The Effect of Mutations in Type II Topoisomerases on Fluoroquinolone Resistance in Clinical Canine Urine *Escherichia coli* Isolates

by

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Abstract

A series of experiments were preformed in order to validate a rapid FRET-PCR based assay for the detection of fluoroquinolone resistance in small animal *Escherichia coli* urinary tract infection (UTI) isolates. Three hundred and six canine UTI *E. coli* isolates from pure culture were subjected to the FRET-PCR assay. Fourty-three of 50 enrofloxacin resistant isolates were detected by FRET-PCR for a sensitivity of 86% and a specificity of 97%. Urine was then spiked with 7 isolates of varying minimum inhibitory concentration for enrofloxacin (MIC_{Enro}) to evaluate sensitivity of detection and resistant isolates were detected at concentrations as small as 10^3 CFUs. Lastly DNA extracted from 438 small animal urine samples was subjected to the FRET-PCR assay. Two hundred and seventy-eight were confirmed to contain *E. coli*, 18 of which were resistant to enrofloxacin based on susceptibility testing. The FRET assay positively identified 15 of 18 enrofloxacin resistant urine samples (sensitivity of 83.33%) and negatively identified 388 of 420 samples (specificity of 92.36%). When compared to FRET run on DNA extracted from isolates, isolates had better specificity and sensitivity than FRET run on DNA extracted from urine samples.

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CHAPTER I

LITERATURE REVIEW: GENETIC FACTORS INFLUENCING FLUOROQUINOLONE RESISTANCE IN *ESCHERICHIA COLI*

Topoisomerases

Topoisomerases are enzymes responsible for controlling the tension of supercoiled DNA by facilitating the winding and unwinding during DNA replication and transcription. Winding and unwinding is especially important to reducing tension in front of the replication fork where the progress of the helicase and DNA polymerase machinery cause large amounts of force on the downstream DNA. Two classes of toposiomerases; Type I and Type II have been described.

Type I topoisomerases are monomer proteins that cut and reanneal single stands of double stranded DNA, allowing for a change in the linking number by +1 or -1 coils to the double helix. Type I can be further broken down into three subclasses: Type IA, Type IB, and Type IC. Type IA's structure resembles a lock and makes a break in the DNA to form a 5' phosphotyrosine intermediate. A strand or duplex of DNA is then passed through the break before reannealing the strands back together, thus introducing a positive or negative coil in an ATP independent process. In *E. coli*, the gene that codes for this enzyme is referred to as *topA*. Type IB and IC both work in a rotary fashion by nicking the double stranded DNA and forming a 3' phosphotyrosine intermediate while allowing the torque of the wound DNA to control the unwinding of the DNA until the single strands are reannealed. This process is also ATP independent. Type IB is coded for by *topB* in *E. coli* (Dean et al, 1983).

Type II topoisomerases are multimer proteins that cut and reanneal double strands of DNA, allowing for a change in the linking number by +2 or -2 coils to the double helix in an ATP dependant process. Type II can also be broken into subclasses Type IIA and Type IIB. Type IIA includes bacterial DNA gyrase and bacterial topoisomerase IV (topo IV) while Type IIB are only found in archaea and higher plants. *E. coli* DNA gyrase is a heterodimer coded for by genes *gyrAB,* while topo IV is of similar design but coded for by *parCE*. Their structure consists of an ATPase domain, a Rossmann fold (a motif that binds nucleotides such as NAD and FMN), a DNA binding domain, and a variable C terminus (Watt et al, 1994).

DNA gyrase is solely responsible for relaxing positive supercoils ahead of the DNA replication fork. Topo IV, however, has an extra function in the cell. In addition to working like DNA gyrase to remove positive supercoils, it also has decatenating activity, being responsible for separating the daughter chromosome from the parent chromosome at the end of replication so that cell division can occur (Kato et al, 1990).

Topoisomerases are the target of the quinolone drug class of antimicrobials. Quinolones interfere with DNA replication and RNA transcription by targeting the DNA/Topoisomerase duplex. Two quinolone molecules bind to the duplex (Yoshida et. al. 1993) and DNA is then cleaved by topoisomerase; however, religation of the double stranded break is inhibited and the unreligated DNA/topoisomerase complex is trapped within a DNA/topoisomerase/quinolone ternary complex (Critchlow and Maxwell, 1996) (Anderson et.al.1998). The Topoisomerase is unable to reanneal and religate the DNA strands back together, causing a lethal SOS response by the cell. Topoisomerase IV is also inhibited similarly in its concatamer releasing activity. In gram-negative bacteria, DNA gyrase is the primary target for quinolones while for gram-positive bacteria topoisomerase IV is the primary target. In *E. coli* the effects of quinolones on

topoisomerase IV appear to be more bacteriostatic as opposed to the bacteriacidal effects associated with DNA gyrase. (Khodursky, 1995)

Quinolones and Drug Development

Development of drugs in the quinolone class began with the discovery of naladixic acid in 1962 (Lesher, et. al, 1962). Discovered while producing chloroquine, an antimalarial drug as a derivative of 1,8-Naphthyridine, naladixic acid was found to be an effective antimicrobial against Enterobacteriaceae. Naladixic acid was followed by oxolinic acid (Turner, et. al., 1967), cinoxacin (Wick, et. al., 1973), and pipemidic acid (Shimizu, et. al.,1975); together, these drugs comprised the first generation of quinolone drugs. By 1963, naladixic acid-induced resistance in patients with *E. coli* urinary tract infections was observed (Barlow, 1963).

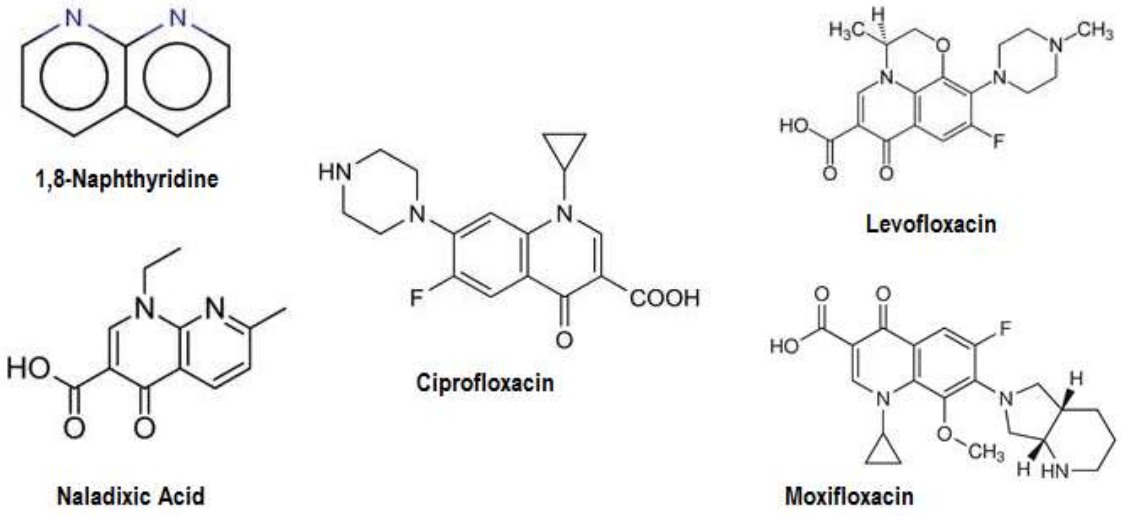

Figure 1: Progression of quinolone structure through generations. 1,8-napthyridine is core molecule leading to the first generation quinolones (Naladixic acid), second generation quinolones (ciprofloxacin), third generation quinolones (levofloxacin) and fourth generation quinolones (moxifloxacin).

The second generation of quinolones marks the advent of fluoroquinolones in which a fluorine atom was added to C6 and the methyl group at C7 was replaced with a piperazine group. These changes increased bactericidal potency by improving cell penetration and binding to the DNA/Gyrase complex (Chu and Fernandes, 1989). This second generation was further divided into two classes. Class 1 includes norfloxacin, the first fluoroquinolone to be approved for use in humans in the United States, (Ito et. al., 1980), lomefloxacin (Hirose et. al. 1987), and enoxacin whose spectrum are similar to first generation. Class two includes ciprofloxacin, enrofloxacin and ofloxacin, each of which is characterized by a broader spectrum of microbial targets, including atypical pathogens (e.g., *Bacillus anthracis*, *Yersinia pestis*, *Vibrio cholerae*) and *Pseudomonas aeruginosa*. Other R group adjustments in the class 2 fluoroquinolones included replacement of the N1 ethyl group with a cyclopropane (figure1), which allowed for ciprofloxacin's increased bioavailability, allowing more convenient usage of these antimicrobial on systemic infections (Domagala, 1994). However, even as second generation quinolones were synthesized, new patterns of resistance began to emerge. These quinolones were shown to cause cross-resistance with each other as well as the original class of quinolones (Barry and Jones, 1984). In 1984, Sanders et al. showed that *Klebsiella pneumoniae* mutant isolates selected with naladixic acid, ciprofloxacin, and norfloxacin also expressed resistance to antibiotics in the beta-lactam class (Sanders et. al, 1984). For gram positive isolates, resistance to second generation quinolones was quickly detected in *Staphylococcus aureus*. This resistance emerged because single nucleotide polymorphism (SNP) mutations increased their MIC to concentrations higher than could be achieved in serum at recommended doses. In *S. aureus*, resistance emerged more quickly in methicillin resistant (MRSA) strains (Blumberg, et. al 1991). In a study conducted at Atlanta Veteran's Medical Center, MRSA was observed within 3 months of introducing

ciprofloxacin as a treatment. In methicillin susceptible (MSSA) strains resistance was observed within 7 months of introducing ciprofloxacin as a treatment.

With the coming of third generation quinolones the antimicrobial properties were extended to *Streptococcus*. Development of sparfloxacin (Nakamura et. al., 1989), levofloxacin (the l- enantiomer form of ofloxacin) (Tanaka et al, 1992), grepafloxacin (Imada et. al., 1992), Marbofloxacin, and temafloxacin involved modifications such as methyl groups to the piperazine ring at C7. These methyl groups reduced central nervous system adverse reactions in the patient and the potential for drug interactions, while improving activity against gram positive organisms (Domagala, 1994). Once again, not long after levofloxacin was introduced in 1992 as a treatment for *Streptococcus pneumoniae*, resistance induced by its use was observed (Laferedo et al., 1993). In addition, cross resistance with ciprofloxacin was also observed.

The fourth generation quinolones currently are a rising group of fluoroquinolones including the drugs gatifloxacin (Hosaka et. al, 1992), moxifloxacin (Dalhoff et. al., 1996) trovafloxacin, and clinafloxacin. These drugs act dually on DNA gyrase as well as topoisomerase IV slowing emerging resistance. Additionally, trovafloxacin's substitution of a difluorophenyl group at N8 and clinifloxacin's addition of a chlorine atom at C8 accounts for their heightened activity against *Bacteroides fragilis* (Ashina et al. 1992) (Hecht et. al 1996).

Mechanisms of Quinolone Resistance

E. coli is a common cause of urinary tract infections (UTI). Antibioctic resistant *E. coli* is increasingly identified in association with both UTI and nosocomial infections in human and veterinary teaching hospitals. An increase in fluroquinolone resistance in particular has been reported and this fluoroquinolone resistance is progressively more associated with MDR (Cohn

et. al. 2003) (Boothe et al., 2006) (Shaheen, et. al., 2010). An important risk factor associated with the emergence of fluroquinolone resistance is use of fluroquinolone antimicrobials (Richard et. al. 1994). Resistant *E. coli* have been documented to emerge during treatment of *E. coli* infections with quinolones, resulting in therapeutic failure. (Webber et. al. 2004) High levels of naladixic acid resistance has been reported from single step exposure with a frequency of 10^{-7} while low level resistance to fluroquinolones have been detected from single step exposure with a frequency of 10^{-9} (Wolfson and Hooper, 1989). Sources of quinolone resistance have been identified such as mutations in the quinolone resistance determining region (QRDR) of *gyrAB* (Yoshida et. al 1988) (Yamagishi et. al. 1986), (Shaheen et al., 2011) and *parCE* (Vila et. al. 1996) (Breines et. al. 1997), plasmid mediated factors *qnrA, qnrB, qnrS, aac(6')-Ib-cr*, and *qepA*, as well as overexpression of efflux pumps, specifically *acrAB*/*tolC.*

Conformational Change of Topoisomerases

Single nucleotide polymorphisms located within *gyrAB* and *parCE* coding for non-synonymous mutations lead to fluroquinolone resistance in both gram-positive and gram-negative isolates. These mutations reside in a region referred to as the quinolone resistance determining region (QRDR). The QRDR is located between nucleotides 199-318 of *gyrA* or *parC* (Yoshida et. al. 1990), and 1276-1392 of *gyrB* or *parE* (Yoshida et. al. 1991, Soussy et. al 1993).

In *E. coli*, mutations in gyrase can increase resistance to fluoroquinolones by a factor of 100 x (ug/ml) (Cullen et. al., 1989), while mutations in topoisomerase IV can contribute to a increase of a factor of 10 in fluoroquinolone resistance (Khodursky et. al 1995). Among the studies providing evidence of the role of mutations in topoisomerase are those which replace

mutations with wild-type sequences. Cullen and co-workers isolated DNA gyrase A from an *E. coli* strain that was cross resistant to several second generation fluoroquinolones, and then complemented the protein with wild-type gyrase B. The supercoiling function of the topoisomerase of the resultant isolate was characterized by an 100 fold increase in resistance to enoxacin. Genetic analysis of gyrase A revealed that an amino acid substitution of S83W was solely responsible for the increase. Subsequent studies revealed that a S83L substitution was more common due to C → T transition in the second position (resulting in a leucine substitution) than a C →G transversion (resulting in a tryptophan substitution). Levofloxacin resistant ParC mutant *E. coli* became susceptible after transformation of plasmids containing wild-type *parC* resulting in an MIC change from 50 to 1.56 ug/ml. It was also observed that resistance could be induced by introducing a multicopy plasmid containing mutated *parC* into a quinolone susceptible *E. coli* (Kumagai et.al 1996).

Further, mutations in GyrA have been demonstrated to affect the supercoiling activity of the protein, not just the protein's susceptibility to quinolones. Barnard and Maxwell conducted a study in which the hypermutable amino acids in GyrA (codon 83 and 87) were substituted with alanine to make 3 different mutant proteins, GyrA S83A, GyrA N87D, and GyrA S83A, N87D. In the GyrA mutant with only the S83A substitution, while the mutation was only responsible for conferring low levels of quinolone resistance and it had little to no affect on the catalytic activity of DNA gyrase. However, in the N87D mutant and the S83A, N87D double mutant, the mutated region appeared to have a higher affinity to DNA therefore resulting in 2.5 fold less supercoiling activity in the N87D mutant and 5 fold less supercoiling activity in the S83A, N87D double mutant resulting in a situation where protein function is compromised in exchange for resistance.

The N87D mutation did account for high level quinolone resistance in both the single mutant and the double mutant.

Pfeiffer and Hiasa addressed the sequelae on norfloxacin resistance when the α4 region of Topoisomerase IV (the region that houses the QRDR for ParC) was replaced with the α4 region of DNA gyrase using overlap extension PCR. The PCR product was cloned into a plasmid vector and transformed into *E. coli* HMS174 (DE3). This vector was expressed with wild type *parE* to create a protein with two mutated ParC subunits and two wildtype ParE subunits. Whereas the substitution of the α4 region of GyrA into ParC didn't affect the quinolone sensitivity of the protein, it significantly and negatively affected the catalytic activity of the protein. Interestingly, the norfloxacin/ParCα4GyrA/DNA ternary complex was found to be more stable, and the inhibition more cytotoxic than the norfloxacin/ParC/DNA ternary complex, but less stable and cytotoxic than the norfloxacin/GyrA/DNA ternary complex. This suggests a stabilizing interaction between the amino acids in the catalytic sites of the topoisomerases with quinolone antibiotics.

The Role of Efflux Systems in Fluroquinolone Resistance

Five major families of efflux pumps exist in *E. coli*: ATP-Binding Cassette (ABC) superfamily, major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE) family, resistance nodulation cell division (RND) family, and small multidrug resistance (SMR) family.

The efflux system which most effects quinolone resistance is AcrAB which belongs to the Resistance Nodulation Cell Division (RND) family. The AcrAB efflux system also includes one copy of the outer membrane protein TolC. TolC is a transmembrane protein channel that

reaches out through the outer membrane to allow the substrate to cross the periplasmic space as part of a RND or MFS efflux pump (Fralick, 1996). Ma et. al. observed that when *acrAB* is deleted, the *E. coli* cell becomes hypersusceptible to bile salts. They also observed that *acrAB* expression was increased in multidrug resistant *E. coli* mutants. AcrB is the portion of the pump located in the inner membrane, deriving energy from proton motive force (Ma et al, 1993). This portion of the protein is believed to be the part of the efflux system that captures the molecule, transferring it to TolC for efflux. In contrast, AcrA is a lipoprotein found in the periplasmic space and the inner membrane; it serves to transport non polar molecules (Zgurskaya and Nikaido, 1999) but also appears to stabilize the TolC-AcrAB complex. All three proteins are needed in order for the AcrAB/TolC efflux system to be functional. Figure 2 shows the structure and mechanism for AcrAB/TolC efflux system.

