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A world inside

Gastrointestinal microbiota in healthy Swedish children at day care centers and aspects on antibiotic resistance, enteric pathogens and transmission

JOHAN KAARME

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Abstract
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Antibiotic resistance is a growing threat to human health and is defined by the World Health Organization as a crisis that must be managed with the utmost urgency. Antibiotic resistant bacteria increase both mortality and morbidity and have a great impact on the global economy. Resistance is not confined to human health care, but is present also among animals and in our environment at large. Indeed, resistant bacterial strains have now been found in virtually all parts of the world, even in locations without direct human contact.

The human gastrointestinal tract is populated by a complex, dynamic, diverse and highly interactive collection of microorganisms, including bacteria, archaea, fungi, yeasts and viruses, which constitutes our gastrointestinal microbiota. This microbiota is an important reservoir of resistance genes (our gastrointestinal *resistome*) and a "melting pot" for transfer of resistance genes between microbes, including potential pathogens.

In this thesis I investigated the prevalences of two clinically important kinds of antibiotic resistance: extended-spectrum β-lactamases (ESBL) and vancomycin-resistant enterococci (VRE), as well as asymptomatic carriage of potential enteropathogens among healthy preschool children in Uppsala. Fecal samples from unidentified, individual diapers were collected in 2010 (125+313 samples) and in 2016 (334 samples). In addition, 204 environmental samples from the children's preschools were collected in autumn 2016.

A prevalence of 2.9% ESBL-producing *Enterobactericeae* was demonstrated in the first samples from 2010. No VRE were found and the occurrence of enteropathogens were reassuringly low. Results on ESBL prevalence in 2016 and transmission of resistance between children will be presented when the manuscript is published and at the dissertation.

Keywords: ESBL, VRE, enteropathogens, antibiotic resistance, children, preschool

Johan Kaarme, Department of Women's and Children's Health, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

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Allt är möjligt

To my magnificent family

Populärvetenskaplig sammanfattning

Antibiotikaresistens, alltså när bakterier blivit motståndskraftiga mot antibiotika, är ett ökande problem i hela världen och betecknas av WHO som ett av de främsta hoten mot vår hälsa. Resistenta bakterier orsakar varje år miljontals dödsfall runtom i världen på grund av att infektioner inte går att behandla. De kan dessutom göra så att sjukdomar får ett svårare förlopp och innebär kraftigt ökade sjukvårdskostnader. I Norden har vi länge haft en jämförelsevis låg förekomst av antibiotikaresistens, även om vi under 2000-talet sett flera utbrott på sjukhus och i samhället.

Många bakterier har en inbyggd motståndskraft mot vissa typer av antibiotika. Problemet uppstår främst då tidigare känsliga bakterier skaffar sig ny resistens och därför inte längre går att behandla med vanliga antibiotika.

Antibiotikaresistens är inte bara en företeelse inom hälso- och sjukvården, utan resistenta bakterier finns även i omgivningen, till exempel bland djur och i vår mat. Detta beror sannolikt på en rad faktorer, där felaktigt användande av antibiotika bland människor och i djurhushållningen, bristande vattenrening och ökat resande är några. Under senare år har forskare påvisat resistenta bakterier i praktiskt taget alla delar av världen, även långt från bebodda platser, som på Arktis och Antarktis. För att stoppa den här utvecklingen behövs samordnade politiska åtgärder och forskningssamarbete mellan många olika vetenskapsområden.

Varje människa bär på drygt 1 kg bakterier i sina tarmar. Dessa bakterier fyller en rad viktiga funktioner för ämnesomsättningen, immunförsvaret, tillväxten, utvecklingen och för vår fysiska och psykiska hälsa i stort. I vår magtarmkanal finns vid sidan av bakterier även en stor mängd virus, svamp och andra mikroorganismer, vilka tillsammans bildar vår *mikrobiota,* en komplex, föränderlig och mycket interaktiv mikromiljö. Tarmen är med sin jämna temperatur, där en ständig tillgång till näringsämnen och en enorm mängd mikroorganismer finns, en smältdegel för uppkomst och spridning av antibiotikaresistens. Då balansen i tarmen rubbas – till exempel under en antibiotikakur – ökar risken för att resistenta bakterier ska växa till. Barns mikrobiota skiljer sig på flera sätt från vuxnas och störs lättare.

I min avhandling har jag studerat hur vanligt det är att friska förskolebarn i Uppsala bär på två viktiga typer av antibiotikaresistens, extended-spectrum β-lactamase (ESBL; betalaktamas med utvidgat spektrum) och vancomycin-resistenta enterokocker (VRE) samt hur vanligt det är att friska barn bär på potentiellt skadliga virus, bakterier och parasiter.

ESBL är grupp ämnen (enzymer) som bildas av bakterier och bryter ner ett antal viktiga antibiotikatyper så att dessa blir verkningslösa. Dessa ämnen sprids ofta bland tarmbakterier, vanligen bland bakteriefamiljen *Enterobactericeae*, förekommer i hela världen och är den vanligaste och snabbast växande resistensen i Sverige.

VRE innebär att ett slags vanligt förekommande tarmbakterier, enterokocker, har blivit resistenta mot vancomycin, ett antibiotikum som används vid flera allvarliga infektioner. VRE är mindre vanligt i Sverige än ESBL, men har under 2000-talet orsakat ett rad utbrott av svårbehandlade infektioner och även dödsfall på sjukhus i Sverige. Såväl *Enterobactericeae* som enterokocker förekommer normalt i våra tarmar men bär då vanligen varken på ESBL eller vancomycinresistens.

Magsjuka är ett välkänt tillstånd bland småbarnsfamiljer och kan orsakas av flera olika virus, parasiter och bakterier.

I mitt arbete har jag under 2010 och 2016 samlat in anonyma avföringsprover via blöjor från friska förskolebarn på kommunala förskolor i Uppsala. 2016 kompletterades insamlingen även med prover från förskolemiljön. I fyra delarbeten har jag tillsammans med forskarkollegor studerat förekomsten av ESBL, VRE och magsjukeorsakande mikroorganismer. Vi har också undersökt om förskolebarn sprider resistenta tarmbakterier mellan varandra på förskolorna.

I den första studien från 2010 kunde vi konstatera att 2,9% av barnen bar på ESBL. Siffran var som förväntat lägre än i många andra delar av världen. Vi kunde emellertid också visa att ett par av barnen sannolikt spridit resistens mellan sig på två av förskolorna.

I studie II konstaterade vi att inget av barnen bar på VRE.

Under studie III jämfördes försommar- och höstprover från 2010 avseende 21 olika magsjukeorganismer. Glädjande nog visade sig förekomsten vara mycket låg.

Under hösten 2016 upprepade jag undersökningen för att se vad som hänt med förekomsten av ESBL under åren som gått. Den här gången samlade jag in både blöjor och prov från förskolemiljön (toaletter, handfat, lunchbord), samt

gjorde en mer djupgående analys av exakt vilka mikroorganismer det var som spridits. Vi fann en oroväckande ökning av ESBL, som nu fanns i hela 20,1% av proverna. Vi kunde även se att de resistenta bakterierna i många fall hade spridits inom de enskilda förskolorna. Förekomsten av resistens var inte kopplad till några speciella delar av Uppsala, utan i princip hela staden hade förskolor med hög mängd ESBL. Vi kunde heller inte se några tydliga tecken på att förskolorna inte skulle följa hygienreglerna. Spridningen sker sannolikt mellan barnen.

Konsekvenserna av fynden återstår att se, men det finns en klar risk för att vi framöver kommer att se fler barn än idag bli sjuka av ESBL-bärande tarmbakterier.

Mer forskning behövs för att se om den höga förekomsten av ESBL bland förskolebarn kan ses även utanför Uppsala. Vi behöver också veta hur länge barnen kan bära på de resistenta bakterierna och hur stor risken är att de blir sjuka av dem.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Kaarme** J, Molin Y, Olsen B, Melhus A. Prevalence of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in healthy Swedish preschool children. Acta Paediatr. 2013;102(6):655-60.

II. **Kaarme** J, Hasan B, Rashid M, Olsen B. Zero prevalence of vancomycin-resistant enterococci among Swedish preschool children. Microb Drug Resist. 2015;21(1):65-8.

III. **Kaarme** J, Hickman RA, Nevéus T, Blomberg J, Öhrmalm C. Reassuringly low carriage of enteropathogens among healthy Swedish children in day care centres. Public Health. 2016;

IV. **Kaarme** J, Riedel H, Nevéus T, Melhus A. Increase in carriage rates of cefotaxime-resistant *Enterobactericeae* in healthy Swedish children. (manuscript)

Contents

List of abbreviations and glossary

AmpC	Ampicillinase C; a type of β-lactamase (enzyme group)
pAmpC	Plasmid mediated Ampicillinase C
AP-PCR	Arbitrary primer polymerase chain reaction
Carbapenemas	β-lactamase that hydrolyse carbapenems (enzyme group)
CITM	An AmpC β-lactamase subtype
CLED	Cysteine-lactose-electrolyte-deficient (agar plate)
CMY	An AmpC β-lactamase subtype
CNS	Central nervous system
CTX-M	Cefotaximase-München (enzyme subgroup)
DCC	Day care center
DDD	Defined daily dose (of antibiotics)
DHAM	An AmpC β-lactamase subtype
Dysbiosis	Microbial imbalance (in the gastrointestinal tract)
EARS-NET	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended spectrum β-lactamase (enzyme group)
ETEC	Enterotoxigenic *Escherichia coli*
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
EUR	Euro
Eubiosis	Microbial balance (in the gastrointestinal tract)
GALT	Gut-associated lymphoid tissue
GI tract	Gastrointestinal tract
ICU	Intensive care unit
In silico	By computer modeling
MALDI-TOF	Matrix-assisted Laser Desorption/Ionization Time of Flight

MFI	Median fluorescence intensity
MLST	Multilocus sequence typing
Multiplex-PCR	PCR for simultaneous amplification of several different DNA sequences with multiple primers
OXA	An AmpC β-lactamase subtype
PCR	Polymerase chain reaction
Plasmid	Extra-chromosomal, double stranded DNA, which replicates independently
Probiotics	Live organism which when administered in adequate amounts confer a health benefit on the host[1]
Resistome	The sum of the microbial resistance genes (in the gastrointestinal tract)
SCFA	Short chain fatty acids
SHV	Sulfhydryl variable β-lactamase (enzyme subgroup)
STEC	Shiga toxin-producing *Escherichia coli*
STRAMA	The Swedish Strategic Programme Against Antibiotic Resistance
TEM	Temoneira β-lactamase (enzyme subgroup)
UTI	Urinary tract infection
VRE	Vancomycin resistant enterococcus/enterococci
WGS	Whole genome sequencing
WHO	The World Health Organization

Introduction

A short introduction to the human gastrointestinal microbiota

The human gastrointestinal (GI) tract is populated by a complex, dynamic, diverse and highly interactive collection of microorganisms. This dense population of bacteria, archaea, fungi, yeasts and viruses constitutes our gastrointestinal microbiota. The microbiota is an important reservoir of resistance genes (the gastrointestinal *resistome*) and serves as a "melting pot" for transfer of resistance genes between microbes as well as a probable reservoir for potential pathogens. The human gut offers nutrients, a stable temperature and an abundance of microbes to interact with. Antibiotic resistance can develop not only through colonization and emergence of resistant bacterial strains, but also by horizontal transfer of resistance genes between bacteria or even between microbes of different kingdoms. The emergence of antibiotic resistance in the gastrointestinal tract is greatly influenced by external factors, such as the surrounding environment and food habits, and of course antibiotic usage. The concept of "One health", where humans, animals and environmental factors are seen as one connected and interactive entity, is well illustrated by the function of the human microbiota.

The importance of the microbiota for the outcome of health and disease has gained increasing attention in research ever since the Nobel laureate Elie Metcnikoff first drew attention to the human GI microbiota in the beginning of the 20th century, with the hypothesis of beneficial microbes and probiotics. The microbiota has digestive, metabolic and trophic functions, and is necessary for the development of the immune system and even for the neuronal development of the host. For many years most attention has been given the bacteria; it is, however, likely that a broader perspective is needed, since viruses, yeasts, fungi and protozoa also influence the balance, function and resistome of the microbiota[2-4].

The composition of the microbiota, its role in the spread of antibiotic resistance and in asymptomatic carriage of pathogens seems to differ considerably between young children and adults. Using more advanced techniques it has become evident that the habitat in our GI tract is far more complex and interactive than previously known. The functions and changes of one species

have implications on the whole system. So far, most studies have focused on adults in health care settings and less is known about healthy children in the community.

In order to understand the colonization pattern in the GI tract, the carriage rate of potential pathogens, the intestinal prevalence of antibiotic resistance and variations between both children and adults, individuals and geographical areas, one must understand the characteristics of the human microbiota and what affects it.

