EPIDEMIOLOGICAL AND MOLECULAR INVESTIGATIONS ON ANTIBIOTIC RESISTANCE IN GROUP B *STREPTOCOCCUS* AND *ESCHERICHIA COLI*

by

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DEDICATION

To my parents, my husband, Ai and my daughter, Mimi

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LIST OF ABBREVATIONS

ATCC	The American Type Culture Collection
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic acid
EB	Ethidium bromide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FQ	Fluoroquinolone
GBS	Group B *Streptococcus*
HR	Hazard Ratio
MIC	Minimal Inhibitory Concentration
OR	Odds Ratio
PCR	Polymerase Chain Reaction
QRDR	Quinolone-Resistance Determining Region
RNA	Ribonucleic acid
RR	Relative Risk
TMP/SMX	Trimethoprim/Sulfamethoxazole
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infections

ABSTRACT

Antibiotic resistance is currently one of the greatest challenges to clinicians and to public health. The detection of resistance mechanisms to antibiotics has become more and more epidemiologically and clinically important. This dissertation investigates rates, mechanisms of antibiotic resistance and adaptation in Group B *Streptococcus* (GBS) and *Escherichia coli* by the combined power of epidemiologic principles and molecular methods.

In chapter 3 and 4, I describe the rates and mechanisms of resistance to fluoroquinolones in GBS. My colleagues and I report a strikingly high frequency of resistance to different fluoroquinolones, especially to norfloxacin (~93%) among a collection of 1075 GBS strains isolated from South Korea between 2006 and 2008. Ciprofloxacin and levofloxacin resistance was higher in invasive than in colonizing GBS isolates (10.6% versus 2.5% ($p < 0.001$) in ciprofloxacin resistance, 9.8% versus 2.1% ($p < 0.001$) in levofloxacin resistance). Mutations in gyrase and topoisomerase were found to be the resistance mechanism to ciprofloxacin, levofloxacin and moxifloxacin while efflux pumps appear to be the predominant resistance mechanism among GBS strains resistant to only norfloxacin. Strain serotypes were not associated with susceptibility to fluoroquinolones.

In chapter 5, I turn my attention to *Escherichia coli* adaptation measured by differences in antibiotic resistance and in the distribution of CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) between commensal *E. coli* and uropathogenic *E. coli* (UPEC) isolates. Recently, CRISPRs were identified as an immune system protecting numerous bacteria against invasion by phages, plasmids or other forms of foreign DNA. In my study on 81 matched pairs of commensal *E. coli* and UPEC strains isolated from UTI women, commensal *E. coli* isolates were found to have more repeats ($p = 0.009$) and more unique spacers ($p < 0.0001$) than UPEC isolates. Additionally, UPEC isolates were more likely to be resistant to the antibiotics tested (cefazolin ($p < 0.0001$), trimethoprim/sulfamethoxazole ($p = 0.05$)) than commensal *E. coli* isolates. Association between CRISPRs and antibiotic resistance was not well identified in this study. These findings support the hypothesis of better adaptability of UPEC and are suggestive of the positive role of *E. coli* CRISPRs as a defense system.

CHAPTER 1

INTRODUCTION

Infectious diseases are among the top leading causes of morbidity and mortality worldwide (19). In the World Health Report 2008 (World Health Organization), lower respiratory tract infection and tuberculosis were two of the ten leading causes of death in low-income and middle-income countries (19). The discovery of antibiotics, the powerful weapon against infectious diseases caused by bacteria, was the breakthrough medical innovation in the twentieth century. However, resistance to antibiotics was recognized immediately after penicillin, the first antibiotic, was introduced in 1946. The spread and increase of disease-causing microbes that have become resistant to antimicrobial therapy is an emerging public health threat. Nowadays, about 70% of the bacteria that cause nosocomial infections are resistant to at least one of the antibiotics most commonly used for treatment (17). Identifying rates and mechanisms of antibiotic resistance is thus of utmost importance in the prevention, treatment and development of new antimicrobial agents against infectious diseases.

There are several mechanisms involved in antibiotic resistance which vary by the mechanisms of action of the antibiotics. These mechanisms destroy the antibiotic, prevent the antibiotic from reaching its intracellular target through

physical removal from the cell, or modify the target site so it is not recognized by the antibiotic (16). Investigating the frequency and mechanisms of antibiotic resistance in Group B *Streptococcus* using the combined power of epidemiologic principles and molecular tools is the first aim of this dissertation.

Group B *Streptococcus* (GBS, *Streptococcus agalactiae*) is considered the most common cause of neonatal sepsis. This beta-hemolytic, gram positive streptococcus also causes meningitis, osteomyelitis, pneumonia and peritonitis (8). During pregnancy, GBS may cause amnionitis, endometritis, urinary tract infection, bacteremia or sepsis and septic abortion (8). Among nonpregnant adults without underlying disease, GBS also causes vaginitis, cervicitis, uthrethritis, cystitis, pyelonephritis, balanitis and pharyngitis (8). In the United States, while the incidence of group B Streptococcal disease in neonates is decreasing as a result of the recommendations issued by the Centers for Disease Control (CDC), the disease incidence in nonpregnant adults has increased by 32% between 1999 and 2005 (13). GBS is not only pathogenic but it is often found in the bowel microbiota as a commensal (8). As a commensal, GBS is exposed to all antibiotics taken by the host and thus may be exposed to selective pressures for resistance. While still sensitive to penicillin, GBS has become resistant to alternative therapies, including erythromycin and clindamycin. In a 2012 study conducted among pregnant women in a hospital in the U.S., resistance rates to clindamycin and erythromycin were 50.7% and 38.4%, respectively (1). GBS resistance to fluoroquinolones was reported in 2005 (6.8% to norfloxacin (15), 4.4% to levofloxacin (18)). Increasing antibiotic

resistance among GBS has been documented in several countries including the U.S., Canada, Taiwan (3, 4, 9) and is a great concern for researchers and healthcare providers.

A second focus of my dissertation is the adaptability of bacteria and how quickly a bacterium can adapt to new environments related to acquisition of antibiotic resistance. This work focuses on *Escherichia coli* (*E. coli*). *E. coli* is the leading cause of community-acquired and nosocomial urinary tract infections (UTI). In the United States, up to 50% of females eventually experience at least one episode of UTI (6). *E. coli* causes 12-50% of nosocomial infections and 4% of all cases of diarrheal disease (12). Similar to GBS, *E. coli* is a commensal that can be exposed to different antibiotics and acquire resistance (7,11). In many countries, *E. coli* is highly resistant to common antibiotics used for the treatment of UTI such as ampicillin, ciprofloxacin and cotrimoxazole (5). In Denmark, the frequency of *E. coli* resistance to ciprofloxacin increased three fold from 2002 to 2005 (10). CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system (14) is an immune system that protects bacteria from bacteriophage infection and plasmid conjugation in *Streptococcus thermophilus* and *Streptococcus mutans* (2). *E. coli* is one of the best-studied organism of CRISPRs (14). However, whether CRISPRs are associated with antibiotic resistance or play a role in the immunity of clinical *E. coli* isolates is still poorly understood.

Therefore, my dissertation has two goals: 1) to investigate the molecular epidemiology of group B *Streptococcus* for deeper understanding of the

frequency and mechanisms of antibiotic resistance, specifically to fluoroquinolones, to contribute to the development of prophylactic and therapeutic strategies; and 2) to investigate the CRISPR distribution and its association with antibiotic resistance in *E. coli* as a novel approach to the origin of antibiotic resistance and immunity.

Specific objectives:

1. Identification of mutations in the fluoroquinolone-resistant determining regions among clinical isolates of GBS from South Korea
2. Identification of efflux-mediated resistance to norfloxacin among clinical isolates of GBS from South Korea
3. Identification of *E. coli* CRISPR structure and the association with sites of colonization and antibiotic resistance among clinical isolates from Michigan.

This dissertation is organized in a multiple manuscript format. Chapter 2 provides current background information on antibiotic resistance profiles of GBS and *E. coli* including rates, mechanisms as well as antibiotic resistance-related adaptation. Chapter 3 is an accepted manuscript that describes the screening of mutations in GBS isolations from South Korea that are resistant to fluoroquinolones (objective 1 above). Chapter 4 is a manuscript that identifies the presence of an efflux pump, a new resistance mechanism to norfloxacin, among clinical GBS isolates from South Korea (objective 2). Chapter 5 identifies

CRISPR structure and the association with sites of colonization and antibiotic resistance among clinical *E. coli* isolates from Michigan (objective 3). Chapter 4 and 5 will be submitted for publication. Chapter 6 concludes with summary and integration of this dissertation's findings with current GBS and *E. coli* research and suggestions for future research.

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CHAPTER 2

BACKGROUND AND SIGNIFICANCE

This chapter reviews some of the general features and antibiotic resistance of GBS and *E. coli* to provide the background information for the studies presented in chapters 3 to 5. I also describe bacterial adaptability observed in these two organisms as measured by differences in antibiotic resistance and CRISPR-mediated immunity.

GBS overview

GBS belongs to the family *Streptococcaceae* and is characterized by the Lancefield group B antigen. The organism is catalase negative and grows in chains. Cultured on sheep blood agar plates, colonies are surrounded by a narrow zone of β-hemolysis (124). Group B *Streptococcus* can be a commensal or a pathogen. The process of GBS colonization and infection varies among different populations depending on the risk factors affecting the disease pathway. In neonatal and maternal infection, maternal colonization and peripartum transmission are the main mechanisms of GBS pathogenesis (101, 124). Overall, maternal genitourinary or gastrointestinal colonization is the first step in the disease process. The organism may then encounter a number of different

barriers (chorioamnionic membranes, amnion cells, placenta basement membrane, pulmonary epithelial cell layer, the vascular endothelial cell layer etc.) (101, 124) or even enter different eukaryotic cells and survive within these cells (133). After entering into sterile body sites, GBS may interfere with host defense mechanisms, causing clinical disease (124). GBS can cause mild to serious human infections, particularly among newborns, pregnant women and those with underlying illness (53). During pregnancy, GBS may cause amnionitis, endometritis, urinary tract infection and bacteremiae (53). Among nonpregnant adults without underlying disease, GBS may cause vaginitis, cervicitis, uthrethritis, cystitis, pyelonephritis, balanitis and pharyngitis (53).

Epidemiology of GBS colonization and infection

Incidence and prevalence of GBS colonization and infection

In the United States, approximately 19,800 cases of GBS occur annually in all age groups, 7,600 of these cases are among newborns (Data from CDC, 2010) (28). Although the GBS transmission rate from mothers to neonates through vaginal delivery is approximately 50%, only 1-2% of colonized neonates develop invasive group B streptococcal disease (97). The rate of early-onset infection has decreased from 1.7 cases per 1,000 live births in 1993 to 0.28 cases per 1,000 live births in the recent years (28). GBS colonization in women varies among age groups depending on study population, sites sampled and method of detection (43). About 25% of pregnant women (28) and 35% young, non pregnant woman (85) carry GBS in the rectum or vagina. While the

incidence of group B streptococcal disease in neonates appears to be decreasing, the disease rate in nonpregnant adults appears to be increasing in the U.S., with an overall increase of 32% between 1999 and 2005, reaching 7.9 per 100 000 in 2005 (104). In one recent study in Pittsburg, up to 40% of non pregnant women were colonized (78).

Distribution of GBS colonization and infection

GBS is the leading cause of neonatal meningitis and sepsis in developed countries including the United States, Western Europe and Australia (9, 64, 113) and in several developing countries in Africa (1.93-3.64 per 1000 live births in Southern Africa (83), 1.40-2.34 per 1000 live births in Malawi (55) and 0.65-1.40 per 1000 live births in Kenya (13). However, lower incidence of neonatal GBS was observed in other developing countries in Africa (0-0.17 per 1000 live birth in Nigeria and Gambia) (33), South Asia and the Middle East (0-0.26 per 1000 live births) (33). In a 2012 meta-analysis of incidence of neonatal GBS in developing countries, variation in incidence was observed between and within geographical regions (33). The primary explanation for this variation is the difference in maternal colonization rates among different geographical sites. However, there is insufficient evidence to support this hypothesis (125). Other explanations include the differences in individual susceptibilities, strain virulence or antibody levels and/or culture techniques (125). Further, underreporting or underestimating of cases of neonatal GBS due to home delivering and poor reporting system might also lead to low incidence of neonatal GBS in developing countries (138).

Previous studies have identified differences in rates of GBS colonization among racial and ethnic groups (5, 24, 117). In a 2000 study on pregnant women, GBS colonization rate was significantly higher among black women than other racial and ethnic groups (OR = 1.7; 95% CI 1.4 – 2.1) (24). In a 2011 study, also on pregnant women, white race (OR 1.89, 95% CI 1.30-2.75), and black race (OR 1.78, 95% CI 1.32-2.41) were independently associated with increased maternal GBS colonization (117).

Risk factors of GBS colonization and infection

- Infants

Infant early-onset disease

Risk factors for infant early-onset GBS infection have been well identified, including maternal colonization at birth, preterm birth < 37 weeks' gestation, rupture of membranes > 18 hours before delivery, lack of maternal antibodies to type specific capsular polysaccharides, chorioamnionitis, multiple gestation, non-white maternal race, intrapartum fever > 38°C, intrauterine monitoring, postpartum maternal bacteremia, and having had previous infant with invasive GBS disease (1, 115). Additionally, previous preterm delivery increases the incidence for invasive neonatal GBS disease compared to term delivery (111).

Infant late-onset disease

Risk factors for late-onset GBS disease have not been well identified as those for early-onset GBS disease. Prematurity was reported to be the major risk factor for late-onset GBS disease in the study conducted by Lin FY et al (79). The risk

of late onset GBS disease is also higher in black infants and in infants with young mothers (115).

- Pregnant women

Asymptomatic GBS colonization among pregnant women is associated with many risk factors, including age group, education, frequency of pregnancy, premature rupture of membrane (73), number of sex partners, frequency of sexual intercourse (107) and smoking (129). GBS infection during pregnancy is correlated with older gestational age, premature rupture of membrane and preterm labor, as reported by Dechen et al. (39). In a 2008 study, time interval between two pregnancies and the intensity of GBS colonization in the index pregnancy were found to be associated with recurrent maternal GBS colonization (29).

- Non-pregnant adults

Adults with underlying illness are at higher risk for GBS infection. The known risk factors include diabetes mellitus, cancer, neurologic impairment, liver disease and other forms of immune deficiency such as HIV infection and steroid use (10). In the study conducted by Schuchat et al. (114), patients with diabetes who were 20 to 64 years old had an 11-30 fold increased risk of GBS infection. Further, older age and black race were found to be correlated with GBS colonization and infection in non-pregnant adults (45). Sexual activity including having multiple sex partners and having frequent sexual intercourse was reported to be an important risk factor for vaginal acquisition of GBS among non-pregnant women (92). This finding was consistent with results from the study

conducted by Foxman B. et al (48) where engagement in any sexual activity during the three weeks prior to sample collection was significantly associated with a higher risk of GBS colonization among college women and men. However, no significant associations with GBS colonization were found with lifetime number of partners, timing of most recent sexual encounter, age at first encounter, type of sexual contact, method of contraception, or history of sexually transmitted diseases in the same study population (16).

