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# **ANTIBIOTIC RESISTANCE IN GRAM-NEGATIVE BACTERIA AFFECTING CHILDREN FROM LEÓN, NICARAGUA**

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*To my dear family*



## ABSTRACT

Annual child mortality has declined in the world from 12.5 million in 1990 to 8.8 million in 2008. Yet, infectious diseases are still the major cause of death in this group (6.97 million); with diarrhoea responsible for the death of 1.3 million and neonatal septicaemia for 0.5 million. On the positive side, Latin America/Caribbean is among the regions with the highest progress in reduction of child mortality. In Nicaragua, nearly 4000 children under 5 years of age died in 2008, with diarrhoea as the cause of death for 343 children and neonatal sepsis for the death of 62 children.

A key problem in the management of diseases such as diarrhoea or septicaemia has been the emergence of antibiotic resistant Gram-negative bacteria. The treatment options for Gram-negative infections affecting children are scarce or with probable toxic effects as for the neonates. Thus, the global burden of antimicrobial resistance requires appropriate interventions. Local surveillance to identify prevalent pathogens and bacterial resistance patterns is necessary for selecting optimal treatment regimens with the aim of a positive outcome in the patient. Furthermore, the evidence that this surveillance can provide with the environmental water is crucial in order to create risk management strategies for these settings. However, in Nicaragua this information is still lacking. Thus the studies presented in this thesis focused in the determination of the prevalence of antibiotic resistant Gram-negative bacteria in children and environmental water.

The results from the first study are: 74% (34/46) of the bacteria related to neonates with septicaemia were Gram-negative bacteria, mainly *Klebsiella pneumoniae*, *Serratia marcescens* and *Serratia liquefaciens*. Interestingly, these pathogens were also isolated in the neonatal intensive care unit (NICU) environment. The *K. pneumoniae* showed clonal similarity among the isolates affecting neonates and those from the NICU's environment. High levels of antibiotic resistance were found in those Gram-negative, e.g. more than 85% of *K. pneumoniae* strains from neonates with septicaemia, and the environment were resistant to ceftazidime, ceftriaxone, and gentamicin. Furthermore, a high prevalence of TEM-1, SVH-11/12 and CTX-M-15-producing Gram-negative bacteria from the neonates with septicaemia and the NICU's environment was found. Thus, implementation of infection control practices, and appropriate empirical therapy should also be considered to reduce the prevalence as well as the dissemination of these organisms in this area.

The results from the second study are: 47% (296 of 727) of the *E. coli* isolates analyzed were resistant to ampicillin and trimethoprim-sulfamethoxazole. Enteropathogenic *E. coli* showed higher resistance levels to most of the tested antibiotics when compared to other *E. coli* categories. In general, the antibiotic resistance level in *E. coli*, from children with/without diarrhoea, have not yet reached the high levels of resistance to the most common antibiotics used for diarrhoea treatment as in other countries, yet CTX-M-5 or CTX-M-15 production was detected in some multi-antibiotic resistant diarrhoeagenic and non-diarrhoeagenic *E. coli* isolates. This suggests the emergence of ESBL in the Nicaraguan community and may indicate future treatments complications.

The results from the third study are: Among all of the *E. coli* isolates included in this study, those from the hospital sewage water showed higher antibiotic resistance levels to ampicillin (100%), nalidixic acid (70%), ciprofloxacin (69%), chloramphenicol (69%) and trimethoprim-sulfamethoxazole (100%) compared to the other *E. coli* isolates. Among the well water samples which represent the contribution of the community in the input of antibiotic resistance bacteria to the aquatic environment, *E. coli* isolates from well water sample P55 were fully resistant to the tested antibiotics which indicated a high contribution to the spread of multi-antibiotic resistant bacteria. Our results suggest that multi-resistant CTX-M-9 and CTX-M-15-producing *E. coli* were widely spread in hospital sewage water and some community water samples.

## LIST OF PUBLICATIONS

- I. **Amaya E**, Cáceres M, Fang H, Ramirez, AT, Palmgren AC, Nord CE, Weintraub A.  
Extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* in a neonatal intensive care unit in León, Nicaragua.  
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- II. **Amaya E**, Cáceres M, Fang H, Ramirez AT, Palmgren AC, Nord CE, Weintraub A.  
Antibiotic resistance patterns in gram-negative and gram-positive bacteria causing septicaemia in newborns in León, Nicaragua: correlation with environmental samples.  
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- III. **Amaya E**, Reyes D, Vilchez S, Paniagua M, Möllby R, Nord CE, Weintraub A.  
Antibiotic resistance patterns of intestinal *Escherichia coli* isolates from Nicaraguan children.  
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- IV. **Amaya E**, Reyes D, Paniagua M, Calderón S, Colque P, Kühn I, Möllby R, Nord CE, Weintraub A.  
Antibiotic resistance patterns of *Escherichia coli* isolates from different aquatic environmental sources in León, Nicaragua.  
In manuscript.

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## LIST OF ABBREVIATIONS

AMPs	Antibacterial peptides
CLSI	Clinical and Laboratory Standards Institute
CSF	Cerebrospinal fluid
DEC	Diarrhoeagenic <i>E. coli</i>
ESBL	Extended spectrum $\beta$ -lactamase
ESBLsKp	ESBL-producing <i>K. pneumoniae</i>
EAEC	Enteropathogenic <i>E. coli</i>
EHEC	Enterohaemorrhagic <i>E. coli</i>
EIEC	Enteroinvasive <i>E. coli</i>
ETEC	Enterotoxigenic <i>E. coli</i>
EPEC	Enteropathogenic <i>E. coli</i>
HEODRA	Hospital Escuela Oscar Danilo Rosales Arguello
ICU	Intensive care unit
MIC	Minimal inhibitory concentrations
NICU	Neonatal intensive care unit
OM	Outer membrane
PBPs	Penicillin-binding proteins
RAPD	Randomly amplified polymorphic DNA
UPGMA	Unweighted Pair Group Method using Arithmetic Averages
WHO	World Health Organization



# 1 INTRODUCTION

Annual child mortality has declined in the world from 12.5 million in 1990 to 8.8 million in 2008 (182). Interestingly, infectious diseases are still the major cause of death in this group (6.97 million); with diarrhoea responsible for the death of 1.3 million and neonatal septicaemia for 0.5 million (17). Moreover, there is an urgent need for the global health community to refocus diarrhoea as one of the three most important causes of death in children under 5 years of age.

On the positive side, Latin America/Caribbean is among the regions with the highest progress in reduction of child mortality (182). In Nicaragua, nearly 4000 children under 5 years of age died in 2008, with diarrhoea as the cause of death for 343 children and neonatal sepsis for the death of 62 children (17).

A key problem in the management of diseases such as diarrhoea or septicaemia has been the emergence of antibiotic (this term will be used in this thesis to cover any substances with antibacterial activity) resistant bacteria (16, 44, 107, 110, 121). Throughout history, humans have continuously struggled against microorganisms that cause infectious diseases (77, 97). Major advances in antibacterial antibiotic development in the 20<sup>th</sup> century and other means of infection control helped to move the balance in favour of humans. For example, the introduction of penicillin from laboratory to bedside heralded a new era in the clinical setting (75, 155). Antibiotic therapy has led to a dramatic decline in mortality and morbidity with the ability to prevent, cure and to curtail the transmission of infectious diseases such as meningitis, tuberculosis, pneumonia; many of which had been untreatable and fatal. However, the common belief in the medical community over the potential conquest of infectious diseases was short lived (75). Bacteria are quite smart and almost as soon as antibiotics were introduced, bacteria responded by manifesting various forms of resistance (146). As antibiotic usage increased, so did the level and complexity of the resistance mechanisms exhibited by bacterial pathogens. The efforts to gain the battle against bacterial infections continue to this day, development pipeline for agents targeting Gram-positive bacteria has compensated to certain extend antibiotic resistance in these bugs. The contrary is seen for Gram-negative bacteria, few, if any, new antibiotics are being introduced into the antibiotic pipeline to compensate the emergence of antibiotic-resistance Gram-negative pathogens (33, 60, 62, 105).

## 2 GRAM-NEGATIVE BACTERIA

The Gram stain (named after Christian Gram, Danish scientist and physician, 1853–1938) is the most useful and widely employed differential stain in bacteriology. This method divides bacteria into two groups: those that possess a thick murein conferring the ability of retaining a complex of a purple dye and iodine when challenged with a brief alcohol wash (Gram-positive) and those that do not (Gram-negative) and can later be counterstained with a red dye, safrinin. The majority of the known Gram-negative bacteria of medical interest are encompassed in the phylum Proteobacteria which constitutes at present the largest and phenotypically most diverse phylogenetic lineage.

Infections due to Gram-negative pathogens may occur in any human organ system, but they occur most frequently in the urinary, gastrointestinal, and respiratory tracts (125, 142, 148). Those that are mild and occur in the community (cystitis, pyelonephritis, cholecystitis, and sinusitis) are usually responsive to oral agents, such as fluoroquinolones, trimethoprim-sulfamethoxazole, cephalosporins, as well as ampicillin and its derivatives (57). Initial therapy should be chosen, in part, on the basis of local antibiotic susceptibility patterns (120). However, during the late 1990s and 2000s, Enterobacteriaceae (mostly *Escherichia coli*) producing novel extended spectrum β-lactamase (ESBL), the CTX-M enzymes, have been identified predominantly from the community as a cause of urinary tract infections (132). Resistance to other classes of antibiotics, especially the fluoroquinolones, is often associated with ESBL-producing organisms (3, 44).

More serious Gram-negative infections occur in patients in a hospital setting or in long-term care facilities. Among inpatients, the most problematic infections occur in intensive care units (ICUs). Patients in the ICU usually have serious comorbid conditions or have conditions compromised by invasive procedures, such as surgery, mechanical ventilation and use of vascular or urinary catheters (125). These infections are often life-threatening and may be caused by Gram-negative organisms that are resistant to multiple antibiotics. Several clinical studies from the past decade or earlier have provided convincing evidence that effective initial empirical antibiotic therapy, defined by ultimate antibiotic-susceptibility results, improves survival. Such studies also indicate that later adjustment of inadequate initial therapy, after antibiotic-susceptibility data become available, does not mitigate the adverse effect of inadequate initial therapy. Thus, an important therapeutic principle has emerged that supports aggressive, broad-spectrum initial empirical therapy of serious infections, followed

by appropriate de-escalation of treatment according to the results of antibiotic-susceptibility data (142).

A heightened awareness of these organisms by clinicians and enhanced testing by laboratories, including molecular surveillance studies, is required to reduce treatment failures, to limit their introduction into hospitals and to prevent the spread of emerging pathogens within the community (132, 179).

## **2.1 ANTIBIOTIC TREATMENT OF INFECTIONS CAUSED BY GRAM-NEGATIVE BACTERIA**

Antibiotic resistant bacteria have emerged as a global health concern; it is such the fear that it has been compared with a perfect storm or with the beginning of a new era of untreatable infections (61, 84). This is not entirely true; new antibiotics have been approved for the treatment of infections caused by Gram-positive bacteria. For example, linezolid, quinupristin/dalfopristin, daptomycin and tigecycline have been approved for the treatment of methicillin-resistant *Staphylococcus aureus* infections (13, 84, 156). However, the opposite is seen for infections caused by Gram-negative bacteria with few new antibiotics in development and with the emergence of Gram-negative bacteria resistant to all of the treatment options (60, 62, 142).

Infections caused by Gram-negative bacteria have features that are of particular concern. These organisms are highly efficient at up-regulating or acquiring genes that code for mechanisms of antibiotic resistance, especially in the presence of antibiotic selection pressure. Furthermore, they have available to them a plethora of resistance mechanisms, often using multiple mechanisms against the same antibiotic or using a single mechanism to affect multiple antibiotics (125). Some of the new antibiotic compounds, for treating infections caused by multi-resistant Gram-negative bacteria, will be described in sections 2.1.1 – 2.1.5.2. Traditional approaches and the newer genomic mining approaches have not yielded novel classes of antibacterial compounds. Instead, improved analogues of existing classes of antibiotics have been developed by improving potency, minimizing resistance and alleviating toxicity (46).

### **2.1.1 β-Lactams and β-lactamase inhibitors**

The β-lactams, which include penicillins, cephalosporins, carbapenems and monobactams, remain the most heavily used of all antibiotic classes. However, increased rates of resistance to this class of antibiotic is of concern. Several novel β-lactams have recently been approved or are in clinical trials. Recently approved agents including ertapenem and doripenem, both of which possess some useful properties compared with earlier carbapenems, have substantially longer half-life (ertapenem) and a slightly better activity (doripenem) against *Pseudomonas aeruginosa* in vitro, coupled with a reduced potential to select spontaneous resistant mutants against other carbapenems (105).

When combined with certain β-lactam antibiotics, β-lactamase inhibitors augment the potency of these against β-lactamase-producing bacteria. Three β-lactamase inhibitors are available (sulbactam, clavulanate and tazobactam). The in vitro and clinical efficacies of β-lactamase inhibitors have been compromised by the emergence of resistant isolates during the last decade. The principal mechanisms of resistance to these compounds are the production of class C β-lactamases, overproduction of the class A penicillinases TEM-1 and TEM-2, and production of low-affinity enzymes such as inhibitor-resistant TEM and the OXA enzymes (1). NXL104 (Novexel), is currently the only inhibitor of class C β-lactamases in clinical trials, mainly for the treatment of nosocomial Gram-negative infections, including those caused by *P. aeruginosa* (105).

### **2.1.2 Antibacterial peptides (AMPs)**

Polymyxins are a good example of the use of old class antibiotics as a treatment option for multi-antibiotic resistant Gram-negative infections. These agents were abandoned in the seventies because of their nephrotoxicity and neurotoxic effects. However, the nephrotoxicity is less pronounced than previously thought, but may still complicate patient's therapy or even require its discontinuation. The toxicity is related to their highly cationic nature (net charge, +5), analogously to aminoglycosides, which are less toxic though (60, 167). The antimicrobial target of colistin is the bacterial cell membrane, where the polycationic peptide ring interacts with the lipid A of lipopolysaccharides, allowing penetration through the outer membrane by displacing Ca<sup>2+</sup> and Mg<sup>2+</sup>. Insertion between the phospholipids of the cytoplasmic membrane leads to loss of membrane integrity and to bacterial cell death (60). Regarding new AMPs, most of them suffer from weak activity, nonspecific cytotoxicity and apparent susceptibility to proteolysis. Few have undergone any systematic toxicity and

efficacy studies. The most promising compounds include ceragenins, and RTA-3 (all new classes) as well as the novel NAB-series of polymyxins (167).

