EXAMINATION OF FACTORS PROMOTING PROGRESSIVE EMERGENCE OF ANTIBIOTIC RESISTANCE AMONG MICROBIOTA STRAINS OF GASTROINTESTINAL TRACT

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Examination of Factors Promoting Progressive Emergence of Antibiotic Resistance Among Microbiota Strains of Gastrointestinal Tract

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CERTIFICATE OF ORIGINALITY

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Dachuan Lin (Name of student)

ABSTRACT

The benefits of antibiotic usage in treatment of human infection, which we have all enjoyed for decades, are being rapidly eroded by the gradual emergence of antibiotic resistance among bacterial pathogens, posing not only a huge economic cost to the society, but also threatening the lives of millions of patients worldwide. To devise a solution to this problem or at least slow down the rate of antibiotic resistance development, more studies need to be performed to investigate the nature and scope of the factors that promote antibiotic resistance development in bacteria, so as to devise novel approaches to enhance the effectiveness of antibiotic usage and prolong the shelf life of the currently used antimicrobial agents. The objective of this study is therefore to better understand the cellular mechanisms that govern expression of antibiotic resistance phenotypes among bacterial pathogens during the treatment process, especially among microbiota strains in the gastrointestinal tract (GI tract).

In order to study the mechanisms underlying the rapid increase in emergence of the antibiotic resistant strains, the first phase of this study involved investigation of various aspects of antibiotic resistance in China, so as to accumulate sufficient material and identify potential targets for subsequent experiments. Currently, antibiotic resistant strains may be divided into two categories: the hospital-acquired pathogens and the community-acquired resistant organisms. The first phase of my work therefore focuses on investigating the current status of the problem, with a particular emphasis on the nosocomial pathogens of Carbapenem-resistant enterobacteriaceae (CRE), and multiple drug resistant (MDR) isolates collected from meat samples. The first chapter of this thesis comprises five separate sections, beginning with studies on clinical CRE isolates, the results of which demonstrate the seriousness of the situation in hospitals. This is followed by three sections on antibiotic resistant *Salmonella* which is a food borne pathogen, in which we revealed not only a high isolation rates of the pathogen in meat products, but also a high resistance rate among those strains. Analysis on isolates recovered from meat samples hints on a close linkage between community acquired strains and the nosocomial strains, suggesting that a significant proportion of drug resistant clinical strains originated from food animals.

In order to delineate mechanisms behind the rapid development of resistance, we then tested the range of mutations that may occur in selected antibiotic target genes,

and factors promoting acquisition of extra antibiotic resistance genes. The second chapter describes the range of antibiotic target mutations detectable among gut microbiota strains subjected to antibiotic selection pressure. The first section of the chapter is on the effect of *in vivo* mutation induction by sub-inhibitory concentrations of antibiotics; we showed that low doses of antibiotic were sufficient to induce mutational changes, often producing identical target mutations. That finding allows us to conclude that a specific cellular mechanism in bacteria is involved in active generation of target mutations. Following this finding, the second section of this chapter demonstrated the role of biofilm formation in resistance development upon *in vivo* ciprofloxacin treatment. Our results indicate that production of a biofilm is essential or a prerequisite for mutational changes.

The third chapter comprises three sections. The first reports result of investigation of CRE strains in food animals China, which allow us to conclude that the carbapenemase genes could readily be transferred without carbapenem selection pressure. The second section reports on analysis of the response of GI tract strains to antibiotic treatment, with results showing that dissemination of the antibiotic resistance genes occur even during the parenteral administration. Based on this finding, we were eventually able to identify factors which play a role in promoting transmission of antibiotic resistance genes among the gut microbiota strains.

PUBLICATIONS RELATED TO CURRENTLY COMPLETED WORKS

Increasing prevalence of hydrogen sulfide negative *Salmonella* in retail meats

Dachuan Lin, Meiying Yan, Song Lin, Sheng Chen

Food Microbiology. Volume 43, October 2014, Pages 1-4.

Selection of target mutation in rat gastrointestinal tract *E. coli* by minute dosage of enrofloxacin

Dachuan Lin, Kaichao Chen, Ruichao Li, Jiubiao Guo, Wen Yao, Sheng Chen

Front Microbiol. Volume 5, September 2014, Pages 468 .

First detection of conjugative plasmid-borne fosfomycin resistance gene *fosA3* in *Salmonella* isolates of food origin

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Antimicrob Agents Chemother. Volume 59(2), February 2015, Page 1381-3.

Increasing prevalence of ciprofloxacin-resistant food-borne Salmonella strains harboring multiple PMQR elements but not target gene mutations

Dachuan Lin, Kaichao Chen, Chan EW-C, Sheng Chen

Scientific reports, Volume 5, October 2015.

IncFII conjugative plasmid-mediated transmission of blaNDM-1 elements among animal-borne E. coli strains

Dachuan Lin, Miaomiao Xie, Ruichao Li, Kaichao Chen, Chan EW-C, Sheng Chen

Antimicrobial Agents and Chemotherapy**,** Volume 61, January 2017, e02285-16.

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In the next phase of my study, I will try my best to complete works of my research programme and not let the people around me down.

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LIST OF ABBREVIATIONS

µl: Microliter

AST: Antimicrobial susceptibility test

BHA: Brain Heart Agar

BP: Base Pair

CLSI: Clinical and Laboratory Standards Institute

ESBLs: Extended-spectrum beta-lactamases

g/L: Grams/Litre

AMRB: Antimicrobial-resistant bacteria

HGT: Horizontal gene transfer

MDR: Multiple-drug resistance

XDR: Extensively drug-resistant

RDR: Pandrug-resistant

mg/L: Milligrams/Liter

MIC: Minimal Inhibitory Concentration

PCR: Polymerase Chain Reaction

PFGE: Pulsed field gel electrophoresis

1 Literature Review

The discovery of antibiotics has brought us a wide range of benefits, among which the most important is their ability to save the lives of people from bacterial infections. In addition, antibiotics have provided us affordable meat products by improving animal feed efficiency. Meanwhile, antibiotic usage also has a negative impact , namely antibiotic resistance. Both the rate of resistance development and dissemination of resistant strains haverisen so dramatically that resistance emerges almost the same time as clinical application of a new antibiotic is launched. The problem of antibiotic resistance has -worsened to a level that it has become a major threat to human survival, with the number of effective antimicrobial agents available for treatment of infections decreasing continuously. Antibiotic resistance costs billions of dollars every year. A number of old “miracle drugs” which were extremely effective years ago -are now ineffective against many kinds of bacteria. Multiple new terminologies have appeared such as the multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (RDR). Even people who have never read any medical literature know the word super bacteria, which have been found in healthcare-associated settings because of the usage of antibiotics [1, 2]. The emergence of resistance results in a corresponding increase in the prevalence of incurable infection not only in the developing countries, but also in the developed countries[3]. Hence the evolution of microbial pathogens, especially those resistant to antibiotics, is one of the most urgent crises that needs to be addressed [4]. It was reported that in the USA alone, antibiotic resistant bacteria cause at least 2 million infections and 23,000 deaths each year [5]. Although antibiotic resistance has been recognized as a major problem in the past decade[6], no solution has been devised, and the discovery of an antibiotic is often followed by the emergence of its resistance[7]. In addition, scientists have shown that antibiotic resistance actually appears much earlier than the discovery and usage of antibiotics[8, 9]. People believe that drug resistance will continue to emerge in a large scale because bacteria are able to adapt to antibiotic selection pressure. Such selection pressure is generated not only by clinical usage of antibiotics, but also through agricultural usage of large amounts of antibiotics, which imposes strong selective pressure on potential human pathogens, promoting dissemination of both resistance genes and resistant strains [10].

The worrying situation that infections are increasingly caused by antimicrobial-resistant pathogens is also associated with a significant and increasing rate of morbidity and mortality, and higher treatment costs and longer stays among hospitalized patients. To date, there are very few ways to deal with the upsurge in multidrug resistant bacteria[11, 12].

1.1 Antibiotic resistance affects everybody in the world

Like climate changes, everyone is affected by antibiotic resistance. It is hard to explain the complex underlying basis for development of antibiotic resistance simply by human behaviour, for example the excess usages of antibiotics, yet everybody should be blamed for [13]. Mutations that can induce antibiotic resistance have been identified long ago, but their relationship with antibiotic usage could not be established. Lederberg in 1952 proved that the mutations happened by chance using the Replica plating method [14]. However, we must also admit that usage of antibiotics has induced or accelerated this process. In the 1940s, patients were treated with penicillin of 8 units per ml [15], but now the amounts being used are in the range of millions of units [16], even for infants who have never encountered any antibiotics [17]. Hence it does not mean that if a person insists not to use antibiotics then he/she will only be infected by sensitive organisms. No one could be excused from being affected by antibiotic resistance. Everyone is threatened by the resistant bacteria that reside in the environment. For example, those recoverable from animal products are derived from animals treated with antibiotics, or those in soil or water which have been polluted by the pharmaceutical industry, even if someone could refuse to use antibiotic himself.

Scientists are working to prevent the risk of a post-antibiotic era in which no antimicrobial treatment options are available for treating infections[18]. This is almost certainly coming because of the rapid decrease in antibiotic efficiency in recent years. We will use concrete examples and statistical data to illustrate the scale of the risk.

1.1.1.1 The concrete example for the emergence of antibiotic resistance

Three rapidly disseminating pathogens are discussed below. USA300 strain: a strain of methicillin resistant *Staphylococcus aureus* (MRSA) is identified as the

primary types that cause community-acquired MRSA infections in USA. According to a previous survey of *Staphylococcus aureus* –mediated skin and soft-tissue infection, MRSA accounted for 72% (279 out of 389 episodes) of the cases[19], and 90% of MRSA that could be typed by PFGE are USA300. The result caused great worries because it means traditional usage of antibiotics will become ineffective in treating serious skin and soft-tissue infections. The study by Matthew *et al* revealed the prevalence of nasal colonization as well as an increase in MRSA colonization, despite the fact that the incidence of total colonization of *S.aureus* decreased[20]. This data caused more worries because this was a nationally representative sample of the USA citizens, that means everyone has the potential to be evolved. The high rate of antibiotic resistance poses a huge concern as the statistical data of Friedrich *et al* show[21], in which a higher mortality of MSSA infections was recorded when the infective agents were accompanied with the MRSA infections.

Serious antibiotic resistance also occurs in Gram negative strains, for example the *Escherichia coli* ST131, which is a classic extended-spectrum -lactamase (ESBL) strain. In a Yearly Susceptibility Test (America), nearly half (54/127) of the test isolates were identified as ST131, of which 52% were multidrug-resistant [22]. The ST131 strain is not only a common MDR strain but also a highly virulent strain that frequently causes death [23]. Studies also showed that, contrary to the sporadic nature of an epidemic, the prevalence of ST131 is now called a 'pandemic', representing emergence worldwide [24]. According to the previous statistics, the most common CTX-M type enzyme worldwide is CTX-M-15; carbapenems would be as the last choice for treating serious infections caused by resistant organisms producing this enzyme [25].

Using new antibiotics was once a good strategy to deal with antibiotic resistance in the past decades. However, new resistant strains would appear almost immediately. Interestingly, previous data indicated that combined usage of third-generation cephalosporins and aztreonam was associated with a significant increase in carbapenem resistance of *Acinetobacter* [26]. Soon after the first detection of KPC-producing *K. pneumoniae* in the USA[27], different types of CRE were reported all over the world, such as *Klebsiella* ST258, which is also a MDR strain with high virulence, and could be found in different countries[28]. Importantly, it is a common

carbepenem resistant isolate[29]. The emergence of resistance is much faster than the discovery of new antibiotics, so people have to choose the old drugs such as colistin, and the colistin resistant *Klebsiella* ST258 strain has appeared as a consequence[30]. What is more, the increasing prevalence of *K. pneumoniae* ST258, interspecies transfer to other *Enterobacteriacae* also increased, consequently reducing the carbapenem efficiency[31].

1.1.1.2 Statistical data of antibiotic resistance

From the examples discussed above, we could see that emergence of new antibiotic resistant strains is so quick that few choices are left for the treatment of bacterial infections. In this section, statistical data was used to illustrate that the old strategy of searching for new miracle drugs is becoming impossible.

Figure 1.1.1 illustrates why the old way to count on new drugs is not suitable. As the number of approved antibiotics decreased significantly in the past three decades, people have to re-discover the old drugs. However, the decreasing number of effective drugs and the increasing number of newly discovered antibiotic resistance genes, such as those encoded for β-lactamases, have made discovery of new antibiotics increasingly difficult (Figure 1.1.2). With the rapid increase in antibiotic resistance, we may face the desperate situation in which treatment of infections completely fails within the coming few years. The good news is that during past decades more and more scientists are working on this field and the number of publications in the Scopus databases are also increasing significantly.

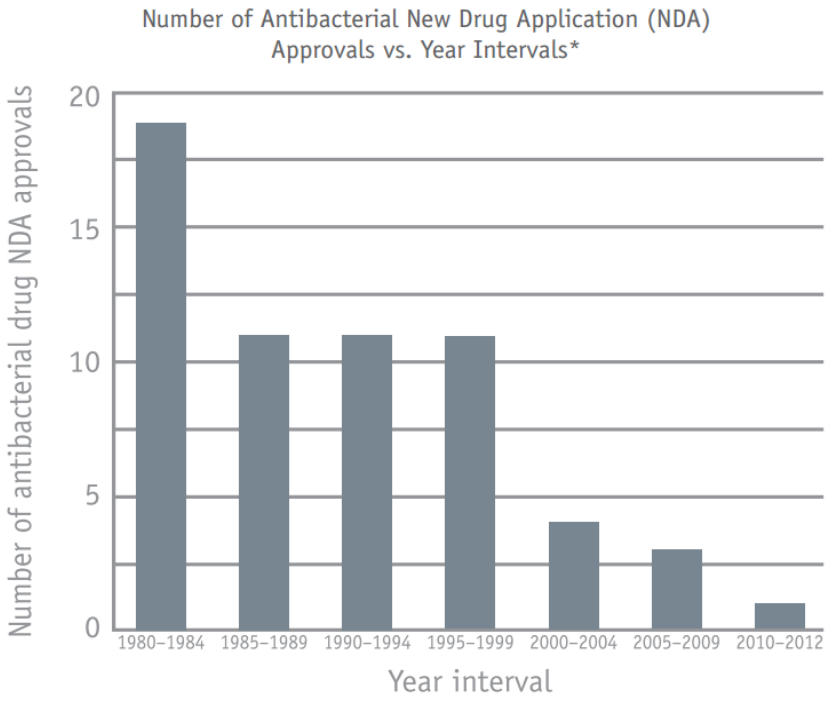

Figure 1.1. 1 The number of approved antibacterial drugs.

*Data courtesy of FDA's Center for Drug Evaluation and Research (CDER)

CDC 2013[32]

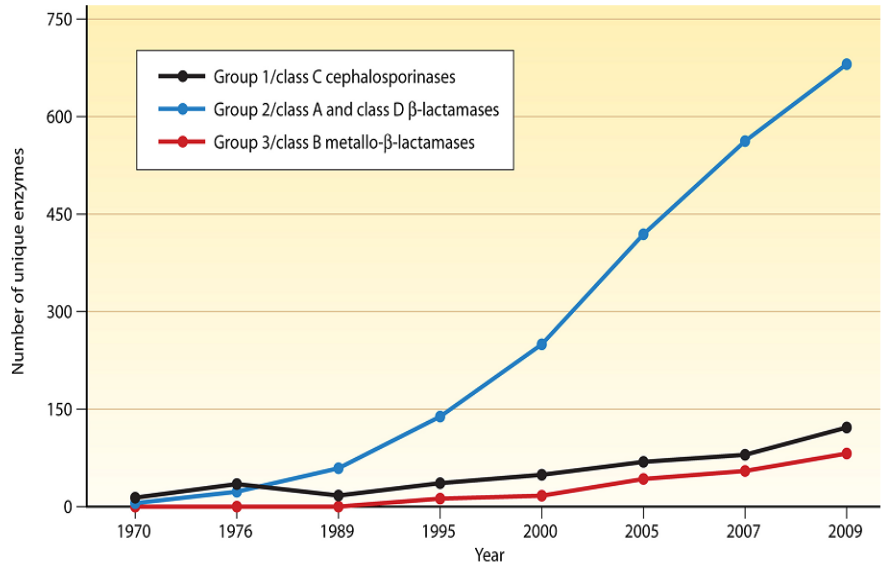

Figure 1.1. 2 Increase in the number of lactamases from 1970 to 2009[33]

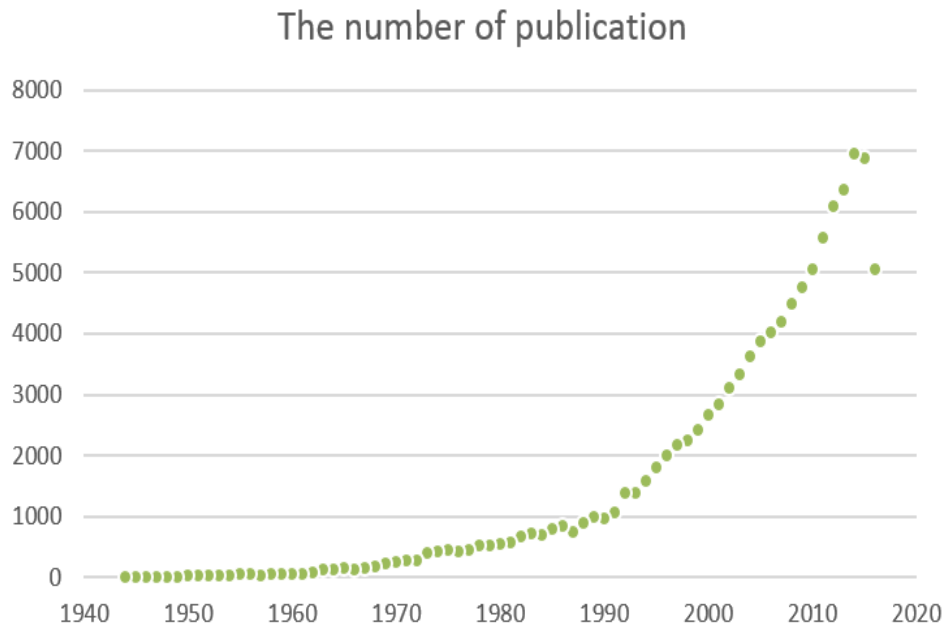

Figure 1.1. 3 The number of publication in Scopus.

Using the TITLE-ABS-KEY “antibiotic resistance”.

1.1.2 Resistance is spreading worldwide

Increasing in antibiotic resistance is being reported not only in the developing countries but also in the developed countries. The most widely distributed CTX-M enzyme was first detected in India in 2001 [34], and the most common carbapenemase NDM was also first found in India in 2008[35]. There are many campaigns in the developed countries to educate the public about the appropriate use of antibiotics, hoping to provide effective interventions [36]. It is a good attempt although the outcomes remain unclear. However, education alone is not enough, because tackling antibiotic resistance needs united actions of the whole world. For example, an estimated 25,000 death are caused by antibiotic resistant isolates in Europe annually[37], and MRSA alone is associated with 19 000 deaths in the USA in 2005[38]. As the resistant strains may reside as a reservoir of normal flora in healthy human and animal populations, their transmission by travellers and migrating animals is extremely fast. Examples include the transmission of $bla_{\text{NDM-1}}$ within in a few years after its first discovery, resulting in its emergence in every country in the world[39, 40].Numerous studies have shown that the resistance levels become unacceptable [41]. There is no safe place in world that allows us to avoid antibiotic resistance. For

example, drug resistant *E. coli* was found among birds in the Arctic, which is one of the last outposts of wilderness [42].

1.1.3 The high burden of antibiotic resistant infections

Infections caused by antibiotic-resistant pathogens are often fatal. The estimated prevalence of morbidity and mortality is high in the developed countries as mentioned above. However, it is even higher in the developing countries. For example, compared with the industrialized countries, the neonatal infections were 3-20 times higher in the developing countries [43]. In addition to the higher mortality rates , the economic loss was even more serious in the developing countries, as the new antibiotics are much more expensive than the old ones. At least 1.5 billion euros each year were lost due to MDR strains in Europe [37]. The same happens in the USA, where it is reported that the annual loss caused by the MDR strains would be 20 billion US dollars, which are even higher than the loss caused by HIV-related diseases [44]. 1.1.4 The usage of human and agriculture antibiotics is increasing

Although there is a lack of the exact number, during the past 60 years at least millions of metric tons of antibiotics have been consumed [10] . In addition, millions of tons of antibiotics have been released to the environment. Lots of antibiotics are used in the veterinary field, for example, 71% of the total antibiotic consumption in Denmark in 2010 was for animal production [45]. This causes many problems because resistance genes can be transferred from bacteria in animals to human pathogens. It was reported that 17.8% to 70% of antibiotics are used in animal production [46] . However, it is hard to calculate the quantity of antibiotics used in animals because some antibiotics are used more often in human. For example, in Chile, the quantity of quinolones used in human is 10 times of that used in the veterinary field. The World Health Organization estimates that nearly half of the total amounts of antibiotics is used in food animals [47]. In fact, most of the antibiotics used in the veterinary field are used for growth promotion [48] .

Despite the fact that in some countries the use of antibiotics increased and in some countries it has decreased [49], the total consumption has increased by 36%, which Includes the last-defense agents cabapenems and Polymixins [50]. Althoughlack of direct evidence that antibiotic resistance is frequently associated with

the usage of antibiotics [51, 52], it is almost certain that increasing usage will result in a rise in antibiotic resistance rates. For example the ceftazidime sensitivity portion has decreased from 83% to 54% accompanied with increasing usage of ceftazidime [53].

1.2 Epidemiology of antibiotic resistant pathogens

Based on the Centers for Disease Control and Prevention (CDC) guidelines, infections could be divided into "Community-onset" and "nosocomial'' . Accordingly, pathogens also can be divided into the community pathogens and nosocomial pathogens [54]. Previous studies have indicated that the antibiotic resistance rate in hospitals is high. For example, the rate of resistance to carbapenems has reached about 50%, and carbapenem resistant strains could be detected in all hospitals [26]. Common belief that nosocomial pathogens caused a high death rate, and the community pathogen caused an even larger number of illnesses. It is difficult to distinguish between the two in recent years. For example, MRSA, a nosocomial pathogen frequently found in hospital-based outbreaks, is now an emerging community pathogen[55]. The distinction between community-acquired and hospital-acquired MRSA is becoming more and more blurred worldwide[56]. Previous studies show, the community has an increasing prevalence of infections by MDR strains, implying that antibiotic resistance is no longer limited in hospital [57] .

1.2.1 The prevalence of CRE

As mentioned above, the ESBL strains have been found around the world and carbapenems are supposed to be the last resort to treat infections caused by such organisms [58]. However, the increasing prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) worldwide strongly reduces the effectiveness of these last defense agents, such as the *Klebsiella pneumoniae* carbapenemase (KPC) in the United States [59].

Infections caused by CRE are big changes because they are difficult-to-treat and with high mortality [60]. A previous study showed that compared with the prevalence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) in 2000, the prevalence

expanded 8 times in 2007 (New York and New Jersey) [61] . Previous studies also indicated that clonal transmission within and between the hospitals occur [62].

Until now CRE is rarely found in the community and only sporadic cases are reported. However, CRE may still contaminate food and the environment. In addition CRE in community exhibits an increasing incidences, for example the detection rate was 5 times higher in 2012 than in 2008 in the south eastern United [63].

1.2.2 The incidence of antibiotic resistant *Salmonella*

It was estimated that 76 million foodborne illnesses and 5000 annual deaths occur merely in the United States [64] , with *Salmonella* being one of the three major food pathogens that caused most of the deaths [65] .Salmonella is not only a zoonotic disease but also a food-borne disease. Salmonella spp have a wide range of hosts including the major livestock species, but colonization in animals is often asymptomatic, therefore contamination of meat is frequent and often overlooked [66]. Frequent reports of outbreaks of food-borne salmonella infections mean the adaptation and evolution of the this pathogen have potential serious consequences [67]. The isolation of antibiotic-resistant Salmonella from retail meat has been reported several decades before, and the derived frequency of antimicrobial resistance in Salmonella has been reported 30 years ago [68]. The frequency of resistance in the 1980s has increased to 24% compared with in the 1970s. Yet variations in resistance types and mechanisms have only been observed recently. In addition, with the decreasing sensitivity of nalidixic acid and ciprofloxacin, extended spectrum beta lactamase (ESBL) resistance has also risen sharply worldwide [66]. Resistant strains of Salmonella has reached such a very high level that according to one survey, 84% of isolates were resistant to at least one antibiotic and 53% were resistant to at least three antibiotics [69] . Previous studies have demonstrated that resistant strains cause higher morbidity and mortality.

1.3 General mechanism of antibiotic resistance

The resistance mechanisms for all the 15 different classes of antibiotics have been reported [70], indicating that appearance of the super strains that are resistant to all the antibiotics is possible. Compared with intrinsic resistance, most resistant

organisms exhibit acquired mechanisms of resistance to antibiotics [71, 72]. Among the four known mechanisms, the most common ones are “changes in the structure and protection of antibiotic targets” and “direct modification or inactivation of antibiotics” [73]. Emergence of antibiotic-resistant bacteria is majorly determined by a combination of de novo mutations and horizontal gene transfer(HGT) of mobile resistance elements [74]. It has been reported that in parts of southeast Asia and China, 60-70% of *E. coli* isolates are resistant to fluoroquinolones [75]. Mutations that confer antimicrobial resistance can occur in different parts of the genome, and fluoroquinolone (FQ) resistance is usually caused by mutations within the quinolone resistance-determining regions(QRDR) [76] .It has been reported that most antibiotic resistance genes reside on conjugative plasmids, regardless of whether they are Gram-negative or gram-positive bacteria [77, 78] . The genome location of resistance genes also can transfer mediated by the mobile genetic elements Including plasmids, integrons and transposable elements [79] . The enterococcal genome sequences proved the role of plasmid in the development of multidrug resistant [80]. Becasue many conjugative plasmids carry beneficial traits, they are generally regarded as the key agents of horizontal gene transfer (HGT) [81] .

1.4 The gut is an important reservoir of antibiotic resistant organisms

The microbiome in the GI tract is normally regarded as a reservoir of antibiotic resistance, because it is the most densely populated microbial ecosystem on earth, facilitating horizontal transfer of antimicrobial resistance genes (AMR)[82]. The broad-spectrum antibiotics can have a significant impact on non-target microbes, enriching the pool of resistance genes available for pathogens[83]. Previous studies have shown not only AMR affects the health of human microbial flora [84], but also honeybees worldwide[85]. To date, however, factors promoting horizontal gene transfer are not well-known.

1.5 Brief introduction of the contents of this thesis

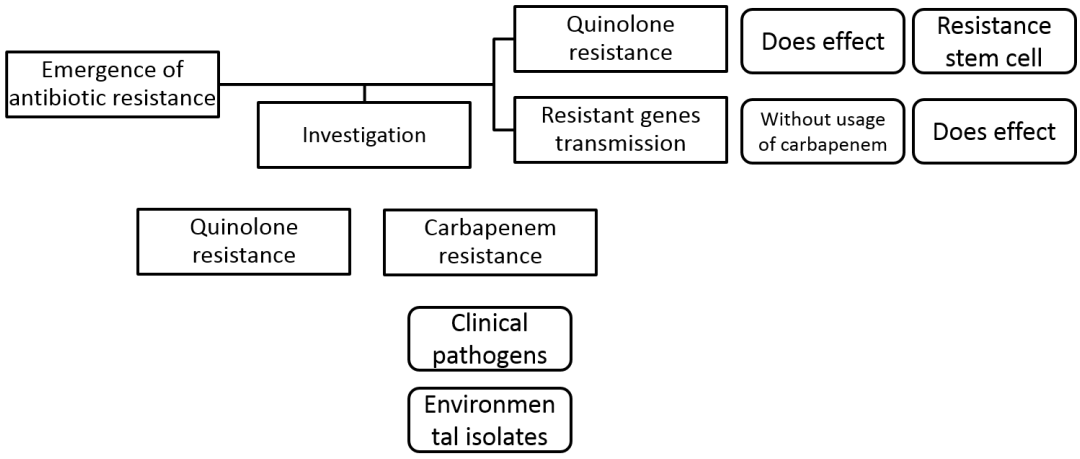

Figure 1.1. 4 Structure of this study

As mentioned previous, there are many kinds of mechanisms for the different antibiotic. This study picked up quinolone and carbapenem as represent antibiotics to do study, because quinolone was recommended to treat low risk infections and carbapenem was recommended to treat high risk infections[86]. Antibiotic-resistant is an old phenomenon that antibiotic-resistant microbes date back to millions years ago[87], this study only focused on the latest situation. To better understand the current resistance situation, this study first investigated the resistance phenomenon of the two representative antibiotics and then the major mechanisms of the two kinds of antibiotics in the GI tract.

For the quinolone resistance, the first section mainly introduces the ciprofloxacin-resistant food-borne Salmonella strains, as quinolone was recommended to treat low risk infections. For the carbapenem resistance surveillance, begin with the carbapenem resistance clinical isolates as carbapenem mainly used to treat high risk infections. These hospital-acquired pathogens cause potentially life-threatening infections [88]. It is known that most of the cases of ICU-acquired bacterial infection are caused by MDR or XDR[89]. In this study, among the 31 of the MDR CRE strains that we received, and we found some of them even carried multiple carbapenemase genes. In recent years the sporadic incidence are not only found in clinical isolates but also the in community[90], especially the novel carbapenemase New Delhi metallo-β-lactamase (NDM). This study analysed the prevalence of

carbapenem resistant strains isolated from different food products to predict the dissemination patterns of the carbapenemase genes, and reveal the genetic characteristics of those isolates in order to identify the antibiotic resistance mechanisms concerned. The worldwide increasing trend of Carbapenem resistance in nosocomial isolates cause serious problem because it dramatically limits the choice of therapeutic method[91, 92]. This study found a high antibiotic resistance prevalence rates in both nosocomial and community isolates. Previous studies had suggested that the antibiotic resistant zoonotic bacteria from food animals could potentially infect human through meat consumption[93].

Previous reported bacteria evolve resistance to antibiotics by one of two routes: spontaneous mutation and horizontal gene transfer[94]. In the second phase of this study, we attempted to probe the origin of the quinolone resistance, and the factors in this process, and third phase is the horizontal gene transfer. This part comprises two sections. Mutations are known to emerge spontaneously in bacteria, but target mutation rates in pathogens appear to be higher when there is antibiotic selection pressure. We need to determine the mechanisms that endow the strains a high potential to develop target mutations and how antibiotic usage stimulates the development of the target mutations. This was achieved by studying the resistance selection effect of minimum dosage of enrofloxacin in vivo. The second section focus the strains factors. This section focus on the strains have higher abilities to generate resistance in GI tract. Many studies mostly focus on the epidemiological surveys of antibiotic resistant strains and new mechanism of antibiotic resistance. Although mutations happen spontaneously in bacteria, whether the underlying mechanisms of the target mutations in pathogens that may help the strains to survive antibiotic treatments share some common functional characteristics are not well defined. A model of SPF rats with and without target mutations was built to discovers the mechanisms that endow the strains the ability to develop target mutations and how antibiotic usage stimulates the development of target mutations. In vivo experiment showed the strains with biofilm and *hipAB* genes performed better performance under quinolone selection pressure. In vitro experiments, the results between different batches and even between different animal experiments were consistent. What is more, the WGS (Whole genome sequencing) data in the NCBI database also proved the

conclusion. We found that very low doses of antibiotics can affect the prevalence of resistant strains in the animal.

Many studies suggest that intestinal MDR colonization can predispose clinical infection, especially among patients concomitantly colonized with multiple antibiotic-resistant pathogens[95]. The third part focuses on the antibiotic resistance genes transmission in the GI tract. Since the discovery of *bla*$_{\text{NDM-1}}$, it has been widely detected in clinical isolates of various species. However, it is rarely detected in Enterobacteriaceae in animals. This study aims to investigate the prevalence, transmission dynamics and genetic characteristics of plasmids harbouring the *bla*$_{\text{NDM-1}}$ gene in *E coli* strains isolated from farmed animals to provide information regarding the origin of this important resistance determinant. Our data showed that *E. coli* harbouring the *bla*$_{\text{NDM-1}}$ gene can only be detected in fattening pigs in one of the ten farms surveyed in the study. In this farm, strains recoverable from different animals often exhibited identical PFGE and MLST patterns. However, *E. coli* strains with different PFGE and MLST patterns were also observed among strains recovered from the same animal, suggesting that clonal expansion and horizontal transfer of mobile resistance elements were both responsible for the widespread dissemination of the *bla*$_{\text{NDM-1}}$ gene in animal E. coli strains. Two IncFII *bla*$_{\text{NDM-1}}$-encoding plasmids with only one MDR region difference were found to be responsible for the transmission of *bla*$_{\text{NDM-1}}$ in these strains. The *bla*$_{\text{NDM-1}}$ gene can be incorporated into plasmids and stably inherited in animal-borne *E. coli* strains that can be maintained in animal gut microflora even without carbapenem selection pressure. Such organisms should be closely monitored for signs of dissemination to human. The second section is about the effect of commercial antibiotics on *E. coli* in the swine GI tract. Antibiotic regimens designed to treat bacterial infection rarely take into account their effects on the population structure of the complex microbiome in the human body, in particular the possibility of selective enrichment of multidrug-resistant organisms. To address this issue, we investigated the changes in the size and antibiotic susceptibility profiles of antibiotic resistant sub-population organisms in the animal gastrointestinal tract upon variation in the level of antibiotic stress imposed onto the gut flora. Most of antibiotics have been applied to patients or animals and the most common way to absorb or excrete them is through the GI tract, hence it is a much-discussed topic to

study antibiotic resistance in the GI tract[96]. The dense microbial community in the gastrointestinal tract represents a necessary yet highly complicated system for analysis of the resistance induction effect of antibiotic treatment. Compared with the important role in the dissemination of antibiotic resistance genes, few studies have identified factors that could regulate this process. This study utilized a range of plasmids which harboured different antibiotic resistance genes to test different factors that might be related to the transmission of such resistance genes.

