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Phenotypic and Genotypic Characterization of Antimicrobial Resistance among Diarrheagenic *Escherichia coli*

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Abstract: Diarrhea caused by multidrug-resistant bacteria is an important public health problem among children in developing countries. *Escherichia coli* is an important cause of disease in animals and humans worldwide. Twenty five *E. coli* isolates with rate 61.2% among human and environments were tested for susceptibility to 10 antimicrobial agents by disk diffusion method. Resistant isolates were screened by molecular methods for resistance genes, *TetA, TetB,* strepA, *MarI* and *MarII*. Molecular result showed that all isolates harbored resistance gene for the *TetA, TetB,* strepA, MarI, and *MarII* even though the genotypic test showed sensitive to the drugs. *E. coli* isolates exhibit a wide repertoire of genetic elements to sustain antimicrobial pressure. The results of this study using pheno-genotypic techniques highlight the distribution of *E. coli* among human, animal, aquatic ecosystems and the potential public health threat of *E. coli* originating from municipal wastewater sources.

Key words: Antimicrobial Resistance (AMR), Polymerase Chain Reactions (PCR), *Escherichia coli* (*E. coli*), public health

INTRODUCTION

Isolates of *Escherichia coli* can be non-pathogenic commensals or human and/or animal pathogen influences several aspects of public health. Although antimicrobial therapy is generally not required, the emergence of strains showing multiresistance to several antimicrobial drugs is a public health concern (White *et al*., 2002). As a matter of fact, *E. coli* from livestock is exposed to a great selective pressure because in some countries, more than half of the antimicrobial agents are used in food-producing animals (Schwarz and Chaslus-Dancla, 2001). Consequently, resistance is increasing and various resistance determinants have been described. Resistance genes can spread on mobile genetic elements like plasmids, transposons and integrons (Carattoli, 2001; Schwarz and Chaslus-Dancla, 2001). Pathogenic subtypes of *E. coli* are known to cause illness around the world (Leclerc *et al*., 2001) and one of the standard indicator organisms for fecal pollution in environmental waters (American Public Health Association, 1998). Knowledge of indicator organism source is necessary for risk assessment and remediation of polluted waters, including application, such as total maximum daily load assessment. Consequently, the field of Microbial Source Tracking (MST), which seeks to determine the origin of fecal material in water, has emerged (Anderson *et al*., 2006; Whitlock *et al*., 2002). Many studies have limited their focus to well-known sources such as agriculture, sewage treatment plants and combined sewer overflows (George *et al*., 2004; Kon *et al*., 2007; Saini *et al*., 2003) and assumed limited bacterial survival between the sources and surface waters. However, high bacterial counts in surface waters along shorelines may also be a result of bacterial survival in beach sand in the absence of fecal input (Alm *et al*., 2006; McLellan *et al*., 2003). Resistant bacteria have been isolated from a variety of sources, including domestic sewage, drinking water, rivers and lakes (Kasper and Burgess, 1990; McKeon *et al*., 1995; Mulamattathil *et al*., 2000). Resistance of a.single bacterial isolate to more than one antimicrobial drug is commonly reported while, multiple antimicrobial drug resistance profiles have been used to identify and differentiate *E. coli* strains from different animal species (Krumperman,

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1983; Troy *et al*., 2002). Recently, multiple resistance profiles have been used to identify sources of fecal contamination in water (Hagedorn *et al*., 1999; Harwood *et al*., 2000; Kasper and Burgess, 1990; Parveen *et al*., 1997, 1999; Wiggins, 1996; Wiggins *et al*., 1999). Characterization of continuous, localized sources, including environmental sources, of microbial indicators is essential to complement current water-monitoring strategies and standards. This study was conducted to determine the prevalence and distribution of antimicrobial-resistant diarrheagenic *E. coli* from various sources genotypic characteristics of antibiotic resistance of tetracycline *(tetA* and *tat*B), streptomycin *(strep*A) and multiple antibiotic resistances *(mar I* and *marI I*) among *E. coli*. It is anticipated that the findings of this study will help to understanding of antimicrobial resistance in among human and environmental isolated from divers sources.