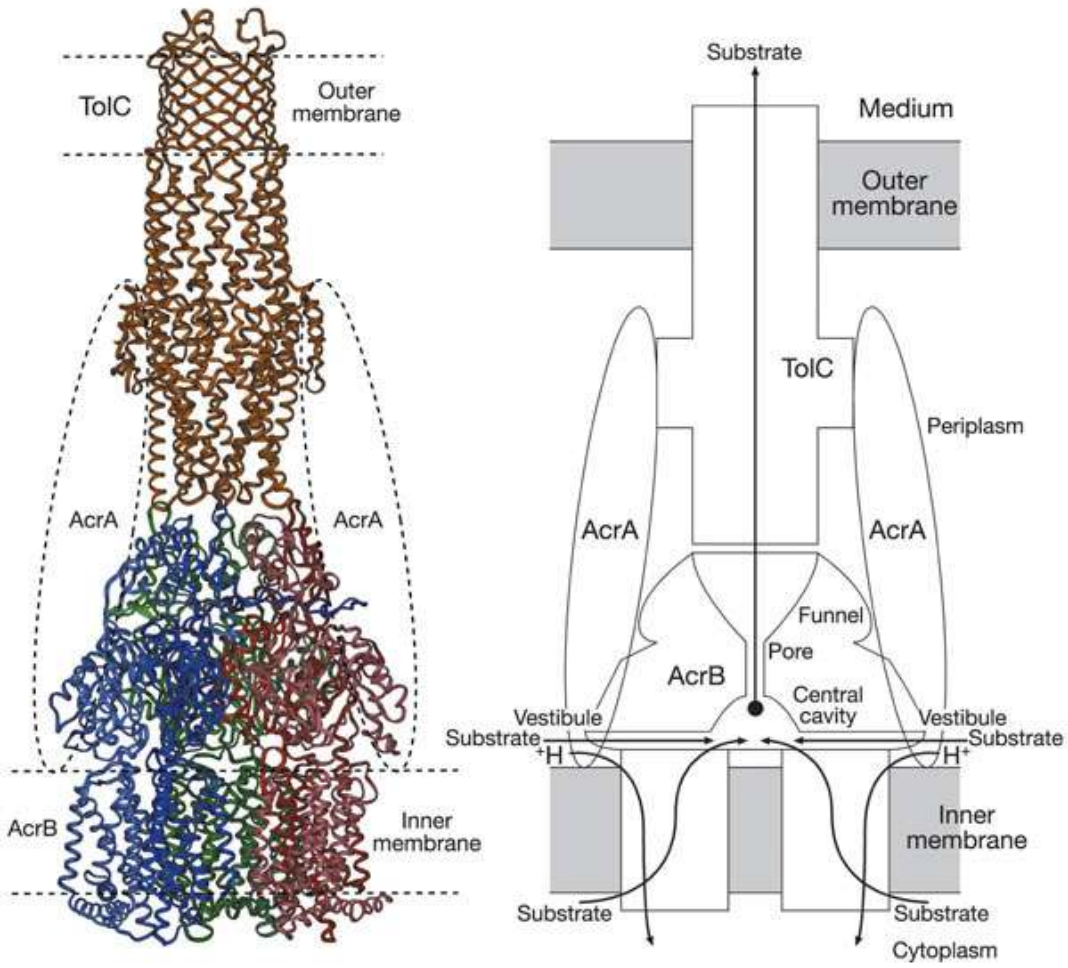

Figure 2: Structure for AcrAB/TolC efflux system in *E. coli*. (Murakami et. al., 2002)

Substrates for the AcrAB/TolC efflux system include such compounds as tetracycline, chloramphenicol, fluoroquinolones, β-lactams, erythromycin, fusidic acid, ethidium bromide, crystal violet, sodium dodecyl sulfate (SDS), and bile acids.

marABR is believed to code for regulators of antimicrobial resistance. The *marABR* operon is located on the chromosome of *E. coli*; when expressed, it increases resistance seen initially to chloramphenicol and tetracycline. The accepted functions of these three genes are as follows: MarA is thought to be a transcriptional activator of antimicrobial resistance genes by

activating *sodA* (a superoxide dismutase), *zwf* (a glucose-6-phosphate dehydrogenase) and *micF* (an antisense RNA regulator of outer membrane porins). The function of MarB is still yet to be determined. MarR is the repressor of the *marABR* operon. (Cohen et. al. 1993) With MarA sharing a pathway with SoxS, MarA is also found to be associated with upregulation of AcrAB, making it also part of the multi-antimicrobial resistance pathway. (Ma et. al, 1996).

Several other genes contribute to efflux pump activity in *E. coli*. *mdfA* encodes for the major facilitator superfamily (MFS) of efflux pumps.. MdfA is a multidrug efflux pump. Originally identified as a chloramphenicol resistance pump, it is now known to efflux other antimicrobial substrates such as tetraphenylphosphonium (TPP+), ciprofloxacin, and ethidium bromide. *norE* was also identified as a multi substrate efflux pump, this time belonging to the multi antimicrobial extrusion (MATE) family. Yang et. al in 2003 compared the roles of AcrAB, MdfA, and NorE in quinolone resistance. Regardless of the efflux pump, expression of each increased resistance (based on magnitude of increase in MIC) only 10 fold. Strains of E. coli studied (n=15) including those with mutations *gyrA* S83L, *parC* E84K, *gyrA* S83L *parC* E84K, and each of those strains with each combinations of deletions: Δ*acrB1*, Δ*norE*, Δ*mdfA* or overexpression by plasmid of *acrB1*, *norE* and *mdfA.* In cells overexpressing *acrB1*, resistance increased up to 6.4for ciprofloxacin and 5.3 for norfloxacin; overexpression of *acrB1* and *norE*, resulted in increases of 9.4 and 16.0 fold, for ciprofloxacin and norfloxacin respectively, and overexpression of *acrB1* and *mdfA*, an increase in 11.8 fold and 16 fold, respectively (Shaheen et al., 2010a).

The deletion of *norE* or *mdfA* alone or in combination had no significant effect compared with the wild type, although deletion of *acrA1* decreased the MIC_{Cip} 8 fold. The combined deletion of *acrA1* and *mdfA* increased MIC_{Cip} by1.1 fold, for *acrA1*, *and norE* as well as

combination of all three deleted the increase was 1.05 fold. When coupled with the MIC's resulting from deletion of the efflux pump genes, the data suggests that overexpression of efflux pumps, and especially combinations of efflux pumps, significantly increases *E. coli* resistance to fluoroquinolones.

Resistance Mechanism	Gene	MIC_{Cipro} Change
Efflux Pump	*acrAB*	10x
	mdfA	10x
	norE	10x
Efflux Pump Gene Overexpression	*acrB1*	6.4x
	acrB1/mdfA	11.8x
	acrB1/norE	9.4x
Efflux Pump Gene Deletion	*acrB1*	.125x
	mdfA	No Change
	norE	No Change
	acrB1/mdfA	1.1x
	acrB1/norE	1.1x
	acrB1/mdfA norE	1.05x

Table1: Fold change in MIC_{Cipro} for *E. coli* cells expressing *acrAB, mdfA* and *norE*, overexpressing acrB1, acrB1/mdfA, acrB1/norE, and with deletions of *acrB1, mdfA, norE, acrB1*/*mdfA, acrB1*/*norE, acrB1*/*mdfA*/*norE* (Yang et al, 2003)

According to a study in 2000 by Maira-Litrán et. al, while in biofilm (in which bacterial cells specialize in their function to form large bacterial communities), *E. coli* resistance to antimicrobials does not appear to be mediated through the upregulation of *mar* or *acrAB* operons. Further, mutations in gyrAB and parCE are ineffective at conferring quinolone

resistance, with *acrAB* being severely down-regulated or deleted. (Oethinger et. al, 2000) These findings exemplify the complexity involved in conferring antimicrobial resistance.

Emerging Factors: Plasmid Mediated Quinolone Resistance and More

Plasmid mediated quiniolone resistance genes termed *qnr* code for pentapeptide repeat proteins located on integron structures (Tran and Jacoby, 2002). First discovered on multi-resistance plasmid pMG252 in *Klebsiella pneumonia*, QnrA was determined to have a broad host range found to exist in many gram negative microorganisms as well as some select gram positive microorganisms (Martínez-Martínez et. al. 1998)**.** Antimicrobial susceptibility testing to an *E. coli* with a plasmid containing *qnrA* gene demonstrated an increased MIC_{Cip} by 4 to 7 fold. Tran et. al. (2005) demonstrated that QnrA is able to cause this increase by binding specifically to DNA gyrase, thus sheltering the target enzyme from fluroquinolones. Although the mode of action may reflect prevention of the ternary complex of DNA gyrase/ DNA/ fluroquinolone from forming, it is not through interference of DNA gyrase/ DNA interaction nor creation of the heterodimer required for DNA gyrase activity. The authors also proposed that QnrA may allow the toxic ternary complex to form, but that the replication fork is preserved by destabilizing the cleavage complex, thus avoiding the lethal double stand break. In a 2005 publication Tran et. al also showed similar patterns for topoisomerase IV and QnrA interaction. .

Other Qnr proteins have also been identified; however the homology by amino acid identity is below 60% across all Qnr proteins. In 2005 Hata *et. al.* isolated QnrS from a clinical strain of *Shigella flexneri* via pulse field gel electrophoresis, and conjugated the wild plasmid carrying the quinolone resistance gene into competent strain *E. coli* HB101. Transconjugant *E. coli* HB101 displayed a MIC_{Cip} of .25mcg/ml compared to baseline 0.06mcg/ml MIC_{Cip}. A

second experiment in which the wild plasmid was conjugated into quinolone susceptible *S. flexneri* resulted in an increase in MIC_{Cip} 4 fold, thus demonstrating that the *qnrS* gene encoded on this wild plasmid was responsible for conferring the observed quinolone resistance. Sequencing showed that QnrS shared an amino acid identity of 59% with QnrA.

The next Qnr protein to be identified was QnrB isolated from *K. pneumonia* clinical isolates in India exhibiting low level fluoroquinolone resistance. (Jacoby et al, 2004) These isolates, however, were QnrA negative. Cloning studies confirmed QnrB protein as responsible for the low level fluroquinolone resistance.

Plasmid in *E. coli* J53	Qnr protein	MIC (µg/ml)				
		Nalidixic acid	Ciprofloxacin	Gatifloxacin	Levofloxacin	Moxifloxacin
R^-		4	0.015	0.03	0.03	0.06
pMG252	QnrA	32	0.5	0.5	1	1
pHSH4-3	QnrA	16	0.25	0.5	0.5	0.5
pMG298	QnrB1	16	1	1	0.5	2
pMG299	QnrB1	16	0.25	0.5	0.5	1
pMG300	QnrB1	16	0.25	0.5	0.25	1

Table 2: Susceptibilities of *E. coli* transformed with plasmids harboring different qnr genes. (Jacoby et al, 2004)

Other variants of *qnr* include, 6 *qnrA*, 20 *qnrB*, 4 *qnrS*, *qnrC* in *Proteus mirabilis*, and *qnrD* in *Salmonella enterica* serovar *Kentucky* with *qnrB*19 being the most common (Rodríguez-Martínez, J. et. al 2010). *qnrB* is thought to be the oldest of the *qnr* genes with the first evidence of *qnrB* being identified from *E. coli* isolated in 1988. (Jacoby et. al. 2009)

Another plasmid mediated mechanism for quinolone resistance is the *cr* variant of *aac(6')-Ib* an aminoglycoside acetyltransferase found to inactivate ciprofloxacin by acetylating the antibiotic at the amino nitrogen on its piperazinyl ring; this change causes an increase of

MIC_{Cip} to 1.0 ug/ml. This was a groundbreaking finding since fluroquinolones are synthetic drugs and it was thought that there was no natural source of modification to them. Through the course of this finding Robicsek et. al. identified two mutations in *aac(6')-Ib* that conferred this *cr* variant. Mutations in Trp102Arg and Asp179Tyr were revealed to be responsible for the ability to modify ciprofloxacin at the piperazyl ring. This claim was strengthened by performing site directed mutagenesis on *aac(6')-Ib-cr:* in the absence of those two mutations the enzyme was no longer linked to ciprofloxacin resistance. (Robicsek et. al., 2007) In a survey of clinical isolates, *aac(6')-Ib-cr* was found in 15 of 47 ciprofloxacin resistant *E. coli* isolates. (Park et. al. 2006) (Shaheen et al, 2010b)

In 2007 a third class of plasmid mediated resistance was discovered in QepA, a plasmid mediated quinolone efflux pump first isolated from an *E. coli* in Japan. An *E. coli* KAM32 was transformed with pSTV with *qepA* as well as pSTV with *qepA* deleted. When subjected to susceptibility testing it revealed that pSTV*qepA* exhibited a 32 fold increase in MIC to ciprofloxacin when compared to pSTV Δ*qepA*. An increase was also observed across all quinolones: naladixic acid, lomefloxacin, and sparfloxacin 2 fold, levofloxacin and pazufloxacin 4 fold, moxifloxacin and gatafloxacin 8 fold, tosufloxacin 16 fold, enrofloxacin 32 fold, and norfloxacin 64 fold, (Yamane et. al. 2007)

The amino acid sequence of QepA was found to be similar to EmrB from the MFS class of efflux pumps and secondary structure and super secondary structure was predicted and is shown in Figure 5.

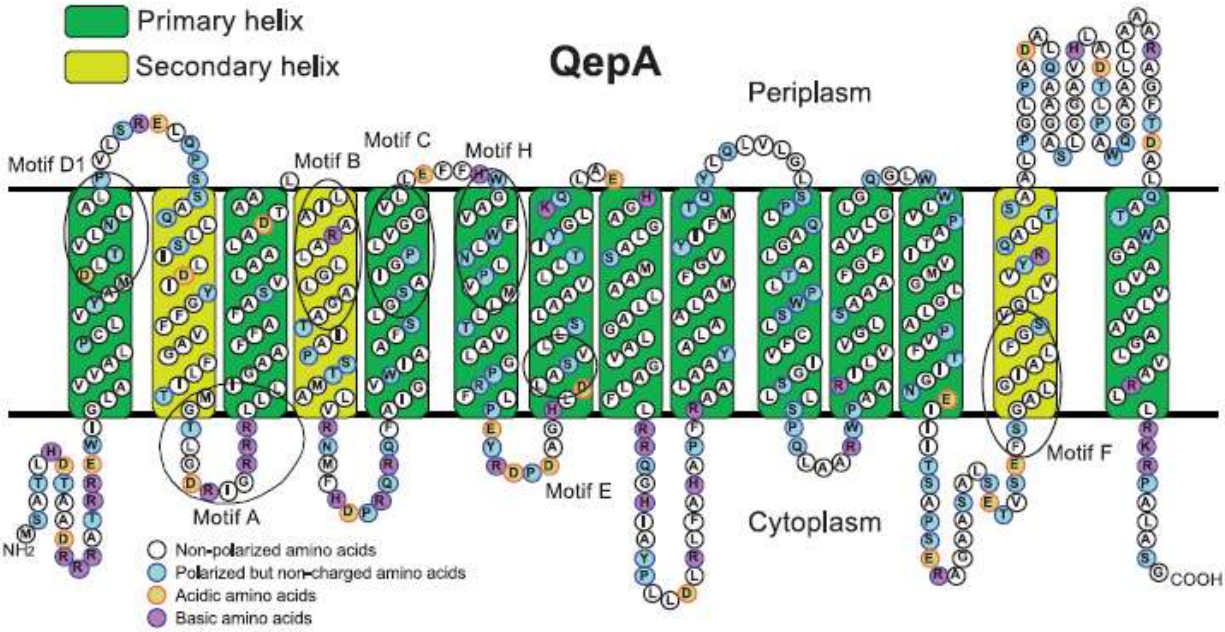

Figure 3: Prediction of the secondary structure for the plasmid mediated quinolone efflux pump QepA. (Yamane et al. 2007)

A second efflux pump, OqxAB conferring quinolone resistance is associated with the pOLA52 plasmid, belonging to the RND superfamily of efflux pumps. Originally associated with resistance to olaquindox, it also confers resistance to ethidium bromide (a DNA mutagen) and chloramphenicol. *E. coli* N43 transformed with a pLOW plasmid with and without *oqxAB* exhibited no change in the MIC of 3 compounds, but an increase of MIC in 16 out of 19 compounds including Chloramphenicol and Sodium doecyl sulfate (128x), Ciprofloxacin and Flumequine (32x), Norfloxacin, Olaquindox and Trimethoprim (64x),. (Hansen et. al. 2007)

An emerging factor contributing to antimicrobial resistance is a state of persistence, a transient physiological state not associated with genetic modification but in which antibiotics are ineffective. Persisters to ciprofloxacin develop randomly, in response to antimicrobial exposure.

However, the mechanisms conferring persistence are not yet understood. Dorr et.al (2006) suggested that persistent bacteria do not experience double strand breaks in the presence of fluoroquinolones, eliminating the signals that induce genetic mutation, or subsequent repair functions, or allow a plasmid mediated response typical of such exposure. RecA and RecBCD are proteins expressed during the SOS response, RecA is responsible for binding to single stranded DNA and RecBCD is responsible for facilitating recombination repair. Mutants with *recA* or *recB* deleted were used to test this hypothesis; the remaining persisters in Δ*recA* mutants were greatly reduced. In Δ*recB* mutants persisters were eliminated within 6 hours of exposure. This suggests that the *recBCD* response is needed to repair double strand breaks in persisters and to induce the SOS response. There is also evidence that persisters undergo at least one site specific recombination event in order to repair damage from the double strand break. Therefore, in order for persistence to occur the SOS response must be induced by RecA binding to damaged DNA. Conversely, this does not rule out spontaneous induction of the SOS response in order to create the physiological state necessary for fluoroquinolone persistence. This study revealed that a certain level of SOS response is necessary for persistence to occur since antimicrobials elicit an SOS response from all bacteria. In the natural environment of a specific bacterium, the bacterium is usually faced consistently with stresses and growth is commonly being inhibited. It is now predicted that in the wild it is not uncommon to find persistent bacteria (Dorr et. al 2009).

Levels of persistence are seen to differ between stages of the bacteria growth curve, with persistence being low during exponential phase and high in stationary phase. This is because in a state of non growth their drug targets are inactivated. (Dorr et. al 2009).

Methods for Detection of Resistance

Methods by which fluoroquinolones resistance can be detected are numerous. The oldest is susceptibility testing by either broth dilution (Donovick et al., 1945) Kirby-Bauer antibiotic testing (Kirby et al., 1956), or Episilometer testing (Bolmstrom et al., 1988). Broth dilution is the original modern susceptibility test and remains as the gold standard today.

The broth dilution method was first introduced in 1945 by Donovick, R., et al., as a solution to standardize susceptibility testing. Previously, antimicrobial susceptibility was measured in dilution units (Waksman, 1943), from agar dilution and diffusion units (Schatz et. al., 1944), from antimicrobial diffused across agar. These practices lead to publishing of *Escherichia coli* units, *Bacillus subtilis* units, *Staphylococcus aureus* units, etc. all of which were incomparable. The lack of standardization was addressed with broth dilution performed using multiple dilutions of each antimicrobial in nutrient broth and inoculating this broth with standardized numbers of colonies of the organism of interest. The inoculated sample is incubated in optimal growth conditions until log phase growth, dilutions are then inspected for growth inhibition. The greatest dilution (or lowest concentration of drug) in which growth is not observed is considered the organism's minimum inhibitory concentration (MIC) toward that drug (Donvick, R. et al, 1945). This procedure has since been updated to such that it is performed using microbroth dilution procedures which is the considered the gold standard by the Clinical Laboratory Standards Institute (CLSI, 2008).

Kirby-Bauer antibiotic testing, also known as the disk diffusion method, is performed using solid agar inoculated with a known amount of bacterial or fungal suspension. A disk infused with the antimicrobial of choice is placed on the agar creating an antimicrobial gradient.