1.6 Limitations of the current study

As a number of complex factors interact to promote development of antibiotic resistance, observation of different phenomena in this study can only partially explain the mechanisms concerned. Overall, this study hopes to deliver the latest antibiotic resistance statistical data to validate the theory that GI tract microflora plays a key role in the development of antibiotic resistance development, and depict the possible mechanisms concerned, so as to allow us to better understand the molecular mechanisms behind the rapid increase in antibiotic resistance among bacterial pathogens.

2 Investigation on antibiotic resistance mechanisms

2.1 Investigation of quinolone resistant Salmonella

Foodborne salmonellosis is one of the leading causes of foodborne illnesses worldwide. Although antimicrobial treatment is usually not necessary due to the self-limiting nature of salmonellosis, it can be lifesaving in cases of invasive infections[97], with ceftriaxone and ciprofloxacin being the key drugs of choice[98]. Resistance to ceftriaxone or other extended spectrum beta-lactams is usually due to intracellular production of extended spectrum β-lactamases (ESBLs) such as the CTX-M group and AmpC β-lactamase, including the CMY-2 enzyme, which are usually located on transmissible plasmids that tend to disseminate among members of Enterobacteriaceae[69, 99]. Prevalence of resistance to ceftriaxone in Salmonella appears to be slowly increasing, reaching a rate of around 3 ~ 4% at present[100]. However, the rate of resistance to ciprofloxacin has increased dramatically both in

clinical and food isolates around the world, in particular in China and its adjacent areas[101]. Ciprofloxacin resistance is mainly attributed to double mutations in the *gyrA* gene and single mutation in the *parC* gene in Salmonella[102, 103]. Efflux pumps and the presence of plasmid-mediated quinolone resistance (PMQR) determinants have also been regarded as contributive factors of development of low level resistance to nalidixic acid. At least three types of PMQRs have been reported including (i) the Qnr types, which are pentapeptide repeat proteins that bind to DNA gyrase by mimicking double stranded DNA to prevent fluoroquinolone from binding to gyrase, (ii) Aac(6′)-Ib-cr, a modified aminoglycoside acetyltransferase that hydrolyzes fluoroquinolones and (iii) the efflux pumps QeqA, and OqxAB. Unlike E. coli and various other members of Enterobacteriaceae, the development of mutations in the gyrA and parC genes in Salmonella is known to be a very slow event, resulting in an unusually low level of ciprofloxacin resistance in Salmonella. On the other hand, although PMQRs were commonly detectable in Enterobacteriaceae, in particular in E. coli, prevalence of PMQRs in Salmonella remains extremely low. To date, a few types of PMQRs Including qnrA, qnrB, qnrD, and qnrS alleles have been reported in a limited number of studies[104-109]. Recently, *oqxAB*, a new PMQR gene which was originally identified on a plasmid (pOLA52) recoverable from *E. coli*, was first reported in Salmonella isolates of food origin. The mobile efflux pump OqxAB 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[110]. Exhibiting the ability to enhance the MICs of olaquindox, ampicillin, quinolones and chloramphenicol[101, 111], this element was subsequently found to be increasingly prevalent among Salmonella isolates recoverable from different sources after the year 2006[101, 112]. The oqxAB operon was suggested to play a functional role which helps accelerate the development of ciprofloxacin resistance in Salmonella, and was hence considered to be responsible for causing the recent dramatic increase of ciprofloxacin resistance in clinical Salmonella strains[113]. The combination of PMQR such as oqxAB and a single target gene mutation, in particular in the gyrA gene, could possibly mediate development of resistance to ciprofloxacin in Salmonella, and dramatically reduced the time required for the development of a resistance phenotype associated with generation of double gyrA mutations and single parC mutation. This idea is supported by the observation of an increasing prevalence

of different PMQR genes in various species of Enterobacteriaceae, and the emergence of ciprofloxacin-resistant E. coli and Salmonella strains carrying multiple PMQRs without target mutations[114]. In this study, we reported a high prevalence of ciprofloxacin resistant Salmonella strains in food samples, most of which were found to harbor either only a single mutation in gyrA, or no mutation in both target genes. However, the strains commonly contained multiple PMQR genes, with the most prevalent being the oqxAB and aac(6′)-Ib-cr elements. This novel phenomenon, in which one bacterial resistance mechanism promotes the onset of another, signals a risk of aggravation of the clinical problem of ciprofloxacin resistance in Salmonella. The current situation warrants a need for continuous surveillance of the prevalent mechanisms of ciprofloxacin resistance in Salmonella in order to better understand the genetic background of this new category of resistant organisms.

A total of 82 Salmonella strains were isolated from chicken and pork samples purchased from supermarkets and wet-markets in Shenzhen, China during the period from November 2012 to June 2013. These Salmonella strains were subjected to further characterization of their antimicrobial resistance to various antibiotics (Table 2.1.1), and the underlying resistance mechanisms. Overall, these strains exhibited a very high rate of resistance to most of the antibiotics tested. The resistance rates of the three most important front line antibiotics (ceftriaxone, ciprofloxacin and azithromycin) were 10%, 39% and 25% respectively, which were significantly higher than that reported in other countries. Salmonella strains isolated from pork samples exhibited a higher rate of resistance to most of the antibiotics tested when compared with Salmonella chicken isolates, in particular ciprofloxacin (Table 2.1.1). Surprisingly, chicken Salmonella isolates exhibited a much higher rate of resistance to ceftriaxone (35%) than the pork isolates (11%). This phenomenon is probably due to the high rate of resistance to ceftriaxone in S. Indiana. Among the different serotypes tested, S. Indiana also exhibited the highest rate of resistance to most antibiotics, including the three front line drugs of ceftriaxone, ciprofloxacin and azithromycin. Such phenotype has only been reported in S. Typhimurium and S. Kentucky previously[101, 115]. S. Typhimurium and S. Derby also exhibited a very high resistance rate except that S. Typhimurium did not exhibit resistance to ceftriaxone. Two serotypes, namely S. Heidelberg and S. Rosentha, exhibited an intermediate rate

of resistance to the tested antibiotics. On the other hand, resistance was less commonly observed among S. Enteritidis. Another important observation was that, among the 32 ciprofloxacin-resistant strains, all were resistant to ampicillin, nalidixic acid, kanamycin, streptomycin, chloramphenicol, tetracycline and sulfamethoxazole; furthermore, the MIC of olaquindox was generally 32 mg/L or higher for these strains. We also observed that among such isolates, up to 84% were resistant to gentamicin, 25% were resistant to azithromycin (MIC≥ 32 mg/L), and 13% were also resistant to ceftriaxone (Table 2.1.2). Mechanisms of resistance in selected strains, in particular those mediating resistance to the front line antibiotics such as ceftriaxone and ciprofloxacin, were investigated. Resistance to ceftriaxone was detectable in 8 Salmonella isolates including 4 S. Indiana, 2 S. Heidelberg, 1 S. Enteritidis and 1 S. Derby. These 8 strains were examined for their ability to produce Extended-spectrum β-lactamases and AmpC β-lactamases, and diverse resistance mechanisms were observable. Three out of the four S. Indiana strains were found to contain *bla*$_{\text{CTX-M-65}}$, with the fourth one harboring the *bla*$_{\text{CMY-2}}$ gene. The *bla*$_{\text{CTX-M-55}}$ gene was detectable in one S. Enteritidis and one S. Derby strain. For the two S. Heidelberg isolates, the *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{CMY-72}}$ genes were each detectable in one strain (Table 2.1.2). A total of 32 ciprofloxacin-resistant Salmonella strains were subjected to investigation of the mechanisms involved. Contrary to the resistance mechanisms commonly observable in clinical ciprofloxacin resistant strains, in which double and single mutations often occur in the gyrA and parC genes respectively, most of the 32 ciprofloxacin resistant Salmonella strains tested in this work were found to contain either only a single mutation in gyrA, with S83T, S83F, and D87N being the most common amino acid changes, or no mutation in either target genes (Table 2.1.2). The few exceptions were all S. Indiana isolates which harbored the double gyrA mutations S83F and D87N, with or without the single parC mutation S80R. It should also be noted that a pair of novel double gyrA mutations which resulted in the H80N and S83T changes, and single parC mutation causing the Q91H substitution, were detectable in a S. Rissen isolate; however, the roles of such mutations in development of Salmonella fluoroquinolone resistance are not well defined at present. Other less common mutations that were detectable include the C72G change in the parC gene product of a S. Indiana strain, and a S83I change in the GyrA protein of a S. Derby isolate. The nature of contribution of these novel mutations to the development of ciprofloxacin

resistance in Salmonella needs further investigation. No mutations were detected in gyrB or parE. The presence of PMQR genes in ciprofloxacin resistant Salmonella isolates were also screened by PCR and sequencing (Table 2.1.2). Surprisingly, all isolates were found to carry PMQRs, with oqxAB and aac(6′)-Ib-cr, the most prevalent elements, reaching a rate of 91% and 75% respectively. Other PMQR genes detectable included qnrS (66%), qnrB (16%) and qnrD (3%). The most common PMQR combination observable was aac(6′)-Ib-cr-oqxAB-qnrS2, which accounted for 50% of all the ciprofloxacin resistant Salmonella strains tested. To determine if other resistance mechanisms such as efflux activities contribute to ciprofloxacin resistance in such isolates, the MIC of ciprofloxacin against these isolates was determined in the presence and absence of the efflux pump inhibitor, Phenylalanine-arginine β-naphthylamide (PAβN). The results showed that PAβN caused a mild reduction in the MIC level, suggesting that drug efflux only played a partial role in ciprofloxacin resistance development in these organisms (Table 2.1.2). Detailed analysis of the relative roles of PMQRs and target gene mutations in conferment of ciprofloxacin resistance phenotypes suggested that several PMQRs including aac(6′)-Ib-cr, oqxAB and qnrS, alone or in combination, could mediate ciprofloxacin resistance development in Salmonella isolates which did not contain target gene mutations. In particular, the presence of four different PMQRs, such as the aac(6′)-Ib-cr-oqxAB-qnrS2-qnrB8 and aac-oqxAB-qnrS2-qnrD combinations, was consistently observable in ciprofloxacin-resistant Salmonella isolates without any target mutations, suggesting that effects of such elements in conferring antibiotic resistance in Salmonella are additive or synergistic in nature (Table 2.1.2). It should also be noted that PMQR-mediated ciprofloxacin resistance is commonly associated with a MIC level of 4 to 8 μg/ml, which is comparable to those conferred by target mutations. Conjugation experiments were performed on these 32 ciprofloxacin-resistant Salmonella isolates to confirm if PMQRs were readily transferable to other Enterobacteriaceae species, using the E. coli J53 strain as recipient. Surprisingly, none of the ciprofloxacin resistance phenotypes tested could be transferred to *E. coli*, suggesting these PMQR genes may be present on a non-conjugative plasmid or the chromosomal DNA of *Salmonella*. To test these possibilities, five representative *Salmonella* strains including *S.* Derby strains S3, S4 and S38, and *S.* Typhimurium strains S7 and S71, all exhibiting different PFGE types and harboring different PMQRs, were selected for

S1-PFGE and southern hybridization analysis (Fig. 2.1.1). Among these isolates, the *aac(6′)-Ib-cr* gene was shown to be located in both chromosomal DNA and a ~200 kb size plasmid of the *S.* Derby strain S38, in the chromosome of the *S.* Derby strain S3 and *S.* Typhimurium strain S71, and in a ~200 kb plasmid in the *S.* Derby strain S4 and *S.* Typhimurium strain S7 (Fig. 2.1.1). The *oqxAB* gene was found to be located in the chromosome of all the three *S.* Derby strains and the *S.* Typhimurium strain S71, as well as in the same ~200 kb plasmid of *S.* Typhimurium strain S7 which also harbored the *aac(6′)-Ib-cr* gene as aforementioned. The *qnrS* element was shown to be located in the chromosomal DNA of all *S.* Derby strains and the *S.* Typhimurium strain S71, but not in strain S7. Such findings are consistent with the PCR screening results (Fig. 2.1.1, Table 2.1.2). Hybridization was also performed to probe the location of *qnrB* that was present in *S.* Typhimurium strain S71. However, the hybridization experiment was not successful even though the presence of *qnrB* in this strain had been confirmed by both PCR and sequencing. *Salmonella* isolates that exhibited resistance to both ceftriaxone and ciprofloxacin included three *S.* Indiana strains, S13, S14 and S16. Again, conjugation experiments failed to transfer either the ciprofloxacin or ceftriaxone resistance phenotype to *E. coli* J53. Southern hybridization was performed on strains S13 and S14 to determine the genetic location of the $bla_{\text{CTX-M-65}}$ element and the PMQR genes. Our data demonstrated that the $bla_{\text{CTX-M-65}}$, *oqxAB* and *aac(6′)-Ib-cr* elements were all located on the same ~200 kb plasmid. However, hybridization experiment performed to confirm the genetic location of the *qnrB* gene in strain S14 was not successful even though it was proven to be present in the isolate by PCR.

Table 2.1 1 Prevalence of antimicrobial resistance in different Salmonella serotypes

Antimicrobials	% of Resistance								
	Overall	Chicken	Pork isolates	*S.* Derby	*S.*	*S.*	*S.*	*S.*	*S.*
Ampicillin	68	62	72	69	100	100	63	100	25
Cefotaxime	10	21	4	3	0	25	0	100	25
Ceftriaxone	10	21	4	3	0	25	0	100	25
Chloramphenicol	74	69	75	83	79	100	50	75	50
Gentamicin	40	19	47	52	43	25	0	100	0
Kanamycin	48	27	55	69	64	25	25	100	0
Streptomycin	50	42	53	59	57	63	25	100	0
Nalidixic acid	63	46	68	72	57	50	38	100	25
Ciprofloxacin	39	17	51	50	57	0	0	75	0
Sulfamethoxazole	100	100	100	100	100	100	100	100	100
Tetracycline	65	42	75	76	79	63	63	100	0
Amikacin	4	4	4	0	0	0	0	75	0
Azithromycin	25	23	31	38	6	13	25	75	0
Olaquidox	51	35	58	90	71	50	25	75	25

Table 2.1 2 Phenotypic and genotypic characteristics of *Salmonella* strains isolated from retail meat products.

Strain #	Isolation	Sources	Serotypes	PFGE	Resistance	CIP	CIP/	PMQRs	Mutations in	Mutations in
S3[a]	12/12/12	P	Derby	DER1	Gen	16	2	aac-*oqxAB-qnrS*2	-	-
S24	01/12/13	P	Derby	DER3	Gen	4	2	*oqxAB*	S83I	
S35	01/26/13	P	Derby	DER5	Gen	4	4	aac-*oqxAB-qnrS*2	S83T	-
S36	01/26/13	P	Derby	DER5	Gen-Azi	8	2	aac-*oqxAB-qnrS*2	-	-
S37	01/26/13	P	Derby	DER5	Gen	4	2	aac-*oqxAB-qnrS*2	-	-
S38[a]	01/26/13	P	Derby	DER5	Gen-Azi	2	2	aac-*oqxAB-qnrS*2	N78H	-
S39	01/26/13	P	Derby	DER5	Gen	8	4	aac-*oqxAB-qnrS*2	-	-
S40	01/26/13	P	Derby	DER5	Gen-Azi	8	4	aac-*oqxAB-qnrS*2	-	-
S41	01/26/13	P	Derby	DER5	Gen-Azi	4	4	aac-*oqxAB-qnrS*2	-	-
S42	01/26/13	P	Derby	DER5	Gen	4	2	aac-*oqxAB-qnrS*2	-	-
S44	01/26/13	P	Derby	DER5	Gen	4	4	aac-*oqxAB-qnrS*2	-	-
S4[a]	12/12/12	P	Derby	DER6	Gen	2	2	aac-*oqxAB-qnrS*2-*qnrB*8	-	-
S9	12/12/12	P	Derby	DER6		4	0.5	aac-*oqxAB-qnrS*2	S83T	-
S54	03/13/13	C	Derby	DER8		2	0.12	*oqxAB-qnrS*8-*qnrB*	S83T	
S49	03/03/13	P	Derby	DER10	Gen-Azi	>32	>8	*oqxAB-qnrS*1-*qnrB*	S83T	
S48	03/03/13	C	Derby	DER11	Gen	2	0.5	*qnrS*1	S83T	-
S7[a]	12/12/12	P	Typhimurium	TR1	Gen	4	0.25	aac-*oqxAB*	D87N	-
S8	12/12/12	P	Typhimurium	TR1		4	0.25	*oqxAB*	D87N	-
S11	12/12/12	P	Typhimurium	TR1	Gen	2	0.25	aac-*oqxAB*	D87N	-
S79	05/17/13	P	Typhimurium	TR2	Gen	4	0.12	*oqxAB*	S83F	-
S65	03/21/13	P	Typhimurium RH2	TRH1	Gen	2	0.5	aac-*oqxAB-qnrS*1	D87N	-
S66	03/21/13	P	Typhimurium RH2	TRH2	Gen	4	0.5	aac-*oqxAB-qnrS*1	-	-
S71[a]	05/01/13	P	Typhimurium RH2	TRH2	Gen	>32	>8	aac-*oqxAB-qnrS*1	D87N	-
S6	12/12/12	P	Typhimurium RH2	TRH2	Gen	4	0.25	aac-*oqxAB-qnrS*1	-	-
S20	01/12/13	C	Typhimurium RH2	TRH3	Gen	2	0.5	aac-*qnrB*	D87N	-
S13	12/25/12	P	Indiana	I1	Gen-Azi-Cro	>32	>8	aac-*oqxAB*	S83F, D87N	-
S14	12/25/12	C	Indiana	I1	Gen-Azi-Cro	>32	>8	aac-*oqxAB-qnrB*	S83F, D87N	S80 R
S16	12/25/12	P	Indiana	I1	Gen-Azi-Cro	>32	>8	aac-*oqxAB*	S83F, D87N	C72G, S80 R
S27	01/19/13	P	Rissen	R1		8	0.12	*oqxAB*	H80N,S83T	Q91H
S59	03/16/13	C	London	L1		2	0.12		D87N	
S2	12/12/12	P	Sanferberg	S1	Gen	4	4	aac-*oqxAB-qnrS*2	S83T	-
S45	02/22/13	P	Virchow	V2	Gen	4	4	aac-*oqxAB-qnrS*8-qnrD	-	-

*All isolates were resistant to the antibiotic profile of Amp-Cip-Nal-Kan-Str-Chl-Tet-Sul-Ola (olaquindox); specific strains were also resistant to Gen, gentamicin; Azi, azithromycin; and Cro, ceftriaxone. PaβN, Phenylalanine-arginine β-naphthylamide. C, Chicken; P, Pork; aac, *aac(6')-Ib-cr*; aselected for S1- PFGE and Southern hybridization.

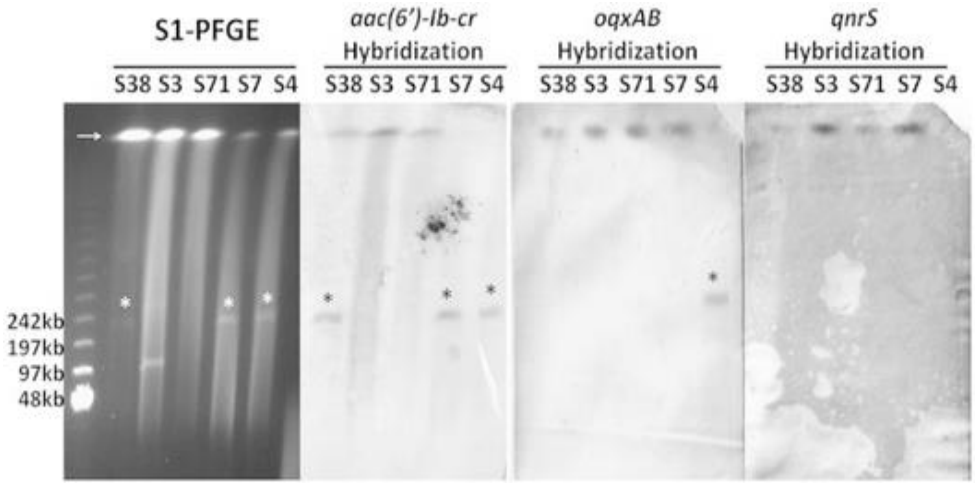

Figure 2.1 1 Analysis of genetic location of specific PMQRs in specific ciprofloxacin resistant strains by S1-PFGE and Southern hybridization.

An asterisk denotes plasmid band in which positive hybridization signal was detectable.

PMQRs play an important role in the development of fluoroquinolone resistance in *Enterobacteriaceae[114]*. These elements have mainly been reported in *E. coli* strains isolated from various sources and their prevalence has been shown to increase dramatically in recent years. In contrast, PMRQs have only been recoverable from *Salmonella* since 2005; nevertheless, their prevalence remains extremely low in *Salmonella* until the emergence of a new PMQR determinant, namely *oqxAB*, which encodes an efflux pump mediating resistance to olaquindox, chloramphenicol, nalidixic acid and elevated MICs of other antimicrobial reagents including ampicillin and gentamicin16. The *oqxAB* operon was first found to be present in an IncX1 type plasmid designated as pOLA52, which was recoverable from swine *Escherichia coli* isolates[116]. More recently, *oqxAB* was reported to be prevalent in organisms isolated from pork as well as pig farms in China[117-119]. In fact, various lines of evidence suggest that this mobile resistance element already existed in poultry *E. coli* isolates as early as 1994[117]. On the other hand, *oqxAB* had not been found in clinical isolates until recently, when it became detectable in clinical strains of *E. coli*

and *Klebsiella pneumoniae*[120-123]. In *Salmonella*, *oqxAB* was first found to be present in the chromosomal DNA of two *S.* Derby strains of food origin in 2013[112]. A retrospective study of clinical isolates of *Salmonella* in China revealed that *oqxAB* could be detected in *Salmonella* as early as 2006[101], and that it was often genetically associated with the *aac(6′)-Ic-br* element, contributing to transmission of drug resistant organisms in clinical setting in both clonal and non-clonal manner. Further studies showed that *oqxAB* and *aac(6′)-Ic-br* could greatly facilitate development of fluoroquinolone resistance by abolishing the requirement of target gene mutations, thereby potentially causing a dramatic increase of fluoroquinolone resistance in *Salmonella*[113]. Our data confirmed that this is indeed the case, and suggested that by further acquiring other PMQRs in *Salmonella* strains which already harbored the *oqxAB* and *aac(6′)-Ib-cr* elements, fluoroquinolone resistance at a level comparable to that conferred by target mutations is consistently achievable in organisms that do not even harbor a single *gyrA* mutation. The finding that multiple PMQR elements can simultaneously or synergistically produce a fluoroquinolone resistance phenotype via the mechanisms of enzymatic inactivation, drug efflux, and competitive inhibition of drug binding is intriguing. Since *oqxAB* or other PMQR determinants that confer reduced susceptibility of the host organism to fluoroquinolones may also enhance the rate of mutational changes in the drug target gene [107], the phenomenon of rapid transmission of PMQR elements among members of *Enterobacteriaceae* is alarming. Although our data showed that the PMQRs detectable in *Salmonella* were often found to be located in chromosome or plasmids that could not be transferred to other bacterial through conjugation, these elements must have been, at some stages, harbored by mobile elements that are capable of transferring their contents to the chromosome of the host strain via transposition events. This was evidenced by the observation that the *oqxAB* element could be recoverable in both chromosome and mobile elements containing the IS*26* element[112, 113]. With the fast progress of *oqxAB*-associated PMQR evolution in *Salmonella,* transmission of plasmids mediating fluoroquinolone resistance among *Salmonella,* or between *Salmonella* and other bacterial species, may become even more efficient, posing a huge threat to *Salmonella* infection control in clinical settings. Plasmids carrying the *bla*CTX-M-65 gene and multiple PMQR cassettes are of particular concern, despite the fact that they are currently restricted to specific strains

such as *S.* Indiana. Olaquindox has been a widely used growth enhancer in the pig-raising industry since the 1970s[124, 125]. Its antibiotic activity can be attributed to its ability to inhibit DNA synthesis. This agent was previously considered safe since it was not structurally related to any human drugs. Findings in this work constitute part of the evidence that the use of olaquindox as growth promoter in the swine industry has resulted in some unexpected consequences, the impact of which has only became evident decades later. First, our recent study confirmed that *oqxAB* actually originated as a chromosomal efflux pump gene of *Klebsiella pneumoniae*, which was picked up and incorporated into a mobile element by IS*26*-mediated transposition, presumably under the selection pressure of olaquindox. These events resulted in constitutive expression of the plasmid-borne *oqxAB* operon. Second, the process of inter-species transmission from *E. coli* to *Salmonella* occurred over a period of at least a decade, during which *oqxAB* was not detectable in clinical *Salmonella* strains until 2006. Third, amplification of an *oqxA* –positive *S.* Typhimurium strain resulted in a sharp increase in the prevalence of *oqxAB*-borne clinical *Salmonella* isolates in subsequent years. Finally, our data demonstrated that co-existence of the *oqxAB* genes with other PMQR elements has become commonplace, leading to emergence of a new category of fluoroquinolone-resistant organisms that exhibit selective advantages in both the environment and clinical settings where antibiotic selection pressure is high. At present, *Salmonella* strains harboring multiple PMQR/*oqxAB* elements appear to be confined to zoonotic organisms but the risk of these strains causing human infections is apparently increasing rapidly. To conclude, findings in this work highlight a need to devise specific infection control measures to halt further transmission of the *oqxAB*/PMQR–borne resistant *Salmonella* strains, and investigate the impact of other animal growth promoters in selection of both bacterial resistance and virulence determinants in a wide range of foodborne and zoonotic pathogens.

***Salmonella* isolation from retail meat products.** *Salmonella* were isolated from retail meat samples including chicken and pork from supermarkets and wet markets in Shenzhen, China from October 2012 to June 201332. Food samples were collected aseptically in plastic bags and transported on ice to the laboratory for isolation of *Salmonella* within 6 h. Twenty-five grams of meat samples were placed in a stomacher bag with 100-ml Buffered Peptone Water (BPW) (Difco, Detroit, MI)

which was subjected to homogenization for 5 min. The homogenate was incubated at 35 °C for 24 h. One ml aliquot of pre-enriched homogenate was transferred to 10 mL of Tetrathionate broth (Difco) and incubated at 42 °C for 24-h. A loopful of the enriched content was streaked on XLT4 agar and incubated for 24 h to 48 h at 37 °C. One typical *Salmonella* strain recovered from each sample was purified and subjected to species identification by detection of the *invA* gene and 16S RNA sequencing. All isolates were serotyped according to the Kauffmann-White scheme, using commercial antiserum (Difco, Detroit).

Antimicrobial susceptibility tests. Confirmed *S.* Typhimurium isolates were subjected to antimicrobial susceptibility testing using the agar-dilution method, and the results were interpreted according to the CLSI guidelines33. Fourteen antimicrobial agents 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 and 35218, *Enterococcus faecalis* strain ATCC 29212, *Staphylococcus aureus* strain ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control.

Screening of target gene mutations, *oqxAB*, and other PMQR genes. The QRDR regions of the *gyrA, gyrB, parC* and *parE* genes were amplified by PCR as previously described[34], followed by determination of their nucleotide sequences and comparison to the wild-type *Salmonella* Typhimurium LT2 strain for identification of target gene mutations in the test strains. The presence of the PMQR genes *qnrA, qnrB, qnrC, qnrD, qnrS, qepA, oqxAB* and *aac(6')Ib-cr*, was determined by PCR using primers described previously[126].

Molecular typing. Clonal relationship between representative *Salmonella* isolates was examined by pulsed-field gel electrophoresis (PFGE) according to the PulseNet PFGE protocol for *Salmonella*36. 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 37 °C 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). Southern blot hybridization was carried out by following the manufacturer's instructions of the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics), using the different PMQR gene and *bla*$_{\text{CTX-M-64}}$ digoxigenin-labeled probes.

2.2 Investigation of Carbapenem resistant isolates

Since the invention of antibiotics people have been fighting against antibiotic resistance. The evolution of both pathogenic and opportunistic microbes in hospital and the environment has caused enormous problem in infection treatment. In particular, Carbapenemase-producing Enterobacteriaceae (CPE) constitute a public health problem regarding both hospital- and community-acquired infections[127], because carbapenems are the last resort used to treat serious infections that may threaten human lives. There are currently more than 450 known antibiotic resistance genes [128], and the number of genes related to the development of antibiotic resistance is even bigger. This study picked up *bla*$_{\text{NDM-1}}$(New Delhi lactamase) to do study. The *bla*$_{\text{NDM-1}}$ gene is a typical resistance determinant of most of the carbapenems. Concurrent resistance to multiple other antibiotic classes in addition to the β lactamase antibiotics cause more panic among people because that has left very few options for infection treatment[129, 130]. Hence the pandemic spread of the *bla*$_{\text{NDM-1}}$ in the last decade is a serious concern. This *bla*$_{\text{NDM-1}}$ was herein chosen as a representative gene to depict the dissemination features of CPE not only because of the broad resistance profile caused by *bla*$_{\text{NDM-1}}$ but also because of the amazing speed of its transmission. The *bla*$_{\text{NDM-1}}$ gene was first reported in 2008 in Sweden after transferral from a New Delhi hospital[35], but the oldest strains that harboured *bla*$_{\text{NDM-1}}$ was isolated in 2007[131]. The *bla*$_{\text{NDM-1}}$ harboured strains can now be found in all the 7 continents within 10 years. Hence, we conducted a investigation about the *bla*$_{\text{NDM-1}}$ harboring strains.

2.2.1 The molecular investigation of *bla*$_{\text{NDM-1}}$ positive clinical isolates

Carriage of multiple β-lactamase genes often confers high level of resistance to the host strain. Examples include co-existence of the *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{CMY-42}}$ genes, or

the *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$ elements in *E.coli*[132]. There has been an increasing number of studies in recent years that reported the simultaneous presence of the *bla*$_{KPC}$ and *bla*$_{NDM}$ carbapenem resistance genes in clinical strains in various countries, and that these plasmid-borne genes exhibit a much higher potential to disseminate than the chromosome-borne genes. Representative examples are the co-expression of the *bla*$_{KPC-2}$ and *bla*$_{NDM-1}$ enzymes in *Citrobacter freundii* [133], the existence of an integron-borne *bla*$_{KPC-2}$ gene that co-exists with the *bla*$_{NDM-1}$ element in a *P. aeruginosa* strain in India[134], the recovery of a *C. freundii* isolate that carries the *bla*$_{KPC-2}$ and *bla*$_{NDM-1}$ elements [135], and similar situation in a *Klebsiella pneumoniae* strain in India[136], as well as in *Enterobacter hormaechei* and *Enterobacter cloacae* strains in Brazil[137, 138]. However, few studies have reported the complete sequence of such co-existing plasmids that harboured the carbapenam resistance genes. Furthermore, to our knowledge, the fitness cost and stability of such plasmids have not been investigated. In particular, despite the fact that *bla*$_{NDM}$ positive IncX3 plasmids are wildly distributed among bacterial pathogens, information regarding their genetic characteristics and stability remains scarce. To fill this knowledge gap, we performed detailed genetic and phenotypic characterization on some representative carbapenamase-encoding plasmids in this work and confirmed that carriage of such mobile resistance elements was associated with negligible fitness cost, and that they could be stably passed onto the future generations.

Three carbapenemases producing strains, 13E163, 13E168 and 13E169, were isolated from puncture fluid, ascites and drainage fluid from a hospital patient in Henan Province, China in 2013 after LB agar supplemented with meropenem 0.5μg/ml was used to screen for carbapenemase producing strains. The three strains were identified by using API20E strips and 16s rRNA sequencing. MICs of 17 antimicrobial agents were determined by the agar dilution method and results were interpreted by CLSI standards[139]. The ESBL genes and carbapenemases genes (*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{KPC}$ and *bla*$_{OXA-48}$) harboured by the test strains were detected by PCR as previously described[140].

To investigate the transferability of the resistance genes, plasmids extracted from the strain 13E169 were transformed into *Escherichia coli* strain TG1 by

electroporation. Transformants were recovered on LB agar containing 4μg/ml of cefotaxime, then purified and subjected to detection of the presence of *bla*$_{NDM}$ or *bla*$_{KPC}$ by PCR. Transformants harbouring *bla*$_{NDM}$ and *bla*$_{KPC}$ respectively were selected for further plasmid analysis.

S1 pulsed-filed gel electrophoresis (S1-PFGE) and Southern hybridization were performed on both the parental strain and transformations carrying the *bla*$_{KPC}$ and *bla*$_{NDM}$ genes to probe the genetic locations of such elements according to the manufacturer's instructions (Roche).

Plasmids were extracted from transformants respectively carrying the *bla*$_{KPC}$ and *bla*$_{NDM}$ genes, using the Plasmid Midi kits (Qiagen, Germany). Both Illumina NextSeq 500 and PacBio RSII single-molecule real-time (SMRT) sequencing platforms were used to sequence the plasmid samples. After obtaining the raw reads, SPAdes was utilized to perform the hybrid-assembly and obtain complete plasmid sequences. Illumina short-reads were then utilized to polish the finished plasmids. The RAST annotation pipeline was chosen to perform rapid annotation of the plasmids[141] . Comparison of the plasmids against the highly homologous plasmids in the NCBI database was performed by BRIG [142]. Alignment of the MDR regions with other similar MDR regions was completed by Easyfig[143]. The sequences of pHN84KPC and pHN84NDM, which harboured the *bla*$_{NDM}$ and *bla*$_{KPC}$ genes, were submitted to GenBank under the accession numbers KY296103 and KY296104, respectively.

Plasmid stability was tested using a previously described method with slight modification[144]. Briefly, bacteria were inoculated into Luria-Beranti (LB) broth culture and grown overnight, then transferred into fresh LB broth diluted 100 times every 12 hours (equivalent to 10 generations). At the time of transfer, the cultures were spread on the MAC plates with or without CTX (8μg/ml) upon serial dilutions. Plasmid stability was determined as the ratio of the number of colonies on MAC containing CTX to the number of colonies on MAC without antibiotics [145].