### **2.1.3 Tigecycline**

Tigecycline, a novel, first-in-class glycylcycline and an analogue of the semisynthetic antibiotic minocycline, is a potent, broad-spectrum antibiotic that acts by inhibition of protein translation in bacteria by binding to the 30S ribosomal subunit and blocking the entry of amino-acyl to RNA molecules into the A site of the ribosome (37). Tigecycline was approved in 2005 by the Food and Drug Administration of the United States and in 2006 by the European Medicines Agency for the treatment of complicated skin and skin structure infections and complicated intra-abdominal infections in adults (60). It exhibits broad-spectrum antibacterial action, including inhibition of Gram-positive, Gram-negative, atypical, anaerobic, antibiotic-resistant organisms and at least in vitro to *Acinetobacter baumannii*. As such, it represents the only new agent launched in recent years capable of circumventing existing resistance mechanisms. Unfortunately, tigecycline does not exhibit useful antibacterial activity against *P. aeruginosa*, since it is extruded from the cell by the MexXY efflux pump (37, 105).

### **2.1.4 Fosfomycin**

Fosfomycin tromethamine is a soluble salt of fosfomycin with improved bioavailability over fosfomycin. It is licensed as single-dose treatment for uncomplicated urinary track infections caused by *E. coli* and *Enterococcus faecalis*. However, it is only poorly active against *P. aeruginosa*, *Proteus* spp. and *Providencia* spp. Fosfomycin inactivates the enzyme pyruvyl-transferase, which is required for the synthesis of the bacterial cell wall peptidoglycan. This antibiotic is bactericidal against a broad spectrum of Gram-positive and Gram-negative pathogens, and possesses a low potential for cross resistance with other classes of antibiotics. Some studies showed that fosfomycin exhibited good in vitro and in vivo antimicrobial activity against antibiotic resistant clinical isolates of Enterobacteriaceae (33, 53, 54, 60). Although, further clinical studies are necessary, fosfomycin is a good candidate for the treatment of infections caused by those organisms.

### **2.1.5 Novel approaches**

As a consequence of the emergence of bacteria resistant to virtually all class of antibiotics, there is a need of the discovery of new antibiotics with novel mechanisms of action as well as

novel unexploited bacterial targets and strategies that may pave the way for combating antibiotic resistance and emerging pathogens.

#### *2.1.5.1 Anti-virulence agents*

A novel approach to the treatment of bacterial infections involves specifically blocking the ability of Gram-negative bacteria to disseminate and cause systemic infection within the body (105). The main goal of targeting virulence factors is to interfere with the process of infection before host damage occurs, rather than bacterial viability (4). Anti-virulence strategies may specifically interfere with the ability of the bacteria to recognize host signals that alert the bacteria that are at the site of infection and/or activate specific virulence traits that are needed to establish infection. By preventing the expression or activity of virulence traits, the bacteria are less able to colonize the host. In addition, as this strategy does not directly kill the bacteria, there is presumably less evolutionary pressure for the development of resistant clones than with traditional antibiotics. It is thought that this inhibition could allow the host immune system, including the normal microbiota, to prevent bacterial colonization or clear any established infection (147). Among the main anti-virulence target approaches are inhibitors of toxins, adhesins, specialized bacterial secretory systems, organism-specific virulence gene expression, and organism-specific cell-to-cell signalling (4, 46, 147).

#### *2.1.5.2 Efflux pump inhibitors*

Efflux pumps play an essential role in Gram-negative bacteria antibiotic resistance. For example, over-expression of efflux pumps that expel unrelated antibiotics conferring a multi-antibiotic resistance profile to the bacteria. Yet some compounds have proven to be selectively interfering with bacterial multi-antibiotic efflux pumps, some of them are under investigation for clinical use and others were not suitable for it (3, 82, 87, 105, 112).

## **2.2 MECHANISMS OF ANTIBIOTIC RESISTANCE IN GRAM-NEGATIVE BACTERIA**

A summary of the mechanisms of antibiotic resistance in Gram-negative bacteria is seen in Fig. 1. In general, Gram-negative bacteria use four mechanisms of resistance to survive to the antibiotic treatment:

### **2.2.1 Efflux of antibiotics from bacteria**

Efflux pumps play a key role in antibiotic resistance and also serve other functions in bacteria such as the uptake of essential nutrients and ions, excretion of metabolic end products and deleterious substances as well as the communication between cells and the environment (81, 138, 142, 164). This mechanism of resistance is a major concern because a single efflux pump can produce a simultaneous resistance to a number of antibiotics.

### **2.2.2 Outer membrane (OM) permeability**

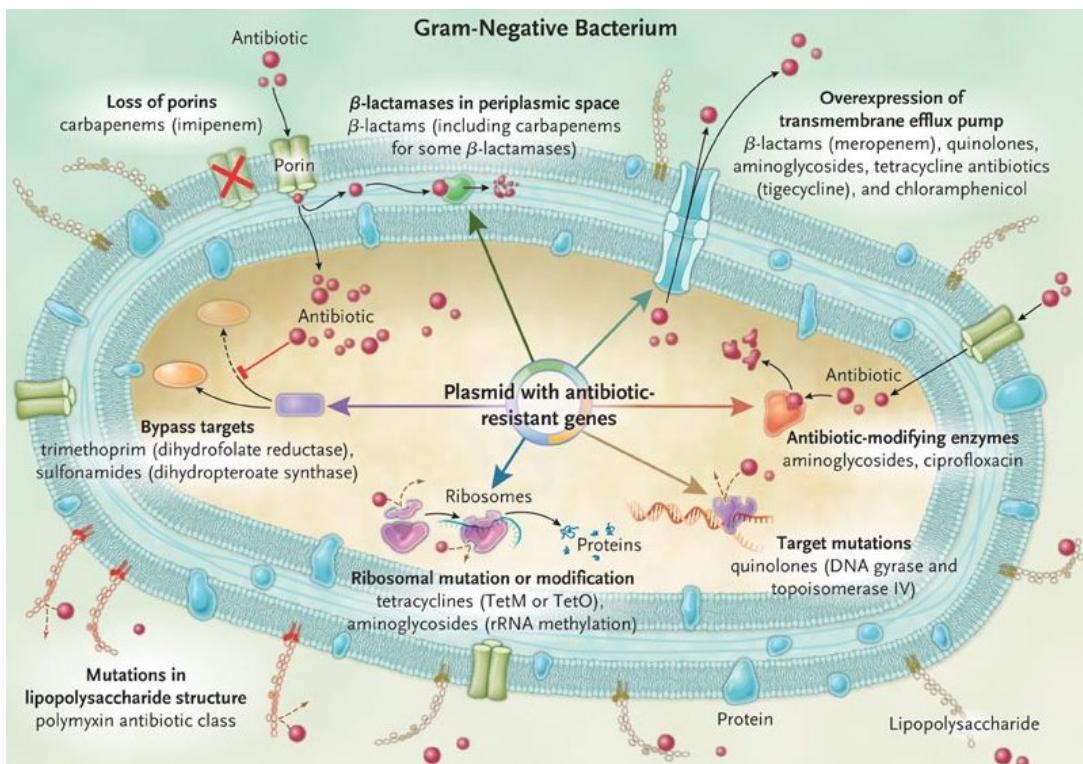
The OM of Gram-negative bacteria is a barrier to both hydrophobic and hydrophilic compounds. By combining a highly hydrophobic lipid bilayer with pore-forming proteins of specific size-exclusion properties, the OM acts as a selective barrier. The permeability properties of this barrier, therefore, have a major impact on the susceptibility of the microorganism to antibiotics, which, to date, are essentially targeted at intracellular processes. Small hydrophilic antibiotics, such as  $\beta$ -lactams, use the pore-forming proteins (water-filled channel proteins embedded in the outer membrane, e.g., OmpF in *E. coli* and OprD in *P. aeruginosa*) to gain access to the cell interior, while macrolides and other hydrophobic antibiotics diffuse across the lipid bilayer. The existence of antibiotic-resistant strains in a large number of bacterial species due to modifications in the lipid or protein composition of the OM indeed highlights the importance of the OM barrier in antibiotic sensitivity (3, 42, 113, 142, 164).

### **2.2.3 Target modifications**

This mechanism is based on alterations of bacterial sites that are targeted by antibiotics and thus preventing the antibiotic from binding to its site of action. For example fluoroquinolone resistance is attributed to mutations within the drug's targets (DNA gyrase and topoisomerase).

### **2.2.4 Enzymatic modification of the antibiotic**

Enzymes that modify antibacterial antibiotics are divided into two general classes: (i)  $\beta$ -lactamases that degrade antibiotics and (ii) others (including the macrolide and aminoglycoside-modifying proteins) that perform chemical transformations to render the antibiotic inefficient. Further information about  $\beta$ -lactamases will be described in section 2.3.1.2.



**Figure 1.** Mechanisms of resistance in Gram-negative bacteria, and the antibiotics affected (reprinted with the permission from The Publishing Division of the Massachusetts Medical Society (125).

Seven mechanisms of resistance are shown in the gram-negative bacterium, with some being mediated by a mobile plasmid. These mechanisms include the loss of porins, which reduces the movement of antibiotic through the cell membrane; the presence of  $\beta$ -lactamases in the periplasmic space, which degrades the  $\beta$ -lactam; increased expression of the transmembrane efflux pump, which expels the antibiotic from the bacterium before it can have an effect; the presence of antibiotic-modifying enzymes, which make the antibiotic incapable of interacting with its target; target site mutations, which prevent the antibiotic from binding to its site of action; ribosomal mutations or modifications, which prevent the antibiotic from binding and inhibiting protein synthesis; metabolic bypass mechanisms, which use an alternative resistant enzyme to bypass the inhibitory effect of the antibiotic; and a mutation in the lipopolysaccharide, which renders the polymyxin class of antibiotics unable to bind this target. Red spheres indicate antibiotics.

## 2.3 ANTIBIOTIC RESISTANCE IN LATIN AMERICA WITH FOCUS ON GRAM-NEGATIVE BACTERIA

Antibiotic resistance is now considered a global health problem that increases the morbidity, mortality and costs of treating infectious diseases (66, 84). However, this issue is particularly serious in developing countries where bacterial infections remain the major causes of morbidity and mortality, especially in childhood (17).

Among the factors that have contributed for this threat are increased antibiotic usage (suboptimal control of the sale, quality, as well as the use of antibiotics in both human and animal medicine), greater movement of people, environmental changes, and poor sewage and

water systems (35, 66, 86). Most of those factors are quite evident in developing countries (109, 110, 152), with poverty as the driving force (28, 109, 133).

The Gram-negative bacteria are among the most important causes of serious nosocomial and community acquired bacterial infections in humans (121, 125, 164) In addition, antibiotic resistance in this group of pathogens has become an increasing relevant health problem worldwide (52, 62, 66, 93, 110, 121, 142). Fluoroquinolone and  $\beta$ -lactam antibiotics are important antibiotic classes used to treat infections caused by Gram-negative bacteria. Emerging resistance mechanisms against these agents have been described in these pathogens (142, 160, 164), and include plasmid mediated quinolone resistance, the production of newer  $\beta$ -lactamases such as plasmid mediated AmpC (e.g. CMY types), CTX-M types, among others. The Antimicrobial Availability Task Force of the Infectious Diseases Society of America has highlighted a triumvirate of Gram-negative organisms that are proving especially problematic: *A. baumannii*, *P. aeruginosa*, and ESBL-producing Enterobacteriaceae (159). The incidence of infection, in particular of severe hospital-acquired infection, caused by these pathogens is increasing (125), and a growing number of isolates exhibit multi-antibiotic resistance, or even pan-antibiotic resistance (52, 93, 121).

The epidemiology of antibiotic resistance can exhibit remarkable geographical variability and rapid evolution over time, due to a complex interplay of factors involved in the selection and spread of different resistant bacteria and resistance genes, which are still only partially understood (61, 62, 66, 84). According to data of the surveillance program for detection of resistance in enteric pathogens established by the Pan-American Health Organization, reports 2004 and 2006 (114, 115) showed that for that period there was a reduction in ampicillin resistance in South American countries. In Brazil, ampicillin resistance in *Salmonella enterica* serotype Enteritidis (*S. enteritidis*) was as low as 3% by 2004, but it decreased to 0.6% by 2006. For *Shigella flexneri* it was as high as 90% by 2004 while in 2006 it decreased to 36%. In Chile, 10% of *Salmonella* spp. were resistant to ampicillin in 2004 versus 7% in 2006; for *Shigella* spp. 68% were resistant to ampicillin in 2004 versus 62% in 2006. The contrary is observed for Central American countries. In Costa Rica, ampicillin resistance in *S. typhimurium* was 44% while in 2006 it showed 67% of resistance to the same antibiotic. In Guatemala, ampicillin resistance in *Salmonella* spp. was of 6% in 2004 and increased to 32% in 2006. For *Shigella* spp. resistance to ampicillin was of 45% in 2004 and of 44% in 2006. Interestingly, in Nicaragua, ampicillin resistance decreased in *Salmonella* spp. from 10% in

2004 to 0% in 2006. But, there was a raise in ampicillin resistance in *Shigella* spp. from 90% in 2004 to 100% in 2006.

Regarding urinary tract infections, the SENTRY Antimicrobial Surveillance Program in its report of *E. coli* causing community-acquired urinary tract infection for year 2003 in Argentina, Brazil, Chile, Mexico and Venezuela, reported an overall resistance to ampicillin of 53%, to ampicillin/sulbactam of 23.3%, to ciprofloxacin of 21.6%, to trimethoprim-sulfamethoxazole of 40.4%, and to nitrofurantoin of 6.9%. The highest resistance to ciprofloxacin was found in Mexico, 72.2%, and the lowest from Brazil, 11.1% (10). In Nicaragua, it was reported that *E. coli* associated with urinary tract infections possess high resistance rates to amoxicillin (82%), trimethoprim-sulphamethoxazole (64%), cephalothin (58%), ciprofloxacin (30%), amoxicillin/clavulanate (21%) and gentamicin (12%) (91).