Susceptible organisms will not grow in the presence of the antibiotic creating a zone of inhibition. Large zones of inhibition indicate organisms that have greater susceptibility to the antimicrobial and therefore smaller MICs. The radius of the zone of inhibition is measured and compared to the time elapsed since exposure to the disk and a MIC is estimated (Bauer et. al, 1966).

Episilometer testing (E-test) is conducted similarly to the Kirby- Bauer method. However the disk is substituted for a metered strip impregnated with the antimicrobial of choice at the top. The antimicrobial diffuses in to the agar creating a gradient. Susceptibility is measured by comparing the area of inhibited growth to the coordinating meter on the strip indicating the amount of antimicrobial present at that point in the gradient (Bolmstrom, et. al., 1988).

Advantages of culture and susceptibility testing is that a clear quantitative susceptibility threshold is acquired; however the disadvantage is for organisms like *M. tuberculosis.* For such organisms, slow growth complicates susceptibility testing due to risk of contamination and extended time between sample collection and sensitivity result. There is a need for development of more rapid assays.

Other novel approaches for detecting quinolone resistance have also been described. These approaches have been molecular approaches aim at creating rapid detection of quinolone resistance. Techniques such as blotting, high performance liquid chromatography, pyrosequencing, mismatch amplification mutation assay, single-strand conformation polymorphism, and quantitative PCR have been utilized to bypass susceptibility testing.

In 1996, a technique for detection of ciprofloxacin resistant *Mycobacterium tuberculosis* was introduced utilizing 16S rRNA precursor. The assay uses slot blots hybridized with

nucleotide probes specific for the sequences found in terminal stems of 16S pre-rRNA which is spliced during RNA maturation. They observed that in rifampin and ciprofloxacin resistant strains when exposed to these drugs in broth, 16S pre-rRNA collected in the cell unprocessed to mature rRNA and was detected in large amounts by the nucleotide probe. However, in susceptible strains pre-RNA was not detected after exposure to the antimicrobials (Cangelosi, et. al., 1996). For *Campylobacter jejuni*, a nonradioisotopic single-strand conformation polymorphism (non-RI SSCP) assay has been described for rapid detection of quinolone resistance. This assay takes advantage of changes in gyrA folding by comparing its mobility in a polyacrylamide gel, and silver staining which produces better resolution bands so that small differences can be detected. (Charvalos, et. al, 1996)

A mismatch amplification mutation assay (MAMA) was developed by Zirnstein et al. to detect ciprofloxacin resistance in *Campylobacter*. MAMA uses a conserved primer coupled with a mutation detection primer for PCR and products are analyzed by gel electrophoresis. Isolates that contain the targeted mutation in *gyrA* are amplified in the PCR reaction while wild type or non targeted mutations are not (Zirnstein et. al, 1999). In a further search for a rapid and sensitive assay, denaturing high performance liquid chromatography was attempted in *Salmonella enterica* to detect a DNA sequence variation indicative of quinolone resistance. This technique consists of temperature dependant denaturation of dsDNA followed by ion pair chromatography. In this study 11 profiles were created; however, the profile for the resistant Asp87Gly mutation was indistinguishable from the wild type (Eaves et al, 2002).

Quantitative PCR based assays were the next frontier for rapid detection of quinolone resistance. Again, in *C. jejuni* a technique was developed to detect quinolone resistance by targeting mutations in *gyrA*. Using Taq-man probe TAQ1 and primers designed specifically to

the QRDR of *C. jejuni* this assay was able to rapidly detect SNPs in *C. jejuni gyrA* responsible for quinolone resistance (Wilson, et al, 2000), a similar assay was also developed for *Salmonella enterica* (Esaki et al, 2004) and using a dual probe approach for *Mycoplasma bovis* (Ben Shabat et al, 2010). Soon FRET-PCR assays detecting SNPs in *gyrA* were developed for *Yersinia pestis* (Lindler et. al, 2001), *Neisseria gonorrhoeae* (Li et al, 2002), *Streptococcus pneumoniae* (Page et. al, 2008) along with a protocol for *Haemophilus influenza* for SNP detection in *gyrA*/*parC* (Nakamura et al, 2009) and *gyrA/gyrB* in *Clostridium difficile* (Spigaglia et al, 2010). Recently a new assay has been developed using qPCR for *M. tuberculosis*. This assay utilizes asymmetric PCR with sloppy molecular beacons (probes with long sequences allowing hybridization with many different species amid mismatched nucleotide pairs) to detect mixed resistance (Chakravorty et al, 2011).

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CHAPTER 2

DEVELOPMENT AND EVALUATION OF A FRET-PCR ASSAY FOR DETERMINING FLUOROQUINOLONE RESISTANCE IN CANINE URINE *ESCHERICHIA COLI* ISOLATES

Abstract

Antimicrobial resistance in *Escherichia coli* particularly that associated with urinary tract infection (UTI) is increasing in both human and veterinary patients. Fluoroquinolones (FQ) such as enrofloxacin are among the drugs of choice for treatment in canines. *E. coli* resistance to FQ, including ENR, includes mutations in topoisomerases, but may involve mechanisms associated with multidrug resistance (MDR). Among the difficulties in effective treatment of *E. coli* UTI is rapid detection of FQ resistance. The purpose of this study was to determine the specificity and sensitivity of a FRET-PCR based assay for the rapid detection of UTI caused by ENR-R *E. coli*. Three hundred and six clinical canine urine *E. coli* isolates were subjected to susceptibility testing for 14 drugs representing 6 drug classes, including ENR at a range of MIC (0.03-512 μg/ml). Isolates were designated (n) NDR (no drug resistance, n=89), SDR (single drug resistance, n=116) and MDR (multi-drug resistant, n=101, including ENR-S [n=51] and ENR-R [n=50]). Extracted DNA was subjected to FRET-PCR targeting single nucleotide polymorphisms in *gyrA*. Further, to determine the sensitivity of the assay, microbial free canine urine was inoculated with 10^6 to 10^1 CFU/ml of 7 *E. coli* isolates characterized by variable susceptibility to ENR (MIC_{Enro}=0.03, 0.06, 0.15, 1, 64, 128, 256 μg/ml). Of 306 isolates, 43/50 ENR-R (MIC_{Enro} >4 μg/ml), were positively identified by FRET-PCR to be enrofloxacin resistant (a sensitivity of 86%; increasing to 97% for isolates expressing

high level resistance (MIC > 8 X breakpoint [64 mcg/ml]), and MDR (n=34). Only 1/50 ENR-R isolate was not detected (specificity = 97%). Colony dilutions of *E. coli* in sterile urine confirmed the assay able to detect enrofloxacin resistance in as few as 10^1 CFU/ml. These results confirm that the assay designed provides the specificity and sensitivity to accurately predict antimicrobial resistance in clinical *E. coli* isolates. Studies now are needed in urine samples from clinical patients.

Introduction

E. coli is a major cause of urinary tract infections (UTI) in canines (Ling et. al. 1979). Of these infections, antimicrobial resistant *E. coli* is increasingly identified. An increase in fluroquinolone resistance in particular has been reported; such isolates invariably express multidrug resistance (MDR) (Hirsch et. al. 1973), (Cook et. al 2002), (Cohn et. al. 2003), (Boothe et. al 2006), (Shaheen et al, 2009). An important risk factor associated with the emergence of FQ resistance is use of FQ antimicrobials (Richard et. al. 1994). Resistant *E. coli* have been documented to emerge during treatment of *E. coli* infections with quinolones, resulting in therapeutic failure (Webber and Paddock, 2001). Culture and susceptibility testing of *E. coli* continues to be the gold standard for the detection of antimicrobial resistance. However, this technique is tedious and costly and far from rapid, requiring 2-5 days from time of sample collection until results are reported to the clinician. This window can contribute to therapeutic failure particularly if treatment is initiated with an antimicrobial to which the infecting isolate is resistant (Bubenik et al., 2007). There is a need for an alternative method that allows rapid and sensitive detection of MDR/FQ resistance in urinary isolates for a clinical setting (Siedner et al., 2007).

Mutations characterized by single nucleotide polymorphisms (SNPs) in the quinolone resistance determining regions (QRDR) of DNA gyrase (*gyrAB*) and topoisomerase IV (*parCE*) are the most common mechanisms causing fluoroquinolone resistance (Oram and Fisher, 1991), (Willmott and Maxwell, 1993), (Everett et. al. 1996), (Villa et. al. 1996), (Piddock, 1999). These SNPs can be easily detected by hybridization probes and quantitative PCR (qPCR). qPCR allows monitoring of PCR amplification with each cycle. This is in contrast to conventional PCR for which only qualitative information is provided and further processing of the amplicon by gel electrophoresis is necessary. Other molecular techniques such as mismatch amplification mutation assay (MAMA) combined with DNA sequencing have been developed for the detection of ciprofloxacin-resistant clinical *E. coli* isolates in human medicine (Qiang, et. al 2002). However, this method also requires gel electrophoresis.

A quantitative PCR (qPCR) system can achieve precise discrimination with utilization of a Fluorescence Resonance Energy Transfer (FRET) assay monitoring the temperature-dependent hybridization of sequence-specific hybridization probes to single stranded DNA while performing melting curve analysis. The melting temperature (T_m) is dependent on the length, GC content, and on the degree of homology between the two DNA strands. Hybridization probes bound perfectly to the matching target DNA require a higher T_m to separate in comparison with those bound to DNA containing destabilizing mismatches.

In this study we evaluate the effectiveness of a FRET-PCR based assay for detection of SNPs in *E. coli gyrA* from pure culture originally isolated from canine urine samples as well as urine inoculated with *E. coli* and its accuracy in predicting FQ resistance.

Materials and Methods

Bacterial Isolate Culture Conditions

Escherichia coli isolates were harvested from canine urine samples submitted to IDEXX laboratories for suspected urinary tract infections. Isolates had been identified by the laboratory and subjected to susceptibility testing before duplicate cultures were transferred by mail on trypticase soy agar (TSA) slants to the Auburn University Clinical Pharmacology Laboratory. Upon receipt, each *E. coli* isolate was re-cultured on BBL CHROMagar Orientation (BD Diagnostics) at 37°C overnight to confirm isolate identification as *E. coli* before transfer to TSA for collection in cryovials. Isolates were stored at -80°C in trypticase soy broth/glycerol cyrovials (mixture Percentage) until testing.

Antimicrobial Susceptibility Testing

Isolates were subjected to antimicrobial susceptibility testing in order to determine their minimum inhibitory concentrations (MIC).The isolates were cultured directly by transfer to a tryptic soy agar (TSA) plate. The colonies collected from TSA plates were subjected to broth microdilution for susceptibility testing as described by CLSI (CLSI, 2008). Fifteen drugs representing 6 classes of antimicrobials were tested: amoxicillin-clavulanic acid, ampicillin, ticlacillin-clavulanic acid, cefotaxime, cefoxitin, cefpodoxime, ceftazidime, cephalothin, chloramphenicol, doxycycline, enrofloxacin, ciprofloxacin, gentamicin, meropenam and trimethoprim-sulfamethoxazole. Inocula were prepared by suspending growth from overnight cultures in sterile normal saline to a turbidity of approximately 0.5 McFarland standards. Final inocula contained 2 to 7 x 10^5 CFU/ml. The suspension was used to inoculate custom prepared microtiter trays (TREK Diagnostic Systems, Cleveland, OH). The trays were incubated at 37°C and read at 18 h with a TREK VIZION System (Trek Diagnostic Systems, Cleveland, OH). The

minimum inhibitory concentration (MIC) of each antimicrobial was recorded. For quality control purposes *E. coli* ATCC® 25922 (American Tissue Cell Culture, Manassas, VA) was included in each sample set. Using CLSI standards, each isolate was designated as resistant (R; MIC ≥ the resistant breakpoint), susceptible (S; MIC ≤ the susceptible breakpoint) or intermediate (I; MIC between the two breakpoints; this designation is not provided by CLSI for each drug) (CLSI, 2008). In this study, intermediate isolates were recorded and analyzed as "resistant". Each isolate was designated as to the presence of no drug resistance to any drug (NDR), single drug resistance (SDR; resistance to one drug class), or multidrug resistance (MDR; resistance to 2 or more of drug classes). All SDR isolates were susceptible to fluoroquinolones (FQ); MDR isolates were further classified as FQ-susceptible (FQ-S), FQ-low level resistant (4 μg/ml $<MIC_{Enro}<32$ μg/ml; FQ-LR) or FQ-high level resistant ($MIC_{Enro}>64$ μg/ml; FQ-HR) (Table 1).

Selection of Clinical Isolates and Sample Preparation

306 *E. coli* isolates (n=101 MDR, 51 MDR-FQ-S, 34 MDR-FQ-HR, and 16 MDR-FQ-LR), 116 SDR and 89 NDR) were revived on TSA plates at 37°C overnight. DNA was extracted using PrepMan ULTRA (Applied Biosystems, Foster City, CA) in preparation for the FRET assay.

Experimentally Inoculated Urine Samples

Canine urine was collected via cystocentesis, and submitted for culture to verify sterility. Urine determined as negative for bacteria was confirmed microbial-free by transfer of 10μl on to TSA and incubated at 37°C for 48h. After confirmation, 4.5 ml aliquots were made for dilutions. 7 *E. coli* isolates representing increasing enrofloxacin susceptibilites were suspended in 9% saline to .5 McFarland standard (~10^9 CFUs) (Table 3). Dilutions were made from 10^6 to 10^1 CFUs in microbial free urine. After dilutions were made, the inoculated urine samples were

applied to Microcep 100K Centrifugal Microconcentrators (Pall Corporation, Port Washington, NY) and centrifuged for 40m at 3000 rpm. After centrifugation, the filter was washed with 150uL of microbial free urine and the wash collected for DNA extraction. DNA was extracted using the Viral RNA Kit (Omega).

Quantitative FRET-PCR

The LightCycler 480 Real-time PCR system (Roche) was used for amplification, detection of quantification and melting curve analysis. Primers and probes were designed to be specific for a consensus QRDR wild-type sequence (Shaheen et. al, 2009). Fluorophores were selected with 3' labeled 6-FAM carboxyfluorescein for the donor probe and 5' labeled, 3' phosporylated LightCycler Red 640 for the reporter probe (Figure 1). LightCycler 480 Genotyping Master (Roche Applied Science, Indianapolis, IN) supplemented with 2.0 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) was used for the FRET-PCR reactions. The thermocycling program was based on a prior study with modifications for 96 well plates: 18 high stringency step down cycles were succeeded by 25 amplification and fluorescence acquisition cycles with a final melting curve (Shaheen et. al., 2009). The high stringency step down cycling program is as follows: 95°C for 5m; 6 cycles at 95 °C for 15 s, 72 °C for 30 s; 9 cycles at 95 °C for 15 s, 70 °C for 30 s; 3 cycles at 95 °C for 15 s, 68 °C for 30 s, 72 °C for 30. Amplification was then achieved by 35 cycles of denaturation at 95 °C 15 s, annealing at 52 °C for 15 s, 66 °C for 30 s, and extension at 72 °C for 30 s. Emittance for the Lightcycler was set at 498nm and absorption at 640nm. Determination of nucleotide sequences was performed by Macrogen USA (Macrogen, Rockville, MD) on the QRDR of *gyrA* locus of 20 isolates in order to determine the specificity of the assay.

Sequencing of Isolates to confirm FRET Results

20 isolates were selected by FRET results to confirm accuracy of the assay. These isolates exhibited low melting temperatures suggesting extreme resistance (T_m<60C), melting temperatures suggesting only one mutation (63C<T_m<68C), and isolates deemed ENR-R producing melting temperatures suggesting susceptibility (T_m>68C) (Table 2).

Results

FRET-PCR on Clinical Isolates in Pure Culture

Of 306 *E. coli* isolates, 50 were confirmed by susceptibility testing to be positive for enrofloxacin resistance (MIC_{Enro} >4 μg/ml). 43 of these isolates were also positively identified by the FRET-PCR assay yielding a sensitivity of 86.00%. However, of the isolates expressing high level enrofloxacin resistance (MIC > 64 μg/ml), and MDR phenotype (n=34), the assay yielded a sensitivity of 97.06%. 247 out of 256 isolates expressing an FQ-S MIC were negatively identified yielding a specificity of 96.66% (Figure 4). Three melting curve profiles for the isolates were produced by the assay (Figure 3). Sequences of the 20 selected isolates revealed a set of synonymous mutations present in 17 of the isolates (Arg91, Tyr100, Ser111). A complete deletion of codon 83 was also observed in one of the isolates creating the only false positive reading.

FRET-PCR on Dilutions of Experimentally Infected Urine

Colony dilutions of *E. coli* were detectable at as low as 10^1 CFU/mL (Figure 4). However, due to background, the melting temperatures could be accurately determined only at

dilutions $\geq 10^3$ CFU/mL. No relationship between CFUs and the peak height of –(d/dt) fluorescence could be discerned. When nucleic acid concentrations were checked with a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) it was concluded that there was no discernable relationship between DNA concentration and colony forming units (CFU/ml) (Figure 6).

Discussion

From the specificity and sensitivity results it is confirmed that the FRET assay is able to detect fluoroquinolone resistance in *E. coli*. The presence of false negatives may have arisen because there are many other genetic and physiological factors linked with FQ resistance other than mutations located in the QRDR of *E. coli gyrA.* Transmembrane factors such as overexpression of efflux pump (most notably AcrAB) and porin modification have been attributed to MDR phenotypes. (Everett et al., 1996), (Giraud et al., 2001), (Mazzariol et al., 2000), (Piddock, 1999). Mutations in *soxS* and additional mutations in the QRDRs of *gyrB*, *parCE* have been identified; these along with presence of plasmids containing *qnr* (quinolone resistance gene) have also been linked to FQ related MDR phenotypes. Since so many other factors are involved in the conformation of FQ resistance it is impossible for an assay targeting *gyrA* to give a completely accurate correlation of mutations to MIC. However, for high level enrofloxacin resistance that is only reported to appear with the occurrence of nonsynonymous mutations in the *gyrA* gene, the FRET-PCR assay is able to specifically discern such isolates in pure culture. The standard deviation (σ) for T_m in isolates expressing extremely susceptible MICs (0.03-0.6 μg/ml, n=225) is 0.991 for this set of isolates while σ for isolates expressing extremely resistant MICs ($x \geq 128$ μg/ml, n=19) is 0.671. Variation is observed in isolates expressing transition type MICs ($0.12 \leq x \leq 32$ μg/ml, n=38) shown by an σ of 4.575.