The growth curves for the original strains TG1 and the transformants harbouring the *bla*$_{KPC}$ and *bla*$_{NDM}$ genes were tested by recording the number of colonies on the MAC plates after serial dilution every two hours. The OD values of the overnight

culture were adjusted to 0.1 at OD595, 100μl of which was then inoculated into 3ml LB broth with shaking (250rpm) at 37℃. The number of colonies was recorded every two hours after serial dilution for 12 hours and the growth curves were plotted with GraphPad software.

The fitness cost of plasmids was tested by pairwise growth competition assays of the two transfomants carrying a plasmid which contained the bla_{NDM} / bla_{KPC} genes, and the parent strain without plasmids, following a previously described method[146]. In brief, pairwise competition between the original strain (TG1) and transformants was carried out in LB broth without antibiotics. The overnight culture was diluted into 0.1 at OD595 with saline, then 100μl diluted culture was inoculated into 3 ml LB broth and incubated at 37 ℃ and 250rmp for 12 hours. The number of colonies on the MAC plates with and without CTX (8μg/ml) after serial dilution was calculated every two hours. Pairwise competition curves were plotted with the GraphPad software.

The species identity of three clinical carbapenamase producing strains were confirmed as *Enterobacter cloacae* (13E169, 13E168) and *Klebsiella pneumoniae* (13E163). The latter was recovered from the puncture fluid, whereas strains 13E168 and 13E169 were recovered from ascites and drainage fluid respectively. Antibiotic susceptibility of these three test strains, as determined by the agar dilution method and shown in Table 2.2.1, showed that they were resistant to most conventional antimicrobial compounds, including β-lactams, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole and fosfomycin, but remained sensitive to colistin. Each of the two *E. cloacae* strains were found to harbour the β-lactamase genes bla_{SHV}, bla_{KPC} and bla_{NDM}; for the *K. pneumoniae* strain, only bla_{KPC} was detectable. The *Enterobacter cloacae* strain 13E169 was selected for further analysis. Transformation result showed that the NDM and KPC encoding-plasmids that it harboured could be transformed into the strain TG1. However, the transformant (TG1-84kpc) which harboured the bla_{KPC} gene was less susceptible to meropenem than the one (TG1-84ndm) that carried the bla_{NDM} gene (Table 2.2.1).

Both bla_{NDM-1} and bla_{KPC-2} recipient strains exhibited resistance to penicillin and cephalosporin, and to a lesser extent carbapenem. On the other hand, both strains were found to remain sensitive to colistin (Table 2.2.1). According to the S1-PFGE and

hybridization results, strain 13E169 contained five plasmids, two of which were positive for *bla*$_{NDM-1}$ and *bla*$_{KPC-2}$ respectively (Figures 2.2.2) and designated as pHN84NDM and pHN84KPC after their complete sequences were obtained. Rapid dissemination of multiple β-lactamase genes in the same strains has become a common phenomenon worldwide. Recent studies suggested that the IncX3 plasmids are common vectors responsible for transmission of the *bla*$_{NDM}$ gene, especially in China[135, 147], resulting in an increasing incidence of co-expression of multiple carbapenemases among the clinical strains [136, 148].

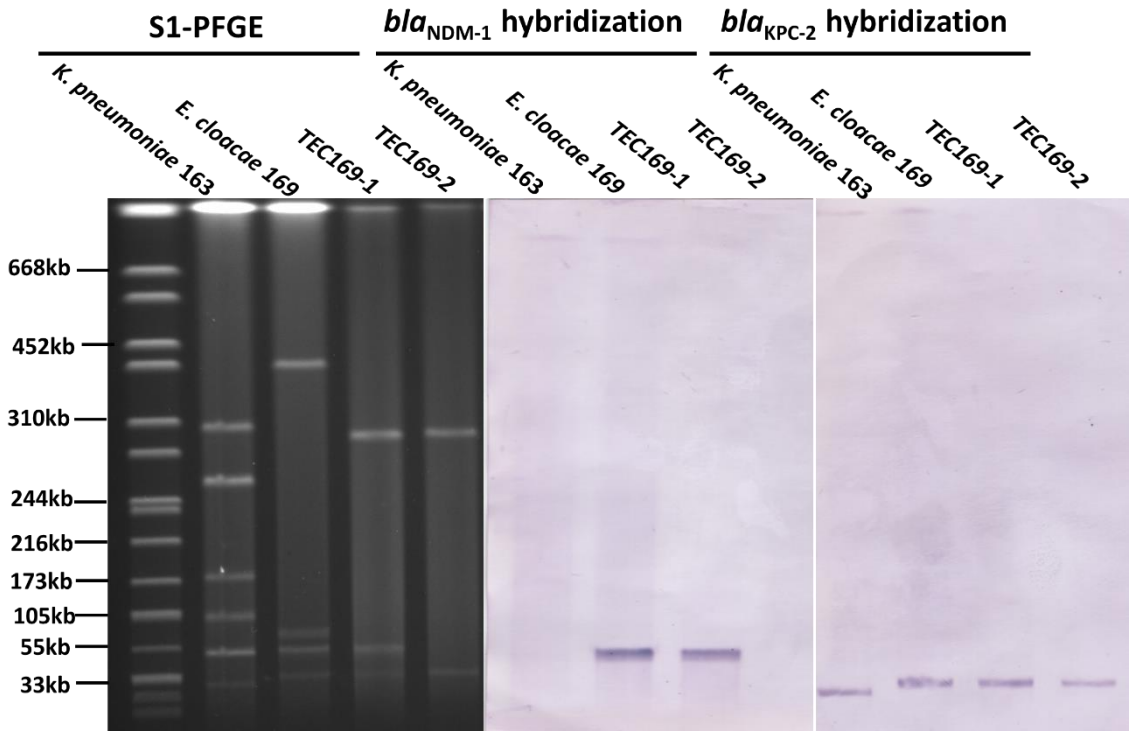

Figure 2.2 1 S1-PFGE and Southern hybridization of CRE strains and their transformants

Table 2.2 1 MICs of the carbapenamase producing strains and their corresponding transformants against 15 antimicrobials.

Strains	β-lactamase	CRO	SXT	GEN	TET	CHL	FOS	IMP	AMP	MRP	TGC	CL	CIP	ATM	CAZ	NAL
E. coli 25922	-	0.5	<0.025	1	1	<0.25	2	<0.25	8	<0.25	<0.5	2	<0.06	<0.5	<0.25	4
E. coli TG1	-	<0.25	<0.025	0.25	0.5	<0.25	1	<0.25	2	<0.25	<0.5	2	<0.06	<0.5	<0.25	4
K. pneumonia	*bla*$_{KPC-2}$	>32	>32	>64	>32	4	64	16	>64	>16	<0.5	2	>16	>32	>64	>64
E. cloacae 169	*bla*$_{KPC-2}$, . .	>32	>32	2	16	8	256	>32	>64	>16	<0.5	1	8	>32	>64	>64
TEC169-1	*bla*$_{KPC-2}$, . .	>32	<0.025	1	1	<0.25	1	4	>64	8	<0.5	2	<0.06	16	>64	4
TEC169-2	*bla*$_{KPC-2}$	>32	<0.025	1	4	<0.25	1	0.5	>64	4	<0.5	1	<0.06	8	>64	4
TEC169-3	*bla*$_{KPC-2}$, . .	>32	<0.025	1	4	<0.25	1	4	>64	8	<0.5	2	<0.06	16	>64	4

CRO: Ceftriaxone; SXT: Trimethoprim-sulfamethoxazole; GEN: Gentamicin; TET: Tetracycline; CHL: Chloramphenicol; FOS: Fosfomycin; IMP: Imipenem; AMP: Ampicillin; MRP: Meropenem; TGC: Tigecycline; CL: Colistin; CIP: Ciprofloxacin; ATM: Aztreonam; CAZ: Ceftazidime; NAL: Nalidixic acid.

Plasmid pHN84NDM, recovered from the transformant TG1-84ndm, was found to contain the *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{SHV-12}}$ genes. This plasmid was 53.77kb in size, encoded 50 hypothetical proteins and belonged to the IncX3 type plasmid (**Figure 2.1.18**). It was almost identical to previously reported IncX3 plasmids such as the pZHDC33 (KX094555) element recovered from a *E.coli* strain in 2016, and the pNDM-ECN49 (KP765744) plasmid harboured by an *Enterobacter cloacae* strain . This finding constitutes evidence that widespread dissemination of the IncX3 plasmid has occurred in China. The *bla*$_{\text{KPC}}$-bearing plasmid, pHN84KPC, was found to belong to the IncR type and harbor 41 open reading frames (ORFs) of 39.37kb in size, and with an average GC content of 55.65% (Fig. 2.2.2). The plasmid contained a wide variety of genetic elements, Including resistance genes, mobile elements, as well as genes essential for replication and stable inheritance. The BLASTN result showed that the backbone of the plasmid comprised several plasmid maintenance and partitioning modules such as *vgaC/vagD* and *resD*, the sequences of which exhibited 99% similarity to the following elements: JM45(NC_022078) with 80% coverage of *Klebsiella pneumoniae* strains in China, KUN4843(LC155908) with 64% coverage of *Klebsiella pneumoniae* strains in Japan, and pWSZBE (LC155909) with 73% coverage of *Klebsiella pneumoniae* strains in China. Two resistance genes, *tetA* and *bla*$_{\text{KPC}}$, were found in the variable regions of the plasmid (Figure 2.2.2). The immediate *bla*$_{\text{KPC}}$ genetic context was identical to pKP048, which was isolated from a clinical *K. pneumoniae* strain but belonged to a different Inc group(IncF)[149]. The *tetA* gene, with a similar genetic context, was also reported in the plasmid pKPS30, which also belonged to IncR. The same genetic context was found in strain 13E163. It should be noted that the transposon Tn*1721* was known to harbor the *tetA* gene in many plasmids [150]. On the other hand, the *tetA* gene was also known to be associated with various intergrons in *Salmonella* or *E.coli*[150]. The BLASTN result shows that the genetic context of *bla*$_{\text{KPC-2}}$ was identical to that of plasmid pKP048(FJ628167) in *Klebsiella pneumoniae* strain KP048, which was formed by integrating a Tn*3*-based transposon with a partial Tn*4401* structure[150], with the Tn*1721* transposons being on both sides of this fragment (Figure 2.2.2). Hence formation of the *bla*$_{\text{KPC}}$-bearing structure in plasmid pHN84KPC may be due to recombination of the *tetA* and *bla*$_{\text{KPC-2}}$ fragments mediated by Tn*1721*. Tn1721-based transposons are frequently described in Asia[151, 152], and IS*Kpn6, korC*, and

klcA are often accompanied by *bla*$_{KPC-2}$ in other plasmids, such as those in plasmid pKP048.

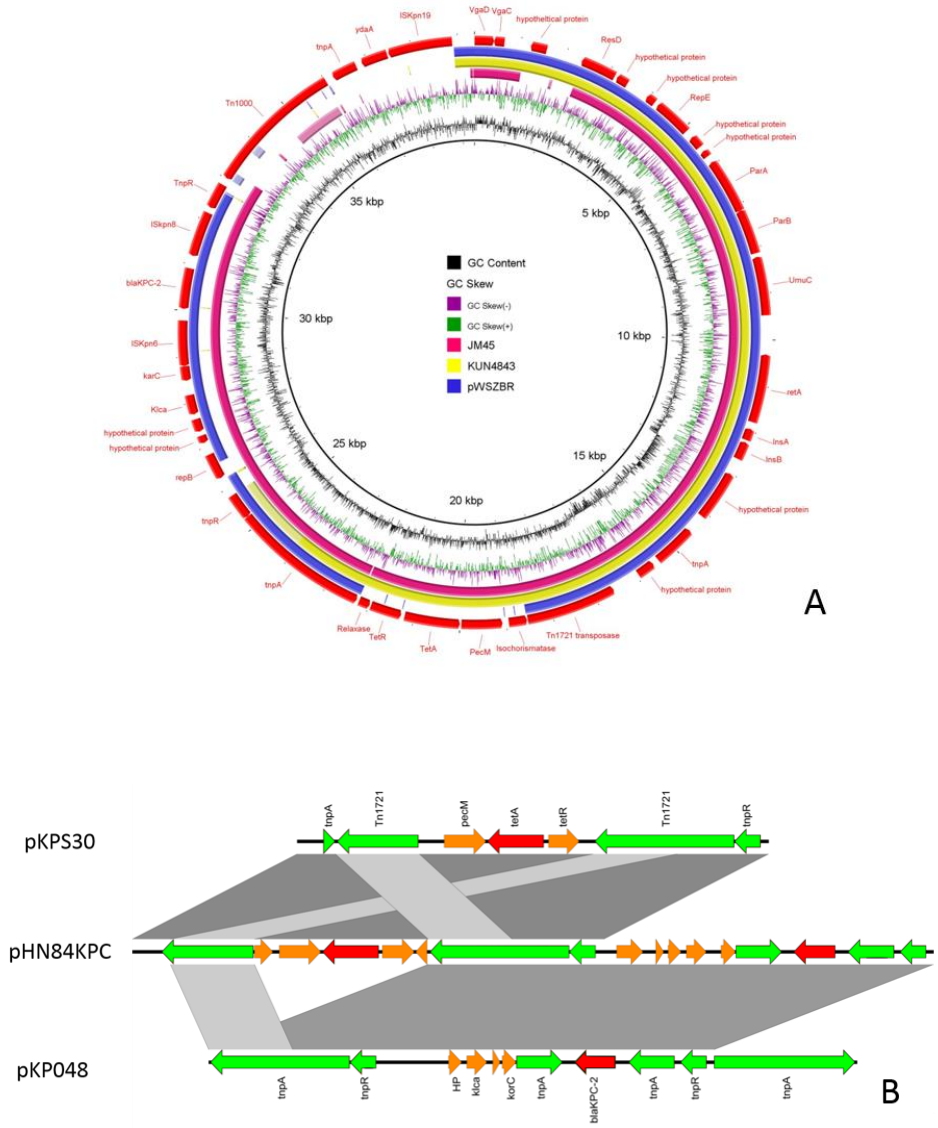

Figure 2.2 2 Comparison between plasmid pHN84KPC and known plasmids detectable by BLSTN.

(A) Comparison between different plasmids. The outside ring represents the ORFs of pHN84KPC which was used as a reference for comparison. The dull red circle represents similar regions detectable between the *Klebsiella pneumoniae* plasmid JM45 and reference plasmid. The Yellow ring represents homologous fragments between the *Klebsiella pneumoniae* plasmid KUN4843 and pHN84KPC. Blue circle represents homologous sequences shared by the *Klebsiella pneumoniae* plasmid pWSZBR. The displayed regions

denote the homologous plasmids with 90% similarity by BLASTN. The innermost black ring shows the GC skew; the green and pure circles depict the GC content. Although these plasmids are all much larger than pHN84KPC, the difference is mainly confined to the MDR region. (B) Comparison between the MDR region and similar fragments retrieved by BLSTN. Genetic alignment of the MDR region of pHN84KPC shows recombination of *bla*$_{KPC-2}$ fragment with the *tetA* fragment, which is also mediated by Tn1721. The green arrows indicate the mobile elements. The red arrows represent the resistance genes. All the other genes are shown in orange. Different shading legends depict the percentages of sequence identity obtained from the BLASTN result.

Plasmids pHN84KPC and pHN84NDM were found to persist in 100% of the subsequent bacterial progenies after *in vitro* growth for 220 generations without antibiotic selection pressure. The MICs against meropenem for the offspring strains collected after 220 generations were identical to their parent strains. These findings showed that plasmids harbouring carbapenemase genes were highly stable even in the absence of antibiotic selection pressure. Nevertheless, it should be noted that transformants harbouring *bla*$_{KPC-2}$ had the same growth rate as those carrying the *bla*$_{NDM}$ gene. Data of growth kinetics showed that they grew more slowly in the presence of cefotaxime or meropenem (Figure 2.2.3). This phenomenon is alarming because it infers that transmission and further acquisition of resistance genes in the plasmids concerned will result in further limitation in treatment options.

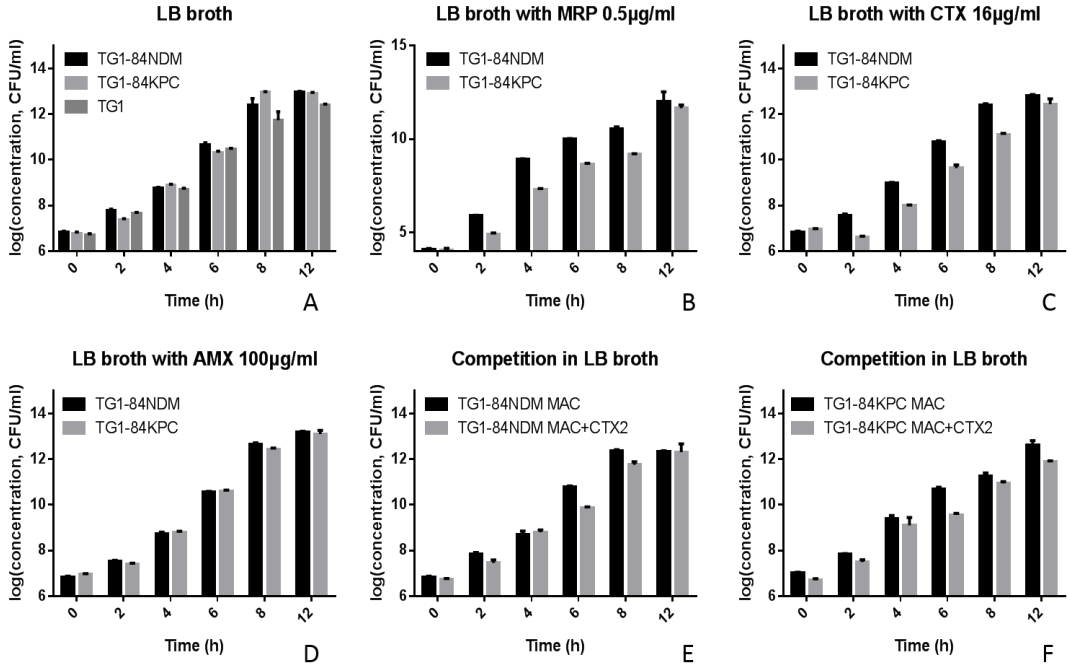

Figure 2.2 3 Growth curve and competition experiment

A: Growth curve of the recipient strain and the corresponding transformants in LB broth; B: Growth curve of the recipient strain and the tranformants in LB broth containing meropenem (0.5μg/ml); C: Growth curve of the recipient strain and the transformants in LB broth containing cefotaxime (16 μg/ml); D: Growth curve of the recipient strains and the transformants in LB broth containing ampicillin (100 μg/ml); E: Result of competition between the original TG1 strain and the TG1 strain harboring the *bla*$_{NDM-1}$ plasmid. F: Result of competition between the original TG1 strain and the TG1 strain harboring the *bla*$_{KPC-2}$ plasmid.

The growth data showed that the transformant TG1-84NDM harbouring *bla*$_{NDM-1}$ grew faster than the one carrying *bla*$_{KPC-2}$ in LB broth supplemented with cefotaxime and meropenem (Figure 2.2.4). In contrast, the strains exhibited the same growth rate in the LB broth with or without amoxicillin. This may be due to the fact that KPC-2 confers higher resistance to ampicillin than to cefotaxime and imipenem[153]. Previous studies also showed that carbapenems are much more efficient in selecting organisms harboring *bla*$_{NDM-1}$ than those harboring *bla*$_{KPC-2}$[134]. We speculated that the acquired IncX3 plasmid can confer to the host strain a competitive edge to grow under the pressure of carbapenems or ceftiofur, as well as a high degree of inheritance stability of the plasmids in bacteria. In view of this finding, we hypothesized that co-existence of two different carbapenemase genes in a plasmid may be due to its ability to acquire such genes in a stepwise manner. Horizontal transfer of antibiotic resistance genes within the same patient was studied recently[154, 155], but the stability and fitness cost of these plasmid after transformation remains unknown. In this work, we confirmed that plasmids that carry carbapenemase genes are highly stable and exhibit little fitness cost in the host strain, facilitating further acquisition of other types of antibiotic resistance genes in the future.

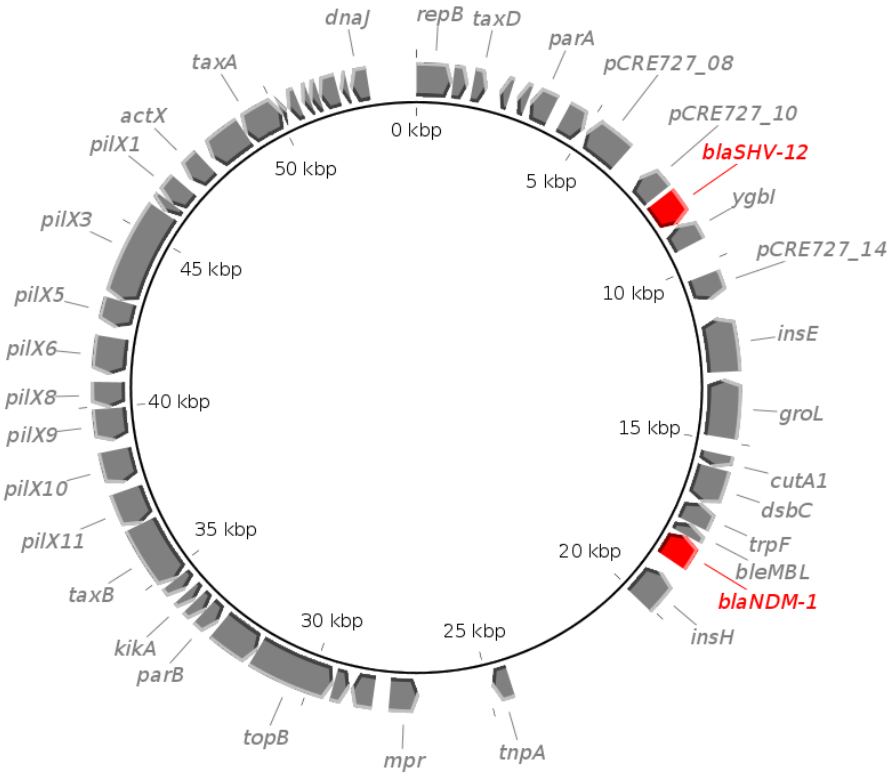

Figure 2.2 4 The circle depicts the genetic content of the IncX3 type plasmid pHN84NDM. The red arrows denote antibiotic resistance genes.

In this study, the *Enterobacter cloacae* strain *13E169 was found to co-harbor the* bla_{KPC-2} and bla_{NDM-1} genes, which encode high level resistance to carbapenams. The plasmids in which these genes were located could be transformed into *E.coli* with little fitness cost, and stably passed onto the offsprings of the transformants. This phenomenon infers that mobile genetic elements harbouring multiple resistance genes can persist among the commensal strains of the patient long after the original donor strain has been eradicated by antimicrobial treatment, and that dissemination of such mobile resistance elements is an efficient event, leading to further increase in the rate of resistance to beta-lactam antibiotics in clinical settings. Future studies should focus on devising new methods to inhibit transmission of such resistance-encoding plasmids among bacterial pathogens.

2.2.2 Fosfomycin resistant isolates

Fosfomycin is a naturally occurring antibacterial agent with a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria, except

Acinetobacter baumannii [156]. Although this agent has been used to treat urinary tract infections, development of bacterial resistance during therapy frequently occurred, rendering it unsuitable for sustained therapy of severe infections [157]. Recently, renewed attention has been paid to fosfomycin for the treatment of both urinary and systemic infections due to the rapid dissemination of multidrug-resistant Gram-negative bacteria, especially members of Enterobacteriaceae that are resistant to traditionally used agents[158]. Compared with other agents, fosfomycin seems to have retained antimicrobial activity against a substantial percentage of clinical isolates, in particular *E. coli*.

Fosfomycin targets cell wall biosynthesis through binding to the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), thereby inhibiting the formation of N-acetylmuramic acid (a precursor of peptidoglycan) from N-acetylglucosamine and phosphoenolpyruvate. Bacteria develop resistance to fosfomycin through several different mechanisms [159]. Firstly, chromosomal mutations altering the structures of fosfomycin transporter, glycerol-3-phosphate transporter (GlpT), as well as the hexose phosphate transporter (UhpT), can result in fosfomycin resistance. Secondly, target mutations or overexpression that lead to fosfomycin resistance may occur. It has been shown that substitution of C115, D369 and L370 in MurA, the drug target, could lead to fosfomycin resistance in E. coli. Lastly, fosfomycin inactivation enzymes have also been found to confer fosfomycin resistance. Specifically, fosA, a glutathione transferase, and fosB, a L-cysteine thiol transferase, are plasmid-borne and commonly found in Gram-negative and Gram-positive bacteria, respectively. FosX, an epoxide hydrolase, is chromosomally encoded in Listeria monocytogenes [159] . A novel integron-mediated fosfomycin-resistance genetic determinant, fosK, was recently described in Acinetobacter soli 27 HK001 strain [160].

Recently, a fosfomycin resistance gene, fosA3, has been reported in *E.coli* and *Klebsiella pneumoniae* isolates [161-165]. This gene is normally plasmid-mediated, surrounded by IS*26* transposases, and often detectable in CTX-M-producing and multidrug-resistant E. coli recovered from animals as well as patients in China, Japan and Korea [161-165]. It has been suggested that the increasing prevalence of fosA3 is due to dissemination of the IncI and IncN plasmids among E. coli isolates rather than

clonal expansion of specific strains [163]. One direct negative effect of antibiotic resistance is the increasingly heavy load of infection control especially in health care facilities. The dramatically increasing prevalence of methicillin-resistant *Staphylococcus aureus*[166], and glycopeptide-resistant enterococci [167], the extended-spectrum β-lactamase-producing *Enterobacteriaceae*, and Gram-negative bacteria resistant to carbapenems have become a cause for concern (e.g. multidrug-resistant carbapenemase-producing *Klebsiella pneumoniae* and *Acinetobacter spp*.). These bacteria are very dangerous because they can be resistant to all currently available antimicrobial agents or remain susceptible only to older, potentially more toxic agents such as the polymyxins, leaving limited and suboptimal options for treatment. This phenomenon is important because re-admitted patients are often colonized with AMRB. The 'feed loop' is dangerous as pathogens can be introduced into the facilities and can infect new patients[168]. Bacteria are isolated from the different patients in the same hospital. Some kinds of bacteria like *Enterobacter cloacae*, the prevalent strains are different (Figure 2.2.5) and contain several types of NDM borne plasmids (Figure 2.2.6). The strains of *Morganella morganii* have different PFGE types but have same-size plasmids. For *Clostridium perfringens*, the PFGE types are nearly the same but have different plasmid types. Recently the rapid spread of fosfomycin resistance genes has caused great worries because fosfomycin was supposed to treat MDR strains[158], the carriage of the fosA3 gene in the carbapenem resistant strains means very limited methods left could be used to treat those kinds of isolates.

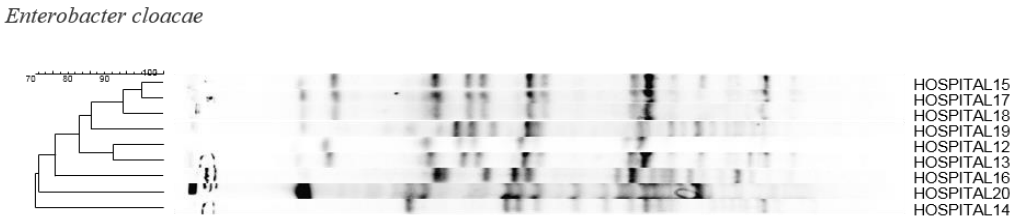

Figure 2.2.5 PFGE analysis of NDM positive Enterobacter cloacae strains isolated from hospital

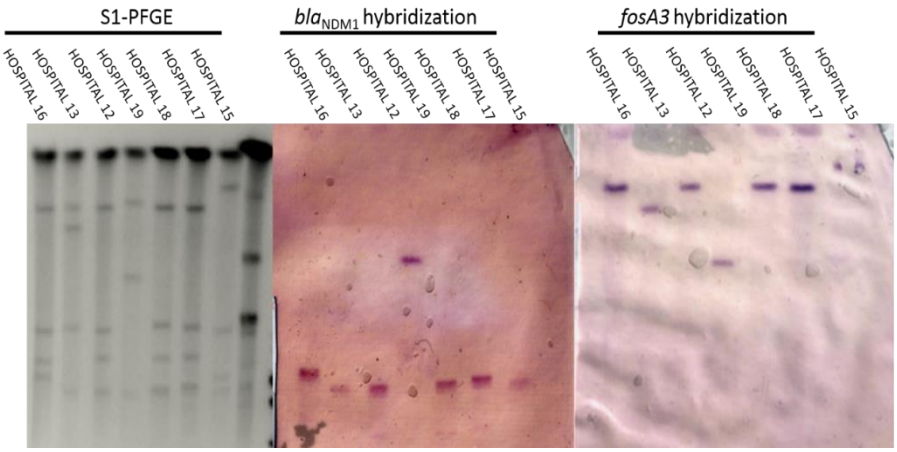

Figure 2.2.6 S1-PFGE and Southern hybridization of NDM positive Enterobacter cloacae

S1-PFGE and Southern hybridization analysis of *bla*$_{NDM-1}$ (middle) and FosA 3 (right) borne plasmids from *Enterobacter cloacae* strains isolated from hospital.

This following part aims to determine whether fosA3-borne plasmids can disseminate among other closely related Enterobacteriaceae species such as *Salmonella*, and the underlying genetic mechanisms regulating its dissemination potential. *Salmonella* was isolated from pork and chicken meat purchased from supermarkets and wet markets in Hong Kong during 2012~2013 as described previously [169]. The genetic identity of all *Salmonella* isolates was confirmed by PCR targeting the invA gene and 16S rRNA sequencing. Specific *Salmonella* isolates were serotyped according to the Kauffmann-White scheme.

Confirmed *Salmonella* isolates were subjected to antimicrobial susceptibility testing using the agar-dilution method, and the results were interpreted according to the CLSI guidelines [170]. Eighteen antimicrobials were tested: ampicillin, amoxicillin/clavulanic acid, ceftazidime, ceftiofur, cefotaxime, ceftriaxone, meropenem, chloramphenicol, gentamicin, kanamycin, streptomycin, nalidixic acid, ciprofloxacin, sulfamethoxazole, tetracycline, trimethoprim, amikacin and azithromycin. Olaquindox and fosfomycin were also tested for certain isolates. *E. coli* strains ATCC 25922 and 35218, *Enterococcus faecalis* strain ATCC 29212, *Staphylococcus aureus* strain ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control.

Fosfomycin resistance gene screening was performed as described previously using the *fosA3* gene of *E. coli* as positive control [163, 171]. Prevalence of ESBLs was determined by PCR targeting known β-lactamase genes as previously described [169]. β-lactamase genes were amplified full length by specific primers as shown in Table 2.2.3, followed by nucleotide sequencing to determine their specific type.

A conjugation experiment was carried out as previously described using a sodium azide-resistant *E. coli* J53 strain as recipient [172]. 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 fosfomycin (16μg/mL) and sodium azide (100μg/mL) to select drug resistant transconjugants. Plasmid replicon typing was performed as previously described [173].

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 2.2 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). Multi Locus Sequence Typing was performed using primer sets as suggested in www.mlst.net. Southern blot hybridization was carried out using DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics) following the manufacturer's instructions using a *fosA3* digoxigenin-labelled probe.

Primer walking and PCR mapping were performed to investigate the *fosA3* gene environment. A set of primers (Table 3) were designed according to the published flanking sequence of the *fosA3* gene [162, 163, 165, 174]. The PCR products were sequenced and aligned together using BioEdit software.

One hundred and forty six non duplicated *Salmonella* strains were isolated from 425 meat poduct samples purchased from supermarkets and wet markets in Hong Kong in 2013. The total isolation rate was about 46% which is similar to our previous study [169]. The *Salmonella* isolation rate for pork was about 52%, slightly higher

than that for chicken (37%). In general, very similar isolation rates from supermarket and wet market were recorded [175-177].

Susceptibilities of the test strains to 18 antimicrobials were determined. Up to 65% of the 146 *Salmonella* isolates exhibited resistance to tetracycline, followed by sulfamethoxazole (53%), nalidixic acid (35%) and chloramphenicol (30%). Around 27% of the isolates were resistant to ampicillin, yet among them, only two (1%) were also resistant to cefotaxime and ceftriaxone. Various *Salmonella* isolates also exhibited resistance to kanamycin (4%) and gentamicin (3%). Suprisingly, two ceftriaxone-resistant isolates were also shown to be resistant to fosfomycin. These two isolates were further characterized for the mechanism of fosfomycin resistance. *Salmonella* strain S76 was typed to be *S. Detby*; such strain was isolated from chicken product purchased in a supermarket on Feburary 22, 2013, whereas strain S79, which was found to be *S. Enteritidis*, was isolated on the same day from different chicken product purchased from a different supermarket in Hong Kong. In additon to being resistant to fosfomycin, these two strains also exhibited resistance to other antibiotics such as nalidixic acid and different β-lactams. Furthermore, strain S79 was also resistant to ciprofloxacin (Table 2.2.2). MLST analysis of these two isolates showed that S76 belonged to ST11, whereas S79 belonged to ST460. ST11 has been reported to be associated with invasive *S. Enteritidis* and ceftriaxone-resistant *S. Enteritidis* strains carrying CTX-M-14 and CTX-M-15 on IncI1 and IncFII plasmids respectively [178-180]. In contrast, ST460 has not been reported to be associated with CTX-M producing *S. Derby* previously.

