The Molecular Epidemiology of Antibiotic Susceptible Versus Antibiotic Resistant North American Urinary *Escherichia coli* Isolates

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Abstract

Urinary tract infections (UTI) are important causes of morbidity, affecting 8-10 million Americans each year. *Escherichia coli* (*E. coli*) is the most common UTI-causing pathogen, responsible for approximately 80% of community-acquired infections. A major concern to the medical community is the increasing occurrence of multi-drug resistant (MDR) *E. coli,* presenting challenges to the empirical treatment of UTI. 347 *E. coli* isolates collected by the North American Urinary Tract Infection Collaborative Alliance (NAUTICA) were selected for investigation based on susceptibility or resistance to ampicillin (Ampi), trimethoprim-sulfamethoxazole (TMP/SMX), and fluoroquinolones (FQ). Pulsed-field gel electrophoresis (PFGE) of *Xba*I digests was performed to determine the genetic similarity amongst isolates susceptible to all three drug classes, resistant to only one or two drugs, or resistant to all three drugs. PFGE analysis showed that the majority of susceptible isolates and TMP/SMX-resistant (R) isolates were genetically unrelated, while Ampi-R and Ampi + TMP/SMX-R isolates each revealed one cluster (similarity of coefficient ≥80%) containing ≥10% of isolates. Genetic homogeneity was greatest among the MDR isolates, with one large epidemiologic cluster containing 32% of all isolates in the phenotype. The spread of MDR *E. coli* in North America appears to involve both clonal dissemination and the *de novo* acquisitions of resistance-conferring mutations/determinants.

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List of Abbreviations Used

Ampi - Ampicillin

Cipro - Ciprofloxacin

CNF-1 - Cytotoxic necrotizing factor 1

DHF - Dihydrofolic acid

DHFR - Dihydrofolic acid reductase

DHP – Dihydropteroic acid

DHPS - Dihydropteroate synthetase

ESBL - Extended-spectrum beta lactamase

FQ - Fluoroquinolone

Levo - Levofloxacin

MDR - Multi-drug resistant

MIC - Minimum inhibitory concentration

MRSA – Methicillin-resistant *Staphylococcus aureus*

NAUTICA - North American Urinary Tract Infection Collaborative Alliance

NCCLS - National Committee for Clinical Laboratory Standards

PABA - p-aminobenzoic acid

PBP - Penicillin-binding proteins

PFGE - Pulsed-field gel electrophoresis

QRDR - Quinolone resistance determining region

SMX - Sulfamethoxazole

THF - Tetrahydrofolic acid

TMP - Trimethoprim

TMP/SMX - Trimethoprim-sulfamethoxazole

UPEC - Uropathogenic *E. coli*

UTI - Urinary Tract Infection

UV - Ultraviolet

Introduction

1. Urinary Tract Infections (UTIs)

Urinary tract infections (UTIs) are among the most common human bacterial infections, with global infection rates nearing 180 million per year (Russo & Johnson, 2003). UTIs are clinically defined as the bacterial colonization of any tissue along the urinary tract, from the urethral opening to the kidneys. Infections of the lower urinary tract may be confined to the urethra (urethritis) or the bladder (cystitis), with symptoms including frequent, painful, and urgent urination. Diagnosis is typically made based upon pyuria of at least 10 leukocytes per high power microscopic field and the presence of at least 10^5 colony forming units of infecting organisms per mL of clean catch urine, though infections with lower numbers of organisms are not infrequent. Upper tract infections frequently occur in the kidneys (pyelonephritis), often accompanied by flank pain and tenderness, fever, chills, nausea, and vomiting and may also include symptoms of lower UTI (Sobel & Kaye, 2004). Based on the presence of physiological or anatomical abnormalities, including obstruction of the bladder outlet, vesicoureteral reflux, enlarged prostate in men, diabetes and pregnancy, UTIs are referred to as complicated, whereas those in an uncompromised host are termed uncomplicated (Sobel & Kaye, 2004; Kunin, 1987). A final level of UTI classification deals with whether these infections are acquired in the general community or hospital setting, and are termed community acquired and nosocomial, respectively.

The incidence of UTIs differs greatly between the sexes, with infections occurring fourteen times more often in females than males (Prescott *et al.,* 2002). The means through which infection begins greatly influences the differing rates between

women and men. UTIs can occur in one of three ways: via the ascending route, the hematogenous route, or the lymphatic route. The ascending route is undoubtedly the most common route of infection. Bacteria from the normal flora of the colon are regularly excreted in fecal matter, and pathogenic strains may travel to the nearby periurethral area where they enter the urinary tract and cause ascending infection. The close proximity of the urethral meatus to the anus puts females at greater risk for UTIs. Sexual intercourse is indicated as a predisposing factor for ascending UTIs, as coliforms that colonize the vaginal introitus may be transferred to the urethral opening during sexual activity (Sobel & Kaye, 2004). The male anatomy greatly reduces the risk of UTI among men; however, infections are seen in small numbers. Rectal intercourse puts men at increased risk for a UTI, while a lack of circumcision may also play a role (Bergan, 1997). The hematogenous route of infection is far less common and limited only to infection of the kidneys, where blood-borne pathogens enter and contribute to the formation of abscesses. These infections are almost exclusively caused by bacteremic *Staphylococcus* species, with *E. coli* infections being virtually non-existent. There is little evidence to indicate the importance of infection via the lymphatic route, however due to the presence of direct lymphatic channels between the intestines and the kidneys, their role in producing upper tract infections cannot be ruled out entirely (Kunin, 1987; Sobel & Kaye, 2004).

2. Microbiological Causes of UTI

There are several organisms known to cause UTIs, including *Pseudomonas aeruginosa, Staphylococcus saprophyticus, Enterococcus spp., Proteus mirabilis,*

Klebsiella spp., Citrobacter spp., and most importantly *Escherichia coli* (*E. coli*), the most common infecting agent (Zhanel *et al.,* 2005; Nicolle, 2002). *E. coli* is an enteric Gram-negative bacillus found in the large intestine of all healthy individuals, and is responsible for 70-95% of episodes of cystitis and more than 90% of cases of pyelonephritis (Hooton, 2003; Russo & Johnson, 2003). Certain serogroups of *E. coli*, based on their ability to adhere to and colonize the urinary tract, are known as uropathogenic *E. coli* (UPEC). UPEC are distinguished from commensals by the presence of special virulence factors, including adhesins and toxins, which enhance their ability to both cause infection and evade host responses.

Adhesins are chromosomally encoded, non-filamentous outer-membrane proteins known as pili or fimbriae (Sobel & Kaye, 2004). UPEC express many types of fimbriae differentiated by their receptor affinity. Type-1 fimbriae, characterized by their ability to bind mannose-containing oligosaccharides on host glycoproteins, are encoded by the *fim* gene cluster, composed of nine genes (Emody, 2000). These are especially well known as important factors for colonization of the bladder. The FimH adhesin, located on the tip of the pilus, recognizes terminal D-mannose moieties on secreted and cell-bound glycoproteins and is responsible for UPEC cell binding to host cells (Bergan, 1997). Single nucleotide differences in the *fimH* gene produce FimH proteins with the UTI-associated mono-mannose binding phenotype as opposed to the commensal type that binds to tri-mannose receptors (Johnson & Russo, 2005). P-fimbriae are known for their enhanced ability to bind to the colon and remain in the gastrointestinal tract for an extended period. Following this, they confer the ability to spread to the perineum and urinary tract, causing ascending infections (Sobel & Kaye, 2004). The allelic variation in

the *papG* gene, which encodes for the adhesin at the tip of these fimbriae, produces variants that preferentially bind to different Gal(α1-4)Gal-containing glycolipids. Because these glycolipids are distributed differentially throughout the body, only certain papG variants contribute to disease in specific anatomical sites (Johnson & Russo, 2005). Two of the four papG adhesin classes are associated with UTIs, with class II mainly in pyelonephritis and class III in strains causing cystitis (Marrs *et al.,* 2005).

In addition to molecules that aid in adherence, UPEC produce toxins that enhance infectivity. Cytotoxic necrotizing factor 1 (CNF-1) is a protein that induces host cell cytoskeleton rearrangement by permanently stimulating the Rho protein. By modifying the permeability of uroepithelium, bacteria are able to invade these cells, avoiding host defenses and antibiotics (Emody, 2000). In addition to CNF-1, UPEC may also produce α–hemolysin, a toxin that facilitates invasion of host tissues and causes damage to renal tubules and epithelial and parenchymal cells (Marrs *et al.,* 2005).

3. Treatment of UTI

The morbidity associated with UTIs makes treatment of these infections a necessity. When choosing an appropriate agent to combat these infections, there are several factors for clinicians to consider. First, the drug of choice should have good *in vitro* and *in vivo* activity against many of the organisms known to cause UTIs, not just against *E. coli*. Additionally, the agent should be able to achieve high and prolonged concentrations in the urine and surrounding urinary tract tissues without loss of activity (Bergan, 1997; Jancel & Dudas, 2002). In terms of the patient's well being, drugs should have the fewest and mildest side effects possible, with minimal effects on normal colonic

and vaginal flora (Sobel & Kaye, 2004). There are numerous antibiotics used to treat UTIs, with the aminopenicillins, fluoroquinolones, and trimethoprim-sulfamethoxazole among the most important classes from a current and historical perspective.

3.1. Penicillins

In 1929, the discovery of penicillin by Alexander Fleming revolutionized the world of infectious diseases. By 1949, penicillin was isolated in a relatively pure form, in sufficient quantities to treat a number of bacterial infections. The crucial point in the history of this drug class came in 1945, when the basic chemical structure of all penicillins was revealed as a bicyclic β–lactam-thiazolidine nucleus with variable side chains. The β–lactam ring holds the key to the drug's antibacterial activity, while the side chain determines the spectrum of activity for each particular penicillin compound (Preston & Drusano, 1999). Penicillins exert their antibacterial properties by interfering with peptidoglycan synthesis, which compromises cell wall integrity, leading to cell death.

Penicillins inhibit the cross-linking of N-acetylmuramic acid residues of adjacent peptidoglycan strands during the final step of peptidoglycan synthesis by acting as structural analogues of acyl-D-alanyl-D-alanine and competing for the active site of the enzyme transpeptidase. In doing so, they prevent the cross-linking of peptidoglycan precursors, thereby disrupting cell wall stability (Mascaretti, 2003; Preston & Drusano, 1999). In addition to transpeptidases, penicillins bind to a variety of other bacterial enzymes, collectively known as penicillin-binding proteins (PBPs). In *E. coli,* eight

PBPs have been described, including carboxypeptidases and endopeptidases, all of which are important in cell wall formation and maintenance (White, 2000).

While the original, natural penicillins demonstrated excellent activity against Gram-positive organisms, they had very limited activity against Gram-negative pathogens, including *E. coli*. The aminopenicillins were synthesized to overcome this problem. With the addition of an amino group to the fused β-lactam-thiazolidine nucleus, aminopenicillins exhibited enhanced penetration into Gram-negative bacteria, such as *E.coli,* while retaining activity against gram-positive UTI pathogens, such as *Enterococcus spp.* and *Staphylococcus saprophyticus,* making them ideal agents for the treatment of UTI (Yao *et al.,* 1999).

3.2. Sulfonamides and Trimethoprim

When coadministered, trimethoprim and sulfamethoxazole (TMP/SMX) are bactericidal agents with excellent antibacterial activity. Used in combination to treat UTI since 1968, TMP/SMX interferes with bacterial folate metabolism, by sequentially blocking the formation of tetrahydrofolic acid (THF). SMX is a structural analogue of p-aminobenzoic acid (PABA), the substrate for the enzyme dihydropteroate synthetase (DHPS), used to make dihydropteroic acid in the first step of THF synthesis. SMX preferentially binds to DHPS over PABA, thereby blocking the formation of dihydropteroic acid (DHP), which is needed to form dihydrofolic acid (DHF) in a later step along the THF synthesis pathway. TMP is a structural analogue of the pteridine moiety of DHF and preferentially binds to dihydrofolic acid reductase (DHFR), the enzyme normally responsible for the conversion of DHF to THF. By creating a

sequential blockade in the formation of THF, TMP/SMX eliminates the thymidine pool required for DNA synthesis, leading to bacterial cell death (Goldstein & Stein, 1999; Mascaretti, 2003). Its low cost, high absorption, oral availability, extended half-life, and activity against Gram-positive and Gram-negative organisms has made TMP/SMX the first choice for empiric treatment of upper and lower UTI (Gupta & Stamm,2002; Goldstein & Stein, 1999).