MATERIALS AND METHODS

Sources of isolates: Twenty five *E. coli* isolates as human, sea water; river, food and animal were studied from five different sources in Malaysia 2005-2006. The human (pus, 2urine and 2 stools) isolates from stock culture of Medical Microbiology Laboratory which provided from Kula Lumpur Hospital (HKL), sea water and river isolates were collected from Costrica beach, Sunggi linggi river Nergeri Sembilan State. The food (milk powder, chess, yogurt and 2 raw meats) isilates were selected randomly from different restaurant in Seri Serdang area, Selangor state. The last.samples of animal source (deer, pig, goat and 2 chickens) were provided from Microbiology Department, Faculty of Veterinary Universiti Putra Malaysia (UPM).

Isolation of *E. coli* from water samples: The membrane filtration method used according to (USEPA, 2005) recommendation to isolate *E. coli* from water samples. Water samples were filtered through a sterile, white, grid-marked, 47-mm-diameter membrane (pore size, 0.45 ± 0.02 μm). After filtration membrane containing bacteria was placed on a selective and differential medium Chromocult Coliform Agar (CCA, Merck, Germany) and incubated at 44°C for 22 h. After overnight incubation *E. coli* colonies turned pink or purple on these media.

Antimicrobial agent susceptibility testing: Standard Kirby-Bauer disk diffusion method used to determine the antimicrobial agent sensitivity profiles of the *E. coli* isolates following recommendation of National Committee for Human Laboratory Standard (2004). Ten antimicrobial agents ampicillin (10 g), Chloramphenicol (30μg), sulfmethaxzol-trimethoprim (5 μg), tetracycline (5 μg), gentamycin (10μg), kanamycin (30 μg), cefataxime (30 μg), norfloxacin (10μg), ciprofloxacin (10 μg), nalidixic acid (5 μg) (Oxoid UK). A 150 mm Mueller-Hinton agar was swabbed with LB inoculated with *E. coli* and incubated to a turbidity of 0.5 McFarland standards.

Data analysis: Using SPSS, version 13.5 software (SPSS, Inc., Chicago).

DNA preparation: *E. coli* isolates grown on Chromoocult Coliform Agar (Merek. Germany) overnight at 37°C. A single colony of each strain was transferred to Luria-Bertani medium (Oxoid. UK) and grown overnight in a 37°C shaking water bath. DNA was prepared with a DNA isolation kit (Qiagen, Germany) DNA extraction according to the manufacturer's instructions. The genomic DNA was checked for the concentrations and purities using spectrophotometer (Shimadzu 1601. Japan).

Polymerase Chain Reaction (PCR): used to amplify a specific region of a genome such as *Tet*A, *Tet*B, *Str*A, *Mar*RI and *Mar*RII genes. Twenty five μl containing 1X BST buffer (Biosynthec Inc. Malaysia), 1.8 mM MgCl2 (Biosynthec Inc. Malaysia), 200um dNTPs (Fermentas Life Sciences), 5 IU taq polymerase (Biosynthec Inc. Malaysia), 10 pmoles of each primer and 200 ng μL^{-1} DNA template. The PCR programmer steps performed were initial denaturation at 94 °C for 2 min, followed by 35 cycles of amplification steps consisting of denaturation at 94°C for 4 min, annealing temperature is depends on published primers as shown in (Table 1) and elongation at 72°C for 2 min. The amplification was ended with final extension at 72°C for 10 min. After amplification, an aliquot of 10 μL reaction mixture was loaded into the wells of 1.4 % agarose gel and electrophoresed, then stained with ethidium bromide and image was captured under UV illumination (Alpha Imager TM 2200, Alpha Innotech Corporation).

Table 1: Primer sequence and annealin g temperature which used in single PCR

Target	5'----------3'	Size (bp)	Annealing Temp
*Mar*RI*1	(F)'GCCAGGCCAAGAAATAACGC3'		
	(R) 'GAGTAACCCGAACGCTCTGA3'	872	57.7°C
*Mar*RII*1	(F) 5'GGTGGTTGTTATCCTGTGTA3'		
	(R) 5'GGTTGTCCTCGATCCAGTC3'	727	54°C
*Str*A*2	(F) 5'AGGAGGAACAGGAGGGTGC3'		
	(R) 'CGGTAAGAAGTCGGGATTGA3'	229	58.9°C
*Tet*A*3	(F) 5'GGCGGTCTTCTTCATCATGC3'		
	(R) 'CGGCAGGCAGAGCAAGTAGA3'	501	64°C
*Tet*B*3	(F) 5'CATTAATAGGCGCATCGCTG3'		
	(R) 'TGAAGGTCATCGATAGCAGG3'	929	64°C.