Information about antibiotic-resistance bacteria causing diseases such as diarrhoea in children or neonatal sepsis is scarce in Nicaragua. Yet such knowledge can be used for an optimal treatment selection, in order to minimize the emergence and to plan an effective infection control-strategy (11, 19, 179). This kind of studies could be of crucial importance to Nicaragua which is part of the Millennium Development Goal for child survival (MDG 4, which calls for a reduction in under-five mortality by two thirds by 2015) (80). The control of antibiotic resistant bacteria can increase the chance of survival in children affected by those pathogens.

### **2.3.1 Non-fermenting Gram-negative bacteria**

Non-fermenting Gram-negative bacteria (unable to ferment sugars to generate energy for cell function) are widespread in the environment and are an increasing cause of nosocomial infections worldwide (45, 49, 93, 125). Many species are notable for their resistance to multiple antibiotics and the facility with which they may acquire further resistances (22, 49, 93, 103). Among the main members of this group with clinical relevance are *P. aeruginosa*, *A. baumannii*, *Stenotrophomonas maltophilia* (49, 93, 128).

*P. aeruginosa* is an opportunistic pathogen that most often infects patients who are immunocompromised, critically ill, burned and/or diabetic (22, 49). The organism also has a propensity to colonise and infect the lungs of cystic fibrosis (CF) patients and is virtually ineradicable in this setting (49). Antibiotic resistance rates differ by geographic region and site of infection. The SENTRY Antimicrobial Surveillance Program, with data reported

between 1997 and 2001, showed that multi-antibiotic-resistant (resistant to piperacillin, ceftazidime, imipenem and gentamicin) *P. aeruginosa* bloodstream isolates were most prevalent in Latin America (12.0–17.6%; average 15.0%), followed by Europe (5.1–14.2%; average 9.3%) and North America (1.6–2.5%; average 2.1%) (70). Of additional concern is the frequent isolation of *P. aeruginosa* resistant to carbapenems, which are often prescribed when bacterial isolates are resistant to cephalosporins and fluoroquinolones. This problem is seen in Latin America, where the resistant rates to carbapenems are higher compared to the United States, with the prevalence of resistance for meropenem doubling from 17% in 1997 to 36% in 2001 (9). The first isolation of carbapenemase VIM2 in Latin America was made from one *Pseudomonas fluorescens* from Chile and three strains of *P. aeruginosa* from Venezuela recovered in year 2002 as part of the SENTRY Antimicrobial Surveillance Program (94). *P. aeruginosa* strains with high rates of resistance to all available antibiotics are now a reality in many hospitals (93). Thus, requiring the introduction of antibiotics such as colistin or tigecycline as a treatment option (22).

*A. baumannii* is usually a commensal in healthy individuals and rarely causes infections in the community setting. However, this pathogen in nosocomial-acquired infections can be associated with pneumonia, skin infections, urinary tract infections, peritonitis, meningitis and often, bacteraemia (22, 49, 59). The antibiotic-resistant nature of the pathogen and its unusual and unpredictable susceptibility patterns make empirical and therapeutic decisions even more difficult (22, 59). According to the SENTRY Antimicrobial Surveillance Program, antibiotic resistance of *Acinetobacter* species isolates from Canada and United States were lower to all recorded antibiotics compared to those from Latin America (59). Another difference is that in Latin America the resistance rates of nosocomial and community isolates were very similar for all antibiotic classes. In Canada and the United States, nosocomial isolates were significantly more resistant to β-lactams than the community-acquired isolates. Production of carbapenemases has also been found in this pathogen, the SENTRY Antimicrobial Surveillance Program reported IMP1 from *Acinetobacter* isolated in Argentina and Brazil, IMP 16 from Brazil, and OXA 23 from *A. baumannii* isolated in Venezuela (56). Dissemination of *A. baumannii* clones with OXA 23 was reported in Colombian hospitals (172).

*S. maltophilia* is related with nosocomial infections such as respiratory infection, bloodstream infection, skin infections, and others (59). The treatment of nosocomial infections caused by *S. maltophilia* is difficult as this pathogen shows high levels of intrinsic or acquired resistance

to different antibiotics, drastically reducing the antibiotic options available for treatment. Intrinsic resistance may be due to reduced outer membrane permeability or to the multi-antibiotic efflux pumps. However, specific mechanisms of resistance such as aminoglycoside modifying enzymes or the heterogeneous production of metallo- $\beta$ -lactamase have contributed to the multi-antibiotic-resistant phenotype displayed by this pathogen (103). Trimethoprim-sulfamethoxazole, ticarcillin-clavulanic acid, gatifloxacin and trovafloxacin should be considered as the empirical choice for clinically suspected *S. maltophilia* infections (59, 103, 151). According to the SENTRY Antimicrobial Surveillance Program, overall rates of resistance to the “antibiotic of choice” (trimethoprim-sulfamethoxazole) ranged from 2% in Canada, in Latin America to 10%, and in Europe (range during study period) 3%–19%. However, strains of *S. maltophilia* isolated in the Asia-Pacific region tended to be more resistant, especially to  $\beta$ -lactams and tetracycline (59). In general, it was found that *S. maltophilia* has emerged a multi-resistant pathogen with trimethoprim-sulfamethoxazole, ticarcillin-clavulanic acid, gatifloxacin and trovafloxacin as some of the agents suitable for treatment of *S. maltophilia* infections.

### **2.3.2 Enterobacteriaceae and $\beta$ -lactamases**

For the ordinary clinician engaged in hospital practice in the late 20th century, choosing therapy for significant infections caused by Enterobacteriaceae was simple. Whether targeted or empirical, cephalosporins and the fluoroquinolones were seen as reliable antibiotic choice. Unfortunately, resistance to cephalosporins, conferred primarily by ESBLs and AmpC enzymes, as well as to fluoroquinolones has increased to levels that now impact on the ordinary clinician’s ability to make confident, reliable choices (44, 69, 121). Many members of the Enterobacteriaceae carry  $\beta$ -lactamases with association of particular genotypes with different geographical regions, for example CTX-M (25, 29, 30, 65). High-level of fluoroquinolone resistance (mainly caused by *gyrA* mutations) has also been shown to be associated with CTX-M and CMY-type enzymes, commonly due to co-carriage on conjugative plasmids of the gene for the aminoglycoside-inactivating enzyme AAC-61-Ib-cr and *qnr* genes (which confer low-level resistance), allowing the easy selection of *gyrA* mutants in the host strain (66).

The use of carbapenems is recommended as therapy option for severe infections caused by Enterobacteriaceae producing ESBL (125, 142). However, this treatment option is also getting compromised since carbapenems resistance has been reported in some members of the Enterobacteriaceae (104).

The members of the Enterobacteriaceae possess many mechanisms of resistance to  $\beta$ -lactam antibiotics such as loss of porin, efflux pumps, etc (157). However,  $\beta$ -lactamases are the most common and clinically significant mechanism of resistance to  $\beta$ -lactam antibiotics among this bacterial group (83, 157, 164). The  $\beta$ -lactamases are enzymes that hydrolyze the  $\beta$ -lactam chemical structure and inactivate the antibiotic. They are typically classified by either the Ambler or the Bush-Jacoby Medeiros classification scheme (8, 26, 27). The Ambler classification scheme is based on the amino acid sequence and divides  $\beta$ -lactamases into class A, C, and D enzymes which utilize serine for  $\beta$ -lactam hydrolysis and class B metallo enzymes which require divalent zinc ions for substrate hydrolysis. The updated functional classification scheme of Bush-Jacoby, takes into account substrate and inhibitor profiles in an attempt to group the enzymes in ways that can be correlated with their phenotype in clinical isolates (26).

ESBLs represent enzymes that have evolved from class A  $\beta$ -lactamases, namely, TEM-1, TEM-2, and SHV-1, which are frequently expressed in Gram-negative bacteria and which confer resistance to ampicillin, amoxicillin, and other penicillins, as well as to early, but not later-generation cephalosporins. ESBLs arose when mutations of the genes encoding TEM-1, TEM-2, or SHV-1 gave rise to new  $\beta$ -lactamases that became able to hydrolyze third-generation cephalosporins and aztreonam (23, 74, 153). TEM- or SHV-type ESBLs are typically not active against cephemycins (e.g., cefotetan, cefoxitin, orceftazidime) or carbapenems (imipenem, ertapenem, and meropenem), and can generally be inhibited by  $\beta$ -lactamase inhibitors such as clavulanate, sulbactam, or tazobactam (122). Enterobacteriaceae may also express ESBLs that are not closely related to TEM- or SHV-related species, including CTX-M- and OXA-type ESBLs, among others (99, 122). CTX-M type ESBLs typically hydrolyze cefotaxime and ceftriaxone more efficiently than ceftazidime, but point mutations around the active site of some enzymes belonging to the CTX-M-1 and CTX-M-9 groups have increased their ability to hydrolyze ceftazidime significantly (129).

Unlike most ESBLs that have been found in *E. coli*, *K. pneumoniae*, and other Enterobacteriaceae, OXA-type ESBLs have been found mainly in *P. aeruginosa* and only rarely in Enterobacteriaceae (99, 122). Another important fact about ESBLs is that they are typically plasmid rather than chromosomally mediated  $\beta$ -lactamases (136). ESBLs should be distinguished from other  $\beta$ -lactamases capable of hydrolyzing extended-spectrum cephalosporins. Examples include AmpC and carbapenemases (69, 85, 137, 140).

Carbapenemases may be further grouped as either metallo- $\beta$ -lactamases (class B) or serine carbapenemases (classes A and D).

Like ESBLs, AmpC  $\beta$ -lactamases hydrolyze third-generation or expanded-spectrum cephalosporins, but unlike ESBLs, they are also active against cephemycins and are resistant to inhibition by clavulanate or other  $\beta$ -lactamase inhibitors (69). Carbapenemases have broader-range activity, covering carbapenems as well as expanded-spectrum cephalosporins. Carbapenemase-producing Enterobacteriaceae are becoming a major threat, since carbapenems are treatment option for Enterobacteriaceae producing ESBL and/or resistant to quinolones (85, 104, 137, 140).

The SHV-type ESBLs may be more frequently found in clinical isolates than any other type of ESBLs (122, 129). The first report of plasmid-encoded  $\beta$ -lactamase that was able to destroy extended-spectrum  $\beta$ -lactam antibiotics was described in Germany in 1983 (74). It was related to the production of a variant of the SHV-1 enzyme, a broad spectrum penicillinase found in *Klebsiella pneumoniae*. In Latin America, the first isolation of an ESBL-producing bacteria was reported from Argentina in 1981 in a strain of *K. pneumoniae* producing SHV-5 (32). Another strain of *K. pneumoniae*, isolated in Santiago, Chile, in 1985, was later proven to be SHV-5 ESBL (64). Currently, SHV-5 and SHV-12 predominate in surveys of resistant isolates in South America (173). In México, Mosqueda-Gómez et al. reported SHV-5 and SHV-2 in ESBL-producing *K. pneumoniae* (ESBLsKp) causing bloodstream infection (98).

Soon after the discovery of the first ESBL, novel  $\beta$ -lactamases were reported which were closely related to TEM-1 and TEM-2, but had the ability to confer resistance to the extended-spectrum cephalosporins (23, 153). The first report of a TEM-type enzyme in Latin America was made by Paterson et al. in 2003 (123), describing TEM-10 and TEM-12 enzymes in bloodstream isolates of *K. pneumoniae*. Yet, TEM-type ESBLs have been very rarely reported from Latin American countries (122). One report showed that TEM-10 and TEM-26 are among the most common TEM enzymes detected in South America (173).

Among the different ESBLs, particular attention should be paid to the worldwide increasing prevalence of the CTX-M types. These enzymes are prevalent not only in nosocomial environment, but also in the community setting. (29, 30, 65, 125, 131, 150, 173) Organisms producing specific CTX-M have been isolated from different countries: CTX-M-9 and CTX-

M-14 are mostly present in Spain, CTX-M-14 in Canada and China, CTX-M-1 in Italy, CTX-M-3 in Poland and CTX-M-2 in several South American countries, Japan and Israel, while CTX-M-15 has been described from all continents except Antarctica (29, 30, 65, 129, 131, 150). The prevalence of CTX-M enzymes in Latin American countries is among the highest in the world (173). The first report of CTX-M in Latin America came from Argentina; an explosive dissemination of nontyphoid *Salmonella* strains resistant to cefotaxime was observed in the beginning of 1989. Starting in a hospital in La Plata, from there, the strains spread to neonatology units of paediatric hospitals in Buenos Aires, and further to neighbouring countries. The  $\beta$ -lactamase CTX-M-2 gene was characterized from a conjugative plasmid of *S. enterica* serovar Typhimurium strain CAS-5 isolated during this outbreak in 1990 (14, 15).

Currently, CTX-M  $\beta$ -lactamases include more than 80 different enzymes that are clustered into six groups based on their amino acid identities and include the CTX-M-1, -2, -8, -9, -25 and -45 groups (129, 150). The members of these clusters exhibit >94% amino acid identity within each group and  $\geq$  90% amino acid identity between the different groups (129). Unlike for most other acquired ESBLs, the original source of genes encoding CTX-M-type  $\beta$ -lactamases is known. The sources of CTX-M determinants are chromosomal genes resident in members of the genus Kluyvera, which includes a number of environmental species with little or no pathogenic activity against humans (150). Multiple genetic mechanisms have apparently been involved in the capture and dissemination of CTX-M enzymes, genes encoding CTX-M  $\beta$ -lactamases have been associated with different genetic platforms, such as the insertion elements IS<sub>Ecp</sub>1, ISCR1 or phage-related sequences that are often located on conjugative plasmids (136). The insertion element IS<sub>Ecp</sub>1 plays an important role in the expression and continuous spread of these  $\beta$ -lactamases (134). The genes responsible for CTXM  $\beta$ -lactamases are encoded by plasmids belonging to both narrow host-range types (IncFII, IncFII, IncHI2 and IncI) or broad host-range types (IncN, IncP-1-a, IncL/M and IncA/C) (31).

Until now SHV, TEM, CTX-M types are the most common  $\beta$ -lactamases that have been reported in Latin America. Yet, new  $\beta$ -lactamases are emerging, e. g. PER-2 which seems to be restricted to Argentina and bordering countries such as Chile, Paraguay and Uruguay (141), and two novel  $\beta$ -lactamases GES-1 (135) and BES-1 (18).