3.3. Fluoroquinolones

A relatively modern class of drugs, fluoroquinolones are becoming increasingly popular in the treatment of UTI due to their broad spectrum of activity and rapid bactericidal effects. The prototype for modern day quinolones, nalidixic acid, was discovered in 1962 as a byproduct of the formation of the anti-malarial drug chloroquine. The 1980s brought about the synthesis of the second-generation quinolones, the fluoroquinolones, including ciprofloxacin and levofloxacin, which have the broad spectrum of Gram-negative activity of the first generation quinolones as well as improved activity against Gram-positive organisms (Zhanel *et al.,* 2002*).*

The fluoroquinolones exhibit their action on bacterial cells by interacting with DNA gyrase and topoisomerase IV, inhibiting their ability to regulate the topological state of DNA during replication, transcription, and repair. The tetrameric DNA gyrase protein is composed of two "A" subunits, which produce and re-seal strand breaks in the chromosome during replication, and two "B" subunits, which introduce negative supercoils into the strand of DNA. Second-generation quinolones act primarily by binding to the DNA-DNA gyrase complex formed during replication, preventing the A

subunits from resealing strand breaks in the bacterial chromosome. Topoisomerase IV plays an integral role in chromosome segregation during bacterial cell division in addition to its role in relieving the DNA molecule of torsional strain. While the details of quinolone interactions with Topoisomerase IV are not as well known, the importance of its loss of function must not be overlooked (Zhanel *et al.,* 2002; Mulholland, 1996). With their excellent bioavailability when orally administrated, good tissue penetration, low incidence of side effects and broad spectrum of activity, fluoroquinolones are prescribed to combat a variety of clinical infections and are especially favored in combating complicated UTI (Mascaretti, 2003; Mulholland, 1996).

4. Antibiotic Resistance in UTI

While a number of drugs are available for UTI management, increasing resistance rates among infecting organisms have limited the use of a number of antibiotics, leading to greater difficulty in treating these infections. Resistance to an antimicrobial agent is the phenomenon whereby an organism is completely resistant or demonstrates decreased susceptibility to a particular drug that normally causes bacterial cell death or hinders cell replication. Acquired resistance occurs when members of a bacterial population initially susceptible to a particular drug undergo a change rendering them less susceptible or completely resistant to the drug, as has been ongoing since the introduction of antibiotics (Mascaretti, 2003). When patients are treated with a given antibiotic, there is strong selective pressure on the targeted pathogens. While the susceptible organisms are eliminated, the small proportion of naturally resistant members of the bacterial population is able to increase and flourish. As such, the same drugs used to eradicate a bacterial

infection are those that promote the propagation of resistant strains (Mascaretti, 2003; de la Cruz *et al,* 2002).

With the exception of bacteria that are naturally resistant to a particular drug, resistance is usually gained via chromosomal mutations or acquisition of foreign DNA through mobile genetic elements. Conjugative plasmids and transposons are easily transferred between genera, contributing to the horizontal transfer of resistance genes (Mascaretti, 2003). Additionally, bacteriophages have been shown to transfer resistance genes into host cells in laboratory experiments, and this phenomenon cannot be ruled out in natural settings (Salyers *et al.,* 2002).

Bacteria resist the actions of antibiotics by three general mechanisms. They may produce cellular enzymes to modify the antibiotic in such a way that it is no longer functional, or alternatively they may decrease the binding affinity of a particular drug by altering the structure of its cellular target. The third way bacteria evade the action of antibiotics is by preventing or reducing their entry into the cell. This may be accomplished by altering membrane permeability, decreasing drug uptake, or actively pumping the drug out of the cell (Mascaretti, 2003).

4.1. Resistance to Aminopenicillins

The aminopenicillins were the longstanding favorites in the treatment of UTI. However due to extraordinary resistance rates in uropathogens their usefulness is diminishing (Mulholland, 1997). In Gram-negative organisms, aminopenicillin resistance is largely due to the production of β–lactamases, secreted enzymes that destroy the β–lactam nucleus of penicillins (Preston & Drusano, 1999). In contrast,

modifications in PBPs play a major role in Gram-positive penicillin resistance, though in Gram-negative organisms such as *E. coli,* this is not a common occurrence (Quintilliani *et al.,* 1999). Presently, more than 35% of *E. coli* isolates in North America are resistant to aminopenicillins, causing these once first-line antibiotics to be demoted to second- or third-line therapy for UTIs (Zhanel *et al.,* 2005).

4.2. Resistance to TMP/SMX

TMP/SMX remains the drug of choice for empirical treatment of uncomplicated UTIs in the U.S. as it has for over 20 years (Hooton, 2003; Burman *et al.,* 2003). However, increases in resistance to this drug combination in recent years may soon render it unsuitable for empiric treatment of UTI (Carrie *et al.,* 2004). In the *Enterobacteriaceae*, mechanisms of acquired resistance to SMX include a) hyperproduction of PABA, which competes with SMX for the active site of the enzyme DHPS; b) altered DHPS with decreased affinity for SMX; c) hyperproduction of DHPS; and d) decreased membrane permeability to SMX. Similarly, resistance to TMP is attributed to either altered or hyperproduction of the enzyme DHFR. The widespread use of these compounds has led to the development of resistance in most bacterial species on a global scale (Goldstein & Stein, 1999). Guidelines set by the Infectious Disease Society of America recommend fluoroquinolones for empiric therapy of UTI once TMP/SMX resistance levels exceed 20%, and such rates have been reported (Zhanel *et al.,* 2005, Hooton, 2003; Burman *et al.,* 2003). This has led to a shift in prescribing for UTI, with TMP/SMX prescriptions dropping from 48 to 24% between 1989 and 1998, and fluoroquinolone use increasing from 19 to 29% over the same period (Hooton, 2003).

4.3. Fluoroquinolone Resistance

While quinolone resistance rates in North America are relatively low, their increased popularity in recent years has led to a rise in resistance. Currently ciprofloxacin resistance rates in North American urinary *E. coli* isolates sit slightly above 5% (Zhanel *et al.,* 2005). The ability of Gram-negative organisms to prevent the cellular accumulation of fluoroquinolones has been documented. Resistant isolates utilize energy-dependent efflux pumps to remove fluoroquinolones from the cell while reducing porin channels to decrease diffusion into the cell (Mascaretti, 2003). Additionally, chromosomal mutations in *gyrA* and *parC* result in altered DNA gyrase and topoisomerase IV, respectively, leading to decreased fluoroquinolone affinity (Zhanel *et al.,* 2002; Mascaretti, 2003).

4.4. Multi-Drug Resistance

A current phenomenon of great concern in the medical community is the rise in multi-drug resistant organisms, defined as bacteria with simultaneous resistance to three or more different classes of antibiotics (Guyot *et al.,* 1999). In a recent survey of North American fluoroquinolone resistant uropathogens, more than 55% of *E. coli* isolates exhibited additional resistance to ampicillin as well as TMP/SMX (Zhanel, Unpublished data). Patients infected with such organisms experience significantly higher degrees of treatment failure, prolonging antibiotic usage and morbidity associated with infection (Sobel & Kaye, 2004; Gupta & Stamm, 2002).

5. Assessment of Genetic Relatedness

In the field of medical microbiology, clinicians are faced with a variety of situations where the knowledge of whether epidemiologically related isolates are also genetically related would be of great use in the treatment and eradication of the disease in question. The term "clone" and "strain" are often used interchangeably, referring to a group of genetically similar or identical cells whose similarity is due to their descent from a common ancestral strain (Selander *et al.,* 1987). This determination can be accomplished through a variety of methods, including genome sequence analysis, gene detection, or scanning of the entire bacterial genome (Johnson & Russo, 2005). Pulsed-field gel electrophoresis (PFGE) is commonly employed to compare epidemiologically related bacterial isolates in order to reveal possible clones or clonal groups of organisms. By utilizing more than 80% of the bacterial genome to produce banding patterns, it is considered to be the most discriminating method for comparison of epidemiologically related isolates (Kaufmann, 1998).

PFGE involves the digestion of unsheared DNA with a rare-cutting restriction endonuclease, followed by separation of fragments on an agarose gel. Negatively charged DNA molecules are pulled through pores in the gel by an electric field, with small fragments being separated from larger ones due to their varying passage rates through the gel (Finney, 2000). After staining, DNA fragments appear as a series of bands, with shortest fragments migrating furthest from the origin. Banding patterns of epidemiologically related isolates may be compared by eye or using a number of software packages. Tenover *et al.* (1995) have set forth criteria for interpretation of banding patterns in organisms such as *E. coli*, whose PFGE profiles consist of more than 10

bands. For epidemiologically related isolates, a difference of up to six bands in PFGE profiles indicates possible genetic relatedness and such isolates may be defined as clonally related. When dealing with a large number of samples, dendrograms are often produced as a rapid means of analyzing the number of clones or clonal groups in a pool of related isolates, since clusters of similar organisms are easily discernable. For software-generated dendrograms, figures of 80-85% similarity have been used to define a strain, which correlates with a difference in up to six bands between two isolates when visually examined (Kaufmann, 1998).

6. Purpose of Study

Ongoing surveillance studies have identified the rise in antibiotic resistance among urinary pathogens in North America in recent years (Zhanel *et al.,* 2005; Burman, 2003). The purpose of this research was to characterize groups of epidemiologically related urinary *E. coli* isolates, based on their resistance phenotypes, in order to evaluate whether current levels of antimicrobial resistance to ampicillin, TMP/SMX, and fluoroquinolones are attributable to clonal spread or *de novo* acquisitions of resistance-conferring mutations. Most notably, this research was intended to determine whether infections caused by multi-drug resistant isolates were caused by the clonal spread of a small number of related isolates, or by many genetically diverse strains of *E. coli*.

Materials and Methods

1. Organism

Three hundred and forty seven *E. coli* isolates (Table 1) were selected from a cohort of organisms collected by the North American Urinary Tract Infection Collaborative Alliance (NAUTICA) study between 2002-2004 from 41 medical centers across Canada and the United States (Appendix I). Isolates were chosen to represent a broad geographic range and were categorically selected based on resistance phenotype (susceptibility or resistance to ampicillin, TMP/SMX, ciprofloxacin, and levofloxacin).

Table 1. Resistance profiles of five categories of *E. coli* isolates used for comparisons of genetic relatedness.

Number of Isolates	Resistance Phenotype	Ampicillin Resistant	Trimethoprim-Sulfamethoxazole Resistant	Fluoroquinolone Resistant
49	Ampi-R[a]	+	-	-
49	TMP/SMX-R[b]	-	+	-
49	Ampi + TMP/SMX-R[c]	+	+	-
100	MDR[d]	+	+	+
100	Susceptible	-	-	-

[a] Ampi-R, ampicillin-resistant
[b] TMP-SMX-R, trimethoprim-sulfamethoxazole-resistant
[c] Ampi + TMP/SMX-R, ampicillin and TMP/SMX-resistant
[d] MDR, multi-drug resistant.

All *E. coli* isolates were stocked in 50% skim milk powder (BD, Sparks, MD) and frozen at -80ºC until required for plug preparation. Unless otherwise indicated, each isolate was run only once on a gel, due to time constraints and the high cost associated with PFGE.

