From Department of Clinical Science, Intervention and Technology
Division of Pediatrics
Karolinska Institutet, Stockholm, Sweden

EVERY BUG COUNTS
NEONATAL COLONIZATION AND INFECTION OF GRAM-NEGATIVE BACILLI - ASPECTS ON ANTIBIOTIC RESISTANCE IN SWEDEN AND ECUADOR

Viveka Nordberg

Stockholm 2020

All previously published papers were reproduced with permission from the publisher.
Cover photo by Christer Roheden.
Published by Karolinska Institutet.
Printed by Universitetsservice US-AB.
© Viveka Nordberg, 2020
ISBN 978-91-7831-879-7

EVERY BUG COUNTS
NEONATAL COLONIZATION AND INFECTION OF GRAM-NEGATIVE BACILLI - ASPECTS OF ANTIBIOTIC RESISTANCE IN SWEDEN AND ECUADOR

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Viveka Nordberg

Principal Supervisor:
Associate professor Lars Navér
Karolinska Institutet
Department of Clinical Science,
Intervention and Technology (CLINTEC)
Division of Pediatrics

Co-supervisor(s):
Professor Christian G Giske
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

PhD Aina Iversen
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Professor Mikael Norman
Karolinska Institutet
Department of Clinical Science,
Intervention and Technology (CLINTEC)
Division of Pediatrics

Opponent:
Professor Jens Schollin
University of Örebro
Faculty of Medicine and Health

Examination Board:
Associate Professor Carl-Johan Treutiger
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Associate Professor, Elisabeth Olhager
University of Lund
Department of Clinical Science

Associate Professor, Christina Åhrén
University of Gothenburg
Department of Infectious Diseases
Institute of Biomedicine, Sahlgrenska Academy

"If you don´t like bacteria, you´re on the wrong planet"

-Steward Brand

To Adam, Moa and Lova

ABSTRACT

Neonatal infections caused by extended-spectrum beta-lactamase (ESBL)-producing Gram-negative bacilli are associated with high morbidity and mortality. In order to perform targeted preventive interventions against the impacts of antibiotic resistance in neonates, it is crucial to study colonization, infection and the spread of EPE-bacteria.

Methods and results

Study I. A prospective observational study where the proportion of intestinal colonization with EPE, their resistance pattern and risk factors of EPE-colonization were assessed in a neonatal intensive care unit in Ecuador. In total, 56% of the neonates were colonized with EPE. The strains found were ESBL-*E. coli* (ESBL-EC, 89%) and ESBL-*K. pneumoniae* (ESBL-KP, 11 %) and the main risk factor for colonization was length of hospital stay. Two of the isolated clones were epidemic and known to disseminate carbapenem-hydrolysing beta-lactamases. These results underline the necessity of implementing colonization surveillance and improved hygiene standards in this and similar neonatal care settings.

Study II. A prospective cohort study where we analysed the ESBL-KP isolates in a neonatal outbreak of EPE and determined the duration of intestinal colonization of EPE in affected neonates (n= 14). The intestinal relative abundance of EPE was determined in each carrier. One fourth of the neonates were still carriers of EPE, two years after a NICU outbreak. The median length of colonization was 12.5 months. The low virulent but highly resistant ESBL-KP strain ST101 persisted in 2/13 children. One patient was colonized with ESBL-EC at five years of age. No infant suffered from an EPE-infection during the 5-year follow-up.

Study III. In this study, the findings in study 2 were extended by characterizing the resistance encoding plasmids using optical DNA mapping (ODM) combined with Cas9-assisted identification of resistance genes. The method detected two plasmids; one small (80 kb) and one large plasmid (220 kb) in all ESBL-KP isolates. We found that the $bla_{\text{CTX-M-15}}$ gene was located on the small plasmid and that this plasmid was stable in the ESBL-KP clone for two years. There was an unrelated acquisition of an ESBL-EC strain, contradicting plasmid transfer between KP and EC in this outbreak. ODM is a promising and rapid tool for surveillance and infection control in clinical settings.

Study IV. In a population-based retrospectively matched cohort study, we investigated the incidence, mortality and bacterial characteristics of neonatal sepsis caused by Gram-negative bacilli (GNB) sepsis in Stockholm County during a 11-year period. The primary outcome was death before discharge and secondary outcomes were death within 5 and 30 days after

sepsis onset. The cumulative incidence of GNB-sepsis was 0.35 cases per 1,000 live born and for early onset sepsis (EOS) 0.11 and late onset sepsis (LOS) 0.24 respectively. Case fatality rate was 5/33 (15%) in GNB-EOS and 26/74 (35%) in GNB LOS. Neonates with GNB-LOS were 3.9 times more likely (adjusted OR, 95% CI: 1.6-9.4) to die before discharge compared to the uninfected matched control group. Overall, 6.5% (7/107) of the isolates were multidrug-resistant. The incidence of both GNB-EOS and GNB-LOS was lower than reported in previous studies from other high-income countries comparable settings. Mortality in GNB-LOS remains high. The occurrence of acquired antibiotic resistance was low and did not change substantially over time.

Conclusions

A high proportion (56%) of the neonates at a NICU in a tertiary hospital in Ecuador, became colonized by EPE. These EPE included two clones of ESBL-KP that are known to disseminate carbapenemases. Infants that were colonized during an EPE-outbreak at two NICUs in Stockholm carried the same ESBL-KP for 26 months. There was no plasmid transfer between bacteria during the outbreak. During a 11-year period in Stockholm, Gram-negative sepsis was rare but a major risk for neonatal mortality.

LIST OF SCIENTIFIC PAPERS

I. **High proportion of intestinal colonization with successful epidemic clones of ESBL-producing Enterobacteriaceae in a neonatal intensive care unit in Ecuador.**
Nordberg V, Quizphe Peralta A, Galindo T, Turlej-Rogacka A, Iversen A, Giske C.G , Navér L.
PLoSOne. 2013 Oct; 8(10):e76697. Doi:10.1371/journal.pone.0076597

II. **Neonatal intestinal colonization with Extended-Spectrum β-Lactamase-producing Enterobacteriaceae- a five year follow-up study.**
Nordberg V, Jonsson K, Giske G. C, Iversen A, Aspevall O, Jonsson B, Camporeale A; Norman M, Navér L.
Journal of Clinical Microbiology and Infection. 2018 Sep;24(9), p.1004-1009. Doi:10.1016/j.cmi.2017.12.028. Epub Jan 8.

III. **Optical DNA mapping combined with Cas9-targeted resistance gene identification for rapid tracking of resistance plasmids in a neonatal intensive care unit outbreak.**
Bikarrarolla K.S**, Nordberg V,** Rajer F, Müller V, Humaun Kabir M, Sriram KK, Dvirnas A, Ambjörnsson T, Giske G. C, Navér L, Sandegren L, Westerlund F.
mBio. 2019 Jul. Doi:10.1128/mBio.00347-19

IV. **A decade of neonatal sepsis caused by Gram-negative bacilli- a retrospective matched cohort study.**
Nordberg V, Iversen A, Tidell A, Giske G. C, Navér L.
Submitted

CONTENTS

1 INTRODUCTION 1
2 BACKGROUND 5
2.1 BACTERIA 5
2.2 ANTIBIOTIC RESISTANCE 7
2.3 ENTEROBACTERIACEAE 10
2.4 METHODS FOR DIAGNOSING EPE 11
2.5 NEONATES 15
2.6 HUMAN MICROBIOTA 16
2.7 NEONATAL SEPSIS 22
2.8 COLONIZATION AND INVASIVE INFECTION OF EPE 28
3 AIMS 31
4 METHODS 32
4.1 ETHICS 32
4.2 PATIENTS AND SETTINGS 32
4.3 STUDY DESIGNS 33
4.4 DATA COLLECTION 33
4.5 SELECTION OF BACTERIA 34
4.6 ESBL-SCREENING, SPECIES IDENTIFICATION, SUSCEPTIBILITY TESTING 35
4.7 VIRULENCE AND RESISTANCE GENE DETECTION 36
4.8 OPTICAL MAPPING IN PLASMIDS 37
4.9 STATISTICAL METHODS 38
5 RESULTS 39
5.1 STUDY 1 39
5.2 STUDY 2 42
5.3 STUDY 3 47
5.4 STUDY 4 50
6 DISCUSSION 56
7 CONCLUSIONS 68
8 FUTURE 69
9 POPULÄRVETENSKAPLIG SAMMANFATTNING 70
10 ACKNOWLEDGEMENTS 71
11 REFERENCES 75

LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
Bla	Gene encoding Beta-Lactamase
BPD	Broncho pulmonary dysplasia
CLSI	Clinical Laboratory Standards Institute
CRISPR	Clustered Reguarly Interspaced Short Palindromic Repeats
CTX-M	Cefotaximase Munich, Beta-lactamase of ESBL-A type
DNA	Deoxiribonucleic acid
EC	*Escherichia coli*
ESBL	Extended Spectrum Beta-Lactamase
EOS	Early onset sepsis
EPE	ESBL-producing Enterobacteriaceae
EUCAST	European Committee of Antimicrobial Susceptibility Testing
GNB	Gram-negative bacteria
GPB	Gram-positive bacteria
GW	Gestational week
HIC	High income country
IVH	Intraventricular hemorrhage
KP	*Klebsiella pneumoniae*
LIC	Low income country
LMIC	Low middle income country
LOS	Late onset sepsis
MDR	Multidrug resistant organism
MLST	Multi-locus sequence typing
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit
ODM	Optical DNA mapping
PFGE	Pulse-Field Gel Electrophoresis
RA	Relative abundance
ROP	Retinopathy of prematurity
VLBW	Very low birth weight

1 INTRODUCTION

The human has evolved together with bacteria over a period of 400 million years.

About one million newborns die due to bacterial infections during the first month of age and out of these infections, about 30% are estimated to be caused by antibiotic resistant bacteria (1). Antibiotics are the most commonly used drugs in neonates worldwide.

Medicine has, in the last decade, undergone a paradigm switch from talking about killer microbes to talking about the microbes that we need for a good health. When a newborn infant needs support directly after birth and is admitted to a neonatal intensive care unit, the antibiotic paradox is present from the first second of life. The high risk of dying from a pathogenic and, in large parts of the world, resistant bacteria versus the negative impact of antibiotics on the normal microbiota is part of the paradox.

Antimicrobial resistance (AMR) and antibiotic resistance (ABR) has affected both low-income countries (LICs), low- and middle-income countries (LMICs) and high-income countries (HICs) for many years. The increase in ABR represents a major threat to child health as well as public health (2). The prevalence of multidrug-resistantorganisms varies geographically in healthcare settings.

In 2015, the World Health Organization (WHO) identified ABR as one of the biggest threats to human health and as a key priority in reducing mortality due to infectious disease (3). MDRO infections are significantly associated with higher economic burden than infections with susceptible bacteria (4). In 2019, a collaboration between leading biopharmaceutical companies, the World Health Organization, the Wellcome Trust and the European Investment Bank resulted in the launch of the AMR action fund. The AMR action fund aims to bridge the funding barriers of late-stage antibiotic development in all countries and to bring 2-4 new antibiotics to the market before 2030.

Outbreaks of MDRO that colonize hospital-admitted neonates, share some common characteristics: the time needed to identify the problem, the difficulties in identifying the source, the time to implement infection control measures, and finally, eradication of the bacteria (5). If it is known that an outbreak of MDRO is taking place in the neonatal intensive care unit (NICU) and what the characteristics of the causing strains are, the chance of treating a septic neonate increases.

When the work with this thesis started, the nature and development of the neonatal intestinal microbiome was not as widely studied as it is now. It was more commonly assumed that the uterus environment and the first fetal meconium were sterile and that the neonate gut was strictly colonized with bacteria after birth. Recently, data on the perinatal and infant microbiome has shown otherwise (6-9). However, the importance of microbial communities at different times in the developing fetus´s and infant´s life, is yet not fully understood (10, 11).

The ongoing research on the spread of ABR-bacteria is broad and making progress. Strategies for how to educate societies, individuals and health care personnel to reduce mortality from ABR-related infections is on the agenda of many countries. Understandingly, some of them have greater difficulty in achieving the set goals. One way to tackle the invisible threat posed by antibiotic resistance infections to neonates, is to look at the way it challenges neonates and their health care workers in different settings. This thesis contributes results from research studies carried out in both a LMIC and a HIC setting.

How prevalent is neonatal colonization of ABR-bacteria, such as ESBL-producing Enterobacteriaeae (EPE), in neonates in a LMIC with high antibiotic use, limited infection-control measures and low awareness of antibiotic resistance? This thesis presents results from a study in a tertiary university hospital in Ecuador, which was done in order to strengthen the indications for preventive work in NICUs and to raise awareness within the country and the Latin American region.

The bacteria in the human microbiome are a highly important reservoir of antibiotic resistant genes. Since intestinal colonization of resistant Gram-negative bacteria is a critical step in the pathogenesis of invasive infections, the issue of duration of colonization is clinically important within NICU-settings. The question of duration of colonization is addressed in this thesis.

Advances in neonatal intensive care in HIC have improved survival of extremely low birth weight infants and other critically ill newborns, who are highly exposed to nosocomial infections (12). These neonates do often have a long duration of hospital stay. We conducted a study to determine the incidence of Gram-negative bacterial (GNB) sepsis and the burden of ABR among neonates in the Stockholm region. We had a hypothesis that there was a low to moderate prevalence of GNB-sepsis but a high in-hospital mortality when the sepsis causing bacteria were EPE. We wanted to bring data into the discussion about whether or not

our sepsis care and our antimicrobial stewardship was efficient during the neonatal period in our region.

For the neonatologist, to make a good assessment of the specific EPE-strain that is causing the NICU outbreak, the new sequencing methods are helpful. It is a fact though, that they take a considerable amount of time and in a situation where the bacteria are already ahead of us, time is an important factor. In this thesis, we address plasmid-mediated spread of resistance in neonatal intensive care settings and optical DNA mapping (ODM) as a tool to rapidly diagnose it.

2 BACKGROUND

2.1 BACTERIA

Bacteria were one of the first living organisms on earth. They are primitive one-celled organisms with no nucleus and free-floating genetic material in their cytoplasm. The bacteria store their DNA in the nucleoid (chromosomal) and in DNA molecules in the cytoplasm (plasmids).

Bacteria reproduce by dividing into two identical daughter cells. They are classified in divisions (phyla), subdivisions (subphyla), subgroups, families and genuses. Another classification of bacteria is whether or not the bacterium has one or two membranes. These two groups have different responses to a color staining procedure called Gram-staining, with some exceptions. The Gram-positive group of bacteria (GPB) has one membrane (monoderm) and the Gram-negative group (GNB) has two (diderm). The Gram-staining technique is used to group bacteria based on how their cell wall is constituted. The thick peptidoglycan layer are stained purple in GPB while the GNB have a thinner peptidoglycan layer in the cell wall and are not stained.). Not all bacteria have a cell wall. Bacteria have different shapes and are as well classified into five groups according to their shapes: rods-shaped (bacilli), spiral (spirilla), comma (vibrios), spherical (cocci) or corkscrew (spirochaetes). Gram-staining and shape description are rapid tools to group species into subdivisions of the bacteria. The cell structure of a bacteria is depicted in **figure 1**.

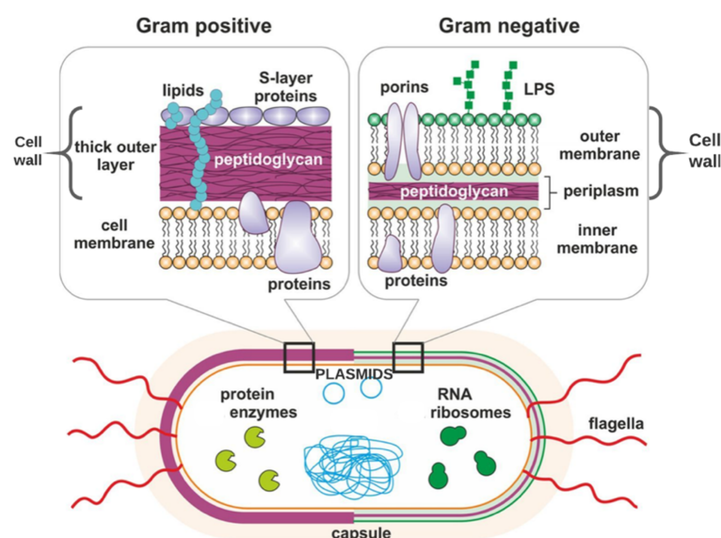

Figure 1. *Cell structure of Gram-positive bacteria and Gram-negative bacteria. Modified and with permission to republish from American Society for Microbiology.*

Bacteria produce bioactive substances, so called “natural products”, which have antibacterial activity. These substances from antibiotic-producing microbes have been used to prevent and treat disease for more than 2,000 years and eventually this knowledge led to the synthetic production of the modern antibiotics (13-16). The Gram-negative (diderm) bacteria have an outer membrane composed of a thick lipopolysaccharide layer. One hypothesis concerning bacterial evolution has proposed that this outer cell membrane has evolved from monoderm bacteria to diderm bacteria because of the natural antibiotic selection pressure (17).Thus, the thick outer membrane of the Gram-negative bacteria provides additional protection from the surrounding environment.

Bacteria have, as well, responded to the introduction of antibiotics by developing several other protective capabilities such as; the production of specific antibiotic efflux pumps, the downregulation of porins to decrease the permeability of antibiotics through the cell membrane and the production of enzymes that degrades the antibiotic structure. The virulence of a bacteria is its ability to damage the host.

In 2008, a group of bacteria were defined as the ESKAPE pathogens. They are more capable of “escaping” the action of antibiotics than others and are problematic in terms of ABR. The ESKAPE group consists of *Enterococcus faecium, Staphylococcus aureus* (SA), *Klebsiella pneumoniae* (KP*), Acinetobacter baumannii* (AB), *Pseudomonas aeruginosa* (PA) and Enterobacter spp (18, 19).

In this thesis, I will address how the clinically relevant Gram-negative bacilli KP and Enterobacter spp, from the family Enterobacteriaceae, can affect the neonate. The family Enterobacteriaceae is a large group within the group Gammaproteobacteria and phylum Proteobacteria.

2.1.1 Plasmids

Plasmids are circular, double-stranded large DNA molecules (1-1,000 kb) in the bacterial cytoplasm. These genetic elements are extrachromosomal and not essential to maintain basic bacterial functions. However, they play a crucial role when it comes to adapting to new environments and to carry and spread resistance genes. The plasmid replicates its DNA autonomously and has the ability to acquire and insert new genetic material into its sequence or drop genes that no longer are necessary. Many of the important human pathogenic bacteria within the *Enterobacteriaceae* family transfer plasmids carrying resistance genes (20).

The process that transfers plasmid DNA between bacteria to another is called conjugation. Conjugation is mediated by a transport system that includes a relaxase protein hooking on to the plasmid DNA on the cytoplasmic side and a 12-protein complex transport system which forms the transport channel and the pilus (fimbriae) in the bacteria. The DNA-relaxase complex is then transported through the pili into the recipient bacterial cell (21). Plasmids are classified in a number of ways. One traditional classification is into incompatibility groups (Inc groups). Plasmids in the same Inc group share the replication machinery and interfere with each other´s replication. Plasmids in different Inc groups can coexist stably (22).

2.2 ANTIBIOTIC RESISTANCE

2.2.1 An historical glance

“The thoughtless person playing with penicillin treatment is morally responsible for the death of the man who succumbs to infection with the penicillin-resistant organism”

-Sir Alexander Fleming 1945

Antibiotic resistance is a natural phenomenon that began to accelerate almost a century ago, when antibiotics came to be the foundation of modern medicine for treating bacterial infections (23). ABR is driven by many factors that range from poverty to poor policy implementations. The golden age of antibiotics was 1950-60. **Figure 2** shows the sources of the origin of the antibiotics, when resistance against them was found and some hot spots in neonatology in the same timeline.

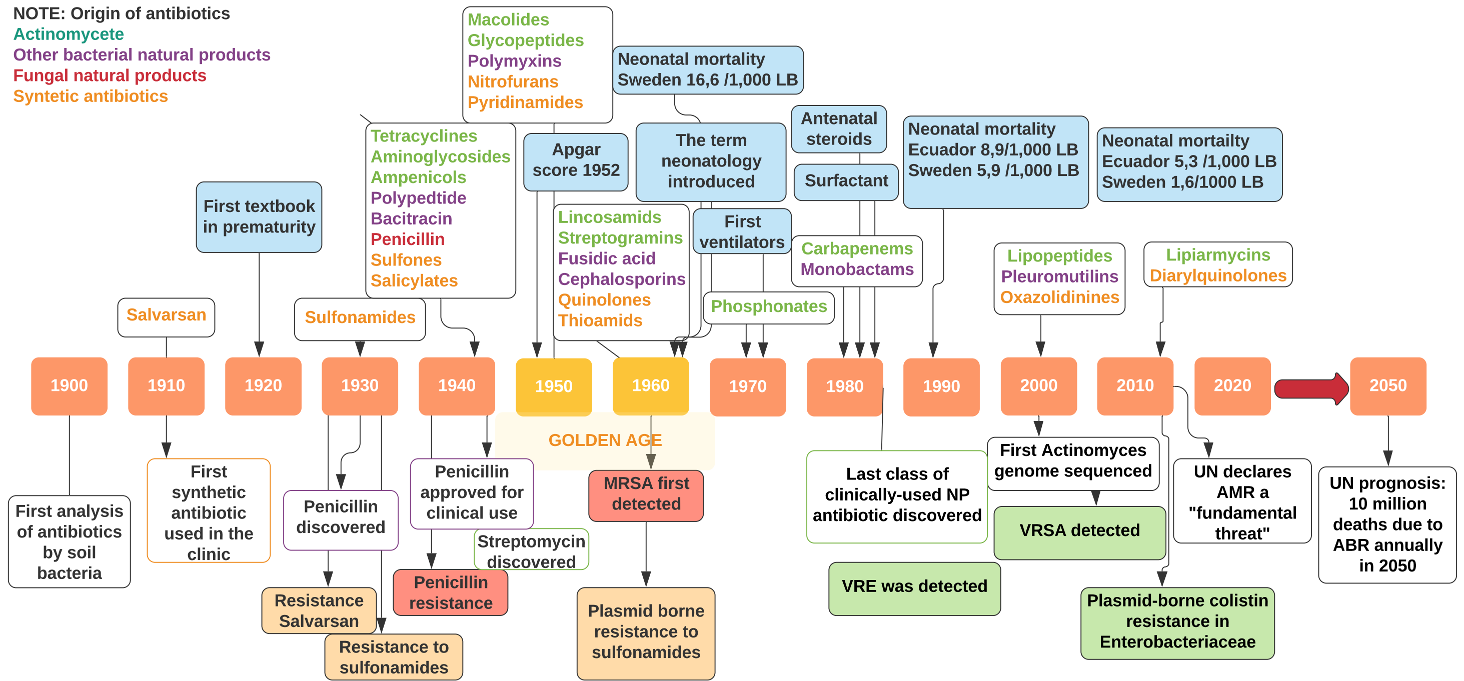

Figure 2. *Timeline showing the decade that new antibiotic was developed and reached the clinic. It shows the source of origin were green=actinomycetes, purple=other bacteria, red=penicillin, orange=synthetic products. The blue boxes indicate some important years in the development of neonatal care. Modified after permission from Current opinion in Microbiology, Hutchings et al.*

The WHO has identified a list of priority pathogens as highly important pathogens for future research on new treatment methods. ESBL-producing and carbapenemase-producing *Enterobacteriaceae* are included in the critical (priority 1) group as highly important pathogens for future research on new treatment methods. The global spread of ABR in Gram-negative bacilli is one of the largest concerns because of their ability to confer resistance to carbapenems which have, until lately, been the drug of choice when treating MDR-infections. At this point in time, 1.5 million people in the world die every year of antibiotic resistant infections. A UN prognosis predicts 10 million deaths due to ABR annually, in 2050.

2.2.2 Antibiotic mechanisms and bacterial mechanisms of defense

All antibiotics have one of the following mechanisms of action against bacteria: inhibiting the bacterial cell wall synthesis; preventing bacteria from synthesizing proteins; or preventing bacteria from duplicating genetic material. Beta-lactam antibiotics include penicillins, cephalopsporins, monobactams and carbapenems. They are bactericidal and inhibit the biosynthesis of the cell wall. Broad-spectrum beta-lactam antibiotics are mainly used to treat

severe Gram-negative bacterial infections. The clinically most significant mechanism of resistance among Gram-negative bacilli is their ability to produce Extended-Spectrum Beta-Lactamases (ESBL). ESBLs are enzymes that, by cleaving the amide bond in the beta-lactam ring inhibit multiple classes of beta-lactam antibiotics, and in some cases all beta-lactams (24). The beta-lactamase in GNB is excreted between the cytoplasmic membrane and the outer membrane, called the periplasmic membrane. The ESBLs are classified into four classes, A to D.

Of all ESBLs, the CTX-M beta-lactamases are the most widespread enzymes (25).
The CTX-M family consists of about 40 enzymes which can be divided into five different phylogroups, or clusters, and each of which contain a number of plasmid-mediated CTX-Ms are included. For example, the CTX-M group 1 includes six plasmid-mediated CTX-Ms (26). In 2008, the New Dehli Metallo beta-lactamase (blaNDM-1) was first found (27). This carbapenemase inactivates the carbapenems, the antibiotic class that was developed to overcome beta-lactamases.

2.2.3 Plasmid-mediated antibiotic resistance

ABR evolves constantly, and traditionally the epidemiology of resistant bacteria has been investigated with strain typing to document clonal spread. One reason for the successful dissemination by EPE, in recent years, is the result of the evolution and horizontal spread of beta-lactamase genes that are located on plasmids. CTX-Ms were originally chromosomally encoded beta-lactamases in the Klyvera spp but did later mobilize on to transferable plasmids in *Enterobacteriaceae*. ESBL genes are the most frequently described genes encoded on plasmids. Various plasmids are associated to different gene classes i.e IncI are mainly associated with ESBL-genes. (22)

Resistance gene transfer via plasmids can result in generation of novel multidrug-resistant strains that are not clonally related and therefore the traditionally used epidemiological methods are not sufficient to detect nosocomial transmission. Traditional characterization of plasmids requires several different, time-consuming techniques, such as S1/PFGE, PCR and Southern blotting (28).

2.3 ENTEROBACTERIACEAE

The family *Enterobacteriaceae* is large and consists of rod-shaped, non-fermenting, facultative anaerobic Gram-negative bacteria. Some of them can be found in soil and water and some of them are a part of the human intestinal microbiome. The most common *Enterobacteriaceae* are the pathogens *Escherichia coli* (EC) and *Klebsiella pneumoniae* (KP) which can cause severe infections but can also be commensals.