Microbiota- more than just bacteria

Using a simplified illustration, the human intestinal microbiota could be compared to a forest with trees, bushes, and undergrowth symbolizing the different microbes. A forest does not only consist of trees, just as the microbiota does not only consist of bacteria (hence the change from "micro*flora*" to "micro*biota*"). Nor does the forest exist as an autonomous unit, but it interacts with the environment, just like the microbiota interacts with the human body and the external environment. Changes in the surroundings have direct implications on the forest/microbiota as do changes in the composition of the different entities. If the balance is disturbed, if for example a certain species of tree disappears, it is likely that another species will take its place. It does not even have to be a tree, but will more probably be the specimen that grows fastest or has the best conditions for occupying the vacated space. Changes in the soil, air or surroundings affect the different species in different ways. The point is that both habitats have to be looked upon as highly interactive, adaptive and constantly evolving systems.

Even though >99% of the mapped DNA sequencing readings from the GI tract derive from bacteria[3,5], the intestinal genome also consists of viruses, fungi, archaea and protozoa[1], which all have impact on the eubiosis (ie the intestinal microbial balance), functions, resistance prevalence and development of different diseases[2-3,6]. One example of interaction between microbes from different kingdoms is the protective effect of probiotics on rotavirus-associated gastroenteritis[6].

Our understanding of the microbiota is increasing rapidly, through individual research, more advanced techniques as well as joint projects, such as the International Human Microbiome Consortium, the Human Microbiome Project (www.hmpdacc.org) and the European Commission's Metagenomics of the Human Intestinal Tract Project (www.metahit.eu).

Mapping of the intestinal bacteria has long been facilitated by using 16S ribosomal RNA gene sequencing or metagenomic analysis. New techniques have now demonstrated that among others picobirnaviruses, adenoviruses,

anelloviruses, astroviruses, bocaviruses, rotaviruses, sapoviruses and enteroviruses are also commonly present in feces from healthy children[3]. Each individual seems to harbor up to 1500 different virotypes in the GI tract, with a notable variation in composition due to age and diet[4]. Some of these viruses have pathogenic potential and their role in the GI tract is yet to be determined.

Even though it lies beyond the direct focus of this thesis, the bacteriophages are of particular interest. Bacteriophages are at least as numerous as the bacteria and most likely of great importance in the balance of the bacterial composition in the gut, transfer of genes (for example antibiotic resistance or other mechanisms of adaptive advantage) and for modification of the functionality of the microbiome[4,7]. There are hypotheses that in the future phages could perhaps also be used as anti-bacterial agents beside antibiotics, although their safety has to be defined[4].

Adlerberth and Wold noted already in 2009 that as many as approximately 20% of healthy adults carry *Candida* spp. and 35% of the study population other yeasts in the GI tract[8]. In a more recent study fungal gene fragments were detected in 100% of the patient fecal samples by using pyrosequencing[3].

Functions of the microbiota

Much attention has hitherto been drawn to the (bacterial) composition of the microbiota. The bacterial population interacts closely with the other kingdoms and overlapping functions can be carried out by different microbes. Function and diversity is probably far more important[5,9] than individual microbial composition.

Five main functions of the microbiota have so far been identified:

1. Metabolic[1,6,10-11]

- Fermentation of non-digestible carbohydrates and production of short-chain fatty acids (SCFA), which are locally used as energy, affect colonic pH, promote growth of the epithelium, inhibit pathogen colonization and activate the immune system
- Salvage of calories
- Production of arginine and glutamine
- Synthesis of vitamins B_1, B_{12}, K_1, K_2 and folic acid
- Absorption of calcium, magnesium and iron
- Deconjugation of bile

2. *Trophic*[10]
    - Epithelial cell proliferation and differentiation

3. *Development and differentiation of the immune system*[6,10,12]
    - Induction of regulatory T-cells and gut-associated lymphoid tissue (GALT)
    - Stimulation of immunoglobulin production (locally and systemically)
    - Development of oral tolerance
    - Tightening of epithelial junctions

4. *Protection*[8,13]
    - Barrier effect and colonization resistance (ie present microbiota inhibits novel colonization of pathogens)
    - Activation and direction of the immune system towards pathogens
    - Production of bacteriocins
    - Promotion of peristalsis

5. *Neuronal development*[14-15]
    - Modulation and maturation of the brain-gut axis and production of neurotransmitters and neuromodulators
    - Promotion of CNS development

One additional example of the systemic effect of the microbiota on the human body is translocation. Translocation of bacteria from the gut to the bloodstream is an obvious and feared cause of septicemia, but according to a groundbreaking, although somewhat controversial, hypothesis the regular translocation of small quantities of bacteria exerts a boosting effect on the systemic immune system[6,8,16].

Establishment and development of the microbiota

The microbiota and the resistome of infants and young children seem to differ from, and exhibit a higher degree of variability than, those of the adult[17]. Furthermore, like fingerprints, the microbiota seems to be highly individual, with no two persons sharing the exact same composition[18]. The microbial composition changes over time and a more stable situation is established only after the age of three[19] or even later[20]. Early adverse influences on the microbiota can lead to the development of diseases later in life[20-21], so the first years of life and perhaps even the intrauterine period may constitute a "window of opportunity" for preventive interventions. Clearly, we need a better understanding of the pediatric microbiota.

The newborn's microbiota is characterized by the domination of facultative bacteria and aerobes, which constitute less than 1% in adults. A large number of staphylococci, streptococci and yeasts are present during the first weeks[8]. As the facultative anaerobic population expands, oxygen is consumed and more anaerobic species are established in the gut. Since most potentially pathogenic bacteria are facultatives, this developing domination of obligate anaerobes provides protection against pathogenic colonization and is a good example of "colonization resistance".

Factors known to influence the microbiota in newborns and infants are listed below[6,9,10,12,16,18,19,21-23].

- Gestational age
- Mode of delivery (caesarean section alters the microbial composition up to 7 years)[16]
- Perinatal or postnatal antibiotic exposure
- Disease (especially viral gastroenteritis)
- Hygiene and environmental factors
- Breastfeeding/formula
- Maternal diet
- Siblings and pets
- Geography and lifestyle

Antibiotic resistance and human health

The rapid emergence of antibiotic resistance is considered one of the main threats to global public health by the World Health Organization (WHO) and a crisis that must be managed with the utmost urgency, as it increases mortality and morbidity worldwide and leads to a drain on the global economy (Figure 1)[24].

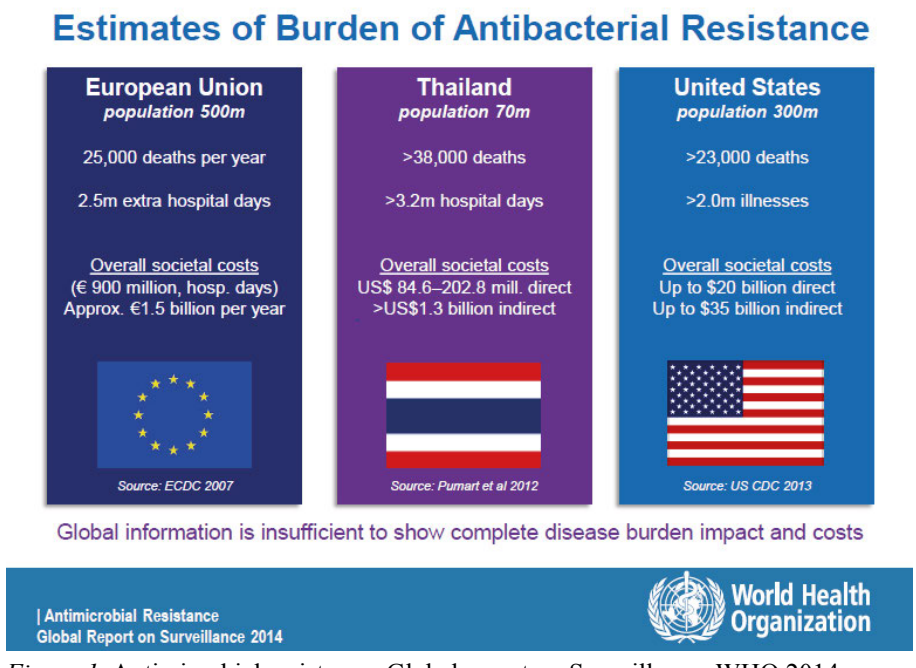


Figure 1. Antimicrobial resistance, Global report on Surveillance, WHO 2014

Antibiotic resistance is now dispersed over the entire globe and resistant bacterial strains have been found even in remote locations without direct exposure to antibiotics, such as in Arctic birds[25] or water in the Antarctic[26].

Antibiotic resistance causes millions of deaths annually, due to lack of effective drugs, and billions in costs in global health expenses. In Sweden alone the extra costs related to antibiotic resistance until 2024 are estimated to almost 0.5 billion EUR, provided that the current, comparably low prevalence of resistance remains[27]. Should the prevalence equal that of Southern Europe, the sum would end at 2.8 billion EUR, or even more if global prevalences are reached.

A "One health" approach is needed to address this threat. The WHO has listed five main objectives in the *Global action plan on Antimicrobial resistance*, adopted at the World Health Assembly in May 2015:

- Improve awareness and understanding
- Strengthen the knowledge through surveillance and research
- Reduce the incidence of infections, focusing on preventive measures
- Optimize the use of antibiotics and antimicrobial agents
- Enable investments and initiatives towards new medicines, diagnostic tools, vaccines etc[24]

The resistome - antibiotic resistance and the gastrointestinal microbiota

The human gut microbiota constitutes one of the most densely populated microbial ecosystems on earth. There are no less than 800-1000 different bacterial species inhabiting our GI tract[23] and about 10 times more microbial cells in the gut lumen than eukaryotic cells in the human body[11]. Gut microbes are an important reservoir of resistance genes and the high density of a variety of microorganisms facilitates transfer of antibiotic resistance between different microbes[28-29]. Recent research indicates that the diversity of this gut resistome has been underestimated and that factors other than exposure to antibiotic treatment also promote and enhance the emergence of resistance[28].

Until recently it was believed that the microbial colonization started at birth and that the intrauterine environment was sterile. As mentioned above, recent studies have on the contrary demonstrated that gut colonization starts already *in utero* as microorganisms have been detected in the amniotic fluid, fetal membranes, umbilical cord, placenta and in the meconium[16,21,29].

Presence of multiple resistance genes have been demonstrated in the GI tract of newborns without prior exposure to antibiotics, and the development of antibiotic resistance in the fetal and infantile gut microbiota seems to be connected to the resistance pattern among maternal and environmental microbes[29-32]. This may indicate that antibiotic resistance can be transferred even before birth (perhaps through translocation of bacteria from the pregnant woman's gut to the fetus). Findings from Zhang *et al*. indicate that the resistome can be significantly amplified within the gut even in the absence of antibiotics[30] and others have demonstrated that resistance genes in the meconium of newborns can persist for several months[29].

Antibiotic treatment in children seems to have a deeper influence on the gastrointestinal eubiosis compared to adults, with longer time of carriage of resistance genes and lesser diversity in GI composition for years in children receiving antibiotic agents[32-33].

Further evidence of transfer of resistance genes to the gut microbiota through food or the environment at large has been presented[23,34] supporting the "One health"-perspective.

Antibiotic resistance can develop in the gut microbiota in three major ways: *first* through selective pressure on commensal bacteria, *second* through horizontal gene transfer between different bacteria and *third* via transfer of resistance genes from viruses (or perhaps other microbes), that infect bacteria.

Many commensal intestinal bacteria harbor natural resistance to different antibiotics. Spread of resistance to former non-resistant strains has a particular clinical relevance, such as extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* or vancomycin-resistant enterococci (VRE).

Even though the focus for long has been on human carriage and spread, both ESBL-producers and VRE are (like methicillin-resistant *Staphylococcus*) present not only among humans, but also in animals, food and the environment, and transmission has been observed between these domains[35]. It is therefore important to obtain a better understanding of all the habitats where these resistance genes exist and how they interact.

Extended-spectrum β-lactamases (ESBL) among *Enterobactericeae*

Definition and classification of ESBL

Extended-spectrum β-lactamases (ESBL) are enzymes that degrade β-lactam antibiotics with extended spectrum (penicillins, cephalosporins and in some cases carbapenems)[36]. ESBL-producing *Enterobacteriaceae* are found among humans, animals, livestock, food and in the environment.

ESBL have so far been found exclusively in gram-negative organisms, primarily among *Enterobactericeae* (such as *Escherichia, Klebsiella, Enterobacter, Citrobacter, Proteus, Shigella, Salmonella, Serratia and Morganella* spp), but also in *Acinetobacter, Burkholderia,* and *Pseudomonas* spp. From a clinical perspective transferable ESBL-resistance among *Enterobactericeae* is of particular interest and focus has usually been on *Escherichia* and *Klebsiella* spp.

The three main ESBL-types are known by the acronyms TEM, SHV and CTX-M[37]. Of these, the CTX-M-type has become the predominant ESBL in humans (especially the CTX-M-15 allele). It is present on every continent and embraces well over 100 different enzymes[38]. The main reason for the rapid spread of ESBL-genes seems to be a highly effective transfer of mobile genetic elements on plasmids[39] (figure 2).