GBS serotypes

GBS is classified by capsular polysaccharide antigens into nine different serotypes (Ia, Ib, II-VIII) whose distribution varies by geographical area and clinical population (75). Serotypes Ia, Ib, II, and III were dominant in neonates in the 1970s-1980s (59, 75) and serotype V has emerged as a frequent cause of GBS infection or colonization in children and adults (50, 116, 132). Virulence, ability to cause invasive diseases, antibiotic resistance and transmission vary by GBS serotype. For instance, the ST-17 complex of serotype III is more likely to be virulent than other serotypes (80). While serotype V has become one of the most common GBS serotypes causing invasive disease in the United States (17, 59), serotype Ia was reported to be the dominant serotype detected in non-pregnant adults in Portugal (88). Antibiotic resistance is more frequently encountered in strains of serotype V than in strains of other serotypes (19, 116). Further, GBS transmission rate was also reported to be higher in serotype V than other serotypes (50).

GBS and antibiotic resistance

Treatment and prevalence of antibiotic resistance

GBS remains susceptible to penicillin, the first-line agent for both prophylaxis and treatment of GBS infection (104). Until recently, for patients with history of beta-lactam agent allergy, clindamycin and erythromycin were recommended as alternatives therapies. However, resistance to these two antibiotics has been remarkably increasing (25, 57, 67) (Table 2.1). Extremely high resistance rates to erythromycin and clindamycin were observed in some studies (50.7% to erythromycin (8) and 54% to clindamycin (67) (Table 2.1). Because of possible resistance problems with erythromycin and clindamycin, vancomycin is now the initial recommended treatment of GBS infection in patients who are allergic to penicillin (54).

Fluoroquinolones, especially the later generations, are active against GBS infections. Third and four-generation fluoroquinolones have been recommended by many health authorities and international organizations to treat pneumonia caused by GBS (15). However, resistance to fluoroquinolones has been detected, even to levofloxacin, a third-generation fluoroquinolone (112, 135, 137) (Table 2.1).

Mechanisms of Group B Streptococcal antibiotic resistance

GBS uses several mechanisms to achieve antibiotic resistance: alteration of penicillin-binding protein by point mutation (in resistance to beta-lactam antibiotics) (34), acquisition of new genes coding for ribosome protection or efflux

pump (60, 84, 106), acquisition of antibiotic resistance chromosomal transposon (106), chromosomal mutation (135), and plasmid-mediated resistance (22) (Table 2.2)

Beta-lactam antibiotics are bactericidal agents that act by inhibiting the peptidoglycan synthesis in bacteria cell wall (128). Although GBS remains susceptible to penicillin, ampicillin and first-generation cephalosporins, cases with increasing MICs in penicillin, ampicillin and cefazolin have been reported with alteration in penicillin-binding proteins (26). Point mutations in *bpb2x, a* gene encoding a region of penicillin-binding protein were identified among GBS strains with elevated MICs to beta-lactam antibiotics tested (34, 74) (Table 2.2).

Erythromycin and other macrolides inhibit bacterial growth by binding to the 23rRNA molecule in the 50S subunit of the bacteria ribosome, blocking the exit of peptide chain (128). Two common resistance mechanisms to erythromycin identified in GBS are methylation of 23S rRNA encoded by *erm* (erythromycin ribosome methylation) genes and drug efflux encoded by *mef* (macrolide efflux) genes (60, 106) (Table 2.2).

Similar to erythromycin, clindamycin and other lincosamides work primarily by binding to the 50S ribosomal subunit of bacteria, interfering the transpeptidation reaction and disrupting protein synthesis (128). Resistance to clindamycin is also caused by acquisition of *erm* genes (60, 106). Recently, the *inu*(B) gene was characterized and identified to be responsible for lincosamide nucleotidylation in GBS resistant isolates (84) (Table 2.2).

Fluoroquinolones exert their antimicrobial effects by inbibiting DNA synthesis through cleavage of bacterial DNA in gyrase and topoisomerase genes (102). Resistance mechanism related to fluoroquinolones among GBS has been mainly associated with target site alterations in the fluoroquinolone-resistance determining region (QRDR) of gyrase (*gyr*A , *gyr*B) and topoisomerase genes (*parC*, *par*E) (112, 135, 137) (Table 2.2). These two enzymes play a role in the replication, transcription, recombination and repair of DNA (66). The *gyr*A mutations typically occur at amino acid positions 81 and 85 and the *par*C mutations typically occur at amino acid positions 79 and 83 (14). Efflux-mediated resistance to fluoroquinolones has also been reported in *Staphylococcus aureus* and some other *Streptococcus spp*. (70, 71, 105).

Group B Streptococcal antibiotic resistance and serotypes

Distribution of GBS serotypes among GBS resistant strains has been analyzed in many studies. Some serotypes are more likely to be resistant to antibiotics than the others, which might relate to better adaptability. In the United States, serotype V strains are more likely than other serotypes to be resistant to erythromycin and clindamycin (25). In one study in Korea, more than three quarters of serotype V was found to be resistant to clindamycin or erythromycin or both (77). Serotype V was identified to be dominant (67%) among GBS isolates with macrolide resistance in pregnant women in Poland (21). In a 2011 study using clinical samples collected from Italy, most erythromycin-resistant GBS strains were of serotype V (56.8%) (38).

Does group B Streptococcal antibiotic resistance vary among colonizing and invasive isolates?

GBS can be a commensal or a pathogen. As a commensal, GBS can still develop resistance to antibiotics taken by the host. It is not yet determined whether there is any significant difference in the point prevalence of resistance to antibiotics between colonizing and invasive GBS isolates. In a 2001 study conducted in Canada, the resistance rate to erythromycin and clindamycin was found to be higher among non-invasive (colonizing) GBS isolates compared to invasive isolates (to erythromycin:18% versus 8%, to clindamycin: 8% versus 4.5%) (37). A similar result was observed in a 2006 study using GBS isolates collected from Wisconsin and Michigan (18). However, more studies are needed to identify the trend in the difference of antimicrobial resistance between commensal and pathogenic GBS.

***Escherichia coli* overview**

E. coli is a facultative anaerobic, gram-negative bacillus that belongs to the family *Enterobacteriaceae*. Most *E. coli* strains are part of the normal microbiota of the gastrointestinal tracts of humans and warm-blooded animals (136). In the human digestive tract, commensal *E. coli* strains predominantly reside in the large intestine, especially in the caecum and the colon (127). Commensal *E. coli* synthesizes vitamin K, an essential vitamin, and protects the host from invasions by pathogenic bacteria (7). The main source of *E. coli* pathogens are commensals that have acquired a virulence gene set (127). However, most

pathogenic *E. coli* have existed as separate pathogenic lineages for a very long time (127). The urinary tract is one of the most common sites of *E. coli* infection. Up to 80%- 85% of uncomplicated UTI and 25% of nosocomial UTI are caused by uropathogenic *E. coli* (UPEC) (100), which might originate in the perianal or vaginal region and are transmitted to the urinary tract via colonization of the vagina and periurethral area (134). It is very likely that UPEC are predominantly selected among different types of *E. coli* for growth in the urinary tract (65).

Epidemiology of UTI

Incidence and prevalence of UTI

Incidence and prevalence of UTI vary by age, gender and depend on many clinical and social risk factors (58, 63). Clinical studies suggest that the prevalence of UTI is higher in females, especially during adolescence and childbearing years, than in males (49). In the United States, UTI account for about 4 million ambulatory-care visits each year, representing about 1% of all outpatient visits (27). According to the report form NHANES (The National Health and Nutrition Examination Survey) in 2003, 53.5% of women and 13.9% of men in the U.S. self-reported to have UTI in the period of 1988-1994 (98*)*. A Practice Bulletin from the American College of Obstetricians and Gynecologists (ACOG) reported that approximately 11% of U.S. women report at least one diagnosed UTI per year, and the lifetime probability for a woman to have an UTI is 60% (4). The prevalence of UTI in children is lower than in adults. Up to 7% of girls and 2% of boys have symptomatic UTI by the age of six (86).

E. coli is responsible for a majority of UTI. In a 2005 study conducted in Turkey, *E. coli* was the causative agent in 90% of the uncomplicated UTI and in 78% of the complicated UTI (6). In another study on incidence of UTI in Nigeria (3), 22.4% and 4.56% of females and males tested, respectively had positive urine culture in which *E. coli* constituted the predominant organism and was responsible for 52.77% of the cases of UTI. *E. coli* was also found to account for 81% of the uropathogens isolated from 1667 patients with clinical suspicion of UTI visiting the University Health Clinic of University of California, Berkeley from 1999 to 2005 (121). In the study conducted by Foxman et al. on rectal-rectal colonization of *E. coli* in UTI and non-UTI women and partners from Michigan (51), 86% of the UTI were caused by *E. coli*. In children, a clear majority of UTI cases are caused by *E. coli* (up to 80%) (2).

Risk factors for UTI

UTI are commonly classified as complicated or uncomplicated. Uncomplicated UTI occur in patients who have normal, unobstructed genitourinary tract, who are not pregnant and who have no history of instrumentation (47, 91). All other cases belong to complicated UTI. Bacterial factors including adherence factors, siderophores, bacteriocins, toxins and biofilm formation are important risk factors for both uncomplicated and complicated UTI (47). However, these two types of UTI are differentiated by other risk factors including host factors and transmission of uropathogens (47).

Host-related risk factors for uncomplicated UTI include female sex, history of UTI, frequency of sexual intercourse, vaginal infection, condom or spermicide use and genetic susceptibility (47). In uncomplicated UTI, uropathogens are predominantly transmitted through sexual intercourse and possibly via food or water (47). Sexual intercourse in the week before study entry (RR = 2.98, 95% CI 1.30 – 6.83) and oral contraceptive use (RR = 2.41, 95% CI 1.12 – 5.17) were reported to be associated with the development of UTI among women with type 1 diabetes (52). In a cohort study conducted between 1998 and 2002 on healthy post-menopausal women (82), recent sexual intercourse was strongly associated with incident UTI (HR = 3.30, 95% CI 1.44 – 7.58).

Quite different from uncomplicated UTI, structural or functional abnormalities of the genitourinary tract are host-related risk factors for a majority of complicated UTI (35, 99). For instance, benign prostatic hyperplasia (BPH) that causes bladder obstruction and prostatitis are risk factors for UTI in men (56). In children, Vesicouteteral Reflux (VUR), the abnormal flow of urine from the bladder to the upper urinary tract can put the children at risk of UTI recurrence (93). Another important complicated UTI is catheter-associated UTI. Up to 40% of hospital-acquired UTI cases are related to urinary catheter insertion, a route of transmission of uropathogens (108). Higher risk of hospital-acquired UTI is associated with long-term use of catheter (120). In the 2008 studies conducted by Smith P.W. et al and Saint S. et al., long-term indwelling urinary catheter with associated bacteriuria was found in 5% - 10% of patients in long-term care facilities (110, 120). Having a catheter *in situ* for ≥7 days was significantly

associated with post-operative UTI (OR = 2.44) as reported in the study conducted by Crossby-Nwaobi RR. et al. (32).

Pathogenesis of E.coli UTI

The pathogenesis of *E. coli* UTI involves many steps including attachment of uropathogenic *E. coli* (UPEC) to the epithelium of the urinary tract, colonization, invasion and stimulation of inflammatory responses (68). UPEC strains initially colonize the perianal or vaginal region, then ascend to the periurethra region and other sites of the urinary tract (the urethra, bladder and kidney) where they subsequently colonize and cause injury by their virulence factors. The virulence factors carried by UPEC include diverse adhesins, siderophores, aerobactin system, toxins, hemolysin, O-antigen and K capsule (68). In general, the disease is associated with a variety of risks factors including gender, sexual behavior, pregnancy or menopause status, diabetes, kidney problem, use of catheter and use of antibiotics (46).

E. coli and antibiotic resistance

Treatment of E. coli UTI and prevalence of antibiotic resistance in E. coli

In general, antibiotics are the main therapy for all types of UTI. Treatment of UTI is not necessarily based on the specific causative bacteria or the actual bacterial count (103). The antibiotics recommended usually have the antimicrobial effect over a variety of organisms commonly found in UTI, including *E. coli*. The choices of antibiotics for the treatment of UTI depend on severity of

infection and characteristics of patients (age, gender, pregnancy status, medical history, etc.) (91) .

In uncomplicated UTI, trimethoprim/sulfamethoxazole (TMP/SMX) is the standard and first-line regimen (4, 27). Due to the emerging resistance to TMP/SMX and the high possibility of allergy to this antibiotic group, ciprofloxacin is recommended as an alternative and, in some cases, as the preferred first-line agent (27). Other alternatives include nitrofurantoin and fosfomycin, amoxicillin (with or without clavulanate) and cephalosporins (4, 27). In cases of frequent recurrences of UTI, continuous prophylaxis with once-daily treatment with nitrofurantoin, norfloxacin, ciprofloxacin, trimethoprim, or TMP/SMX is recommended (4).

Antibiotic resistance in *E. coli* is observed not only in pathogenic isolates but also in commensal strains isolated from fecal specimens. High rates of resistance to TMP/SMX, ampicillin and ciprofloxacin among *E. coli* isolates has been reported in several countries (up to 95% to ampicillin (11) and 94% to TMP/SMX in Bolivia and Peru (11) and 59% to ciprofloxacin in Lebanon (36)) (Table 2.3), thus limiting the effectiveness of UTI treatment with these empirical antibiotics. Resistance to other broad spectrum antibiotics (aminoglycosides (36, 81), cephalosporins (36)) has also been documented in several studies (Table 2.3).

Mechanisms of antibiotic resistance in E. coli

Resistance to antibiotics in *E. coli* are mediated by different mechanisms, depending on the mechanisms of action of each antibiotic. In this section, I summarize the mechanisms of resistance to three groups of antibiotics that are commonly recommended for the treatment of UTI (sulfonamides, fluoroquinolones and beta-lactams). Plasmid-mediated resistance is found against all these three groups of antibiotics.

TMP/SMX and other sulfonamides gain their bacteriostatic activity by competing with para-aminobenzoic acid (PABA) in the synthesis of dihydrofolic and tetrahyfrofolic acid (THF), resulting in inhibiting DNA synthesis. *E. coli* may develop resistance to TMP/SMX by over production of PABA (90) or by acquisition of R-plasmid specifying a dihydrofolate reductase which was resistant to trimethoprim (119).

Three different mechanisms have been reported to be responsible for resistance to fluoroquinolones among *E. coli* isolates: i) chromosomal mutations in gyrase (*gyr*A, *gyr*B) or topoisomerase genes (*par*C, *par*E) (61), ii) efflux-mediated (61) and iii) plasmid-mediated resistance associated with *qnr*, *aac*(6')*Ib-cr* or *qep*A genes (44). In 2011, resistance to fluoroquinolones through multi mechanisms was identified in fecal *E. coli* strains isolated from Accra, Ghana (96).

The most common resistance mechanism to beta-lactam antibiotics in *E. coli* is the production of beta-lactamases, enzymes that destroy beta-lactam ring and inactivate the antibiotics. In *E. coli*, this mechanism of resistance is mediated by

the acquisition of plasmid-encoded beta-lactamases TEM-1, TEM-2, SHV-1 (109) or AmpC (126).