2.3.1 *Escherichia coli*

There is a great diversity of pathogenicity within the EC-species. EC is usually a non-pathogenic member of the commensal intestinal microbiome and the majority of the EC have low-virulence. In the last 15 years, resistant strains of EC have become increasingly prevalent. These strains are more virulent and give EC the ability to overcome host defenses and cause infections like sepsis, urinary tract infections and diarrhea. EC are classified by their serotypes (O, K, H), their phylo groups (A, B1, B2, D) and their sequence types (STs) (35, 36). Bacterial characteristics and behavior differ between each phylo group. The phylogroups A and B1 are assumed to be low-virulent and therefore commensals. Extra intestinal infections (ExPEC) are mainly caused by phylogroup B2 and D and are also predominant in neonatal sepsis. Phylogroup B2 is associated with multiple virulence genes and longer duration of intestinal colonization compared to the other phylogroups (37, 38). The virulence factors in ExPEC are capsules (K1), toxins *(hemolysin),* toxin transporters *(sat, vat)* adhesins *(pap, fimbriae)* and iron invasion of endothelial brain protein *(ibeA).* When ExPEC are described by multi-locus sequence typing (MLST), it has been shown that ST131, ST 69 and ST 117 seem to be responsible for a large proportion of the invasive infections. The leading serogroup is O1, which is the leading cause of sepsis in pediatric patients (38).

In EC, blaCTX-M 15 is the most common and globally disseminated ESBL-variant.

2.3.2 *Klebsiella pneumoniae*

KP was first described in 1882 and is the clinically most important Klebsiella species. KP is a colonizer in mammalian mucosal surfaces (mostly in the gastrointestinal tract and the respiratory tract) and is also found in water, plants and soil. Immunocompromised patients are particularly susceptible to KP. KP is, after EC, the second leading cause of GNB- sepsis in a pediatric population (29). The pathogenicity of KP is linked to the presence of virulence factors, such as the capsule, the lipopolysaccharides (LPS), the siderophores and the fimbriae

(30). These virulence factors appear to facilitate the initial colonization of the mucosal surface of the host, and then to persist and infect the host. KP have 78 different capsular types, where K1, K2, K5, K54 and K57 are associated with invasive infections of KP. The capsule contributes to the formation of biofilms and biofilm formation is an important strategy among KP strains to resist antibiotic treatment (31, 32).

Most antimicrobial resistance in KP results from resistance carrying plasmids, but this species is also intrinsically resistant to ampicillin due to the presence of SHV beta-lactamase. Nosocomial isolates of KP often carry plasmids with resistance genes and not so often virulence plasmids. Nosocomial KP-strains are therefore more often resistant to antibiotics, compared to the KP-strains that are spread in the community (33). Some high-risk clones (ST 15,48, 101, 147 and 383) of KP, have been described carrying a "hybrid resistance" which means that they feature both resistance -and virulence genes of major clinical concern (34).

2.4 METHODS FOR DIAGNOSING EPE

2.4.1 Phenotypic confirmation

Rectal swabbing is a well-known and often used technique to collect samples to confirm intestinal ESBL-colonization. Detection of the ESBL enzyme in *Enterobacteriaceae* involves two phases, namely screening and confirmation.

Firstly, the bacterial samples are plated on selective cephalosporin agar to screen for reduced sensitivity. Cefpodoxime or cefotaxime and ceftazidime are normally used in this first step. (39). Secondly, there is a phenotypic confirmatory test, such as combination disk testing or double disk synergy testing, both of which demonstrate that the beta-lactamase can be inhibited by a beta-lactamase inhibitor, such as clavulanic acid. (40). The confirmation can as well be automated by VITEK 2 (bioMérieux).

2.4.1 Genotypic confirmation

To determine the relatedness between pathogenic bacteria, pulsed-field gel electrophoresis (PFGE) was for a long time considered the gold standard method. In later years, more automated sequence typing methods to investigate evolutionary relationships among bacteria have been introduced, such as multi-locus sequence typing (MLST) or core gene MLST (cgMLST). These methods are normally applied on sequence data from whole-genome sequencing (WGS). MLST is a widely used method that is based on characterization of bacterial strains by their unique allelic profile in the DNA sequence variations in 7 housekeeping genes. The principle of MSLT is PCR amplification and DNA sequencing. The MLST technique was, in 2014, extended to a cgMLST protocol for KP that targets 694 core genes. This method is used to define clones with higher resolution and how they aggregate into clonal groups (CGs) (41). WGS is increasingly used in place of PCR-based sequencing. In WGS, the sequence data includes both chromosomal and plasmid DNA. Single nucleotide polymorphism (SNP) genotyping is a typing method of to measure genetic variations. A SNP is a mutation of a single base pair in the bacterial DNA.

2.4.3 Optical DNA-mapping of plasmids

In optical DNA mapping (ODM), sequence information is obtained for single large DNA molecules, with a resolution of around 1 kb. This method is well suited to determine the number of all large plasmids in the bacterial cell and to characterize them by size and by their unique barcode.

The plasmid barcode can be used to identify spread of previously unknown plasmids between different strains and species of bacteria (42, 43). Furthermore, previously sequenced plasmids can be identified by comparing experimental barcodes with theoretical barcodes of thousands of plasmids within the NCBI public database (44).

Labeling of the DNA molecules for ODM can be done in different ways (45). We here use competitive binding of fluorescent molecules, where fluorescent YOYO-1 and non-fluorescent netropsin are added to the plasmid DNA. The YOYO-1 molecule can bind to both AT-rich and GC-rich regions of the DNA-molecule while netropsin is AT-specific and therefore competes with YOYO-1 for the AT-rich regions (46). This results in an emission intensity variation along the DNA molecule that reflects the underlying sequence, where AT-rich regions are dark and GC-rich are bright, a DNA barcode **(figure 3)**.

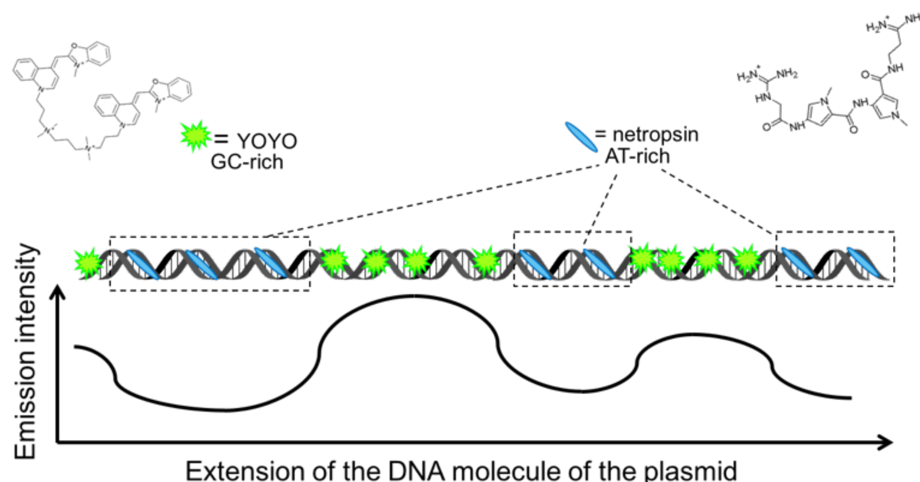

Figure 3. *The principle of optical DNA mapping via competitive binding of YOYO and Netropsin to plasmid DNA.*

An extension of the ODM assay is to use the CRISPR/Cas9 system to determine on which plasmid a specific (resistance) gene is located (47). Clustered Reguarly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein 9 (Cas 9) is a adapted from the genome editing system in bacteria (216). The Cas9 enzyme is guided to the specific DNA site by a guide RNA g(RNA) and produces a double-stranded break at the site of interest, in our case where the resistance gene is located. The cuts will occur at the same position along the sequence and is therefore site-specific. If the double-stranded break is caused by light, it will appear at random sites along the DNA sequence **(figure 4)**.

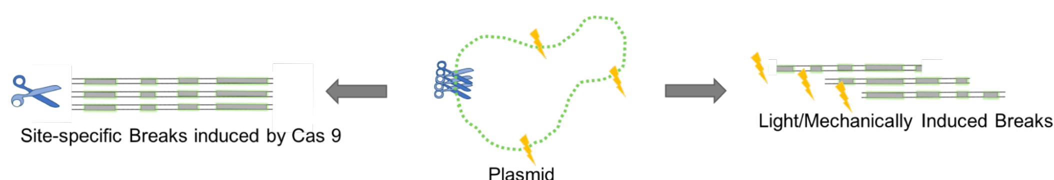

Figure 4. *Cleavage of the plasmid by Cas9 or light induced.*

To visualize the variation in emission intensity along the plasmid, the plasmids need to be stretched and linearized. This can be done by in several ways (48), but here we stretch the plasmids by forcing them into nanochannels by pressure. Using a fluorescence microscope, an image is then taken of the linearized plasmid in the nanochannels. The images of these one-dimensional intensity profiles are stacked underneath each other to form a kymograph. The kymograph is then aligned and when the mean value of each column has been calculated,

an average emission intensity profile for the plasmid is obtained, the DNA barcode. To obtain a consensus barcode, an average barcode from many plasmids are averaged. A flowchart of ODM can be seen in **figure 5**.

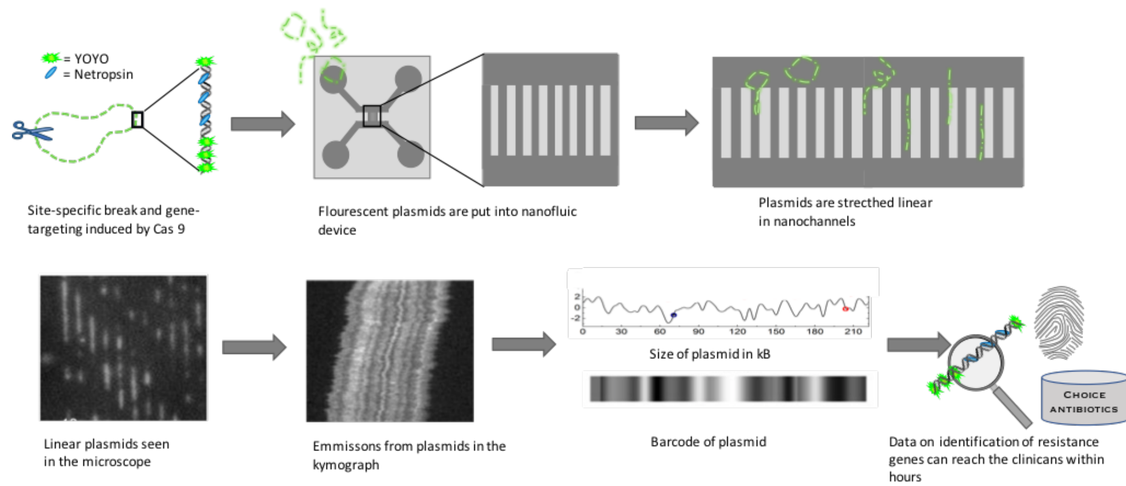

Figure 5. *Optical mapping as a rapid method for characterization of bacterial plasmids. The plasmids are cut by Cas9 at the site of the resistance gene and labeled with fluorescent and non-fluorescent molecules. The plasmids are forced into in nanochannels and stretched linear. The underlying DNA sequence of the plasmids is imaged as emission intensity profiles and time traces (kymograph) represent the optical maps and gives each plasmid a unique barcode and ID.*

2.5 NEONATES

The neonatal period is defined as the first 28 days after birth. A normal pregnancy lasts about 40 weeks. Preterm birth is classified, based on gestational age in weeks (GW), into extremely preterm (less than 28 GW), very preterm (less than 32 GW) and moderate to late preterm (32-36 GW). Infants born extremely preterm and very preterm need, to a variable degree, supportive care at a NICU.

Globally, around 15 million babies are born preterm and complications due to prematurity are one of the leading causes of death under five years of age (49). The worldwide incidence of preterm birth is estimated to be 11%, ranging between 18% in Malawi and 5% in northern Europe (50). In Sweden 2018, the incidence of preterm born infants was 6,1% which was about 7000 births in total. The same year in Ecuador, preterm infants were 6.8% of all born and registered infants.

2.5.1 The neonatal defense against infections

The preterm neonate has an immature skin barrier and an intestine with a lower barrier function and underdeveloped peristalsis compared to term neonates. This makes the skin easier to perforate and the intestine a potential site of infection and inflammation. Bacterial translocation, from the gastrointestinal tract to extraintestinal sites, is more common in the premature infant than the term born infant (51, 52).

The most immature neonates have an immune system that is characterized by a low pool of neutrophils and during sepsis there is rapid exhaustion of the bone marrow reserve (53). Transplacental antibodies (IgG) play an important role in the first line defense against bacteria and low IgG levels are associated with late onset sepsis (54). Opsonisation, coating bacteria with antibody and complement, is an important step in the process to clear bacteria from blood and tissue. Impaired opsonisation is associated with low gestational age (55, 56). Administration of immunoglobulins (IVIG) that can stimulate complement activation, opsonic activity and antibody-dependent cytotoxicity, have had no prophylactic effect on neonatal sepsis (57). IVIG treatment in sepsis and suspected sepsis in neonates did not reduce mortality in a large multi-center trial (58).

The results of recent general discussions on the topic whether the neonatal immune system is defective or not, have indicated that the neonatal immune system responses are fully competent in some instances and lower in others (59). Neonatal immunity is suggested to not be simply immature, but actually regulated for the early stage of postnatal life. The first months of life are set to be an important time window for the immune system, and the gut microbiota and the immune development in infancy are tightly linked together (60-62).

2.6 HUMAN MICROBIOTA

The human microbiota is highly diverse and includes all microbial organisms that live in and on the human body. The number of bacterial cells in this complex ecosystem, is often estimated as 10 times as many microbial cells compared to human cells. This estimation has lately been questioned and the ratio has been approximated to be closer to 1:1 (63). The human microbiota consists of many phyla of bacteria and the most common are Bacteroidetes, Firmicutes, Actinobacteria, Verrucomicrobria, Fusobacteria and Proteobacteria.

The microbial communities (i.e. microbiota) in different parts of the body differ widely and play a functional role in the physiology of humans. The balance between protective commensals and pathogens is important. The commensals inhibit pathogen growth, metabolize drugs to active metabolites, digest carbohydrates, produce vitamins, stimulate and modulate immune function, stimulate gut motility, and maintain a barrier function and stimulate epithelial repair. There is a specific microbiome for the mouth, skin, urogenital tract, respiratory system, intestinal tract, breast milk and placenta (64). Most of the intestinal microbes are confined to the distal ileum and colon. The abundance and the species change over the gastrointestinal tract.

Changes in the composition of the microbiota occurs naturally during life, such as from infancy to adulthood and in pregnancy. Many external factors can change the microbiota which can be harmful (called dysbiosis) and result in long-term consequences like auto-immune diseases. Recent studies suggest that dysbiosis in infant and child gut microbiota is associated with childhood asthma, coeliac disease, type 1-diabetes mellitus and atopy (65-68).

2.6.1 Neonatal gut microbiota

The hypothesis that the fetus, uterus and placenta are germ-free environments has been challenged and further studied in recent years. Diagnostic methods such as next-generation sequencing (NGS), have established that the fetus does not reside in a totally sterile environment and subsequently that the meconium of neonates is not sterile. Recent studies have shown the presence bacterial DNA in placenta, meconium and amniotic fluid (6-9).

One study characterized a unique placental microbiome and showed a non-commensal microbiome with Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes and Fusobacteria. Antenatal urinary tract infection (UTI) in the first trimester and preterm birth <37 GW was associated with the same bacteria later found in the placental microbiome(7). Another study showed that the features of the bacteria in the microbiome of the placenta and amniotic fluid were the same as in the infant´s first meconium. This suggests a transfer of microbes in the foeto-maternal interface. At three days of age, the infant´s intestinal microbiota resembled the bacteria detected in colostrum (8). Gosalbes et al implies that the neonatal gut can be a reservoir of antibiotic resistant genes even before birth (69). It is more common to find bacteria that colonize the basal plate of the placenta from preterm deliveries than term deliveries (70).

These small recent studies suggest that there is a pre-colonization phase of the fetal intestine in utero, but the presence of bacteria is only an association and large perspective studies have not been done. The importance of this is so far is not known. However, there is strong evidence that the initial colonization (phase two) is clinically important and influences immune development in the neonatal gut (71). The colonization phases of the infant are presented in **table 1**.

The development of the gut microbiota in preterm infants shows that neonates can change the abundance of dominating bacteria in their gut microbiota within days and that the microbiota composition and bacterial load is strongly associated with postnatal age (72). Extremely preterm-born neonates often spend a long time in the NICU. A study of the neonatal microbiome in the NICU shows that the specific microbiome of one neonate was found in environmental bacterial samples, despite regular cleaning, and suggests that hospitalized neonates and their caregivers shape the microbiome of the NICU. In other words, each room/caring area has its own unique neonatal microbial fingerprint by the time the patient has been discharged (73).

Phases of intestinal colonization of the infant intestine	Normal conditions	Clinical conditions with an atypical (dysbiotic) intestinal colonization in the perinatal period
Phase one Intrauterine period	Some studies suggest that the fetus can become exposed to maternal microbiota by trans- placental passage into amniotic fluid.	Dysbiosis in maternal intestine (use of antibiotics, obesity) can affect transplacental passage of microbiota to the amniotic fluid. *So far, only associations in mice. More studies needed.*
Phase two 1st week of life	The neonate ingests vagina/colonic microbiota in birth canal. (Full term, vaginal delivery)	Sparse colonization due to dysbiosis inmaternal intestine, premature delivery, perinatal prophylactic antibiotics, ceaseran delivery
Phase three 2 weeks- 4 months	Introduction of oral feeding	The colonization process modifies slightly by introduction of feeding.
Phase four 2 weeks- 4 months	The period of weaning to solid foods	
Phase five 1 to 3 years	Infant recieves table-food and the gut microbiome resembles an adult intestine. Diversity of bacteria >1000 species	The colonization is delayed and incomplete until four to six years

Table 1. *Phases of the intestinal colonization of the infant. Modified from Walker et al "The importance of appropriate bacterial colonization of the intestine in newborn, child and adult health, Pediatric Research 2017.*

In a cohort, were the median gestational age of the study participants was 25-31, the most common bacteria (>50% of bacteria) in the gut microbiota were Staphylococcus, Enterococcus, Enterobacter and Bifidobacteria. With increasing postnatal age, the pattern changed from Staphylococcus-Enterococcus domination to the Enterobacter and later Bifidobacteria- dominated microbiota (72). A gut microbiome without dysbiosis stabilizes around 2-3 years of age (74). Premature neonates in the NICU have an intestinal bacterial composition consisting of more GNB compared to full-term breast-fed neonates who mainly harbor less harmful Bifidobacteria in their intestines (75).

2.6.2 Factors that influence the neonatal gut microbiota

Maternal microbiome

The early neonatal gut microbiota mirrors the microbiome of the mother. In the normal pregnancy, the maternal intestinal microbiome changes most significantly between first and third semester. The species diversity decreases and the counts of specific microbes increase in the third trimester, Actinobacteria and Proteobacteria being the species most commonly increased. In a healthy mother, the vaginal microbiome increases the abundance of the lactic acid producing bacteria, Bifidobacteria, towards the end of the pregnancy. A healthy intestinal microbiome has a high diversity of species and a healthy vaginal microbiome has less diversity (76). Dysbiosis in the vaginal microbiome, with a increased vaginal diversity and decreased intestinal diversity, is associated with preterm birth, but no specific vaginal species have been clearly associated with premature birth (76).

Feeding

The Bifidobacteria from the mother play an important role in the healthy microbial ecosystem of the neonatal intestine. Human and bacterial evolution have produced the most perfect composition of the breast milk for the newborn infant. Bifidobacteria metabolize the human milk oligosaccharides (HMO) into lactate and acetate to lower the pH in the intestine. This provides a “colonization resistance”, a strategy to limit opportunistic pathogens from growing (77, 78). The HMO are oligosaccharides that have no nutritional value for the baby as they are only digested by the gut bacteria. These HMOs interact and inhibit the binding of pathogenic bacteria to the host cells receptors (79, 80).

Human milk also contains microbes, such as Streptococcus spp, Staphylococcus spp, Cutibacterium and lactic acid bacteria. Less risk for colonization by EPE- strains has been shown to be associated with breastmilk and HMO formula feeding (77). Cow´s milk formula, on the other hand, is associated with a higher abundance of GNB in the neonatal gut.

In addition to Bifidobacteria, the gut microbiome of a healthy infant is also dominated by Lactobacillus species and Enterobacteriaceae species (11). These species stimulate specific IgA-antibodies in in the intestinal lumen and also inhibit pathogens from adhering to the intestinal wall, becoming a part of the intestinal “barrier effect” against invasive infection (81, 82).

Lactobacilli with antimicrobial properties in the oral microbiota are more common in breastfed infants compared to formula-fed infants (83, 84). A strategy of feeding probiotics

containing Bifidobacteria to prevent neonatal late onset sepsis (LOS) has been suggested, in theory, to increase the intestinal mucosal barrier in order to prevent bacterial translocation into the blood and exclude potential pathogens (78, 85). Early enteral feeding is also proposed to prevent atrophy of the intestinal mucosa and therefore establish a faster healthy flora and mucosal immunity (86).

Probiotics with *Bacteroides infantis* have shown to decrease the abundance of *Enterobacteriaceae* and the bacterial antibiotic resistant gene load in the infant gut in breastfed infants (87). The low pH is suggested to be the key to colonization resistance. A meta-analysis of 20 randomized controlled trials show that probiotics decrease severe (88). It is also stated that probiotics alone are not enough to maintain the complex homeostasis of the neonatal gut microbiota. Indeed, a holistic approach to the microbiome is needed (89).

When breastfeeding ends, the intestinal microbiota shifts to a more mature gut microbiota and the abundance of Firmicutes increases (90). Later the microbiome changes to include bacteria that facilitate the digestion of nutrients from solid food (90, 91).

Birth mode

Birth mode is a factor that strongly affects the gut microbiota in neonates (92). The gut microbiome differs in composition and diversity between infants born by caesarean section (CS) and vaginal delivery (52). The lack of colonization by the fecal and vaginal microbes results in a lower diversity and a decreased abundance of Bacteroides, Bifidobacterium and Lactobacillus in neonates born by cesarean section (93-95). Full term neonates are more dominated by oral and skin microbes in the gut microbiota after CS and Lactobacillus spp is, as mentioned, more often seen in the vaginally-born neonates. CS is correlated with a higher abundance of Staphylococcus spp early in life (96).

The gut microbiota of extremely preterm neonates with a long hospital stay, clinically delayed oral feedings, prolonged parenteral nutrition and long duration of antibiotic therapy, have shown to predominantly consist of KP, EC, *Staphylococcus epidermidis, Enterococcus fecalis*, and *Enterobacter cloacae* (97). Some of these species are the most common pathogens in LOS (98).

Antibiotic treatment

Studies show, that neonates that do not receive antibiotics develop a more diverse microbiota (91). Even a treatment period with antibiotics of less than 3 days has shown decreased the abundance of Bifidobacterium in the three subsequent weeks. After 5 days of antibiotic treatment, the Bifidobacteria abundance remains decreased until 6 weeks postnatally. After anti-fungal treatment, there were lower levels of Bifidobacterium as well (93).

Two studies have showed an association between intra partum antibiotic prophylaxis (IAP) for Group B Streptococcus (GBS) and delayed in colonization of Actinobacteria and decreased in abundance of Bifidobacteria (99, 100).

Early antibiotic treatment is, due to dysbiosis, associated with an increased risk of the feared neonatal complication of necrotizing enterocolitis (NEC) (101). A high abundance of Enterobacter, has been shown in the gut microbiota in premature born neonates after antibiotic treatment. A common belief is that the impaired intestinal barrier leads to a translocation of gut bacteria and bacteremia. Exposure to antibiotic treatment of more than 10 days, has been associated with an three-fold risk of developing NEC (102).

Studies on adult microbiota and antibiotic treatment have shown a correlation between antibiotic treatment and changes in glucose metabolism, development of diabetes type 2 and bodyweight regulation and as well a change in abundance of microbiota for several months after the treatment period (103-105).

2.7 NEONATAL SEPSIS

2.7.1 The neonatal sepsis definition

Neonatal sepsis is the inflammatory response of a systemic invasive bacterial, viral or fungal infection that affects the neonate during the first 28 days of life. Early-onset sepsis (EOS) is defined as sepsis presenting within 72 h after birth and late-onset sepsis (LOS) occurs between 72 h and 28 days of life (106). To make a definite sepsis diagnosis can be difficult in the neonatal period (107). The balance between the commensal intestinal bacteria and the invasive pathogen is complex. The clinical signs of sepsis are non-specific and other non-infectious symptoms can be similar to those of sepsis.

At the International Pediatric Sepsis Consensus Conference in 2005, a definition of neonatal sepsis was set. It included the systemic inflammatory response syndrome (SIRS)-criteria together with proven or suspected infection (108). Another hypothetical sequential organ failure assessment (nSOFA)-score has suggested a definition neonatal sepsis in 2018. The nSOFA algorithm includes six items with scores from 0 to 3; respiratory status, cardiovascular status, renal function CNS function, platelet counts and absolute neutrophil counts.

The inclusion of white blood cells has been questioned because of its poor predictive value (109). There are ongoing discussions regarding a definition of neonatal sepsis, but at this moment there is no consensus defined (110, 111). Neonates with clinical symptoms as SIRS and no detected bacteria in the blood-culture are diagnosed as "suspected sepsis". Most data on neonatal sepsis only include culture-confirmed cases of neonatal sepsis.

2.7.2 Incidence and mortality of neonatal sepsis

In 2018, 2.5 million children died in the first month of life. The numbers of these neonatal deaths are approximately 7,000 deaths every day. Neonatal deaths account for almost half (47%) of all global under-five deaths (112). Neonatal infections cause more than one third (36%) of neonatal deaths worldwide and neonatal sepsis remains the leading cause in LIC and LMIC (113). Neonatal sepsis due to EPE is associated with a high morbidity and mortality and the number of deaths due to all antibiotic resistant bacteria is estimated as 214 000 cases per year (1, 114-116).

Approximately 58,000 of these neonates die in India (117). One of the Sustainable Development Goals, promulgated in 2015, aims to decrease preventable deaths of newborns and children under 5 years of age by 2030. All countries are aiming to reduce neonatal mortality to at least 12 per 1,000 live births, and under-5 mortality to at least 25 per 1,000 live births (118).

The incidence of neonatal sepsis is 1-4 per 1,000 live births in HIC (119). In a review of 32 studies that reported neonatal bloodstream infections in LIC the neonatal sepsis rate is 3-20 times higher than in HIC. For example, the incidence of neonatal sepsis is 30-40/1,000 in the African region and 12/1,000 in the regions of the Americas.

