HORIZONTAL TRANSFER OF EXTENDED-SPECTRUM β-LACTAMASE- AND CARBAPENEMASE-ENCODING GENE IN *KLEBSIELLA PNEUMONIAE* IN RAW FOODS

by

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ABSTRACT OF THE DISSERTATION

Horizontal transfer of extended-spectrum β-lactamase- and carbapenemase-encoding gene in *Klebsiella pneumoniae* in raw foods

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Klebsiella pneumoniae is ubiquitous in nature as a commensal bacterium, but can also cause nosocomial infections as an opportunistic pathogen. Extended-spectrum β-lactamase (ESBL)- or carbapenemase-producing bacteria or ESBL- or carbapenemase-encoding genes in raw foods have been more prevalent in recent years; however, limited studies have been conducted on the transfer of ESBL- or carbapenemase-encoding genes in raw foods. Therefore, the goal of this research was to elucidate the transfer of ESBL- or carbapenemase-encoding genes in *K. pneumoniae* under actual food-related conditions.

ESBL-producing *Klebsiella pneumoniae* ATCC 700603 served as a donor and three recipients (KP342$^{\Delta KM}$, KP4$^{\Delta KM}$, KP8$^{\Delta KM}$) isolated from seed sprouts were used for the mating experiments in TSB, pasteurized milk, unpasteurized milk, alfalfa sprouts, and chopped lettuce at 4˚C, 15˚C, 24˚C, and 37˚C. An investigation on the potential transfer of carbapenemase-encoding gene (bla_{KPC}) in *Klebsiella pneumoniae* to *Salmonella* and *E.*

coli O157:H7 was also performed in TSB and alfalfa sprouts.

In mating experiments to identify ESBL-encoding gene (bla_{SHV18}) transfer, no transconjugants were detected at 4°C in liquid media and chopped lettuce, but detected in all media at 15°C, 24°C, and 37°C. At 24°C, the transfer of the bla_{SHV18} gene occurred more frequently in alfalfa sprouts (5.15E-04 transconjugants per recipient) than in liquid media (1.08E-05) ($p<0.05$). On chopped lettuce, no transconjugant was detected at day 1 post-mating at 15°C, but it was observed on day 2 (1.43E-05). Transconjugants carried the bla_{SHV18} gene transferred from the donor and virulence gene harbored by the recipient. Moreover, a class 1 integrase gene and resistance to tetracycline, trimethoprim/sulfamethoxazole were co-transferred during the mating. No transfer of bla_{KPC} from *Klebsiella pneumoniae* to *Salmonella* and *E. coli* O157:H7 was confirmed under the conditions evaluated.

These quantitative results suggest that the transfer of this antibiotic resistance gene under laboratory conditions underestimates its transfer frequency in fresh produce. Importantly, fresh produce exposed to temperature abuse may serve as vehicles for the spread of antibiotic resistance having a potentially negative impact on human health. While we could not confirm the transfer of bla_{KPC} both in TSB and alfalfa sprouts, more studies aimed at determining favorable conditions for the transfer of carbapenemase-encoding gene in foods should be conducted.

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Dedication

In memory of my parents, Jaehyun Jung, Minsook Song,
my husband, Seho, and my son, Jayden…

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List of Abbreviations

AS	Alfalfa sprouts
BS	Broccoli sprouts
CPS	Capsular polysaccharides
CRE	Carbapenem-resistant Enterobacteriaceae
CDC	Center for Disease control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CS	Clover sprouts
E. coli	*Escherichia coli*
IMP	Imipenemase
KPC	*K. pneumoniae* carbapenemase
K. pneumoniae	*Klebsiella pneumoniae*
LPS	Lipopolysaccharides
LB broth	Luria-Bertani broth
MAC	MacConkey agar
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MRSA	Methicillin-resistant *S. aureus*
NDM	New Delhi metallo

OXA	Oxacillinases
PM	Pasteurized milk
UPM	Unpasteurized milk
SHV	Sulfhydryl variable
SPP	Species
SPW	Sterile peptone water
S. aureus	*Staphylococcus aureus*
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VRSA	Vancomycin-resistant *S. aureus*
VIM	Verona imipenemase

Chapter 1. Introduction

1.1.Rationale and Significance

The genus *Klebsiella* in the *Enterobacteriaceae* family is ubiquitous in nature as harmless natural flora; in contrast, *Klebsiella pneumoniae* can cause nosocomial infections as opportunistic pathogens (Podschun and Ullmann, 1998 and Lau et al., 2008). In foods, commensal bacteria acquired from the environment (soil, water, air) may facilitate the spread of antibiotic resistance genes, serving as recipients and donors of antibiotic resistance genes (Wang et al., 2006).

Klebsiella spp. are known to be intrinsically resistant to β-lactam antibiotics due to the production of β-lactamase (Bradford, 2001). The extensive use of third-generation cephalosporins which are in the category of β-lactam antibiotics has led to the evolution of β-lactamases. These new β-lactamases were then categorized as extended-spectrum β-lactamases (ESBLs) (Pitout and Laupland, 2008). ESBLs are predominantly encoded on conjugative self-transferable plasmids, which are responsible for the rapid spread of the antibiotic resistance genes (Gniadkowski et al., 1998). Due to the widespread use of carbapenems to treat infections caused by ESBL(s)-producing bacteria, increased resistance to carbapenems in *Enterobacteriaceae* has been reported worldwide during the past 10 years (Zhanel et al., 2007). Resistance to carbapenems in *Enterobacteriaceae* has mainly arisen due to the production of carbapenemase(s) by bacteria. ESBL or carbapenemase-producing bacteria have now spread into community settings, threatening public health.

Since antibiotics are directly used for growth promotion and prevention of

diseases in food-producing animals, considerable attention has been given to the prevalence of antibiotic-resistant bacteria associated with food-producing animals and their environment, including farms, feedlots, processing plants, or packing plants (Aslam and Service, 2006, Santos et al., 2007, Aslam et al., 2009, Edrington et al, 2009, and Jouini et al., 2009). Raw foods such as fresh produce are now implicated as vehicles of transfer of antibiotic resistance since raw foods often harbor high populations of antibiotic-resistant bacteria (Boehme et al., 2004 and Falomir et al., 2010). Most people consume fresh produce without applying a process (e.g., cooking) to inactivate microorganisms that may be present, thereby facilitating the transfer of antibiotic resistance gene(s) into the human gastrointestinal tract (Macovei and Zurek, 2007 and Schjorring and krogfelt, 2011).

The literature is rich with papers on the prevalence of antibiotic-resistant bacteria associated with fresh produce, but there are few studies on the transfer of antibiotic resistance genes among bacteria on fresh produce or to bacteria in the human gastrointestinal tract (Ruimy et al., 2010, Hassan et al., 2011, Walia et al., 2013, and Blaak et al., 2014). The limitation of previous studies on the transfer of antibiotic resistance genes was that often liquid laboratory media was used for the mating experiments and model organisms such as *E. coli* K12 or *E. coli* DH5α as recipients (Mc Mahon et al., 2007a, Walsh et al., 2008, and Schjorring and Krogfelt, 2011). Furthermore, to the best of our knowledge and based on an extensive review of the literature, the transfer of carbapenemase-encoding genes in food-related conditions has not been investigated. In fact, there is a paucity of studies on the prevalence of carbapenemase producers or carbapenemase-encoding genes related to food, although

some carbapenemase-producers have been found in non-clinical sources including food-producing animals (Woodford et al., 2013).

Therefore, the goal of this research was to understand and elucidate the transfer of ESBL- or carbapenemase-encoding genes in *K. pneumoniae* under actual food-related conditions.

1.2.Objectives

Several hypotheses were developed and are addressed in this research: 1) seed sprouts contain high populations of bacteria resistant to β-lactam antibiotics and serve as competent vehicles of antibiotic resistance gene transfer, 2) ESBL-encoding genes transfer occurs more frequently in fresh produce than in a laboratory liquid medium, and 3) transfer of carbapenemase-encoding genes in *K. pneumoniae* to foodborne pathogens takes place in raw foods.

We tested these hypotheses by addressing the following specific objectives.

1) Evaluate the microbiological quality of seed sprouts and screen for sprout associated *K. pneumoniae*.
2) Identify and characterize K. *pneumoniae* isolated from seed sprouts.
3) Conduct mating experiments to determine the transfer frequency of bla_{SHV18} genes in liquid media, alfalfa sprouts, and chopped lettuce at defined temperatures and to characterize transconjugants obtained from mating experiments.
4) Investigate whether the carbapenemase-encoding genes in *K. pneumoniae* could transfer to foodborne pathogens in liquid media and alfalfa sprouts.

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Chapter 2. Literature Review

2.1. Antibiotic resistance

An “antibiotic” may be defined as a drug to treat infectious diseases in humans, animals, or plants (Doyle et al., 2006). Cloete (2003) described resistance as a transient or permanent capability of microorganisms to survive and replicate in the presence of antibiotic(s) when compared to original isolates. Bacterial resistance may be caused by a mutation or horizontal acquisition of genetic materials (Courvalin, 2005, and Doyle et al., 2006). Since penicillin was mass-produced in the 1940s, antibiotics have been crucial in the treatment of bacterial infections worldwide. In 1945, Alexander Fleming, who won a Nobel Prize for his discovery of penicillin, warned that inappropriate use of penicillin might cause a “mutant” form of bacteria, leading to antibiotic resistance (Misccky, 1996). After four years of mass production of penicillin, penicillin-resistant *Staphylococcus aureus* (*S. aureus*) began to emerge in 1947 (Rosenblatt-Farrell, 2009). Consequently, a new antibiotic, methicillin, was introduced to treat penicillin-resistant *S. aureus* in 1959, and the first methicillin-resistant *S. aureus* (MRSA) was subsequently reported in 1961 (Ippolito et al., 2010). Due to the increase of MRSA around the world, vancomycin, one of the glycopeptide antibiotics, is now used as the “last resort” antibiotic to treat MRSA infection (Perez et al., 2008). In 2002, vancomycin-resistant *S. aureus* (VRSA) appeared, leading to a failure in the treatment of severe infectious *S. aureus* diseases (Chang et el., 2003). Along with MRSA and VRSA, extended-spectrum β-lactamases (ESBLs) or carbapenemases-producing *Enterobacteriaceae* are now emerging public health issues due to the evolution of bacterial β-lactamases against a new generation of β-lactam antibiotics. The vicious cycle of antibiotic discovery and antibiotic resistance continues.

Currently, antibiotic-resistant bacteria cause at least two million illnesses and 23,000 deaths each year in the United States (The White House, 2015). In 2014, President Obama signed an executive order to combat antibiotic-resistant bacteria, which leads international collaborative efforts with governments, academia, and industry for the surveillance, prevention, and control of antibiotic resistance (Nathan et al., 2014).

2.2.Mechanisms and dissemination of antibiotic resistance

In general, several bacterial mechanisms for resistance to antibiotics have been proposed (Figure 2.1): activation of efflux pumps, production of enzymes to degrade antibiotics, modification of cell wall proteins to inhibit antibiotic uptake, and alteration of antibiotic targets in bacteria (Doyle et al., 2006). Efflux pumps are a primary mechanism by which bacteria transport antibiotic substances that have entered the cell to the extracellular milieu (Khachatourian, 1998). It is known that some bacteria that are resistant to nalidixic acid or ciprofloxacin modify specific receptor sites, mutating *gyrA* and *gyrB* genes (Heddle and Maxwell, 2002). Moreover, Gram-negative bacteria alter membrane permeability as an intrinsic mechanism to confer resistance to the action of specific types of antibiotics. For example, *E. coli* replaces its original porins (OmpF) to narrower porins (OmpC) in the outer membrane when they are exposed to β-lactam antibiotics (Nikaido et al., 1983).

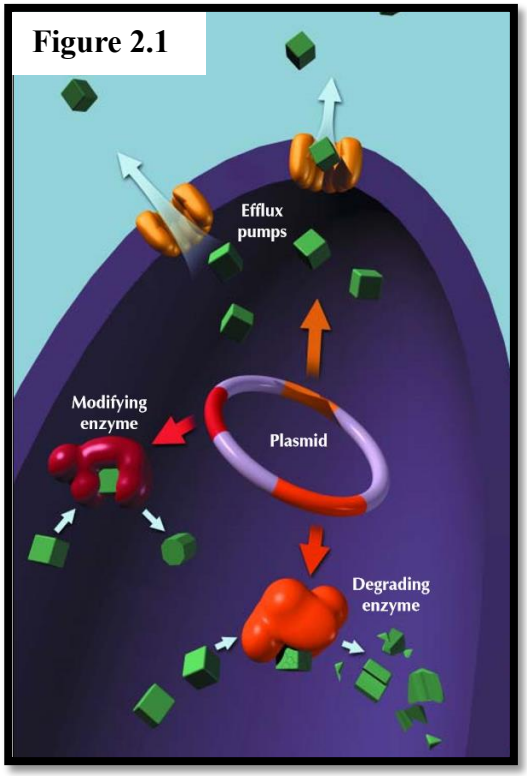
Dissemination of antibiotic resistance occurs at three levels: clonal dissemination (bacteria), replicons (plasmid), or genes (transposons) (Courvalin et al., 2005). Antibiotic resistance genes are horizontally transferred to other bacteria by transduction (Figure 2.2A), conjugation (Figure 2.2B), and transformation (Figure 2.2C) (Khachatourian, 1998).

Conjugation is the non-taxonomic related transfer of genetic materials between bacterial cells (Rossi et al., 2014). In Gram-negative bacteria, conjugation occurs by cell-to-cell direct contact via pili, while Gram-positive bacteria use pheromone-induced or aggregation-mediated plasmid transfer as an alternative mechanism of direct cell contact (Verraes et al., 2013). It is widely recognized that the spread of antibiotic resistance is associated with horizontal gene transfer rather than from a random natural mutation in bacteria (Marshall et al., 2009). More specifically, antibiotic resistance genes are predominantly encoded on mobile elements, such as gene cassettes (integrons), transposons (mobility of genes and operons), and plasmids (mobility of operons and regulons) in bacteria, which play a major role in the dissemination of antibiotic resistance (Cruz and Davies 2000 and Mathur and Singhand, 2005). According to a summary on mobile genetic elements involved in horizontal gene transfer of antibiotic resistance, gene cassettes are transferred by constitutive expression, while plasmids are transferred by regulated expression (Cruz and Davies, 2000). Moreover, horizontal gene transfer occurs among different and the same genera and species of bacteria.

Transformation is a process in which bacteria take up naked DNA from the environment (Kelly et al., 2009). Some bacteria including *Campylobacter* and *Bacillus* exist as naturally competent cells (Verraes et al., 2013). Transduction is performed by bacteriophages, leading the mobility of regulons. Transduction, however, is unlikely to naturally occur due to a narrow host range (Cruz and Davies 2000).

The horizontal transfer of virulence determinants also becomes a concern with the potential co-transfer of antibiotic resistance. Tansfer of virulence associated genetic material from pathogenic to commensal bacteria may lead to the occurrence of “new”

emerging pathogens (Kelly et al., 2009). In 2011, a deadly *E. coli* O104:H4 outbreak linked to sprouts occurred in Germany. This outbreak was caused by an enteroaggregative *E. coli* O104:H4 strain that had acquired virulence genes encoding for Shiga-toxins and exhibited resistance to expanded-spectrum cephalosporins (Frank et al., 2011 and Muniesa et al., 2012). Bacteria continue evolving under changing environmental conditions and survive by modifying characteristics to adapt to new environmental conditions.



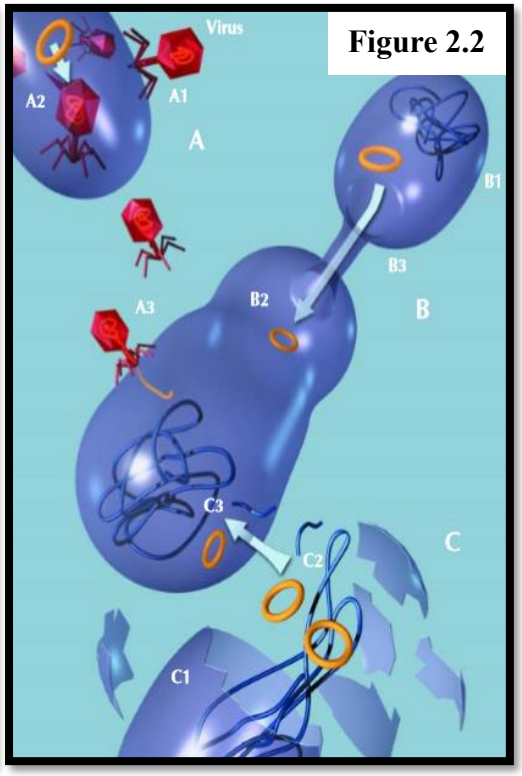


Figure 2.1. Transfer of antibiotic resistance from one bacterium to another can occur by means of bacterial plasmids. Plasmid-mediated resistance mechanisms include efflux pumps, which remove antibiotics (represented as cubes) from the cell; modifying enzymes, which render the antibiotic ineffective by changing its conformation; and degrading enzymes, which degrade the antibiotic altogether.

