ORIGIN OF *OQXAB* AND ITS CONTRIBUTION IN FLUOROQUINOLONE RESISTANCE IN *SALMONELLA* SPP.

WONG HO-YIN MARCUS

Ph.D

The Hong Kong Polytechnic University

2016

The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

Origin of *OqxAB* and Its Contribution in Fluoroquinolone Resistance in *Salmonella* spp.

WONG Ho-yin Marcus

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

August 2015

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree of diploma, except where due acknowledgement has been made in the text.

_____(Signed)

_____WONG, Ho-yin Marcus_____(Name of Student)

ABSTRACT

Bacterial infections have become a serious public health issue worldwide. Antimicrobial agents may not be necessary for treatment of mild cases, they can be life-saving in systemic infections or diseases affecting immunocompromised patients. Fluoroquinolones have been regarded as one of the frontline antimicrobial drugs. Resistance to this category of antibiotic is mediated by a variety of mechanisms, amongst them, acquisition of Plasmid Mediated Quinolone Resistance (PMQR) determinants is a major route by which bacteria evolve to become fluoroquinolone resistant. A PMQR, *oqxAB*, which encodes a mobile Resistance-nodulation-division family efflux pump, has become increasingly prevalent amongst fluoroquinolone resistant Gram negative bacteria including *Escherichia coli* and *Klebsiella pneumoniae*. The works described in this thesis aimed to assess the prevalence of *oqxAB* in environmental and clinical *Salmonella* isolates, determine the relative functional role of this element in fluoroquinolone resistance development, identify its evolutionary origin, and elucidate the mechanisms by which expression of PMQR genes are regulated in *Salmonella* spp..

Prevalence and antimicrobial resistance profile of *Salmonella* spp. in meat products being sold in Hong Kong was investigated. The *oqxAB* element,which was encoded in transposon Tn*6010*, was detectable in two *Salmonella* isolates for the first time. The study was then expanded to include clinical *S.* Typhimurium isolates recovered in Hong Kong and China during the period of

2005-2011, with results showing that clinical *S.* Typhimurium isolates were increasingly resistant to ciprofloxacin and antibiotics of the ACSSuT group. Importantly, we found that this trend of increasing resistance rate correlated with an increasing prevalence of *oqxAB* and another PMQR gene *aac(6')-Ib-cr* amongst clinical *Salmonella* isolates; in particular, *oqxAB* became detectable only from 2006 onwards. In addition, these two PMQR determinants exhibited strong linkage with the ACSSuT resistance phenotype and were mainly confined to the *S.* Typhimurium ST34 strain. It was also found that *oqxAB* and *aac(6')-Ib-cr* contributed significantly to the formation of ciprofloxacin resistance in *S.* Typhimurium by facilitating acquisition of an additional single mutation in the *gyrA* gene. Both *oqxAB* and *aac(6')-Ib-cr* were encoded on plasmids of various sizes, which upon transformation into a *oqxAB*-negative *Salmonella* host led to a 4-fold increase in CIP MIC. Furthermore, the presence of *oqxAB* and *aac(6')-Ib-cr* in *Salmonella* caused an dramatic increase in the mutation prevention concentration (MPC) of ciprofloxacin.

Attempt was then made to probe the origin of the mobile RND-type efflux pump *oqxAB*. It was found that *oqxAB* had been harboured by *K. pneumoniae* isolates recovered before the year 1984. Sequence and phylogenetic analysis confirmed that the *oqxAB* operon in *K. pneumoniae* was genetically closest to their Tn*6010* counterparts recoverable from other *Enterobacteriaceae* since 2003. *K. pneumoniae* strains generally did not exhibit a typical *oqxAB*-mediated phenotype despite harboring *oqxAB* chromosomally. The data therefore

suggests that *oqxAB* originated from *K. pneumoniae* and does not necessarily function as a PMQR determinant in this bacterial species under normal circumstances. Contrary to the chromosomally-encoded *oqxAB* element, the activator gene *rarA* was not captured by Tn*6010*. However, we demonstrated in *S.* Typhimurium by gene knockout study that the global regulator *ramA* has a role in activating *oqxAB* expression, unveiling the role of global regulatory mechanisms in controling the expression of foreign resistance genes.

PUBLICATIONS PRODUCED DURING THE COURSE OF THIS STUDY

Wong, M.H., Kan, B., Chan, E.W., Yan, M., Chen, S. (2016). IncI1 Plasmids Encoding Various *bla*$_{\text{CTX-Ms}}$ Contributed to Ceftriaxone Resistance in *Salmonella* Enteritidis in China. *Antimicrob Agents Chemother, 60*(2), 982-989.

Wong, M. H., Liu, L.Z., Chan, E.W., Chen, S. (2015). Dissemination of IncI2 plasmids that harbour blaCTX-M element amongst clinical *Salmonella* isolates. *Antimicrob Agents Chemother, 59*(8), 5026-5028.

Wong, M. H., Chan, E. W., Chen, S. (2015). Isolation of carbapenem-resistant Pseudomonas spp. from food. *J Glob Antimicrob Resist, 3*(2), 109-114.

Li, R., Lin, D., Chen, K., **Wong, M.H.**, Chen, S. (2015). First detection of AmpC β-lactamase blaCMY-2 on a conjugative IncA/C plasmid in Vibrio parahaemolyticus of food origin. *Antimicrob Agents Chemother, 59*(7), 4106-4111.

Wong, M. H., Chan, E. W., Li, Y., Chen, S. (2015). Functional categorization of carbapenemase-mediated resistance by a combined genotyping and two-tiered Modified Hodge Test approach. *Front Microbiol, 6*, 293.

Wong, M. H., Chan, E. W., Chen, S. (2015). Evolution and Dissemination of OqxAB-like Efflux Pumps, an emerging Quinolone Resistance Determinant amongst members of Enterobacteriaceae. *Antimicrob Agents Chemother, 59*(6), 3290-3297.

Li, R., **Wong, M.H.**, Zhou, Y., Chan, E.W., Chen, S. (2015). First complete nucleotide sequence of a conjugative plasmid carrying blaPER-1. *Antimicrob*

Agents Chemother, 59(6), 3582-3584.

Po, K.H., **Wong, M.H.**, Chen, S. (2015). Identification and characterisation of a novel plasmid-mediated quinolone resistance gene, qnrVC7, in Vibrio cholerae of seafood origin. *Int J Antimicrob Agents, 45*(6), 667-668.

Wong, M. H., Chan, E. W., Liu, L. Z., Chen, S. (2014). PMQR genes oqxAB and aac(6')Ib-cr accelerate the development of fluoroquinolone resistance in Salmonella typhimurium. *Front Microbiol, 5*, 521.

Wong, M.H., Yan, M., Chan, E.W., Biao, K., Chen, S. (2014). Emergence of Clinical Salmonella Typhimurium with Concurrent Resistant to Ciprofloxacin, Ceftriaxone and Azithromycin. *Antimicrob Agents Chemother, 58*(7), 3752-3756.

Liu, M., **Wong, M. H.**, Chen, S. (2013). Molecular characterisation of a multidrug resistance conjugative plasmid from Vibrio parahaemolyticus. *Int J Antimicrob Agents, 42*(6), 575-579.

Liu, M., **Wong, M. H.**, Chen, S. (2013). Mechanisms of fluoroquinolone resistance in Vibrio parahaemolyticus. *Int J Antimicrob Agents, 42*(2), 187-188.

Wong, M. H., Yan, M., Chan, E. W., Liu, L. Z., Kan, B., Chen, S. (2013). Expansion of Salmonella Typhimurium ST34 clone carrying multiple resistance determinants in China. *Antimicrob Agents Chemother, 57*(9), 4599-4601.

Wong, M. H., Chen, S. (2013). First detection of oqxAB in Salmonella spp. isolated from food. *Antimicrob Agents Chemother, 57*(1), 658-660.

Wong, M. H., Zeng, L., Liu, J. H., Chen, S. (2013). Characterization of Salmonella food isolates with concurrent resistance to ceftriaxone and

ciprofloxacin. *Foodborne Pathog Dis, 10*(1), 42-46.

Wong, M. H., Wan, H. Y., Chen, S. (2013). Characterization of multidrug-resistant Proteus mirabilis isolated from chicken carcasses. *Foodborne Pathog Dis, 10*(2), 177-181.

Wong, M. H., Liu, M., Wan, H. Y., Chen, S. (2012). Characterization of extended-spectrum-beta-lactamase-producing Vibrio parahaemolyticus. *Antimicrob Agents Chemother, 56*(7), 4026-4028.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Dr Sheng CHEN for his supervision throughout my PhD study. I have been working with Dr Chen since my undergraduate project, the time when I did not even know how to perform Polymerase Chain Reaction. I do really appreciate the opportunity to work in his laboratory, otherwise I would have become another ordinary person living a mundane life. But now, since I have been greatly motivated by the way he perform scientific research, and have learned how to overcome the hardship that I have encountered from time to time during my study, I believe I am equipped with the ability to tackle something extraordinary.

I am extremely pleased to have the support given by my family and friends. They have been kind to me, they have endured my "unrealistic" student status for four years, and have to bear my financial burden by increasing their own. I could not imagine how I can finish my study without their support. I understand that I have to embark on another journey after my PhD study. For the time being, I would like to dedicate this thesis to them, before I am able to reward them with anything.

I am greatly thankful to Dr Jiachi CHIOU, Dr Jiubiao GUO, Ms Echo WAN and Dr Edward CHAN, who have been working alongside with me in the lab. They are extraordinary and have helped me a lot not only in research techniques and manuscripts writing, but have also given me some invaluable advice

regarding personal matters. I am also thankful to my fellow colleagues Mr Sam HUI, Dr Chris WONG and Ms Kathy PO who often shared fun and joy with me in the lab. The working atmosphere built by them, and other members of the lab, was warm and motivating. It has been a privilege to be able to work and study in such a peaceful and fascinating laboratory environment.

Lastly, I would like to thank Dr K.P. HO, Dr Susan HO, Dr S.W. CHAN as well as all technical staff of the Department for their assistance and encouragement during my study. I would also like to thank the lecturers who have taught me when I was in Hong Kong Institute of Vocational Education (Chai Wan), the place where I obtained my higher diploma and developed interests in microbiology.

It is a privilege to have finishing my PhD study at my college Alma-mater. The completion of this thesis concludes a chapter in my life, yet I know there will be another more challenging one ahead, and the experience that I have gained in the past years has certainly enabled me to face up to the future challenges .

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I - INTRODUCTION

BACKGROUND

Bacterial infections are becoming a serious public health issue worldwide, with foodborne illnesses constituting a substantial proportion of all infection cases. There are two main categories of foodborne illnesses: toxicoinfection and intoxication. The former refers to direct ingestion of foodborne pathogen, such as *Salmonella*-contaminated lettuce, whereas intoxication indicates the intake of bacterial toxin-contaminated food, for instance, *Staphylococcus aureus* toxins in sandwiches. The symptoms caused by food poisoning include diarrhea, vomiting, abdominal cramp and dysentery. In some cases fever could also be manifested especially when systematic infections occur. Most cases of foodborne illnesses are self-limiting, except those caused by invasive pathogens such as *Escherichia coli* O157:H7, *Salmonella* Typhi as well as *Vibrio cholera*. Although it is not always necessary, prescription of antimicrobials for treating bacterial infections has been an essential option for certain groups of patients, particularly for those who are immunocompromised, as well as for children and the elderly. Nevertheless, antimicrobials will always be given to patients who encountered invasive infection since consequences of treatment failure could be fatal.

Since the discovery and introduction of penicillin into clinical usage in 1928 and 1943 respectively, several classes of antimicrobial drugs, have been discovered or developed (Demain and Sanchez, 2009). The efficacy of

antimicrobials ranged from narrow to extended spectrum, and varied between different cellular targets, thus they provide a full coverage from Gram positive to Gram negative pathogens, and also allow alternative treatment options towards infections caused by different bacteria. Due to their satisfactory efficacy and relative low price, antimicrobials has sometimes been prescribed to patients regardless of the nature of infections. They may be prescribed to patients suffering various kinds of infections, ranging from those diagnosed of mild foodborne illnesses to those hospitalised for life-threatening infections. Antimicrobials are also particularly important for patients who are immuno-suppressed, such as those undergoing organ transplant operations. Although antimicrobials has been so essential in terms of saving lives and maintaining human health, development of antimicrobial compounds is a long process. From initial researches to clinical trials and subsequently final approval, the average time for the development of an antimicrobial to the stage of marketing could be as long as ten years (Piddock, 2012). Although development of new antimicrobials on the basis of modification of current drug classes is still taking place, the novel antimicrobials development pipeline has been dried up in recent years, until the latest identification and discovery of Teixobactin (Ling et al., 2015). The pace of emergence of antimicrobial resistant and multidrug resistant pathogens is so rapid in modern times that current drug development process fails to catch up (Figure 1.1).

Bacteria are capable of developing resistance to antimicrobial pressure by both

intrinsic and extrinsic mechanisms. Intrinsically, the development of mutations in genes related to drug binding targets and their regulatory systems have been frequently observed in various bacterial species. For instance, mutated DNA gyrase genes have always been linked to fluoroquinolone resistance in virtually all Gram negative bacteria. An altered outer membrane porin OprD coupled with increased efflux expression in *Pseudomonas aeruginosa* is also a major resistance mechanism towards carbapenems (Quale et al., 2006). Conversely, acquiring extracellular genetic materials, such as plasmids, integrons and transposons encoding drug-modifying enzymes are extensively associated with β-lactams and aminoglycoside resistance. It is believed that the emergence of antibiotic resistant strains would become more efficient and rapid when the organisms are subjected to antimicrobial selection pressure. Thus the impact of usage of antimicrobials in various settings has been evaluated by scientists and practitioners in recent decades due to the concern on resistance development.

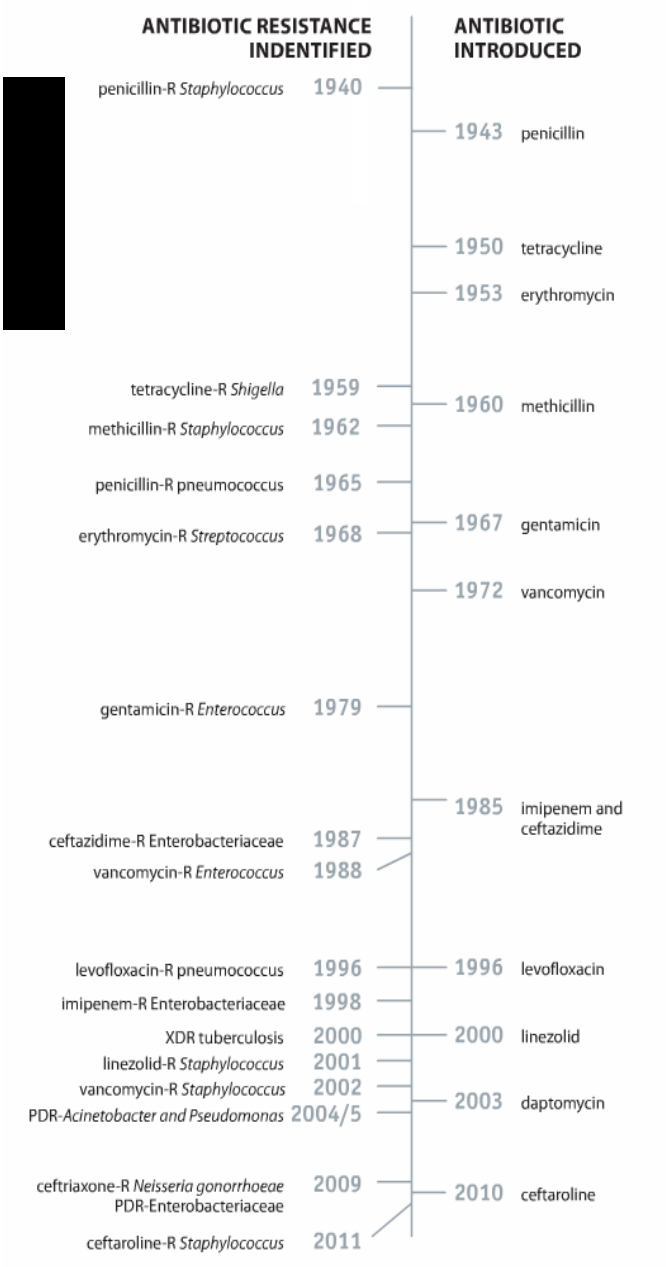

Figure 1.1. Timeline of key antimicrobial resistance events.

(CDC, 2013).

The administration of drugs into animal husbandry is mainly for diseases management, prophylaxis as well as promoting growth of animals. It is

estimated about 24.6 million pounds of antibiotics are used in the United States for animal farming annually (Oliver et al., 2011). It is evident that the use of antibiotics in animal husbandry could subsequently reduce the amount of feed necessary to maintain the growth of animals. Growth rate could also be enhanced by 1-10% and the quality of meat could be improved by giving antibiotic-supplemented feed. Due to these benefits, the use of antibiotics in animal farming has become a global practice. Though the drugs being used to feed animals were not listed as medicine options for human, it has long been speculated that using extensive amount of antibiotics in animal farming could promote the emergence of antimicrobial-resistant bacteria. The rationale behind this theory is that some antibiotics used in animals are homologues of those being used in human. For instance, the use of the glycopeptide avoparcin in animals has been shown to facilitate the emergence of bacteria that exhibited cross resistance to vancomycin, which is also a glycopeptide antimicrobial used in humans (Marshall and Levy, 2011). Another possible route of emergence of antimicrobial resistant bacteria in farm setting is the elimination of normal competitor flora due to constant selective pressure imposed by non-therapeutic application of drugs, which consequently lead to establishment of a natural reservoir of resistant bacteria. In the People's Republic of China, the high prevalence of quionlone resistant *Salmonella* spp. and *E. coli* of animal origins can be attributed to heavy usage of quinolones in farming practices. In the United States, use of enrofloxacin, a fluoroquinolone antibiotic, had been approved by the Food and Drug Administration in 1996 for treating infection in

poultry. A surveillance conducted in 1998 showed that fluoroquinolone-resistant *Camplylobacter* spp. accounted for 18% of human isolates (Gupta et al., 2004). The potential spread of such bacteria from animal origins to clinical environment has been a concern. In 1999, the European Union banned the use of animal growth promoters, including avoparcin, in animal husbandry. Subsequently, the isolation rate of vancomycin-resistant enterococci from human dropped from 13% to 4% and 5.7% to 0.7% in Germany and Belgium respectively (Klare et al., 1999;Ferber, 2002). In 2005, USFDA withdrew the approval for using enrofloxacin in poultry industry (Nelson et al., 2007).

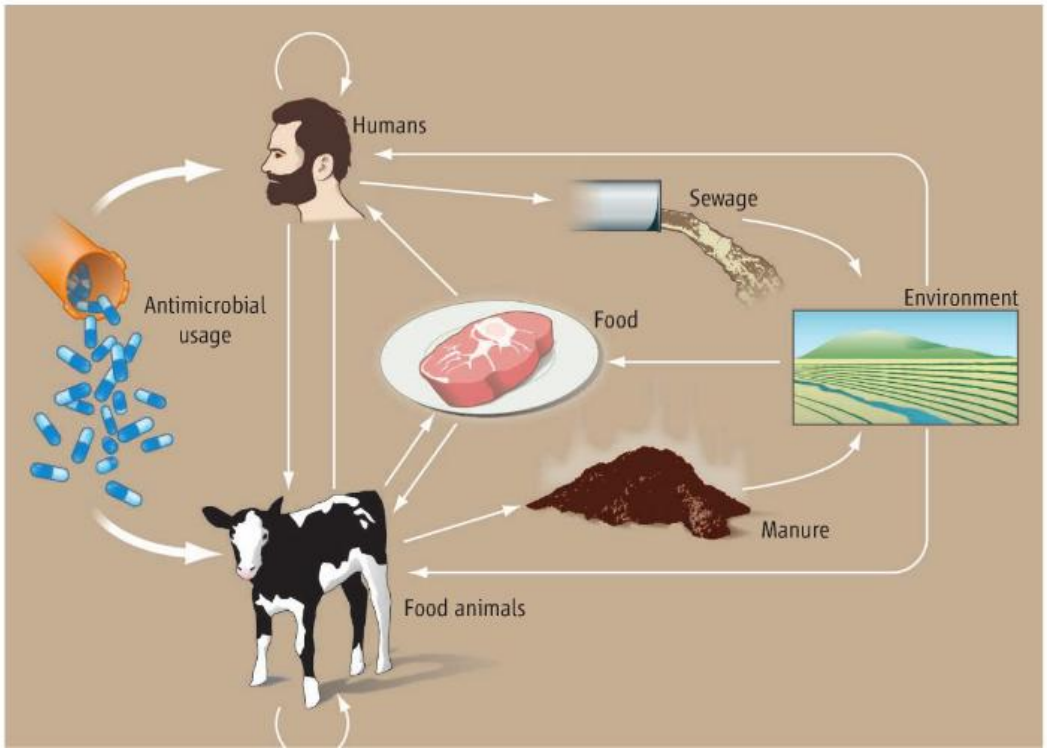

Figure 1.2. Pathways to antimicrobial resistance.

(Woolhouse and Ward, 2013). Spread of antimicrobial resistance may occur through direct and indirect pathways. The connection between different pathways differs greatly depends on location, types of anitmicrobial resistance and microbes.

Apart from being used in animals, antimicrobials are also extensively used as human medicine. Although the prescription of antimicrobials is sometimes essential, especially for patients undergoing surgeries, it was reported that in the United States about 20-50% of acute hospital cases were unnecessarily prescribed with antimicrobials (Dellit et al., 2007). The improper use of antimicrobial drugs in clinical settings may not only pose risks to patients, but also favour the emergence of resistant bacteria. According to a report published by the Centres for Disease Control and Prevention of the United States, at least 2 million people suffered from infections caused by multi-drug resistant bacteria each year, causing 23,000 death even after excluding patients who died of other medical conditions or complications due to antimicrobial resistant bacterial infections (USCDC, 2013). Antibiotics stewardship programme (ASP), or so called "Prudent Use" of antimicrobials, has been promoted by the Society for Healthcare Epidemiology of America, the Infectious Disease Society of America and the Paediatric Infectious Diseases Society, and has been implemented in different countries (Society for Healthcare Epidemiology of et al., 2012). It is believed that the reduction and/or optimisation of antimicrobial use could suppress resistance development. Consequently, a study conducted in a hospital in the People's Republic of China revealed a ~56% reduction of antimicrobial usage upon implementation of an antibiotic stewardship programme, and the resistance rate of clinical important bacterial species significantly decreased or remained stable (Zou et al., 2015). In addition to antibiotic stewardship, it has been proposed to investigate the possibility of

reusing antimicrobials which were previously abandoned. For instance, the use of daptomycin, a drug being dropped from clinical usage due to its toxicity, has been reinstalled as an alternative treatment option for certain infections (Bush et al., 2011).

ANTIMICROBIAL RESISTANCE IN *SALMONELLA*

Salmonella spp. is an important foodborne pathogen worldwide. In the United States, Non-typhoidal salmonellosis is the first leading cause of foodborne-associated hospitalisation and foodborne-associated death, and is the second leading cause of foodborne illnesses. It was estimated that *Salmonella* caused more than one million of foodborne illness cases each year in the US, resulting in about 20,000 hospitalisation and about 400 deaths (USCDC, 2014). *Salmonella* is also the second most common causative agent of foodborne diseases in Australia and responsible for 35% of food-poisoning cases (OzFoodNet Working, 2012). In Hong Kong, a report published by the Centre for Health Protection in 2011 stated that non-typhoidal salmonellosis accounted for 28% of confirmed food poisoning outbreaks (CHP, 2011). Due to its relative high virulence, antimicrobials are usually prescribed to hospitalised patients. In addition, *Salmonella* spp. is also a member of microflora in poultry. The emergence of resistant bacteria in animal farming as mentioned in previous section is becoming more serious. Hence resistance to antimicrobials in this bacterial species is of significant concern.

Multidrug resistant *Salmonella* spp. can be exemplified by the infamous *S.* Typhimurium phage type DT104. This strain was first isolated from the United Kingdom in 1980s from a clinical isolate and subsequently emerged to cause an endemic by spreading into animal husbandry and food industry facilities throughout the UK. In the 1990s, *S.* Typhimurium DT104 infections became epidemic and this strain eventually spread to the United States, France and Germany, and became the most prevalent *S.* Typhimurium phage type around the world (Helms et al., 2005). More importantly, the integration of resistance genes into its chromosome has rendered the host organism multi-drug resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines (Threlfall et al., 1994). Moreover, additional phenotypic resistance to trimethoprim and ciprofloxacin has been observed in DT104 clinical isolates since 1992 (Threlfall et al., 1997). The situation continued to worsen in 1998 when 13% and 16% of DT104 in Britain were found to exhibit resistance to trimethoprim and reduced susceptibility to ciprofloxacin respectively (Threlfall et al., 2000). The spread of this virulent and resistant clone globally in both clinical and environmental settings has been a major public health concern worldwide.

In the United States, drug-resistant foodborne bacteria are monitored through the National Antimicrobial Resistance Monitoring System (NARMS). The system gathers and analyses data collected from national-wide public health centers on the prevalence of drug resistant *Salmonella* spp, *Shigella* spp., *E. coli*

O157, *Campylobacter* spp. as well as *Vibrio* spp.. From the 2012 NARMS report, antimicrobial drugs for which the highest level of prevalence of resistance in *Salmonella* Typhimurium recorded were respectively Tetracycline (26.8%), Sulfasoxazole (26.8%), Streptomycin (23.7%), Ampicillin (23.4%) and Chloramphenicol (18%). Worse still, amongst all non-typhoidal *Salmonella* which were resistant to all the above drugs (the ACSSuT phenotype), 50% of them were *S.* Typhimurium. In addition, about 5% of *S.* Typhimurium tested were resistant to 1st and 2nd generation of cephalosporins, including cefalotin and cefuroxime. A low but increasing trend was observed in number of *S.* Typhimurium resistant to fluoroquinolone, from 0% in 2003 to 0.3% in 2012 (USCDC, 2012). However, a different situation was observed in the People's Republic of China. A study conducted in Guangdong Province analysed 1764 non-typhoidal *Salmonella* isolates, including 523 *S.* Typhimurium. Except the ACSSuT phenotype, it was found that about 11% and 13% of *S.* Typhimurium were resistant to ciprofloxacin and cefepime (Ke et al., 2014). Another study analysed 4483 non-typhoidal *Salmonella* isolates including 1174 *S.* Typhimurium from Shanghai city and the result was comparable with the one from Guangdong province, in which 21% and 5% of *S.* Typhimurium were resistant to ciprofloxacin and cefepime (Zhang et al., 2015). Although a national-wide study on antimicrobial resistant *Salmonella* spp. is still lacking, it is obvious that amongst *Salmonella* spp., resistance to cephalosporins and fluoroquinolone, the frontline antimicrobials used to treat salmonellosis, has become commonplace in China.

KEY ANTIMICROBIAL CLASSES AND MECHANISMS OF RESISTANCE TO THESE AGENTS IN MAJOR BACTERIAL PATHOGENS

β-lactams

β-lactams comprise a group of antimicrobials sharing the β-lactam ring core structure. This class of antimicrobials inhibit bacterial cell wall synthesis, by binding to Penicillin-Binding Proteins (PBPs). PBPs are important elements in bacterial cell-wall formation by playing a role in the synthesis of peptidoglycan, an essential component in bacterial cell wall. The β-lactam ring mimics the structure of D-alanine-D-alanine terminal of the stem peptide of the building block of cell wall, enabling it to bind to PBPs (Schneider and Sahl, 2010). Once it is bound to PBPs, it sterically blocks the activity of PBPs and the subsequent transpetidation process, resulting in termination of cell wall synthesis (Llarrull et al., 2010). Since the discovery of penicillin, numerous derivatives have evolved under the β-lactam class antimicrobials, including cephalosporins, monobactams and carbapenems. These β-lactams are referred as extended-spectrum β-lactams and initially known to possess good activity towards various kinds of bacteria. However, resistance to β-lactams has soon become frequently reported in numerous bacterial pathogens. Resistance mechanism of β-lactams is multi-factorial which can result from altered membrane permeability by closure of outer membrane porin and overexpression of efflux systems. Resistance can also arise by modification of PBPs and acquisition of exogenous genetic elements that encode β-lactamases (Figure 1.3). Amongst these factors, the ability to produce β-lactamases is considered a major β-lactam resistance mechanism.

Currently, four classes of β-lactamases have been identified, with class A serine β-lactamases being the most prevalent group of enzymes. Class A β-lactamases consist of various sub-groups, including *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{CMY}$ and others. The past few decades have seen the expansion and evolution of β-lactamases. Of particular importance is the emergence of broad/extended-spectrum β-lactamases (ESBLs), including variants derived from narrow spectrum enzymes such as those encoded by *bla*$_{TEM}$ and *bla*$_{SHV}$. ESBLs possess good hydrolysing activity towards various kinds of β-lactams, such as third/fourth generation of cephalosporins. Moreover, ESBLs are always plasmid-encoded and are able to be disseminated within different bacterial species in environmental and clinical settings, resulting in rapid expansion of the size of ESBLs-carrying *Enterobacteriaceae* population.

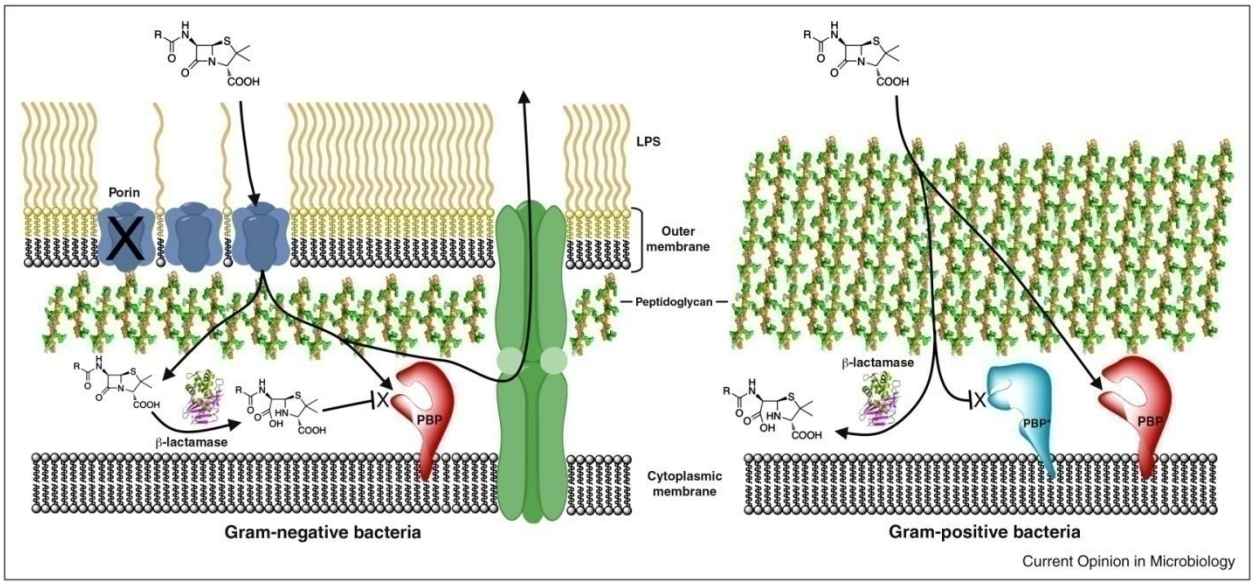

Figure 1.3. A schematic representation of the entities involved in the antibacterial mechanism of the β-lactams, and the β-lactam resistance mechanisms exploited by Gram-negative and Gram-positive bacteria.
(Llarrull et al., 2010)

Carbapenems, including imipenem and meropenem, are considered antimicrobial agents of the last resort. They are usually prescribed to treat nosocomial infections caused by *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* which are often multi-drug resistant. However, the emergence of ESBL-carrying pathogens has catalysed the adoption of carbapenems as treatment options in other bacterial infections. Unfortunately, the occurrence and emergence of carbapenemases including those belonging to Class A serine β-lactamases (*bla*$_{KPC}$) and Class B metallo-β-lactamases (*bla*$_{NDM}$, *bla*$_{IMP}$ and *bla*$_{VIM}$) has posed a serious public health threat due to the possibility that the current treatment options may eventually run out.

Aminoglycosides

Aminoglycosides belong to a group of natural or semi-synthetic amino sugars including amikacin, gentamicin, tobramycin, streptomycin and kanamycin. This group of antibiotics inhibit the protein translation process by binding to 30S subunit of the ribosome, thus leading to cell death (Le Goffic et al., 1979). Aminoglycosides exhibit inhibitory activity to both Gram-positive and Gram-negative bacteria. Resistance towards aminoglycosides is mainly due to inactivation of antimicrobials by aminoglycosides modifying enzymes. Due to the presence of exposed hydrosyl and amide groups, aminoglycosides are vulnerable to enzyme modifications. Currently, aminoglycoside modifying enzymes can be categorised into three main groups: acetyltransferases (AACs), nucleotidyltransferases (ANTs) and phosphotransferases (APHs). Each of them possess different characteristics and mechanisms (Norris and Serpersu, 2013).

Nevertheless, it has been shown that all the three groups of aminoglycoside modifying enzymes are able to bind to aminoglycosides due to the presence of analogous active site that structurally resembles the ribosomal binding cleft (Romanowska et al., 2013). Numerous variants of aminoglycoside modifying enzymes have been identified to date, amongst which the AAC(6')-Ib is of clinical significance as it was found in more than 70% of clinical Gram-negative isolates (Vakulenko and Mobashery, 2003). Aminoglycoside modifying enzymes are always transferrable between bacterial species as genes encoding such enzymes can be part of transposons, integrons as well as other integrative conjugative elements being carried on plasmids (Ramirez and Tolmasky, 2010). For instance, the AAC(6')-Ib-encoding element and its close variants are usually found in integrons as a part of gene cassette, or can be seen associated with insertion sequence IS*26* on transposons (Sarno et al., 2002;Woodford et al., 2009). As a result, aminoglycoside modifying enzymes are prevalent and detectable in bacteria especially Gram negative pathogens including *Klebsiella* spp., *Salmonella* spp., *E. coli* and *Vibrio* spp.(Ramirez et al., 2013). The expansion of these enzymes results in a reduction in the efficacy of aminoglycosides in treating various infections.