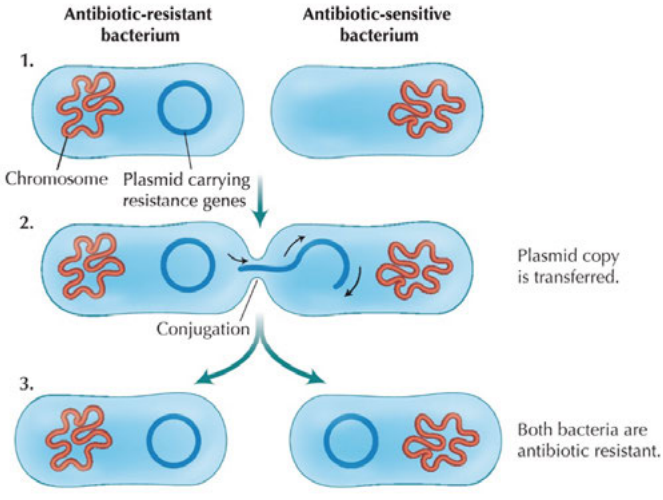


FIGURE 7.19. Lateral transfer of antibiotic resistance via plasmids. (*1*) Antibiotic-resistant and -sensitive bacteria are shown. (*2*) Bacteria "mate" via conjugation, during which a copy of the plasmid carrying antibiotic resistance genes is transferred. (*3*) Both bacteria are now antibiotic resistant.

7.19, adapted from Collignon P.J., *Med. J. Australia* **177:** 325–329, © 2002 Australasian Medical Publishing Co.

Evolution © 2007 Cold Spring Harbor Laboratory Press

Figure 2. Illustration of plasmid mechanism

Historically, two different classification schemes have been used for β-lactamases: the Ambler structural classification and the Bush-Jacoby functional classification, which both have been updated[40-41]. In the older classification schemes some acquired and important β-lactamases were excluded, such as plasmid-borne AmpC and some carbapenemases. In this thesis I have used the definition for extended-spectrum β-lactamases recommended by the Public Health Agency of Sweden, based on the classification proposed by Giske *et al*.[36,42], in concordance with international practice:

1. A β-lactamase with phenotypic resistance to cefotaxime and/or ceftazidime and/or carbapenems (imipenem, meropenem, ertapenem or doripenem)

2. The gene coding for resistance has transferable capacity between different strains of the same species and between different species within the *Enterobactericeae* family

This definition is broader and more clinically orientated and includes "classical ESBL" ($ESBL_A$) as well as plasmid-borne AmpC ($ESBL_M$) and carbapenemases ($ESBL_{CARBA}$):

$ESBL_A$	inhibited by clavulanic acid (ex CTX-M, SHV, TEM)
$ESBL_M$	inhibited by cloxacillin (ex CMY, DHA and some OXA)
$ESBL_{CARBA}$	hydrolytic activity against carbapenems (ex metallo- β-lactamases and OXA-48)

Clinical and economic implications of ESBL

Infections caused by ESBL-producing bacteria are associated with higher morbidity, mortality and healthcare costs than the corresponding non ESBL-producing organisms[43-44]. Especially in neonates and younger children, bloodstream infections caused by ESBL-producing *Enterobacteriaceae* can be fatal[45]. In Europe, >2700 deaths per year and 18 million EUR in additional health care costs are estimated to be due to ESBL-producing bacteria[46].

In addition to resistance to β-lactams, these bacteria often harbor transferable resistance to many other kinds of antibiotics located on transferable plasmids, such as fluoroquinolones and aminoglycosides, which further limits the therapeutic options.

There is today a relatively comprehensive evidence base for therapeutic options in adults against $ESBL_A$. Guidelines are scarce for $ESBL_{CARBA}$, whereas the number of studies is limited and often limited to *in vitro*-observations[36]. Evidence based guidelines for children are lacking.

ESBL in a global perspective

For long it was assumed that the spread of ESBL-producing bacteria in the community was caused by ESBL-producers originating from hospitalized patients. However, with time it has become clear that an independent and rapid dispersal of ESBL-producers is taking place in the community as well[39]. Foreign travel has in numerous studies been demonstrated to constitute an important source of ESBL-producing bacteria[47-49].

There is also evidence that ESBL- and CMY-producers might spread within families[44,50-52] or from farm animals and pets[53-56] and that there is a transfer of community-acquired ESBL-producing strains into hospitals through colonized or infected patients[51,57]. Some researchers have indicated a route of transmission from animals to humans through the food chain[58-59], but these observations have been questioned by de Been *et al*., demonstrating similar, but not identical resistant genes, using whole genome sequencing[60].

Most studies have focused on ESBL-producing bacteria among hospitalized patients and fewer have explored the prevalence of ESBL-producers in community settings and among healthy individuals, especially among children, which can represent a reservoir for ESBL genes.

Studies on fecal carriage of ESBL-producers in healthy adult populations have given widely divergent results, with prevalences ranging from 2.1 to >90%[49,61]. There are clear geographical and enzyme-type differences in prevalences around the world[39,46], but a general increase over time of particularly successful clones[38-39].

In Sweden, infections with ESBL-producing bacteria seem to be most common among older individuals, particularly elderly women[36].

The average carriage rate of ESBL-producers among Swedish healthy adults is estimated to range between 2 and 5%. In a recent, multidisciplinary study, conducted by the Swedish Civil Contingencies Agency, The National Food Agency, the National Veterinary Institute and the Public Health Agency of Sweden, comprising healthy individuals, patients, animals and environment samples, a carriage rate of 4.8% was demonstrated among 2134 participating, healthy individuals[62]. Andersson *et al*. demonstrated an ESBL prevalence of 3% among asymptomatic elderly residents of nursing homes in Sweden in 2012, some of whom carried ESBL resistance genes for more than 2 years[63]. In 2014, the prevalence had increased to 11% among 91 nursing home residents and 8.7% among 69 elderly living independently[64].

Less is known about the duration of intestinal ESBL colonization and its consequences for potential transmission. From travelers' studies the colonization time have been demonstrated to vary from a few percent after 3 months[49] to 25% after six months[65], although Alsterlund has demonstrated the presence of ESBLs in the same individuals for as long as 5 years[46].

Even though the number of studies are significantly lower outside the field of human medicine, intestinal carriage of ESBL-genes seems to be even more common in animals than in humans. 39% of Czech livestock have been demonstrated to harbor ESBL-resistance genes, as well as 63.4% of chickens in Switzerland[35]. In a comprehensive study on 3158 gulls in nine European countries, Stedt *et al.* demonstrated a carriage rate of ESBL ranging from 0 to 74.8% (the corresponding figure for Swedish gulls was 20.7%), with similarity between gull and human enzyme-types[66]. In 2015 only 1% of Swedish samples from pigs and cattle carried ESBL-producing bacteria, while ESBL-producers were detected in 39% of intestinal samples from poultry[67]. Sewage

water and wastewater plants have also been well documented as major reservoirs for ESBL dissemination[68-70] and ESBL have been demonstrated even in water from the Antarctic[26], highlighting the amplitude of antibiotic resistance outside the human medical domain.

ESBL and children

In the first study on healthy community-dwelling children, Pallecchi *et al.* demonstrated an increase from 0.1% to 1.7% in the carriage rate of ESBL-producing *Escherichia coli* in Bolivian and Peruvian children between 2002 and 2005[71]. Soon thereafter Guimarães and co-workers gave a corresponding figure of 2.7% in healthy Portuguese children[72]. Since then studies from the Netherlands, France, Spain, Laos, Lebanon and Germany have yielded carriage rates ranging between 4.6 and 49.6% with the highest prevalence in Laotian preschool children (24.8%) and Lebanese infants (49.6%)[73-80]. Still, the number of studies is limited and the populations are not fully comparable.

Vancomycin-resistant enterococci (VRE)

Definition and classification of VRE

Enterococci (formerly known as group D streptococci) are enteric, facultative anaerobic gram-positive organisms that constitute a natural part of the bacterial flora of humans, animals and insects, and are frequently found in the community[81-82].

The genus *Enterococcus* includes more than 15 species, many of which are harmless commensals in the gastrointestinal microbiota. Only two are responsible for the majority of clinically important infections among humans: *E. faecalis* and *E. faecium*.

The glycopeptide vancomycin has been in clinical use since the 1950s and is one of the most important antibiotics against enterococci, staphylococci and clostridia. Vancomycin interferes with the cell wall synthesis, making the bacterial wall instable and causing lysis.

Vancomycin-resistant enterococci (VRE) carry one of eight identified resistance genes, coding for an enzyme (a ligase, which modifies the bacterial cell wall), rendering the bacteria resistant to vancomycin. These genes are classified by the prefix *van*: *vanA, B, C, D, E, G, L*, and *M*[83]. Bacteria with a *van*-genotype have a different cell wall construction, which makes it more difficult for vancomycin to adhere to the bacteria. Some enterococci, such as

Enterococcus gallinarum, Enterococcus casseliflavus, and *Enterococcus flavescens*, are intrinsically resistant to vancomycin due to the chromosomal presence of *vanC*. The genes *vanA, B, G, L* and *M* can be transferred between bacteria, while *vanC, D*, and *E* are non-transferable.

Following a strict definition, VRE include all enterococci that exhibit resistance to vancomycin, but the term is in practice normally limited to the two genotypes with the highest clinical importance: *vanA* and *vanB* of *Enterococcus faecium* and *Enterococcus faecalis*.

Clinical and economic implications

Enterococci are intrinsically resistant to a variety of antibiotics such as β-lactams, clindamycin, fluoroquinolones, trimethoprim–sulfamethoxazoles and aminoglycosides, and possess a high capability of transfer and acquisition of resistance genes[81].

Another troublesome property of the enterococci is that they easily adhere to foreign materials. They can survive for long periods (>3 months) in the environment outside humans or animals and tolerate desiccation and high temperatures well, which makes them difficult to eradicate and more prone to disperse[83]. Enterococci can colonize intact skin and have been demonstrated to cross-contaminate via the hands of health care staff, despite the use of disposable gloves[84].

Vancomycin-resistant enterococci cause urinary tract infections, wound infections, surgical infections, cholecystitis, endocarditis, bacteremia and sometimes meningitis, and have thus emerged as a growing problem, associated with high morbidity and mortality[85-87]. The ratio of infected to colonized patients in clinical wards has been demonstrated to be 1:10[84], which emphasizes the importance of screening, tracing and preventive measures when VRE is demonstrated in a patient. Health care personnel have been demonstrated to have a higher degree of colonization and are more likely to transfer resistant strains to their household members[81,84,88].

Apart from horizontal gene transfer between enterococci species, transfer of vancomycin resistance from enterococci to the clinically much more feared staphylococci has been confirmed, with the first case described in 2002 in the United States[89].

VRE in a global perspective

The growth promoting glycopeptide avoparcin may induce cross-resistance to vancomycin. The previous use of avoparcin in food additives among livestock

in Europe is suspected to be one of the explanations for the relatively high community prevalence of VRE on the European continent, compared to other parts of the world where VRE is usually more prevalent in health care settings[90-91]. Avoparcin was banned in Sweden in 1986 and in the European Union in 1997, causing a decline in prevalence among humans, but not a disappearance in the environment[92-93].

In most European countries the community prevalence of VRE is estimated to be around a few percent[81,91]. Nursing homes[94-95] and hospitals[96-98] have been pinpointed as hubs for transmission[81].

The exact duration of intestinal carriage depends on factors such as length and frequency of hospital care visits, antibiotic treatment, presence of tubes or catheters, medication, age, and health status[96,99]. Karki *et al.* have demonstrated a high rate of re-colonization in adults after negative screening samples, but indicate that carriage beyond 4 years is unlikely[99]. To the best of my knowledge no comparable studies on children have been published.

As already stated, VRE are not confined to human medicine. The microbes have previously been reported in as much as 40% of Swedish broilers[100], although the occurrence of 11% VRE in Swedish poultry in 2015 (all of which were *E. faecium vanA*) was the lowest, since the screening begun in 2001[67] and in 79% of sludge samples from wastewater sewage plants[101]. In other parts of the world the carriage rate of VRE in the environment is even higher, exemplified by 70% *vanA*-genes in Dutch poultry products[102] and an older study from England which revealed that 90% of all uncooked chicken specimens contained VRE[103]. The risk of transmission of VRE to humans from food, water or the environment is still debated, but may constitute a growing problem[35,67,104].

VRE and children

There are very few studies on the prevalence of VRE among healthy, community-dwelling children. In a study on twins from the US, Gurnée *et al.* found a very low prevalence[105], while VRE was detected in 11.6% of fecal samples from healthy Portuguese children in 2009[72].

Enteric pathogens in preschool children

Preschools and child day care centres (DCCs) represent a high risk environment for infections. Studies have demonstrated an up to sevenfold increased risk for infectious diseases among young children in DCCs[106] and a threefold

risk for gastroenteritis[107] compared to children who are cared for in their homes.

Asymptomatic carriage of potential enteric pathogens among young children is more common than among adults, and preschool children may thus act as a reservoir for spread of disease to other children, families and to the community in general[107-109]. This is of particular interest in environments such as DCCs where many children congregate.

There are still comparably few studies using molecular methods assessing silent carriage of potential enteric pathogens in DCC-settings[77,107,110-114]. To the best of my knowledge no study has previously been performed in Sweden.