Antibiotic resistance among commensal and uropathogenic E.coli

Although commensal *E. coli* is not pathogenic, it can still become resistant to the antibiotics consumed by the host. The difference in antibiotic resistance profile might be an important signal predicting the difference in adaptability between commensal and pathogenic *E. coli*. Resistance was more common in infecting (pathogenic) than commensal *E. coli* strains isolated from Southern India (89). However, in many studies, extremely high resistance rates to TMP/SMX, ciprofloxacin and ampicillin are observed in both commensal (e.g. 96% to ampicillin, 94% to TMP/SMX, 33% to ciprofloxacin) (11) and UPEC (e.g. 77% to ampicillin, 56% to TMP/SMX, 59% to ciprofloxacin) (36). More studies, especially those targeting at commensal and uropathogenic *E. coli* in the same population, are needed to accurately estimate if there is a difference.

Uropathogenic E. coli and the hypothesis of adaptation

UPEC is more transmissible than commensal *E. coli* between heterosexual sex partners (51). Although it is not clear whether the resistance rate to antibiotics is higher among UPEC strains compared to commensal strains, the emergence of UPEC resistance to antibiotics, recently to fluoroquinolones, suggests the speedy acquisition of antibiotic resistance among UPEC (23, 69). Additionally, high frequency of mutations was more likely to be observed in UPEC strains than in other *E. coli* (40). One suggestive hypothesis is that UPEC is more adaptable than commensal *E. coli* to the changes in the environment, possibly referring to horizontal gene transfer of mobile genetic elements (pathogenicity islands, plasmids, phages, transposons, insertion elements).

In 1987, CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) structure was first discovered in *E. coli* (87). Recently, this system has been identified as an immune system against undesirable genetic elements in bacteria and archea (62). The CRISPR region consists of several direct repeats separated by spacers (122). The DNA sequences of spacers are derived from phages or plasmids to which bacteria are exposed (130, 131). RNAs transcribed from spacer sequences recognize their target bacteriophage or plasmid and protect the host bacteria from phage infection or plasmid conjugation (87), thus limiting horizontal gene transfer. Reasoning that CRISPR might act as a defense system in *E. coli*, we hypothesized that the better adaptability in UPEC including acquisition of antibiotic resistance might be associated with a lower level of CRISPR-based immunity.

Significance

Antibiotic resistance is a serious health threat all over the world. High prevalence of resistance to antibiotics, and increasing trends in resistance have associated with many bacteria, including GBS and *E. coli*. Resistance rates and antibiotic resistance mechanisms in each organism often vary greatly among different populations and geographic sites. New mechanisms of resistance are also emerging. Findings from our investigations could contribute not only to the choice of appropriate therapeutic strategies but also to the development of new molecules that are effective against the resistant organisms. In addition to the mechanisms of resistance, comparison of antibiotic resistance rates between colonizing and invasive strains of GBS and *E. coli* is helpful to better understand the adaptability of these two organisms.

Adaptability among *E. coli* strains can also be studied through the recently-described CRISPR system (87). Molecular epidemiologic research on the association among CRISPR distribution, sites of colonization and antibiotic resistance will give laboratory evidence for the hypothesis of better adaptation in UPEC and provide deeper insight into the relationship between antibiotic resistance and immunity. Moreover, this research is useful in leading the way to similar studies in GBS and other bacteria. Finally, in the perspectives of public health and clinical practice, results from this research could contribute to the prediction of outbreaks or antibiotic resistance emergence, to the prevention of transmission among high risk individuals and to the development of CRISPR-intergrated antimicrobial agents or vaccines.

Table 2.1. Literature review on prevalence and trends of Group B Streptococcal resistance to erythromycin, clindamycin and quinolones (1998-2011)

Antibiotic	Location	Study population	Sites sampled	Prevalence and trend of GBS resistance	Time	Source
Erythromycin	USA & Canada	Neonates, adults, pregnant women	Blood; CFS, vaginal, anal, throat, ear specimens	7% - 25%	1998 - 2001	26
	USA	Adults	Rectal and vaginal specimens	38%	Dec. 2004 - Jun. 2005	57
	USA	Neonates, men, pregnant and non-pregnant women	Blood, amniotic fluid, placentas and other normally sterile sites	25.6% Trend: increase (15.8%- 32.8%)	1996 - 2003	25
	USA	Pregnant women	Perinatal, rectovaginal specimens	50.7%	Jan. 2011 – Oct. 2011	8
	South Korea	Pregnant women	Urine, vaginal and rectal specimens	25.6%	Jan. 2006 - May 2008	77
	Taiwan	Adults	Wound, pus, urine, blood, female genito urinary tracts	44%	Jun. 2001- Apr. 2007	67
Clindamycin	USA & Canada	Neonates, adults, pregnant women	Blood; cerebro spiral fluid (CSF), vaginal, anal, throat, ear specimens	3% - 15%	1998 - 2001	26

	USA	Adults	Rectal and vaginal specimens	21%	Dec. 2004-Jun. 2005	57
	USA	Neonates, men, pregnant and non-pregnant women	Blood, amniotic fluid, placentas and other normally sterile sites	25.6% Trend: increase (10.5% - 15%)	1996-2003	25
	USA	Pregnant women	Perinatal, rectovaginal specimens	38.4%	Jan. 2011 – Oct. 2011	8
	Taiwan	Adults	Wound, pus, urine, blood, female genito urinary tracts	39 %	Jun. 2001-Apr. 2007	67
	South Korea	Pregnant women	Urine, vaginal and rectal specimens	54%	Jan. 2006-May 2008	77
Quinolones	New York, USA	Clinical patients	Urine, blood, skin and soft tissue, respiratory tract, body fluids.	4.4% (levofloxacin)	1999-2002	135
	Italy	Pregnant women	Vaginal and rectal specimens	6.8% (norfloxacin) 2.7% (ciprofloxacin) 6.8% (ofloxacin) 10.9% (pefloxacin) 1.3% (levofloxacin)	2008	112
	Taiwan	Adults	Wound, pus, urine, blood, female genito urinary tracts	1.3% (levofloxacin)	2008	137

Table 2.2. Literature review on mechanisms of resistance to antibiotics among Group B *Streptococcus* isolates (1980-2008)

Antibiotic	Mechanisms of resistance	Year(s) detected	Location	Source
Beta-lactams	Point mutation in *pbp2x* gene	2008	USA	34
Fluoroquinolones	Mutations in gyrase (*gyr*A, *gyr*B) and topoisomerase (*par*C, *par*E)	2005	USA	135
Erythromycin	Ribosomal methylase (acquisition of *erm*(A), *erm*(B), *erm*(C), *erm*(TR) genes)	2003	France	106
		2004	USA	60
	Efflux pump (acquisition of *mef*(A), *mef*(E) genes)			
Tetracyclin	Ribosome protection (acquisition of *tet*(M), *tet*(O), *tet* (S), *tet* (T) genes)	2003	France	106
	Efflux by proton antiporters (acquisition of *tet* (L), *tet* (K) genes)			
	Plasmid-mediated	1980	USA	22
Streptomycin Kanamycin Gentamicin	Acquisition of *aphA*-3 gene	2003	France	106
	Acquisition of chromosomal gentamicin resistance transposon Tn3706			
Lincosamides	Ribosomal methylase (acquisition of *erm*(A), *erm*(B), *erm*(C), *erm*(TR) genes)	2004	USA	60
	Lincosamide nucleotidylation (acquisition of *lnu* (B) gene)	2004	New Zealand	84

Table 2.3. Literature review on prevalence and trends of *Escherichia coli* resistance to trimethoprim/sulfamethoxazole, fluoroquinolones, beta-lactams and aminoglycosides (1998-2010)

Antibiotic	Location	Study population	Site sampled	Prevalence and trend of *E. coli* resistance	Time	Source
Trimethoprim/ Sulfamethoxazole	USA	Outpatients	Urine	18%	1998	72
	USA	Women with cystitis	Urine	14.6%	2002-2006	31
	Japan	Patients with UTI	Urine	10.3%	2001-2002	94
	Lebanon	In- and out-patients with UTI	Urine	Trend: increase 49% - 56%	2000-2009	36
	UK	Community and hospitalized patients	Urine	40% (trimethoprim)	Jan 2005-Dec 2005	12
	Peru and Bolivia	Children	Feces	94%	Sep 2005-Dec 2005	11
Fluoroquinolones	USA	Outpatients	Urine	2.2% (ciprofloxacin)	1998	72
	USA	Men underwent Transrectal Ultrasound	Rectum	22%	Jan 2009-Mar 2010	81

		Guided Prostate Needle Biopsy				
	Japan	Patients with UTI	Urine	7.9% (levofloxacin)	2001-2002	94
	Lebanon	In- and out-patients with UTI	Urine	Trend: increase 25%-65% (norfloxacin) 22%-59% (ciprofloxacin)	2000-2009	36
	France	Hospitalized patients	Urine	5.3%	Nov. 1998-Feb. 1999	123
Beta-lactams	USA	Outpatients	Urine	38.5% (ampicillin)	1998	72
	Lebanon	In- and out-patients with UTI	Urine	Trend: increase 63%-77% (ampicillin) 42%-47% (amoxicillin/clavulanic) 4%-26% (cefotaxime) 3%-20% (cefepime)	2000-2009	36
	France	Hospitalized patients	Urine	48.1% (amoxicillin) 46.9% (ticarcillin) 40.6% (piperacillin)	Nov. 1998-Feb. 1999	123
	UK	Community and hospitalized patients	Urine	55% (ampicillin)	Jan 2005-Dec 2005	12
	Peru and	Children	Feces	95% (ampicillin)	Sep 2005-	11

	Bolivia				Dec 2005	
Aminoglycosides	USA	Men underwent Transrectal Ultrasound Guided Prostate Needle Biopsy	Rectum	33% (gentamicin) 45% (tobramycin)	Jan 2009-Mar 2010	81
	Lebanon	In- and out-patients with UTI	Urine	Trend: increase 11%-24%(gentamicin)	2000-2009	36

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CHAPTER 3

EMERGING FLUOROQUINOLONE RESISTANCE IN GROUP B *STREPTOCOCCUS* FROM SOUTH KOREA[1]

Abstract

GBS strains collected from 221 asymptomatic pregnant women (35 - 37 weeks of gestation) and clinical strains collected from 838 patients with GBS infection in Korea from 2006-2008 were tested for susceptibility to four fluoroquinolones: norfloxacin, ciprofloxacin, levofloxacin and moxifloxacin. 8.9 % 8.1 % and 0.8 %, of GBS were resistant to ciprofloxacin, levofloxacin and moxifloxacin respectively and ~ 93% of GBS strains were resistant to norfloxacin using EUCAST 2009 and 2011 guidelines. Resistance to ciprofloxacin and levofloxacin increased between 2006 to 2008. All strains were susceptible to penicillin. Ciprofloxacin and levofloxacin resistance were higher in clinical strains of GBS isolated from infections compared to colonizing strains isolated from pregnant women. Mutations in the quinolone resistance determining regions of gyrase and topoisomerase were detected in strains resistant to ciprofloxacin,

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levofloxacin and moxifloxacin; no mutation in gyrase or topoisomerase were found with strains resistant only to norfloxacin. We found a strong correlation between MIC values and presence of mutations in gyrase and topoisomerase genes. Strain serotypes were not associated with susceptibility to fluoroquinolones.

Introduction

GBS causes neonatal sepsis, and soft tissue infections, bacteraemia and endocarditis in immuno-compromised adult populations (9). Although GBS is a common bowel and vaginal inhabitant in many countries, GBS colonization rates in South Korea are low. Among pregnant women, vaginal colonization ranges from 1- 6%. However, the incidence of neonatal disease attributable to GBS has increased in recent years (15). GBS remains sensitive to penicillin; erythromycin or clindamycin are recommended alternatives for patients who are beta-lactam intolerant (9).

Fluoroquinolones (FQs) were introduced into clinical treatment for bacterial infections in the mid 1980s. The first and second generation FQs, including norfloxacin and ciprofloxacin are primarily active against gram-negative and some gram-positive organisms, while third generation FQs, levofloxacin, moxifloxacin and gatifloxacin, demonstrate improved gram-positive activity (23). GBS resistance to FQs emerged in 2002 (13), and continues to increase (4). In one hospital in Taiwan, FQ resistance among GBS increased from 0.33% in 2004 to 5.04% in 2006 (31). GBS serotypes most implicated in disease are also

associated with antibiotic resistance (1, 26). FQs primarily inhibit bacterial growth by binding to enzymes involved in DNA replication: DNA gyrase and DNA topoisomerase IV. Specific mutations found in a region of the *par* and *gyr* genes, called the quinolone resistance determining region (QRDR), alter the amino acid compositions of these enzymes resulting in decreased binding and decreased activity of FQs (6,13).

We studied the prevalence and serotype distribution of FQ resistance, and QRDR mutations among colonizing and clinical GBS strains collected from South Korea between 2006-2007. Since serotype V and III are the dominant circulating GBS serotypes in this population (24), we were interested in whether serotype V and III are also prevalent among FQ resistant strains. We report the association of FQ resistance phenotype with mutations in the QRDR region of the *gyr* and *par* genes and with GBS serotype.

Materials and methods

Study collection

The study collection included 333 colonizing isolates collected from 221 asymptomatic pregnant women (35 - 37 weeks of gestation) and 838 clinical isolates collected from 838 patients with GBS infection as described previously (23). Among 333 colonizing isolates, 237 unique isolates (based on unique serotypes from each individual) were selected for analysis. GBS isolates were collected from pregnant women receiving prenatal care at four hospitals, Eulji Hospitals in Seoul and Daejeon, Cheil Hospital in Seoul, and Motae Women's

Hospital in Daejeon. The isolates from clinical patients were sent to the Seoul Clinical Laboratories & Seoul Medical Science Institute (SCL) for microorganism culture from hospitals and clinics throughout the country between January 2006 and December 2008 (24). Written informed consent was obtained from all pregnant women, and the study protocol was approved by the Institutional Review Board of Eulji University Hospital (04 - 08 and 06 - 25), and Cheil Hospital (SCH – IRB – 2005 - 24).

Sample collection and isolation of GBS

GBS was isolated from urine, vaginal and anal swabs using selective media as previously described (24). We used a catalase test followed by a latex agglutination assay (Streptex; Murex Biotech Ltd., Dartford, England) to confirm the isolate was GBS.

Minimal Inhibitory Concentration (MIC) determination

All confirmed GBS isolates were tested for sensitivity to norfloxacin, ciprofloxacin, levofloxacin and moxifloxacin using microtiter broth determinations with VITEK II (www.biomerieux-usa.com) based on the protocols of Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (17). Using the MIC test, 1075 isolates were evaluated for ciprofloxacin and levofloxacin susceptibility, and 1027 isolates for moxifloxacin susceptibility. For the remaining 48 strains moxifloxacin, susceptibility was determined using disk diffusion test. To interpret MIC results, we used the two

different guidelines available. EUCAST 2009 is used by clinicians in Korea to guide treatment of GBS infections; the 2011 EUCAST guidelines are the most recent and increase the MIC cutoffs for levofloxacin and moxifloxacin. The EUCAST 2009 and EUCAST 2011 guidelines are shown in Table 3.1 (www.eucast.org).

Serotyping of GBS isolates

Commercially available GBS–kits (Essum, Sweden) was initially used to determine serotypes. Dot blot capsular type (5) in a microarray format (32, 33) or polymerase chain reaction (PCR) amplification was performed for isolates that were categorized as non-typeable by the kit, using protocols described previously (16). The following isolates were used as positive controls for dot blot capsular typing: CNCTC 1 / 85(Ia), DK14(Ib), DK23(II), M781(III), CNCTC 1 / 82(IV), CNCTC 10 / 84(V), NT6 (VI), 87-603(VII), and JM9(VIII) (C.E. Rubens collection) (10).