Sulfonamides

Sulfonamides are synthetic antimicrobials and the first drugs exhibiting selective effect on bacteria which can be used systemically. The first sulfonamide antimicrobial was used in 1932 (Skold, 2000). Sulfonamides were

always coupled with trimethoprim for synergistic efficacy (Bushby and Hitchings, 1968). Sulfonamides and trimethoprim impair bacterial folate synthesis pathways by inhibiting dihydropteroate synthase (DHPS), which is essential for catalytic synthesis of dihydropteroic acid. Trimethoprim inhibits the dihydrofolate reductase (DHFR). Mammalians and eukaryotes do not have to synthesise folic acid thus they lack DHPS. Use of sulfonamides has been reduced drastically in recent decades due to the introduction of new antimicrobials of greater efficacy and rapid expansion of resistant sub-population since its introduction. Resistance towards sulfornamides can be due to chromosomal mutations and acquisition of plasmid borne resistance determinants. Mutations in DHPSs (*folP*) have been reported in *E. coli*, *Campylobacter* spp., as well as *Staphylococcus* and *Streptococcus* (Skold, 2000). Although it has been documented that a mutated *folP* gene had a lower efficiency in producing dihydropteroic acid, sulfonamides have lost binding affinity to such gene product, eventually leading to resistance (Swedberg et al., 1979). In contrast, plasmid mediated sulfonamide resistance is prevalent in clinical Gram negative enteric bacteria. The plasmids concerned carry additional DHPSs which are drug-resistant variants. Two types of plasmid-mediated DHPSs, namely *sulI* and *sulII*, were identified (Swedberg and Skold, 1983). The former is always associated with other resistance genes located in an integron, whereas the latter is frequently located on IncQ plasmids (van Treeck et al., 1981).

Macrolides

Macrolides are a group of natural and synthetic compounds having a core structure of a polyketide macrolactone ring substituted with non-nitrogenous /amino sugar. The clinically useful macrolides have 14-membered or 16 membered lactone rings (Bryskier, 1993). Major macrolide antibiotics include the naturally occurring 14-membered erythromycin and its synthetic derivative clarithromycin and the 15-membered azithromycin. Macrolides exert their bactericidal effects through inhibiting bacterial protein translation, by binding to the ribosomal peptide exit tunnel. The polypeptides assembled in the peptidyl transferase centre pass through this narrow conduit tunnel during their synthesis by ribosome (Dunkle et al., 2010). This resulted in inhibition of polypeptides synthesis and subsequently immature drop-off of peptidyl-tRNA (Tenson et al., 2003). Macrolides, in particular azithromycin, have been considered potent antimicrobials for treating infections arisen by Gram-positive cocci, as well as those by *Enterobacteriaceae*, including *Shigella* and *Salmonella* infections (Gordillo et al., 1993). However, resistance towards macrolide antimicrobials has been increasingly reported in *E. coli*, *Enterobacter*, *Shigella* as well as *Salmonella* (Arthur et al., 1990;Boumghar-Bourtchai et al., 2008;Wong et al., 2014b). Like resistance mechanisms of other antimicrobials, macrolides resistance can be attributed to acquisition of exogenous DNA, target mutations and efflux extrusion. Macrolide resistance genes including the *mph* (phosphotransferase), *ere* (esterase) and *erm* (methylase) types, whose products are enzymes modifying 23SrRNA target sites, thereby protecting them from

being bound by macrolides (Phuc Nguyen et al., 2009). Another type of resistance genes, *mef*, encodes a membrane-bound efflux protein and was first identified in Gram-positive organisms (Luna et al., 2000). These resistance genes are frequently identified in both Gram negative and positive bacteria, conferring full resistance towards erythromycin and azithromycin (Phuc Nguyen et al., 2009). Apart from the exogenous resistance genes, mutational events have been documented at position 2058 and 2059 of 23SrRNA in macrolide-resistant isolates. Mutations could also occur in ribosomal proteins L4 and L22 (Zaman et al., 2007). Similar to the modifying enzymes, these mutations alter the binding affinity of macrolides to ribosome, thus attenuating the bactericidal effect (Pfister et al., 2004;Caldwell et al., 2008). In addition to these mechanisms, a ATP-binding cassette transporter (ABC), *macAB*, has been identified to be a macrolide efflux pump which confers reduced macrolide susceptibility in *E. coli* (Kobayashi et al., 2001).

Fluoroquinolones

Fluoroquinolones are derivatives of synthetic quinolones antibiotics (Lesher et al., 1962). The addition of a fluorine atom into quinolones enhances the potency and broadens the activity spectrum of the drug (Ball, 2000). Fluoroquinolones exhibited efficacy towards Gram-positive and Gram-negative bacteria by inhibiting bacterial type II topoisomerases (Hooper, 2001). These enzymes are essential in bacterial DNA replication. Bacterial chromosomal DNA is largely condensed due to the supercoiling process that enables this large and bulky

genetic material to be fitted into a tiny bacterial cell (Drlica, 1990). DNA gyrases (GyrA and GyrB) and Topoisomerase IV (ParC and ParE) are type II topoisomerases which resolve over-twisted DNA into a relaxed state or conversely introduces supercoils to under-twisted DNA (Baranello et al., 2012). This process is important in maintaining cell functions and is essential in DNA replication in the circumstances of cell multiplication (Cameron et al., 2011). Inhibition of type II topoisomerases by fluoroquinolones thus leads to fragmentation of DNA due to disrupted enzymatic process and subsequently impaired DNA synthesis, resulting in cell death (Drlica et al., 2009). The effectiveness of fluoroquinolones in treating bacterial infections infers that these drugs are extensively used in human and veterinary medicine. Inevitably, resistance to this class of antimicrobials is increasing and has become a major public health concern. Fluoroquinolone resistance is multi-factorial. Mutations and subsequent amino acid substitutions, which occur at the drug target sites, alter the protein structure and reduce drug binding affinity. The site of mutations has always been found within the Quinolone Resistance Determining Region (QRDR)(Yoshida et al., 1990;Yoshida et al., 1991). The frequently observed mutations are S83 and D87 in GyrA and S80 in ParC (Everett et al., 1996). Double mutations in *gyrA* and a single one in *parC* in *S.* Typhimurium could lead to a ciprofloxacin MIC of ≧16μg/ml (Wong et al., 2014b). In addition, over-expression of endogenous RND-type efflux pump AcrAB, which resulted from mutational events in its regulatory proteins, has been demonstrated to confer fluoroquinolone resistance (Webber and Piddock,

2001;Baucheron et al., 2002). High MIC (64μg/ml) to ciprofloxacin was observed in bacteria with concurrent occurrence of target gene mutations and efflux pump over-expression(Garvey et al., 2011). Apart from these bacterial self-physiological mechanisms, acquisition of external genes could also reduce bactericidal effects by fluoroquinolones. These genes are termed Plasmid Mediated Quinolone Resistance (PMQR) determinants, which inferred from their names that they are often carried by plasmids and are capable of disseminating between bacteria. *QnrA* was the first identified PMQR gene in *K. pneumoniae* (Martinez-Martinez et al., 1998). Since then, several *qnr* variants including *qnrB, qnrC, qnrD* and *qnrS* have been described (Robicsek et al., 2006). Qnr proteins exhibit a pentapeptide-repeat characteristic and bind to topoisomerases and prevent them from inhibition by fluoroquinolones (Xiong et al., 2011). Another frequently identified PMQR is *aac(6')-Ib-cr*. This gene is an aminoglycoside acetyl transferase variant which differs from other members in the family and is capable of acetylating the amino nitrogen on the piperazinyl substituent in ciprofloxacin and norfloxacin (Martinez-Martinez et al., 1998;Robicsek et al., 2006). Other determinants that belong to the PMQR family include *qepA* and *oqxAB,* which encode efflux systems that are able to extrude fluoroquinolones. *QepA* was firstly identified in 2007 from an *E. coli* clinical isolate. This gene encodes a major facilitator superfamily efflux and confers a 16 fold change of ciprofloxacin MIC (Yamane et al., 2007). In contrast, *oqxAB* is a RND-type efflux identified in 2004 which has become prevalent in *Salmonella* clinical isolates since 2006 (Hansen et al., 2004;Li et

al., 2013). Positve detection of *oqxAB* in fluoroquinolone resistant Gram negative bacterial species, including *E. coli* and *Salmonella,* is becoming prevalent, especially amongst isolates of clinical and environmental origins (Chen et al., 2012;Wong et al., 2013).

EFFLUX SYSTEMS IN GRAM NEGATIVE BACTERIA

Multi-drug resistant bacteria are currently a growing threat to human health. The endogenous and ubiquitous active efflux systems in bacteria have been demonstrated to play a key role in mediating antimicrobial resistance. The interplay between efflux systems and other cellular mechanisms such as enzymatic drug hydrolysis, alteration of membrane permeability as well as drug target site mutations, greatly enhance the ability of bacteria to survive under antimicrobial pressure. Given their existence in bacteria long before the introduction of antimicrobial therapy, it is well suggested that efflux functions do not just confer bacteria resistance, but are also involved in multiple cell physiological functions including stress-response and virulence. Efflux systems are highly conserved in different Gram-negative bacteria species. For instance, homologues of the well characterised RND type efflux gene *acrAB* could be found in *E. coli*, *Salmonella*, *K. pneumoniae* as well as other members in the *Enterobacteriaceae* family.

Currently the classification of efflux systems in bacteria mainly depicts the amino acid phylogenetic grouping. The most important efflux systems are the

Major Facilitator Superfamily (MFS), Resistance Nodulation Division family (RND), ATP-binding cassette (ABC), Multidrug and toxic compound extrusion (MATE) and Small multidrug resistance family (SMR) (Figure 4). They are different from each other in terms of protein structure, extrusion mechanisms as well as substrate specificity. In this section, structure and contribution to antimicrobial resistance of three main classes of efflux system, MFS, ABC and RND will be briefly reviewed.

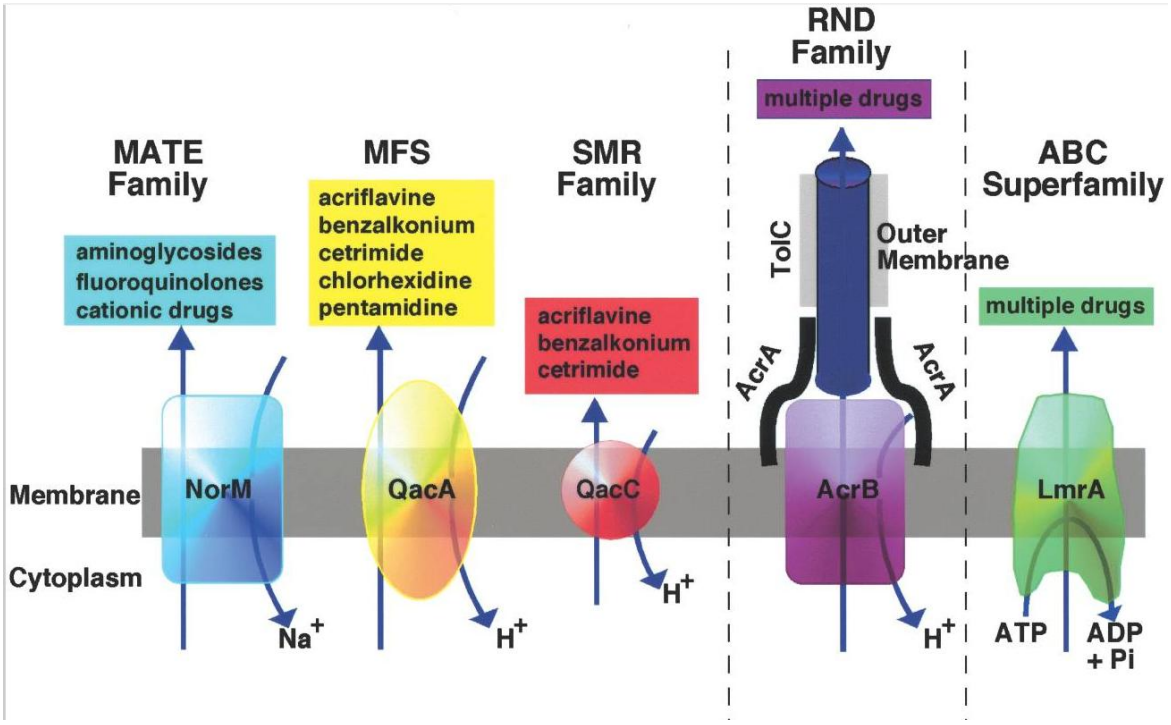

Figure 1.4. Diagrammatic comparison of the five families of efflux pumps.

(Piddock, 2006)

Major Facilitator Superfamily (MFS)

MFS transporters are standalone transporters that utilise proton-motive force to

expel substrates (Paulsen et al., 1996). Most of the drug-related MFS transporters are composed of members of 12-transmembrane segment (TMS) and 14-TMS, and can be categorised into more than 70 families based on sequence homology. Fifteen out of seventy MFS in the laboratory workhorse *E. coli* K-12 are considered drug exporters (Reddy et al., 2012). MFS transporters are positioned in the inner membrane, and pump out drugs or other substrates from the cytosol to periplasm. Due to the relative lipophilic properties of most antimicrobials, drugs which have been pumped out in the periplasm by MFS may diffuse across the phospholipid-bilayer and enter the cytosol again. Therefore, MFS transporters alone are not considered to be able to confer high-level resistance (Li et al., 2015). However, it has been demonstrated that other transporters in the periplasm, including RND-type efflux systems, may extrude the MFS-pumped substances from the periplasm into extra-cellular environment (Lee et al., 2000). This phenomenon suggests the synergistic effect of MFS transporters and the RND efflux system, which could lead to a certain level of resistance. In addition to the standalone form, some of the MFS transporters are capable of forming a tripartite structure with the endogenous periplasmic adaptor proteins and outer membrane proteins. Examples include the products of *emrB*/*emrA* and *ermY*/*emrK*, which are often coupled with outer membrane proteins (Lomovskaya and Lewis, 1992;Nishino and Yamaguchi, 2001).

One typical example of clinically important MFS transporter is the plasmid

encoded TetA pump. It was the first efflux system found to be related to drug extrusion in bacteria (McMurry et al., 1980). TetA and its closely related variants are able to confer tetracycline-specific resistance in both Gram positive and negative pathogens (Levy, 1992). Currently there are 13 phylogenetic groups of 12-TMS TetA pumps, which are abundant in Gram-negative bacteria (Sapunaric and Levy, 2005). Apart from TetA, there are several MFS transporters that are plasmid encoded and known to mediate reduced antimicrobial susceptibility. The macrolide efflux gene *mef*(A) and *mef*(B) were identified in *Streptococcus pneumoniae* and *E. coli* and found to confer reduced susceptibility towards erythromycin and azithromycin (Liu et al., 2009). *Mef*(A) was located on conjugative transposons and *Mef*(B) was found to be plasmid-borne, suggesting that they are capable of being disseminated horizontally (Zhong and Shortridge, 2000). Another frequently identified mobile MFS gene is *qepA*. This determinant was first identified in an *E. coli* clinical isolate in Japan in 2007, and was shown to mediate fluoroquinolone and quinolone susceptibility (Yamane et al., 2007). Since its discovery, *qepA* has been frequently detected in *E. coli* and *Salmonella* from various countries (Kim et al., 2009a;Chen et al., 2012;Wong et al., 2014b).

ATP Binding Cassettes

ATP binding cassettes, or ABC transporter systems, are widespread in various organisms including bacteria, yeast and mammalian cells. The sequence of ABC transporters are highly conserved in various hosts (Holland and Blight,

1999). ABC systems make use of the energy derived from ATP hydrolysis to accomplish various cellular functions. Based on the functions they perform, ABC systems are classified into three main categories (Davidson et al., 2008). Importers are responsible for nutrients intake. Exporters secrete drugs, lipids, toxin and others. Some members of ABC system may also be involved in protein translation and DNA repair (Davidson et al., 2008). ABC transporters are the major drug efflux system in Gram-positive bacteria, including LmrA in *Lactococcus lactis* (van Veen et al., 1996), Msr(A) in *Staphylococcus epidermidis* (Ross et al., 1990) and Lsa(A) in *Enterococcus faecalis* (Singh et al., 2002). On the contrary, only a handful of drug efflux pumps in Gram-negative bacteria are found to belong to ABC family. One of the well characterised drug-related ABC transporters is the MacAB complex (macrolide-specific ABC-type efflux carrier) first identified in *E. coli* in 2001 (Kobayashi et al., 2001). It is composed of the ABC transporter MacB and its periplasmic adaptor MacA. MacAB functions by coupling to the outer memebrane TolC and form a tripartite structure across the bacterial membrane. A *E. coli* Δ*acrAB* mutant carrying *macAB* plasmid construct exhibited 8-fold reduced susceptibility to 14- and 15-membered macrolides, including erythromycin and azithromycin (Kobayashi et al., 2001). However, changes of macrolide susceptibility were not observed in a Δ*macAB* mutant in *E. coli* and *Salmonella* Typhimurium, which may be due to interplay between the products of *acrAB* and *macAB* (Sulavik et al., 2001) (Nishino et al., 2006).

Resistance Nodulation Division

The RND efflux systems are ubiquitous in Gram-negative bacteria. In their functional form, they are located in the inner membrane and coupled with a periplasmic adaptor protein and an outer membrane protein, and manifested as tripartite components which span across the bacterial membrane. Similar to MFPs, RND systems employ proton motive force to expel substrates (Li et al., 2015). The wide range of substrate specificity of RND pumps conferred them with various functions. Mediation of antimicrobial susceptibility by RND efflux pumps have been demonstrated in multiple Gram-negative pathogens. AcrAB-TolC in *E. coli* and *Salmonella* has been extensively linked with reduced susceptibility to fluoroquinolone, chloramphenicol as well as tetracycline (Piddock et al., 2000;Sulavik et al., 2001). Similarly, overexpression of MexAB-OprM, MexCD-OprJ and MexXY-OprM in *Pseudomonas aeruginosa*, which are homologues of AcrAB-TolC, has been found to exhibit substrate specificities similar to those of AcrAB-TolC (Li et al., 1994;Poole et al., 1996;Masuda et al., 2000). Reduced carbapenem susceptibility and even carbapenem resistance were also observed when efflux activity was coupled with chromosomally-encoded AmpC overexpression (Tomás et al., 2010). A mobile RND efflux pump OqxAB identified in *E. coli* in 2004 isolated from swine manure was found to mediate olaquindox (an animal growth promoter) and chloramphenicol resistance (Sørensen et al., 2003). Subsequent studies reported that this mobile efflux determinant could be disseminated through plasmid transmission and conferred reduced susceptibility

towards quinolones and fluoroquinolones in *E. coli*, *Klebsiella pneumoniae* and *Salmonella* spp. isolated from animals husbandry environment as well as clinical setting (Kim et al., 2009b;Wong et al., 2014a).

AcrAB-TolC

AcrAB-TolC is highly conserved in various Gram-negative bacteria including *E. coli*, *Salmonella an*d *Klebsiella* spp.. It is composed of the inner membrane RND pump AcrB, a periplasmic adaptor AcrA and an outer membrane channel TolC. The structure of AcrAB-TolC in *E. coli* has been resolved (Figure 1.5)(Murakami et al., 2002). AcrB is known to comprise 1049 amino acid residues. In its functional form, AcrB is a homotrimer with a large periplasmic domain. Connection between AcrB and TolC is facilitated by a funnel-like opening at the apex of AcrB, which exhibits a similar diameter to the TolC entrance. Each monomer of AcrB is tightly connected to each other in the periplasmic domain, and constitutes a closed central pore. Contrary to MFP and ABC transporters, AcrB has 12 membrane-spanning α-helices. In addition to its large cavity facing the cytosol, the internal central cavity facing the periplasm could also capture free substrates in the periplasmic environment (Eswaran et al., 2004). This structure allows the pump to serve as a "vacuum cleaner" to constitutively pump out substrates from the cytosol and periplasm into extracellular environment (Piddock, 2006). Although AcrB and TolC are close enough to be structurally linked *in-vivo*, they are not able to interact with each other stably. The adaptor protein AcrA serves as a facilitator to establish an

environment favourable for connecting AcrB and TolC in the periplasmic space and stabilising the complex (Touze et al., 2004). The mechanism of drug extrusion has been elucidated by crystallography. AcrB is composed of three protomers which assemble into a trimer, yet each protomer exhibits a different conformation. This suggests a rotating mechanism in which conformational changes occur in each protomer sequentially and expel substrates bound to the binding protomer (one of the three AcrB protomers) in the periplasmic space (Nikaido and Takatsuka, 2009). Recently, it is found that a small protein composed of 49 amino acids, AcrZ (formerly named ybhT), interacted closely with AcrB at the inner membrane. A mutant lacking AcrZ exhibited reduced susceptibility to most of the antimicrobials tested compared to wild-type strain, thus it has been suggested that AcrZ may play a role in enhancing the ability of AcrAB-TolC to pump out specific substrates (Hobbs et al., 2012).

Similar to *E. coli*, although five RND systems have been identified currently in *Salmonella enterica* (AcrAB-TolC, AcrAD-TolC, AcrEF-TolC, MdtABC-TolC and MsdAB-TolC), AcrAB-TolC is regarded as the major RND efflux system (Nishino et al., 2007;Nishino et al., 2009). AcrAB-TolC exhibits a wide spectrum of substrate specificity. It has been demonstrated that almost all types of antimicrobial agents, such as fluoroquinolones, β-lactams, sulfonamides and tetracyclines, are substrates of AcrB (Piddock, 2006). In addition, detergents, fatty acids, dyes as well as microbicides are also expelled by AcrB (Ma et al., 1995;Thanassi et al., 1997;Elkins and Mullis, 2006). Thus RND efflux systems

have been postulated to play a role in various bacterial physiological functions. It has been demonstrated that in *Salmonella* and *E. coli*, deletion of RND efflux systems, including AcrB, AcrD and AcrEF, impaired the ability of bacteria to form biofilm (Baugh et al., 2012;Baugh et al., 2014). Furthermore, expression of AcrAB-TolC could be induced by bile salts, ethanol and fatty acids, which are substances that can be found in the intestinal environment (Lennen et al., 2013;Sylvie et al., 2014). The induction of expression of AcrAB-TolC by these substances suggests a role of efflux systems in bacterial stress responses. Furthermore, a clear relationship between virulence and AcrAB-TolC has been elucidated in *Salmonella* Typhimurium: studies showed that *S.* Typhimurium with knocked out AcrB/TolC functions would lose the ability to colonise in chicken intestines, and failed to invade macrophages (Buckley et al., 2006).

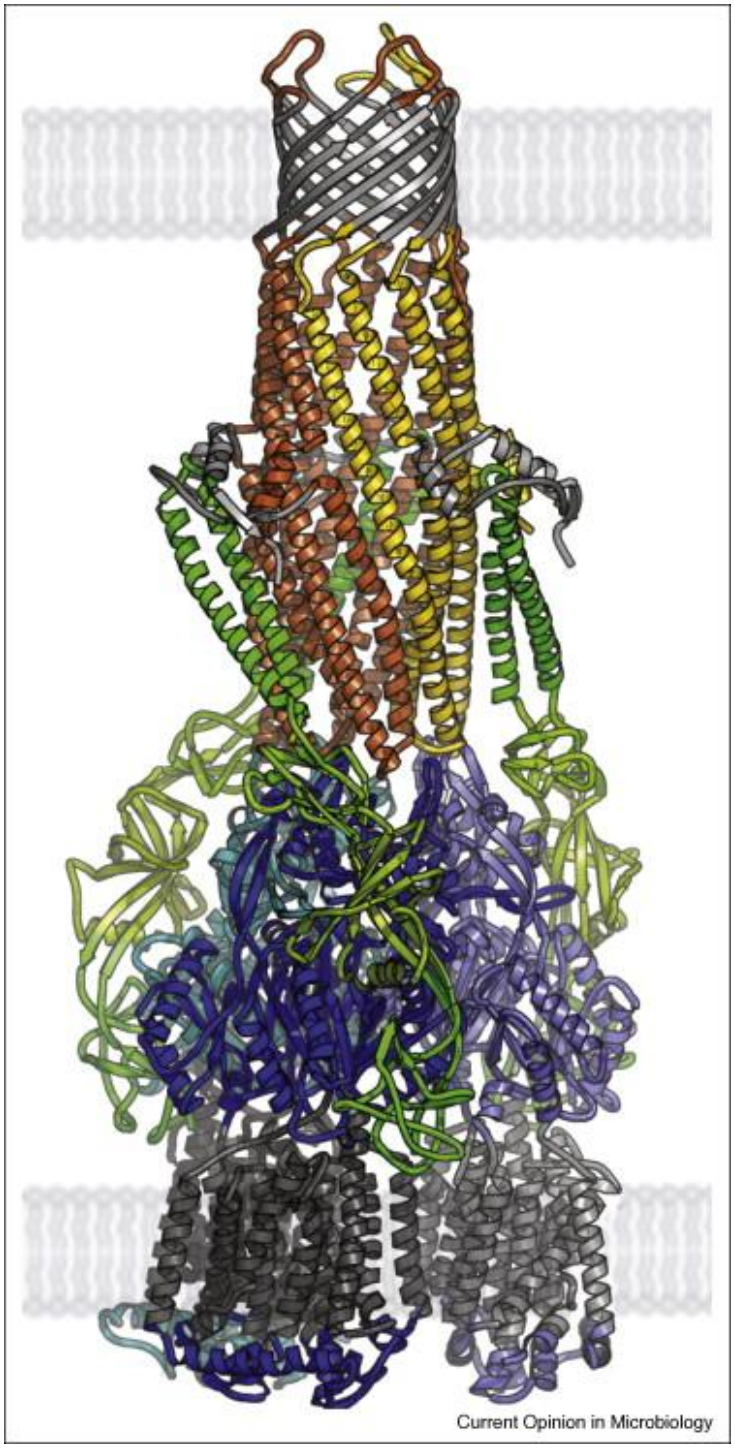

Figure 1.5. Crystal structure of AcrAB-TolC of *E. coli*.
The AcrB subunits are shown in shades blue, TolC subunits in orange/yellow and AcrA is shown in green. Membrane exposed surfaces and the TolC equatorial domain are shown in grey.(Blair and Piddock, 2009)

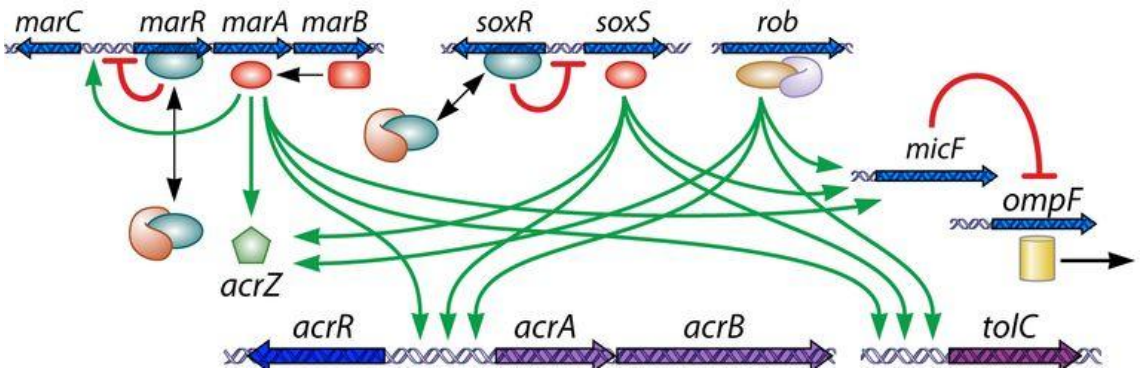

Figure 1.6. Local and global regulators mediating *acrAB* expression.

(Li et al., 2015). Efflux gene *acrAB* is locally regulated by its repressor *acrR*. Global regulators *marA*, *soxS* and *rob* up-regulate *acrAB*, *tolC* and *micF*. The elevated transcription of *micF* leads to repressed *ompF* production. *MarB* modulates transcription of *marA*, whereas expression of the *marRAB* operon is repressed by *marR*. Mutations within *marR* and *soxR* render the loss of their repressive effects.

It has been suggested that AcrAB is constitutively expressed in *E. coli* (Li et al., 2015). To-date, several regulation mechanisms of AcrAB expression have been proposed (Figure 1.6). It is believed that regulation of AcrAB relies on a series of complex but related pathways, and can be affected by both local and global regulators. The local regulator AcrR represses expression of AcrAB by binding to the promoter region of AcrAB (Ma et al., 1996). Nevertheless the binding of AcrR is not stable enough to fully repress AcrAB expression. In contrast, global regulators including MarA, SoxS and Rob exhibit a significant effect on the expression level of AcrAB and TolC. The genetic locus encoding MarA, together with two neighbouring genes encoding MarR and MarB, are located in the multiple antibiotic resistance (*mar*) locus (Alekshun and Levy, 1997). MarA controls the expression of its own operon as well as *acrAB* transcription positively (Barbosa and Levy, 2000). The regulatory mode of MarA is

dependent on its repressor MarR, which binds to promoter region of *marRAB* (Alekshun et al., 2001). Amino acid changes in MarR greatly reduce its ability as repressor (Oethinger et al., 1998). Another global regulator SoxS positively regulates AcrAB (McMurry et al., 1998). Similarly, mutational events in *soxR* could lead to reduced DNA binding affinity and even inactivation of the protein, leading to loss of repression and subsequent over-expression of AcrAB. Apart from positively regulating AcrAB, these global regulators are found to elevate *micF* transcription, which subsequently represses the translation of OmpF porin. The combination of the above regulatory pathways leads to increased efflux activity by *acrAB* overexpression, and decreased influx activity by shutting down OmpF outer membrane porin (Li et al., 2015). Other than these regulators, the histone-like nucleoid structuring protein (H-NS), the AcrS repressor regulating the AcrEF RND pump, as well as the SdiA quorum sensing protein, are all found to be involved in *acrAB* regulation (Rahmati et al., 2002;Nishino and Yamaguchi, 2004).

OqxAB

RND-type efflux pumps are encoded on chromosomes of different Gram-negative bacteria endogenously. A mobile RND efflux pump gene *oqxAB* was first identified in 2004 in an *E. coli* strain isolated from swine manure (Sørensen et al., 2003). The strain exhibited resistance towards olaquindox (quinoxaline-di-*N*-oxide olaquindox), which is widely used as an animal growth promoter (Bronsch et al., 1976). The *oqxAB* gene,together with its putative

regulator, were flanked by the Insertion sequence IS*26* and found to be located on a plasmid, in which *oqxA* and *oqxB* encoded an adaptor protein and RND transporter respectively (Norman et al., 2008). The OqxAB pump was found to exhibit 30% and 77% homology with the MexE and MexF proteins in *Xanthomonas axonopodis* respectively. It is believed that OqxAB recruits endogenous outer membrane protein TolC in order to assemble a tripartite structure similar to that of other RND systems (Hansen et al., 2004). Similar to AcrAB, it was later on discovered that OqxAB had a wide substrate specificity, and conferred reduced susceptibility towards chloramphenicol, ciprofloxacin, nalidixic acid, norfloxacin, triclosan and trimethoprim (Hansen et al., 2007). Since then, *oqxAB* had been regarded as a PMQR determinant and was frequently detected in various Gram-negative pathogens including *K. pneumoniae*, *Enterobacter cloacae* and *E. coli* (Kim et al., 2009b;Yuan et al., 2012). Noteworthy, a prevalence rate of *oqxAB* in *K. pneumoniae* as high as 100% has been reported in certain studies (Rodríguez-Martínez et al., 2013). Nevertheless, only some of the *K. pneumoniae* isolates exhibited resistance antibiogram similar to those consistently observed amongst *E. coli* strains carrying *oqxAB.* Furthermore, it has been found that a homologue of *oqxAB* named *hlyD* was located in the chromosome of *K. pneumoniae*. Although some studies conducted in *K. pneumoniae* had designated this chromosomal gene as *oqxAB*, concrete evidence showing that *oqxAB* originated from this bacterial species is still lacking (Figure 1.7).

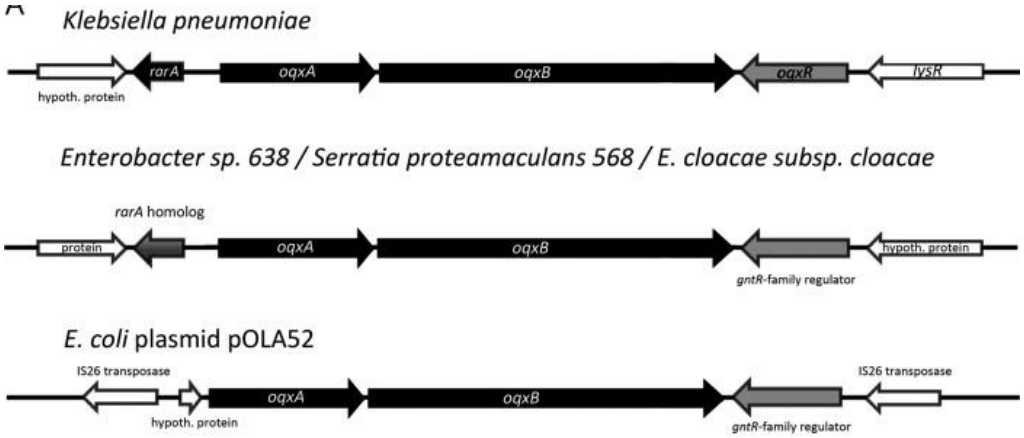

Figure 1.7 Genetic configuration of *oqxAB* and its homologues in different bacteria.
(Veleba et al., 2012)

The regulatory mechanisms of the chromosomally encoded *oqxAB* homologue in *K. pneumoniae* have been elucidated. The operon is flanked by two putative transcriptional regulators, namely *rarA* and *oqxR* (Figure 1.7). The *rarA* gene is a homologue of other well-characterised transcriptional regulators including *marA*, *ramA*, *soxS* and *rob*, whereas *oqxR* encodes a GntR type transcriptional regulator (Veleba et al., 2012). Increased susceptibility towards olaquindox (substrate of *oqxAB*) was observed in Δ*rarA K. pneumoniae* strain, and this phenotype was diminished upon complementation. It is therefore believed that *rarA* shares similar function as *ramA* and *soxS*, and activates expression of endogenous efflux systems. Elevated expression of *oqxAB* has been described in *K. pneumoniae* strain with mutations in their *oqxR* gene (Bialek-Davenet et al., 2015). Introduction of a wild-type *oqxR* gene into these strains generally normalised *oqxAB* expression, demonstrating that the *oqxR* gene product is a repressor for the operon.