Figure 2.2. Resistance genes are transferred to other bacteria in the following ways. A: A virus infects a plasmid-bearing cell (A1), where it replicates, picking up a plasmid in the process (A2). The new viruses, which now carry the plasmid and the associated resistance mechanisms, go on to infect other cells (A3). B: A donor bacterium (B1) may transfer plasmid DNA (B2) to another cell through a pilus (B3). C: When bacteria are lysed (C1), they release their plasmids and chromosomal DNA (C2), which can then enter other bacteria (C3).

Figure 2.1 and 2.2 were adopted from Khachatourian (1998).

2.3.Commensal bacteria

Horizontal transfer of antibiotic resistance genes among pathogenic bacteria, commensal bacteria, or between pathogenic and commensal bacteria is of great concern for the broad dissemination of antibiotic resistance (Wang et al., 2006). Much attention has been focused on pathogenic bacteria rather than commensal bacteria due to their impact on human health. As shown in Figure 2.3, major reservoirs of resistance genes in the ecosystem are commensal bacteria (Marshall et al., 2009). The influx of antibiotic resistance genes from commensal bacteria to pathogenic bacteria or other commensal bacteria leads to the dissemination of antibiotic resistance in clinical or community settings. Recent findings indicate that ready-to-eat food contains abundance of antibiotic-resistant bacteria or genes; therefore, food consumption may serve as an important avenue in the dissemination of antibiotic-resistant bacteria to humans (Wang et al., 2006 and Zhang et al., 2011). Wang et al. (2006) detected 10^2-10^7 CFU of antibiotic-resistant bacteria per gram of food, including cheese, deli turkey, mushrooms, spinach, shrimp, and yogurt. Bezanson et al. (2008) assessed the occurrence of antibiotic-resistant bacteria associated with fresh produce grown in Canada. Oxidase positive and Gram-negative bacteria were recovered predominately from a single sampling of retail purchased packaged savory spinach and alfalfa sprouts and non-packaged whole romaine lettuce. Of the 205 isolates collected, approximately 72% were resistant to one or more antibiotics; 93.5 and 90.0% of isolates were resistant to ampicillin and cephalothin, respectively; 35.7% to chloramphenicol, 10.0% to streptomycin, 4.2% to nalidixic acid, 4.2% to kanamycin and 2.8% to gentamicin. Most antibiotic-resistant isolates carried mobile elements, integron-specific DNA sequences, and conjugative R-plasmids, facilitating the

possibility of the horizontal transfer of antibiotic resistance. A study on saprophytic bacteria in spinach found that Gram-negative bacteria associated with spinach harbored ESBL-encoding genes, providing evidence that microbiota associated with organic and inorganic spinach serve as a reservoir for mobile antibiotic resistance genes (Raphael et al., 2011).

It is well established that environmental commensal bacteria are a major reservoir of antibiotic resistance genes, and their genes are closely related to clinical pathogenic bacteria. Njage and Buys (2015) reported high prevalence (90% of isolates) of ESBL/AmpC β-lactamase positive bacteria associated with lettuce and revealed a close phylogenetic relatedness of *E. coli* isolates from water and lettuce. Bhutani et al. (2015) also observed that the SHV11-encoding gene sequence of *Enterobacteriaceae* from retail lettuce was 98% homologous with the ESBL sequence of clinical *Enterobacteriaceae* isolates. Reuland et al. (2014) investigated the prevalence of ESBL-producing *Enterobacteriaceae* in raw retail vegetables in the Netherlands, reporting that the ESBL-encoding genes were most closely associated with genes found in enterobacteria strains of human origin. Falomir et al. (2013) stressed that in addition to commensal bacteria, opportunistic pathogens (*Enterobacter* and *Klebsiella*), which are frequently associated with fresh produce, should be considered as important food safety concerns since they may play a major role as vehicles of antibiotic resistance via the food chain (Falomir et al., 2013).

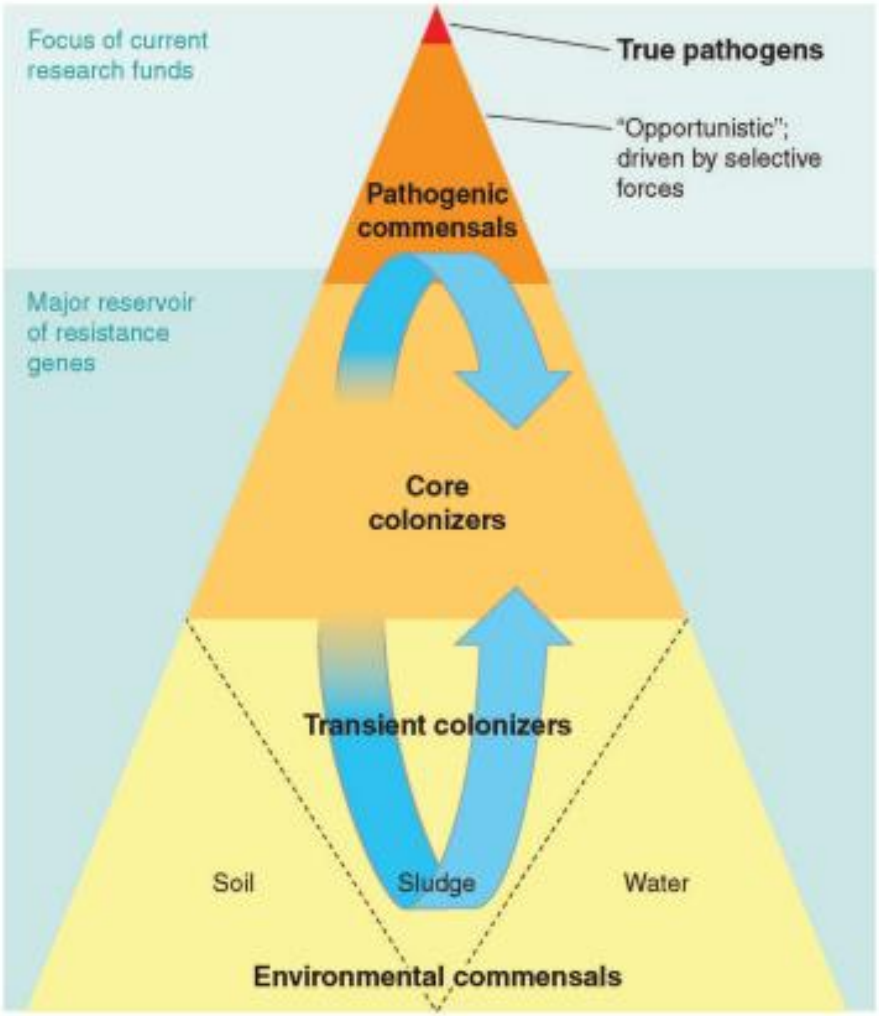


Figure 2.3. Hierarchy of commensals (not to scale). While commensal bacteria are the major reservoirs of antibiotic resistance genes, core and transient colonizers are greatly understudied. Commensals may play a critical role in the relationships with their hosts within the environment. Most environmental commensals may become transient colonizers of humans and animals through the food chain and other routes of exposure.

Adopted from Marshall et al., 2009

2.4.*Klebsiella pneumoniae*

The genus *Klebsiella* is a member of the *Enterobacteriaceae* family. *Klebsiella* species are ubiquitous in nature as harmless commensal bacteria and they are also known as opportunistic pathogens (Brisse et al., 2009). Nosocomial infections caused by *Klebsiella* spp. are mostly associated with the urinary tract, respiratory tract or blood infections (Podschun and Ullmann, 1998). The virulence factors associated with *K. pneumoniae* include serum resistance, adhesins, lipopolysaccharides (LPS), capsular polysaccharides (CPS), and siderophores (Podschun and Ullmann, 1998 and Brisse et al., 2009). In North America, Europe, and Australia, there were few cases of severe community-acquired pneumonia by *K. pneumoniae* over the past two decades, yet*K. pneumoniae* is considered an important cause of severe community-acquired pneumonia in Asia and Africa (Ko et al., 2002 and Yu et al., 2007). *K. pneumoniae* that is responsible for nosocomial epidemics usually produce extended-spectrum β-lactamase(s) and exhibit multiple-antibiotic resistance profiles (Champs et al., 1989 and Robin et al., 2012). For several decades, *Klebsiella* species have been studied from a clinical perspective as opportunistic pathogens. There have been only a few cases where *Klebsiella* spp. were implicated as enteroinvasive foodborne pathogens (Sabota et al., 1998). Nonetheless, *Klebsiella* spp. should be dealt with as commensal bacteria in foods since commensal bacteria may play a crucial role in the evolution and transmission of antibiotic resistance. Researchers have investigated the prevalence of antibiotic-resistant bacteria on fresh produce and have shown that *Klebsiella* spp. were some of the predominant commensal bacteria associated with agricultural products and were multiple-antibiotic-resistant. Patterson and Woodburn (1980) investigated bacterial flora

on alfalfa and bean sprouts at the retail level and reported that there were 5.4E+06 and 8.6E+06 cells/g of *Klebsiella* on alfalfa and bean sprouts, respectively. Boehme et al. (2004) screened 20 vegetables from German markets for antibiotic-resistant coliform bacteria and enterococci: 9 of 92 isolates recovered were *Klebsiella* spp., having more than five multiple-antibiotic-resistant phenotypes. For example, resistance to ampicillin, cefoxitin, tetracycline, streptomycin, and chloramphenicol was observed. More recently, multiple-antibiotic-resistant *Klebsiella* spp. were isolated from alfalfa sprouts (Loui et al., 2008). A study on the occurrence of enteric bacteria in raw vegetables was conducted in Saudi Arabia (Hassan et al., 2011). In that study, *Klebsiella* spp. were recovered from 5.9% of samples, and a *K. pneumoniae* isolate recovered from tomatoes exhibited resistance to ampicillin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole and cephalothin.

2.5.β-lactamases and extended-spectrum β-lactamases (ESBLs)

Antibiotics work by targeting specific sites or functions of a bacterium. β-lactam and glycopeptide antibiotics interrupt bacterial cell wall synthesis, whereas other antibiotics act on cell membranes (polymyxins), protein synthesis (aminoglycosides, tetracyclines, and macrolides phenicols), RNA (rifamycins), or DNA (fluoroquinolones and trimethoprim–sulfamethoxazole (Kohanski et al., 2010). β-lactam antibiotics contain a β-lactam ring in their chemical structure, and are the primary choice of treatment for infections caused by Gram-negative bacteria (Sandegren et al., 2012). Enzymatic degradation via hydrolysis of the β-lactam ring is the primary mechanism of resistance to β-lactam antibiotics (Massova and Mobashery, 1998). β-lactamases catalyze the hydrolysis of the β-lactam ring by splitting the amide bond, thus disrupting the chemical

structure of the antibiotic and preventing inhibition of bacterial cell wall synthesis and cell lysis (Williams, 1999). β-lactamases can be present as chromosome-encoded enzyme(s) or plasmid-encoded enzyme(s) (Livermore, 1995). β-lactamases have been classified by their hydrolytic spectrum, susceptibility to inhibitors, or whether they are chromosome- or plasmid-encoded (Livermore, 1995). Bush and Syke (1983) proposed a classification of β-lactamases by their substrate preference. Ambler developed an alternative scheme; a sequence-based classification that is recognized as a simple grouping, and designated A to D (Ambler, 1980 and Waley, 1992). Ambler Class A enzymes, including the SHV and TEM β-lactamases, are usually plasmid-mediated enzymes and are prevalent among Gram-negative bacteria. These enzymes hydrolyze penicillins and some cephalosporins, but are unable to hydrolyze extended-spectrum cephalosporins such as ceftazidime and azteronam (Bush at al., 1995). Ambler Class B enzymes are metallo-β-lactamases and require zinc ions for their activities. Class B β-lactamases can hydrolyze carbapenems, cephalosporins, and penicillins (Walsh et al., 2005). Ambler Class C enzymes are known as AmpC-β-lactamases. They tend to be encoded on the chromosome although several plasmid-borne classes have been observed (Hanson, 2003). These enzymes are widely distributed among *Enterobacteriaceae* and mediate resistance to cephalosporins, oxyminocephalosporins, and aztreonam (Jacoby, 2009). Ambler Class D enzymes are located both on plasmids and on chromosomes of a wide range of Gram-negative bacteria, and they are able to hydrolyze oxacillin and penicillin. These enzymes are relatively resistant to clavulanic acid inactivation (Nass et al., 1998).

Extended-spectrum β-lactamases (ESBLs) are plasmid-mediated and mainly

derived from Ambler Class A enzymes such as TEM or SHV (Sirot, 1995). Recently, CTX-M β-lactamases have been recognized as a newly emerging group of ESBLs. In the early 1980s, third-generation cephalosporins were used to control β-lactamase(s)-producing bacteria in clinical practice; however, by 1983, the first report of plasmid-encoded β-lactamases, which were able to hydrolyze the extended-spectrum cephalosporins, appeared (Knothe et al., 1983). The gene encoding the new β-lactamase showed a mutation of a single nucleotide compared to the gene encoding SHV1, Ambler Class A β-lactamases. ESBL enzymes were initially reported in *K. pneumoniae* and *Serratia marcescens* strains in 1983 in Europe and in *K. pneumoniae* and *Escherichia coli* strains in 1989 in the United States (Knothe et al., 1983, Quinn et al., 1989, and Gupta et al., 2003). Historically, *Klebsiella* spp. or *E. coli* producing either TEM or SHV type enzymes were the predominant ESBL-producing bacteria in the United States (Lewis et al., 2007). These new β-lactamases, ESBLs, conferred resistance to penicillin, first-, second-, and third-generation cephalosporins, such as ceftriaxone, cefotaxime, ceftazidime, and aztreonam, but were not active against carbapenems (Paterson and Bonomo, 2005). The plasmids encoding ESBLs are also able to carry other antibiotic resistance genes, including aminoglycosides, chloramphenicols, trimethoprim, and tetracyclines, showing properties of multiple-antibiotic-resistance (Jacoby and Sutton, 1991). Since the genes are encoded on plasmids they can be readily transferred between bacterial species. Since ESBL was first identified, ESBL-producing *Enterobacteriaceae* have rapidly disseminated encompassing a larger geographic regions and are now globally recognized together with MRSA and VRSA as major contributors to the serious antibiotic resistance problem (Gniadkowski, 2001). To detect ESBLs phenotypically, the

US Clinical and Laboratory Standards Institute (CLSI) has published guidelines for *Enterobacteriaceae* especially for *E. coli, Klebsiella* spp., and *Proteus* spp. (National Committee for Clinical Laboratory Standards, 2012). The principle of this guideline is that most ESBLs are able to hydrolyze third-generation cephalosporins, although they are inhibited by clavulanic acid. An initial screening for *K. pneumoniae* and *E. coli* can be performed with 4 μg/ml of cefpodoxime or 1 μg/ml each of cefotaxime, ceftazidime, ceftriaxone, or aztreonam, followed by phenotypic confirmatory tests using cefotaxime and ceftazidime, in combination with 4 μg/ml of clavulanic acid (National Committee for Clinical Laboratory Standards, 2012). For the genotypic detection, molecular methods can be used to amplify ESBL-encoding genes, such as bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ that can then be sequenced, serving as the gold standard method (Pitout and Laupland, 2008).