Detection of resistance genes with polymerase chain reaction (PCR) and sequencing

The QRDR regions of the *gyr* and *par* genes were amplified using primer CGAGTTTTATCGATTACGCC and TCACCAAGGCACCAGTAGG (PCR product size 511 bp), *gyrB* using primers CTGCTTCCAAAACAGGTCGC and GGAGAAGATGTTCGTGAAGG (PCR product size 644 bp); *parC* using primers AAGGGATTTCGCAAATCTGC and TCCTTGAATGATAGCGCCAG

(PCR product size 494 bp) and *parE* using CGTAAGGCATAAAAGCACG and CTATATCCGTCCAAGCATAC (PCR product size 547 bp). Reactions were carried out using a Bio-Rad MyCyclerTM under the following conditions: denaturation at 94^0C for 3 minutes, annealing at 45^0C, and elongation at 68^0C for 1 minute for 30 cycles, followed by a final elongation at 68^0C for 6 minutes. A total of 10 µl of PCR product was analyzed by electrophoresis on a 1% agarose gels in Tris-acetic acid/EDTA buffer (pH 7.5) with GelRed as post gel staining agent, and visualized by UV transillumination. PCR products were sequenced at the University of Michigan sequencing core and the DNA sequences were analyzed using the Lasergene DNA STAR software package.

Statistical Analysis

All statistical analyses were done using SPSS 18.0 statistical software (SPSS Inc., IL, USA) or SAS 8.0 statistical software (The SAS Institute, NC, USA). Changes in GBS resistance by year and serotype were tested for statistical significance using the chi-square test.

Results

Colonizing and clinical GBS strains (*n* =1075) were collected over a three-year period from different geographic areas in South Korea. All colonizing isolates were obtained from pregnancy screening, whereas clinical isolates were from 'test requested' samples to the Seoul Clinical Laboratory from throughout Korea. Clinical isolates were from urine (61.8%), vagina (10.4 %), surgical wound (6.0 %), cervix (5.9 %), pus or abscess (5.1 %), prostate (2.9 %), blood (0.8 %), sputum (0.8 %), and other (6.3 %). Colonizing isolates were from the urine (43.2 %), vagina (29.1 %), rectum (21.9 %) or were combined vaginal / rectal specimens (5.7 %).

Cross-resistance to other fluoroquinolones

Among the 1075 strains, 92 strains (8.6%) were resistant to newer quinolones, levofloxacin or moxifloxacin (Table 3.2). Among these 92 strains, 9 strains were resistant to all four FQs, 81 strains were resistant to norfloxacin, ciprofloxacin and levofloxacin and 2 strain was resistant to norfloxacin, ciprofloxacin and levofloxacin. Cross resistance amongst FQs was high; all ciprofloxacin resistant strains were norfloxacin resistant, all levofloxacin resistant were ciprofloxacin resistant, and all but one moxifloxacin resistant strain was also levofloxacin resistant (Table 3.2).

Fluoroquinolone resistance in GBS strains

Applying the EUCAST 2009 guidelines for antimicrobial susceptibility to results from microdilution testing, 8.9 % 8.1 % and 0.8 %, of GBS were resistant to ciprofloxacin, levofloxacin and moxifloxacin respectively (Table 3.3). 93% of strains were resistant to norfloxacin. With the exception of moxifloxacin resistance, prevalence of resistance to FQs was higher among clinical than colonizing isolates; ciprofloxacin resistance: 10.6% versus 2.5% ($p < 0.001$); levofloxaxin resistance 9.8% versus 2.1% ($p < 0.001$); moxifloxacin resistance: 0.2% versus 2.1% to ($p = 0.002$, Table 3.3). Between 2006 and 2008, prevalence of ciprofloxacin and levofloxacin increased significantly ($P = 0.009$ and $P = 0.009$, Table 3.3).

Serotype distribution

Strains resistant only to norfloxacin were less likely to be serotype Ia than strains resistant to both norfloxacin and ciprofloxacin ($p < 0.0001$, chi square test) and those sensitive to norfloxacin and ciprofloxacin ($p = 0.0004$). Strains resistant to norfloxacin and ciprofloxacin had a similar serotype distritbution to norfloxacin and ciprofloxacin sensitive strains (Table 3.4).

Genetic mutations

We anticipated that all FQ resistant strains would have mutations in one or more of the *gyrA* and *gyrB* subunits of gyrase and the *parC* and *parE* subunits of topoisomerase. We randomly selected strains resistant only to norfloxacin

(n=20), resistant to norfloxacin and ciprofloxaxin (n=36), resistant to levofloxacin (n=29 (EUCAST 2009 cutoff or n=45 (EUCAST 2011 cutoff)), resistant to moxifloxacin (n=9 (EUCAST 2009 cutoff or n=12 (EUCAST 2011 cutoff)), and sensitive to all FQs (n=2) for screening (Table 3.6). We also included five sequenced GBS strains that were FQ susceptible as controls for sequencing the *gyr* and *par* genes. Overall, we observed mutations in *gyr*A and *par*C in the resistant strains; none of the susceptible strains harbored mutations in *gyr* or *par* genes. We also observed silent mutations in *gyr*B, *par*C and *par*E (Table 3.5). Only mutations with amino acid changing were taken into analysis. Only three ciprofloxacin resistant strains harbored the Ser407Leu mutation in *gyrB*; these strains were also intermediate resistant to levofloxacin (data not shown). No mutations were detected in *parE*. We observed no *gyr* or *par* mutations among the 20 strains tested that were resistant only to norfloxacin. All ciprofloxacin resistant strains tested (n = 36) were also resistant to norfloxacin; all but one strain had a known mutation in either *par*C or *gyr*A (Table 3.6). Mutations in both the *parC* and *gyr* were found in 90% (27/29) of the levofloxacin resistant strains; the remaining two levofloxacin resistant strains harbored mutations only in *gyr.* All moxafloxacin resistant strains, had mutations in both *par*C and *gyr*A (table 3.6).

Among 15 strains that were either susceptible or intermediate resistant to norfloxacin and susceptible to other FQs (ciprofloxacin, or levofloxacin), we observed no mutation that occurred in both the *gyr* and *par* regions. However, the story for moxifloxacin is more confusing. Using the 2009 EUCAST guidelines

for resistance, 25% of the moxifloxacin susceptible strains had *gyr* and *par* mutations (data not shown). In the EUCAST 2011 guidelines, the MIC level for moxifloxacin was made more sensitive. Using this cutpoint for susceptibility, none of the moxifloxacin susceptible strains harbored mutations in both *gyr* and *par* genes.

Table 3.7 shows the association of MIC values for ciprofloxacin, levofloxacin and moxifloxacin with *par* and *gyr* mutations. There was a statistically significant association between the presence of both *par* and *gyr* mutations and high MIC values; this was especially true for ciprofloxacin and levofloxacin resistance where > 90% of the strains had MIC ≥ 8 ug/ml when both *par* and *gyr* mutations were present. Mutation in *par* alone or *gyr* alone were not significantly associated with high MIC values.

Among 95 GBS strains tested, more gyrase and topoisomerase mutations were observed in clinical isolates than in colonizing isolates (mutations in both *gyr* and *par* : 33.8% versus 29.6% (p=0.88), mutations in either *gyr* or *par* : 29.4% versus 18.5% (p=0.41) (Table 3.8)).

Discussion

We report an unexpectedly high prevalence of fluoroquinolone resistance among 1075 colonizing and clinical GBS strains from South Korea. In the entire collection, 93% of strains were resistant to norfloxacin, 8.9 % to ciprofloxacin, 8.1% to levofloxacin and 0.8% for moxifloxacin. A subset of nine GBS strains were resistant to all FQs tested but remained susceptible to ampicillin, penicillin,

cefazolin, and vancomycin; these antibiotics remain the preferred choice to treat GBS infections (4). With the exception of one strain, all FQ resistant strains were susceptible to erythromycin and clindamycin. In *Streptococus pneumoniae,* increased fluoroquinolone use is associated with increasing MIC and resistance to levofloxacin; this is likely true for other infections including GBS where fluoroquinolones are recommended for treatment (3, 26).

The resistance levels we observed are greater than that reported by the Antimicrobial Surveillance Program (1997-2004), where levofloxacin resistance was 0.7% in North America and 0% in Europe (4). Case reports of levofloxacin resistance have been reported in France (26) and Japan (13). In Taiwan, 5.0% of GBS strains collected from a single hospital were resistant to levofloxacin; an increase from 0.33% in 2004 to 1.3% in 2006 (31). The highest resistance rate to norfloxacin in GBS we have found so far was 6.8% in clinical GBS isolates from Italy, 2008 (22). This is particularly striking as 94% of the GBS strains in our collection were resistant to norfloxacin. FQ resistance was not concentrated in any one serotype, and the serotype distribution of ciprofloxacin resistant strains were not significantly different than that observed among susceptible strains.

Point mutations in the QRDR regions of *gyr* and *par* genes are known to confer FQ resistance in *Staphylococci* and *Streptococci* (13, 30, 6). We identified single and double mutants in FQ resistant GBS, where *par* mutations were found only in *parC* subunit, and *gyr* mutations were mainly found in *gyrA*. Three strains that were ciprofloxacin resistant and levofloxacin intermediate resistant harbored

mutations in *gyrB* but not in *gyrA*. Mutations in *gyrB* are uncommon, but have been previously reported in FQ resistant *Streptococcus pneumoniae* (20). We did not find triple mutations in *gyrA*/*parC*/*parE*, which was previously reported for moxifloxacin resistance in GBS from Taiwan (31). A few FQ resistant strains harbored mutations only in *par* or only in *gyr*; thus mutations in both *par* and *gyr* do not seem to be necessary for resistance to ciprofloxacin, levofloxacin or moxifloxacin. However, the MICs for resistant strains were higher when mutations in both *gyr* and *par* were present. The presence of both *par* and *gyr* mutations was associated with MIC levels ≥ 8 ug/ml. This is a level considerably higher than reportedly present in the serum during treatment (3- 5 ug/ml), although tissue concentrations may be higher. Using the EUCAST 2009 cutpoints to determine resistance, two strains with intermediate moxifloxacin resistance harbored mutations only in amino acid 81 in *gyrA.* None of the intermediate moxifloxacin resistant strains harbored mutations in *parC*. Seven strains with intermediate resistance to ciprofloxacin and levofloxacin harbored a mutation only in *parC* either at amino acid 79 or at 83. These findings are consistent with previous reports that hydrophilic and hydrophobic quinolones differ in their primary targets. In *Streptococcus pneumoniae* and *Staphylococcus aureus* the more hydrophopic quinolone moxifloxacin targets gyrase, while the hydrophilic quinolones, ciprofloxacin and levofloxacin, primarily target topoisomerase (*par* genes) (7, 18, 25).

We detected a very high prevalence of norfloxacin resistance in GBS (~93%), however we did not find QRDR mutations in GBS strains resistant only

to norfloxacin. Studies were thus conducted to determine if an active efflux pump was operational in the strains resistant only to norfloxacin, which is also the content of the next chapter of the dissertation. Efflux pumps have been reported for other *Streptococci* and *Staphylococcus aureus* (2,12,19). Other mechanisms, including plasmid encoded FQ resistance, has been reported for gram negative organisms (27) but their association with FQ resistance in gram positive organisms has not been demonstrated (21). Mutations outside the QRDR regions may also contribute to expression of resistance in *Streptococci*. In a lab generated FQ resistant strain, mutations in the promoter region of the *parE* gene(s) had lowered transcript levels of *par* and increased ciprofloxacin resistance even when no mutations were present in the QRDR region (11).

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Table 3.1. MIC breakpoints for determining antimicrobial susceptibility of Group B *Streptococcus* to fluoroquinolones.

Guidelines *	MIC Breakpoint (µg/ml)			
	EUCAST 2011		EUCAST 2009	
	S	R	S	R
Norfloxacin	na	na	≤1	≥ 4
Ciprofloxacin	- **	- **	≤1	≥ 4
Levofloxacin	≤ 1	>2	≤ 2	≥ 8
Moxifloxacin	≤ 0.5	> 1	≤ 1	≥ 4

* S : Susceptible, R: Resistant

** Susceptibility testing not recommended as species is a poor target for therapy with drug.

na: Zone diameter breakpoints not available.

Table 3.2. Cross resistance among fluoroquinolones in Group B *Streptococus* strains collected in South Korea (2006-2008)

Norfloxacin (NF)	Ciprofloxacin (CF)	Levofloxaci (LF)	Moxifloxacin (MF)	N	Number of strains tested *
S	S	S	S	2	**2**
I	S	S	S	54	**13**
R	S	S	S	886	**20**
R	I	S	S	23	**14**
R	I	I	S	10	**10**
R	R	S	R	1	**1**
R	R	I	S	7	**6**
R	R	R	S	81	**18**
R	R	R	I	2	**2**
R	R	R	R	9	**9**
			Total	1075	**95**

* Strains tested for mutations in the QRDR of *gyr* and *par* genes.
I: intermediate, S: susceptible, R: resistant

Table 3.3. Fluoroquinolone resistance in Group B *Streptococcus* from South Korea (2006-2008)

	Resistance prevalence %				Resistance by year %			
Fluoroquinolone (n)	Total (1075)	Clinical (838)	Colonizing (237)	p-value*	2006	2007	2008	p-value**
Norfloxacin* **	92.7	90.1	89.0	**0.002**	94.1	93.1	98.1	0.215
Ciprofloxacin	8.9	10.6	2.5	**<0.001**	5.6	10.6	11.2	**0.009**
Levofloxacin	8.1	9.8	2.1	**<0.001**	5.1	9.6	10.6	**0.009**
Moxifloxacin	0.8	0.2	2.1	**0.002**	1.0	0.4	0.6	0.414

p-values obtained by chi-square test * and chi-square test for trend**. Values in bold were statistically significant

*** 1058 strains were tested for norfloxacin susceptibility.

Table 3.4. Comparison of serotype distribution of Group B *Streptococcus* among norfloxacin and ciprofloxacin susceptible and resistant strains from South Korea (2006-2008)

Serotype (n)	NF only resistant strains (n) %	NF &CF resistant strains (n) %	NF &CF susceptible strains (n) %
Ia (108)	62 (14)	32 (34)	14(39)
Ib (54)	35 (8)	14 (15)	5 (14)
III (175)	148 (33)	17 (18)	10 (28)
V (152)	126 (28)	21 (22)	5 (14)
Other * (85)	72 (16)	11 (12)	2 (6)
Total (574)	443	95	36

NF: norfloxacin, CF: ciprofloxacin, LF: levofloxacin, MF: moxifloxacin

* Other serotypes include serotypes II, VI, VIII and non –typeable strains.
Serotype data available for 574 out of 1075 strains.

Table 3.5. Gyrase and topoisomerase mutations identified among 95 group B *Streptococcus* strains from South Korea (2006 – 2008) that were screened for mutations

Quinolone resistance determining region	**Nucleotide substitution that altered one aminoacid**	**Silent mutations**
gyrA	Ser81Leu Glu85Lys	-
gyrB	Ser407Leu	1101 T-->A
parC	Ser79Phe Ser79Tyr Ser79Ile Asp83Gly Asp83Tyr	1873 G-->A; 1936 T-->C; 1981 G -->A; 2026 G-->A; 2035 G-->T or G -->C; 2213 T-->C; 2214 C-->T; 2218 G-->A; 2225 G-->A or G-->T
parE	-	1302 C-->T; 1356 A-->G

Table 3.6 . Mutations in Quinolone Resistance Determining Region QRDR regions of *gyr* and *par* genes in FQ resistant strains of Group B *Streptococcus* from South Korea (2006-2008). Minimal inhibitory concentration (MIC) cutoffs for antimicrobial susceptibility were determined using EUCAST 2009 guidelines except where indicated.