BRIEF OVERVIEW OF THIS THESIS

The work presents in this thesis describes fluoroquinolone resistance mechanisms in *Salmonella* spp.. Particularly, the effect and contribution of the PMQR element *oqxAB* in *Salmonella enterica* Serovar Typhimurium fluoroquinolone resistance development is evaluated.

Chapter I provides a brief literature review on the current knowledge on antimicrobial resistance in Gram-negative bacteria, with a focus being placed on *Salmonella* spp.. Data on the prevalence of *Salmonella* and resistance rate from other studies are summarised. Major antimicrobial classes and latest findings on resistance mechanisms thereof are re-examined. Functions and classification of major bacterial efflux systems, particularly the Resistance-Nodulation-Division (RND) type efflux and their regulation mechanisms are revisited.

Chapter II describes the first ever study on the prevalence of and antimicrobial resistance in *Salmonella* in fresh meat products sold in Hong Kong. Pork and chicken meats were bought from various locations for *Salmonella* isolation. Antimicrobial resistance profile was determined for isolates collected. Resistant isolates were tested for the putative mechanism. Importantly, a PMQR element, *oqxAB*, was detected for the first time in two *Salmonella* isolates. Most of the materials described in this chapter have been published in *Antimicrobial Agents and Chemotherapy*.

Chapter III describes the investigation of the cellular mechanisms underlying antimicrobial resistance development in *Salmonella* Typhimurium isolated from clinical setting, and their contribution to reduced fluoroquinolone susceptibility to this organism. Antimicrobial susceptibility test was performed on a large number of clinical *S.* Typhimurium isolates collected during the period of 2005-2011 from Hong Kong, and various provinces in People's Republic of China; efforts on elucidating resistance mechanisms were described. Remarkably, *oqxAB* was detected in the isolates from 2006 onwards and exhibited an increasing trend year by year. Target gene mutations in *gyrA* and *parC* of isolates were screened by PCR. At certain ciprofloxacin MIC, *S.* Typhimurium carrying *oqxAB* generally contained less mutation than those without this determinant. Expectedly, *S.* Typhimurium transformed with *oqxAB*-plasmid exhibited a four-fold increase in ciprofloxacin MIC and was able to develop mutations in an environment with high ciprofloxacin concentration. Most of the materials presented in this chapter have been published in *Antimicrobial Agents and Chemotherapy* and *Frontiers in Microbiology*.

Chapter IV describes the study on identifying the origin of *oqxAB* efflux pump. More than three hundred clinical isolates of various bacterial species were screened for the presence of *oqxAB* by PCR using a total of 5 sets of primers. All 93 *K. pneumoniae* isolates were positive in PCR screening. Sequence analysis based on *K. pneumoniae* isolates collected from three decades ago

revealed a 97-99% homology to the plasmid encoded *oqxAB*, confirming that *oqxAB* originated from this bacterial species and was disseminated into other organisms through transposase capture. Most of the materials contained in this chapter have been published in *Antimicrobial Agents and Chemotherapy*.

Chapter V describes the study on elucidating regulatory mechanisms of expression of the plasmid-borne *oqxAB* operon in *S.* Typhimurium. Constructs targeting different portions of the *oqxABR* locus were generated and transformed into *E. coli* DH5α as well as the *S.* Typhimurium wild type strain 14028s and its Δ*ramA*, Δ*ramR* and Δ*soxS* mutants. Western blotting and qRT-PCR were adopted to determine the expression level of *oqxAB* and its adjacent repressor gene *oqxR*. It was found that the global regulator RamA was an essential factor that overcomes the repressive effects of OqxR, thus allowing high expression of *oqxAB* encoded in Tn*6010* cassette in *S.* Typhimurium. Importantly, we confirmed that elevated level of RamA also suppressed *oqxR* expression.

Chapter VI summarises the findings in the thesis and concludes that the *K. pneumoniae* endogenous RND efflux pump gene *oqxAB* has been disseminated to other organisms in the *Enterobacteriaceae* family in recent decades. The expansion of *S.* Typhimurium carrying this determinant could be explained by its constitutively expressed nature and the reduced susceptibility that it confers towards fluoroquinolones. Our main conclusion is that the spread of *Salmonella*

spp. carrying *oqxAB* has significantly compromised the effectiveness of treatment of bacterial infections, causing great public health concern.

CHAPTER II - FIRST DETECTION OF *OQXAB* IN *SALMONELLA* SPP. ISOLATED FROM FOOD

ABSTRACT

Foodborne salmonellosis is an important public health problem worldwide. In Hong Kong, *Salmonella* is the second leading cause of foodborne illnesses. *Salmonella* resistance to cephalosporins and fluoroquinolones is of major concern since it can lead to treatment failure. In this study, the prevalence of *Salmonella* in meat products in Hong Kong was investgated for the first time. *Salmonella* isolation rate is very similar to that reported in mainland China, but much higher than other countries. However, the rate of antimicrobial resistance in *Salmonella* isolates in food samples in Hong Kong was generally lower than other regions. Interestingly, a PMQR gene, *oqxAB*, which mediates resistance to nalidixic acid, chloramphenicol and olaquindox, was detectable in two isolates for the first time. Due to its putative role in mediating quinolone resistance in *Salmonella*, further surveillance of *oqxAB* will be needed to reveal its prevalence in *Salmonella* food and clinical isolates.

INTRODUCTION

Foodborne salmonellosis is an important public health problem worldwide. More than 1.4 million cases of salmonellosis occur in the U.S. each year, causing 300,000 hospitalization events and around 500 deaths (Scallan et al., 2011). In Hong Kong, *Salmonella* is the second leading cause of foodborne illnesses (CHP, 2011). There were over 3000 salmonella infection cases reported to Department of Health within last several years, but this rate is regarded as underestimated because of the self-limiting nature of the disease. Most human *Salmonella* infections occur through the consumption of contaminated food of animal origin, such as poultry, beef, pork, eggs and milk (Gomez et al., 1997). Although antibiotics are not essential for the treatment of most cases of salmonellosis, they can be lifesaving in invasive infections, especially among children and elderly people (White et al., 2001). Resistance of *Salmonella* to conventional drugs including amplicillin, chloramphenicol and tetracycline has been frequently reported (Chen et al., 2004). Fortunately, the resistance rate of fluoroquinolones and broad spectrum cephalosporins, which have been the choices of treatment for multi-drug resistant (MDR) non-typhoidal *Salmonella* infection in adult and children, respectively (Chiu et al., 2004), remains extremely low.

Nevertheless, cephalosporin-resistant *Salmonella* food isolates have also been reported recently, with ceftiofur and cefoxitin resistance being the most common (Rodriguez et al., 2009;Zhao et al., 2009). Resistance to these

extended-spectrum β-lactams are mainly due to the acquisition of genes encoding extended-spectrum β-lactamases (ESBLs)(Chen et al., 2004;Lewis et al., 2007;Rodriguez et al., 2009). Various classes of ESBLs have been discovered to date. AmpC type ESBLs are known to be associated with cephalosporin-resistant *Salmonella*, whereas CTX-M types of ESBLs have also been detected in both food and clinical *Salmonella* isolates (Lewis et al., 2007;Yu et al., 2011). Fluoroquinolone resistance is relatively rare amongst *Salmonella* isolates when compared to other food-borne pathogens such as *E. coli* and *Campylobacter*. The mechanism of fluoroquinolone resistance in *Salmonella* is mainly due to the development of double and single mutations in the *gyrA* and *parC* genes respectively. To date, the underlying basis of the low frequency of recovery of mutations in *gyrA* alone, which accounts for the less common, low level fluoroquinolone resistance phenotype in *Salmonella*, is not clear (Chen et al., 2004). In recent years, sporadic cases of quinolone resistance in *Salmonella* clinical isolates, especially amongst the clinically significant serotypes such as *S.* Typhimurium and *S.* Enteritidis, have been reported (CDC, 2009). Quinolone resistance in *Salmonella* may not cause direct treatment failure, yet it may lead to longer hospital stay, higher treatment cost, higher treatment failure rate, and hence a potential increase in mortality rate (Slinger et al., 2004;Broughton et al., 2010;Parry et al., 2011). Quinolone resistance in *Salmonella* may be attributed to the prevalence of plasmid-mediated quinolone resistance (PMQR) determinants, including derivatives of quinolone resistance proteins (Qnr), aminoglycoside acetyltransferase AAC(6′)-Ib-cr, and the

quinolone efflux pump QepA (Nordmann and Poirel, 2005;Robicsek et al., 2006). This study describes the prevalence and antimicrobial resistance phenotypes of *Salmonella* isolated in retail meats sold in Hong Kong, and the spectrum of molecular mechanisms responsible for quinolone and cephalosporins resistance.

MATERIALS AND METHODS

Bacterial isolation and confirmation

Retail pork and chicken products were collected from chain supermarkets and wet markets at different locations in Hong Kong (Hong Kong Island, Kowloon and New Territories) on six different sampling days between June and September 2010. Upon collection, the food samples were delivered to the laboratory immediately for processing. Sample processing was carried out following the procedures described by US Food and Drug Administration (USFDA, 2004) for *Salmonella*, with modification. Briefly, 25g of meat sample were homogenised with 225ml peptone water by stomacher. The homogenate was enriched for 12h at 37℃. A loopful of enriched content was streaked on XLT4 agar and incubated for 24h at 37℃. Two colonies were sub-cultured on another XLT4 agar and incubated for 24h at 37℃. Two purified colonies were picked for further identification and stock preparation. Isolates obtained were subjected to biochemical confirmation by the API20E kit (Biomeriux). The efforts from Hoi-ying WAN, Chun-yip CHEUNG, Ming-lai CHOW, Pui-yan LAW and Hoi-ting WONG for participating in sampling were acknowledged.

Antimicrobial susceptibility testing

Confirmed *Salmonella* isolates were streaked on Mueller-Hinton Agar (Oxoid, Basingstoke, UK) prior to antimicrobial test. Antimicrobial susceptibility testing of *Salmonella* was carried out by the agar-dilution method and the result was interpreted according to the CLSI guidelines (CLSI, 2010). Thirteen

antimicrobials were tested: ampicillin, cefotaxime, ceftriaxone, sulfamethoxazole, kanamycin, amikacin, gentamicin, tetracycline, chloramphenicol, ciprofloxacin, nalidixic acid, streptomycin. *E.coli* ATCC25922 was used as quality control. Susceptibility towards olaquindox was also examined and resistance phenotype was defined by >4 fold increase in MIC compared to *E. coli* ATCC25922.

β-lactamase and PMQR genes screening and analysis

β-lactamase gene screening was conducted according to an established multiplex PCR method (Dallenne et al., 2010). PMQR genes, including *qnrA, qnrB, qnrC, qnrD, qnrS, qepA, aac(6')-Ib-cr* and *oqxAB*, were screened by PCR as described previously (Wong et al., 2012) (Table 2.1). Genomic DNA was prepared by the boiling method. Briefly, overnight culture was spun down and resuspended in phosphate buffered saline, boiled for 5 minutes and centrifuged at 13,000g. 2μl of supernatant were used for PCR reaction.

Conjugation experiments and plasmid typing

A conjugative experiment was carried out as previously described (Carattoli et al., 2005) using sodium azide-resistant *E. coli* J53 strain as recipient. Briefly, overnight culture of donor and recipient strains were mixed and collected on a filter, which was subjected to overnight incubation on a blood agar plate. The mixture was then spread on double selective blood agar plates containing ceftriaxone (16μg/ml) and sodium azide (100μg/ml).

Molecular typing

Clonal relationships between representative *Salmonella* isolates were examined by pulsed-field gel electrophoresis (PFGE) following the PulseNet PFGE protocol for *Salmonella* (Ribot et al., 2006). Briefly, agarose-embedded DNA was digested with 50 U of XbaI (New England Biolab) for least 4 h in a water bath at 37°C. The restriction fragments were separated by electrophoresis in 0.5 Tris-borate-EDTA buffer at 14°C for 18 h using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA) with pulse times of 2.16 to 63.8 s. Phage Lambda PFGE ladder (New England Biolab) was used as DNA size markers. The gels were stained with GelRed, and DNA bands were visualized with UV transillumination (Bio-Rad).

Gene	Primer	Sequence	Product Size
qnrA	qnrAF	ATTTCTCACGCCAGGATTTG	516
	qnrAR	GATCGGCAAAGGTTAGGTCA	
qnrB	qnrBF	GATCGTGAAAGCCAGAAAGG	476
	qnrBR	ATGAGCAACGATGCCTGGTA	
qnrC	qnrCF	GGGTTGTACATTTATTGAATCG	307
	qnrCR	CACCTACCCATTTATTTTCA	
qnrD	qnrDF	TTTTCGCTAACTAACTCGC	1085
	qnrDR	GAAAGGATAAACAGGCAAAT	
qnrS	qnrSmF	GCAAGTTCATTGAACAGGGT	428
	qnrSmR	TCTAAACCGTCGAGTTCGGCG	
aac(6')-Ib-cr	aacIbF	TTGCGATGCTCTATGAGTGGCTA	482
	aacIbR	CTCGAATGCCTGGCGTGTTT	
qepA	qepAF	AACTGCTTGAGCCCGTAGAT	596
	qepAR	GTCTACGCCATGGACCTCAC	
oqxA	oqxA-F	CTCGGCGCGATGATGCT	394

	oqxA-R	CCACTCTTCACGGGAGACGA	
oqxB	oqxB-F	TTCTCCCCCGGCGGGAAGTAC	512
	oqxB-R	CTCGGCCATTTTGGCGCGTA	

Table 2.1. PCR primers for PMQR screening.

RESULTS

A total of 150 meat samples, which included eighty and seventy pork and chicken samples respectively, were collected. For the pork samples, forty-five were collected from supermarket and thirty-five were from wet market, whereas for the chicken meat, thirty-six and thirty-four samples were from supermarket and wet market, respectively. The *Salmonella* isolation rate for meat samples collected from supermarket (37%) was similar to that of samples recovered from wet market (45%). *Salmonella* isolates were recovered from forty-five pork (56%) and sixteen chicken (23%) samples. Since some of the samples contained two *Salmonella* isolates, a total of 112 *Salmonella* strains were eventually collected for further characterization; amongst these 112 strains, 86 were isolated from pork and 26 were isolated from chicken.

Antimicrobial susceptibilities to 13 antimicrobials were determined for these *Salmonella* isolates. Eighty-four (75%) *Salmonella* strains isolated from retail meats exhibited resistance to at least one antimicrobial agent. Resistance to tetracycline (55%), sulfamethoxazole (46%), nalidixic acid (35%) and chloramphenicol (30%) was commonly observed. Around 24% of the isolates were resistant to ampicillin, yet amongst them, only one isolate was also resistant to cefotaxime and ceftriaxone. Various *Salmonella* isolates also exhibited resistance to kanamycin (4%) and gentamicin (3%). Two isolates were found to exhibit cross resistance to chloramphenicol, tetracycline and nalidixic acid, and olaquindox (MIC=256μg/ml). All *Salmonella* isolates recovered from retail foods were susceptible to amikacin and ciprofloxacin. The

resistance profile of *Salmonella* isolates was shown in Table 2.2.

No. of Isolates (n=84)	Resistance Profile	Resistance Genes
14	NA	*qnrS* (n=1)
12	AMP-SUL-TET-CHL-NA	*qnrS* (n=1),*aac(6')-Ib-cr* (n=1)
11	SUL-TET	
9	SUL-TET-CHL	
7	TET	
4	AMP-SUL-TET	
3	SUL	
2	AMP-SUL-KAN-GEN-TET-NA	
2	AMP-SUL-TET-CHL	
2	AMP-SUL-TET-NA	
2	CHL-NA	
2	SUL-TET-CHL-NA	
2	TET-CHL-NA-OLA	*oqxAB* (n=2)
2	TET-CHL	
1	AMP	
1	AMP-CEF-AXO-SUL-TET	$bla_{\text{CMY-2}}$(n=1)
1	AMP-SUL	
1	AMP-SUL-CHL	
1	AMP-TET	
1	GEN-NA	
1	KAN-TET	
1	SUL-KAN-TET-CHL	
1	SUL-TET-NA	

Table 2.2. Antimicrobial resistance profiles and resistance genes of *Salmonella* isolated from food.

AMP, ampicillin; CTX, cefotaxime; CRO, ceftriaxone; SUL, sulfamethoxazole; KAN, kanamycin; GEN, gentamicin; TEL, tetracycline;; CHL, chloramphenicol; NAL, nalidixic acid; OLA, olaquindox.

β-lactamase gene screening was performed on the isolate which exhibited resistance to ceftriaxone and cefotaxime. A PCR product carrying partial

bla$_{CMY-2}$ sequence was detectable. Insertion sequences (ISs) were frequently detected upstream of ESBLs and was regarded as being responsible for the capture and mobilization of the antibiotic resistance genes. Forward primers targeting insertion sequences IS*CR1* (5'AGACGCCGTGGAAGCGTGTG), IS*Ecp1*(5'CTGCAAACGGTGCTGCGGAA) and IS*903*(5' CGCAGCGTCAGTGAACCCCC) and reverse primer CMY-2R(5'AGCGGTTATTGCAGCTTTTCAAGAA) were used to amplify the whole length of CMY variant. A ~2kb fragment was amplified by primers targeting to IS*Ecp1* and CMY-2R. DNA sequencing of the whole DNA fragment revealed that the CMY variant was CMY-2. IS*Ecp1* was 248bp upstream of CMY-2 and the ~2kb fragment was identical to a sequence encoded on pNF4656 plasmid in *Salmonella*. Conjugation experiment was performed for the *Salmonella* carrying *bla*$_{CMY-2}$ using *E. coli* J53 as recipient strain and no transconjugant was obtained, suggesting that *bla*$_{CMY-2}$ was not harboured by self-transmissible plasmid. These data suggested that IS*Ecp1* mediated CMY-2 transmission between different plasmids in *Salmonella*.

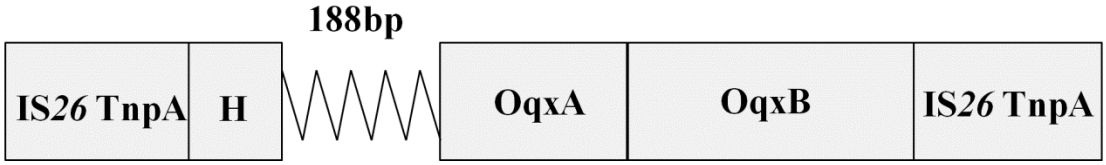

Figure 2.1.Genetic environment of *oqxAB.*

IS*26* transpoase together with a hypothetical protein (H) were located upstream of the *oqxA* gene, separated by 188 nucleotides. The sequence exhibited 100% identity to pOLA52, the plasmid where *oqxAB* was firstly discovered.

PMQR screening of *Salmonella* isolates showed that two *Salmonella* isolates which exhibited nalidixic resistance harbored the *qnrS* gene. Both of them had the same MIC for nalidixic acid (≥256 μg/ml) and ciprofloxacin (0.25μg/ml), although their resistance profiles were different (**Table 2.2**). Two olaquindox-resistant isolates were found to contain the *oqxAB* gene, which was further confirmed to be associated with IS*26* by PCR analysis (**Fig 2.1**). The *oqxAB* gene could not be transferred to *E. coli* through conjugation. Chromosomal and plasmid DNA were isolated from the *oqxAB* positive strain and subjected for PCR amplification. Sequences of *oqxAB* were amplifiable from the chromosomal DNA of *Salmonella*.

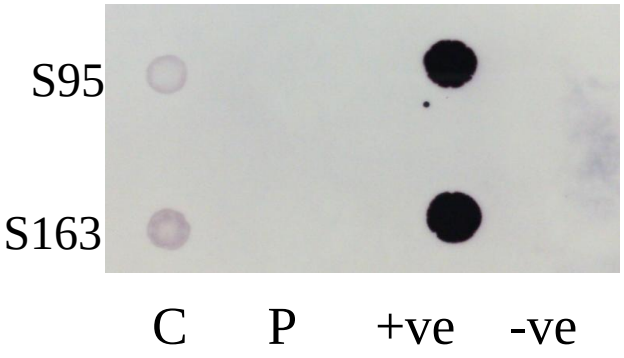

Figure 2.2. Southern hybridization of *oqxA*.

OqxA on chromosome and plasmid extracted from two *oqxAB*-positive isolates, S95 and S163. C, chromosomal DNA; P, plasmid DNA; +ve, PCR product of *oqxA*; -ve, H_2O.

DISCUSSION

This study described the prevalence of *Salmonella* in retail pork and chicken in Hong Kong. The isolation rate of *Salmonella* was 56% for pork meat and 23% for chicken meat, which is much higher than that of other countries (Khaitsa et al., 2007;Schwaiger et al., 2012). Yet this finding corroborated with a recent study on the prevalence of *Salmonella* in poultry products in China, which showed that the isolation rate was as high as 52% (Yang et al., 2011). Since a large proportion of pork and chicken sold in Hong Kong is imported from China, the high prevalence of *Salmonella* observed on chicken in this study is not unexpected. However, the rate of resistance to antibiotics in *Salmonella* from Hong Kong is much lower than that of isolates from mainland China, reflecting the fact that meat products sold in Hong Kong originated from multiple sources. In particular, there is a dramatic difference between the prevalence of ESBLs and rate of resistance to ciprofloxacin. *Salmonella* isolates from Hong Kong exhibited very low rates of resistance to the antibiotics concerned. On the other hand, the resistance pattern of *Salmonella* food isolates to different antibiotics is very similar as that of the clinical isolates reported recently except for a slightly higher rate of resistance to ciprofloxacin amongst the clinical isolates (0% and 1.6% for food and clinical isolates, respectively), suggesting that clinical use of fluoroquinolone may promote the development of ciprofloxacin resistance (Jin and Ling, 2009). Interestingly, the higher antimicrobial resistance rate and pattern in *Salmonella* food isolates in mainland China is comparable to that of the clinical isolates in Hong Kong

(Yang et al., 2010;Lu et al., 2011).

Various resistance profiles were observed in *Salmonella* isolates recovered in this study, in which a larger majority exhibited co-resistance to tetracycline, sulfamethoxazole and nalidixic acid. In addition, a large portion (46/112) of *Salmonella* strains in this study exhibited reduced susceptibility to ciprofloxacin, with the MIC range of 0.1-0.5 μg/ml. These results are not surprising since *Salmonella* isolates were often reported to be resistant to these antimicrobials (Beutlich et al., 2010;de Jong et al., 2012). Indeed quinolone resistance is becoming more common among *Salmonella* clinical and food isolates. This trend is consistent with findings of this study. To better understand the molecular mechanisms of this resistance phenotype, PMQR gene screening was performed; with results showing that only two isolates, both with a ciprofloxacin MIC of 0.25 μg/ml, harbored either one or both of the PMQR genes *qnrS* and *aac(6')-Ib-cr*, both of which have previously been reported in *Salmonella* (Ahmed et al., 2009;Jeong et al., 2011). Importantly, the two *oqxAB*-positive isolates, which were recovered from different food samples, displayed identical PFGE profile. Our data also suggested that nalidixic acid resistance in *Salmonella* was not due to widespread dissemination of PMQR genes but single mutation in their *gyrA* gene (data not shown).

This study identified OqxAB, a plasmid mediated resistance-nodulation-division (RND) family efflux pump in *Salmonella* food

isolates for the first time. This efflux pump was first identified in 2004 and was reported to confer resistance to the porcine growth promoter olaquindox (Hansen et al., 2004). Further studies revealed that OqxAB was able to mediate resistance to quinolones and chloramphenicol and reduced susceptibility to other antibiotics by several folds (Hansen et al., 2007). The *oqxAB* gene was found to mediate nalidixic resistance and possess the potential to be integrated into the chromosome of *Salmonella*. In our work, *oqxAB* was found to be flanked by the insertion element IS26 and the genetic sequence of such elements was found to be identical to those harbored by pOLA52, the first plasmid known to harbor the *oqxAB* gene (Norman et al., 2008). It should be noted, however, that apart from the insertion elements and the *oqxAB* functional gene, no other similar sequences between pOLA52 and our *Salmonella* isolates were found. Further research will be needed to address how IS26 mediated *oqxAB* is transferred from pOLA52 to the chromosome of *Salmonella* where it becomes stably integrated. OqxAB was recently found in environmental and clinical *Enterobacteriaceae* isolates, including *E.coli* and *Klebsiella pneumoniae* (Kim et al., 2009b). No *oqxAB* has been detected in *Salmonella* to date and further investigation will be needed to investigate the dissemination features of *oqxAB* amongst *Salmonella* since quinolone resistance in *Salmonella* has important clinical implications.

Acquisition of genes encoding AmpC β-lactamases through horizontal gene transfer is a common way for *Salmonella* spp. to develop resistance to β-lactam antimicrobials. More than sixty variants of CMY have been reported so far, in

which CMY-2 is the most commonly found variant in cephalosporin-resistant *Salmonella*. ESBL is not common in *Salmonella* recovered from food since only one *Salmonella* isolate was found to carry CMY-2 ESBL. In Hong Kong, in addition to CMY-2, CTX-M-14 was also reported in *Salmonella* clinical isolates (Jin and Ling, 2006). This suggests that clinical *Salmonella* infection may be associated with other sources such as consumption of contaminated egg, which accounts for 60% of *Salmonella* infection in Hong Kong. Further surveillance of the prevalence and the corresponding resistance mechanisms of *Salmonella* in eggs in Hong Kong is necessary.

CHAPTER III - PREVALENCE AND CONTRIBUTION OF *OQXAB* IN REDUCED FLUOROQUINOLONE SUSCEPTIBILITY IN *SALMONELLA* TYPHIMURIUM

AND

ABSTRACT

Emergence of multidrug-resistant *S.* Typhimurium strains, especially the Ampicillin-Chloramphenicol-Streptomycin-Sulfonamides-Tetracycline (ACSSuT) and nalidixic acid resistance (R) types, has significantly compromised the effectiveness of current strategies to control *Salmonella* infections, resulting in increased morbidity and mortality. Clinical *S.* Typhimurium isolates recovered in Hong Kong during the period of 2005-2011 were increasingly resistant to ciprofloxacin and antibiotics of the ACSSuT group. Our data revealed that a transferable efflux determinant *oqxAB* was for the first time detectable in clinical *S.* Typhimurium isolates in 2006 in both Hong Kong and China and its prevalence increased steadily in subsequent years, rising to around 40% in both regions in 2011. Interestingly, *oqxAB* exhibited strong linkage with a specific sequence type ST34 and PFGE type CN0006 and was commonly found to co-exist with *aac(6')-Ib-cr*. In addition, *oqxAB* exhibited a strong linkage with the ACSSuT resistance type. *OqxAB* and *aac(6')-Ib-cr* were harboured by plasmids of various sizes and the presence of these two elements together with a single *gyrA* mutation in *S.* Typhimurium were sufficient to mediate resistance to ciprofloxacin. Acquisition of the *oqxAB* and *aac(6')-Ib-cr* -borne plasmids by *S.* Typhimurium caused a 4-fold increase in CIP MIC. Furthermore, the presence of *oqxAB* and *aac(6')Ib-cr* in *Salmonella* dramatically increased the mutation prevention concentration (MPC) of ciprofloxacin, which may be due to mutational changes in the drug target genes. In conclusion, possession of *oqxAB* and *aac(6')-Ib-cr*-borne plasmid

facilitates the selection of ciprofloxacin resistant *S.* Typhimurium, thereby causing a remarkable increase of ciprofloxacin resistance amongst clinical *Salmonella* strains in Hong Kong. The quick expansion of *oqxAB* positive, ciprofloxacin-resistant *S.* Typhimurium will pose huge threat to clinical *Salmonella* infection control.

INTRODUCTION

Non-typhoidal *Salmonellae* are amongst the principal bacterial pathogens implicated in food-borne gastroenteritis worldwide(Gomez et al., 1997). Antimicrobial agents are not usually required for treatment in salmonellosis but can be lifesaving in cases of severe or systemic infections (Hohmann, 2001). Multidrug resistance in *Salmonellae* has been documented since 1980, a representative class of resistant organisms being the ACSSuT resistance type of *S.* Typhimurium DT104, which originated in the United Kingdom and spread rapidly to the US and other parts of the world (MMWR, 1997;Glynn et al., 1998;Markogiannakis et al., 2000). The increasing prevalence of multidrug resistant *Salmonellae* has undermined the usefulness of older antimicrobial agents such as ampicillin, chloramphenicol, and tetracycline, hence fluoroquinolones and the extended-spectrum cephalosporins have become the drugs of choice for treatment of acute gastroenteritis caused by *Salmonellae* and other enteric pathogens. Although high level fluoroquinolone-resistant *Salmonellae* are known to be associated with specific serotypes of *Salmonella* and have been reported in scattered regions around the world, their prevalence remains low. Nevertheless, several lines of evidence have suggested that emergence of multidrug resistant non-typhoidal *Salmonella* strains has significant impact on the effectiveness of current treatment options to control and manage diseases associated with food-borne infections. These include reduced efficacy of early empirical treatment, limited choice of treatment, and increased bacterial transmission rate due to horizontal transfer of resistance

genes. Importantly, multidrug-resistant *Salmonellae* are also known to be associated with increased morbidity and mortality. A comprehensive review of data from the literature indicated that *S.* Typhimurium produced up to 3 fold higher mortality rate than an average *Salmonella* infection (Helms et al., 2002). Worse still, the ACSSuT R type, nalidixic acid (Nal) R type and ACSSuT-Nal R type were respectively associated with 4.8, 10.3 and 13.1 fold higher mortality rate (Molbak, 2005).

Plasmid mediated quinolone resistance (PMQR) genes such as *qnrA, qnrB, qnrC, qnrD, qnrS, qepA* and *aac(6')-Ib-cr* have been increasingly reported in bacterial pathogens, and postulated to contribute to the development of quinolone resistance in these organisms (Cattoir and Nordmann, 2009). Recently, a novel transmissible RND efflux pump OqxAB, which mediated resistance to olaquindox, chloramphenicol, nalidixic acid and elevated MICs of other antimicrobial reagents including ampicillin and gentamicin and ciprofloxacin (MIC between 0.06~0.25μg/ml), has been identified (Hansen et al., 2007). More recently, OqxAB was reported to be prevalent in organisms isolated from pork and pig farms in China (Zhao et al., 2010;Liu et al., 2011;Chen et al., 2012), as well as from human food (18). On the other hand, the *oqxAB* gene has not been found in clinical isolates until recently, when it became detectable in clinical strains of *E. coli*, *Enterobacter* spp. and *Klebsiella pneumoniae* (Kim et al., 2009b;Park et al., 2012;Ruiz et al., 2012;Yuan et al., 2012). Though it is detectable in *Enterobacteriaceae*, its prevalence in

Salmonella spp. has not been evaluated. It is hypothesised that spread of *oqxAB* in *S.* Typhimurium within clinical setting may contribute to fluoroquinolone resistance. The aim of this study is to evaluate the prevalence of *oqxAB* in *Salmonella* Typhimurium collected from hospitals in Hong Kong and the People's Republic of China, and to elucidate the actual role played by *oqxAB* in fluoroquinolone susceptibility in this important foodborne pathogen.

MATERIALS AND METHODS

Bacterial isolates and serotyping

Human clinical *S.* Typhimurium isolates were collected from two study sites during the period of 2005 through 2011. In the first study site, clinical *S.* Typhimurium strains were recovered in hospitals of the New Territorial East cluster region of Hong Kong (covering approximately 20% of the total population in HK). The second site refers to the State Key Laboratory of the National Institute for Communicable Disease Control and Prevention (ICDC), Beijing, China. Human clinical *S.* Typhimurium isolates were collected from hospitals in eight participating cities and provinces in China including Guangdong, Guangxi, Henan, Fujian, Sichuan, Beijing, Shanghai and Chongqing. All isolates were serotyped according to the Kauffmann-White scheme.

Antimicrobial susceptibility testing

Confirmed *S.* Typhimurium isolates were subjected to antimicrobial susceptibility testing using the agar-dilution method and the results were interpreted according to the CLSI guidelines(CLSI, 2010). Fourteen antimicrobials were tested: ampicillin, cefotaxime, ceftriaxone, amoxicillin/clavulanic acid, sulfamethoxazole, kanamycin, amikacin, gentamicin, tetracycline, chloramphenicol, ciprofloxacin, nalidixic acid, streptomycin and olaquindox. *E. coli* strains ATCC 25922 was used as quality control.

PMQR and Target gene mutation screening in *S.* Typhimurium

The presence of PMQR genes, *qnrA, qnrB, qnrC, qnrD, qnrS, qepA,oqxAB* and *aac(6')-Ib-cr*, was determined by PCR using primers described previously (20, 21). The Quinolone Resistance Determining Regions (QRDRs) of *gyrA* and *parC* were amplified by PCR as previously described (Chen et al., 2007), followed by determination of their nucleotide sequences and comparison to the wild-type *Salmonella* Typhimurium LT2 strain to identify target gene mutations in the test strains. The *gyrA* and *parC* sequences of four *Salmonella* isolates, S08-52, S10-9, S05-23 and S05-30, were submitted to GenBank with the accession numbers for *gyrA*, KM504240, KM504241, KM504242 and KM504243 and *parC*, KM513651, KM513652, KM513653 and KM513654. The nature of association between Insertion sequence IS*26* and *oqxAB* was determined by PCR using primers IS*26*-F(5'GCTGTTACGACGGGAGGAG) and oqx-R (5' GGAGACGAGGTTGGTATGGA).

Molecular typing

Clonal relationship between representative salmonella isolates was examined by Pulsed-field gel electrophoresis (PFGE) according to the PulseNet PFGE protocol for *Salmonella* spp.. Briefly, agarose-embedded DNA was digested with XbaI nuclease (New England Bio-Lab) at 37ºC for 1 hr. The restriction fragments were separated by electrophoresis in 0.5 Tris-borate-EDTA buffer at 14°C for 18 h using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA) with pulse times of 2.16 to 63.8 s. Phage Lambda PFGE ladder (New

England Biolab) was used as DNA size marker. The gels were stained with GelRed, and DNA bands were visualized with UV transillumination (Bio-Rad). Band patterns were analysed by BioNumerics. Multi locus sequence typing (MLST) was also adopted according to Oxford protocol listed for *Salmonella* spp. in www.mlst.net. **(Table 3.1)**.