Day care centers and preschools in Sweden

In Sweden all children between 1-5 years of age are offered preschool daycare. Attendance is voluntary, but financially subsidized by the municipalities, with an equal sum per child given for municipal and private preschools. Most children attend preschools 6-8 hours per day during weekdays and the activities follow a national curriculum, defined in *The Education Act (2010:800),* which stipulates that preschools shall provide conditions for acquisition of knowledge and democratic values.

In 2015 83.2% (n=493 609) of Swedish children aged 1-5 years attended preschools, and 80.5% of these children were enrolled in municipal preschools, with an average of 16.8 children per group and 5.3 attendees per preschool employee.

The corresponding figure for children attending preschools in Uppsala County in 2015 was 80%, with 74% enrolled in municipal preschools and an average of 16.7 children per group and 5.8 attendees per employee[115].

Aims of thesis

The overall aim of this thesis was to assess prevalences of extended-spectrum β-lactamases and vancomycin-resistant enterococci as well as common enteric pathogens, in asymptomatic Swedish preschool children, to examine changes over time and to evaluate whether transmission of resistance genes took place between the children or not.

The study-specific aims were:

Study I
To measure the prevalence of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* in feces and to establish if transmission took place between children within the preschools.

Study II
To measure the prevalence of VRE in the same study population.

Study III
To estimate the prevalence of asymptomatic carriage of multiple enteropathogens among the children by means of molecular techniques, and thus to investigate whether this population constitutes a reservoir for gastrointestinal pathogens.

Study IV
The aim of this follow-up study was to investigate if the prevalence of ESBL-producers in our community based pediatric population showed an increase between 2010 and 2016, similar to the one seen in patients, and to reassess whether our previous indications of interpersonal transmission of resistance between children at preschools in the same area could be confirmed or not, using more advanced methods.

Ethical considerations and ethical approval

According to a contact with the Regional Ethics Committee prior to studies I-III no ethical consent was needed, since the samples were classified as biological waste and none of the participants could be identified.

Regardless of this, prior to study IV an advisory statement was formally demanded, and obtained, from the Regional Ethics Committee, which established diapers as biological waste, seeing no ethical obstacles for the study (*dnr 2016/216*).

Consent to the studies was given by the director of the municipal preschools in Uppsala County, as well as by the respective heads in each participating preschool. Written information about the study with attached contact details was sent to all participating preschools for further distribution to the staff and parents, with an option for a meeting with the staff and parents if requested.

Even though all samples were unidentifiable and the handling itself did not result in any (additional) inconvenience for the participants or staff, the unexpected and pronounced increase in prevalence of ESBL-producing *Enterobactericeae* between 2010 and 2016 imposes an ethical dilemma.

Firstly, there is a risk that the results may be presented incautiously in media, since both antibiotic resistance and children are "hot topics" in the press and an interpretation of the research where DCCs are being discredited would be unfortunate. Media and the public could also demand to get information about preschools with high prevalences and suspected transmission, which could have direct consequences for the children, employees and families.

Secondly, there might be a moral obligation from a clinical point of view to identify the carriers and the attendees at risk for acquiring resistance genes in the preschools.

We intend to address these problems by first informing the director of the municipal preschools in the County about the results, and thereafter the County Medical Officer, in order to prepare a unified strategy.

Materials and methods

Study populations and setting in general

Studies I-II

In September and October 2010 a prospective study was performed, addressing all 63 municipal preschools within Uppsala city. 41 preschools chose to participate. Information about the study was sent to the preschool directors for further distribution to the staff and parents. One diaper per child, marked with the child's age, was collected from the participating preschools.

Statistics on antibiotic use among all children in Uppsala County were provided by the Swedish Strategic Programme for the Rational Use of Antimicrobial Agents and Surveillance of Resistance, comprising antibiotics on prescription within Uppsala County and on requisition to Uppsala University Children's Hospital in 2010.

Study III

In addition to the previously described autumn collection, 125 samples were collected with similar setup in June. The first collection included 17 preschools, among which 15 were also included in the study in September and October.

Study IV

During two weeks in the end of August 2016, a prospective follow-up study was conducted with collection of fecal samples from individual diapers from attendees in municipal preschools in the city of Uppsala. The procedure was identical to that in 2010. Approximately one month later the preschools were revisited, and environmental samples were collected from the water trap of washbasins, the toilet flush buttons and the lunch tables.

All 71 municipal preschools within the central parts of Uppsala were invited to participate in the study. The number of attendees at the participating preschool units were similar in size, following the national standard. All preschools had outdoor sandpits and none had pets.

Study I

Bacteria and media

All diapers were transported to the Department of Clinical Microbiology, Uppsala University Hospital. Fecal samples from the diapers were collected by swabs. The swabs were immediately inoculated into Luria-Bertani broth supplemented with cefpodoxime. After incubation at 37°C for 24-36 h in room atmosphere, the samples were plated onto CLED agar plates with discs containing cefotaxime and ceftazidime. Colonies were identified to the species level.

Antibiotic susceptibility testing

Phenotypic determination of ESBL activity was performed by the double disc approximation method on isolates with reduced susceptibility to cefotaxime (<21 mm) and/or ceftazidime (<22 mm). Isolates with no ESBL activity and resistance to cefoxitin were subjected to a boronic acid test.

Additional antimicrobial susceptibility was determined for all isolates with ESBL or AmpC phenotype by the disc diffusion method.

Molecular analyses of β-lactamases with extended spectrum

Isolates positive in the ESBL test were screened for genes encoding *bla*TEMs, *bla*SHVs and *bla*CTX-M. Presence of plasmid-mediated AmpC was determined with a multiplex-PCR. Amplified products from isolates carrying TEM, SHV, CTX-M group I or plasmid-mediated AmpC were purified and sequenced on both strands at Uppsala Genome Center.

Epidemiological typing

To explore the genetic relatedness between isolates harboring the same ESBL type, a combined rep-/AP-PCR was performed. DNA bands were analysed visually, and isolates differing by one or more bands were assigned to different types.

Study II

Sample handling

Fecal samples from the diapers were collected by sterile swabs, immediately inoculated with the sample into 1 ml bile azide esculin broth and stored at -70˚ for analysis.

Screening for VRE was performed with a selective bile azide esculin broth, supplemented with vancomycin and aztreonam, followed by PCR for *vanA* and *vanB,* conducted according to the Swedish Institute for Infectious Disease Control and using the methods described previously.

Study III

xTAG® Gastrointestinal pathogen panel (GPP)

The xTAG® GPP targets the following potentially pathogenic viruses, bacteria, and parasites: adenovirus 40/41, *Campylobacter* (*C. jejuni, C. coli, C. laris*), *Clostridium difficile* toxin A and toxin B, *Cryptosporidium* (*C. parvum, C. hominis*), *Entamoeba histolytica*, *E. coli* O157, enterotoxigenic *E. coli* (ETEC) LT and ST, *Giardia* (*G. lamblia* also known as *G. intestinalis* and *G. duodenalis*), norovirus G1 and GII, rotavirus A, *Salmonella* (two different probes), shiga-like toxin producing *E. coli* (STEC) stx1 and stx2, *Shigella*, *Vibrio cholera* and *Yersinia enterocolitica*.

According to the manual of xTAG® GPP has the following procedural limitations: *i.* primer for *Salmonella* may recognize *S. subterranean*. *ii.* primers for *Shigella* may also recognize enteroinvasive *E. coli* (EIEC). *iii.* a target call of STEC stx1/stx2 may be from *Shigella dysenteriae* type 1 or STEC. In total the xTAG® GPP contains 21 different types of specific probes on beads targeting the different microorganisms, including an internal control; *E. coli* phage MS2. An early version of the xTAG® GPP was used.

Nucleic acid extraction

The samples were pre-treated by mixing the sample, internal control *E.coli* phage MS2 and NucliSENS easyMag Lysisbuffer. Nucleic acid extraction was performed by the nucliSENS easyMAG platform, and the extracts were stored at -70°C until use in the multiplex PCR.

Multiplex PCR

Extracted nucleic acid was used in a One-step PCR according to the manufacturer's protocol. The PCR amplimers range from 58 to 293 base pairs + a unique 24-mer tag for each pathogen-amplicon.

Hybridization

The xTAG® GPP bead mix contained 21 different types of fluorescent beads, representing the different pathogens and the internal control. The hybridization was preformed according the manufacturers protocol. During the analysis in the Luminex-instrument each bead is detected, counted, identified and the amount of biotinylated target is quantified by measuring the fluorescence of the SAPE conjugate bound to the biotins of the amplicons.

Data analysis

xPONENT 3.1 software (Luminex Corporation) was used to analyze the samples. A minimum of 100 beads per target were analyzed and the sample volume was set to 50 µL. For each bead population a median fluorescence intensity (MFI) was generated. The raw data of each bead type and pathogen was

subsequently subtracted with its specific threshold, spanning from 150 to 1400 MFI.

Confirming PCR methods

xTAG® GPP samples with MFI above threshold were reanalyzed with a second PCR assay whenever possible.

Study IV

Bacteria and media

After collection, the diapers were transported to the Department of Clinical Microbiology at Uppsala University Hospital. The feces was sampled with cotton swabs. The samples were thereafter streaked onto MacConkey agar with a cefotaxime disc and inoculated into two tubes with Luria-Bertani broth supplemented with cefpodoxime or ertapenem. After incubation the growth on the MacConkey agar was semi quantified. The broth was plated onto CLED agar plates with discs containing cefotaxime and ceftazidime for selection of producers of ESBLs and AmpC or imipenem and ceftazidime for detection of carbapenemases. Colonies growing in the inhibition zones after 24 h of incubation were identified to the species level, matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS).

The environmental samples were collected with cotton swabs and inoculated on site in Luria-Bertani broth. The subsequent handling was identical to the fecal isolates.

Antibiotic susceptibility testing

Phenotypic confirmation of ESBL-production was performed (figures 3 and 4).

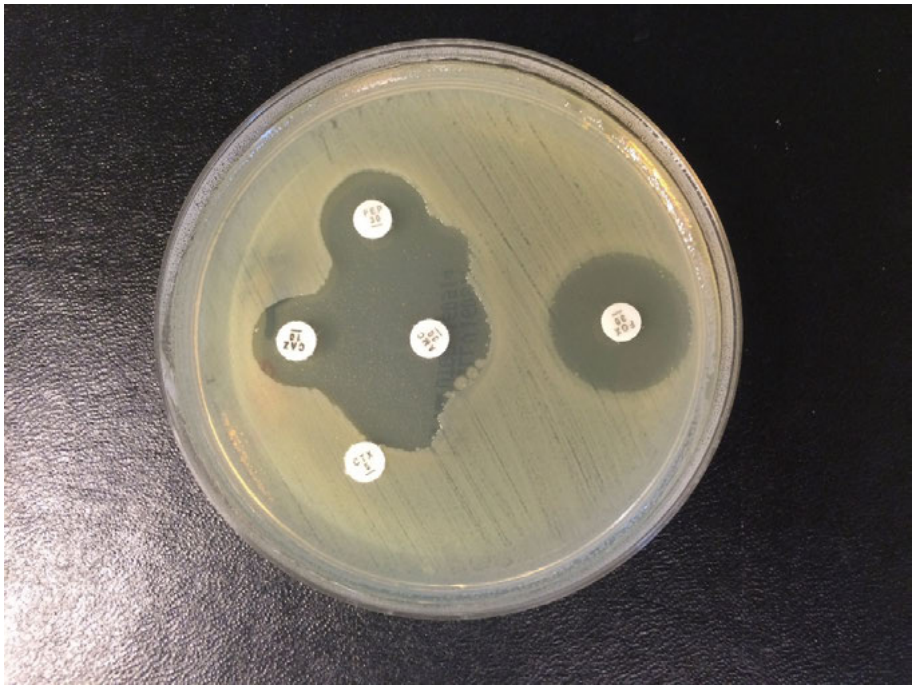


Figure 3. Phenotypic detection of $ESBL_A$

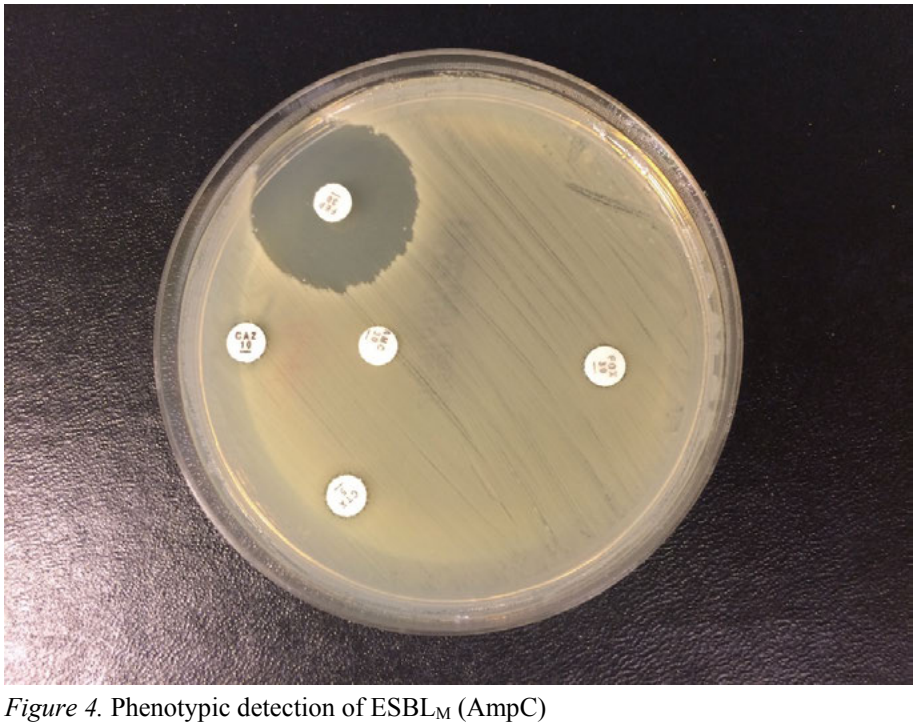


Figure 4. Phenotypic detection of $ESBL_M$ (AmpC)

Additional antibiotic susceptibility was determined for all isolates with either ESBL or AmpC production, using the disc diffusion method and the break-points recommended by EUCAST. Multidrug resistance was defined as resistance to three or more antimicrobial classes in addition to resistance to β-lactams.