Sepsis related mortality in neonates also depends on the causative agent. The mortality for GPB- LOS has in large studies been approximated to 6-8% and is suggested not to differ from the infants with suspected sepsis (8%)(120). GNB-sepsis is less prevalent than GPB-sepsis, but is associated with higher mortality (19-36%) (120-122). The case fatality rate of GNB-LOS in very low birth weight (VLBW) infants that were admitted to 313 NICUs in North America between 1997 and 2010 was 21% (123). Treatment failure of neonatal sepsis is associated with increased mortality, longer hospital stay and higher costs (12, 124, 125).

Neonatal sepsis (both GNB and GPB) is associated with neonatal morbidities like white matter injuries and subsequent neurodevelopmental sequele, bronchopulmonary dysplasia (BPD), NEC and retinopathy of prematurity (ROP) (98,119,121,131)

Incidence and mortality of neonatal sepsis in Sweden and Ecuador

Two studies from the western area of Sweden between the years 1975-1986 and 1987-1996, show the incidence of neonatal sepsis as 2.8-3.7/1,000 live births. The case fatality decreased in the second study to 9% from the previous 15% (126, 127). A recent study from the same area shows a significant decreased incidence of EOS from 1,4/1,000 live births (1997-2007) to 0,9/1,000 live births (2008-2017). The case-fatality rate of all patients with EOS was unchanged at 7%. The proportion of neonates with gestational age <28 rose during the study period but the incidence of GNB- sepsis remained unchanged (128). There are no present studies of incidence and mortality of GNB-LOS in Sweden.

The neonatal mortality in Ecuador has increased from 5.3 to 6.0/1,000 live born between the years 2010-2018. The total under five-mortality has decreased from 12.6 to 12.2/1,000 born during the same years (129) There are no available data on mortality due to neonatal sepsis in Ecuador.

2.7.3 Causative pathogens of early and late onset sepsis

The distribution of pathogens causing neonatal sepsis varies between regions and over time. It varies even within hospitals due to the demographic characteristics of the patients, the microflora colonization of the environment and the local antibiotic stewardship (130).

Hospital-based studies suggest that EC*, Streptococcus agalactiae* (group B streptococci, GBS), *Staphylococcus aureus* (SA) and KP are the most common pathogens causing neonatal sepsis globally. Etiological data for neonatal sepsis is challenging to collect and there are few community based etiological studies from LICs.

EOS is often caused by GBS, EC and *Listeria monocytogenes* while LOS is more often associated with SA, *coagulase-negative staphylococci* (CoNS), and Gram-negative bacilli. In the Gram-negative bacilli group, EC and KP, are the most clinically relevant species but CoNS is the most common pathogen group causing LOS in the extremely preterm born neonates (121, 131). In **figure 6**, the most common causative pathogens of neonatal LOS are presented. The data is from a review article of 11 LOS- studies and presents the incidence by geographical areas.

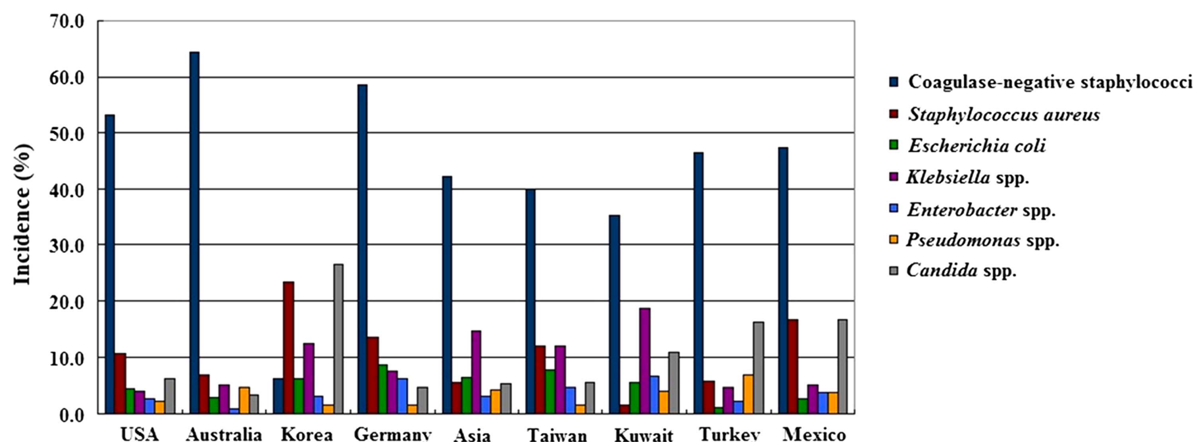

Figure 6: *The most common causative pathogens of neonatal LOS. Copyright: Dong et al, 2014. With permission to publish from Arch Dis Child Fetal Neonatal ED.*

CoNS accounts for approximately 53-78 % of LOS in HICs and 36-47% in LICs. Neonatal sepsis caused by anaerobic bacteria is uncommon. In the rare cases this occurs, the risk factors for be affected by anaerobic bacteria infection have been shown to be necrotizing enterocolitis, multiple abnormalities, and repeated steroid use (132).

2.7.4 Diagnostic methods and their challenges

The basics of bacterial diagnostics are culture approaches and the gold standard of the sepsis diagnosis is the blood culture. These laboratory investigations are time-consuming processes and generally takes around 48 hours (106, 133). Insufficient sample volumes, low-density bacteremia and antibiotic treatment prior to culture can reduce the performance of the culture and post mortem cultures have shown to be falsely sterile (134, 135). One milliliter (1 mL) is the recommended minimum volume for a blood culture. When the volume is sufficient, this method has shown to have a good sensitivity even when the level of bacteremia is as low as 1-4 colony forming units (CFU) per mL.

The sensitivity decreases by 10-40% when sampling 0.5 mL of blood (136). PCR techniques are not widely used as a diagnostic tool in neonatal sepsis, but it has been suggested that a positive 16S PCR can be used to support initiation of antibiotic treatment in culture-negative suspected neonatal sepsis (137). **Figure 7** illustrates the existing and desired workflow in diagnosing neonatal sepsis.

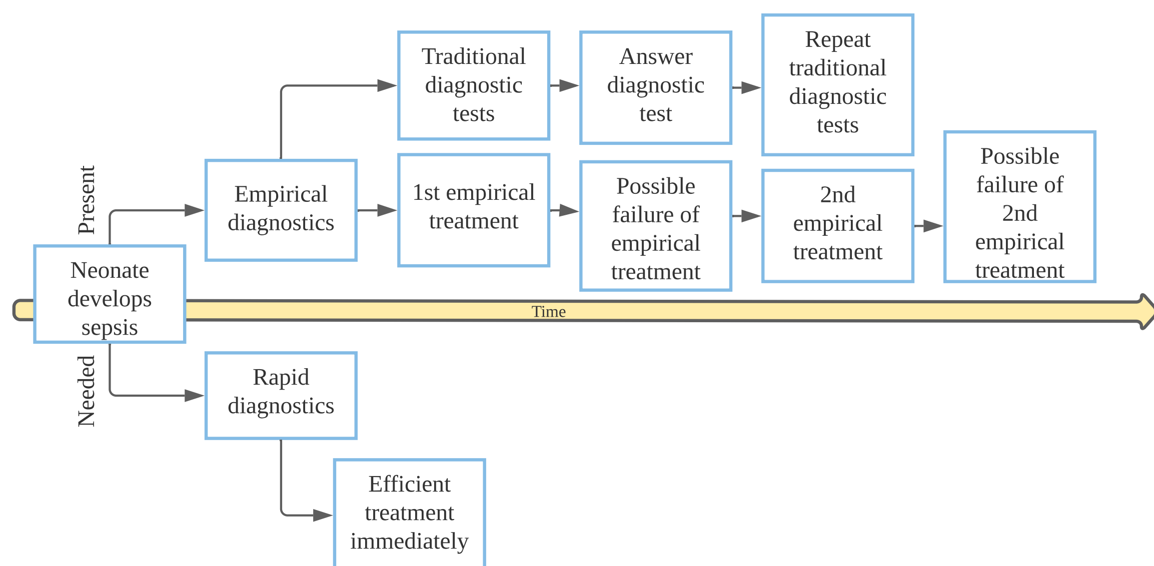

Figure 7. *Existing and desired workflow in diagnosing neonatal sepsis.*

Positive predictive value and sensitivity of biomarkers like C-reactive protein (CRP), Pro-calcitonin (PCT) and interleukins are at the onset of EOS suboptimal. This is due to perinatal non-infectious conditions such as meconium aspiration, complicated labor and intraventricular hemorrhage, that gives a rise in CRP and PCT (138). These methods are, nevertheless, used as diagnostic tools for sepsis in neonates whereas CRP is the most studied

biomarker. CRP rises physiologically in full term healthy neonates during the first 48 first hours of life.

After a vaginal delivery, CRP in a healthy term infant can reach CRP values between 40-50 mg/L (139, 140). PCT and CRP levels are affected by gestational age and preterm neonates are not responding with as high CRP and PCT levels, as full term neonates do (141, 142). CRP and PCT does not pass the placenta and is therefore not affected by maternal fever in labour(143). PCT, CRP and total blood count (CBC) is alone inaccurate to define neonatal sepsis unless the blood culture is positive (110).

2.6.5 Antimicrobial management of confirmed EOS/LOS and suspected EOS/LOS

Antibiotics are the most commonly used drugs in neonates worldwide. The antibiotic treatment is can be given either divided into when pathogens are known, or when suspected (empirical treatment).

Due to many risk factors for neonatal sepsis among preterm born infants, many of them are exposed to empirical or prophylactic antimicrobial treatment. The regimen, including duration of prophylactic antibiotic treatment, probably varies between different NICUs and even between neonatologists. The choice of empirical therapy should be decided by the antibiotic resistance susceptibility pattern of the bacteria often found in the specific NICU or in the community.

In international sepsis networks, the empirical treatment of culture confirmed EOS, is suggested to be ampicillin and an aminoglycoside. A cephalosporin (cefotaxime) should be used if meningitis is suspected. If no pathogen is isolated, the consideration should be given to ending. For EOS, 10 days of treatment is suggested and for GBS or uncomplicated meningitis the treatment should last 14 days. If the infection is complicated, the treatment period can be extended up to 21-28 days. The empirical treatment for LOS is vancomycin and an aminoglycoside (106).

To reduce the selective pressure from the cephalosporin usage, a penicillin and aminoglycoside combination is recommended. When GNB- sepsis is proven or strongly suspected a third-generation cephalosporin should replace the penicillin. Piperzillin-tazobactam combination covers the Gram-negative bacteria better than the penicillin-aminoglycosides combination with lower risk than cephalosporins of driving resistance, but is not commonly used in neonates (144).

Nowadays in Sweden, the recommended empiric treatment for EOS is benzylpenicillin in combination with an aminoglycoside. For LOS with mild clinical symptoms of sepsis the recommended empiric treatment is isoxazolylpenicillin and an aminoglycoside, and for severe clinical symptoms, the empiric treatment is cefotaxime plus an aminoglycoside. Vancomycin is chosen when resistant CoNS is suspected.

2.7.5 Suspected sepsis

Neonates with culture-negative sepsis but suspected contribute to a high antibiotic consumption in NICUs. Studies conducted in HICs report a significant higher use of antibiotics in suspected sepsis than culture-proven cases (109, 145, 146). The clinical symptoms and the pathophysiological response to bacterial infection differ in term and pre-term neonates due to age-dependent maturity.

If the antibiotic treatment is continued longer than 36 h despite negative blood-cultures in EOS, the National Institute for Health and Care Excellence (NICE) guidelines from the United Kingdom, suggest that the neonate should be reviewed, for continuation of treatment every 24 h (147). There are limited data on the difference in outcome between suspected sepsis and confirmed sepsis but effective interventions to reduce antibiotic consumption have been successful (145).

2.8 COLONIZATION AND INVASIVE INFECTION OF EPE

Most studies on EPE-colonization are conducted in adults. A high rate of EPE-acquisition have been showed in European travellers that returns from Asia (148), sub-Saharan Africa and Latin America (149). The duration of the EPE-colonization is relatively short, since in healthy persons, the EPE are most commonly outcompeted by other species in the intestinal microbiome (149). Contrarily, when the pathogen has colonized or infected patients in a hospital setting, the abundance of ESBL-KP declines over time when the patients have reached a healthy state outside of the hospital setting (30).

Specific virulence factors of the bacterial strain play a role for EPE-colonization and the colonization rate has been presented as (43%) in adult patients 12 months after the ESBL-EC infection (149, 150). A hypothesis on the dynamics of intestinal EPE-colonization is seen in **figure 8**.

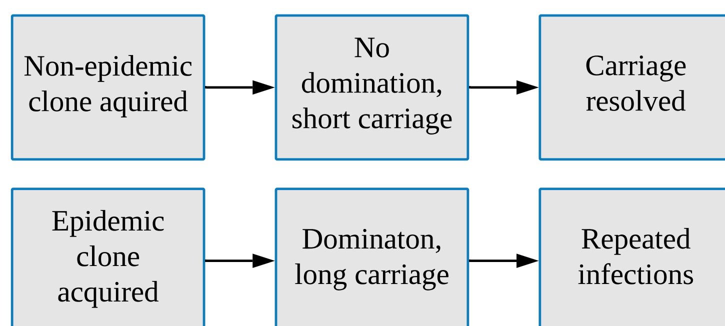

Figure 8: *A hypothesis on the dynamics of EPE-colonization.*

2.8.1 EPE in neonatal intensive care units

EPE-colonization in neonates is clinically important since colonization of EPE is a critical step in the pathogenesis of invasive infections. It has been shown that EPE-colonization is associated with subsequent invasive infections caused by the same strain of colonized neonates (151-153). The progression to infection is not only reliant on the immunocompetence in the neonate, but also on the genes the bacteria possess (154).

In a Swedish study from a NICU in Lund 2013, the prevalence of the EPE-colonization was 1.77% in a non-outbreak situation. This was considered low in a global perspective. Although

there is relatively low level of spread of antimicrobial resistant bacterial strains in Swedish NICUs, there have been a number of outbreaks of intestinal EPE-colonization in recent years.

A number of factors have been associated with neonatal intestinal EPE-colonization, such as low birth weight, low gestational age, long hospital stay, early-onset pneumonia, CS, maternal to neonatal transmission and prior treatment with antibiotics (155, 156). The maternal-neonatal transmission of EPE is high, in both LIC and HIC, and maternal colonization is proposed to be the most important risk factor for intestinal EPE-colonization in neonates. Vertical transmission of EPE from mother's breast milk to the neonate has been shown during an outbreak (157). Treatment of intestinal EPE-colonization is not recommended (155, 158, 159).

Carbapenems are the drugs of choice in neonatal EPE-infections and are recommended to be used as a monotherapy. The use of older antimicrobial agents, such as colistin, has been reintroduced because of the emergence of multi-drug resistant Gram-negative bacilli. This drug has earlier been associated with adverse effects such as nephrotoxicity. Colistin is effective and reasonably safe for treatment in EPE-sepsis in preterm infants, but unfortunately the colistin resistance is reported to emerge as well (160, 161).

The experience of using fosfomycin in neonates is low and this treatment was for a long time considered as the last option to treat carbapenem resistant EPE(162). In later years, new antibiotics such as, ceftazidime-avibactam, ceftozolane/tazobactam and meropenem, have been introduced. Limited data exist on use in children and especially neonates (163, 164).

2.8.2 Outbreaks of EPE in NICUs

NICU settings are particularly vulnerable due to high antibiotic use, immunocompromised patients and high transmission of bacteria between patients or between patients and staff and parents. A systematic review of 276 NICUs and 453 other ICUs showed that Enterobacteriaceae significantly more often caused outbreaks in NICU than in other ICUs (165).

Some specific clones of resistant EPE, i.e. E. coli ST131 have reached pandemic spread and carbapenem-resistant producing Enterobacteriaceae (CPE) have been detected worldwide in recent years (166, 167).

The spread of EPE-bacteria in NICUs may also occur in non-outbreak periods and may be the result of diffuse cross-transmission related to poor infection control procedures (168).

In Scandinavia, the first described EPE-outbreak at a NICU was an outbreak of CTX-M-15 KP in 2008-2009 in Norway. This was at the same time that a EPE-outbreak occurred at two NICUs at Karolinska in Stockholm. The situation regarding neonatal EPE-colonization or EPE-outbreaks in NICUs in Ecuador was unknown before our study. Reports from other parts of the region have shown a high frequency of ESBL-producing bacteria as well as limited resources for infection control and antibiotic resistance surveillance (156, 169, 170).

However, in contexts where the resistant bacteria have reached endemic levels, the dynamics of transmission seem to depend more on environmental factors in the NICUs. The first $bla_{\text{NDM-1}}$ -producing KP in a NICU in South America was found in Colombia in 2013 (171).

LICs and LMICs face large challenges in preventing MDR-outbreaks e.g overcrowded and under-staffed nurseries, lack of essential equipment (soap, clean water, sterilizers), lack of aseptic techniques and lack of limited possibilities to follow existing guidelines to optimize infection control (168). In an analysis of the global strategies to tackle antimicrobial resistance it seems that LIC and LMIC more difficulties in following the guidelines published by the Centers for Disease Control and Prevention and WHO (172).

In Sweden, successful action to contain ABR has included monitoring antibiotic use, active surveillance of resistance, educational activities and an achieved commitment at all levels of health and medical care (173).

3 AIMS

The overall aim of this Thesis is to improve health of infants and the older children by presenting how and to what extent ESBL-producing Enterobacteriaceae (EPE) colonize and infect newborn infants in an era of emerging antibiotic resistance.

The specific aims are:

Study I

- to highlight and determine the extent of the problem of ABR by showing the proportion of intestinal EPE- colonization among neonates and identifying the risk factors of aquistition of EPE in a tertiary NICU in Ecuador.

Study II and III

- to characterize Klebsiella pneumoniae isolates in an outbreak of ESBL-producing Enterobacteriaceae in a NICU in Sweden.
- to determine the duration of colonization of EPE after discharge.
- to investigate possible horizontal resistance gene transfer between bacterial species and between neonates during the outbreak, by using an optical DNA mapping technique.
- to highlight the usefulness of optical DNA mapping for revealing details about resistance carrying plasmids in an EPE-outbreak in a NICU-setting.

Study IV

- to determine the incidence, mortality and bacterial characteristics of neonatal sepsis caused by Gram-negative bacilli (GNB) sepsis in Stockholm County during 2006-2016.
- to present to show the extent to which antibiotic resistant Gram-negative strains have been invasive in this patient group.

4 METHODS

4.1 ETHICS

The Swedish Ethical Review Authority approved all studies in this thesis.

In study I, written information about the study was given to the parents in Spanish and verbal consent was obtained and documented in the patient´s medical record. The Regional Ethical Committee of Cuenca University, Ecuador approved the part of the study that was conducted in Hospital Vicente Corral Moscoso. In study II and III, written informed consent was obtained from all parents of the included neonates.

Study IV is a retrospective register study and spans a large time interval, which is why it was not considered reasonable or possible to obtain approval from each individual that participated in the study. Other similar surveys have shown that parents generally have great satisfaction in being able to contribute to current research issues. No identifiable data can be linked to any study subject.

4.2 PATIENTS AND SETTINGS

Paper	Inclusion criteria	Setting
I	All admitted neonates>24 h of age	One third level NICU, 24-beds, 400 admissions/year Hospital Vicente Corral Moscoso, Ecuador Patient-nurse ratio 7:1 Shortest space between incubators 75 cm
II-III	All neonates colonized with ESBL-KP at discharge from NICU after EPE-outbreak	Two third level NICUs, 46-beds, 1,000 admissions/year Karolinska University Hospital, site Solna and HuddingePatient-nurse ratio 3:1
IV	All neonates with culture-confirmed Gram-negative sepsis between January 2006-December 2016.	Four 2-3 level NICUs, 80-beds, 2,900 admissions/year Karolinska University Hospital, site Solna, Huddinge, Danderyd and Sach´s Children hospital, Stockholm Patient-nurse ratio 3:1

Table 2. *Characteristics of each study population and setting in the four different papers*

4.3 STUDY DESIGN

Study I, was a prospective cohort surveillance study of EPE-colonization at a third level NICU at the Hospital Vicente Corral Moscoso, Cuenca, Ecuador. In **study II-III**, we followed 14 neonates, colonized with EPE after a 5-months long outbreak, prospectively for five years after their discharge from two NICUs in Stockholm. The follow-up period was every second month for two years after discharge and the children were cultured again three times during 6 successive weeks at age five years. **Study IV**, a matched cohort study, based on registry data and medical records, was undertaken where all neonates with GNB-sepsis at Stockholm´s four NICUs between January 2006 and December 2016 were included.

4.4 DATA COLLECTION

4.4.1 Study I

In study I, data on patient characteristics of each neonate was retrieved from the medical records in Hospital Vicente Corral Moscoso, Cuenca, Ecuador. Stool samples were collected from admitted neonates every two weeks during a three-month period. We collected data on risk factors for vertical transmission of EPE from the mother and as well for acquired colonization during NICU stay.

4.4.2 Study II and III

In study II-III, we collected data on patient characteristics from the electronic medical record system Take Care. We collected stool samples from hospitalized neonates every two weeks until discharge, thereafter every two months until two years of age. A long-term follow-up was conducted. This follow-up consisted of three subsequent sample collections, every other week, during a six-week period five years after discharge from the NICU. After discharge, the caregivers filled in a form about antibiotic therapy, international travel, hospital visits and the use of probiotics.

4.4.3 Study IV

All data for study IV was collected from the Swedish Neonatal Quality Register (SNQ)(174), the Medical Birth Register (MBR) and from the electronic medical record systems Take Care and Clinisoft. To identify the patients to be included we used the ICD-10 codes for bacterial sepsis We defined sepsis as: a positive blood-culture, at least two clinical signs (fatigue,

respiratory instability, temperature instability, poor feeding, vomiting, cyanosis) and antibiotic therapy for at least five days.

All patients with GNB-sepsis were matched with two groups of controls. The three groups were matched by gestational age, the close birth date (the nearest birth date as the case) and born at the same hospital. The first control group were matched with a 1:1 ratio and consisted of patients with suspected sepsis with clinical symptoms, a negative blood culture and subsequent antibiotic therapy for at least 5 days.

The second control group consisted of uninfected controls that were alive at 72 hours of age. The uninfected controls were matched with a ratio of 1:2.6 (74 LOS cases:196 uninfected controls). The uneven We excluded the cases with Gram-positive sepsis in the regression analysis. The age at infection of the case became the index age for the controls, in the mortality analysis.

4.5 SELECTION OF BACTERIA

All EPE was screened for in study **(I)**, and we found and analyzed KP-ESBL and EC-ESBL. In **study II-III**, the main focus was on ESBL-KP but ESBL-EC isolates were as well thoroughly studied. In study **(IV)**, all Gram-negative bacteria were analyzed with susceptibility testing.

We retrieved 33/107 Gram-negative strains from the study period for further molecular characterization. These strains were collected in the calendar year of 2010 and from 2013-2016. Other strains were only frozen for 5 years and thus not available.

The Gram-negative bacteria study **(IV)** refers to are the following species: EC, KP, *K. oxytoca* (KO), *K. aerogenes* (KA*), Enterobacter cloacae* (ECL), *Citrobacter koseri* (CK*), Serratia marcescens* (SM), *Proteus mirabilis, Pseudomonas aeruginosa* (PA), *Acinetobacter baumannii* (AB) and *Haemophilus influenzae* (HI).

The Gram-positive organisms that were considered as pathogens were *Streptococcus agalactiae* (group B streptococci; GBS), SA, CoNS, *Streptococcus pyogenes* (group A streptococci; GAS), *Enterococcus faecalis* and *Streptococcus pneumoniae*. The following pathogens were considered to be likely contaminants: Neisseria species, Rothia mucilaginosa, viridans group streptococci and *B. cereus* (175). Blood cultures with contaminants are not presented herein.

4.6 ESBL-SCREENING, SPECIES IDENTIFICATION AND SUSCEPTIBILITY TESTING

In Ecuador **(I)**, the first screening for ESBL-producing isolates was done on MacConkey agar containing cefotaxime and ceftazidime 1 mg/L. The phenotyping and the ESBL-confirmation were performed by the disk diffusion method on Müller Hinton agar plates with ceftazidime and cefotaxime +/- clavulanic acid (Beckton Dickinson, BLL) at two different laboratories; the laboratory at the Medical University of Cuenca and the PAHO-certified microbiology laboratory of SOLCA in Cuenca. The ESBL-screening in study **II-III** was performed on ChromID agar (bioMérieux).

Species identification and susceptibility testing, of all ESBL-isolates in this thesis was performed at the Department of Clinical Microbiology at Karolinska University Hospital, Stockholm. The methods used were Vitek2 (bioMérieux) and disc diffusion (Oxoid, Basingstoke, UK). and interpreted according to the guidelines using EUCAST breakpoints **(study I- IV)(**176).

Antibiotic susceptibility testing was performed by the disk diffusion method and interpreted according to the guidelines of the Swedish Reference Group of Antibiotics before 2011 **(I)**, and between 2011-2016 **(II-IV)** by guidelines from the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org).

In study **II**, we determined the relative dominance of the ESBL-KP and ESBL-EC at each sampling occasion. We used a culture approach on MacConkey agar to perform this and considered the dominance as high if at least one of the two most dominating colonies was the EPE-strain.

4.7 VIRULENCE AND RESISTANCE GENE DETECTION

The molecular identification of blaCTX-M and the assignment to distinct CTX-M phylogroups was performed by a probe-based PCR-assay **(study I-IV*)*** (177).

In paper I, the microbial typing system of DiversiLab (DL) was used in order to show relatedness between isolates (bioMérieux, Marcy l´Etoile, France) (178). Multi-locus sequence typing (MLST) was performed according to the guidelines available on the Pasteur Institute MLST website, on selected CTX-M producing KP-isolates.

In order to present genetic information regarding the antibiotic resistance genes and the phylogenetic relation between the isolates, we performed WGS at the Science for Life Laboratory (SciLife, Solna, Sweden) **(study II-IV),** multi-locus sequence typing (MLST) **(study II-IV),** core-genome MLST (cgMLST) **(study II-III)** and pulse field gel electrophoresis (PFGE) **(study II-III).**

In **study III**, complete genomic DNA (chromosomal DNA and plasmids) was isolated used to extract DNA. The methods to perform long read sequencing of the plasmid is further presented in the method part of paper III. We used the resources ResFinder, PlasmidFinder, and VirulenceFinder to detect plasmid replicons, resistance genes and virulence genes, by submitting de novo assembled contigs for each bacterial isolate to these online resources found at the Center for Genomic Epidemiology, DTU, Denmark (179-181).