	NF only resistance (n) %	**NF and CF resistance (n) %**	**LF resistance (n) %**		**MF resistance (n) %**	
Number of resistant strains	20	36	29	45 *	9	12*
parC and gyrA	0 (0)	31 (86)	27 (90)	31 (69)	9(100)	10(83)
parC or gyrA	0 (0)	11 (4)	2 (7)	11 (24)	0 (0)	1 (8)
No mutation detected**	20 (100)	3 (1)	0 (0)	3 (7)	0 (0)	1 (8)

NF: norfloxacin, CF: ciprofloxacin, LF: levofloxacin, MF: moxifloxacin

MIC cutoffs with EUCAST 2009 guidelines: MIC ≥ 4 ug/ml for strains resistant to norfloxacin, ciproflocacin and moxifloxacin, MIC ≥ 8 ug/ml for levofloxacin resistance.

*MIC cutoffs with EUCAST 2011 guidelines: MIC > 1 ug/ml for moxifloxacin and MIC >2 for levofloxacin resistance. Guidelines for ciprofloxacin and norflocacin were not available.

** Two strains susceptible to all FQs tested did not harbor any mutation in *gyr* or *par*. Strains with intermediate resistance to FQs were not included for analysis.

Table 3.7. Association of fluoroquinolone minimal inhibitory concentration (MIC) (µg /ml) in 95 group B *Streptococcus* study strains with mutations in *par* and *gyr* for ciprofloxacin, levofloxacin and moxifloxacin

	MIC (µg/ml)					
	≤ 0.5	**≤ 1**	**2**	**4**	**≥ 8**	**p-value***
Ciprofloxacin						
par and *gyr* *(n(%))*	0 (0%)	0 (0%)	0 (0%)	4 (57%)	27 (93%)	**0.001**
par or *gyr* *(n(%))*	1 (4%)	0 (0%)	15 (62%)	2 (29%)	2 (7%)	0.63
Levofloxacin						
par and *gyr* *(n(%))*	0 (0%)	0 (0%)	0 (0%)	4 (25%)	27 (93%)	**0.001**
par or *gyr* *(n(%))*	0 (0%)	1 (4.5%)	8 (44%)	9 (56%)	2 (7%)	0.39
Moxifloxacin						
par and *gyr* *(n(%))*	0 (0%)	20 (61%)	1 (50%)	9 (91%)	0 (0%)	**0.001**
par or *gyr* *(n(%))*	10 (20%)	9 (27%)	1 (50%)	0 (0%)	0 (0%)	0.38

*p-value generated using Cochran Mantel Haenszel statistic for the association of *par* and *gyr* mutations together with MIC levels.

Table 3.8. Comparison of gyrase and topoisomerase mutations between invasive and colonizing Group B *Streptococcus* strains from South Korea (2006-2008)

	Clinical (n=68)	**Colonizing** (n=27)	**p value*** (α=0.05)	**95% CI**
par and *gyr* (%(n))	33.8% (23)	29.6% (8)	0.88	-0.2 - 0.3
par or *gyr* (%(n))	29.4% (20)	18.5% (5)	0.41	-0.1 - 0.3
Total	63.2% (43)	48.1% (13)	0.26	-0.1 - 0.4

*p-values obtained by chi-square test

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CHAPTER 4

EFFLUX-MEDIATED RESISTANCE IDENTIFIED AMONG NORFLOXACIN RESISTANT CLINICAL STRAINS OF GROUP B *STREPTOCOCCUS* FROM SOUTH KOREA[2]

Abstract

Group B *Streptococcus* (GBS) is a major cause of neonatal sepsis and an emerging cause of infection in immune-compromised adult populations. GBS is also a commensal commonly found in the vaginal and gastrointestinal tract. While still sensitive to penicillin, GBS is increasingly resistant to secondline therapy, including erythromycin and clindamycin. In 2005, GBS resistance to fluoroquinolones was identified; resistance was caused by mutations in the quinolone- resistance determining regions (QRDRs) of gyrase and topoisomerase genes. We selected 146 GBS strains out of which 88 strains were resistant only to norfloxacin to screen for evidence of efflux-mediated resistance to norfloxacin. This study collection was randomly selected from 221 asymptomatic pregnant women (35 - 37 weeks of gestation) and 838 patients with GBS infection from South Korea from 2006-2008. Susceptibility to

[2]This study will be submitted with the following authors: Dang, N.D.T, Z. Britt, U. Srinivasan, S. Ponnaluri , C.F. Marrs, M. Ki and B. Foxman.

norfloxacin, ciprofloxacin, levofloxacin and moxifloxacin was identified using VITEK II automatic system (Biomerieux) with the MIC breakpoints from EUCAST 2009 and 2011 guidelines. Fifty-three percent of the study collection was previously screened for mutations in the quinolone resistance regions of gyrase and topoisomerase genes. To identify the efflux phenotype, we used two different susceptibility tests: one with norfloxacin and the other with ethidium bromide as substrates in the presence of reserpine. With both substrates, evidence of the efflux phenotype was found in half of GBS strains resistant only to norfloxacin with no known mutations. However, isolates classified as the efflux phenotype using norfloxacin as a substrate agreed only moderately with isolates classified using ethidium bromide as a substrate (Kappa = 0.6). No evidence of efflux phenotype was detected in GBS strains that were resistant to moxifloxacin or levofloxacin or both. Additionally, no difference in the proportion of efflux phenotype between colonizing and clinical strains was found among GBS strains resistant only to norfloxacin. To our knowledge, this is the first report of efflux-mediated resistance to fluoroquinolones among GBS. Since the two methods used proved inconsistent, future studies are needed, especially those aiming at characterizing genes responsible for efflux resistance to fluoroquinolones in GBS.

Introduction

Group B Streptococcus (GBS, *Streptococcus agalactiae*) is a major cause of neonatal sepsis and an emerging cause of infection in immune-compromised adult populations (6). It is also a common member of the bowel microbiota. As a commensal, GBS is exposed to all antibiotics taken by the host. Although fluoroquinolones are not a firstline GBS therapy - GBS remains sensitive to penicillin - fluoroquinolone resistance in GBS has been reported worldwide (7, 17, 23, 26, 27).

Fluoroquinolone resistance in GBS is usually attributed to mutations in the gyrase and topoisomerase genes. However, we previously observed a high level of resistance to norfloxacin among a collection of GBS isolates from South Korea that could not be explained by these mutations (14) (see Chapter 3). One possible alternative mechanism is an efflux pump. Efflux-mediated resistance to fluoroquinolones has been observed in *Staphylococcus aureus* (5, 11, 12, 19), *Streptococcus pneumoniae* (10, 18, 22, 29), *Streptococcus pyogenes* (16) and *Streptococcus suis* (16).

Efflux can result from several mechanisms, with the mechanism varying by substrate. One antibiotic may be the substrate of one or more efflux pumps from the same or different efflux families (15,16) (Appendix A). Two possible ways of detecting efflux systems involved in norfloxacin resistance included (i) identifying changes in fluoroquinolone minimum inhibitory concentration (MIC), and (ii) measurement of susceptibilities to efflux pump substrates (21). While there are other methods, these two lend themselves to screening because of ease of use

and low cost.

We screened invasive and colonizing norfloxacin-resistant GBS isolates from South Korea with and without known mutations in the gyrase and topoisomerase genes for the efflux type. Over half of the isolates with no known mutations expressed an efflux phenotype.

Materials and methods

Study collection

Sample collection and GBS isolation were described previously (25). Briefly, invasive isolates were collected throughout South Korea, and colonizing isolates were collected from the urine, vagina and rectum of healthy pregnant women receiving prenatal care at four hospitals in South Korea (Eulji Hospitals in Seoul and Daejeon, Cheil Hospital in Seoul, and Motae Women's Hospital in Daejeon) between January, 2006 and December, 2008. Susceptibility to quinolones was tested using VITEK II at Seoul Clinical Laboratories & Seoul Medical Science Institute (SCL) with the MIC levels set according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) 2009 and 2011 guidelines (www.eucast.org) (Table 3.1). Among the 1075 GBS strains collected, 9.3%, 9.5% and 0.8% were resistant to ciprofloxacin, levofloxacin and moxifloxacin, respectively and 82% (886 strains) were resistant only to norfloxacin. Ninety-five strains were randomly selected to represent the observed resistance patterns, including 20 strains resistant only to norfloxacin, for screening for mutations in the quinolone resistance regions of gyrase and topoisomerase genes. Mutations

were identified using the polymerase chain reaction with specific primers followed by sequencing (14).

For the current study, we selected 88 strains resistant only to norfloxacin including the 20 strains resistant only to norfloxacin previously screened for mutations mentioned and 68 strains randomly selected from the remaining 866 strains resistant only to norfloxacin. For comparison, we included 58 strains previously screened for mutations from other fluoroquinolone resistance patterns (Table 4.1). For the analysis, the 146 GBS strains tested for the efflux phenotype were classified into three different categories: i) no mutations in *gyr*A and *par*C, ii) mutations in *par*C only and iii) mutations in both *gyr*A and *par*C (Table 4.1). Among the selected strains, 71% were invasive and the remaining strains were colonizing.

Identification of Efflux Phenotype

We used two tests for the efflux phenotype. First, we screened for a change in the MIC of norfloxacin in the presence of reserpine (20 µg/mL), an efflux pump inhibitor. As a confirmation, we repeated the experiments using ethidium bromide as the substrate. For both tests, GBS were grown in Todd Hewitt broth at 37^0C for 18 hours. The suspension was adjusted to a 0.5 Mc Farlane standard suspension and transferred into a 96-well plate of Todd Hewitt medium in the presence of different concentrations of norfloxacin or ethidium bromide (EB). The final bacterial concentration in each well was ~$5*10^5$ CFU/ml. The plate was incubated at 37^0C and MIC values were read after 18 hours of

incubation (24). Norfloxacin was obtained from Sigma Aldrich, Co., Missouri, U.S.A; reserpine was obtained from MP Biomedicals, LLC, Ohio, U.S.A and ethidium bromide was obtained from Fisher Scientific, New Jersey, U.S.A.

We used *Staphylococcus aureus* strain 1199B cloned with *nor*A efflux pump (provided by Dr. Glenn W. Kaatz, Wayne State University) as a positive control and GBS strains ATCC 12403 and A909 as negative controls.

Data analysis

All data analyses were performed using SAS ® software (SAS Version 9.2 for Windows; SAS Institute Inc., Cary, NC).

Results

In over half of the GBS strains resistant only to norfloxacin that had no known mutations, we observed an approximately fourfold reduction in the mean of norfloxacin in the presence of reserpine (Table 4.2). This reduction suggests the presence of an efflux pump. There was also weak evidence of efflux among GBS strains with mutations in *par*C gene only, where we observed a 1.6 fold reduction in mean of norfloxacin MIC in the presence of reserpine (Table 4.2). Among GBS strains with mutations in both *par*C and *gyr*A, no change in norfloxacin MIC was detected (Table 4.2). Additionally, we found no statistically significant difference in the proportion of efflux phenotypes between colonizing and clinical isolates among GBS strains resistant to only norfloxacin (data not shown).

In the similar susceptibility test using EB as the substrate, 29.4% of these isolates showed a fourfold reduction in mean of EB MIC (data not shown). In contrast to the results from the susceptibility test using norfloxacin where the prevalence of efflux phenotype increased only slightly with a twofold cutoff (from 52.2% to 59.1%), a remarkable difference was found in the prevalence of efflux phenotype between fourfold and twofold cutoff (29.4% and 54.1%, respectively). EB MICs of two negative controls, GBS strains ATCC 12403 and A909, were both 4 μg/mL and no difference in EB MIC was found when reserpine was added.

A comparison of the results of the two detection methods, using the fourfold cutoff for norfloxacin and twofold cutoff for EB showed only a moderate agreement (kappa = 0.6) (Table 4.3).

Discussion

We found evidence of the efflux phenotype in half of 88 GBS strains resistant only to norfloxacin with no known mutations using both norfloxacin and EB as the substrates in the presence of reserpine. To our knowledge, this study is the first to screen for evidence of efflux-mediated resistance to norfloxacin among clinical strains of GBS. This study is also the first report of the efflux phenotype among norfloxacin resistant strains of GBS, although efflux-mediated resistance to other antibiotics (macrolides (3, 4), tetracyclin (2)) has been reported in GBS. The efflux phenotype was observed in 45.4% of 273 norfloxacin- and ciprofloxacin-resistant clinical isolates of *Streptococcus pneumoniae* in the studies conducted by Brenwald (1) and in all norfloxacin-

resistant clinical isolates of *Streptococcus pneumoniae* with no QRDR mutations in Iraurgui's study (8).

We found no evidence of the efflux phenotype in GBS strains resistant to levofloxacin, moxifloxacin, ciprofloxacin and norfloxacin when *gyr*A and *par*C mutations were present. However, we did detect the efflux phenotype in two strains with mutations in *par*C and no *gyr*A mutation. This result is consistent with previous reports in other gram-positive bacteria (12, 20) that suggest that efflux is the first step of low-level resistance to hydropholic compounds like norfloxacin and ciprofloxacin, while target alteration (i.e, mutations in *gyr*A and *par*C) accounts for higher levels of resistance. Hydrophobic fluoroquinolones (e.g. moxifloxacin and levofloxacin) were poor substrates for efflux pumps in *Streptococcus pneumoniae* (1) *and Staphylococcus aureus* (12, 28).

We observed overall agreement of 80% (kappa = 0.6) when comparing efflux prevalence detected by norfloxacin (using a fourfold reduction cutoff) to EB MIC (using a twofold reduction cutoff). An efflux pump may operate differently depending on the substrate or we may have detected two different efflux pumps. In *Streptococcus pneumoniae*, fluoroquinolones are the substrates of three different efflux systems (PatA/PatB, SP2073/SP2075, PmrA) while SP2073/SP2075 is the only efflux system associated with EB (16). In our collection, the pump (or pumps) detected were less effective when using EB as a substrate than norfloxacin.

A fourfold reduction in MIC in the presence of reserpine is considered the appropriate cutoff for most fluoroquinolone MIC screening methods (13). We

found only modest differences in the prevalence of the efflux phenotype using the fourfold and twofold cutoffs with norfloxacin as a substrate. By contrast, using EB as a substrate, the cutpoint choice dramatically changed the estimate of the prevalence of the efflux phenotype (29.4% with fourfold cutoff and 54.1% with twofold cutoff). Previous studies using EB as a substrate have used a fourfold cutoff for laboratory mutants of *Staphylococcus aureus* (13) and *Streptococcus pyogenes* (9) but a twofold for clinical strains of *Staphylococcus aureus* (21). Our experience suggests that more than one substrates should be used to detect the efflux phenotype.

In summary, our study suggests the presence of an efflux phenotype in GBS using norfloxacin as a substrate. Future studies are needed to identify and characterize the mechanism leading this phenotype.