Table 3.1. Primers for *Salmonella* MLST typing

Primer	Sequence (5'-3')	Product size
thrA-F	GTCACGGTGATCGATCCGGT-	852 bp
thrA-R	CACGATATTGATATTAGCCCG	
thrA-Sq	GTGCGCATACCGTCGCCGAC	
purE-F	GACACCTCAAAAGCAGCGT	510 bp
purE-R	AGACGGCGATACCCAGCGG	
sucA-F	CGCGCTCAAACAGACCTAC	643 bp
sucA-R	GACGTGGAAAATCGGCGCC	
hisD-F	GAAACGTTCCATTCCGCGC	894 bp
hisD-R	GCGGATTCCGGCGACCAG	
aroC-F	CCTGGCACCTCGCGCTATAC-	826 bp
aroC-R	CCACACACGGATCGTGGCG	
hemD-F	GAAGCGTTAGTGAGCCGTCTGCG	666 bp
hemD-R	ATCAGCGACCTTAATATCTTGCCA	
dnaN-F	ATGAAATTTACCGTTGAACGTGA	833 bp
dnaN-R	AATTTCTCATTCGAGAGGATTGC	
dnaN-Sq	CCGCGGAATTTCTCATTCGAG	

Conjugation experiments

A conjugation experiment was carried out as previously described (Jacoby et al.,

2003) using sodium azide-resistant *E. coli* J53 strain as recipient. Briefly, overnight culture of donor and recipient strains were mixed and collected on a filter, which was subjected to overnight incubation on a blood agar plate. The mixture was then spread on double selective blood agar plates containing olaquindox (128μg/ml) and sodium azide (100μg/ml).

S1-PFGE and hybridization

S1-PFGE was conducted to determine the size of large plasmids. Briefly, agarose-embedded DNA was digested with S1 nuclease (New England Bio-Lab) at 37ºC for 1 hr. The restriction fragments were separated by electrophoresis in 0.5 Tris-borate-EDTA buffer at 14°C for 18 h using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA) with pulse times of 2.16 to 63.8 s. Phage Lambda PFGE ladder (New England BioLab) was used as DNA size marker. The gels were stained with GelRed, and DNA bands were visualized with UV transillumination (Bio-Rad). Chromosomal and plasmid DNA of *S.* Typhimurium strains were transferred and cross-linked onto nylon membrane and hybridized with a DIG-labeled *oqxAB* probe using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche) following manufacturer's instructions to determine the localization of *oqxAB* and *aac(6')-Ib-cr* genes in *S.* Typhimurium genetic materials.

***oqxAB* cloning, plasmid transformation and plasmid curing**

Cloning of *oqxAB* into pTrcHisB (Life Technologies) vector was done by PCR using primers pTrc-oqxAB-F (5'TTACTACTCGAGAATGAGCCTGCAAAAAAC) and pTrcoqxAB-R (5'AGGATCGAATTCCTAGGCGGGCAGATCCTC). pTrc-*oqxAB* was transformed into *S.* Typhimurium LT2. Plasmids from clinical strains were extracted by Qiagen Mini-prep kit, electroporated into *S.* Typhimurium LT2 and a nalidixic acid and ciprofloxacin susceptible *S.* Typhimurium clinical strain 11-28, and selected on plates containing 32µg/ml olaquindox. Plasmid curing was performed on clinical *S.* Typhimurium strain 10-63 as previously described with slight modification (Sato et al., 2013). The strain was grown in 3ml LB at 43℃ for 2 weeks and selected on plates containing 0, 8, 16, 32µg/ml olaquindox.

Mutation prevention concentration (MPC)

Mutation prevention concentration of *oqxAB, aac(6')-Ib-cr* positive and negative strains was determined as described previously (Gebru et al., 2011;Gebru et al., 2012). Briefly, MPC was determined by spreading 1 x 10^9 cells on LB agar plates containing a range of concentration of ciprofloxacin (CIP): 0, 0.05, 0.1, 0.25, 0.5, 1, 2, 4, 8, 16, 32µg/ml. Plates containing ciprofloxacin were incubated for up to 72 h, whereas ciprofloxacin-free plates were incubated for 24hr. Viable counts on each plate were recorded. MPC was defined as the lowest antibiotic concentration at which no colonies were

observed. For each strain, MPC was determined on the basis of the results of at least three independent experiments.

RESULTS

High prevalence of fluoroquinolone resistance in *S.* Typhimurium

A total of 239 human clinical *S.* Typhimurium isolates recovered in hospitals in Hong Kong during 2005~2011 were selected for this study. In addition, a total of 546 human clinical *S.* Typhimurium isolates, accounting for 50% of the total *S.* Typhimurium and 12% of *Salmonella* strains recovered from ICDC in China during 2005~2011 were also included in this work. *S.* Typhimurium from both HK and China exhibited high level resistance to quinolone, fluoroquinolones and other antibiotics such as ACSSuT and trimethoprim. In particular, the resistance rate to nalidixic acid of *S.* Typhimurium from HK and China was 73% and 67%, respectively. The corresponding rate to ciprofloxacin (MIC≥2μg/ml) was 34% and 36%, respectively.

Increasing prevalence of *oqxAB*

The mechanisms mediating the high prevalence of fluoroquinolone resistance in *S.* Typhimurium was investigated. We previously found that *oqxAB*, a plasmid-mediated RND efflux pump conferring resistance to multiple antibiotics, existed in *Salmonella* isolates recovered from food samples. We then assessed the prevalence of *oqxAB* in human clinical *S.* Typhimurium isolates, with results showing that the prevalence of *oqxAB* increased markedly in *S.* Typhimurium from 2005-2011. Amongst the Hong Kong isolates, the overall *oqxAB* positive rate was about 28%. Yet this element was not detectable in *Salmonella* in 2005 and an increasing trend was only observable from 2006

onwards, with 12%, 24%, 34%, 34%, 37% and 43% positive rate detectable amongst the *S.* Typhimurium isolates recovered in 2006 through 2011, respectively (**Fig 3.1**). A similar trend was observable amongst the ICDC isolates. The overall *oqxAB* positive rate in *S.* Typhimurium was 29 % (159 out of 546), and the yearly rate was 0%, 13%, 26%, 32%, 36%, 39% and 42%, respectively, during the period of 2005~2011(**Fig 3.1**). By testing 300 randomly selected strains recovered within the period of 1988~2004 in Hong Kong, we confirmed that *oqxAB* had not been introduced into clinical *S.* Typhimurium strains prior to 2006 (results not shown). Screening of other PMQR genes in these *S.* Typhimurium isolates revealed the high prevalence of *aac(6')-Ib-cr* in 85% and 83% of the oqxAB-positive *S.* Typhimurium in Hong Kong and China, respectively, while none of the *oqxAB*-negative *S.* Typhimurium were positive for *aac(6')-Ib-cr*. No other PMQR genes were detected in these *S.* Typhimurium isolates.

Table 3.2. Rate of resistance of *oqxAB* positive and negative salmonella isolates to fourteen antimicrobial agents

Antibiotics	Break-point (μg/ml)	% of resistance (% of Intermediate Resistance)					
		HK isolates (n=239)			ICDC isolates (n=546)		
		Overall	oqxAB + (n=67)	oqxAB – (n=172)	Overall	oqxAB + (n=159)	oqxAB – (n=387)
Ampicillin	32	67	100	55	47(3)	96(1)	27
Amoxicillin/ Clavulanic acid	16/8	6(18)	15(40)	3(9)	6(20)	17(45)	2(10)
Cefotaxime	4	3	3	3	4	3	5
Ceftazidime	16	3	3	3	2	1	2
Chloramphenicol	32	50	100	30	43	100	20
Gentamicin	16	23	42	15	35	70	21
Nalidixic acid	32	73	100**	63	63	100**	48
Ciprofloxacin	4	11(23)	18(76)*	9(2)	20(16)	48(50)*	9(2)
Streptomycin	-	52	61	49	28	42	22
Sulfamethoxazole	512	70	100	59	55	100	36
Tetracycline	16	68	88	60	49	96	30
Kanamycin	32	36	63	26	44	85	27
Trimethoprim	16	38	73	25	42	100	18
Olaquindox	32	28	100**	0	29	100**	0

$\chi 2$ test was performed for nalidixic acid, ciprofloxacin and olaquindox susceptibilty of isolates from Hong Kong and China with/ without *oqxAB*. *p<0.05; **p<0.001.

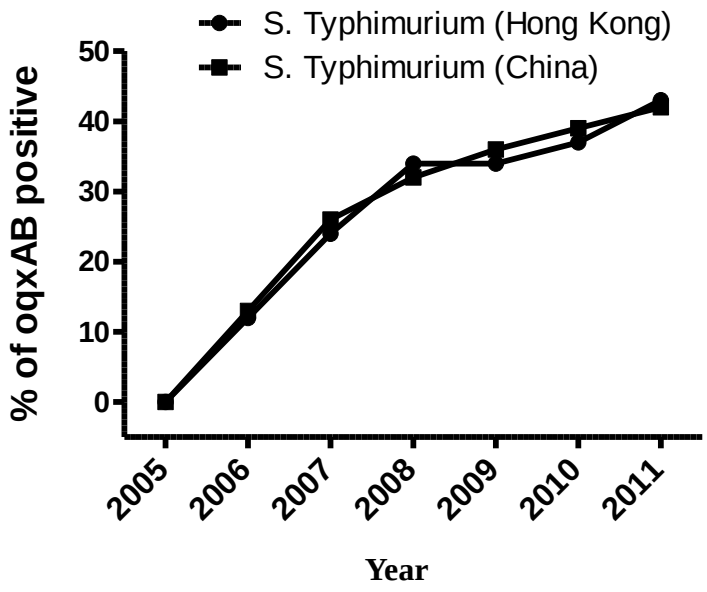

Figure 3.1. Percentage of *S.* Typhimurium isolates carrying *oqxAB* from 2005-2011.

Role of OqxAB in mediating multi-drug resistance

In Hong Kong, *oqxAB* positive *S.* Typhimurium strains were significantly more resistant to all antimicrobials tested when compared to their *oqxAB* negative counterparts, an exception being the extended-spectrum cephalosporins (**Table 3.2**). Strikingly, all positive strains were resistant to chloramphenicol, yet only a 30% resistance rate was recorded amongst the negative group. Likewise, amongst the *oqxAB* positive group, 94% and 98% of *S.* Typhimurium were resistant to ciprofloxacin (MIC≥2μg/ml) respectively from HK and ICDC, while the corresponding resistance rate in *oqxAB* negative *S.* Typhimurium HK and ICDC was only 9% (**Table 3.2**). Since the emergence of *oqxAB* in *S.* Typhimurium in HK, ciprofloxacin-resistant rate in *oqxAB* positive *S.*

Typhimurium remained at 90~100% from 2006~2011, whereas ciprofloxacin-resistance rate in *oqxAB* negative S. Typhimurium gradually decreased to 0% in 2010 and 2011(**Fig 3.2A**). The linkage between *oqxAB* and ciprofloxacin resistance appears to be even stronger amongst the ICDC isolates. Amongst the *oqxAB* positive group of these isolates, the ciprofloxacin resistance rate reached a level between 90% and 100% in each of the calendar year during the study period, and as much as 80% of the *oqxAB* positive *S.* Typhimurium strains exhibited CIP MIC≥4μg/ml in 2011. However, within the *oqxAB* negative group, the ciprofloxacin resistance rate decreased from 20% to 2% in 2010 and 2011(**Fig 3.2B**). These data showed that *oqxAB*-mediated ciprofloxacin-resistant *S.* Typhimurium became more prevalent than *oqxAB*-negative ciprofloxacin-resistant *S.* Typhimurium strains.

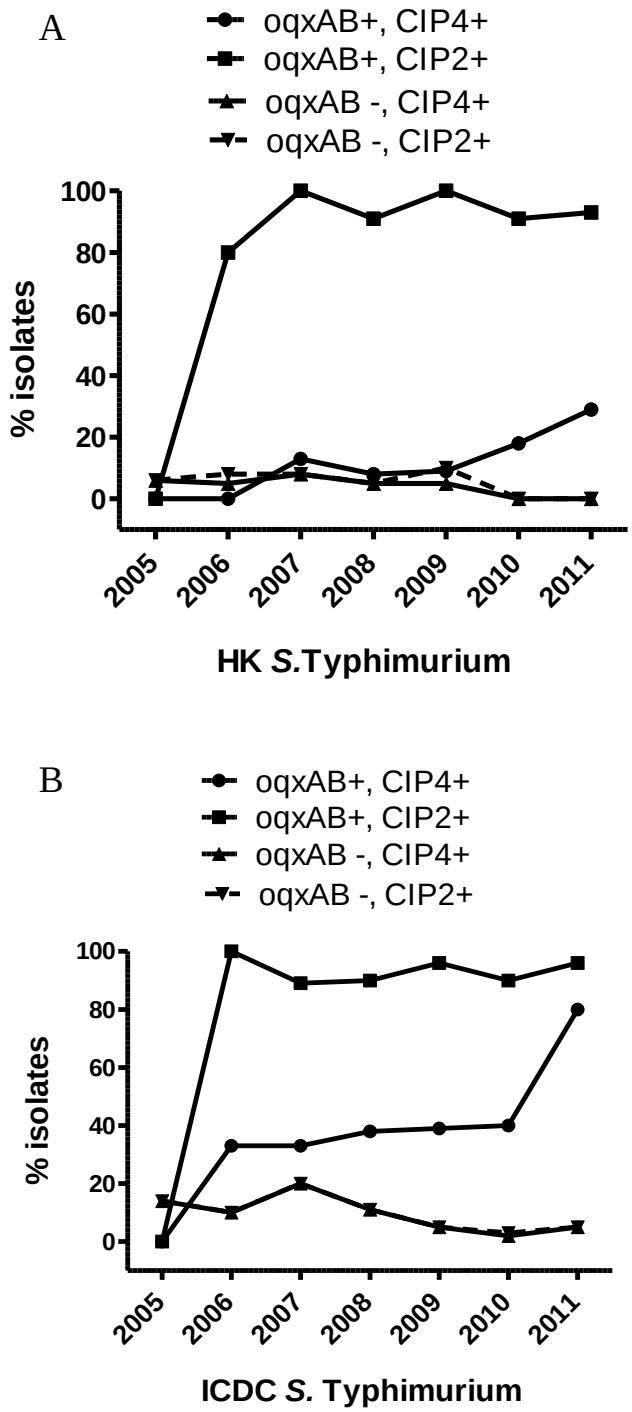

Figure 3.2. Relationship between the *oqxAB* status and fluoroquinolone resistance in *S.* Typhimurium.
Percentages of *oqxAB*-negative and -positive *S.* Typhimurium isolates that exhibit different ciprofloxacin MIC (CIP2, CIP MIC=2μg/ml, CIP2+, CIP MIC ≧2μg/ml, CIP4+, CIP MIC≧4μg/ml) in Hong Kong (A) and China (B).

Role of *oqxAB* in the development of ACSSuT resistance phenotypes

Amongst the *oqxAB* positive *S.* Typhimurium isolates in Hong Kong, the proportion of strains which exhibited the four major resistance types, ACSSuT, ACSSuT plus Tri (Trimethoprim), ACSSuT plus CIP(MIC ≥2μg/ml) and ACSSuT plus Tri and CIP, was 58%, 44%, 41% and 39% respectively. In the *oqxAB* negative group, however, the respective proportions of strains exhibiting these resistance types ranged between 4~11% (**Fig 3.3A**). For the ICDC strains, a very similar pattern was observed. The proportion of *oqxAB* positive isolates that displayed the four major resistance types, ACSSuT, ACSSuT plus Tri (Trimethoprim), ACSSuT plus CIP(MIC ≥2) and ACSSuT plus Tri and CIP, was 53%, 49%, 50% and 47% respectively; these numbers were much higher than those of the negative group, in which the respective rates for these resistance types were all below 13% (**Fig 3.3B**)

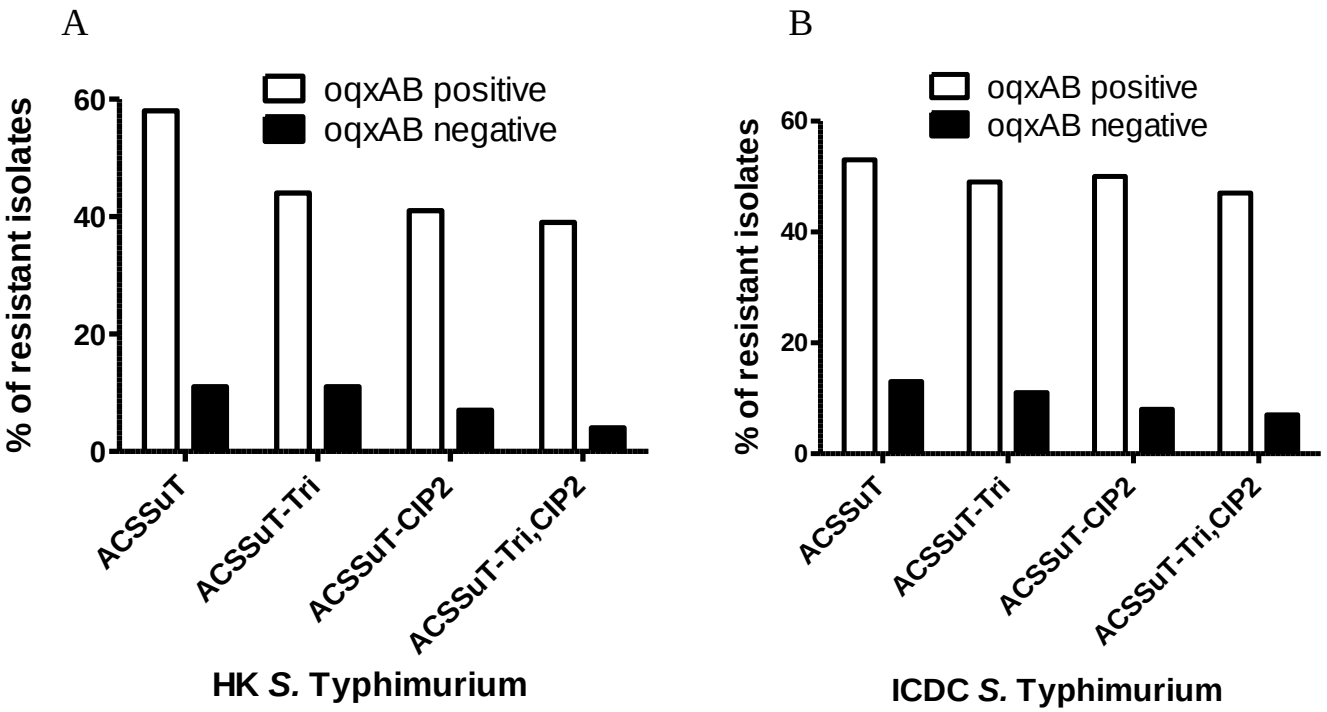

Figure 3.3. Percentage of *S.* Typhimurium isolates exhibited ACSSuT phenotype.

(A), Hong Kong isolates; (B) China isolates; Tri, resistant to Trimethoprim; CIP2, ciprofloxacin MIC=2μg/ml.

Unique clonal features of *oqxAB* positive strains

The clonal features of *oqxAB-positive* salmonella isolates were examined by pulsed-field gel electrophoresis (PFGE). Amongst the 159 *oqxAB*-positive ICDC isolates, a total of 61 PFGE patterns were observed amongst the *oqxAB*-positive strains. Interestingly, up to 37% of *oqxAB*-positive isolates belonged to one single PFGE type, which we designated as CN0006 (Figure 4). Amongst 67 *oqxAB*-positive Hong Kong isolates, 18 PFGE patterns were detectable; again 57% of the *oqxAB*-positive strains belonged to the CN0006 type **(Figure 3.4)**. Using an 80%-cutoff Dice coefficient index for the PFGE profiles, about 83% and 66% of *S.* Typhimurium isolates from Hong Kong and China, respectively, were related to the CN0006 clone. This finding suggested that CN0006 and related clones are responsible for the expansion of the ACSSuT-ciprofloxacin-*oqxAB*-*aac(6')Ib-cr* type of *S.* Typhimurium in clinical settings in China. Results of MLST showed that all of the 20 randomly selected *oqxAB*-positive strains from Hong Kong and 16 out of the 20 randomly selected *S.* Typhimurium strains from the ICDC belonged to a specific sequence type, ST34, a sequence type that was frequently associated with the ACSSuT resistance type of *S.* Typhimurium, which is also frequently reported in the European Union (Antunes et al., 2011).

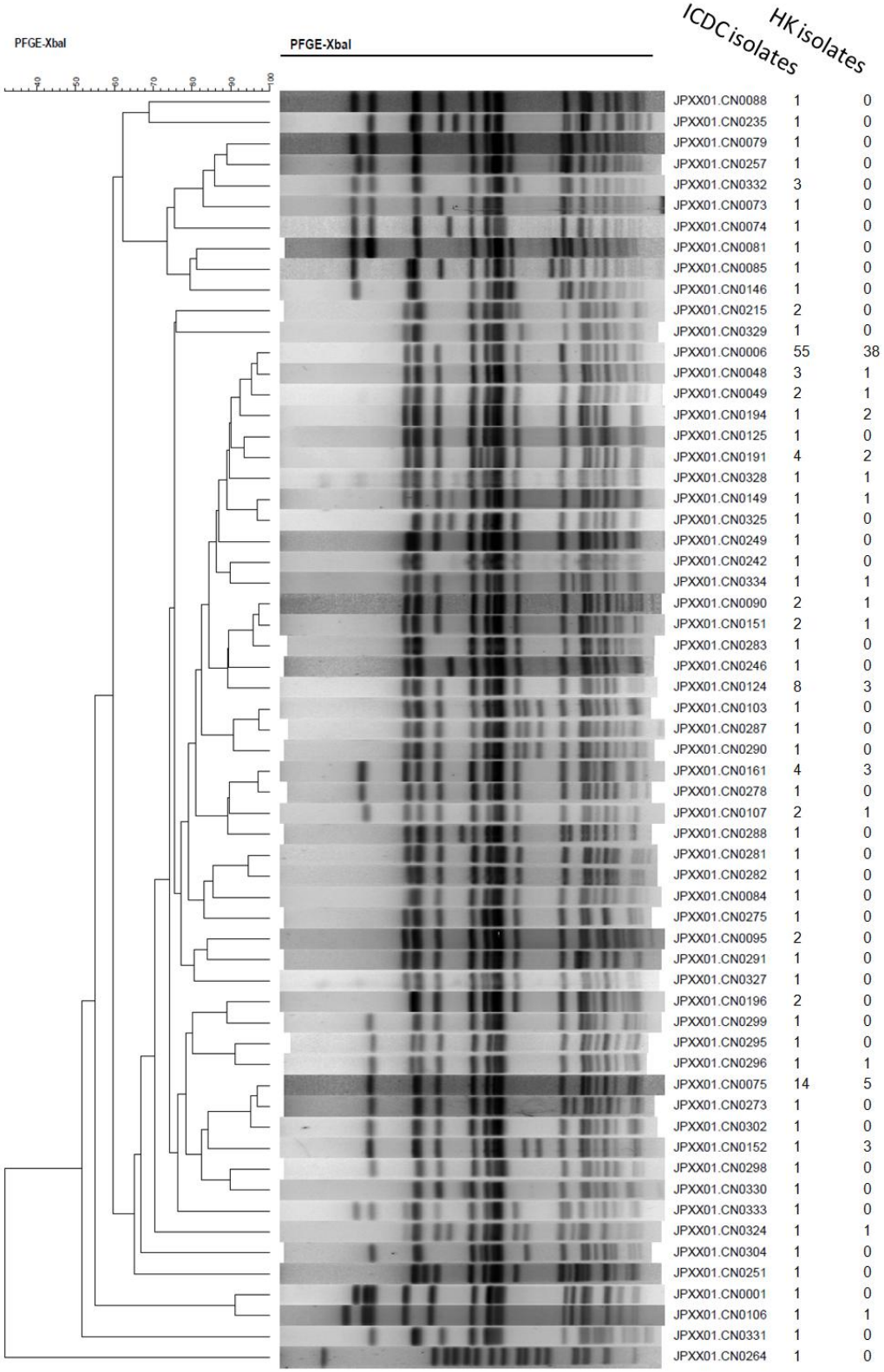

Figure 3.4. Dendrogram of XbaI-digested PFGE patterns of *oqxAB*-positive *S.* Typhimurium clinical isolates from Hong Kong and China.

PMQRs and single *gyrA* mutation mediate development of fluoroquinolone resistance in *S.* Typhimurium

To determine if *oqxAB* alone or the combination of *oqxAB* and *aac(6')-Ib-cr* can contribute to fluoroquinolone resistance, the effect of interplay between *oqxAB*, *aac(6')-Ib-cr* and target mutations in mediating fluoroquinolone resistance phenotypes in *Salmonella* was studied. Amongst all *oqxAB* negative *S.* Typhimurium organisms, the vast majority of those which exhibited CIP MIC≦0.05μg/ml had no mutation in the *gyrA* and *parC* genes. Single amino acid substitution (D87Y or D87N) in GyrA was often detected in strains with CIP MIC between 0.1μg/ml and 1μg/ml. Interestingly, two ciprofloxacin-resistant isolates (CIP MIC =1μg/ml) were found to harbour only single amino acid substitution (D87Y). Double amino acid substitution in GyrA (S83F and D87Y or N) and a single substitution in ParC (S80I) were consistently detectable in strains with CIP MIC ≧2μg/ml (**Table 3.3**). Amongst all *oqxAB* positive *S.* Typhimurium strains, no mutation was detected in *gyrA* and *parC* in strains with CIP MIC≦0.05μg/ml; single amino acid change in GyrA (D87Y or D87N), but not in ParC, was detected in strains whose CIP MIC was between 0.25μg/ml and 2μg/ml. Amongst all isolates which were positive to both *oqxAB* and *aac(6')-Ib-cr*, single amino acid substitution in GyrA (D87Y or D87N), but not in ParC, was detected in strains whose CIP MIC was between 0.25μg/ml and 2μg/ml, whereas most of the strains from this category exhibited CIP MIC≧1μg/ml. Comparative analysis of mutational and drug susceptibility data of *oqxAB* negative, *oqxAB* positive, and *oqxAB*,

aac(6')-Ib-cr positive strains showed that similar mutational profiles could result in drastically different CIP MIC, depending on whether the organism harbored the *oqxAB* or *oqxAB, aac(6')-Ib-cr* genes. Strikingly, simultaneous presence of a single *gyrA* mutation and *oqxAB*, or both *oqxAB* and *aac(6')-Ib-cr* genes, was sufficient to produce ciprofloxacin resistance (CIP MIC=1μg/ml); however, double mutations in *gyrA* plus a single mutation in *parC* were required to mediate CIP MIC ≧2μg/ml when *oqxAB* was absent. Importantly, around 98% of *oqxAB* positive *S.* Typhimurium strains harbored mutations in the *gyrA* or *parC* genes, whereas less than 60% of *oqxAB* negative *S.* Typhimurium strains had mutations in either or both of these two genes (Data not shown). Taken together, these findings suggest that acquisition of *oqxAB*, or *oqxAB* alongside *aac(6')-Ib-cr* by *S.* Typhimurium could mediate selection of fluoroquinolone resistance in *S.* Typhimurium.

Transferability and genetic location of *oqxAB*

Thirty randomly selected *oqxAB* positive *S.* Typhimurium isolates were subjected to conjugation experiment to determine the transferability of the *oqxAB* gene that they harbored. Surprisingly, none of the *S.* Typhimurium strains tested was able to transfer this resistance element to *E. coli* J53 recipient strain through conjugation. S1-PFGE and Southern hybridization were performed on 4 *S.* Typhimurium isolates and the results showed that *oqxAB* and *aac(6')-Ib-cr* were concurrently present on plasmids of various sizes in these *S.* Typhimurium isolates, hybridization results of two of the isolates are shown in

Figure 3.5. In all the tested *S.* Typhimurium isolates, the *oqxAB* gene was found to be flanked by the IS*26* fragment in a manner similar to that of the pOLA52 plasmid as previously reported (Norman et al., 2008), suggesting that the *oqxAB* gene that was becoming prevalent in *S.* Typhimurium could have been derived from the original transferable element located in pOLA52. To test this possibility, we performed PCR screening to determine if pOLA52 specific DNA sequences were prevalent amongst the test plasmids. To our surprise, however, none of the plasmids that carried *oqxAB* and *aac(6')-Ib-cr* contained such sequences of pOLA52 (Data not shown).

Table 3.3. Presence of target mutations in different level of ciprofloxacin MIC of *oqxAB* positive and negative *S.* Typhimurium isolates.

CIP MIC	**Clinical *S. Typhimurium***								
	***oqxAB* -**			***oqxAB* +**			***oqxAB*+, *aac(6')-Ib-cr* +**		
	# of isolates	***gyrA***	***parC***	**# of isolates**	***gyrA***	***parC***	**# of isolates**	***gyrA***	***parC***
≦0.05	104	WT/D87N	WT/S80R	1	WT	WT	0		
0.1	24	D87N	WT	0			2	WT	WT
0.25	14	D87N	WT	1	D87Y	WT	1	D87Y	WT
0.5	18	D87N	WT	3	D87Y	WT	3	D87Y	WT
1	2	D87Y	WT	2	D87Y	WT	6	D87Y	WT
2	2	S83F, D87G	S80R	3	D87Y/ D87N	WT	35	D87Y/ D87N	WT
4	0			0			10	D87Y	WT
8	0			0			0		
≧16	8	S83F, D87G	S80R	0			0		
Total	172			10			57		

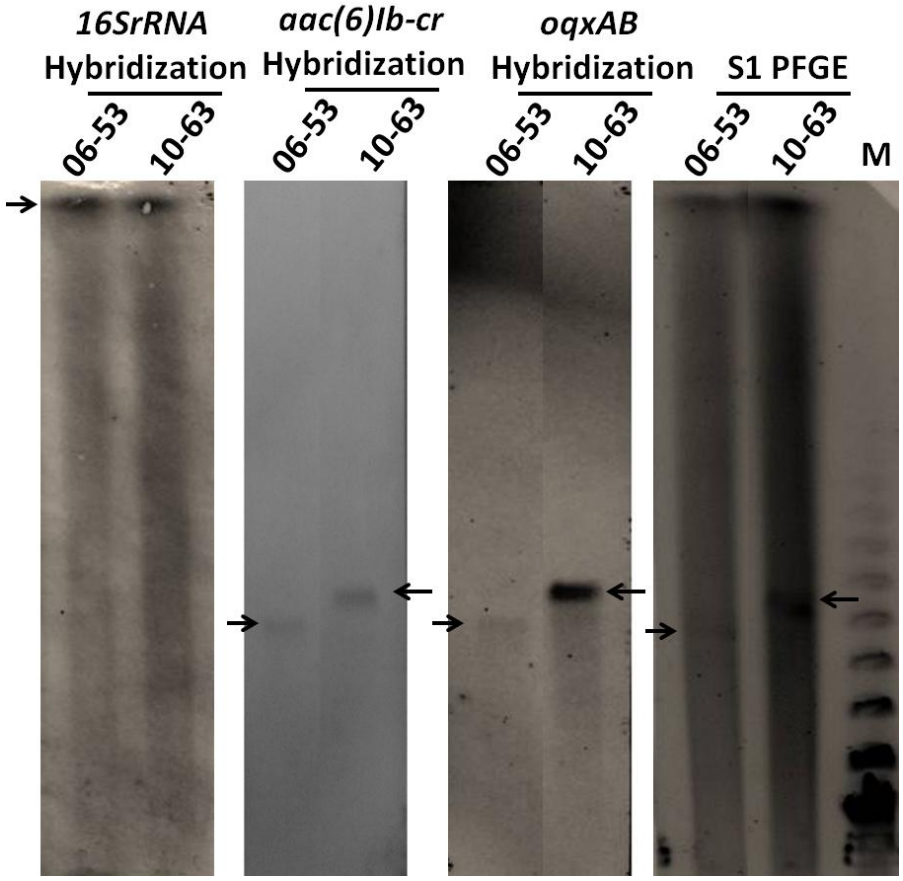

Figure 3.5. S1-PFGE and southern hybridization of 16SrRNA, *oqxA* and *aac(6')Ib-cr* on two oqxAB-positive isolates.

Arrows indicated chromosomal DNA or plasmids harboring *oqxAB* and *aac(6')Ib-cr*. 06-53 and 10-63 are *oqxAB*-positive *S.* Typhimurium clinical isolates; M, Lambda PFGE marker.

Contribution of *oqxAB* and *aac(6')-Ib-cr* to the elevated ciprofloxacin MIC in *S.* Typhimurium

To directly prove the degree of contribution of *oqxAB* and *aac(6')-Ib-cr* to the development of fluoroquinolone resistance in *S.* Typhimurium, *oqxAB* was cloned into a pTrc expression vector and transformed into *S.* Typhimurium LT2 strain. Compared to the original *oqxAB* negative *S.* Typhimurium LT2 strain, pTrc-*oqxAB*- carrying *S.* Typhimurium LT2 exhibited a CIP MIC of 0.25μg/ml, with a 20 fold increase. However, *S.* Typhimurium LT2 carrying pTrc-*oqxAB* showed much weaker growth than its parental counterpart, which was presumably due to the fitness cost caused by the over-expression of *oqxAB* in the host strain. To overcome this problem, the plasmids that carried *oqxAB* and *aac(6')-Ib-cr* were extracted from different clinical *S.* Typhimurium isolates and electroporated into *S.* Typhimurium LT2 with no success. The plasmids were then electroporated into an *oqxAB*-negative *S.* Typhimurium strain 11-28. Upon acquisition of such plasmid, the CIP MIC of this *S.* Typhimurium strain increased by ~4-fold (**Table 3.4**). To further prove the contribution of *oqxAB* and *aac(6')-Ib-cr* to *S.* Typhimurium fluoroquinolone resistance, the plasmid carrying such genes in a clinical *S.* Typhimurium strain 10-63 was cured and it showed that the curing of the plasmid in 10-63 decreased the CIP MIC by ~4-fold (**Table 3.4**). Taken together, our data had proven that *oqxAB* and *aac(6')-Ib-cr* contributed to about 4-fold increase of CIP MIC in *S.* Typhimurium. The MICs of other antibiotics were also determined for *S.* Typhimurium that acquired *oqxAB, aac(6')-Ib-cr* encoding plasmids. In addition,

it was shown that acquisition of *oqxAB* and *aac(6')-Ib-cr* borne plasmids conferred resistance to ampicillin, chloramphenicol, streptomycin, nalidixic acid, sulfamethoxazole, tetracycline, trimethoprim, and olaquindox, in addition to the elevated CIP MIC (**Table 3.4**). This is also consistent to our previous finding that the presence of *oqxAB* in *S.* Typhimurium was associated with the ACSSuT R phenotype. As much as 56% of *oqxAB*-positive *S.* Typhimurium clinical isolates were resistant to ACSSuT, whereas only 14% of *oqxAB*-negative isolates were resistant to ACSSuT (Wong et al., 2013).