## **2.4 NEONATAL SEPTICAEMIA AND GRAM-NEGATIVE BACTERIA**

The division between developed and developing regions – particularly the least developed countries – is perhaps greater on children mortality than on almost any other issue. The proportion of deaths occurring in the neonatal period (aged 0–27 days) has increased from 37% in 2000–03 (24), to 41% in 2008 (17), particularly affecting neonates of developing countries. In this age-group, the greatest single causes of death were preterm birth complications and birth asphyxia, but collectively infectious causes were also important, especially septicaemia and pneumonia (17, 79).

Neonatal infection in developing countries can occur in hospital and community settings (163, 183) and possess a common denominator Gram-negative bacteria as the major cause of neonatal infections (36, 154). Yet, neonatal infections and common pathogens are traditionally divided in early onset (those occurring in the first 72 hours of life, major pathogens: Group B *Streptococcus*, *E. coli* and *Listeria monocytogenes*) and late onset (those occurring after 72 hours of life, major pathogens: coagulase-negative staphylococci, *Staphylococcus aureus*, and Gram-negative organisms such as *Klebsiella*, *Pseudomonas* and *Serratia* spp.) infections (100, 169).

Some special characteristics found in developing countries such as lack of appropriate hygiene during labour and delivery, postnatal care and feeding have made Gram-negatives pathogens the major cause of infections in those settings (36, 111, 169, 183, 184).

Selection of empiric antibiotic therapy for neonates should depend on the target organisms and their antibiotic susceptibility, spectrum of antibiotic activity, association with emergence of resistance, antibiotic distribution, therapeutic index, cost of therapy and ease of use. Furthermore, the pharmacokinetics of antibiotics in neonates differ from those in older children. Pre-term neonates have additional limitations due to organ system immaturity. In particular, gastro-intestinal absorption of oral antibiotics is reduced and varies considerably among neonates (175).

Empiric antibiotic therapy of a β-lactam antibiotic with an aminoglycoside (commonly ampicillin and gentamicin) is initiated after obtaining cultures from patients with suspected septicaemia (168, 175, 176). Ampicillin is preferred to penicillin for the treatment of neonatal septicaemia as it is also active against some Gram-negative pathogens, especially *E. coli*, one of the three most frequent causes of neonatal septicaemia in developing countries. It acts

synergistically with aminoglycosides, has good penetration in the cerebrospinal fluid (CSF) and can be administered parenterally as well as orally. In view of its safety and efficacy, most experts recommend it as a first line empiric therapy for serious neonatal infections in combination with an aminoglycoside. Amoxicillin is similar to ampicillin in its spectrum. Twice daily oral dosing is theoretically adequate in neonates. Addition of clavulanate broadens the spectrum against many  $\beta$ -lactamase producing strains. However, absorption varies and in very low birth-weight neonates amoxicillin-clavulanate increases the risk of necrotizing enterocolitis (175).

Aminoglycosides form the sheet anchor of antibiotic therapy of serious neonatal infections because of the broad-spectrum activity against Gram-negative bacteria as well as staphylococci. Amikacin is active against many nosocomial Gram-negative organisms and it is used in case of gentamicin resistance (168, 175). Tobramycin has especially low minimum inhibitory concentrations (MIC) against *Pseudomonas*. Therapeutic plasma aminoglycoside concentration is achieved equally well by intravenous as by intramuscular routes of administration. There is enough data to show that once daily dosing of gentamicin is effective and safe for treating neonates. Once daily dosing leads to a higher and quicker peak serum level resulting in prolonged efficacy and greater initial bacterial killing. There is a lower risk of ototoxicity and nephrotoxicity with single daily dosing regimens because of the longer phase of sub-toxic antibiotic levels (175).

Cephalosporins are not recommended for routine use as first-line agents for the treatment of neonatal septicaemia. A third generation cephalosporin, often in combination with an aminoglycoside, is commonly used as the second line of treatment of newborn septicaemia (175). A third generation cephalosporin is used if there is evidence of meningitis, in view of cephalosporin's excellent CSF penetration compared to aminoglycosides. For example, ceftriaxone has excellent CSF penetration and requires only single daily dosing (168, 175).

Although carbapenems are stable against most  $\beta$ -lactamases (especially ESBL)-producing Enterobacteriaceae, their safety profiles have not been established in neonates. Yet not approved, meropenen is the most frequently antibiotic used for the treatment of neonatal infections (168).

Aztreonam is a monobactam ( $\beta$ -lactam agent with side chains) agent that is effective against aerobic Gram-negative rods, including Enterobacteriaceae, *Haemophilus* and *Neisseria*. It is

synergic with aminoglycosides against *Pseudomonas* and antibiotic-resistant Gram-negative rods in vitro and appears to be well tolerated in neonates (168).

Other antibiotics such as chloramphenicol, quinolones, trimethoprim-sulphamethoxazole are not recommended due to insufficient safety data. trimethoprim-sulphamethoxazole has the most extensive evidence base for community-based treatment of serious neonatal bacterial infections, although emergence of resistance threatens the utility of this agent (39, 175). Ciprofloxacin is also increasingly accepted as safe in neonates and warrants further investigation for treatment of infections in neonates. Because of the emergence of multiple antibiotic-resistant strains of Gram-negative organisms, ciprofloxacin is being used with increasing frequency in the treatment of serious infections among paediatric patients and neonates (39). The potential for significant life-threatening toxicity among neonates associated with chloramphenicol makes it the least preferred of the agents for empiric therapy (40).

The concern about antibiotic resistant bacteria is also found in neonatal infections (162, 169, 183). It has been estimated that 70% of the bacteria causing neonatal infections in hospitals of developing countries may not be covered by the World Health Organization's (WHO) recommended empiric regimen of ampicillin and gentamicin for neonatal septicaemia (175-177). Furthermore, there has been an increase of resistance to more expensive second and third line antibiotics. For example, the high rates of resistance to cefotaxime found among *E. coli* (46%) and among *Klebsiella* spp. (51%) suggest a widespread dissemination of resistant strains in hospital settings (183).

Although antibiotic resistance data from neonatal septicaemia in the community is scarce, Thaver et al. searched the literature published since 1990 for studies carried out in developing countries reporting resistance among serious community-acquired infections in neonates. The main finding showed a high proportion of *E. coli* resistant to ampicillin (72%) and trimethoprim-sulfamethoxazole (78%); in addition, 19% were resistant to third generation cephalosporins. Among *Klebsiella* spp., almost all were resistant to ampicillin, 45% to trimethoprim-sulfamethoxazole and 66% to third generation cephalosporins. Resistance to gentamicin was low among *E. coli* (13%), but much higher among *Klebsiella* spp. (60%).

The emergence of resistant pathogens is a problem for both institutions and communities (183, 184) of developing countries. A lack of control of antibiotic use, limited legislation on

prescription, over-the-counter sale of antibiotics, poor sanitary conditions, a lack of basic facilities that would help hand-washing as well as the lack of surveillance for standards of health-care facilities are the key factors for the emergency and transmission of antibiotic resistant Gram-negative bacteria in neonatal intensive care unit (NICU) (111, 183).

## **2.5 DIARRHOEA IN CHILDREN, *E. COLI* AND ANTIBIOTICS**

Despite that global mortality rate in children less than 5 years of age has declined from 10.6 million per year during 2000–03 (24) to 8.795 million deaths occurred in 2008 (17), diarrhoea still remains the second leading cause of death in children younger than 5 years globally (17, 90).

Diarrhoeal illness rarely requires antibiotic treatment and can be prevented by improving living conditions. However, in few cases antibiotics are used to treat severe diarrhoea caused by bacterial pathogens, especially in cholera and shigellosis (178, 181) and to prevent diarrhoea in travellers (48, 106). Antibiotics are also indicated to help reduce severity and duration of disease, to manage or prevent serious extraintestinal complications, to shorten the period of pathogen excretion and thus the dissemination of the infection (58, 107). It is important to remember that antibiotics are widely available over the counter and through other unregulated outlets in a number of developing countries, thus probably use as empirical therapy for diarrhoea may be common in the community setting (96).

A diversity of bacteria, viruses and parasites can cause diarrhoea. Diarrhoeagenic *E. coli* (DEC) are among the bacteria most frequently associated with diarrhoea in children from developing countries (106, 107, 161). DEC remains an important aetiological agent of infantile diarrhoea in Nicaragua (92, 118, 171), yet, there are no recent studies regarding the antibiotic resistance among DEC.

Mayatepek et al. reported in 1993 high levels of resistance to ampicillin (>82%), chloramphenicol (44%) and trimethoprim-sulfamethoxazole (>62%) among DEC affecting Nicaraguan children (92). Report of antibiotic resistance in DEC is also found in other Latin American countries, resistance in DEC isolated from children less than 5 years was demonstrated in Mexico with more than 70% of resistance to ampicillin and trimethoprim-sulfamethoxazole in hospitalized patients (51). In Peru, in infants followed-up from 2 to 12 months of age, it was found that diarrhoeagenic *E. coli* as a group exhibited high levels of antibiotic resistance in diarrhoeal cases to ampicillin (85%), trimethoprim-sulfamethoxazole

(79%), tetracycline (65%), and nalidixic acid (28%). Among individual *E. coli* groups in patients with diarrhoea, diffusely adherent *E. coli* and enteroaggregative *E. coli* (EAEC) exhibited significant higher frequencies of resistance to ampicillin, trimethoprim-sulfamethoxazole, tetracycline and nalidixic acid than enteropathogenic *E. coli* and enterotoxigenic *E. coli*. Antibiotic resistance to ampicillin and trimethoprim-sulfamethoxazole were more frequent in *E. coli* isolated from diarrhoeal samples than controls, which reflected greater antibiotic exposure in patients with gastroenteritis (108).

Management of diarrhoea is relatively simple: appropriate hydration with fluid and electrolyte therapy. Yet, the use of other measures is recommended when necessary, for example probiotics, zinc supplements and antisecretory antibiotics that may have a mild favourable impact (106, 166, 176, 178). Thus, the use of antibiotics for treatment of diarrhoea should be considered only in extreme cases and, whenever possible, based on stool cultures.

## **2.6 ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT**

The importance of characterization of the prevalence of antibiotic resistant bacteria in an environmental reservoir such as water is that the environmental source is not only a way of dissemination of antibiotic resistant microorganisms among human and animal populations, but also the route by which resistance genes are introduced in natural bacterial ecosystems. Kümmerer showed in a review about antibiotics in aquatic environment that bacteria which become resistant through the use of antibiotics in medical treatment are an important source for resistance material found in hospital effluents, municipal sewage and sewage treatment plant (78). Kim et al. showed in a review about the impact of antibiotic and antibiotic resistant bacteria from wastewater treatment plants that these sites provide favourable conditions for the proliferation of antibiotic resistant bacteria and spread of resistance genes to non-resistant bacteria (72).

### **3 AIMS OF THE THESIS**

The major aims of this thesis were:

- To determine the prevalence of antibiotic-resistant Gram-negative bacteria causing neonatal septicaemia in a neonatal intensive care unit in León, Nicaragua and their relations with the neonatal intensive care unit environment.
- To determinate the antimicrobial resistance profile and mechanisms of resistance of diarrhoeagenic and non-diarrhoeagenic *Escherichia coli* isolated from Nicaraguan children.
- To investigate the antibiotic resistance patterns of *Escherichia coli* isolates from different aquatic environmental sources in León, Nicaragua.

## **4 METHODOLOGY**

A brief description of the methodology used in this thesis is shown below. Further details can be found in individual papers.

### **4.1 STUDY AREA**

All clinical samples investigated in the present thesis came from subjects living in León, Nicaragua. Nicaragua is a country situated in the middle of the Central American isthmus that bridges the North and South America subcontinents, bordered by both the Caribbean Sea and the North Pacific Ocean, between Costa Rica and Honduras (68). Nicaragua is made up of 15 departments and two autonomous regions, all of them located in three macro-regions: the Pacific region (with León city being part of it), the Central region, and the Atlantic region (68, 180). Nicaragua has an estimated population of ~ 5.1 million inhabitants and 11.8 % are children less than 5 years of age (67). The climate of this country is tropical in both the Pacific and the Atlantic regions, while cooler in the Central region. Two seasons are found in this country, summer (December to May) and winter (June to November) (68). The municipality of León has an area of 820 km<sup>2</sup>. It is the second largest city of Nicaragua. León municipality is located within 90 km of the capital Managua. The population is estimated to be 177,000 inhabitants. The health system is organized on two levels: primary healthcare is provided by health posts serving a population of about 30,000, and secondary-level care is provided mainly by the regional hospital (126).

### **4.2 STUDY POPULATION**

#### **4.2.1 Papers I and II**

Blood samples were taken from 135 neonates, 95 males and 40 females, admitted to the NICU of the Hospital Oscar Danilo Rosales Arguello (HEODRA) in León, Nicaragua between August and October 2005 as a prospective surveillance study. Patients enrolled in the study were neonates (less than 28 days of age) with clinical symptoms of septicaemia and had a positive blood culture. In addition, 98 samples from the NICU environmental screening were collected once per month during the study period. The sites for environmental sampling were selected from all objects (i. e., beds, incubators, sinks) that were located in the NICU's room and suspected of possible source of infection.

Further analyses were carried out in 34 Gram-negative bacterial strains isolated from 28 neonates with clinical symptoms of septicaemia and in 38 Gram-negative bacterial strains from the NICU environment at the hospital.

#### **4.2.2 Paper III**

The total number of *E. coli* isolates, analyzed in this study, was 727. Three hundred and ninety-five isolates were non-DEC (270 from children with diarrhoea and 125 from children without diarrhoea, respectively), and 332 were DEC (241 from children with diarrhoea and 91 from children without diarrhoea, respectively). The distribution of the DEC isolates herein analyzed was as follows: 203 enteroaggregative *E. coli* isolates (EAEC), 73 enterotoxigenic *E. coli* isolates (ETEC), 47 enteropathogenic *E. coli* isolates (EPEC), 8 enterohaemorrhagic *E. coli* isolates (EHEC) and 1 enteroinvasive *E. coli* isolate (EIEC).