2.6.Carbapenemase(s)

Carbapenems (imipenem, ertapenem, and meropenem) are commonly recommended to treat infections caused by ESBLs-producing *Enterobacteriaceae* and multiple-antibiotic-resistant Gram-positive bacteria (Nordmann et al., 2009 and Guerra et al., 2014). The detection of carbapenem-resistant *Enterobacteriaceae* (CRE) was infrequent in the United States before 1992 (Gupta et al., 2011). In the past 10 years, however, increasing resistance of *Enterobacteriaceae* to carbapenems has been reported worldwide (Zhanel et al., 2007). Resistance to carbapenems is mediated by carbapenemase enzymes and modification of the outer membrane permeability and the cell efflux system. So far, nine families (KPC, SME, NMC-A, IMI, PER, GES, SFO, SFC, IBC) in Ambler Class A, six families (VIM, GIM, SIM, NDM, IMP, SPM) in Ambler Class B, and two families (OXA, PSE) in Ambler Class D have been identified

(Stuart et al., 2010). Among them, *K. pneumoniae* carbapenemase (KPC; Ambler Class A), verona imipenemase (VIM; Ambler Class B), imipenemase (IMP; Ambler Class B) and oxacillinases (OXA; Ambler Class D) have been identified more frequently in *Enterobacteriaceae* than others (Nordmann et al., 2009). The KPC enzymes are mostly encoded on plasmids in *K. pneumoniae* and recognized as the most frequent Class A carbapenemases. Since KPC-producing *K. pneumoniae* was firstly identified in North Carolina in 1996, the spread of KPC-producing *Enterobacteriaceae* has been rapid (Nordmann et al., 2011). The spread of CRE into both community and health care settings underscores the limited therapeutic options to combat CRE (Gupta et al., 2011). The detection of resistance to carbapenems cannot be determined with a simple test due to the heterogeneity of carbapenemases. Carbapenemase(s) can be detected by genotypic and/or phenotypic methods, including the MIC test and/or the modified Hodge test with meropenem, imipenem and ertapenem and detection and sequencing of carbapenemase-encoding gene(s), according to the guidelines of CLSI (Stuart et al., 2010). Prevalence studies on carbapenemase-producing bacteria in non-clinical settings remain low, and the source of bacteria or colonization with carbapenemase-producers has not yet occurred (Woodford et al., 2014 and Guerra et al., 2014). Table 2.1 shows the list of acquired carbapenemases in bacteria from non-human sources (Woodford et al., 2014). KPC-, NDM-, and OXA-48-encoding genes are mostly encoded on transferable plasmids, so these carbapenemase-encoding genes are easily transferred between different *Enterobacteriaceae* species (Walsh, 2010). For example, KPC is located on variably sized plasmids with other resistance gene(s), conferring multiple-antibiotic-resistance (Quale, 2008). Seiffert et al. (2014) screened 30 pet foods for ESBL and carbapenemase-

encoding genes and found *bla*$_{CTX\text{-}M\text{-}15}$, one of the ESBL-encoding genes, in 53.5% of samples and the *bla*$_{OXA\text{-}48\text{-}like}$ carbapenemase-encoding gene in 13.3% of collected samples.

Table 2.1. Acquired carbapenemases (by order of first report) in bacteria from non-human sources or in the zoonotic species *S. enterica. Adopted from Woodford et al., 2013.*

Enzyme	Species	Source	Country	Year
KPC (class A)	*S. enterica subsp. enterica serovar* Cubana	human(feces)	USA	2003
	Multiple genera	hospital sewage, effluent	Brazil	2011, 2013
	E. coli	river	Portugal	2012
	Citrobacter freundii, Enterobacter cloacae	hospital sewage	China	2012
IMI (class A)	*Enterobacter asburiae*	river	USA	2005
	Pseudomonas pseudoalcaligenes, Pseudomonas aeruginosa	river, sewage	Portugal	2005, 2006
VIM (class B)	PCR amplicons only	sewage, effluent	Germany	2009
	Salmonella Kentucky	human (feces)	Morocco	2010
	E. coli, Salmonella Infantis	pig and poultry farm, flies, mice, manure	Germany	2012, 2013
	K. pneumoniae, Helicobacter pylori	river	Tunisia	2013
	K. pneumoniae	river	Switzerland	2013
IMP (class B)	PCR amplicons only	sewage, effluent	Germany	2009
	K. pneumoniae	river	Tunisia	2013
OXA-48 (class D)	PCR amplicons only	sewage, effluent	Germany	2009
	Salmonella Kentucky and Saintpaul	human (faeces)	Egypt	2010
	E. coli, K. pneumoniae	dogs	Germany	2013
NDM (class B)	Multiple genera	water	India	2011
	K. pneumoniae	river	Vietnam	2012
	Acinetobacter lwoffii	poultry	China	2012
	A. baumannii	pig	China	2013
	E. coli	dogs/cat	USA	2013
	Salmonella Senftenber	human (perirectal screen)	USA/India	2012
	Salmonella Westhampton	human (urine, faeces)	Reunion Island/India	2012
	Salmonella Corvallis	black kite (wild raptor)	Germany	2013
	A. baumannii	water, hospital sewage	China	2013
OXA-23 (class D)	*Acinetobacter* 15TU	dairy cows	France	2012
	Acinetobacter spp.	horses	Belgium	2012
BIC-1 (class A)	*Pseudomonas fluorescens*	river	France	2012

2.7.Vehicles of antibiotic resistance; focusing on ESBL-producers and ESBL-encoding genes

As shown in Figure 2.4, reservoirs of antibiotic resistance are connected in various ways (Witte, 2000). More specifically, administering antibiotics to animals for treatment of infections that may precipitate in the development of antibiotic-resistant bacteria (Figure 2.5). Animal feces containing antibiotic-resistant bacteria may subsequently enter the environment. Consequently, humans are exposed to antibiotic-resistant bacteria from agricultural products, including crops and meat. On the other hand, in clinical or community settings such as hospitals or nursing homes, foods prepared in food service facilities located in those operations can serve as a vehicle for the transfer of antibiotic resistance genes between clinical isolates originating from patients or residents in the facility and food commensal bacteria (Kluytmans et al., 1995 and Jones et al., 2002). Additionally, patients or healthcare providers may spread antibiotic-resistant bacteria in healthcare facilities or into the community (Figure 2.5). Calbo et al. (2011) reported a foodborne nosocomial outbreak of SHV1 on March 2008, and CTX-M-15-producing *K. pneumoniae* where 165 colonized and/or infected patients were identified. Based on a phenotypic and genotypic analysis of all clinical, environmental, and fecal carrier isolates, the investigation revealed that the outbreak was caused by a single strain of SHV1 and CTX-M-15-producing *K. pneumoniae* from handmade fruit puree. This was the first outbreak by ESBL-producing *K. pneumoniae* via food, providing evidence that food can be a vehicle of the dissemination of ESBL-encoding gene(s) in a clinical setting. Since the rapid global dissemination of Gram-negative bacteria harboring ESBL-encoding gene(s), the transfer of ESBL-encoding

genes between different genera have also been documented. Schjorring et al. (2008) investigated the horizontal spread of the *bla*$_{SHV}$ and *bla*$_{\text{TEM}}$ in the mouse intestine and they observed the transfer of ESBL-encoding genes from *K. pneumoniae* to *E. coli*. One study also showed the horizontal transfer of ESBL-encoding genes between *Salmonella* and *K. pneumoniae* (Sarowska et al., 2009). In this research, after conjugation, plasmids encoding ESBL harbored by *K. pneumoniae* were successfully transferred to three recipients, *Salmonella* Enteritidis, *S.* Typhimurium, and *S.* Hadar.

Since antibiotics are directly used for growth promotion and the prevention of diseases in food-producing animals, a number of published reports have focused on commercial farms, feedlots, processing plants, or packing plants in animal husbandry as sources of antibiotic-resistant bacteria (Aslam and Service, 2006, Edrington et al, 2009, Aslam et al., 2009, Jouini et al., 2009, and Santos et al., 2007). However, raw foods such as raw milk and vegetables, consumed without thermal processing, may contain bacteria harboring antibiotic resistance genes, increasing the potential for the transfer of those genes among indigenous and pathogenic bacteria (Schjorring and Krogfelt, 2011). In general, a dairy cow with mastitis or an unhygienic environment of milk production (e.g., milking parlor) can be possible sources of bacterial contamination of milk (Carter, 1995). Li et al. (2011) reported antibiotic-resistant bacteria were not detected in properly pasteurized liquid milk. Conversely, Manuzon et al. (2007) reported a high prevalence of antibiotic-resistant bacteria in retail cheeses made from pasteurized milk. Gundogan and Yakar (2006) investigated the prevalence in milk and milk products of ESBL-producing *Klebsiella* spp., their virulence factors and antibiotic resistance. *Klebsiella* isolates were recovered from 53.8% of 80 raw milk, pasteurized milk, ice cream and white cheese

samples. Approximately 35% of the isolates were ESBL-producing *Klebsiella* spp. and their resistance to the range of antibiotics evaluated was greater than non-ESBL-producing isolates from those products. Locatelli et al. (2010) isolated 140 *Klebsiella* spp. from milk samples obtained from bovine clinical mastitis and determined that 9 of the *K. pneumoniae* isolates produced ESBL enzymes, SHV, TEM, and/or CTX-M. Moreover, Straley et al. (2006) found that bulk tank milk can be an important source of antibiotic-resistant Gram-negative bacteria. They pointed out that some people consume raw milk and raw milk products that may contain antibiotic-resistant bacteriaare exposed to a potential public health risk (Straley et al., 2006 and Jayarao and Wang, 1999).

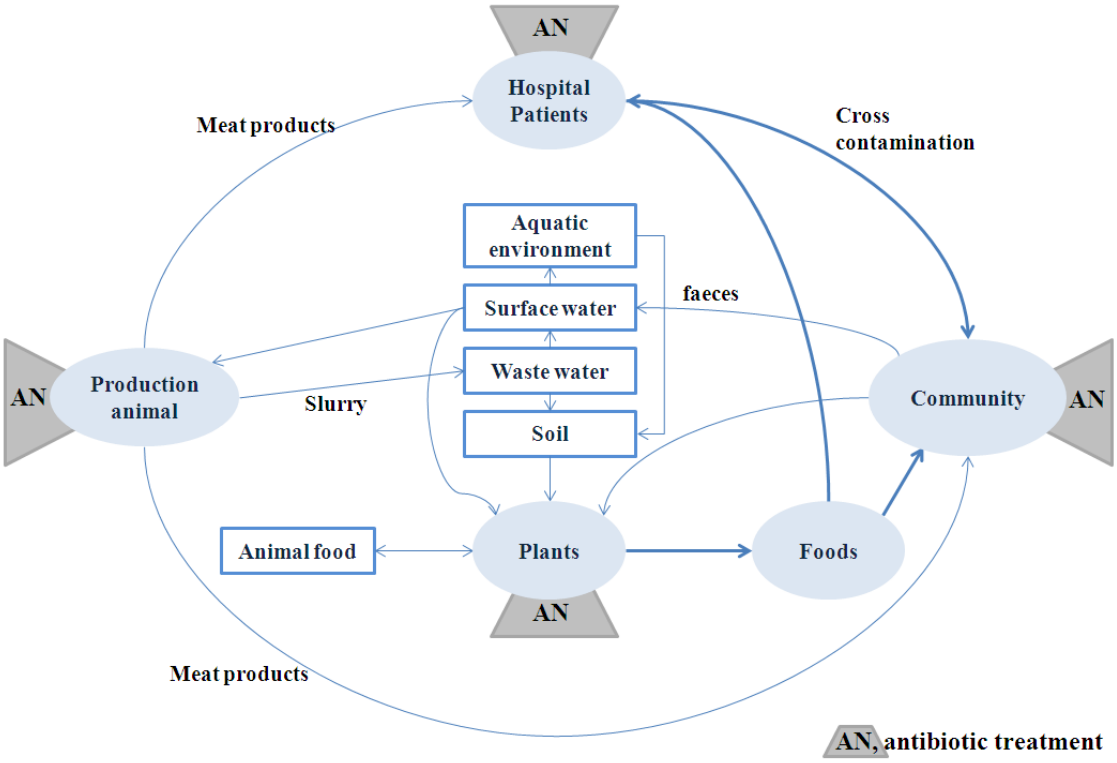


Figure 2.4. Possible routes of dissemination of antibiotic resistance, *adopted from, Witte, 2000.*

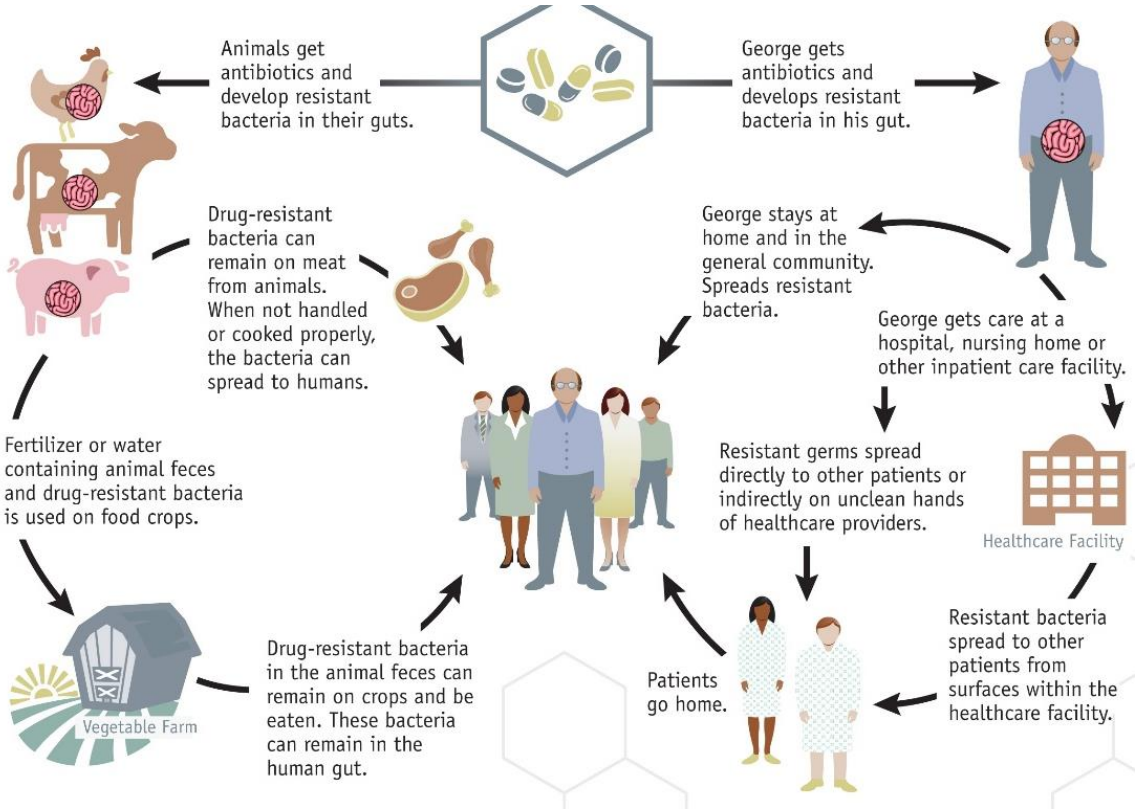


Figure 2.5. Examples of how antibiotic resistance spreads, *adapted from CDC, 2013.*

2.8.What factors may affect the transfer of antibiotic resistance gene?

Food may serve as a medium for the dissemination of antibiotic resistance genes between clinical and food origin bacteria in clinical settings. Although antibiotic resistance and virulence factors share characteristics involved in adaptive mechanisms, the connection between virulence factors and antibiotic resistance gene transfer remains unclear (Martinez and Baquero, 2002). Arias et al. (2009) recently reported that a large transferable plasmid containing the hyl_{EFM} gene (encoding a putative hyaluronidase) could be co-transferred with antibiotic resistance genes. Some virulence genes encoding aerobactin, the ferric aerobactin receptor, and adhesive factors in *K. pneumoniae* were also found on R-plasmids (Darfeuille-Michaud et al., 1992). Carlson et al. (2007) reported the co-localization of virulence and antibiotic resistance genes on the same mobile genetic elements, suggesting enhanced virulence due to the co-transfer of genes encoding antibiotic resistance and virulence factors. However, there is a lack of research on whether virulence factors affect antibiotic resistance gene transfer or vice versa. In other words, although many of virulence factors have been characterized in terms of their role in clinical pathology, their functions in the horizontal transfer of mobile antibiotic resistance elements of *Klebsiella* spp. are largely unknown.

A number of studies have reported the prevalence of antibiotic-resistant bacteria in raw foods such as fresh vegetables and raw milk. However, limited research has been focused on the transfer of antibiotic resistance genes in actual raw foods. For example, considering commensal bacteria associated with fresh produce are exposed to various stresses including antimicrobial agents (e.g., sanitizers) during food production, it is warranted to delineate the characteristics of antibiotic resistance gene transfer depending

on the sub-lethal stresses that donors and recipients may face under actual production and processing conditions. Research suggests that exposure to chlorine in a food-processing plant can confer cross-resistance to antibiotics via induction of the multiple-antibiotic-resistant operon (Potenski et al., 2003). However, effects of sub-lethal stresses on antibiotic resistance gene transfer of bacteria in foods are fundamentally unknown. Environmental stresses, such as pH extremes, anaerobiosis, heat shock, osmotic shock and starvation have been reported to induce the development of antibiotic resistance by genomic reorganizations or mutations in bacterial cells (McMahon et al., 2007a). Bacterial conjugation is considered to be an active process and thus regulated by the physiological state of recipient cells and donor cells. McMahons et al. (2007b) reported that sub-lethal stresses (high/low temperature, osmotic, and pH stresses) increased the rate of horizontal transmission of antibiotic resistance plasmids between *E. coli* strains and between *E. coli* and *S.* Typhimurium. Muela et al. (1994) observed that starvation stress affected the ability of donor cells to transfer plasmids, but had no effect on recipient cells. Furthermore, Frischer et al. (1993) showed that the natural plasmid transformation rate of *Vibrio* was reduced at 37˚C, compared to 4˚C, suggesting that temperature and physiology of the donor and the recipient may affect the transfer of genetic material during horizontal gene transfer. On the other hand, lack of resistance gene transfer at 4˚C in *E. coli* and *Salmonella* may be associated with low metabolic rates of the donor and the recipient (Walsh et al., 2008). Interestingly, Christensen et al. (2011) investigated the secondary transfer of *vanA* gene in *Enterococcus*; demonstrating an increase in the transfer efficiency resulted from secondary conjugation.