The isolates that were closely related in the MLST analysis **(study IV)** were further analyzed with single nucleotide polymorphism (SNP) analysis in CLC Workbench (182).

4.8 OPTICAL MAPPING IN PLASMIDS

In study **III**, we used optical mapping to get an overview of the plasmid content in all ESBL-KP samples and some of the ESBL-EC samples. We considered the ESBL-KP outbreak as the clinically most important due to KP´s ability to spread in hospital environments.

All plasmids were extracted from the ESBL-KP and the ESBL-EC strain and separated from chromosomal DNA. Netropsin and YOYO in a molar ratio of 100:1 were used to form the emission intensity variation along the DNA and obtain the barcode. In order to compare plasmid length, a lambda-DNA which has a known number of base pairs (48,502) was simultaneously stained together with the plasmids.

Cas9 and gRNA targeting blaCTX-M group 1 was added to the plasmid solution following a previous published protocol (47). The methods used to suppress photo nicking and photo bleaching of the DNA molecules are described in the method part of study III. The nanochannels used to stretch the plasmids linearly were fabricated, dimensioned and cleaned according to previous methods (44, 183). The plasmids were forced into the nanochannels by applying pressure with nitrogen gas.

To image the DNA molecules, an inverted microscope equipped with a 100 x oil immersion objective and an EMCCD camera was used. The DNA molecules were imaged for 200 frames with 100 ms exposure time. The underlying DNA sequence of the plasmid was reflected by the barcode and therefore each plasmid given a specific ID. All unique barcodes, sizes and numbers of the plasmids were determined in each sample.

The barcode of the plasmid was compared with long-read PacBio sequencing of the plasmid to see if the prediction of the location of blaCTX-M-15 gene was correct by Cas9.

4.9 STATISTICAL METHODS

Comparisons between variables	Study I	Study II	Study III	Study IV
Dichotomous variables				
Pearson´s X^2 test	x			x
Fischer´s exact test	x	x		
Continous variables				
Wilcoxon rank-sum				x
Two sample t-test				x
Pearson´s correlation			x	
Multivariable analysis				
Logistic regression	x			x
Cox-regression				x

Table 3*, all the statistical methods used in the four studies are summarized. For further details, see the section on statistical methods in the specific studies.*

5 RESULTS

5.1 STUDY 1

"High proportion of intestinal colonization with successful epidemic clones of ESBL-producing Enterobacteriaceae in a neonatal intensive care unit in Ecuador"

We collected 123 EPE isolates from 73 admitted neonates, during the study period. Forty-one neonates (56%) were colonized with EPE at some point during the hospital stay. The majority of the EPE-strains were ESBL-EC (89%) and the rest were ESBL-KP (11%). No other EPE was found. Multiple colonization, meaning colonization with two or more DL-types, was found in 27% (11/41) of the neonates. Most neonates patients harbored the bacteria until discharge.

The gestational age of the neonates varied between 29-41 weeks (mean 35.5 w) and their birth weight between 500 and 4900 g (mean 1972 g). Of those colonized, 74% (31/41) had a positive stool culture already at study entry (the first rectal swab after admission).

Forty-five infants (62%) received antibiotics during their NICU stay. Gentamicin resistance occurred in 98.2% of EC and 100% of KP. Ciprofloxacin resistance occurred in 98.2% of EC and 0% of the KP. All EC were susceptible to amikacin, unlike the KP strains which were all resistant to this antimicrobial. All strains were susceptible to meropenem and imipenem.

The results from this study show that a large proportion of the admitted neonates became colonized with EPE and that they were resistant to the empirical first- and second-line antibiotics for neonatal sepsis. The first line empirical antibiotics were ampicillin and gentamycin. Ceftriaxone in combination with cloxacillin was the most common second line alternative.

All but two patients receiving ceftriaxone also received cloxacillin, which is why impossible to analyze them separately. Regarding the high level of resistance to ciprofloxacin, normally not used in the neonate, we cannot draw any conclusions other than it reflects a general high consumption of quinolones within the hospital or/and in society.

The results of the epidemiological typing divided the ESBL-KP into two clusters and the ESBL-EC isolates into two clusters and one singleton. All ESBL-KP and ESBL-EC isolates harboured $bla_{\text{CTX-M}}$ of phylogroup 1, except for the singleton ESBL-EC isolate that harboured

bla$_{CTX\text{-}M}$ of phylogroup 9.

The different strain types of ESBL-EC and ESBL-KP isolated from various sampling occasions can be seen in **study I** (184). We performed MLST on three representative KP-isolates of each DL-type (n=7) and could thereafter showe that five (n=5) belonged to the ST897 lineage and two (n=2) to the ST855 lineage.

Characteristics of the infants and risk factors for colonization with ESBL-producing Gram-negative bacteria during NICU care are summarized in **table 4.** In the univariate model birth weight $\geq$ 2,500 g, shorter NICU stay, no use of ceftriaxone was significantly associated with less ESBL colonization. In a stepwise logistic regression model, a length of NICU stay longer than 21 days and enteral feeding with a combination of breastfeeding and formula feeding were significantly associated with ESBL colonization.

Characteristics	No (%)	Colonized (n=41)	Not colonized (n=32)	P- Values Univariate model	P-values Forward step logistic regression
Sex (male)	53.4	23	16	NS	
Gestatonal weeks					
>36	31 (42.5)	13	18	NS	
35-36	20 (27.4)	11	9	NS	
32-34	15 (20.5)	11	4	NS	
<32	7 (9.6)	6	1	NS	
Birth weight					
>2,500	15 (20.5)	4	11	0.02**	
2,000-2,499	10 (13.7)	5	5	NS	
1,500-1,999	33 (45.2)	19	14	NS	
<1,500	15 (20.5)	13	2	0.009*	
Mode of delivery (Ceasarean section)	40 (54.8)	24	16	NS	
Length of stay in NICU, days					
1-10	20 (27.4)	6	14	0.0081**	
11-20	22 (30.1)	8	14	0.039**	
21-30	15 (20.5)	12	3	0.045*	0.003*
>30	16 (21.9)	15	1	0.0005*	0.001*
Endotracheal tube	20 (27.4)	13	7	NS	
Central venous catheter	5 (6.8)	3	2	NS	
Parenteral nutrition	21 (28.8)	16	5	0.038*	
Breast milk feeding only	28 (38.4)	13	15	NS	
Formula feeding only	19 (26.0)	9	10	NS	
Both breast milk and formula feeding	26 (35.6)	19	7	0.048*	0.006*
Ampicillin/Gentamycin)		28	17	NS	
Ceftriaxone, days					
0	51 (69.9)	23	28	0.0046**	
1-5	5 (6.8)	4	1	NS	
6-10	10 (13.7)	7	3	NS	
>10	7 (9.9)	7	0	0.016*	
Malformations	2 (2.7)	1	1	NS	
APGAR at 5 min <	2 (2.7	1	1	NS	

Table 4. *Characteristics of the patients and risk factors for colonization with EPE during NICU care in a tertiary hospital in Ecuador Note: In the univariate analysis, when more than two categories were represented within a factor, each category was compared to all other categories. *Associated with colonization, **Associated with less colonization. NS- Not significant.*

The length of hospitalization was ranged from3 to 61 days (mean 20.6 days). The mean time between admission and first surveillance swab was 6.4 days. One limitation of this study is the short study period of only three months, but we considered it to be enough to address the main question. Other limitations are that we did not screen the mothers for EPE-colonization and we did not distinguish between EPE acquisition (acquired during ICU stay) and those imported (EPE identified directly at admission). Indeed, risk factors associated with importation are different tothose for acquisition.

5.2 STUDY 2

"Neonatal intestinal colonization with extended spectrum beta-lactamase producing Enterobacteriaceae- a 5- year follow up study"

Seventeen neonates became colonized by EPE during the five-month long EPE-outbreak. Fourteen out of these 14 survived and were discharged from the NICU. The index case in the EPE-outbreak was a neonate with a blood-culture confirmed ESBL-KP.

Three of the ESBL-KP colonized infants also had invasive infections and died during their hospital stay. One infant, extremely preterm born in GA 24 w had clinical sepsis signs at two days of age, a perforated intestine when three days old and died on the fourth day after birth.

A second infant GA 23 w had clinical signs of sepsis when 7 days old, a clinically suspected NEC, an ESBL-KP confirmed blood-culture and died 8 days after birth. A third, a full-term infant died at two weeks of age, after clinical signs of sepsis and renal failure. The peritoneal dialysate and tracheal cultures were positive for ESBL-KP but the blood culture was negative. In **figure 9**, a flowchart on the included neonates is depicted

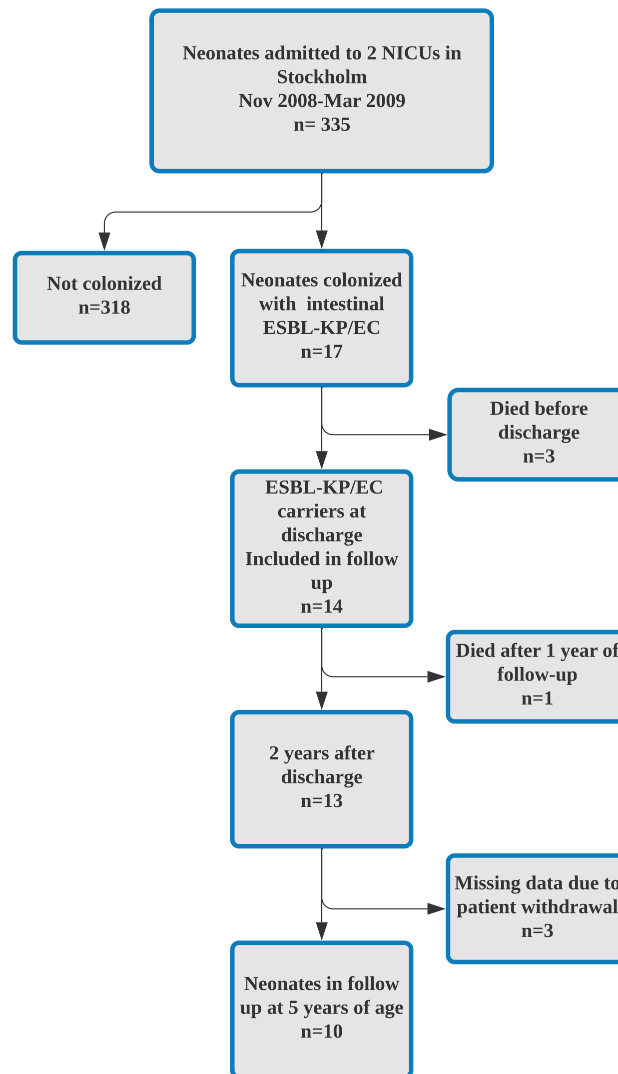

Figure 9: *Seventeen children became colonized during the outbreak and 14 of them were included in the study. Fecal sampling was conducted every month from discharge until 26 months after discharge. There was no sampling between 26 months and 5 years after discharge. Sampling was performed on three occasions during a 6-week period, 5 years after discharge.*

Fourteen neonates colonized with ESBL-KP/EC at discharge, were included in the follow-up study. ESBL-KP was seen in 13 patients and ESBL-EC in one patient. One patient in the study died due to severe bronchopulmonary dysplasia (BPD) 13 months after discharge. The patients were born at different gestational ages and were between 2-95 days old (median 15,5 days) when they were diagnosed with ESBL-KP/EC colonization. The median length of ESBL-KP/EC colonization was 12.5 months (range 5-68 months).

A co-colonization with ESBL-EC was later seen in 7/13 patients during the follow-up period. Among surviving children 5/13 (38%) remained ESBL-KP/EC positive 20 months after

discharge. Two of 13 children (15%) remained ESBL-KP positive and 1 of 13 children carried an ESBL-EC at 23-26 months after discharge. Notably, only one child had two negative cultures before the sampling at 23-26 months of age. The duration of ESBL-KP colonization and how many of the patients that were sampled on each occasion can be seen in figure 1 in the published paper of study II (185).

In **table 5**, characteristics of the fourteen study participants are presented.

	EPE at 2 y	GA (w)	BW (g)	Birth mode	Antenatal AB	First intestinal EPE (age in days)	Hospital days	MV in total (days)	NEC	Feed type at NICU	LOS at NICU	AB in NICU	AB after discharge	Travel between 1-5 y after discharge	Hospital care after discharge	Probiotics after discharge	Morbidity after discharge
Pat 1	No	25+2	698	VD	No	64	101	30	Yes	BM,F	CoNS	47	No	Singapore	Yes	No	ARI, GE
Pat 2	No	25+2	761	VD	No	95	101	16	Yes	BM,F	CoNS	34	No	Singapore	No	No	ARI
Pat 3	**Yes**	**24+2**	**756**	**CS**	**Yes**	**30**	**160**	**24**	**No**	**BM**	**CoNS**	**38**	**Yes**	**No**	**Yes**	**No**	**UTI, ARI**
Pat 4	No	27+0	1011	CS	No	15	52	0	No	BM	CoNS	8	Yes	No	Yes	No	ARI
Pat 5	No	32+5	2214	CS	Yes	16	16	0	No	BM	GBS	7	No	No	No	No	ARI
Pat 6	**Yes**	**27+1**	**1080**	**CS**	**No**	**11**	**71**	**8**	**No**	**BM**	**CoNS**	**36**	**Yes**	**Turkey**	**Yes**	**Yes**	**GE, ARI**
Pat 7	No	37+6	2814	VD	No	2	38	0	No	BM	No	16	Yes	Finland	No	No	ARI
Pat 8	No	26+6	1054	CS	Yes	54	134	19	No	BM,F	CoNS	17	No	No	Yes	No	ARI
Pat 9	No	40+6	3060	VD	No	4	45	7	Yes	BM	No	21	No	No	No	No	ARI
Pat 10	No	33+5	1330	CS	No	3	24	0	No	BM	No	5	Yes	No	No	No	OM
Pat 11	**Yes**	**31+1**	**1935**	**CS**	**No**	**5**	**20**	**0**	**No**	**BM,F**	**No**	**0**	**Yes**	**Albania**	**Yes**	**No**	**GE, OM, ARI**
Pat 12	No	34+5	2745	VD	No	5	8	0	No	BM	No	0	No	No	No	Yes	ARI
Pat 13	No	38+3	2785	VD	No	32	31	0	No	BM,F	No	0	No	No	Yes	No	No
Pat 14	No	29+0	1300	VD	No	38	50	1	No	BM	No	6	No	No	No	No	No

***Table** 5. ESBL-KP/EC colonized children two years after discharge from NICU are marked in bold text. Pat 8 died 13 months after discharge. Pat 1,2 and 4 were followed 26 months after discharge.*

At two years after NICU discharge, seven patients had three or more subsequent negative cultures and remained culture negative on the last sampling occasion. Ten children were followed-up at five years of age and, at that point, one child was colonized by ESBL-EC.

The characterization of the *K. pneumoniae* outbreak strain showed that the resistome and the virulome of the KP-ESBL strains were identical. The MLST type was ST101 and carried the *bla* $_{\text{CTX-M-15}}$ gene. Clonal relatedness was shown by PFGE between the initial isolate and the last isolate taken after two years after discharge and was thereafter confirmed by cgMLST typing.

Within the ST101 clone we could detect no allelic differences with cgMLST (694 genes). The virulence-associated genes found in were *kfu, iutA, mrk*, *irp1* and *irp2*. These genes encode the synthesis of proteins needed for bacterial iron uptake and transportation (*kfu, iutA, irp1, irp2*) and the attachment to host cells by fimbriae (*mrk*). The capsule type was K29. The antibiotic susceptibility pattern and the resistant genes are presented in **table 5**.

Drug	Susceptibilty	Antibiotic class	Resistance gene in index strain of ESBL-KP	
Cefotaxime	R	Beta-lactams Cell wall inhibitors	*bla*$_{TEM1A}$	Plasmid-mediated Beta-lactamase Hydrolyses beta-lactams
Ceftazidime	R		*bla*$_{CTX-M-15}$	Plasmid-mediated Beta-lactamase Hydrolyses beta-lactams
Ceftibuten	R		*bla*$_{SHV-1}$	Chromosomal Beta-lactamase Hydrolyses beta-lactams
Ertapenem	S			
Meropenem	S			
Imipenem	S			
Piperacillin- Tazobactam	S	Beta-lactam/ beta lactamase inhibitor		
Gentamicin	R	Protein synthesis (30S inhibitors)	*aac(3)-Iid*	Plasmid-mediated Altered ribosomal binding
Amikacin	S		*strB*	Chromosomal Altered ribosomal binding
			srtA	
Ciprofloxacin	R	Nucleic acid synthesis	*qnrB1*	Plasmid-mediated stops inhibition DNA synthesis
			oqxB	Plasmid-mediated Efflux pump gene
			oqxA	Plasmid-mediated Efflux pump gene
Trimethoprim-Sulfamethoxazole	R	Metabolic pathways (folate)	*dfrA1A*	Plasmid mediated stops inhibition DNA synthesis

Table 5*. Antimicrobial susceptibility and resistance of the* $_{bla}$*CTX-M-15 positive index strain (ESBL-KP) in the long-term follow up study.*

When we studied how the EPE-strain dominated the microbiome we saw that half of the children (7/14) had a dominant EPE-strain on at least one occasion during the study period. Continuing EPE-colonization was demonstrated in the subsequent culture of these children following initial identification.

Compared to the 2 of 7 children with subsequent positive culture in the group without EPE dominance, EPE-dominance is suggested as a predictor for prolonged carriage ($p = 0.02$). The patient questionnaire addressed risk factors for change in diversity of the microbiota but no statistical conclusions were drawn due to low patient numbers.

A summary of the duration of EPE-carriage, the characteristics of the colonizing EPE-strains and the relative dominance of the strain at each sampling occasion during follow-up is shown below in **figure 10.**

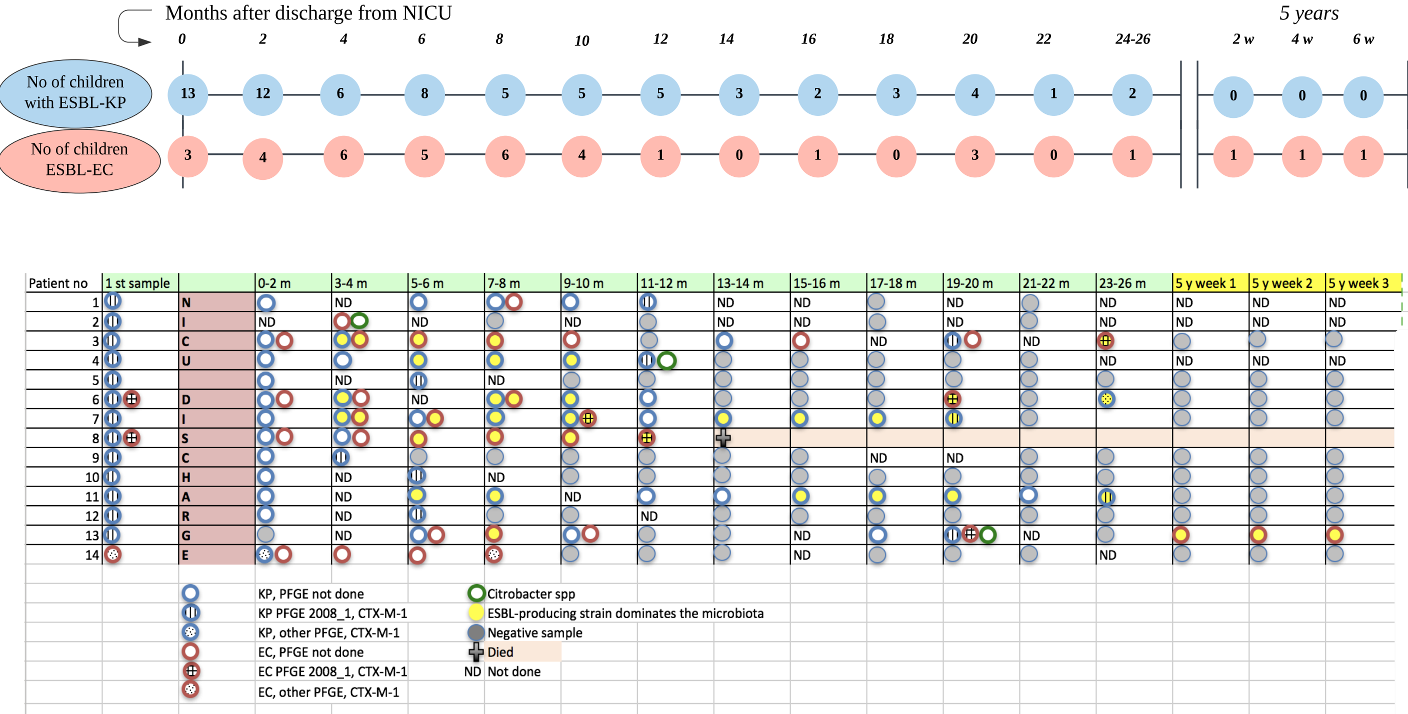

Figure 10. *Duration of EPE-carriage in neonates after discharge from the NICU. The relative dominance of the strain at each sampling occasion during follow up.*

5.3 STUDY 3

"Optical DNA mapping combined with Cas9-targeted resistance gene identification for rapid tracking of resistance plasmids in a neonatal intensive care unit outbreak"

In **study III**, we present results from Optical DNA mapping of plasmids from the EPE-outbreak at two NICU sites at Karolinska 2008-2009. Seventeen neonates were colonized during the outbreak, as mentioned above. At the time of plasmid analysis, 6 years later, one isolate was not viable, unfortunately. So, our first set of strains were 16 isolates of the ESBL-KP-outbreak strain. The follow-up cohort and study period was the same as in **study II**.

In **figure 11**, an overview of the patients and the ESBL- KP/EC isolates that were analyzed with OM is presented.

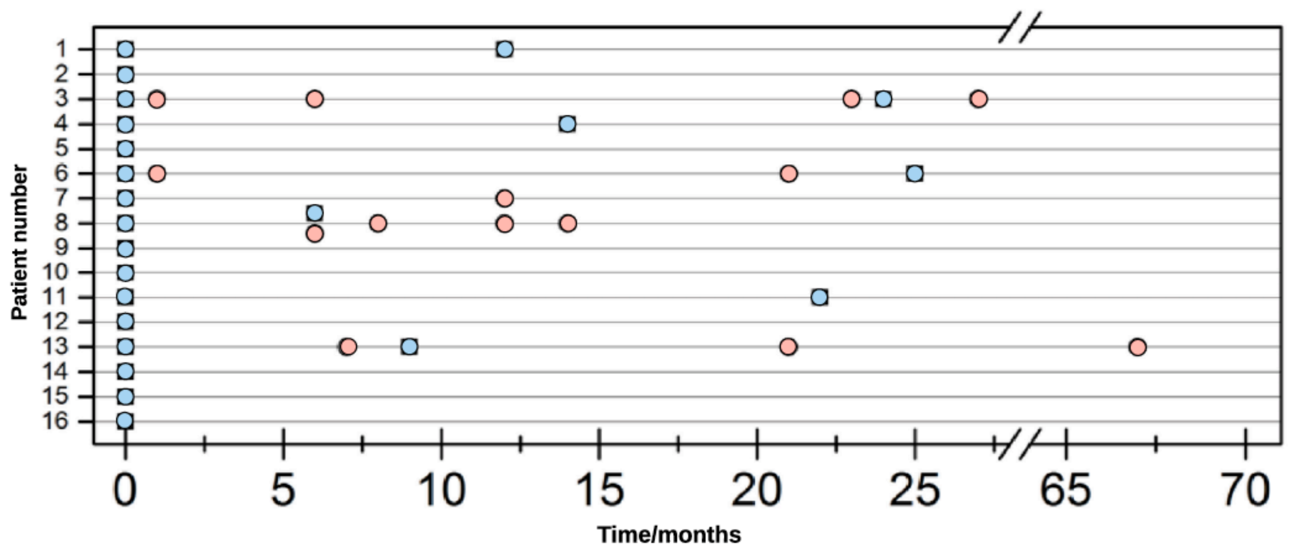

Figure 11. This is the same cohort as in study II, but here we show which isolates were analyzed with OM. The patient number is listed on the vertical axis. ESBL-KP are presented as blue circles and ESBL-KP as pink circles.

When analyzing the KP-outbreak strain, each isolate contained two plasmids. One small plasmid with a size of 80 kb and one large with a size of approximately 220 kb. In the main ESBL-EC strain we found a 130 kb plasmid. The barcodes from the two plasmids that were found in the 16 KP-isolates are shown in **figure 12.** The position cut by Cas 9 is indicated by the grey area and means that the small plasmid is carrying the *bla*$_{CTX-M}$ gene (53).

k

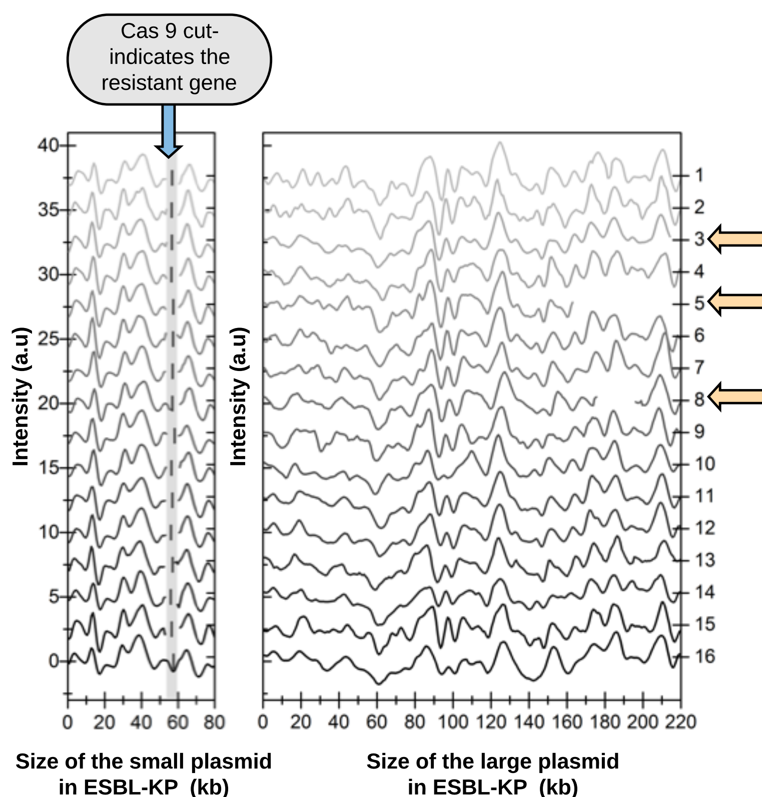

Figure 12: *The barcodes from two plasmids, the small (80 kb) and large (220 kb) plasmid, in the ESBL-KP outbreak strain from 16 neonates. The grey area indicates the position cut by Cas9. The small plasmid carried the resistance gene. For clarity, all barcodes are shifted vertically. The yellow arrows indicate deletions in pat 3,5, 8.*

By comparing the barcodes of the small 80 kb plasmid in all patients, we could confirm that all these plasmids were identical with a p-value <0.01. The p-values were determined using a set of 1000 random barcodes with sizes corresponding to the two compared barcodes, as described in detail in reference (43).