#### **4.2.3 Paper IV**

Water environmental sampling was carried out through October 2008 to May 2009 from different localities of León, Nicaragua. Samples were collected once in each (i) household drinking water (n=20); (ii) wells water (n=87) used for consumption; (iii) sewage water from two municipal sedimentation treatment plants (two samples of each influent and effluent, n=8) and (iv) from the sewage effluents of the main hospital of León (n=3).

In this study, a total of 142 *E. coli* isolates were analyzed from 8 of the well water samples, 87 *E. coli* isolates were analyzed from 7 sewage water samples from the two municipal sedimentation treatment plants, and 96 *E. coli* isolates from 3 water samples of the sewage effluents of the main hospital of León.

### **4.3 ANTIBIOTIC SUSCEPTIBILITY TESTING**

Minimal inhibitory concentrations (MICs) were determined by the agar dilution method according to the Clinical and Laboratory Standards Institute guidelines (34). *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as control strains. Further description of the antibiotics tested can be found in individual papers.

### **4.4 PHENOTYPIC DETECTION OF ESBL**

ESBL production in the Gram-negative bacteria that showed resistance to any of the third generation cephalosporins tested (cefotaxime, ceftriaxone, and ceftazidime) were analyzed

using the Etest® system (Biomérieux); cefotaxime/cefotaxime + clavulanic acid, ceftazidime/ceftazidime + clavulanic acid, and cefepime/cefepime + clavulanic acid.

**Table 1.** List of antibiotics tested in this thesis.

Antibiotic name	Breakpoints	Paper 1	Paper 2	Paper 3	Paper 4
Ampicillin	S<=8 R>=32			*	*
Amoxicillin/Clavulanic acid	S<=8 R>=32	*	*	*	*
Ceftazidime	S<=8 R>=32	*	*	*	*
Ceftriaxone	S<=8 R>=64	*	*	*	*
Cefotaxime	S<=8 R>=64				*
Ciprofloxacin	S<=1 R>=4	*	*	*	*
Chloramphenicol	S<=8 R>=32			*	*
Gentamicin	S<=4 R>=16	*	*	*	*
Imipenem	S<=4 R>=16	*	*	*	
Nalidixic acid	S<=16 R>=32				*
Trimethoprim/Sulfamethoxazole	S<=2 R>=4	*	*	*	*

#### 4.5 DETECTION OF $\beta$ -LACTAMASE GENES BY PCR AMPLIFICATION

Papers I to IV: ESBL positive Gram-negative strains were screened for the resistance genes encoding SHV, TEM, CTX-M and OXA enzymes by a multiplex PCR assay using universal primers following the procedure described by Fang et al. (55).

#### 4.6 TYPING OF ESBL-PRODUCING *E. COLI* ISOLATES BY RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

Papers I, II, and IV: The epidemiological relationships were analyzed by RAPD as described by Touati et al. (165) with some modifications. Total DNA was prepared with QIAamp® DNA mini Kit (Qiagen, Solna, Sweden) and used for RAPD typing. The experiment was performed using puReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK) together with the following primers: primer 4 (5'-AAGAGCCCGT-3') and primer 5 (5'-AACGCGCAAC -3') (Thermo Fisher Scientific, Limburg, Germany). PCR amplification was carried out as follows: 1 cycle at 94 °C for 5 min, 35 (primer 4) and 31 (primer 5) cycles at 94°C for 5 sec, 42°C for 30 sec and 72°C for 1 min, with a final extension period at 72 °C for 5 min. After amplification, the banding pattern of randomly amplified DNA was visualized and analyzed on 1.5% agarose gel in Tris-acetate buffer 1X. A negative control was included in each PCR run with no target DNA. Reproducibility of the amplification results was evaluated in parallel experiments by the repetition of the PCR reactions three times. Electrophoresed agarose gels were analysed in paper I and II using the program Molecular Analyst/PC Fingerprint Version 1.12 (Bio-Rad Laboratories, Hercules, CA, USA) and in

paper IV using the BioNumerics® version 6 software (Applied Maths, Sint-Martens-Latem, Belgium). Dendograms based on Jaccard coefficient and Unweighted Pair Group Method using Arithmetic Averages (UPGMA) were generated.

#### **4.7 FURTHER ANALYSIS OF THE CTX-M-PRODUCING GRAM-NEGATIVE BACTERIA**

The following analyses were not included in papers I to III:

- Detection of CTX-M groups 1, 2, 9, 8 and 25 was performed using a multiplex PCR assay as described by Dallenne et al. (38) and a single PCR assay as described by Pitout et al. (130).
- To identify the  $\beta$ -lactamase genes detected in the PCR assays for SHV, TEM, OXA and CTX-M groups, DNA sequence analyses of the amplicons were performed. Based on the RAPD analysis, representative isolates (mostly, those isolates that were positive for CTX-M) from each clonal group were selected for sequencing analysis for the paper I to IV. Amplified PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Solna, Sweden) and bidirectional sequencing was performed. Each sequence was then compared with the known  $\beta$ -lactamase genes sequences (<http://www.lahey.org/studies/>) by multiple-sequence alignment using the BLAST program.

## 5 RESULTS

### 5.1 NEONATAL SEPTICAEMIA (PAPERS I AND II)

#### 5.1.1 Patients and microbial identification

Thirty-four Gram-negative bacterial strains were isolated from 40 neonates with clinical symptoms of septicaemia and 38 Gram-negative bacterial strains from the NICU environment at the hospital. The gender distribution was as follow: 28 neonates were males and 12 females. One, or more than one, antibiotics were used in 24 of 40 neonates before sampling. The antibiotics used were: amikacin (21/24), ceftriaxone (12/24), ampicillin (10/24), dicloxacillin (4/24), ampicillin-sulbactam (3/24), ceftazidime (3/24), penicillin (2/24), gentamicin (2/24), chloramphenicol (1/24), and clindamycin (1/24).

Seven species of Gram-negative bacteria were isolated from neonates with septicaemia and eight species from the NICU's environment. The most common Gram-negative bacteria affecting neonates were *Klebsiella pneumoniae*, *Serratia marcescens* and *S. liquefaciens*.

Gram-negative bacteria were related to the death of 10 of the neonates. Moreover, two of these deaths occurred in neonates where more than one Gram-negative species was isolated. Regarding the isolation of more than one species from a neonate, *S. liquefaciens* together with *S. marcescens* were isolated in four neonates (1 of 4 died) and *Chryseomonas luteola*, *K. pneumoniae*, and *S. marcescens* in one neonate that died. The rest of the deaths were related to *K. pneumoniae* (5 of 10) and coagulase-negative staphylococci (3 of 10).

*E. cloacae*, *E. coli*, *K. pneumoniae* and *S. liquefaciens* were isolated from both neonates with septicaemia and the NICU's environment. *A. baumannii*, *Enterobacter sakazakii*, *P. aeruginosa*, and *S. maltophilia* were isolated only in the NICU's environment and *C. luteola*, *Pantoea* spp. and *S. marcescens* only from neonates with septicaemia (Table 2).

#### 5.1.2 Antibiotic susceptibility testing

The antibiotic susceptibility testing data are shown in Table 2. More than 85% of *K. pneumoniae* strains from neonates with septicaemia and the environment were resistant to ceftazidime, ceftriaxone, gentamicin, and trimethoprim-sulfamethoxazole. Resistance to amoxicillin-clavulanic acid was low in both groups (28.6 and 16.7%), resistance to ciprofloxacin (25%) and imipenem (8.3%) was found only in *K. pneumoniae* strains from the environment. Ciprofloxacin and imipenem were the most active antibiotics against *K.*

*pneumoniae* strains isolated from the neonates with septicaemia (MIC90 of 0.064 mg/L for ciprofloxacin and 0.25 mg/L for imipenem).

**Table 2.** Characteristics and percent of resistance of the Gram-negative bacteria from the neonates with septicaemia and from the NICU's environment.

Organism	No. of isolates	Percent of resistance						No. of isolates harboring $\beta$ -lactamase gene				
		AMC	CAZ	CRO	CIP	GEN	IPM	SXT	SHV	TEM	CTX-M	E-test ESBL
<i>C. luteola</i> <sup>n</sup>	2	100	50	50				100		2		
<i>E. cloacae</i> <sup>n</sup>	1	100	100	100		100		100		1		1
<i>E. coli</i> <sup>n</sup>	1			100						1		
<i>K. pneumoniae</i> <sup>n</sup>	14	28.6	100	100		100		100	13	13	12	14
<i>Pantoea</i> spp. <sup>n</sup>	1	100		100				100		1		
<i>S. liquefaciens</i> <sup>n</sup>	6	100	50	100		100		100		6	6	6
<i>S. marcescens</i> <sup>n</sup>	9	77	22	100		100		100		9	9	9
<i>A. baumannii</i> <sup>e</sup>	10	70	70	80	60	80		80		8		
<i>E. cloacae</i> <sup>e</sup>	5	80	80	80	20	80		60	2	5	3	3
<i>E. coli</i> <sup>e</sup>	2	50	100	100	50	50		100		2	2	2
<i>E. sakazakii</i> <sup>e</sup>	1			100								
<i>K. pneumoniae</i> <sup>e</sup>	12	16.7	91.7	91.7	25	91.7	8.3	91.7	10	11	10	11
<i>P. aeruginosa</i> <sup>e</sup>	3	100	33	100	100	100		100		2		
<i>S. maltophilia</i> <sup>e</sup>	2	100		100	50	100	100	50				
<i>S. liquefaciens</i> <sup>e</sup>	3	100	66	66		66		66		2	2	2

<sup>n</sup>, Bacteria isolated from a neonate with septicaemia; <sup>e</sup>, bacteria isolated from NICU's environment; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CRO, ceftriaxone; CIP, ciprofloxacin; GEN, gentamicin; IPM, imipenem; and STX, trimethoprim-sulfamethoxazole.

Different resistance patterns were defined in both groups of *K. pneumoniae* strains. The most prevalent multi-resistance pattern (resistance to at least two antibiotics) was ceftriaxone, ceftazidime, gentamicin, and trimethoprim-sulfamethoxazole, in 58% of the *K. pneumoniae* strains from the environment and 57% from neonates with septicaemia. Multi-antibiotic resistance was detected in 96% of the *K. pneumoniae* strains.

The other Gram-negative bacteria, from the neonates with septicaemia, showed high resistance to amoxicillin-clavulanic acid (85%), ceftazidime (35%), ceftriaxone (95%), gentamicin (80%) and trimethoprim-sulfamethoxazole (95%). The most prevalent multi-antibiotic resistance patterns in this group of strains were (i) amoxicillin-clavulanic acid, ceftriaxone, gentamicin, trimethoprim-sulfamethoxazole (40%) and (ii) amoxicillin-clavulanic acid, ceftriaxone, ceftazidime, gentamicin, trimethoprim-sulfamethoxazole (30%).

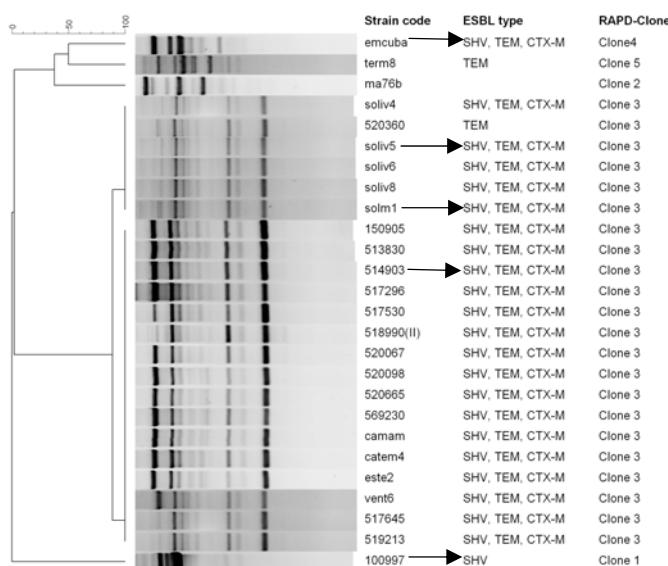
Regarding the NICU's environment, the rest of the Gram-negative bacterial strains in this group were resistant to amoxicillin-clavulanic acid (80%), ceftazidime (62%), ceftriaxone (85%), ciprofloxacin (46%), gentamicin (77%), imipenem (8%) and trimethoprim-sulfamethoxazole (73%). The most prevalent multi-antibiotic resistance patterns in this group of strains were (i) amoxicillin-clavulanic acid, ceftriaxone, ceftazidime, ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole (23%); (ii) amoxicillin-clavulanic acid, ceftriaxone, ceftazidime, gentamicin and trimethoprim-sulfamethoxazole (15%).

### 5.1.3 ESBL detection

The E-test for ESBL detection showed that 88% (30/34) of the Gram-negative bacteria isolated from neonates with clinical symptoms of septicaemia and 47% (18/38) from the NICU's environment produced ESBL.

### 5.1.4 Typing of ESBL-producing Gram-negative bacteria by RAPD

Typing by RAPD revealed that the 26 *K. pneumoniae* strains could be separated into five clones. Among the five clones, clone 3 encompasses nine of the *K. pneumoniae* strains isolated from NICU's environment and 13 of the *K. pneumoniae* strains isolated from the neonates with septicaemia (Fig. 2). Twelve of the clone 3 strains and single clone 4 harboured genes coding for SHV, TEM and CTX-M enzymes. One of the strains from clone 3 and single clones 1 and 5 harboured only genes coding for SHV or TEM enzymes.



**Figure 2.** Clonal similarity of the *K. pneumoniae* strains from the neonates with septicaemia and from the NICU's environment by RAPD-PCR.

*E. cloacae*, *E. coli* and *S. liquefaciens*, isolated from both neonates with septicaemia and the NICU's environment, were also subjected to RAPD analysis. No clonal similarity among these organisms was observed. The prevalence of the genes encoding for the different  $\beta$ -lactamases in the rest of Gram-negative strains were: 0 for SHV, 100% for TEM and 75% for CTX-M in strains isolated from the neonates with septicaemia and 8% for SHV, 73% for TEM, and 27% for CTX-M in strains isolated from the NICU's environment. None of these strains harboured the gene for the OXA enzymes.