Acknowledgements

We thank Dr. Glenn W. Kaatz, Wayne State University for providing the positive control strain, *Staphylococcus aureus* strain 1199B cloned with *nor*A efflux pump. This study was financially supported by MAC-EPID (The Center for Molecular and Clinical Epidemiology of Infectious Diseases), The University of Michigan.

Table 4.1. Distribution of resistance to selected fluoroquinolones in isolates tested for efflux phenotype and screened for mutations among 1075 invasive and colonizing clinical Group B Sreptococcus isolates from South Korea (2006-2008)

Norfloxacin (NF)	Ciprofloxacin (CF)	Levofloxacin (LF)	Moxifloxacin (MF)	N	Number of strains tested for efflux	Number screened for mutations*	Mutations identified
S	S	S	S	2	2	2	No mutation
I	S	S	S	54	9	9	
R	S	S	S	886	88	20	
R	I	S	S	23	8	8	Mutations in parC only
R	I	I	S	10	8	8	
R	R	S	R	1	1	1	
R	R	I	S	7	6	6	Mutations in both *gyr*A and *par*C
R	R	R	S	81	13	13	
R	R	R	I	2	2	2	
R	R	R	R	9	9	9	
			Total	1075	146	78	

Table 4.2. Minimum inhibitory concentration (MIC) to norfloxacin in the presence/absence of reserpine of 146 clinical Group B *Streptococcus* strains from South Korea (2006-2008)*

Category	Count	Mean of MIC (μg/mL)		Average (range) of fold reduction in MIC between -/+ reserpine	% detected with reduction in MIC between -/+ reserpine	
		- Reserpine	+ Reserpine		≥ 4 fold difference	≥ 2 fold difference
No mutations						
Susceptible	2	4	4	1 (1-1)	0	0
Intermediate resistant to norfloxacin	9	13.3	7.6	2.2 (1-4)	33.3	55.6
Resistant to norfloxacin	88	33.9	12.8	3.6 (1-16)	52.2	59.1
Mutations in *par*C	17	41	31.5	1.6 (1-4)	18.8	25.0
Mutations in both *gyr*A and *par*C	30	128	128	1 (1-1)	0	0

* Norfloxacin MICs of two negative controls, GBS strains ATCC 12403 and A909, were both 4 μg/mL; no difference in norfloxacin MICs was found when reserpine was added.

Table 4.3. Agreement in detection of the efflux phenotype using norfloxacin and ethidium bromide (EB) as substrates among 88 clinical Group B *Streptococcus* strains resistant only to norfloxacin from South Korea (2006-2008)

		Norfloxacin MIC-based method (using fourfold reduction in norfloxacin MIC as cutoff)		
		+	-	
EB MIC-based method (using twofold reduction in EB MIC as cutoff)	+	38	9	47
	–	8	33	41
		46	42	88

Kappa = 0.6

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CHAPTER 5

IDENTIFICATION OF THE ASSOCIATIONS AMONG *ESCHERICHIA COLI* CRISPR STRUCTURE, UROPATHOGENICITY AND ANTIBIOTIC RESISTANCE AMONG CLINICAL ISOLATES FROM MICHIGAN[3]

Abstract

CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) are short fragments of DNA first discovered in *E. coli* that act as a sort of immune system protecting bacteria against invasion by phages, plasmids or other forms of foreign DNA. Using 81 pairs of *E. coli* strains derived from urinary and fecal specimens from women with UTI, we conducted a matched case-control study to investigate the association among CRISPR structure, uropathogenicity and antibiotic resistance. Each pair includes one uropathogen and one fecal sample from the same female patient.

Compared to uropathogens, fecal isolates had more repeats at ≥ 2 CRISPR loci (44.4% versus 28.6%, p=0.048), more repeats (p = 0.009) and more unique

[3] This study will be published with the following authors: Dang N.D.T., L. Zhang, A. Khadija, S. Zöllner, C.F. Marrs, B. Foxman.

spacers ($p < 0.0001$) at four CRISPR loci. Uropathogens also had higher prevalence of resistance to three antibiotics tested (ampicillin, cefazolin or trimethoprim/sulfamethoxazol) but no association between CRISPRs and antibiotic resistance was identified. To our knowledge, this study is the first to compare CRISPR organization and antibiotic resistance of *E. coli* by uropathogenicity. Our results from our study support the hypothesis that uropathogenic *E. coli* are more adaptable and suggest a positive role of *E. coli* CRISPRs in adaptive immunity.

Introduction

Bacteria can quickly adapt to changes in the environment by acquiring genetic material from related or unrelated species through horizontal or lateral gene transfer (19, 20). Horizontal gene transfer occurs by direct uptake of exogenous DNA (transformation) or by the incorporation of heterologous DNA carried on mobile genetic elements, such as plasmids and bacteriophages (32). However, horizontal gene transfer may cause deleterious effects that drive the host to extinction (27). To avoid these effects, bacteria have developed different mechanisms to limit horizontal gene transfer, one such process is through the use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs).

CRISPR sequences protect bacteria against bacteriophage infection and plasmid conjugation (2, 16, 21, 29). This ability, referred to as CRISPR immunity or CRISPR interference, relies on the spacers that intercalate between

CRISPRs, which are of bacteriophage or plasmid origin. RNAs transcribed from spacer sequences recognize their target bacteriophage or plasmid and prevent bacteriophage infection and plasmid conjugation (20). The CRISPR/Cas system, formed by the combination of CRISPRs and Cas proteins, is considered an adaptive immune system in many bacteria and archae (14). Approximately 40% of bacterial genomes contain at least one CRISPR locus (11). CRISPR clusters consist of direct repeats of 24–47 bp, separated by spacers of 25–72 bp in length (30) (Figure 5.1). CRISPRs and spacers are generally flanked by CRISPR-associated (*cas*) genes, and transcribed into small RNAs that can guide other Cas proteins to silence exogenous genetic elements at the RNA or DNA level (14, 18).

E. coli CRISPRs are found in two pairs of loci, CRISPR1 and -2 and CRISPR3 and -4, located at 62 and 20 min on the chromosome, respectively (5, 33, 34). The role of CRISPRs in *E. coli* immunity against phage infection and plasmid conjugation is still controversial. In the studies conducted by Brouns S.J. et al (4) and Edgar R. et al (6), laboratory strains of *E. coli* demonstrated the function of CRISPRs in phage resistance. However, phage resistance due to CRISPR system function was not observed in one natural strain of *E. coli* in the study conducted by Pougach K. et al (26). Touchon M. et al analyzed 263 *E. coli* strains isolated from humans and animals from various regions of France (34) and found *E. coli* CRISPRs small and unchanged for long periods of time. The

role in of *E. coli* CRISPRs in natural adaptation, therefore, remains poorly understood.

To better understand the role of CRISPRs in *E. coli* immunity and adaptability including acquisition of antibiotic resistance, we conducted a matched pair study on fecal (commensal) and uropathogenic strains of *E. coli* (UPEC) from the same UTI female patients, comparing the associations among CRISPR distribution, uropathogenicity (via site of colonization) and antibiotic resistance. UPEC differs from commensal *E. coli* in that it colonizes the urethra, periurethra and vagina (15). UPEC is also more likely than commensal *E. coli* to be shared between heterosexual sex partners (10). Reasoning that UPEC might be more adaptable to the changes in environment than commensal *E. coli*, we hypothesized that UPEC would contain fewer CRISPR loci and spacers when CRISPRs are present than fecal isolates. Since CRISPR sequences prevent plasmid conjugation (2) and plasmids often carry antibiotic resistance genes (17), we also hypothesized that the number of CRISPRs would be inversely correlated with antibiotic resistance.

Material and methods

Study design

We compared 81 matched pairs of fecal (commensal) and urinary *E. coli* (UPEC) isolates, each pair was from the same UTI female patient. We quantified the number of CRISPR loci, repeats and spacers; all isolates were phenotyped for resistance to selected antibiotics.

Study population

The 81 *E. coli* pairs were randomly selected from an existing collection (10). This collection was acquired from 166 women with physician-diagnosed, culture confirmed UTI visiting the University of Michigan Health Service in Ann Arbor, Michigan between September 1996 and April 1999. Demographic data and data on risk factors including sex history were collected through a self-administered questionnaire. Most of the 81 women selected were age 20-24 (70.9%) and were Caucasian (76.5%) (Table 5.1).

Bacteria identification

Urinary specimens were self-collected from clean-catch midstream urine; fecal specimens were self-collected using rayon-tipped swabs and were immediately placed in transport media (Cultureswab Transport System; Difco, East Molesey, UK). Specimens collected were inoculated on both trypticase soy agar with 5% sheep blood and MacConkey agar, then incubated for 18-24h at

37^0C (8, 9). *E. coli* was identified by a manual test system (API 20E, Biomeriex-Vitek, Hazelwood, MO) and frozen glycerol stocks were made.

CRISPR amplification and sequencing

CRISPR loci were amplified by polymerase chain reaction (PCR) and sequenced. The primers and PCR conditions used were derived from Touchon M. et al (34) (Figure 5.2, Table 5.2). *E. coli* strains K12 and CFT 073 were used as controls.

E.coli genomic DNA was extracted and purified using Wizard Genomic DNA Purification Kit (Promega, U.S.A). PCR was performed using Accuprime Supermix II (Invitrogen Corporation, USA) at 1 μmol/L concentrations. PCR conditions included an initial denaturation step for 2 minutes at 94^0C, followed by 10 cycles of 94^0C for 30 seconds, 56^0C for 30 seconds, and 72°C for 1 min 30 seconds. Twenty-five cycles of the same condition were followed with the addition of a 10-second elongation step at the end of each successive cycle. PCR products were visualized using gel electrophoresis on 1% agarose gels in Tris-acetic acid/EDTA buffer (pH 7.5) with GelRed as the post gel staining agent. Purified PCR products were subsequently sequenced on both strands using paired-end Illumina sequencing technology (3) by University of Michigan DNA Sequencing Core, Michigan, USA.

CRISPRs and spacers analysis

CRISPR1 and -2 have the same repeat sequence of 29 bp (CGGTTTATCCCCGCTGGCGCGGGGAACAC or CGGTTTATCCCCGCTGGCG CGGGGAACTC), while CRISPR3 and -4 have the same repeat of a different sequence of 28 bp (GTTCACTGCCGTACAGGCAGCTTAGAAA) (34). By identifying these repeat patterns, the CRISPR groups were identified using the Lasergene DNA STAR software package (DNASTAR, Inc., Madison, Wisconsin).

Spacers were aligned and analyzed for similarity with sequences from all annotated phages and plasmids available in the NCBI (National Center for Biotechnology Information) database between April 5th and April 10th, 2012, using BLASTn (Basic Local Alignment Search Tool). Since the spacers were very short sequences (27-42 bp), an E (Expect) value of 0.05 was used to identify matches. We used the criteria from Touchon M. et al. (34) that two spacers were considered similar if their sequences were at least 95% identical and there was less than 10% difference in sequence length.

Antibiotic susceptibility test

Urinary isolates were tested for susceptibility to 6 antibiotics and fecal isolates were tested for susceptibility to 17 antibiotics including 6 antibiotics tested on urinary isolates (ampicillin, cefazolin, gentamicin, ciprofloxacin, nitrofurantoin and trimethoprim/sulfamethoxazole). Both tests were conducted at the University of Michigan Health System Laboratory by means of VITEK II

(Biomerieux, System Version 05.01, www.biomerieux-usa.com), using Clinical and Laboratory Standards Institute (CLSI) M100-S18 (2008) Guidelines (24).

Data analysis

We used the Mann-Whitney and chi-square test, respectively, to compare the average number and proportion of CRISPRs and spacers between fecal and urinary isolates or between susceptible and resistant isolates. Wilcoxon Signed-rank test was used to compare the number of CRISPRs between matched pairs.

All analyses and data management was conducted using SAS software (SAS Version 9.2 for Windows; SAS Institute Inc., Cary, NC) or R software (R Version 1.14.1, The R Foundation for Statistical Computing).

Results

Analysis of repeats at CRISPR loci

Three quarters of commensal *E. coli* and 58% of UPEC isolates had repeats at any CRISPR locus (table 5.3). The range of repeats was remarkably wide (Figures 5.3), especially at C1 and C3 (Table 5.3). For every locus and overall, there were more repeats in commensal *E. coli* than UPEC isolates ($p = 0.009$ for overall, Wilcoxon Signed-Rank test) (Table 5.3)

Not all isolates had repeats at CRISPR loci. Among all isolates tested, 33.3% (24.6% commensal *E. coli* and 42.0% UPEC) had no CRISPR loci or no repeats at CRISPR loci, 30.2% (30.8% commensal *E. coli* and 29.6% UPEC) had

repeats at only one locus, 27.2% (32.0% commensal *E. coli* and 22.4% UPEC) had repeats at two loci, 6.8% (8.6% commensal *E. coli* and 5.0% UPEC) had repeats at three loci and 2.5% (3.8% commensal *E. coli* and 1.2% UPEC) had repeats at all four CRISPR loci. Among strains with repeats observed in two CRISPR loci, the combinations of C1-C2 and C3-C4 were the most frequent in both commensal *E. coli* and UPEC samples (data not shown).

There were UPEC isolates that had no repeats at any CRISPR locus than commensal *E. coli* isolates (42.0% versus 24.6%; p = 0.03). By contrast, more commensal *E. coli* isolates had repeats at two or more CRISPR loci than UPEC isolates (44.4% versus 28.6%; p = 0.048).

Analysis of spacers

In order to get further insight into the role of CRISPRs in immunity and adaptation, we analyzed the characteristics and distribution of spacers at the four CRISPR loci. The length of spacers varied from 27 bp to 42 bp, with more than 80% of spacers being 32 bp in length, regardless of the position in the CRISPR loci (Table 5.4). Among the spacers identified at each CRISPR locus, we detected unique or single spacers that occurred only once in the whole collection and other spacers that existed in different *E. coli* strains ("repeating spacers"). The spacers within one *E. coli* strain were quite distinctive and no spacers were shared across CRISPR loci.

Approximately one-fifth of the spacers identified were unique spacers.

However, this proportion tremendously increased at C2 (58.7%) and decreased at C3 (8.3%). Interestingly, at each of the four CRISPR loci, the proportion of unique spacers was significantly higher among commensal *E. coli* than UPEC isolates ($p = 0.003$, $p < 0.0001$, $p < 0.0001$ and $p = 0.02$ at C1, C2, C3 and C4, respectively) (Table 5.4). Unique spacers were subsequently analyzed by BLASTn to identify the level of matching to known phages and plasmids from NCBI database. Taking an E-value of 0.05 as the cutoff to determine matched sequences, only 12 spacers (5.3%) and 31 spacers (13.7%) were homologous with sequences of phages and plasmids, respectively. Most of these spacers were detected in commensal *E. coli* isolates (88%).

Among four CRISPR loci, C1 had the greatest number of both unique and repeating spacers (87 and 83, respectively) (Table 5.4). At locus C1, the distribution of repeating spacers also differed between fecal and urinary isolates. Spacers that occurred less frequently (≤ 5 times) were more likely to be shared among fecal isolates while spacers that occurred at a higher frequency (> 6 times) were more common among urinary isolates (Figure 5.4).