Rep-PCR

To explore the genetic relatedness between ESBL-producing isolates from the same preschool, a rep-PCR was performed, including 46 isolates from 13 different preschools. To control if transmission only occurred when ESBL-producers were involved, four preschools with no ESBL-positive isolates and one with a single ESBL-positive isolate was also included.

Whole genome sequencing (WGS)

WGS was performed on ESBL-producers. In addition, a representative of each DNA pattern with rep-PCR was fully sequenced to determine ESBL, localize single nucleotide polymorphisms, and carry out multilocus sequencing (MLST) analysis *in silico*.

Results and discussion

ESBL-producing *Enterobactericeae* in 2010 and 2016

Our collection in 2016, comprising 334 individual fecal samples and 204 environmental samples, resulted in a surprisingly high prevalence of ESBL-producing *Enterobactericeae* of 20.1%, among which 17.4% was $ESBL_A$ and 2.7% $ESBL_M$/AmpC. Twelve of the isolates carried more than one ESBL-enzyme and 19% were defined as multi resistant.

The corresponding figure in 2010 was 2.9% and only one isolate was multiresistant at that time.

Our data shows that ESBL is evenly distributed in all parts of the city, regardless of socioeconomic level or predominant ethnical composition. Seventeen of the participating preschools had no ESBL-producing *Enterobactericeae*, while in four the prevalence exceeded 45%. In the most affected preschool the prevalence reached 80%.

Furthermore, the transmission of resistant strains between children in the same preschools, suggested in study I in 2010, was confirmed in 2016. Rep-PCR and WGS demonstrated dispersal of identical ESBL-producing clones between attendees in 7 of 13 preschools, and spread of more than one clone in two of the preschools. A similar pattern was observed for non-ESBL-producing *E. coli*, where 4 out of 5 analyzed preschools had identical *E. coli*-strains shared between two or more children. In one of these preschools three non-resistant *E. coli*-strains were shared among the children. Interestingly, we could not see any correlation between the semi quantification on agar plates (rating the number of resistant colonies growing in the inhibition zones) and tendency for the same strains to disperse within the preschools. It would have been easy to assume that a high degree of growth in the inhibition zones would correlate with a higher tendency for transmission, but this was not the case.

Among the clones detected with WGS were ST58 and ST354, both epidemic clones, proven to have high virulence[116-117].

Person-to-person transmission seems to be the most probable route for dissemination of resistance, since several identical clones were demonstrated among the children, but only one non-related AmpC from the 204 environmental samples, taken from assumed “hot spots”.

$ESBL_{CARBA}$ was not detected in either of the studies I or IV. This was perhaps not unexpected, since the prevalence in Sweden is still low (see below) and according to data from the Public Health Agency of Sweden, mostly restricted to hospital care[118].

The reasons for the steep increase are not known, nor investigated, in this thesis. A similar, but not as pronounced tendency has been demonstrated among healthy French children between 2010 and 2015, where the prevalences increased from 4.8% to 10.2%[74]. Moreover, according to the reports from the Public Health Agency of Sweden the national prevalence of ESBL-producing *Enterobactericeae* has almost doubled between 2010 and 2015 (Figure 6). Still, this is far from the sevenfold increase we demonstrated.

It is not unlikely that changing travel habits and the increased number of refugees, both known to be correlated to a higher prevalence[47-49,119-120], may have influenced the results, but this is merely a hypothesis. On a study on 420 Finns (34 of which were children 0-17 years old) travelling outside the Nordic countries, Kantele *et al.* however indicated that older age was associated with a higher risk for ESBL acquisition and that only 2 of 34 children were colonized after return[121].

Eighteen percent of the inhabitants of Uppsala County in 2015 were born outside Sweden[122] compared to a corresponding average of 17% for Sweden at large. In Uppsala County, 77.8% of the children 1-5 years old of families born abroad attend preschools, as compared to 80.5% for ethnic Swedish children of the same age[115].

According to statistical data on ESBL-producing *Enterobactericeae*, Uppsala County has a slightly higher prevalence than the country at large (Figure 5)[67].

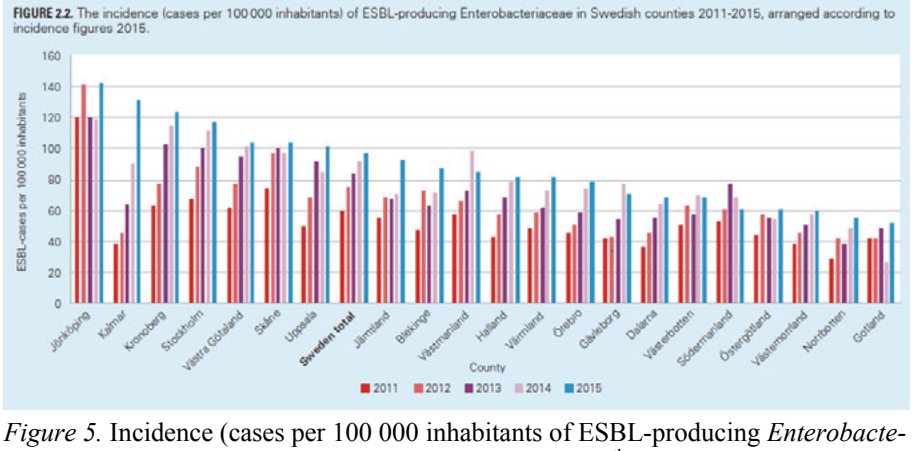


Figure 5. Incidence (cases per 100 000 inhabitants of ESBL-producing *Enterobactericeae* in Swedish Counties 2011-2015); Uppsala is the 7th from the left; Statistics from SWEDRES/SWARM 2016

Apart from travels, factors demonstrated to increase the risk for acquisition of ESBL-producers in preschool settings are:

- Large groups
- Age below 24 months
- Chronic disease[73]

And risk factors for colonization among children in general are:

- Broad-spectrum antibiotics (especially 3rd generation cephalosporins)[50,123]
- Hospital care (especially in ICUs)
- ESBL-carriage among household members
- Medical devices/catheters[123]
- Underlying neurological disease[44]

We can to a large extent rule out medical devices and catheters in our population, but cannot state how the other factors have influenced our results.

Trends in ESBL-producing bacteria

The prevalence of ESBL-producers increases worldwide. In Sweden dissemination of ESBL-producers is the fastest growing bacterial resistance, with an annual growth of 8-40% since they became mandatorily notifiable in 2007[124].

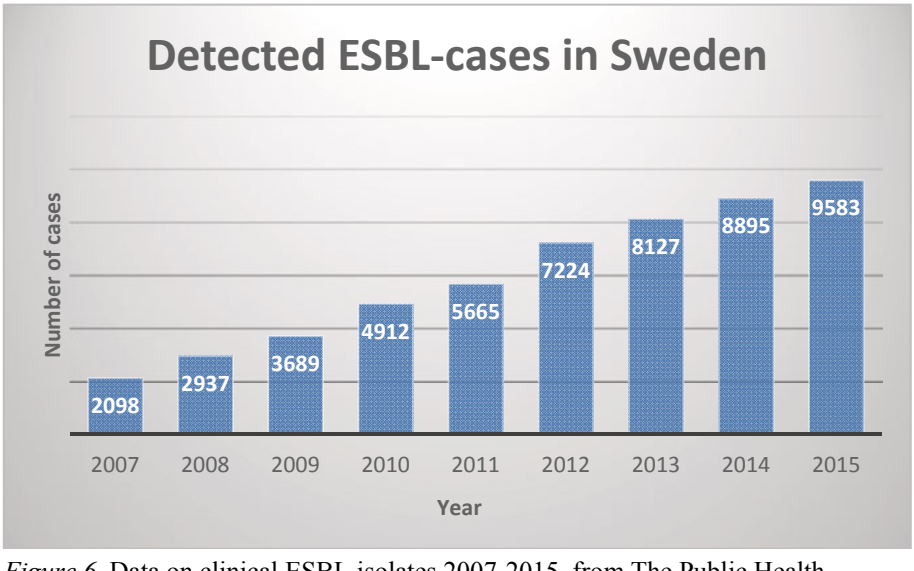


Figure 6. Data on clinical ESBL isolates 2007-2015, from The Public Health Agency of Sweden 2016.

In 2015 86% of the clinical ESBL-producing isolates were *E. coli* and 9% *K. pneumoniae*.

The sample locations were:

- Urine 56%
- Feces 21%
- Rectum 13%
- Blood 4%
- Wounds 2%

578 cases of invasive ESBL isolates were reported in Sweden in 2015 (576 from blood and 2 from cerebrospinal fluid), which is an increase of 11% since 2014.

The increase of $ESBLc_{ARBA}$ is even steeper (Figure 7). Even though the relative numbers are smaller, infections with $ESBLc_{ARBA}$ are considerably more difficult to treat, since therapeutic options are even scarcer.

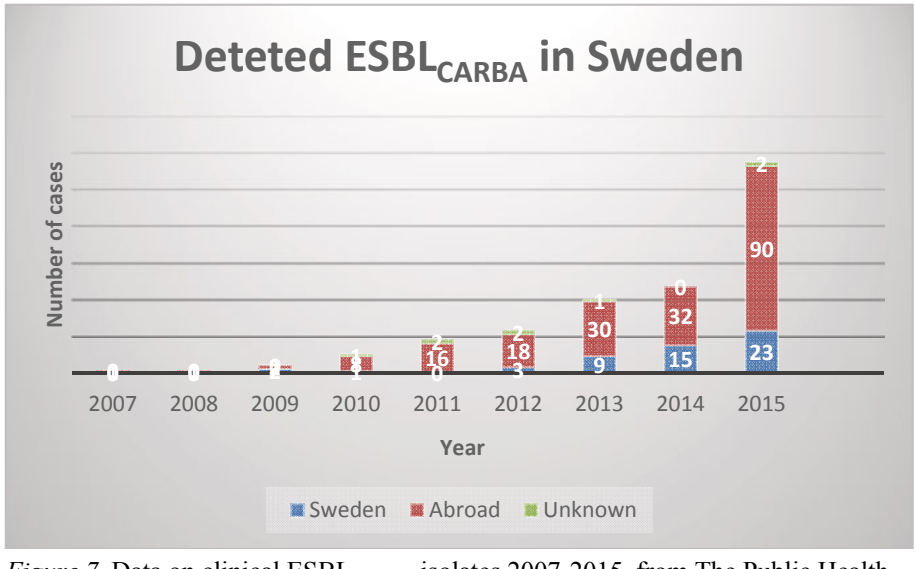


Figure 7. Data on clinical $ESBL_{CARBA}$-isolates 2007-2015, from The Public Health Agency of Sweden 2016.

Data on global trends for ESBL-producers are scarce, since there is no common international surveillance system and many countries and regions do not report prevalences of antibacterial resistance. In Europe the EARS-NET, a network of national surveillance systems supervised by the ECDC, provides European reference data on selected antimicrobial resistance for public health purposes since the beginning of the 21st century[125].

A graphic illustration of resistance to 3rd generation cephalosporins among invasive *E. coli*-isolates in 2005 and 2015 respectively is presented in Figures 8-9, demonstrating an increasing prevalence in most European countries.