The large plasmids all varied somewhat in size between patient isolates. They were all shown to originate from the same plasmid, since they harbored a large region (~160 kb) that was identical in all isolates and were therefore related. The variation in size was due to deletions in three of the plasmids from the ESBL-KP outbreak. The deletions were seen in patient 3 (5 kb), patient 5 (55 kb) and patient 8(31 kb).

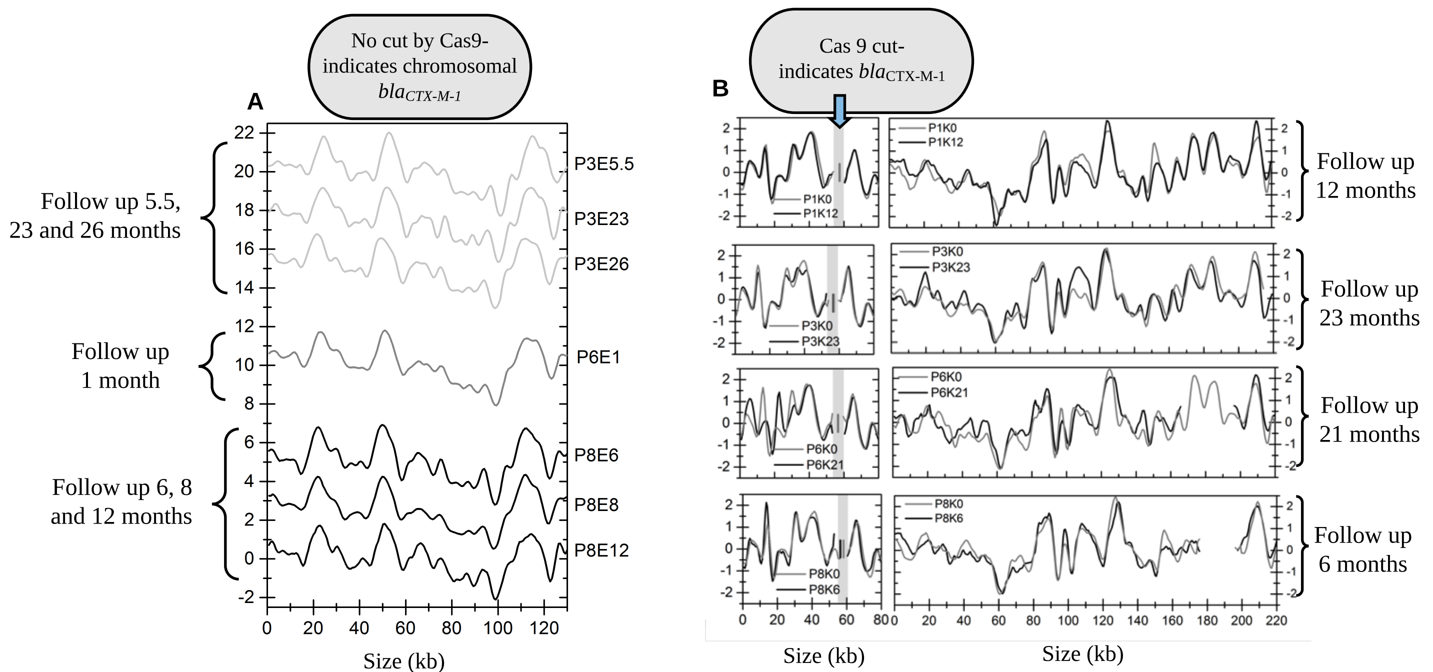

***Figure 13 A)** This figure shows the plasmid barcode of follow-up samples of the EC samples in patient 3,6 and 8. **B)** This illustrates how the barcodes from the outbreak KP-strain (gray lines) and the follow-up strain (black lines) match. The samples taken 6,12,21 and 23 months after the first samples show that the same plasmids are still present. The names of each isolates are PXKY where P is patient, X is the patient number, Y is the month the isolates was collected relative to the initial isolate and K stands for K. pneumoniae.*

In the follow up samples we analyzed ESBL-KP from seven of the children. The barcodes from all follow-up KP- plasmids can be seen in figure 3 in paper III (186). The PacBio sequencing data also allowed us to investigate the genetic rearrangements found in several of the ESBL-KP ST101 isolates.

The sequencing results excellently confirmed the optical mapping data regarding both position and size of the deletions. The deletions seen were most likely an effect of transposase activity. Figure 4 in paper III illustrates how the barcodes from optical mapping and the theoretical barcodes predicted from the PacBio sequencing, were compared and that the overlap is excellent (186).

In the ESBL-EC strain we found a 130-kb plasmid. The plasmid barcodes of the EC-isolates and some of the follow up samples of the ESBL-KP strains are presented in **figure 13.** PCR analysis of the ESBL-EC strain revealed that this isolate also carries a *bla* $_{\text{CTX-M}}$-group 1 gene, but the Cas9 assay did not show that the gene was not present on the 130-kb plasmid. We performed PacBio sequencing also on one of these isolates (Patient 3 after 5.5 months), to support the optical DNA mapping data. The barcode of this plasmid did perfectly overlap

with the theoretical barcode predicted from the sequencing. The resistance gene was located on the chromosome of this EC.

Finally, we investigated whether or not there had been plasmid transfer between KP and EC due to conjugation. We compared all plasmid content of all EPE-isolates by optical mapping and ODM demonstrated identical 130 kb plasmids in all the ESBL-EC and they did not resemble any of the barcodes of the plasmids from the ESBL-KP strain, which indicates that the ESBL-EC plasmid had a different origin. We can, with this result, state that there was no plasmid transfer between KP and EC in this outbreak.
ODM can rapidly characterize plasmids directly from fecal samples. After a short cultivation step in broth (< 4h), the plasmids can be extracted and typed using ODM and the genetic analysis can be completed on the same day that the sample was collected.

5.4 STUDY 4

"A decade of neonatal sepsis caused by Gram-negative bacilli- a retrospective matched cohort study"

During the study period 310,091 infants were born alive at the obstetric departments of the delivery units in the Stockholm area. Of these, 31,878 (10.2%) neonates were admitted to the neonatal units of Karolinska Danderyd (n=10,418), Karolinska Solna (n=5,828), Karolinska Huddinge (n=6,904) and Södersjukhuset (n=8,728).

A total of 804 admitted infants (table X) had at least one positive blood-culture, which corresponds to a total incidence of culture-confirmed neonatal sepsis of 2.6/1,000 live born.

The incidence of GNB sepsis in neonates in the Stockholm area between the years 2006-2016 was 0.35 cases per 1,000 live born. Divided into GNB-EOS and GNB-LOS, the incidence was 0.11 and 0.24/1,000 live births respectively. GPB-sepsis was 6.3 times more common than GNB-sepsis with an incidence of 2.2 per 1,000 live born. Among the infants admitted to the neonatal unit, 1,026/31,878 (3.2%) had suspected sepsis (not culture-verified sepsis) with a cumulative incidence of 3.3 per 1,000 live born. Described as NICU-admissions, EC-EOS was 0.75/1,000 admissions and EC-LOS was 1.4/1,000 admissions. We did comparisons between neonates with GNB-sepsis and two other groups of matched controls.

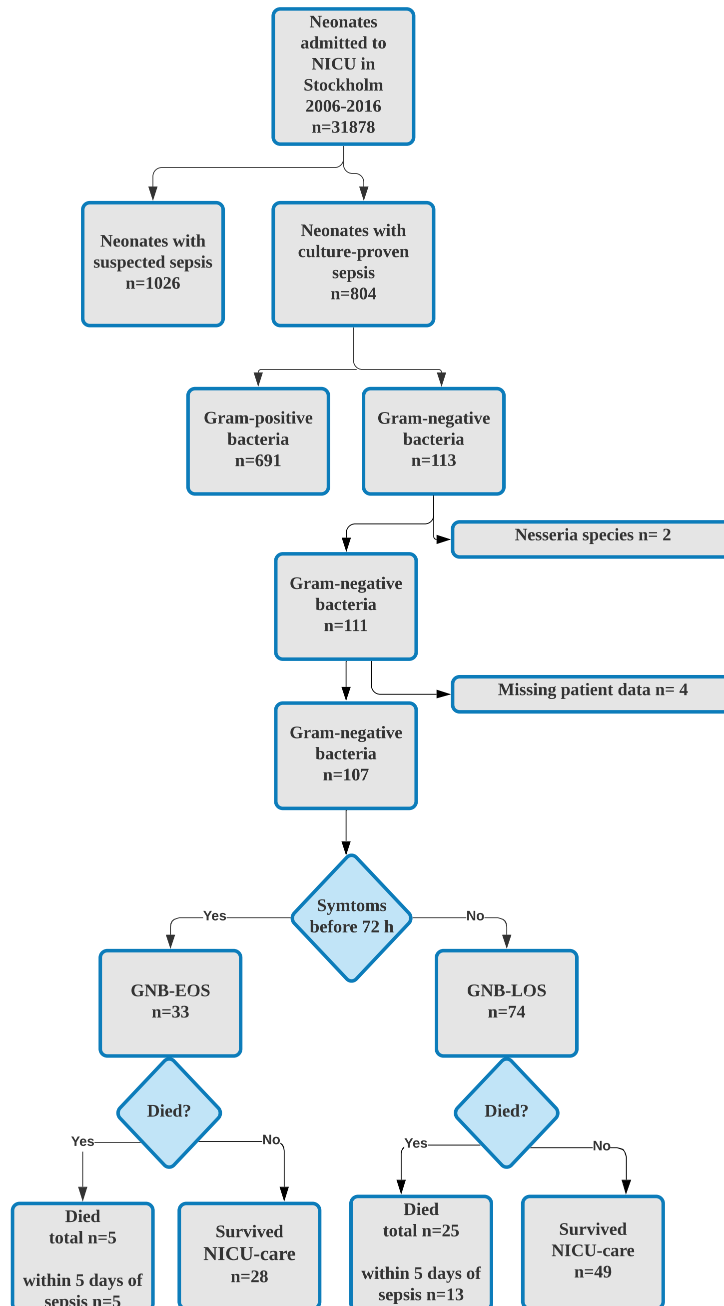

Figure 14**: Flow chart of all patients included in **study IV

The median gestational age at delivery in patients acquiring GNB-EOS was 34 weeks (IQR 26-38) and in GNB-LOS 27 weeks (IQR 25-29). The median age at diagnosis was 1 day (IQR 0-1) for EOS and 19 days (IQR 11-31) for LOS. Median onset for suspected EOS was day 0, day 19 (11-31) for GNB-LOS and day 9 (IQR 4-17) for suspected–LOS. The difference between the GNB-LOS and suspected was significant ($p<0.001$)

Maternal antibiotics were more commonly given to neonates that developed GNB-EOS as well as GNB-LOS compared to the uninfected group. The difference was significant and the

proportions were (52% vs 16%) and (49% vs 40%) respectively.
Birth mode did not differ in the GNB-EOS group, but was in the GNB-LOS group more common (42%) than in both suspected-LOS (15%) and uninfected controls (22%).

The characteristics of all 107 GNB cases (GNB-EOS and GNB-LOS) and the pair-wise comparisons with suspected sepsis controls and uninfected controls are seen in **table 8.**

The primary outcome in **study IV**, was to present the in-hospital mortality of neonates with GNB-sepsis. We showed that 30 of all 107 neonates with GNB-sepsis died before discharge and present it as the case fatality rate (28%). The mortality of GNB-LOS was compared with the suspected-LOS and uninfected control groups. The proportion of deaths before discharge was 35% (25/74), 17.5% (13/74) and 7.6% (15/196).
The secondary outcome, the 5-day case fatality rate, was 5/33 (15%) in GNB-EOS and 13/74 (17.5%) in GNB-LOS.

The logistic regression showed that the adjusted odds ratio (OR) to die before discharge in the GNB-LOS groups was 4.7 compared to the uninfected matched control group, and 2.2 when compared to the suspected sepsis group. No difference was seen between GNB-LOS versus suspected sepsis or suspected sepsis versus controls

	GNB-EOS n=33	Susp-EOS n=33	Uninfected control n=99	P* EOS	P** EOS	P*** EOS	GNB-LOS n=74	Susp-LOS n=74	Uninfected control n=196	P* LOS	P** LOS	P*** LOS
Gest age (w)	34 (26-38)	34 (26-38)	34(26-38)	0.94	0.97	0.91	27 (25-29)	27 (25-29)	27 (25-29)	0.98	0.60	0.58
Gender, male	16 (48)	21 (64)	57 (58)	0.16	0.36	0.42	45 (61)	53 (72)	99 (51)	0.16	0.13	0.002
BW (g)	2,225 (994-2950)	2,013 (686-3500)	2087 (995-3325)	0.88	0.81	0.89	885 (750-1395)	812 (627-1259)	960 (747-1315)	0.17	0.69	0.04
Apgar at 5 min <7	13 (39)	5 (15)	26 (26)	0.027	0.16	0.18	23 (31)	29 (39)	63 (32)	0.33	0.83	0.33
Caesarean section	16 (48)	16 (48)	50 (22)	0.90	0.96	0.84	31 (42)	11 (15)	44 (22)	<0.001	<0.001	0.17
Prenatal steroids	11 (33)	14 (42)	34 (34)	0.72	0.72	0.40	52 (70)	64 (87)	146 (75)	0.17	0.61	0.034
Antenatal antibiotics	17 (52)	11 (33)	16 (16)	0.11	<0.001	0.037	36 (49)	31 (42)	79 (40)	0.048	<0.01	<0.81
Onset sepsis (d)	1 (0-1)	0 (0)	No sepsis	0.023			19 (11-31)	9 (4-17)	No sepsis	<0.001		
Days of MV	1 (0-7)	0 (0-6)	0 (0)	0.77	<0.001	0.57	8 (2-24)	9 (2-16)	0 (0-7)	0.43	<0.001	<0.001
Days of CPAP	2 (0-8)	1 (0-4)	3 (0-20)	0.31	0.05	0.24	16 (3-36)	18 (2-38)	6 (1-25)	0.77	0.003	0.03
Days of TPN	8 (2-13)	2 (0-10)	1 (0-8)	0.06	0.001	0.001	22 (11-37)	13 (8-24)	9 (5-14)	0.001	<0.001	<0.001
Days of UAC	1 (0-6)	0 (0-2)	0 (0-4)	0.038	0.046	NA	6 (3-8)	5(0-7)	3(0-6)	0.43	<0.001	0.036
Days of UVC	1 (0-5)	0 (0-1)	0 (0-0)	0.062	0.015	NA	1 (0-4)	2 (0-5)	0 (0-5)	0.40	0.72	0.20
Days of pCVC	0 (0-9)	0 (0-9)	0 (0-0)	0.39	<0.001	0.55	15(8-28)	7 (1-20)	2 (0-8)	0.009	<0.001	<0.001
BPD discharge	4 (12)	6 (18)	13 (13)	0.64	0.67	0.78	32 (43)	35 (47)	64 (33)	0.48	0.12	0.083
ROP 1-2 discharge	4 (12)	6 (18)	2 (2)	0.48	0.004	0.059	9 (12)	20 (27)	26 (13)	0.11	0.99	0.017
ROP 3-4 discharge	4 (12)	0 (0)	1(1)	0.095	0.01	0.85	10 (14)	4 (5)	9 (5)	0.18	0.019	0.83
IVH 1-2 discharge	7 (21)	4 (12)	5 (5)	0.32	0.005	0.059	16 (22)	14 (19)	19 (10)	0.55	0.008	0.039
IVH 3-4 discharge	5 (15)	3 (9)	2(2)	0.48	0.004	0.059	6 (8)	6 (8)	11 (6)	0.60	0.20	0.45
All NEC	NA	NA	NA	NA	NA	NA	27 (36)	15 (20)	5 (3)	0.09	0.059	0.39
Surgical NEC	NA	NA	NA				11 (15)	5 (7)	0 (0)	0.11	0.003	0.78
Mortality before discharge	NA	NA	NA				25(34)	12 (16)	33 (17)	0.04	<0.001	0.008

Table 8. *The characteristics of all 107 GNB cases (EOS and LOS) and pair-wise comparisons with suspected sepsis controls and uninfected controls.*

**Comparison between case and suspected sepsis*

*** Comparison between case and uninfected control,*

**** Comparison between suspected case and uninfected control*

Continuous variables are presented with means, SD, medians and interquartile range. Categorical variables are presented as proportions and %. BW=birth weight, MV= mechanical ventilation, CPAP= continuous positive airway pressure, TPN=total parenteral nutrition, pCVC=peripheral central venous catheter, UVC=umbilical venous catheter, UAC= umbilical arterial catheter, BPD= bronchopulmonary dysplasia, ROP= retinopathy of the newborn, IVH=intraventricular hemorrhage, NEC= necrotizing enterocolitis

From the survival analysis, the cumulative survival rate of GNB-LOS and the matched controls was presented as Kaplan-Meier curves of 5- and 30-days survival (**figure 15*).*** The adjusted cox-regression model show that the hazard ratio of dying 5 days after GNB-onset was 4.3 (p=0.005) compared to uninfected controls and in GNB-LOS versus suspected LOS the HR was 2.8, but not significant (p=0.08).

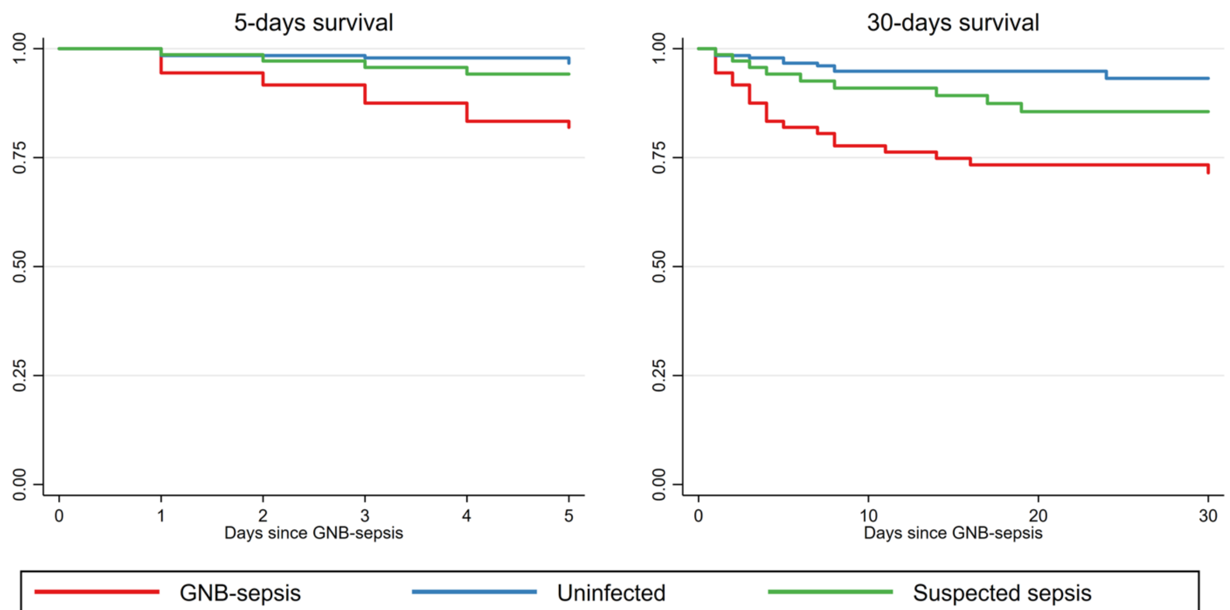

Figure 15. *The cumulative survival rate after GNB-LOS onset (index day) in Kaplan-Meier of GNB-LOS.*

EC was the most common Gram-negative pathogen causing GNB-sepsis in both GNB-EOS and GNB-LOS, followed by Klebsiella spp and Enterobacter spp. The Enterobacter spp showed the highest mortality due to a specific pathogen. All culture-confirmed pathogens and the proportions of death due to specific GNB are shown in **table 9**. The Gram-positive pathogens predominated with 691/804 (86%) vs Gram-negative 113/804 (14%).

Confirmed Gram-negative pathogens	No of GNB-sepsis per pathogen	No of GNB-EOS per pathogen	EOS 5 days case fatality rate per pathogen (%)	EOS mortality before discharge per pathogen (%)	No of GNB-LOS per pathogen	LOS 5 days case fatality rate per pathogen (%)	LOS mortality before discharge per pathogen (%)
Escherichia coli	47	24	2/24 (8.3)	3/24 (12.5)	23	2/23 (9)	7/23 (30)
Klebsiella species	24	5	1/5 (20)	1/5 (20)	19	3/19 (15.7)	5/19 (26.3)
Enterobacter species	16	1	0	0	15	5/15 (33.3)	7/15 (46.7)
Serratia marcescens	10	0	-	-	10	2/10 (20)	4/10 (40)
Acinetobacter baumannii	3	0	-	-	3	0	0
Pseudomonas aeruginosa	4	1	1 (100)	1	**3**	0	1/3 (33)
Hemofilus influenzae	2	**2**	0 (0)	0	0	-	
Citrobacter koserii	1	0	-	-	1	1/1 (100)	- 1/1 (100)
Total[a]	107	33	5/33 (15%)	5/33 (15%)	74	13/74 (18%)	25/74 (34 %)

Confirmed Gram-positive pathogens	Numbers of neonatal sepsis episodes with casued by this pathogen
Coagulase-negative Staphylococcus	412
Staphylococcus aureus	149
Group B streptococcus	85
Enterococcus species	21
Alfa-streptococcus	12
Other[b]	12
Total	691

Table 9*: A total of 804 episodes of neonatal sepsis was confirmed by blood-culture.* ***Left****: All confirmed Gram-negative pathogens and the case fatality by pathogen is here presented.* [a] *Due to missing data from four neonates and two apathogenic Neisseria, 107 of 113 confirmed Gram-negative pathogens is shown here.* ***Right****: Here all confirmed Gram-positive pathogens during the study period are presented.* [b] *Other includes S. pneumoniae, B. cereus, S. pyogenes and S. salivarius.*

We retrieved only 33 of the 107 Gram-negative strains from the study period for further characterization. This was due to the fact that the strains were only kept for 5 years in the freezers of the microbiology laboratory. These strains were collected in the calendar year of 2010 and from 2013-2016. In these 33 isolates, we performed molecular and genetic characterization.

In the EC group, we found that three patients had the same sequence type (EC ST95) and two patients had EC ST357 and therefore SNP-analysis was performed on these isolates. They were not correlated in time and NICU-site. No genetic relation was found in the SNP-analysis. A high level of genetic similarity was seen in a group of *S. marcescens* infecting four patients closely related in time and NICU-site. The *S. marcescens* strain differed with 1-3 SNPs and was then confirmed as a small outbreak. Other from this, the data from MLST showed a high diversity of STs in the infecting strains *E. cloacae* (n=4), *K. pneumoniae* (n=4) *and E. coli* (n=12).

6 DISCUSSION

Neonatal sepsis is the leading cause of mortality in the newborn. The main challenge of ABR in NICUs has been the Gram-negative bacterial species, where GNB-sepsis is associated with higher mortality compared to GPB-sepsis. The NICU is an environment with high antibiotic use. Antibiotics are essential for treating life-threatening infections, but antibiotic exposure induces unwanted early disturbances in the neonatal microbiota (100, 101). As described, dysbiosis of the neonatal gut microbiome has multifactorial causes and the early colonization of EPE is a risk factor for subsequent invasive infection (93, 97, 99, 100). A complication in studies regarding neonates and ABR is the difficulty of conducting clinical trials for rare resistant infections in neonates. The duration of EPE-colonization and the characteristics of different EPE bacteria, have in terms of virulence and resistance factors, great clinical importance as well as understanding the dynamics of an EPE-outbreak. Therefore, rapid and reliable methods for diagnostics and efficient treatment are important when caring for the septic neonate.

Herein, discussion points regarding colonization and infection of Gram-negative bacteria in neonates, with focus on ABR, are presented.

6.1 How did this thesis contribute to the knowledge of EPE-outbreaks in NICU setting in Sweden?

There is an increasing prevalence of nosocomial outbreaks caused by EPE in NICUs worldwide, but to our knowledge not yet in Sweden. A small number of EPE-outbreaks occurede in Swedish NICUs during the period 2006-2009. The National Board for Health and Welfare did thereafter recommend screening programs, but sporadic small EPE-outbreaks still occur (187).

Spread of EPE-bacteria in neonatal care may also occur in non-outbreak periods and depends on the characteristics of the strain and the cross transmission between neonates by health care personnel in the NICU. Cross transmission is often related to poor infection control procedures. Hygiene precautions are the key to infection control in all settings. Local surveillance is crucial to prevent and control EPE-outbreaks in neonatal ICUs.

In two studies **(II-III)**, we identified and characterized the EPE-strains that caused a four months long outbreak in two of the NICUs in Stockholm. The EPE-outbreak affected 17

neonates . We can state that three of these children also had invasive infection with the same ESBL-KP strain and died before discharge. We characterized this highly resistant ESBL-KP strain in terms of virulence and resistance factors. We concluded that the ESBL-KP-strain spread to a greater extent than the co-colonizing ESBL-EC strain, that we also found in a small number of patients. We showed that infants can carry the same ESBL-KP strain with the intact resistance carrying plasmid for up to 26 months.

In addition to the traditional methods of characterizing of the bacterial strains we could confirm by the ODM method that the ESBL-KP outbreak strain carried an identical plasmid and therefore indicated an outbreak. We show that the ODM method can become an important tool in surveillance of resistant bacteria in a NICU setting.
Studies show that low-risk interventions such as screening of intestinal EPE in neonates, enhanced control measures in the NICU, such as continuous long term surveillance with cohorting are associated with a decrease of ESBL-KP acquisition within the NICU (188, 189).

6.2 What are the risk factors for EPE-carriage in newborns?

We showed that the main risk factors for EPE-colonization a tertiary NICU in Ecuador were length of stay in hospital care settings and mixed feeding with breastmilk and formula **(I)**. That a combination of formula and breast milk, but not exclusive formula feeding, was a risk factor is interesting and were at the time of our study **(I)**, not in line with previous reports.

Nutrition has been shown to affect abundance of EPE. Human milk has been demonstrated to be a protective factor to reduce colonization and infection with resistant *Enterobacteriaceae* in preterm infants and formula milk made of cow milk is associated with a higher abundance of the same (77, 190). Mixed feeding has lately been shown to be a risk factor for viral infections such as HIV in infants, probably due to increased inflammation in the gut mucosa (191). The finding is interesting but repeated studies are needed before any conclusions can be drawn.