These two strains were screened for the presence of fosfomycin resistance genes, *fosA, fosB, fosX* and *fosK*. The gene *fosA3* was detectable in both strains. Conjugation experiment showed that this fosfomycin resistance determinant could be transferred to *E. coli* J53. Plasmid replicon typing confirmed that plasmids from strains S76 and S79 were IncFII and IncFIV respectively. Conjugative plamisd in *E coli* J53 that encoded *fosA3* was shown to be IncFII. S1 PFGE confirmed that plasmids of two different sizes (~80kb and ~45kb) were detectable in both isolates, with the conjugative plasmid being a size of ~80kb. Plasmids from S79 were slightly smaller in size than those from S76, presumably due to the migration variation in the gel (Fig 2.2.7).

Southern hybridization showed that the *fosA3* gene were present in the ~80kb conjugative plasmid.

Since the *fosA3* gene is commonly associated with IS*26* transposases, primers targeting IS*26* and *fosA3* were used to amplify the flanking regions of the *fosA3* gene. It was shown that the *fosA3* gene harbored by the conjugative plasmid in *Salmonella* was surrounded by two IS*26* elements with a genetic structure in the order of IS*26*-*fosA3*-*orf1*-*orf2*-*orf3*-IS*26*. An identical structure was first reported in plasmids from clincial *E. coli* isolates in Japan [165]. The *orf1*-*orf2*-*orf3* cassette shares 78% nucleotide identity with a region in the chromosome of *Klebsiella pneumoniae* strain 342 [165]. Since then, similar IS*26* flanked cassettes have also been reported in clinical *E. coli* isolates in Korea and Hong Kong [162, 163, 174]. In addition, the *fosA3* borne plasmid was also shown to contain a ceftaxime resistance determinant. Importantly, PCR assay detected a β-lactamase gene, $bla_{\text{CTX-M-55}}$, on the conjugative plasmids from strains S76 and S79. We predicted that the genetic environement of $bla_{\text{CTX-M-55}}$ may be the same as the $bla_{\text{CTX-M}}$ casseette present in pHK23a. Primers targeting IS*26* mediated $bla_{\text{CTX-M-4}}$ cassette were used to depict the genetic structure of the $bla_{\text{CTX-M-55}}$ genes, with results showing that a gene cassette $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-55}}$ flanked by IS26, in the format of IS*26*-$bla_{\text{TEM-1}}$-*orf20*-$bla_{\text{CTX-M-55}}$-IS*26*, was also detectable in the pS76 and pS79 (Table 2.2.3, Fig 2.2.8). The genetic structures of *fosA3* and $bla_{\text{CTX-M-55}}$ were very similar to a previously identified plamsid, pHK23a, except that the $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-4}}$ elements were found in pHK23a and pS76/pS79 respectively [174]. However, the linkage between *fosA3* and $bla_{\text{CTX-M-55}}$ genetic structures in pS76 and pS79 was different from that of pHK23a since attempt to amplify the linkage region between the *fosA3* and $bla_{\text{CTX-M-55}}$ structures in pS76 and pS79 was not sucessful, suggesting that the linkage may be too wide to be amplified by PCR. Further confirmation tests were performed to check the similarity between these two plasmids. Restriction map analysis of pHK23a showed 4 EcoR I and 3 BamH I restriction sites in pHK23a respectively, resulting in 5 and 4 bands under Restriction Fragment Length Polymorphism (RFLP) analysis. However, RFLP analysis of ~80kb pS76 and pS79 showed very different patterns (Data not shown). Further amplification of other regions of pS76 and pS79 using primers designed on the basis of pHK23a sequence did not result in successful amplification in pS76,

suggesting that the plamsid backbones of pHK23a was different from pS76 and pS79 (Data not shown).

So far, four different genetic strutures of typical *fosA3* cassette IS*26-fosA3-orf1-orf2-orf3*-IS*26* and ceftaxime resistance determinants have been identified (Fig 2.2.8). In pHK23a, the IS*26-blaTEM-1-orf20-blaCTX55*-IS*26* cassette is located downstream of the *fosA3* cassette, whereas in the other two genetic structures, the $bla_{\text{CTX-Ms}}$ casseette was found to be upstream of the *fosA3* cassette. In one of the genetic structures, the $bla_{\text{CTX-M}}$ cassette is a truncated one. These data may suggest that a typical *fosA3* cassette could be randomly inserted into the adjacent area of the $bla_{\text{CTX-Ms}}$ casseette, forming different genetic structures. In pS76 and pS79, the two cassettes were seperated by a certain distance (Fig 2.2.8). This suggests that IS*26*-flanked antimicrobial resistance cassette can be inserted into the hot spots in plasmids forming multiple resistance gene clusters in the plasmid. In addition to the typical *fosA3* cassette, IS*26-fosA3-orf1-orf2-orf3*-IS*26*, several C-terminal truncated forms of this cassettes, IS*26-fosA3-orf1-orf2*-IS*26,* IS*26-fosA3-orf1-Äorf2*-IS*26* and IS*26-fosA3-Äorf1*-IS*26,* have been detected in plamsids recovered from *E. coli* isolates, suggesting that IS*26* transposition activity mediated the formation of *fosA3* casseette [161, 171].

In conclusion, this study has for the first time identified and characterized a conjugative FII plasmid carrying both IS*26* mediated $bla_{\text{CTX-M-55}}$ and *fosA3* gene cassettes in two *Salmonella* isolates. The association of the *fosA3* gene with $bla_{\text{CTX-Ms}}$ could eliminate the possiblity of using fosfomycin as an alternative treatment approach, which may be effective for treatment of multidrug-resistant *Salmonella* infection. Further monitoring of transmission of the *fosA3* gene in *Salmonella* is necessary.

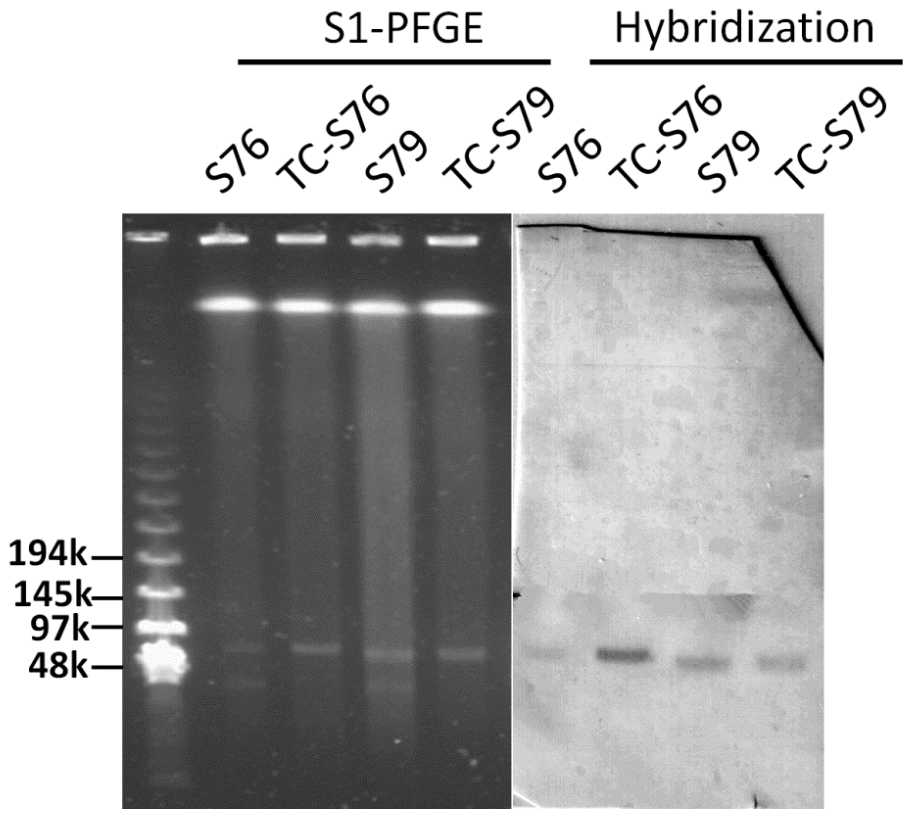

Figure 2.2. 7 S1-PFGE and Southern hybridization analysis of *fosA3* borne plasmids from *Salmonella* strains S76 and S79.

Salmonella isolates S76, S79, and their transconjugants TC-S76, TC-S79, were subjected to S1-PFGE and Southern hybridization analysis using the *fosA3* probe.

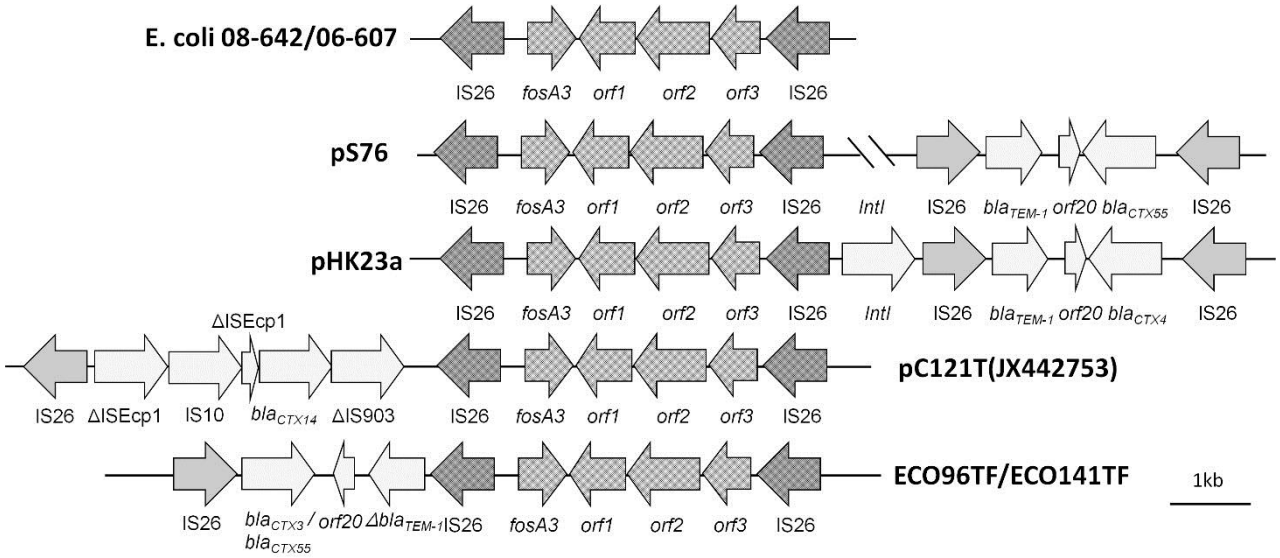

Figure 2.2. 8 Representative genetic structures of *fosA3* and $bla_{\text{CTX-Ms}}$ in different conjugative plasmids

Table 2.2 2 Characteristics of *Salmonella* S76, S79 and their transconjugants.

Strains	Isolation date	Source	serotype	MLST	Replicon typing	MICs(mg/L)										
						FOS	CIP	ENR	NAL	OLA	AMP	CTX	CRO	CAZ	CTO	FOX
S76	22/02/2013	Chicken	Derby	ST11	FII, FIV	>512	0.25	≤0.25	>64	16	>64	>16	>16	>64	>128	2
TC-S76					FII	>512	0.015	≤0.25	16	8	>64	>16	>16	≤0.5	32	2
S79	22/02/2013	Chicken	Enteritidis	ST460	FII, FIV	>512	1	1	>64	>256	>64	>16	>16	32	>128	2
TC-S79					FII	>512	0.06	≤0.25	>64	128	>64	>16	>16	4	32	2

FOS, Fosfamycin; CIP, Ciprofloxacin; ENR, Enrofloxiacin; NAL, Nalidixic acid; OLA, Olaquindox; AMP, Ampicillin; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime; CTO, ceftiofur; FOX, cefotaxime.

Table 2.2 3 Primers used in market antibiotic resistance study

Primer name	Sequence (5'-3')	Expected size (bp)	Target regions
FosA3-F	GCGTCAAGCCTGGCATTT	282	fosA3 gene
FosA3-R	GCCGTCAGGGTCGAGAAA		
FosC2-F	TGGAGGCTACTTGGATTTG	217	fosC2 gene
FosC2-R	AGGCTACCGCTATGGATTT		
FosA-F	ATCTGTGGGTCTGCCTGTCGT	271	fosA gene
FosA-R	ATGCCCGCATAGGGCTTCT		
IS26R	TTACATTTCAAAAACTCTGCTTACC	1416	Upstream IS26-fosA3
FosA3-R	GCCGTCAGGGTCGAGAAA		
FosA3-F	GCGTCAAGCCTGGCATTT	2797	fosA3-IS26 Downstream
IS26F	GGCATCAGTTACCGTGAGC		
CTX-M Group 1-F	ATGGTTAAAAAATCACTGCG	876	CTX-M group 1
CTX-M Group 1-R	TTACAAACCGTCGGTGAC		
CTX-M Group 2-F	ATGATGACTCAGAGCATTCGC	876	CTX-M group 2
CTX-M Group 2-R	TCAGAAACCGTGGGTTACGAT		
CTX-M Group 9-F	ATTCAGAGCTCATGGTGACAAAGAGAGT	876	CTX-M group 3
CTX-M Group 9-R	TAGTAGGATCCTTACAGCCCTTCGGCGAT		
F-pHK23a7725	CTTCGATAATGACCTCGGGA	729	Region between fosA and
R-pHK23a8454	CGCTAATATTCGCCCTGTTC		
F-pHK23a12260	GTCAAAATTCAGCACCACGA	2233	CTX-M gene
R-pHK23a14493	CGGCATCAGTTACCGTGAG		
F-pHK23a11652	TGGGTCTCGCGGTATCATTG	1084	CTX-M Upstream region
R-pHK23a12736	GGTGACTATGGCACCACCAA		
IS26F	GGCATCAGTTACCGTGAGC	1411	TEM gene
TEMR	CGTTCATCCATAGTTGCCTGAC		

2.2.3 The investigation of meropenem resistant isolates from meat products

Hence it is not surprising that Enterobacteriaceae especially the Gammaproteobacteria are commonly found in the hospital patients[181, 182], and other Enterobacteriaceae (Citrobacter freundii)[183]. Carbapenem-resistant Enterobacteriaceae (CRE) is largely confined to hospital, but has been recently also reported in community infections too[59], indicating that it has become an emerging pathogen in the community. Carbapenems are one of the most critically important antimicrobials in the clinical field[184]. There are also many reports about the residue of antibiotic in the environment[185, 186], it is not surprising to find *bla*$_{\text{NDM-1}}$ isolates in the environment.

To analyse the prevalence of carbapenemase resistant strains, isolates from different food products were collected and analysed in order to predict route the carbapenemase dissemination, and the character of those isolates for potential antibiotic resistance mechanisms.

From september 2012 to september 2013, 515 carbapenem resistant strains were isolated from Shenzhen, China. The MIC for 17 antibiotics Including imipenem, meropenem, ertapenem, ceftazidime, colistin, tigecycline were tested by agar dilution according to CLSI standards[139]. The carbapenemases genes (*bla*$_{\text{NDM}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{KPC}}$ and *bla*$_{\text{OXA-48}}$) harboured by the test strains were detected by PCR as previously described[140]. Among the 318 meat samples, 81.2% were positive for carbapenemase strains, although only 8 isolates belonged to Enterobacteriaceae. Among all the collected meat samples, carbapenem resistant isolates were more commonly found in pork(98.8%) than beef (64.4%). The positive rate from shrimp (77.6%) and chicken (77.4%) are nearly the same. Carbapenem resistant isolates were prevalent among the fresh meat samples. Among the 113 *bla*$_{\text{NDM-1}}$ positive isolates, most of the resistance genes failed to conjugate to the receipient strain J53. Only 42 isolates could transfer the resistance genes to J53. The *bla*$_{\text{NDM-1}}$ gene and IncX3 type plasmid could be found in the transconjugants. The S1 PFGE and Southern blot showed the *bla*$_{\text{NDM-1}}$ gene was located at the 54kb plasmid. Most of the wild type strains failed to obtain the PFGE type, other methods like WGS was using to obtain the exact gene context of these isolates.

To understand the molecular characters of those isolates, plasmids were extracted with the Qiagen plasmid midi kit (Qiagen) then sequenced with Illumina NextSeq 500 combined with PacBio RSII single-molecule real-time (SMRT) sequencing platform. After de novel assemble using SPADES[187], the sequence was annotated by RAST[141] and IS Finder (https: //www-is.biotoul.fr). Characterization of the plasmids that could be conjugated were tested by plasmids typing, S1 PFGE and Southern hybridization. In addition, all the conjugative plasmids belonged to IncX3. This studies also found other types of *bla*$_{\text{NDM-1}}$ positive plasmids such as the IncN plasmid. Using entire plasmid DNA sequence to do the homology and phylogenetic analysis, 98% identity with the plasmids pNDM-BTR (GenBank: KF534788.1, harbouring *bla*$_{\text{NDM-1}}$).

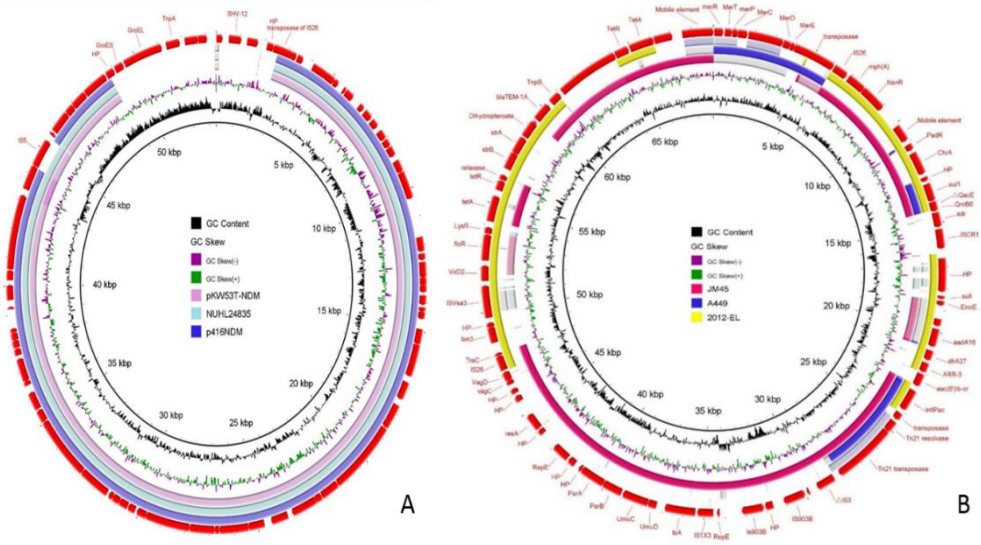

Figure 2.2. 9 Two plasmids recovered from *Citrobacter freundii* 415tsp5 from a pork sample from Shenzhen, Guangdong ,China, 2014.

The size of the plasmid is 44.962kb, and there are 60 coding regions in it. This plasmid was 53.77kb in size, encoded 50 hypothetical proteins and belonged to the IncX3 type plasmid. This plasmid belongs to the most common *bla*$_{\text{NDM1}}$ harbored *IncX3* type plasmid. The major differences between p416NDM and common 54kb plasmid such as pNDM-ECN49 (KP765744.1) is the missing shv cluster. There are two plasmids pKW53T-NDM (KX214669.1, isolated from the Arabian Peninsula, urine, E.coli); NUHL24835 (CP014006.1, Klebsiella pneumoniae , urine, China) that are even more similar to p416NDM than these two plasmids p416NDM only lack of Is mobile element. B The other plasmid which also carried multi-drug resistance

genes belongs to IncR type. It is a 69,088 circular plasmid with 70 predicted ORFs. There are Aminoglycoside， Beta-lactam， Fluoroquinolone， MLS - Macrolide, LIncosamide and Streptogramin B， Phenicol， Rifampicin， Sulphonamide， Tetracycline，Trimethoprim resistance genes in this plasmid. The closest plasmids are JM45(CP006657), which is a Klebsiella pneumoniae isolated from a 72-year-old male patient, and a clinical Vibrios cholera plasmid 2012EL-2176(CP007636). This plasmid is a recombination of multiple mobile elements.

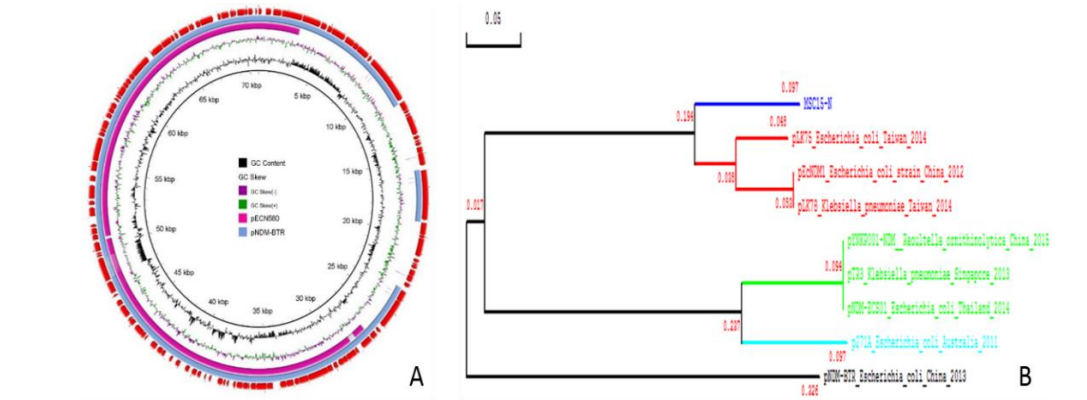

Figure 2.2. 10 A This plasmid is from Escherichia coli strain isolated from chicken sample Shenzhen, China.

The size of this IncN plasmid is 75,018 bp with 152 predicted ORFs. The PMLST typing for this plasmid is ST-7, and the most similar plasmid in NCBI database is Morganella morganii pMR3-OXA181. B Phylogenetic tree. After translating the DNA sequences to amino acid sequences, using maximum-like algorithm to do alignments about msc15-1NDM with other IncR NDM positive plasmid (acquried in NCBI database). Phylogenetic tree was drawn using MEGA 6[188], and the evolution distance shown by the nodes got by bootstrap consensus using 1,000 relicates.

In addition to intrinsic resistance, there are many mechanisms that cause resistance phenotypes such as changes in the permeability of cell[189] and overexpression of efflux pumps[190]. Further study should be done to study the porin mutation(ompK35, ompK36, and ompK37) as previously reported[191], and efflux pumps functions in the strains without carbapenemase genes but still not sensitive to

carbapenems[192].In this study, no carbapenemase resistance genes were found in the rest of the Enterobacteriaceae. That phenomenon was also observabled in clinical isolates called resistance- nodulation-division (RND) efflux pumps such as AcrAB-TolC system[193, 194].

3 Antibiotic target mutations in GI tract

From the above investigation, we can conclude that although in medical field the antibiotic resistant problem is a relative recent problem, the abundant and diversity resistant genes existed in the environment and have the potential ability to transmit the resistance determinants to clinical-pathogen before the broad usage of antibiotics. In the following section, we design experiment to study the situation in bodies and after test those observed phenomena *in vitro*. Finally, we retested those hypotheses in animal models again. There are many scenarios in which bacteria can generate antibiotic resistance, but few studies about this progress in body especially in GI tract. Although people have been studying the evolution of antibiotic resistance for decades, the true processes that occur in the human or animal body have not been fully interpreted. The resistance phenomenon has appeared almost at the same time as the appearance of antimicrobials, but people most care about the spread of resistance in human related microbes such as the pathogen populations or the potential pathogen which can threaten human lives. We propose that to control antibiotic resistance we need to at least slow down the distribution rate by obtaining a better understanding of how resistance evolves in the real situation.

After testing the survival in the market, food animal farms and the clinical isolates, we then used animal models to test the development of resistance and the constraints on bacterial adaptation in the animal bodies. Despite the complex process of antibiotic resistance development, it is now known that there are generally two major molecular mechanisms by which resistance develops: de-novo mutations under clinical antibiotic selection pressure and the acquisition of resistance genes from the environment strains[3]. This studies selected two kinds of antibiotics: fluoroquinolones and β-lactams, to study these two most common ways by which resistance develops. In this chapter, we first studied the target mutations generations in GI tract.

3.1 Emergence of antibiotic resistance in SPF animals

3.1.1 Brief introduction on the current mutation studies

The use of antibiotics in treatment of bacterial infections represents one of the most important inventions in human history. Since its discovery in the 1940s, antibiotics have saved millions of human lives and have also been widely used in the fields of veterinary medicine and agriculture in the past 70 years. However, due to the extensive use and misuse of antibiotics in various settings, most agents have lost their efficacy to bacteria as a result of emergence and spread of multiple-drug-resistant bacterial pathogens [195]. In the last decade, resistance to human first line drugs has increased significantly and the choices of treatment for serious bacterial infections have become extremely limited, threatening to take medicine back into the pre-antibiotic era [195-199].

The mechanisms of antimicrobial resistance in bacteria have been intensively investigated [200]. To date, the mechanisms of bacterial resistance to any antibiotic has been known to a certain extent. However, the driving forces mediating the selection and development of antibiotic resistance in bacteria as well as how resistance genes spread between different organisms and environment niches are complex and are still not completely understood. Since resistance to antibiotics in bacteria has been evolving over a long period of time, a complex repertoire of resistance mechanisms have emerged. At this stage, it is still not clear whether mutation development or acquisition of antimicrobial resistance genes is the primary mechanism which is required to initiate the development of antimicrobial resistance in a bacterial population. Nevertheless, a wide range of transferable elements harbouring antimicrobial resistance genes is known to be responsible for the rapid dissemination of resistance traits in bacteria [201-204]. Regardless of the nature of resistance mechanism or genetic mutations responsible for bacterial antibiotic resistance, constant antibiotic pressure is commonly accepted to be the driving force of expansion of the resistant population. It has been suggested that resistant bacteria were selected at the concentration of antibiotics within the mutation selection window, a concentration between the MIC (minimal inhibitory concentration) and MPC (minimal mutation prevention concentration) [205-207]. However, recent studies have

shown that resistant bacteria can also be selected at very low concentration of antibiotics, which is even lower than that used for growth promotion in animals, and those of environmental antibiotic residues due to natural production by microorganisms and human contamination [208]. However, these studies were conducted in *in vitro* model and could not explain how animal GI tract organisms respond to different concentrations of antibiotics, since growth promotional usage of antibiotics in animals is considered as the most important practice that selected for both antibiotic-resistant genes and bacterial pathogens [209-211]. Animal GI tract is considered a more complicated environment than any other ecosystems. Recent studies have demonstrated the role of bacterial stress responses in antibiotic resistance development [212-214]. The environmental stresses that the bacteria encounter may lead to variation in physiological functions of the organisms, eliciting changes in the intrinsic mutation rate and abilities to survive drug action [215]. Animal GI tract imposes a mixture of stresses to bacteria including low pH, oxidative stress, starvation stress, antimicrobial compounds and interactions between microbiota, which may influence the development of antibiotic resistance in GI tract flora. However, in contrast to *in vitro* condition, the physiological changes and fitness costs arisen during the response to these stresses and the development of mutations may in turn put the GI track bacteria under adverse conditions which may affect the survival fitness of such organisms in the GI tract environment. The effect of these factors on the development of antimicrobial resistance in GI track bacteria is not fully understood. In this study, a rat model was used to test the effect of different concentrations of antibiotics on the resistance development in *E. coli* in animal GI tract.

Enrofloxacin was purchased from the Sangon Biotech company (Shanghai, China). The antibiotic was dissolved in 0.1 M NaOH at 10 mg/mL stock solution on the day of use. 11– 15 weeks old, specified pathogen-free (SPF) male SD rats with body weight 250 -350g were used in all experiments. Animals were purchased from Guangdong Medical Laboratory Animal center and were housed individually and allowed free access to food and water. They were examined twice every day for any clinical signs such as behaviour, gastrointestinal function, respiratory distress, food and water intake. The experimental protocol was approved by the Research Animal Care and Use Committee of the Hong Kong Polytechnic University.

3.1.2 Antimicrobial treatment

Since fluoroquinolones are concentration-dependent antibiotic[216], we checked the effect of different concentrations of fluoroquinolone on the development of resistance to enrofloxacin in *E. coli* in animal GI tracts. Thirty male rats were equally divided into six groups: one group was treated with therapeutic dose of enrofloxacin (10mg/kg body weight), one was treated with saline as control and other groups were treated with doses of 10 times, 1/10 time, 1/100 time and 1/1000 time of the therapeutic dose.

The antibiotic treatment regimen lasted about 1 month including three antibiotic treatments and three cessation gaps between treatments. All the rats were subjected to different doses of enrofloxiacin treatment for 5 days, then cessation of antibiotic treatment for 4 days, another enrofloxiacin retreatment for 5 days, followed by another cessation of antibiotic treatment for 8 days, then enrofloxiacin retreatment for another 5 days, and tracing for 3 more days without antibiotic treatment.

At the indicated time intervals, fresh feces (250 –500 mg) were collected and resuspended in 1 ml of saline. The suspension was mixed and diluted by 10-fold in saline. 100µl of suspension was plated on MacConkey agar containing 0mg/L, 0.125mg/L, 0.5mg/L and 2mg/L of enrofloxacin. The plates were incubated at 37ºC for 12h and the total colony counts were recorded. The CFUs (colony forming units) per gram of feces were determined. Colonies that showed typical morphology of *E. coli* on MacConley plate (pink to rose-red, large regular colonies) were considered as *E. coli* and some of which were confirmed by 16S rRNA sequencing using primers.

3.1.3 Antimicrobial susceptibility

Antimicrobial susceptibilities to enrofloxacin were determined by the agar dilution method in accordance with Clinical and Laboratory Standards Institute guidelines [217] using *E. coli* strain ATCC 25922 as quality control. The minimal inhibitory concentrations (MICs) of enrofloxacin were determined following CLSA guidelines[217].

3.1.4 Determination of the target mutations in *E. coli*

Presence of target mutations of the Quinolone-Resistance Determining Regions (QRDR) of the *gyrA* and *parC* genes in *E. coli* were determined by PCR as previously described[217].

Thirty SPF SD rats were selected for this experiment. Resistance background of gastrointestinal tract *E. coli* of these rats were checked by plating 200~300mg of fecal samples of the rats on MacConkey plates with 0, 0.125, 0.5 and 2mg/L of enrofloxacin. All rats were found to contain a similar amount of *E. coli* in their gastrointestinal tract (Data not shown). Five rats exhibited a background of less susceptible *E. coli* which can grow on MacConkey plate containing 0.5mg/L of enrofloxain. No fecal *E. coli* was able to grow on MacConkey plate with 2mg/L concentration of enrofloxain (Data not shown). Twenty colonies with typical morphology of E. coli from MacConkey plates were randomly selected and confirmed to be *E. coli* by 16S rRNA sequencing. Similar *E. coli* confirmation was performed for the following each group of experiment by randomly selecting 20~40 *E. coli* for 16S rRNA sequencing. All the checked colonies were confirmed to be *E. coli* (Data not shown).

Upon background check, these rats were separated into six experimental groups. All five rats with a low background of *E. coli* with reduced susceptibility to enrofloxacin were grouped into one category and treated with a high dose of enrofloxacin (10X therapeutic dose). The rest of the 25 rats were randomly separated into 5 groups with one control group and 4 different treatment groups including therapeutic dose, 1/10, 1/100 and 1/1000 times of therapeutic doses treatments.

3.1.5 Effect of high dose of antibiotic treatment on rat GI tract *E. coli*

For the 10X therapeutic dose treatment group, the application of enrofloxaicn caused gradual eradication of rat GI tract *E. coli*. The numbers of GI tract *E. coli* decreased gradually during the first three days of treatment; the reduction became dramatic at the fourth day of treatment and the number of *E coli* recoverable remained

at a low level for the first treatment period. After withdrawing the antibiotics, *E. coli* appeared again at the 2nd day after the cessation of antibiotic treatment and increased on the 3rd and 4th day. At the beginning of the second course of treatment period, the numbers of GI tract *E. coli* almost returned back to the normal level. During the whole 5-days antibiotic treatment, the numbers of GI tract *E. coli* were not affected, and remained at the normal level, which lasted throughout the second antibiotic cessation period. The GI tract *E. coli* gradually decreased again upon the start of the third antibiotic treatment. The number of *E. coli* became dramatically reduced again at the 3rd day of the third course of the treatment and then gradually recovered at the 4th and 5th days of the antibiotic treatment. The number of *E. coli* kept recovering upon cessation of antibiotic treatment (**Fig 3.1.1 A**).

The effect of antibiotic treatment to the development of fluoroquinolone-resistant *E. coli* was recorded. The number of less susceptible *E. coli*, which can grow at MacConkey plate with 0.125mg/L was high before antibiotic treatment and slightly decreased on the 2nd and 3rd days of antibiotic treatment. The number of less susceptible *E. coli* became dramatically decreased on the 4th and 5th days of treatment. After withdrawing the antibiotic, the number of less susceptible *E. coli* reverted back to the normal level and remained so until the beginning the third course of the antibiotic treatment. The less susceptible *E. coli* slightly decreased at the first day of the third course of the antibiotic treatment and became undetectable at the 3rd day of the treatment, then recovered to normal level throughout the rest of the treatment and non-treatment period (**Fig 3.1.1 B**).

The *E. coli* that can grow on MacConkey with 0.5mg/L enorfloxacin exhibited different response to high dose enrofloxacin treatment. The numbers of *E. coli* that can grow on MacConkey with 0.5mg/L enorfloxacin reduced on the 2nd day of treatment and became almost undetectable throughout the rest of the first, second and third treatments and non-antibiotic treatment period except for a period of brief appearance during the later course of the 3rd treatment and antibiotic cessation period (**Fig 3.1.1 C**). Throughout the whole course of treatment, no *E. coli* isolates could be recovered on MacConkey plate with 2mg/L of enorfloxacin, suggesting that high dose antibiotic could not select for fluoroquinolone-resistant GI tract *E. coli*.