2. Susceptibility Testing

All isolates previously underwent susceptibility testing in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution method (NCCLS, 2003). Microdilution panels were prepared using 100µL cation-adjusted Mueller-Hinton Broth (Ca^{2+}, 25 µg/ml; Mg^{2+}, 12.5µg/ml) and appropriate doubling concentrations of the antibiotics of interest per well. Wells were inoculated with the appropriate amount of bacteria to give a final concentration of approximately $5x10^5$ colony forming units per mL, and incubated at 35ºC in ambient air for 24 hours. The ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, and levofloxacin minimum inhibitory concentrations (MICs) for each isolate were determined upon visual examination of microdilution panels and defined as the lowest concentration of antibiotic required to prevent the growth of the bacterium. The MIC breakpoints for *E. coli,* as published by the NCCLS, were used to classify an isolate as either susceptible or resistant as follows: ampicillin (susceptible $\leq$8µg/ml, resistant $\geq$32µg/ml), TMP/SMX (susceptible $\leq$2µg/ml, resistant $\geq$4µg/ml), ciprofloxacin (susceptible $\leq$1µg/ml, resistant $\geq$4µg/ml), and levofloxacin (susceptible $\leq$2µg/ml, resistant $\geq$8µg/ml) (NCCLS, 2002).

3. Pulsed-Field Gel Electrophoresis

3.1. Agarose plug preparation

Frozen bacterial samples were plated onto Blood Agar (Oxoid, Nepean, ON), incubated at 37ºC for 14-20 hours in ambient air, and checked for purity following incubation. Providing plates were free of external bacterial contamination, a sterile swab was used to transfer approximately $^1/_8$ - ¼ plate of confluent growth of each isolate into a

sterile test tube containing 2mL cell suspension buffer (Appendix II) to produce a desired optical density of 0.3-0.4 absorbance units at 610nm. Suspensions with optical densities above or below this range were adjusted accordingly, and 100µl of the adjusted cell suspension was then combined with 100µl of melted 1.6% low-melt:1% SDS agarose in a 0.6mL microfuge tube. This was then quickly vortexed, and immediately dispensed into Bio-Rad disposable plug molds (Bio-Rad laboratories, Hercules, CA).

After cooling for 15 minutes at room temperature, solidified plugs were transferred from plug molds into 1.5mL microfuge tubes containing 1mL cell lysis buffer (Appendix II) with 10µL of 25 µg/mL proteinase K, and incubated in a 50ºC water bath for 2 hours. Following incubation, the lysis buffer was carefully aspirated from each tube and plugs underwent two distilled water-washes as follows: 1mL of distilled H_20, preheated to 50ºC, was added to all tubes, which were then incubated for 15 minutes at 50ºC. Plugs were then removed from the water bath, distilled water was aspirated, and this water wash step was repeated. After the second water wash, 1mL of pre-heated (50ºC) TE buffer (Appendix II) was added to all tubes, which were then incubated for 15 minutes at 50ºC. Three additional TE buffer washes were performed and following the final wash, 1mL of fresh (room temperature) TE buffer was placed into each tube and plugs were stored at 4ºC for up to 3 months until needed for electrophoresis.

3.2. Plug Digestion

On the day of electrophoresis, plugs were removed from the TE buffer and cut in half. One half of the plug was returned to the TE buffer and placed back in storage at 4ºC, while the other half was placed into a 0.6mL microfuge tube to undergo restriction

endonuclease digestion. Approximately 195µL of freshly prepared *Xba*1/buffer mixture (Appendix II) was added to each tube, ensuring that plug slices were completely submerged, and plugs were incubated in a 37ºC water bath for 4 hours. Following incubation, the enzyme/buffer solution was removed from each tube and replaced with 200µL of 0.5X TBE (Appendix II). After sitting for 5 minutes at room temperature the 0.5X TBE buffer was aspirated from each tube and plug slices were melted at 65-70ºC for 20 minutes before loading onto the gel.

3.3. Agarose Gel Preparation

While plugs underwent restriction endonuclease digestion in the 37ºC water bath, 0.5X TBE buffer was prepared by diluting 100mL 10X TBE in 1900mL distilled H_20. A 1% Pulsed Field Certified (Bio-Rad Laboratories, Hercules, CA) large agarose gel (containing 20 wells) was made by heating 1.5g agarose in 150mL 0.5X TBE while stirring until it reached a rapid boil. After cooling for about 3 minutes, the newly prepared agarose gel was carefully poured onto the casting surface and left to solidify for at least one hour before combs were removed. After this point a sterile scalpel was used to cut 0.5-1mm-thick sections of lambda ladder (New England Biolabs, Mississauga, ON), which were loaded into the wells of the outermost lanes as molecular size standards. This was followed by the loading of experimental isolates, where 35µL of melted plug was loaded into each well. Melted plugs were left to solidify in the gel for 5 minutes, after which it was placed in the electrophoresis chamber.

3.4. Electrophoresis

Approximately twenty minutes prior to the start of electrophoresis, 2mL of 50mM thiourea was added to the remaining volume of the 0.5X TBE buffer, which was poured into the electrophoresis chamber. The pump was turned on and the cooling module was set to 14ºC in order to circulate and cool the buffer before placing the gel in the chamber. Following the addition of lambda ladder and digested plugs, the gel was placed into the CHEF (contour-clamped homogenous electric field) chamber (CHEF DRIII: Bio-Rad Laboratories, Hercules, CA). Gels were run for 18 hours under the following electrophoresis parameters: 2.2-second initial switch time, 54.2-second final switch time, voltage of 200V delivered at 6 V/cm, and an induced angle of 120 degrees.

3.5. Staining and Documentation of Gels

Staining procedures were carried out within one hour of the completion of each run. 40µL of SYBR® Green (Molecular Probes, Eugene, OR) stain was combined with 300mL TE buffer in a rectangular Tupperware container. Gels were removed from the CHEF apparatus and placed in the Tupperware to stain for 50 minutes in the dark. Following visualization under ultra violet (UV) light to ensure bands were sufficiently stained, gels were destained with distilled H_20 for 2-4 hours as necessary. Gels were visualized and photographed under UV light using a Gel Doc 1000®, and were digitized for analysis using Molecular Analyst (Fingerprinting Plus, version 1.12) software. An example of a digitized gel can be found in Appendix III.

For isolates that showed poor banding resolution upon analysis, new plugs were made and run on a new gel. This was done to eliminate the possibility that the remaining

half of their original plug would also produce a poor banding image if ran on a new gel. In addition, three isolates were randomly selected to undergo a second run. This was a quality control measure to ensure consistency was maintained in terms of plug preparation and restriction endonuclease digestion.

3.6. Analysis of Genetic Relatedness

After all isolates underwent PFGE and their banding profiles digitized, analysis of genetic relatedness was carried out using Bionumerics® software to generate dendrograms, one for each of the five categories of isolates (Table 1). Only bands of 48.5kb - 679kb, falling between the first and fourteenth genomic length concatamers resolved from the lambda ladder, were used for comparison. This ensured that fragments smaller than those generated by the lambda ladder would not be included, as they cannot undergo the proper normalization. Additionally this served to avoid the inclusion of large fragments that may have been inadequately digested. The Dice coefficient, which uses probability theory to determine whether bands shared by isolates at specific migration points are distinct or similar, was used to compare PFGE patterns of isolates in each category (Yokayama & Uchimura, 2006). Dendrograms were created using the unweighted group pair method with arithmetic averages, and isolates displaying $\geq$80% similarity were deemed genetically related, in accordance with criteria set forth by Tenover *et al.* (1995). Within each resistance phenotype, a single cluster of isolates comprising $\geq$10% of their respective category was considered significant.

Results

1. Antibiotic Susceptibility

The susceptibility levels of each category of *E. coli* isolates (ampicillin + TMP/SMX + fluoroquinolone-susceptible, ampicillin-resistant, TMP/SMX-resistant, ampicillin + TMP/SMX-resistant, and multi-drug resistant) to ampicillin, TMP/SMX, ciprofloxacin, and levofloxacin are provided in Table 2.

2. PFGE Results

Dendrogram analysis of the 100 isolates belonging to the susceptible phenotype (ampicillin + TMP/SMX + fluoroquinolone-susceptible) revealed a high level of genetic diversity. Ninety-eight unique genotypes were generated based on PFGE profiles, and no large clusters of isolates sharing $\geq$ 80% banding pattern homology were present (Figure 1).

From the 49 isolates of the ampicillin resistance phenotype, 49 unique resistance genotypes were revealed by PFGE analysis. Dendrogram analysis revealed four small clusters of three isolates each, and one large epidemiologic cluster containing 18.4% of isolates in the resistance category (Figure 2).

As seen in the ampicillin-resistant group, the TMP/SMX-resistant isolates revealed 100% unique genotypes based on PFGE profiles. Further, dendrogram analysis revealed no significant clustering of isolates with $\geq$ 80% similarity in banding patterns, with only one small cluster of three isolates being seen (Figure 3).

PFGE analysis of the ampicillin + TMP/SMX-resistant phenotype revealed 47 unique genotypes from the 49 total isolates within the group. Three small clusters of 3 isolates each, as well as 1 large epidemiologic group containing 14.3% of isolates in the category were illustrated by dendrogram analysis (Figure 4).

Finally, PFGE analysis of 100 MDR isolates revealed 98 unique genotypes. A single clonal group predominated within this resistance phenotype, as demonstrated by dendrogram analysis, consisting of 32% of the total number of isolates. Additionally, five small clusters of isolates were revealed, all containing less than 10% of isolates from this group (Figure 5).

Table 2. Antibiotic susceptibilities of 347 urinary *E. coli* isolates according to resistance phenotype interpreted following incubation at 35ºC for 24 hours.

Resistance Phenotype	Number of isolates	Ampicillin		TMP/SMX		Ciprofloxacin		Levofloxacin	
		% R[g]	MIC range[h]	% R	MIC range	% R	MIC range	% R	MIC range
Ampi[a] + TMP/SMX[b] + FQ[c] – S[d]	100	0	0.5→8	0	0.25→1	0	0.03→0.25	0	0.03→0.5
Ampi-R[e]	49	100	32→ 1024	0	0.25→2	0	0.03→1	0	0.03→1
TMP/SMX-R	49	0	0.5→8	100	4→1024	0	0.03→1	0	0.03→1
Ampi + TMP/SMX-R	49	100	32→1024	100	64→1024	0	0.03→1	0	0.03→1
Ampi + TMP/SMX + FQ-R (MDR)[f]	100	100	32→1024	100	4→1024	100	4→256	100	8→128

[a] Ampi, ampicillin
[b] TMP/SMX, trimethoprim-sulfamethoxazole
[c] FQ, fluoroquinolone
[d] S, susceptible
[e] R, resistant
[f] MDR, multi-drug resistant
[g] %R, percentage of isolates resistant to a given drug in each resistance phenotype
[h] MIC range, range of minimum inhibitory concentrations to a given drug for isolates in each resistance phenotype, expressed in μg/mL