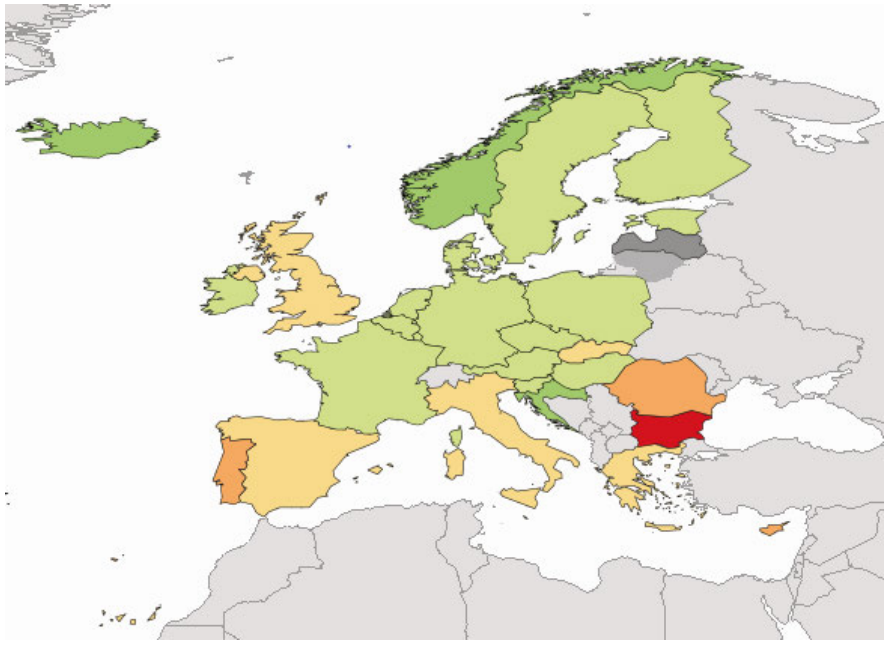


Figure 8. Surveillance atlas of antimicrobial resistance; Resistant invasive *E. coli* isolates to 3rd generation cephalosporins 2005; ECDC/EARS-NET.

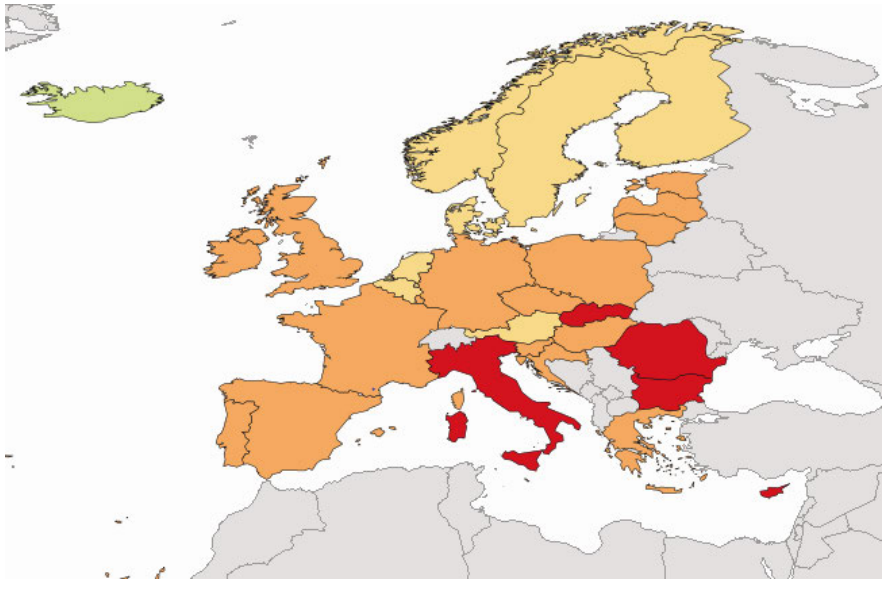
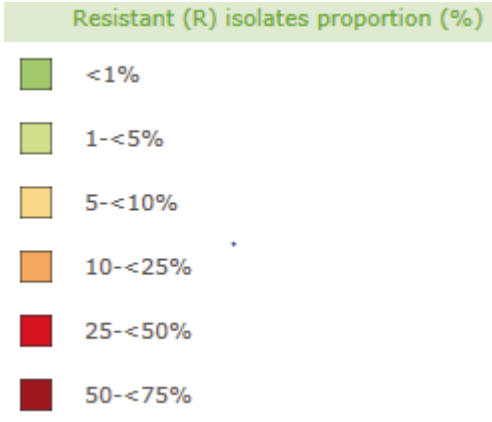


Figure 9. Surveillance atlas of antimicrobial resistance; Resistant invasive *E. coli* isolates to 3rd generation cephalosporins 2015; ECDC/EARS-NET.



Hygiene measures in hospitals

Most studies are based on experiences and observations from outbreaks of ESBL-producing bacteria, but the conclusions are generally valid. Mere colonization without risk factors has been considered less associated with acquisition. Risk factors for transmission and/or acquisition of ESBL-producers are:

- Diarrhea
- Fecal or urine incontinence
- Catheters
- Abdominal drains or gastrostomies
- Tracheostomies
- Open wounds
- Shared patient rooms
- Antibiotic treatment
- ICU-care
- Underlying disease

There are national and local guidelines in Sweden for hygiene routines for ESBL-producing bacteria[36,126]. Screening from rectum or feces for ESBL in a patient on admission to a ward is recommended if the patient during the last 6 months has received health care abroad or in a health care setting in Sweden where there is an ongoing outbreak. Screening should also be performed if the patient is known to be, or have been, infected or colonized with $ESBL_{CARBA}$.

For care of ESBL-colonized or infected patients, the local guidelines in Uppsala University Hospital states:

- Complete adherence to hygiene routines
- Single room with personal toilet obligatory if patient has $ESBL_{CARBA}$ or risk factors
- Single room recommended for $ESBL_{A\ and\ M}$
- Information and meticulous hygiene among relatives
- Restriction for food handling and meals – preferably meals in the room
- Limited transfer between rooms and wards

In cases with $ESBL_{CARBA}$ contact is warranted with the hospital infectious control unit, for guidance.

$ESBL_A$ – therapeutic options in children[36]

Carbapenems is the antibiotic class of choice against most serious infections caused by $ESBL_A$- or $ESBL_M$–producing bacteria, even though the susceptibility pattern, site of infection and individual health considerations all influence the applicable treatment choice. Imipenem, ertapenem, meropenem or the newer carbapenem doripenem have all been proven effective against $ESBL_A$-producing *Enterobactericeae*, even if decreased susceptibility (up to 22% in some studies) has been demonstrated for ertapenem in *K. pneumoniae* and a few percent in *E. coli.* Doripenem is not yet registered for use in Sweden. In conclusion, meropenem should be the first line agent in children[123], followed by imipenem.

Carbapenems should be administered in combination with a single dose aminoglycoside (preferably amikacin, since a diminished susceptibility has been observed for both *K. pneumoniae* and *E. coli* against gentamicin and tobramycin).

An oxymino-β-lactam cephalosporin, such as cefepime or ceftriaxone (in higher doses), or a fluoroquinolone can be considered as a second line intravenous option against serious infections, provided that the isolate has susceptibility *in vitro*, according to the Public Health Agency of Sweden. However, like doripenem, cefepime is not available on the Swedish market and international studies have dissuaded from the use of these agents, with reference to questionable efficacy[127-129]. Cephalosporin-β-lactamase inhibitors (such as ceftolozane-tazobactam and ceftazidime-avibactam) are novel drugs, not yet in clinical use, but with promising applicability[130]. Piperacillin/tazobactam can be a last resort alternative against urinary tract infections in children from 2 years of age, but it has been associated with higher mortality for other infections by ESBL-producing bacteria.

Oral antibiotic alternatives for non-invasive infections in children include amoxicillin/clavulanic acid, nitrofurantoin (from 1 month of age), pivmecillinam (from the age of 5 years) and fosfomycin (not registered in Sweden)[131].

$ESBL_{CARBA}$ – therapeutic options in children

Infections due to $ESBL_{CARBA}$ represent a great challenge in general and among children in particular, due to few antibiotic options and limited evidence from studies. The treatment needs to be tailored, based on the susceptibility data, co-morbidity, age and other medications. Pediatric experience of using many of the agents is limited and further studies are needed to evaluate efficacy, toxicity and dosage.

According to Swedish recommendations, therapeutic options for serious infections in adults caused by $ESBL_{CARBA}$-producing bacteria include colistin, tigecycline and fosfomycin. Aztreonam and ceftazidime-avibactam have been proposed in combination with other agents, even though the observations are yet very limited on humans and *Enterobactericeae*[132-133]. Yet other options may include combinations with fluoroquinolones or aminoglycosides[123].

From a pediatric point of view the drug of choice against $ESBL_{CARBA}$-producers should be colistin, although resistance has been demonstrated[134-135]. Fosfomycin is associated with a rapid emergence of resistance when used, and tigecycline is restricted to adolescents and adults.

To conclude, data on therapeutic alternatives among children are limited, but a useful clinical summary has been presented by Moxon *et al*. (Table 1)[123].

Table 1. Pediatric treatment options against infections by ESBL-producing bacteria 2016; reproduced with courtesy of drs Christopher Moxon and Stéphane Paulus.

Table 2 Available agents for the treatment of beta-lactamase producing bacteria in children.

Agent	Class	Evidence for efficacy in ESBL, AmpC, CPE.	Notes
Commonly used agents			
Piperacillin—Tazobactam	Extended spectrum penicillin (beta-lactam) + beta-lactamase inhibitor.	**ESBL:** Not for bacteraemia as evidence of decreased efficacy compared to carbapenems.[42] **AmpC:** Not recommended. AmpC poorly inhibited by tazobactam. **CPE:** Ineffective.	
Cefepime	4th Generation Cephalosporin (beta-lactam)	**ESBL:** Not recommended for bacteraemia/ severe infection as reduced efficacy compared with Carbapenems.[45] **AmpC:** Possible choice but not if high bacterial inoculum likely or incomplete source control.[12] **CPE:** Not recommended	
Ciprofloxacin	Fluoroquinolone	**ESBL, AmpC:** Reasonable choice if susceptible *in vitro* although less evidence for effectiveness than carbapenems for serious infection in children. **CPE:** Not recommended — generally resistant.	
Carbapenems — Meropenem, Imepenem, Ertapenem	Carbapenem (beta-lactam)	**ESBL, AmpC:** Good efficacy data (unless additional resistance mutations). **CPE:** Some *in vitro* resistance (by definition) but may be of benefit if used in combination with a second agent with activity against CPE if MIC $\leq$ 8 ug/mL.[8]	
Alternative agents			
Temocillin	Extended spectrum penicillin, derivative of ticarcillin (beta-lactam)	**ESBL, AmpC:** Good option for urinary tract infections with efficacy data in children.[46] May be useful for sepsis/pneumonia but limited data in adult studies only. **CPE:** Has *in vitro* activity against KPC. Limited clinical efficacy data.	No CNS penetration. Higher breakpoint for systemic *vs.* urinary tract infections. Twice daily IV dosing facilitates outpatient therapy.
Fosfomycin	Unique class, inhibits synthesis of MurA, disrupting cell wall synthesis[47]	**ESBL, AmpC:** Limited clinical data. Highly effective against certain strains but resistance emerges rapidly. Most likely to be useful in combination with carbapenems **CPE:** May be useful in combination with second agent. Often active *in vitro* but resistance develops rapidly. Limited clinical data but anecdotal efficacy in critically ill adults and children when other drugs have failed.	IV preparation only in some countries, oral preparation more widely available. Good penetration in Bone, lung and CNS.
Pivmecillinam	Extended spectrum penicillin (beta-lactam)	**ESBL:** Option in uncomplicated UTI. Effective in some studies[48] but others show high failure rates.[49] **AmpC:** Good *in vitro* susceptibility, limited clinical efficacy data. **CPE:** Sometimes susceptible *in vitro*. Very limited clinical data.	Achieves high concentration in urine. Significant experience with use for uncomplicated UTIs in several Scandinavian countries. No anti-pseudomonal activity.
Colistin	Unique class: displaces $Ca2+$ and Mg + leading to increased cell wall permeability	**CPE:** Used in combination therapy - may be the only agent to which some NDM-1 strains are effective.	Initial concerns of nephrotoxicity probably overstated. Well tolerated in children in recent studies[50,51]

Tigecycline	Synthetic tetracycline	CPE: Limited clinical efficacy data in children. Good efficacy in adult studies in combination with a second agent.	Not in UTI (limited penetration); Suboptimal as monotherapy in bacteraemia (large volume of distribution). Not recommended in children <8 yrs of age due to risk of dental staining/hypoplasia.

VRE prevalence in 2010

In Study II, on 313 samples from autumn 2010, we did not detect any *E. faecium* or *E. faecalis* with *vanA* or *vanB*-genes. This was reassuring, but perhaps not surprising, since the incidence in Sweden at the time was still comparably low, as demonstrated in Figure 10. VRE are mandatorily notifiable in Sweden since 2000, and until 2007 only sporadic cases were reported. This trend was interrupted in 2008, due to an outbreak with a single strain *E. faecium vanB* in hospitals in Stockholm, which spread to other parts of the country and persisted until 2011[83]. The increase in 2012-2014 related to another hospital-based outbreak in the County of Gävleborg.

There has been no outbreak in Uppsala and during 2006-2013 zero to two clinical cases annually were reported from Uppsala county (two cases in 2010), which indicates that our zero-prevalence result was not unexpected.

The majority of the Swedish cases of VRE is *E. faecium* (92-99%) and with the exception of 2012 and 2015 *vanB* is the most common gene type. The median age for the patient or carrier is 72 years for males and 74 years for females. Most of the cases are domestic, and in 2015 only 38% were traced to foreign sources[136].

