055 Strep^R.

A) Liver, B) lung, and C) spleen pathologies were scored at 24 h.p.i. in two-day-old C57BL/6 mice born to dams immunized with either heat-killed *K. pneumoniae* B5055 Strep^R, O1:rFlaB + adjuvant, or adjuvant alone, and subsequently challenged perorally with $2.75 - 3.3 \times 10^5$ CFU of *K. pneumoniae* B5055 Strep^R. Degree of pathology was based on Table 2.2, with a maximum score of 4 for each parameter. Data points represent an individual mouse from a single experiment. Bar at GMT.

Discussion

In this study, we investigated the suitability of a maternal immunization model to effectively protect against *K. pneumoniae* neonatal sepsis with two distinct *Klebsiella* vaccine candidates: Heat-killed *K. pneumoniae* B5055 Strep^R and the O1:rFlaB glycoconjugate vaccine. We demonstrated that both the heat-killed *K. pneumoniae* B5055 Strep^R and O1:rFlaB glycoconjugate vaccine induced robust anti-O1 and anti-K2 serum IgG responses in immunized dams, leading to their transfer to neonates. However, only maternal immunization with heat-killed *K. pneumoniae* conferred protection in one of two litters of neonates from lethal challenge with *K. pneumoniae* B5055 Strep^R. This inconsistency in protection between the litters may be attributed to a number of factors. The litter that succumbed to infection had neonates that were notably smaller in size, which may have resulted in a reduction of maternally transferred antibodies at time of infection. Additionally, the bacterial dose may have been overwhelming for the smaller neonates, even in the presence of protective antibodies. However, protection was observed for three dams using our preliminary immunization schedule consisting of three 10 µg doses, administered two weeks apart (**Appendix Figure 10A**), confirming that maternally administered heat-killed *K. pneumoniae* B5055 Strep^R can confer protection against mortality to their offspring.

Protection against colonization was observed; heat-killed *K. pneumoniae* B5055 neonates had reduced bacterial counts in the liver, lungs and spleen as compared to adjuvant alone neonates. Additionally, O1:rFlaB neonates had reduced bacterial counts in the liver but not the lungs nor the spleen as compared to adjuvant alone neonates. It is important to note that the small group size ($n = 3$) for the O1:rFlaB group may have hindered our ability to detect statistical significance, and a larger group size may have revealed significant differences in bacterial burden compared to our adjuvant alone group. Conversely, neither reduced the histopathological effects associated with infection.

The successful induction of antigen-specific serum IgG titers in adult C57BL/6 female mice with either heat-killed *K. pneumoniae* B5055 Strep^R or O1:rFlaB + adjuvant, highlights the immunogenicity of both vaccines. The ability of O1:rFlaB to induce anti-O1 IgG titers in adult mice was previously demonstrated. CD-1 mice were immunized i.m. with 2.5 μg per dose of O1:rFlaB without adjuvant, eliciting robust anti-O1 IgG titers (GMT = $\sim 10^4$ EU/mL) after three doses (62). In this study, O1:rFlaB + adjuvant successfully induced a $\sim \log_{10}$ higher GMT of 1.81×10^5 EU/mL in adult C57BL/6 mice after two i.p. 20 μg doses; however, this was ~ 2 log lower than titers induced by heat-killed *K. pneumoniae* B5055 (GMT = 1.73×10^7 EU/mL). As expected, heat-killed B5055 elicited robust anti-K2 IgG titers. Notably, mice immunized with O1:rFlaB + adjuvant presented elevated anti-K2 serum IgG titers which were subsequently transferred to neonates, an unexpected result which may be attributed to *K. pneumoniae* exposure. Overall, our data indicate that although O1:rFlaB + adjuvant activates a humoral response, it is not as efficient as heat-killed *K. pneumoniae*.

The principle of maternal immunization hinges on the effective transfer of protective antibodies to vulnerable neonates. Indeed, several studies have demonstrated the successful transplacental and/or trans-mammary transfer of maternally-derived antibodies, specifically IgG, to neonates and their subsequent ability to confer protection following lethal challenge (104, 165–168). We observed a GMT anti-O1 IgG titer of 1.25×10^6 EU/mL in neonates from dams immunized with heat-killed B5055 and 5.83×10^4 EU/mL in neonates from dams immunized with O1:rFlaB prior to lethal challenge. We observed 80% survival in neonates born to one heat-killed B5055-immunized dam (anti-O1 IgG titer = 1.08×10^7 EU/mL), with 0% survival observed across all other groups, including neonates from dams immunized with O1:rFlaB. In our previous study, heat-killed B5055 maternal immunization resulted in 60-100% survival (**Appendix Figure 10A**) in neonates challenged with a similar dose (1.0×10^6 – 1.82×10^6 CFU/mouse) of *K. pneumoniae* B5055 Strep^R. While this data did not include a control group, the lethality of this challenge dose was confirmed (**Appendix figure 11**), making the observed survival significant. Therefore, it is conceivable that although the three-dose regimen of O1:rFlaB generates a humoral response in adult C57BL/6 mice, the specific titers and/or functionality of maternally transferred IgG may not be sufficient to confer protection against *K. pneumoniae* neonatal sepsis. Additionally, the diverse antibody repertoire elicited by the heat-killed B5055 preparation likely contributes to its protective effect. This could explain why the O1:rFlaB immunization did not translate into improved neonatal survival in our model.