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Chapter 3. Transfer of extended-spectrum β-lactamase-encoding gene, *bla*$_{SHV18}$ gene, between *Klebsiella pneumoniae* in temperature abused raw foods

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3.1. Abstract

This study investigated the transfer frequency of the extended-spectrum β-lactamase-encoding gene (*bla*$_{SHV18}$) among *Klebsiella pneumoniae* in tryptic soy broth (TSB), pasteurized milk, unpasteurized milk, alfalfa sprouts and chopped lettuce at defined temperatures. All transconjugants were characterized phenotypically and genotypically. KP04 and KP08 were isolated from seed sprouts and used as recipients in mating experiments with *K. pneumoniae* ATCC 700603 serving as the donor. In mating experiments, no transconjugants were detected at 4˚C in liquid media or chopped lettuce but were detected in all media tested at 15˚C, 24˚C, and 37˚C. At 24˚C, the transfer of *bla*$_{shv18}$ gene occurred more frequently in alfalfa sprouts (5.15E-04 transconjugants per recipient) and chopped lettuce (3.85E-05) than in liquid media (1.08E-05). On chopped lettuce, transconjugants were not detected at day 1 post-mating at 15˚C, but observed on day 2 (1.43E-05). Transconjugants carried the *bla*$_{SHV18}$ gene transferred from the donor and virulence gene(s) harbored by the recipient. Importantly, a class 1 integrase gene and resistance to tetracycline, trimethoprim/sulfamethoxazole were co-transferred during mating. These quantitative results suggest that fresh produce exposed to temperature abuse may serve as a competent vehicle for the spread of genes encoding for antibiotic

resistance, having a potential negative impact on human health.

3.2. Introduction

Annually, more than two million people in the United States suffer from illnesses caused by antibiotic-resistant bacteria (CDC, 2013). To combat antibiotic-resistant bacteria, studies have been conducted to elucidate mechanisms of antibiotic resistance and transfer of genes that encode for antibiotic resistance. Research indicates that foods may mediate the transfer of antibiotic resistance, serving as a route connecting the environment and humans (Witte, 2000 and Rossi et al., 2014). Moreover, commensal bacteria harboring antibiotic resistance genes in food may play a role in the dissemination of antibiotic resistance (Wang et al., 2006 and Marshall et al., 2009). Since antibiotics are directly used for growth promotion and the prevention of diseases in food-producing animals and crops, considerable attention has focused on the prevalence of antibiotic-resistant bacteria associated with food-producing animals and their environment, including commercial farms, feedlots, processing plants, and packing plants (Aslam and Service, 2006, Santos et al., 2007, Aslam et al., 2009, Edrington et al, 2009, and Jouini et al., 2009). Raw foods such as fresh produce often harbor high populations of antibiotic-resistant bacteria, serving as a reservoir of resistance genes that can potentially be transferred to bacteria of animal, human or environmental origin (Boehme et al., 2004 and Falomir et al., 2010). Most people consume fresh produce without applying a process (e.g., cooking) to inactivate bacteria before consumption; this may facilitate the transfer of antibiotic resistance gene(s) in the human gastrointestinal tract (Macovei and Zurek, 2007 and Schjorring and krogfelt, 2011). The literature is rich with papers on the prevalence of antibiotic-resistant bacteria associated with fresh produce, but there are few

studies on the transfer of antibiotic resistance genes among bacteria on fresh produce or to bacteria in the human intestine (Ruimy et al., 2010, Hassan et al., 2011, Walia et al., 2013, and Blaak et al., 2014).

Resistance to third-generation cephalosporins in *Enterobacteriaceae* is one of the major health concerns in human beings. The production of extended-spectrum β-lactamases (ESBLs) and/or AmpC β-lactamases confers resistance to almost all β-lactam antibiotics including third-generation cephalosporins, but not to carbapenem and cephamycins (Pitout and Laupland, 2008 and Blaak et al., 2014). While AmpC β-lactamases are chromosomal- or plasmid-mediated enzymes, ESBL-encoding genes are typically located on plasmids (Rawat and Nair, 2010). Due to the mobile capability of plasmids, ESBL-producing *Enterobacteriaceae* have rapidly disseminated, encompassing larger geographic regions. ESBL-producing *Enterobacteriaceae* are globally recognized as one of the major contributors of the serious antibiotic resistance problem together with methicillin-resistant staphylococci and vancomycin-resistant enterococci (Gniadkowski, 2001). Among the ESBL-producing *Enterobacteriaceae*, *Klebsiella* spp. or *E. coli* producing either TEM, SHV, CTX-M type enzymes are distributed worldwide and are found in clinical and non-clinical settings (Lewis et al., 2007). In connection with the emerging issue in clinical settings, recent research has focused on the prevalence of ESBL-producing *Enterobacteriaceae* and/or ESBL-encoding genes in fresh produce (Blaak et al., 2014, Reuland et al., 2014, Kim et al., 2015, and van Hoek et al., 2015). However, there is a paucity of research on the transfer of ESBL-encoding gene(s) by *Enterobacteriaceae* in agricultural foodstuffs.

The genus *K. pneumoniae*, a member of the *Enterobacteriaceae* family, is

prevalent in nature and is an important opportunistic pathogen that can cause nosocomial infections (Brown and Seidler, 1973, Podschun and Ullmann, 1998, Gupta et al., 2003, and Gundogan and Yakar, 2007). Researchers have shown that *Klebsiella* spp. are one of the predominant commensal bacteria associated with fresh produce. Österblad et al. (1999) found that *Klebsiella* spp. were second to *Enterobacter* spp. among 535 different isolates found in vegetables in Finland. Twenty vegetables collected from German markets were screened to determine the prevalence of antibiotic-resistant coliform bacteria and enterococci; 9 of 92 isolates recovered were *Klebsiella* spp., having more than five multiple-antibiotic-resistant phenotypes (Boehme et al., 2004). For example, resistance to ampicillin, cefoxitin, tetracycline, streptomycin, and chloramphenicol was exhibited. Populations of 5.4E+06 and 8.6E+06 cells/g of *Klebsiella* were observed in alfalfa and bean sprouts, respectively (Patterson and Woodburn, 1980). Approximately, 10% of 189 samples of ready-to-eat vegetables collected in South Korea harbored ESBL-producing *E. coli* or *K. pneumoniae* (Kim et al., 2015). A study in Saudi Arabia was conducted to determine the occurrence of enteric bacteria in raw vegetables (Hassan et al., 2011). *Klebsiella* spp. were isolated from 5.9% of samples, and a *K. pneumoniae* isolated from tomatoes exhibited resistance to ampicillin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole and cephalothin. The phylogenetic relatedness of *K. pneumoniae* isolated from meat and human clinical isolates has been reported (Davis et al., 2015). Kim and his colleagues (2015) showed that ESBL-producing *E. coli* and *K. pneumoniae* isolated from ready-to-eat vegetables exhibited similar genotypic and phenotypic patterns to clinical and other food isolates.

Given the numerous studies demonstrating antibiotic resistance genes in foods

such as fresh produce and animal products, it is clear that food matrices may serve as avenues of dissemination of antibiotic resistance genes. Predicting the transfer rate of antibiotic resistance genes in different food matrices may contribute to a better understanding of the dissemination of antibiotic resistance. Walsh et al. (2008) investigated the transfer rates of ampicillin resistance from *Salmonella* Typhimurium DT104 to *E. coli* K12 in different food matrices, showing a higher transfer rate in ground beef than in a laboratory nutrient broth and milk. Wright (2010) also emphasized that quantitative studies on horizontal gene transfer rate and factors that affect horizontal gene transfer would be needed to predict the emergence and spread of antibiotic resistance. A wealth of studies has been conducted in microbiological media in an attempt to understand the transfer of antibiotic resistance genes. Typically, a model organism such as *E. coli* K12 or *E. coli* DH5α serves as a recipient to overcome the limitations of identifying transconjugants following mating (Mc Mahon et al., 2007, Walsh et al., 2008, and Schjorring and Krogfelt, 2011). Unfortunately, such studies may fail to reflect "real" conditions where the transfer of antibiotic resistance gene(s) occurs.

In the present study, non-ESBL-producing *K. pneumoniae* isolated from seed sprouts were used as recipients to investigate the transfer of an ESBL-encoding gene in different food matrices and at various temperatures. The molecular and phenotypic characteristics of transconjugants were determined.

3.3. Materials and methods

3.3.1. Seed sprouts and its microbiological analysis

Different brands and varieties of seed sprouts packed in clearclam shell packages were purchased from local supermarkets in Northern and Central New Jersey during

Spring (March to May) and Fall (October to November) months. All experiments were performed within the sell-by-dates of a given package. A total of twenty samples were collected, including alfalfa (n=8), broccoli (n=3), radish (n=2), clover (n=1), and mixed sprouts (n=6). For microbiological analysis, a 25 g of sample sprouts was weighed into a sterile filter bag, diluted with 75 ml 0.1% sterile peptone water (SPW: Difco, Becton Dickinson, Sparks, MD) and homogenized for 5 min using a stomacher lab blender 400 (Dynatech laboratories, Alexandria, VA). A 100 µl aliquot of the homogenized sample was serial diluted (1:10), and a 100 µl aliquot was spread on tryptic soy agar (TSA: Difco, Becton Dickinson, Sparks, MD) plates. To determine the viable population of bacteria resistant to ampicillin and ceftazidime in seed sprouts; 32 µg/ml of ampicillin (Sigma-Aldrich, St. Louis MO) and 16 µg/ml of ceftazidime (hydrate, Sigma-Aldrich, St. Louis MO) were added to the TSA plates. Plates were incubated at 37°C for up to 24 h, and colonies were enumerated.

3.3.2. Isolation and Identification of *Klebsiella pneumoniae*

A homogenized sprout sample (100 µl aliquot), prepared as described, was spread on MacConkey agar plates (Difco, Becton Dickinson, Sparks, MD). A pink to red colony was picked from MacConkey agar to confirm a single strain. Additionally, oxidase and motility tests were performed, as *K. pneumoniae* is non-motility and oxidase negative. Confirmation was performed using the API® 20E strip (bioMerieux, Basingstoke, UK). Confirmed *K. pneumoniae* isolates were additionally screened for the presence of an internal transcribed spacer (ITS) region using a polymerase chain reaction (PCR). *K. pneumoniae* specific primers (ITS Pf, 5′-ATTTGAGAGGTTGCAAACGAT-3′ and ITS Pr1, 5′ -TTCACTCTGAAGTTTTCT TGTGTTC-3′) were used (Liu et al., 2008). Two *K.*

pneumoniae strains, ATCC 13882 and ATCC 33495 were used as control strains. Stocks of identified *K. pneumoniae* were made and stored at -80°C to be used in mating experiments as recipients.

3.3.3. Antimicrobial susceptibility test

Antimicrobial susceptibility tests were performed by the Kirby-Bauer disk diffusion method or the micro-broth dilution method according to the US Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute (CLSI), 2012). Antibiotic disks (Sensi-Disc, Becton, Sparks, MD) for the Kirby-Bauer disk diffusion test were placed on cation-adjusted Mueller-Hinton Agar (Difco, Becton Dickinson, Sparks, MD). Antibiotic disks included gentamicin (GEN, 10 µg), streptomycin (STR, 10 µg), imipenem (IMP, 10 µg), tetracycline (TET, 30 µg), nalidixic acid (Na, 30 µg), amoxillin/clavulanic acid (AMC, 20 µg/10 µg), ampicillin/sulbactam (SAM, 10 µg/10 µg), cefepime (FEP, 30 µg), cefotaxime (CTX, 30 µg), trimethoprim/sulfamethoxazole (SXT, 1.25 µg/23.75 µg), and chloramphenicol (C, 30 µg). Resistance to kanamycin, ciprofloxacin, ampicillin, and ceftazidime was determined by the micro-broth dilution method. The *K. pneumoniae* strain ATCC 700603 was used as a reference strain. To determine whether isolates were ESBL-producers, phenotypic confirmation was performed based on the US CLSI standard method (Clinical and Laboratory Standards Institute, 2010). Briefly, initial screening was conducted by a disk diffusion method with 30 µg of cefotaxime and ceftazidime, followed by phenotypic confirmatory tests using both cefotaxime and ceftazidime, in combination with 10 µg/ml of clavulanic acid.

3.3.4. PCR protocol

The amplification of the ESBL-encoding gene (*bla*$_{SHV18}$) and four virulence genes associated with *K. pneumoniae* was performed according to the manufacture's instruction (GoTaq® Flexi, Promega, Madison, WI). Crude genomic DNA was prepared by either using a boiling method or picking an isolated colony directly from a plate for use in PCR cocktail with selected primer sets (Table 3.1). The total volume of the PCR reaction mixture was 50 µl including 10 µl of 5X green Go taq Flexi buffer, 4 µl of 25 mM $MgCl_2$, 4 µl of 10 mM dNTPs, 5 µl (each) of primer, and 0.25 µl Go taq Flexi DNA polymerase (5 U/µl). PCR reactions were performed in a Perkin-Elmer GeneAmp PCR system 2400 (Perkin-Elmer Corp., Foster City, CA) for 1 cycle of 2 min at 95°C (initial denaturation); followed by 30 cycles of 30 sec at 95°C (denaturation), 45 sec at 60°C (annealing) and 1 min/kb at 72°C (extension); followed by 1 cycle of 5 min at 72°C (final extension). Amplified products were separated on 1% agarose gels (IBI Scientific, Peosta, IA) by electrophoresis, and visualized following ethidium bromide staining (Bio-Rad Laboratories Hercules, CA).

3.3.5. *In vitro* and *In vivo* mating experiments

3.3.5.1. Donor and recipients

K. pneumoniae ATCC 700603 harboring the *bla*$_{SHV18}$ gene, one of the characterized ESBL-encoding genes, was used as a donor strain. Recipients included *K. pneumoniae* 342 (KP342) and two *K. pneumoniae* isolates from seed sprouts. KP342 was originally isolated from maize and is known to colonize subsurface regions of alfalfa sprout seedlings (Dong et al., 2003 and Fouts et al., 2008). *K. pneumoniae* isolated from

seed sprouts were spontaneously mutated for resistance to kanamycin (step-up exposure from 2 µg/ml to128 µg/ml) to facilitate differentiation from the donor. Table 3.3 shows the antibiotic resistance profile and genotypic characteristics of both the donor and the recipients.

3.3.5.2. Mating procedure

In vitro mating was done in triplicate, according to a slightly modified version of the procedure by Willetts (1988). Briefly, overnight cultures of the donor and recipient cells grown in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) were harvested, and mixed as a ratio of 1:1. A 100 µl aliquot of the mixed preparation was dispensed into 5 ml of fresh TSB and incubated at 4°C, 15°C, 24°C (ambient room temperature), and 37°C without shaking. After 18-20 h, an aliquot of the mixed cultures was spread on TSA agar containing appropriate antibiotics. Antibiotic concentration was based on the profile of antibiotic susceptibility for selective recipients, 128 µg/ml of kanamycin or 100 µg/ml of streptomycin was added to the TSA. For transconjugants, 16 µg/ml of ceftazidime and 100 µg/ml of streptomycin or 128 µg/ml of kanamycin were added to the TSA.