Table 3.4. MIC profiles for *Salmonella* strains with various *oqxAB* and *aac(6')-Ib-cr* -borne plasmids.

Strain	MIC (μg/ml)											
	AMP	CRO	CIP	NA	TET	CHL	SUL	TRI	AMK	GEN	OLA	STE
11-28*	≤4	≤1	0.012	4	2	≤4	≤128	≤4	≤4	≤1	8	8
P06-57#	≥128	≤1	0.05	16	64	≥128	≥1024	32	≤4	32	128	≥128
P07-43#	≥128	≤1	0.05	32	64	≥128	≥1024	32	≤4	32	64	≥128
P08-11#	≥128	≤1	0.05	16	64	≥128	≥1024	32	≤4	32	64	≥128
P10-9#	≥128	≤1	0.05	16	64	≥128	≥1024	32	≤4	32	128	≥128
10-63*	≥128	≤1	1	≥128	64	≥128	≥1024	32	≤4	≤1	512	32
10-63C	16	≤1	0.25	≥128	2	≥128	≥1024	≤4	≤4	≤1	16	8

AMP, ampicillin; CRO, ceftriaxone; CIP, ciprofloxacin; NA, Nalidixic acid; TET, tetracycline; CHL, chloramphenicol; SUL, sulfamethoxazole; TRI, trimethoprim; AMK, amikacin; GEN, gentamicin; STE, streptomycin; OLA, olaquindox.

**Salmonella* clinical isolates with various *oqxAB, aac(6')-Ib-cr* background;

transformants with the transformation of *oqxAB, aac(6')-Ib-cr* encoding plasmid from different clinical *Salmonella* isolates to parental *Salmonella* strain 11-28; C, *oqxAB, aac(6')-Ib-cr* encoding plasmid cured strain.

Contribution of *oqxAB* and *aac(6')-Ib-cr* to elevated MPC of fluoroquinolone in *S.* Typhimurium

To validate the hypothesis that *oqxAB* and *aac(6')-Ib-cr* contributed to mutation development, mutation prevention concentrations (MPC) of ciprofloxacin were determined for *S.* Typhimurium with and without *oqxAB.* As shown in **Table 3.5**, *oqxAB, aac(6')-Ib-cr* positive clinical *Salmonella* isolates, 06-57, 07-43 and 08-11, exhibited much higher MPC of ciprofloxacin than the *oqxAB, aac(6')Ib-cr*-negative *Salmonella* strains, 05-41, 07-54 and 10-25 (**Table 3.5**). Furthermore, although the *Salmonella* 11-28 strain exhibited MPC for ciprofloxacin of about 0.1ug/ml, transformation of plasmids from other clinical *Salmonella* isolates carrying *oqxAB, aac(6')-Ib-cr* to *Salmonella* 11-28 dramatically increased its MPC to 2~4µg/ml. On the other hand, *Salmonella* 10-63 exhibited MPC of 8µg/ml, yet the curing of the *oqxAB, aac(6')-Ib-cr* encoding plasmid led to a slightly decreased MPC (4µg/ml). The minimal effect of curing of *oqxAB, aac(6')-Ib-cr* encoding plasmid on the MPC of 10-63 may be due to the fact that the long-term starvation stress used to cure the plasmid may have caused stress response to develop in the isolate, thereby indirectly contributing to the elevated MPC for strain 10-63C. It has been shown that long-term starvation stress stimulates the stringent SOS response in bacteria, which is essential in bacteria for acquisition of mutations leading to resistance to some antibiotic drugs (Fung et al., 2010). Most importantly, compared to *Salmonella* 11-28 alone, which did not develop *gyrA* mutation in MPC assay, *Salmonella* 11-28 transformed with *oqxAB* and *aac(6')Ib*-cr

encoding plasmids from *Salmonella* 06-57 and 08-11 developed single mutation in *gyrA*, which may partly contributed to the increase in CIP MPC (**Table 3.5**). It is probably due to the fact that the presence of *oqxAB* and *aac(6')Ib*-cr may enable *S.* Typhimurium to survive under fluoroquinolone stress and facilitate subsequent development of target mutations. Nevertheless, these data confirm that *oqxAB* and *aac(6')-Ib-cr* play a key role in elevated CIP MIC and MPC, and hence resistance to fluoroquinolone in *S.* Typhimurium.

Table 3.5. MICs of nalidixic acid (NA) and ciprofloxacin (CIP), and Mutation Prevention Concentration (MPC) toward ciprofloxacin of *Salmonella* isolates with various background of *oqxAB* and *aac(6')-Ib-cr*.

Salmonella Isolate	*oqxAB, aac(6')-Ib-cr*	QRDR Mutations		MIC (μg/ml)		MPC (μg/ml)	GyrA mutation†	MPC/MIC
		GyrA	ParC	NA	CIP	CIP		
06-57*	+	WT	WT	32	0.1	2	NT	20
07-43*	+	WT	WT	16	0.05	0.5	NT	10
08-11*	+	WT	WT	32	0.1	1	NT	10
05-41*	-	D87N	WT	32	0.025	0.1	NT	4
07-54*	-	WT	WT	16	0.012	0.1	NT	8
10-25*	-	WT	WT	4	0.025	0.1	NT	4
11-28*	-	WT	WT	4	0.012	0.1	WT	8
p06-57#	+	WT	WT	16	0.05	4	D87N	80
p07-43#	+	WT	WT	32	0.05	2	WT	40
p08-11#	+	WT	WT	16	0.05	2	D87G	40
p10-9#	+	WT	WT	16	0.05	2	WT	40
10-63*	+	D87N	WT	≥128	1	8	D87Y	8
10-63c	-	D87N	WT	≥128	0.25	4	D87Y	16

NA, Nalidixic acid; CIP, ciprofloxacin.

**Salmonella* clinical isolates with various *oqxAB, aac(6')-Ib-cr* background;

transformants recovered from transformation experiments in which *oqxAB, aac(6')-Ib-cr*-borne plasmids extracted from different clinical *Salmonella* isolates were transformed into parental *Salmonella* strain 11-28;

† *gyrA* mutation from strains that were selected after MPC assay and exhibited CIP MIC between 0.5~4μg/ml

C, *oqxAB, aac(6')-Ib-cr* encoding plasmid cured strain

NT, Not test.

DISCUSSION

An important finding in this work is that the *oqxAB* and *aac(6')-Ib-cr* gene products not only directly contribute to the elevated CIP MIC of *S.* Typhimurium, but also enhance the ability of the host organism to survive in an environment with high dose of ciprofloxacin, which may in turn facilitate the development and selection of fluoroquinolone resistance. The mechanism of fluoroquinolone resistance in *Salmonella* has conventionally been attributed to double mutations in *gyrA* with or without a single *parC* mutation (Casin et al., 2003;Chu et al., 2005). Unlike *E. coli* and *Campylobacter*, double *gyrA* mutations in *Salmonella* were rare and presumably difficult to acquire, therefore fluoroquinolone remained an effective treatment of choice for severe *Salmonella* infections. In this study, we demonstrated that acquisition of the *oqxAB or oqxAB, aac(6')-Ib-cr* genes in *S.* Typhimurium could mediate development of resistance to ciprofloxacin (CIP MIC$\geqq$1μg/ml). We postulate that the pump activities and enzymatic hydrolysis of fluoroquinolones enable the organisms to withstand antibiotic pressure for a prolonged period, during which mutational changes can occur. Elevation of the antibiotic resistance potential of *Salmonella* is one way by which *oqxAB* can help the host strain to successfully launch clinical infection in human, leading to a dramatic increase in the proportion of *oqxAB* positive strains observable amongst clinical *Salmonella* isolates recovered in recent years (Wong et al., 2014b). The increased prevalence of *oqxAB* positive *S.* Typhimurium in clinical isolates also contributes directly to a higher percentage of fluoroquinolone resistance in

clinical salmonella strains. In 2011, the proportion of the *oqxAB* positive *S.* Typhimurium in Hong Kong and Peoples' Republic of China that were found to be resistant to ciprofloxacin reached 34% and 36% respectively.

The fact that *oqxAB* could not be found in *S.* Typhimurium until 2006 may be due to its poor ability to replicate in *Salmonella* initially; this notion is supported by the fact that transformation of *oqxAB*-borne plasmid to *S.* Typhimurium did not elevate MIC of CIP in these strains and that direct expression of *oqxAB* in *S.* Typhimurium had a fitness cost (Hansen et al., 2007;Wong et al., 2013). Nevertheless, our data indicate that the *oqxAB* gene has adapted to co-exist in *S.* Typhimurium. In this study, *oqxAB* were found to be associated with IS*26* but not carried by pOLA52-like plasmids, suggesting that *oqxAB* was excised from pOLA52 and integrated into other plasmids mediated by IS*26* transposase. Since no *oqxAB* encoding plasmid in *Salmonella* has been sequenced, the mechanism underlying the co-existence of *oqxAB* and *aac(6')-Ib-cr* in over 80% of the *oqxAB*-positive strain is not clear. The quick expansion of *oqxAB* and *aac(6')-Ib-cr* positive, ciprofloxacin-resistant *S.* Typhimurium will pose a huge threat to efforts of infection control of *Salmonella* infections. Urgent actions are required to halt further transmission of the *oqxAB* positive strains in both environmental and clinical settings. In addition, it remains to be seen if *oqxAB* has been taken up by other bacterial species and whether it plays a role in the evolution of resistance and virulence traits of various bacterial pathogens. Findings in this work also highlight a need

to investigate the impact of *oqxAB* in a wide range of foodborne and zoonotic pathogens.

CHAPTER IV - Origin of *oqxAB* efflux pump

ABSTRACT

The OqxAB efflux pump, a Plasmid-Mediated Quinolone Resistance (PMQR) determinant, has become increasingly prevalent amongst members of *Enterobacteriaceae* over the past decade. To investigate the evolution and dissemination routes of the *oqxAB* operon, we assessed the prevalence of *oqxAB*-like elements amongst various Gram negative bacterial species and analyzed the genotypic and phenotypic characteristics of organisms harboring such elements. Using a comprehensive genotyping approach, a chromosome-based *oqxAB* operon was detectable in all *K. pneumoniae* strains tested, including organisms isolated before the year 1984. Sequence and phylogenetic analyses confirmed that the *oqxAB* operon in *K. pneumoniae* was genetically closest to their plasmid-borne counterparts recoverable only from *E. coli* and *Salmonella* isolates collected from the year 2003 onwards. Chromosomal elements with much lower sequence homology were also found amongst the *Enterobacter spp*. but not other Gram negative species. Contrary to the quinolone resistance phenotypes which were consistently observable amongst organisms harboring *oqxAB*-borne plasmids, chromosomal *oqxAB* elements generally did not confer quinolone resistance, except for *K. pneumoniae* strains which exhibited a typical *oqxAB*-mediated phenotype characterized by cross resistance to olaquindox, chloramphenicol and the quinolones. Gene expression analysis illustrated that such phenotypes were due to elevated expression of the chromosomal *oqxAB* operon. Furthermore, transposition of the *oqxAB* operon from bacterial chromosome to plasmids was

found to result in more than 80–fold increase in the level of expression of the OqxAB pump, confirming its status as the first constitutively expressed efflux system located in bacterial mobile elements.

INTRODUCTION

The mobile efflux pump OqxAB, first identified in *Escherichia coli* in 2003, belongs to the RND-family and shares up to 40% homology with other RND-type efflux systems such as AcrAB in *E. coli* and MexAB in *Pseudomonas aeruginosa* (Hansen et al., 2004). At the time of its discovery, the gene encoding this pump was located in a conjugative plasmid designated as pOLA52 and was found to contribute to phenotypic resistance towards nalidixic acid and chloramphenicol, as well as reduced susceptibility to ciprofloxacin, in *Escherichia coli* (Hansen et al., 2007;Norman et al., 2008). Since then, *oqxAB* has been frequently detected as a Plasmid Mediated Quinolone Resistance (PMQR) determinant amongst members of *Enterobacteriaceae* (Kim et al., 2009b;Zhao et al., 2010;Wong and Chen, 2013). Sequencing analysis of pOLA52 initially showed that *oqxAB*, together with an open reading frame *orf68* of unknown function, was flanked by the insertion sequence IS*26* (Norman et al., 2008). A set of corresponding genes which shared 99% nucleotide homology with the *oqxAB* operon in pOLA52, including an *oqxR* gene which was genetically identical to the plasmid-borne *orf68* element, was subsequently detectable in the genome of *K. pneumoniae*, which did not exhibit phenotypic resistance to either nalidixic acid or chloramphenicol (Yuan et al., 2012). More recently, Bialek *et al*. (Bialek-Davenet et al., 2015) showed that mutations in *oqxR* induced over-expression of not only *oqxAB*, but also *rarA*, which encoded the *oqxAB* transcriptional activator in *K. pneumoniae*. These findings infer that a mutated *oqxR* gene is required to elicit over-expression of

oqxAB and cross-resistance to quinolone and chloramphenicol in *K. pneumoniae*. Despite these findings, however, the evolutionary origin of *oqxAB*–borne plasmids**,** and the molecular basis of the differential phenotypes observable in organisms harboring the chromosomal and plasmid-borne *oqxAB* genes, remain ill-defined. First, although *oqxAB* was detected frequently in *K. pneumoniae*, concrete evidence showing that *oqxAB* is intrinsic to this bacterial species is not available as failure of *oqxAB* detection in *K. pneumoniae* is common (Perez et al., 2013;Rodriguez-Martinez et al., 2013). In addition, *oqxAB* homologues were also identified in other members of *Enterobacteriaceae*, including *Enterobacter aerogenes* and *Enterobacter cloacae*, as well as some other *Klebsiella* spp. (Yuan et al., 2012), prompting a need to perform cross-species analysis of the pattern of distribution for both chromosomal and plasmid-borne *oqxAB*-like elements, and the respective roles of such elements in conferring phenotypic resistance. Second, whether translocation of the *oqxAB* genes from chromosome to plasmid results in over-expression of this efflux pump is not clear. Currently, data regarding the expression level of *oqxAB* in pOLA52 and the regulatory mechanisms concerned are not available.

We hypothesised that *oqxAB* is an endogenous gene in *K. pneumoniae* and it serves as an intrinsic RND-type efflux system similar to the role of *acrAB* in other *Enterobacteriaceae*. To address the above issues, we performed a comprehensive assessment of the prevalence of the *oqxAB* genes in various members of *Enterobacteriaceae* recovered from different time periods and

regions to map the evolution and dissemination routes of this antibiotic resistance determinant. We then performed genetic analysis of *oqxAB*-like elements recoverable from the test strains to obtain evidence which suggests that the plasmid-borne *oqxAB* operon originated from the chromosome of *K. pneumoniae* and evolved to become even more functionally active than their chromosomal counterparts.

MATERIAL AND METHODS

Bacterial isolates

Eighty-five clinical *K. pneumoniae* isolates were collected from The Prince of Wales Hospital, Hong Kong, amongst which 15 were isolated in or before 1984. Another eight *K. pneumoniae* isolates were obtained from the *Salmonella* Genetic Stock Center (SGSC) in the University of Calgary, Canada (http://people.ucalgary.ca/~kesander/), including one *K. pneumoniae* type strain MGH78578. Fifty-seven isolates of other bacterial species collected from SGSC, including *K. oxytoca* (n=8), *E. cloacae* (n=27), *E. aerogenes* (n=15), *Serratia marcescens* (n=3), *Serratia odorifera* (n=2) and *Serratia liquefaciens* (n=2), and thirty clinical isolates each of *Salmonella* spp, *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Vibrio parahaemolyticus*, other *Vibrio* spp., *Staphylococcus aureus* and *Enterococcus* spp. were also included in this study. A *oqxAB*-positive *Salmonella* Typhimurium clinical isolate, ST07-37, isolated in 2007 at The Prince of Wales Hospital, Hong Kong, was used in gene expression analysis.

PCR and sequence analysis

Prevalence of *oqxAB* amongst different bacterial species was performed by utilizing multiple PCR primer sets (*oqxA*, *oqxB*, *hae*, *oqxAB2* and *oqxAB4*) targeting different yet overlapping regions of the *oqxAB* operon in pOLA52 (**Fig 4.1**). Presence of the IS*26* element upstream of *oqxA* was detected by the primer set IS*26*-*oqxA*. All PCR amplicons were subjected to nucleotide

sequencing for confirmation. Primers used in this study are listed in **Table 4.1**. Full length of the *oqxAB* gene was amplified from four oldest *K. pneumoniae* strains (QE137, QE319, QE321 and QE324) isolated in or before the year 1984, followed by nucleotide sequencing and comparison to known *oqxABR* homologues recovered from *K. pneumoniae*, *K. oxytoca*, *E. aerogenes* and *E. cloacae*, as well as plasmid-encoded elements recorded in the Genbank. Sequencing of PCR products was performed using Sanger Sequencing service provided by BGI Hong Kong. Nucleotide and protein BLAST were performed by utilizing the NCBI BLAST services. Sequence alignment and Maximum likelihood phylogenetic analysis were conducted by means of the MEGA6.06 software (Tamura et al., 2013).

Antimicrobial susceptibility testing

Minimal Inhibitory Concentration (MIC) of five antimicrobials (ciprofloxacin, norfloxacin, nalidixic acid, chloramphenicol and olaquindox) was determined for all test strains and interpreted according to CLSI guidelines (CLSI, 2013). *E. coli* ATCC25922 and ATCC35218 were used as quality control.

Southern Hybridization

S1-PFGE was performed to determine the location of *oqxAB* in selected strains. Briefly, agarose-embedded DNA was digested with S1 nuclease (New England Bio-Lab) at 37ºC for 1 hr. The restriction fragments were separated by electrophoresis in 0.5 Tris-borate-EDTA buffer at 14°C for 18 h using a Chef

Mapper electrophoresis system (Bio-Rad, Hercules, CA) with pulse times of 2.16 to 63.8 s. Phage Lambda PFGE ladder (New England Biolab) was used as DNA size marker. The gels were stained with GelRed, and DNA bands were visualized with UV transillumination (Bio-Rad). Chromosomal and plasmid DNA of *S.* Typhimurium strains were transferred and cross-linked onto nylon membrane and hybridized with DIG-labeled 16SrRNA and *oqxAB2* probes using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche), following the manufacturer's instructions.

RNA extraction and qRT-PCR

Total RNA was extracted by the Qiagen Protect Bacteria Minikit, followed by DNase treatment. The quality and quantity of RNA was determined by the Nanodrop spectrophotometer. One µg of RNA was subjected to reverse transcription using Life technologies Superscript III reverse-transcriptase; qRT-PCR was performed by using the Bio-rad iQ5 iCycler and the Life technologies SYBR Select Master mix. *K. pneumoniae* strain MGH78578 was used as control and expression levels of the test genes were normalized with that of 16SrRNA.

Nucleotide Sequence Accession

Full length *oqxAB* sequences from 4 *K. pneumoniae* isolates (QE137, QE319, QE321 and QE324) were deposited to GenBank (Accession numbers: KJ875814, KJ875815, KJ875816, and KJ875817 respectively). Various

nucleotide and genome sequences were retrieved from the Genbank and used in assessment of genetic relatedness of *oqxAB*-like elements. *Klebsiella pneumoniae* Genome Sequences: MGH78578, CP000647.1; XH209, CP009461.1; PMK1, CP008929.1; PittNAM01, CP006798.1; CG43, CP006648.1; KPNIH31, CP009876.1; blaNDM-1, CP009114.1; ATCC BAA-2146, CP006659.1; 342, CP000964.1; 1084, CP003785.1; JM45, CP006656.1; KCTC2242, CP002910.1. *Klebsiella variicola* strain AT22, NC_013850.1. *Klebsiella oxytoca* strain KCTC1686, NC_016612.1. *Serratia marcescens* Genome Sequences: WW4, CP003959.1; SM39, AP013063.1. *Enterobacter aerogenes* strain KCTC2190, CP002824.1. *Enterobacter cloacae* Genome Sequences: ATCC13047, CP001918.1; EcWSU1, CP002886.1; ENHKU01, CP003737.1. Plasmid sequences: pOLA52, NC_010378.1; pSDB58, KF840373.1; pHXY, NG_041556.1; E16, GQ497565.1.

Table 4.1. Primers used in this study.

Primer set	Forward 5' - 3'	Reverse 5' - 3'	Nucleotide position in *oqxAB* operon[a]	Reference
16SrRNA	CTCCTACGGGAGGCAGCAG	GWATTACCGCGGCKGCTG	-	(Turner et al., 1999)
oqxB-RT	TATCTCATTGGCGGCGTGAA	CGCGATTTTGGCGTTGATCT	-	This study
rarA-RT	GCAGGTGCCACTTCGAATA	GCGCCATCATTCAGGATCT	-	(Veleba et al., 2012)
oqxR-RT	TAACGAAGCCTGCTCTGCTT	AATGGTTCCGCTAACTCGTG	-	This study
IS*26*-oqxA	GCTGTTACGACGGGAGGAG	GGAGACGAGGTTGGTATGGA	-	(Zhao et al., 2010)
OqxA	CTCGGCGCGATGATGCT	CCACTCTTCACGGGAGACGA	43-435	(Kim et al., 2009b)
OqxB	TTCTCCCCCGGCGGGAAGTAC	CTCGGCCATTTTGGCGCGTA	1632 - 2144	(Kim et al., 2009b)
HAE	GCCTGGTAAGTCGAGATCGG	CTCGAACGGCTATCAGGGAC	2792 - 3357	This study
OqxAB2[b]	ACGGTGTACGTCTACTTTGA	GTCTCGGCAATCACTTTCG	640 -1384	(Sato et al., 2013)
OqxAB4	ATCGAGATGGGTTCCGGTAG	TAAACGGACGGAAAATCCAG	2010 - 2772	(Sato et al., 2013)

[a] Nucleotide position was based on those of the *oqxAB* operon in plasmid pOLA52. (Accession: NC_010378.1). [b] Also used as hybridization probe.

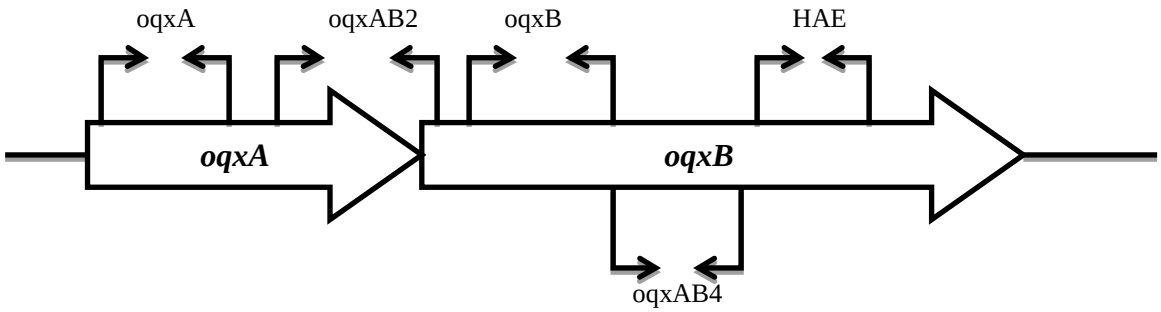

Figure 4.1. Target regions of the *oqxAB* operon in pOLA52 subjected to PCR genotyping with 5 primer sets.

RESULTS

K. pneumoniae chromosome as origin of o*qxAB*

To test the idea that *oqxAB* originated from *K. pneumoniae* where it exists as a chromosomally-encoded membrane transporter, five primer sets were used to determine the relative prevalence of *oqxAB* in different bacterial species. To obtain convincing evidence on the evolutionary origin of *oqxAB*, we included 15 *K. pneumoniae* clinical isolates collected in The Prince of Wales Hospital, Hong Kong in or before the year 1984, which was 10 years earlier than the earliest date when *oqxAB* was first detected in a plasmid in *E. coli* (Chen et al., 2012). This *oqxAB* PCR detection was regarded as positive if one or more of the five primer sets resulted in successful amplification of *oqxAB*–like fragments. Based on this criterion, all *K. pneumoniae*, *K. oxytoca* and *E. aerogenes* isolates, including the 30 years-old strains, were found to be positive, whereas twenty-six out of twenty-seven *E. cloacae* strains were also found to contain *oqxAB*-related genes. The *oqxAB* positive rate for *Salmonella* Typhimurium was 29%. However, no *oqxAB*-like elements were detectable in *Serratia* spp., *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Vibrio parahaemolyticus*, other *Vibrio* spp. and *Staphylococcus aureus*.

It should be noted that highly variable result patterns of the genotyping tests with five primer sets were observed amongst the test isolates; hence only fourteen *K. pneumoniae* strains were positive to all primer sets tested, including ten collected after 2008. Alignment of primer sequences in known *K.*

pneumoniae genomes confirmed that the negative genotyping test results were due to sequence variations rather than a lack of the priming regions in the respective genomes (results not shown). This idea is supported by our observation that, for each isolate which we define as *oqxAB*-positive by our *oqxAB*-genotyping approach, at least one primer set targeting the *oqxA* gene and one targeting the *oqxB* gene produced positive result. On the other hand, association between IS*26* and *oqxA* was not observable in all isolates tested, suggesting that this gene was not introduced into the chromosome of *K. pneumoniae* by transposition events. To further confirm the chromosomal location of *oqxAB,* S1-PFGE southern hybridization was performed on eight *K. pneumoniae* isolates for which the positive response rate to different primer sets varied, with results showing that the *oqxAB* genes were consistently detectable in the chromosomal DNA of these isolates (results not shown).

To assess the validity of the genotyping tests, the original *oqxAB* operon in pOLA52 (Accession: NC_010378.1) was subjected to BLASTN homology search in the NCBI database. All identical hits were plasmid-borne *oqxAB* operon in *E. coli* and *Salmonella*. Chromosomal high homology hits (≥97%) were also identified, but all such elements were membrane transporter in *K. pneumoniae* and *K. variicola*. The rest were intermediate homology hits (81~88%) involving *K. oxytoca*, *E. cloacae, E. aerogenes* and *Serratia* spp. Nucleotide alignment data showed that the *oqxAB* operon in pOLA52 was 99% and 97% identical to the *K. pneumoniae* MGH78578 strain and *K. variicola*

At-22 strain respectively, but exhibited only 88%, 87%, 87% and 81% identity to *K. oxytoca*, *E. cloacae*, *E. aerogenes* and *Serratia marcescens* respectively; *oqxAB*-like elements were not found in organisms which do not belong to the family of *Enterobacteriaceae*. On the other hand, amino acid sequences of the OqxA, OqxB and transcriptional regulator Orf68 proteins encoded by genes located in pOLA52, were 99~100% identical to those of *K. pneumoniae* and *K. variicola*, but only 91-97% to *K. oxytoca*, *E. cloacae*, *E. aerogenes* and *Serratia marcescens* (**Fig 2)**. Interestingly, the OqxB protein in different bacterial species shared 97-100% amino acid homology with pOLA52, indicating that it was more conserved than OqxA. Taken together, the genotyping and sequence alignment data suggest that *K. pneumoniae* is genetically most related to the plasmid-borne *oqxAB* genes detectable in *E. coli* and *Salmonella* clinical isolates in recent years.

To further investigate the genetic characteristics of the chromosomal *oqxAB*–like element in *K. pneumoniae*, the entire *oqxAB* operon in four of the oldest *K. pneumoniae* isolates, namely QE137, QE319, QE321 and QE324, which were isolated in the year 1984 or before, was sequenced and compared to various plasmid-borne and chromosomal *oqxAB* operons. Consistent with the sequence alignment data, the *oqxAB* operon in these 4 isolates were found to share 97-99% homology at the nucleotide level, and 99-100% homology at the amino acid level with pOLA52 (**Fig 4.2**). Importantly, pockets of identical sequence variations or nucleotide polymorphism were observable amongst the

chromosomal and plasmid-borne elements (**Fig 4.3**). Amongst specific regions of genetic polymorphism, the plasmid-borne element was found to share a higher level of sequence identity with the 30 years' old *K. pneumoniae* isolates than the more recent strains, suggesting that the plasmid-borne genes originated from the earlier *K. pneumoniae* strains. Results of phylogenetic analysis depicting the genetic relationship between various chromosomal and plasmid-borne *oqxAB* operons lend support to this idea (**Fig 4.4**).

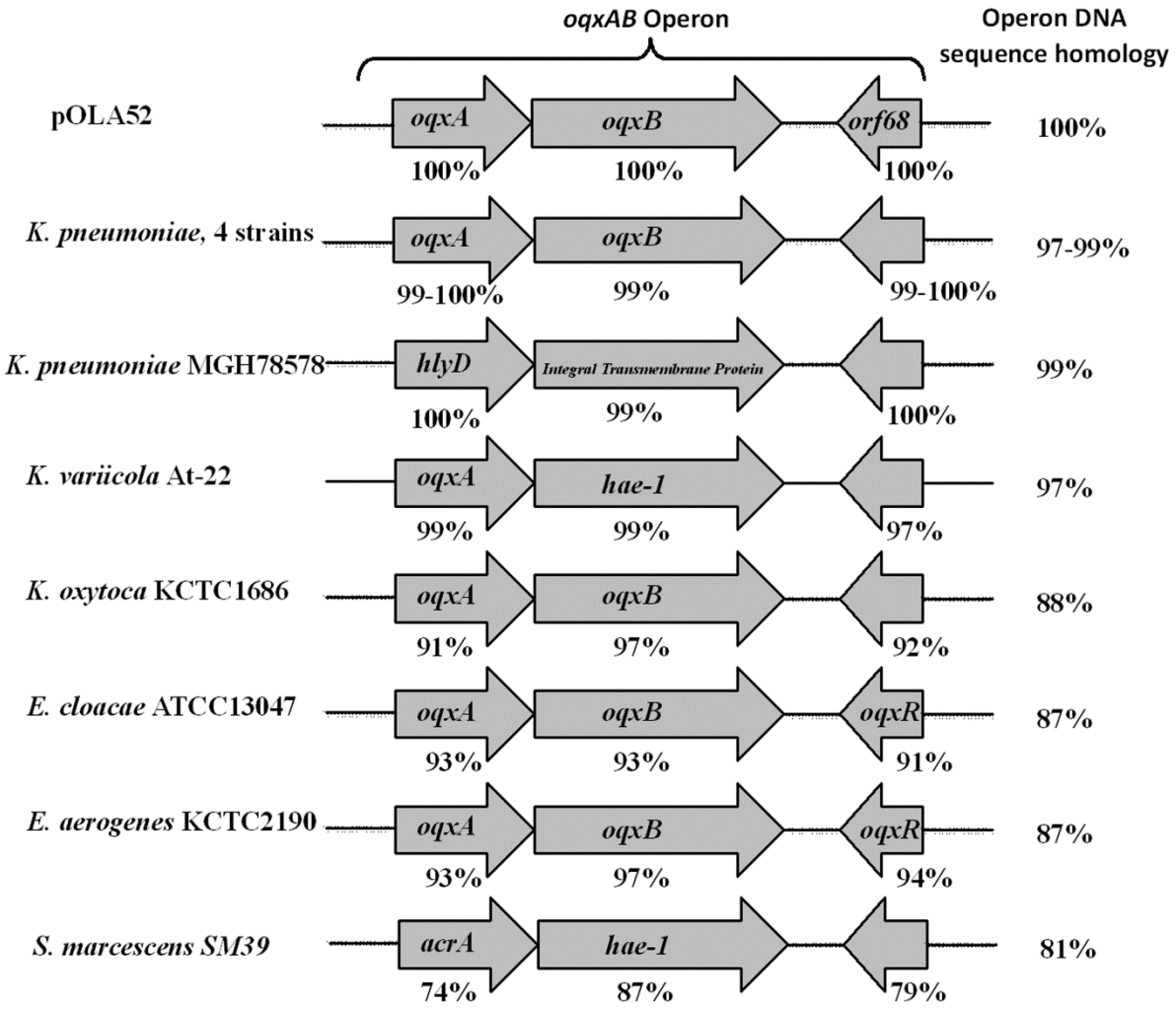

Figure 4.2. Amino acid sequence alignment of *oqxAB* operon (or its synonyms) from genomes of various control strains and four *K. penumoniae* strains recovered in Hong Kong in or before the year 1984, against *E. coli* plasmid pOLA52.

The arrows depict open reading frames and their respective orientation. Percentage below each ORF depicts amino acid sequence homology to specific gene in the *oqxAB* operon in pOLA52. Overall nucleotide homology with pOLA52 is shown on the right.