Maternal IgG plays a crucial role in providing passive immunity to neonates. These antibodies can opsonize bacteria, coating the bacterial membrane targeting them

for phagocytic uptake (169, 170). Additionally, IgG can directly neutralize bacterial toxins, specifically LPS, preventing cellular damage and dampening the inflammatory response (169, 170). We observed decreased bacterial loads in the liver, lungs, and spleen of neonates born to both heat-killed and O1:rFlaB immunized dams compared to adjuvant alone neonates. This data suggests that the maternally transferred IgG, though not 100% protective against lethal challenge, was functionally active in reducing bacterial proliferation and dissemination to these tissues. However, this reduction in bacterial burden did not correlate with reduced histopathological effects, as there was no significant difference in damage between neonates born to dams immunized with heat-killed *K. pneumoniae* and O1:rFlaB when compared to adjuvant alone. These results indicate that tissue damage may precede effective bacterial clearance. Further investigation of the protective mechanisms of maternally transferred IgG is warranted.

This study had limitations. Our antibody assessment focused strictly on anti-O1 and anti-K2 serum IgG titers. Heat-killed *K. pneumoniae*, a whole bacteria preparation, would have elicited a much broader and more diverse antibody repertoire against other surface antigens. Therefore, this limited scope prevents a full understanding of the protective response conferred by heat-killed B5055. Additionally, timing of conception in the dams led to variability in the interval between the final immunization and the birth of the neonates, introducing experimental variation. Furthermore, our sample sizes were small, as they were contingent on litter size, meaning the study was sometimes underpowered to detect statistically significant differences between the groups. Future studies should encompass larger, more controlled groups to limit these discrepancies and provide a more comprehensive assessment of the protective mechanisms at play.

In summary, maternal immunization with heat-killed *K. pneumoniae* B5055 Strep^R induced robust humoral immunity, conferred protection against lethal challenge and colonization in our neonatal sepsis model, but failed to hinder tissue damage. Similarly, maternal immunization with O1:rFlaB glycoconjugate vaccine with adjuvant induced modest humoral immunity, conferred protection against *K. pneumoniae* colonization, but failed to protect against lethal challenge and tissue damage. These results emphasize the complex and multifaceted nature of eliciting protection against *K. pneumoniae* neonatal sepsis, as hindering colonization did not consistently correlate with improved survival or reduced pathology. Future directions should focus on optimizing vaccine design, through the use of a more potent adjuvant or exploring the combined effect of different antigens, to enhance antibody quantity and functionality. Furthermore, these data raise important questions about the characteristic of maternal antibodies that confer protection against *K. pneumoniae* neonatal sepsis in humans. Understanding how maternal antibodies behave in our neonatal sepsis model could provide valuable insight into which protective readouts, survival or bacterial burden, are more relevant to vaccine efficacy studies.

Chapter 5 Discussion and Conclusion

The overarching goal of this thesis project was two-fold: 1) To develop and characterize a *K. pneumoniae* neonatal sepsis rodent model to aid in the development and evaluation of novel vaccines and, 2) To identify and evaluate a suitable immunization model for evaluating vaccine efficacy against *K. pneumoniae* neonatal sepsis.

Well-established rodent models for neonatal bacterial sepsis, particularly those involving GBS and *E. coli*, have consistently demonstrated an age-dependent susceptibility to infection, increased bacterial burden, and/or disseminated infection in neonates (105, 107, 171–175). Building upon these studies, we hypothesized that neonatal mice would exhibit a similar heightened susceptibility to *K. pneumoniae* infection, with increased mortality when compared to older animals, accompanied by increased bacterial burden and bacterial dissemination in infected neonates. Our findings in Chapter 2 supported this hypothesis: neonatal mice were significantly more susceptible to *K. pneumoniae* infection, demonstrating increased mortality upon lethal challenge when compared to adults. Furthermore, neonatal mice were bacteremic as early as 2-hours post infection, with *K. pneumoniae* infection leading to severe pathology in the liver and lungs of infected neonates.

The work described in Chapter 3 explores the utility of passive immunization within the neonatal sepsis model to protect against *K. pneumoniae* infection. I showed that passive administration of heat-killed CVD 3001 rabbit antisera conferred protection against *K. pneumoniae* through improved survival rates. However, it was not able to confer protection against *K. pneumoniae* bacterial burden and pathology. Passive

administration of qKPPA rabbit antisera failed to improve survival rates, reduce bacterial burden, or hinder pathology against *K. pneumoniae* infection. This differential outcome, in regard to survival rates, between the two antisera suggests that protective elements present in heat-killed CVD 3001 rabbit antisera are either insufficient or absent in the qKPPA rabbit antisera. As a polyclonal preparation, heat-killed CVD 3001 antisera is expected to have a diverse antibody repertoire targeting multiple antigens including LPS, OMPs and fimbriae. The presence of multiple antibody specificities in heat-killed CVD 3001 allows for synergistic protective mechanisms that are likely insufficient in qKPPA rabbit antisera. Based on the results from Chapter 3, I hypothesize a broad antibody response is necessary to confer protection against *K. pneumoniae* neonatal sepsis.