## **5.2 INTESTINAL *E. COLI* FROM CHILDREN (PAPER III)**

### **5.2.1 Antibiotic susceptibilities in the *E. coli* isolates**

In general, resistance to ampicillin was found in 67.7% (225/332) of the DEC isolates and in 53.2% (210/395) of the non-DEC isolates. For trimethoprim-sulfamethoxazole, resistance was found in 71.6% (238/332) of the DEC isolates and 57.7% (228/395) of the non-DEC isolates. Furthermore, resistance to chloramphenicol was found in 9.3% (31/332) of the DEC isolates and in 13% (51/395) of the non-DEC isolates. No resistance to imipenem was observed, and for the other agents the level of resistance was low in all *E. coli* isolates ( $\leq$  2.6%).

### **5.2.2 Resistance patterns in the *E. coli* isolates**

Different resistance patterns were defined in all the *E. coli* isolates with and without diarrhoeagenic virulence markers. The two most prevalent multi-resistance patterns (resistance to at least two antibiotics), among the 727 *E. coli* isolates included in this study, were (i) ampicillin and trimethoprim-sulfamethoxazole (41%), and (ii) ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole (7.2%).

### **5.2.3 Antibiotic susceptibility differences among *E. coli* categories**

The differences in the distributions of resistance and MICs were seen for individual antibiotics and for each category of *E. coli* (Table 3). The multiple comparisons showed a significant difference in resistance to amoxicillin-clavulanic acid ( $P=0.031$ ), ampicillin ( $P<0.001$ ) and trimethoprim-sulfamethoxazole ( $P<0.001$ ).

#### *5.2.3.1 Enteropathogenic *E. coli* (EAEC)*

When the comparisons of antibiotic resistance of two groups of *E. coli* were performed, EAEC from children with diarrhoea showed significant difference in resistance when compared to other *E. coli* categories isolated from children with and without diarrhoea. The

EAEC were significantly more resistant ( $P<0.005$ ) to ampicillin and trimethoprim-sulfamethoxazole as compared to EHEC and EPEC. Interestingly, EAEC from children with diarrhoea also showed to be significantly more resistant ( $P<0.005$ ) to amoxicillin-clavulanic acid, ampicillin and trimethoprim-sulfamethoxazole as compared to non-DEC from children with and without diarrhoea. In addition, EAEC were significantly more resistant ( $P=0.021$ ) to amoxicillin than ETEC from children with diarrhoea.

EAEC strains from children without diarrhoea were significantly more resistant ( $P<0.005$ ) to ampicillin and trimethoprim-sulfamethoxazole than EPEC and to amoxicillin-clavulanic acid, ampicillin and trimethoprim-sulfamethoxazole ( $P<0.05$ ) as compared to non-DEC from children without diarrhoea. Yet EAEC from children without diarrhoea showed significantly lower resistance ( $P<0.05$ ) to chloramphenicol than EHEC from children with diarrhoea and non-DEC from children without diarrhoea.

#### 5.2.3.2 *Enterotoxigenic E. coli (ETEC)*

ETEC from children with diarrhoea were significantly more resistant ( $P<0.005$ ) to ampicillin as compared to EPEC from children with diarrhoea and to non-DEC from children with diarrhoea. ETEC from children with diarrhoea showed also significantly more resistance ( $P<0.005$ ) to ampicillin and trimethoprim-sulfamethoxazole as compared to EPEC from children without diarrhoea and non-DEC from children without diarrhoea.

#### 5.2.3.3 *Non-diarrhoeagenic E. coli (non-DEC)*

In relation to non-DEC, the group of *E. coli* from children with diarrhoea were more resistant ( $P=0.018$ ) to ampicillin compared to EPEC from children without diarrhoea. When comparing, non-DEC from children with diarrhoea versus non-DEC from children without diarrhoea, the former were significantly more resistant ( $P<0.005$ ) to amoxicillin-clavulanic acid, ceftazidime and trimethoprim-sulfamethoxazole, whereas non-DEC from children without diarrhoea were significantly more resistant ( $P<0.005$ ) to chloramphenicol and ceftriaxone.

### 5.2.4 Detection of $\beta$ -lactamase genes

The gene coding for TEM enzyme was detected in most isolates, with higher prevalence in EAEC isolates from children with diarrhoea (12.7%), and in non-DEC from children without diarrhoea (13%). The gene CTX-M was more commonly detected in EAEC (4.3%) and in

**Table 3.** Distribution of resistance and MICs for individual antibiotics and each *E. coli* positive for a characteristic DEC marker.

Children with diarrhoea				Children without diarrhoea				
Organism and agent	Minimal inhibitory concentration (mg/L)			Organism and agent	Minimal inhibitory concentration (mg/L)			
	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC Range	n (% R)	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC Range	n (% R)
EAEC (134)				EAEC (69)				
AMC	8	16	2 - 32	3 (2.2)	AMC	8	16	2 - 32
AMP	128	128	2 - 128	105 (78.4)	AMP	128	128	2 - 128
CAZ	0.25	0.5	0.032 - 32	2 (1.5)	CAZ	0.25	0.5	0.032 - 16
CHL	4	128	2 - 128	16 (11.9)	CHL	4	8	2 - 128
CRO	0.064	0.125	0.03 - 128	2 (1.5)	CRO	0.064	0.125	0.03 - 128
CIP	0.032	0.064	0.016 - 8	2 (1.5)	CIP	0.032	0.032	0.008 - 0.5
GEN	1	2	0.25 - 32	3 (2.2)	GEN	1	1	0.064 - 1
IMP	0.125	0.5	0.094 - 2		IMP	0.125	0.5	0.032 - 2
SXT	4	4	0.032 - 4	108 (80.6)	SXT	4	4	0.016 - 4
ETEC (64)				ETEC (9)				
AMC	8	16	2 - 16		AMC	8	16	2 - 16
AMP	128	128	2 - 128	45 (70.3)	AMP	128	128	2 - 128
CAZ	0.25	0.5	0.016 - 16		CAZ	0.25	0.25	0.12 - 0.25
CHL	4	8	2 - 128	6 (9.4)	CHL	4	128	2 - 128
CRO	0.064	0.125	0.032 - 0.5		CRO	0.064	0.064	0.032 - 0.064
CIP	0.032	0.064	0.016 - 2	1 (1.6)	CIP	0.032	0.032	0.016 - 0.032
GEN	0.5	1	0.25 - 16	3 (4.7)	GEN	1	1	0.5 - 1
IMP	0.125	0.5	0.064 - 1		IMP	0.25	0.5	0.12 - 0.5
SXT	4	4	0.032 - 4	44 (68.8)	SXT	4	4	0.032 - 4
EPEC (34)				EPEC (13)				
AMC	8	16	2 - 32	2 (5.9)	AMC	4	8	2 - 16
AMP	2	128	2 - 128	16 (47.1)	AMP	2	128	2 - 128
CAZ	0.25	0.25	0.032 - 5		CAZ	0.25	0.25	0.032 - 0.5
CHL	4	8	2 - 128	3 (8.8)	CHL	4	8	40217
CRO	0.064	0.25	0.032 - 0.25		CRO	0.064	0.25	0.032 - 0.25
CIP	0.032	0.064	0.016 - 1		CIP	0.032	0.032	0.016 - 0.5
GEN	1	2	0.5 - 2		GEN	1	1	0.5 - 2
IMP	0.25	0.5	0.12 - 1		IMP	0.125	0.5	0.12 - 0.5
SXT	4	4	0.032 - 4	21 (61.8)	SXT	0.064	4	0.016 - 4
EHEC (8)								
AMC	2	16	2 - 16					
AMP	2	128	2 - 128	3 (37.5)				
CAZ	0.125	1	0.125 - 1					
CHL	4	128	2 - 128	2 (25)				
CRO	0.064	0.5	0.032 - 0.5					
CIP	0.032	0.032	0.016 - 0.032					
GEN	1	2	0.5 - 2					
IMP	0.25	0.5	0.125 - 0.5					
SXT	0.064	4	0.064 - 4	4 (50)				

non-DEC from children without diarrhoea (5.6%). Among the isolates harbouring CTX-M gene, 13/13 were resistant to ampicillin, 13/13 to ceftazidime, 10/13 to chloramphenicol, 13/13 to ceftriaxone, 5/13 to ciprofloxacin, 8/13 to gentamicin, and 13/13 to trimethoprim-sulfamethoxazole. The gene encoding for OXA enzyme was detected in EAEC isolates from children with diarrhoea and in non-DEC from children with/without diarrhoea.

### **5.3 ENVIRONMENTAL WATER (PAPER IV)**

#### **5.3.1 Selection of the *E. coli* isolates for antibiotic susceptibility testing**

Antibiotic resistant bacteria were detected in 3/3 of the hospital sewage water samples, 8/37 of the well water samples and in 7/8 sewage water samples from the municipal sedimentation treatment plants. A total of 325 *E. coli* isolates that fulfilled the selection criteria were included in this study.

#### **5.3.2 Antibiotic susceptibilities in the selected *E. coli* isolates**

The results of the antibiotic susceptibility testing performed on 325 *E. coli* isolates are shown in Table 4. In general, the *E. coli* isolates showed high level of resistance to ampicillin (60%), trimethoprim-sulfamethoxazole (57%) and chloramphenicol (45%). Furthermore, the *E. coli* isolates also showed resistance to nalidixic acid (35.7%), ciprofloxacin (34%), all of the third generation cephalosporin tested (14.2%) and yet low, to amoxicillin-clavulanic acid (0.3%).

High levels of antibiotic resistance were found in the *E. coli* isolates from the hospital sewage water and the well water samples (Table 4). Among the hospital sewage water samples, *E. coli* isolates from samples H1B and H1C showed 100% of resistance to ampicillin, nalidixic acid, ciprofloxacin, trimethoprim-sulfamethoxazole and chloramphenicol but were sensitive to amoxicillin-clavulanic acid, ceftazidime, ceftriaxone, cefotaxime, and gentamicin. On the contrary, *E. coli* isolates from sample H1A showed resistance levels not only to trimethoprim-sulfamethoxazole, chloramphenicol, nalidixic acid and ciprofloxacin but also to the β-lactam antibiotics (except for amoxicillin-clavulanic acid) and gentamicin.

Among the well water samples (Table 4), *E. coli* isolates from sample P55 were fully resistant to the tested antibiotics except for amoxicillin-clavulanic acid. Antibiotic resistance levels in the *E. coli* isolates from the remaining well water samples were low or very infrequent. For example, low levels of resistance to ampicillin, ceftazidime, ceftriaxone, cefotaxime, ciprofloxacin, chloramphenicol, gentamicin, nalidixic acid, and trimethoprim-sulfamethoxazole were found in the *E. coli* isolates from samples P04, P11, P13, P17 and

WW9. In addition, *E. coli* isolates from samples WW10 and WW37 showed only low level of resistance to ampicillin, gentamicin, trimethoprim-sulfamethoxazole, and chloramphenicol.

Among the *E. coli* isolates (Table 4), from the sewage water samples from the municipal sedimentation treatment plants most were sensitive to gentamicin, chloramphenicol and the tested  $\beta$ -lactams, except for ampicillin. Interestingly, antibiotic resistance levels to both nalidixic acid and ciprofloxacin were found in sample SUA1 and SUB2.

**Table 4.** Distribution of resistance and MICs for individual antibiotics and each water source where *E. coli* was isolated.

Sample code	No. <i>E. coli</i> isolates tested	% Resistance									
		AMP	AMC	CAZ	CRO	CTX	GEN	NAL	CIP	SXT	CHL
<b>H1A<sup>h</sup></b>	32	100		37.5	37.5	37.5	68.8	9.4	6.2	100	96.9
<b>H1B<sup>h</sup></b>	32	100						100	100	100	100
<b>H1C<sup>h</sup></b>	32	100						100	100	100	100
<b>P04<sup>w</sup></b>	24	16.7		16.7	16.7	16.7	16.7	16.7	16.7	16.7	16.7
<b>P11<sup>w</sup></b>	16	6.2		6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2
<b>P13<sup>w</sup></b>	16	12.5		6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2
<b>P17<sup>w</sup></b>	24	12.5		12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
<b>P55<sup>w</sup></b>	24	100		100	100	100	100	100	100	100	100
<b>WW09<sup>w</sup></b>	4	25		25	25	25		25	25	25	
<b>WW10<sup>w</sup></b>	14	85.7						14.3		78.6	78.6
<b>WW37<sup>w</sup></b>	20	30						35		30	30
<b>SIA1<sup>s</sup></b>	8	37.5	12.5							25	
<b>SIA2<sup>s</sup></b>	10	50								60	
<b>SIB1<sup>s</sup></b>	5	60								40	
<b>SIB2<sup>s</sup></b>	8	50						37.5	37.5		
<b>SUA1<sup>s</sup></b>	24	91.7								8.3	
<b>SUA2<sup>s</sup></b>	8	75						12.5		50	
<b>SUB2<sup>s</sup></b>	24	12.5						45.8	29.2	91.7	

h: Hospital sewage water samples

w: Well water samples

s: Sewage water samples from the municipal sedimentation treatment plants

AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; CIP, ciprofloxacin; CHL, chloramphenicol; GEN, gentamicin; NAL, nalidixic acid; and STX, trimethoprim-sulfamethoxazole

### 5.3.3 Resistance patterns of the *E. coli* isolates

The two most common multi-resistance patterns among the 325 *E. coli* isolates were (i) ampicillin, chloramphenicol, ciprofloxacin, nalidixic acid and trimethoprim-sulfamethoxazole and ampicillin; and (ii) cefotaxime, ceftriaxone, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid and trimethoprim-sulfamethoxazole in 20% and 10% of the *E. coli* isolates, respectively.