Each of 20 *E. coli* isolates that grew on MacConkey with 0.125mg/L and 0.5mg/L enrofloxacin respectively were selected to check their MIC and target mutation profiles. *E. coli* that grew on MacConkey with 0.125mg/L enrofloxacin exhibited a MIC range of enrofloxacin of 0.006~0.03 with no mutation in any of the four target genes *gyrA*, *gyrB*, *parC* and *par*E; yet all *E. coli* strains that grew on MacConkey with 0.5mg/L enrofloxacin exhibited MIC of enrofloxacin of 0.25~1mg/L with single mutation inn *gyrA* (S83L) (**Table 3.1.1**). Therefore, the MacConkey plate with 0.5mg/L enrofloxacin could be used to check for the mutation rate of GI tract *E. coli* upon antibiotic treatment.

3.1.6 Effect of therapeutic dose of antibiotic treatment on rat GI tract *E. coli*

For the therapeutic treatment group, the GI tract *E. coli* exhibited slightly less susceptible *E. coli* background. Hence the therapeutic treatment dose of enrofloxacin did not have any effect on the numbers of *E. coli* in rat GI tract (**Fig 3.1.2 A**). However, after antibiotic treatment, the number of less susceptible *E. coli* decreased slightly and then increased to high level throughout the first course of antibiotic treatment and antibiotic cessation periods. During the second course of treatment, the numbers of less susceptible *E. coli* reduced gradually during treatment and remained at a lower level during the second antibiotic withdrawal period. During the third course of antibiotic treatment, the number of less susceptible *E. coli* increased to a high level and remained so until the end of the experiment (**Fig 3.1.2 B**).

The numbers of *E. coli* that grew on MacConkey agar with 0.5mg/L enrofloxacin (mutation rate) increased to around half of the total *E. coli* at the 2nd day upon the treatment of enrofloxicin and remained at this level during the first course of the treatment. *E. coli* strains with mutation disappeared during the antibiotic withdrawal period and reappeared during the 2nd course of treatment albeit at a lower level. *E. coli* strains with mutation increased to a high level at the 2nd day of the third course of treatment, then decreased and reappeared again during the antibiotic cessation period (**Fig 3.1.2 C**). The data showed that mutation in *E. coli* might mainly triggered by antibiotic treatment and release of the antibiotic pressure can reduce the numbers of *E. coli* with mutation, which suggested that without antibiotic pressure, the *E. coli* with

mutation may be less competitive than other normal *E. coli* in animal GI tract. The long term antibiotic treatment may make *E. coli* with mutation to adapt to animal GI tract, which can be seen from the increased number of *E. coli* with mutation at the end of third course of antibiotic treatment and the following cessation period.

3.1.7 Effect of sub-therapeutic and lower doses of antibiotic treatment on rat GI tract *E. coli*

Similar to the effect of the therapeutic dose of antibiotic treatment, sub-therapeutic dose of enrofloxacin did not have any impact on the overall numbers of *E. coli* in rat GI tract (**Fig 3.1.3 A**). The less susceptible *E. coli* strains emerged upon antibiotic treatment and remained at a stable level throughout the course of the experiment (**Fig 3.1.3 B**). The numbers of GI tract *E. coli* with target mutation slightly increased upon antibiotic treatment and disappeared during the antibiotic cessation period for the first two courses of treatments. The mutation rate was significantly increased in *E. coli* upon the third course of antibiotic treatment (**Fig 3.1.3 C**).

For the lower-level antibiotic treatment groups, namely 1/100 and 1/1000 of the therapeutic doses, no change in the number of GI tract *E. coli* could be observed throughout the experiment (**Fig 3.1.4 A, 3.1.5 A**). The less susceptible *E. coli* could be selected during the second and third courses of antibiotic treatment and the *E. coli* with mutation could also be selected during 2nd and 3rd courses of the antibiotic treatments, with a higher rate at the end of the 3rd course of the treatment, suggesting long term antibiotic treatment is the major trigger for the development of resistance in GI tract *E. coli* (**Fig 3.1.4 B,C; 3.1.5 B, C**). For the control group, neither less susceptible *E. coli* nor *E. coli* with target mutation could be selected (**Fig 3.1.6**). Throughout the whole experiment, no *E. coli* that can grow on 2mg/L of enrofloxacin could be obtained (Data not shown).

Each of 20 *E. coli* isolates that grew on MacConkey with 0.125mg/L and 0.5mg/L enrofloxacin from different groups of experiments were selected to check their MIC and target mutation profiles. Different from the results obtained from the high dose treatment group, *E. coli* that grew on MacConkey with 0.125mg/L enrofloxacin showed MIC range of enrofloxacin of 0.015~0.5, a little bit higher MIC

than E. coli from high dose treatment group, with no mutation on any of the four target genes *gyrA*, *gyrB*, *parC* and *parE* for most of the strains, but not all strains; on the other hand, *E. coli* that grew on MacConkey with 0.5mg/L enrofloxacin exhibited MIC of enrofloxacin of 0.25~1mg/L with a single mutation on GyrA (S83L) and no mutation at other target genes for all test strains, similar to the results from high dose treatment group (**Table 3.1.1**). The results further confirmed the use of MacConkey plate with 0.5mg/L enrofloxacin as a tool to check for the mutation rate of GI track *E. coli* upon antibiotic treatment.

Improper uses of antibiotics from clinical applications and promotion of animal growth are the main causes for high prevalence of antimicrobial resistance in bacterial pathogens[209-211]. Evidences have shown that in-feed use of antibiotics could dramatically lead to an increase in the number of antimicrobial resistant genes and the size of microbial flora pool in anima GI tract[209]. Oral usage of antibiotics may be the direct route that facilitates the selection of antimicrobial resistant gene and bacteria pool in animal GI tract [218]. These studies reinforced the concept that the antibiotic pressure in animal GI tract favoured the amplification of antibiotic-resistant bacterial pool and cause it to become dominant in the animal GI tract, therefore increasing both antibiotic-resistant gene and bacteria pool. This study focuses mainly on the understanding of how GI tract bacteria, in particular *E. coli*, develop resistance upon encountering different levels of antibiotic pressure.

To obtain meaningful interpretation of the data, all rats were checked for the initial load of GI tract *E. coli* and their background level of susceptibility to enrofloxacin. Five rats with a predominant background of low level resistance to enrofloxacin (grow at MacConkey with 0.5mg/L, but not with 2mg/L) were arranged into one group and treated with high dose of enrofloxacin to check whether high dose of antibiotic could eradicate organisms with intermediate enrofloxacin resistance, while other groups of mice with lower or no background of less resistant *E. coli* were treated with different doses of antibiotic. This study has come up with several conclusions that may be useful for the understanding of bacterial resistance development in animal GI tract. Firstly, high dose of antibiotic treatment can eradicate less susceptible *E. coli* and prevent mutation even with long term treatment; therapeutic dose of enrfloxacin could select for the less susceptible *E. coli* and those

containing target mutations. It is commonly accepted that intake of therapeutic dose of antibiotics for the whole course of treatment could prevent the development of resistance in bacterial pathogens. Our data showed that only high dose of antibiotic could effectively eliminate *E. coli* with target mutation, which are present in rat GI tract before treatment. However, therapeutic dose, 1/10 and 1/100 of therapeutic doses of enrfoloxacin could select for less susceptible *E. coli* without target mutation and *E. coli* with de novo target mutation. Extremely low dose of enrofloxacin treatment, such as 1/1000 of therapeutic dose, can select for less susceptible *E. coli* without target mutation and *E. coli* with de novo target mutation, but with lower efficiency. From the data obtained, we can also see that most of the target mutations in *E. coli* were selected during the later course of antibiotic treatment and antibiotic cessation periods. Another interesting finding for this study is that antibiotic treatment, except for those with high dosage, did not affect the total number of GI tract *E. coli*. Even under high dosage antibiotic treatment, the number of GI tract *E. coli* decreased upon treatment and then became normal even during the second treatment. The normal GI tract *E. coli* may be reflected by the healthy status and normal growth rate of rats which received different doses of antibiotic treatment.

Recent studies have reported the selection of resistant bacteria under very low concentration of antibiotics *in vitro* [208]. However, these studies mainly focus on determining the concentration of antibiotics that allow the resistant bacteria to compete with their drug-susceptible counterparts favorably. In other word, we wish to better understand how concentration of antibiotics lower than the MIC of susceptible cells, namely sub-MIC levels, promotes the enrichment of resistant bacteria. One previous study also showed how low concentration of antibiotic selects for de novo resistant mutants. The selection process in such study was antibiotic dependent. At 1ug/ml concentration of streptomycin, *Salmonella* could develop resistance to up to 128ug/ml of streptomycin after 700 generations, whereas at 2.3ng/ml of ciprofloxacin, *E. coli* could be selected at 184ng/ml, but not resistant to ciprofloxacin after 600 generations. Similar to *in vitro* studies, our data showed that regardless of the concentrations of the enrofloxacin within three courses of treatment and cessation regimen, no norofloxacin-resistant (MIC≥4mg/L) *E. coli* could be selected. In addition, no double target mutations in *gyrA* or *parC* could be detected in *E. coli* that

could grow on MacConkey with 0.5mg/L enrofloxacin. Our data is consistent with an early study in chicken in that treatment with enrofloxacin at doses routinely prescribed (50 ppm) rapidly reduced the fecal counts of colonized *E. coli* below the detection limit and did not induce resistance, whereas high frequencies of fluoroquinolone-resistant colonized *C. jejuni* were selected due to the *de novo* mutation at the target genes [219]. These data suggested that the generation of double target mutations in GyrA and/or ParC, which is the major mechanism of fluoroquinolone resistance in *E. coli*, may be associated with a fitness cost that hampers its survival in animal GI tract [220, 221]. The development of enrofloxacin-resistant *E. coli* in animal GI tract may require long term selection under antibiotic selective pressure in animal gut or may be due to colonization of enrofloxacin-resistant *E. coli* that has been selected outside the animal gut. Lastly, the development of enrofloxacin-resistant *E. coli* in animal GI tract may require the acquisition of plasmid mediated resistance determinant, which may further facilitate the development of target mutation and therefore development of enrfloxacin resistance in *E. coli*. Recent study has shown the high carriage of different PMQR genes in animal *E. coli* isolates, which contribute to the development of enrofloxacin resistance in *E. coli* [201, 204]. Different hypotheses of how GI tact *E. coli* development resistance to fluoroquinolone require further investigations.

In conclusion, this study demonstrated the response of rats GI track *E. coli* to different concentrations of enrofloxaicin treatment and provided insights into the rational use of antibiotics in animal husbandry.

Table 3.1. 1 MIC of enrofloxacin and target mutation profiles of rat GI track *E. coli*

Animal group	Origin of E. coli (enrofloxacin selective plates)	Number of isolates	MIC range of enrofloxacin (mg/L)	GyrA(No. of E. coli)	ParC
A*	0.125mg/L	20	0.015~0.6	Wt (20/20)	Wt
	0.5mg/L	20	0. 25~1	S83L (20/20)	Wt
B	0.125mg/L	20	0.015~0.6	Wt (20/20)	Wt
	0.5mg/L	20	0.25~1	S83L (20/20)	Wt
C	0.125mg/L	20	0.015~0.5	Wt (19/20), S83L (1/20)	Wt
	0.5mg/L	20	0.25~1	S83L (20/20)	Wt
D	0.125mg/L	20	0.06~0.5	Wt (17/20), S83L (3/20)	Wt
	0.5mg/L	20	0.125~1	S83L (20/20)	Wt
E	0.125mg/L	20	0.06~1	Wt (15/20), S83L (5/20)	Wt
	0.5mg/L	20	0.125~1	S83L (20/20)	Wt

*A, high dose (10-fold therapeutic dose) enrofloxacin treatment; B, therapeutic dose of enrofloxacin treatment; C, sub-therapeutic dose (1/10 of therapeutic dose) of enrofloxacin treatment; D, low dose (1/100 of therapeutic dose) enrofloxacin treatment; E, very low dose (1/1000 of therapeutic dose) of enrofloxacin treatment.

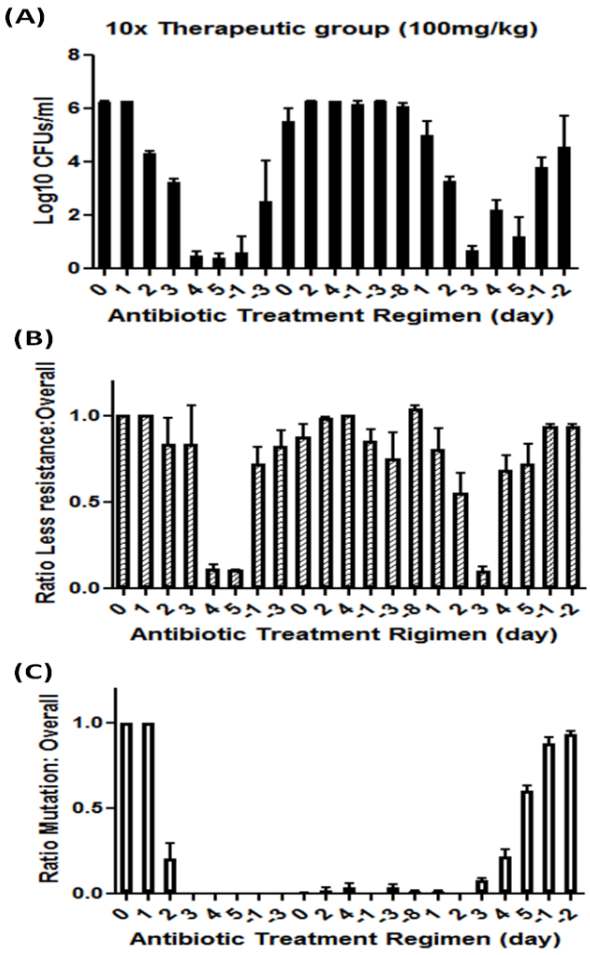

Figure 3.1. 1 Effect of high dose (10 X therapeutic dose) enrofloxacin treatment on mutation development in rat GI tract *E. coli*.

(A) Levels of rat GI tract *E. coli* during enrofloxacin treatment; (B) levels of less susceptible (grown on MacConkey supplemented with 0.125mg/L of enrofloxacin) GI tract *E. coli* during enrofloxacin treatment; (C) Levels of rat GI tract *E. coli* with target mutation (grown on MacConkey supplemented with 0.5mg/L of enrofloxacin) during enrofloxacin treatment. The number is the average of data from 5 rats in the group.

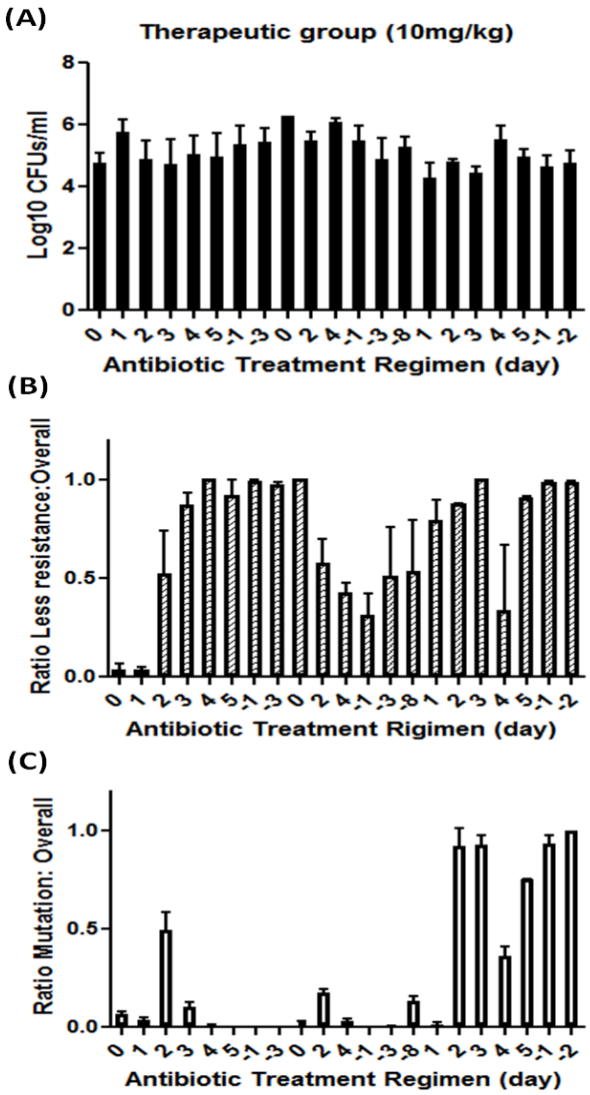

Figure 3.1. 2 Effect of therapeutic dose of enrofloxacin treatment on the mutation development in rat GI tract *E. coli*.

(A) Levels of rat GI tract *E. coli* during enrofloxacin treatment; (B) levels of less susceptible (grown on MacConkey supplemented with 0.125mg/L of mg/L of enrofloxacin) GI tract *E. coli* during enrofloxacin treatment; (C) Levels of rat GI tract *E. coli* with target mutation (grown on MacConkey supplemented with 0.5mg/L of enrofloxacin) during enrofloxacin treatment. The number is the average of data from 5 rats in the group.

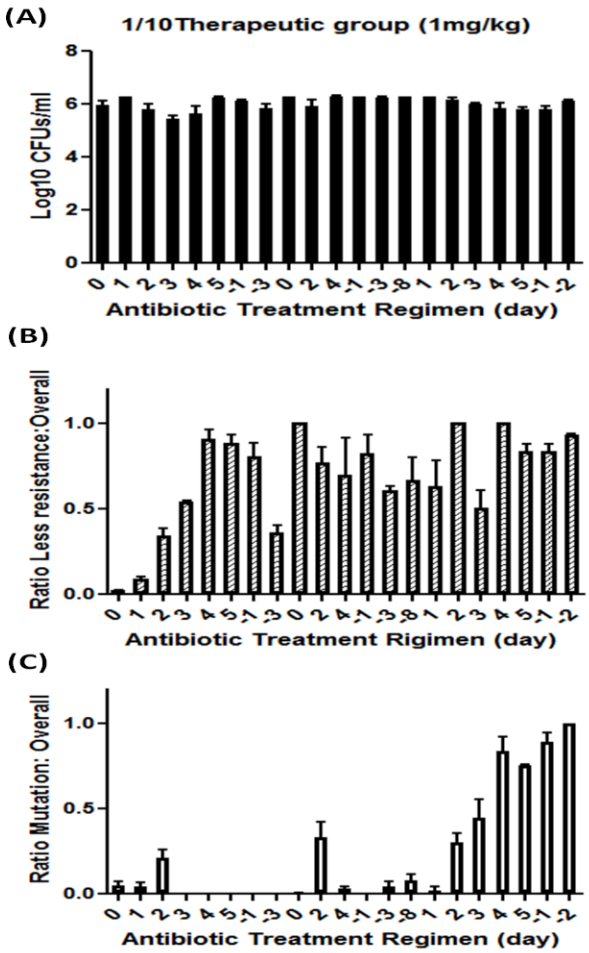

Figure 3.1. 3 Effect of sub-therapeutic dose (1/10 of therapeutic dose) of enrofloxacin treatment on mutation development in rat GI tract *E. coli*.

(A) Levels of rat GI tract *E. coli* during enrofloxacin treatment; (B) levels of less susceptible (grown on MacConkey supplemented with 0.125mg/L of enrofloxacin) GI tract *E. coli* during enrofloxacin treatment; (C) Levels of rat GI tract *E. coli* with target mutation (grown on MacConkey supplemented with 0.5mg/L of enrofloxacin) during enrofloxacin treatment. The number is the average of data from 5 rats in the group.

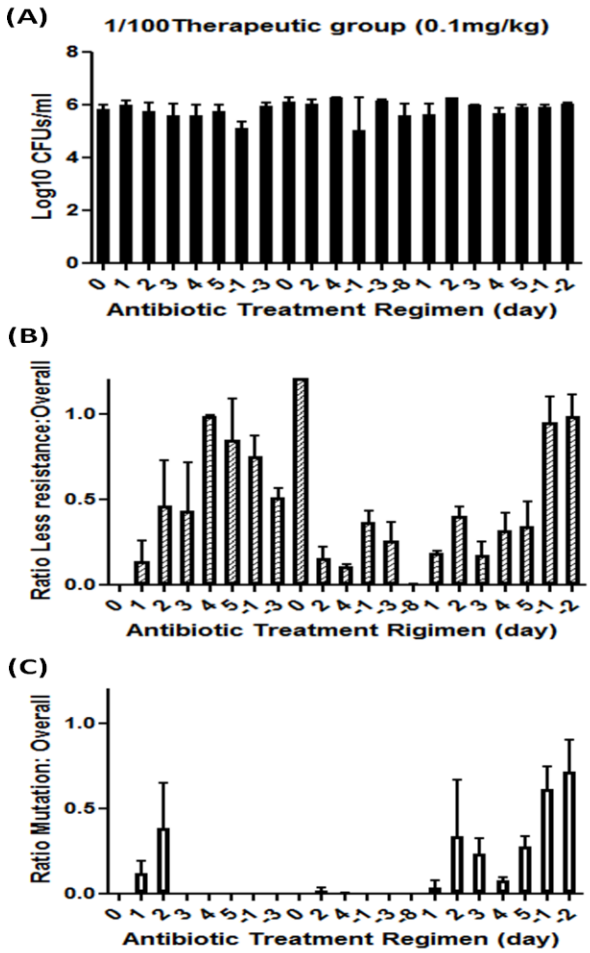

Figure 3.1. 4 Effect of low dose (1/100 of therapeutic dose) enrofloxacin treatment on mutation development in rat GI tract *E. coli*.

(A) Levels of rat GI tract *E. coli* during enrofloxacin treatment; (B) levels of less susceptible (grown on MacConkey supplemented with 0.125mg/L of enrofloxacin) GI tract *E. coli* during enrofloxacin treatment; (C) Levels of rat GI tract *E. coli* with target mutation (grown on MacConkey supplemented with 0.5mg/L of enrofloxacin) during enrofloxacin treatment. The number is the average of data from 5 rats in the group.

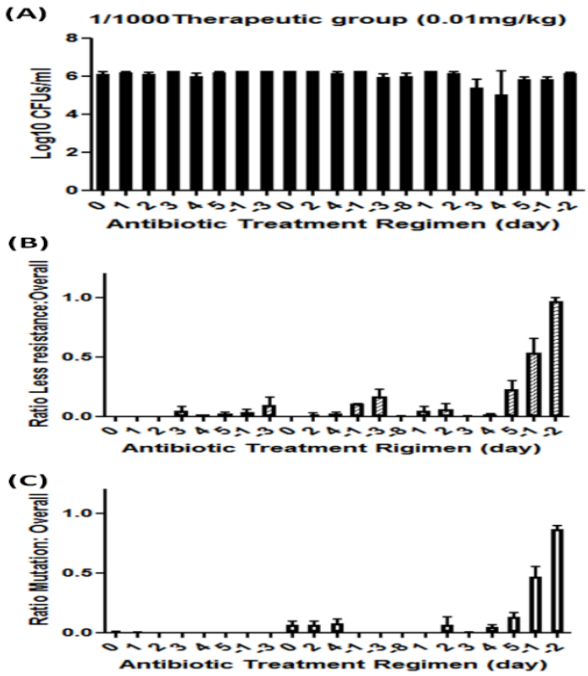

Figure 3.1. 5 Effect of very low dose (1/1000 of therapeutic dose) of enrofloxacin treatment on mutation development in rat GI tract *E. coli*.

(A)Levels of rat GI tract *E. coli* during enrofloxacin treatment; (B) levels of less susceptible (grown on MacConkey supplement with 0.125mg/L of enrofloxacin) GI tract *E. coli* during enrofloxacin treatment; (C) Levels of rat GI tract *E. coli* with target mutation (grown on MacConkey supplemented with 0.5mg/L of enrofloxacin) during enrofloxacin treatment. The number is the average of data from 5 rats in the group.

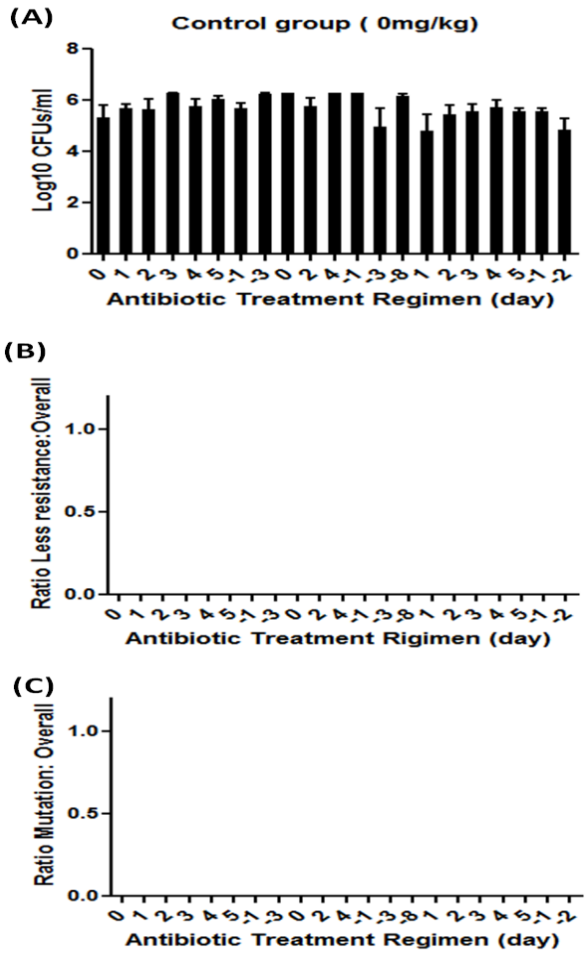

Figure 3.1. 6The mutation development in rats GI tract *E. coli* in control group.

(A) Levels of rat GI tract *E. coli* during enrofloxacin treatment; (B) levels of less susceptible (grown on MacConkey supplemented with 0.125mg/L of enrofloxacin) GI tract *E. coli* during enrofloxacin treatment; (C) Levels of rat GI tract *E. coli* with target mutation (grown on MacConkey supplemented with 0.5mg/L of enrofloxacin) during enrofloxacin treatment. The number is the average of data from 5 rats in the group.

3.2 Predicted mechanism for generation mutation in vivo

Although the number of deleterious mutations are usually larger than that of beneficial mutations, the mutations may promote adaptation in adverse environments[222, 223]. Since antibiotic pressure is the most common selecting pressure for pathogens, mutated strains are more often associated with antibiotic resistance[224-226]. A previous study noticed that bacteria may use different ways to

reduce the fitness cost brought about by antibiotic resistance within and outside host[224]. As the gut microbiome is affected by various factors such as the nature of feed[227], we envisage the need to build more models to decipher the evolutionary forces that shape changes within the population of commensal strains.

Representative strains were cultured and collected from Mac Conkey (MAC) agar plates before and after antibiotic treatment, and identified by MALDI-TOF MS. A total of 300 *E. coli* isolates recovered before treatment and 40 strains collected after the treatment were subjected to pulsed-field gel electrophoresis (PFGE) upon Xbal digestion. The commensal strains isolated from rats in this study and the standard strain BW25113 which harboured mutations were further studied and listed in Table 3.2.1.

Three SD male rats were obtained from the Guangdong Medical Laboratory Animal Center where the animals had free access to feed and water. The cages, food and water had been freshly autoclaved. After a week of observation, fecal samples were collected and the number of *E.coli* strains that was present per unit weight of each fecal sample was recorded after serial dilutions. A single dosage of ciprofloxacin (Sheng gong, Shanghai) at 1mg/kg body weight was administered each day through oral gavage. After 5 days of ciprofloxacin treatment, a 5-day cessation period was introduced before the next treatment. Such process was repeated 4 times. The total plate count method was used to identify the changes in the number of isolates collected during the process of ciprofloxacin treatment. Samples were collected every 2 days, and fecal samples were homogenized, serially diluted before spreading onto plates. Up to 300 *E. coli* isolates were randomly collected from the fecal samples of both rats. Four colonies were picked from each sampling. Pulsed-field gel electrophoresis (PFGE) typing were performed for all the isolates, following the PulseNet protocol (http://www.pulsenetinternational.org/protocols/). Dendrogram of cluster analysis of the isolates was created by BioNumerics.

Table 3.2. 1 Strains used in this studies

Escherichia coli	Genotype	
E2	Wildtype	This study
E3	Wildtype	This study
E15	Wildtype	This study
E21	Wildtype	This study
E27	Wildtype	This study
E62	Wildtype	This study
BW25113	Wildtype	[228]
ΔdsdC	ΔdsdC::kan (KanR)	This study
ΔpgaA	ΔpgaA::kan (KanR)	This study

28 male SD rats were treated with streptomycin (5mg/ml) which was administered in the drinking water of the rats as described previous [229] ; a 1:1 mixture of wild type strains (BW25113) and D-serine locus knockout strains (ΔpgaA) were added into the drinking water bottles and the rats were fed for 12 hours. Ciprofloxacin treatment began at 24 hours (12 hours later than the withdraw streptomycin). All the procedures had been approved by the Research Animal Care and Use Committee of the Hong Kong Polytechnic University.

Bioinformatic analysis of different representative *E.coli* strains

Following PFGE analysis, genomic DNA was extracted from the six representative isolates after overnight culture using Invitrogen PureLink genomic DNA mini kit, following the manufacturer's instruction. Libraries for the sequencing using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®(NEB) were obtained so that 2×150 bp paired-end reads could be sequenced by the NextSeq 500 Illumina. After *de novo* assembly by SOAPdenovo2[230], contigs were uploaded to CSI Phylogeny 1.2 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). The *Escherichia*

coli strain K-12 substr. MG1655(NC_000913.3) was chosen as the reference genome[231]. The SNP profile was obtained using iTOL to construct the Phylogenetic tree (http://itol.embl.de/index.shtml)[232].

To obtain the complete genome of strains E3 and E62, PacBio RSII single-molecule, real-time (SMRT) sequencing was performed to create libraries of 20 kb at the Wuhan Institute of Biotechnology, Wuhan, China. SPAdes was utilized to perform hybrid-assembly to complete sequencing of the two isolates[233].

PM tests were performed following the instruction of the manufacturer, using R2A agar for bacterial pre-growth. In order to measure the ability to utilize specific nutrients, this study chose a nutrient limit agar for pre-growth [234, 235].

A total of 12 pathogen-free chicken (500g each) which had never been treated with antibiotics before were chosen for the experiment. These animals were randomly divided into 3groups, 4 within each group, and given free access to food and water. Groups One, Two, and Three were treated with ciprofloxacin-containing saline (10mg/kg and 1mg/kg respectively) through gavage every 5 days, followed by a 5-day interval without antibiotic treatment. The biofilm genes in bacterial strains residing in the GI tract of the chicken were detected by determining the existence of the *pgaA* and *pgaC* genes by PCR as described previously [236]. This process was repeated until resistant strains were recovered. Prior to treatment, 50 *E.coli* strains were selected from each animal and subjected to testing of the portion of carriage of both *hipAB* and biofilm genes. At the beginning of the experiment, the portion of carriage of both genes in every group was respectively 0.765±0.2087263, 0.5275±0.1241974 and 0.635±0.1652271. Fecal samples were collected every two days, and after serial dilution the samples were spread on the MAC agar with 0, 0.5 μg/ml of Ciprofloxacin. After two consecutive treatment, the resistant strains were recovered from the second and third groups. At this stage, all the recovered strains were found to harbor the *hipAB* and biofilm genes. No resistant strains were found in the Group One until the last day of experiment.

To probe the underlying basis of growth advantage of strains E3 and E62 in normal GI condition and antibiotic selection pressure respectively, we first looked for genes that were uniquely by strains E 3 and E62, based on the Kegg annotation data.

There were a total of 50 unique genes harbored by strain E3. 21 of them were phage and plasmid replication genes, one was involved in synthesis of the folate, which was beneficial to the host, one is involved in utilization of L-rhamnonate, even though strain E62 grew better in this substrate, 3 genes were involved in synthesis of cell wall and capsule, 6 genes were found to belong to the pathway of D-allose utilization, which is extremely rare in nature[237], one gene was related to antibiotic synthesis, 5 were found to encode hypothetical protein, and the rest were D-serine utilization genes. The growth advantages of E62 when compared with E3 was revealed by the comparing the core genes of the two strains. Comparing the core genes of 54 strains which harbored QRDR mutations in the NCBI complete *E.coli* genome database, we identified a total of eight core genes that were harbored by strain E62 and the 54 strains carrying QRDR mutations, but not the E3 strain. Among the eight genes, the *gpr*, *rhas*, *yjdJ* were involved in nutrient utilization, however, the MPC of strains in which these genes had been deleted did not exhibit changes when compared to the strain BW25113. Another two genes, namely *dkgB* and *yhbX* were also found to exhibit little effects on MPC according to the gene knockout data. Additionally, most of the strains in the database were found to harbor these genes, only few (156/4513,440/4513) strains lack those genes. Another gene, *manZ*, is even more common in the database, with only 60/4513 genomes lacking this gene. On contrast, the biofilm gene *pgaB* and other known biofilm genes were found in 80% of the *E. coli* genomes in the NCBI database.

All the 4513 *E.coli* genome sequences were downloaded from the NCBI genome(https://www.ncbi.nlm.nih.gov/genome/) 2016.05.01. Among the 4513 genomes, there were 2413 genomes with known isolation source. A total of 1608 *Acinetobacter baumannii* genomes in the database were also examined to test our hypothesis that biofilm plays a very important role in the development of antibiotic resistance in the human body.

Total RNA was isolated from strain E62 cultured in LB broth with and without ciprofloxacin (0.5μg/ml) for 10 hours upon inoculation of a 1:100 dilution of overnight culture. Total RNA was isolated using Rneasy kit (QIAGEN) following the manufacturer's instructions. The total RNA sample was then treated with the Turbo DNA free kit (Ambion, Austin, TX) following the manufacturer's instruction. Reverse

transcription was performed using the SuperScript III quantitative one-step kit (Invitrogen).16s rRNA was used to normalize the expression level of the test genes in order to compare the expression levels of biofilm genes under different test conditions. The 16s rRNA primers and biofilm gene primers were used as previously described [238, 239]. SYBR Green master mix (Applied Biosystems) was used to perform Real-time PCR. Results were analyzed by the iQ5 optical system software.