Chapter 4 examines the suitability of a maternal immunization model for evaluating protection against *K. pneumoniae* neonatal sepsis. Similar to the results shown in Chapter 3, heat-killed B5055 conferred protection through improved survival rates in neonates born to immunized dams. Conversely, O1:rFlaB, a component of the qKPPA vaccine, did not improve neonatal survival when administered via maternal immunization. Interestingly, the maternal immunization model revealed protective phenotypes not observed with the passive administration of rabbit antisera in Chapter 3. Specifically, neonates born to dams immunized with heat-killed B5055 exhibited a significant reduction in bacterial burden within the blood and tissues following lethal challenge with *K. pneumoniae*. Furthermore, O1:rFlaB significantly reduced bacterial burden in the liver of infected neonates. These results suggest maternally derived antibodies generated via heat-killed B5055 and passively transferred to neonates effectively improved survival, which is in alignment with the results from Chapter 3.

Additionally, these maternally derived antibodies promote bacterial clearance, indicated by reduced bacterial loads, likely through opsonization and/or complement activation. Conversely, maternally derived antibodies generated via O1:rFlaB primarily promoted bacterial clearance, evidenced by the reduced bacterial burden. Collectively, these results highlight bacterial clearance as a critical component of protection following active immunization with whole cell and conjugate *K. pneumoniae* vaccines.

In the passive immunization experiments, we utilized antisera from rabbits immunized with either heat-killed CVD 3001 or the qKPPA vaccine. The heat-killed CVD 3001, a capsule-deficient *K. pneumoniae* B5055 strain, served as a positive control known to elicit high anti-O1 IgG titers, thus providing a strong indicator of the protective role of O1 antibodies against *K. pneumoniae* neonatal sepsis. However, the presence of other antigens, such as OMPs, MrkA, and unexpectedly K2, means we cannot rule out a synergistic effect within this antiserum. The qKPPA vaccine is a vaccine candidate designed to elicit antibodies against the four major O-serotypes of clinically relevant *K. pneumoniae*. While it generated high anti-O1 IgG titers, the narrow breadth of its antigenic targets likely led to its inability to confer protection in our neonatal sepsis model.

The maternal immunization studies utilized heat-killed *K. pneumoniae* B5055 and O1:rFlaB, a component of the qKPPA glycoconjugate vaccine. Similar to Chapter 3, heat-killed B5055 served as a positive control. As a wild-type strain we expected it to elicit a diverse antibody repertoire capable of conferring protection in our neonatal sepsis model. The O1:rFlaB vaccine, in contrast, allowed us to specifically assess the role of O1 in protection against *K. pneumoniae* neonatal sepsis. Interestingly, though O1 antibody

titers transferred to O1:rFlaB immune neonates were lower (albeit not statistically significant) than those passively administered qKPPA (**Table 3.1 and 4.2**), maternal immunization did protect against bacterial burden in our neonatal sepsis model.

The differential outcomes between the passive immunization and maternal immunization study outcomes are likely attributable to the sustainability of the antibody response. While passive immunization provides an immediate, high-titer dose of antibodies, this protection is short-lived. In contrast, maternal immunization provides a continuous, prolonged transfer of antibodies throughout both the perinatal and postnatal periods. The impact of this sustained defense, through the transplacental and/or trans-mammary transfer of antibodies is well documented (176–179). Studies have demonstrated maternally-transferred IgG and secretory IgA (sIgA) from the placenta and/or breast milk are required for prolonged protection against infectious diseases in neonates (180–186). Therefore, the ability of maternal immunization to provide this sustained, dual route antibody support may be crucial for protection against *K. pneumoniae* infection in our neonatal sepsis model.

Furthermore, the data from these studies suggests a broad antibody response is essential for comprehensive protection against *K. pneumoniae* neonatal sepsis. Improved survival was only demonstrated in our passive immunization model following administration of heat-killed CVD 3001 and in our maternal immunization model with heat-killed B5055. Conversely, the O1:rFlaB conjugate vaccine in our maternal immunization model elicited an immune response that was sufficient to significantly reduce bacterial burden in the liver of infected neonates, a phenotype not observed in our passive immunization model with qKPPA rabbit antisera. Previous studies have shown

the passive transfer of antibodies elicited from conjugate vaccines can confer protection following lethal challenge and reduced bacterial burden in infected mice (16, 62, 156). The outcome from Chapter 4 highlights a more nuanced role for O1:rFlaB: O1-specific antibodies elicited by the conjugate vaccine may be highly effective at bacterial clearance but insufficient in combating other aspects of disease, such as the inflammatory response or tissue damage. This suggests that while bacterial clearance is a key component of protection, it may not be the sole determinant of survival in neonatal mice. A more comprehensive immune response, targeting multiple antigens, may be necessary to fully protect against the severity of *K. pneumoniae* neonatal sepsis.

Future directions of this project should focus on determining the correlates of protection against *K. pneumoniae* neonatal sepsis. Both heat-killed CVD 3001 and heat-killed B5055 provided similar protective effects in our neonatal sepsis model, though the breadth of antibodies they elicited remains a key area of investigation. Evaluating the antibody repertoire beyond O- and K-antigens would help pinpoint which specific antigens are critical for protection. Once identified, examination of antibody class and subclasses elicited would further elucidate their protective mechanisms. Human and mouse IgG subclasses perform analogous roles in immune defense (187–192). Evaluation of which subclasses are more abundant and their specific functions, i.e., opsonization, neutralization, or complement activation, would provide insight into how protection is mediated. Additionally, sIgA has also demonstrated a protective role against neonatal infections (193–196), thus its presence and function should also be assessed. To determine whether maternal protection in neonates is mediated via transplacental transfer

or breast milk, a cross-fostering experiment could be conducted to evaluate the primary mechanism of antibody protection.