The use of cephalosporins, parenteral nutrition and a low birth weight differed significantly in the univariate comparisons of EPE-colonized and non-colonized admitted neonates **(I)**. Unfortunately, the groups were not matched by gestational age but the association is interesting since they all have been shown earlier to be risk factors for EPE-colonization and as well to be factors that can modify the diversity of the neonatal gut microbiome (12, 77, 91,

124). Vertical transmission from the mother has been shown repeatedly as the most common mode of acquisition for EPE colonization in the neonate (155, 192, 193). In our studies **(I-II)** we did not have the possibility to screen the mothers for intestinal EPE. Other limitations of study **(I)** is the small number of study participants and the short period of observation.

6.3 How long do children carry the EPE bacteria after being colonized in the newborn period?

The length of EPE colonization in adults is associated with the virulence and resistance of the strain and the EPE-colonization have been shown to persist for at least 12 months (148, 150). When neonates are EPE-colonized in the NICU, the absolute majority remain colonized during hospital stay **(I, II)**.

To answer the question of duration of EPE-colonization we need to know the abundance of the resistant bacteria has in the intestine. The EPE-abundance depends on the existing commensal bacteria that can balance the resistant strains. As stated before, the dysbiosis in the NICU-cared neonatal microbiota is multi-factorial and associated with gestational age, birth mode, feeding type and antibiotic treatment. The development of bacterial load and neonatal microbiota follows a pattern that depends on both the gestational age and the postnatal age when colonized and early gut colonization influences the composition of the later microbiota (10, 72, 194). That is why the question of duration of colonization is not easy to answer since we need to take that in account together with the virulence factors in the colonizing strain. Since we did not characterize the entire intestinal microbiome in each child, we cannot present possible explanations for why each child at each sampling moment had a dominating EPE-strain.

The study **(II)** included 14 patients, which leaves us with sparse possibilities to draw any statistical conclusions of possible risk factors for a long duration of EPE-colonization. It is likely that the long-term hospitalized neonates that have acquired an epidemic clone in the NICU, at early postnatal age is more likely to remain colonized for a longer period than a healthy newborn infant that becomes a carrier of a community-acquired EPE-strain. When planning study **(II)** there was a sparse knowledge on whether acquired EPE-colonization could persist longer than two years. We describe the median carriage length with ESBL-KP as one year after discharge, which is similar to a recent study(195). The longest ESBL-KP duration of colonization we can show was 26 months after discharge, which was somewhat

longer than previously described. Two years after discharge, there were three children colonized with ESBL-KP and ESBL-EC. These were all born by caesarean section and were 5 (KP)and 11 (KP) and 30 (EC) days old, respectively.

The child in the long-term follow-up **(II)**, that harboured a dominating strain of ESBL-EC at five years after discharge from NICU, had during the two first years of follow up, been co-colonized with ESBL-KP, ESBL-EC and ESBL-Citrobacter. The child was admitted to the paediatric ward due to gastroenteritis and later received a gastrostomy. This was interpreted as a re-colonization of ESBL-EC, since EC is a more natural part of the intestinal microbiota compared to KP.

The interpretation of re-colonization was later verified with ODM **(III)** that confirmed that the ESBL-EC strain at 68 months had not acquired any resistance gene by plasmid transfer from the outbreak ESBL-KP strain. A hypothesis is that this child had a dysbiotic gut microbiome due to other morbidities, antibiotic treatment and hospital stay and therefore was susceptible to ESBL-EC acquisition.

The strengths of study **(II)** are the five year follow up period and the analyses of relative dominance of EPE and duration of EPE-colonization. The study has limitations such as the lack of maternal-, breast milk-, and environmental samples and samples from the health care workers in order to anlyse the dynamics of the outbreak in more detail. The small number of included study participants together with four (n=4) missing at follow-up at age five in combination with no culture information between 2 and 5 years after discharge make the results less strong than we would have liked.

6.4 What impact did a prevalence study of EPE-colonization in neonates have in Ecuador?

At the time when the study **(I)** was initiated, Ecuador was affected by frequent cases of sepsis with a high mortality in many neonatal wards. Ecuador faced great difficulties in treating the septic neonates. There was little experience among health professionals of managing ABR, and further characterization of the causative agents was not possible. The healthcare system was provided with a lot of technical equipment to solve this problem, but few or no interventions were made in terms of optimizing the microbial diagnostics and clinical approaches. There was at the time, in 2010, no accumulated experience that could contribute to a comprehensive and efficient action plan against the spread of ABR bacteria and the high sepsis-related mortality in the NICUs of Ecuador. As long as no data exists on colonization or resistance patterns, it is less probable that targeted interventions can be initiated.

Our study, is the first published study of intestinal colonization of EPE in Ecuador and therefore the first study on neonatal acquisition of EPE in the country. It showed a high prevalence of EPE-colonization, in more than half of the admitted neonates, and the presence of a highly successful clone of ESBL-KP known to disseminate carbapenemases was presented. We found five epidemic bacterial clusters which can indicate several ongoing outbreaks. These results can also be explained by an endemic situation with a high prevalence of EPE in the society and the hospital settings. This indicates that the problem with ABR in the NICU was deeply rooted, while the awareness among health professionals was low.

Together with the new knowledge of highly virulent and resistant EPE-strains and the local capacity building measures that came out of the collaborative work with the NGO ReAct Latin America, the study results and conclusions were presented to the regional health care authorities. Medical protocols were changed and the regulations of the hospital were strengthened.

ReAct is short for Action on Antibiotic resistance and is an international network, working from five continents, to find global, regional and local solutions that counteract antibiotic resistance. React is funded by SIDA and the European head quarter is based at Uppsala university.

The main impact of the study, however, was not on policy-makers but on health care workers, students and teachers who, as a result of ReACt´s sharing of the results of this study at local and regional meetings, became motivated to initiate other studies and to develop new ideas to deal with resistant microbes within local and regional contexts. While the Ministry of Health

organized a national meeting with over thousand participants to discuss ABR (196, 197), political problems unfortunately intervened, and the activities were interrupted for some years. In general, countries that lack specific ABR-policies may provide little guidance on how LIC and LMIC can fill in gaps in surveillance policies (1, 172).
Ecuador started to work with their National Action plan in 2015 and as in many countries, it closely reflects the Global Action Plan developed by WHO. A major challenge in these settings will be to find key priorities and sustainable measures that are feasible in terms of capacity and costs.

6.5 What is the importance of bacterial characterizing of the resistant bacteria?

Species determination and detecting resistance factors of the bacterial strain are the important first steps in order to know how to treat the infection. The epidemiological typing of EPE is necessary when characterizing a colonizing or infecting isolate. It is an important tool both in routine diagnostics and it provides valuable information about spreading routes in an EPE-outbreak (114). If a clone of bacteria with a certain successful ST is detected in the NICU, the clinician should be prepared for a bacterial strain harder to eradicate, an infection more difficult to treat and longer duration of the intestinal colonization of the bacteria. Bacterial characterization is also important when it comes to find new sequence type, in the continuous evolution of bacteria and their ability to resist antibiotics.

6.6 Are the characterized bacterial strains found in the neonates in these studies local or global?

In Ecuador **(I)**, we found ESBL-EC and ESBL-KP that featured $bla_{\text{CTX-M}}$ of phylogroup 1. The ESBL-KP belonged to two epidemically successful clones of KP known for rapid transmission between patients. The first one, ST855 is a single-locus variant of ST11, and a member of the clonal complex CC258, a complex that is known to disseminate carbapenemases. The second, ST897 is a single-locus variant of ST14, and hence part of CC14. Both CC11 and CC14 are clonal complexes known to rapidly spread carbapenemases. These clones had not been shown before in Ecuador but had earlier been detected in Brazil (ST 855) and Canada (ST 897).

In the two NICUs in Stockholm **(II)**, the *bla* $_{CTX-M-15}$ producing ST101 KP-strain that was characterized had not been shown before in NICUs in Sweden. However, the strain is globally spread and has been observed and presented in studies from southern Europe and northern Africa (198-200).

In the culture confirmed neonatal sepsis cases in Stockholm between 2006-2016 **(IV)**, GPB predominated causing 86% of the sepsis vs GNB that caused 14%. The three most common organisms causing GPB-sepsis were CoNS, *S. aureus* and GBS. The most common pathogens for GNB-sepsis were EC, KP and Enterobacter spp. The sepsis-causing pathogens are similar to the pathogens that were presented in an observational retrospective study about EOS in the western part Sweden from 2008-2017 (128).

We show the presence of EC ST95 in some of the invasive isolates. EC ST95 belongs to a globally disseminated clone of EC that is highly associated with neonatal sepsis and meningitis. The levels of drug resistance in EC ST95 have been defined as low among clinical isolates, but recently a highly virulent EC ST95 lineage has been detected. This lineage has, as well, been shown to easily acquire the plasmid-born colistin resistance gene (*mcr-1*). In our material, none of the patients with invasive EC ST95 suffered from meningitis. These strains are highly worrisome in clinical practices, and in particularly in intensive care units and NICUs where the antibiotic pressure is high (201).

6.7 How can diagnostics with ODM play a role in clinical settings?

In NICU-settings there is a need for epidemiological tools to detect rapid spread of EPE. Traditionally, characterizing plasmids requires several time-consuming techniques. ODM can, within a couple of hours, detect and characterize plasmids and as well confirm whether or not they harbor the resistance gene. The results can be retrieved in one single experiment. In a presumptive outbreak, it is enough to perform long read sequencing on only one isolate and thereafter the rest of the plasmids in the isolates can be identified by ODM(186). ODM is therefore a suitable method to use in EPE-outbreak situations.

In study **(III)**, we used the ODM method to determine whether or not the presence of resistance genes in different bacterial species was due to plasmid transfer between bacteria in the infant and also between neonates. The ODM method showed that there was no conjugation of plasmids between bacteria, in contrast to another Swedish NICU where ODM at a EPE-outbreak revealed plasmid conjugation (43). ODM confirmed that it was the same

strain in the outbreak strain and the multiple follow up strains, since the plasmid, even though there had been deletions and insertions in the DNA-sequence, had an identical barcode. By ODM we can state that the ESBL-EC in one patient at 68 months was not carrying the same plasmid as the KP-ESBL outbreak chain and therefore was therfor a re-colonization.

By using ODM we confirmed that the location of the CTX-M enzyme of the EC isolates, occurring in three of the patients, did not exist in the plasmid and revealed that it was located on the chromosome. We also analyzed one additional ESBL-KP-isolate that harbored three different plasmids. This strain did not carry the resistance gene in the plasmid, and we could therefore conclude that this strain did not belong to the actual outbreak.

By ODM, we could conclude that this outbreak was plasmid-mediated in the sense that resistance genes were carried on a plasmid. Furthermore, we could conclude that the ESBL-KP outbreak chain was not spread by plasmid transmission within the NICU environment, rather it was caused by a classical clonal outbreak. The horizontal spread of the ESBL-KP strain was by the health care personnel and underlines the importance of maintaining strict infection control measures and guidelines in the NICU setting. ODM has emerged as a powerful tool and shown to be a useful method as well in detecting bacterial clonal spread (202).

Promising results from a recent study show that the ODM method is capable of identifying bacteria from urine samples directly, without the culturing step, by matching large bacterial DNA molecules to a bacterial genome database (203). A method like ODM is an important part of the toolbox in the ongoing fight to stop local and global spread of ABR and a step forward in targeting the WHO´s Sustainable Development Goals for reducing neonatal mortality.

6.8 Does the neonatologist need to think differently about neonatal sepsis in low- and high-income countries?

Treatment failure of neonatal sepsis is associated with increased mortality and higher costs, which is similar in both settings (12, 124, 125). In LIC settings compared to HIC, where a lack of reliable microbiological laboratories and competency regarding infection control is a fact, the physician might tend to prolong the duration of antibiotic treatment (168). The availability of empirical antibiotic treatment varies highly in LIC and LMIC settings. Purchase of over-the-counter antibiotics can lead to inappropriate use of antibiotics (204). What is decisive for the clinician is the knowledge of the local burden of ABR, which is a practical challenge if there are limited laboratory resources. The clinician needs to think about whether or not the neonate has received previous antibiotics, and what type of antibiotic, since studies show that antibiotic treatment is a risk factor for subsequent sepsis and that a less diverse microbiota is a profound risk factor for neonatal LOS (51). The use of cephalosporins in NICU settings significantly increases the incidence of ESBL-producing bacteria in neonatal blood cultures (205, 206).

In **study I**, it would have been interesting to have gathered data on the association between colonization and invasive infection in the LMIC setting. However, the standard of the existing microbiological laboratory at the hospital did not allow this and therefore the possibilities of this project were restricted. We assume, though, that such a high frequency of EPE-carriers indicates that there was a large proportion of invasive cases as well. Since we lack data from clinical cultures we cannot comment specifically on the local empirical choice of antimicrobial treatment, other than stating that EPE seem to be very common in the NICU setting we studied in Ecuador.

6.9 Do we need to study suspected sepsis more?

The culture confirmed GNB-cases are rare in the Stockholm region, and corresponding to 0.8 cases per month in the four NICUs **(IV)**. We found that the incidence of culture confirmed sepsis with suspected EOS/LOS in admitted neonate was low (ratio of 1:1,3) compared to other studies (1:6-16)(109, 145, 207). Most studies on suspected neonatal sepsis in HIC are performed on EOS and not suspected LOS. A conclusion from this result might be that we have underreported the cases with suspected sepsis in the patients journals and in (SNQ). It can also be influenced by the antibiotic stewardship programs set up between 2006-2016 in our region.

A study on neonatal mortality following LOS has shown that the in-hospital mortality of GNB-LOS was 19%. The in-hospital mortality in GBP-LOS caused by CoNS was (8%), other GPB (6%) and was similar to in-hospital mortality in patients with a negative blood culture (8%). The survival analysis by Cox hazard regression in the same study showed that neonates with bacteremia with CoNS (HR 0.92) and other GPB (HR 0.74) had even a lower hazards as neonates with negative blood cultures (120). That suggests that there is a slightly higher hazard of dying at a specific time point in the suspected sepsis group compared to the groups with CoNS-sepsis or other GPB-sepsis. However, the selection basis for including patients with negative blood cultures may have differed from our study and therefore the comparison might not be correct.

In our study, the in-mortality of GNB-LOS was 25/34 (34%) and for the suspected –LOS group it was 12/74 (16%). The adjusted odds of dying before discharge were 3.9 times higher in the GNB-LOS group compared to the uninfected group. Due to the limited number of patients, the comparisons between GNB-LOS and the suspected sepsis group was not statistically significant.

Recent research show that prolonged antibiotic treatment in preterm neonates without confirmed infection increases the risk for NEC, ROP, periventricular white matter damage and mortality (208). As long as a sufficient blood volume has been taken for the blood culture to ensure reliable results, it is of great importance that the neonatologist analyze the benefits and risk of every dose of antibiotics in situations when the blood-culture is negative.

So, yes, suspected sepsis, and especially suspected-LOS, need further studies to determine whether it is a different entity and whether it might be treated with a different antibiotic strategy from culture proven sepsis. Many studies on EOS and treatment guidelines suggest stopping antibiotics within 36-48 hours in a culture-negative situation, unless there are strong clinical and biochemical indications not to do so (109, 209). The studies in this thesis do not oppose the recommendations from earlier studies that clinicians should trust negative blood cultures (109, 209).

6.10 How important is the clinical burden of ABR in NICUs in the Stockholm area?

The invasive EPE in neonates were rare during the study period and not as high as we had expected **(IV)**. During the EPE-outbreak 2008-2009, there were three neonates that died due to invasive ESBL-KP in combination with abdominal symptoms, intestinal perforation/necrotizing enterocolitis other major neonatal risk factors for mortality. Of all 107 GNB isolates that have caused neonatal sepsis in the Stockholm area during an eleven year period, the proportion of multidrug-resistant invasive isolates strains is considered very low. This may well be the result of long-standing efforts in infection control and antimicrobial stewardship.

A study of EPE carriage in Swedish pre-school children (Uppsala-region), shows a rapid increase in ESBL-carrier rates during the last decade. The prevalence of EPE-carriers in this group of societies increased from 2.6% to 16.8% during the time period from 2010 to 2016. Of all ESBL isolates, 58% were multi-resistant. No differences in antibiotic use in the area was seen (210). Even though the prevalence of invasive neonatal EPE-infections in Sweden is still rather low, there is no reason to believe that the EPE-prevalence will not increase among children. Travelling and immigration can explain the increase of ESBL-prevalence in Sweden (211).

6.11 Which interventions can be done and to prevent LOS by ABR bacteria in neonates?

Prevention of drug-resistant infection requires strict infection control protocols, avoidance of unnecessary use of broad-spectrum antibiotics, and also less invasive procedures in neonatal care (98, 122). Successful prevention protocols like the *Matching Michigan* initiative, with evidence-based strategies such as full-barrier precautions, avoidance of using the femoral route, prompt removal of catheters, hand hygiene, use of chlorhexidine skin antiseptics, did together with behavioral and cultural support, decreased the LOS due to venous catheters in 19 pediatric ICUs in Great Britain (212). Surfactant administration and prenatal steroids reduce the risk for invasive procedures, such as mechanical ventilation, and therefore also reduces the risk for LOS (212, 213).

An good exemplary model for reducing antibiotic use in a NICU in a HIC setting can be found in a recently published study in Lancet were the researchers conducted antibiotic stewardship strategies using surveillance and assessments of all antibiotic use. The overall

antibiotic use in the NICU decreased by 27% and there was no statistically significant differences in the safety outcomes between the baseline and intervention period (145).

When the new intervention “Scrub the hub” was introduced in many of the Swedish neonatal wards whereby the membrane of the intravenous catheters were scrubbed with alcohol for 15 seconds, LOS caused by CoNS was shown to be prevented (214). Respiratory viruses were, in contrast to gastro intestinal viruses, detected in a considerable number of suspected-LOS cases and should therefore not be forgotten in diagnosing the neonate with late-onset symptoms of infection (215).

7 CONCLUSIONS

- A high proportion (56%) of the neonates, in a NICU in a tertiary hospital in Ecuador, were colonized with EPE. Two highly successful clones of KP, that are associated with spread of carbapenem resistance, were found. This finding was highly worrisome. The study was a good contribution to the collaborative work within the international and Latin American networks on antibiotic resistance.

- *Bla*$_{\text{CTX-M 15}}$ was the genotype of the ESBL spread at two NICUs at Karolinska University hospital (2008-2009). This is the ESBL that has been shown to be the most common genotype in Stockholm.

- The children that were colonized during their NICU stay carried the same ESBL-KP for up to 26 months. The loss of EPE-colonization after two years seems to be related to the strain virulence, the environmental factors and the unique programming and development of the newborn microbiome.

- There was no plasmid transfer between bacteria during the outbreak.

- EPE-outbreaks need to be diagnosed rapidly. Optical DNA mapping is a reliable diagnostic tool in EPE-outbreaks in NICU-settings.

- During a 11-year period, Gram-negative sepsis was rare but a great risk for neonatal mortality. GNB-LOS was associated with a higher neonatal mortality compared to uninfected controls, but not compared to suspected sepsis.

8 FUTURE

There is currently a lot of new knowledge in the research field of infectious biology especially addressing the microbiome. In the field of neonatology, a clear definition of neonatal sepsis, taking into account the different age-dependent pathophysiologic responses preterm neonates, is required as a first step to enable the neonatologist to align to an adequate antibiotic treatment strategy.

Antibiotic therapy for truly infected neonates is crucial and prospective clinical trials are needed to evaluate efficacy and safety of new antibiotics to treat ABR infections in neonates. Striving to achieve rational use of antibiotics in suspected sepsis, taking into account better rapid diagnostics and supporting the physicians to provide the most appropriate antibiotics, must continue.

Therefore, studies on suspected sepsis need to be addressed. Prospective cohort studies, with an objective to initiate a surveillance of the microbiota before starting empiric antibiotics and during NICU-stay might guide the neonatologists to better understand how the changes in microbiota can precede onset of disease in the neonate.

Within the neonatal sepsis study **(IV)**, we also identified all neonates with confirmed GPB-sepsis and the characteristics of these strains in terms of antibiotic susceptibility. This group would as well be interesting to study further in terms of pathogen specific LOS-mortality and resistance epidemiology.

As a result of the multidisciplinary collaboration in the studies presented in this thesis, a project on antibiotic resistance in a NICU setting in Tanzania will be undertaken in the upcoming years in which ODM will be implemented in a format that is suitable for LMICs.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Neonatala infektioner utgör mer än en tredjedel av alla dödsfall i nyföddhetsperioden globalt sett. Blodförgiftning (sepsis) står för den största dödligheten och motsvarar ungefär en miljon neonatala dödsfall per år. Världshälsoorganisationen (WHO) prioriterar utvecklingen av antibiotikaresistens högt och betraktar det som ett av de största hälsohoten mot människors hälsa. Bakteriers förmåga att vara motståndskraftiga mot antibiotika kan ärvas men även överföras mellan bakterier om resistensgenen är lokaliserad på fria cirkulära DNA-molekyler (plasmider) i bakterien. Resistens-mekanismen hos de Gram-negativa tarmbakterier, som kallas Enterobacteriaceae, är förmågan att bilda Extended Spectrum Betalactamase (ESBL). ESBL är en grupp enzym som inaktiverar många typer av bred-spektrum antibiotika. Plasmid-medierad antibiotikaresistens är en stor del av ESBL-bildande Enterobacteriaceae (EPE)-stammars framgångsrika spridning över världen. Det finns anledning att vara oroad över antibiotikaresistensens utveckling och spridning i den del av vården där prematurfödda och nyfödda vårdas. De utgör är en grupp med särskild utsatthet och känslighet för bakteriella infektioner. Kolonisation innebär att bakterien bärs runt av sin värd men har inte gått över kroppens skyddsbarriär och orsakat en infektion. Infektioner orsakade av EPE hos både vuxna och barn är associerade med ökad dödlighet och sjuklighet. Lång sjukhusvård på neonatalavdelning är en riskfaktor för att utveckla bärarskap av EPE i de nyfödda barnets tarm, även om det vanligaste sättet för barnet att få EPE är att det överförs från modern i samband med förlossningen. Vetskapen är bristfällig avseende sambandet mellan kolonisation av EPE som man drabbats av under nyföddhetsperioden och senare infektioner orsakade av dessa. Erfarenheten kring hur kolonisation av EPE samt hur länge kolonisationen varar hos nyfödda är relativt liten. De studier som redovisat data avseende duration av EPE-kolonisation i samhället är oftast utförda på vuxna patienter.

Antibiotika är det mest använda läkemedlet på neonatalavdelningar. Antibiotikas verkan är livsnödvändig för att behandla ett nyfödd med sepsis men antibiotikas sidoeffekter på det nyfödda barnets tarmflora har visats kunna ge en obalans som. Detta kan i sin tur kan spela en icke gynnsam roll avseende det tidiga utvecklandet av barnets immunförsvar samt senare även i den framtida utvecklingen av kroniska sjukdomar.

I detta avhandlingsarbete presenteras hur vanligt förekommande EPE-kolonisation i tarmen är hos nyfödda i Ecuador **(studie I).** Vi påvisade att 56 % av alla inneliggande nyfödda på neonatalavdelningen koloniserades med EPE under sin tid på avdelningen. Utav dessa EPE fanns en bakteriestam känd för att driva karbapenemresistens. I **studie II-III** följde vi barn som blivit EPE-koloniserade under ett fyra månader långt EPE-utbrott på två neonatalavdelningar i Stockholm under åren 2008-2009. Avföringsodlingar togs på 14 barn varannan månad upp till 26 månader och vid 5 år efter utskrivning gjordes ytterligare provtagning. Vi såg att 2/14 barn fortsatt bar samma bakteriestam vid 23 och 26 mån. Vi har använde Optimal DNA mapping, för att se att plasmiderna var identiska efter två års tid. Vid 5 års ålder hade ett barn hade blivit återkoloniserat av en annan stam av EPE. Plasmidöverföring mellan bakterierstammar undersöktes men förekom ej. I **studie IV** undersöktes Gram-negativ sepsis med förmåga att bilda ESBL i Stockholm mellan åren 2006-2016. Dödligheten var dock hög, 30 av 107 fall avled innan utskrivning men en låg andel av sepsisfallen berodde på EPE.

10 ACKNOWLEDGEMENTS

To all colleagues working with newborns and in the laboratories; We need to use a thin sharp sword to kill the pathogens infecting our small patients and a big spoon to feed the good bacteria that will make our newborns thrive and develop into healthy children.

This PhD-journey has led me to many places I never thought I would go. Both in a physical and emotional way. Now I love this field of nature and science. I am overwhelmed by all generous and stimulating colleagues within this field and I am truly grateful of all new friendships that has edged my journey.

I would like to thank:

Lars Navér, my principal supervisor. Head of the Neonatology department. My deepest gratitude for supporting and encouraging me in all times of my PhD-studies. I have truly enjoyed every minute of our collaboration. Your straight forward approach to science, pragmatism, humor and accuracy are amazing. Most of all, thank you for a great friendship.

Christian Giske, my co-supervisor. For your positive energy and passion for bacteria and science. Thank you for sharing your vast experience of this field and supporting me during this research school. You seem to be one of those that have 30 hours per day. You are a great inspiration.

Aina Iversen, my co-supervisor. For the invaluable support during these years and for sharing your great knowledge of all the bugs within this thesis with me. I am so grateful that you brought me into the microbiology laboratory so I got to know the actual bacteria that I am writing about in this thesis.

Mikael Norman- my co-supervisor. For bringing me good and supportive advices along the way. You inspired me to choose the field of neonatology and as well that research could be a natural part of working as a neonatologist.

Arturo Quizphe- My sincere gratitude for a wonderful support and for a unique collaboration. Your passion for humans, microbes and how everything is connected is fascinating. Your leadership is unique and inspiring.

Mary Murray- my research mentor. I could not have found a better research mentor than you. I wish everyone would have the opportunity to get to know such a colorful, extraordinary and sharp woman like you are. My deepest thanks for all that you have done for me during my PhD-studies.

Georgina Munoz and **Diana Iniguez Rodriguez**. My warmest thanks to you both for your generous way when you introduced me into the neonatal ward and the into microbiology lab in Cuenca, Ecuador. Without you, no Ecuador study!

Fredrik Westerlund- For your supportive and educative way of introducing me into the world of plasmids. My deepest thanks for all your support. You have an impressive energy and I think also, an growing interest in neonatology.

Björn Fischler-Head of division of Pediatrics. Thank you for your supportive way and whenever there is time, a nice chat. **Mats Blennow**-the head of department CLINTEC. Thank you for letting me be a PhD student at CLINTEC and for sharing your great experience in clinical neonatology.