Antibiotic susceptibilities of commensal E. coli and UPEC isolates

UPEC isolates were tested for susceptibility to 6 antibiotics and commensal *E. coli* isolates were tested for susceptibility to 17 antibiotics including 6 antibiotics tested on urinary isolates. Fecal samples were resistant to ampicillin (19.8%) and trimethoprim/sulfamethoxazole (TMP/SMX) (7.4%) and completely

susceptible to all other antibiotics tested. Urinary isolates were resistant to ampicillin (25.9%), cefazolin (51.9%), TMP/SMX (16%) and completely susceptible to the remaining antibiotics. Therefore, we compared susceptibilities to ampicillin, cefazolin and TMP/SMX between commensal *E. coli* and UPEC isolates. The prevalence of strains resistant to ampicillin, cefazolin or TMP/SMX was higher in UPEC isolates than in commensal *E. coli* isolates (25.9% versus 19.8% for ampicillin ($p = 0.34$), 16% versus 1.2% for cefazolin ($p < 0.0001$) and 16% versus 7.4% for TMP/SMX ($p = 0.05$), chi square test). A statistically significant difference in the prevalence of resistance to cefazolin and TMP/SMX between UPEC and commensal *E. coli* isolates was also observed using McNemar test for matched pair analysis ($p < 0.0001$ and $p = 0.05$, respectively).

Association between CRISPRs and antibiotic resistance

We estimated the relationship between the number of repeats and antibiotic resistance to ampicillin, cefazolin and TMP/SMX, using Mann-Whitney test. For cefazolin, more repeats were observed in susceptible strains than in resistant strains ($p = 0.046$). By contrast, for TMP/SMX, more repeats were observed in resistant strains than in susceptible strains ($p = 0.046$). This difference still remained true when stratified by commensal *E. coli* and UPEC ($p = 0.09$ for commensal *E. coli* and $p = 0.03$ for UPEC). For ampicillin, we saw little difference in the number of repeats between resistant and susceptible strains (Table 5.5).

Discussion

In a comparison of 81 matched pairs of fecal (commensal) and uropathogenic *E. coli* isolates (UPEC), we observed several significant differences in CRISPR organization and antibiotic resistance. The number of repeats at C2, C4 and the total repeats at all four CRISPR loci is significantly higher in commensal *E. coli* isolates than in UPEC isolates. More commensal *E. coli* isolates were found with repeats at ≥ 2 CRISPR loci than UPEC isolates. Further, the proportion of unique spacers was much higher among commensal *E. coli* isolates than UPEC isolates, at all four CRISPR loci. Finally, the prevalence of strains resistant to each of the three antibiotics tested (ampicillin, cefazolin or TMP/SMX) was higher among UPEC isolates. These findings support our hypothesis that UPEC is more adaptable than commensal *E. coli.* The difference between commensal *E. coli* and UPEC isolates is suggestive of the positive role of *E. coli* CRISPRs as a defense system, which is in contrast to the results reported by Touchon et al. (34).

The fact that the proportion of unique spacers was much higher among commensal *E. coli* isolates raises a question on the role of unique spacers in CRISPR-associated defense system in *E. coli*. However, only 19% of the unique spacers, i.e. 4.1% of the total spacers had matches in NCBI database. Although this percentage is pretty close to what has been reported (18, 20, 22), it is too low to identify the actual role of unique spacers in particular and spacers in general. This percentage could be increased when the database is enriched with

more sequences, such as community genomic data from bacteria and phages in a particular microbial niche (1, 12).

The negative association between the presence of CRISPR/cas system and acquisition of antibiotic resistance was reported in *Enterococcus faecalis* in the study conducted by Palmer K.L et al (25). However, in our study, the association between CRISPRs and antibiotic resistance differed by types of antibiotics (resistance to TMP/SMX, cefazolin, and ampicillin was positively, negatively and not associated with the number of CRISPRs, respectively). This result is explainable since resistance to each of these antibiotics are caused by different mechanisms, one of which is plasmid-mediated (7, 13, 23, 28, 31). If genes responsible for resistance to each of these antibiotics are carried by plasmids and if *E. coli* CRISPRs can prevent plasmid conjugation, it will still be difficult to predict the association between the number of repeats and antibiotic resistance.

In previous studies, *E. coli* strains were isolated from multiple collections (humans, animals) from different time periods (5, 34). This diversity provided a general view on CRISPR distribution and function but there were a variety of factors that could potentially bias results. In this study, the population was restricted to female UTI patients with each pair of urinary and fecal isolate derived from the same individual. This matched-pair design can reduce possible confounders and thus facilitate interpretations of our findings.

In summary, this study is the first to compare CRISPR organization and antibiotic resistance of clinical *E. coli* isolates by sites of colonization. Findings

from the study suggest the better adaptability of UPEC compared to commensal *E. coli* and the positive role of *E. coli* CRISPRs in adaptive immunity.

Acknowlegement

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Table 5.1. Characteristics of 81 women visiting the University of Michigan Health Service in Ann Arbor, Michigan between Sep. 1996 and Apr.1999 with physician-diagnosed, culture-confirmed urinary tract infection due to *E. coli* from whom study strains were derived[$]

Characteristic	No.	%
Age (years)		
18-19	9	11.1
20-24	57	70.4
25-29	10	12.3
30-34	2	2.5
≥35	1	1.2
Race/ethnicity		
African America	3	3.7
Asian	9	11.1
Caucasian	62	76.5
Other	5	6.2

$ Numbers do not sum to totals because of missing values and percentages do not sum to 100 because of rounding.

Table 5.2. Primers used to amplify CRISPR loci (33)

Primer	Sequence (5' – 3')*	PCR target
CRISPR1 Fw	GTTATGCGGATAATGCTACC	*iap*
CRISPR1 Rev	CGTAYYCCGGTRGATTTGGA	*cas 2*
CRISPR2 Fw	AAATCGTATGAAGTGATGCAT	*ygcE*
CRISPR2 Rev	GTCGATGCAAACACATAAATA	*ygcF*
CRISPR3 Fw	GCGCTGGATAAAGAGAAAAAT	*clpA*
CRISPR3 Rev	GCCCACCATTCACCTGTA	*cas1*
CRISPR4 Fw	CTGAACAGCGGACTGATTTA	*cys4*
CRISPR4 Rev	GTACGACCTGAGCAAAG	*infA*

* Y: C or T; R: A or G; M: A or C

Table 5.3. Distribution of repeats at four CRISPR loci among 81 matched pairs of commensal *E. coli* and uropathogenic *E.coli* (UPEC) isolates from women from Michigan with physician-diagnosed, culture-confirmed urinary tract infection due to *E. coli* (1996-1999)

	Location (CRISPR locus)	**C1**	**C2**	**C3**	**C4**	**All**
Commensal *E. coli* (n=81)	Number (%) of strains with repeats at the locus	32 (39.5)	21 (25.9)	24 (29.6)	20 (24.7)	61 (75.3) *
	Number of repeats					
	Average	3.34	1.54	2.58	1.24	8.69
	Median	0	0	0	0	7
	Range	0-14	0-10	0-13	0-7	0-27
UPEC (n=81)	Number (%) of strains with repeats at the locus	28 (34.5)	19 (23.4)	18 (22.2)	7 (8.6)	47 (58.3) *
	Number of repeats					
	Average	2.75	0.9	2.01	0.4	6.1
	Median	0	0	0	0	0
	Range	0-15	0-9	0-12	0-6	0-23
p value**		0.34	**0.04**	0.47	**<0.001**	**0.009**

* The number (%) of strains that had repeats at any locus. This number is not equal to the sum of strains that had repeats at loci C1, C2, C3 and C4 because one strain could have repeats at one or more loci.

** p value obtained by Wilcoxon Signed-Rank test comparing the number of repeats within matched pairs of fecal and urinary *E. coli* isolates. Values in bold are statistically significant.

Table 5.4. Main characteristics of spacers at four CRISPR loci among 81 matched pairs of commensal *E. coli* and uropathogenic *E.coli* (UPEC) isolates from women from Michigan with physician-diagnosed, culture-confirmed urinary tract infection due to *E. coli* (1996-1999)

	CRISPR 1	**CRISPR 2**	**CRISPR 3**	**CRISPR 4**	**Total**
Number of strains with spacers	60	39	42	27	
Total number of spacers	446	143	339	130	1058
Urinary	193 (43.3%)	63 (44.1%)	149 (44.0%)	33 (25.4%)	438 (41.4%)
Fecal	253 (56.7%)	80 (55.9%)	190 (56.0%)	97 (74.6%)	620 (58.6%)
Number of different spacers observed	170	104	66	38	378
Number of spacers occurring only once (unique spacers)*	87 (19.5%)	84 (58.7%)	28 (8.3%)	28 (21.5%)	227 (21.5%)
Urinary**					
Fecal***	22 (11.4%)	24 (38.0%)	1 (0.7%)	2 (6.0%)	49 (11.2%)
	65 (25.7%)	60 (75.0%)	27 (14.2%)	26 (26.8%)	178 (28.7%)
p-value[$]	**0.0003**	**<0.0001**	**< 0.0001**	**0.02**	**<0.0001**
Length of spacers (median, range) (bp)	32; 31-37	32; 28-42	32; 31-35	32; 27-34	

, **, ** Percentage calculated over the total number of spacers, the total number of spacers in fecal isolates and the total number of spacers in urinary isolates, respectively

[$] p-value obtained by chi-square test comparing the proportion of unique spacers between fecal and urinary isolates. Values in bold are statistically significant.

Table 5.5. Comparison of the number of repeats at four CRISPR loci between resistant and susceptible *E.coli* isolates from 81 matched pairs of commensal *E. coli* and uropathogenic *E.coli* (UPEC) isolates from women from Michigan with physician-diagnosed, culture-confirmed urinary tract infection due to *E. coli* (1996-1999)

	Resistant		**Susceptible**			
	N	**Average number of repeats**	**N**	**Average number of repeats**	**p[$] (α =0.05)**	**95% CI**
Ampicillin						
Commensal *E. coli*	16	9.1	65	8.6	0.72	-4.0 , 5.0
UPEC	21	6.0	60	6.2	0.96	-2 .0 , 3.0
Total	37	7.4	125	7.4	0.95	-2.0 , 2.0
Cefazolin*						
Commensal *E. coli***	1	26	80	8.4		
UPEC	42	5.2	39	7.3	0.25	-6.0 , 0.0
All	43	5.5	119	8.1	**0.046**	**-5.0 , -7.3e-07**
TMP/SMZ						
Commensal *E. coli*	6	14.3	75	8.2	0.09	-1.0 , 13.0
UPEC	13	10.3	68	5.3	**0.03**	**5.7e-05 , 10.9**
All	19	11.6	143	6.9	**0.02**	**4.1e-05, 9.9**

* R includes all strains with resistance and intermediate resistance to cefazolin

** Not tested because of small sample size

[$] p value obtained by Mann-Whitney test. Values in bold are statistically significant.

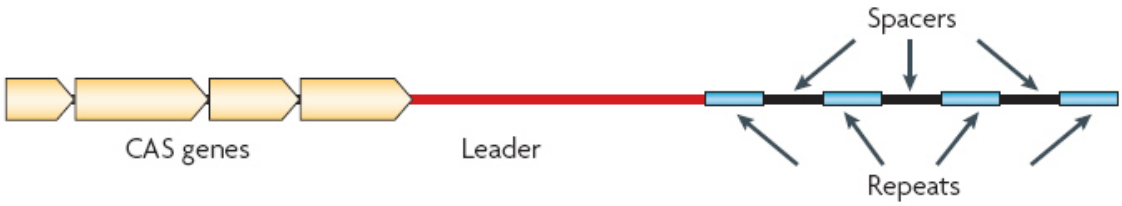

Figure 5.1. Diagram of a CRISPR array (Sorek R., 2008) (30)

(Used with the agreement from Nature Publishing Group,
license number 2877720031476)

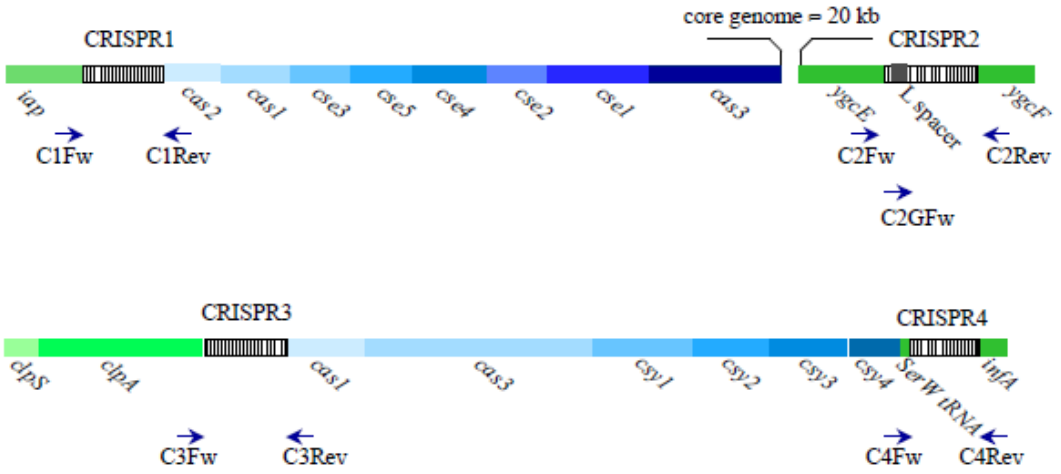

Figure 5.2. Schematic representation of the primers used for the PCRs and sequencing

(Touchon M., 2010) (34)

(Used with the agreement from American Society for Microbiology,

license number 2877720296181)

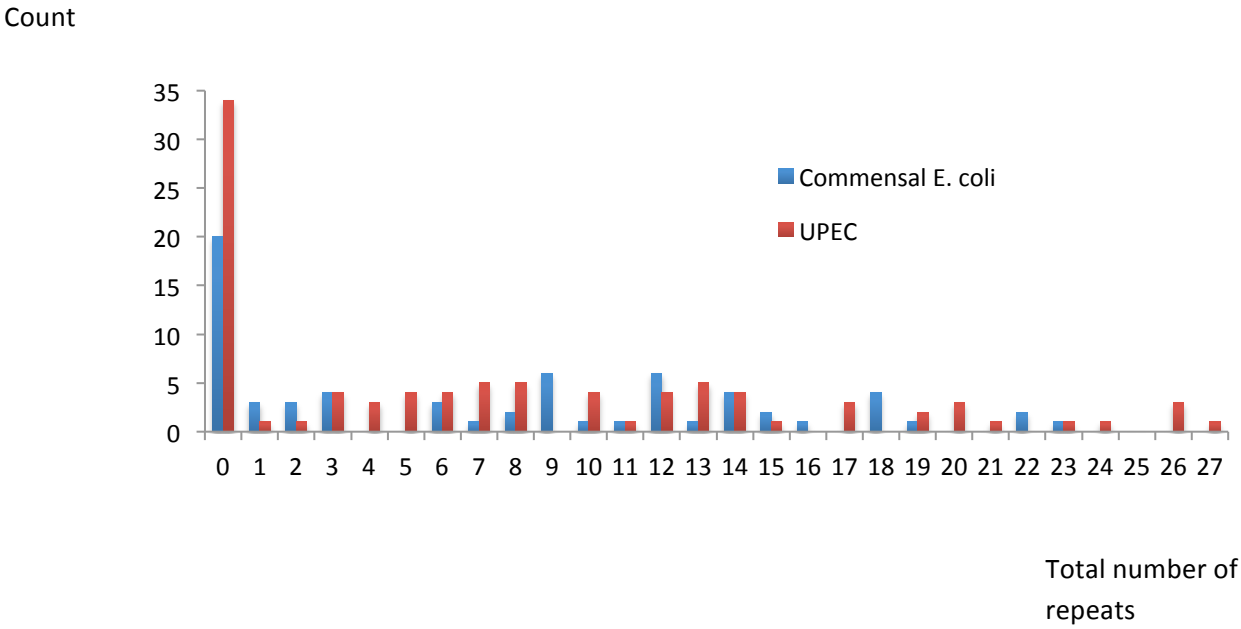

Figure 5.3. Distribution of total repeats at four CRISPR loci among 81 matched pairs of commensal *E. coli* and uropathogenic *E.coli* (UPEC) isolates from women from Michigan with physician-diagnosed, culture-confirmed urinary tract infection due to *E. coli* (1996-1999)