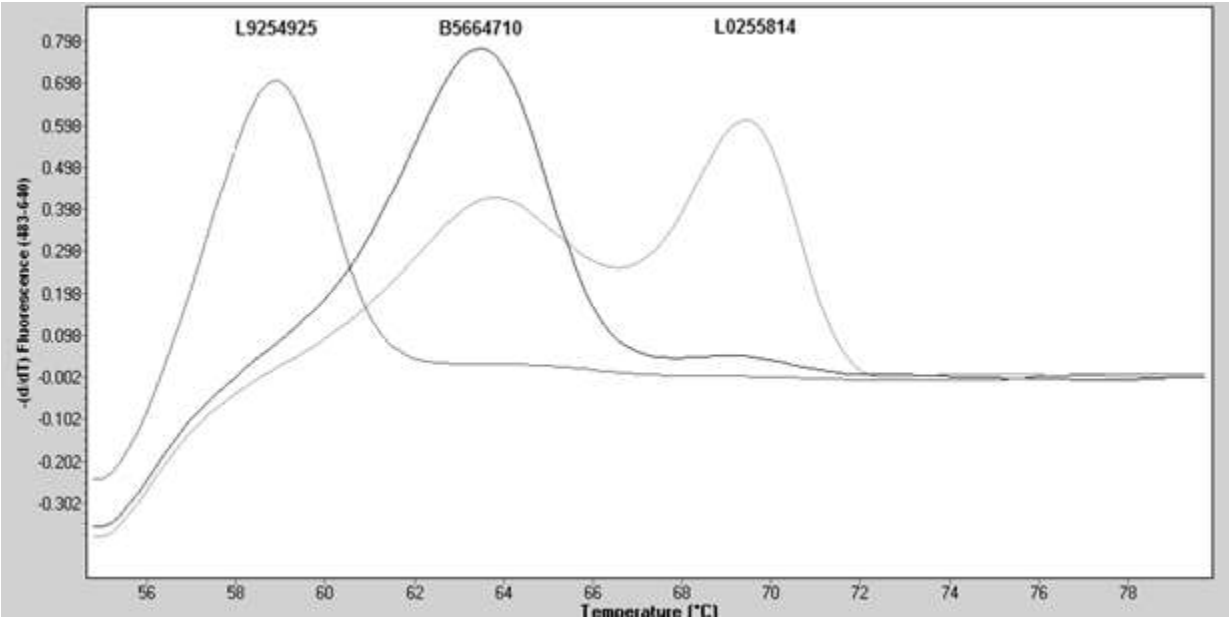
From sequencing of the amplified regions in isolates exhibiting unexpected FRET results, a set of synonymous mutations (Arg91, Tyr100, Ser111) were present in 17 of the 20 isolates (Table 2). The mutations were always found together with Arg91 (C →T), Tyr100 (T →C) located within the QRDR where as Ser111 (T →C) was located 5 residues outside the QRDR. The possibility of Arg91 and Tyr100 to mutate is a cause of concern since Arg91 is involved with ciprofloxacin binding when Asp87 has been mutated to Tyr or Gly (Black et al, 2008). Tyr100 is a site of interaction with ciprofloxacin and gatafloxacin and should nonsynonomous mutation occur may cause instability in drug binding. Another interesting finding was a deletion of Ser 83 in an isolate that exhibited an MIC of 0.06 μg/ml, thus supporting the conclusion that Ser 83's interaction with the fluoroquinolone (e.g, Naladixic acid or Ciprofloxacin) is not what confers suceptability but the overall conformation of the gyrase protein that when changed (i.e. Ser 83 Leu causing hydrophobic interaction) results in high level quinolone resistance .

Results of the experimentally infected urine reveal that the FRET assay is sensitive enough to detect E. coli at 10^1 CFUs. However, if less than 10^3 CFU's, background interference may affect interpretation of results. Never the less since most urinary tract infections are diagnosed with greater than 10^5 CFUs, the FRET assay proves to be sensitive enough to distinguish in pure culture. Further research will have to be preformed to determine the efficacy of the FRET assay for *E. coli* FQ resistance in mixed culture clinical isolates.

Drug Class	Antimicrobial
Beta-Lactamases (1)	Ampicillin (A) Tricarcillin/Clavulanic Acid (R) Amoxicillin/Clavulanic Acid (X) Cephalothin (C) Cefoxitin (O) Cefpodoxime (P) Cefotaxime (T) Ceftazidime (Z)
Tetracyclines (2)	Doxycycline (D)
Chloramphenicol (3)	Chloramphenicol (H)
Fluoroquinolones (4)	Enrofloxacin (E) Ciprofloxacin (F)
Aminoglycosides (5)	Gentamicin (G)
Sulfonamides (6)	Trimethoprim/ Sulfamethoxazole (S)

Table 1: Notation for the 14 Antimicrobials used and their respective drug classes.

Figure 1: Primer and Probe set designed for FRET-PCR. Shown is the alignment of Wild-Type and FQ-R E. coli QRDR regions, boxes outline the placement of described oligonucleotides. The 3' end of the reporter probe is labeled with LightCycler Red 640, while 5' end of the donor probe is labeled with 6-FAM fluorescein.



Sample ID	MDRx	MIC_{Enro} (µg/ml)	Phenotype	Mutations
L9254925	MDR^{124}	128	XATOPZCDER	S83L, D87N , R91, Y100, S111, I112L, A123V, E139A, V146F
B5664710	SDR	0.25	C	D87N, R91, Y100, S111
L0255814	NDR	0.03	N/A	N/A

Figure 3: Melting curves from 3 different *gyrA* mutation profiles encountered in the clinical isolates. Melting temperature and MIC are negatively correlated. MDR indicates the isolate is Multi-drug resistant while SDR and NDR indicate single drug resistance and no drug resistance respectively. Phenotype describes the antimicrobials of which the isolate expressed resistance.

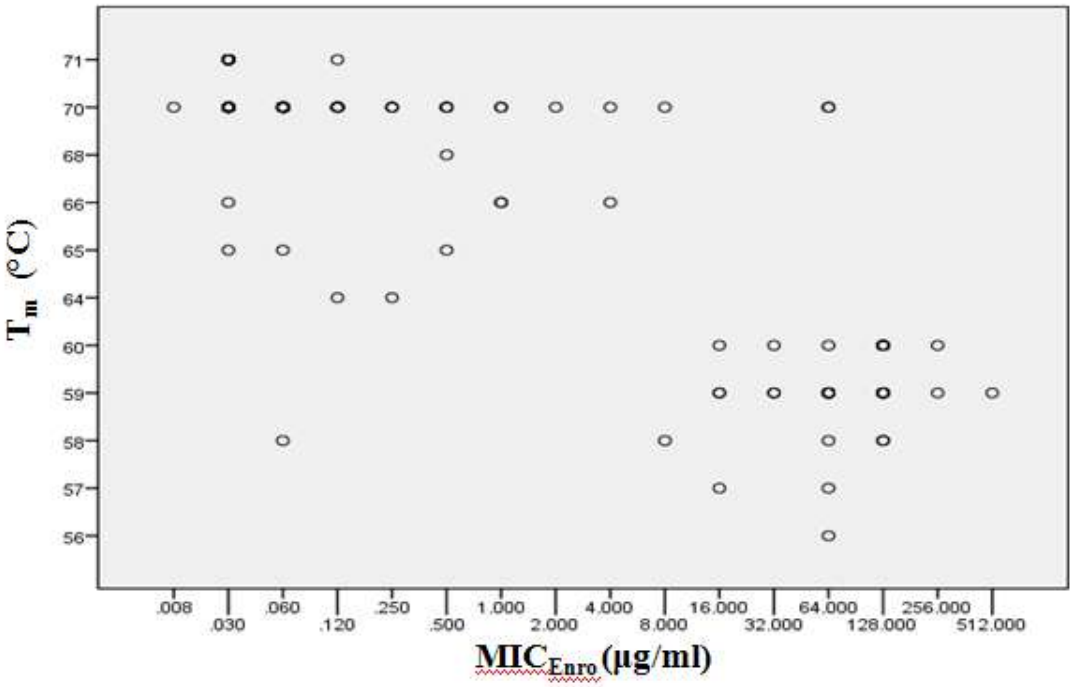


Figure 4: Scatter plot of isolate MIC_{Enro} respective to T_m. MIC and Tm are negatively correlated (R=-0.688). 4 (µg/ml) is the resistant break point for enrofloxacin after which a clear distinction is made between melting temperature.

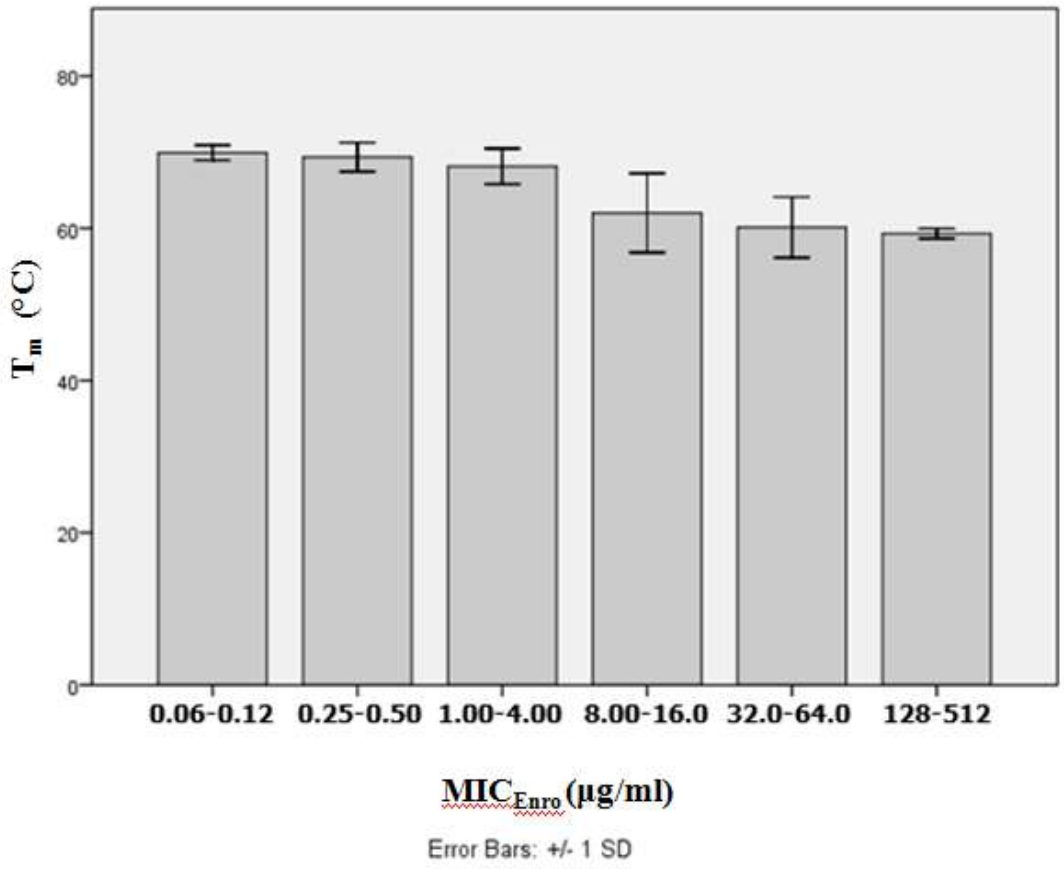


Figure 5: Mean and Standard Deviation of T_m for isolates grouped by MIC_{Enro} class. Variability is not observed in base susceptibility where no mutations in QRDR exist, or in extreme resistance which can only be conferred by coexistence of S83L and D87N. All variablilty is observed in mid-range MICs which may be conferred through methods of resistance other than QRDR mutations alone.

Sample	Location	Melt temp	MDR_X	PHENOTYPE	ENROFL (E) MIC µg/ml	Resistance	Forward Sequence comments
M1896780	Box 3-13	56	MDR^{13456}	XAFOPZCHEGRS	64	R	Ser83Leu C->T Asp87Asn G->A
C8994648	Box 4-66	57	MDR^{12345}	AFHDES	16	R	Ser83Leu C->T Asp87Asn G->A
D8481203	Box 1-78	58	MDR^{16}	CG	0.06	S	Ser83 deleted Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
D8632999	Box 2-45	58	MDR^{12456}	ACDEGRS	8	R	Ser83Leu C->T Asp87Asn G->A Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
K5919720	Box 1-2	58	MDR^{12456}	XAFCDEGRS	64	R	Ser83Leu C->T Asp87Asn G->A Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
L9254925	Box 1 -89	58	MDR^{124}	XATOPZCDER	128	R	Ser83Leu C->T Asp87Asn G->A Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C Ile112Leu A->C Ala123Val C->T Glu139Ala A->C Val146Phe G->T
M1671888	Box 1-1	58	MDR^{124}	XAFTOPCDER	128	R	Ser83Leu C->T Asp87Asn G->A Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
B5664710	Box 1-26	64	SDR	C	0.25	S	Asp87Asn G->A Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
C6393086	Box 1-22	64	SDR	C	0.12	S	Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
B5710554	Box 1-27	65	SDR	D	0.06	S	No Mutations

I9323218	Box 3-82	65	NDR	N	0.5	S	Ser83Leu C->T Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C Ala136Ala C->T
I9874054	Box 3-33	65	NDR	N	0.03	S	Ser83Leu C->T Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C Ala136Ala C->T
M1213579	Box 1-36	66	MDR[12346]	XAOPZCHDEGR	4	R	Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
R6559423	Box 1-46	68	MDR[13]	CH	0.5	S	Asp87Gly A->G Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
I0960311	Box 4-23	70	MDR[1245]	XAFTOPZCDERS	64	R	Ser83Leu C->T Asp87Asn G->A Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
I3180001	Box 2-46	70	MDR[14]	EM	8	R	Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
I7862967	Box 3-27	70	MDR[134]	HEM	64	R	Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C Ala136Ala C->T
K5693300	Box 2-50	70	MDR[14]	CE	1	I	Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
L2020568	Box 3-7	70	MDR[1234]	HDEMR	4	R	Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C
L9245953	Box 2-49	70	SDR	E	2	I	Arg91Arg C->T Tyr100Tyr T->C Ser111Ser T->C

Table 2: Results of Nucleotide Sequences for Determination of Assay Specificity

Sample	MDRX	MIC_{Enro} (μg/ml)	Phenotype
K5262419	NDR	0.006	N
J8067928	SDR	0.12	C
M1840309	MDR14	1	XAOPZCER
I0960311	MDR1245	64	XAFTOPZCDERS
M1671888	MDR124	128	XAFTOPCDER
N0728888	MDR123456	256	XAFTOPZCHDEGRS
ATCC	SDR	0.015	C

Table 3: 7 Escherichia coli isolates of increasing MIC_{Enro} used in inoculating urine. MDRX represents is isolate expresses no drug resistance (NDR), single drug resistance (SDR), or multi-drug resistance (MDR) and to which antimicrobial class resistance is observed. Phenotype represents the individual antimicrobials which the isolate expresses resistance.

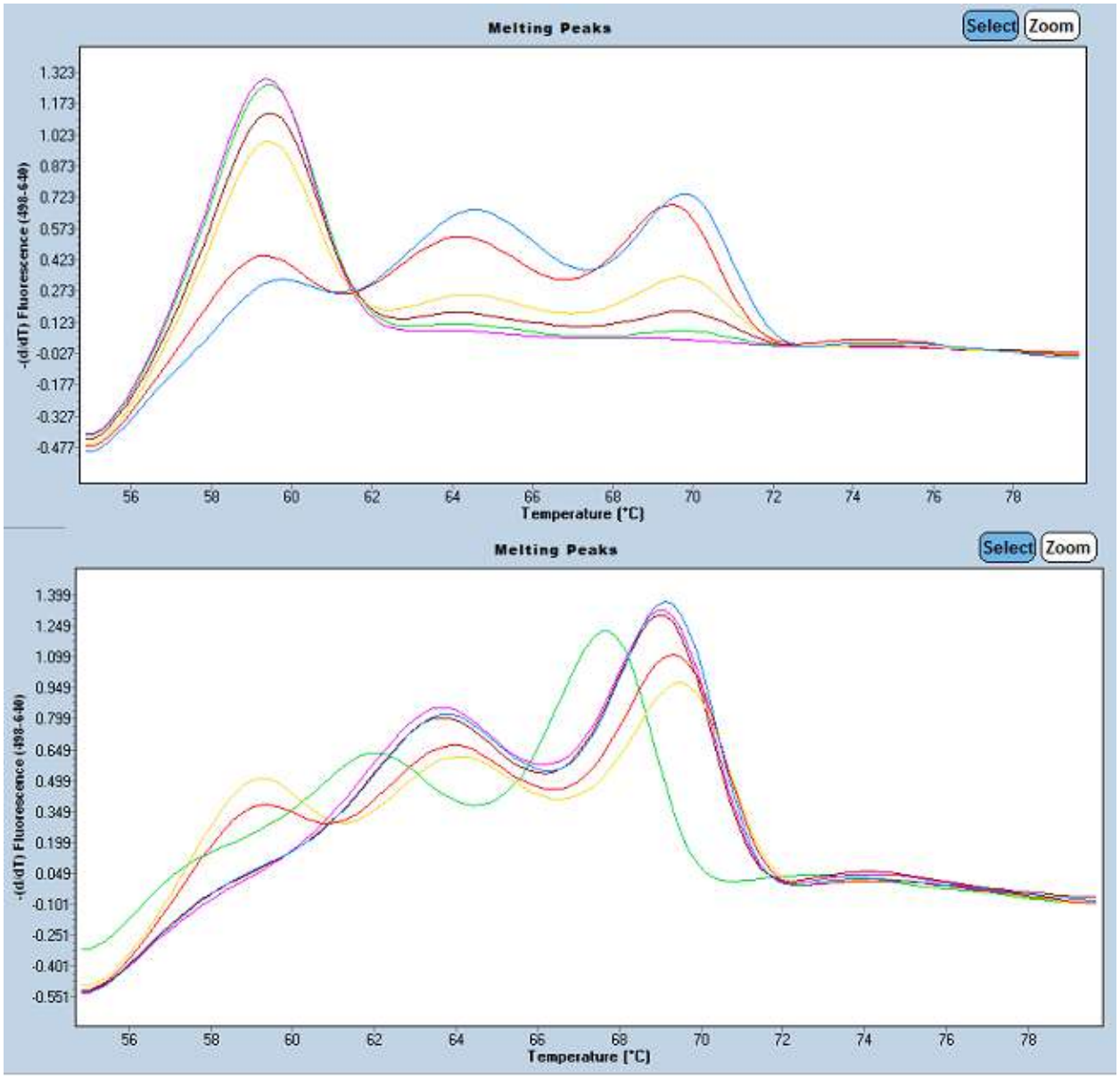


Figure 6: Melting Curves of Canine Urine Inoculated with Dilutions of *E. coli*. Colors denote the following inoculation dilutions (CFU/ml): Green 10^6, Pink 10^5, Brown 10^4, Yellow 10^3, Red 10^2, Blue 10^1. Top: Isolate N0728888 MDR1234 MIC_{Enro} 256 µg/ml Phenotype: XAFTOPZCHDEGRS, Bottom: ATCC25922 SDR MIC_{Enro} 0.015 µg/ml Phenotype:C.