Kajsa Bohlin- my clinical boss. Thank you for all your support over these years and helping me to balance my clinical work with research activities. You are one of the skilled physicians that guided me into the field of neonatology!

Svante Norgren - The Head of Astrid Lindgrens Children Hospital. Thank you for all your support over the years while we formed the international group of ALB and later on PHI that has been done in parallel to my PhD-studies.

Karolina H, Karolina I- Thank you for assistance in discussions blood cultures and for me, advanced microbiology analyses.

My co-authors- A. Tidell, M. Kabir, S. Bikarolla, L. Sandegren, F. Rajer, V. Müller, S. KK, A. Dvirnas, T. Galindo, A. Turlej-Rogacka. K. Jonsson, B. Jonsson. O. Aspevall. A. Camporeale. Thank you for good collaborations!

Jessi S, Mikaela W and **Gabriella L** – for doing a great job with the data collection and for being such wonderful persons to collaborate with. You are the best!

To ALL colleagues in the Neonatology ward and the Pediatric section at Karolinska University hospital. I can´t mention you all by name. The patients and their parents are lucky to have you all around. I am lucky to have such a great team around me. Plus I can go and have a drink with you after work, as well. Lovely!

Therese W: For your kindness and your support with SNQ. I am so grateful. **Marie H**- For always being so nice and helping me with the administrativ issues around the clinics and research.

Christer R- for making the coverpage of this book. Art is science. Thank you!

Jenny S-For supporting, inspiring and comforting me in all situations. Thank you for being such a great colleague and friend.

Ewa H- Such a clever and lovable person you are. Thanks for many laughs and often long talks by the copy-machine.

To all collegues in Pediatric Health Initiative, especially **Jessi, Jonas, Helena, Maggan, Martino, Hampus, Katarina, Sara, Maggan, Camilla –** I am so glad to be a part of what we accomplish within PHI. You make me a better version! Thank you for all laughs and tears over the years.

Dragan and **Istvan**, thank you for all the laughs and that you never say no, even though the job is tough. I am so grateful of your great cooperation. I owe you.

Sarah S, Karin F, Lisa F, Agnes L, Karin N, Panteha H, Lena L, Karin H, Åsa K, Sara dH, Dirk W, Carro M, Mona-Lisa E, Olle J, Kalle L, Jenny A, Sara L, Kalle H, Helga N, Martha M – Each one of you are special to me and have during the years shared important periods of my life and as well given me tons of energy to start research school and as well finish this book. I want to thank you for that!

Ingrid, Atlas, Hektor, Sofia, Luna, Nina, Thea, Ebbe, Joel- how could I have survived without your tremendous support and love during these years. Thank you, with all of my heart.

Ville, Anton, Robert: my fantastic brothers. Thank you for the love you give and the moments we share. Some times often, some times more seldom. But still, the best moments.

Olle, Camilla, Maja, Johan, Anders, Karin, Bill- All summernights and laughs we have had together. From now on the computer will stay closed during summer breaks. See you at Austre!

My parents in law- **Anne-Chatrine**- You have since day one been a truly support to me and a such a caring mother in law and loving grandmother to our children. Thank you! -**Olle**- For being such a lovable father in law and for sharing so many of your live experiences and memories.

Kjell N, Ann E, Karin dH, Jocke P, Chris, Marit- For being the persons you are. I appreciate you all a lot!

Monica, Conny, Jiunndyi, Violet- My amazing set of parents. Thank you for giving me the love, time and challenges to develop my courage to always dare to take another step.
Mom- I couldn´t have done this without your endless and loving support.

My wonderful children; **Adam, Moa, Lova**- Ni är bäst! Thank you for all the help writing this book! Your energy, curiosity and wisdom amaze me daily. I love you to the moon and back!

Per- The love of my life. My deepest thanks for your support, your interest and your patience during so many years while I have been working with different projects. You are really one of a kind. Thanks for being such a wonderful person to hang around. I love you endlessly.

11 REFERENCES

1. Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, Vlieghe E, Hara GL, Gould IM, Goossens H, Greko C, So AD, Bigdeli M, Tomson G, Woodhouse W, Ombaka E, Peralta AQ, Qamar FN, Mir F, Kariuki S, Bhutta ZA, Coates A, Bergstrom R, Wright GD, Brown ED, Cars O 2013 Antibiotic resistance-the need for global solutions. Lancet Infect Dis 13:1057-1098.
2. Agency; EM Antimicrobial resistance 2019. .
3. World Health Organisation 2014 Antimicrobial resistance: global report on surveillance
4. Zhen X, Lundborg CS, Sun X, Hu X, Dong H 2019 Economic burden of antibiotic resistance in ESKAPE organisms: a systematic review. Antimicrob Resist Infect Control 8:137.
5. Zingg W, Posfay-Barbe KM, Pittet D 2008 Healthcare-associated infections in neonates. Curr Opin Infect Dis 21:228-234.
6. Rautava S, Collado MC, Salminen S, Isolauri E 2012 Probiotics modulate host-microbe interaction in the placenta and fetal gut: a randomized, double-blind, placebo-controlled trial. Neonatology 102:178-184.
7. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J 2014 The placenta harbors a unique microbiome. Sci Transl Med 6:237ra265.
8. Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S 2016 Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Sci Rep 6:23129.
9. Donnet-Hughes A, Perez PF, Dore J, Leclerc M, Levenez F, Benyacoub J, Serrant P, Segura-Roggero I, Schiffrin EJ 2010 Potential role of the intestinal microbiota of the mother in neonatal immune education. Proc Nutr Soc 69:407-415.
10. Chu DM, Seferovic M, Pace RM, Aagaard KM 2018 The microbiome in preterm birth. Best Pract Res Clin Obstet Gynaecol 52:103-113.
11. Moles L, Gomez M, Heilig H, Bustos G, Fuentes S, de Vos W, Fernandez L, Rodriguez JM, Jimenez E 2013 Bacterial diversity in meconium of preterm neonates and evolution of their fecal microbiota during the first month of life. PLoS One 8:e66986.
12. Boo NY, Ng SF, Lim VK 2005 A case-control study of risk factors associated with rectal colonization of extended-spectrum beta-lactamase producing Klebsiella sp. in newborn infants. J Hosp Infect 61:68-74.

13. Brunel J 1951 Antibiosis from Pasteur to Fleming. J Hist Med Allied Sci 6:287-301.
14. Newman DJ, Cragg GM 2016 Natural Products as Sources of New Drugs from 1981 to 2014. J Nat Prod 79:629-661.
15. Embley TM, Stackebrandt E 1994 The molecular phylogeny and systematics of the actinomycetes. Annu Rev Microbiol 48:257-289.
16. Seipke RF, Kaltenpoth M, Hutchings MI 2012 Streptomyces as symbionts: an emerging and widespread theme? FEMS Microbiol Rev 36:862-876.
17. Gupta RS 2011 Origin of diderm (Gram-negative) bacteria: antibiotic selection pressure rather than endosymbiosis likely led to the evolution of bacterial cells with two membranes. Antonie Van Leeuwenhoek 100:171-182.
18. Pendleton JN, Gorman SP, Gilmore BF 2013 Clinical relevance of the ESKAPE pathogens. Expert Rev Anti Infect Ther 11:297-308.
19. WHO 2017 World Health Organization, Geneva. List of bacteria for which new antibiotics are urgently needed.
20. Carattoli A 2009 Resistance plasmid families in Enterobacteriaceae. Antimicrob Agents Chemother 53:2227-2238.
21. Ilangovan A, Connery S, Waksman G 2015 Structural biology of the Gram-negative bacterial conjugation systems. Trends Microbiol 23:301-310.
22. Rozwandowicz M, Brouwer MSM, Fischer J, Wagenaar JA, Gonzalez-Zorn B, Guerra B, Mevius DJ, Hordijk J 2018 Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J Antimicrob Chemother 73:1121-1137.
23. Fleming A 2001 On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. 1929. Bull World Health Organ 79:780-790.
24. Iredell J, Brown J, Tagg K 2016 Antibiotic resistance in Enterobacteriaceae: mechanisms and clinical implications. BMJ 352:h6420.
25. Canton R, Gonzalez-Alba JM, Galan JC 2012 CTX-M Enzymes: Origin and Diffusion. Front Microbiol 3:110.
26. Bonnet R 2004 Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother 48:1-14.
27. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR 2009 Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in

Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother 53:5046-5054.

28. Brolund A, Sandegren L 2016 Characterization of ESBL disseminating plasmids. Infect Dis (Lond) 48:18-25.
29. Lake JG, Weiner LM, Milstone AM, Saiman L, Magill SS, See I 2018 Pathogen Distribution and Antimicrobial Resistance Among Pediatric Healthcare-Associated Infections Reported to the National Healthcare Safety Network, 2011-2014. Infect Control Hosp Epidemiol 39:1-11.
30. Podschun R, Ullmann U 1998 Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 11:589-603.
31. Clegg S, Murphy CN 2016 Epidemiology and Virulence of Klebsiella pneumoniae. Microbiol Spectr 4.
32. Hall-Stoodley L, Costerton JW, Stoodley P 2004 Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2:95-108.
33. Bagley ST 1985 Habitat association of Klebsiella species. Infect Control 6:52-58.
34. Turton J, Davies F, Turton J, Perry C, Payne Z, Pike R 2019 Hybrid Resistance and Virulence Plasmids in "High-Risk" Clones of Klebsiella pneumoniae, Including Those Carrying blaNDM-5. Microorganisms 7.
35. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG 1998 Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A 95:3140-3145.
36. Kaper JB, Nataro JP, Mobley HL 2004 Pathogenic Escherichia coli. Nat Rev Microbiol 2:123-140.
37. Clermont O, Bonacorsi S, Bingen E 2000 Rapid and simple determination of the Escherichia coli phylogenetic group. Appl Environ Microbiol 66:4555-4558.
38. Nojoomi F, Ghasemian A 2019 The relation of phylogroups, serogroups, virulence factors and resistance pattern of Escherichia coli isolated from children with septicemia. New Microbes New Infect 29:100517.

39. Hope R, Potz NA, Warner M, Fagan EJ, Arnold E, Livermore DM 2007 Efficacy of practised screening methods for detection of cephalosporin-resistant Enterobacteriaceae. J Antimicrob Chemother 59:110-113.
40. Drieux L, Brossier F, Sougakoff W, Jarlier V 2008 Phenotypic detection of extended-spectrum beta-lactamase production in Enterobacteriaceae: review and bench guide. Clin Microbiol Infect 14 Suppl 1:90-103.
41. Bialek-Davenet S, Criscuolo A, Ailloud F, Passet V, Jones L, Delannoy-Vieillard AS, Garin B, Le Hello S, Arlet G, Nicolas-Chanoine MH, Decre D, Brisse S 2014 Genomic definition of hypervirulent and multidrug-resistant Klebsiella pneumoniae clonal groups. Emerg Infect Dis 20:1812-1820.
42. Frykholm K, Nyberg LK, Lagerstedt E, Noble C, Fritzsche J, Karami N, Ambjornsson T, Sandegren L, Westerlund F 2015 Fast size-determination of intact bacterial plasmids using nanofluidic channels. Lab Chip 15:2739-2743.
43. Muller V, Karami N, Nyberg LK, Pichler C, Torche Pedreschi PC, Quaderi S, Fritzsche J, Ambjornsson T, Ahren C, Westerlund F 2016 Rapid Tracing of Resistance Plasmids in a Nosocomial Outbreak Using Optical DNA Mapping. ACS Infect Dis 2:322-328.
44. Nyberg LK, Quaderi S, Emilsson G, Karami N, Lagerstedt E, Muller V, Noble C, Hammarberg S, Nilsson AN, Sjoberg F, Fritzsche J, Kristiansson E, Sandegren L, Ambjornsson T, Westerlund F 2016 Rapid identification of intact bacterial resistance plasmids via optical mapping of single DNA molecules. Sci Rep 6:30410.
45. Jo K, Schramm TM, Schwartz DC 2009 A single-molecule barcoding system using nanoslits for DNA analysis : nanocoding. Methods Mol Biol 544:29-42.
46. Nilsson AN, Emilsson G, Nyberg LK, Noble C, Stadler LS, Fritzsche J, Moore ER, Tegenfeldt JO, Ambjornsson T, Westerlund F 2014 Competitive binding-based optical DNA mapping for fast identification of bacteria--multi-ligand transfer matrix theory and experimental applications on Escherichia coli. Nucleic Acids Res 42:e118.
47. Muller V, Rajer F, Frykholm K, Nyberg LK, Quaderi S, Fritzsche J, Kristiansson E, Ambjornsson T, Sandegren L, Westerlund F 2016 Direct identification of antibiotic resistance genes on single plasmid molecules using CRISPR/Cas9 in combination with optical DNA mapping. Sci Rep 6:37938.

48. Dorfman KD, King SB, Olson DW, Thomas JD, Tree DR 2013 Beyond gel electrophoresis: microfluidic separations, fluorescence burst analysis, and DNA stretching. Chem Rev 113:2584-2667.
49. Liu L, Oza S, Hogan D, Chu Y, Perin J, Zhu J, Lawn JE, Cousens S, Mathers C, Black RE 2016 Global, regional, and national causes of under-5 mortality in 2000-15: an updated systematic analysis with implications for the Sustainable Development Goals. Lancet 388:3027-3035.
50. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, Adler A, Vera Garcia C, Rohde S, Say L, Lawn JE 2012 National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet 379:2162-2172.
51. Madan JC, Salari RC, Saxena D, Davidson L, O'Toole GA, Moore JH, Sogin ML, Foster JA, Edwards WH, Palumbo P, Hibberd PL 2012 Gut microbial colonisation in premature neonates predicts neonatal sepsis. Arch Dis Child Fetal Neonatal Ed 97:F456-462.
52. Westerbeek EA, van den Berg A, Lafeber HN, Knol J, Fetter WP, van Elburg RM 2006 The intestinal bacterial colonisation in preterm infants: a review of the literature. Clin Nutr 25:361-368.
53. Edmond K, Zaidi A 2010 New approaches to preventing, diagnosing, and treating neonatal sepsis. PLoS Med 7:e1000213.
54. Fanaroff AA, Korones SB, Wright LL, Verter J, Poland RL, Bauer CR, Tyson JE, Philips JB, 3rd, Edwards W, Lucey JF, Catz CS, Shankaran S, Oh W 1998 Incidence, presenting features, risk factors and significance of late onset septicemia in very low birth weight infants. The National Institute of Child Health and Human Development Neonatal Research Network. Pediatr Infect Dis J 17:593-598.
55. Forman ML, Stiehm ER 1969 Impaired opsonic activity but normal phagocytosis in low-birth-weight infants. N Engl J Med 281:926-931.
56. Kallman J, Schollin J, Schalen C, Erlandsson A, Kihlstrom E 1998 Impaired phagocytosis and opsonisation towards group B streptococci in preterm neonates. Arch Dis Child Fetal Neonatal Ed 78:F46-50.
57. Fanaroff AA, Korones SB, Wright LL, Wright EC, Poland RL, Bauer CB, Tyson JE, Philips JB, 3rd, Edwards W, Lucey JF, et al. 1994 A controlled trial of

intravenous immune globulin to reduce nosocomial infections in very-low-birth-weight infants. National Institute of Child Health and Human Development Neonatal Research Network. N Engl J Med 330:1107-1113.

58. Group IC, Brocklehurst P, Farrell B, King A, Juszczak E, Darlow B, Haque K, Salt A, Stenson B, Tarnow-Mordi W 2011 Treatment of neonatal sepsis with intravenous immune globulin. N Engl J Med 365:1201-1211.

59. Adkins B, Leclerc C, Marshall-Clarke S 2004 Neonatal adaptive immunity comes of age. Nat Rev Immunol 4:553-564.

60. Kollmann TR, Levy O, Montgomery RR, Goriely S 2012 Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly. Immunity 37:771-783.

61. Olin A, Henckel E, Chen Y, Lakshmikanth T, Pou C, Mikes J, Gustafsson A, Bernhardsson AK, Zhang C, Bohlin K, Brodin P 2018 Stereotypic Immune System Development in Newborn Children. Cell 174:1277-1292 e1214.

62. Martin R, Nauta AJ, Ben Amor K, Knippels LM, Knol J, Garssen J 2010 Early life: gut microbiota and immune development in infancy. Benef Microbes 1:367-382.

63. Sender R, Fuchs S, Milo R 2016 Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. Cell 164:337-340.

64. Nuriel-Ohayon M, Neuman H, Koren O 2016 Microbial Changes during Pregnancy, Birth, and Infancy. Front Microbiol 7:1031.

65. Arrieta MC, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, Kuzeljevic B, Gold MJ, Britton HM, Lefebvre DL, Subbarao P, Mandhane P, Becker A, McNagny KM, Sears MR, Kollmann T, Investigators CS, Mohn WW, Turvey SE, Finlay BB 2015 Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med 7:307ra152.

66. Dedrick S, Sundaresh B, Huang Q, Brady C, Yoo T, Cronin C, Rudnicki C, Flood M, Momeni B, Ludvigsson J, Altindis E 2020 The Role of Gut Microbiota and Environmental Factors in Type 1 Diabetes Pathogenesis. Front Endocrinol (Lausanne) 11:78.

67. Fujimura KE, Sitarik AR, Havstad S, Lin DL, Levan S, Fadrosh D, Panzer AR, LaMere B, Rackaityte E, Lukacs NW, Wegienka G, Boushey HA, Ownby DR, Zoratti EM, Levin AM, Johnson CC, Lynch SV 2016 Neonatal gut microbiota

associates with childhood multisensitized atopy and T cell differentiation. Nat Med 22:1187-1191.

68. Marild K, Ye W, Lebwohl B, Green PH, Blaser MJ, Card T, Ludvigsson JF 2013 Antibiotic exposure and the development of coeliac disease: a nationwide case-control study. BMC Gastroenterol 13:109.

69. Gosalbes MJ, Valles Y, Jimenez-Hernandez N, Balle C, Riva P, Miravet-Verde S, de Vries LE, Llop S, Agerso Y, Sorensen SJ, Ballester F, Francino MP 2016 High frequencies of antibiotic resistance genes in infants' meconium and early fecal samples. J Dev Orig Health Dis 7:35-44.

70. Stout MJ, Conlon B, Landeau M, Lee I, Bower C, Zhao Q, Roehl KA, Nelson DM, Macones GA, Mysorekar IU 2013 Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations. Am J Obstet Gynecol 208:226 e221-227.

71. Walker WA 2017 The importance of appropriate initial bacterial colonization of the intestine in newborn, child, and adult health. Pediatr Res 82:387-395.

72. Korpela K, Blakstad EW, Moltu SJ, Strommen K, Nakstad B, Ronnestad AE, Braekke K, Iversen PO, Drevon CA, de Vos W 2018 Intestinal microbiota development and gestational age in preterm neonates. Sci Rep 8:2453.

73. Brooks B, Olm MR, Firek BA, Baker R, Geller-McGrath D, Reimer SR, Soenjoyo KR, Yip JS, Dahan D, Thomas BC, Morowitz MJ, Banfield JF 2018 The developing premature infant gut microbiome is a major factor shaping the microbiome of neonatal intensive care unit rooms. Microbiome 6:112.

74. Bokulich NA, Chung J, Battaglia T, Henderson N, Jay M, Li H, A DL, Wu F, Perez-Perez GI, Chen Y, Schweizer W, Zheng X, Contreras M, Dominguez-Bello MG, Blaser MJ 2016 Antibiotics, birth mode, and diet shape microbiome maturation during early life. Sci Transl Med 8:343ra382.

75. Schwiertz A, Gruhl B, Lobnitz M, Michel P, Radke M, Blaut M 2003 Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. Pediatr Res 54:393-399.

76. Stout MJ, Zhou Y, Wylie KM, Tarr PI, Macones GA, Tuuli MG 2017 Early pregnancy vaginal microbiome trends and preterm birth. Am J Obstet Gynecol 217:356 e351-356 e318.

77. Underwood MA, German JB, Lebrilla CB, Mills DA 2015 Bifidobacterium longum subspecies infantis: champion colonizer of the infant gut. Pediatr Res 77:229-235.
78. Duar RM, Kyle D, Casaburi G 2020 Colonization Resistance in the Infant Gut: The Role of B. infantis in Reducing pH and Preventing Pathogen Growth. High Throughput 9.
79. Manthey CF, Autran CA, Eckmann L, Bode L 2014 Human milk oligosaccharides protect against enteropathogenic Escherichia coli attachment in vitro and EPEC colonization in suckling mice. J Pediatr Gastroenterol Nutr 58:165-168.
80. Musilova S, Rada V, Vlkova E, Bunesova V 2014 Beneficial effects of human milk oligosaccharides on gut microbiota. Benef Microbes 5:273-283.
81. Lievin V, Peiffer I, Hudault S, Rochat F, Brassart D, Neeser JR, Servin AL 2000 Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut 47:646-652.
82. Yasui H, Kiyoshima J, Ushijima H 1995 Passive protection against rotavirus-induced diarrhea of mouse pups born to and nursed by dams fed Bifidobacterium breve YIT4064. J Infect Dis 172:403-409.
83. Holgerson PL, Vestman NR, Claesson R, Ohman C, Domellof M, Tanner AC, Hernell O, Johansson I 2013 Oral microbial profile discriminates breast-fed from formula-fed infants. J Pediatr Gastroenterol Nutr 56:127-136.
84. Stewart CJ, Ajami NJ, O'Brien JL, Hutchinson DS, Smith DP, Wong MC, Ross MC, Lloyd RE, Doddapaneni H, Metcalf GA, Muzny D, Gibbs RA, Vatanen T, Huttenhower C, Xavier RJ, Rewers M, Hagopian W, Toppari J, Ziegler AG, She JX, Akolkar B, Lernmark A, Hyoty H, Vehik K, Krischer JP, Petrosino JF 2018 Temporal development of the gut microbiome in early childhood from the TEDDY study. Nature 562:583-588.
85. Garland SM, Tobin JM, Pirotta M, Tabrizi SN, Opie G, Donath S, Tang ML, Morley CJ, Hickey L, Ung L, Jacobs SE, ProPrems Study G 2011 The ProPrems trial: investigating the effects of probiotics on late onset sepsis in very preterm infants. BMC Infect Dis 11:210.
86. Morgan J, Bombell S, McGuire W 2013 Early trophic feeding versus enteral fasting for very preterm or very low birth weight infants. Cochrane Database Syst Rev:CD000504.

87. Casaburi G, Duar RM, Vance DP, Mitchell R, Contreras L, Frese SA, Smilowitz JT, Underwood MA 2019 Early-life gut microbiome modulation reduces the abundance of antibiotic-resistant bacteria. Antimicrob Resist Infect Control 8:131.
88. AlFaleh K, Anabrees J 2014 Probiotics for prevention of necrotizing enterocolitis in preterm infants. Evid Based Child Health 9:584-671.
89. Kim CS, Claud EC 2019 Necrotizing Enterocolitis Pathophysiology: How Microbiome Data Alter Our Understanding. Clin Perinatol 46:29-38.
90. Backhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, Li Y, Xia Y, Xie H, Zhong H, Khan MT, Zhang J, Li J, Xiao L, Al-Aama J, Zhang D, Lee YS, Kotowska D, Colding C, Tremaroli V, Yin Y, Bergman S, Xu X, Madsen L, Kristiansen K, Dahlgren J, Wang J 2015 Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. Cell Host Microbe 17:852.
91. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE 2011 Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A 108 Suppl 1:4578-4585.
92. Martin R, Makino H, Cetinyurek Yavuz A, Ben-Amor K, Roelofs M, Ishikawa E, Kubota H, Swinkels S, Sakai T, Oishi K, Kushiro A, Knol J 2016 Early-Life Events, Including Mode of Delivery and Type of Feeding, Siblings and Gender, Shape the Developing Gut Microbiota. PLoS One 11:e0158498.
93. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, van den Brandt PA, Stobberingh EE 2006 Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118:511-521.
94. Levin AM, Sitarik AR, Havstad SL, Fujimura KE, Wegienka G, Cassidy-Bushrow AE, Kim H, Zoratti EM, Lukacs NW, Boushey HA, Ownby DR, Lynch SV, Johnson CC 2016 Joint effects of pregnancy, sociocultural, and environmental factors on early life gut microbiome structure and diversity. Sci Rep 6:31775.
95. Magne F, Puchi Silva A, Carvajal B, Gotteland M 2017 The Elevated Rate of Cesarean Section and Its Contribution to Non-Communicable Chronic Diseases in Latin America: The Growing Involvement of the Microbiota. Front Pediatr 5:192.
96. Goldenberg RL, Culhane JF, Iams JD, Romero R 2008 Epidemiology and causes of preterm birth. Lancet 371:75-84.

97. Fanaro S, Chierici R, Guerrini P, Vigi V 2003 Intestinal microflora in early infancy: composition and development. Acta Paediatr Suppl 91:48-55.
98. Dong Y, Speer CP 2015 Late-onset neonatal sepsis: recent developments. Arch Dis Child Fetal Neonatal Ed 100:F257-263.
99. Stearns JC, Simioni J, Gunn E, McDonald H, Holloway AC, Thabane L, Mousseau A, Schertzer JD, Ratcliffe EM, Rossi L, Surette MG, Morrison KM, Hutton EK 2017 Intrapartum antibiotics for GBS prophylaxis alter colonization patterns in the early infant gut microbiome of low risk infants. Sci Rep 7:16527.
100. Aloisio I, Quagliariello A, De Fanti S, Luiselli D, De Filippo C, Albanese D, Corvaglia LT, Faldella G, Di Gioia D 2016 Evaluation of the effects of intrapartum antibiotic prophylaxis on newborn intestinal microbiota using a sequencing approach targeted to multi hypervariable 16S rDNA regions. Appl Microbiol Biotechnol 100:5537-5546.
101. Greenwood C, Morrow AL, Lagomarcino AJ, Altaye M, Taft DH, Yu Z, Newburg DS, Ward DV, Schibler KR 2014 Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of Enterobacter. J Pediatr 165:23-29.
102. Alexander VN, Northrup V, Bizzarro MJ 2011 Antibiotic exposure in the newborn intensive care unit and the risk of necrotizing enterocolitis. J Pediatr 159:392-397.
103. Mikkelsen KH, Allin KH, Knop FK 2016 Effect of antibiotics on gut microbiota, glucose metabolism and body weight regulation: a review of the literature. Diabetes Obes Metab 18:444-453.
104. Sullivan A, Edlund C, Nord CE 2001 Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect Dis 1:101-114.
105. Jernberg C, Lofmark S, Edlund C, Jansson JK 2010 Long-term impacts of antibiotic exposure on the human intestinal microbiota. Microbiology 156:3216-3223.
106. Shane AL, Sanchez PJ, Stoll BJ 2017 Neonatal sepsis. Lancet 390:1770-1780.
107. Ohlin A 2011 What is neonatal sepsis? Acta Paediatr 100:7-8.
108. Goldstein B, Giroir B, Randolph A, International Consensus Conference on Pediatric S 2005 International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics. Pediatr Crit Care Med 6:2-8.