Food matrices used in *in vivo* mating experiments to assess antibiotic resistance gene transfer included unpasteurized milk (UPM), pasteurized milk (PM), alfalfa sprouts (AS), and chopped lettuce. Pasteurized milk, alfalfa sprouts seeds, and heads of romaine lettuce were purchased from a local supermarket in Central New Jersey. Half-gallon bottles of unpasteurized milk were purchased from a local food cooperative in Pennsylvania. For mating in UPM and PM the method was based on mating in TSB. The procedure by Thompson et al. (1999) was followed for the mating experiments in alfalfa

sprouts. Five grams of seeds (The Sprout House Certified Organic Sprouting Seeds, Lake Katrine, NY) were inoculated with 5 ml of the donor and recipient overnight cultures and then held at room temperature overnight. The inoculated seeds were placed in sterile mesh trays with cheesecloth and watered once a day. After 7 days, 10 g of sprouts were aseptically transferred into a sterile filter bag and homogenized with 90 ml of 0.1% SPW. A 100 µl aliquot of serial diluted sample was plated onto TSA containing appropriate antibiotics. For mating on chopped lettuce, outer leaves and the core were removed, and the remaining leaves were cut into 3 cm^2 pieces with a sterile knife. The chopped lettuce was then dipped into 2 L of sterile tap water containing approximately 10^8 CFU ml^{-1} mixture of donor and recipient for 1 min. Inoculated chopped lettuce was placed on a stainless screen to drain excessive inoculum and allowed to dry for 1 hour. Lettuce (25 g) was placed into a sterile sampling bag and stored at 4°C, 15°C, and 24°C. After 1 and 2 days, each lettuce sample was homogenized with 75 ml of 0.1% SPW and spread plated on TSA containing appropriate antibiotics. Each mating experiment for each food was conducted three separate times. Transfer frequency was calculated by enumeration of transconjugants and recipients on TSA (plus antibiotics) and expressed as a number of transconjugant per recipient. The mean transfer frequency was compared statistically (one-way analysis of variance, Duncan's post hoc analysis) using SAS software (SAS Institute Inc., USA).

3.3.5.3. Verification and characterization of transconjugants

Typically, three to five presumptive transconjugant and recipient colonies were randomly picked from TSA (containing antibiotics) and verified with PCR amplification of the *bla*$_{SHV18}$ gene, virulence determinants (*kfu* or *alls*), and the class 1 integrase gene

(*intl1*). Subsequently, randomly amplified polymorphic DNA (RAPD) typing was performed with randomly selected suspect colonies to differentiate donor, recipient, and transconjugant based on discriminatory banding patterns. RAPD PCR conditions included: 1 cycle of 3 min at 94°C (initial denaturation); followed by 40 cycles of 20 sec at 94°C (denaturation), 1 min at 36°C (annealing) and 1 min at 72°C (extension); followed by 1 cycle of 7 min at 72°C (final extension). Hi-RP (5′-AACTCGGCGACCAGCTACAA-3′) was used as a primer (Wong et al., 1994).

Antimicrobial susceptibility of the transconjugant was compared with the recipient and based on the CLSI method. Stability of the transferred bla_{SHV18} gene was evaluated by transferring a transconjugant colony into TSB (without antibiotics), incubating overnight, and then streaking for isolation on TSA. An isolated colony from the TSA plate was picked and used in PCR to amplify the bla_{SHV18} gene. This procedure was repeated up to three times to confirm the presence of transferred bla_{SHV18} gene.

3.4. Results

3.4.1. *Enterobacteriaceae* resistant to β-lactam antibiotics isolated from seed sprouts

Microbiological analysis of 20 different packages of retail seed sprouts, including broccoli, clover, and alfalfa sprouts, was conducted by plating on TSA and MacConkey agar plates. Figure 3.1 shows the total aerobic plate count of 20 seed sprout samples. Figure 3.1A shows the distribution of total bacteria associated with different retail seed sprout samples plated on TSA, TSA containing ampicillin (32 µg/ml), or TSA containing ceftazidime (16 µg/ml). On TSA, the average total bacterial population was 7.9 log CFU/g of seed sprouts. The average bacterial population on TSA plates containing

ampicillin or ceftazidime was 7.0 and 5.5 log CFU/g of seed sprouts, respectively. The average population of *Enterobacteriaceae* from seed sprouts on MacConkey agar (used to differentiate *Enterobacteriaceae*) containing no antibiotic, ampicillin, or ceftazidime was 7.4, 6.5, and 4.9, respectively (Figure 3.1B).

3.4.2. *K. pneumoniae* isolated from seed sprouts

A total of eight *K. pneumoniae* strains were isolated from 20 different seed sprouts samples and identified using an API® 20E system and by PCR specific for the ITS region. The eight isolates were identified as KP01 to KP08. Triplicate testing with 15 different antibiotics was performed to determine the antimicrobial susceptibility of each *K. pneumoniae* isolate by broth microdilution or disk diffusion methods. Table 3.2 shows the antibiotic susceptibility profile and associated virulence determinants for each isolate. All isolates were resistant to ampicillin and intermediately resistant to streptomycin, with four isolates intermediately resistant to nalidixic acid. Most isolates were susceptible to the remaining antibiotics tested. Based on screening and confirmatory tests for ESBLs, none of the 8 isolates were ESBL-producers. Six of eight isolates carried more than one of the four virulence genes targeted. The *rmpA* gene regulates mucoid phenotype (Nadasy et al., 2007), while the *wcaG* gene is involved in capsular fucose synthesis, which may increase the ability of the bacteria to escape phagocytosis (Turton et al., 2010). The *alls* gene is an activator of the allantoin regulon, and a marker of the K1 serotype and production of aerobactin (Yu et al., 2008), while the *kfu* gene is responsible for an iron uptake system (Yu et al., 2008). Virulence gene screening was performed to aid in the differentiation of isolates. Mating pairs were established based on the genotypic and antimicrobial susceptibility patterns of the recipient and donor. Among the 8 *K.*

pneumoniae isolates, KP04 and KP08, were spontaneously mutated for resistance to kanamycin and designated as KP04$^{\Delta KM}$ and KP08$^{\Delta KM}$. Table 3.3 shows the antimicrobial susceptibility and genotypic profiles of KP04$^{\Delta KM}$ and KP08$^{\Delta KM}$. Following induction of spontaneous resistance to kanamycin, KP04$^{\Delta KM}$ and KP08$^{\Delta KM}$, exhibited resistance to all aminoglycosisde antibiotics including gentamicin and streptomycin.

3.4.3. The transfer frequency of *bla$_{SHV18}$* gene in different food matrices

Mating experiments with donor *K. pneumoniae* ATCC 700603 and three recipients (KP04$^{\Delta KM}$, KP08$^{\Delta KM}$, and KP342) were performed in liquid media including TSB, pasteurized milk (PM), and unpasteurized milk (UPM) at 4˚C, 15˚C, 24˚C (room temperature) and 37˚C. In all liquid media, transconjugants were detected at 15˚C, 24˚C, and 37˚C, but not at 4˚C (Figure 3.2A). Because the transfer rates of *bla$_{SHV18}$* gene to the three recipients were not significantly different, the average transfer rate of all recipients was compared statistically to confirm the effect of temperature and medium on the transfer rate (Figure 3.2B). As a result, the mean transfer rate at 37˚C was significantly higher than at 15˚C and 24˚C in TSB (P<0.05). In PM and UPM, the transfer rate at 37˚C was significantly higher than at 15˚C, but not at 24˚C. The transfer frequency, however, was less affected by differences in the type of liquid media. Under all conditions, the highest transfer rate was 4.69E-05 at 37˚C in PM while the lowest rate was 8.23E-07 at 15˚C in TSB for mating experiments with recipient KP04$^{\Delta KM}$. Regardless of different recipients and media, the *bla$_{SHV18}$* gene of *K. pneumoniae* ATCC 700603 was transferred at an average frequency of 2.19E-06, 1.08E-05, and 3.07E-05 at 15˚C, 24˚C, and 37˚C, respectively.

Transfer frequency was compared among TSB, PM, UPM, and alfalfa sprouts

(AS) at 24°C. As shown in Figure 3.3, the transfer rate in AS was significantly higher than in TSB, UPM and PM at 24°C (P<0.05). Among the recipients, there was no significant difference (P>0.05) detected in the transfer rate. On day 7 post-inoculation of donor and recipient to alfalfa seeds, the average population of recipients (KP04$^{\Delta KM}$, KP08$^{\Delta KM}$, and KP342) and transconjugants (TC603KP04, TC603KP08, and TC603KP342) were 1.36E+08 and 7.09E+04 CFU/g of alfalfa sprouts, indicating a transfer rate of 5.15E-04 transconjugants per recipient.

Transfer of the *bla$_{SHV18}$* gene was observed on chopped lettuce. After dip inoculation of chopped lettuce in the donor and recipient cocktail (10^8 CFU ml^{-1}), the chopped lettuce was stored at 4°C, 15°C, and 24°C for 2 days. No transconjugants were detected at 4°C in any of the liquid media tested. The average transfer frequency among the 3 different recipients was not distinguishable in liquid media and alfalfa sprouts (Figure 3.4). At 15°C, no transconjugants were detected at day 1 post-mating. On day 2 at 15°C, transformation frequency averaged 1.43E-05 based on the transfer of the *bla$_{SHV18}$* gene. At room temperature, the average transfer rate of the *bla$_{SHV18}$* gene among recipients was 3.85E-05 and 1.44E-04 at day 1 and day 2 post-mating, respectively. Notably, the transfer rate on day 2 at 24°C was significantly higher than day 1 at 24°C and day 2 at 15°C (P<0.05).

3.4.4. Verification and characterization of transconjugants

In this study, a transconjugant is defined as a recipient acquiring the *bla$_{SHV18}$* gene from the donor and retaining the recipient's virulence determinants. PCR for detection of the *bla$_{SHV18}$* gene was performed on presumptive transconjugants followed by RAPD analysis to compare the donors, recipients, and transconjugants. As shown in Figure

3.5A, transconjugants, TC603KP4, TC603KP8 and TC603342 carried both the transferred *bla*$_{SHV18}$ gene and their own virulence gene(s). Based on RAPD analysis, each transconjugant and recipient exhibited a similar banding pattern that was distinct from the donor (Figure 3.5B). Furthermore, this study found that a Class 1 integrase gene (*intl1*) was transferred from the donor to the recipient during mating, demonstrating that transconjugants harbor the *intl1* gene (Figure 3.5C).

To characterize the transconjugants phenotypically, their antimicrobial susceptibility profiles were compared with the recipient and donor (Table 3.3). Resistance to nalidixic acid (NA) and chloramphenicol (C) was not co-transferred, but resistance to tetracycline (TET), trimethoprim/sulfamethoxazole (SXT), ceftazidime (CFZ) and cefotaxime (CTX) was co-transferred when the *bla*$_{SHV18}$ gene was transferred. Moreover, transconjugants were shown to be ESBL-producers. Importantly, following 2-3 subcultures in antibiotic-free culture media not all transconjugants retained the acquired *bla*$_{SHV18}$ gene.

3.5.Discussion

The current study demonstrated a high abundance of *Enterobacteriaceae* resistant to β-lactam antibiotics (ampicillin and ceftazidime) in seed sprouts and the transfer of the *bla*$_{SHV18}$ gene in different food matrices including unpasteurized milk, alfalfa sprouts, and chopped lettuce at defined temperatures. RAPD analysis, which is a faster, less labor intensive and more cost effective method than pulsed-field gel electrophoresis (PFGE) was used to differentiate a donor, recipient, and transconjugant (Gori et al., 1996). The stability of transferred *bla*$_{SHV18}$ gene was quite poor without antibiotic pressure. The use of antibiotics during the production of fresh produce should thus be carefully considered

in order to reduce the spread of antibiotic-resistant bacteria.

It is known that most *Enterobacteriaceae* including *Enterobacter* spp. and *Klebsiella* spp. are intrinsically resistant to ampicillin through the production of β-lactamase(s) (Livermore, 1995 and Leclercq et al., 2013). Bezanson et al. (2008) assessed the occurrence of antibiotic-resistant bacteria associated with romaine lettuce, savory spinach, and alfalfa sprouts purchased from markets in Canada. More than 90% of isolates (n=205) from those vegetables were resistant to ampicillin and cephalothin (first-generation cephalosporin). Campos et al. (2013) found that 43 of 50 ready-to-eat salads purchased at retail outlets in Portugal had populations of more than 10^6 CFU/g of aerobic mesophilic microorganisms, and *E. coli* among recovered bacteria. The bacteria were resistant to tetracycline streptomycin, sulfamethoxazole, trimethoprim, ampicillin, nalidixic acid, ciprofloxacin or chloramphenicol. Our findings that approximately 5 log CFU/g of *Enterobacteriaceae* isolated from the seed sprouts were capable of growth in the presence of 16 µg/ml of ceftazidime was unexpected; total *Enterobacteriaceae* recovered from MacConkey agar was 7.4 log CFU/g of seed sprouts. Ceftazidime is one of the third-generation cephalosporin antibiotics and used for determination of the ESBL-producer phenotypically. Of course, this result does not necessarily mean that all 5 log CFU/g of *Enterobacteriaceae* are ESBL-producing *Enterobacteriaceae* because every single colony was not tested to determine ESBL-production by phenotypic and genotypic methods. However, considering the importance of third-generation cephalosporins in clinical therapeutic use, the high prevalence of *Enterobacteriaceae* resistant to ceftazidime is worrisome.

Few studies on the prevalence and characteristics of ESBL-encoding genes

associated with bacteria isolated from fresh produce have been conducted. Njage and Buys (2015) reported a high prevalence of ESBL/AmpC β-lactamase positive bacteria (90% of isolates) isolated from lettuce. They also found a close phylogenetic relatedness of *E. coli* isolates from water and lettuce, suggesting water as a possible route of dissemination. Bhutani et al. (2015) observed that the SHV11-encoding gene sequence of *Enterobacteriaceae* from retail lettuce was 98% homologous with the ESBL sequence of clinical *Enterobacteriaceae* isolates. The prevalence of ESBL-producing *Enterobacteriaceae* associated with raw retail vegetables in the Netherlands was investigated (Reuland et al., 2014). Six percent of samples (n=119) yielded ESBL-producing *Enterobacteriaceae,* and most ESBL-encoding genes were associated with samples from sprouts or organically grown vegetables. The ESBL-encoding genes identified were most closely associated with genes found in enterobacteria strains of human origin.

Calbo et al. (2011) reported on a foodborne nosocomial outbreak of SHV1 and CTX-M-15-producing *K. pneumoniae*. This was the first outbreak by ESBL-producing *K. pneumoniae* via food, providing evidence that food can be a vehicle of dissemination of the ESBL-encoding gene in a clinical setting. Poirel and his colleagues (2002) proposed that CTX-M, one of the emerging extended-spectrum β-lactamases, developed in soil before becoming a prevalent clinical problem (Cantón and Coque, 2006). In this respect, the role of commensal bacteria as a vehicle of antibiotic resistance gene transfer under certain favorable conditions may partially explain why antibiotic resistance genes such as $bla_{CTX\text{-}M}$ are prevalent in both clinical and non-clinical settings. There are several reasons why commensal bacteria associated with raw food are important in the dissemination of

antibiotic resistance: 1) commensal bacteria are a major reservoir of antibiotic resistance genes, 2) raw foods contain high populations of diverse communities of commensal bacteria, and 3) the transfer of antibiotic resistance genes goes back and forth between commensal and pathogenic bacteria. In the present study, *K. pneumoniae* was selected as a target organism because of its role as an opportunistic pathogen in nosocomial and community settings, and *K. pneumoniae* is also a commensal organism in the environment and gastrointestinal tracts of animals and humans. The conditions in foods or other matrices that facilitate the transfer of antibiotic resistance genes by *K. pneumoniae* have yet to be determined.

This study highlighted the different transfer rate of the bla_{SHV18} gene in TSB, PM, and UPM at 4, 15, 24, and 37°C. UPM was utilized as a medium for the mating experiment because UPM typically contains a high population of commensal bacteria ($\geq 10^3$ CFU/ml). Straley et al. (2006) emphasized that raw bulk tank milk can be a reservoir of antibiotic-resistant Gram-negative bacteria. Moreover, consumption of raw milk and raw milk products poses a potential public health risk by exposing the antibiotic-resistant bacteria to the human gastrointestinal tract (Jayarao and Wang, 1999, Van den Bogaard and Stobberingh, 1999 and Straley et al., 2006). Gundogan and Yakar (2007) investigated the prevalence of ESBL-producing *Klebsiella* spp. and their virulence factors and antibiotic resistance profiles in milk and milk products. *Klebsiella* spp. were isolated from 53.8% of 80 raw milk, pasteurized milk, ice cream and white cheese samples; approximately 35% of the isolates were ESBL-producers. In this study, there was a significant difference in the transfer rate of the bla_{SHV18} gene at 24°C compared to 37°C in TSB, but not in PM and UPM. This is in partial agreement with Walsh et al.