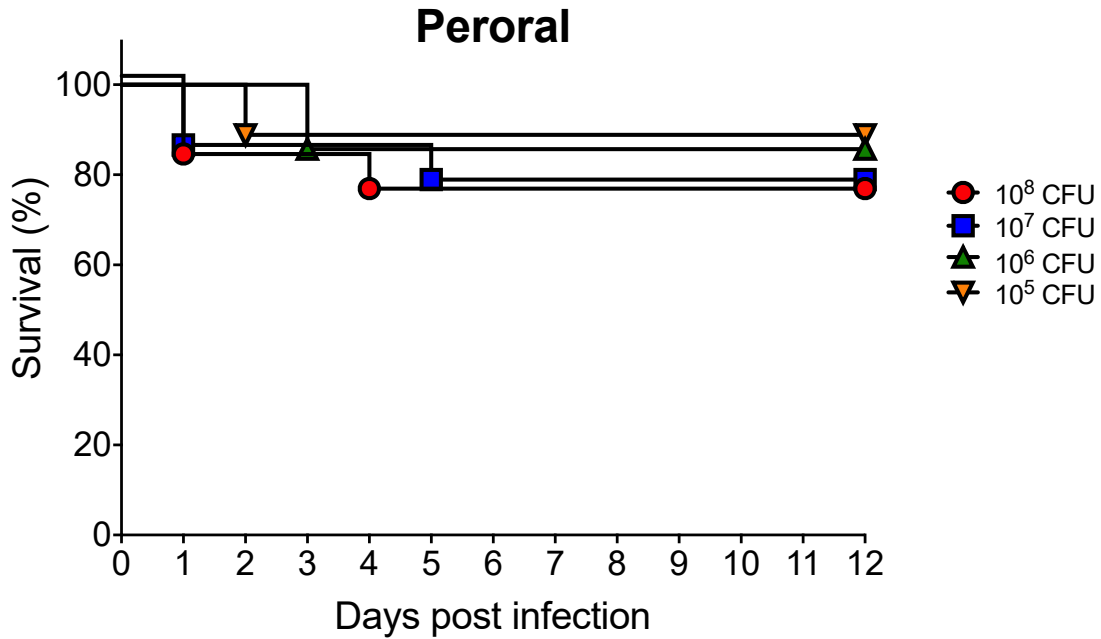
Furthermore, the suitability of our maternal immunization model was limited to a single *K. pneumoniae* strain (O1:K2). It is essential to expand the scope of this model to other clinically relevant O- and K-serotypes, including neonatal sepsis isolates, to determine if a particular serotype is associated with enhanced virulence in neonates. Another key area of research should decipher the factors mediating susceptibility to *K. pneumoniae* infection in neonates. Characterization of the immune response elicited in neonates, i.e., cytokine profiles, following *K. pneumoniae* challenge should be explored. Identification of specific inflammatory pathways that are either protective or detrimental would provide insights into host-pathogen interactions. Additionally, the role of the maternal and neonatal microbiome in influencing susceptibility and/or resistance to infection should be investigated. Understanding this complex interplay between host, pathogen, and microbiome would aid in the development of more targeted interventions.

Although the heat-killed *K. pneumoniae* vaccine conferred protection against neonatal sepsis in our mouse model, this vaccine would not be approved by regulatory authorities due to its potential for reactogenicity. Therefore, the development of a more defined, safer vaccine, such as our glycoconjugate vaccine, would be ideal for use in pregnant women. So to potentially improve the efficacy of our O1:rFlaB glycoconjugate vaccine, the use of a more potent adjuvant should be explored. Aluminum hydroxide is a widely used adjuvant with a Th2-biased immune response (197–199). However, limitations, such as weak induction of cell-mediated immunity and antibody responses, give way for more potent alternatives. Emulsions or saponin-based adjuvants promote

robust T-cell dependent responses and are considered safe for use in pregnant women (200–202). We hypothesize that co-formulation of O1:rFlaB with a more potent adjuvant would enhance antibody quality and titers, resulting in improved bacterial clearance and neonatal survival outcomes.

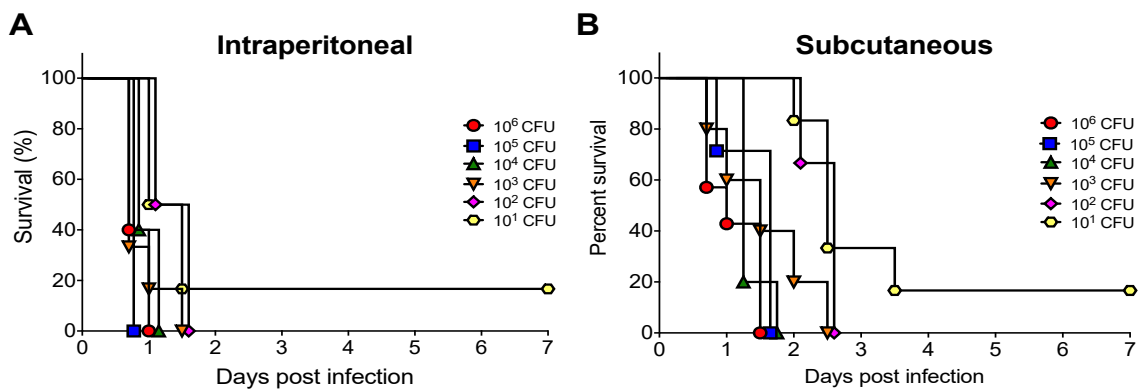
Taken together, I have developed and characterized a *K. pneumoniae* neonatal sepsis mouse model. Through this model I identified various protective readouts to aid in evaluating vaccine efficacy against *K. pneumoniae* neonatal sepsis. Based on this work, I recommend the use of an active immunization model, such as maternal immunization, to evaluate protective effects of OPS-based vaccines against neonatal sepsis. Furthermore, to accurately assess vaccine efficacy, bacterial burden should be a protective readout in conjunction with survival rates. The combination of reduced bacterial burden and improved survival are the most desirable outcomes, as they could also reduce potential transmission of *K. pneumoniae*. Overall, this work provides a platform for the preclinical development and assessment of novel vaccine candidates against *K. pneumoniae* neonatal sepsis, offering critical insights as MDR strains continue to rise globally.