(a)

(b)

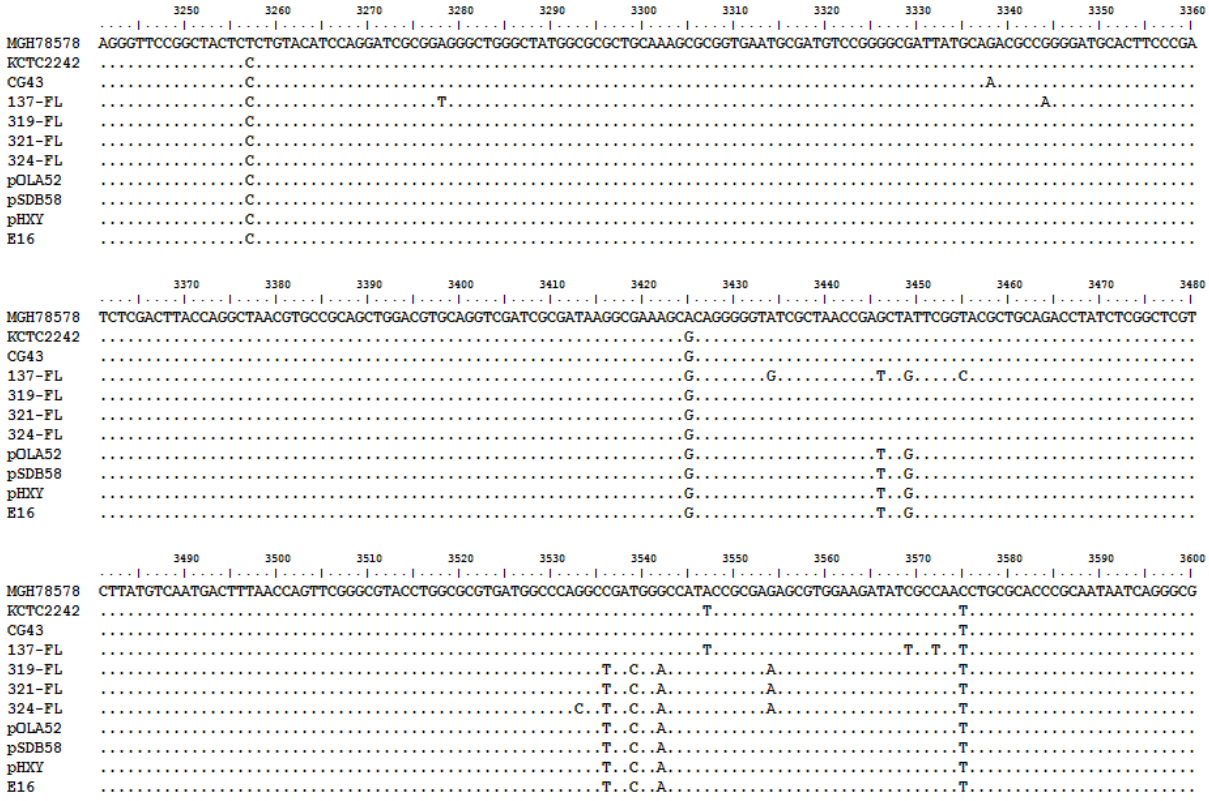

Figure 4.3. Nucleotide sequence alignment depicting identical sequence variations in two regions (a and b) of the chromosomal and plasmid borne *oqxAB* operon recoverable from *K. pneumoniae* and *Salmonella / E. coli* respectively.
MGH78578, KCTC2242, CG43: Control *K. pneumoniae* strains. 137-FL, 319-FL, 321-FL and 324-FL: Clinical *K. pneumoniae* strains isolated in or before the year 1984. pOLA52, pSDB58, pHXY and E16: plasmids harboring the *oqxAB* operon, recoverable from *Salmonella / E. coli*. Nucleotide sequence of strain MGH78578 was used as the reference sequence. Sequence data of the four clinical *K. pneumoniae* strains were generated in this study. All other data are retrievable from Genbank.

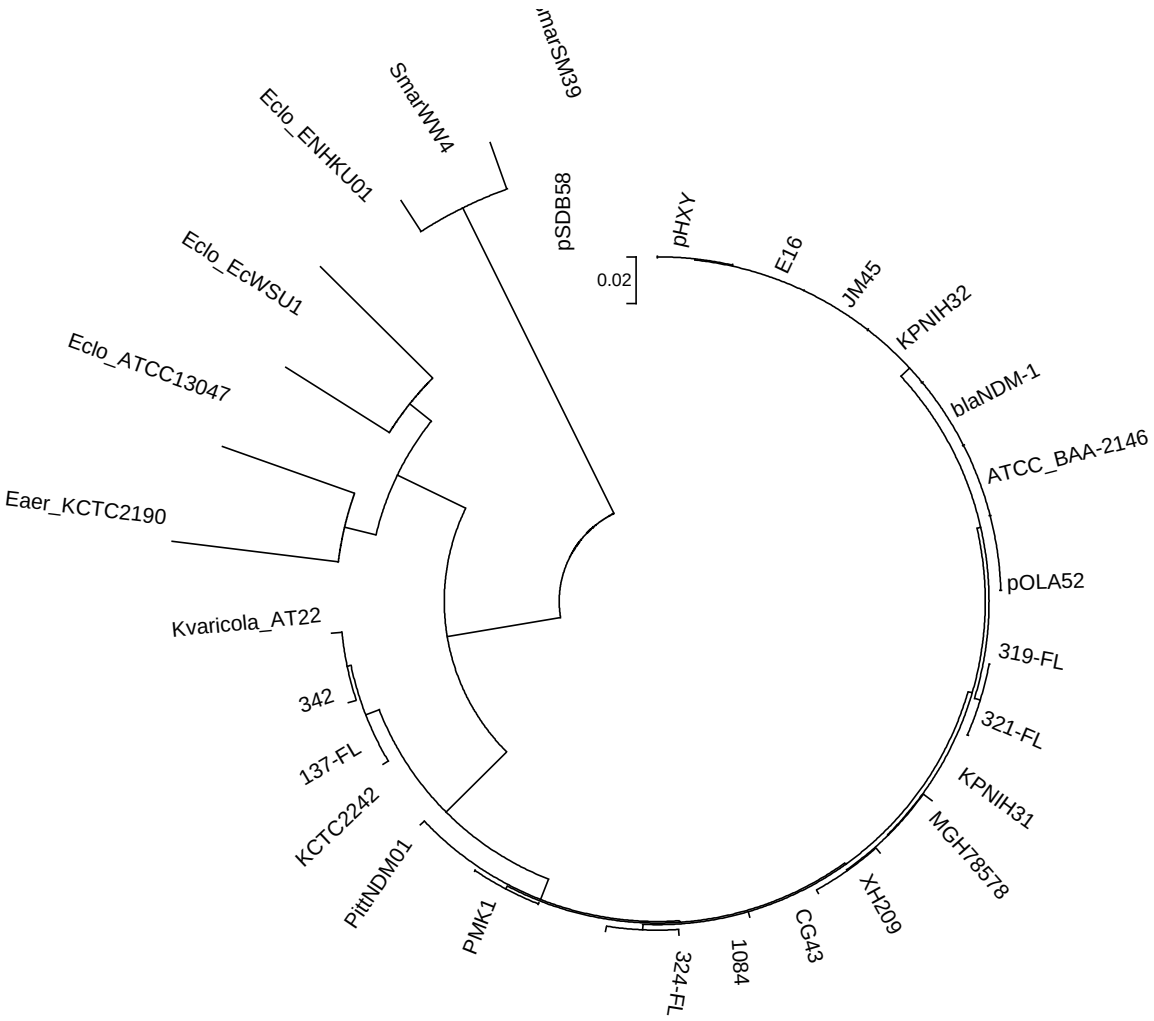

Figure 4.4. Phylogenetic tree depicting the genetic relatedness of *oqxAB* operons retrieved from various sources.

OqxAB operons were extracted from the chromosome of *K. pneumoniae* (JM45, CG43, MGH78578, 1084, KPNIH31, KPNIH32, blaNDM-1, ATCC BAA-2146, PMK1, PittNDM01, KCTC2242, 342, 137-FL, 319-FL, 321-FL and 324-FL), *K. variicola* (AT22), *Enterobacter cloacae* (EcWSU1 and ENHKU01), *Enterobacter aerogenes* (KCTC2190 and ATCC13047), and *Serratia marcescens* (WW4 and SM39), and plasmids recoverable from *E. coli* and *Salmonella* (pSDB58, pHXY, E16 and pOLA52).

Relative antimicrobial susceptibility and *oqxAB* expression profiles of *K. pneumoniae* and organisms harbouring the pOLA52-like plasmid

With *oqxAB* being consistently detectable in *K. pneumoniae*, drug susceptibility phenotypes were checked for the 85 clinical isolates tested in this study, with results being consistent with previous findings that *K. pneumoniae* clinical isolates were generally susceptible to quinolones (**Table 4.2**). Amongst these 85 *K. pneumoniae* clinical isolates, only 20 were resistant to chloramphenicol, and 28 were resistant to nalidixic acid (MIC≥32μg/ml), 14 of which were also resistant to ciprofloxacin (MIC≥4μg/ml). The majority of the isolates (51 out of 85) had a nalidixic acid MIC of <4μg/ml, whereas 49 strains strains had a ciprofloxacin MIC of <0.006μg/ml. However, high level resistance to olaquindox, chloramphenicol and nalidixic acid, and reduced susceptibility to ciprofloxacin, a typical resistance phenotype conferred by *oqxAB*-borne plasmid in *Salmonella*, was observable in only two *K. pneumoniae* strains isolated during or after the 1990s (strain 94-3 and GN53, **Table 4.2**). We confirmed, by S1-PFGE, that such phenotype was not caused by extra-chromosomal *oqxAB* elements. On the other hand, bacterial species harbouring chromosomal *oqxAB* homologues such as *E. cloacae* and *E. aerogenes* were mostly susceptible to the test agents, suggesting that these homologues did not contribute to drug resistance under the test conditions.

Table 4.2. Summary of genotypic and phenotypic characteristics of organisms harboring *oqxAB*-like elements.

Bacterial Species: test population / specific strain	**Place / year of isolation**	**Location of *oqxAB****	**Nucleotide sequence homology with pOLA52**	***oqxAB* genotyping with 5 primer sets**						**MIC (mg/L)**				
				oqxA	**oqxB**	**HAE**	**oqxAB2**	**oqxAB4**	**Overall**	**CIP**	**NAL**	**NOR**	**CHL**	**OLA**
K. pneumoniae (HK, *n*=85)	HK /1984-2011	-	-	67	35	32	83	73	85/85 (100%)	-	-	-	-	-
(SGSC, n=8)	SGSC /1996	-	-	8	4	8	8	8	8/8 (100%)	-	-	-	-	-
MGH78578	-	chromosome	99%	+	-	+	+	+	+	1	≥128	4	≥128	16
KPNE QE319	HK /1984	chromosome	99%	-	-	+	+	-	+	<0.006	<4	<2	<4	<16
KPNE 94-3$^{\Delta}$	HK /1994	chromosome	ND	+	-	-	+	-	+	0.25	≥128	≤2	64	512
KPNE GN53$^{\Delta}$	HK /2006	chromosome	ND	+	-	+	+	+	+	4	≥128	≥64	≥128	512
KPNE 06-2	HK /2006	chromosome	ND	+	-	-	+	+	+	≤0.006	≤4	≤2	≤4	≤16
K. oxytoca (SGSC, n=8)	SGSC / 1996	-	88%$^{\#}$ -	0	0	4	8	8	8/8 (100%)	<0.012 -0.05	<4-> 128	<4	<4	8-32
E. aerogenes (SGSC, n=15)	SGSC/1996-1999	-	87%$^{\#}$ -	0	10	15	15	4	15/15 (100%)	<0.012	<4 - 8	<4	<4	8-64
E. cloacae (SGSC, n=27)	SGSC/1996-2000	-	87%$^{\#}$ -	3	15	9	14	15	26/27 (96%)	<0.012 -0.05	<4-16	<4	<4-16	16-128
*S.*Typhimurium (n=17)	HK/2007	-	-	5	5	3	4	4	5/17 (29%)	-	-	-	-	-
STYP 1792	HK/2007	Plasmid	100%	+	+	-	-	+	+	2	≥128	ND	≥128	≥512
STYP 2005	HK/2007	Plasmid	100%	+	+	-	-	+	+	2	≥128	ND	≥128	≥512
Other species§	HK/2006-2010	-	-	-	-	-	-	-	-	-	-	-	-	-

#: Based on nucleotide sequences of the standard strains KCTC1686, KCTC2190 and ATCC13047 for *K. oxytoca*, *E. aerogenes* and *E. cloacae* respectively. Δ: Strains KPNE94-3 and KPNEGN53 exhibited typical *oqxAB*-mediated antibiotic resistance profile (resistance to olaquindox, chloramphenicol and the quinolones) without harboring *oqxAB*-borne plasmid.*: Based on results of S1-PFGE and Southern hybridization studies. §: All other bacterial species tested including *Serratia spp* (n=7) *Pseudomonas aeruginosa* (n=30), *Acinetobacter* spp.(n=30), *Vibrio parahaemolyticus* (n=30), other *Vibrio* spp. (n=30), *Staphylococcus aureus* (n=30) and *Enterococcus* spp (n=30) were *oqxAB* negative. CIP, ciprofloxacin; NAL, nalidixic acid; NOR, norfloxacin; CHL, chloramphenicol; OLA, olaquindox. HK, Hong Kong; SGSC, *Salmonella* Genetic Stock Center, Calgary, Canada. KPNE, *K. pneumoniae* ; EAER, *E. aerogenes* ; ECLO, *E. cloacae* ; STYP, *Salmonella* Typhimurium.

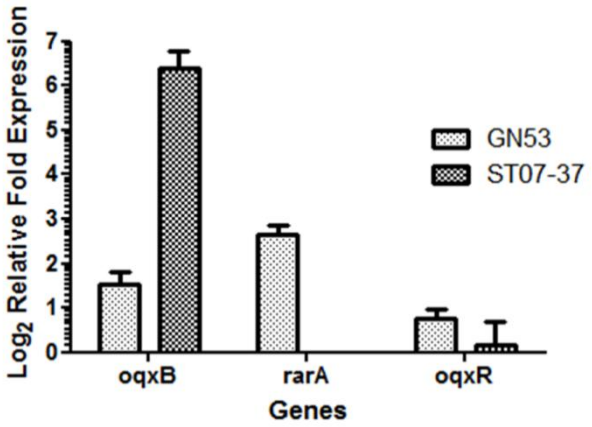

Figure 4.5. Relative expression levels of the *oqxB*, *rarA* and *oqxR* genes.
The relative expression levels of oqxB, rarA and oqxR genes in the olaquindox-resistant *K. pneumoniae* strain GN53, and a *Salmonella* Typhimurium strain ST07-37 harboring a pOLA52-like plasmid. The *K. pneumoniae* strain MGH78578 was used as control.

To probe the molecular basis of the discrepancy in susceptibility phenotypes observable amongst various *oqxAB*-borne organisms, *K. pneumoniae* strain GN53, and a *Salmonella* strain harbouring the pOLA52 plasmid (ST07-37), which were both olaquindox resistant, were subjected to quantitative RT-PCR analysis, with results showing that the expression level of both *rarA* and *oqxB* in strain GN53 was significantly higher than that of the wild type strain MGH78578 (**Fig 4.5**). This finding suggests that the drug resistance phenotypes of this strain were at least partially due to up-regulated expression of *oqxAB*. Interestingly, expression of *oqxR* was also moderately elevated, inferring that the effect of its gene product as *oqxAB* repressor might have been counteracted by the extraordinary large amount of the RarA protein produced in this strain. On the other hand, the expression level of *oqxB* in the *Salmonella* strain

ST0737 was elevated as much as 85 fold increase when compared to the wild type, drug sensitive *K. pneumoniae* strain. Whether such high level expression of the plasmid-borne *oqxAB* genes was due to de-repression of the *oqxAB* operon as a result of lack of OqxR binding site remains to be elucidated.

DISCUSSION

This study highlighted several important issues regarding the evolutionary origin and dissemination features of the PMQR determinant *oqxAB.* First, although this resistance determinant has become increasingly prevalent amongst Gram negative pathogens, our study showed that they are mainly confined to members of *Enterobacteriaceae*. Second, *oqxAB* was most prevalent amongst the *Enterobacter* spp. and *Klebsiella* spp.. In particular, both the detection rate and level of sequence homology with the *oqxAB* operon in pOLA52, the original plasmid in which *oqxAB* was first recovered, approached 100% even in *K. pneumoniae* strains recovered ten years earlier than the time of discovery of pOLA52. Such finding has important implication on the origin of mobile *oqxAB* elements. Third, organisms containing chromosome-based and plasmid-borne elements exhibited drastically different levels of gene expression and susceptibility to the quinolones; in particular, the expression level of the plasmid-borne *oqxAB* operon was more than 80 fold higher than the chromosomal genes.

We postulate that the *oqxAB* operon, together with a transcriptional regulator *orf68* in the chromosome of *K. pneumoniae*, were captured by IS*26* transposase and transferred to foreign plasmids, which were subsequently disseminated to other bacterial species that do not harbour *oqxAB*–like elements in the chromosome. It should be noted that, although the *oqxAB* operon is also prevalent amongst other species such as *Enterobacter* spp., a significantly lower

degree of sequence homology with the plasmid-borne element was observed, suggesting that these *oqxAB* homologues were not as readily captured by transposition activities as the *K. pneumoniae* genes. The underlying principle by which structural differences between various *oqxAB* homologues affect transposition efficiency remains to be elucidated. In addition, although factors limiting horizontal transfer of existing *oqxAB*-borne plasmids to non-*Enterobacteriaceae* species are not understood, the possibility that the *oqxAB* operon may be captured and transferred to other types of plasmids that can be taken up by other Gram negative pathogens should be investigated.

E. coli and *Salmonella* isolates carrying *oqxAB*-borne plasmids were found to consistently exhibit reduced susceptibility to ciprofloxacin and olaquindox, with MIC of these two agents reaching as high as 2-8μg/ml and 512μg/ml respectively (Yuan et al., 2012;Wong et al., 2013;Wong et al., 2014b). Conversely, clinical *K. pneumoniae* isolates in this and a previous study were mostly susceptible to ciprofloxacin and olaquindox (Rodriguez-Martinez et al., 2013). We confirmed that phenotypic resistance to these agents in two *K. pneumoniae* clinical isolates observable in this work was due to over-expression of the chromosomal *oqxAB* genes. It has previously been demonstrated that *oqxAB* over-expression in *K. pneumoniae* is triggered by elevated expression of *rarA,* which encodes an activator of the *oqxAB* operon, or point mutations of the gene encoding its repressor, *oqxR* (synonym of *orf68* in pOLA52) (Veleba et al., 2012;Bialek-Davenet et al., 2015). This concept is generally consistent with

our findings, in which a highly up-regulated *rarA* gene product was found to offset the effect of the OqxR suppressor in strain GN53, leading to over-expression of the *oqxAB* genes. On the other hand, the extremely high level expression of the plasmid-borne *oqxAB* genes allows us to conclude that the OqxR protein indeed plays a role in suppressing the expression of the original *oqxAB* operon that resides in the chromosome of *K. pneumoniae*.

Taken together, *oqxAB* or its homologues represent one of the many endogenous efflux systems in *K. pneumoniae* and *Enterobacter* spp., which is functionally similar to *acrAB* in other members of *Enterobacteriaceae*. Yet a major difference between *oqxAB* and other chromosomal efflux genes, as illustrated by findings in this work, is that the *oqxAB* operon can become plasmid-borne via transposition events, during which the *oqxAB* genes become over-expressed, presumably as a result of loss of the OqxR repressor function. The dissemination patterns of mobile elements harbouring over-expressed efflux pumps should be closely monitored

CHAPTER V - Regulation of plasmid-borne *oqxAB* in *Salmonella* Typhimurium

ABSTRACT

The Plasmid-mediated Quinolone Resistance (PMQR) determinant *oqxAB* is becoming more prevalent in members of *Enterobacteriaceae*, particularly in *Salmonella* spp.. This mobile Resistance-Nodulation-Division (RND) type efflux pump confers reduced susceptibility towards fluoroquinolones, quinolones and chloramphenicol antimicrobials. Like any other bacterial efflux systems, *oqxAB* is tightly regulated in *Klebsiella pneumoniae*, where the pump was originated. The regulatory components involve its adjacent repressor OqxR and the activator RarA. Nonetheless, the *rarA* gene and part of *oqxR* upstream region, were not captured by IS*26* in the Tn*6010*-mediated *oqxAB*, which is the form being carried by plasmids and circulated amongst bacterial pathogens. In addition, when *oqxAB* is carried by a host, the role of its global regulators on the expression of this foreign gene is not understood. In this study, constructs targeting different portions of the *oqxABR* locus were generated and transformed into *E. coli* DH5α as well as *S.* Typhimurium wild type strain 14028s and the Δ*ramA*, Δ*ramR* and Δ*soxS* mutants. Western blotting revealed production of OqxAB from all strains transformed with *oqxAB* construct lacking *oqxR*. Loss of OqxAB production was seen in strains carrying *oqxABR* with complete *oqxR* upstream sequence. Remarkably, high level *oqxAB* expression was observable in strains transformed with *oqxAB* whose sequence was identical to the Tn*6010* cassette. Quantitative RT-PCR showed that the

expression level of *oqxR* in the Tn*6010* cassette was higher than its chromosomal counterpart, despite the fact that repression of *oqxAB* was not observed. In addition, production of OqxAB was obstructed in *S.* Typhimurium strains lacking *ramA*, whereas high level *ramA* expression was found to suppress expression of *oqxR*. Findings in this work therefore revealed the role of *ramA* in regulation of *oqxAB*, and demonstrated that foreign genes could be controlled by host global regulation mechanisms in a fashion similar to the endogenous efflux systems.

INTRODUCTION

Transmission of the mobile RND-type efflux pump *oqxAB* has been observed amongst various members of *Enterobacteriaceae* family, including *E. coli* and *Salmonella* (Chen et al., 2012). Recently, *oqxAB* has emerged as a key Plasmid-mediated Quinolone Resistance (PMQR) determinant in these pathogens. The pump was found to be one of the endogenous efflux systems in *Klebsiella pneumoniae* (Wong et al., 2015). Dissemination of the whole operon is possibly due to transposition to conjugative plasmids by IS*26* transposase. Resistance to olaquindox (substrate of *oqxAB*) and reduced susceptibility towards nalidixic acid and ciprofloxacin has been consistently observed in organisms harbouring plasmid-borne Tn*6010*-mediated *oqxAB* (Liu et al., 2013). However, this correlation can only be barely established in *K. pneumoniae*, which may be due to differential expression of *oqxAB* in various hosts (Kim et al., 2009b). In *K. pneumoniae*, expression of *oqxAB* is regulated at both local and global level, which is similar to *acrAB* regulatory mechanisms in other Gram negative bacteria. Genetically, *oqxAB* in *K. pneumoniae* is flanked by two local transcriptional regulators, namely *rarA* and *oqxR*, the former is an activator for *oqxAB*, whereas the latter is a repressor (Veleba et al., 2012). Expression of *oqxAB* is also subjected to regulation by the global regulator RamA (De Majumdar et al., 2015). Contrary to its chromosomal counterpart, *rarA* is missing from the plasmid-borne *oqxAB*, probably due to the excision process of IS*26* (Norman et al., 2008). Nevertheless, it has been previously shown that plasmid-borne *oqxAB* is constitutively expressed in *Salmonella*

Typhimurium (Wong et al., 2015). Global regulators in *S.* Typhimurium, such as *ramA*, *marA* and *soxS*, are playing integral role in regulating the host's endogenous efflux *acrAB*. However, whether *oqxAB* is under regulation when it is located on a plasmid, and the putative regulatory mechanism unde the control of the cellular signals of the host, are largely unknown. We hypothesised that *oqxR* in plasmid-borne *oqxABR* locus may not be fully functional and subsequently leads to overexpression of *oqxAB*. As a RND efflux, it is hypothesised that *oqxAB* may also be subjected to global regulatory signals of the hosts. The aim of this study is to evaluate the effect of *oqxR* in its plasmid-borne state, and elucidate the role of various global regulators in *Salmonella* Typhimurium in *oqxAB* regulation.

MATERIALS AND METHODS

Bacteria strains and vectors

Bacterial strains used in this study are listed in Table 5.1. *Salmonella* Typhimurium 14028s (PY1) and *Escherichia coli* DH5α were obtained from laboratory collection. Propagation of bacteria was conducted in LB with/without suitable antibiotics at 37 degree Celsius.

Generation of *ramA*, *ramR* and *soxS* knockout mutants

S. Typhimurium PY1 knockout mutants were generated by pKD46 homologous recombination system. Helper plasmid pKD46 was electroporated into PY1 competent cells. Expression of λ recombinase was induced by addition of L-arabinose. PCR products with 50bp homologous sequence were generated by primers listed in Table 5.2 and were subsequently electroporated into recombinase-induced cells. Mutants were selected on LB agar plate supplemented with 50mg/L kanamycin. Verification of the identity of knockout mutants was performed by PCR using primers listed in Table 5.2.

Generation of *oqxABR* operon constructs

Three constructs, namely ABR, ABR0 and AB, targeting different regions of the *oqxABR* operon were generated by using pET15B as cloning vector. ABR was constructed by cloning the *oqxABR* operon together with ~400bp upstream region of *oqxR* present in *Klebsiella pneumoniae* MGH78578. ABR0 was constructed by cloning the *oqxABR* locus together with ~100bp upstream region

of *oqxR*, resembling the configuration encoded in Tn*6010*. AB was constructed by cloning the *oqxAB* region with ~200bp sequence upstream of *oqxA* present in *Klebsiella pneumoniae* MGH78578 (Figure 5.1). Primers used are listed in Table 5.2..

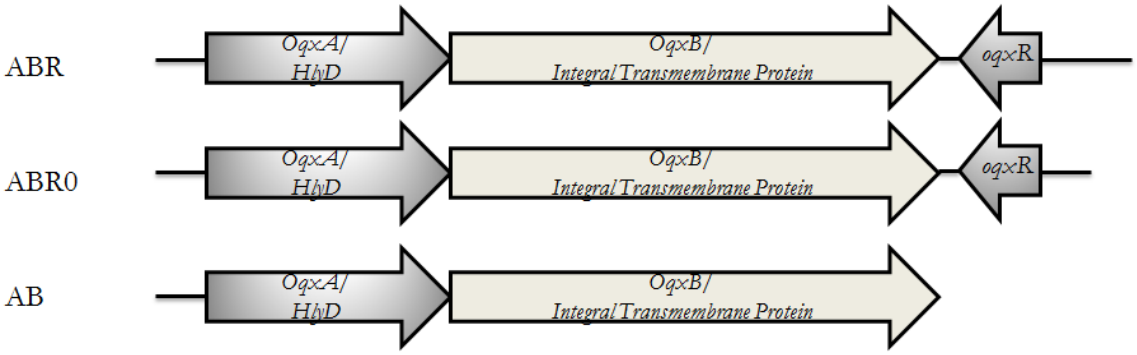

Figure 5.1.Diagramatic illustration of three constructs generated in this study.

Antimicrobial susceptibility testing

Minimal Inhibitory Concentration (MIC) of four antimicrobials (ciprofloxacin, nalidixic acid, chloramphenicol and olaquindox) was determined for all test strains and interpreted according to CLSI guidelines (CLSI, 2013). *E. coli* ATCC25922 and ATCC35218 were used as quality control.

Outer membrane preparation and Western blotting

0.5ml of culture was spin down and resuspended in SDS loading buffer, followed by boiling for 10 minutes. Solubilised proteins were separated by SDS-PAGE and were subsequently transferred to PVDF membrane through semi-dry transfer apparatus. Western blotting was carried out by probing the

membrane with *oqxA*-specific antibody and the signal was visualised by addition of HRP-substrate. *Salmonella* ompC-specific antibody was used as internal loading control.

RNA extraction and qRT-PCR

Total RNA was extracted by the Qiagen Protect Bacteria Minikit, followed by DNase treatment. The quality and quantity of RNA was determined by the Nanodrop spectrophotometer. One μg of RNA was subjected to reverse transcription using Life technologies Superscript III reverse-transcriptase; qRT-PCR was performed by using the Bio-rad iQ5 iCycler and the Life technologies SYBR Select Master mix. Expression levels of the test genes were normalized with that of 16SrRNA.

Table 5.1. Strains and plasmids used in this study.

Strain	Description
K. pneumoniae MGH78578	Wt strain as template
S. Typhimurium 0653	Hong Kong clinical isolate as template, *oqxAB*+
E. coli BW25113/pKD4	*E. coli* K-12 + pKD4 template vector, Kan^R
E. coli BW25113/pKD46	*E. coli* K-12 + pKD46 helper vector, Amp^R
E. coli DH5α	Laboratory strain
DH5α/pET15B	Vector control, Amp^R
DH5α/pAB	*oqxAB* cloned into pET15B [NdeI,SacI], Amp^R
DH5α/pABR	*oqxABR* from MGH78578 cloned into pET15B[NdeI,SacI], Amp^R
DH5α/pABR0	*oqxABR* from 0653 cloned into pET15B[NdeI,SacI], Amp^R
S. Typhimurium PY1	*S.* Typhimurium 14028s Type strain
PY1/pET15B	Vector control, Amp^R
PY1/pAB	*oqxAB* cloned into pET15B [NdeI,SacI], Amp^R
PY1/pABR	*oqxABR* from MGH78578 cloned into pET15B[NdeI,SacI], Amp^R
PY1/pABR0	*oqxABR* from 0653 cloned into pET15B[NdeI,SacI], Amp^R
PY1Δ*ramA*	*ramA*-deleted strain, Kan^R
PY1Δ*ramA*/pET15B	Vector control, Kan^R, Amp^R
PY1Δ*ramA*/pAB	*oqxAB* cloned into pET15B [NdeI,SacI] , Kan^R, Amp^R
PY1Δ*ramA*/pABR	*oqxABR* from MGH78578 cloned into pET15B[NdeI,SacI] , Kan^R, Amp^R
PY1Δ*ramA*/pABR0	*oqxABR* from 0653 cloned into pET15B[NdeI,SacI], Kan^R, Amp^R
PY1Δ*ramR*	*ramR*-deleted strain, Kan^R
PY1Δ*ramR*/pET15B	Vector control, Kan^R, Amp^R
PY1Δ*ramR*/pAB	*oqxAB* cloned into pET15B [NdeI,SacI], Kan^R, Amp^R
PY1Δ*ramR*/pABR	*oqxABR* from MGH78578 cloned into pET15B[NdeI,SacI], Kan^R, Amp^R
PY1Δ*ramR*/pABR0	*oqxABR* from 0653 cloned into pET15B[NdeI,SacI], Kan^R, Amp^R
PY1Δ*soxS*	*soxS*-deleted strain, Kan^R
PY1Δ*soxS*/pET15B	Vector control, Kan^R, Amp^R
PY1Δ*soxS*/pAB	*oqxAB* cloned into pET15B [NdeI,SacI], Kan^R, Amp^R
PY1Δ*soxS*/pABR	*oqxABR* from MGH78578 cloned into pET15B[NdeI,SacI], Kan^R, Amp^R
PY1Δ*soxS*/pABR0	*oqxABR* from 0653 cloned into pET15B[NdeI,SacI], Kan^R, Amp^R

Table 5.2. Primers used in this study.

Primer	Sequence (5'-3')
Knockout	
soxRS-KP1	CGCGGCGTTCAGTATTGTCAGGGATGGCACTTTGCGAAG **GTGTGTAGGCTGGAGCTGCTTCG**
soxRS-KP2	ATACAACCGTCCAGCTCATCGCGCAACGCCACCAGC **TCCATATGAATATCCTCCTTAG**
ramA-KP1	GAGCCGCTGACGAGTTTGATAGAGGGGAGAGCACGATGACT **GTGTAGGCTGGAGCTGCTTCG**
ramA-KP2	GTTGTTTTGTTTATGGTTTCTGTTGCTCGGCGCGCTGGAA **TCCATATGAATATCCTCCTTAG**
ramR-KP1	TCGAATCCCAGCGCAATATATTCGCCAGCGCGAGCGGGATCGCGC **GTGTAGGCTGGAGCTGCTTC**
ramR-KP2	AAGCATTACTGGAAGCGGCAACCCAGGCGATAGCGCAATCCGGTA **TCCATATGAATATCCTCCTTAG**
Knockout Verification	
V-soxRS-F	GCGGCTAAAAATCATTGC
V-soxRS-R	CAAACCGGAACCTCCACCAC
V-ramA-F	GCGATAAGCTGTCTCACAAT
V-ramA-R	TGCTGATGGCGTTGCTCTCC
V-ramR-F	CCGTCCATTATTGCTCCTCG
V-ramR-R	GGGTAACGTGTAGTGGCTCG
pKD4-k1	CAGTCATAGCCGAATAGCCT
pKD4-k2	CGGTGCCCTGAATGAACTGC
Cloning	
oqxAB-ABR0-F	GGATACATATGAGTGCAACAATTATTCTTG
oqxAB-ABR0-R	CGTCAGAGCTCGGCCTTTGAATAAGACAAA
oqxAB-ABR-F	GGATACATATGGCGGCCGGTTAAAAGCATC
oqxAB-ABR-R	CGTCAGAGCTCGGGGGTATGTCCCCTGTTC
oqxAB-AB-F	GGATACATATGGCATCTTTGTCAGTTGCAT
oqxAB-AB-R	CGTCAGAGCTCTCATTTTCTGGTGACGAAA
qRT-PCR	
rrsG-F	GTTACCCGCAGAAGAAGCAC
rrsG-R	CACATCCGACTTGACAGACC
oqxB-RT-F	TATCTCATTGGCGGCGTGAA
oqxB-RT-R	CGCGATTTTGGCGTTGATCT
oqxR-RT-F	CGAACCCGAGCTTTATCCGT
oqxR-RT-R	CGGACGGCCAAGATGAATTG

RESULTS

Elevated expression of *oqxAB* and *oqxR* in plasmid portion

Sequences upstream of *oqxR* was found to be truncated by IS*26* elements in the *oqxABR* locus carried by plasmids compared with its chromosomal counterparts. Compared to *K. pneumoniae* chromosomal sequence which has ~400bp upstream of *oqxR* ahead of the next ORF, in the plasmid-borne *oqxABR* locus there were about 100bp nucleotides intergenic region between *oqxR* and IS*26*, within which only 50bp in the neighbourhood of *oqxR* were identical to the chromosomal sequence. This discrepancy may lead to differential expression level of *oqxR* between chromosomal/plasmid due to altered promoter activity. To evaluate the expression level and effect of *oqxR* in the *oqxABR* locus encoded on chromosome and plasmids, three constructs, namely pAB, pABR and pABR0, targeting to different regions of *oqxABR* locus, were cloned into pET15B vector. These constructs were transformed into *E. coli* DH5α and *S.* Typhimurium PY1 respectively. Expression of *oqxAB* was determined by western blotting using anti-OqxA antibody, whereas expression of *oqxR* was determined by qRT-PCR. To ensure that *oqxAB* in synthetic constructs was expressed and located in cell membrane portion, the sub-cellular location where it functions, western blotting on outer membrane preparations from *S.* Typhimurium PY1 carring different constructs was performed. Antimicrobial susceptibility assay was performed on these strains with host strains carrying empty vector as control, and the results are shown in Table 5.3.

Result of western blotting revealed that *oqxAB* was expressed in both *E. coli* and *S.* Typhimurium PY1 carrying constructs pAB and pABR0, but not pABR. Noteworty, the level of *oqxAB* expression was much higher in pAB than pABR0 in both host strains, and that production of OqxAB was more abundant in *E. coli* **(Figure 5.3)**. Consistently, when compared to the vector control, *E. coli* pAB exhibited a reduced susceptibility to substrates of OqxAB, including nalidixic acid (>2 fold), ciprofloxacin (16 fold), olaquindox (16 fold) and chloramphenicol (16 fold) **(Table 5.3)**. In contrast, although expression was observed in western blotting, almost no changes in MIC were observed in *S.* Typhimurium PY1 regardless of which constructs they carried, suggesting that the observed phenotypes are not necessarily mediated by basal expression of *oqxAB*.