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CHAPTER 3

EVALUATION OF A FRET-PCR ASSAY FOR DETERMINING FLUOROQUINOLONE RESISTANT *ESCHERICHIA COLI* IN CLINICAL URINE ISOLATES FROM COMPANION ANIMALS

Abstract

Antimicrobial resistance in *Escherichia coli* is becoming of increasing concern in public health affecting patients of both human and veterinary hospitals. A commonly selected antimicrobial for treatment in small animals is enrofloxacin, a second generation fluoroquinolone (FQ). Among the difficulties in effective *E. coli* treatment is rapid detection of fluoroquinolone resistance. The purpose of this study was to determine the specificity and sensitivity of a FRET based assay for the rapid detection of urinary tract infections caused by fluoroquinolone associated multi-drug resistant *E.coli*. Two hundred and thirty-eight clinical urine samples were collected via cystocentesis or free catch, and screened for presence of aerobic bacteria. Isolates were subjected to susceptibility testing for enrofloxacin and the FRET assay, while DNA was also collected directly from urine samples and subjected to the FRET assay. Of 438 urine samples, 278 were confirmed to contain *E. coli* 18 of which were confirmed to be resistant to enrofloxacin by susceptibility testing. The FRET assay positively identified 15 of the 18 enrofloxacin resistant *E. coli* urine samples for sensitivity of 83.33% and negatively identified 406 samples for specificity of 92.36%.

Introduction

Escherichia coli is a major cause of urinary tract infections (UTI) in canines (Ling et. al. 1979). Of these infections, antimicrobial resistant *E. coli* is increasingly identified. In particular, an increase in fluoroquinolone resistance has been reported, and it is frequently associated with multidrug resistant phenotypes (Hirsch et. al. 1973), (Cook et. al 2002), (Cohn et. al. 2003), (Boothe et. al 2006). A previous study demonstrated that a FRET-PCR based assay could discriminate between fluroquinolone resistant and susceptible *E. coli*. (Shaheen et al, 2009). Such an assay might facilitate early treatment decisions in the clinical patients infected with *E. coli* by minimizing the inappropriate use of an FQ if the *E. coli* already is resistant or by detecting resistance that emerges in the face of therapy (Richard et. al. 1994),(Webber and Paddock, 2001). While culture and susceptibility testing of *E. coli* continues to be the gold standard for the detection of antimicrobial resistance, time becomes an issue, requiring 2-5 days to obtain results. There is a need for an alternative method that allows rapid and sensitive detection of MDR/FQ resistance in urinary isolates for a clinical setting (Siedner et al., 2007). Development of rapid diagnostic tools for *E. coli* have been attempted in DNA microarray (Yu et al, 2007), (Barl et al, 2008), pyrosequencing (Guillard et al, 2010), and mismatch amplification mutation assay; however these techniques can be costly, cumbersome, and require specialty equipment. A FRET-PCR based assay for detection directly from urine sample would decrease the window between the collection and susceptibility result. This type of technique has been developed for many other pathogens with success while maintaining cost effectiveness (Lindler et. al, 2001), (Qiang et al, 2002), (Page et. al, 2008), (Nakamura et al, 2009), and (Spigaglia et al, 2010).

In this study we evaluate the ability of a FRET-PCR based assay to discriminate fluroquinolone resistant *E. coli* in clinical urine samples from companion animal patients.

Materials and Methods

Collection of Urine Samples and Isolation of Bacteria

Urine samples collected from dogs and cats and submitted to Auburn University Small Animal Teaching Hospital (AUSATH) through Clinical Pathology and Clinical Microbiology, and IDEXX Laboratory were studied. Samples had been collected either by cystocentesis or free catch. Upon receipt at Auburn University, samples were stored at 4C. 10uL of urine was transferred to CHROMagar (BD Diagnostics, Franklin Lakes, NJ) and incubated at 37C overnight for isolation, detection, and speciation of bacteria. Individual colonies from each present species were transferred to trypticase soy agar (TSA) in order to grow for cryogenic storageIsolates were preserved in brucella broth/ glycerol cyrovials (70% brucellla broth/30% glycerol); these samples were held in reserve (Table 1).

Susceptibility Testing for Enrofloxacin Resistance

Urine samples collected through AUSMTH Clinical Microbiology and IDEXX Laboratories were subjected to susceptibility testing appropriate to organism via CLSI guidelines (CLSI, 2008) and results forwarded to Auburn University Veterinary Clinical Pharmacology Laboratory. Isolates obtained through Clinical Pathology were subjected to susceptibility testing for enrofloxacin via E-test Epsilometer testing (bioMérieux, Marcy l'Etoile, France).

Preparation of Urine Samples and Isolates for FRET-PCR

Urine samples were concentrated using Microsep 100k Centrifugal Devices (Pall Corporation, Port Washington, NY). Samples were centrifuged at 1900 x g for 40m, precipitate was collected along with 150 uL of urine supernatant for DNA extraction. DNA was extracted with the E.Z.N.A. Viral RNA Kit (Omega Bio-tek,) using the extracting bacterial DNA from urine protocol. DNA was eluted to 50uL and stored at 4C. For the bacterial isolates, one bacterial colony was selected from TSA plates and DNA was extracted using 200 uL of PrepMan ULTRA sample preparation reagent (Applied Biosystems, Foster City, CA). Isolated DNA was then stored at 4C. Gyrase A FRET-PCR primers and probes (Shaheen et al, 2009) and LightCycler 480 Genotyping Master (Roche Applied Science, Indianapolis, IN) supplemented with 2.0 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) were used for the FRET-PCR reactions.

Determination of FRET-PCR Results

A result was considered a true positive if FRET-PCR $T_m \leq 60°C$ and if the sample contained an enrofloxacin resistant *E. coli* as determined by culture and susceptibility testing. A result was designated a true negative if FRET-PCR revealed $60°C < T_m$ despite the absence of enrofloxacin resistant *E. coli*. False positive samples yielded FRET-PCR $T_m \leq 60°C$ in samples containing no enrofloxacin resistant *E. coli* whereas false negative results reflected FRET-PCR $60°C < T_m$ in samples containing resistant *E. coli.*

Results

Collection of Urine Samples and Susceptibility Testing From Each Origin

Out of 438 urine samples collected, 327 were positive for aerobic bacterial growth. Of these, 31 contained multiple species accounting for a total of 362 isolates. 280 of these isolates were identified as *E. coli*, 21 of which exhibited intermediate (1 ug/ml ≤ MIC_{Enro} < 4 ug/ml, n=5) or resistant MIC_{Enro} (MIC_{Enro} ≥ 4 ug/ml, n=16) For the rest of the isolates, 26 were identified as *Enterococcus sp.*, 22 were identified as *Klebsiella sp.*, 13 were identified as *Staphylococcus sp*., 12 were identified as *Proteus sp.*, 7 were identified as *Streptococcus sp.*, and 4 were identified as *Pseudomonas sp.* (Table 1) (Figure 2) (Figure 3). The 64 isolates from Clinical Microbiology were unable to be cultured in the Clinical Pharmacology Laboratory due to hospital regulations on holding samples. Their isolates were unable to be compared to the urine sample results.

Anaylsis of Urine Samples by FRET-PCR

Of 438 urine samples, 17 were confirmed by culture and susceptibility testing to be positive for enrofloxacin resistant *E. coli*. 14 of these isolates were also positively (true positives) identified by the FRET-PCR assay yielding a sensitivity of 83.33%. 33 urine samples not containing *E. coli* FQ-R were detected yielding a specificity of 92.36% (false positives).

Out of 298 aerobic bacterial isolates that were cultured from the urine, 8 were confirmed by culture and susceptibility testing to be positive for ENR-R *E. coli.* All of these isolates were positively identified by the FRET-PCR assay yielding a sensitivity of 100%. 278 isolates not containing *E. coli* expressing an FQ-R MIC were detected yielding a specificity of 95.86% (Table 2). When sensitivity and specificity is determined for isolates collected from AUSMTH by collection method cystocentesis has lower sensitivity (70.00%) but higher specificity (94.11%) compared to voided (sensitivity = 100%, specificity = 89.52%) (Table 3).

Discussion

The results of this study confirm our findings from the previous study that the FRET assay is able to detect enrofloxacin resistant *E. coli*. While we do have discrepancy between specificity and sensitivity among the DNA extraction methods, it has been seen previously that extraction method can influence results (Behringer et al, 2011) (Figure 1). Sources of this discrepancy could be unculturable organisms found in the urine that would be lost when collecting isolates, interfering DNA from canine endothelial cells, or residual reagents from the different extraction methods may be enough to disrupt PCR reaction chemistry. In addition by products from the urine may contaminate the DNA elution or the extraction from urine may be too rigorous causing some damage to the DNA (Figure 4) (Figure5).

Nucleic acids from these organisms would be present in the extracted DNA sample from urine and may interfere with probe specificity which is seen in the melting peaks in figure 5 there is noticeable background.

Urine samples containing confirmed mixed cultures resulted in 4 false positive profiles (3 of which contained E.coli/Enterococcus) and 1 false negative profile. Urine samples containing *Staphylococcus sp.* (n= 12) accounted for 5 false positive profiles along with uninfected urine (n= 110) Urine samples containing *Streptococcus pseudointermedius* (n= 7) accounted for 4 false positive profiles while only 3 samples containing *Enterococcus* (n= 26) and 1 sample containing each *Klebsiella sp.* (n= 22) and *Proteus sp*. (n= 13) gave false positive profiles. For the *Klebsiella* isolate the MIC_{Enro} >32 ug/ml, while for the *Proteus* isolate the MIC_{Enro} = .12ug/ml. After examination of alignments of the reporter probe and laboratory strains of each organism, this is probably due to the greater homology between the front of the reporter probe and regions in *Staphylococcus* and *Streptococcus gyrA* thus giving the probe a stronger anchor near the

fluorophore allowing it to become excited creating a low T_m melting curve. Adjustments in annealing temperature during themocycling may allow the primers to be more specific and avoid producing template that the probes could bind to causing inaccurate results.

Origin	Source	Species	Number of Strains
Clinical Pathology	Cystocentesis	Escherichia coli	17
		Enterococcus sp.	8
		Klebsiella sp.	4
		Proteus sp.	3
		Staphylococcus sp.	1
	Void	Escherichia coli	22
		Enterococcus sp.	14
		Klebsiella sp.	15
		Proteus sp.	4
		Streptococcus sp.	5
		Staphylococcus sp.	1
	Catheter	Escherichia coli	4
		Enterococcus sp.	1
		Klebsiella sp.	3
Clinical Microbiology	Cystocentesis	Escherichia coli	53
		Enterococcus sp.	3
		Pseudomonas sp.	2
		Staphylococcus sp.	1
	Void	Escherichia coli	11
		Proteus sp.	1
		Streptococcus sp.	1
	Catheter	Escherichia coli	1
		Streptococcus sp.	1
IDEXX		Escherichia coli	171
		Proteus sp.	4
		Pseudomonas sp.	2
		Staphylococcus sp.	9
Total			362

Table 1: Species, source, and origin of organisms isolated from urine samples

Source of DNA Extraction	Result of FRET Assay	Totals
Urine	True Positive	15
	True Negative	387
	False Positive	32
	False Negative	3
	Sensitivity	**82.35%**
	Specificity	**92.36%**
Isolates	True Positive	8
	True Negative	278
	False Positive	12
	False Negative	0
	Sensitivity	**100.00%**
	Specificity	**95.86%**

Table 2: Comparison of sensitivity and specificity of FRET assay by DNA extraction method

Collection Method	Result of FRET Assay	Number of samples expressing result
Cystocentesis	True Positive	13
	True Negative	214
	False Positive	15
	False Negative	3
	Sensitivity	**81.25%**
	Specificity	**93.44%**
Voided	True Positive	5
	True Negative	98
	False Positive	14
	False Negative	0
	Sensitivity	**100.00%**
	Specificity	**87.50%**
Catheter	True Positive	0
	True Negative	13
	False Positive	0
	False Negative	0
	Sensitivity	**0.00%**
	Specificity	**100.00%**

Table 3: Sensitivity and Specificity of FRET Assay by Collection Method; IDEXX samples are omitted because collection method was not disclosed.

Sample #	ID#	FRET Result	Lab	Collection Method	Species 1	Phenotype	Species 2	Phenotype
111	1091631	+	Clin. Path	Voided	*E. coli*	E		
160	1082748	+	Clin. Path	Voided	*E. coli*	E	*Klebsiella*	E
249	1092349	-	Clin. Path	Cystocentesis	*E. coli*	XAVOYHDE *GMBRS	*Proteus*	
312	1092349	+	Clin. Micro	Cystocentesis	*E. coli*	XAVOYHDE GMBRS	*Pseudomonas*	H
319	1061672	-	Clin. Micro	Cystocentesis	*E. coli*	XAYDEGM BRS		
323	1092933	+	Clin. Micro	Cystocentesis	*E. coli*	AVYEMBRS	*E. coli*	N
325	Bac 2399	+	Clin. Micro	Voided	*E. coli*	XAVOYHDE MBRS		
341	1080953	+	Clin. Micro	Cystocentesis	*E. coli*	E		
344	1079634	+	Clin. Micro	Cystocentesis	*E. coli*	XAVOYHEG MBR		
345	1091476	-	Clin. Micro	Cystocentesis	*E. coli*	XAVOYHDE MBR		
346	1061672	+	Clin. Micro	Cystocentesis	*E. coli*	XAVOYD	*E. coli*	XAVOYH DEMBRS
347	1082748	+	Clin. Micro	Cystocentesis	*E. coli*	ADEMBRS		
1024	S5749049	+	Idexx		*E. coli*	H*EGMS		
1065	A8435917	+	Idexx		*E. coli*	XVYH*EMB		
1090	L1553660	+	Idexx		*E. coli*	XVYEGM		
1094	L1590805	+	Idexx		*E. coli*	H*EM		
1112	L1604320	+	Idexx		*E. coli*	XVYHEMBS		

Table 4: FRET results for urine samples containing enrofloxacin resistant *E. coli*. For 17 resistant isolates, 14 were identified by the FRET assay. Species 1 designates primary infective species; Species 2 designates co infective species. Phenotype describes the antimicrobials to which the isolate expressed resistance.

Sample #	ID#	FRET Result	Lab	Collection Method	Species 1	Phenotype	Species 2	Phenotype
10	1090596	+	Clin. Micro	Cystocentesis	*E. coli*			
23	1085460	+	Clin. Path	Voided	*Klebsiella*	KHE*G	*Streptococcus*	E
33	1091169	+	Clin. Path	Voided	-			
62	1091330	+	Clin. Path	Voided	*Enterococcus*			
66	1069127	+	Clin. Path	Voided	*Streptococcus*			
85	1076465	+	Clin. Path	Cystocentesis	*E. coli*			
88	1082748	+	Clin. Path	Cystocentesis	*Enterococcus*	E*		
116	1091653	+	Clin. Path	Voided	-			
119	1070105	+	Clin. Path	Voided	-			
125	1082748	+	Clin. Micro	Cystocentesis	*Enterococcus*	XAEM		
134	1091717	+	Clin. Path	Voided	*E. coli*		*Enterococcus*	E*
136	1073104	+	Clin. Path	Voided	*Streptococcus*	E*		
153	1091783	+	Clin. Path	Voided	-			
213	1061672	+	Clin. Path	Cystocentesis	*E. coli*			
235	1090519	+	Clin. Path	Voided	*Proteus*			
240	1080953	+	Clin. Path	Cystocentesis	*Enterococcus*			
284	116514	+	Clin. Path	Cystocentesis	*Klebsiella*	E		
321	Bac 2395	+	Clin. Micro	Voided	*E. coli*	N	*Streptococcus*	N
330	1090008	+	Clin. Micro	Cystocentesis	*E. coli*	AH	*Enterococcus*	N
1079	A8407897	+	Idexx		*Staphylococcus*	N		
1080	K2009631	+	Idexx		*E. coli*	H*		
1081	A8481393	+	Idexx		*Staphylococcus*	N		
1083	L1536014	+	Idexx		*Staphylococcus*	XVYEG*MS		
1084	C0551061	+	Idexx		*E. coli*	N		
1085	A8436315	+	Idexx		*Proteus*	N		
1086	A8480046	+	Idexx		*Staphylococcus*	N		
1089	A8312185	+	Idexx		*Staphylococcus*	N		
1096	A8547029	+	Idexx		*E. coli*	N		
1102	A8563514	+	Idexx		*E. coli*	N		

1105	L1607528	+	Idexx		*E. coli*	N		
1110	A8559398	+	Idexx		*E. coli*	N		
1111	L1610766	+	Idexx		*E. coli*	H*		
1114	L1602432	+	Idexx		*E. coli*	N		

Table 5: Urine samples falsely identified by FRET to have enrofloxacin resistant *E. coli.* Species 1 designates primary infective species; Species 2 designates co infective species. Phenotype describes the antimicrobials to which the isolate expressed resistance.