Appendix: Supplementary material



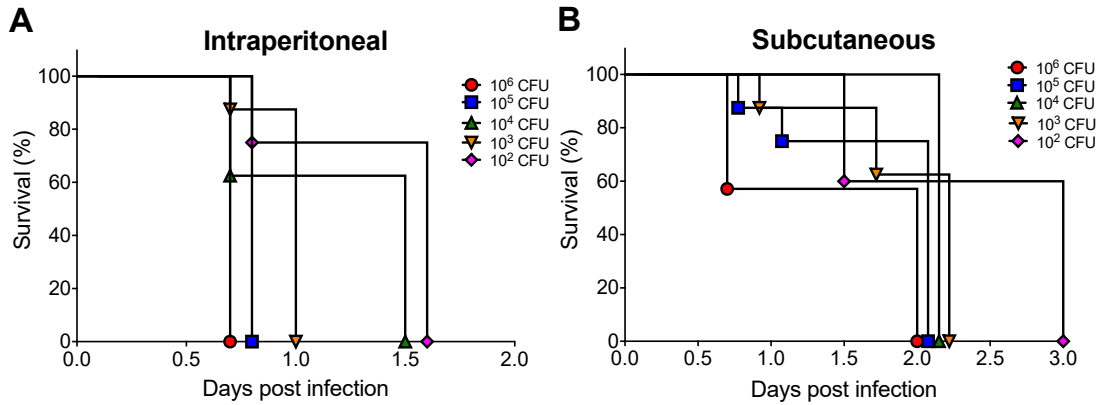
Appendix Figure 1. The 50% lethal dose of *K. pneumoniae* B5055 Strep^R following peroral administration in neonatal CD-1 mice.

Whole litters of two- and three-day-old CD-1 mice (n = 6-15 per litter) were infected perorally with the indicated inocula of *K. pneumoniae* B5055 Strep^R and monitored daily for survival. The Kaplan-Meier survival curves shown are one representative experiment performed at least two times.



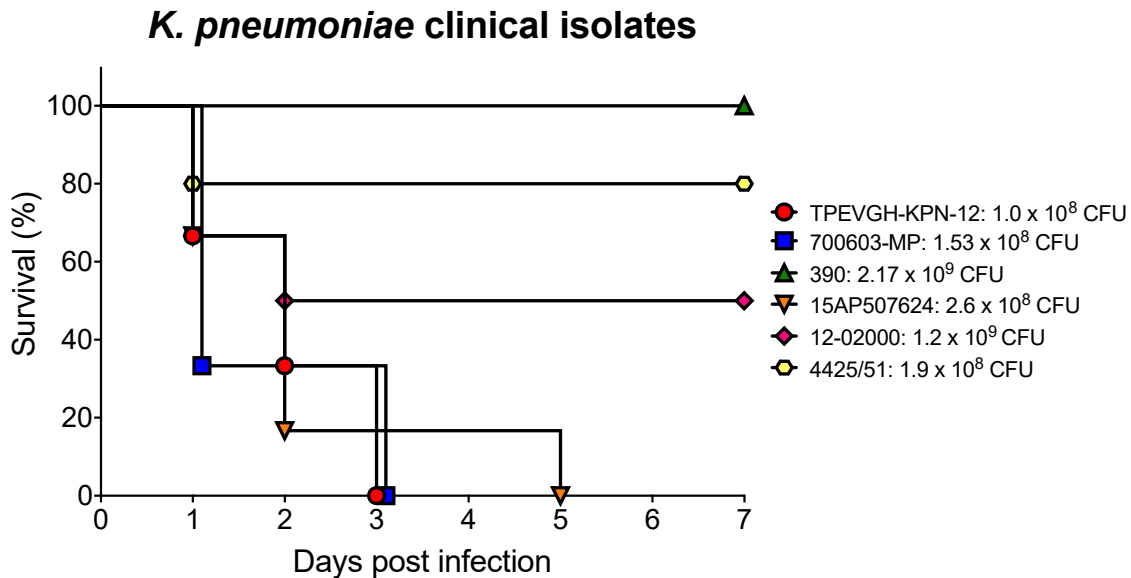
Appendix Figure 2. The 50% lethal dose of *K. pneumoniae* B5055 Strep^R following intraperitoneal or subcutaneous administration in neonatal CD-1 mice.