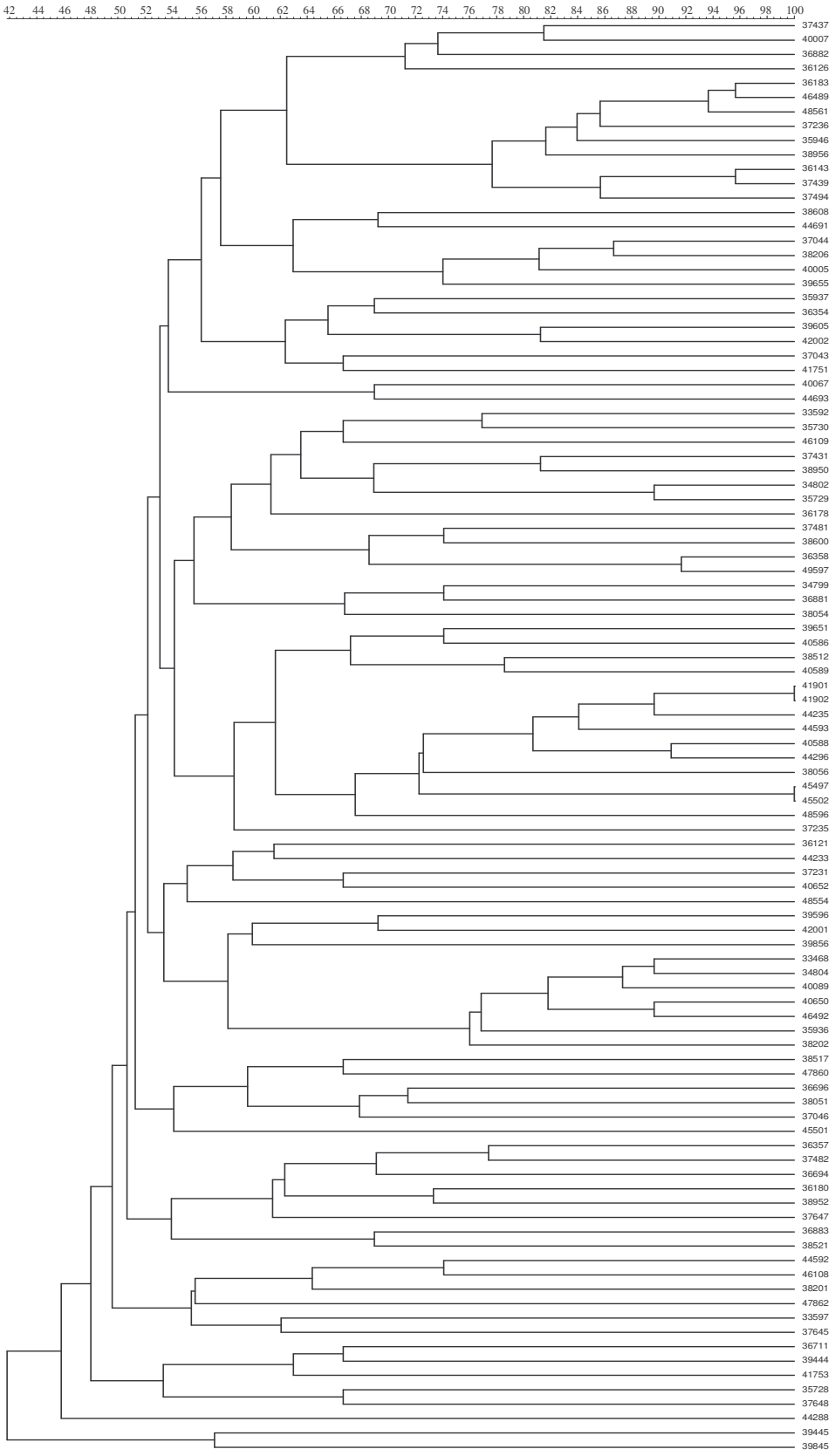

Figure 1. Dendrogram depicting the percent similarity of *Xba*1 restriction patterns of 100 *E. coli* isolates susceptible to ampicillin + TMP/SMX + fluoroquinolones.

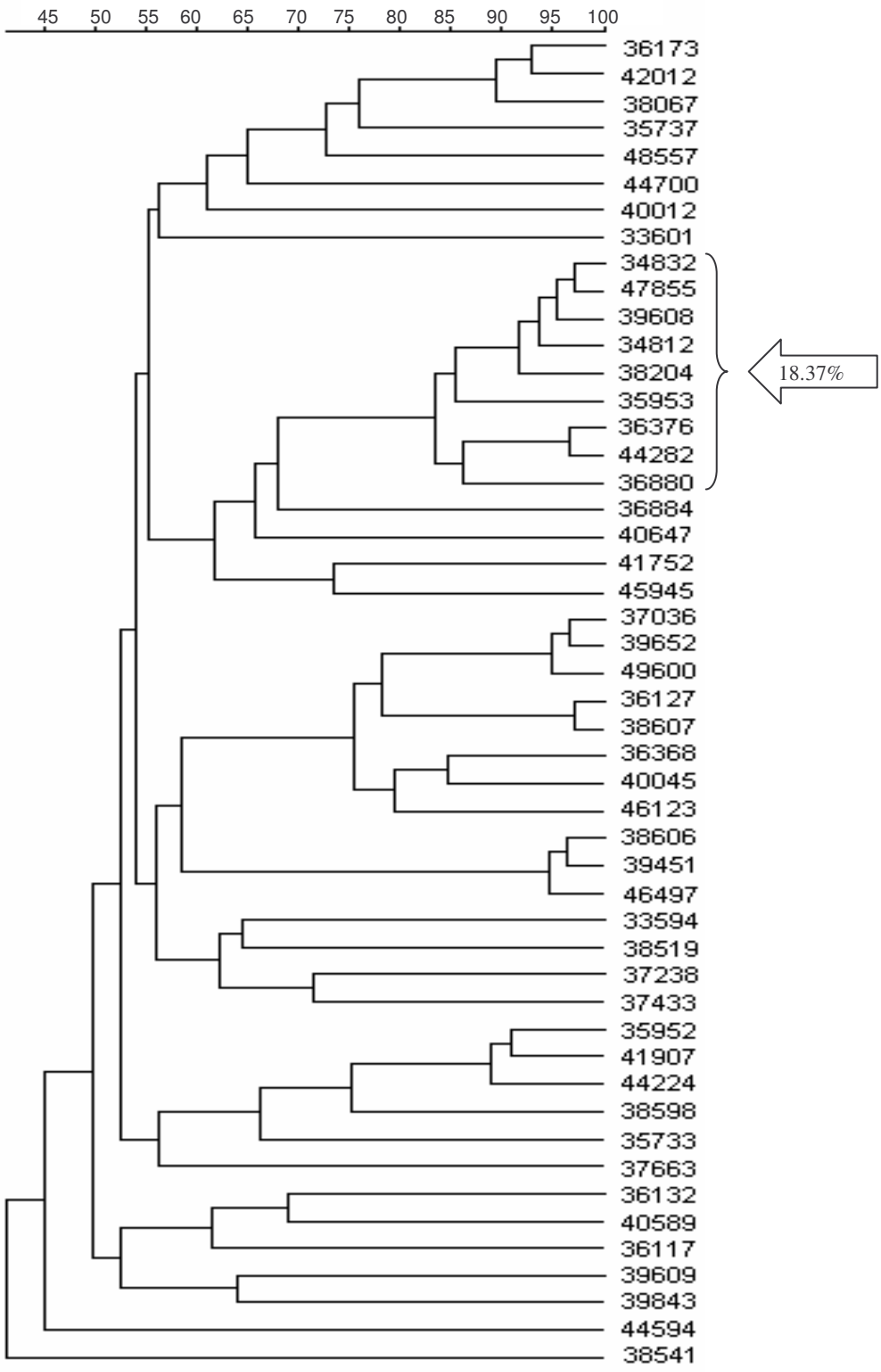

Figure 2. Dendrogram depicting the percent similarity of *Xba*1 restriction patterns of 49 *E. coli* isolates resistant to ampicillin and susceptible to TMP/SMX and fluoroquinolones

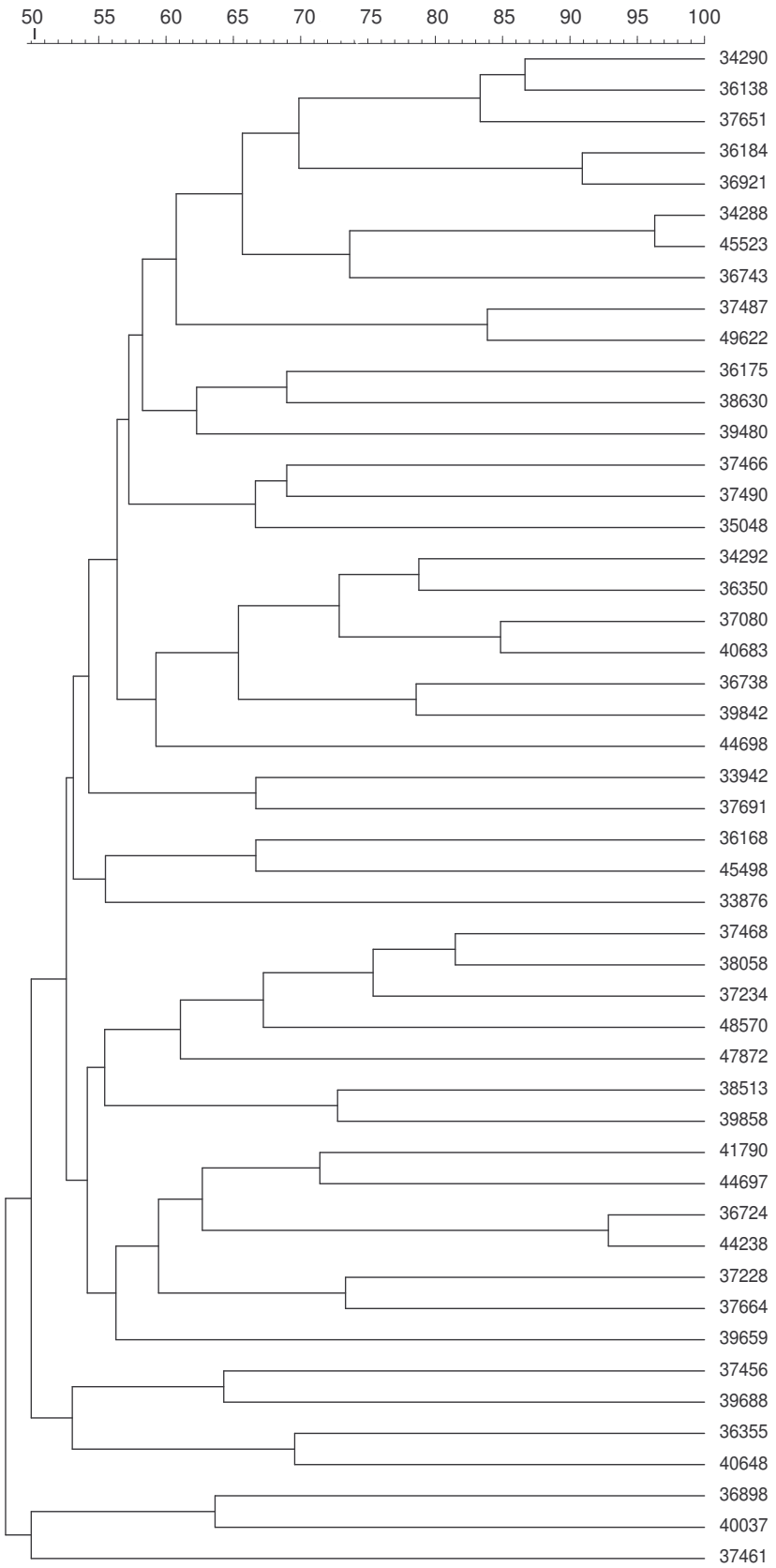

Figure 3. Dendrogram depicting the percent similarity of *Xba*1 restriction patterns of 49 *E. coli* isolates resistant to TMP/SMX and susceptible to ampicillin and fluoroquinolones.