*1 (Lindgren *et al*., 2003), *2 (This study) and *3 (Boerlin *et al*., 2005)

RESULTS

Antimicrobial resistance phenotype characteristics: All *E. coli* isolates from different sources showed (Table 2) variant resistance patterns to the 10 antibiotics tested, (61.2%) *E. coli* isolates were retrieved for antimicrobial agent resistance profiling. Antibiotic resistance was more prevalent among *E. coli* isolates from food (64.0%) commonly resistant to sulphathiazole-trimethoprime and kanamycin (80.0%) human and animals (62.5%) resistant to chloramphenicol and kanamycin respectively (95.0% and 85.0%), sea water (62.0%) resistant to chloramphenicol (90.0%) and river (55.0%) resistant to gentamycin and nalidixic acid (80.0%). Tetracycline and kanamycin the most commonly reported antimicrobial agent (81.0%), followed by chloramphenicol (76.0%), gentamycin (72.0%), ampicillin (73.0%). Resistances to ciprofloxacin (24.0%), norfloxacin (27.0%) and cefatoxin (40.0%) were the least prevalent in all types of samples.

Detection of *Escherichia coli* specific fragment using PCR: The *E. coli* isolates used in this study after various optimizations showed a single band at position 502, 929,229, 727 and 872 bp respectively from different gene TetA, TetB, StrA, MarRI and *Mar RII,* respectively. The single band pattern observed (Fig. 1-5) for each isolates were located between 100-1000 bp based on the 100 bp DNA ladder marker (MBI Fermentas).

E. coli causes diverse infections in animals and humans and the most common cause of urinary tract infections, community-acquired bacteremia and sepsis (Russo and Johnson, 2000). In the present study, human, surface water, food and animal isolates were analyzed for the distributions of related acquired antimicrobial resistance genes, their prevalence profiles, from different sources. Our study demonstrates that similar antibiotic resistance patterns could be observed in all isolates. *E. coli* isolates were resistant to ampicillin, tetracycline and sulfonamides. It is noteworthy that although ampicillin

Table 2: Percentage of antimicrobial resistance in *E. coli* isolated from various sources (Human, sea water, river, foods and animals) Abbreviations: A, Ampicillin; C, Chloramphenicol; STX, Sulphathiazole-Trimethoprime; T, Tetracycline; S, Streptomycin; GEN, Gentamycin. K, Kanamycin; CEF, Cefatoxin, CIPX, Ciprofloxacin, NORX, Norfloxacin, NALX, Nalidixic Acid

Test/ Source	AMP	CHL	STX	T	GEN	KAN	CEF	CIPX	NORX	NALX	%R
Human	75	95	60	80	70	85	40	30	30	60	62.5
S.water	70	90	60	90	80	80	50	20	20	60	62.0
River	60	70	60	70	80	70	40	10	10	80	55.0
Food	70	70	80	90	70	80	50	30	30	70	64.0
Animals	80	55	60	80	80	85	50	25	35	75	62.5
Total	73	76	60	81	72	81	40	24	27	69	61.2.

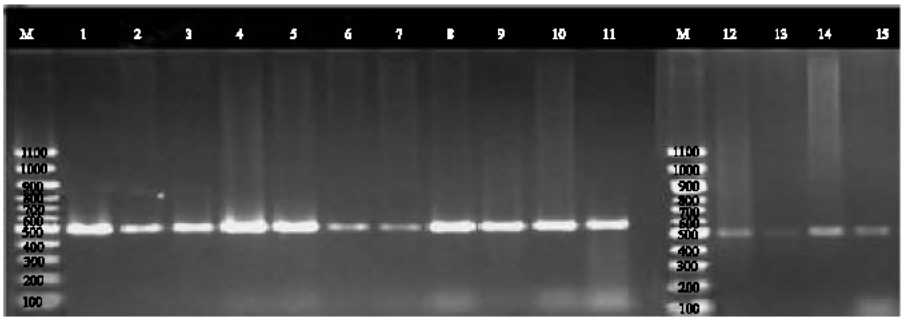

Fig.1: The detection of *TetA* fragment by PCR. Lane M represents 100 bp DNA marker ladder. Lanes1-3 from human, Lane 4-6 sea water, Lane 7-9 river, Lane 10-12 food and Lane 13-15 animal