Whole litters of two-day-old CD-1 mice (n = 5-7 per litter) were split and either infected (A) intraperitoneally or (B) subcutaneously with the indicated inocula of *K. pneumoniae* B5055 Strep^R and monitored daily for survival. The Kaplan-Meier survival curves shown are a single experiment for each administration route.



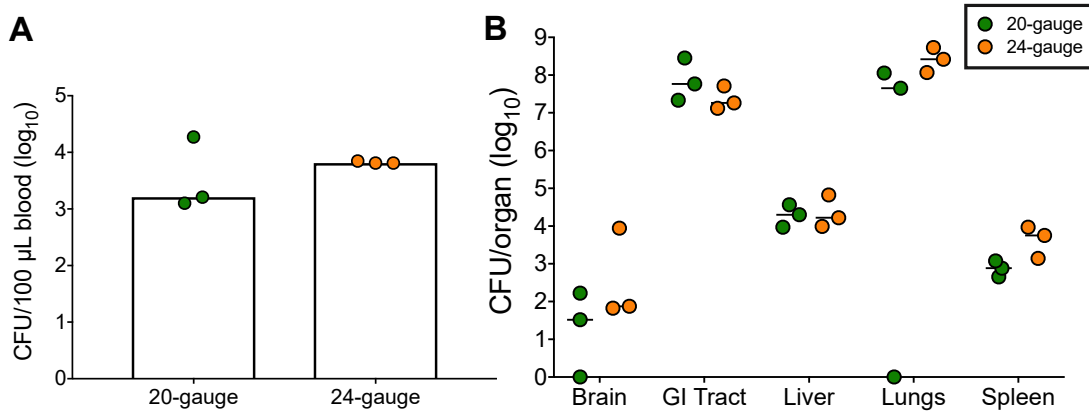
Appendix Figure 3. The 50% lethal dose of *K. pneumoniae* B5055 Strep^R following intraperitoneal or subcutaneous administration in neonatal C57BL/6 mice.

Whole litters of two-day-old C57BL/6 mice (n = 4-8 per litter) were infected (A) intraperitoneally or (B) subcutaneously with the indicated inocula of *K. pneumoniae* B5055 Strep^R and monitored daily for survival. The Kaplan-Meier survival curves shown are a single experiment for each administration route.



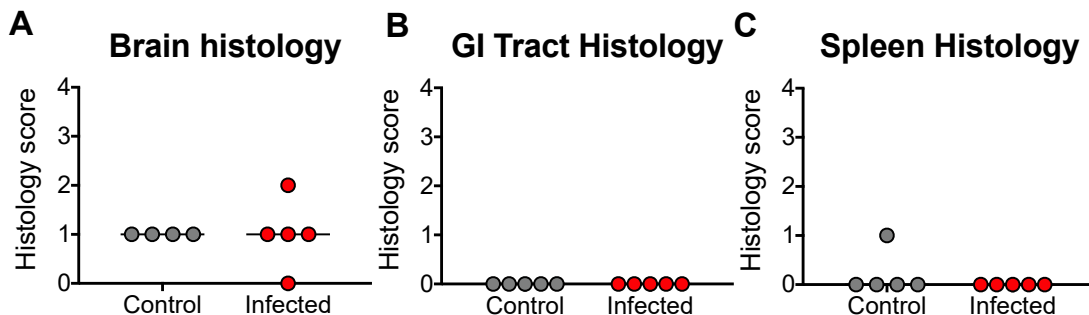
Appendix Figure 4. Survival comparison of C57BL/6 neonatal mice infected with clinical isolates of *K. pneumoniae*.

Two-day-old C57BL/6 (n = 3-6 per group) were infected perorally with the indicated inocula of *K. pneumoniae* TPEVGH-KPN-12, 700603-MP, 390, 15AP507624, 12-02000, or 4425/51 and monitored daily for survival. The Kaplan-Meier survival curves shown are a single experiment for each clinical isolate.



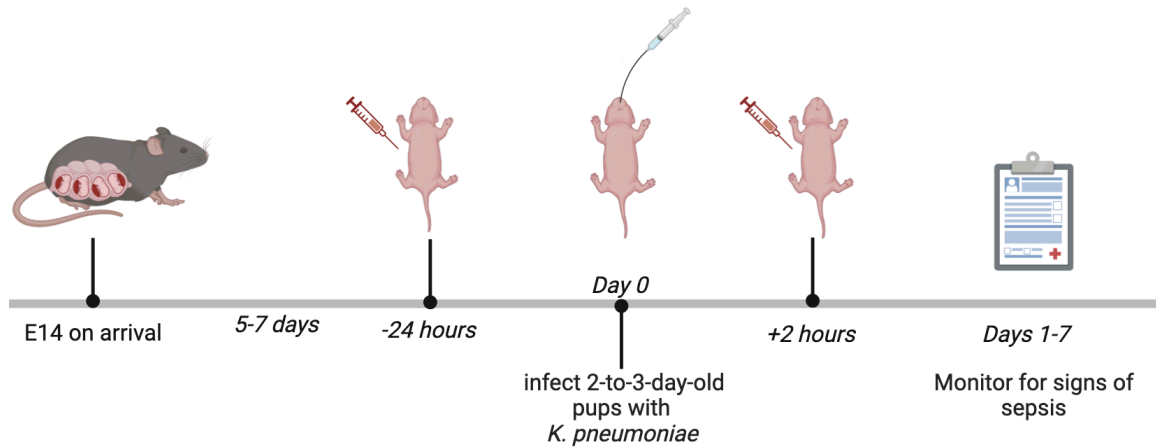
Appendix Figure 5. Assessment of infection methodology on bacterial burden in neonatal mice.