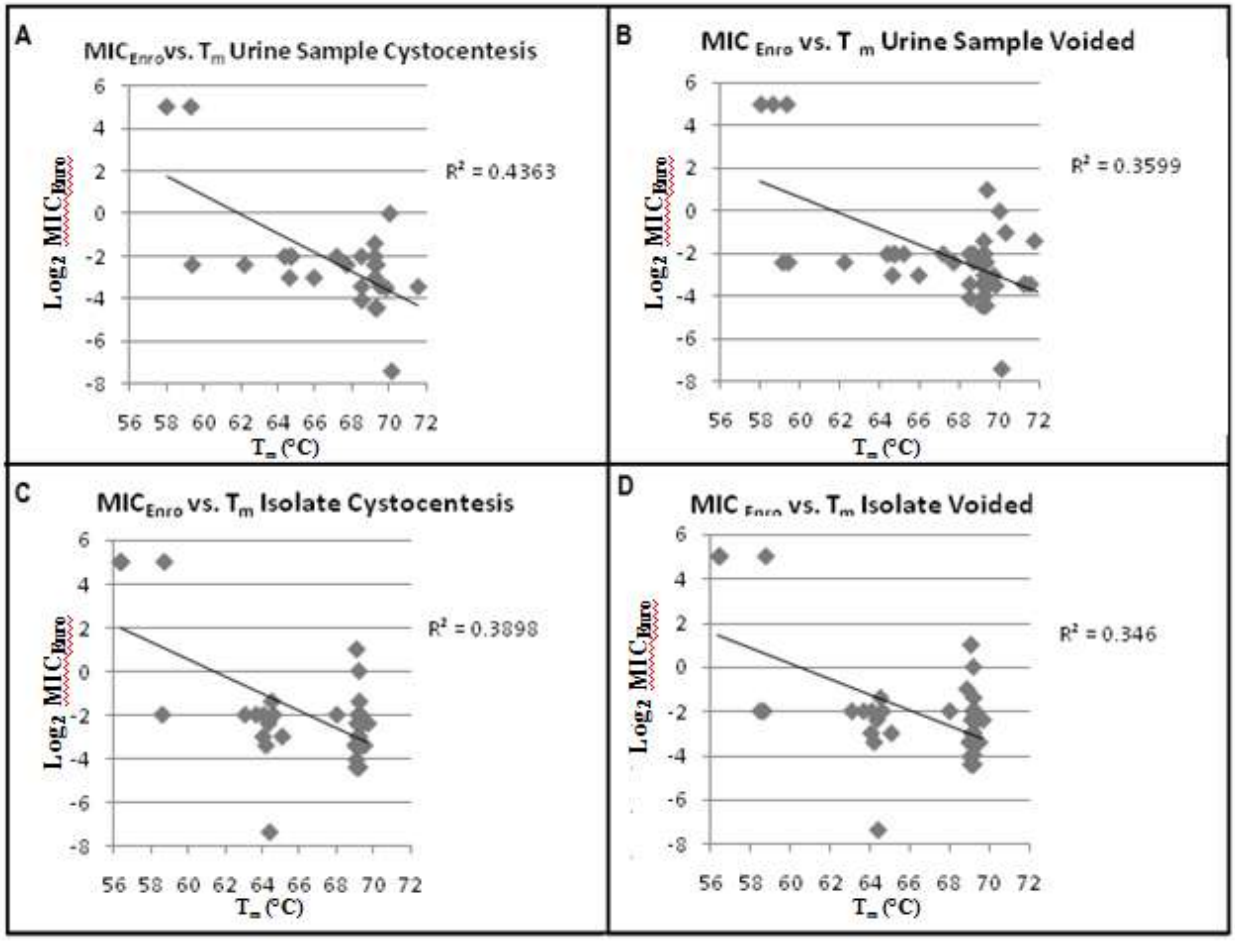

Figure 1: $\log_2$ MIC_{Enro} vs. T_m for urine samples containing *E. coli* and *E. coli* isolates. A) Urine samples containing *E. coli* collected by cystocentesis. B) Urine samples containing *E. coli* collected by void. C) *E. coli* isolated from urine collected by cystocentesis. D) *E. coli* isolated from urine collected by void. R^2 represents the correlation between MIC_{Enro} and T_m.

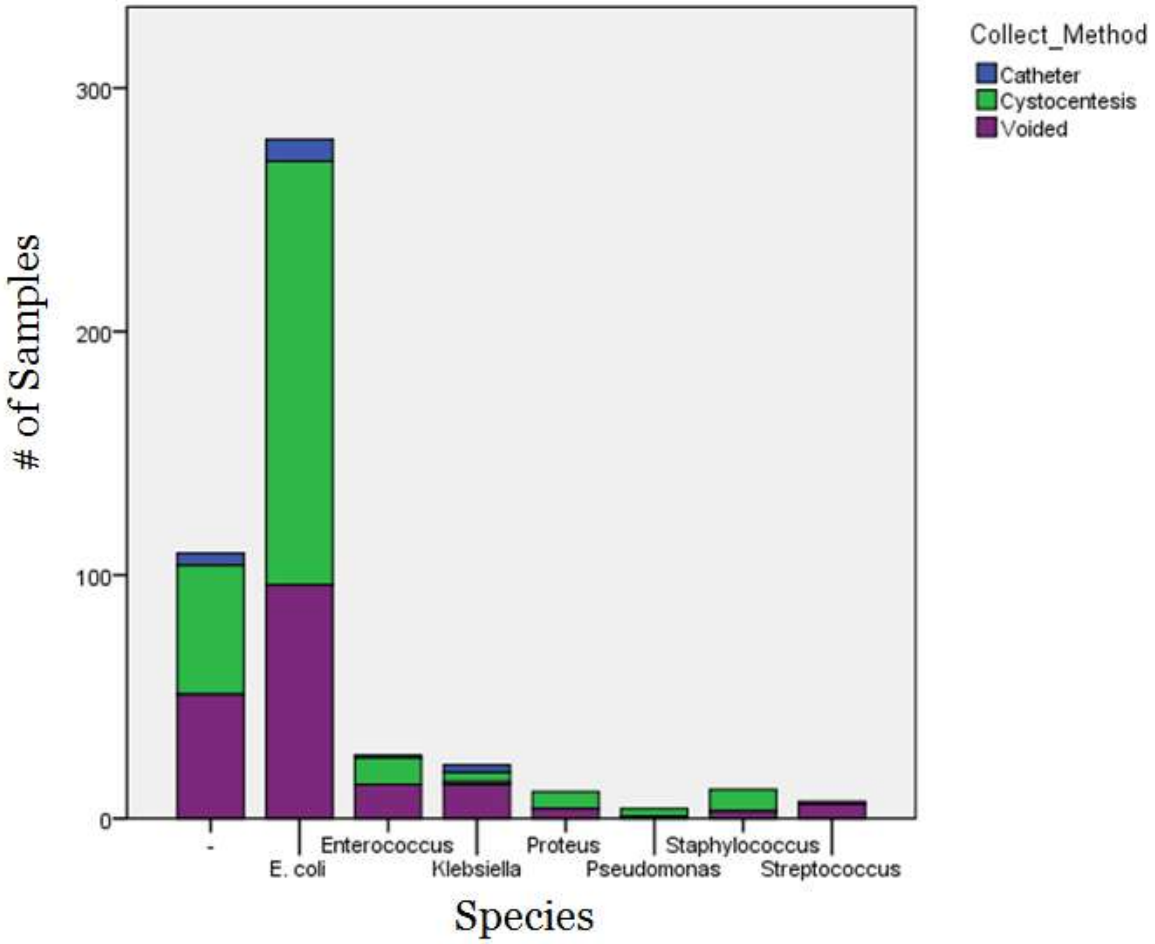

Figure 2: Contents of urine samples by collection method. Urine samples negative for bacteria were most prominent overall and for voided urine. For cystocentesis, *E. coli* infection was most prominent.

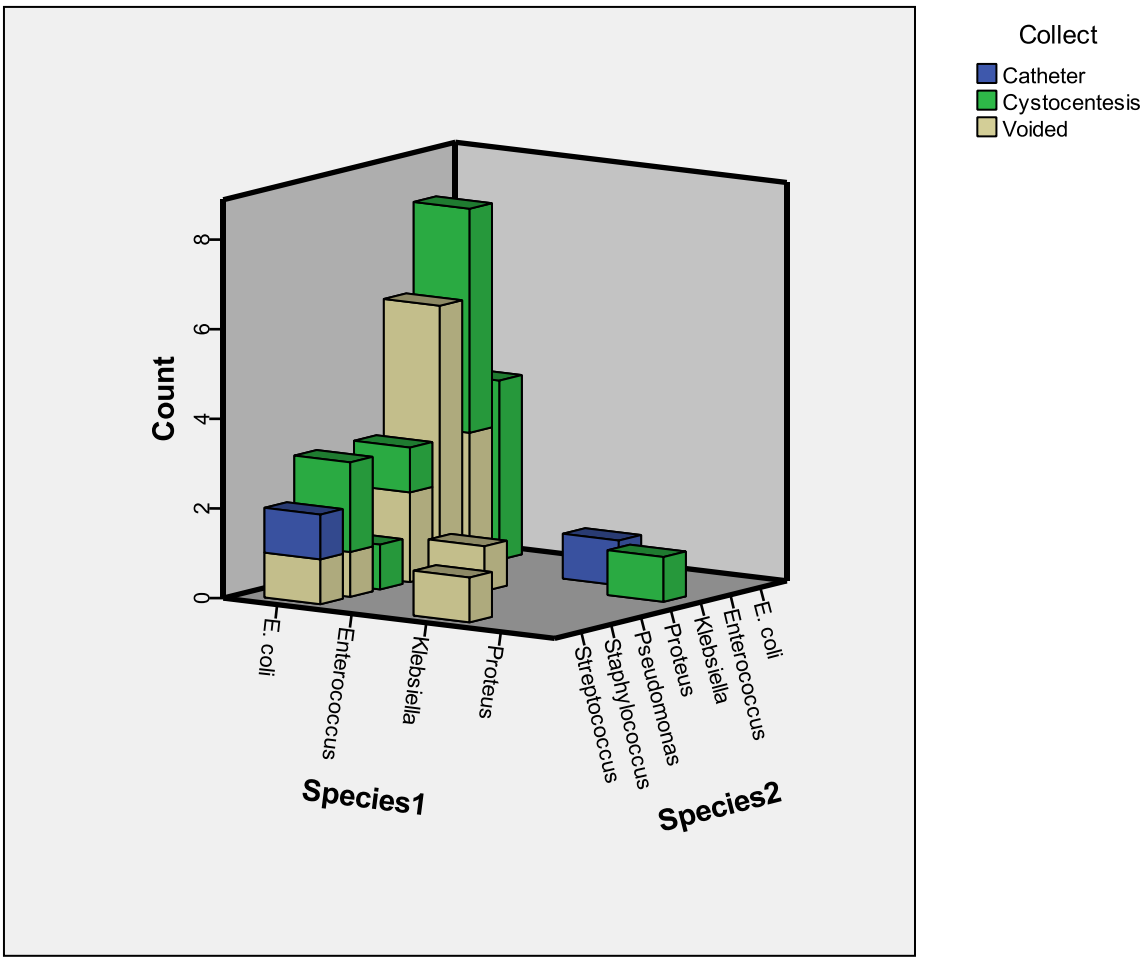

Figure 3: Distribution of Urine samples containing multiple organisms by species and collection method. Species1 represents primary infective organism while Species 2 represents secondary infective organism. This was designated by concentration of each organism.

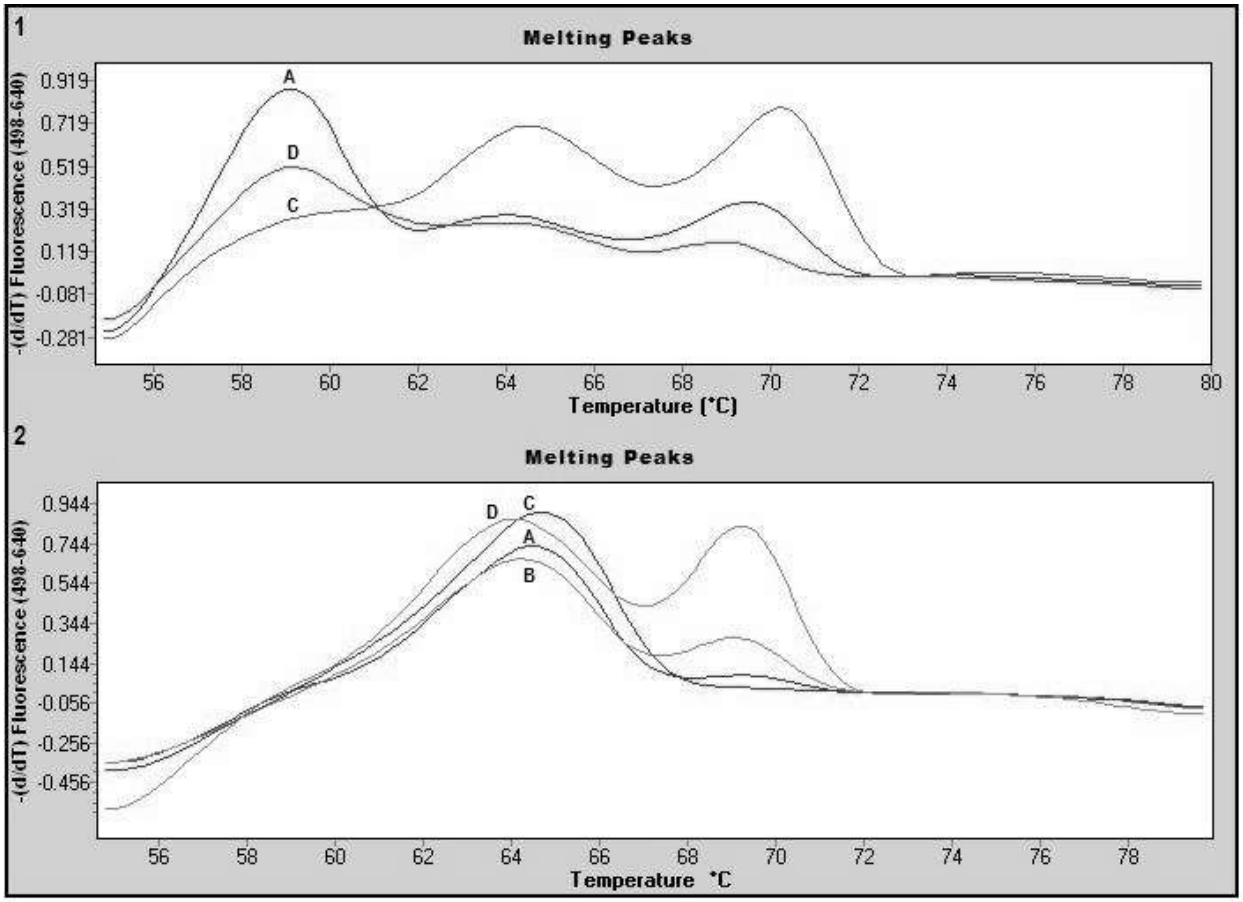

Figure 4: Representative Melting curve analysis of DNA from both extraction methods. 1) DNA extracted from urine sample A) 1085460 *Klebsiella sp.*/*S. agalactiae*, C) 1091182 *E. coli*, D) 1091330 *Enterococcus sp.* 2) DNA extracted from isolated colonies A) 1085460-1 *Klebsiella sp.*, B) 1085460-2 *S. agalactiae* (Isolated from A in urine sample), C) 1091182 *E. coli*, D) 1091330 *Enterococcus sp.* The urine sample T_m is shifted left compared to isolate $T_{m,}$ this may be due to other DNA contaminants found in the urine, such as host DNA.

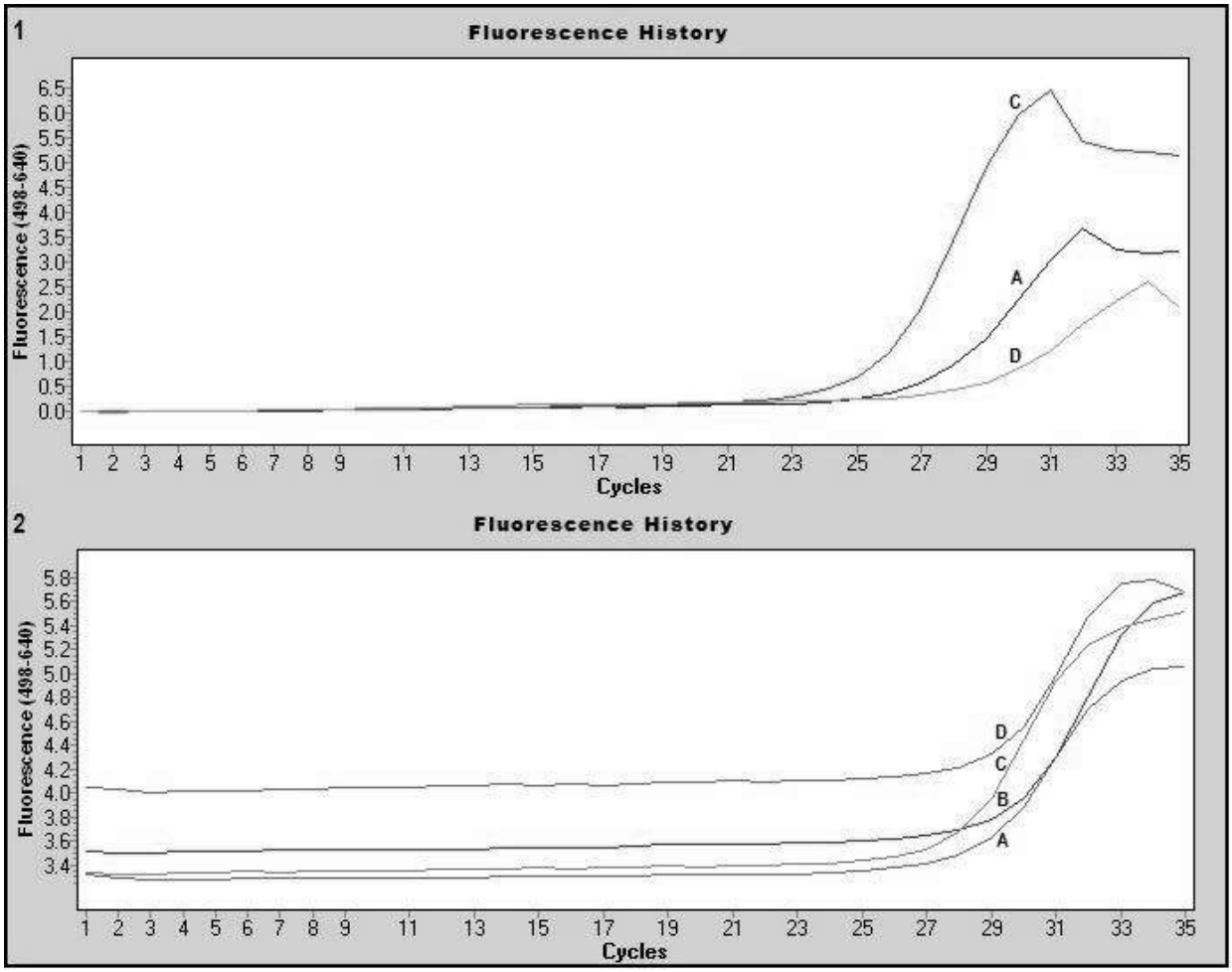

Figure 5: Amplification curves of DNA from both extraction methods. 1) DNA extracted from urine sample A) 1085460 *Klebsiella sp./S. agalactiae*, C) 1091182 *E. coli*, D) 1091330 *Enterococcus sp.* 2) DNA extracted from isolated colonies A) 1085460-1 *Klebsiella sp.*, B) 1085460-2 *S. agalactiae*, C) 1091182 *E. coli*, D) 1091330 *Enterococcus sp.* The urine sample amplification is not smooth; this may be due to different extraction methods.