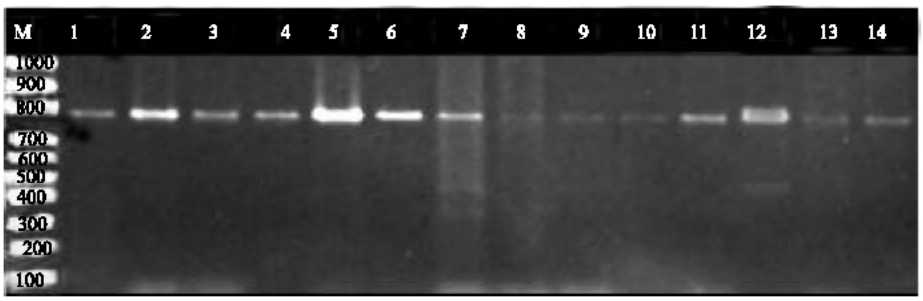

Fig. 2: The detection of *TetB* fragment by PCR. Lane M represents 100 bp DNA marker ladder. Lanes1-3 from human, Lane 4-6 sea water, Lane 7-9 river, Lane 10-11 food and Lane 12-14 animal

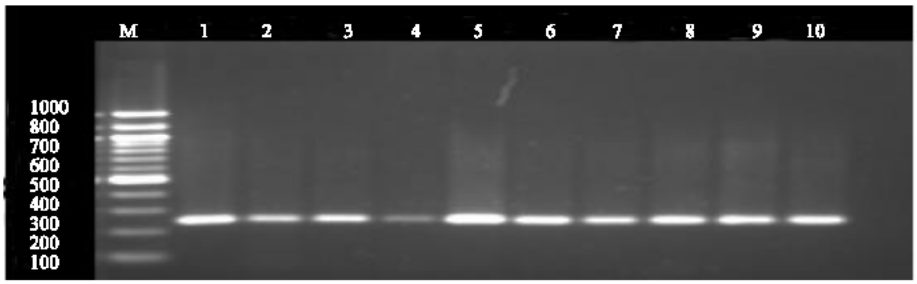

Fig. 3: The detection of *StrA* gene by PCR. Lane M represents 100 bp DNA marker ladder. Lanes1-2 from human, Lane3-4 sea water, Lane 5-6 river, Lane 7-8 food and Lane9-10 animal

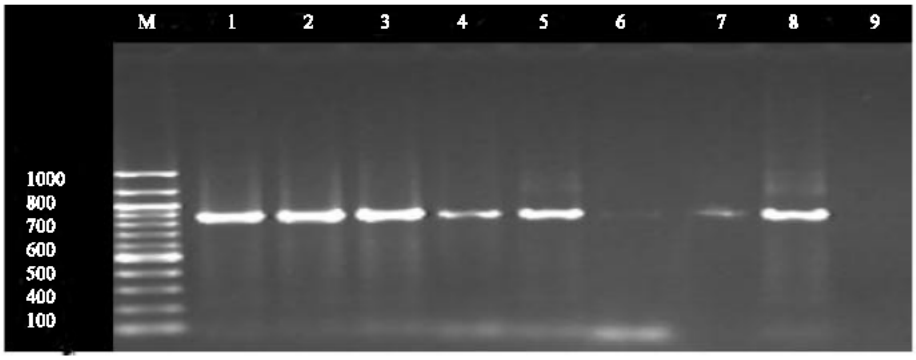

Fig. 4: The detection of *MarRI* fragment by PCR. Lane M represents 100 bp DNA marker ladder. Lanes1-3 from human, Lane4 sea water, Lane 5 river, Lane 6 food and Lane7-8 animal