Two-day-old C57BL/6 mice (n = 3 per group) were infected perorally with 1.5×10^7 CFU of *K. pneumoniae* B5055 Strep^R using either a PTFE 20-gauge or a stainless steel 24-gauge feeding needles. Bacterial loads were determined in the (A) blood and (B) tissues (brain, GI tract, liver, lungs, and spleen) at 2 h.p.i. Each point represents an individual mouse. Median burden is represented by a bar or line.

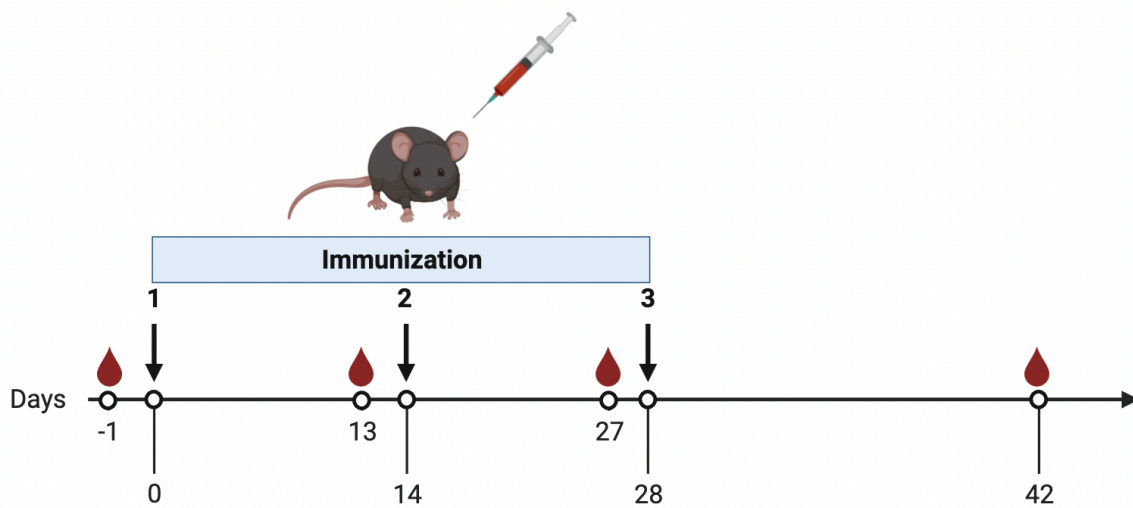


Appendix Figure 6. Pathology following peroral infection with *K. pneumoniae* B5055 Strep^R in neonatal mice.

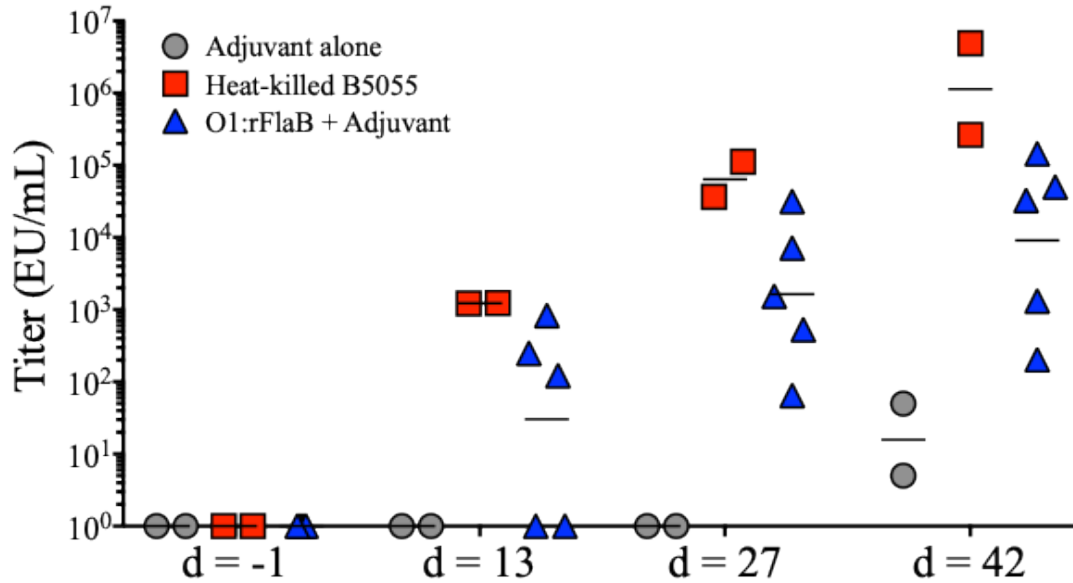
(A) Brain, (B) GI tract, and (C) spleen pathology were scored in *K. pneumoniae* B5055 Strep^R-infected and control two-day-old C57BL/6 mice (n = 5 per group) at 18 h.p.i. Degree of pathology was based on Table 2, with a maximum score of 12. Data points represent an individual mouse from a single experiment.



Appendix Figure 7. Passive immunization schematic to assess neonatal survival rates and bacterial burden following lethal challenge.