Table 5.3. Minimal Inhibitory Concentrations (MIC) of bacterial strains towards different antimicrobials.

Strain	**Constructs**	**MIC (μg/ml)**			
		NA	**CIP**	**OLA**	**CHL**
DH5α	VC	64	0.0078	1	2
DH5α	AB	**≥128**	**0.1248**	**16**	**32**
DH5α	ABR	32	0.0078	1	2
DH5α	ABR0	64	0.0156	1	2
PY1	VC	2	0.0156	4	4
PY1	AB	2	0.0078	4	4
PY1	ABR	2	0.0156	4	2
PY1	ABR0	4	0.0078	8	4
PY1Δ*ramA*	VC	2	0.0078	8	4
PY1Δ*ramA*	AB	2	0.0039	2	1
PY1Δ*ramA*	ABR	2	0.0039	4	1
PY1Δ*ramA*	ABR0	4	0.0039	4	1
PY1Δ*ramR*	VC	4	0.0156	8	8
PY1Δ*ramR*	AB	8	0.03125	8	4
PY1Δ*ramR*	ABR	8	0.0156	8	8
PY1Δ*ramR*	ABR0	4	0.0156	16	8
PY1Δ*soxS*	VC	4	0.0078	16	4
PY1Δ*soxS*	AB	1	0.0039	8	2
PY1Δ*soxS*	ABR	2	0.0039	8	4
PY1Δ*soxS*	ABR0	4	0.0078	32	4

VC, Vector control; NA, nalidixic acid; CIP, ciprofloxacin; OLA, olaquindox; CHL, chloramphenicol.

It has been suggested that OqxR is a repressor of the *oqxAB* operon. Western blotting results revealed that diminished *oqxAB* expression was observed in PY1 strain carrying pABR, confirming the repressor function of OqxR. Interestingly, for pABR0, where upstream sequence of *oqxR* was truncated, expression of *oqxAB* resembled that of pAB. Results of qRT-PCR showed that expression of *oqxR* was higher in ABR0 than in ABR by ~0.5 fold, suggesting a stronger promoter sequence may be created in plasmid-borne *oqxR*, or the loss of self-regulation mechanism due to truncation of upstream region resulted from IS*26* transposition. Remarkably, consistently elevated expression of *oqxAB* and *oqxR* were observed in both ABR and ABR0 in qRT-PCR experiments, suggesting that the repressive effect of the *oqxR* gene product is independent of its expression level **(Figure 5.2)**.

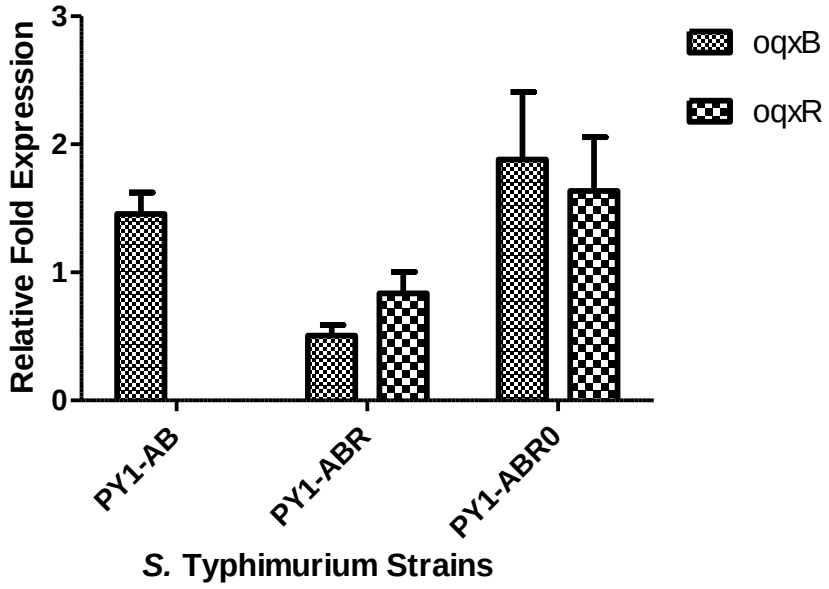

Figure 5.2. Relative expression level of *oqxB* and *oqxR* from *S.* Typhimurium PY1 carrying different constructs.

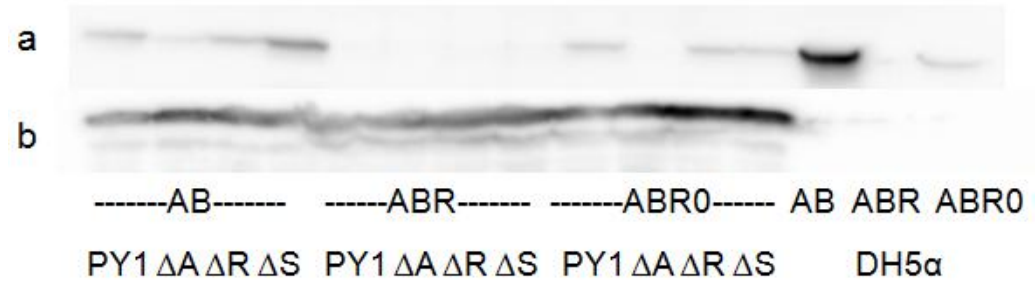

Figure 5.3. Western blotting result using oqxA antibody.
(a) OqxA blotting; (b) *Salmonella* specific OmpC blotting. AB, strains carrying pAB; ABR, strains carrying ABR; ABR0, strains carrying ABR0; PY1, *S.* Typhimurium 14028s wild type strain; ΔA, PY1Δ*ramA*; ΔR, PY1Δ*ramR*; ΔS, PY1Δ*soxS*; DH5α, *E. coli* lab strain.

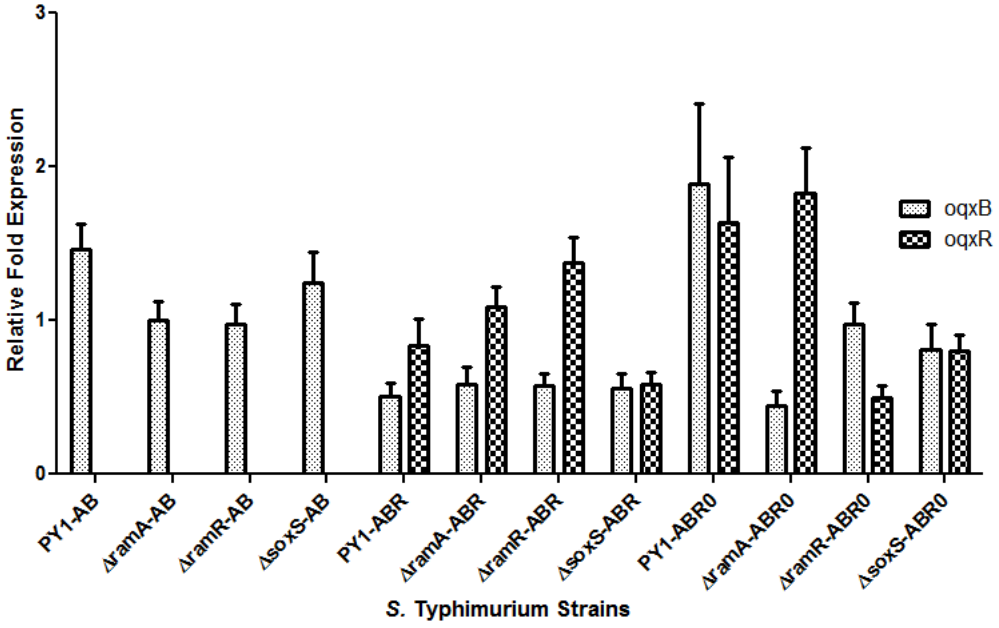

Figure 5.4. Relative expression level of *oqxB* and *oqxR* in *S.* Typhimurium PY1 and its corresponding mutants carrying different constructs.

Effect of global regulators in *oqxAB* regulation in *S.* Typhimurium

To evaluate the potential role played by global regulators in *oqxAB* expression, *S.* Typhimurium PY1 and its Δ*ramA*, Δ*ramR* and Δ*soxS* mutants were created followed by transformation of pET-*oqxAB* constructs. No significant changes were noted in antimicrobial susceptibility amongst all *S.* Typhimurium strains. Western blotting result showed that production of OqxAB was detected in all host strains transformed with pAB, and qRT-PCR data displayed a comparable *oqxAB* expression level from these strains **(Figure 5.3)**. The data are consistent with that obtained from the wild-type strain and suggestive of the validity of the theory that *oqxAB* is constitutively expressed when *oqxR* is lost.

For *S.* Typhimurium carrying *oqxABR* with *oqxR* intergenic region in *K. pneumoniae*, production of OqxAB was not observed in western blotting irrespective of whether any regulators had been disrupted. Expression of *oqxAB* in qRT-PCR analysis displayed no major change in expression level between different strains. Similar expression level of *oqxR* was detected in all mutants carrying pABR except the one in which *soxS* has been inactivated, whose expression level was reduced by half when compared with wild-type **(Figure 5.4)**. Based on the above result, we surmise *oqxAB* is tightly repressed by *oqxR* when intact *oqxR* upstream sequence is in place, and that under such situation, key global regulators in *S.* Typhimurium have no role in *oqxAB* expression. The role of *soxS* in *oqxR* regulation is yet to be determined.

Contrary to the observation on strains carrying pABR, production of OqxAB was observed in PY1 wild type strain, the Δ*ramR* and Δ*soxS* mutant harbouring pABR0 construct but not from Δ*ramA* mutant, suggesting that the *ramA* gene product may have a role in *oqxAB* activation when this PMQR is carried by plasmids **(Figure 5.3)**. Result of qRT-PCR showed that the expression level of *oqxR* in Δ*ramA* mutant was comparable to that of the wild type strain. Nevertheless, *oqxR* expression from pABR0 was found to be reduced by ~1 fold in Δ*ramR* mutant when compared to Δ*ramA*, and the level of production of OqxAB reverted back to that of the wild-type **(Figure 5.4)**. These data again confirmed that repressive effect of OqxR does not necessarily correlate with its own expression level. In addition, it has been suggested that RamA plays a role in *oqxAB* expression activation under normal condition by attenuating the effect of OqxR repressor. In the case in which a high level of RamA protein is present (loss of ramR function), expression of *oqxR* may also be suppressed

DISCUSSION

Multidrug efflux systems are one of the major contributors of antimicrobial resistance in bacteria. In *Salmonella* spp., resistance towards fluoroquinolones, which is a key antimicrobial compound for treating salmonellosis, is found to be attributed to its endogenous RND-type efflux pump AcrAB-TolC. Several regulation mechanisms of the *acrAB-tolC* genes have been elucidated. First, the *acrAB* is regulated by its adjacent repressor *acrR* located upstream of *acrA.* The *oqxABR* locus has a similar genetic configuration as *acrABR*, with the exception that *oqxR* is located downstream of *oqxB.* The results presented in this work corroborates with previous findings, claiming that the *oqxR* gene product is a repressor of *oqxAB* in *K. pneumoniae*, the function of which can be demonstrated by the loss of OqxAB production upon integration with *oqxR* in the pABR construct (Veleba et al., 2012). The purpose of building the construct pABR0 was to simulate the genetic environment of *oqxABR* being carried by plasmids, in which the whole locus is flanked by IS*26* and under the control of an altered *oqxR* upstream region. Considering the fact that organisms harbouring plasmid-borne *oqxAB* are always non-susceptible to ciprofloxacin and resistant to olaquindox, we hypothesised that this may be due to the loss of repressive effect of OqxR resulting from the loss of *oqxR* promoter. Nevertheless, unexpected elevated expression of *oqxR* together with *oqxAB* in pABR0 was observed, providing evidence that *oqxR* being encoded on plasmid is expressible. The GntR type transcriptional regulator OqxR shares common structures with other members in the family. It contains a typical

helix-turn-helix (HTH) domain and a ligand-binding domain (Hoskisson and Rigali, 2009). Thus it is believed that OqxR exhibits its repressive effects in a fashion similar to other transcription factors such as AcrR, in which its HTH domain binds to the inverted repeats (IR) nucleotide sequences located within the promoter region of *oqxAB* in the form of dimer to avoid transcription by RNA polymerase. It has been reported that *acrR* is self-regulated. The increased expression of *oqxR* may be explained by the fact that the truncation of its upstream region leads to changes in nucleotide sequences, thus resulting in loss of regulatory binding sites. The reason why expression level of *oqxAB* and *oqxR* increased in-line was not known. A similar phenomenon has been observed in *acrAB* and *acrR* in *E. coli*, in which simultaneously elevated expression was observed in both genes upon being challenged by stress (Ma et al., 1996). The actual interactive activities between *oqxR* and *oqxAB* remains to be elucidated.

In addition to local regulation, expression of bacterial multidrug efflux systems is to a greater extent regulated at global level. Various global transcriptional regulators have been identified. A considerable number of studies have been conducted for determining the effects of these transcriptional factors on expression of *acrAB* in *Salmonella* spp. and *E. coli*. Regulators such as the *ramA*, *marA* and *soxS* gene products are found to be activators of *acrAB*. It has been reported that in *K. pneumoniae*, where endogenous *oqxAB* resides, loss of *ramR* (which resulted in overproduction of RamA) would lead to

overexpression of *oqxAB* (De Majumdar et al., 2015). Our data showed in *Salmonella* spp., *ramA* could counteract the effect of OqxR in plasmid-carried *oqxABR*. Over-production of RamA could also suppress the expression of *oqxR*. Another important finding is that when Tn*6010*-associated *oqxABR* was introduced into wild-type *S.* Typhimurium, the repressive effect of OqxR was diminished despite the fact that the protein was expressed in high level. The reason why *oqxR* was expressed in high level when it was carried by plasmid is not known. In the case of *acrAB* regulation, it has been reported that *acrR* in *E. coli* is self-regulated (Ma et al., 1996). In the current study, the fact that the upstream region of *oqxR* in plasmid-borne *oqxABR* is truncated may constitute the basis of loss of *oqxR* consensus binding sequence, and subsequent autoregulation feedback.

Based on the partial sequences of plasmids deposited into Genbank, the PMQR determinant *oqxABR* are all found to be mediated by the transposon Tn*6010*, which was first sequenced along with an *E. coli* IncX plasmid carrying *oqxAB* in 2008 (Norman et al., 2008). It is believed that the IS*26* transposition event captured the whole *oqxABR* locus from *K. pneumoniae*, but not the *rarA* gene located upstream of *oqxA*. A previous study has demonstrated that *rarA*, a homologue of *ramA*, may exhibit induction effect on *oqxAB* expression and subsequently confer reduced antimicrobial susceptibility in *K. pneumoniae* (Veleba et al., 2012). Considering the fact that the *rarA* product binds to the promoter region of *oqxAB*, and also its genetic homology with *ramA*, we

surmise that in *Salmonella* spp., functional defect due to a lack of the *rarA* gene can be compensated by the effect of the product of the homogenous *ramA* gene, which acts as an transcriptional factor to overcome the repressive effect of OqxR in a stronger fashion than the *rarA* gene product This may further explain the phenomenon of correlation with olaquindox resistance upon acquisition of *oqxAB*-plasmids in *Salmonella*. Taken together, our data suggested that intrinsic transcriptional factors, which have distal regulatory effects, could not only regulate endogenous genes, but also those acquired through plasmid uptake or transposition. Interestingly, the fact that the absence of *ramA* in *E. coli* does not hinder the substrate extrusion capacity of OqxAB implies that this PMQR may be controlled by different regulatory mechanisms in this bacterial species.

To conclude, this work revealed the constitutively expressed nature of the plasmid encoded PMQR element *oqxAB*. The transcriptional factor RamA in *S.* Typhimurium is involved in plasmid-borne *oqxAB* regulation and can help overcome the repressive effect of the local repressor OqxR, demonstrating that global regulatory mechanisms are capable of controlling expression of resistance genes located in extra-chromosomal elements. Further studies should be conducted to elucidate the actual events regarding the interaction between the products of *oqxAB, oqxR* and *ramA* in *Salmonella* spp., as well as mechanisms regulating the expression of PMQR genes in other members of *Enterobacteriaceae*.

CHAPTER VI - CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

The emergence of antimicrobial resistant bacteria, some of which have begun to threaten the long term survival of mankind, has drawn attention of world leaders. The United States Federal Government and The United Kingdom Parliamentary Government have shown their commitment to combat against the worsening situation by budgeting more resources into researches related to antimicrobial resistance. In this day and age the eyes of the world mainly focus on the carbapenem-resistant *Enterobacteriaceae*, as there is virtually no effective means to treat infections caused by these organisms. However, attention should also be paid on the development of resistance towards other antimicrobials before it is too late, particularly infections caused by foodborne pathogens. Antimicrobial resistance in *Salmonella* spp. has been marked as "Serious" level by the Center of Disease Control and Infection of the United States, especially the fluoroquinolone and ceftriaxone resistant strains which do not respond to the frontline drugs used to cure salmonellosis. Emergence of resistance to the treatment choices may result in not only treatment failure, but also inevitably the increased usage of drugs of the last resort including the carbapenems.

Acquisition of PMQR genes is one of the major modes by which bacteria utilise to develop fluoroquinolone resistance. Amongst various PMQR, *oqxAB* has become increasingly prevalent in members that belong to *Enterobacteriaceae*,

and possibly plays a key role in mediating fluoroquinolone resistance. Based on the findings in this work, several conclusions could be made: (i) This mobile RND-type efflux pump was one of the endogenous efflux systems in *K. pneumoniae*, which was then captured by transposase IS*26* and became the plasmid-mediated transposon Tn*6010* identifiable for the first time in an *E. coli* swine isolate. (ii) Starting from this first batch of isolates, Tn*6010*-encoded *oqxAB* migrated into other Gram negative bacteria in clinical setting. From 2006 onwards, *oqxAB* could be detected from *Salmonella* Typhimurium isolated from patients hospitalised in Hong Kong and the People's Republic of China. Since then, the prevalence of this determinant in *S.* Typhimurium exhibited an increasing trend by year and correlated well with ciprofloxacin susceptibility. (iii) *OqxAB* was plasmid-encoded and in most cases co-existed with another PMQR *aac(6')-Ib-cr* in *S.* Typhimurium clinical isolates. This combination was shown to confer immense capacity for *S.* Typhimurium to develop ciprofloxacin resistance, and to tolerate high ciprofloxacin concentration as demonstrated by MPC assay. The benefits brought by *oqxAB* may explain its increasing detection rate in *S.* Typhimurium. (iv) As one of the intrinsic efflux systems, *oqxAB* was subjected to stringent control in its host *K. pneumoniae*. Thus the reduced ciprofloxacin susceptibility phenotype was not always observed in this bacterial species; whereas in *S.* Typhimurium, *oqxAB* was constitutively expressed. Through comparing with its chromosomal counterpart, the local activator, encoded by *rarA*, is missing in Tn*6010*-encoded *oqxAB*, its role is taken by the global regulator RamA in *S.* Typhimurium. RamA itself attenuated the

repressing effect of *oqxR* and even suppressed *oqxR* expression level when it was present at high level, demonstrating the role of global regulators in controlling foreign resistance genes. This work concludes that *oqxAB* has been adopted as a major PMQR element in *S.* Typhimurium. The spread of strains harbouring this determinant will pose a threat on future *Salmonella* infection control effort. Further studies should be conducted to monitor the prevalence of *oqxAB* in other *Salmonella* serotypes, such as the infamous *S.* Enteritidis. In addition, efflux pumps are always involved not only in development of multidrug resistance but also various cell physiological activities, including virulence and stress response. Thus, the role by which *oqxAB* may play in enhancement of virulence and chance of survival under stress in *S.* Typhimurium should also be studied. Furthermore, understanding the actual interaction events between the products of *oqxAB, oqxR* as well as *ramA* may provide valuable information that help elucidate the complete spectrum of regulatory mechanisms of *oqxAB* in other Gram negative bacteria.

REFERENCES

Ahmed, A.M., Younis, E.E., Ishida, Y., and Shimamoto, T. (2009). Genetic basis of multidrug resistance in Salmonella enterica serovars Enteritidis and Typhimurium isolated from diarrheic calves in Egypt. *Acta Trop* 111**,** 144-149.

Alekshun, M.N., and Levy, S.B. (1997). Regulation of chromosomally mediated multiple antibiotic resistance: the mar regulon. *Antimicrobial agents and chemotherapy* 41**,** 2067-2075.

Alekshun, M.N., Levy, S.B., Mealy, T.R., Seaton, B.A., and Head, J.F. (2001). The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 A resolution. *Nat Struct Biol* 8**,** 710-714.

Antunes, P., Mourao, J., Pestana, N., and Peixe, L. (2011). Leakage of emerging clinically relevant multidrug-resistant Salmonella clones from pig farms. *J Antimicrob Chemother* 66**,** 2028-2032.

Arthur, M., Molinas, C., Mabilat, C., and Courvalin, P. (1990). Detection of erythromycin resistance by the polymerase chain reaction using primers in conserved regions of erm rRNA methylase genes. *Antimicrob Agents Chemother* 34**,** 2024-2026.

Ball, P. (2000). Quinolone generations: natural history or natural selection? *J Antimicrob Chemother* 46 Suppl T1**,** 17-24.

Baranello, L., Levens, D., Gupta, A., and Kouzine, F. (2012). The importance of being supercoiled: how DNA mechanics regulate dynamic processes. *Biochim Biophys Acta* 1819**,** 632-638.

Barbosa, T.M., and Levy, S.B. (2000). Differential expression of over 60 chromosomal genes in Escherichia coli by constitutive expression of MarA. *J Bacteriol* 182**,** 3467-3474.

Baucheron, S., Imberechts, H., Chaslus-Dancla, E., and Cloeckaert, A. (2002). The AcrB multidrug transporter plays a major role in high-level fluoroquinolone resistance in Salmonella enterica serovar typhimurium phage type DT204. *Microb Drug Resist* 8**,** 281-289.

Baugh, S., Ekanayaka, A.S., Piddock, L.J., and Webber, M.A. (2012). Loss of or inhibition of all multidrug resistance efflux pumps of Salmonella enterica serovar Typhimurium results in impaired ability to form a biofilm. *J Antimicrob Chemother* 67**,** 2409-2417.

Baugh, S., Phillips, C.R., Ekanayaka, A.S., Piddock, L.J., and Webber, M.A. (2014). Inhibition of multidrug efflux as a strategy to prevent biofilm formation. *The Journal of antimicrobial chemotherapy* 69**,** 673-681.

Beutlich, J., Rodriguez, I., Schroeter, A., Kasbohrer, A., Helmuth, R., and Guerra, B. (2010). A predominant multidrug-resistant Salmonella enterica serovar Saintpaul clonal line in German turkey and related food products. *Appl Environ Microbiol* 76**,** 3657-3667.

Bialek-Davenet, S., Lavigne, J.P., Guyot, K., Mayer, N., Tournebize, R., Brisse, S., Leflon-Guibout, V., and Nicolas-Chanoine, M.H. (2015). Differential contribution of AcrAB and OqxAB efflux pumps to multidrug resistance and virulence in Klebsiella pneumoniae. *J Antimicrob Chemother* 70**,** 81-88.

Blair, J.M., and Piddock, L.J. (2009). Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. *Curr Opin Microbiol* 12**,** 512-519.

Boumghar-Bourtchai, L., Mariani-Kurkdjian, P., Bingen, E., Filliol, I., Dhalluin, A., Ifrane, S.A., Weill, F.X., and Leclercq, R. (2008). Macrolide-resistant Shigella sonnei. *Emerg Infect Dis* 14**,** 1297-1299.

Bronsch, K., Schneider, D., and Rigal-Antonelli, F. (1976). [Olaquindox - a new growth promoter in animal nutrition. 1. Effectiveness in raising piglets]. *Z Tierphysiol Tierernahr Futtermittelkd* 36**,** 211-221.

Broughton, E.I., Ip, M., Coles, C.L., and Walker, D.G. (2010). Higher hospital costs and lengths of stay associated with quinolone-resistant Salmonella enterica infections in Hong Kong. *J Public Health (Oxf)* 32**,** 165-172.

Bryskier, A. (1993). *Macrolides : chemistry, pharmacology, and clinical uses.* Paris ; Boston: Arnette Blackwell.

Buckley, A.M., Webber, M.A., Cooles, S., Randall, L.P., La Ragione, R.M., Woodward, M.J., and Piddock, L.J. (2006). The AcrAB-TolC efflux system of Salmonella enterica serovar Typhimurium plays a role in pathogenesis. *Cellular microbiology* 8**,** 847-856.

Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., Jacoby, G.A., Kishony, R., Kreiswirth, B.N., Kutter, E., Lerner, S.A., Levy, S., Lewis, K., Lomovskaya, O., Miller, J.H., Mobashery, S., Piddock, L.J., Projan, S., Thomas, C.M., Tomasz, A., Tulkens, P.M., Walsh, T.R., Watson, J.D., Witkowski, J., Witte, W., Wright, G., Yeh, P., and Zgurskaya, H.I. (2011). Tackling antibiotic resistance. *Nat Rev Microbiol* 9**,** 894-896.

Bushby, S.R., and Hitchings, G.H. (1968). Trimethoprim, a sulphonamide potentiator. *Br J Pharmacol Chemother* 33**,** 72-90.

Caldwell, D.B., Wang, Y., and Lin, J. (2008). Development, stability, and molecular mechanisms of macrolide resistance in Campylobacter jejuni. *Antimicrobial agents and chemotherapy* 52**,** 3947-3954.

Cameron, A.D., Stoebel, D.M., and Dorman, C.J. (2011). DNA supercoiling is differentially regulated by environmental factors and FIS in Escherichia coli and Salmonella enterica. *Mol Microbiol* 80**,** 85-101.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K.L., and Threlfall, E.J. (2005). Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 63**,** 219-228.

Casin, I., Breuil, J., Darchis, J.P., Guelpa, C., and Collatz, E. (2003). Fluoroquinolone resistance linked to GyrA, GyrB, and ParC mutations in Salmonella enterica typhimurium isolates in humans. *Emerg Infect Dis* 9**,** 1455-1457.

Cattoir, V., and Nordmann, P. (2009). Plasmid-mediated quinolone resistance in gram-negative bacterial species: an update. *Curr Med Chem* 16**,** 1028-1046.

Cdc (2009). National Enteric Disease Surveillance:

Salmonella Annual Summary. *http://www.cdc.gov/nationalsurveillance/PDFs/NationalSalmSurveillOverview_508.pdf*.

Chen, S., Cui, S., Mcdermott, P.F., Zhao, S., White, D.G., Paulsen, I., and Meng, J. (2007). Contribution of target gene mutations and efflux to decreased susceptibility of Salmonella enterica serovar typhimurium to fluoroquinolones and other antimicrobials. *Antimicrob Agents Chemother* 51**,** 535-542.

Chen, S., Zhao, S., White, D.G., Schroeder, C.M., Lu, R., Yang, H., Mcdermott, P.F., Ayers, S., and Meng, J. (2004). Characterization of multiple-antimicrobial-resistant salmonella serovars isolated from retail meats. *Appl Environ Microbiol* 70**,** 1-7.

Chen, X., Zhang, W., Pan, W., Yin, J., Pan, Z., Gao, S., and Jiao, X. (2012). Prevalence of qnr, aac(6')-Ib-cr, qepA, and oqxAB in Escherichia coli isolates from humans, animals, and the environment. *Antimicrob Agents Chemother* 56**,** 3423-3427.

Chiu, C.H., Su, L.H., and Chu, C. (2004). Salmonella enterica serotype Choleraesuis: epidemiology, pathogenesis, clinical disease, and treatment. *Clin Microbiol Rev* 17**,** 311-322.

Chp (2011). "Review of Nontyphoidal Salmonella Food Poisoning in Hong Kong". Centre for Health Protection, Government of Hong Kong).

Chu, C., Su, L.H., Chu, C.H., Baucheron, S., Cloeckaert, A., and Chiu, C.H. (2005). Resistance to fluoroquinolones linked to gyrA and par C mutations and overexpression of acr AB efflux pump in Salmonella enterica serotype Choleraesuis. *Microb Drug Resist* 11**,** 248-253.

Clsi (2010). *Performance Standards for Antimicrobial Susceptibility Testing. CLSI document M100-S20*. Wayne, PA: Clinical and Laboratory Standards Institute.

Clsi (2013). *Performance Standards for Antimicrobial Susceptibility Testing. CLSI document M100-S23*. Wayne, PA: Clinical and Laboratory Standards Institute.

Dallenne, C., Da Costa, A., Decre, D., Favier, C., and Arlet, G. (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. *J Antimicrob Chemother* 65**,** 490-495.

Davidson, A.L., Dassa, E., Orelle, C., and Chen, J. (2008). Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol Mol Biol Rev* 72**,** 317-364, table of contents.

De Jong, A., Thomas, V., Simjee, S., Godinho, K., Schiessl, B., Klein, U., Butty, P., Valle, M., Marion, H., and Shryock, T.R. (2012). Pan-European monitoring of susceptibility to human-use antimicrobial agents in enteric bacteria isolated from healthy food-producing animals. *J Antimicrob Chemother* 67**,** 638-651.

De Majumdar, S., Yu, J., Fookes, M., Mcateer, S.P., Llobet, E., Finn, S., Spence, S., Monaghan, A., Kissenpfennig, A., Ingram, R.J., Bengoechea, J., Gally, D.L., Fanning, S., Elborn, J.S., and Schneiders, T. (2015). Elucidation of the RamA Regulon in Klebsiella pneumoniae Reveals a Role in LPS Regulation. *PLoS pathogens* 11.

Dellit, T.H., Owens, R.C., Mcgowan, J.E., Jr., Gerding, D.N., Weinstein, R.A., Burke, J.P., Huskins, W.C., Paterson, D.L., Fishman, N.O., Carpenter, C.F., Brennan, P.J., Billeter, M., Hooton, T.M., Infectious Diseases Society Of, A., and Society for Healthcare Epidemiology Of, A. (2007). Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America guidelines for developing an institutional program to enhance antimicrobial stewardship. *Clin Infect Dis* 44**,** 159-177.

Demain, A.L., and Sanchez, S. (2009). Microbial drug discovery: 80 years of progress. *J Antibiot (Tokyo)* 62**,** 5-16.

Drlica, K. (1990). Bacterial topoisomerases and the control of DNA supercoiling. *Trends Genet* 6**,** 433-437.

Drlica, K., Hiasa, H., Kerns, R., Malik, M., Mustaev, A., and Zhao, X. (2009). Quinolones: action and resistance updated. *Curr Top Med Chem* 9**,** 981-998.

Dunkle, J.A., Xiong, L., Mankin, A.S., and Cate, J.H. (2010). Structures of the Escherichia coli ribosome with antibiotics bound near the peptidyl transferase center explain spectra of drug action. *Proc Natl Acad Sci U S A* 107**,** 17152-17157.

Elkins, C.A., and Mullis, L.B. (2006). Mammalian steroid hormones are substrates for the major RND- and MFS-type tripartite multidrug efflux pumps of Escherichia coli. *J Bacteriol* 188**,** 1191-1195.

Eswaran, J., Koronakis, E., Higgins, M.K., Hughes, C., and Koronakis, V. (2004). Three's company: component structures bring a closer view of tripartite drug efflux pumps. *Curr Opin Struct Biol* 14**,** 741-747.

Everett, M.J., Jin, Y.F., Ricci, V., and Piddock, L.J. (1996). Contributions of individual mechanisms to fluoroquinolone resistance in 36 Escherichia coli strains isolated from humans and animals. *Antimicrob Agents Chemother* 40**,** 2380-2386.

Ferber, D. (2002). Antibiotic resistance. Livestock feed ban preserves drugs' power. *Science* 295**,** 27-28.

Fung, D.K., Chan, E.W., Chin, M.L., and Chan, R.C. (2010). Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. *Antimicrob Agents Chemother* 54**,** 1082-1093.

Garvey, M.I., Baylay, A.J., Wong, R.L., and Piddock, L.J. (2011). Overexpression of patA and patB, which encode ABC transporters, is associated with fluoroquinolone resistance in clinical isolates of Streptococcus pneumoniae. *Antimicrob Agents Chemother* 55**,** 190-196.

Gebru, E., Choi, M.J., Lee, S.J., Damte, D., and Park, S.C. (2011). Mutant-prevention concentration and mechanism of resistance in clinical isolates and enrofloxacin/marbofloxacin-selected mutants of Escherichia coli of canine origin. *J Med Microbiol* 60**,** 1512-1522.

Gebru, E., Damte, D., Choi, M.J., Lee, S.J., Kim, Y.H., and Park, S.C. (2012). Mutant prevention concentration and phenotypic and molecular basis of fluoroquinolone resistance in clinical isolates and in vitro-selected mutants of Escherichia coli from dogs. *Vet Microbiol* 154**,** 384-394.

Glynn, M.K., Bopp, C., Dewitt, W., Dabney, P., Mokhtar, M., and Angulo, F.J. (1998). Emergence of multidrug-resistant Salmonella enterica serotype typhimurium DT104 infections in the United States. *N Engl J Med* 338**,** 1333-1338.

Gomez, T.M., Motarjemi, Y., Miyagawa, S., Kaferstein, F.K., and Stohr, K. (1997). Foodborne salmonellosis. *World Health Stat Q* 50**,** 81-89.

Gordillo, M.E., Singh, K.V., and Murray, B.E. (1993). In vitro activity of azithromycin against bacterial enteric pathogens. *Antimicrob Agents Chemother* 37**,** 1203-1205.

Gupta, A., Nelson, J.M., Barrett, T.J., Tauxe, R.V., Rossiter, S.P., Friedman, C.R., Joyce, K.W., Smith, K.E., Jones, T.F., Hawkins, M.A., Shiferaw, B., Beebe, J.L., Vugia, D.J., Rabatsky-Ehr, T., Benson, J.A., Root, T.P., Angulo, F.J., and Group, N.W. (2004). Antimicrobial resistance among Campylobacter strains, United States, 1997-2001. *Emerg Infect Dis* 10**,** 1102-1109.

Hansen, L.H., Jensen, L.B., Sorensen, H.I., and Sorensen, S.J. (2007). Substrate specificity of the OqxAB multidrug resistance pump in Escherichia coli and selected enteric bacteria. *J Antimicrob Chemother* 60**,** 145-147.