(2008), who investigated the transfer of ampicillin resistance from *Salmonella* Typhimurium DT104 to *E. coli* K12 in LB broth, milk, and ground beef. The transfer frequency was not affected when evaluated in liquid media at optimum (37˚C) or sub-optimum conditions (25˚C) (Walsh et al., 2007). They reported that transfer at 15˚C occurred only in ground beef, but not in LB and milk. In the current study, transconjugants were observed in TSB, UPM, and PM at 15˚C, although the average transfer frequency was significantly lower (P<0.05) than 24˚C and 37˚C. McMahon et al. (2007) observed the transfer of antibiotic resistance genes between *E. coli* in LB broth at 5˚C. No antibiotic resistance gene transfer occurred at 4˚C in the present study or in research conducted by others (Walsh et al., 2008). Lack of resistance gene transfer at 4˚C may be associated with low metabolic rates of donors and recipients (Walsh et al., 2008). The notion that temperature can influence the transfer of genetic material by bacteria has been known for decades (Frischer et al., 1993). The natural plasmid transformation rate of *Vibrio* was reduced at 37˚C compared to 4˚C. Collectively, the research suggests that the rate or occurrence of genetic material transfer may be associated with the physiological characteristics of donors and/or recipients and the matrices in which the gene transfer occurs.

Fresh produce contains high populations (up to 10^7CFU/g) of bacteria, which can persist from the production environment to the consumer. During this journey, bacterial interaction on a commodity may result in the transfer of antibiotic resistance genes. Under favorable conditions for the transfer of genetic material, the population of antibiotic-resistant bacteria may increase and change in diversity. There are few studies that have investigated factors that influence antibiotic resistance gene transfer among

bacteria associated with fresh produce. In this study, the rate of *bla*$_{SHV18}$ gene transfer between bacteria on alfalfa sprouts and lettuce was investigated.

Raw seed sprouts are recognized as a potentially hazardous food due to concerns over microbiological safety. Given the way seed sprouts are produced and handled, they often contain high populations of commensal bacteria making them a good choice for inclusion in this study. Mølbak et al. (2003) observed transconjugants at the frequency of 3.4E-04 to 2.0E-06 from *Pseudomonas putida* to commensal bacteria on alfalfa sprouts. In a separate study, the transfer rate of erythromycin and tetracycline resistance determinants between lactic acid bacteria ranged from 4.7E-04 to 3.9E-01 (Toomey et al., 2009). In the present study, the transfer of *bla*$_{SHV18}$ gene occurred at a frequency of 1.03E-03 to 1.05E-04 in alfalfa sprouts, which was a significantly higher rate than in the liquid media evaluated. Chopped lettuce was also used in mating experiments; this may be the first study using lettuce. On lettuce at 15°C, no transconjugants were detected on day 1, but 2.2 log CFU transconjugants/g of lettuce were detected on day 2 post-inoculation of the donor and the recipient. The transfer frequency at 24°C on day 2 was significantly higher than on day 1. Although the mating matrices were different, these results agree with Walsh and colleagues who reported a higher transfer rate of ampicillin resistance from *S.* Typhimurium DT104 to *E. coli* K12 in ground beef at 36 hours compared to 18 hours after co-inoculation of the donor and recipient (Walsh et al., 2008). This may be a result of the adaptation of recipients and donors against stress conditions such as low temperatures and limited nutrients. Research suggests that the transfer of mobile genetic elements and transfer efficiency are affected by bacterial metabolic activity, ecological factors, and environmental stresses (Muela et al., 1994, Van Elsas and

Bailey, 2002, and Johnsen and Kroer, 2007). The transfer frequency in alfalfa sprouts and chopped romaine lettuce was higher than in the liquid food matrices and in the laboratory medium, suggesting that *in vitro* research may underestimate antibiotic resistance gene transfer, highlighting the need for quantitative studies in a variety of food matrices.

The donor strain *K. pneumoniae* ATCC 700603 was selected for use in the present study since the SHV18 enzyme carried by the strain was characterized, and the strain was used in conjugal transfer of bla_{SHV18} to *E. coli* HB101 in LB (Rasheed et al., 2000). Transfer efficiency was not determined in the study mentioned. The hypothesis of this study was not whether the transfer of bla_{SHV18} occurs, but whether the temperature and food matrices influence transfer. During the conjugal transfer of bla_{SHV18} from *K. pneumoniae* ATCC 700603 to *E. coli* HB101, resistance to cefoxitin, chloramphenicol, and tetracycline was not transferred with ceftazidime resistance. The results of this study confirm those findings since co-transfer of resistance to chloramphenicol did not occur. Importantly, the present study demonstrated co-transfer of the class 1 integron (*intl1)* from *K. pneumoniae* ATCC 700603 to recipient strains. Integrons are associated with mobile DNA elements such as transposons and conjugative plasmids, which play an important role in the spread of antibiotic resistance genes (Mazel, 2006 and Raphael et al., 2011).

Collectively, the present study demonstrates that the antibiotic resistance gene bla_{SHV18} can be transferred to commensal bacteria (*K. pneumoniae*) more frequently in alfalfa sprouts and chopped romaine lettuce than in liquid media at room temperature. No transconjugants were detected at 4°C in liquid media and chopped lettuce. Considering the abundance of ceftazidime-resistant *Enterobacteriaceae* as well as the

average transfer frequency of *bla*$_{SHV18}$ gene in alfalfa sprouts (5.15E-04) and chopped lettuce (3.85E-05), the overall reduction of commensal bacteria in agricultural products may reduce exposure to antibiotic-resistant bacteria. These results suggest that the transfer of antibiotic resistance genes on fresh produce, exposed to temperature abuse during storage, may permit the accelerated spread of antibiotic resistance genes into the community having a negative impact on human health.

3.6.Reference

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Table 3.1. PCR primer sequences used for the verification of transconjugants

Target Gene	Primer Name	Oligonucleotide Sequence (5' to 3')	Product Size (bp)	Tm (°C)	Reference
rmpA	rmpAF rmpAR	ACTGGGCTACCTCTGCTTCA CTTGCATGAGCCATCTTTCA	516	63.9 64.0	Nadasy et al., 2007
wcaG	wcaGF wcaGR	GGTTGGKTCAGCAATCGTA ACTATTCCGCCAACTTTTGC	169	65.1 62.9	Turton et al., 2010
alls	allSF allSR	CCGAAACATTACGCACCTTT ATCACGAAGAGCCAGGTCA C	508	63.6 64.3	Yu et al., 2008
kfu	kfuB-F1179 kfuC-R649	GAAGTGACGCTGTTTCTGGC TTTCGTGTGGCCAGTGACTC	797	65.0 66.4	Yu et al., 2008
bla_{SHV18}	SHV18_F SHV18_R	AGAATAGCGCTGAGGTCTG AGCGCGAGAAGCATCCTG	1369	59.8 66.9	Rasheed et al., 2000
intl1	Int1F Int1R	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	280	63.5 58.8	Raphael et al., 2011

Table 3.2. Antibiotic susceptibility and virulence determinant profiles of *K. pneumoniae* isolates

Isolates	Source[§]	Resistance profile[*]	Virulence gene (s)[#]
KP01	AS	AMP^R, STR^I, NAL^I	*wcaG, alls*
KP02	AS	AMP^R, STR^I, NAL^I, CTX^I	*wcaG, alls*
KP03	AS	AMP^R, STR^I	-
KP04	AS	AMP^R, STR^I	*kfu*
KP05	CS	AMP^R, STR^I	*kfu*
KP06	CS	AMP^R, STR^I	-
KP07	BS	AMP^R, STR^I, NAL^I	*kfu*
KP08	BS	AMP^R, STR^I, NAL^I	*alls*

[§] AS: alfalfa sprouts, CS: clover sprouts, BS: broccoli sprouts

*Antimicrobial susceptibility test and interpretive criteria was followed by CLSI. reference; ^{S}: susceptible, ^{I}: intermediate, ^{R}: resistant, AMP: ampicillin, STR: streptomycin, NAL: nalidixic acid, CTX: cefotaxime

[#]: Four virulence genes (*rmpA, wcaG, alls, kfu)* associated with *K. pneumoniae* were screened by PCR.

Table 3.3. Phenotypic and genotypic characteristics of donor, recipient, and transconjugants

			Donor	Recipients			Transconjugants		
			700603	KP4$^{\Delta KM}$	KP8$^{\Delta KM}$	KP342	TC603 KP04	TC603 KP08	TC603 KP342
Phenotypic	Aminoglycosides	GEN	I	R	R	S	R	R	S
		KM	S	R	R	S	R	R	S
		STR	S	R	R	R	R	R	R
	Tetracycline	TET	R	S	S	R	R	R	R
	Fluoroquinolones	CIP	S	S	S	S	S	S	S
	Quinolones	NA	R	S	S	R	S	S	R
	Carbapenems	IMP	S	S	S	S	S	S	S
	Penicillins	AM	R	R	R	R	R	R	R
	β-lactam /β-lactamase	AMC	S	S	S	S	S	S	S
		SAM	I	S	S	S	I	I	S
	Cephems	FEP	S	S	S	S	S	S	S
		CTX	R	S	S	I	R	R	R
		CFZ	R	S	S	S	R	R	R
	Folate pathway inhibitors	SXT	R	S	S	I	R	R	R
	Phenicols	C	R	S	S	R	S	S	R
	ESBL producer[§]		+	-	-	-	+	+	+
Genotypic	*bla*$_{SHV18}$		+	-	-	-	+	+	+
	class 1 integrase gene (*intl1*)		+	-	-	-	+	+	+
	virulence gene [#]		-	*kfu*	*alls*	*kfu*	*kfu*	*alls*	*kfu*

Antimicrobial susceptibility test and interpretive criteria was followed by CLSI reference; S=susceptible, I=intermediate, R=resistant
*GEN: gentamicin (10 µg), STR: streptomycin (10 µg), IMP: imipenem (10 µg), Tet: tetracycline (30 µg), Na: Nalidixic acid (30 µg), AMC: amoxillin/clavulanic acid (20 µg/10 µg), SAM: ampicillin/sulbactam (10 µg/10 µg), FEP: cefepime (30 µg), CTX: cefotaxime (30 µg), SXT: trimenthoprim/sulfamethoxazole (1.25 µg/23.75 µg), and C: chloramphenicol (30 µg). Tests for kanamycin ciprofloxacin, ampicillin, and ceftazidime were performed by the microdilution method. [§]: ESBL producer was confirmed phenotypically based on CLSI reference (+: producer, -: non-producer). [#]: Four virulence genes (*rmpA, wcaG, alls, kfu)* associated with *K. pneumoniae* were screened by PCR.

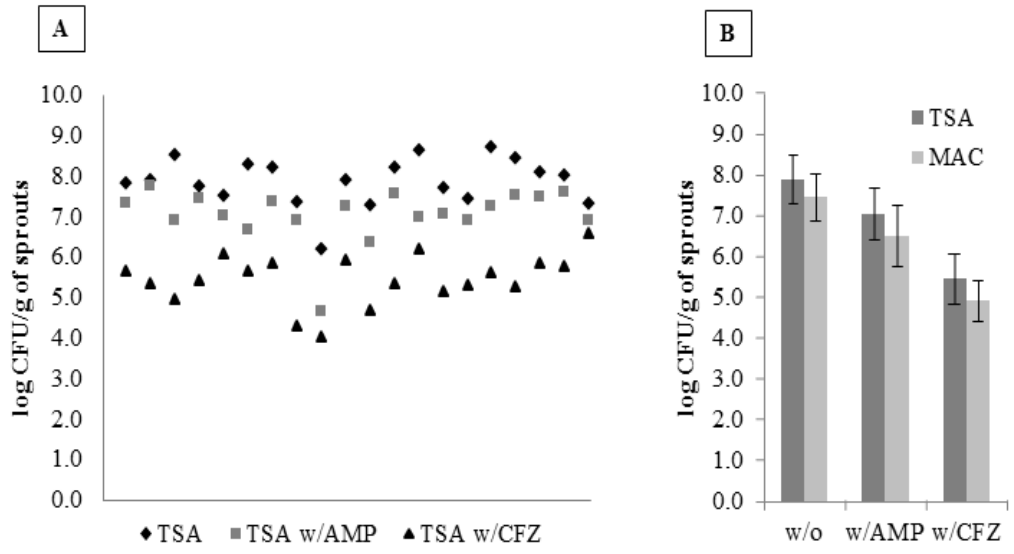

Figure 3.1. Total bacterial population from collected seed sprouts on TSA or MacConkey agar (MAC) plates containing no antibiotic, ampicillin (AMP, 32 µg/ml), or ceftazidime (CFZ, 16 µg/ml). A: distribution of total microbial population of different retail seed sprouts sample on TSA, TSA containing ampicillin or TSA containing ceftazidime. B: The comparison of the population between TSA and MAC with or without antibiotic. The data is the mean of 20 seed sprouts samples, indicating standard deviations.

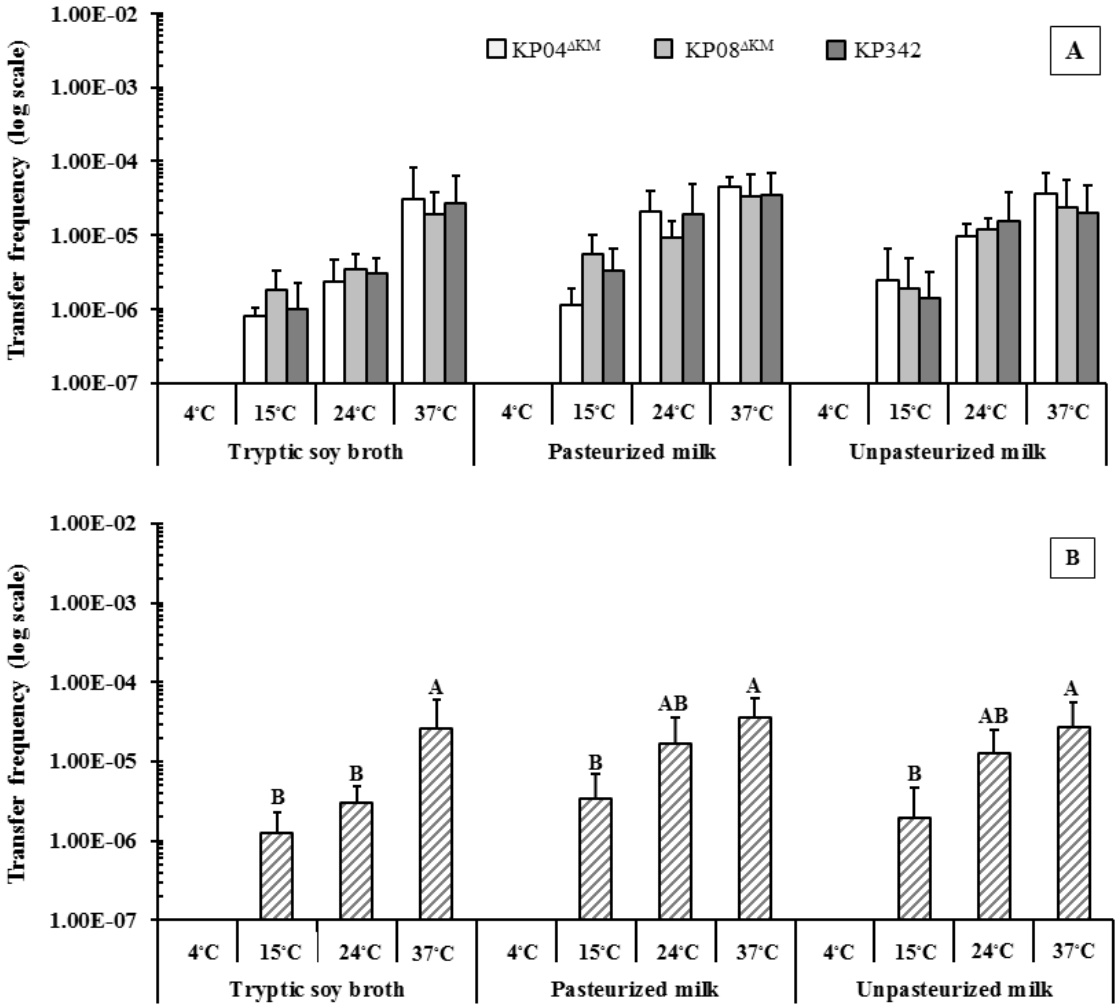

Figure 3.2. Transfer frequency of *bla*$_{SHV18}$ A) to each of the three recipients (KP04$^{\Delta KM}$, KP08$^{\Delta KM}$, and KP342) and B) of the mean of all recipients in liquid media at 4, 15, 24, and 37˚C. Transfer frequency was calculated by enumeration of transconjugants and recipient on selective TSA and presented as the number of transconjugants per recipient. Y-axis was converted to log-scale. Values are the averages of three replications; error bar indicates the positive standard deviations.