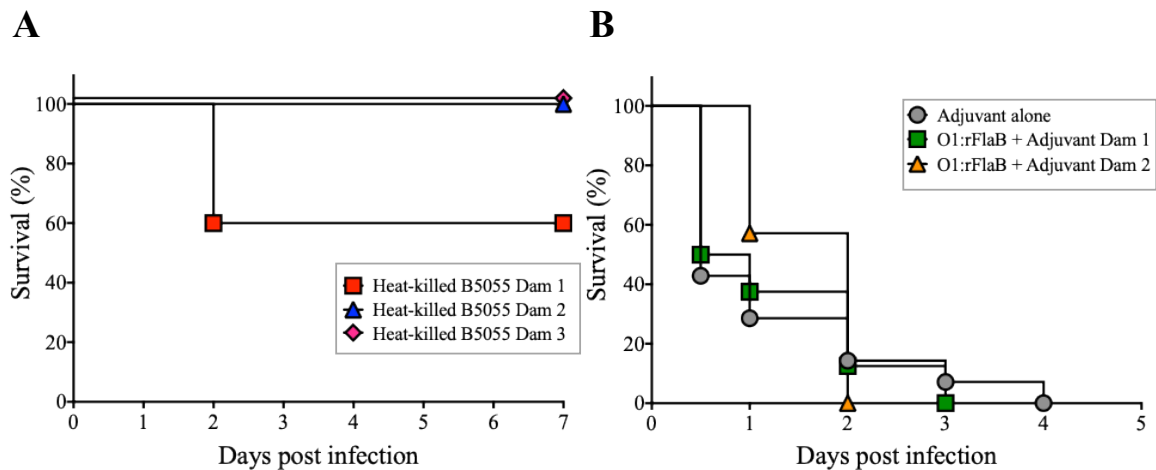


Appendix Figure 8. Initial immunization schematic to assess protection in *K. pneumoniae* neonatal sepsis model following maternal immunization.



Appendix Figure 9. Maternal antibody responses following immunization with Heat-killed *K. pneumoniae* B5055 Strep^R, O1:rFlaB glycoconjugate vaccine + Adjuvant, or Adjuvant alone.

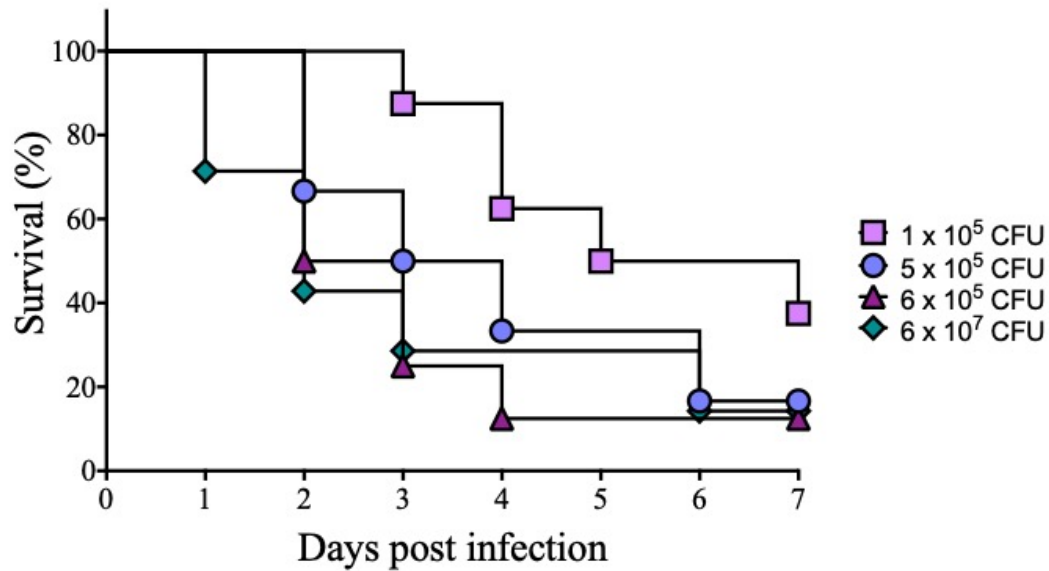
Five-week-old female mice received three doses of either vaccine, spaced 2 weeks apart, with serum collected 1 day prior to immunization, and a final collection two following that third dose (day -1, 13, 27, and 42). Anti-O1 serum IgG titers from dams utilized to assess seroconversion prior to mating. Bar at GMT.



Appendix Figure 10. Survival curves of neonates born to immunized dams following lethal challenge with *K. pneumoniae* B5055 Strep^R.

Survival of neonates born to dams immunized with either A) Heat-killed *K. pneumoniae* B5055 Strep^R ($1.0 - 1.82 \times 10^5$ CFU), B) O1:rFlaB glycoconjugate vaccine + Adjuvant, or Adjuvant alone ($1.5 \times 10^7 - 1.62 \times 10^8$ CFU), and challenged perorally at two days post birth with *K.*

pneumoniae B5055 Strep^R. (*, $p \leq 0.05$; **, $p \leq 0.01$, by log-rank analysis). The Kaplan-Meier survival curves shown are a single experiment for each immunization.



Appendix Figure 11. Evaluation of *K. pneumoniae* challenge dose in neonatal mice.

Two-day-old C57BL/6 (n = 6-8 per group) were infected perorally with the indicated inocula doses of *K. pneumoniae* monitored daily for survival. The Kaplan-Meier survival curves shown are a single experiment for each infection dose.

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