Hansen, L.H., Johannesen, E., Burmolle, M., Sorensen, A.H., and Sorensen, S.J. (2004). Plasmid-encoded multidrug efflux pump conferring resistance to olaquindox in Escherichia coli. *Antimicrob Agents Chemother* 48**,** 3332-3337.

Helms, M., Ethelberg, S., Molbak, K., and Group, D.T.S. (2005). International Salmonella Typhimurium DT104 infections, 1992-2001. *Emerg Infect Dis* 11**,** 859-867.

Helms, M., Vastrup, P., Gerner-Smidt, P., and Molbak, K. (2002). Excess mortality associated with antimicrobial drug-resistant Salmonella typhimurium. *Emerg Infect Dis* 8**,** 490-495.

Hobbs, E.C., Yin, X., Paul, B.J., Astarita, J.L., and Storz, G. (2012). Conserved small protein associates with the multidrug efflux pump AcrB and differentially affects antibiotic resistance. *Proceedings of the National Academy of Sciences of the United States of America* 109**,** 16696-16701.

Hohmann, E.L. (2001). Nontyphoidal salmonellosis. *Clin Infect Dis* 32**,** 263-269.

Holland, I.B., and Blight, M.A. (1999). ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *J Mol Biol* 293**,** 381-399.

Hooper, D.C. (2001). Mechanisms of action of antimicrobials: focus on fluoroquinolones. *Clin Infect Dis* 32 Suppl 1**,** S9-S15.

Hoskisson, P.A., and Rigali, S. (2009). Chapter 1: Variation in form and function the helix-turn-helix regulators of the GntR superfamily. *Adv Appl Microbiol* 69**,** 1-22.

Jacoby, G.A., Chow, N., and Waites, K.B. (2003). Prevalence of plasmid-mediated quinolone resistance. *Antimicrob Agents Chemother* 47**,** 559-562.

Jeong, H.S., Kim, J.A., Shin, J.H., Chang, C.L., Jeong, J., Cho, J.H., Kim, M.N., Kim, S., Kim, Y.R., Lee, C.H., Lee, K., Lee, M.A., Lee, W.G., and Lee, J.N. (2011). Prevalence of plasmid-mediated quinolone resistance and mutations in the gyrase and topoisomerase IV genes in Salmonella isolated from 12 tertiary-care hospitals in Korea. *Microb Drug Resist* 17**,** 551-557.

Jin, Y., and Ling, J.M. (2006). CTX-M-producing Salmonella spp. in Hong Kong: an emerging problem. *Journal of medical microbiology* 55**,** 1245-1250.

Jin, Y., and Ling, J.M. (2009). Prevalence of integrons in antibiotic-resistant Salmonella spp. in Hong Kong. *Jpn J Infect Dis* 62**,** 432-439.

Ke, B., Sun, J., He, D., Li, X., Liang, Z., and Ke, C.W. (2014). Serovar distribution, antimicrobial resistance profiles, and PFGE typing of Salmonella enterica strains isolated from 2007-2012 in Guangdong, China. *BMC Infect Dis* 14**,** 338.

Khaitsa, M.L., Kegode, R.B., and Doetkott, D.K. (2007). Occurrence of antimicrobial-resistant salmonella species in raw and ready to eat turkey meat products from retail outlets in the midwestern United States. *Foodborne Pathog Dis* 4**,** 517-525.

Kim, H.B., Park, C.H., Kim, C.J., Kim, E.-C.C., Jacoby, G.A., and Hooper, D.C. (2009a). Prevalence of plasmid-mediated quinolone resistance determinants over a 9-year period. *Antimicrobial agents and chemotherapy* 53**,** 639-645.

Kim, H.B., Wang, M., Park, C.H., Kim, E.C., Jacoby, G.A., and Hooper, D.C. (2009b). oqxAB encoding a multidrug efflux pump in human clinical isolates of Enterobacteriaceae. *Antimicrob Agents Chemother* 53**,** 3582-3584.

Klare, I., Badstubner, D., Konstabel, C., Bohme, G., Claus, H., and Witte, W. (1999). Decreased incidence of VanA-type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. *Microb Drug Resist* 5**,** 45-52.

Kobayashi, N., Nishino, K., and Yamaguchi, A. (2001). Novel macrolide-specific ABC-type efflux transporter in Escherichia coli. *Journal of bacteriology* 183**,** 5639-5644.

Le Goffic, F., Capmau, M.L., Tangy, F., and Baillarge, M. (1979). Mechanism of action of aminoglycoside antibiotics. Binding studies of tobramycin and its 6'-N-acetyl derivative to the bacterial ribosome and its subunits. *Eur J Biochem* 102**,** 73-81.

Lee, A., Mao, W., Warren, M.S., Mistry, A., Hoshino, K., Okumura, R., Ishida, H., and Lomovskaya, O. (2000). Interplay between efflux pumps may

provide either additive or multiplicative effects on drug resistance. *J Bacteriol* 182**,** 3142-3150.

Lennen, R.M., Politz, M.G., Kruziki, M.A., and Pfleger, B.F. (2013). Identification of transport proteins involved in free fatty acid efflux in Escherichia coli. *J Bacteriol* 195**,** 135-144.

Lesher, G.Y., Froelich, E.J., Gruett, M.D., Bailey, J.H., and Brundage, R.P. (1962). 1,8-Naphthyridine Derivatives. A New Class of Chemotherapeutic Agents. *J Med Pharm Chem* 91**,** 1063-1065.

Levy, S.B. (1992). Active efflux mechanisms for antimicrobial resistance. *Antimicrob Agents Chemother* 36**,** 695-703.

Lewis, J.S., 2nd, Herrera, M., Wickes, B., Patterson, J.E., and Jorgensen, J.H. (2007). First report of the emergence of CTX-M-type extended-spectrum beta-lactamases (ESBLs) as the predominant ESBL isolated in a U.S. health care system. *Antimicrob Agents Chemother* 51**,** 4015-4021.

Li, L., Liao, X., Yang, Y., Sun, J., Li, L., Liu, B., Yang, S., Ma, J., Li, X., Zhang, Q., and Liu, Y. (2013). Spread of oqxAB in Salmonella enterica serotype Typhimurium predominantly by IncHI2 plasmids. *J Antimicrob Chemother* 68**,** 2263-2268.

Li, X.-Z.Z., Plésiat, P., and Nikaido, H. (2015). The Challenge of Efflux-Mediated Antibiotic Resistance in Gram-Negative Bacteria. *Clinical microbiology reviews* 28**,** 337-418.

Li, X.Z., Livermore, D.M., and Nikaido, H. (1994). Role of efflux pump(s) in intrinsic resistance of Pseudomonas aeruginosa: resistance to tetracycline, chloramphenicol, and norfloxacin. *Antimicrob Agents Chemother* 38**,** 1732-1741.

Ling, L.L., Schneider, T., Peoples, A.J., Spoering, A.L., Engels, I., Conlon, B.P., Mueller, A., Schäberle, T.F., Hughes, D.E., Epstein, S., Jones, M., Lazarides, L., Steadman, V.A., Cohen, D.R., Felix, C.R., Fetterman, K.A., Millett, W.P., Nitti, A.G., Zullo, A.M., Chen, C., and Lewis, K.

(2015). A new antibiotic kills pathogens without detectable resistance. *Nature*.

Liu, B.T., Wang, X.M., Liao, X.P., Sun, J., Zhu, H.Q., Chen, X.Y., and Liu, Y.H. (2011). Plasmid-mediated quinolone resistance determinants oqxAB and aac(6')-Ib-cr and extended-spectrum beta-lactamase gene blaCTX-M-24 co-located on the same plasmid in one Escherichia coli strain from China. *J Antimicrob Chemother* 66**,** 1638-1639.

Liu, B.T., Yang, Q.E., Li, L., Sun, J., Liao, X.P., Fang, L.X., Yang, S.S., Deng, H., and Liu, Y.H. (2013). Dissemination and characterization of plasmids carrying oqxAB-bla CTX-M genes in Escherichia coli isolates from food-producing animals. *PLoS One* 8**,** e73947.

Liu, J., Keelan, P., Bennett, P.M., and Enne, V.I. (2009). Characterization of a novel macrolide efflux gene, mef(B), found linked to sul3 in porcine Escherichia coli. *J Antimicrob Chemother* 63**,** 423-426.

Llarrull, L.I., Testero, S.A., Fisher, J.F., and Mobashery, S. (2010). The future of the beta-lactams. *Curr Opin Microbiol* 13**,** 551-557.

Lomovskaya, O., and Lewis, K. (1992). Emr, an Escherichia coli locus for multidrug resistance. *Proc Natl Acad Sci U S A* 89**,** 8938-8942.

Lu, Y., Wu, C.M., Wu, G.J., Zhao, H.Y., He, T., Cao, X.Y., Dai, L., Xia, L.N., Qin, S.S., and Shen, J.Z. (2011). Prevalence of antimicrobial resistance among Salmonella isolates from chicken in China. *Foodborne Pathog Dis* 8**,** 45-53.

Luna, V.A., Cousin, S., Jr., Whittington, W.L., and Roberts, M.C. (2000). Identification of the conjugative mef gene in clinical Acinetobacter junii and Neisseria gonorrhoeae isolates. *Antimicrob Agents Chemother* 44**,** 2503-2506.

Ma, D., Alberti, M., Lynch, C., Nikaido, H., and Hearst, J.E. (1996). The local repressor AcrR plays a modulating role in the regulation of acrAB genes of Escherichia coli by global stress signals. *Mol Microbiol* 19**,** 101-112.

Ma, D., Cook, D.N., Alberti, M., Pon, N.G., Nikaido, H., and Hearst, J.E. (1995). Genes acrA and acrB encode a stress-induced efflux system of Escherichia coli. *Mol Microbiol* 16**,** 45-55.

Markogiannakis, A., Tassios, P.T., Lambiri, M., Ward, L.R., Kourea-Kremastinou, J., Legakis, N.J., and Vatopoulos, A.C. (2000). Multiple clones within multidrug-resistant Salmonella enterica serotype Typhimurium phage type DT104. The Greek Nontyphoidal Salmonella Study Group. *J Clin Microbiol* 38**,** 1269-1271.

Marshall, B.M., and Levy, S.B. (2011). Food animals and antimicrobials: impacts on human health. *Clin Microbiol Rev* 24**,** 718-733.

Martinez-Martinez, L., Pascual, A., and Jacoby, G.A. (1998). Quinolone resistance from a transferable plasmid. *Lancet* 351**,** 797-799.

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., and Nishino, T. (2000). Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy* 44**,** 3322-3327.

Mcmurry, L., Petrucci, R.E., Jr., and Levy, S.B. (1980). Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in Escherichia coli. *Proc Natl Acad Sci U S A* 77**,** 3974-3977.

Mcmurry, L.M., Oethinger, M., and Levy, S.B. (1998). Overexpression of marA, soxS, or acrAB produces resistance to triclosan in laboratory and clinical strains of Escherichia coli. *FEMS Microbiol Lett* 166**,** 305-309.

Mmwr (1997). Multidrug-resistant Salmonella serotype Typhimurium--United States, 1996. *MMWR Morb Mortal Wkly Rep* 46**,** 308-310.

Molbak, K. (2005). Human health consequences of antimicrobial drug-resistant Salmonella and other foodborne pathogens. *Clin Infect Dis* 41**,** 1613-1620.

Murakami, S., Nakashima, R., Yamashita, E., and Yamaguchi, A. (2002). Crystal structure of bacterial multidrug efflux transporter AcrB. *Nature* 419**,** 587-593.

Nelson, J.M., Chiller, T.M., Powers, J.H., and Angulo, F.J. (2007). Fluoroquinolone-resistant Campylobacter species and the withdrawal of fluoroquinolones from use in poultry: a public health success story. *Clin Infect Dis* 44**,** 977-980.

Nikaido, H., and Takatsuka, Y. (2009). Mechanisms of RND multidrug efflux pumps. *Biochim Biophys Acta* 1794**,** 769-781.

Nishino, K., Hayashi-Nishino, M., and Yamaguchi, A. (2009). H-NS modulates multidrug resistance of Salmonella enterica serovar Typhimurium by repressing multidrug efflux genes acrEF. *Antimicrob Agents Chemother* 53**,** 3541-3543.

Nishino, K., Latifi, T., and Groisman, E.A. (2006). Virulence and drug resistance roles of multidrug efflux systems of Salmonella enterica serovar Typhimurium. *Molecular microbiology* 59**,** 126-141.

Nishino, K., Nikaido, E., and Yamaguchi, A. (2007). Regulation of multidrug efflux systems involved in multidrug and metal resistance of Salmonella enterica serovar Typhimurium. *Journal of bacteriology* 189**,** 9066-9075.

Nishino, K., and Yamaguchi, A. (2001). Analysis of a complete library of putative drug transporter genes in Escherichia coli. *J Bacteriol* 183**,** 5803-5812.

Nishino, K., and Yamaguchi, A. (2004). Role of histone-like protein H-NS in multidrug resistance of Escherichia coli. *J Bacteriol* 186**,** 1423-1429.

Nordmann, P., and Poirel, L. (2005). Emergence of plasmid-mediated resistance to quinolones in Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy* 56**,** 463-469.

Norman, A., Hansen, L.H., She, Q., and Sorensen, S.J. (2008). Nucleotide sequence of pOLA52: a conjugative IncX1 plasmid from Escherichia coli which enables biofilm formation and multidrug efflux. *Plasmid* 60**,** 59-74.

Norris, A.L., and Serpersu, E.H. (2013). Ligand promiscuity through the eyes of the aminoglycoside N3 acetyltransferase IIa. *Protein Sci* 22**,** 916-928.

Oethinger, M., Podglajen, I., Kern, W.V., and Levy, S.B. (1998). Overexpression of the marA or soxS regulatory gene in clinical topoisomerase mutants of Escherichia coli. *Antimicrob Agents Chemother* 42**,** 2089-2094.

Oliver, S.P., Murinda, S.E., and Jayarao, B.M. (2011). Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. *Foodborne Pathog Dis* 8**,** 337-355.

Ozfoodnet Working, G. (2012). Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: annual report of the OzFoodNet network, 2010. *Commun Dis Intell Q Rep* 36**,** E213-241.

Park, K.S., Kim, M.H., Park, T.S., Nam, Y.S., Lee, H.J., and Suh, J.T. (2012). Prevalence of the plasmid-mediated quinolone resistance genes, aac(6')-Ib-cr, qepA, and oqxAB in clinical isolates of extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli and Klebsiella pneumoniae in Korea. *Ann Clin Lab Sci* 42**,** 191-197.

Parry, C.M., Vinh, H., Chinh, N.T., Wain, J., Campbell, J.I., Hien, T.T., Farrar, J.J., and Baker, S. (2011). The influence of reduced susceptibility to fluoroquinolones in Salmonella enterica serovar Typhi on the clinical response to ofloxacin therapy. *PLoS Negl Trop Dis* 5**,** e1163.

Paulsen, I.T., Brown, M.H., and Skurray, R.A. (1996). Proton-dependent multidrug efflux systems. *Microbiol Rev* 60**,** 575-608.

Perez, F., Rudin, S.D., Marshall, S.H., Coakley, P., Chen, L., Kreiswirth, B.N., Rather, P.N., Hujer, A.M., Toltzis, P., Van Duin, D., Paterson, D.L., and Bonomo, R.A. (2013). OqxAB, a quinolone and olaquindox efflux pump, is widely distributed among multidrug-resistant Klebsiella pneumoniae isolates of human origin. *Antimicrob Agents Chemother* 57**,** 4602-4603.

Pfister, P., Jenni, S., Poehlsgaard, J., Thomas, A., Douthwaite, S., Ban, N., and Böttger, E.C. (2004). The structural basis of macrolide-ribosome binding assessed using mutagenesis of 23S rRNA positions 2058 and 2059. *Journal of molecular biology* 342**,** 1569-1581.

Phuc Nguyen, M.C., Woerther, P.-L.L., Bouvet, M., Andremont, A., Leclercq, R., and Canu, A. (2009). Escherichia coli as reservoir for macrolide resistance genes. *Emerging infectious diseases* 15**,** 1648-1650.

Piddock, L.J. (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin Microbiol Rev* 19**,** 382-402.

Piddock, L.J. (2012). The crisis of no new antibiotics--what is the way forward? *Lancet Infect Dis* 12**,** 249-253.

Piddock, L.J., White, D.G., Gensberg, K., Pumbwe, L., and Griggs, D.J. (2000). Evidence for an efflux pump mediating multiple antibiotic resistance in Salmonella enterica serovar Typhimurium. *Antimicrob Agents Chemother* 44**,** 3118-3121.

Poole, K., Tetro, K., Zhao, Q., Neshat, S., Heinrichs, D.E., and Bianco, N. (1996). Expression of the multidrug resistance operon mexA-mexB-oprM in Pseudomonas aeruginosa: mexR encodes a regulator of operon expression. *Antimicrobial agents and chemotherapy* 40**,** 2021-2028.

Quale, J., Bratu, S., Gupta, J., and Landman, D. (2006). Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of Pseudomonas aeruginosa clinical isolates. *Antimicrob Agents Chemother* 50**,** 1633-1641.

Rahmati, S., Yang, S., Davidson, A.L., and Zechiedrich, E.L. (2002). Control of the AcrAB multidrug efflux pump by quorum-sensing regulator SdiA. *Mol Microbiol* 43**,** 677-685.

Ramirez, M.S., Nikolaidis, N., and Tolmasky, M.E. (2013). Rise and dissemination of aminoglycoside resistance: the aac(6')-Ib paradigm. *Front Microbiol* 4**,** 121.

Ramirez, M.S., and Tolmasky, M.E. (2010). Aminoglycoside modifying enzymes. *Drug Resist Updat* 13**,** 151-171.

Reddy, V.S., Shlykov, M.A., Castillo, R., Sun, E.I., and Saier, M.H., Jr. (2012). The major facilitator superfamily (MFS) revisited. *FEBS J* 279**,** 2022-2035.

Ribot, E.M., Fair, M.A., Gautom, R., Cameron, D.N., Hunter, S.B., Swaminathan, B., and Barrett, T.J. (2006). Standardization of pulsed-field gel electrophoresis protocols for the subtyping of Escherichia coli O157:H7, Salmonella, and Shigella for PulseNet. *Foodborne Pathog Dis* 3**,** 59-67.

Robicsek, A., Jacoby, G.A., and Hooper, D.C. (2006). The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect Dis* 6**,** 629-640.

Rodríguez-Martínez, J.M., Díaz De Alba, P., Briales, A., Machuca, J., Lossa, M., Fernández-Cuenca, F., Rodríguez Baño, J., Martínez-Martínez, L., and Pascual, (2013). Contribution of OqxAB efflux pumps to quinolone resistance in extended-spectrum-β-lactamase-producing Klebsiella pneumoniae. *The Journal of antimicrobial chemotherapy* 68**,** 68-73.

Rodriguez-Martinez, J.M., Diaz De Alba, P., Briales, A., Machuca, J., Lossa, M., Fernandez-Cuenca, F., Rodriguez Bano, J., Martinez-Martinez, L., and Pascual, A. (2013). Contribution of OqxAB efflux pumps to quinolone resistance in extended-spectrum-beta-lactamase-producing Klebsiella pneumoniae. *J Antimicrob Chemother* 68**,** 68-73.

Rodriguez, I., Barownick, W., Helmuth, R., Mendoza, M.C., Rodicio, M.R., Schroeter, A., and Guerra, B. (2009). Extended-spectrum {beta}-lactamases and AmpC {beta}-lactamases in ceftiofur-resistant Salmonella enterica isolates from food and livestock obtained in Germany during 2003-07. *J Antimicrob Chemother* 64**,** 301-309.

Romanowska, J., Reuter, N., and Trylska, J. (2013). Comparing aminoglycoside binding sites in bacterial ribosomal RNA and aminoglycoside modifying enzymes. *Proteins* 81**,** 63-80.

Ross, J.I., Eady, E.A., Cove, J.H., Cunliffe, W.J., Baumberg, S., and Wootton, J.C. (1990). Inducible erythromycin resistance in staphylococci is encoded by a member of the ATP-binding transport super-gene family. *Mol Microbiol* 4**,** 1207-1214.

Ruiz, E., Saenz, Y., Zarazaga, M., Rocha-Gracia, R., Martinez-Martinez, L., Arlet, G., and Torres, C. (2012). qnr, aac(6')-Ib-cr and qepA genes in Escherichia coli and Klebsiella spp.: genetic environments and plasmid and chromosomal location. *J Antimicrob Chemother* 67**,** 886-897.

Sørensen, A.H., Hansen, L.H., and Johannesen…, E. (2003). Conjugative plasmid conferring resistance to olaquindox. *Conjugative plasmid conferring resistance to olaquindox*.

Sapunaric, F.M., and Levy, S.B. (2005). Substitutions in the interdomain loop of the Tn10 TetA efflux transporter alter tetracycline resistance and substrate specificity. *Microbiology* 151**,** 2315-2322.

Sarno, R., Mcgillivary, G., Sherratt, D.J., Actis, L.A., and Tolmasky, M.E. (2002). Complete nucleotide sequence of Klebsiella pneumoniae multiresistance plasmid pJHCMW1. *Antimicrob Agents Chemother* 46**,** 3422-3427.

Sato, T., Yokota, S., Uchida, I., Okubo, T., Usui, M., Kusumoto, M., Akiba, M., Fujii, N., and Tamura, Y. (2013). Fluoroquinolone resistance mechanisms in an Escherichia coli isolate, HUE1, without quinolone resistance-determining region mutations. *Front Microbiol* 4**,** 125.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., and Griffin, P.M. (2011). Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis* 17**,** 7-15.

Schneider, T., and Sahl, H.G. (2010). An oldie but a goodie - cell wall biosynthesis as antibiotic target pathway. *Int J Med Microbiol* 300**,** 161-169.

Schwaiger, K., Huther, S., Holzel, C., Kampf, P., and Bauer, J. (2012). Prevalence of antibiotic-resistant enterobacteriaceae isolated from chicken and pork meat purchased at the slaughterhouse and at retail in Bavaria, Germany. *Int J Food Microbiol* 154**,** 206-211.

Singh, K.V., Weinstock, G.M., and Murray, B.E. (2002). An Enterococcus faecalis ABC homologue (Lsa) is required for the resistance of this

species to clindamycin and quinupristin-dalfopristin. *Antimicrob Agents Chemother* 46**,** 1845-1850.

Skold, O. (2000). Sulfonamide resistance: mechanisms and trends. *Drug Resist Updat* 3**,** 155-160.

Slinger, R., Desjardins, M., Mccarthy, A.E., Ramotar, K., Jessamine, P., Guibord, C., and Toye, B. (2004). Suboptimal clinical response to ciprofloxacin in patients with enteric fever due to Salmonella spp. with reduced fluoroquinolone susceptibility: a case series. *BMC Infect Dis* 4**,** 36.

Society for Healthcare Epidemiology Of, A., Infectious Diseases Society Of, A., and Pediatric Infectious Diseases, S. (2012). Policy statement on antimicrobial stewardship by the Society for Healthcare Epidemiology of America (SHEA), the Infectious Diseases Society of America (IDSA), and the Pediatric Infectious Diseases Society (PIDS). *Infect Control Hosp Epidemiol* 33**,** 322-327.

Sulavik, M.C., Houseweart, C., Cramer, C., Jiwani, N., Murgolo, N., Greene, J., Didomenico, B., Shaw, K.J., Miller, G.H., Hare, R., and Shimer, G. (2001). Antibiotic susceptibility profiles of Escherichia coli strains lacking multidrug efflux pump genes. *Antimicrob Agents Chemother* 45**,** 1126-1136.

Swedberg, G., Castensson, S., and Skold, O. (1979). Characterization of mutationally altered dihydropteroate synthase and its ability to form a sulfonamide-containing dihydrofolate analog. *J Bacteriol* 137**,** 129-136.

Swedberg, G., and Skold, O. (1983). Plasmid-borne sulfonamide resistance determinants studied by restriction enzyme analysis. *J Bacteriol* 153**,** 1228-1237.

Sylvie, B., Kunihiko, N., Isabelle, M., Sylvie, C., Marie-Christine, C.M., Franck, C., Alain, R., Axel, C., and Etienne, G. (2014). Bile-mediated activation of the acrAB and tolC multidrug efflux genes occurs mainly through transcriptional derepression of ramA in Salmonella enterica serovar Typhimurium. *The Journal of antimicrobial chemotherapy*.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30**,** 2725-2729.

Tenson, T., Lovmar, M., and Ehrenberg, M. (2003). The mechanism of action of macrolides, lincosamides and streptogramin B reveals the nascent peptide exit path in the ribosome. *J Mol Biol* 330**,** 1005-1014.

Thanassi, D.G., Cheng, L.W., and Nikaido, H. (1997). Active efflux of bile salts by Escherichia coli. *J Bacteriol* 179**,** 2512-2518.

Threlfall, E.J., Frost, J.A., Ward, L.R., and Rowe, B. (1994). Epidemic in cattle and humans of Salmonella typhimurium DT 104 with chromosomally integrated multiple drug resistance. *Vet Rec* 134**,** 577.

Threlfall, E.J., Ward, L.R., Frost, J.A., and Willshaw, G.A. (2000). The emergence and spread of antibiotic resistance in food-borne bacteria. *Int J Food Microbiol* 62**,** 1-5.

Threlfall, E.J., Ward, L.R., and Rowe, B. (1997). Increasing incidence of resistance to trimethoprim and ciprofloxacin in epidemic Salmonella typhimurium DT104 in England and Wales. *Euro Surveill* 2**,** 81-84.

Tomás, M., Doumith, M., Warner, M., Turton, J.F., Beceiro, A., Bou, G., Livermore, D.M., and Woodford, N. (2010). Efflux pumps, OprD porin, AmpC beta-lactamase, and multiresistance in Pseudomonas aeruginosa isolates from cystic fibrosis patients. *Antimicrobial agents and chemotherapy* 54**,** 2219-2224.

Touze, T., Eswaran, J., Bokma, E., Koronakis, E., Hughes, C., and Koronakis, V. (2004). Interactions underlying assembly of the Escherichia coli AcrAB-TolC multidrug efflux system. *Mol Microbiol* 53**,** 697-706.

Turner, S., Pryer, K.M., Miao, V.P., and Palmer, J.D. (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J Eukaryot Microbiol* 46**,** 327-338.

Uscdc (2012). "National Antimicrobial Resistance Monitoring System: Enteric Bacteria. 2012 Human Isolates Final Report.". Center for Disease Control and Prevention).

Uscdc. 2013. Antibiotic Resistance Threats in the United States, 2013.

Uscdc (2014). *CDC 2011 Estimates: Findings* [Online]. Available: http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html [Accessed 23 Mar 2015].

Usfda (2004). Bacteriological Analytical Manual on line Chapter 9.

Vakulenko, S.B., and Mobashery, S. (2003). Versatility of aminoglycosides and prospects for their future. *Clin Microbiol Rev* 16**,** 430-450.

Van Treeck, U., Schmidt, F., and Wiedemann, B. (1981). Molecular nature of a streptomycin and sulfonamide resistance plasmid (pBP1) prevalent in clinical Escherichia coli strains and integration of an ampicillin resistance transposon (TnA). *Antimicrob Agents Chemother* 19**,** 371-380.

Van Veen, H.W., Venema, K., Bolhuis, H., Oussenko, I., Kok, J., Poolman, B., Driessen, A.J., and Konings, W.N. (1996). Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1. *Proc Natl Acad Sci U S A* 93**,** 10668-10672.

Veleba, M., Higgins, P.G., Gonzalez, G., Seifert, H., and Schneiders, T. (2012). Characterization of RarA, a novel AraC family multidrug resistance regulator in Klebsiella pneumoniae. *Antimicrobial agents and chemotherapy* 56**,** 4450-4458.

Webber, M.A., and Piddock, L.J. (2001). Absence of mutations in marRAB or soxRS in acrB-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of Escherichia coli. *Antimicrob Agents Chemother* 45**,** 1550-1552.

White, D.G., Zhao, S., Sudler, R., Ayers, S., Friedman, S., Chen, S., Mcdermott, P.F., Mcdermott, S., Wagner, D.D., and Meng, J. (2001). The isolation of antibiotic-resistant salmonella from retail ground meats. *N Engl J Med* 345**,** 1147-1154.

Wong, M.H., Chan, E.W., and Chen, S. (2015). Evolution and dissemination of OqxAB-like efflux pumps, an emerging quinolone resistance determinant among members of Enterobacteriaceae. *Antimicrob Agents Chemother* 59**,** 3290-3297.

Wong, M.H., Chan, E.W., Liu, L.Z., and Chen, S. (2014a). PMQR genes oqxAB and aac(6')Ib-cr accelerate the development of fluoroquinolone resistance in Salmonella typhimurium. *Frontiers in microbiology* 5**,** 521.

Wong, M.H., and Chen, S. (2013). First detection of oqxAB in Salmonella spp. isolated from food. *Antimicrob Agents Chemother* 57**,** 658-660.

Wong, M.H., Liu, M., Wan, H.Y., and Chen, S. (2012). Characterization of Extended Spectrum beta-lactamase Producing Vibrio parahaemolyticus. *Antimicrob Agents Chemother*.

Wong, M.H., Yan, M., Chan, E.W., Biao, K., and Chen, S. (2014b). Emergence of clinical Salmonella enterica serovar Typhimurium isolates with concurrent resistance to ciprofloxacin, ceftriaxone, and azithromycin. *Antimicrobial agents and chemotherapy* 58**,** 3752-3756.

Wong, M.H., Yan, M., Chan, E.W., Liu, L.Z., Kan, B., and Chen, S. (2013). Expansion of Salmonella Typhimurium ST34 clone carrying multiple resistance determinants in China. *Antimicrob Agents Chemother* 57**,** 4599-4601.

Woodford, N., Carattoli, A., Karisik, E., Underwood, A., Ellington, M.J., and Livermore, D.M. (2009). Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-M enzymes in three major Escherichia coli lineages from the United Kingdom, all belonging to the international O25:H4-ST131 clone. *Antimicrob Agents Chemother* 53**,** 4472-4482.

Woolhouse, M.E., and Ward, M.J. (2013). Microbiology. Sources of antimicrobial resistance. *Science* 341**,** 1460-1461.

Xiong, X., Bromley, E.H., Oelschlaeger, P., Woolfson, D.N., and Spencer, J. (2011). Structural insights into quinolone antibiotic resistance mediated by pentapeptide repeat proteins: conserved surface loops direct the

activity of a Qnr protein from a gram-negative bacterium. *Nucleic Acids Res* 39**,** 3917-3927.

Yamane, K., Wachino, J., Suzuki, S., Kimura, K., Shibata, N., Kato, H., Shibayama, K., Konda, T., and Arakawa, Y. (2007). New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an Escherichia coli clinical isolate. *Antimicrob Agents Chemother* 51**,** 3354-3360.

Yang, B., Qu, D., Shen, J., Xi, M., Zhi, S., Cui, S., Ji, B., and Meng, J. (2010). [Antimicrobial susceptibility and related genes of Salmonella serovars from retail food in Shaanxi province]. *Wei Sheng Wu Xue Bao* 50**,** 788-796.

Yang, B., Xi, M., Wang, X., Cui, S., Yue, T., Hao, H., Wang, Y., Cui, Y., Alali, W.Q., Meng, J., Walls, I., Wong, D.M., and Doyle, M.P. (2011). Prevalence of Salmonella on raw poultry at retail markets in China. *J Food Prot* 74**,** 1724-1728.

Yoshida, H., Bogaki, M., Nakamura, M., and Nakamura, S. (1990). Quinolone resistance-determining region in the DNA gyrase gyrA gene of Escherichia coli. *Antimicrob Agents Chemother* 34**,** 1271-1272.

Yoshida, H., Bogaki, M., Nakamura, M., Yamanaka, L.M., and Nakamura, S. (1991). Quinolone resistance-determining region in the DNA gyrase gyrB gene of Escherichia coli. *Antimicrob Agents Chemother* 35**,** 1647-1650.

Yu, F., Chen, Q., Yu, X., Li, Q., Ding, B., Yang, L., Chen, C., Qin, Z., Parsons, C., Zhang, X., Huang, J., Luo, Y., Wang, L., and Pan, J. (2011). High prevalence of extended-spectrum beta lactamases among Salmonella enterica Typhimurium isolates from pediatric patients with diarrhea in China. *PLoS One* 6**,** e16801.

Yuan, J., Xu, X., Guo, Q., Zhao, X., Ye, X., Guo, Y., and Wang, M. (2012). Prevalence of the oqxAB gene complex in Klebsiella pneumoniae and Escherichia coli clinical isolates. *J Antimicrob Chemother* 67**,** 1655-1659.

Zaman, S., Fitzpatrick, M., Lindahl, L., and Zengel, J. (2007). Novel mutations in ribosomal proteins L4 and L22 that confer erythromycin resistance in Escherichia coli. *Molecular microbiology* 66**,** 1039-1050.

Zhang, J., Wang, F., Jin, H., Hu, J., Yuan, Z., Shi, W., Yang, X., Meng, J., and Xu, X. (2015). Laboratory monitoring of bacterial gastroenteric pathogens Salmonella and Shigella in Shanghai, China 2006-2012. *Epidemiol Infect* 143**,** 478-485.

Zhao, J., Chen, Z., Chen, S., Deng, Y., Liu, Y., Tian, W., Huang, X., Wu, C., Sun, Y., Zeng, Z., and Liu, J.H. (2010). Prevalence and dissemination of oqxAB in Escherichia coli isolates from animals, farmworkers, and the environment. *Antimicrob Agents Chemother* 54**,** 4219-4224.

Zhao, S., Blickenstaff, K., Glenn, A., Ayers, S.L., Friedman, S.L., Abbott, J.W., and Mcdermott, P.F. (2009). beta-Lactam resistance in salmonella strains isolated from retail meats in the United States by the National Antimicrobial Resistance Monitoring System between 2002 and 2006. *Appl Environ Microbiol* 75**,** 7624-7630.

Zhong, P., and Shortridge, V.D. (2000). The role of efflux in macrolide resistance. *Drug Resist Updat* 3**,** 325-329.

Zou, Y.M., Ma, Y., Liu, J.H., Shi, J., Fan, T., Shan, Y.Y., Yao, H.P., and Dong, Y.L. (2015). Trends and correlation of antibacterial usage and bacterial resistance: time series analysis for antibacterial stewardship in a Chinese teaching hospital (2009-2013). *Eur J Clin Microbiol Infect Dis* 34**,** 795-803.