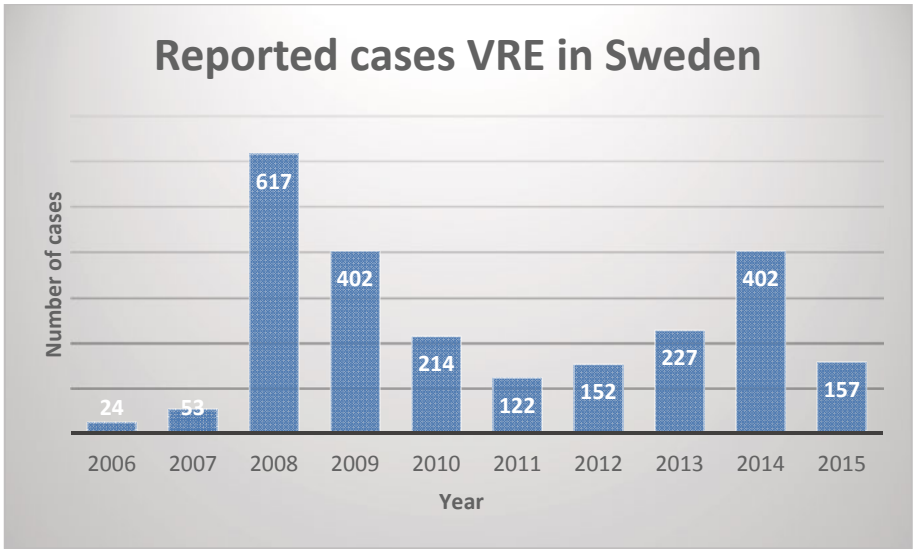


Figure 10. Data on clinical VRE-isolates 2006-2015, from The Public Health Agency of Sweden 2016.

The methods used in Study II, with inoculation in selective broth, followed by PCR, were in concordance with the guidelines from the Public Health Agency of Sweden and international standards. Since no positive isolates were detected, enterococcal species identification was not needed and the known risk for false positive results, for example *vanB*-detection from *Clostridium* spp or *Eggerthella lenta*, with PCR was not relevant[137].

Trends in VRE

The prevalence of VRE varies across the world, but the bacteria are found in most countries. The Scandinavian countries have for a long time experienced a low VRE prevalence among humans, even though occasional outbreaks have occurred[83]. A limited number of studies conducted in Sweden reveal that no VRE was reported in healthy individuals in 1996-97 and only 0.4% of patients with diarrhea carried VRE after travelling abroad[138]. In the previously mentioned study in Swedish nursing homes 2012, no VRE were found[63].

In Europe *E. faecalis* has been causing the vast majority of invasive enterococcal infections among humans (isolates from blood or cerebrospinal fluid) since 2003. However, although the numbers of invasive VRE infections are low, the proportion of *E. faecium* remains higher (with a European average of 8.3%) than vancomycin-resistant *E. faecalis*. No participating country in EARS-NET reports >10% invasive, vancomycin-resistant *E. faecalis* and only three countries demonstrate figures in excess of 3%, while as comparison 10

out of 30 reporting European countries has a proportion invasive vancomycin-resistant *E. faecium* >10%, with the highest prevalences in Ireland and Eastern/South-Eastern Europe)[139].

Hygiene measures

As mentioned, enterococci can survive for long periods of time in the environment and hospital outbreaks are feared consequences of dispersal from colonized or infected patients.

Health care staff have been demonstrated to carry and spread VRE to patients via hands, gloves, aprons and equipment. Despite the use of disposable gloves almost 50% of hospital personnel carried VRE on their hands for more than 60 minutes, even though just 15 seconds of disinfection removed 97% of the bacteria[84].

Adherence to national and local guidelines are hence essential in the management of colonized or infected patients. An unconditional 100% compliance to hygiene routines, the use of disinfectants for everyone (personnel and relatives) after and before contact with the carrier, meticulous information and labeling of medical records, careful and extended cleaning of the room, single-room care and restriction of patient transfer between rooms are vital measures[83,140].

The use of *Lactobacillus rhamnosus GG* has been proposed to decrease the risk for transmission, but sufficient evidence is lacking to recommend this for colonized patients[83,141]. From a personal point of view I would however prescribe it, weighting the potential risks and benefits.

Pediatric risk factors for acquisition of VRE are[88]:

- Antibiotic treatment (especially with vancomycin or 3rd generation cephalosporins)
- Long hospital care or ICU-care
- Invasive or mechanical devices and catheters
- Underlying disease
- Young age (in contrast to adults, where old age has been associated with higher colonization rates)

VRE - Therapeutic options in children

Therapeutic options against serious infections caused by VRE in adults comprise linezolid, daptomycin, tigecycline, teicoplanin and quinupristin-dal-

fopristin[83]. Oral agents for less severe infections include fosfomycin and nitrofurantoin. However, daptomycin is not registered for usage against invasive disease and has been associated with a high degree of development of resistance in enterococci, as is the case for tigecycline. Teicoplanin is not effective against VRE carrying *vanA* and quinupristin-dalfopristin is ineffective against *E. faecalis* and is furthermore not available on the Swedish pharmaceutical market.

For pediatric use ampicillin could have been an option, although resistance among especially *E. faecium* is a considerable problem, reaching up to 80-95%. For severe infections linezolid seems to be the most versatile option in the pediatric population[142]. Even though toxicity remains a concern in prolonged use, with several adverse effects such as thrombocytopenia, anemia, lactic acidosis, neuropathy and serotonin syndrome, the agent has the best outcomes, effectiveness against both *E. faecalis* and *E. faecium*, less resistance problems compared to other alternatives and can be administered both orally and intravenously.

Suggested doses for linezolid are

Preterms < 7 days	10 mg/kg twice daily
Preterms > 7 days	10 mg/kg three times daily
Neonates from w 34	10-15 mg/kg three times daily
Children < 12 years	10 mg/kg three times daily (maximum 600 mg/dose)
Adolescents > 12 years	600 mg/dose twice daily[142]

Quinupristin/dalfopristin has been used in a few cases against severe VRE-infections in children including neonates, using adult doses without serious adverse effects. The outcomes are however poorer and administration of the agent requires a central venous catheter.

Daptomycin is not recommended as a routine agent, due to poorer efficacy, higher resistance and skeletal muscle toxicity.

In conclusion, further studies are warranted.

Carriage of enteropathogens

In Study III we investigated 21 different enteropathogens with multiplex-PCR, using the xTAG® GPP (Gastrointestinal Pathogen Panel) targeting: adenovirus 40/41, *Campylobacter* (*C. jejuni, C. coli, C. laris*), *Clostridium difficile* toxin A and toxin B, *Cryptosporidium* (*C. parvum, C. hominis*), *Entamoeba histolytica*, *E. coli* O157, enterotoxigenic *E. coli* (ETEC) LT and ST, *Giardia* (*G. lamblia* also known as *G. intestinalis* and *G. duodenalis*), norovirus G1 and GII, rotavirus A, *Salmonella* (two different probes), shiga-like toxin producing *E. coli* (STEC) stx1 and stx2, *Shigella*, *Vibrio cholera* and *Yersinia enterocolitica*.

Altogether, our study material comprised 125 spring and 313 autumn samples.

Since studies published with the xTAG® GPP when we started our work were lacking, we chose to reanalyze positive isolates, whenever established alternative molecular methods were available. Only isolates positive with both xTAG® GPP and the second assay were considered confirmed, with the results listed below (Table 2).

Table 2: Confirmed and detected enteropathogens in Study III

Microorganism	Confirmed (number samples)	Not confirmed (number samples)
Enteric adenovirus 40/41	1.6% (7)	
***Campylobacter* spp**	0.7% (3)	
Norovirus GI/II	0.7% (3)	
Dientamoeba fragilis		0.7% (3)
***Clostridium difficile* toxin A**		2.5% (11)
***Clostridium difficile* toxin B**		0.7% (3)
Enterotoxigenic *E. coli* (ETEC)		1.1% (5)

In the most extensive study on children in DCCs so far, Enserink *et al.* demonstrated a significantly higher prevalence among Dutch preschool attendees than in our study. Their group sampled 3% of all preschools in the Netherlands during three years, obtained a total of 5197 samples, and found at least one of 16 enteropathogens in 78% of the samples. Even though the majority of the children were asymptomatic by the time of collection, 4.6% of the children had fever or signs of gastroenteritis, which might partly explain the high detection rate. The included enteropathogens differed from ours and the most common finding was enteropathogenic *E. coli* (EPEC) which was present in 19.9% of the sample, followed by *Clostridium* spp in 16.5% and noroviruses in 9.6%. Another difference between our study and Enserink's is that the Dutch preschool staff were asked to randomly choose 10 samples every month, while we took all diapers produced in one defined day. The authors, however, noted a clear seasonality and conclude that "infections with enteropathogens is not a rare event and that gastroenteritis is only one expression of their presence"[107].

An even higher prevalence of enteropathogenic viruses has been demonstrated among Japanese DCC attendees by Akihara *et al*., who performed a similar one-year prospective study on viral enteric pathogens among 44 symptomatic and asymptomatic children (in total 921 samples, of which 88 originated from diarrheal feces). The children were younger than our study group, with ages ranging from 1 month to 2 years[110]. The Japanese team demonstrated a 20% prevalence of noroviruses GII and 41.7% of adenoviruses in the asymptomatic group. However, primers for all six subgenera of adenovirus A-F were used, and not specifically for the enteric subtypes 40 and 41, which probably explains the high prevalence of adenovirus. Furthermore, 9.6% of the samples were positive for sapovirus and 23% for astrovirus, which were not included in our assay. Like us, Akihara did not detect any rotaviruses, but conclude that enteric viruses are more common among younger children and that a prolonged shedding (up to 56 days for noroviruses) might hamper the interpretation of the results.

A recent study on viruses and *Giardia lamblia* from kindergartens in Gaza, Palestine, among children 3-5 years old demonstrates a prevalence of *Giardia lamblia* of 11.1% among 54 asymptomatic children, with the highest prevalence in the older age group, similar to the findings of Enserink. No enteric viruses (rotavirus, norovirus or adenovirus) were found among the asymptomatic children, but the utilization of enzyme immunoassay and microscopy instead of molecular methods may not have been sufficiently sensitive. It is also noteworthy that the study group was older than ours[111].

De Moura *et al.* demonstrated a 19.1% prevalence of diarrheic *E. coli* among 94 children between 4 and 14 months old, at an infant day care center in Brazil, but found no rotaviruses while using polyacrylamide gel electrophoresis[112]. If the number of studies on asymptomatic enteropathogen carriage among children in preschools is limited, there are more studies on asymptomatic children in general. Our findings are comparably modest, but consistent with data from Denmark from a study on children between 0 and 5 years old[143] as well as a study conducted in our Childrens' Hospital in Uppsala in 1981 on enteric adenoviruses, rotavirus and enteropathogenic bacteria[144].

The highest detected carriage rates of enteropathogens from studies on asymptomatic children with molecular methods are listed below (Table 3), for a comparison with our results and to illustrate the variation in different countries.

Table 3: Comparable studies using PCR for asymptomatic children and location where the study has been conducted, with the highest reported prevalence in percent for each pathogen

Pathogen	Maximum prevalence (%)	Study and location
Rotavirus A	29	Amar 2007; England[145]
Enteric adenoviruses 40/41	6.8	Kabayiza 2014; Rwanda[146]
Norovirus GI/II	35.9	Zhang 2011; China[147]
Campylobacter spp.	33	Elfving 2015; Zanzibar[148]
Clostridium difficile toxin A/B	24	Leibowitz 2015; US[149]
Enterotoxigenic *E. coli* (ETEC) LT/ST	69.7	Elfving 2015; Zanzibar[148]
E. Coli (STEC) stx1/stx2 (including O157)	14.6	Urdahl 2013; Norway[150]
Shigella spp.	33	Elfving 2015; Zanzibar[148]
Giardia lamblia	51.2	Frickmann 2015; Madagascar[151]
Cryptosporidium spp.	17	Samie 2006; South Africa[152]
Entamoeba histolytica	43.7	Paniagua 2007; Mexico[153]
Dientamoeba fragilis	34.6	Bruijnesteijn 2015; Netherlands[154]

A shared difficulty in interpreting the results from all studies using modern molecular techniques is the fact that PCR, even though very sensitive, does not discriminate between viable and non-viable microorganisms[151]. Pre- or postinfectious detection may occur and the prolonged shedding can in some cases be considerable[155]. Although usually of short duration, some examples of very long shedding have been demonstrated: norovirus 100 d, rotavirus 57 d[156], adenovirus 14 d[110], EHEC 46 d[157].

Quantifying the pathogen load in the samples, expressed for example in the number of amplification cycles, is a proposed option to reach a higher clinical specificity[146,154-155], but this method has not been put to use in general practice.