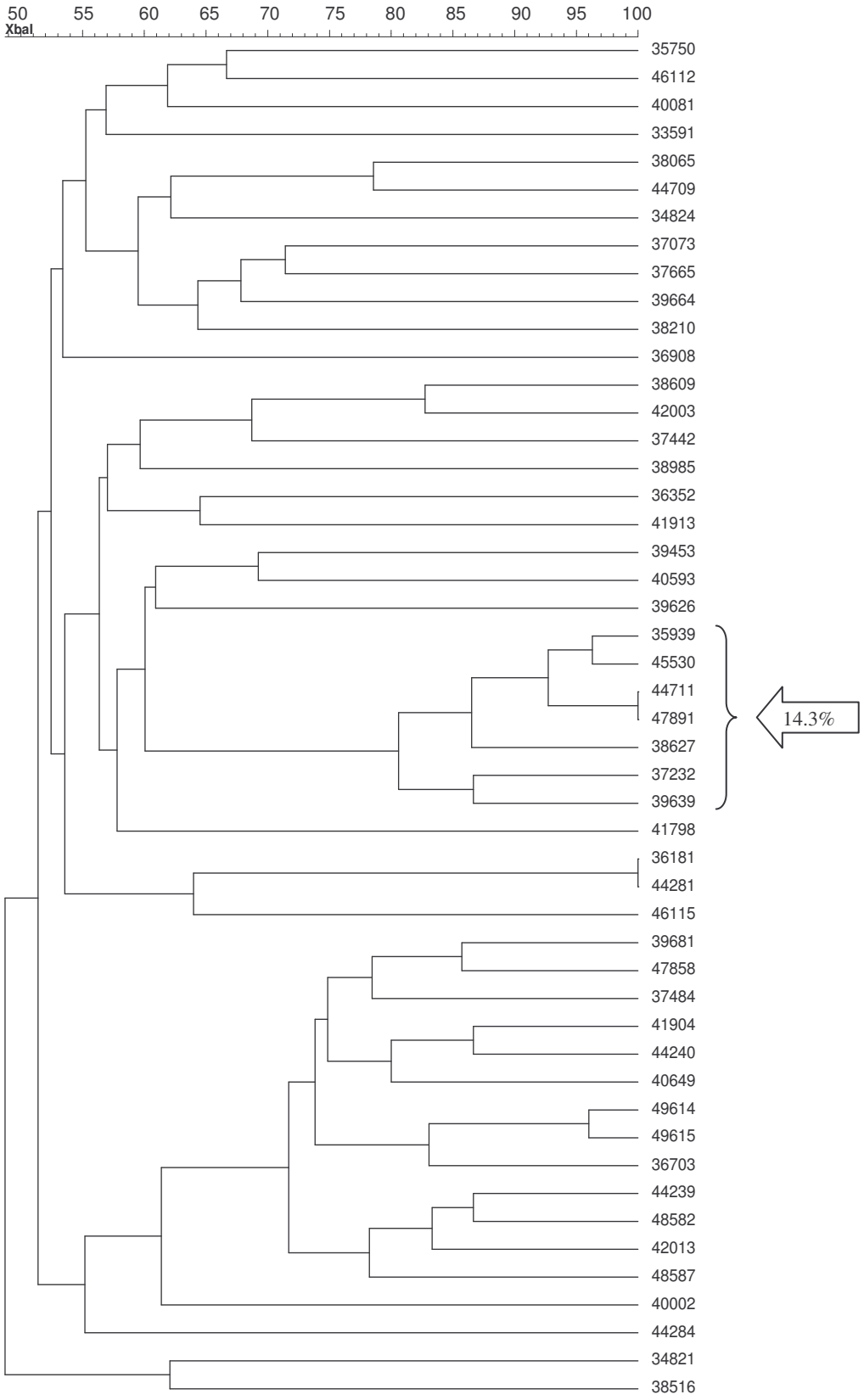

Figure 4. Dendrogram depicting the percent similarity of *Xba*1 restriction patterns of 49 *E. coli* isolates resistant to ampicillin + TMP/SMX and susceptible to fluoroquinolones.

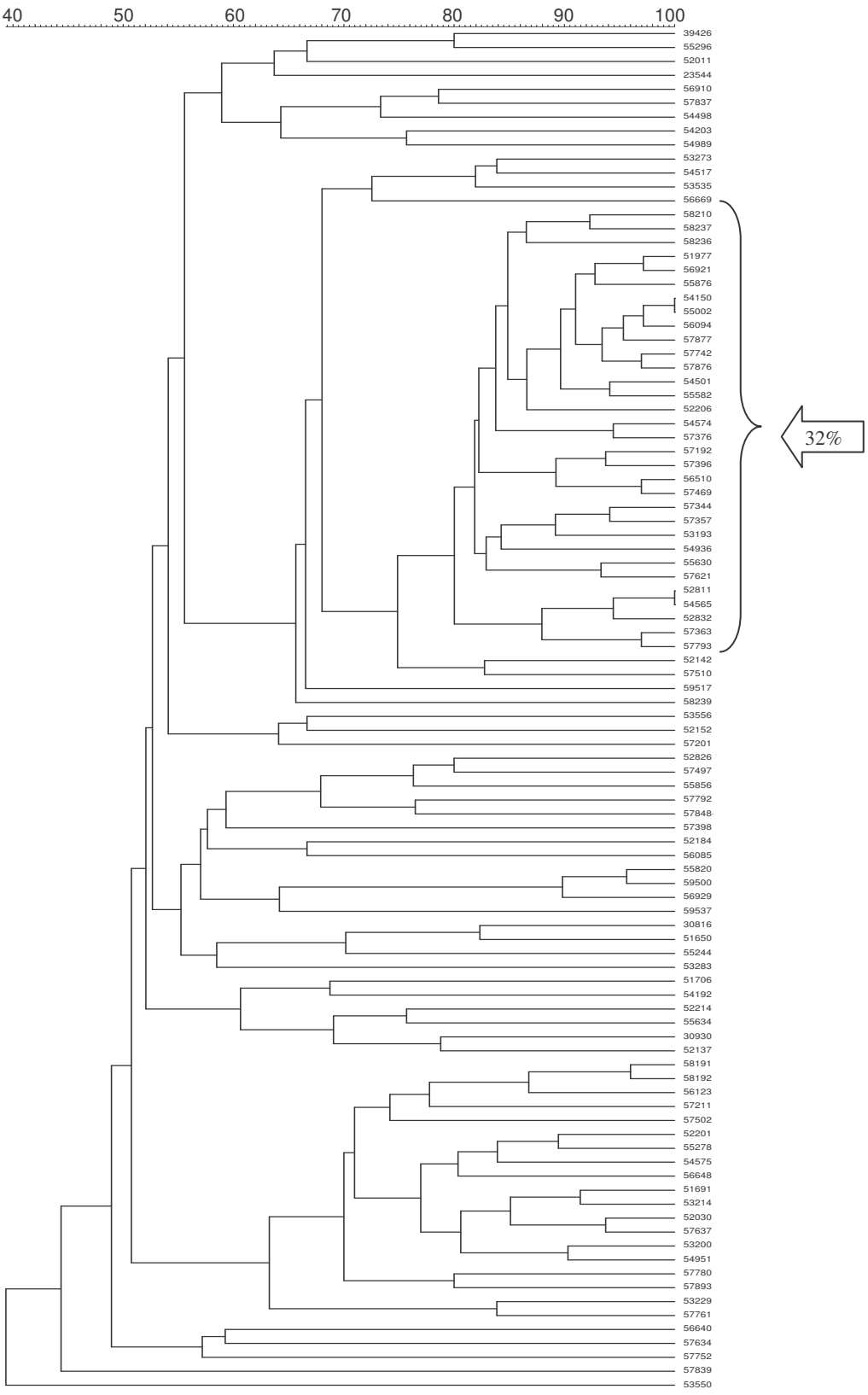

Figure 5. Dendrogram depicting the percent similarity of *Xba*1 restriction patterns of 100 *E. coli* isolates resistant to ampicillin + TMP/SMX + fluoroquinolones.

Discussion

Results from the present research indicate a higher degree of clonal spread amongst multi-drug-resistant isolates. When compared to isolates of the susceptible, ampicillin-resistant, TMP/SMX-resistant, and ampicillin + TMP/SMX-resistant phenotypes, MDR isolates showed a much greater level of genetic homogeneity, with 32% belonging to a single genetic clone.

PFGE revealed that though one large cluster of isolates was indicative of clonal spread, the majority of ampicillin resistant isolates were genetically diverse. This finding suggests that resistance to ampicillin in the community setting arose as either independent response of bacterial strains to the selective pressure of ampicillin exposure or through horizontal plasmid-mediated spread of ampicillin resistance genes, most likely those encoding for β–lactamase production. The spread of ampicillin resistance on plasmids is a well-documented phenomenon in *E. coli* and numerous other genera (Quintilliani *et al.,* 1999). Though the exact ampicillin resistance mechanism in the present isolates can only be speculated, as plasmid analysis was not conducted, the level of genetic diversity among the ampicillin resistant isolates indicated by the present results is not surprising.

The degree of genetic heterogeneity observed in the TMP/SMX-resistant isolates correlates well with the high rates of TMP/SMX resistance in *E. coli* isolates in North America (Burman *et al.,* 2003; Zhanel *et al.,* 2005). Since 1972, plasmid-mediated resistance to TMP/SMX has been documented. Transferable *dhfr* genes (DHFR - dihydrofolic acid reductase, the binding site for TMP), conferring high-level resistance to TMP, as well as altered *dhps* genes (DHPS - dihydropteroate synthetase, the binding site for SMX), which result in greatly decreased sensitivity to SMX, are well known in the

Enterobacteriaceae (Goldstein & Stein, 2002; Mascaretti, 2003). Though acquisition of chromosomal mutations cannot be ruled out, the horizontal transfer of these transposable elements is a likely explanation for the genetic diversity observed in TMP/SMX resistant isolates seen in the present study. For combination resistance phenotypes in *E. coli*, an ampicillin + TMP/SMX-resistant phenotype was found to be most common in several North American and European studies (Kahlmeter & Menday, 2003; Johnson *et al.,* 2005a; Zhanel 2005, unpublished data). Extended-spectrum beta lactamase (ESBL)-producing organisms typically have additional resistance genes, including those against TMP/SMX, encoded on the ESBL-associated plasmid (Branger *et al.,* 2005). Because isolates in this study did not undergo susceptibility testing against a β–lactam drug + β–lactamase inhibitor, it is impossible to determine whether the observed ampicillin resistance was caused by an ESBL or a simple penicillinase. However, the close association of plasmid-mediated TMP/SMX and ampicillin resistance provides evidence for horizontal transfer of this resistance phenotype in genetically diverse isolates, as was the overwhelming trend in the current study.

The increase in clonality seen in the MDR group from the ampicillin + TMP/SMX-R phenotype is very likely because the third drug is a fluoroquinolone. As opposed to plasmid-associated resistance against ampicillin and TMP/SMX, fluoroquinolone resistance is almost exclusively linked to acquired chromosomal mutations related to efflux or target modification under selective pressure of fluoroquinolone exposure. Further, high-level resistance to fluoroquinolones requires two consecutive mutations in the quinolone resistance-determining region (QRDR) of the bacterial genome (Komp Lindgren *et al.,* 2003). Therefore, resistance associated with

fluoroquinolones is indicative of over-exposure to these drugs, either at low levels or on multiple occasions to allow consecutive mutations to accrue. For these reasons, a higher degree of clonal spread would be expected among these isolates due to the difficulty of acquiring such resistance.

While QRDR mutations provide the greatest contributions to the acquisition of fluoroquinolone resistance in susceptible isolates, a novel gene, *qnr*A (formerly *qnr*), was recently discovered as a plasmid-mediated conveyer of quinolone resistance (Tran *et al.,* 2005a). By simultaneously protecting DNA gyrase and topoisomerase IV, the two bacterial targets of fluoroquinolones, the *qnr*A gene product can generate an 8- to 32-fold increase in quinolone MICs in the *Enterobacteriaceae.* Though the presence of the plasmid alone is insufficient to convert a susceptible strain into a resistant one, its presence facilitates the selection of isolates with single chromosomal mutations in the QRDR, allowing them to survive and acquire higher level resistance (Robicsek *et al.,* 2005; Tran *et al.,* 2005b). Additionally, the presence of *qnrA* is highly associated with resistance to additional antimicrobial classes, likely due to its location on an integron. Though the prevalence of *qnrA* in North America is quite low at present, its strong presence in countries where fluoroquinolone resistance rates have surpassed 50% is indicative of the increasing role it will play in the horizontal spread of fluoroquinolone and multi-drug resistance in North America (Wang *et al.,* 2003).

While UTIs are not typically thought of as outbreak type infections, dissemination of clonal groups of *E. coli* have been implicated in community-wide outbreaks of cystitis and pyelonephritis on a number of occasions. This is especially notable in resistant clonal groups. From 1987-1988, the *E. coli* clonal group

O15:K52:H1 caused a major community-wide outbreak of cystitis and pyelonephritis in London, England (Johnson & Russo, 2005, Manges *et al.,* 2006). More recently, the multi-drug resistant *E. coli* clonal group A was found responsible for 49% of TMP/SMX-resistant UTIs in a California university setting, and was additionally found in UTI patients from select Minnesota, Michigan, and Colorado communities (Manges *et al.,* 2006; Johnson *et al.,* 2005b). Similar trends have also been observed in Canadian sites, such as the outbreaks of clonally related β-lactamase producing CTX-M-14 strains of *E. coli* in the Calgary area from 2000-2002 (Pitout *et al.,* 2005).