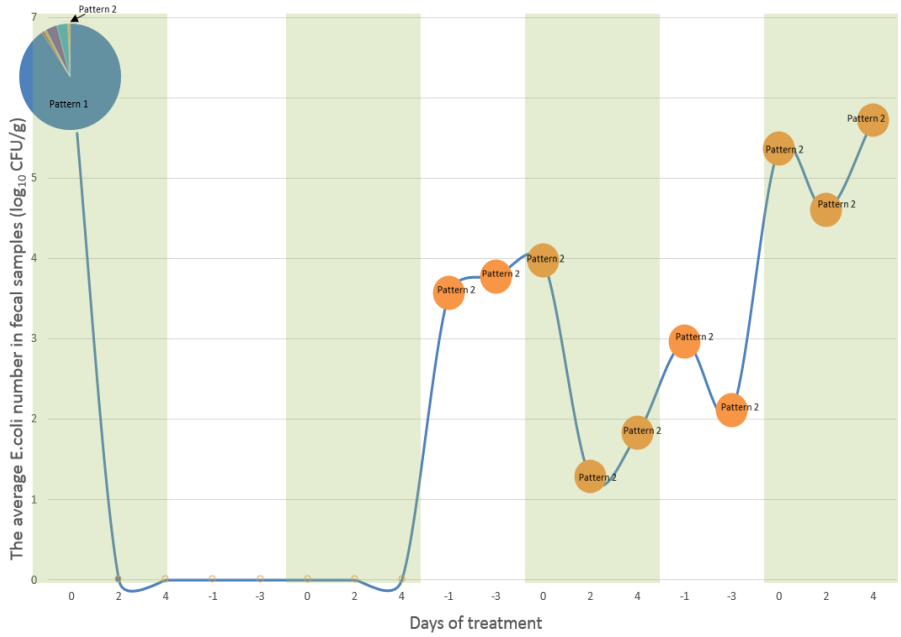

Figure 3.2. 1: Dynamic changes of the E. coli number

Dynamic changes in the average number of *E. coli* strains in the faecal samples of the three rats (log $_{10}$ CFU/g). 0, 2, 4, -1, -3 represent the day of treatment, -1 and -3 respectively denote 1 and 3 days after withdrawal of treatment. The pie chart at each time point represents the portion of *E. coli* strains of different PFGE patterns isolated from the faecal samples. The Illumina and Pacbio platforms were subsequently used to obtain whole genome sequences of representative strains of each PFGE type.

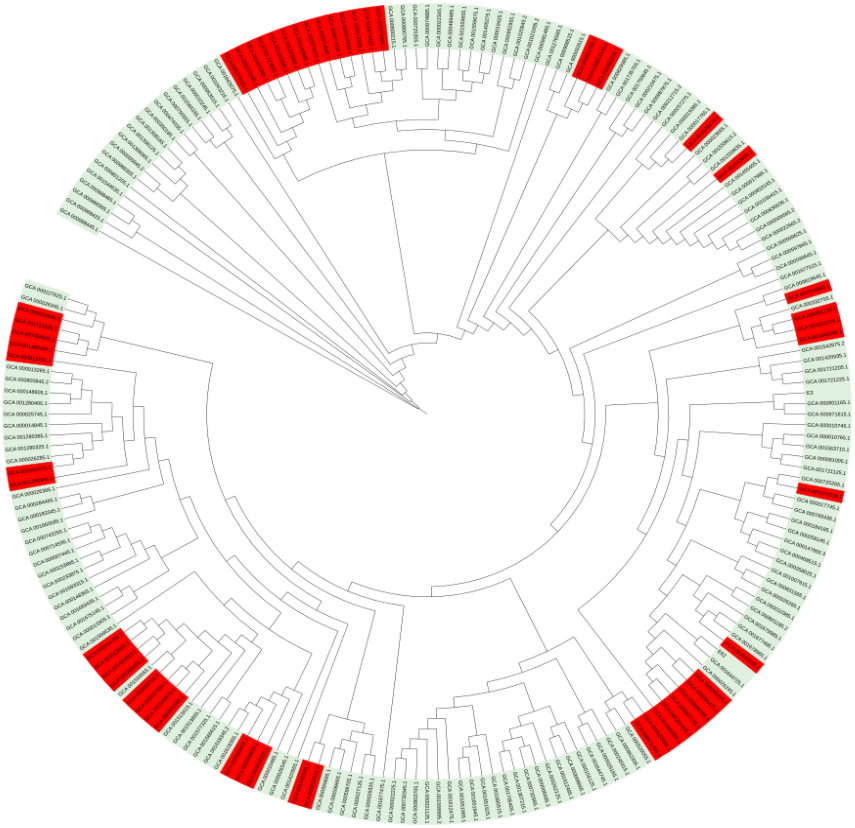

Figure 3.2. 2 Phylogenetic tree of 208 complete *E.coli* genomes in the NCBI genome database (2016.10.01).

Strains E3 and E62 were isolated in this study. Light green represents strains without QRDR target mutations and red denotes organisms harboring target gene mutations.

After annotating all the CDS fasta by prokka(*http://vicbioinformatics.com/*), the phylogenetic tree was obtained through comparison between the core and pan genes using the Roary approach [240].The mutations were identified by BLASTX with reference as described above.

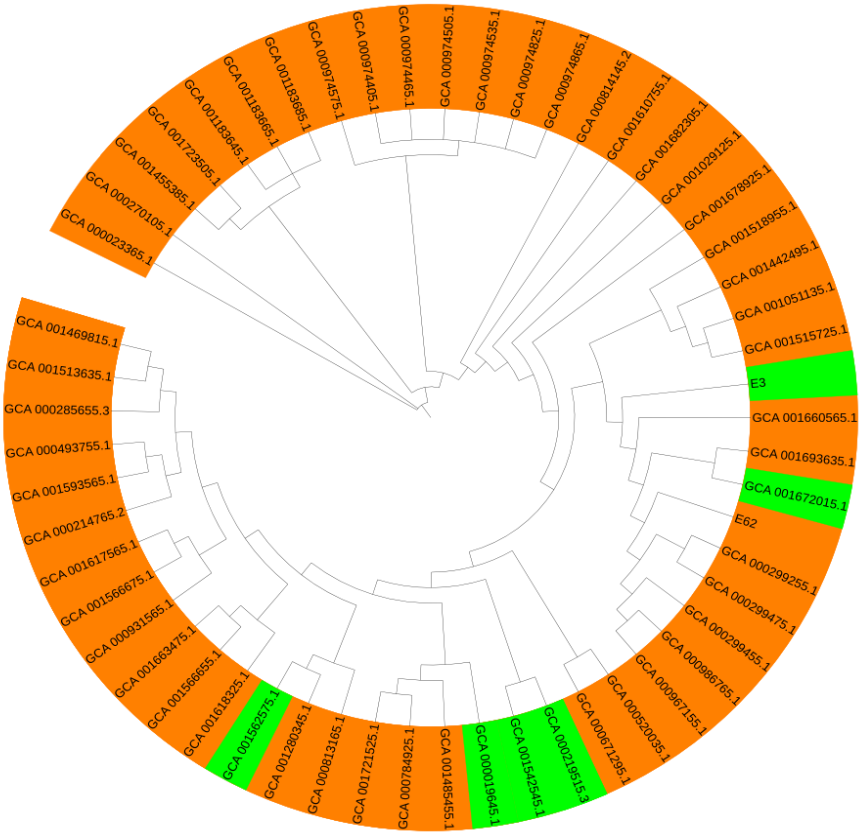

Figure 3.2. 3 The Phylogenetic tree of strains harboring QRDR target mutations as well as strains E3 and E62.

The orange color depicts genomes which harbor biofilm genes whereas the green color denotes genomes lacking biofilm genes. Existence of biofilm genes was defined by the Prokka annotation results.

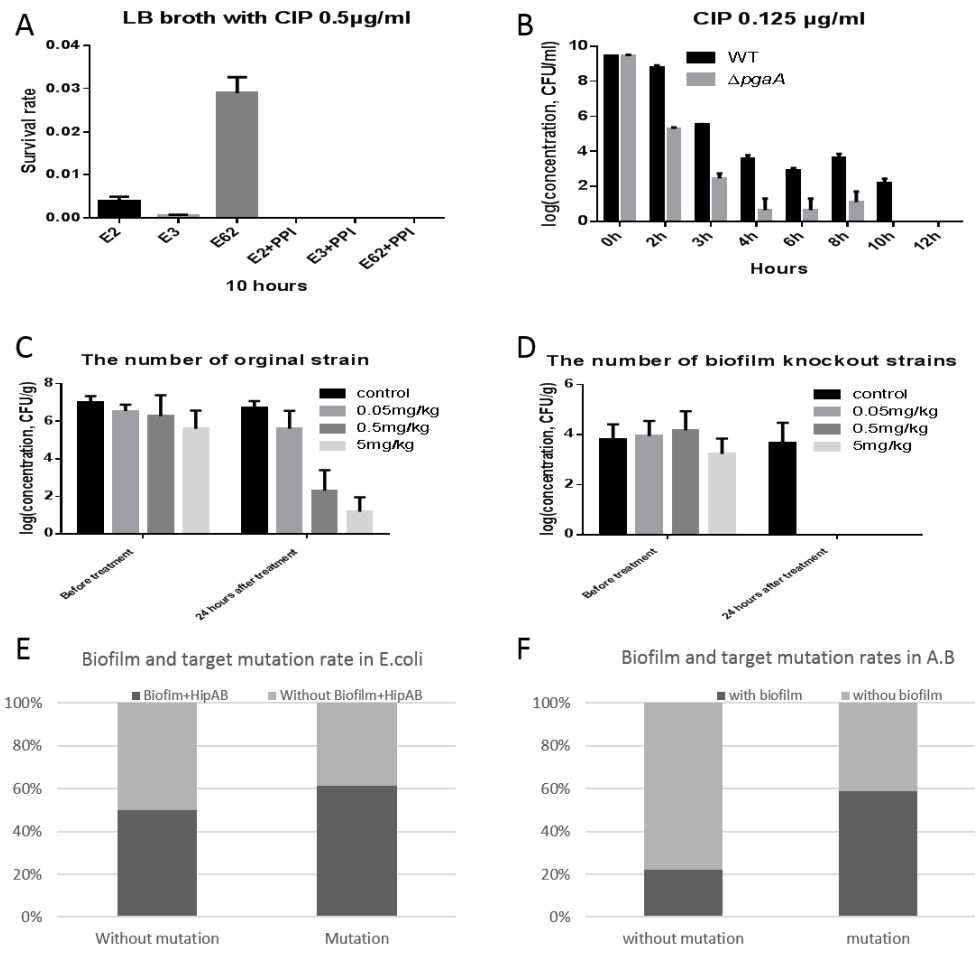

Figure 3.2. 4 : The relationship between biofilm formation, survival under ciprofloxacin selection, and development of QRDR mutations

A. Survival rate of the test strains with and without Proton pump inhibitors (PPI) at a concentration of 0.5μg/ml. PPI: represents the Proton pump inhibitor esomeprazole, which was reported to inhibit formation of biofilm[241] . It was added to the assay to produce a final concentration of 32 μg/ml, to test the effect of biofilm-mediated survival of strains E2, E3 and E62. B. Kill curve of the wildtype and biofilm knockout strain in the presence of ciprofloxacin. The number of the surviving organisms was recorded every two hours under the test condition of (CIP 0.125 μg/ml, incubation at 37℃,shaking at 250rmp). WT: Wildtype strain BW25113, *ΔpgaA*: biofilm knockout strain.

C and D. Survival of the wildtype and biofilm knockout strain in different groups of rats. 28 male SD rats (200 gram each) were dived into 4 groups, 7 rats per group. Upon streptomycin treatment, 10^{14} CFU wildtype and biofilm knockout strains were

added into drinking water. The number of WT strain and biofilm knockout strains that could grow on the MAC ager with and without kanamycin (100ìg/ml) was recorded.

E and F. Relationship between quinolones target mutations and carriage of biofilm and *hipAB* genes in *E.coli* and *Acinetobacteria baumannii.* In addition to acquisition of exogenous resistance genes, *de-novo* mutations that occur under clinical antibiotic selection pressure is another major way to generate antibiotic resistance [3]. The QRDR region of *E.coli* [242] and *Acinetobacter baumannii*[243] was selected as the query sequence; BLASTP (Evalue=1e-10) was used to identity the target mutations. The results show that among the 3403 strains without QRDR mutation, the proportion of organisms carrying the biofilm and *hipAB* genes is around 50%. In contrast, among the 1110 strains harboring target mutations, more than 60% were found to harbor both biofilm and *hipAB* genes. The difference between the proportion of target mutation strains among strains with and without biofilms and *hipAB* is statistically significant (P<0.0001;Chi-square test). Among the 3403 *E.coli* strains without QRDR mutations, there were 1702 genomes without biofilm and *hipAB* genes. On the contrary, among the 1110 *E.coli* genomes in which QRDR mutations were detectable, a total of 679 were found to contain the biofilm and *hipAB* genes. A similar phenomenon was also found in *Acinetobacteria baumannii*. In all the 56 genomes without biofilm genes, there were 35 genomes in which QRDR target mutations were found, whereas among the 1552 genomes with biofilm genes, 1385 genomes were found to harbor QRDR mutations. The difference between the portion of QRDR target mutation in the two groups of strains are statistical significant for both *E.coli* and acinetobacteria baumannii (P<0.0001;Chi-square test).

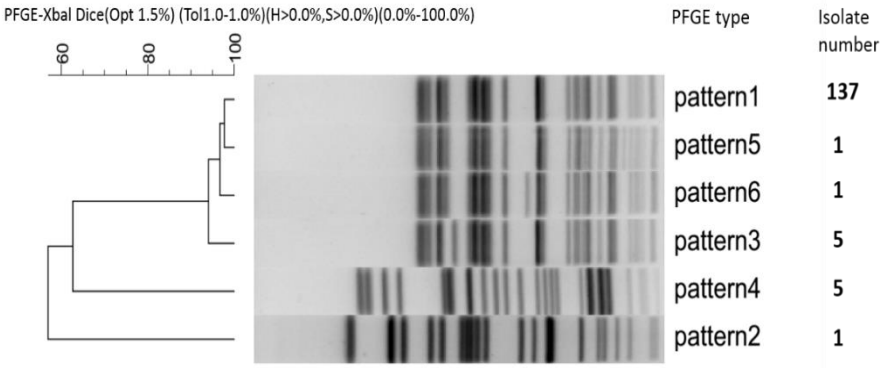

Figure 3.2. 5. Dendrogram showing results of cluster analysis of the XbaI-PFGE profile of 150 *E.coli* strains collected before antibiotic treatment.

The degree of similarity of the PFGE patterns of the test strains was calculated by BioNumerics 5.1 (Applied Maths, Kortrijk, Belgium), using the conventional criteria, with Dice coefficient being at 1.5% optimization (Opt) and 1% position tolerance (Tol). In this comparison analysis, the H (minimum height), S (minimum surface) were set to 0%; and the coverage using 0.0%-100% for the entire length. Three major patterns of the test isolates were observable. Remarkably, a total of 44 strains collected after treatment were found to belong to pattern 2.

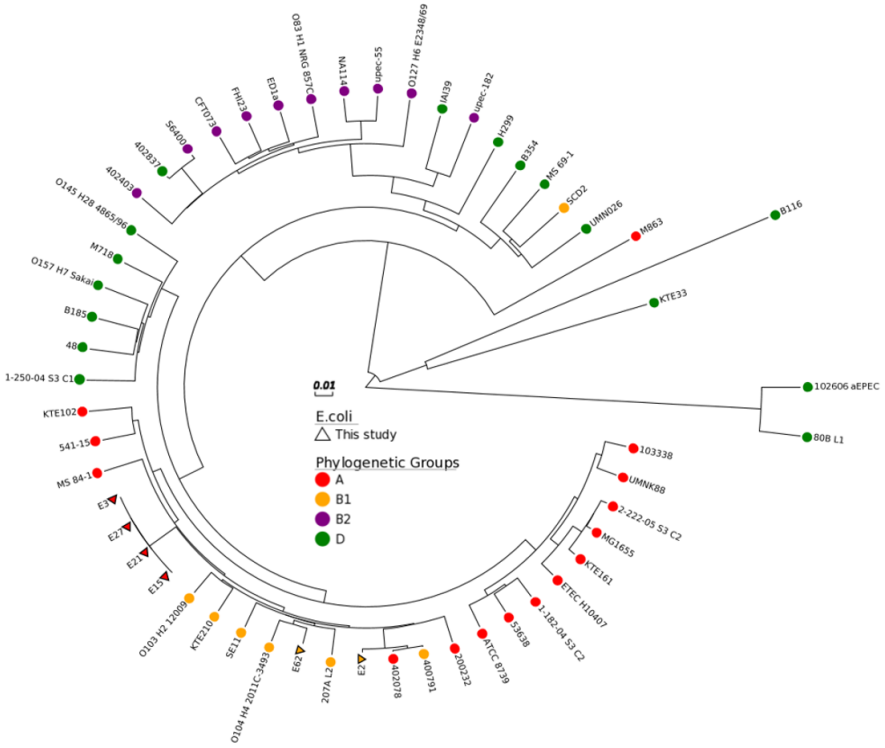

Figure 3.2. 6**.** Phylogenetic tree of six representative *E. coli* strains and specific strains in the NCBI database selected by genomic BLAST

After De dovo-assembly, contigs were uploaded to CSI Phylogeny 1.2 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). *Escherichia coli* strain K-12 substr. MG1655(NC_000913.3) was chosen as the reference genome[231]. The iTOL software was used to create the Phylogenetic tree based on the SNP profiles of the test strains (http://itol.embl.de/index.shtml)[232].

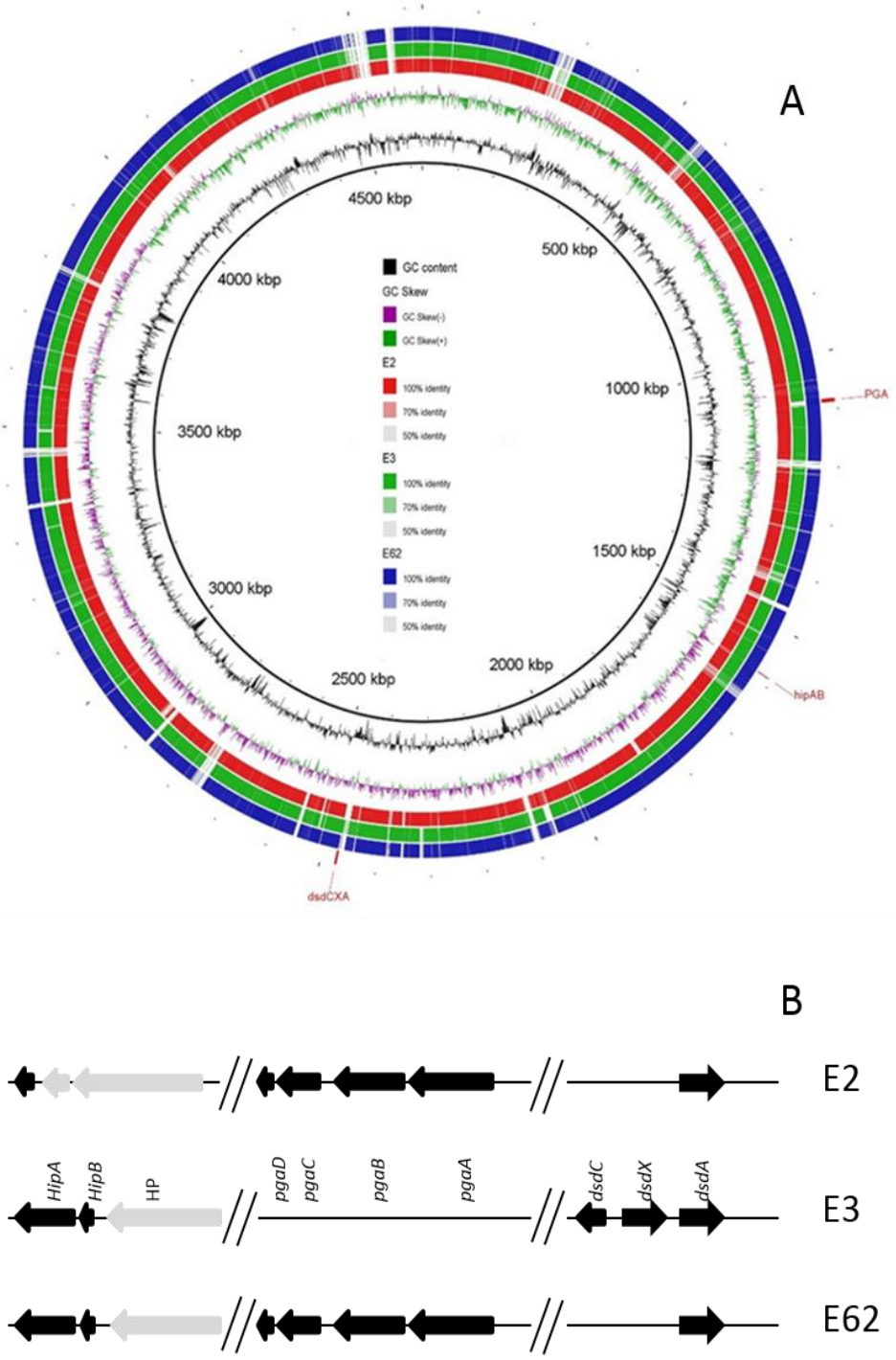

Figure 3.2. 7. Comparative genomic analysis of strains E2, E3 and E62.

The rings represent assembled draft genomes subjected to Blastn similarity matches with BW25113 as the reference sequence. Figure was produced using BRIG**[142]**. B, genetic clusters in which significant differences among the three test strains were observed.

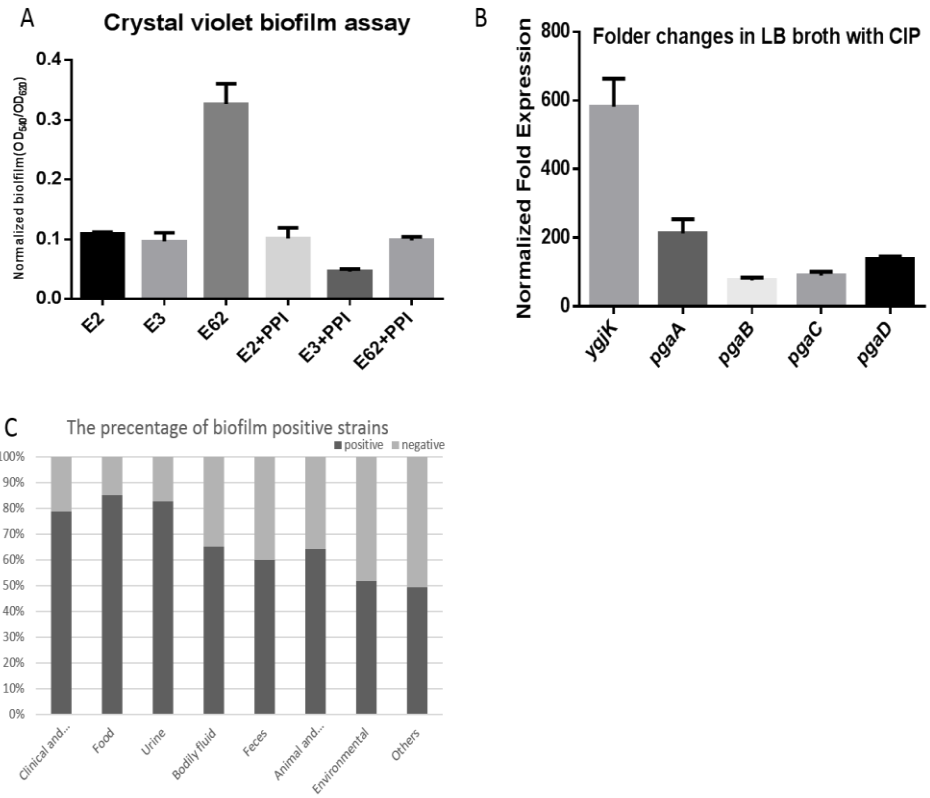

Figure 3.2. 8 The role of biofilm

A. The biofilm assay was conducted in 96-well polystyrene plates containing 100µl LB broth. Bacteria were inoculated into the wells and subjected to incubation at 37℃ for 24h without shaking. The cell density (turbidity at 595) of the bacterial culture and formation of biofilm (absorbance at 540 nm) in the wells were measured by staining with 0.1% crystal violet. Biofilm formation potential was determined by normalizing the total amount of detectable biofilm with the degree of bacterial growth of each culture. Proton pump inhibitors, PPI esomeprazole[241] ,was added to produce a final concentration of 32 *µg/*ml for testing the effect of biofilm on the bacterial survival rate.

B. Changes in expression levels of biofilm related genes with and without ciprofloxacin selection pressure. Fold changes were calculated after normalization with 16s rRNA. The ygjK gene, which encodes a glucosidase[244], was found to be a biofilm modulator regulated by various toxins[238].

C. The proportion of biofilm-forming strains detectable in different sources. Out of a total of 4513 genome sequences in the NCBI genome database, the sources of 2413 genomes could be found, among which 610 were recovered from clinical and blood samples, 20 from food samples, 86 from urine, 80 from body fluid, 1400 from

faeces, 95 from animals and farms, 27 from environmental samples, and 95 were obtained from other sources.

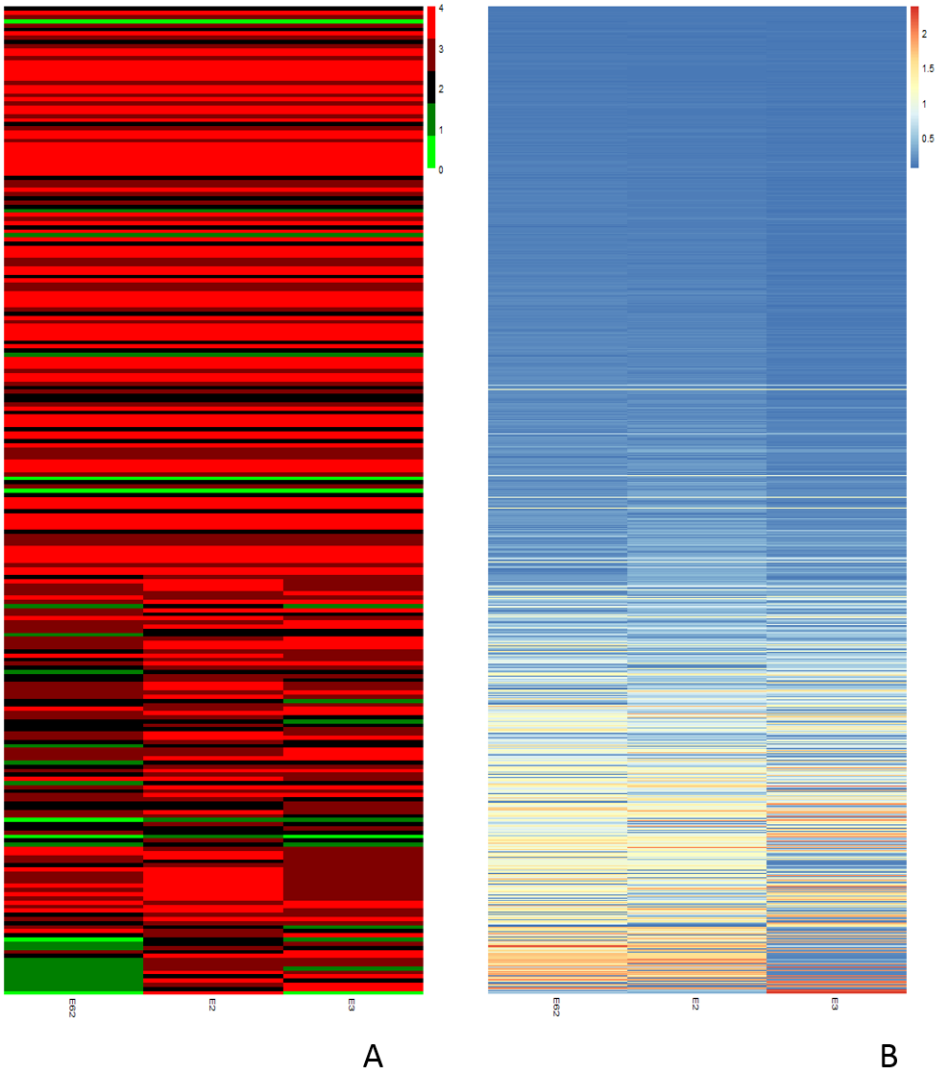

Figure 3.2. 9. Phenotype microarray analysis of strains E2, E3 and E62.

A: The result of phenotype microarray result of phenotype array PM11-20 (Biolog). A total of 240 different kinds of compounds (4 concentrations each) were tested. Failure of the test organisms to grow in none of 4 concentrations resulted in a record of 0; 1 indicates that the test strain only could only grow at the lowest concentration; 3 indicates that the test isolate could grow in 3 of the 4 concentrations; 4 means growth in all the concentration. B The result of phenotype microarray result of the PM 1-10. OD 595 (595 nm) was recorded at 18 hours upon incubation at 37℃ in the presence of different nutrients. The number indicates growth efficiency of the

test organisms. The heat map is drawn by R.(https://www.r-project.org/) using the R ggplot2 package.

Functional expression of a putative HipA toxin also increased persisters, while deletion of the *hipBA* module caused a sharp decrease in persisters in both stationary and biofilm populations. HipA is thus the first validated persister-MDT gene. We suggest that random fluctuation in the levels of MDT proteins leads to the formation of rare persister cells. The function of these specialized dormant cells is to ensure the survival of the population in the presence of lethal factors[245].

4 The acquisition of antibiotic resistance genes in GI tract

The United States of America alone consumed more the 15 million kilograms of antibiotics every year[246]. Many of the antibiotics have been used in agriculture. People reported that at least half of all the antibiotics were used in animal trials in the United States as well as EU, indicating the significance of antibiotics for agriculture[93, 247]. In recent years the rate of antibiotic used in farm animals may decrease but most countries still allow the agricultural use of antibiotics for the purpose of disease treatment. The U.S even allows the use for disease prevention. It is believed that in Gram negative strains antibiotic resistance is mainly caused by acquisition of plasmids that harbor resistance genes [248]. Identifying the factors that affect transmission of such plasmids is an important research direction. In the previous chapter, we investigated the factors that can enable bacteria generate target mutations under antibiotic pressure. In this chapter, we surveyed the antibiotic resistance gene transmission and the factors which can promote this process.

4.1 Carbapenem resistant isolates in food animals which had no carbapenem usage history

Carbapenems are one of the last-line antibiotics used to treat serious Gram negative bacterial infections [249, 250]. The increasing rate of carbapenem resistance due to dissemination of the $bla_{\text{NDM-1}}$ element has raised serious public health concern [131, 251]. The Metallo-β-lactamase (MBL)-encoding $bla_{\text{NDM-1}}$ gene was first reported in 2009 [35], yet evidence of worldwide dissemination of this resistance determinant had emerged soon after [248, 252]. This gene has been known to spread

to different species of *Enterobacteriaceae* and other Gram negative bacteria [253]. The rapid increase in prevalence of carbapenem resistant *Enterobacteriaceae* (CRE) may be due to both transmission of *bla*$_{\text{NDM-1}}$-harboring elements among the *Enterobacteriaceae* species as well as clonal spread of strains containing such elements [254, 255]. Current evidence, however, suggests that transmission of specific mobile resistance elements among CRE is species or strain-dependent. For example, the *bla*$_{\text{NDM-1}}$ like genes are predominantly detected in the ST131 and ST101 types of *E. coli*, and the ST11 strains of *Klebsiella pneumoniae* [255-258]. These types of *E. coli* and *K. pneumoniae* are commonly associated with human infections, but rarely recovered from animals. Therefore, most of the bacterial strains that are known to harbor *bla*$_{\text{NDM-1}}$ have been recovered from human clinical samples. Organisms carrying *bla*$_{\text{NDM-1}}$ have also been isolated from the environment, but remain extremely rare [259, 260].

Animals have been considered a potential reservoir of multidrug-resistant (MDR) Gram-negative organisms due to the extensive use of antibiotics in animals as growth promoters and for treatment purposes. Up to 72% of antimicrobials sold in US, including the clinically important compounds such as the β-lactam antibiotics, have been used as animal feeds [261]. In addition, there are reports suggesting that pig farms consume 60% of antibiotics that are used in animals[262]. However, detection and transmission of *bla*$_{\text{NDM-1}}$ in animals have rarely been reported. The molecular basis of the low prevalence of *bla*$_{\text{NDM-1}}$-positive bacteria in animals is currently not understood. It is possible that there is a lack of selective pressure due to carbapenems in animals since this category of antimicrobial agents are not used in veterinary medicine. Nevertheless, several recent studies began to report detection and isolation of *bla*$_{\text{NDM-1}}$ -positive strains in animals [259, 263]. These reports have raised serious public health concern since settlement of the *bla*$_{\text{NDM-1}}$ elements or strains carrying such elements in animals may result in an enhanced rate of transmission of both resistance genes/resistant strains among farmed animals, which in turn pose an increased risk of human infections. This study aims to investigate the prevalence and genetic characteristics of mobile elements harbouring the *bla*$_{\text{NDM-1}}$ -like genes that were transmissible among *E. coli* strains isolated from food animals in China, and assess their threat to human.

Sample collection and bacterial isolation

Animal fecal samples from ten swine farms located in seven different provinces in China including Jilin, Henan, Shandong, Jiangsu, Zhejiang, Fujian and Guangdong were collected. These farms were chosen on the basis that carbapenems had not been used in both humans and animals for any purposes within the farms. One farm from each province was selected except for Jiangsu province, where four farms were recruited for the study. These swine farms are located along the eastern coast of China and each was separated from the others geographically by a distance of at least 100 km. Twenty to thirty pigs were randomly selected from each farm for sample collection. Each farm was sampled once during the period of May 2014 to May 2015. For farrow farms, samples were collected both from piglets in the delivery room, and adult pigs in the finishing room.