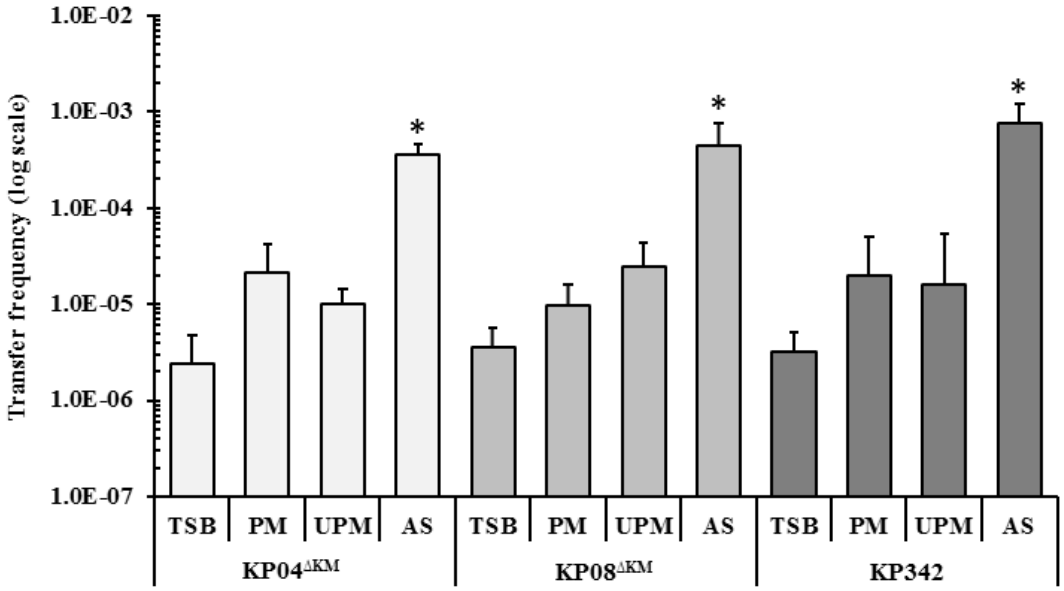

Figure 3.3. The comparison of transfer frequency of *bla*$_{SHV18}$ at 24°C. Transfer frequency was calculated by enumeration of transconjugants and recipient on TSA containing selective antibiotics and presented as the number of transconjugants per recipient. Y-axis was converted to log-scale. Values are the mean of three replications; error bar indicates the positive standard deviations. The star indicates the significance (P<0.05) among tryptic soy broth (TSB), pasteurized milk (PM), unpasteurized milk (UPM), and Alfalfa sprouts (AS).

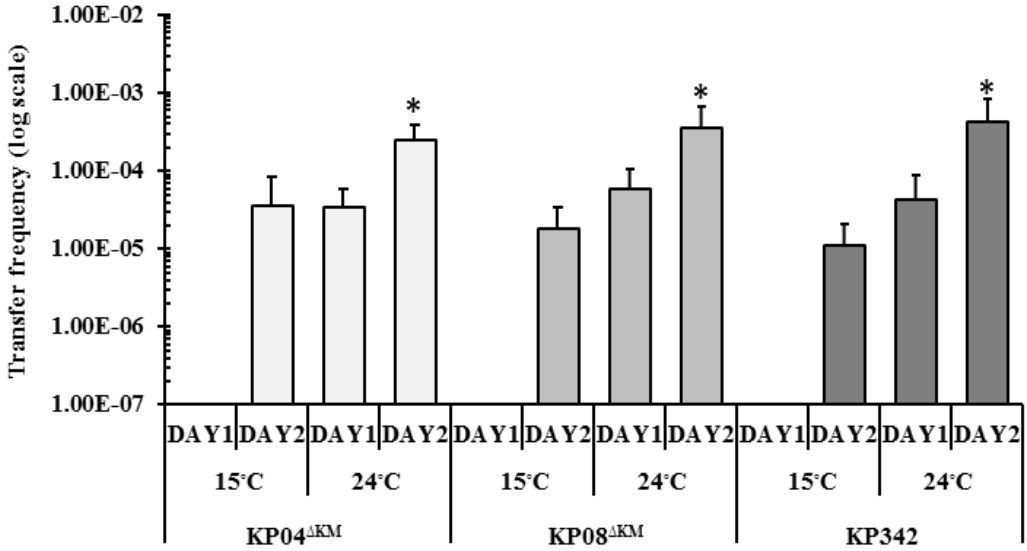

Figure 3.4. The transfer frequency of *bla*$_{SHV18}$ in chopped lettuce at 15°C and 24°C during 2 days. Transfer frequency was calculated by enumeration of transconjugants and recipient on selective TSA and presented as the number of transconjugants per recipient. Y-axis was converted to log-scale. Values are the mean of three replications; error bar indicates the positive standard deviations. The star indicates statistical differences among day 1 and day 2 at 15 and 24°C ($p<0.05$). Results for 4°C not shown since transconjugants were not detected.

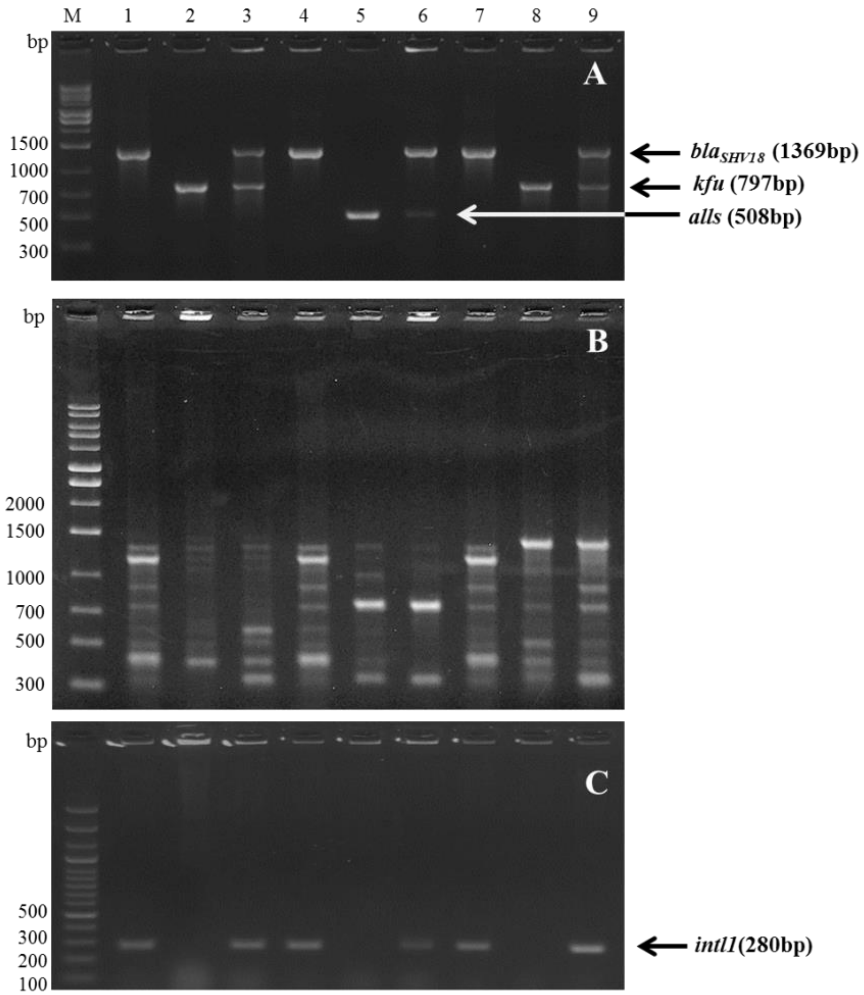

Figure 3.5. Representative gels verifying transconjugants by detection of transferred *bla*$_{SHV18}$ gene (A) and randomly amplified polymorphic DNA (RAPD) typing (B). Class 1 integrase gene (*intl1*) was detected by PCR (C). M: marker (1kb), lane 1, 4, 7: donor, ATCC700603; lane 2: recipient, KP04$^{\Delta KM}$; lane 3: transconjugants, TC603KP04; lane 5: recipient, KP08$^{\Delta KM}$; lane 6: transconjugants, TC603KP08; lane 8: recipient, KP342; lane 9: transconjugants, TC603KP342

Chapter 4. Potential transfer of *bla*$_{KPC}$ from *Klebsiella pneumoniae* to *Salmonella* and *E. coli* O157:H7 in liquid media and alfalfa sprouts.

4.1. Abstract

This study investigated the potential transfer of the carbapenemase-encoding gene (*bla*$_{KPC}$) from *Klebsiella pneumoniae* to *Salmonella* and *E. coli* O157:H7 in TSB and alfalfa sprouts at 24°C and 37°C. In mating experiments, two clinical strains harboring the *bla*$_{KPC}$ gene served as donors. Carbapenem resistance was determined by antimicrobial susceptibility, modified Hodge tests with meropenem and the screening of several carbapenemase-encoding genes. Transconjugants, which are recipients (*Salmonella* or *E. coli* O157:H7) harboring *bla*$_{KPC}$ gene from the donor, were differentiated from the donor using immuno-magnetic beads coated with *Salmonella* or *E. coli* O157:H7 antibody and selective media containing meropenem. For the verification of transconjugants, *bla*$_{KPC}$ of the donor and the virulence gene (*hly* for *E. coli* O157:H7 or *invA* for *Salmonella*) of recipients were amplified by PCR. In TSB and alfalfa sprouts, no transconjugants were detected even when high numbers (6-7 log CFU population) of recipients per milliliter were used. Under the conditions evaluated in the present study, the transfer of *bla*$_{KPC}$ could not be confirmed. The dissemination of carbapenemase-encoding gene(s) in raw food or under food production/processing conditions should be investigated under a range of conditions beyond those of the present study.

4.2. Introduction

Carbapenems are regarded as a last resort antibiotic to treat severe infections

caused by multiple-antibiotic-resistant or carbapenem-resistant Gram-negative bacteria. A growing number of *Enterobacteriaceae* as well as *Pseudomonas* spp. and *Acinetobacter* spp., have shown resistance to carbapenems during the last few years (Canton et al., 2012). So far, 9 families (KPC, SME, NMC-A, IMI, PER, GES, SFO, SFC, IBC) in Ambler class A, 6 families (VIM, GIM, SIM, NDM, IMP, SPM) in Ambler class B, and 2 families (OXA, PSE) in Ambler class D have been identified (Stuart et al., 2010). Among them, the "big five" carbapenemases (KPC, OXA-48, non-metallo-carbapenemase, IMP, NDM, and VIM metallo-carbapenemases) have received the greatest attention (Woodford et al., 2014). In particular, KPC-producers, mostly *Enterobacteriaceae*, have rapidly spread worldwide. It is known from genetic analysis of bla_{KPC} that self-transferable plasmids and transposons (Tn4401) play a role in the dissemination of the bla_{KPC} gene in *Enterobacteriaceae* and *Acinetobacter* spp. (Nordmann et al., 2009 and Cuzon et al., 2011).

Concern associated with the dissemination of carbapenemase-producers and/or carbapenemase-encoding genes may not be limited to clinical settings. Guerra et al. (2014) reviewed recent reports on the prevalence of carbapenemase-producing bacteria in food-producing animals and their environments, companion animals, and wild birds. Although carbapenems are not permitted for veterinary use in the United States and throughout most of the world, New Delhi metallo-β-lactamase (NDM-1)-producing *E. coli* were isolated from companion animals in the United States. Moreover, OXA-48 producing *E. coli* and *K. pneumoniae* were isolated from dogs in Germany (Shaheen et al., 2013 and Stolle et al., 2013). Under specific conditions, carbapenems may be prescribed by veterinarians for companion animals in the UK (Woodford et al., 2014).

Of interest, Seiffert et al., (2014) screened 30 pet foods for the presence of the carbapenemase-encoding genes and found that the *bla*$_{OXA\text{-}48\text{-}like}$ carbapenemase-encoding gene was present in 13.3% of the collected samples. This may not be surprising considering that VIM-1-producing *Salmonella enterica* subsp. *enterica* was isolated from pig and poultry farms in Germany in 2012 (Fischer et al., 2012). The detection of acquired carbapenemase-producing bacteria in food-producing and companion animals is worrisome to say the least. Dissemination into community settings through the food chain could lead to the possible spread of carbapenemase-encoding genes to humans. If a person were to consume food containing carbapenemase-producing bacteria, this could alter the human gastrointestinal microbiome and subsequently generate a public health concern.

Until recently, very few studies focused on carbapenemase-producing bacteria and carbapenemase-encoding genes linked to foods. Rubin et al. (2014) recently isolated VIM-2 carbapenemase-producing *Pseudomonas fluorescens* from squid. Others reported that 3.3% of 121 seafood (squid, sea squirt, clams, and seafood mix) product samples evaluated harbored the *bla*$_{OXA\text{-}48}$ gene (Morrison and Rubin, 2015). The authors emphasized that even though *Pseudomonas fluorescens* is not a foodborne pathogen, the findings were important due to the potential of horizontal gene transfer. Zurfluh et al. (2015) reported the presence of carbapenemase-producing *Enterobacteriaceae*, including an OXA-181-producing *Klebsiella variicola*, isolated from fresh vegetables imported to Switzerland from Asia.

The prevalence of carbapenemase-producing bacteria and their transfer in raw foods, including fresh produce, has not been reported. Some researchers have observed

interspecies transfer of carbapenemase-encoding genes in patients and under *in vitro* conditions. Patron et al. (2011) reported that conjugative plasmids of five NDM-1-producing clinical *E. coli* and *K. pneumoniae* were successfully transferred to *E. coli*, *Salmonella* Typhimurium, *Proteus mirabilis*, and *K. pneumoniae* in LB broth with a frequency from 1.0E-04 to 6.0E-08 transconjugants per donor. In addition, Goren et al. (2010) described interspecies transfer of a carbapenemase (KPC-3)-encoding plasmid from *K. pneumoniae* to *E. coli* in the same patient in Israel, even though the transfer of the KPC-3-encoding plasmid by conjugation experiment was not successful. Additionally, the transfer of KPC-2-encoding gene from *K. pneumoniae* to *E. coli* in a patient was reported in a patient in Italy (Ritcher et al., 2011). The interspecies transfer of KPC-encoding gene has been confirmed in clinical settings; however, there is insufficient research on carbapenemase transfer in non-clinical settings. The transfer of KPC-encoding genes to foodborne pathogens in food may cause further dissemination to the human gut microbiome. The objective of this research was to investigate the potential spread of bla_{KPC}, the most prevalent carbapenemase-encoding gene in *K. pneumoniae*, to foodborne pathogens in liquid media and alfalfa sprouts.

4.3. Materials and methods

4.3.1. Bacterial strains

Clinical *K. pneumoniae* isolates were provided by Dr. Kirn, Pathology and Laboratory Medicine department, Robert Wood Johnson University Hospital (RWJUH). The strains were named RWJ 1 to RWJ 3 (Table 4.1). The collection of isolates was approved by the University of Medicine and Dentistry of New Jersey Institutional Review Board (UMDNJ IRB). The antimicrobial susceptibility of clinical strains

provided by Dr. Kirn was tested using a MicroScan® (Siemens). The foodborne pathogens, *Salmonella* Stanley ATCC 7308 and *E. coli* O157:H7 86-24, were used.

4.3.2. Determination of carbapenem resistance

4.3.2.1. Modified Hodge test

The modified Hodge test procedure was conducted according to the standard method of CLSI (CLSI, 2012). An inoculum of *E. coli* ATCC 25922 grown in Mueller-Hinton broth was adjusted to OD_{600}=0.1, and evenly spread on Mueller-Hinton agar with a cotton swab, per the protocol for the routine disk diffusion method. After 5 min of drying, a meropenem (10 µg) disk (Sensi-Disc, Becton, Sparks, MD) was placed at the center of a Mueller-Hinton agar plate. A target test strain was streaked in a straight line (20-25 mm in length) from the edge of the disk using a 10 µl loop. The plate was incubated at 35˚C for 16-20 h.

4.3.2.2. PCR detection of carbapenemase-encoding gene

The PCR methods were described previously in Chapter 3. Briefly, the amplification of target carbapenemase-encoding genes (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{OXA}$, *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{TEM}$, *bla*$_{SPM}$, *bla*$_{SIM}$, *bla*$_{GIM}$) was performed according to the manufacture's instructions (GoTaq® Flexi, Promega, Madison, WI). Crude genomic DNA was prepared using a boiling method, or an isolated colony on a plate was picked and used directly in the PCR cocktail containing appropriate primer sets (Table 2). The total volume of the PCR reaction mixture was 50 µl including 10 µl of 5X green Go taq Flexi buffer, 4 µl of 25 mM $MgCl_2$, 4 µl of 10 mM dNTPs, 5 µl (each) of primer, and 0.25 µl Go taq Flexi DNA polymerase (5 U/µl). PCR reactions were performed in a Perkin-

Elmer GeneAmp PCR system 2400 (Perkin-Elmer Corp., Foster City, CA) for1 cycle of 2 min at 95˚C (initial denaturation); followed by 30 cycles of 30 sec at 95˚C (denaturation), 45 sec at 60˚C (annealing) and 1 min/kb at 72˚C (extension); followed by 1 cycle of 5 min at 72˚C (final extension). Amplified products were separated on 1% agarose gels (IBI Scientific, Peosta, IA) by electrophoresis and stained with ethidium bromide (Bio-Rad Laboratories Hercules, CA), and bands were visualized under UV light.