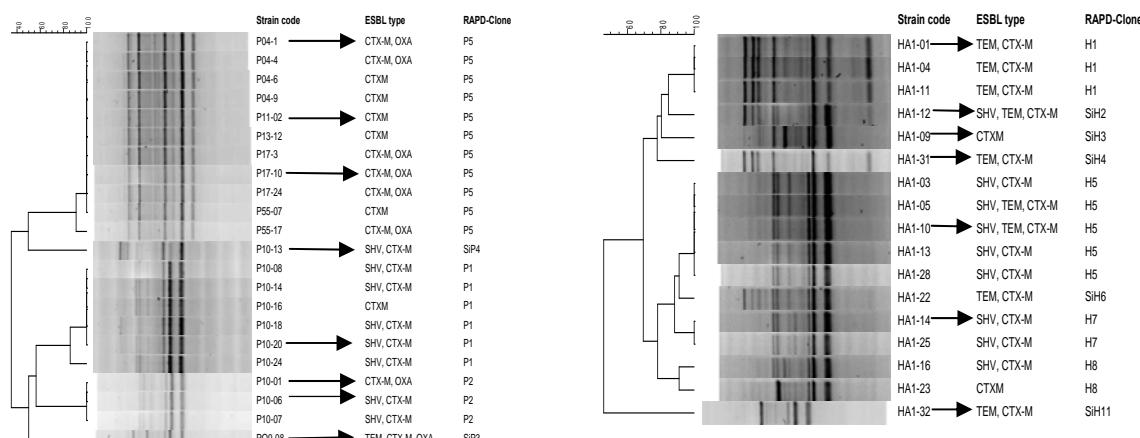
Resistance patterns in the *E. coli* isolates were different among the water samples from the hospital sewage, wells and the municipal sedimentation treatment plants. While 100% of the *E. coli* isolates from the hospital sewage water samples were resistant to two or more of the tested antibiotics, only 36% of the *E. coli* isolates from well water and 32% of the *E. coli* isolates from the municipal sedimentation treatment plants were also resistant to at least two of the tested antibiotics. Interestingly, the *E. coli* isolates from well water samples showed higher frequency of multi-resistance to ampicillin, cefotaxime, ceftriaxone, ceftazidime, chloramphenicol, ciprofloxacin and gentamicin, nalidixic acid, and trimethoprim-sulfamethoxazole as compared to the rest of the isolates.

### 5.3.4 ESBL and β-lactamase genes detection

ESBL-producing *E. coli* included in this study were identified in 33% of the isolates from the hospital sewage water and in 32% of the isolates from the well water samples. The gene encoding for SHV enzyme was more commonly detected in *E. coli* isolates producing ESBL from the hospital sewage water (53%) than from the well water samples (22%). The gene TEM was only detected in *E. coli* isolates producing ESBL from the hospital sewage water samples (14%). In contrast, the gene OXA was only detected in *E. coli* isolates producing ESBL from the well water samples (57%).

### 5.3.5 Typing of ESBL-producing *E. coli* isolates by RAPD

Twenty-two *E. coli* isolates from the well water samples and 17 from the hospital sewage water samples were selected for RAPD analysis. The analysis revealed that *E. coli* isolates from well water samples could be separated into five clones (Fig. 3). In the case of the *E. coli* isolates from the hospital sewage water samples, RAPD analysis showed that they could be separated in 11 clones (Fig. 4).



**Figure 3.** Clonal similarity of *E. coli* from wells water by RAPD-PCR.

**Figure 4.** Clonal similarity of *E. coli* from the hospital sewage water by RAPD-PCR.

The RAPD analysis did not show any clonal similarity between the *E. coli* isolates from the wells and the hospital sewage water samples.

#### 5.4 CTX-M-PRODUCING GRAM-NEGATIVE BACTERIA

Papers I and II: The multiplex PCR for detection of the CTX-M-1, 2, 9, 8, and 25 in those *K. pneumoniae* isolates positive for CTX-M showed that all these isolates were positive for CTX-M-1 group. Based on the previous results and the RAPD analysis, representative isolates from each clonal group (mostly *K. pneumoniae* isolates positive for CTX-M-1) were selected for sequencing analysis. The selected *K. pneumoniae* isolates are marked by arrows (Fig. 2). The results showed that  $\beta$ -lactamase SHV-11/12 and TEM-1 were the specific  $\beta$ -lactamases harboured in the *K. pneumoniae* isolates that were positive in the multiplex PCR assay for SHV or TEM. For the CTX-M groups, the  $\beta$ -lactamase CTX-M-15 was the specific  $\beta$ -lactamase harboured in the *K. pneumoniae* isolates that was positive in the multiplex PCR assay for CTX-M-1 group.

One strain of *S. liquefaciens* and one of *S. marcescens* from the neonates with septicaemia, both harbouring TEM and CTX-M  $\beta$ -lactamase genes, were analyzed by sequencing (Table 3); among the environmental strains, one *E. cloacae* strain harbouring SHV, TEM and CTX-M  $\beta$ -lactamase genes, and one *E. coli* and one *S. liquefaciens* both harbouring TEM and CTX-M  $\beta$ -lactamase were analyzed by sequencing. After sequencing, it was found that the SHV-11/12 and TEM-1 enzymes were present in the selected bacterial isolates positive in the PCR assay for SHV or TEM. For the CTX-M groups, it was found that CTX-M-15 was the specific  $\beta$ -lactamase harboured in the bacterial isolates that were positive in the PCR assay for CTX-M-1 group.

Paper III: Thirteen *E. coli* isolates were selected for sequencing, all of them were positive in the PCR for CTX-M and TEM, and 1/13 for OXA enzyme. The multiplex PCR for detection of the CTX-M-1, 2, 9, 8, and 25 showed that 2/13 *E. coli* isolates were positive for CTX-M-2 group and 11/13 *E. coli* isolates were positive for CTX-M-1. After sequencing, it was found that TEM-1, OXA-1/-30 were present in the *E. coli* isolates positive in the PCR assay for TEM or OXA enzymes. For the CTX-M groups, it was found CTX-M-5 as the specific enzyme in 2/13 *E. coli* (one EAEC and one non-DEC) isolates positive for CTX-M-2 group and CTX-M-15 as the specific enzyme in 11/13 *E. coli* (four EAEC and 7 non-DEC) isolates positive for CTX-M-1 group.

Paper IV: The multiplex PCR showed that CTX-M-9 group was more prevalent in ESBL producing *E. coli* isolates from the hospital sewage water samples. On the contrary, CTX-M-1 group was more prevalent in ESBL producing *E. coli* isolates from the well water samples. The genes encoding for CTX-M-2, CTX-M-8, and CTX-M-25 groups were not detected in any of the *E. coli* isolates studied.

Among RAPD clones from well water samples, P1 and P5 encompass most of the isolates (6 and 11 *E. coli* isolates, respectively). Interestingly, all of the *E. coli* isolates from clone P1 harboured the gene encoding for CTX-M-9 group and most of the combination of genes encoding for SVH, CTX-M enzymes. In contrast, all of the *E. coli* isolates from clone 5 harboured the gene encoding for CTX-M-1 group and most of the combination of genes encoding for TEM or OXA plus CTX-M enzymes.

In the case of the *E. coli* isolates from the hospital sewage water samples, RAPD analysis showed that they could be separated in 11 clones (Fig. 4). Among them, clone H5 encompassed the major number of *E. coli* isolates, and all of them harboured the gene encoding for CTX-M-9 group and most of the combination of genes encoding for SVH, CTX-M enzymes. The CTX-M-1 group was found mostly in *E. coli* isolates that belonged to clone H1.

Based on the RAPD analysis, representative isolates from each clonal group were selected for further analysis by sequencing. The selected *E. coli* isolates from the well water and the hospital sewage water samples are marked by arrows (Fig. 3 and 4). After sequencing, it was found that the enzymes SHV-11/12, TEM-1, and OXA-1/30 were present in the *E. coli* isolates positive in the PCR assay for SHV, TEM or OXA enzymes. For the CTX-M groups, it was found that CTX-M-15 and CTX-M-9 were the specific  $\beta$ -lactamases harboured in the *E. coli* isolates that were positive in the PCR assay for CTX-M-1 and CTX-M-9 groups.

## **6 DISCUSSION**

### **6.1 NEONATAL SEPTICAEMIA (PAPERS I AND II)**

Neonatal infections are estimated to cause more than one million of annual deaths in developing countries (24). Reports from the Nicaragua Ministry of Health indicated that in 2005, when the study was carried out, there were more than 800 neonatal deaths attributable to infections (95). Limitation of resources in these regions implies a lack of information regarding the causative pathogen in these infections.

Although we did not cover all the possible risk factors associated with the acquisition of Gram-negative bacteria in neonates such as invasive procedures, underlying diseases, etc., our results show that neonates were prompt to undergo cyanosis when they are infected by Gram-negative bacteria. Venkatesh et al. showed in their review about the management of neonatal sepsis by Gram-negative pathogens that the risk factors significantly associated with Gram-negative causing septicaemia are the use of central venous catheters, catheterization for more than 10 days, nasal continuous positive airway pressure, the use of H<sub>2</sub>-blockers or proton-pump inhibitors, gastro-intestinal tract pathology including necrotizing enterocolitis, feeding difficulties, administration of total parenteral nutrition, premature (less than 28 weeks) at birth, and less than 1000 g at birth (168).

Our data show that 74% (34/46) of the bacteria related to neonates with septicaemia were Gram-negative. This finding is supported by previous studies showing that neonates in developing countries are commonly affected by Gram-negative bacteria (154, 183, 184). This fact is mainly due to a lack of infection control measures before and after parturition (183). Our data also show that 48% of the bacteria isolated from the NICU's environment were Gram-negative. Gram-negative bacteria causing neonatal septicaemia belong to two general groups, Enterobacteriaceae and non-fermenting bacteria (168). In the present study the Enterobacteriaceae group was the most common bacteria affecting neonates (Table 1) mainly *K. pneumoniae*, *Serratia marcescens* and *S. liquefaciens*. *E. cloacae*, *E. coli*, *K. pneumoniae* and *S. liquefaciens* were isolated from both neonates with septicaemia and the NICU's environment. However, only *K. pneumoniae* showed clonal similarity among the isolates affecting neonates and those from the NICU's environment. *A. baumannii*, *Enterobacter sakazakii*, *P. aeruginosa*, and *S. maltophilia* were isolated only in the NICU's environment and *C. luteola*, *Pantoea* spp. and *S. marcescens* only from neonates with septicaemia.

*K. pneumoniae* has become important neonatal pathogen in developing countries in both nosocomial and community acquired infections (154, 183, 184). Incidence of neonatal *Klebsiella* infection varies between 4.1 and 6.3 per 1000 livebirths, with case fatality rates of 18–68% (183). Our results support these findings; *K. pneumoniae* was the Gram-negative bacterium most frequently isolated from neonates with clinical symptoms of septicaemia in the present study (Table 2) and was related to the death of six neonates, yet in one of this neonates *K. pneumoniae* was co-infecting with *C. luteola* and *S. marcescens*. The most common habitats of *Klebsiella* are the natural environment (e.g., water and soil) and mucosal surfaces of mammals (e.g., humans) (63). It is believed that the main source of antibiotic resistant *Klebsiella* infections is the heavily contaminated environmental reservoirs identified in developing countries rather than acquired from the gastrointestinal and vaginal normal flora (183). The ability of this organism to spread rapidly often leads to hospital-acquired outbreaks; in this context asymptomatic-colonized patients, and/or the contaminated healthcare environment can serve as reservoirs for *Klebsiella* spp. that can be spread by transient hand carriage by healthcare workers (63).

Other Gram-negative bacteria, *S. marcescens* and *S. liquefaciens* were isolated in 15 neonates with septicaemia. Interestingly, these pathogens were co-infecting four neonates (1 of 4 died). *S. marcescens* has been described as an important opportunistic pathogen in NICU (174). Al Jarousha et al. found in a case control study carried out at a NICU from Gaza city, Palestine that *S. marcescens* was the cause of septicaemia in 159 neonates (of them 70 died and 89 recovered) (2). Other Gram-negatives such as *E. cloacae* and *E. coli* were scarcely isolated from the neonates with septicaemia, yet they are a common cause of neonatal septicaemia in developing countries (183, 184). As described above for *K. pneumoniae*, the main source of infection is attributed to NICU's environmental contaminations. *E. cloacae*, *E. coli* and *S. liquefaciens* but not *S. marcescens* were found in the NICU's environment.

Antibiotic resistance have emerged as a global health problem in which bacteria causing neonatal infections are included (162, 169, 183). It has been reported that the WHO recommended that empiric regimen of ampicillin and gentamicin may not be an effective treatment against 70% of the pathogens causing neonatal infections in developing countries where members of the Enterobacteriaceae family, including *E. coli*, *Enterobacter*, *Klebsiella*, *Citrobacter* and *Serratia* species, are often resistant to at least one of these antibiotics (16, 154, 183). Our results support these findings, β-lactams and aminoglycosides were the most common antibiotics used for treatment of the neonates included in this study. It is probable

that the use of such treatments have not provided a positive effect in the neonates since our results show a high prevalence of antibiotic resistance to the  $\beta$ -lactams and gentamicin. More than 85% of *K. pneumoniae* strains from neonates with septicaemia and the environment were resistant to ceftazidime, ceftriaxone, gentamicin (Table 2). These data could explain the treatment failure in the neonates who died during our study.

## **6.2 INTESTINAL *E. COLI* FROM CHILDREN (PAPER III)**

Diarrhoeal disease is one of the most important causes of illness and death in young children from developing countries (21, 76, 119). The use of antibiotics for diarrhoea treatment is not recommended, except in cases in which the pathogens have been identified in order to shorten the course and spread of the disease, as in shigellosis (58, 107, 178, 181). The health workers in developing countries rarely have access to sufficient high quality diagnostic laboratory facilities to analyse stool samples for all children with diarrhoea. In addition, there is a high risk of overuse of antibiotics for treatment of diarrhoea in developing countries since it is not necessary to have a medical prescription to have access to the drugs (96). Thus, the use of antibiotics for treatment of diarrhoea needs to be approached with caution due to potential problems of antibiotic resistance and possible reactions of some micro-organisms, for example the induction of toxin synthesis by EHEC when a person is treated with certain antibiotics, potentially causing severe illness (73).

Diarrhoea can be caused by a wide variety of microorganisms such as diarrhoeagenic *E. coli* (DEC). In developing countries, DEC is commonly associated with diarrhoea in children (102, 106, 107, 171). Furthermore, these pathogens have in several studies been reported to be resistant to several antibiotics. Villa et al., in their study on antibiotic resistance in DEC in children from Ifakara, Tanzania (101, 139, 158), found that 38% of the cases of diarrhoea were due to multi-resistant ETEC, EPEC and EHEC (170).