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APPENDIX A

DATA FOR URINES CONTAINING NEGATIVE AND SINGLE CULTURES

Sample	ID	T_m C	FRET Result	Lab	Collection Method	Species 1	Phenotype
4	1090460	70	-	Clin. Micro	Cystocentesis	*E. coli*	
5	1080784	70	-	Clin. Micro	Cystocentesis	*E. coli*	
6	1088931	70	-	Clin. Micro	Cystocentesis	*E. coli*	
7	1072801	70	-	Clin. Micro	Cystocentesis	*E. coli*	ABS
8	1085623	70	-	Clin. Micro	Cystocentesis	*E. coli*	
9	1090093	70	-	Clin. Micro	Cystocentesis	*E. coli*	
10	1090596	59	+	Clin. Micro	Cystocentesis	*E. coli*	
14	1090885	70	-	Clin. Micro	Cystocentesis	*E. coli*	N
15	1090777	70	-	Clin. Micro	Cystocentesis	*E. coli*	N
16	1091157			Clin. Path	Voided	-	
17	1091149	69	-	Clin. Path	Voided	-	
18	1067405	70	-	Clin. Path	Voided	-	
19	1088390	64.5	-	Clin. Path	Voided	-	
20	1091151	64.5	-	Clin. Path	Cystocentesis	*E. coli*	N
21	1089306	69.5	-	Clin. Path	Voided	-	
22	1091170	69	-	Clin. Path	Voided	-	
23	1085460	59	+	Clin. Path	voided	*Klebsiella*	KHE*G
24	1091152	69.2	-	Clin. Path	Voided	-	
25	1091174	69.5	-	Clin. Path	Cystocentesis	-	
26	1091161	62.6	-	Clin. Path	Cystocentesis	-	
27	1091158	x	-	Clin. Path	Cystocentesis	*Klebsiella*	HE*

28	1082748	69.6	-	Clin. Path	Cystocentesis	*Klebsiella*	E*
29	1091184	68.6	-	Clin. Path	Voided	-	
30	1076465	68.5	-	Clin. Path	Cystocentesis	-	
31	1066184	69.6	-	Clin. Path	Cystocentesis	-	
32	1091182	70.1	-	Clin. Path	Voided	*E. coli*	
33	1091169	60	+	Clin. Path	Voided	-	
34	1091152	64.25	-	Clin. Path	Voided	-	
35	1091211	70	-	Clin. Path	Voided	-	
36	1091210	70.3	-	Clin. Path	Voided	-	
37	1090550	69.5	-	Clin. Path	Cystocentesis	-	
38	1091258	70.8	-	Clin. Path	Cystocentesis	-	
39	1091256	70.1	-	Clin. Path	Voided	-	
40	1081036	70.48	-	Clin. Path	Voided	-	
41	1091249	70	-	Clin. Path	Voided	-	
42	1090899	69.6	-	Clin. Path	Catheter	-	
43	1085302	64.7	-	Clin. Path	Voided	-	
44	1091263	69.8	-	Clin. Path	Cystocentesis	-	
45	1091261	70.2	-	Clin. Path	Cystocentesis	-	
46	1091232	70	-	Clin. Path	Voided	*E. coli*	E*
47	1090805	69.5	-	Clin. Path	Cystocentesis	-	
48	1091281	69.7	-	Clin. Path	Voided	-	
49	1091278	68.7	-	Clin. Path	Cystocentesis	-	
50	1091291	69.9	-	Clin. Path	Catheter	-	
51	1091293	69.7	-	Clin. Path	Cystocentesis	-	
52	1091301	69.8	-	Clin. Path	Cystocentesis	-	
53	1091314	69.7	-	Clin. Path	Cystocentesis	-	
54	1091324	69.3	-	Clin. Path	Voided	-	
55	1091344	69.6	-	Clin. Path	Cystocentesis	-	
56	1076465	69.4	-	Clin. Path	Catheter	*Klebsiella*	
57	1091378	69.7	-	Clin. Path	Voided	*Klebsiella*	E*

58	1091373	69.1	-	Clin. Path	Voided	-	
59	1091391	69.2	-	Clin. Path	Voided	*E. coli*	
60	1091306	69.6	-	Clin. Path	Cystocentesis	-	
61	1091387	69.4	-	Clin. Path	Voided	*Enterococcus*	
62	1091330	59.9	+	Clin. Path	Voided	*Enterococcus*	
63	1091375	69.4	-	Clin. Path	Voided	-	
64	1091157	69.5	-	Clin. Path	Catheter	-	
65	1091401	68.7	-	Clin. Path	Voided	*Klebsiella*	E
66	1069127	59.1	+	Clin. Path	Voided	*Streptococcus*	
67	1091403	68.9	-	Clin. Path	Voided	-	
68	1091394	69.2	-	Clin. Path	Cystocentesis	-	
69	1091439	68.8	-	Clin. Path	Voided	-	
70	1091417	69.5	-	Clin. Path	Cystocentesis	-	
71	1091468	69.1	-	Clin. Path	Cystocentesis	-	
72	1091411	69.4	-	Clin. Path	Cystocentesis	-	
73	1091412	68.5	-	Clin. Path	Voided	*E. coli*	
74	1076465	69.2	-	Clin. Path	Voided	*E. coli*	ADB
75	1091402	69.4	-	Clin. Path	Voided	-	
76	126524	69.1	-	Clin. Path	Voided	-	
77	1091414	69.5	-	Clin. Path	Voided	-	
78	1082592	69.4	-	Clin. Path	Voided	*Klebsiella*	
79	1088390	69.2	-	Clin. Path	Voided	-	
80	1091477	69.3	-	Clin. Path	Cystocentesis	*E. coli*	
81	1091475	69.1	-	Clin. Path	Voided	*Enterococcus*	
82	1091478	69.2	-	Clin. Path	Cystocentesis	*E. coli*	
83	1082899	69.3	-	Clin. Path	Cystocentesis	-	
84	1091508	68.9	-	Clin. Path	Cystocentesis	-	
85	1076465	59.1	+	Clin. Path	Cystocentesis	*E. coli*	
86	1091473	69.3	-	Clin. Path	Voided	*E. coli*	
87	1090121	69	-	Clin. Path	Cystocentesis	*E. coli*	

88	1082748	59.3	+	Clin. Path	Cystocentesis	*Enterococcus*	E*
89	1091474	69.4	-	Clin. Path	Cystocentesis	-	
90	1091492	68.7	-	Clin. Path	Cystocentesis	*E. coli*	
91	1091521	68.9	-	Clin. Path	Voided	-	
92	1091522	68.9	-	Clin. Path	Cystocentesis	-	
93	1091523	69.3	-	Clin. Path	Cystocentesis	-	
94	1091528	69.1	-	Clin. Path	Cystocentesis	-	
95	1091531	69.1	-	Clin. Path	Voided	*Enterococcus*	
96	1091518	69.1	-	Clin. Path	Voided	-	
97	118704	68.9	-	Clin. Path	Cystocentesis	-	
98	1091524	62.2	-	Clin. Path	Voided	*E. coli*	
99	1091540	69.2	-	Clin. Path	Voided	*Enterococcus*	
100	1091542	68.9	-	Clin. Path	Cystocentesis	-	
101	1089908	69.1	-	Clin. Path	Cystocentesis	*E. coli*	N
102	1086044	69.1	-	Clin. Path	Voided	*Enterococcus*	
103	1076341	69.1	-	Clin. Path	Voided	*Klebsiella*	
104	1091559	69	-	Clin. Path	Cystocentesis	*Enterococcus*	
105	1091542	68.4	-	Clin. Path	Catheter	*Klebsiella*	
106	127374	67.6	-	Clin. Path	Cystocentesis	*Proteus*	D
107	1091258	68.7	-	Clin. Path	Cystocentesis	*E. coli*	
108	1091585	68.3	-	Clin. Path	Cystocentesis	-	
109	1083914	68.7	-	Clin. Path	Voided	-	
110	1088573	68.5	-	Clin. Path	Voided	*E. coli*	
111	1091631	58	+	Clin. Path	Voided	*E. coli*	E
112	1091630	68.5	-	Clin. Path	Cystocentesis	-	
113	1091391	68.5	-	Clin. Path	Voided	-	
114	1088785	68	-	Clin. Path	Cystocentesis	-	
115	1091648	66.7	-	Clin. Path	Cystocentesis	-	
116	1091653	58.4	+	Clin. Path	Voided	-	
117	1091644	68.6	-	Clin. Path	Voided	-	

118	1085164	68.4	-	Clin. Path	Cystocentesis	-	
119	1070105	57.8	+	Clin. Path	Voided	-	
120	1091649	66.6	-	Clin. Path	Voided	*Enterococcus*	
121	1618	66.9	-	Clin. Micro	Voided	*E. coli*	N
122	1059042	69.1	-	Clin. Micro	Cystocentesis	*E. coli*	XADBRS
123	1091559	61.18	-	Clin. Micro	Cystocentesis	*E. coli*	
124	1579	67.45	-	Clin. Micro	Voided	*E. coli*	N
125	1082748	58.65	+	Clin. Micro	Cystocentesis	*Enterococcus*	XAEM
126	1564	70	-	Clin. Micro	Voided	*E. coli*	N
127	1089908	69.31	-	Clin. Micro	Cystocentesis	*E. coli*	N
128	1091151	69.27	-	Clin. Micro	Cystocentesis	*E. coli*	N
129	1090828	69.51	-	Clin. Path	Voided	*E. coli*	
130	1068074	69.86	-	Clin. Path	Voided	-	
131	1085481	69.47	-	Clin. Path	Voided	-	
132	1088368	69.98	-	Clin. Path	Voided	-	
133	1091666	69.48	-	Clin. Path	Voided	-	
134	1091717	59.38	+	Clin. Path	Voided	*E. coli*	
135	1069127	69.5	-	Clin. Path	Voided	*E. coli*	
136	1073104	59.47	+	Clin. Path	Voided	*Streptococcus*	E*
137	1081614	69.58	-	Clin. Path	Cystocentesis	-	
138	1089682	68.45	-	Clin. Path	Cystocentesis	-	
139	1085563	68.67	-	Clin. Path	Voided	-	
140	1091739	68.99	-	Clin. Path	Voided	-	
141	1091742	69.04	-	Clin. Path	Cystocentesis	-	
142	127082	68.86	-	Clin. Path	Voided	-	
143	1088527	69.46	-	Clin. Path	Catheter	*Klebsiella*	E*
144	1061738	69.18	-	Clin. Path	Catheter	-	
145	1091764	68	-	Clin. Path	Cystocentesis	-	
146	1091766	68.56	-	Clin. Path	Voided	-	
147	1091767	68.09	-	Clin. Path	Cystocentesis	-	

148	1091754	x	-	Clin. Path	Voided	*Enterococcus*	M
149	1087129	68.67	-	Clin. Path	Cystocentesis	-	
150	99480	69.08	-	Clin. Path	Voided	-	
151	1091761	62.71	-	Clin. Path	Cystocentesis	-	
152	1091796	67.8	-	Clin. Path	Catheter	*E. coli*	
153	1091783	58	+	Clin. Path	Voided	-	
154	1091791	66.19	-	Clin. Path	Voided	-	
155	122913	67.15	-	Clin. Path	Voided	*E. coli*	
156	1091805	68.77	-	Clin. Path	Voided	-	
157	1091823	66.49	-	Clin. Path	Voided	-	
158	1070473	67.95	-	Clin. Path	Voided	-	
159	1091828	66.29	-	Clin. Path	Catheter	-	
160	1082748	67.69	-	Clin. Path	Voided	*E. coli*	E
161	1091852	68.48	-	Clin. Path	Voided	*E. coli*	
167	1091874	x	-	Clin. Path	Voided	-	
168	1091876	69.8	-	Clin. Path	Voided	*E. coli*	
174	1091426	71.75	-	Clin. Path	Cystocentesis	*E. coli*	
175	1081776	71.5	-	Clin. Path	Cystocentesis	-	
176	1091791	72.26	-	Clin. Path	Cystocentesis	-	
177	1067612	71.35	-	Clin. Path	Cystocentesis	-	
178	1090232	71.85	-	Clin. Path	Voided	-	
179	1084679	71.68	-	Clin. Path	Cystocentesis	-	
180	1087054	71.35	-	Clin. Path	Cystocentesis	-	
181	1091876	71.27	-	Clin. Path	Cystocentesis	*E. coli*	N
182	1091742	70.89	-	Clin. Path	Cystocentesis	-	
183	1088573	71.22	-	Clin. Path	Cystocentesis	*E. coli*	N
184	1091968	70.98	-	Clin. Path	Cystocentesis	-	
186	1091970	70.81	-	Clin. Path	Cystocentesis	-	
187	1091966	x	-	Clin. Path	Cystocentesis	-	
188	1091972	72.36	-	Clin. Micro	Cystocentesis	-	

189	1082592	72.48	-	Clin. Path	Voided	-	
190	1091940	72.2	-	Clin. Path	Cystocentesis	*E. coli*	
191	1090805	71.71	-	Clin. Path	Cystocentesis	-	
192	1091941	71.56	-	Clin. Path	Voided	*Enterococcus*	
193	1091942	71.53	-	Clin. Path	Voided	*E. coli*	
196	1091952	x	-	Clin. Path	Cystocentesis	*Proteus*	D
197	1090111	72.02	-	Clin. Path	Voided	-	
199	1075569	71.87	-	Clin. Path	Cystocentesis	-	
200	1088194	71.58	-	Clin. Path	Cystocentesis	-	
201	1779	72.08	-	Clin. Micro	Cystocentesis	*E. coli*	AB
202	1715	71.95	-	Clin. Micro	Voided	*E. coli*	N
203	1716	x	-	Clin. Micro	Voided	*E. coli*	N
204	1085353	x	-	Clin. Micro	Cystocentesis	*E. coli*	N
205	1091258	x	-	Clin. Path	Cystocentesis	-	
206	1091850	72.35	-	Clin. Path	Voided	*E. coli*	
207	1091395	72.05	-	Clin. Path	Cystocentesis	*E. coli*	
208	1091991	69.22	-	Clin. Path	Voided	-	
209	1865	72.6	-	Clin. Micro		-	
210	1088573	68.94	-	Clin. Micro	Cystocentesis	*E. coli*	N
211	1091876	69.75	-	Clin. Micro	Cystocentesis	*E. coli*	N
212	1091868	69.44	-	Clin. Micro	Cystocentesis	*E. coli*	N
213	1061672	58.61	+	Clin. Path	Cystocentesis	*E. coli*	XAVYDEGMBRS
214	1092223	69.37	-	Clin. Path	Catheter	*E. coli*	
215	113914	69.64	-	Clin. Path	Voided	*Klebsiella*	
221	1085353	69.7	-	Clin. Path	Voided	*E. coli*	
227	1092227	69.53	-	Clin. Path	Voided	*Enterococcus*	
235	1090519	57.83	+	Clin. Path	Voided	*Proteus*	
240	1080953	59.19	+	Clin. Path	Cystocentesis	*Enterococcus*	
241	1092328	70.3	-	Clin. Path	Cystocentesis	*E. coli*	N
249	1092349	69.25	-	Clin. Path	Cystocentesis	*E. coli*	XAVOYHDE*GMBRS

253	1092356	69.42	-	Clin. Path	Voided	*E. coli*	
256	1092263	68.91	-	Clin. Path	Cystocentesis	*E. coli*	XAVOYDGBRS
267	1092677	69.23	-	Clin. Path	Voided	*Klebsiella*	
271	1092762	69.76	-	Clin. Path	Voided	*E. coli*	
272	1092764	69.56	-	Clin. Path	Catheter	*E. coli*	
274	1092700	x	-	Clin. Path	Voided	*Streptococcus*	
275	1092755	x	-	Clin. Path	Cystocentesis	*Enterococcus*	E*
280	1092797	69.12	-	Clin. Path	Voided	*E. coli*	
284	116514	58.72	+	Clin. Path	Cystocentesis	*Klebsiella*	E
285	1092361	68.1	-	Clin. Path	Voided	*Streptococcus*	
290	1092764	70.13	-	Clin. Path	Catheter	*E. coli*	
291	1092753	68.95	-	Clin. Path	Voided	*Klebsiella*	
296	1090008	69.41	-	Clin. Path	Voided	*Klebsiella*	
312	1092349	58.64	+	Clin. Micro	Cystocentesis	*E. coli*	XAVOYHDEGMBRS
313	1092328	69.42	-	Clin. Micro	Cystocentesis	*E. coli*	N
314	1092223	69.51	-	Clin. Micro	Catheter	*E. coli*	XAVOYDBRS
315	1088549	69.54	-	Clin. Micro	Cystocentesis	*E. coli*	XAVOYDBR
316	1092542	69.3	-	Clin. Micro	Cystocentesis	*E. coli*	N
317	1092263	69.19	-	Clin. Micro	Cystocentesis	*E. coli*	XAVOYDGBRS
318	1092192	69.28	-	Clin. Micro	Cystocentesis	*E. coli*	D
319	1061672	69.15	-	Clin. Micro	Cystocentesis	*E. coli*	XAYDEGMBRS
320	Bac 2240	69.08	-	Clin. Micro	Voided	*E. coli*	N
321	Bac 2395	58.68	+	Clin. Micro	Voided	*E. coli*	N
322	Bac 2235	69.26	-	Clin. Micro	Voided	*E. coli*	AB
323	1092933	58.89	+	Clin. Micro	Cystocentesis	*E. coli*	AVYEMBRS
324	1092552	69.26	-	Clin. Micro	Cystocentesis	*E. coli*	N
325	Bac 2399	58.85	+	Clin. Micro	Voided	*E. coli*	XAVOYHDEMBRS
326	1092893	67.72	-	Clin. Micro	Cystocentesis	*E. coli*	N
327	1080924	69.63	-	Clin. Micro	Cystocentesis	*E. coli*	N
328	107958	68.85	-	Clin. Micro	Cystocentesis	*E. coli*	N