Hence, our low detection rate, especially of rotaviruses, is somewhat surprising since these microorganisms are not rare among Swedish children[158]. However, the mentioned risks for "false positive" results are to be considered also when interpreting our present study, but may at the same time indicate an even lower clinically relevant carriage rate of enteropathogens among preschool children in Uppsala.

Transmission within preschools

In Study I we demonstrated genetically identical rep-PCR isolates from attendees within two preschools, indicating a possible transmission of ESBL-producing strains.

In the follow-up Study IV this observation was confirmed, using rep-PCR and whole genome sequencing. Sharing of *E. coli* strains seems to occur in both preschools with and without ESBL-producing *E. coli*. Only one of the studied preschools had no transmission of *E. coli*.

Transmission of pathogens as well as outbreaks of gastroenteritis in preschool settings have been demonstrated in numerous studies[107,109,113]. Risk factors for transmission of enteropathogens are poor hygiene or cleaning routines[73], sandpits, pets, water pools and crowding[113,159]. Enserink estimates that every additional attendee in a preschool group increases the risk for enteropathogen transmission with +0.1%[159].

Although many conclusions may be universally applicable, it should be noted that there are great differences as to how preschools and DCCs function in different parts of the world. In the Netherlands only 4% of young children attend preschools more than 30 hours/week, which stands in contrast to the Swedish context[159]. It is intuitively appealing to assume that the more hours a child spends in a DCC, the higher would be the risk for acquisition of pathogens or resistance genes. In Sweden the average child spends just as many

waking hours in the preschool as it does at home. However, the average personnel-to-attendee ratio in the two countries seems to be identical.
In many other countries the child groups are notably larger than in Sweden, such as Laos[76], which makes crowding there more likely. In Sweden the preschool child groups have been rather constant during the last decade, as illustrated in Figure 11.

År	Genomsnittligt antal barn per grupp	per årsarbetare
1985	13,4	4,3
1990	13,8	4,4
1995	16,7	5,5
2003	17,2	5,4
2004	17,2	5,4
2005	17,0	5,2
2006	16,7	5,1
2007	16,7	5,2
2008	16,9	5,3
2009	16,8	5,3
2010	16,9	5,4
2011	16,8	5,3
2012	16,9	5,3
2013	16,8	5,3
2014	16,9	5,3
2015	**16,7**	**5,2**

Figure 11. Average number of preschool children per group and average number of children per staff member; The Swedish National Agency for Education 2016.

A handful of studies have demonstrated a spread of ESBL-producers from preschool children to their household members, but to the best of my knowledge only two have indicated a possible transmission of ESBL-producing bacteria between children in the same preschool a part from our study I[76,80]. Study IV is hence the most extensive work on transmission of ESBL-producers within DCCs so far.

Among the participating preschools in Study IV all had outdoor sandpits, none had water pools or pets and with two minor exceptions where hand-disinfectant was lacking in the diaper changing areas, all participating preschools have uniformly high hygiene standards with separate locations for diaper changing, equipped with disinfection, separate hand-washing basin and paper towels. Even though the general impression is that there is a high awareness of hygiene among the staff at the DCCs, we were not able to ascertain how hygiene guidelines are actually followed.

In conclusion, preschools in Uppsala seem to constitute a limited reservoir for enteropathogens.

Antibiotic usage in Sweden and Uppsala

During the last decades there has been a considerable decrease in antibiotic use in Sweden, in both humans and animals. The trend has been particularly pronounced among children 0-4 years old, with a 74% decrease in prescriptions since 1992 (data from STRAMA).

Regardless of this, Uppsala County still had the second highest number of prescriptions and sales on hospital requisition in Sweden in 2015, with a total of 13.9 defined daily doses (DDD) per 1 000 inhabitants and day (compared to the national average of 12.6 DDD/person-days). 4.87 DDD/1000 person-days were prescribed to children 0-17 years old in Uppsala County, with 5.58 DDD/1000 person-days to children 1-5 years old (STRAMA, Uppsala 2016). The majority (93.5%) of the antibiotics were prescribed for outpatients, against upper respiratory tract infections, with the most commonly used drug among children being penicillin V with 2.44 DDD/1000 person-days, followed by amoxicillin with or without clavulanic acid (0.79 DDD/person-days) and flucloxacillin (0.56 DDD/1000 person-days). The two most commonly used cephalosporins were cefadroxil and ceftibuten, which together yielded 0.22 DDD/1000 person-days.

The differences in antibiotic use in Uppsala compared to other parts on Sweden are difficult to explain from a medical point of view, but are most likely due to a combination of misguided expectations from the patients and an inappropriate use of antibiotic drugs, probably emanating from habits and ignorance among doctors.

There are easily accessible antibiotic guidelines for children, aiming for a prudent and adequate use, both locally and nationally. Broad spectrum antibiotic agents are normally avoided. One important difference between adult and pediatric clinics is however the frequent use of cephalosporins (not seldom 3rd generation agents) against urinary tract infections and upper respiratory tract infections among young children, due to fewer approved alternatives. For example, ceftibuten remains the first line option against urinary tract infections in children.

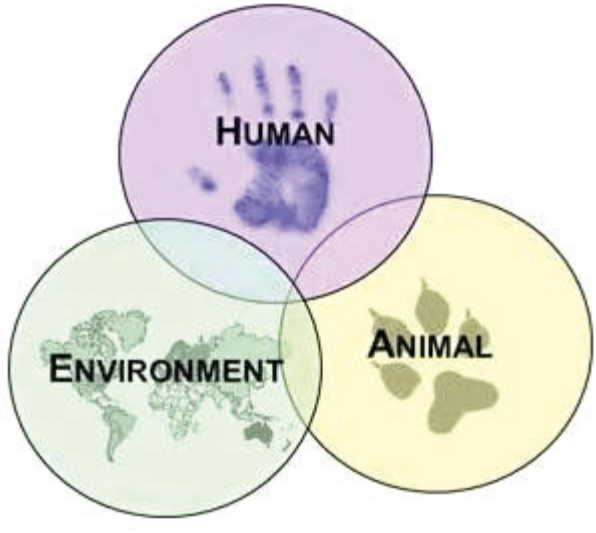
We have no data on antibiotic use in animals in Uppsala County, but Sweden has an internationally comparable low usage of antibiotics in livestock, with 10.4 tons of antibiotics prescribed in 2015 (the corresponding figure for humans is 61.2 tons) with 90% of the amount given to individual animals and

55% of the antibiotics being benzylpenicillin. The use of cephalosporins is minimal in animals. Furthermore, the amount of antibiotics to animals in Sweden has decreased, from 16.4 tons/year 2005 to 10.4 tons 2015 (37%)[67].

One health perspective

Microorganisms and antimicrobial resistance know no boundaries. During the last decades humans have gained historically inequivalent possibilities to travel, bringing back new influences, experiences and – microbes. Today, substantial parts of the world and of our lives are globalized. This brings us obvious possibilities but also challenges, not least when it comes to microbes and resistance. In order to maintain and further improve human health we will need to co-operate over professional, political and geographical borders. Antibiotics are still being mis- and overused in both humans and animals, including the food industry, and waste water treatment standards are often inadequate or lacking. Resistance genes might spread through a multitude of routes, often with our gastrointestinal microbiota as the natural endpoint.

A “One health perspective” is needed.



Strengths and limitations of the studies

The results in this thesis emanate from three different collections of fecal samples from diapers of preschool children in Uppsala in 2010 and 2016. A strength in the methodology is that all municipal preschools in the city were invited to participate and a majority of them chose to take part. The participating preschools were evenly distributed around Uppsala and represented all socioeconomic level and ethnically diverse parts of the town.

Another strength is that the actual collections were done in a way that minimized selection bias. All diapers produced in one predetermined day were collected. Since the procedure of changing diapers did not differ in any substantial way from normal routines, the risk of not obtaining all relevant diapers was small.

Information was provided to all personnel and parents and none declined to participate.

The methodology enables a quick and painless collection of a large number of fecal samples.

As for our two comparative ESBL-Studies I and IV, there is no possibility that any child was sampled twice, due to the time interval elapsed.

The methods applied followed established guidelines, except for Study III, in which a novel multiplex-PCR was used. In this study confirmatory assays were however added and only results positive in both analyses were considered in the published work.

In the ESBL-studies phenotypical ESBL-production was demonstrated using both selective broth and agar, followed by genotypic and species typing.

The obvious limitation of the methodology is the fact that all samples were collected unidentified. Because of this we cannot evaluate risk factors, such as health status, usage of antibiotics, recent hospital care, travel history and family conditions. It is hence conceivable that the attendees sharing identical clones are siblings and that the transmission could have occurred outside the DCC, even though the age span between the attendees in question makes this less likely.

It is not probable that the health status or antibiotic exposure in general should differ between 2010 and 2016, but there is a possibility that travel habits have increased since our last study in 2010 and it is also plausible that the increased number of refugees to Sweden and Uppsala could have contributed to the

higher prevalence in 2016. However, since the objectives were to establish prevalences and possible transmission, the underlying causes for the increase lie beyond the aim of this thesis, although penetrating this would naturally be of interest in a future study.

Another limitation is the point prevalence study design. By collecting samples during only one defined period of the year, there is a risk that we capture only the very maximal (ESBL) or minimal (enteropathogens) prevalences. This is one reason for including pre-summer samples in Study III. Apart from studies already referred to, ESBL-carriage has been demonstrated vary between only a few percent after 3-12 months[49,160-161] to levels around 20% or more after 6-12 months[47,50,65]. Seasonality in ESBL-carriage has not been extensively studied. One study from Madagascar on newborns and mothers demonstrated the highest prevalences from October to March[162], but it is likely that a possible seasonal variation looks different in different parts of the world.

During the three-year surveillance study on Dutch preschool children by Enserink *et al.*[107] there was a clear winter peak (October-April) for viral enteropathogens, while bacterial pathogens were more common during the summer and parasites showed no clear seasonality.

In Study III there is a possibility that the same children were included in the two collections in June and in September-October, since 15 of the 17 preschools in June were also participating in the autumn. The statistical risk has not been calculated, but ought to be minor.

The risk of "false high" results, due to detection of non-viable pathogens in Study III has been discussed previously. The clinical consequence ought to be more pronounced if the prevalences had been higher.

Limitations for PCR and multiplex-PCR analyses, with risks of for example lower detection rates or false negative results, are not specific for this thesis. Hopefully these risks have been reduced by using well-established and/or complementary methods.

Clinical implications

The main finding in this thesis is that the prevalence of ESBL-producing *Enterobactericeae* has reached unexpectedly high levels in six years and that an extensive transmission of resistance genes seems to occur between children at the preschool.

- The result underscores the necessity to be more watchful for the appearance of resistant strains when treating children with infections, and stresses the need for pediatric guidelines for the management of ESBL-producing bacteria. It is likely that resistant strains will be more noticeable in the near future among pediatric patients, and having exceeded a prevalence level of 10% suggests that further dispersal of resistance will not be easily stopped. Rectal or fecal ESBL screening in all children admitted to hospital could be considered if our data are confirmed in more pediatric studies in Sweden.

- Further studies among children, and perhaps adults, are needed to establish whether our observations are reproducible. If so, treatment recommendations will have to be changed, taking into account that every fifth child with for example UTI will carry bacteria resistant to cephalosporins.

- Finally, national pediatric guidelines are needed on doses and suitable antimicrobial agents against serious infections with ESBL-producing bacteria, and desirable against VRE as well.

Conclusions

- A prevalence of 20.1% Extended-spectrum β-lactamase producing *Enterobactericeae* among healthy preschool children in Uppsala was established in 2016.
- Between 2010 and 2016 the prevalence of ESBL-producers has increased almost sevenfold.
- Transmission of resistant clones and non-resistant *E. coli* between children in the same preschools is not an unusual event.
- No $ESBL_{CARBA}$ or VRE were detected among the preschool children in our studies.
- Carriage of potential enteropathogens among attendees in preschools in Uppsala is lower than in comparable studies.

Future perspectives

Our work demonstrates a notable increase in ESBL-producing *Enterobactericeae* between 2010 and 2016 among preschool children in Uppsala, and transmission of resistant clones has been demonstrated within several of the preschools. Two obvious questions to be answered in future projects are firstly if these findings are reproducible in other preschool settings, or limited to Uppsala, and secondly what may be the causes of the increase.

A perspective for future work is consequently to make a similar study with identified participants and enquiries on risk factors, health status, family structure, medical treatment etc. A prospective study design over longer time would also add to the knowledge on colonization time and risks for infections of resistant *Enterobactericeae*, morbidity, later health outcomes and possible seasonality pattern in ESBL-colonization.

As mentioned several studies have been conducted on travelers, but few studies on children. Results from Lebanese infants and children indicate that there is a higher degree of resistance prevalence in younger individuals[78-79], possibly due to a less diversified and resilient microbiota. This leads to the question whether young children acquire resistant clones more easily than adolescents and adults.

It would furthermore be of value to establish carriage prevalences among household members and, desirably, preschool personnel.

Although perhaps not a task primarily for research groups, but treatment guidelines for ESBL-producing bacteria and VRE are needed.

In a broader perspective a prospective collection of identified and repeated samples over time could also add to our knowledge on microbiota development and possible associations to later disease.

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