Examples of clonal spread of pathogenic organisms are by no means limited to *E. coli*. Methicillin-resistant *Staphylococcus aureus* (MRSA) are highly antibiotic resistant pathogens implicated in a wide variety of nosocomial and community acquired infections, such as skin and soft tissue infections, which are not typically considered as highly communicable. Similar to the above examples of clonal spread of resistant *E. coli,* clonal groups of MRSA isolates have been well documented from community acquired infections in several European and North American studies (Hansen *et al.,* 2005; King *et al.,* 2006).

Important to the discussion of clonal spread of resistant isolates is an understanding of *E. coli*'s population genetics. Natural populations of *E. coli* are essentially clonal in arrangement, with a limited number of clones existing together in a given environment. Under low stress conditions, the level of chromosomal recombination is restricted in natural populations to such a degree that distinct cell lines remain as stable clones over long periods of time. This has been demonstrated in long-term studies surveying the fecal flora of healthy individuals, where successive groups of

transient strains of *E. coli* demonstrated unique genotypes, with no indicated relatedness to each other or to the resident strains. Further, the rate of phage-mediated and conjugative plasmid transfer of genes has been experimentally demonstrated to occur at very low rates in continuous cultures, at levels comparable to the mutation rate (10^{-8}/hr). Therefore, due to the low rates of recombination, natural populations are mixtures of more or less independent clonal groups. Human studies and experimental animal models have demonstrated that in general, transient strains of *E. coli* introduced from environmental sources are typically unable to establish themselves as resident members of the flora due to competition with resident strains. However, certain pathogenic strains may be better able to compete for internal adhesion sites or nutrients, granting them longer durations in the intestinal tract and extending the opportunity to induce extraintestinal infections. Such strains are associated with widespread sharing in unassociated hosts, spanning broad geographic locations (Selander *et al.,* 1987).

Resistance was once thought to impose a decreased fitness on a bacterium where in the absence of selective pressure it would be out-competed by susceptible strains. For antibiotic resistant strains, the loss of fitness has been interpreted as a decreased rate of transcription and translation due to the production of resistance gene products. This thereby slows the growth rate of such isolates, which would provide a competitive advantage for the faster-growing susceptible strains in the absence of the antibiotic (Hoyle *et al.,* 2006; Andersson, 2003). However, some resistance mutations seem to convey no direct cost to the bacterium, such as specific forms of streptomycin resistance in *E. coli* and fluoroquinolone resistance caused by *gyrA* and *parC* mutations in *Streptococcus pneumoniae* (Andersson, 2003). Additionally, many resistance genes are

under transcriptional regulation, expressed only in the presence of antibiotics, while others are linked with resistance genes for other antimicrobial classes, therefore their maintenance is ensured when exposed to any of the drugs whose resistance genes are closely linked (Salyers *et al.,* 2002). For these reasons the high proportion of genetically unique isolates with resistance to more than one drug, as observed in the present research, is more acceptable from a fitness point of view.

E. coli is among a select group of bacterial species known as mutators, which exhibit elevated mutation frequencies due to deficiencies in various DNA repair mechanisms. Mutators are able to achieve low and high-level antibiotic resistance at a greatly enhanced rate as they more readily undergo multiple chromosomal mutations. After analyzing *E. coli* isolates of various pathologic backgrounds, Denamur *et al.* (2002) demonstrated that UTI-causing isolates had the highest mutation frequencies. The ability of *E. coli* to undergo high levels of mutation leading to antibiotic resistance may shed some light into the great proportion of single drug and multi-drug resistant isolates examined in this research with no evidence of clonal dissemination.

Though nearly one third of MDR isolates evolved from a common ancestral strain, the fact that the remaining 68% of isolates were not of the same type is indicative of multiple or prolonged fluoroquinolone exposure experienced by these isolates. These findings indicate that strains of MDR *E. coli* will continue to be central in the causation of UTIs based on current trends of antibiotic usage. This solidifies the need for prudent use of antimicrobial agents in order to minimize the evolution of resistant strains. Further, these results indicate the requirement for new classes of antimicrobials with novel mechanisms of action to successfully combat uropathogens in future.

Conclusions

1. Isolates susceptible to ampicillin + trimethoprim-sulfamethoxazole + fluoroquinolones as well as those resistant to trimethoprim-sulfamethoxazole only showed the greatest levels of genetic diversity among all urinary *E. coli* isolates examined, providing no evidence of clonal dissemination.
2. Clonal spread of urinary *E. coli* isolates was responsible for a small percentage of infections caused by isolates resistant to ampicillin only and those resistant to ampicillin + trimethoprim-sulfamethoxazole.
3. Clonal dissemination played the greatest role in multi-drug resistant urinary *E. coli* infections, with 32% of isolates generated by a single ancestral strain.
4. These findings suggest that while clonal spread is involved in multi-drug resistant *E. coli*-mediated urinary tract infections, *de novo* acquisitions of resistance-conferring mutations plays a major role in the production of MDR isolates, presumably as a result of the overuse of antibiotics across North America.

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Appendix I - Stock number, city of origin, resistance phenotype, and resistance profiles against ampicillin, TMP/SMX, and fluoroquinolones for 347 *E. coli* isolates analyzed.

STOCK #	City of origin	Resistance Phenotype	Ampicillin MIC (μg/ml)	Ciprofloxacin MIC (μg/ml)	Levofloxacin MIC (μg/ml)	TMP/SMX MIC (μg/ml)
33468	Winnipeg	susceptible	2	0.03	0.03	0.25
33592	Winnipeg	susceptible	4	0.03	0.06	0.25
33597	Winnipeg	susceptible	4	0.03	0.03	0.25
34799	Edmonton	susceptible	4	0.03	0.03	0.25
34802	Edmonton	susceptible	4	0.03	0.03	0.25
34804	Edmonton	susceptible	4	0.03	0.03	0.25
35728	Moncton	susceptible	4	0.03	0.03	0.25
35729	Moncton	susceptible	2	0.03	0.06	0.25
35730	Moncton	susceptible	8	0.03	0.03	0.25
35936	Tempe	susceptible	4	0.03	0.03	1
35937	Tempe	susceptible	4	0.03	0.06	0.25
35946	Tempe	susceptible	4	0.03	0.03	0.25
36121	Cleveland	susceptible	4	0.03	0.03	0.25
36126	Cleveland	susceptible	4	0.03	0.03	0.25
36143	Cleveland	susceptible	4	0.03	0.03	0.25
36178	Wilmington	susceptible	4	0.12	0.5	0.25
36180	Wilmington	susceptible	4	0.03	0.03	0.25
36183	Wilmington	susceptible	4	0.03	0.03	0.25
36354	Jacksonville	susceptible	4	0.03	0.06	0.25
36357	Jacksonville	susceptible	4	0.03	0.06	0.25
36358	Jacksonville	susceptible	1	0.03	0.03	0.25
36694	Valhalla	susceptible	4	0.03	0.06	0.25
36696	Valhalla	susceptible	1	0.03	0.03	0.25
36711	Valhalla	susceptible	4	0.03	0.06	0.5
36881	Rochester	susceptible	4	0.03	0.03	0.25
36882	Rochester	susceptible	4	0.03	0.06	0.25
36883	Rochester	susceptible	4	0.03	0.06	0.25
37043	Portland	susceptible	4	0.03	0.03	0.25
37044	Portland	susceptible	4	0.03	0.03	0.25
37046	Portland	susceptible	4	0.03	0.06	0.25
37231	Los Angeles	susceptible	4	0.03	0.03	0.5
37235	Los Angeles	susceptible	4	0.03	0.03	0.25
37236	Los Angeles	susceptible	4	0.03	0.03	1
37431	Akron	susceptible	0.5	0.03	0.03	0.25
37437	Akron	susceptible	4	0.03	0.06	0.25
37439	Akron	susceptible	4	0.03	0.06	0.25
37481	New York	susceptible	2	0.03	0.03	0.25
37482	New York	susceptible	8	0.03	0.03	0.25
37494	New York	susceptible	4	0.03	0.06	0.25
37645	Galveston	susceptible	2	0.03	0.03	0.25
37647	Galveston	susceptible	4	0.03	0.06	0.25
37648	Galveston	susceptible	8	0.03	0.03	0.25

Appendix I - *Continued*

STOCK #	City of origin	Resistance Phenotype	Ampicillin MIC (μg/ml)	Ciprofloxacin MIC (μg/ml)	Levofloxacin MIC (μg/ml)	TMP/SMX MIC (μg/ml)
38051	Stanford	susceptible	4	0.03	0.03	0.25
38054	Stanford	susceptible	2	0.03	0.03	0.25
38056	Stanford	susceptible	4	0.03	0.03	0.25
38201	San Francisco	susceptible	4	0.25	0.5	0.25
38202	San Francisco	susceptible	4	0.03	0.03	0.25
38206	San Francisco	susceptible	4	0.03	0.03	0.25
38512	Toronto	susceptible	4	0.03	0.03	0.25
38517	Toronto	susceptible	4	0.03	0.03	0.25
38521	Toronto	susceptible	2	0.03	0.03	0.25
38600	Montreal	susceptible	2	0.03	0.03	0.25
38608	Montreal	susceptible	2	0.03	0.03	0.25
38950	Kansas City	susceptible	1	0.03	0.03	0.25
38952	Kansas City	susceptible	4	0.03	0.06	0.25
38956	Kansas City	susceptible	4	0.03	0.03	0.25
39444	San Antonio	susceptible	8	0.03	0.03	0.25
39455	San Antonio	susceptible	4	0.03	0.06	0.25
39596	Saskatoon	susceptible	4	0.03	0.03	0.25
39605	Saskatoon	susceptible	4	0.03	0.03	0.25
39651	Victoria	susceptible	4	0.03	0.06	0.25
39655	Victoria	susceptible	4	0.03	0.03	0.25
39845	Minneapolis	susceptible	4	0.03	0.03	1
39856	Minneapolis	susceptible	8	0.03	0.06	0.25
40005	Salt Lake City	susceptible	4	0.03	0.03	0.25
40007	Salt Lake City	susceptible	2	0.03	0.03	0.25
40067	New York	susceptible	2	0.06	0.03	0.25
40089	New York	susceptible	4	0.03	0.03	0.25
40586	St. Louis	susceptible	0.5	0.03	0.03	0.25
40588	St. Louis	susceptible	8	0.03	0.03	0.25
40589	St. Louis	susceptible	8	0.03	0.03	0.25
40650	Des Moines	susceptible	2	0.03	0.03	0.25
40652	Des Moines	susceptible	4	0.03	0.03	0.25
41751	Chapel Hill	susceptible	2	0.03	0.06	0.25
41753	Chapel Hill	susceptible	1	0.03	0.03	0.25
41901	Denver	susceptible	2	0.03	0.03	0.25
41902	Denver	susceptible	2	0.03	0.03	0.25
42001	Omaha	susceptible	8	0.03	0.06	0.25
42002	Omaha	susceptible	4	0.03	0.03	0.25
44233	Danville	susceptible	4	0.03	0.03	0.25
44235	Danville	susceptible	4	0.03	0.06	0.25
44288	Chicago	susceptible	4	0.03	0.06	0.25
44296	Chicago	susceptible	4	0.03	0.06	0.25