4.3.3. Mating procedure

In vitro mating was done in triplicate according to a modified version of the procedure by Willetts (1988). Overnight cultures of donor and recipient cells grown in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) were harvested and mixed at a ratio of 1:1. A 100 µl aliquot of the mixed culture was dispensed into 5 ml of fresh TSB and incubated at 24˚C (ambient room temperature) and 37˚C without shaking for 24 h. Five grams of alfalfa seeds (The Sprout House Certified Organic Sprouting Seeds, Lake Katrine, NY) were inoculated with 5 ml of donor and recipient overnight cultures and held at room temperature overnight. The inoculated seeds were placed in a sterile mesh tray with cheesecloth and watered once a day. After 7 days, 10 g of sprouts were aseptically transferred into a sterile filter bag and homogenized with 90 ml of 0.1% SPW. One milliliter of homogenized sample was subjected to immuno-magnetic bead separation for the collection of the bacteria of interest.

4.3.4. Separation of donor and recipient using immuno-magnetic beads

In order to separate *Salmonella* Stanley or *E. coli* O157:H7 from the donor (*K.*

pneumoniae), magnetic beads coated with *Salmonella* or *E. coli* O157:H7 antibodies (BacTrace®, KPL, MD, USA) were utilized according to manufacturer's instructions. One milliliter of a homogenized sample was incubated with 20 µl of immuno-magnetic beads for 15 min and then placed on a magnetic separator. After washing with sterile buffered peptone water containing bovine serum albumin and Tween 20, the separated samples (beads plus *Salmonella* or *E. coli* O157:H7) were plated onto XLT_4 or Sorbitol MacConkey agar with meropenem (1 µg/ml). The presumptive transconjugants should have similar colony appearance to the recipient and be capable of growth on selective media (meropenem positive).

4.3.5. Verification and characterization of transconjugants

Three to five presumptive transconjugant and recipient colonies were randomly picked from selective agar plates and verified using PCR amplification of the bla_{KPC} gene and virulence determinants of recipients. Two sets of primers, bla_{KPC} and *hly* for *E. coli* O157:H7 and bla_{KPC} and *invA* for *Salmonella,* were added to the PCR master mix. PCR conditions were: 1 cycle of 2 min at 95°C (initial denaturation); followed by 30 cycles of 30 sec at 95°C (denaturation), 45 sec at 60°C (annealing) and 1 min/kb at 72°C (extension); followed by 1 cycle of 5 min at 72°C (final extension). Target genes, *hly* (5′-GTAGGGAAGCGAACAGAG-3′ and 5′AAGCTCCGTGTGCCTGAA-3′) and *invA* (5′-TATCGCCACGTTCGGGCAA-3′ and 5′-TCGCACCGTCAAAGGAACC-3′), were used as primers (Wang et al., 1997). Transconjugants will be positive for bla_{KPC} and virulence gene of the recipient.

4.4. Results and discussion

Based on antimicrobial susceptibility testing, RWJ2 and RWJ3 were resistant to carbapenems (Ertapenem, Imipenem and Meropenem) (Table 4.1). The modified Hodge test confirmed that RWJ2 and RWJ3 are carbapenemase producers (Figure 4.1). Black arrows (Figure 4.1) indicate the enhanced growth of ATCC 25922 via carbapenemase production of tested strains, indicating that RWJ2 and RWJ3 were carbapenemase-producers. KP16 and KP26, which are zoonotic *K. pneumoniae*, served as a negative control for carbapenemase production. In addition, the bla_{KPC} gene was detected in RWJ2 and RWJ3 (Figure 4.2A). Based on this analysis, RWJ2 and RWJ3, both of which harbor the bla_{KPC} gene, were utilized as donors in mating experiments.

It is known that carbapenem resistant *Enterobacteriaceae* commonly exhibit resistance to different classes of antibiotics, including carbapenem, cephalosporins, fluoroquinolones, and aminoglycosides (Gupta et al., 2011). RWJ2 and RWJ3 exhibited resistance to most antibiotics tested in this study with the exception of tobramycin, one of the aminoglycoside antibiotics. Since the donors (RWJ2 and RWJ3) were resistant to multiple antibiotics, it was difficult to differentiate the donor from transconjugants and recipients through resistance to select antibiotics. In this study, immuno-magnetic beads were utilized to separate the donor from recipient and transconjugants. Meropenem (1 µg/ml) was added to selective media (XLT_4 for *Salmonella* and Sorbitol MacConkey for *E. coli* O157:H7) to differentiate transconjugants from recipients.

Several presumptive transconjugant colonies following mating experiments were detected on selective media containing meropenem, following immuno-magnetic bead separation. These presumptive colonies were picked for PCR to amplify the bla_{KPC} gene and virulence gene. Figure 4.2B shows bands associated with amplified genes of

presumptive transconjugants and the donor. The results showed that all presumptive colonies were identified as the recipient or the donor, which do not carry the bla_{KPC} gene.

Therefore, under conditions evaluated in the present study, mating was not successful. In other words, the bla_{KPC} gene of the clinical *K. pneumoniae* (RWJ2 and RWJ3) was not transferred to *E. coli* O157:H7 or *Salmonella* at the 6-7 log CFU population of recipients per milliliter of TSB at 24°C and 37°C. In our research previously, the transfer frequency in alfalfa sprouts of the ESBL-encoding gene was significantly higher than in TSB. As a result, alfalfa sprouts were used as a mating medium to increase the possibility of bla_{KPC} gene transfer; however, transconjugants carrying the bla_{KPC} gene as well as the recipient's virulence gene were not detected in TSB. While we could not find any transconjugants in this study, we cannot conclude with complete certainty that it is impossible to transfer the bla_{KPC} gene of *K. pneumoniae* to foodborne pathogens. This may be because immuno-magnetic beads were not able to capture all target *E. coli* O157:H7 or *Salmonella*, potentially missing transconjugants, or the donors and recipients selected for in this study were not perfectly compatible with each other for the transfer of the bla_{KPC} gene under conditions evaluated. Goren et al. (2010) also failed to mimic the natural conjugation of a KPC-encoding plasmid, but from the clinical cases of KPC-encoding gene transfer, we believe that carbapenemase-encoding genes can in fact be transferred in food under favorable conditions (Goren et al., 2010 and Ritcher et al., 2011). Overuse and misuse of carbapenem antibiotics in clinical or agricultural settings may facilitate the selection of carbapenem resistant bacteria in the environment, subsequently increasing human

exposure. In the cycle of the spread of carbapenemase-encoding genes, food may play an important role or at a minimum provide a link to humans. There is a dearth of research on the prevalence of the carbapenemase-encoding gene or carbapenem resistant bacteria in food and no studies have examined carbapenemase-encoding gene transfer in food systems. Accordingly, we must track the dissemination of the carbapenemase-encoding gene and carbapenemase-producing bacteria in food. More importantly, we also need to identify and understand conditions that are favorable for carbapenemase-encoding gene transfer to occur in food matrices or the food processing system.

4.5. Reference

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Table 4.1. Antimicrobial susceptibility profile of clinical isolates

	A/S*	AK	AM	AZT	CAX	CAZ	CAZ/CA	CFT	CFT/CA	CFX	P/T	T/S
RWJ1	>16/8	>32	>16	>16	>32	>16	<=0.25	>32	<=0.5	<=8	64	<=2/38
	R#	R	R	R	R	R		R		R	I	S
RWJ2	>16/8	>32	>16	>16	>32	>16	>2	>32	>4	>16	>64	>2/38
	R	R	R	R	R	R		R		R	R	R
RWJ3	>16/8	>32	>16	>16	>32	>16	>2	>32	>4	>16	>64	>2/38
	R	R	R	R	R	R		R		R	R	R
	CFZ	CP	CPE	CRM	ETP	FD	GM	IMP	LVX	MER	TGC	TO
RWJ1	>16	>2	>16	>16	<=1	>64	>8	<=1	<=2	<=1	<=2	>8
	R	R	R	R	S	R	R	S	S	S	S	R
RWJ2	>16	>2	>16	>16	>4	>64	>8	>8	>4	>8	<=2	>8
	R	R	R	R	R	R	R	R	R	R	S	R
RWJ3	>16	>2	>16	>16	>4	>64	>8	>8	>4	>8	<=2	>8
	R	R	R	R	R	R	R	R	R	R	S	R

Antimicrobial agents' abbreviations

*Amikacin, AK; ampicillin, AM; ampicillin/sulbactam, A/S; aztreonam. AZT; cefazolin, CFZ; cefepime, CPE; cefotaxime, CFT; cefotaxime/CA, CFT/CA; cefoxitin, CFX; ceftazidime, CAZ; ceftaxidime/CA, CAZ/CA; ceftriaxone, CAX; cefuroxime, CRM; ciprofloxacin, CP; ertapenem, ETP; gentamicin, GM; imipenem, IMP; levofloxacin, LVX; meropenem, MER; nitrofurantoin, FD; piperacillin/tazobactam, P/T; tigecycline, TGC; tobramycin, TO; trimethoprim/surfamethoxazol, T/S

R, Resistant; S, Susceptible; Blank, not determined

Table 4.2. PCR primer sequences used for screening carbapenemase-encoding genes

Target Gene	Primer Name	Oligonucleotide Sequence (5' to 3')	Product Size(bp)	Tm (^{o}C)	Reference
bla$_{IMP}$	IMP-F	GGAATAGAGTGGCTTAAYTCTC	232	62.9	Ellington et al., 2007
	IMP-R	GGTTTAAYAAAACAACCACC		55.1	
bla$_{VIM}$	VIM-F	GATGGTGTTTGGTCGCATA	390	62.0	Ellington et al., 2007
	VIM-R	CGAATGCGCAGCACCAG		68.3	
bla$_{OXA}$	OXA-48-F	GCGTGGTTAAGGATGAACAC	438	62.0	Nordmann et al., 2011
	OXA-48-R	CATCAAGTTCAACCCAACCG		65.2	
bla$_{NDM}$	NDM-F	GGTTTGGCGATCTGGTTTTC	621	65.5	Nordmann et al., 2011
	NDM-R	CGGAATGGCTCATCACGATC		67.8	
bla$_{KPC}$	KPC-F	CGTCTAGTTCTGCTGTCTTG	798	57.8	Nordmann et al., 2011
	KPC-R	CTTGTCATCCTTGTTAGGCG		62.2	
bla$_{SPM}$	SPM-F	AAAATCTGGGTACGCAAACG	271	63.6	Ellington et al., 2007
	SPM-R	ACATTATCCGCTGGAACAGG		63.8	
bla$_{SIM}$	SIM-F	TACAAGGGATTCGGCATCG	570	65.8	Ellington et al., 2007
	SIM-R	TAATGGCCTGTTCCCATGTG		65.6	
bla$_{GIM}$	GIM-F	TCGACACACCTTGGTCTGAA	477	64.5	Ellington et al., 2007
	GIM-R	AACTTCCAACTTTGCCATGC		63.9	

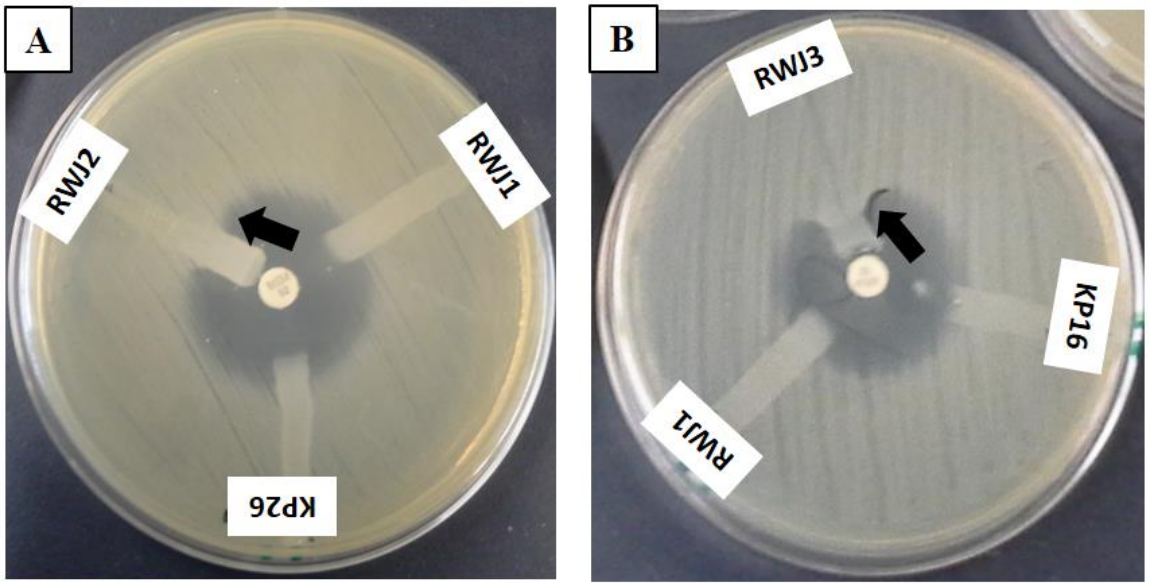

Figure 4.1. The result of modified Hodge test. A black arrow indicated the enhanced growth of ATCC 25922 by RWJ2 (A) or RWJ3 (B), confirming RWJ2 and RWJ3 are carbapenemase-producer. KP26 and KP16 were used for negative controls.

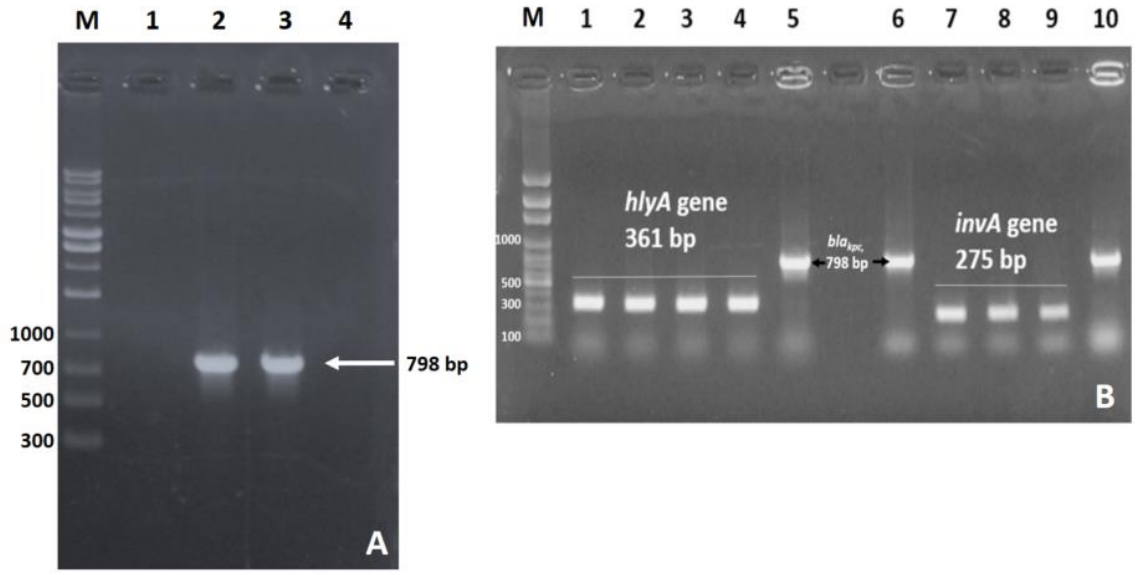

Figure 4.2. Representative gel electrophoresis images to verify the transfer of *bla*$_{KPC}$ gene. A: detection of *bla*$_{KPC}$ gene of RWJ1 (lane 1), RWJ2 (lane 2), RWJ3 (lane3), and ATCC 700603 as a negative control (lane 4). B: verification of transconjugants by detection of transferred *bla*$_{KPC}$ gene (M: marker (100bp), lane 1-3: presumptive transconjugants; lane 4: recipient (*E. coli* O157:H7); lane 5: donor (RWJ2); lane 6-8: presumptive transconjugants; lane 9: recipient (*S.* Newport); lane 10: donor (RWJ2)