Antibiotic resistance patterns in *E. coli* isolates differ among countries. In Peru, Ochoa et al. reported multi-resistance to ampicillin and trimethoprim-sulfamethoxazole as the second most common pattern in DEC (108), whereas in Vietnam, Nguyen et al. reported that ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole was the most common resistance pattern among DEC (101). In the present study, the two most prevalent multi-resistance patterns (resistance to at least two antibiotics), among the 727 *E. coli* isolates included in this study, were (i) ampicillin and trimethoprim-sulfamethoxazole (41%), and (ii) ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole (7.2%).

To the best of our knowledge, there are no studies in Latin America that have included antibiotic resistance analysis in both DEC and non-DEC from children with and without diarrhoea. However, there are few studies comparing antibiotic resistance in *E. coli* isolates positive for a DEC virulence marker. The study carried out in Mexican children (51) showed that among all of the DEC categories, EPEC was significantly less resistant to ampicillin and trimethoprim-sulfamethoxazole compared to ETEC and EAEC. In a recently published study carried out in Peru (108), the prevalence of resistance to ampicillin, trimethoprim-sulfamethoxazole, tetracycline and nalidix acid was significantly higher ( $P<0.05$ ) in DAEC followed by EAEC, EPEC, ETEC. The authors hypothesised that the higher resistances levels in DAEC and EAEC, compared to other groups of DEC, could be due to frequent use of antibiotic since these diarrhoeagenic pathotypes often cause persistent diarrhoea and/or are present in asymptomatic carriers. This hypothesis could be supported by the findings by Vilchez et al., who showed that EAEC was the most frequently isolated pathotype of *E. coli*, with a high level of asymptomatic carriers (171). It is clear, at least in Latin America, that EAEC isolated from children possess high levels of resistance to ampicillin and trimethoprim-sulfamethoxazole and could become a serious health problem.

Although amoxicillin-clavulanic acid, ceftazidime, gentamicin, ceftriaxone, and imipenem are not indicated to treat diarrhoea in children, we have tested the susceptibilities to these antibiotics in all isolates, since they could be empirically or incidentally used. A recent publication showed that outpatients with diarrhoea of presumed bacterial origin at the emergency department of the University Hospital of León, Nicaragua, (50% being children <6 years of age) received trimethoprim-sulfamethoxazole (18.5%) and ciprofloxacin (8.5%) to shorten the course of the disease (43). As shown above, some EAEC and non-DEC isolates from children with/without diarrhoea show low level of resistance to amoxicillin-clavulanic acid, ceftazidime and/or ceftriaxone, and ciprofloxacin. Resistance to ceftazidime and/or ceftriaxone was related to the ESBL production. A pattern of multi-resistance (ampicillin, chloramphenicol, ceftazidime and/or ceftriaxone, gentamicin, and ciprofloxacin) was also observed in those *E. coli* isolates. The resistance to amoxicillin-clavulanic acid could be due to the presence of other enzymes such as AmpC (44, 69). Contrary to our results, the studies carried out in Peru and Mexico showed that DEC were sensitive to ceftazidime and/or to ceftriaxone (51, 108).

The problems of antibiotic resistance require appropriate interventions with special regards to multi-resistant Gram-negative pathogens (61, 62). The rate of resistance to the most common antibiotics used for treatment of diarrhoea is increasing in Latin America. In *E. coli* isolates the resistance is seen in both pathogenic and non-pathogenic isolates (51, 92, 108, 116, 117).

### **6.3 ENVIRONMENTAL WATER (PAPER IV)**

Many studies have shown the presence of antibiotic resistant bacteria or resistance genes in water environments (12, 88, 89). Interestingly, this is found even in countries with high control in the use of antibiotics, e.g. the presence of genes encoding resistance to aminoglycosides,  $\beta$ -lactams and tetracyclines as well as the presence of methicillin-resistant *Staphylococcus aureus* have been found in waste water environments from Sweden (20). In our study, the presence of *E. coli* resistant to at least one of the tested antibiotics was found in 18/48 water environmental samples.

Although many studies have shown the relation of DEC with diarrhoea in Nicaragua (118, 171), we could not detect the presence of DEC in the environmental water samples. However, the presence of antibiotic resistant *E. coli* was frequent. Perhaps a long-term environmental water study covering the diarrhoea season in Nicaragua could show the roll of contaminated water in the diarrhoea disease burden in Nicaragua. Ram et al. in their studies carried out in surface water samples (drinking water, for irrigation, or other purpose) from the Indian rivers have shown the presence of antibiotic resistant shiga toxin and enterotoxin producing *E. coli* (143-145); the authors hypothesize that such a finding are an important health concern due to risk of developing waterborne outbreaks.

In many developing countries, the unregulated sales and dispensing of antibiotics is very common (28, 109). Thus, it is important not only to consider the contribution of hospital effluents but also the contribution of the general community in the input of antibiotic resistance bacteria to the aquatic environment (78). Our results show that among all of the *E. coli* isolates included in this study, those from the hospital sewage water showed higher antibiotic resistance levels to ampicillin (100%), nalidixic acid (70%), ciprofloxacin (69%), chloramphenicol (69%) and trimethoprim-sulfamethoxazole (100%) compared to the other *E. coli* isolates. The similarities in antibiotic resistance percent found in the *E. coli* isolates from hospital sewages samples H1B and H1C could be due to those sewages that collect the water effluent from hospital units with a high use of antibiotics.

Among the well water samples which represent the contribution of the community in the input of antibiotic resistance bacteria to the aquatic environment, *E. coli* isolates from well water sample P55 were fully resistant to the tested antibiotics which indicated a high contribution to the spread of multi-antibiotic resistant bacteria, perhaps due to the high use of antibiotics in those settings. Kümmerer showed that there is a surprisingly high incidence of antibiotic-resistant *E. coli* in rural groundwater, perhaps due to run-off from farms or leakage from septic tanks (78). It has been shown that improper sanitations, e.g. improper excreta management, can prompt to infectious diseases such as diarrhoea. In Nicaraguan rural areas, as in many developing countries, the use of household latrine is very common and perhaps the presence of antibiotic-resistant bacteria in well water samples could be due to improper construction of the latrines and hence leakage to the well water.

Resistance to these antibiotics, yet lower, was also found in the *E. coli* isolates from the sewage water samples from the municipal sedimentation treatment plants. Similar finding were made by Doung et al. in their study on the occurrence of fluoroquinolones agents and the number of *E. coli* resistant to them, in hospital wastewater in Hanoi, Vietnam (47). They found higher level on antibiotic resistant bacteria in waste water from hospital as compared to wastewater treatment plants. It has been reported that resistant bacteria are eliminated quite well in the sewage treatment plants which can explain the low level of resistance found in our study (78). However, it is important to consider that there are factors that could have influenced our results, such as dilution effect and the viability of antibiotic resistance bacteria in the environment.

Nosocomial and community diseases, caused by bacteria producing ESBL, have become a challenge for clinicians due to the limited treatment options against these pathogens (85, 149). Furthermore, it has long been acknowledged that the cephalosporin breakpoints used in most European countries and the USA fail to detect many of the ESBLs in Enterobacteriaceae and that all ESBLs are clinically important (71). In order to detect the major number of *E. coli* producing ESBL, we decided to include in the present study cephalosporin breakpoints following recent CLSI revisions (CLSI breakpoints will not be operative until other  $\beta$ -lactam breakpoints have also been revised) (71). By doing so, we were able to detect 30 *E. coli* isolates that are considered resistant using the recently CLSI revised breakpoints (resistant if  $>2$  mg/L for cefotaxime or ceftriaxone, and  $>8$  mg/L for ceftazidime) but considered susceptible by the still-in-use CLSI breakpoints. Our results support the necessity of the

implementation of the updated CLSI cephalosporin breakpoints in order to identify clinical important ESBL producing pathogens.

In the present study, *E. coli* producing ESBL were detected in 36% of the hospital and in 32% of the well water *E. coli* isolates. None of the *E. coli* isolates from sewage water samples from the municipal sedimentation treatment plants were ESBL positive.

Beta-lactamase enzymes are the main mechanism of resistance to  $\beta$ -lactam antibiotics in Enterobacteriaceae (121, 164). CTX-M group of enzymes have become one of the main public health concerns due to their ability to be involved in nosocomial and community acquired infections. *E. coli* is most often responsible for producing CTX-M  $\beta$ -lactamases and seems to be a true community ESBL pathogen (131). In addition, the ESBL genes are often present together with other resistance genes in plasmids, conferring multi-resistance patterns that in clinical setting result in decrease of treatment options. Since 2000, ESBL-producing *E. coli* positive for the CTX-M enzymes have emerged worldwide as important causes of community-onset urinary tract infections (124, 127).

In previous studies we have reported on the emergence of bacteria producing ESBL causing infection in Nicaraguan children (5-7). In those isolates, the gene encoding for CTX-M was the most commonly detected. In the present study we show that genes encoding for CTX-M enzymes were detected in 100% of the ESBL producing *E. coli* from both hospital sewage and wells water of the community. On the contrary, the gene encoding for TEM enzymes was only found in the hospital samples (14%) and the gene encoding for OXA enzymes in the well water samples (57%). The gene encoding for SHV enzyme was more often detected in hospital samples (53%).

Furthermore, *E. coli* isolates producing ESBL were found to be resistant to most of the tested antibiotics. Thirty-three of the *E. coli* isolates producing ESBL from well water samples were multi-resistant to ampicillin, cefotaxime, ceftriaxone, ceftazidime, chloramphenicol, ciprofloxacin and gentamicin, nalidixic acid, and trimethoprim-sulfamethoxazole. As mentioned above, ESBL and other resistance genes are carried in plasmids that confer multi-resistance profile (50, 132), thus detecting bacteria producing ESBL in clinical setting could indicate the presence of a multi-resistant bacteria.

RAPD analysis did not show any clonal similarities between the ESBL producing *E. coli* isolated from the hospital and well water samples. However, we did find some dominant clones intra sample, e.g. clone P5 encompass most of the ESBL producing *E. coli* from well water samples and clone H5 most of the ESBL producing *E. coli* in the hospital samples. In addition, the carriage of specific CTX-M groups was more common in some clones, e.g. all of the *E. coli* isolates from clone P5 harboured the gene encoding for CTX-M-1 group.

#### **6.4 CTX-M-PRODUCING GRAM-NEGATIVE BACTERIA**

It has been shown that CTX-M enzymes are not only limited to nosocomial setting but also have a potential for spread beyond the hospital environment (131, 132). Since 1990s, CTX-M enzymes have become the most prevalent type of ESBLs described around the world in both nosocomial and community settings. The prevalence of CTX-M enzymes in Latin American countries are among the highest in the world (173). Some of the CTX-M enzymes are widely present in specific countries, such as CTX-M-9 and CTX-M-14 in Spain, CTX-M-1 in Italy, and CTX-M-2 in most South American countries, Japan, and Israel, whereas others such as CTX-M-15 have been detected worldwide (29, 150). CTX-M-15 has often been associated with the co-production of other  $\beta$ -lactamases such as TEM-1, OXA-1, and the aminoglycoside modifying enzyme aac(60)-Ib-cr which has the additional ability to acetylate fluoroquinolones with an unprotected amino nitrogen on the piperazine ring, including norfloxacin and ciprofloxacin but not levofloxacin (124, 129). Our results are in accordance with that information; the production of CTX-M-15 together with the co-production of SHV-11/12 and TEM-1 was found in the Gram-negative bacteria producing ESBL isolated from neonate with septicaemia and from the environment. Similar results were presented by Dashti et al., they highlighted the importance of the NICU's environment in the transmission of Gram-negative bacteria harbouring TEM-1, CTX-M-15 and SHV-112 in NICU in a Kuwaiti hospital. The authors suggested a nurse transmission since they found this clone on the nurses' hands (41). It is interesting to mention that the first report of CTX-M in Latin America was about nontyphoid *Salmonella* strains affecting NICUs (14, 15). In Nicaragua, this is the first report about CTX-M-producing Gram-negative bacteria.

For the intestinal *E. coli* study, CTX-M-5 was detected in 2/13 *E. coli* (1 EAEC and 1 non-DEC) isolates and CTX-M-15 in 11/13 *E. coli* (4 EAEC and 7 non-DEC) isolates. The CTX-15 was also found together with TEM-1 and OXA-1/-30. Other studies in Latin American countries showed a high rate of CTX-M-2 and CTX-M-15 and multi-resistance in non-DEC which supports our finding (116, 117).

In the environmental water study, CTX-M-15 and CTX-M-9 were the specific  $\beta$ -lactamases harboured in the *E. coli* isolates that were positive in the PCR assay for CTX-M-1 group (more prevalent in *E. coli* isolates from the well water samples) and CTX-M-9 group (more prevalent in *E. coli* isolates from the hospital sewage water samples). In addition, CTX-M-15 was also found together with the enzymes SHV-11/-12, TEM-1 and OXA-1/-30.

In general, CTX-M-15 was detected in the three studies which is in accordance with the reports of CTX-M-15 as the most prevalent  $\beta$ -lactamase worldwide. Yet, we did not study the production of aminoglycoside modifying enzymes, it is also evident in the results that these Gram-negative strains were also resistant to those antibiotics.

## 7 CONCLUSIONS

This thesis shows a high prevalence of TEM-1, SVH-11/12 and CTX-M-15-producing Gram-negative bacteria affecting neonates in the NICU's HEODRA, mainly due to cross-contamination with the NICU's environment.

On the positive side, the antibiotic resistance level in *E. coli*, from children with/without diarrhoea, have not yet reached the high levels of resistance to the most common antibiotics used for diarrhoea treatment as in other countries, yet CTX-M-5 or CTX-M-15 production was detected in some multi-antibiotic resistant DEC and non-DEC isolates, which suggests the emergence of ESBL in the Nicaraguan community and may indicate future treatments complications.

Even though we did not perform a longitudinal study of environmental water samples, our results suggest that multi-resistant CTX-M-9 and CTX-M-15-producing *E. coli* were widely spread in hospital sewage water and some community water samples.

The treatment options for Gram-negative infections affecting children are scarce or with probable toxic effects as for the neonates. Thus, the global burden of antimicrobial resistance requires appropriate interventions. Local surveillance to identify prevalent pathogens and bacterial resistance patterns is necessary for selecting optimal treatment regimens with the aim of a positive outcome in the patient. Furthermore, the evidence that this surveillance can provide with the environmental water is crucial in order to create risk management strategies for this settings. Implementation of infection control practices, appropriate empirical therapy should also be considered to reduce the prevalence and dissemination of these organisms in the NICU.

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