Appendix I - *Continued*

STOCK #	City of origin	Resistance Phenotype	Ampicillin MIC (µg/ml)	Ciprofloxacin MIC (µg/ml)	Levofloxacin MIC (µg/ml)	TMP/SMX MIC (µg/ml)
44592	Cleveland	susceptible	4	0.03	0.03	0.25
44593	Cleveland	susceptible	4	0.06	0.03	0.25
44691	Milwaukee	susceptible	2	0.03	0.03	0.25
44693	Milwaukee	susceptible	4	0.03	0.03	0.25
45497	Halifax	susceptible	1	0.03	0.03	0.25
45501	Halifax	susceptible	8	0.03	0.03	0.25
45502	Halifax	susceptible	8	0.03	0.03	0.25
46108	Gainesville	susceptible	4	0.03	0.06	0.25
46489	Sherbrooke	susceptible	4	0.5	0.25	0.25
46492	Sherbrooke	susceptible	8	0.03	0.03	0.25
47860	Macon	susceptible	2	0.03	0.03	0.25
47862	Macon	susceptible	2	0.06	0.03	0.25
48554	Atlanta	susceptible	2	0.03	0.03	0.25
48561	Atlanta	susceptible	4	0.03	0.03	0.25
48596	Atlanta	susceptible	4	0.06	0.03	0.25
49597	Ottawa	susceptible	2	0.03	0.03	0.25
33876	Winnipeg	TMP/SMX-R	4	0.03	0.03	1024
33942	Winnipeg	TMP/SMX-R	4	0.03	0.03	128
34288	Winnipeg	TMP/SMX-R	8	0.03	0.06	1024
34290	Winnipeg	TMP/SMX-R	4	0.03	0.03	256
34292	Winnipeg	TMP/SMX-R	4	0.03	0.06	256
35048	Winnipeg	TMP/SMX-R	4	0.03	0.03	512
36138	Cleveland	TMP/SMX-R	0.5	0.12	0.12	8
36168	Cleveland	TMP/SMX-R	4	0.03	0.03	1024
36175	Wilmington	TMP/SMX-R	4	0.03	0.03	512
36184	Wilmington	TMP/SMX-R	8	0.03	0.03	128
36350	Jacksonville	TMP/SMX-R	8	0.03	0.06	256
36355	Jacksonville	TMP/SMX-R	4	0.03	0.03	64
36724	Valhalla	TMP/SMX-R	4	0.03	0.03	512
36738	Valhalla	TMP/SMX-R	4	0.03	0.03	256
36743	Valhalla	TMP/SMX-R	4	0.03	0.03	64
36898	Rochester	TMP/SMX-R	4	0.03	0.06	4
36921	Rochester	TMP/SMX-R	4	0.03	0.03	64
37080	Portland	TMP/SMX-R	4	0.03	0.03	256
37228	Los Angeles	TMP/SMX-R	4	0.03	0.03	256
37234	Los Angeles	TMP/SMX-R	4	0.03	0.03	1024
37456	Akron	TMP/SMX-R	4	0.03	0.06	64
37461	Akron	TMP/SMX-R	1	0.03	0.03	256
37466	Akron	TMP/SMX-R	8	0.03	0.06	1024
37468	Akron	TMP/SMX-R	4	0.03	0.03	256
37487	New York	TMP/SMX-R	4	0.03	0.03	1024
37490	New York	TMP/SMX-R	4	0.03	0.06	64
37651	Galveston	TMP/SMX-R	2	0.03	0.03	128
37664	Galveston	TMP/SMX-R	4	0.03	0.03	1024

Appendix I - *Continued*

STOCK #	City of origin	Resistance Phenotype	Ampicillin MIC (μg/ml)	Ciprofloxacin MIC (μg/ml)	Levofloxacin MIC (μg/ml)	TMP/SMX MIC (μg/ml)
37691	Galveston	TMP/SMX-R	2	0.03	0.03	128
38058	Stanford	TMP/SMX-R	2	0.03	0.03	64
38513	Toronto	TMP/SMX-R	2	0.03	0.03	256
38630	Montreal	TMP/SMX-R	2	0.03	0.03	256
39480	San Antonio	TMP/SMX-R	4	0.03	0.03	512
39659	Victoria	TMP/SMX-R	4	0.03	0.06	256
39688	Victoria	TMP/SMX-R	4	1	1	1024
39842	Minneapolis	TMP/SMX-R	8	0.03	0.03	1024
39858	Minneapolis	TMP/SMX-R	4	0.03	0.03	256
40037	Salt Lake City	TMP/SMX-R	2	0.03	0.03	1024
40683	Des Moines	TMP/SMX-R	4	0.03	0.03	1024
41790	Chapel Hill	TMP/SMX-R	4	0.03	0.03	256
44238	Danville	TMP/SMX-R	4	0.03	0.03	256
44697	Milwaukee	TMP/SMX-R	4	0.03	0.06	512
44698	Milwaukee	TMP/SMX-R	1	0.03	0.03	256
45498	Halifax	TMP/SMX-R	1	0.12	0.25	4
45523	Halifax	TMP/SMX-R	8	0.03	0.03	1024
47872	Macon	TMP/SMX-R	8	0.03	0.06	128
48570	Atlanta	TMP/SMX-R	4	0.03	0.06	128
49622	Ottawa	TMP/SMX-R	4	0.03	0.03	64
33594	Winnipeg	Ampi-R	512	0.03	0.03	0.25
33601	Winnipeg	Ampi-R	512	0.03	0.03	0.5
34812	Edmonton	Ampi-R	512	0.03	0.06	0.25
34832	Edmonton	Ampi-R	512	0.03	0.03	0.25
35733	Moncton	Ampi-R	256	0.03	0.03	0.25
35737	Moncton	Ampi-R	256	0.03	0.03	0.25
35952	Tempe	Ampi-R	512	0.03	0.06	0.25
35953	Tempe	Ampi-R	1024	0.03	0.06	0.25
36127	Cleveland	Ampi-R	512	0.03	0.06	0.25
36132	Cleveland	Ampi-R	512	0.03	0.06	0.25
36173	Wilmington	Ampi-R	512	0.03	0.03	0.25
36177	Wilmington	Ampi-R	512	0.03	0.06	0.25
36368	Jacksonville	Ampi-R	>1024	0.03	0.06	0.5
36376	Jacksonville	Ampi-R	512	0.03	0.03	0.25
36880	Rochester	Ampi-R	256	0.03	0.03	0.25
36884	Rochester	Ampi-R	512	0.03	0.03	0.25
37036	Portland	Ampi-R	512	0.03	0.06	0.25
37238	Los Angeles	Ampi-R	512	0.03	0.03	2
37433	Akron	Ampi-R	512	0.03	0.06	0.25
37483	New York	Ampi-R	64	0.25	0.5	0.25
37663	Galveston	Ampi-R	32	1	1	0.25
38067	Stanford	Ampi-R	1024	0.03	0.03	0.25
38204	San Francisco	Ampi-R	256	0.03	0.03	0.25

Appendix I - *Continued*

STOCK #	City of origin	Resistance Phenotype	Ampicillin MIC (μg/ml)	Ciprofloxacin MIC (μg/ml)	Levofloxacin MIC (μg/ml)	TMP/SMX MIC (μg/ml)
38519	Toronto	Ampi-R	32	0.03	0.06	0.5
38541	Toronto	Ampi-R	32	0.03	0.06	0.25
38606	Montreal	Ampi-R	256	0.03	0.03	0.25
38607	Montreal	Ampi-R	1024	0.03	0.03	0.25
38958	Kansas City	Ampi-R	512	0.03	0.03	2
39451	San Antonio	Ampi-R	128	0.03	0.03	0.25
39608	Saskatoon	Ampi-R	256	0.03	0.06	0.5
39609	Saskatoon	Ampi-R	>1024	0.03	0.03	0.5
39652	Victoria	Ampi-R	256	0.03	0.03	1
39843	Minneapolis	Ampi-R	1024	0.03	0.03	0.5
40012	Salt Lake City	Ampi-R	512	0.03	0.06	0.25
40054	New York	Ampi-R	1024	0.03	0.03	0.25
40587	St. Louis	Ampi-R	32	0.03	0.03	0.25
40647	Des Moines	Ampi-R	512	0.03	0.06	0.5
41907	Denver	Ampi-R	1024	0.03	0.06	0.25
42012	Omaha	Ampi-R	1024	0.03	0.06	0.25
44245	Danville	Ampi-R	512	0.03	0.06	0.25
44282	Chicago	Ampi-R	512	0.03	0.06	0.25
44594	Cleveland	Ampi-R	512	0.03	0.06	0.25
44700	Milwaukee	Ampi-R	128	0.03	0.03	0.25
45495	Halifax	Ampi-R	512	0.03	0.03	0.5
46123	Gainesville	Ampi-R	512	0.03	0.06	0.25
46497	Sherbrooke	Ampi-R	512	0.03	0.03	0.25
47855	Macon	Ampi-R	512	0.03	0.03	0.25
48557	Atlanta	Ampi-R	>1024	0.03	0.03	0.25
49600	Ottawa	Ampi-R	256	0.03	0.06	1
33591	Winnipeg	Ampi + TMP/SMX-R	512	0.03	0.03	1024
34821	Edmonton	Ampi + TMP/SMX-R	>1024	0.03	0.03	256
34824	Edmonton	Ampi + TMP/SMX-R	1024	0.03	0.03	1024
35750	Moncton	Ampi + TMP/SMX-R	1024	0.03	0.03	1024
35939	Tempe	Ampi + TMP/SMX-R	>1024	0.03	0.06	1024
36181	Wilmington	Ampi + TMP/SMX-R	128	0.03	0.06	256
36352	Jacksonville	Ampi + TMP/SMX-R	1024	0.03	0.06	128
36703	Valhalla	Ampi + TMP/SMX-R	256	0.03	0.03	1024
36908	Rochester	Ampi + TMP/SMX-R	128	0.03	0.06	1024
37073	Portland	Ampi + TMP/SMX-R	128	0.03	0.03	512

Appendix I - *Continued*

STOCK #	City of origin	Resistance Phenotype	Ampicillin MIC (μg/ml)	Ciprofloxacin MIC (μg/ml)	Levofloxacin MIC (μg/ml)	TMP/SMX MIC (μg/ml)
37232	Los Angeles	Ampi + TMP/SMX-R	32	0.5	0.5	128
37442	Akron	Ampi + TMP/SMX-R	128	0.03	0.06	128
37484	New York	Ampi + TMP/SMX-R	1024	0.03	0.03	512
37665	Galveston	Ampi + TMP/SMX-R	256	0.25	0.25	1024
38065	Stanford	Ampi + TMP/SMX-R	1024	0.03	0.03	128
38210	San Francisco	Ampi + TMP/SMX-R	256	0.03	0.03	1024
38516	Toronto	Ampi + TMP/SMX-R	>1024	0.03	0.06	128
38609	Montreal	Ampi + TMP/SMX-R	1024	1	1	1024
38627	Montreal	Ampi + TMP/SMX-R	512	0.03	0.03	1024
38985	Kansas City	Ampi + TMP/SMX-R	256	0.03	0.03	256
39453	San Antonio	Ampi + TMP/SMX-R	>1024	0.03	0.06	1024
39626	Saskatoon	Ampi + TMP/SMX-R	>1024	0.03	0.03	1024
39639	Saskatoon	Ampi + TMP/SMX-R	512	0.03	0.03	1024
39664	Victoria	Ampi + TMP/SMX-R	512	0.03	0.03	256
39681	Victoria	Ampi + TMP/SMX-R	256	0.06	0.06	1024
39844	Minneapolis	Ampi + TMP/SMX-R	>1024	0.03	0.03	1024
40002	Salt Lake City	Ampi + TMP/SMX-R	512	0.03	0.03	64
40081	New York	Ampi + TMP/SMX-R	1024	0.03	0.03	1024
40593	St. Louis	Ampi + TMP/SMX-R	512	0.03	0.06	1024
40649	Des Moines	Ampi + TMP/SMX-R	64	1	0.5	1024
41798	Chapel Hill	Ampi + TMP/SMX-R	1024	0.03	0.06	1024
41904	Denver	Ampi + TMP/SMX-R	128	1	1	1024
41913	Denver	Ampi + TMP/SMX-R	1024	0.03	0.03	1024
42003	Omaha	Ampi + TMP/SMX-R	128	0.03	0.03	1024
42013	Omaha	Ampi + TMP/SMX-R	512	0.03	0.03	1024