Fresh fecal samples were stored at 4ºC and transported to the laboratory within 24h. Approximately 200mg of each sample were resuspended in 2 ml of saline, which were then subjected to three serial dilutions; 100μl suspension from each dilution was spread on MacConkey agar containing 0.5μg/ml meropenem to select for *E. coli* strains producing carbapenemases. A maximum of four colonies on each positive plate were picked, purified, and subjected to species identification by MALDI-MS and 16SrRNA gene sequencing [264]. The presence of carbapenemase genes was screened as previously described using strains containing different carbapenemase genes as positive control [140].

Antimicrobial drug susceptibilities were determined for strains carrying carbapenemase genes following the CLSI guidelines [265]. Nineteen antimicrobials were tested including colistin, polymyxin B, aztreonam, nalidixic acid, ciprofloxacin, amikacin, gentamicin, tetracycline, ampicillin, cefoxitin, ceftazidime, meropenem, imipenem, ertapenem, biapenem, trimethoprim-sulfamethoxazole, chloramphenicol, azithromycin, fosfomycin and tigecycline. *Escherichia coli* strain ATCC 25922 was included as the control strain.

Conjugation experiments

Conjugative experiments were carried out as previously described using sodium azide-resistant *E. coli* J53 strain as recipient to test the transferability of the carbapenemase genes harboured by the test strains (19). Briefly, overnight culture of donor and recipient strains were mixed and collected on a filter, which was then subjected to overnight incubation on a blood agar plate. The mixture was then spread on double selective blood agar plates containing meropenem (0.5μg/mL) and sodium azide (100μg/mL) to select drug resistant transconjugants.

Molecular typing

S1-PFGE was conducted to determine the size of $bla_{\text{NDM-1}}$ –bearing plasmids as previously described [266]. 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 by UV transillumination (Bio-Rad).

Plasmid sequencing and analysis

Plasmids were extracted using the QIAGEN Plasmid Midi Prep Kit and sequenced by the whole-genome shotgun approach, utilizing the NextSeq 500 Illumina platform, with 2×150bp paired-end reads. De novo assembly of the reads was conducted with SOAPdenovo2,[267]. To obtain the complete plasmid map of these two plasmids, PacBio RSII single-molecule, real-time (SMRT) sequencing was performed to create libraries of 20Kbp in Wuhan Institute of Biotechnology, Wuhan, China. The annotations of the plasmids sequences were conducted by RAST [268]. and edited manually. The comparison of different MDR regions was performed with Easyfig[143].

PCR assays

The prevalence of $bla_{\text{NDM-1}}$ –like plasmids in *E. coli* strains collected from the test animals was determined by PCR assays targeting to different conservative genes

of the plasmid using primers listed in Table 1. The PCR assays were performed as previously described [266].

Clonal association of different carbapenem-resistant *E. coli* strains

A total of 220 pig fecal samples were collected from ten farms located in seven provinces of China including Liaoning, Shandong, Jiangsu, Zhejiang, Fujian, Guangdong and Henan. Carbapenem-resistant *E. coli* isolates were only detectable in one farm in Henan province. A total of 20 fecal samples recovered from different animals were screened in this farm including 10 each collected from weaning piglets and finishing pigs. Eleven carbapenem-resistant *E. coli* strains were isolated from 5 finishing pigs (Pig 1~5), yet none of the 10 weaning piglets was found to harbor carbapenem-resistant *E. coli*. Three carbepenem-resistant *E. coli* strains, namely HNEC46, HNEC47 and HNEC62, were isolated from faeces of Pigs 3, 4 and 5 respectively. The other eight carbapenem-resistant *E. coli* strains were isolated from two fecal samples, with strains HNEC55, HNEC56, HNEC57 and HNEC58 being isolated from Pig 1 and HNEC67, HNEC68, HNEC69 and HNEC70 from Pig 2 (**Fig 4.1.1**). PFGE analysis was performed on these isolates and four different patterns were identified in ten isolates and one isolate was untypeable (**Fig 4.1.1**). Interestingly different carbapenem-resistant *E. coli* strains could emerge from the same animal: strains HNEC55, HNEC56, HNEC57 and HNEC58 were from Pig 1, but with different patterns, strains HNEC67, HNEC68, HNEC69 and HNEC70 from Pig 2, but with different PFGE patterns. exhibited identical pattern, which was different from that of HNEC70. On the other hand, several strains isolated from different samples were found to share the same PFGE pattern (**Fig 4.1.1**). Interestingly, strain HNEC46 exhibited a unique PFGE pattern compared to others and HNEC47 was untypeable. The data suggested that transmission of carbapenem-resistant *E. coli* strains in this pig farm was due to both clonal and non-clonal transmission. All carbapenem-resistant *E. coli* strains were subjected to MLST typing following Wirth method[269]. The four different PFGE types belonged to ST1695, ST1585, ST1721 and ST359.

Except for ST359, which was commonly associated with extended-spectrum β-lactamases-producing human clinical isolates [270] or animal isolates [271], the other

three ST types were rarely reported; they were shown to be associated with *bla*$_{NDM-1}$ and production of a carbapenem phenotype for the first time.

Transferability of carbapenemase genes

Screening of common extended-spectrum β-lactamase resistance genes was performed in these isolates[272], with results showing that all test organisms harboured the *bla*$_{NDM-1}$ and *fosA3* genes. Some of the isolates were also found to harbour different *bla*$_{CTX-M}$ variants (HNEC68 harboured *bla*$_{CTX-M-1}$)(Data not show in this study). To further determine the genetic location of these antibiotic-resistance genes, S1-PFGE and Southern hybridization were performed. Strains HNEC55, 56,57, 58, 68 and 69 exhibited similar plasmid profile, which comprised two elements of 50 and 100kb in size, with the 100kb plasmid being the one which harbored the *bla*$_{NDM-1}$ gene. Strains HNEC62 and 70 were found to harbour two plasmids of 135k and 100kb, with the *bla*$_{NDM-1}$ gene being consistently located in the 100kb plasmid. On the other hand, strain HNEC46 was found to harbour two plasmids of 105kb and 95kb, with the *bla*$_{NDM-1}$ gene being located in the latter (**Fig 4.1.2**). To check the transferability of these *bla*$_{NDM-1}$- bearing plasmids, conjugation experiments were performed. All test strains were found to be able to transfer their carbapenem resistance phenotypes to the *E. coli* J53 recipient strain, with all plasmids containing *bla*$_{NDM-1}$ being successfully transferred to J53 (**Table 4.1.2**).

Characterization of conjugative *bla*$_{NDM-1}$ bearing plasmids

To further investigate the genetic characteristics of the animal-borne, carbapenem resistance-encoding plasmids, we chose two plasmids with different sizes, namely plasmids recovered from transconjugants T-HNEC55 and T-HNEC46, for complete sequence analysis using both illumina and PacBio Single Molecular Realtime Sequencing (SMRT) technologies. The plasmid from T-HNEC55 was found to be 81498 in size, contain 110 open reading frames and designated as pHNEC55-NDM. On the other hand, the plasmid recovered from transconjugant T-HNEC46, designated as pHNEC46-NDM, was found to be 74046bp in size and contain 101 open reading frames. Both plasmids belonged to the IncFII type and share almost identical backbone, with the only difference being the structure of multidrug-resistance (MDR) encoding mobile element that they harbored, which was the only

mobile element located in these two plasmids (**Fig 4.1.3**). The pHNEC55-NDM plasmid was found to comprise with four gene cassettes each of which contained different antibiotic resistance genes, and was flanked by multiple transposase genes, with IS*26* being the most common. These gene cassettes included the *mphA*, *bla*$_{\text{NDM-1}}$, *fosA3* and *rmtB* gene clusters. Compared to the MDR mobile element in pHNEC55-NDM, the one in pHNEC46-NDM was found to contain identical *mphA* and *bla*$_{\text{NDM-1}}$ gene cassettes but lacked those of *fosA3* and *rmtB*.

BLAST analysis of the pHNEC55 sequence revealed that the backbone of pHNEC55-NDM (plasmid excluding the MDR element) exhibited >99.9% homology to that of pHN7A8(62.3 kb), a plasmid carried by *E. coli* strain 7A8, which originated from animal in China [273]. The MDR elements of these two plasmids were found to contain several identical gene cassettes such as the upstream tranposase gene, and the downstream *rmtB* and *fosA3* cassettes. A major structural difference was that the *bla*$_{\text{CTX-M-65}}$ cassette in pHN7A8 was replaced by the *mphA* and *bla*$_{\text{NDM-1}}$ genes in pHNEC55-NDM (**Fig 4.1.3, 4.1.4**). Plasmids with similar backbones, but carrying different *bla*$_{\text{CTX-M}}$ gene cassettes, were also reported previously, including plasmid pKP1034 (GenBank: KP893385.1) which was carried by a clinical *K. pneumoniae* isolate, and pHNFP460-1(KJ020575), which was recovered from a *E. coli* strain isolated from animals.

The MDR region of pHNEC55 is a 19 kb fragment comprising three major antimicrobial resistance gene cassettes flanked by four IS*26* elements. These included an 11kb cassette harbouring the *mphA* and *bla*$_{\text{NDM-1}}$ genes, and an 8 kb cassette containing the *fosA3*, *bla*$_{\text{TEM}}$ and *rmtB* genes. In contrast, the MDR region of pHNEC46 only contained the 11kb cassette which comprised the *bla*$_{\text{NDM-1}}$gene (**Fig 4**). BLAST analysis showed that the MDR region of pHNEC55 was similar to the one found in plasmid pKP1034 (KP893385.1), which was carried by a clinical *K. pneumoniae* stain isolated from China in 2015, as well as to plasmid pHN7A8 [273]. The major difference is that the gene cassette containing *bla*$_{\text{CTX-M-65}}$ in these two plasmids was replaced by the *bla*$_{\text{NDM-1}}$ gene fragment (**Fig 4.1.4**). The mobile element harbouring the *bla*$_{\text{NDM-1}}$gene in pHNEC55 and pHNEC46 shared identical structure with the one found in *Acinetobacter* sp. [274] , *Citrobacter freundii* [275] , *Providencia rettgeri* [276] and *klebsiella* sp. (GenBank: CP010390.1) of human origin.

A two-nucleotides deletion was observed in the *aphA6* gene located in pHNEC55 and pHNEC46, resulting in early termination of the protein and inactivation of this gene product; this observation was therefore consistent with the loss of Aminoglycosides resistance phenotype in *E. coli* strains carrying these two plasmids (**Table 4.1.2**).

To characterize the genetic features of conjugative plasmids harboring bla_{NDM-1} recovered from other transconjugants, a set of primers targeting various regions of pHNEC55 and pHNEC46, as shown in table 1, was used to screen for plasmids harboured by other transconjugants. The results showed that plasmids from T-HNEC47, T-HNEC62 and T-HNEC70 contained a plasmid structurally similar to pHNEC46, while other transconjugants carried plasmids resembling pHNEC55 (**Table 4.1.1**).

This study surveyed the prevalence of carbapenem-resistant *E. coli* in different animals in China and characterized the genetic features of conjugative plasmids carrying bla_{NDM-1} in these animal *E. coli* isolates. A number of bla_{NDM-1}-bearing plasmids have previously been found in Henan province [130, 277]. This previous study reported that, in one of the *E. coli* strains which also carried the *rmtB* and *fosA3* genes, the bla_{NDM-1} gene was harboured by an IncA/C type plasmid whereas the *fosA3* gene was located in another element [130]. The IncFII-type plasmid pMC-NDM (87,619 bp) was also reported to harbour the resistance genes bla_{TEM-1} and *rmtB*, but lack *fosA3*, when compared to pHNEC55 [278]. A recent report also found that the *fosA3* and bla_{NDM-1} genes were detectable in the same strain but on different plasmids (31), suggesting that the *fosA3* gene is often co-transmitted with bla_{NDM-1}. This situation is analogous to that observed in a previous report in that transmission of the *rmtB* gene was often accompanied by bla_{TEM-1} in the transposon Tn2 [279]. The co-transmission of bla_{NDM-1} and *fosA3*, as well as other resistance elements, represents a serious threat to human health, as it infers that options for treatment of infections caused by organisms harbouring bla_{NDM-1}-1 strains will be further limited.

Unlike other resistance elements such as the ESBL genes that may evolve in animals, bla_{NDM-1} is less likely originated from farmed animals which are not exposed to carbapenems, the selecting agent of such resistance element. Possible origin(s) of bla_{NDM-1}have been suggested to be microflora of human or wild animals [259]. Data

in this work showed that, as soon as *bla*$_{NDM-1}$ is captured by conjugative plasmids that circulate among animal *E. coli* isolates, it may be disseminated easily within an animal farm, and further transmitted to human. Since animal isolates may serve as a vector for rapid transmission of carbapenemase genes, future studies should focus on depicting factors that determine the efficiency of carriage and transmission of carbapenemase genes in such organisms.

The presence of different mobile MDR elements in plasmids harbored by *E. coli* isolates collected from animal farms provided strong evidence of dynamic evolution of mobile resistance elements. The integration of multiple gene cassettes to form a mobile MDR element is a cause for concern since such element could mediate resistance to all clinically significant antibiotics used in treatment of clinical infections caused by Gram negative bacteria. Transmission of this mobile element among bacterial pathogens is expected to result in serious public health burdens. Recently, a mobile element known as *mcr-1*, which encodes resistance to colistin, one of the last-line agents used to treat Gram negative bacterial infections, was detectable in animal-borne *E. coli* isolates [280]. Recent studies have reported that *bla*$_{NDM}$ and *mcr*-1 may coexist [281], even within the same plasmid[282]. Interestingly, the patient from whom the super bacteria has been isolated has not been treated with colistin[283] .In this study we observe the same phenomenon that although no carbapenem antibiotic has been treated in those animals, plasmids carrying the carbapenemase gene *bla*NDM-1 are still found. Some other studies also question the role of antibiotic in horizontal gene transer[284].Findings in this work suggest a need to investigate the impact of various antimicrobial agents in dissemination of clinically important resistance elements such as bla$_{NDM-1}$ gene and *mcr*-1among animals, and possible transmission to human.

Nucleotide sequence accession number. The completed plasmid sequences for pHNEC55 and pHNEC46 were deposited in NCBI with the accession number KT879914.1 and KX503323 respectively.

Table 4.1. 1. Assessment of genetic similarity of plasmids recovered from different *E. coli* strains using specific primers.

Primer ID*	Primer sequence	Target	HNEC *E. coli* Strains										
			46	47	55	56	57	58	62	67	68	69	70
P1-F	AGTAAAGCAGGGCCAAACTG	pHNEC46	+	+					+				+
P1-R	TAGCCCCAAAGCGCAGTAAA												
P2-F	CTCGGGTGAAGTCGGGAAAA	pHNEC46/p	-	-	+	+	+	+	-	+	+	+	-
P2-R	TGGAACAGCTCATGACACCC	HNEC55											
CopB-F	CGGCATCCAGGTTAAGGCAT	*copB*	+	+	+	+	+	+	+	+	+	+	+
CopB-R	CAGCGTCACACATCTCCTGT												
YgFA-F	ACACCCCACGCAAAAACAAG	*ygFA*	+	+	+	+	+	+	+	+	+	+	+
YgfA-R	GTCGCCATAGGGTCGTCAAC												
FII- F	CTGTCGTAAGCTGATGGC	FII plasmid	+	+	+	+	+	+	+	+	+	+	+
FII- R	CTCTGCCACAAACTTCAGC												
rmtB-F	CCCAAACAGACCGTAGAGGC	*rmtB[286]*			+	+	+	+		+	+	+	
rmtB-R	CTCAAACTCGGCGGGCAAGC												
FosA3-F	GCGTCAAGCCTGGCATTT	*fosA3[287]*			+	+	+	+		+	+	+	
FosA3-R	GCCGTCAGGGTCGAGAAA												
bla_{NDM}-F	ACTTGGCCTTGCTGTCCTT	$bla_{NDM[140]}$	+	+	+	+	+	+	+	+	+	+	+
bla_{NDM}-R	CATTAGCCGCTGCATTGAT												

*Primers for P1, P2 were specifically designed to detect the pHNEC46 and pHNEC55 plasmids.

Table 4.1. 2. PFGE and antibiotic susceptibility profiles of *bla*$_{NDM-1}$-bearing *E. coli* strains

PFGE and antibiotic susceptibility profiles of *bla*$_{NDM-1}$-bearing *E. coli* strains and the corresponding transconjugants which have acquired the *bla*$_{NDM-1}$ –bearing plasmid by conjugation (denoted by the prefix T).

E. coli	Pig	PFGE	CRO	SXT	GEN	TET	CHL	FOS	IMP	MRP	CT	CIP	ATM
TG1			<1	<0.025	<0.25	4	<0.25	1	<0.025	<0.025	2	<0.06	<0.5
HNEC46	Pig 3	3	>256	2	<0.25	>128	>32	8	4	1	<0.25	<0.06	<0.5
T-HNEC46			>256	<0.025	<0.25	4	<0.25	<0.25	4	4	1	<0.06	<0.5
HNEC55	Pig 1	1	>256	<0.025	>128	>128	1	>256	4	1	<0.25	<0.06	<0.5
T-HNEC55			>256	<0.025	>128	4	<0.25	256	4	2	1	<0.06	<0.5
HNEC57	Pig 1	2	>256	2	<0.25	>128	>32	32	4	1	1	<0.06	<0.5
HNEC70	Pig 2	4	>256	>32	2	>128	>32	2	0.5	2	1	8	<0.5

CRO: Ceftriaxone; SXT: Trimethoprim- sulfamethoxazole; GEN: Gentamicin; TET: Tetracycline; CHL: Chloramphenicol; FOS: Fosfomycin; IMP: Imipenem; AMP: Ampicillin; MRP: meropenem; TGC: Tigecycline; CT: Colistin; BPM: Biapenem; PB: Polymyxin B; CIP: Ciprofloxacin; AZI: Azithromycin; ATM: Aztreonam; ETP: Ertapenem; CAZ: Ceftazidime

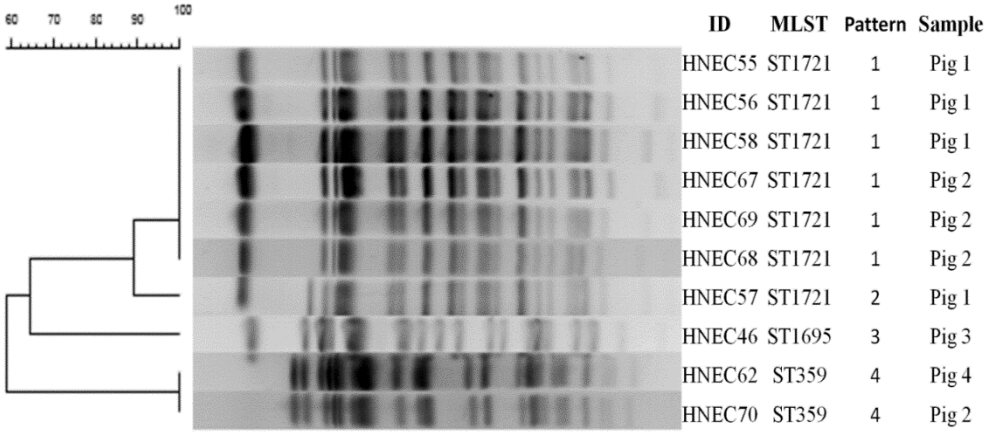

Figure 4.1. 1. Source of isolation, ST types and PFGE patterns of 11 carbapenem-resistant *E. coli* strains isolated from pigs. *E. coli* strain HNEC47 was not typable by PFGE; ST type of HNEC was ST1695.

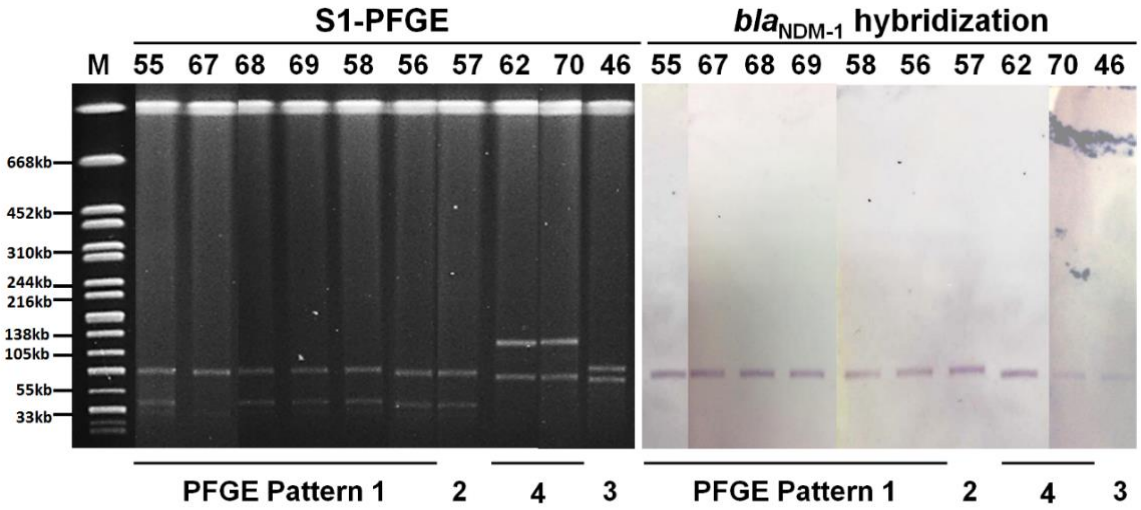

Figure 4.1. 2. S1-PFGE and Southern Hybridization of bla_{NDM-1} in 11 carbapenem-resistant *E. coli* strains isolated from pigs. *E. coli* strain HNEC47 was not typable by S1-PFGE

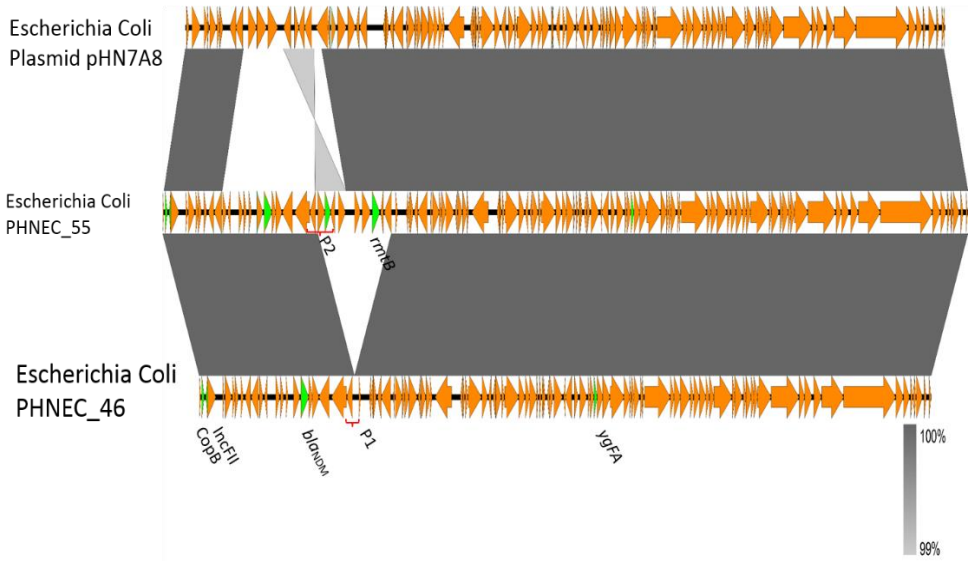

Figure 4.1. 3. Comparative analysis of plasmids pHNEC55, PHENC46 and Phn7A8.

Dark grey shading denotes 100% nucleotide identity; light grey indicates more than 99% nucleotide identity. The location of the primers used to identify the plasmid were labelled in the figure.

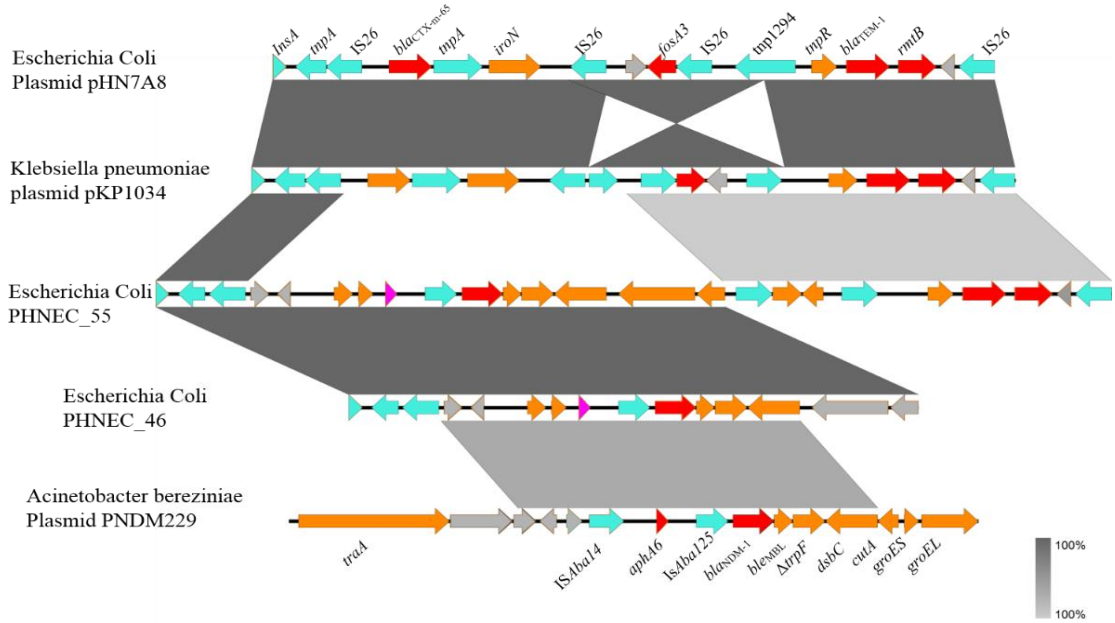

Figure 4.1. 4. Comparative analysis of the MDR region in different plasmids.

Dark grey shading represents 100% nucleotide identity; the light grey region depicts more than 99 % nucleotide identity. Light green represents the mobile elements; Red represents the antibiotic resistant genes; Lavender represents the non-functional genes; Grey represents the Hypothetical protein; and all other genes are labeled with orange.

4.2 The Preliminary investigation of antibiotic resistance transmission in pig farms

Antibiotic regimens designed to treat bacterial infection rarely take into account their long term effects on the complex microbiome, especially the composition of the gut flora which is known to confer a range of immunological, metabolic and neurological benefits to the human host [288]. How the human microbiome structure varies during the course of antibiotic treatment has therefore become a subject of intense research interest in the past few years [289, 290]. To date, the extended spectrum beta-lactams, especially the third and fourth-generation cephalosporins, are the mainstay antibiotics used for treatment of various life-threatening infections such as bacteraemia and meningitis. Due to the prevalence of organisms resistant to these agents, an increasing amount of newer and more expensive drugs such as the carbapenems is being used for treatment of patients who do not respond to cephalosporins [291, 292]. However, few studies have investigated the range of biological responses exhibited by the complex human microbiome during and after treatment with such agents, especially among human hosts who already contained a high proportion of antibiotic resistant strains in the intestine. In particular, it is not clear whether drug resistant organisms commonly colonize the gastrointestinal tract of individuals who have not been exposed to antibiotics for a prolonged period, and how the population structure of gut flora changes in response to different treatment regimens. Likewise, although a number of third-generation cephalosporins have been developed strictly for veterinary use [293], their long term effects on the animal gut flora are unknown. Apart from a recent study which demonstrated that usage of ceftiofur resulted in reduced susceptibility of *E. coli* to third generation cephalosporins [294], few attempts have been made to examine the changes in the population structure of drug-sensitive / resistant organisms in animals upon antibiotic treatment.

Currently, the prevalence rate of ESBL producing *E .coli* strains in China was reported to be 60-70% [295]. Despite this high background resistance rate, however, the use of cephalosporins and penicillins to promote animal growth and treat human infections remains a common practice in China, accounting for over two thirds of total

antibiotic sales in the country [296]. A lack of comprehensive data on the structural characteristics of microbiome in both human or animals, which are expected to evolve from time to time as a result of variation in antibiotic selection pressure and the health status of the host, has hampered the design of antimicrobial strategies that exhibit minimal resistance selection potential on the gut flora. The objective of this study was therefore to evaluate the phenotypic and genotypic changes in the symbiotic bacterial population in animals with a high resistance background upon encountering antibiotic stress.

The initial phase of this study involved investigation of the prevalence of resistant organisms in the normal flora of healthy animals which have not been exposed to antibiotics for a prolonged period. *E. coli* was chosen as the test organism because it is one of the most common and representative organisms in the gut flora. Since the major mechanism of resistance to β-lactam antibiotics in gram-negative bacteria is attributed to production of β-lactamases, with the CTX-M group being the most important enzymes [297-299], the β lactamase gene bla_{CTX} was used as a marker to monitor variation in prevalence rate of resistant organisms in the gut flora. Findings in this work have enormous implications in the design of treatment strategies that take into account the resistance selection potential of antimicrobial drugs.

Changes in resistance rate and population structure of GI tract bacteria in piglets before and after weaning with antibiotic-containing food

A preliminary study was first conducted to investigate the prevalence of antibiotic-resistant microflora in the GI tract of piglets before and after weaning with commercially available animal feed which contained an unknown composition of antimicrobial compounds. A total of 15 piglets which were born and raised in a commercial pig farm and supposed to have never been exposed to antibiotics, were examined for the presence of antibiotic-resistant organisms before and after 10 days of feeding with antibiotic-containing food. Results of this preliminary experiment showed that all new-born animals, which were up to 40 days old, already harboured a significant size of *E. coli* sub-population which was resistant to both cefotaxime and ciprofloxacin (**Fig 4.3.1**). After 10 days' weaning with food which contained antimicrobial compounds, the rate of *E. coli* that were resistant to cefotaxime or

ciprofloxacin were found to further increase, even though the overall population size of *E. coli* in the intestinal tract of these piglets remained unchanged (**Fig 4.3.1**). The cefotaxime resistance rate in the Gram-positive species of *S. aureus* and *Lactobacillus* was also checked before and after the piglets were fed with antibiotic-containing food for 10 days. The overall population size of *S. aureus* and *Lactobacillus* was also found to remain at the same level. Contrary to *E. coli*, however, the cefotaxime resistance rate in these two bacterial species, which was already in the range of 70%, was not found to alter significantly after the 10 days weaning period (**Fig 4.3.1**).

To investigate the detailed drug susceptibility phenotypes of resistant isolates recoverable from the GI tract of the test animals after antibiotic treatment, 66 cefotaxime-resistant *E. coli* strains collected from the faecal samples of weaning piglets (WP) immediately prior to the weaning process, and 108 cefotaxime-resistant *E. coli* strains obtained from faecal samples of piglets after the 10 days weaning period (10PW), were randomly selected from MacConkey plates containing 2 µg/ml cefotaxime for further characterization. Prior to weaning, the resistant organisms were already found to exhibit a very high rate of prevalence of the ESBL marker gene $bla_{\text{CTX-Ms}}$, with more than half (54%) of cefotaxime resistant *E. coli* strains being positive for $bla_{\text{CTX-Ms}}$ and some strains containing more than one $bla_{\text{CTX-Ms}}$ gene (**Table 4.3.1**). On the other hand, data of phylotyping indicated that the population structure of drug resistant *E. coli* did not change significantly 10 days' post weaning with antibiotic treatment, with Group A being the dominant group both at the beginning of weaning and 10-days after (**Table 4.3.1**). Another important observation is that all cefotaxime-resistant *E. coli* isolates tested were resistant to multiple antibiotics, including ciprofloxacin and kanamycin. The isolates obtained after the 10 days weaning period exhibited a higher rate of resistance to other antibiotics such as amikacin, ciprofloxacin, kanamycin and nalidixic acid when compared to organisms isolated before the weaning period. It is not surprising to detect a high rate of resistance to tetracycline (**Table 4.3.2**) because of the frequent usage of oxytetracycline, but the finding that chloromycetin resistance rate was also very high was unexpected as this antibiotic was not added to animal feed. This phenomenon may be due to the effect of cross induction by florfenicol, which was used as an animal feed additive in the farm from which the test piglets were obtained.

Resistance rate and population structure of GI tract *E. coli* in piglets before and after antibiotic treatment

To further test the response of the GI tract microbial flora of piglets to antibiotic treatment under controlled conditions, 20 weaning piglets, purchased from the same pig farm where the piglets examined in the preliminary experiments as described above were obtained, were first subjected to a 10 days period of no antibiotic exposure through feeding with food that did not contain any antibiotics, followed by segregation into different antibiotic treatment groups. Resembling results of the preliminary tests, these 20 piglets were also found to harbor cephalosporin resistant and ciprofloxacin resistant *E.coli* at the beginning of the experiments (isolation date of 4.23, **Fig 4.3.2**). Interestingly, the number of resistant organisms significantly decreased, but still remained detectable in all animals after antimicrobial stress was totally relieved for 10 days (isolation date of 5.4). On the other hand, however, the population size of GI tract *E. coli* remained unchanged (**Fig 4.3.2A, C**). After the 10 days antibiotic withdrawal period, the 20 piglets were randomly separated into 5 groups to examine the effect of renewed antibiotic stress on the remaining resistant sub-population of gut flora. In the ceftiofur sub-therapeutic groups, the sub-population of cefotaxime-resistant *E.coli* rapidly expanded to a higher proportion than the ceftiofur - therapeutic group, which in turn exhibited a higher proportion of resistant organisms than the control group (**Fig 4.3.2B**). The same phenomenon was observable when enrofloxacin was used as the treatment agent (**Fig 4.3.2B, D**). In addition, phenotypic resistance was not confined to cefotaxime, as a significantly higher count of ciprofloxacin resistant *E. coli* was observable after treatment with either ceftofur or enrofloxacin, with the sub-therapeutic group consistently exhibiting a higher resistance rate than the therapeutic group in each case (**Fig 4.3.2C, D**).