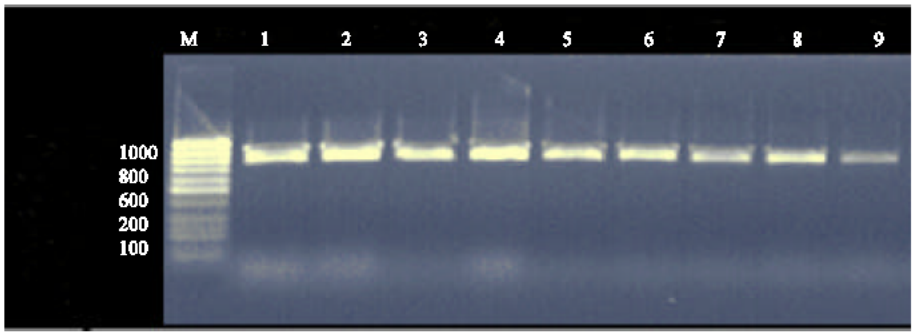

Fig. 5: The detection of *MarRII* fragment by PCR. Lane M represents 100 bp DNAmarker ladder. Lanes1-2 from human, Lane3-4 sea water, Lane 5 river, Lane 6-7 food and Lane8-9 animal

and sulfonamides are old antimicrobials, they are still widely used. Although this antimicrobial agent is more widely used for human therapy than for animal therapy, more animal isolates exhibited resistance for tetracycline, kanamycin, ampicillin respectively. The latter observation can be explained by the fact that the tetracycline resistance gene *tetA, tet*B, which was found among *E. coli* isolates from various sources. Therefore, the selective pressure exerted by the use of neomycin in animals, would have simultaneously selected for neomycin- and kanamycin-resistant strains. Therefore, *E. coli* isolates from animals and humans could not be discriminated on the basis of their phenotypic patterns of antimicrobial resistance, which did not extend to their genotypic resistance patterns may be due to the small number of isolates was tested. When the fact that cephalosporins are used more in human medicine than in animal medicine is considered, it was interesting that resistance to cefatoxin, a cephalosporin used in food-producing animals in Canada and the United States, was found higher rate among sea water, food and animal isolates.

The resistance of animal isolates to cefatoxin was shown to be associated with acquired beta-lactam resistance genes such as the cephamycinase *bla*CMY genes that were found in *E. coli* isolates transfer from *salmonella* sp (Zhao *et al*., 2001).

Resistance genes can be associated with mobile DNA plasmids, transposons and.integrons, which are known to facilitate their distribution (Jacoby, 1994). The molecular investigations on the underlying resistance mechanisms showed that identical resistance were based on different genes, streptomycin *str*A/*B* genes, tetracycline *(tet*(A*)* and *tet*(B)genes) and multiple antibiotic resistant genes (MarI and *MarII*). In the case of *E. coli* resistance to tetracycline and kanamycin was the most prevalent (Guerra *et al*., 2003; Boerlin *et al*., 2005). The lowest levels of resistance (increased susceptibility) found in this study were the levels of resistance ciprofloxacin norfloxacin and cefatoxin that had been restricted uses in veterinary medicine since 1990s after the rapid emergence of resistance to fluoroquinolone (Engberg *et al*., 2001). Chromosomal mutations confer resistance to fluoroquinolone (Prescott *et al*., 2000) and the development of resistance to one agent results in cross-resistance to other fluoroquinolone. All the isolates that were resistant phenotypically carried the respective antibiotic resistant genes; this indicates that all isolates resistant by phenotypic were also genetic resistant. A number of recent studies have attempted to assess the distribution of the resistance genes for these major antimicrobial agents in *E. coli* populations of animal origin, but much remains to be done to draw valid comparisons between *E. coli* isolates from different animal populations (Guerra *et al*., 2003; Boerlin *et al*., 2005). Resistant *E. coli* isolates present in water sources used for drinking or recreation could be an important tool in the development of strategies to better protect public health (Donald and Valerie, 2007). Molecular techniques such as PCR provide alternative means of resistance identification, there are several advantages to the utilization of antimicrobial agent resistance profiles as an alternative means of source determination. Finally, the differences in the distribution of antimicrobial resistance genes in bacteria from different host populations. These include differences in antimicrobial use, the clonal nature of some pathogenic *E. coli*.isolates, a lack of epidemiological and ecological links between *E. coli* isolates of different origin and sampling bias.

CONCLUSION

Further investigations are therefore, required to further explore acquiring resistance, particularly among animals and human isolates to establish whether any similarities exist. *E. coli* isolates between animal and human groups can possess relatively distinct profiles. This suggests that the number and diversity of genes driving phenotypic resistance are dynamic and have evolved through selection by antimicrobial use. Therefore, our study suggested furthering investigating the occurrence of pathogenic *E. coli*, in source waters used for drinking, recreation and irrigation in order to better understand the implications for public health.

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