329	89045	69.23	-	Clin. Micro	Cystocentesis	*E. coli*	N
330	1090008	58.93	+	Clin. Micro	Cystocentesis	*E. coli*	AH
331	1071579	68.99	-	Clin. Micro	Cystocentesis	*E. coli*	N
332	Bac 2175	69.05	-	Clin. Micro	Voided	*E. coli*	A
333	1092930	69.07	-	Clin. Micro	Voided	*E. coli*	N
334	1093128	68.95	-	Clin. Micro	Cystocentesis	*E. coli*	AB
335	1093627	68.92	-	Clin. Micro	Cystocentesis	*E. coli*	A
336	1093584	68.92	-	Clin. Micro	Cystocentesis	*E. coli*	XAHDEMBRS
337	1093357	69.46	-	Clin. Micro	Cystocentesis	*E. coli*	A
338	1092952	69.95	-	Clin. Micro	Cystocentesis	*E. coli*	
339	1093217	69.47	-	Clin. Micro	Cystocentesis	*E. coli*	N
340	1093368	69.62	-	Clin. Micro	Cystocentesis	*E. coli*	A
341	1080953	58.93	+	Clin. Micro	Cystocentesis	*E. coli*	
342	1061672	69.36	-	Clin. Micro	Cystocentesis	*E. coli*	
343	1093457	69.32	-	Clin. Micro	Cystocentesis	*E. coli*	N
344	1079634	59.06	+	Clin. Micro	Cystocentesis	*E. coli*	XAVOYHEGMBR
345	1091476	69.26	-	Clin. Micro	Cystocentesis	*E. coli*	XAVOYHDEMBR
346	1061672	59.02	+	Clin. Micro	Cystocentesis	*E. coli*	XAVOYD
347	1082748	59.06	+	Clin. Micro	Cystocentesis	*E. coli*	ADEMBRS
348	1093680	69.25	-	Clin. Micro	Cystocentesis	*E. coli*	A
349	1093196	69.35	-	Clin. Micro	Cystocentesis	*E. coli*	A
1001	L1511445	63.95	-	Idexx		*Proteus*	N
1002	D1213201-1	69.04	-	Idexx		*E. coli*	N
1004	L1514671	68.94	-	Idexx		*E. coli*	N
1005	L1519471	69.11	-	Idexx		*E. coli*	XHS
1006	L1513280	68.97	-	Idexx		*E. coli*	N
1007	A8204277	64.1	-	Idexx		*Staphylococcus*	N
1008	L1510448	68.86	-	Idexx		*E. coli*	H*
1009	L1510822	69.37	-	Idexx		*Staphylococcus*	N
1010	C0525802	64.25	-	Idexx		*Staphylococcus*	N
1011	L1513163	64.36	-	Idexx		*Proteus*	H*
1014	A8469156	69.18	-	Idexx		*E. coli*	N
1015	A8465237	64.27	-	Idexx		*E. coli*	XVH*

1016	A8451563	69.16	-	Idexx		*E. coli*	XH*
1017	F1887207	69.14	-	Idexx		*E. coli*	N
1018	L1553187	64.43	-	Idexx		*E. coli*	H*E*
1019	A8448441	69.2	-	Idexx		*E. coli*	H*
1020	A8448942	69.17	-	Idexx		*E. coli*	H*
1021	L1533818	69.17	-	Idexx		*E. coli*	H*S
1022	L1514975	69.25	-	Idexx		*E. coli*	H*
1023	A8451222	69.31	-	Idexx		*E. coli*	H*
1024	S5749049	58.95	+	Idexx		*E. coli*	H*EGMS
1025	A8468319	69.67	-	Idexx		*E. coli*	N
1026	F1888287	69.28	-	Idexx		*E. coli*	XH*
1027	A8455320	69.37	-	Idexx		*E. coli*	H*
1028	A8451803	69.16	-	Idexx		*E. coli*	X*H*
1029	S5749076	69.19	-	Idexx		*E. coli*	H*
1030	C0532173	69.16	-	Idexx		*E. coli*	H*
1031	A8484466	69.14	-	Idexx		*E. coli*	XVYH*
1032	K2007047	69.21	-	Idexx		*E. coli*	N
1033	A8464230	69.19	-	Idexx		*E. coli*	XV*H*
1034	L1533827	69.08	-	Idexx		*E. coli*	H*
1036	D0242530	69.17	-	Idexx		*E. coli*	H*
1038	L1519612	64.42	-	Idexx		*E. coli*	H*
1039	S5747966	69.11	-	Idexx		*E. coli*	H*
1040	F1836351	64.09	-	Idexx		*E. coli*	H*E*
1041	A8440514	64.18	-	Idexx		*E. coli*	H*
1042	F1872330	68.65	-	Idexx		*E. coli*	N
1043	L1548900	64.11	-	Idexx		*E. coli*	N
1044	L1544061	68.73	-	Idexx		*E. coli*	H*
1045	A8450341	68.77	-	Idexx		*E. coli*	H*
1046	A8469728	68.85	-	Idexx		*E. coli*	E*
1047	L1522153	69.03	-	Idexx		*E. coli*	H*
1048	L1514385	68.9	-	Idexx		*E. coli*	H*
1050	A8478781	68.99	-	Idexx		*E. coli*	H*
1051	L1555109	68.95	-	Idexx		*E. coli*	N
1052	A8474989	69.12	-	Idexx		*E. coli*	H*
1053	A8491381	69.12	-	Idexx		*E. coli*	N
1054	A8437466	64.12	-	Idexx		*Proteus*	N
1055	A8445833	69.23	-	Idexx		*E. coli*	H*
1056	L1522162	69.2	-	Idexx		*E. coli*	N
1058	A8461480	69.15	-	Idexx		*E. coli*	H*
1059	A8484143	69.26	-	Idexx		*E. coli*	N
1060	C0532281	69.17	-	Idexx		*E. coli*	H*

1061	A8491273	69.28	-	Idexx		*E. coli*	XVYH*
1062	L1551413	69.19	-	Idexx		*E. coli*	N
1063	A8483665	69.27	-	Idexx		*E. coli*	N
1064	A8503759	68.69	-	Idexx		*E. coli*	N
1065	A8435917	58.47	+	Idexx		*E. coli*	XVYH*EMB
1066	S5745282	68.81	-	Idexx		*E. coli*	H*
1067	L1551431	68.57	-	Idexx		*E. coli*	N
1068	A8468721	63.59	-	Idexx		*Staphylococcus*	N
1069	L1567048	68.67	-	Idexx		*E. coli*	N
1070	L1537746	68.65	-	Idexx		*E. coli*	N
1071	A8480153	68.82	-	Idexx		*E. coli*	N
1072	A8469791	64.09	-	Idexx		*Pseudomonas*	HE*G*
1074	A8453666	68.76	-	Idexx		*E. coli*	H*
1075	A8469782	69.22	-	Idexx		*E. coli*	H*
1076	A8469488	69.16	-	Idexx		*E. coli*	XVYH*
1077	A8456720	69.07	-	Idexx		*E. coli*	N
1078	L1544491	68.94	-	Idexx		*E. coli*	N
1079	A8407897	58.61	+	Idexx		*Staphylococcus*	N
1080	K2009631	58.54	+	Idexx		*E. coli*	H*
1081	A8481393	58.58	+	Idexx		*Staphylococcus*	N
1082	D0242567	68.85	-	Idexx		*E. coli*	N
1083	L1536014	58.45	+	Idexx		*Staphylococcus*	XVYEG*MS
1084	C0551061	58.51	+	Idexx		*E. coli*	N
1085	A8436315	58.58	+	Idexx		*Proteus*	N
1086	A8480046	58.5	+	Idexx		*Staphylococcus*	N
1087	L1551450	67.52	-	Idexx		*E. coli*	N
1088	L1551422	69.27	-	Idexx		*E. coli*	N
1089	A8312185	58.83	+	Idexx		*Staphylococcus*	N
1090	L1553660	58.77	+	Idexx		*E. coli*	XVYEGM
1091	A8312194	62.45	-	Idexx		*E. coli*	H*
1092	A8469531	69.24	-	Idexx		*E. coli*	N
1093	A8551451	67.33	-	Idexx		*E. coli*	XVY
1094	L1590805	58.13	+	Idexx		*E. coli*	H*EM
1095	K2016494	69.21	-	Idexx		*E. coli*	XVY
1096	A8547029	58.67	+	Idexx		*E. coli*	N
1097	L1598296	69.3	-	Idexx		*E. coli*	N
1098	A8545651	69.36	-	Idexx		*E. coli*	N
1099	F1913568	69.11	-	Idexx		*E. coli*	N
1100	S5758692	69.18	-	Idexx		*E. coli*	H*
1101	S5756259	67.9	-	Idexx		*E. coli*	XVY*H*
1102	A8563514	58.35	+	Idexx		*E. coli*	N

1103	A8559683	67.96	-	Idexx		*E. coli*	N
1104	A8557455	68.8	-	Idexx		*E. coli*	H*
1105	L1607528	58.35	+	Idexx		*E. coli*	N
1106	A8561396	68.84	-	Idexx		*E. coli*	N
1107	F1879911	69.24	-	Idexx		*E. coli*	N
1108	L1603921	67.57	-	Idexx		*E. coli*	H*
1109	L1600204	68.97	-	Idexx		*E. coli*	N
1110	A8559398	58.38	+	Idexx		*E. coli*	N
1111	L1610766	58.06	+	Idexx		*E. coli*	H*
1112	L1604320	58.7	+	Idexx		*E. coli*	XVYHEMBS
1113	L1611585	69.26	-	Idexx		*E. coli*	N
1114	L1602432	58.74	+	Idexx		*E. coli*	N
1115	A8514055	69.16	-	Idexx		*E. coli*	N
1116	A8520132	69.4	-	Idexx		*E. coli*	N
1117	C0570497	69.27	-	Idexx		*E. coli*	N
1118	F1897956	69.46	-	Idexx		*E. coli*	N
1119	A8510717	69.26	-	Idexx		*E. coli*	H*
1120	A8510735	69.27	-	Idexx		*E. coli*	N
1121	A8510726	63.94	-	Idexx		*E. coli*	N
1122	A8519365	69.39	-	Idexx		*E. coli*	N
1123	S5751038	64.78	-	Idexx		*E. coli*	XH*
1124	A8510708	64.23	-	Idexx		*E. coli*	X*H*
1125	L1570277	69.92	-	Idexx		*E. coli*	N
1126	A8509494	69.16	-	Idexx		*E. coli*	N
1127	A8516050	69.08	-	Idexx		*E. coli*	H*
1128	K2012539	69.05	-	Idexx		*E. coli*	H*
1129	L1582278	69.14	-	Idexx		*E. coli*	H*
1130	T3604679	68.9	-	Idexx		*E. coli*	H*
1131	A8510691	68.98	-	Idexx		*E. coli*	N
1132	K2014570	69.32	-	Idexx		*E. coli*	N
1133	A8502171	69.26	-	Idexx		*E. coli*	H*
1134	A8530101	69.3	-	Idexx		*E. coli*	H*
1135	F1850744	69.08	-	Idexx		*E. coli*	N
1137	A8528462	69.07	-	Idexx		*E. coli*	H*
1138	L1592748	69.56	-	Idexx		*E. coli*	N
1140	A8538290	69.51	-	Idexx		*E. coli*	N
1141	L1606084	69.38	-	Idexx		*E. coli*	N
1143	A8518500	69.31	-	Idexx		*E. coli*	N
1144	L1604473	69.48	-	Idexx		*E. coli*	XVYH*B
1145	L1579639	69.36	-	Idexx		*E. coli*	H*
1147	L1578435	69.49	-	Idexx		*E. coli*	N

1148	A8528168	68.53	-	Idexx		*E. coli*	N
1149	K1071535	69.06	-	Idexx		*E. coli*	H*
1150	A8469352	68.18	-	Idexx		*E. coli*	N
1152	C0553146	69.32	-	Idexx		*E. coli*	N
1153	A8518484	68.99	-	Idexx		*E. coli*	N
1154	A8518466	68.73	-	Idexx		*E. coli*	N
1155	D1280606	68.32	-	Idexx		*E. coli*	H*
1156	D0243420	68.64	-	Idexx		*E. coli*	N
1157	K2012520	69.25	-	Idexx		*E. coli*	N
1158	C0566609	69.06	-	Idexx		*E. coli*	N
1159	L1614184	69.3	-	Idexx		*E. coli*	N
1160	K1548688	64.43	-	Idexx		*E. coli*	H*
1161	L1614219	69.8	-	Idexx		*E. coli*	H*
1162	L1617248	69.44	-	Idexx		*E. coli*	N
1163	L1716264	69.37	-	Idexx		*E. coli*	H*
1166	C0572633	69.1	-	Idexx		*E. coli*	N
1167	A8564269	69.5	-	Idexx		*E. coli*	XH*
1168	C0559041	68.75	-	Idexx		*E. coli*	N
1169	D0244169	69.43	-	Idexx		*E. coli*	N
1170	A8580293	69.44	-	Idexx		*E. coli*	N
1171	D0244571	69.46	-	Idexx		*E. coli*	H*
1172	F1970967	69.3	-	Idexx		*E. coli*	H*
1173	D0243850	68.9	-	Idexx		*E. coli*	N
1174	D0244024	69.04	-	Idexx		*E. coli*	N
1175	A8574026	69	-	Idexx		*E. coli*	N
1176	L1614827	69.49	-	Idexx		*E. coli*	H*
1177	S5761419	69.38	-	Idexx		*E. coli*	H*
1178	C0559060	69.36	-	Idexx		*E. coli*	N
1179	A8582298	x	-	Idexx		*E. coli*	N
1180	D0247115	69.05	-	Idexx		*E. coli*	N
1181	L1626004	69.12	-	Idexx		*E. coli*	N
1182	L1617883	68.92	-	Idexx		*E. coli*	N
1183	D0243887	69	-	Idexx		*E. coli*	XVY*
1184	C0730831	69.32	-	Idexx		*E. coli*	N
1186	A8567916	68.86	-	Idexx		*E. coli*	N
1187	C0731408	69.3	-	Idexx		*E. coli*	H*
1188	A8567264	69.3	-	Idexx		*E. coli*	N
1189	L1720131	69.29	-	Idexx		*E. coli*	H*
1190	A8697812	69.29	-	Idexx		*E. coli*	N
1191	K1563906	69.34	-	Idexx		*E. coli*	H*
1192	S5765329	68.96	-	Idexx		*Pseudomonas*	H

1035-1	L1535616	69.55	-	Idexx		*E. coli*	H*
1037-1	F1847471	69.27	-	Idexx		*E. coli*	H*
1049-1	F1886999	69.03	-	Idexx		*E. coli*	H*
1057-1	A8481532	69.08	-	Idexx		*E. coli*	HS
1073-1	A8469719	69.06	-	Idexx		*E. coli*	N
1136-1	L1595786	69.09	-	Idexx		*E. coli*	H*
1139-1	A8526459	69.46	-	Idexx		*E. coli*	N
1142-1	F1850477	69.78	-	Idexx		*E. coli*	H*
1146-1	F1878923	69.39	-	Idexx		*E. coli*	H*
1151-1	L1582779	69.02	-	Idexx		*E. coli*	XVY

(*) denotes antibiotics that intermediate resistance was observed according to CLSI standards.

APPENDIX B

DATA FOR URINES CONTAINING MULTIPLE CULTURES

Sample #	ID#	T_m C	FRET Result	Lab	Collection Method	Species 1	Phenotype	Species 2	Phenotype
23	1085460	59	+	Clin. Path	Voided	*Klebsiella*	KHE*G	*Streptococcus*	E
46	1091232	70	-	Clin. Path	Voided	*E. coli*	E*	*Klebsiella*	E*
59	1091391	69.2	-	Clin. Path	Voided	*E. coli*		*Klebsiella*	E*
73	1091412	68.5	-	Clin. Path	Voided	*E. coli*		*Proteus*	
74	1076465	69.2	-	Clin. Path	Voided	*E. coli*	ADB	*Klebsiella*	E*
80	1091477	69.3	-	Clin. Path	Cystocentesis	*E. coli*		*Enterococcus*	
82	1091478	69.2	-	Clin. Path	Cystocentesis	*E. coli*		*Enterococcus*	
90	1091492	68.7	-	Clin. Path	Cystocentesis	*E. coli*		*Enterococcus*	
98	1091524	62.2	-	Clin. Path	Voided	*E. coli*		*Klebsiella*	
101	1089908	69.1	-	Clin. Path	Cystocentesis	*E. coli*	N	*Enterococcus*	
105	1091542	68.4	-	Clin. Path	Catheter	*Klebsiella*		*Enterococcus*	
106	127374	67.6	-	Clin. Path	Cystocentesis	*Proteus*	D	*Klebsiella*	
110	1088573	68.5	-	Clin. Path	Voided	*E. coli*		*Enterococcus*	
120	1091649	66.6	-	Clin. Path	Voided	*Enterococcus*		*Proteus*	
134	1091717	59.38	+	Clin. Path	Voided	*E. coli*		*Enterococcus*	E*
135	1069127	69.5	-	Clin. Path	Voided	*E. coli*		*Klebsiella*	E*
160	1082748	59.3	+	Clin. Path	Voided	*E. coli*	E	*Klebsiella*	E
161	1091852	68.48	-	Clin. Path	Voided	*E. coli*		*Staphylococcus*	
190	1091940	72.2	-	Clin. Path	Cystocentesis	*E. coli*		*Staphylococcus*	E
193	1091942	71.53	-	Clin. Path	Voided	*E. coli*		*Enterococcus*	E*
249	1092349	69.25	-	Clin. Path	Cystocentesis	*E. coli*	XAVOY HDE*GM BRS	*Proteus*	
312	1092349	58.64	+	Clin. Micro	Cystocentesis	*E. coli*	XAVOY HDEGM BRS	*Pseudomonas*	H
314	1092223	69.51	-	Clin. Micro	Catheter	*E. coli*	XAVOY DBRS	*Streptococcus*	KG
320	2240	69.08	-	Clin. Micro	Voided	*E. coli*	N	*Proteus*	HD
321	2395	58.68	+	Clin. Micro	Voided	*E. coli*	N	*Streptococcus*	N
323	1092933	58.89	+	Clin. Micro	Cystocentesis	*E. coli*	AVYEM BRS	*E. coli*	N
324	1092552	69.26	-	Clin. Micro	Cystocentesis	*E. coli*	N	*E. coli*	N
327	1080924	69.63	-	Clin. Micro	Cystocentesis	*E. coli*	N	*Staphylococcus*	N
330	1090008	58.93	+	Clin. Micro	Cystocentesis	*E. coli*	AH	*Enterococcus*	N

336	1093584	68.92	-	Clin. Micro	Cystocentesis	*E. coli*	XAHDE MBRS	*E. coli*	N
346	1061672	59.02	+	Clin. Micro	Cystocentesis	*E. coli*	XAVOY D	*E. coli*	XAVOY HDEMB RS

(*) denotes antibiotics that intermediate resistance was observed according to CLSI standards.