To further investigate the nature of changes in the antimicrobial susceptibility phenotype in each treatment group, 50 *E. coli* isolates were randomly selected from one representative piglet of each treatment group at different time points (beginning of weaning (4.23), end of 10 days period of no antibiotic treatment (5.4), and after treatment with different antibiotics (5.8/5.11)) and subjected to antimicrobial susceptibility testing and phylogenetic typing. As shown in **Table 4.3.3**, resistance rate to several antimicrobials such as cefotaxime, ciprofloxacin, kanamycin and

ceftriaxone were found to decrease in the control group in the absence of antibiotic selective pressure, whereas the rate of resistance to several agents such as ampicillin, chloramphenicol, sulfamethoxazole, and tetracycline remained extremely high throughout the test period. A similar trend was observable in strains recoverable from other test groups prior to antibiotic treatment. In the ceftiofur sub-therapeutic group, for example, resistance rate to kanamycin was lower after 10 days' relief of antibiotic selection pressure, but increased again after three days of treatment. In this group, however, resistance rate to fluoroquinolones was found to decrease after the treatment process (**Table 3**). In the ceftiofur therapeutic group, resistance rate to fluoroquinolone first decreased in the absence of antibiotic, and then increased again upon ceftiofur treatment. The effect of enrofloxacin was also found to be similar to that of ceftiofur, resulting in an elevated resistance rate at the end of the treatment process. Taken together, these results suggest that treatment with sub-therapeutic antibiotic concentration can cause an even more rapid increase in resistance rate than treatment with therapeutic concentrations. On the other hand, it should be stressed that a significant difference between the proportion of cefotaxime and ciprofloxacin resistant strains recovered at different time points was observed, indicating that phenotypic resistance to β-lactams and the quinolones were selected at different rates.

Phylogenetic analysis showed that type A was consistently the most dominant type of cefotaxime-resistant *E. coli* in both the control group and other treatment groups, although minor variation in the prevalence rate before and after treatment was observable (**Table 4.3.4**). Carriage of different CTX-M genes in the cefotaxime-resistant *E. coli* strains was also examined, with results showing that the M9 type of CTX-M genes was the most prevalent, and that M1 was less common, and M2 was not detectable (**Table 4.3.4**).

Mechanisms underlying the change in population structure of cefotaxime-resistant *E. coli* in the GI tract of piglet during antibiotic treatment

To check whether the increase in prevalence of cefotaxime-resistant *E. coli* after ceftiofur treatment was due to clonal expansion of specific strains or transmission of CTX-M encoding plasmids, five cefotaxime-resistant *E. coli* strains recoverable from one animal at different treatment time points in each of the ceftiofur therapeutic and

sub-therapeutic group were subjected to PFGE analysis. In addition, 5 cefotaxime-sensitive *E. coli* strains collected at each treatment time point from the same animal in ceftiofur sub-therapeutic group were also subjected to PFGE analysis to determine if the structure of gut microflora altered significantly even under sub-inhibitory concentrations of antibiotic. Our data revealed that drug resistant *E. coli* isolates collected at different time points in the control group exhibited genetically similar PFGE profiles (Group G1, **Fig 4.3.3**). However, such profile became diverse among strains which had been subjected to treatment with sub-therapeutic concentration of ceftiofur (Group 2, **Fig 4.3.3**), indicating that emergence of resistant strains under such condition was likely due to transmission of CTX-M-encoding plasmids to drug sensitive organisms. On the other hand, treatment with therapeutic concentrations of ceftiofur was found to result in emergence of resistant strains which exhibited identical PFGE profiles (Group G3a, **Fig 4.3.3**), suggesting that clonal expansion of specific strains occurred under strong antibiotic selection pressure. Interestingly, PFGE profiles of cefotaxime-susceptible *E. coli* strains recovered at different time points in the ceftiofur sub-therapeutic group also displayed a genetically related profile which differed significantly from the range of diverse genetic profiles detectable among strains collected prior to ceftiofur treatment (Group3b, **Fig 4.3.3**), suggesting that the cefotaxime-susceptible *E. coli* population are also highly sensitive to ceftiofur selection pressure even though the drug concentration was sub-inhibitory.

Mutational changes in the target genes are the major underlying mechanism of antibiotic resistance in bacteria recoverable from the site of infection [300], yet few previous studies have investigated the accompanied changes in the composition of microflora in other body parts during or after the process of infection and antimicrobial treatment [301]. In an attempt to assess the effect of the antimicrobial agent ceftiofur on animal gut flora, we showed that antimicrobial treatment could potentially result in a marked increase in the number of antibiotic resistant organisms in the gut flora of piglets. Our data confirmed that this phenomenon was due to the fact that resistant organisms commonly constituted a significant proportion of the gut flora of piglets even prior to antibiotic exposure, so that subsequent rounds of antimicrobial treatment, regardless of the type of antimicrobial agents used and route of administration, produced an enrichment effects on the drug resistant sub-population

of the gut flora. The resistant organisms were believed to have been transmitted congenitally [302], or through intake of water and food contaminated with faecal particles since ceftiofur has long been used in the veterinary field for treatment of infections in animals [293]. Results of detailed investigation of the composition and phenotypic features of this resistant population by determining the antimicrobial susceptibility profiles of individual organisms indicated that induction of cross-resistance to multiple antibiotics during the treatment process is a common event.

Another important finding of this study is that the physiological fitness of resistant organisms in the intestine of the test animals was not significantly compromised when compared to the drug sensitive organisms of the normal gut microbial flora. Several previous studies on animals with a low resistance background showed that the overall population size of *E.coli* might shrink during the process of treatment (16, [303], and that intramuscular administration of β-lactams such as ampicillin or ceftiofur could alter the structure the microbiota in animal GI tract [289, 304]. However, these works were carried out under artificially controlled conditions, in which a single antibiotic resistant bacterial strain was tested for the effects of beta-lactam antibiotic on the selection of cephalosporin resistance genes during the treatment process [294]. In fact no studies have been performed to elucidate the dynamic changes in the structure or composition of sub-population antibiotic-resistant organisms as a result of antibiotic treatment, especially in animals with a high background of resistant organisms. Our works on animals simulate the real life situation in the human GI tract, where a large proportion of normal flora has been identified as ESBL-producing *E. coli* through analysis of human faecal samples [305, 306]. Data regarding the response of human GI tract flora to commonly used antibiotics such as cephalosporins are currently not available [307, 308].

In a recent study, Fleury *et al* [309] showed, by using a pig model, that the size of cephalosporin resistant *E. coli* sub-population in the gut microbiota expanded substantially upon encountering ceftiofur selection pressure despite the fact that the overall *E. coli* population shrank transiently. Such findings are therefore highly consistent with our observation that the cefotaxime-resistant *E. coli* sub-population in the GI tract did not disappear upon relief of antibiotic stress. Taken together, these phenomena suggest that these resistant organisms could well adapt to the GI tract

environment and could not be out-competed readily by antibiotic susceptible bacteria even in the absence of antibiotic stress. Such resistant organisms are therefore expected to exhibit growth advantage during the next episode of antibiotic treatment. This theory is confirmed by our findings and that of Fleury *et al* (23), which further showed that the proportion of resistant *E. coli* strains in the normal gut flora increased again upon initiation of antibiotic treatment, regardless of the type of antimicrobial agents used and methods of administration. We also showed that this sudden increase in the size of the resistant bacterial sub-population was mainly due to efficient transmission of CTX-M-encoding plasmids from existing resistant organisms to drug sensitive organisms rather than clonal expansion of a specific strains under selection pressure, as evidenced by the observation of diverse PFGE profile of resistant *E. coli* at different treatment time points. This finding is again consistent with that of Fleury *et al* (23). In other words, clonal expansion of the resistant strains under antibiotic selection pressure is not as efficient as the mechanism of horizontal transfer of resistance-determining extra-chromosomal elements in causing an expansion of the resistant sub-population under antibiotic selection pressure.

A surveillance was first performed on a commercial swine farm in China to assess the prevalence of resistant organisms in the intestine of newly born animals. Faecal samples of fifteen 30-days old piglets which began to wean on the same day, and fifteen 40-days old piglets which have weaned for 10 days, were collected for analysis. The piglets were fed according to a commercial protocol, in which oxytetracycline, aminophenazoneco and VB were injected to piglets immediately after the weaning procedure to reduce the level of stress associated with weaning.

To test the effects of the antibiotic treatment regimens on the gut microflora of the test piglets, another batch of twenty 30-day-old piglets which were weaned at the same time and supposed to have never been treated with antibiotic previously were obtained from the same pig farm and housed in our animal facility throughout the process of animal experiments. Every two animals were allocated to one cage to minimize contact, and the degree of stress experienced by these animals during the experiments. Faecal samples from each piglet were collected on the first day of experiment. The animals were then fed with animal feed which did not contain any antimicrobial compounds. The faecal samples were then collected again after a 10

days-period during which antibiotic was completely absent. These piglets were then randomly divided into 5 groups, with 4 animals in each group. Group 1 was the control group which was treated with saline only. Group 3 was treated parenterally with a therapeutic dosage of ceftiofur at 5mg/kg BW once a day for 3 days by intramuscular injection. Group 2 was the sub-therapeutic group in which the piglets were treated in the same way as Group 3 except that ceftiofur was administered parenterally once every three days. The treatment procedure was repeated for 3 consecutive days and faecal samples from each piglet were collected on the fourth day. Enrofloxacin (Sangon Biotech, China) was administered for piglets in Group 4 and 5. Group 4 was gavaged with therapeutic dosage of enrofloxacin at 10 mg/kg of body weight once per day for 7 days [310, 311]. Group 5 was gavaged with sub therapeutic dosage of enrofloxacin at 1 mg/kg of body weight once per day for 7 days. The treatment procedure was repeated for 7 consecutive days and faecal samples from each piglet were collected on the eighth day. The animal experiments only involved administration of antibiotics to the pigs according to approved veterinary regulations. Animal ethical approval was obtained from the Nanjing Agriculture University.

Sample collection and microbiological analysis

Faecal samples were collected directly from the rectum of piglets on the first day (Day 1), ablation day (Day 11) and post treatment day. The therapeutic treatment and sub-therapeutic treatment with ceftiofur lasted for three days, therefore the post treatment samples were collected on Day 14. Therapeutic and sub-therapeutic treatment with enrofloxacin lasted for 7 days, therefore the post treatment samples were collected at Day 18. Faeces (5 g) collected from each piglet were homogenized with 50 ml of saline. One hundred µl of the suspension were added into a new tube with 900 µl saline buffer, mixed and subjected to 10-fold serial dilution. One hundred µl of diluent at 10^{-4}, 10^{-5} and 10^{-6} dilution were inoculated onto MacConkey agar plates containing 0, 0.5 and 2 µg/ml of cefotaxime or ciprofloxacin for selection of lactose fermenting *Escherichia coli* [312].

To check for the presence of *Lactobacillus*, fresh faecal samples were serially diluted as described above under anaerobic conditions and inoculated onto *Lactobacillus* MRS Agar alone and *Lactobacillus* MRS agar containing 2 µg/ml of

cefotaxime, followed by incubation at 35°C for 24h [313]. To check for the presence of *Staphylococci*, fresh faecal samples were serially diluted as described above and inoculated by spreading onto Baird-Parker agar, which was then incubated at 35°C for 24 to 48 h to select for *Staphylococci*. [314].

Assessment of resistance rate

The total number of *E. coli* recoverable per unit weight faecal samples of piglets were determined and compared to that obtained from plates supplemented with 0.5 μg/ml cefotaxime or 2 μg/ml ciprofloxacin to estimate the proportion of resistant organisms in the samples. Cephalosporin resistant *E. coli* strains were selected on MacConkey agar containing cefotaxime (2 μg/ml), whereas enrofloxacin resistant *E. coli* were selected on MacConkey agar containing ciprofloxacin (2 μg/ml). Organisms that were able to grow in the presence of 2 μg/ml of cefotaxime or ciprofloxacin were classified as resistant. The breakpoint MIC values (4μg/ml) were chosen according to the CLSI guidelines [315].

Antimicrobial susceptibility tests

Susceptibilities to cefotaxime, ciprofloxacin, kanamycin, tetracycline, chloramphenicol, nalidixic acid, ampicillin, gentamicin, sulfamethoxazole were assessed by agar dilution and disc diffusion methods according to the CLSI guidelines [315].

Detection of resistance genes, phylotyping and pulsed field gel electrophoresis

The major mechanism of resistance to β-lactam antibiotics in Gram-negative bacteria could be attributed to the production of β-lactamases. The presence of Extended-Spectrum β-Lactamases genes, $bla_{\text{CTX-Ms}}$, in *E. coli* strains isolated from faecal samples of the test piglets were detected using a PCR approach as previously described [298]. To observe the changes in the *E. coli* phylogenetic types and population structure in the GI tract of piglets during and after antibiotic treatment, fifty antibiotic resistant colonies collected from antibiotic-containing MacConkey agar plates at different time points were subjected to analysis of their phylogenetic

linkages using multiplex PCR method as previously described [316]. *E. coli* isolates of the same phylogenetic group were further characterized by PFGE as previously described [317]. *Salmonella Braenderup* H9812 strain was used as molecular marker. PFGE profiles were analysed by the BioNumerics fingerprinting software using a threshold of 95% in Dice similarity coefficient (Applied Maths, St.-Martens-Latem, Belgium).

Table 4.2. 1**.** Assessment of relative prevalence of representative *bla*$_{CTX-M}$ types and phylogenetic types among cefotaxime resistant *E. coli* strains collected from piglets before and after 10 days weaning with antibiotic-containing food.

Piglet grou p*	**No. of *E.coli* strains tested#**	***bla*CTX-M subgroups (%)**			**Phylogenetic groups (%)**			
		***bla*CTX-M-1**	***bla*CTX-M-2**	***bla*CTX-M-9**	**A**	**B1**	**B2**	**D**
WP	66	54	0	2	73	15	9	3
10 PW	108	47	28	29	77	13	4	6

*WP, Weaning piglets prior to being fed with commercially available antibiotic –containing food; 10PW, 10 days post weaning with antibiotic –containing food.

Table 4.2. 2. Rate of resistance to other classes of antibiotic in cefotaxime-resistant *E.coli* strains collected from piglets before and after 10 days weaning with antibiotic-containing food.

Animals	**AMK**	**CIP**	**KAN**	**TET**	**CHL**	**NAL**
WP	26%	71%	72%	100%	100%	44%
10PW	56%	97%	100%	100%	100%	100%

*WP, Weaning piglets prior to being fed with commercially available antibiotic –containing food; 10PW, 10 days post weaning with antibiotic –containing food.

AMK, amikacin; CIP, ciprofloxacin; KAN, kanamycin; TET, tetracycline; CHL, chloramphenicol; NAL, nalidixic acid.

Table 4.2. 3. Rate of resistance to different antibiotics among *E. coli* strains subjected to ceftiofur and enrofloxacin treatment before and after a 10 days period during which the animals had been fed with food which did not contain antibiotics.

Groups[#]	Isolation time[*]	% Resistance									
		CTX	CIP	KAN	CRO	TET	CHL	NAL	AMP	SXT	GEN
1	WP	25	10	100	25	100	100	100	100	100	15
	Pre	0	10	5	0	100	100	100	100	100	5
	Tre	0	0	15	0	100	75	80	100	100	5
2	WP	30	80	85	30	100	75	100	100	100	5
	Pre	15	20	25	20	100	65	45	95	95	15
	Tre	20	5	65	10	95	15	40	100	90	0
3	WP	30	55	70	30	70	60	100	80	100	25
	Pre	5	10	35	5	75	65	70	90	100	25
	Tre	25	55	65	25	75	55	95	100	100	50
4	WP	10	40	85	10	95	55	90	85	95	45
	Pre	5	0	30	5	100	20	15	100	100	0
	Tre	5	40	60	5	100	100	100	100	100	20
5	WP	0	55	45	0	95	90	100	95	95	15
	Pre	0	40	40	0	100	35	45	100	100	0
	Tre	10	50	40	10	80	85	100	100	100	15

[#]Group 1, Control group; Group 2, ceftiofur sub-therapeutic group; Group 3, ceftiofur therapeutic group; Group 4, enrofloxacin therapeutic group; Group 5, enrofloxacin sub-therapeutic group.[*]4.23, April 23, 2014, the date on which weaning with food which did not contain antibiotic started; 5.4, May 4, 2014, the date on which antibiotic treatment started; 5.8 / 5.11, May 08 / 11, 2014, the date on which antibiotic treatment ended. 50 *E. coli* isolated were randomly selected at each time point. CTX, cefotaxime; CIP, ciprofloxacin; KAN, kanamycin; CRO, ceftriaxone; TET, tetracycline; CHL, chloramphenicol; NAL, nalidixic acid; AMP, ampicillin; GEN, gentamicin; SUL, sulfamethoxazole.

Table 4.2. 4. Variation in prevalence of different phylogenetic types and the blaCTX-M gene profile among E. coli strains collected from different treatment groups on three strain isolation dates.

Groups[#]	WP*					Pre					Tre*				
	PGs	**No.**	**M1**	**M2**	**M9**	**PGs**	**No.**	**M1**	**M2**	**M9**	**PGs**	**No.**	**M1**	**M2**	**M9**
1	A	48			46	A	50			35	A	41			41
	B1	2			2	B1					B1	9	6		2
2	A	45	3		38	A	50	9		34	A	50			35
	B1	5			5	B1					B1				
3	A	45	6		28	A	44	9		28	A	23	8		8
	B1	3			3	B1	3			3	B1	27	7		16
	D	2	2			D	3	2			D				
4	A	17	-	ND	-	A	49	-	ND	-	A	50	-	ND	-
	B1	8	-	ND	-	B1	1	-	ND	-	B1		-	ND	-
5	A	39	-	ND	-	A	37	-	ND	-	A	30	-	ND	-
	B1	10	-	ND	-	B1	13	-	ND	-	B1	7	-	ND	-
	B2	1	-	ND	-	B2		-	ND	-	B2		-	ND	-
	D		-	ND	-	D	3	-	ND	-	D	13	-	ND	-

[#]Group 1, Control group; Group 2, ceftiofur sub-therapeutic group; Group 3, ceftiofur therapeutic group; Group 4, enrofloxacin therapeutic group; Group 5, enrofloxacin sub-therapeutic group.

[*]4.23, April 23, 2014, the date on which weaning with food which did not contain antibiotic started; 5.4, May 4, 2014, the date on which antibiotic treatment started; 5.8 / 5.11, May 08 / 11, 2014, the date on which antibiotic treatment ended.

PGs, Phylogenetic groups; M1, M1 *bla*$_{\text{CTX-Ms}}$; M2, M2 *bla*$_{\text{CTX-Ms}}$; M9, M9 *bla*$_{\text{CTX-Ms.}}$

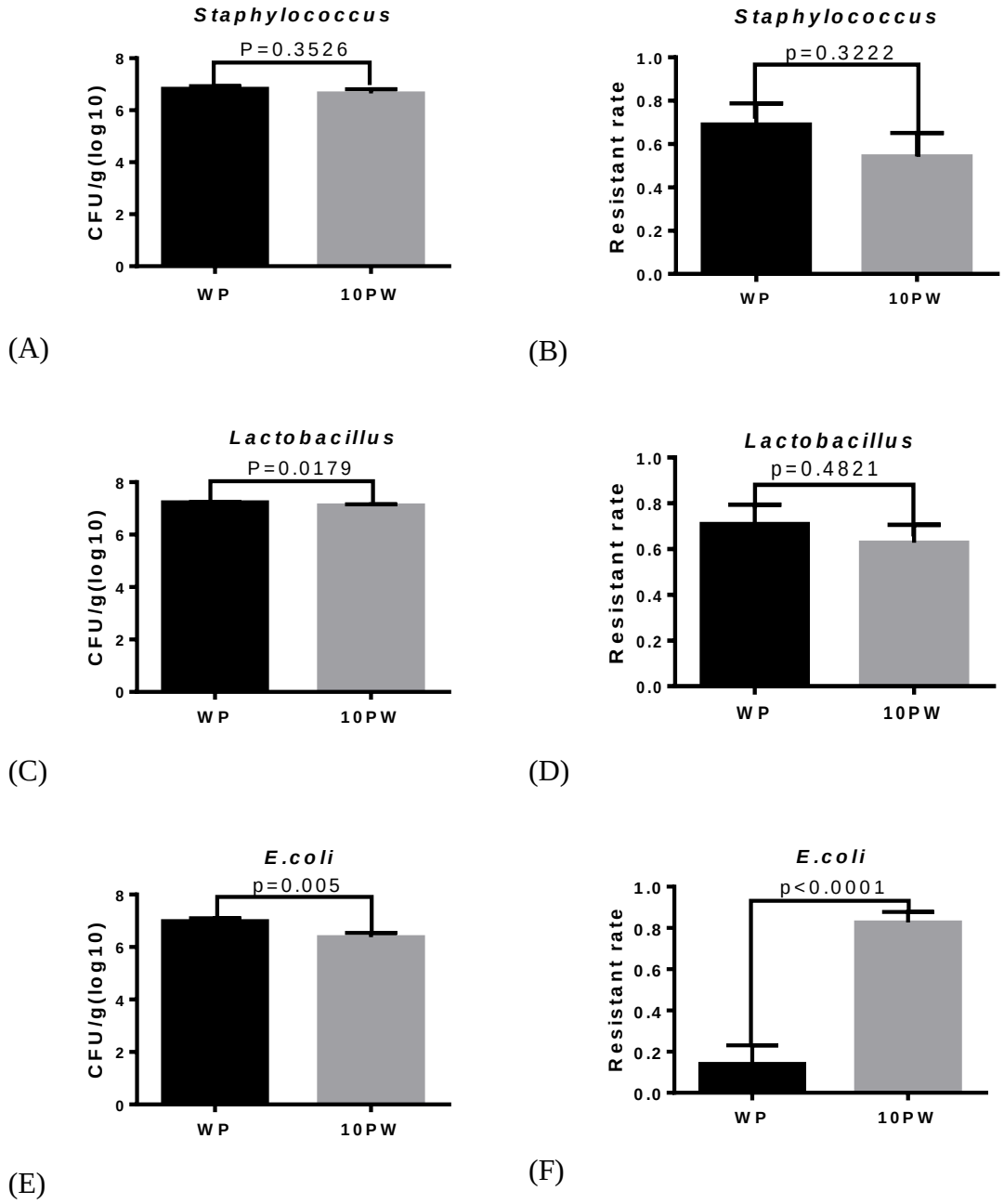

Figure 4.2. 1. Cefotaxime resistance rate of *Lactobacillus*, *Staphylococcus* and *E. coli* population in the GI tract of piglets before and after weaning.

(A), (C) and (E), total GI tract bacterial counts of *Lactobacillus*, *Staphylococcus* and *E. coli*, respectively, prior to weaning (WP) and 10 days' after weaning (10PW); (B), (D) and (F), rate of resistance to cefotaxime in GI tract *Lactobacillus*, *Staphylococcus* and *E. coli*, respectively, before (WP) and 10 days' after weaning (10PW). Data are means ± s.e.m. Using the unpaired T test to compare the different between WP and 10 PW.

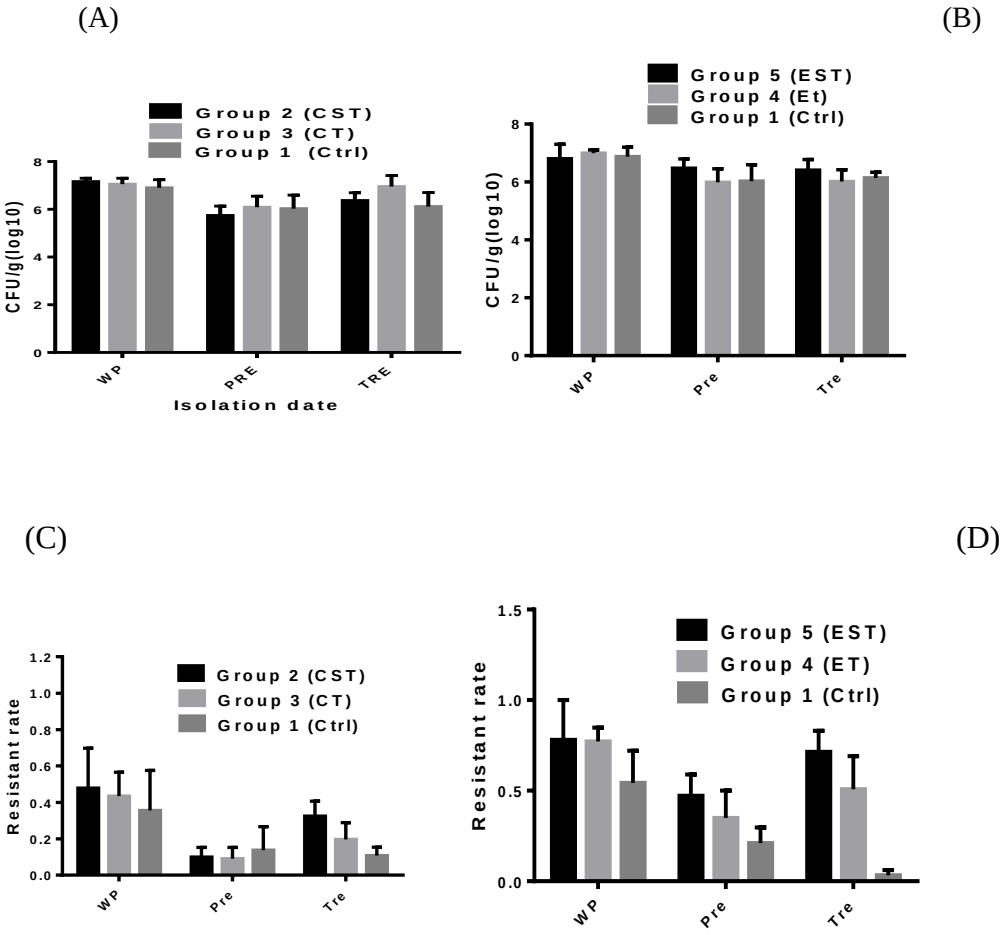

Figure 4.2. 2. Change in the size and resistance rate of GI tract *E. coli* population in piglets upon treatment with ceftiofur and enrofloxacin.

(A) and (C), population size of *E. coli* and rate of resistance to cefotaxime before and after ceftiofur treatment; (B) and (D), population size of *E. coli* and rate of resistance to ciprofloxacin before and after enrofloxacin treatment. A: Comparing the CFU (Colony-forming Units) on the MAC plates show no significant difference among the three groups (ANOVA, F= 0.3001, P>0.05). C: Comparing the CFU (Colony-forming Units) on the MAC plates show no significant difference among the three groups (ANOVA, F= 0.2546, P>0.05). WP, weaning point, before which the piglet were not fed with any commercial feed; Pre, pre-treatment, before which the antibiotic treatment started (10 day no antibiotic treatment); Tre, antibiotic treatment, antibiotic treatment endpoint. CT, Ceftiofur therapeutic group; CST, Ceftiofur sub therapeutic group; EST, Enrofloxacin Sub therapeutic group; ET, Enrofloxacin therapeutic group; Ctrl, Control group. Data are means ± s.e.m.

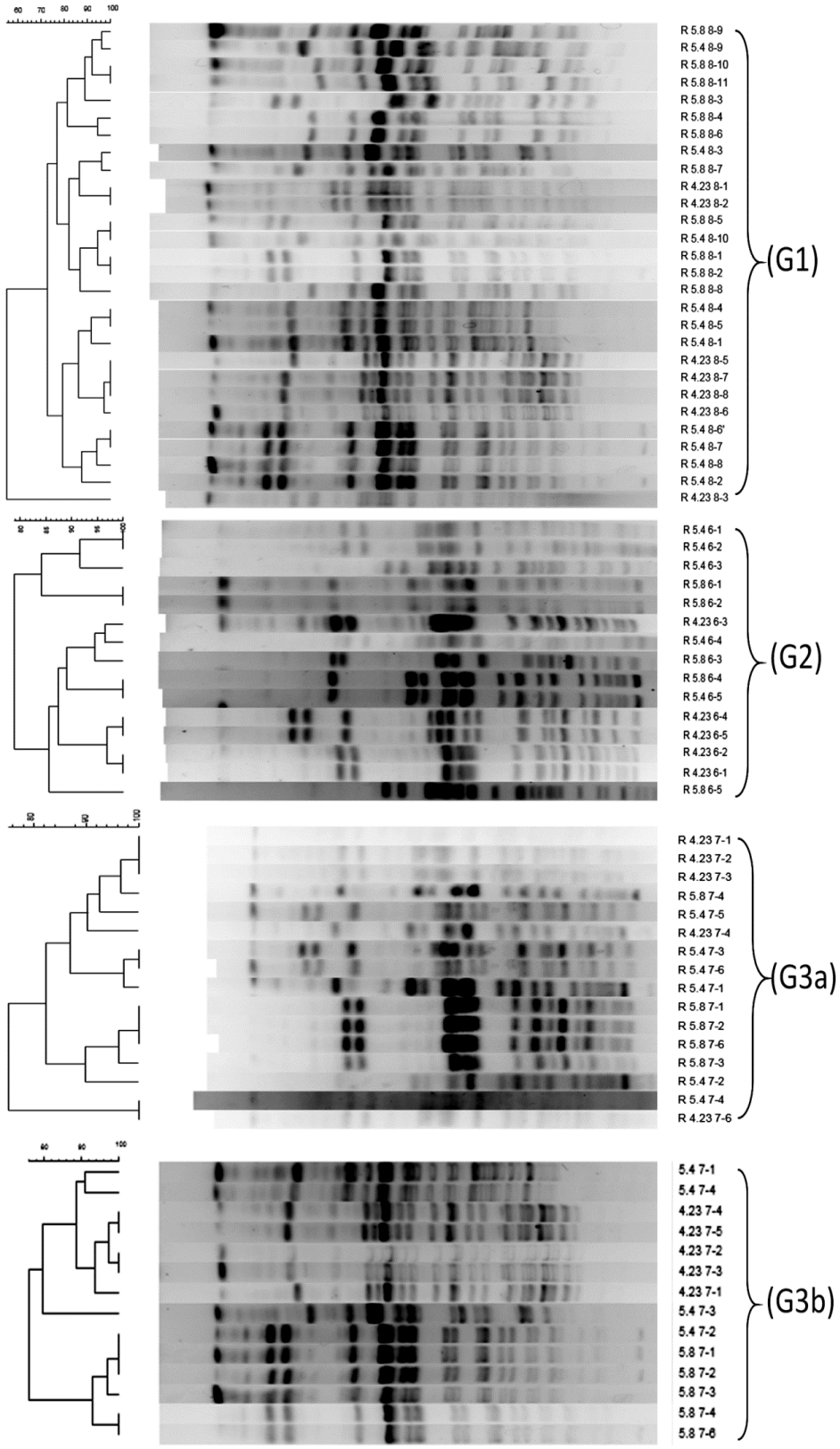
60 70 80 90 100
R 5.8 8-9
R 5.4 8-9
R 5.8 8-10
R 5.8 8-11
R 5.8 8-3
R 5.8 8-4
R 5.8 8-6
R 5.4 8-3
R 5.8 8-7
R 4.23 8-1
R 4.23 8-2
R 5.8 8-5
R 5.4 8-10
R 5.8 8-1
R 5.8 8-2
R 5.8 8-8
R 5.4 8-4
R 5.4 8-5
R 5.4 8-1
R 4.23 8-5
R 4.23 8-7
R 4.23 8-8
R 4.23 8-6
R 5.4 8-6'
R 5.4 8-7
R 5.4 8-8
R 5.4 8-2
R 4.23 8-3
(G1)
R 5.4 6-1
R 5.4 6-2
R 5.4 6-3
R 5.8 6-1
R 5.8 6-2
R 4.23 6-3
R 5.4 6-4
R 5.8 6-3
R 5.8 6-4
R 5.4 6-5
R 4.23 6-4
R 4.23 6-5
R 4.23 6-2
R 4.23 6-1
R 5.8 6-5
(G2)
R 4.23 7-1
R 4.23 7-2
R 4.23 7-3
R 5.8 7-4
R 5.4 7-5
R 4.23 7-4
R 5.4 7-3
R 5.4 7-6
R 5.4 7-1
R 5.8 7-1
R 5.8 7-2
R 5.8 7-6
R 5.8 7-3
R 5.4 7-2
R 5.4 7-4
R 4.23 7-6
(G3a)
5.4 7-1
5.4 7-4
4.23 7-4
4.23 7-5
4.23 7-2
4.23 7-3
4.23 7-1
5.4 7-3
5.4 7-2
5.8 7-1
5.8 7-2
5.8 7-3
5.8 7-4
5.8 7-6
(G3b)

Figure 4.2. 3. PFGE profile of cefotaxime resistant and susceptible *E.coli* strains recovered from one piglet in each of group 1~3 at different isolation dates.

G1, control group; G2, ceftiofur sub-therapeutic group; G3a, cefotaxime resistant *E. coli* strains recovered from the ceftiofur therapeutic group ; G3b, cefotaxime susceptible *E. coli* strains recovered from the ceftiofur therapeutic group. 4.23, April 23, 2014, the date when weaning with food which did not contain antibiotic started; 5.4, May 4, 2014, the date when weaning with food which did not contain antibiotic ended and antibiotic treatment started; 5.8, May 08, 2014, the date on which antibiotic treatment ended.

5 Future plan

There is still a long way to go for understanding the complicated mechanisms of the appearance of antibiotic resistance in GI tract. In the future we will further continue to perform research in three major areas. First we plan to finish the survilance on carbapenem resistant gram negative bacteria, especially the community-acquired MDR isolates. For example, the discovery of antibiotic resistance (AR) genes in all human microbiota [318], even the new born baby also harboured those ARG genes[319]. Based on the finished study we will further carry out comparison between the nosocomial and community isolates in order to find the linkage between community-acquired pathogen and hospital-acquired pathogen. Secondly, the comparisons between the *in vivo* and *in vitro* induced stains under the same antibiotic selecting pressure, and the potential mechanism for that. Using the models in this study to test new hypothesis.

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