Appendix I - *Continued*

STOCK #	City of origin	Resistance Phenotype	Ampicillin MIC (μg/ml)	Ciprofloxacin MIC (μg/ml)	Levofloxacin MIC (μg/ml)	TMP/SMX MIC (μg/ml)
44240	Danville	Ampi + TMP/SMX-R	256	0.03	0.03	1024
44281	Chicago	Ampi + TMP/SMX-R	256	0.03	0.03	1024
44284	Chicago	Ampi + TMP/SMX-R	256	0.03	0.03	512
44709	Milwaukee	Ampi + TMP/SMX-R	512	0.03	0.03	128
44711	Milwaukee	Ampi + TMP/SMX-R	256	0.03	0.03	1024
45530	Halifax	Ampi + TMP/SMX-R	256	0.03	0.03	1024
46112	Gainesville	Ampi + TMP/SMX-R	>1024	0.03	0.03	64
46115	Gainesville	Ampi + TMP/SMX-R	128	0.03	0.03	64
47858	Macon	Ampi + TMP/SMX-R	256	0.03	0.03	64
47891	Macon	Ampi + TMP/SMX-R	256	0.03	0.06	128
48582	Atlanta	Ampi + TMP/SMX-R	1024	0.03	0.03	64
48587	Atlanta	Ampi + TMP/SMX-R	512	0.03	0.06	128
49614	Ottawa	Ampi + TMP/SMX-R	256	0.03	0.03	64
49615	Ottawa	Ampi + TMP/SMX-R	512	0.03	0.06	64
23544	Winnipeg	MDR	512	8	8	1024
30930	Winnipeg	MDR	1024	64	64	1024
39426	Winnipeg	MDR	512	64	32	8
30816	Winnipeg	MDR	> 1024	16	8	256
48201	Winnipeg	MDR	> 1024	32	16	1024
49947	Winnipeg	MDR	> 1024	64	32	1024
51650	New York	MDR	256	32	16	256
51691	New York	MDR	512	16	8	512
51706	New York	MDR	128	32	16	512
51997	Rochester	MDR	1024	32	16	1024
52011	Rochester	MDR	> 1024	16	16	1024
52030	Rochester	MDR	> 1024	16	16	1024
52137	Tempe	MDR	256	16	16	1024
52142	Tempe	MDR	256	8	8	1024
52152	Tempe	MDR	256	64	16	1024
52184	Tempe	MDR	256	8	8	1024
52201	Montreal	MDR	> 1024	16	8	1024
52206	Montreal	MDR	1024	32	16	512
52214	Montreal	MDR	512	32	32	1024
52811	Victoria	MDR	1024	32	16	512

Appendix I - *Continued*

STOCK #	City of origin	Resistance Phenotype	Ampicillin MIC (μg/ml)	Ciprofloxacin MIC (μg/ml)	Levofloxacin MIC (μg/ml)	TMP/SMX MIC (μg/ml)
52826	Victoria	MDR	256	64	16	512
52832	Victoria	MDR	1024	32	16	512
53193	New York	MDR	1024	32	16	1024
53200	New York	MDR	> 1024	128	32	128
53214	New York	MDR	512	16	16	1024
53229	Los Angeles	MDR	256	32	16	512
53273	Los Angeles	MDR	1024	32	16	256
53283	Los Angeles	MDR	512	64	32	1024
53535	Saskatoon	MDR	512	64	16	256
53550	Saskatoon	MDR	1024	128	64	512
53556	Saskatoon	MDR	256	32	16	512
54150	Galveston	MDR	512	32	16	1024
54192	Galveston	MDR	> 1024	64	32	512
54498	San Antonio	MDR	256	128	32	256
54501	San Antonio	MDR	512	32	16	1024
54517	San Antonio	MDR	1024	32	16	1024
54565	Edmonton	MDR	512	32	16	1024
54574	Edmonton	MDR	256	32	16	1024
54575	Edmonton	MDR	512	16	8	1024
54936	Akron	MDR	512	32	16	1024
54951	Akron	MDR	512	16	8	1024
54989	Akron	MDR	512	32	16	512
55002	Akron	MDR	256	64	32	1024
55244	Stanford	MDR	256	32	16	256
55278	Stanford	MDR	> 1024	32	16	256
55296	Stanford	MDR	> 1024	256	128	256
55582	Knoxville	MDR	> 1024	64	32	256
55630	Knoxville	MDR	> 1024	32	16	256
55634	Knoxville	MDR	1024	32	16	256
55820	Des Moines	MDR	512	64	32	256
55856	Des Moines	MDR	512	64	32	512
55876	Des Moines	MDR	1024	64	16	256
56085	Danville	MDR	512	128	32	128
56094	Danville	MDR	512	32	8	512
56123	Danville	MDR	512	16	8	256
56510	Moncton	MDR	512	64	32	256
57469	Moncton	MDR	256	32	16	256
56640	Jacksonville	MDR	> 1024	32	16	256
56648	Jacksonville	MDR	256	32	16	256
56669	Jacksonville	MDR	512	64	16	4
56910	St. Louis	MDR	512	32	16	256
56921	St. Louis	MDR	> 1024	32	16	256
56929	St. Louis	MDR	128	128	32	512
57192	Oklahoma City	MDR	512	32	16	256

Appendix I - *Continued*

STOCK #	City of origin	Resistance Phenotype	Ampicillin MIC (μg/ml)	Ciprofloxacin MIC (μg/ml)	Levofloxacin MIC (μg/ml)	TMP/SMX MIC (μg/ml)
57201	Oklahoma City	MDR	>1024	32	64	512
57211	Oklahoma City	MDR	256	32	16	256
57344	Pawtucket	MDR	512	32	16	256
57357	Pawtucket	MDR	256	32	16	512
57363	Pawtucket	MDR	256	32	16	256
57376	Halifax	MDR	512	64	32	256
57396	Halifax	MDR	256	32	16	256
57398	Halifax	MDR	512	64	32	256
57469	Moncton	MDR	256	32	16	256
57497	Toronto	MDR	64	128	32	256
57502	Toronto	MDR	256	16	8	256
57510	Toronto	MDR	512	256	32	128
57621	Salt Lake City	MDR	> 1024	64	16	256
57634	Salt Lake City	MDR	256	64	32	256
57637	Salt Lake City	MDR	256	16	8	256
57742	San Francisco	MDR	1024	32	16	256
57752	San Francisco	MDR	512	64	32	256
57761	San Francisco	MDR	1024	64	64	512
57780	Denver	MDR	512	16	8	256
57792	Denver	MDR	128	64	32	256
57793	Denver	MDR	512	64	32	256
57837	Laebanon	MDR	512	128	16	256
57839	Laebanon	MDR	512	> 256	64	256
57849	Laebanon	MDR	256	64	16	256
57876	Omaha	MDR	512	32	16	256
57877	Omaha	MDR	512	64	16	256
57893	Omaha	MDR	> 1024	32	16	256
58191	Lexington	MDR	1024	32	16	256
58192	Lexington	MDR	512	32	16	256
58210	Lexington	MDR	256	64	16	256
58236	Chicago	MDR	512	32	16	256
58237	Chicago	MDR	256	32	32	256
58239	Chicago	MDR	128	128	64	256
59500	Burlington	MDR	1024	64	16	256
59517	Burlington	MDR	1024	128	16	256
59537	Burlington	MDR	1024	> 256	64	256

Appendix II - Compounds and solutions required for preparation of all buffer mixtures used in experimental procedures.

10X TBE

108.0g Tris Base (Lot # 0633B7, EMD Chemicals, INC)
55.0g Boric Acid (Lot # 2592B27, EMD Chemicals, INC)
8.3g EDTA (Lot # 1278944, Invitrogen)
distilled H_20 – QS to 1L

The final pH should be 8.3 $\pm$ 0.3, which cannot be adjusted. The buffer must be remade if the pH falls beyond this range. Autoclave following preparation.

0.5X TBE

Dilute 10X TBE 1:20 (100ml 10X TBE in 1900ml distilled H_20)

Cell Lysis Buffer

25ml 2M Tris-HCl (pH 7.6)
100ml 0.5M EDTA (pH 7.5)
50ml 20% Sacrosyl
825ml Sterile distilled H_20

This buffer does not require autoclaving if prepared using sterile glassware.

Cell Suspension Buffer

50ml 2M Tris-HCl (pH 7.6)
200ml 0.5M EDTA (pH 7.5)
750ml sterile distilled H_2O

This buffer does not require autoclaving if prepared using sterile glassware.

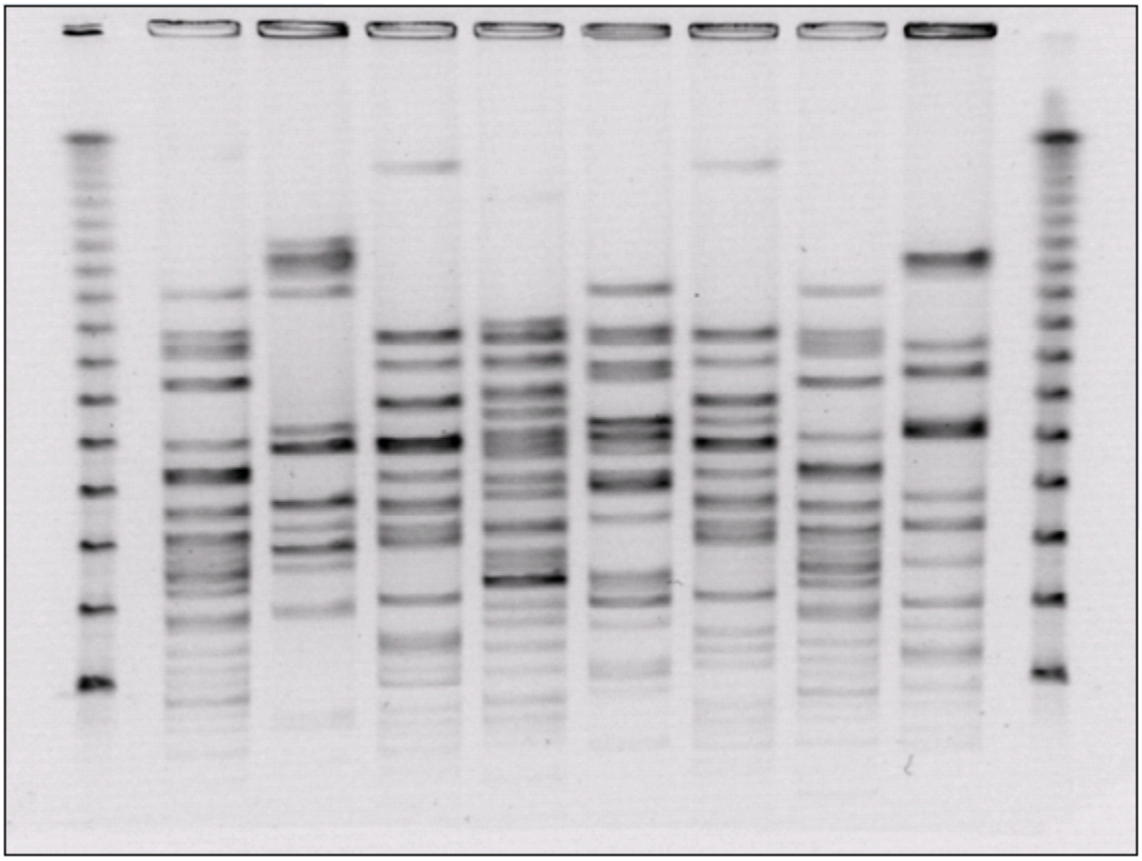
$T_{10}E_1$ (TE) Buffer

1.57g Tris-HCl (Lot # 9328F, MP Biomedicals)
0.37g EDTA (Lot # 1278944, Invitrogen)
distilled H_20 – QS to 1L

The final pH should be 8.0 and may require adjusting. Autoclave following preparation.

Xba1 Buffer mixture

The following restriction enzyme/buffer mixture was prepared fresh, immediately prior to plug digestion on the day of electrophoresis.

2800 µL sterile distilled water
320 µL 10X NEBuffer 2 (New England Biolabs Inc., Missasauga, ON)
32 µL of 10mg/mL Purified BSA (New England Biolabs Inc., Missasauga, ON)
26.4 µL of 15U/µL *Xba*I

Appendix III - One of the 47 Gel Doc 1000®-photographed ten-well gels used for comparison of banding patterns among experimental isolates. Outer lanes represent lambda ladders/molecular weight markers, while the eight inner lanes represent eight unique experimental isolates.