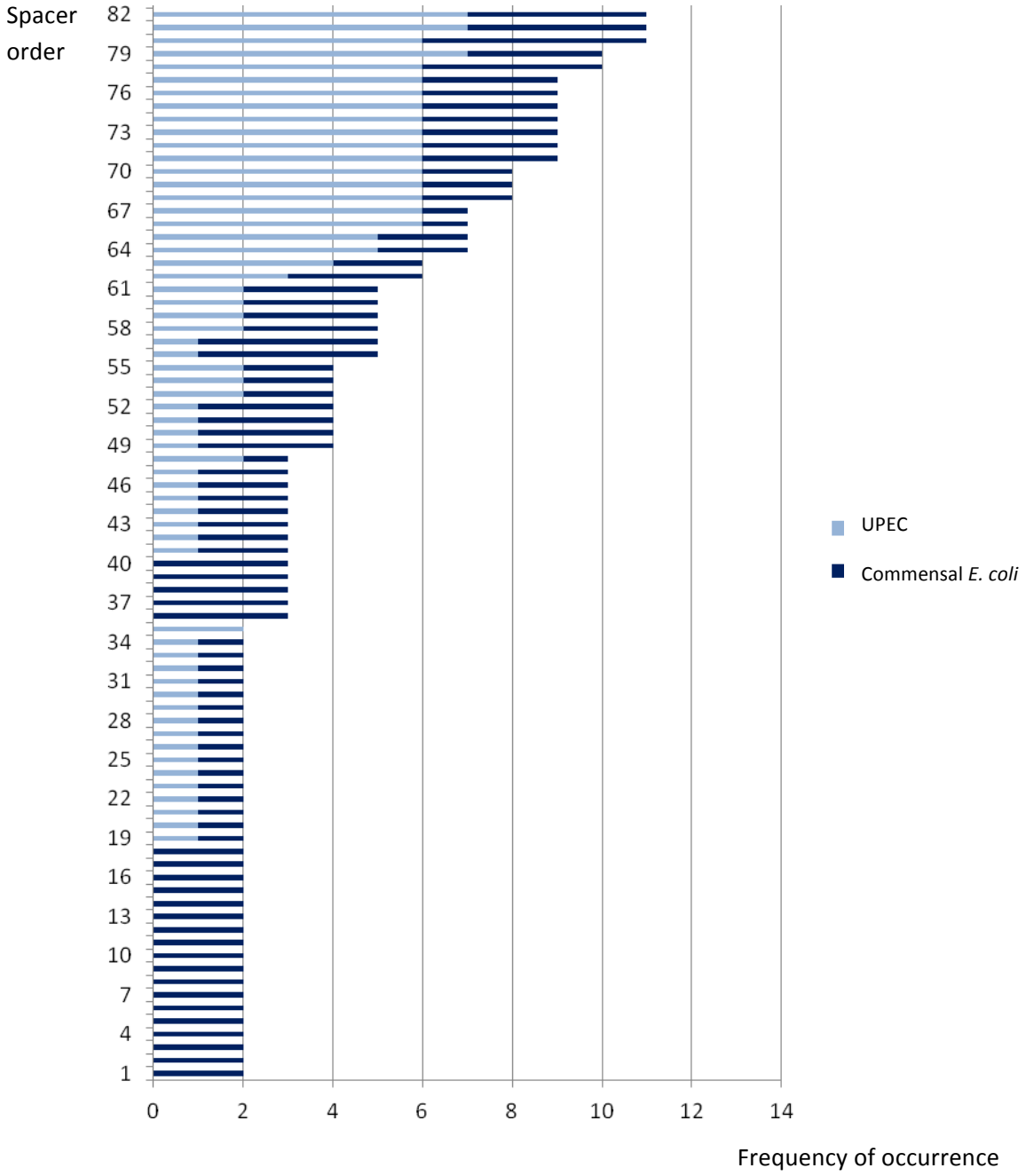

Figure 5.4. Frequency of occurrence of CRISPR spacers that occurred ≥ 2 times at CRISPR1 locus among 81 matched pairs of commensal *E. coli* and uropathogenic *E.coli* (UPEC) isolates from women from Michigan with physician-diagnosed, culture-confirmed urinary tract infection due to *E. coli* (1996-1999)

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Summary of results

This dissertation addresses different features of antibiotic resistance and adaptibility in GBS and *E. coli*. Findings from the studies are summarized below.

Rates and mechanisms of resistance to FQs among GBS strains isolated from South Korea

We observed a strikingly high prevalence of GBS strains resistant to FQs, especially to norfloxacin (~93%). This prevalence was much higher than the resistance rates previously reported (7, 9, 11, 12). An increasing trend in the prevalence of GBS strains resistant to ciprofloxacin and levofloxacin was also identified between 2006 and 2008 (ciprofloxacin: from 5.6% to 11.2%, p = 0.009; levofloxacin: from 5.1% to 10.6%, p = 0.009). GBS serotypes were not associated with susceptibility to fluoroquinolones.

We screened for mutations in the quinolone resistance-determining regions and evidence of efflux phenotype to identify the mechanisms of resistance to FQs among GBS isolates. Overall, we found mutations in GBS strains resistant to ciprofloxacin, levofloxacin and moxifloxacin. We observed double mutations (*gyr*A and *par*C) in strains resistant to levofloxacin and moxifloxacin that were

also cross-resistant to norfloxacin and ciprofloxacin. No evidence of efflux was identified in strains with double mutations. On the contrary, evidence of efflux pumps was observed in half of GBS strains that were resistant only to norfloxacin with no known mutations.

While resistance to FQs among a majority of GBS strains could be explained by either mutations or efflux, resistance to approximately half of GBS strains resistant only to norfloxacin remains unknown, thus needing further investigation.

Comparison of resistance rates and mechanisms between invasive and colonizing GBS isolates

Our data indicated that the prevalence of resistance to ciprofloxacin and levofloxacin was higher among invasive than colonizing GBS isolates (ciprofloxacin resistance: 10.6% versus 2.5% ($p < 0.001$); levofloxaxin resistance: 9.8% versus 2.1% ($p < 0.001$)). Results from the mutations study seemed to support this finding since more gyrase and topoisomerase mutations were observed in clinical than in colonizing isolates (mutations in both *gyr* and *par* : 33.8% versus 29.6% ($p = 0.88$), mutations in either *gyr* or *par* : 29.4% versus 18.5% ($p = 0.41$). However, no difference in the proportion of efflux phenotypes was found between colonizing and clinical strains among GBS strains resistant only to norfloxacin. These findings suggest that efflux might be the first step of low-level resistance to hydropholic FQs (norfloxacin, ciprofloxacin) that can be evenly acquired in both colonizing and invasive GBS

isolates. At a higher level of resistance to other hydrophobic compounds (levofloxacin, moxifloxacin), invasive isolates are more likely to acquire mutations and hence are more resistant than colonizing isolates. Our findings are in contrast to the reports from some previous studies (2, 3), thus providing new insight into the acquisition of antibiotic resistance and adaptability in GBS.

CRISPR distribution and comparison on the number of CRISPR repeats between commensal E. coli and UPEC isolates

Data from our study confirmed the existence of two pairs of CRISPR loci (C1 and C2, C3 and C4). The highest total number of repeats observed in fecal isolates was 27 and in urinary isolates was 23. Although CRISPRs were found in 75% of *E. coli* strains tested, the proportion of each CRISPR locus detected was much lower than results previously reported (4, 10). In general, commensal *E. coli* isolates had more CRISPR loci and more repeats ($p = 0.009$) than UPEC isolates. This finding supports our hypothesis, suggesting the role of CRISPRs as a defense system among *E. coli* isolates.

Distribution and content of CRISPR spacers among commensal E. coli and UPEC isolates

There were more spacers in commensal *E. coli* isolates than in UPEC isolates. Approximately one-fifth of the spacers identified were unique spacers - spacers that occurred only once in the whole collection. The proportion of unique spacers was significantly higher among commensal *E. coli* than UPEC isolates at

all four CRISPR loci ($p = 0.003$, $p < 0.0001$, $p < 0.0001$ and $p = 0.02$ at C1, C2, C3 and C4, respectively), suggesting the role of the unique spacers in CRISPR-associated immunity. Since a very low proportion of unique spacers were homologous with sequences of annotated phages (5.3%) and plasmids (13.7%) from NBCI database, future studies are needed to better identify the origin and roles of these spacer regions.

Antibiotic resistance among commensal E. coli and UPEC isolates and association with CRISPRs

The prevalence of strains resistant to three antibiotics tested (ampicillin, cefazolin or TMP/SMX) was higher among UPEC isolates than commensal *E. coli* isolates (25.9% versus 19.8% for ampicillin ($p = 0.34$), 16% versus 1.2% for cefazolin ($p < 0.0001$) and 16% versus 7.4% for TMP/SMX ($p = 0.05$)). Since commensal *E. coli* and UPEC isolates were in matched pairs, we had more power to address the hypothesis of increased acquisition of antibiotic resistance, which might be related to the increased adaptability in UPEC. However, the association between CRISPRs and antibiotic resistance varied by types of antibiotics and was complicated to interpret, possibly due to different resistance mechanisms involved.

Conclusions

In addition to the resistance mechanisms identified among GBS strains, arising from the whole dissertation are the significant differences observed between invasive and colonizing isolates (commensals) that suggest the difference in adaptability, in both GBS and *E. coli.* Overall, in both organisms, invasive isolates were more likely to be resistant to antibiotics than colonizing isolates. Among clinical *E. coli* strains, the difference between invasive and colonizing isolates was also observed when comparing the number of repeats and unique spacers at the CRISPR loci (chapter 5). However, more evidence is needed to determine whether invasive strains of GBS and *E. coli* are more adaptable than colonizing strains. If invasive strains are more resistant and more adaptable, they should be the predominant target of intervention and treatment.

Future directions

The studies in this dissertation could be expanded into different directions, including further investigations on antibiotic resistance mechanisms in GBS, deeper analysis on CRISPR spacers of *E. coli* or more studies to identify the differences in adaptability between invasive and colonizing strains of GBS and *E. coli*.

Further studies to identify resistance mechanisms to FQs

In our studies in chapter 3 and chapter 4, mutations in the quinolone resistance-determining regions and efflux-mediated resistance were identified as two resistance mechanisms to FQs among GBS. Since two methods applied for efflux screening proved inconsistency, more studies with high sensitivity and specificity are needed. Some applicable methods are measurement of the level of EB accumulation by a fluorometric assay and whole genome sequencing to identify resistance genes in norfloxacin-resistant strains of GBS. The advantage of fluorometric assay is its high accuracy, but it is time-consuming and could not provide genotypic information relating to resistance (8). Whole genome sequencing appears to be a promising approach. This analysis might detect not only genes associated with efflux but also novel genes contributing to norfloxacin resistance in GBS. Such novel genes, if identified, might explain resistance to norfloxacin in strains with neither mutations nor evidence of efflux phenotype. In whole genome sequencing, sequences are generated using paired-end Illumina sequencing or other sequencing technologies. The sequences will subsequently be mapped to the GBS reference genome using specific alignment tools and gene identities are obtained by the analysis with a reciprocal best match strategy (5). One putative challenge of this approach is the choice of appropriate GBS strains for sequencing in order to be able to capture all genetic elements responsible for resistance to FQs in GBS.

Analysis on CRISPR spacers of E. coli isolates

Results from chapter 5 suggest the role of unique and repeating spacers in *E. coli* immunity. To better determine the origin of these spacers, we could continue the BLAST search with the expanded and updated database from different resources including community genomic data from bacteria and phages (1, 6) or from *cas* genes previously identified. Once the origin of the spacers are well identified, an analysis on the relationship between antibiotic resistance and origin of spacers should be carried out to determine the role of specific spacers in antibiotic resistance, if any.

Future studies to identify the differences in adaptability between invasive and colonizing strains of GBS and E. coli

To determine whether invasive strains of GBS and *E. coli* are more adaptable than colonizing strains, more studies are needed. Some potential ones are identifying and comparing the CRISPR structure between invasive and colonizing GBS strains, comparing the resistance rates to antibiotics over time and comparing the virulence factors between invasive and colonizing strains of GBS and *E. coli*.

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APPENDIX

Table A. Review on drug efflux pumps of gram positive bacteria

Organism	Family	Efflux system	Substrate(s)
Bacillus subtilis	MFS	Blt	AD, EB, DO, FQ, RD, TPP
		Bmr3	AD, EB, DO, FQ, RD, SD, TPP
	SMR	EbrAB	AC, EB, PY, SO
S. aureus			
	ABC	MsrA	ML
	MFS	MdeA	BC, DQ, EB, FU, HO, MU, NO, QAC, TPP, VM
		NorA	FQ
		NorB	CT, EB, FQ
		NorC	FQ
		SdrM (Ec)	AC, EB, NF
		Tet38	TC
		QacA*	AC, CH, CV, DD, EB, QAC
	MATE	MepA	CT, EB, FQ, MDB
	SMR	SepA	AC, BC, CH
S. pneumoniae	ABC	PatA, PatB	FQ
		SP2073/SP2075	AC, EB, FQ, NO
	MFS	PmrA	FQ
		MefE	ML
S. pyogenes	MFS	MefA	ML
S. suis	MFS	SmrA	FQ
GBS	MFS	MreA	CL, ML
		MefB, MefG	ML
		Tet42	TC

* The genes encoding these pumps are plasmid-borne.

ABC: adenosine triphosphate-binding cassette superfamily; **AC:** acriflavine; **AD:** acridine dyes; **BC:** belzalkonium chloride; **CH:** chlorhexidine; **CL**:clindamycin; **CV** : crystal violet; **CT:** cetrimIde; **DD:** diamidines; **DO:** doxorubicin; **DQ:** dequalinium chloride; **EB**: ethidum bromide; **FQ**: fluoroquinolones;**FU:** fusidic acid; **HO:** Hoechst 33342; **MATE:** Multidrug and Toxic Compound Extrusion Family; **MDB:** monovalent and divalent biocides; **MFS:** major facilitator superfamily; **ML:** macrolides; **MU:** mupirocin; **NO:** novobiocin; **NF:** norfloxacin; **PY**: pyronine Y; **QAC:** quaternary ammonium compounds; **RD:** rhodamine; **SD**:spermidine; **SMR:** small multidrug resistance; **SO**:safranin O; **TC:** tetracycline; TG: **TPP**: tetraphenyphosphonium; **VM:** virginiamycin

(Source: **Li, X-Z, H. Nikaido.** 2009. Efflux-Mediated Drug Resistance in Bacteria: an Update. Drugs. **69**(12): 1555–1623).