109. Klingenberg C, Kornelisse RF, Buonocore G, Maier RF, Stocker M 2018 Culture-Negative Early-Onset Neonatal Sepsis - At the Crossroad Between Efficient Sepsis Care and Antimicrobial Stewardship. Front Pediatr 6:285.
110. Wynn JL, Wong HR, Shanley TP, Bizzarro MJ, Saiman L, Polin RA 2014 Time for a neonatal-specific consensus definition for sepsis. Pediatr Crit Care Med 15:523-528.
111. Wynn JL, Polin RA 2018 Progress in the management of neonatal sepsis: the importance of a consensus definition. Pediatr Res 83:13-15.
112. UNICEF 2019 Neonatal mortality. UNICEF Data: Monitoring the situation of children and women https://data.unicef.org/topic/child-survival/neonatal-mortality/.
113. Wright S 2014 Ending newborn deaths: Ensuring every baby survives. Save the Children.
114. Sharma D, Kumar C, Pandita A, Pratap OT, Dasi T, Murki S 2015 Bacteriological profile and clinical predictors of ESBL neonatal sepsis. J Matern Fetal Neonatal Med:1-4.
115. Schwab F, Zibell R, Piening B, Geffers C, Gastmeier P 2015 Mortality due to bloodstream infections and necrotizing enterocolitis in very low birth weight infants. Pediatr Infect Dis J 34:235-240.
116. Tzialla C, Borghesi A, Pozzi M, Stronati M 2015 Neonatal infections due to multi-resistant strains: Epidemiology, current treatment, emerging therapeutic approaches and prevention. Clin Chim Acta 451:71-77.
117. Laxminarayan R, Matsoso P, Pant S, Brower C, Rottingen JA, Klugman K, Davies S 2016 Access to effective antimicrobials: a worldwide challenge. Lancet 387:168-175.
118. World Health Organization 2020 Sustainable Development Goals (SDGs)3: Ensure healthy lives and promote wellbeing for all at all ages. https://www.who.int/sdg/targets/en/.
119. Stoll BJ 2007 Infections of the neonatal infant. Nelson Textbook of Pediatrics. 18th ed. Philadelphia: Saunders; 2007.794-811.
120. Benjamin DK, DeLong E, Cotten CM, Garges HP, Steinbach WJ, Clark RH 2004 Mortality following blood culture in premature infants: increased with Gram-negative bacteremia and candidemia, but not Gram-positive bacteremia. J Perinatol 24:175-180.

121. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, Lemons JA, Donovan EF, Stark AR, Tyson JE, Oh W, Bauer CR, Korones SB, Shankaran S, Laptook AR, Stevenson DK, Papile LA, Poole WK 2002 Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. Pediatrics 110:285-291.
122. Downey LC, Smith PB, Benjamin DK, Jr. 2010 Risk factors and prevention of late-onset sepsis in premature infants. Early Hum Dev 86 Suppl 1:7-12.
123. Hornik CP, Fort P, Clark RH, Watt K, Benjamin DK, Jr., Smith PB, Manzoni P, Jacqz-Aigrain E, Kaguelidou F, Cohen-Wolkowiez M 2012 Early and late onset sepsis in very-low-birth-weight infants from a large group of neonatal intensive care units. Early Hum Dev 88 Suppl 2:S69-74.
124. Pessoa-Silva CL, Meurer Moreira B, Camara Almeida V, Flannery B, Almeida Lins MC, Mello Sampaio JL, Martins Teixeira L, Vaz Miranda LE, Riley LW, Gerberding JL 2003 Extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in a neonatal intensive care unit: risk factors for infection and colonization. J Hosp Infect 53:198-206.
125. Kaufman D, Fairchild KD 2004 Clinical microbiology of bacterial and fungal sepsis in very-low-birth-weight infants. Clin Microbiol Rev 17:638-680, table of contents.
126. Tessin I, Trollfors B, Thiringer K 1990 Incidence and etiology of neonatal septicaemia and meningitis in western Sweden 1975-1986. Acta Paediatr Scand 79:1023-1030.
127. Persson E, Trollfors B, Brandberg LL, Tessin I 2002 Septicaemia and meningitis in neonates and during early infancy in the Goteborg area of Sweden. Acta Paediatr 91:1087-1092.
128. Johansson Gudjonsdottir M, Elfvin A, Hentz E, Adlerberth I, Tessin I, Trollfors B 2019 Changes in incidence and etiology of early-onset neonatal infections 1997-2017 - a retrospective cohort study in western Sweden. BMC Pediatr 19:490.
129. 2019 NINdEyC Tasa de mortalidad neonatal https://www.ecuadorencifras.gob.ec/defunciones-generales-2019/.
130. Shim GH, Kim SD, Kim HS, Kim ES, Lee HJ, Lee JA, Choi CW, Kim EK, Choi EH, Kim BI, Lee HJ, Choi JH 2011 Trends in epidemiology of neonatal sepsis in a

tertiary center in Korea: a 26-year longitudinal analysis, 1980-2005. J Korean Med Sci 26:284-289.

131. Ohlin A, Bjorkman L, Serenius F, Schollin J, Kallen K 2015 Sepsis as a risk factor for neonatal morbidity in extremely preterm infants. Acta Paediatr.

132. Messbarger N, Neemann K 2017 Role of Anaerobic Blood Cultures in Neonatal Bacteremia. J Pediatric Infect Dis Soc.

133. Livermore DM, Wain J 2013 Revolutionising bacteriology to improve treatment outcomes and antibiotic stewardship. Infect Chemother 45:1-10.

134. Connell TG, Rele M, Cowley D, Buttery JP, Curtis N 2007 How reliable is a negative blood culture result? Volume of blood submitted for culture in routine practice in a children's hospital. Pediatrics 119:891-896.

135. Squire E, Favara B, Todd J 1979 Diagnosis of neonatal bacterial infection: hematologic and pathologic findings in fatal and nonfatal cases. Pediatrics 64:60-64.

136. Schelonka RL, Chai MK, Yoder BA, Hensley D, Brockett RM, Ascher DP 1996 Volume of blood required to detect common neonatal pathogens. J Pediatr 129:275-278.

137. Ohlin A, Backman A, Ewald U, Schollin J, Bjorkqvist M 2012 Diagnosis of neonatal sepsis by broad-range 16S real-time polymerase chain reaction. Neonatology 101:241-246.

138. Hofer N, Zacharias E, Muller W, Resch B 2012 An update on the use of C-reactive protein in early-onset neonatal sepsis: current insights and new tasks. Neonatology 102:25-36.

139. Chiesa C, Osborn JF, Pacifico L, Natale F, De Curtis M 2011 Gestational- and age-specific CRP reference intervals in the newborn. Clin Chim Acta 412:1889-1890.

140. Ishibashi M, Takemura Y, Ishida H, Watanabe K, Kawai T 2002 C-reactive protein kinetics in newborns: application of a high-sensitivity analytic method in its determination. Clin Chem 48:1103-1106.

141. Chiesa C, Natale F, Pascone R, Osborn JF, Pacifico L, Bonci E, De Curtis M 2011 C reactive protein and procalcitonin: reference intervals for preterm and term newborns during the early neonatal period. Clin Chim Acta 412:1053-1059.

142. Turner MA, Power S, Emmerson AJ 2004 Gestational age and the C reactive protein response. Arch Dis Child Fetal Neonatal Ed 89:F272-273.

143. Robinson P, De SK 2018 How to use... Procalcitonin. Arch Dis Child Educ Pract Ed 103:257-262.
144. Gray JW, Ubhi H, Milner P 2014 Antimicrobial treatment of serious gram-negative infections in newborns. Curr Infect Dis Rep 16:400.
145. Cantey JB, Wozniak PS, Pruszynski JE, Sanchez PJ 2016 Reducing unnecessary antibiotic use in the neonatal intensive care unit (SCOUT): a prospective interrupted time-series study. Lancet Infect Dis 16:1178-1184.
146. Fjalstad JW, Stensvold HJ, Bergseng H, Simonsen GS, Salvesen B, Ronnestad AE, Klingenberg C 2016 Early-onset Sepsis and Antibiotic Exposure in Term Infants: A Nationwide Population-based Study in Norway. Pediatr Infect Dis J 35:1-6.
147. (NICE) NIfHaCE 2012 Antibiotics for Early-onset Neonatal Infection: Antibiotics for the Prevention and Treatment
of Early-Onset Neonatal Infection. London: Clinical guideline Available online at: https://www.nice.org.uk/guidance/cg149.
148. Tangden T, Cars O, Melhus A, Lowdin E 2010 Foreign travel is a major risk factor for colonization with Escherichia coli producing CTX-M-type extended-spectrum beta-lactamases: a prospective study with Swedish volunteers. Antimicrob Agents Chemother 54:3564-3568.
149. Ruppe E, Armand-Lefevre L, Estellat C, Consigny PH, El Mniai A, Boussadia Y, Goujon C, Ralaimazava P, Campa P, Girard PM, Wyplosz B, Vittecoq D, Bouchaud O, Le Loup G, Pialoux G, Perrier M, Wieder I, Moussa N, Esposito-Farese M, Hoffmann I, Coignard B, Lucet JC, Andremont A, Matheron S 2015 High Rate of Acquisition but Short Duration of Carriage of Multidrug-Resistant Enterobacteriaceae After Travel to the Tropics. Clin Infect Dis 61:593-600.
150. Titelman E, Hasan CM, Iversen A, Naucler P, Kais M, Kalin M, Giske CG 2014 Faecal carriage of extended-spectrum beta-lactamase-producing Enterobacteriaceae is common 12 months after infection and is related to strain factors. Clin Microbiol Infect 20:O508-515.
151. Mammina C, Di Carlo P, Cipolla D, Giuffre M, Casuccio A, Di Gaetano V, Plano MR, D'Angelo E, Titone L, Corsello G 2007 Surveillance of multidrug-resistant gram-negative bacilli in a neonatal intensive care unit: prominent role of cross transmission. Am J Infect Control 35:222-230.

152. Li X, Xu X, Yang X, Luo M, Liu P, Su K, Qing Y, Chen S, Qiu J, Li Y 2017 Risk factors for infection and/or colonisation with extended-spectrum beta-lactamase-producing bacteria in the neonatal intensive care unit: a meta-analysis. Int J Antimicrob Agents.

153. Ruiz E, Rojo-Bezares B, Saenz Y, Olarte I, Esteban I, Rocha-Gracia R, Zarazaga M, Torres C 2010 Outbreak caused by a multi-resistant Klebsiella pneumoniae strain of new sequence type ST341 carrying new genetic environments of aac(6')-Ib-cr and qnrS1 genes in a neonatal intensive care unit in Spain. Int J Med Microbiol 300:464-469.

154. Martin RM, Cao J, Brisse S, Passet V, Wu W, Zhao L, Malani PN, Rao K, Bachman MA 2016 Molecular Epidemiology of Colonizing and Infecting Isolates of Klebsiella pneumoniae. mSphere 1.

155. Rettedal S, Lohr IH, Bernhoff E, Natas OB, Sundsfjord A, Oymar K 2015 Extended-spectrum beta-lactamase-producing Enterobacteriaceae among pregnant women in Norway: prevalence and maternal-neonatal transmission. J Perinatol 35:907-912.

156. Cassettari VC, da Silveira IR, Dropa M, Lincopan N, Mamizuka EM, Matte MH, Matte GR, Menezes PR 2009 Risk factors for colonisation of newborn infants during an outbreak of extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in an intermediate-risk neonatal unit. J Hosp Infect 71:340-347.

157. Rettedal S, Lohr IH, Natas O, Giske CG, Sundsfjord A, Oymar K 2012 First outbreak of extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in a Norwegian neonatal intensive care unit; associated with contaminated breast milk and resolved by strict cohorting. APMIS 120:612-621.

158. Bulabula ANH, Dramowski A, Mehtar S 2017 Maternal colonization or infection with extended-spectrum beta-lactamase-producing Enterobacteriaceae in Africa: A systematic review and meta-analysis. Int J Infect Dis 64:58-66.

159. Denkel LA, Schwab F, Kola A, Leistner R, Garten L, von Weizsacker K, Geffers C, Gastmeier P, Piening B 2014 The mother as most important risk factor for colonization of very low birth weight (VLBW) infants with extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E). J Antimicrob Chemother 69:2230-2237.

160. Cagan E, Kiray Bas E, Asker HS 2017 Use of Colistin in a Neonatal Intensive Care Unit: A Cohort Study of 65 Patients. Med Sci Monit 23:548-554.
161. Al-Lawama M, Aljbour H, Tanash A, Badran E 2016 Intravenous Colistin in the treatment of multidrug-resistant Acinetobacter in neonates. Ann Clin Microbiol Antimicrob 15:8.
162. Tzialla C, Borghesi A, Serra G, Stronati M, Corsello G 2015 Antimicrobial therapy in neonatal intensive care unit. Ital J Pediatr 41:27.
163. Iosifidis E, Chorafa E, Agakidou E, Kontou A, Violaki A, Volakli E, Christou EI, Zarras C, Drossou-Agakidou V, Sdougka M, Roilides E 2019 Use of Ceftazidime-avibactam for the Treatment of Extensively drug-resistant or Pan drug-resistant Klebsiella pneumoniae in Neonates and Children <5 Years of Age. Pediatr Infect Dis J 38:812-815.
164. Rodriguez BA, Girotto JE, Nicolau DP 2018 Ceftazidime/Avibactam and Ceftolozane/Tazobactam: Novel Therapy for Multidrug Resistant Gram Negative Infections in Children. Curr Pediatr Rev 14:97-109.
165. Gastmeier P, Loui A, Stamm-Balderjahn S, Hansen S, Zuschneid I, Sohr D, Behnke M, Obladen M, Vonberg RP, Ruden H 2007 Outbreaks in neonatal intensive care units - they are not like others. Am J Infect Control 35:172-176.
166. Prevention CfDCa 2006 Management of Multidrug-Resistant Organisms in Healthcare Settings
167. Riley LW 2014 Pandemic lineages of extraintestinal pathogenic Escherichia coli. Clin Microbiol Infect 20:380-390.
168. Zaidi AK, Huskins WC, Thaver D, Bhutta ZA, Abbas Z, Goldmann DA 2005 Hospital-acquired neonatal infections in developing countries. Lancet 365:1175-1188.
169. Amaya E, Caceres M, Fanc H, Torres Ramirez A, Palmgren AC, Nord CE, Weintraub A 2010 Antibiotic resistance patterns in gram-negative and gram-positive bacteria causing septicemia in newborns in Leon, Nicaragua: correlation with environmental samples. J Chemother 22:25-29.
170. Desimoni MC, Esquivel GP, Merino LA 2004 [Fecal colonization by extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in a neonatal intensive care unit]. Enferm Infecc Microbiol Clin 22:507-511.
171. Escobar Perez JA, Olarte Escobar NM, Castro-Cardozo B, Valderrama Marquez IA, Garzon Aguilar MI, Martinez de la Barrera L, Barrero Barreto ER, Marquez-

Ortiz RA, Moncada Guayazan MV, Vanegas Gomez N 2013 Outbreak of NDM-1-producing Klebsiella pneumoniae in a neonatal unit in Colombia. Antimicrob Agents Chemother 57:1957-1960.

172. Adeniji F 2017 Global analysis of strategies to tackle antimicrobial resistance. Int J Pharm Pract.
173. Molstad S, Lofmark S, Carlin K, Erntell M, Aspevall O, Blad L, Hanberger H, Hedin K, Hellman J, Norman C, Skoog G, Stalsby-Lundborg C, Tegmark Wisell K, Ahren C, Cars O 2017 Lessons learnt during 20 years of the Swedish strategic programme against antibiotic resistance. Bull World Health Organ 95:764-773.
174. Norman M, Kallen K, Wahlstrom E, Hakansson S, Collaboration SNQ 2019 The Swedish Neonatal Quality Register - contents, completeness and validity. Acta Paediatr 108:1411-1418.
175. Kjellander C, Bjorkholm M, Cherif H, Kalin M, Giske CG 2012 Hematological: Low all-cause mortality and low occurrence of antimicrobial resistance in hematological patients with bacteremia receiving no antibacterial prophylaxis: a single-center study. Eur J Haematol 88:422-430.
176. EUCAST 2013 Breakpoint tables for interpretation of MICs and zone diameters. Version 3.1, valid from 2013-02-11.
177. Birkett CI, Ludlam HA, Woodford N, Brown DF, Brown NM, Roberts MT, Milner N, Curran MD 2007 Real-time TaqMan PCR for rapid detection and typing of genes encoding CTX-M extended-spectrum beta-lactamases. J Med Microbiol 56:52-55.
178. Brolund A, Haeggman S, Edquist PJ, Gezelius L, Olsson-Liljequist B, Wisell KT, Giske CG 2010 The DiversiLab system versus pulsed-field gel electrophoresis: characterisation of extended spectrum beta-lactamase producing Escherichia coli and Klebsiella pneumoniae. J Microbiol Methods 83:224-230.
179. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV 2012 Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640-2644.
180. Carattoli A, Zankari E, Garcia-Fernandez A, Voldby Larsen M, Lund O, Villa L, Moller Aarestrup F, Hasman H 2014 In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 58:3895-3903.

181. Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM, Aarestrup FM 2014 Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic Escherichia coli. J Clin Microbiol 52:1501-1510.
182. Froding I, Hasan B, Sylvin I, Coorens M, Naucler P, Giske CG 2020 Extended-Spectrum beta-Lactamase- and plasmid AmpC- producing Escherichia coli causing community-onset bloodstream infection: the association of bacterial clones and virulence genes with septic shock, source of infection and recurrencies. Antimicrob Agents Chemother.
183. Persson F, Tegenfeldt JO 2010 DNA in nanochannels--directly visualizing genomic information. Chem Soc Rev 39:985-999.
184. Nordberg V, Quizhpe Peralta A, Galindo T, Turlej-Rogacka A, Iversen A, Giske CG, Naver L 2013 High proportion of intestinal colonization with successful epidemic clones of ESBL-producing Enterobacteriaceae in a neonatal intensive care unit in Ecuador. PLoS One 8:e76597.
185. Nordberg V, Jonsson K, Giske CG, Iversen A, Aspevall O, Jonsson B, Camporeale A, Norman M, Naver L 2018 Neonatal intestinal colonization with extended-spectrum beta-lactamase-producing Enterobacteriaceae-a 5-year follow-up study. Clin Microbiol Infect.
186. Bikkarolla SK, Nordberg V, Rajer F, Muller V, Kabir MH, Kk S, Dvirnas A, Ambjornsson T, Giske CG, Naver L, Sandegren L, Westerlund F 2019 Optical DNA Mapping Combined with Cas9-Targeted Resistance Gene Identification for Rapid Tracking of Resistance Plasmids in a Neonatal Intensive Care Unit Outbreak. mBio 10.
187. Socialstyrelsen 2011 Smittspridning inom svensk neonatal-sjukvård. Probleminventering och åtgärdsförslag. Stockholm, Sweden: Stockholm; 2011.Available at:www.socialstyrelsen.se (accessed 25 May 2011).
188. Benenson S, Levin PD, Block C, Adler A, Ergaz Z, Peleg O, Minster N, Gross I, Schaffer K, Moses AE, Cohen MJ 2013 Continuous surveillance to reduce extended-spectrum beta-lactamase Klebsiella pneumoniae colonization in the neonatal intensive care unit. Neonatology 103:155-160.
189. Demir S, Soysal A, Bakir M, Kaufmann ME, Yagci A 2008 Extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in paediatric wards: a nested case-control study. J Paediatr Child Health 44:548-553.

190. Duman M, Abacioglu H, Karaman M, Duman N, Ozkan H 2005 Beta-lactam antibiotic resistance in aerobic commensal fecal flora of newborns. Pediatr Int 47:267-273.
191. Njom Nlend AE, Motaze ACN, Sandie A, Fokam J 2018 HIV-1 transmission and survival according to feeding options in infants born to HIV-infected women in Yaounde, Cameroon. BMC Pediatr 18:69.
192. Dubois V, De Barbeyrac B, Rogues AM, Arpin C, Coulange L, Andre C, M'Zali F, Megraud F, Quentin C 2010 CTX-M-producing Escherichia coli in a maternity ward: a likely community importation and evidence of mother-to-neonate transmission. J Antimicrob Chemother 65:1368-1371.
193. Rybczynska H, Melander E, Johansson H, Lundberg F 2014 Efficacy of a once-a-week screening programme to control extended-spectrum beta-lactamase-producing bacteria in a neonatal intensive care unit. Scand J Infect Dis 46:426-432.
194. Eggesbo M, Moen B, Peddada S, Baird D, Rugtveit J, Midtvedt T, Bushel PR, Sekelja M, Rudi K 2011 Development of gut microbiota in infants not exposed to medical interventions. APMIS 119:17-35.
195. Lohr IH, Rettedal S, Natas OB, Naseer U, Oymar K, Sundsfjord A 2013 Long-term faecal carriage in infants and intra-household transmission of CTX-M-15-producing Klebsiella pneumoniae following a nosocomial outbreak. J Antimicrob Chemother 68:1043-1048.
196. ReAct 2018 React Latinoamérica, Catálogo de Publicaciones 2018.
197. ReAct 2016 React Latinoamérica, Taller International, Investigación y formación de talentos humanos en uso racional y resistencia a los antibióticos, Ambato-Ecuador, June 2016.
198. Donati V, Feltrin F, Hendriksen RS, Svendsen CA, Cordaro G, Garcia-Fernandez A, Lorenzetti S, Lorenzetti R, Battisti A, Franco A 2014 Extended-spectrum-beta-lactamases, AmpC beta-lactamases and plasmid mediated quinolone resistance in klebsiella spp. from companion animals in Italy. PLoS One 9:e90564.
199. Frasson I, Lavezzo E, Franchin E, Toppo S, Barzon L, Cavallaro A, Richter SN, Palu G 2012 Antimicrobial treatment and containment measures for an extremely drug-resistant Klebsiella pneumoniae ST101 isolate carrying

pKPN101-IT, a novel fully sequenced bla(KPC-2) plasmid. J Clin Microbiol 50:3768-3772.

200. Loucif L, Kassah-Laouar A, Saidi M, Messala A, Chelaghma W, Rolain JM 2016 Outbreak of OXA-48-Producing Klebsiella pneumoniae Involving a Sequence Type 101 Clone in Batna University Hospital, Algeria. Antimicrob Agents Chemother 60:7494-7497.

201. Forde BM, Zowawi HM, Harris PNA, Roberts L, Ibrahim E, Shaikh N, Deshmukh A, Sid Ahmed MA, Al Maslamani M, Cottrell K, Trembizki E, Sundac L, Yu HH, Li J, Schembri MA, Whiley DM, Paterson DL, Beatson SA 2018 Discovery of mcr-1-Mediated Colistin Resistance in a Highly Virulent Escherichia coli Lineage. mSphere 3.

202. Lin YL, Sewunet T, Kk S, Giske CG, Westerlund F 2020 Optical maps of plasmids as a proxy for clonal spread of MDR bacteria: a case study of an outbreak in a rural Ethiopian hospital. J Antimicrob Chemother.

203. Muller V, Nyblom M, Johnning A, Wrande M, Dvirnas A, Kk S, Giske CG, Ambjornsson T, Sandegren L, Kristiansson E, Westerlund F 2020 Cultivation-Free Typing of Bacteria Using Optical DNA Mapping. ACS Infect Dis 6:1076-1084.

204. Vergnano S, Sharland M, Kazembe P, Mwansambo C, Heath PT 2005 Neonatal sepsis: an international perspective. Arch Dis Child Fetal Neonatal Ed 90:F220-224.

205. Murki S, Jonnala S, Mohammed F, Reddy A 2010 Restriction of cephalosporins and control of extended spectrum beta-lactamase producing gram negative bacteria in a neonatal intensive care unit. Indian Pediatr 47:785-788.

206. Tsai MH, Chu SM, Hsu JF, Lien R, Huang HR, Chiang MC, Fu RH, Lee CW, Huang YC 2014 Risk factors and outcomes for multidrug-resistant Gram-negative bacteremia in the NICU. Pediatrics 133:e322-329.

207. Drageset M, Fjalstad JW, Mortensen S, Klingenberg C 2017 Management of early-onset neonatal sepsis differs in the north and south of Scandinavia. Acta Paediatr 106:375-381.

208. Ting JY, Synnes A, Roberts A, Deshpandey A, Dow K, Yoon EW, Lee KS, Dobson S, Lee SK, Shah PS, Canadian Neonatal Network I 2016 Association Between Antibiotic Use and Neonatal Mortality and Morbidities in Very Low-Birth-

Weight Infants Without Culture-Proven Sepsis or Necrotizing Enterocolitis. JAMA Pediatr 170:1181-1187.

209. Cantey JB, Baird SD 2017 Ending the Culture of Culture-Negative Sepsis in the Neonatal ICU. Pediatrics 140.

210. Kaarme J, Riedel H, Schaal W, Yin H, Neveus T, Melhus A 2018 Rapid Increase in Carriage Rates of Enterobacteriaceae Producing Extended-Spectrum beta-Lactamases in Healthy Preschool Children, Sweden. Emerg Infect Dis 24:1874-1881.

211. Woerther PL, Andremont A, Kantele A 2017 Travel-acquired ESBL-producing Enterobacteriaceae: impact of colonization at individual and community level. J Travel Med 24:S29-S34.

212. Bion J, Richardson A, Hibbert P, Beer J, Abrusci T, McCutcheon M, Cassidy J, Eddleston J, Gunning K, Bellingan G, Patten M, Harrison D, Matching Michigan C, Writing C 2013 'Matching Michigan': a 2-year stepped interventional programme to minimise central venous catheter-blood stream infections in intensive care units in England. BMJ Qual Saf 22:110-123.

213. Bersani I, Speer CP 2012 Nosocomial sepsis in neonatal intensive care: inevitable or preventable? Z Geburtshilfe Neonatol 216:186-190.

214. Bjorkman L, Ohlin A 2015 Scrubbing the hub of intravenous catheters with an alcohol wipe for 15 sec reduced neonatal sepsis. Acta Paediatr 104:232-236.

215. Kidszun A, Klein L, Winter J, Schmeh I, Grondahl B, Gehring S, Knuf M, Weise K, Mildenberger E 2017 Viral Infections in Neonates with Suspected Late-Onset Bacterial Sepsis-A Prospective Cohort Study. Am J Perinatol 34:1-7.

216 Redman M, King A, Watson C, et Al 2016 What is CRISP/Cas 9, Arch Dis Child Educ Pract Ed 2016; **101** 213-215