J. Vet. Malaysia (1996) 8(1):7-13

PLASMID AND ANTIMICROBIAL RESISTANCE TRANSFER FROM POULTRY STRAINS TO AQUATIC STRAINS OF *ESCHERICHIA COLI*

R. Son[1], G. Rusul[2] and M.I.A. Karim[1]

[1]Department of Biotechnology and [2]Department of Food Science, Universiti Pertanian Malaysia, 43400 Serdang, Selangor, Malaysia.

SUMMARY

Plasmid DNA analysis and antibiotic susceptibility were used to study strains of *Escherichia coli* isolated from poultry and aquatic sources. Nineteen of 25 and 22 of 30 among the aquatic and poultry strains respectively were found to contain plasmid DNA bands ranging in size from 2.3 to 102 kilobases (kb). Resistances to five or more of the antimicrobial agents tested were predominant among the poultry strains. However, none were susceptible to all antimicrobial agents tested. More isolates containing plasmid were resistant to ampicillin, erythromycin, streptomycin and tetracycline. The potential of the transfer of natural plasmids between poultry and aquatic strains has been studied. Four antibiotic-multiresistant poultry isolates (EP201, EP205, EP210 and EP211) possessing potentially conjugative large plasmids were mated with two aquatic isolate (EA101 and EA106) and a laboratory strain *E. coli* K12 C600 Na^r in sterilised river water at 37°C and 25°C, and LB medium at 37°C. Conjugation occurred in all system tested with *en bloc* transfer of the donors' antibiotic resistance and plasmid DNA bands. In *in vitro* transfer conducted at 25°C in sterile river water, transfer frequency values decreased by 2 to 3 logs from the optimum conditions at 37°C.

Keywords: *Escherichia coli*, aquatic, poultry, plasmid, antimicrobial resistance, conjugal transfer.

INTRODUCTION

With the advent of antimicrobial agents, antibacterial therapy gained very promising means for treating bacterial infection in humans and animals; although drug-resistant micro-organisms have been known from the early days of chemotherapy (Anon., 1980). Whilst the use of drugs does not cause microbes to become resistant, widespread use does provide a selective pressure in favour of organisms possessing genes that code for resistance (Hinton *et al.*, 1986). Generally, hospital and hospital sewage effluent, commercial fisheries and abattoirs are main contributors of contamination by antimicrobial agents in a variety of aquatic and terrestrial habitats. However, resistant bacteria have also been isolated from apparently nonselective environments, including plants, estuaries, deep ocean water and sediment and drinking water (Fontaine and Hoadley, 1976; Goyal and Hoadley, 1979).

Transfer of genes for resistance by R plasmid occur via all three types of recombination (transformation, viral transduction, and conjugation), although conjugation appears to be the most common method for *in vivo* Transfer is known to occur widely among Gram-negative bacteria, especially the coliform (Linton *et al.*, 1981). To assess the potential risk associated with the release of novel organisms to the environment, it is necessary to determine the capacity of the indigenous community to receive DNA from vehicles for recombinant DNA, and their transfer in natural habitats (Trevors *et al.*, 1987). Thus, plasmid-mediated gene transfer was the focus of this study. Furthermore, when the transfer phenomenon in an aquatic system is assessed, the majority of assays have been made *in vitro*, under conditions which differ greatly from nature (Alcaide and Garay, 1984; McPherson and Gealt, 1986). A study was therefore made on the transfer of R plasmids from poultry to aquatic *E. coli* strains under conditions which approach those found in the environment.

MATERIALS AND METHODS

Collection of samples

The results of plasmid profile and antimicrobial resistance pattern in two general situations for isolates of *Escherichia coli* were compared. Aquatic strains of *E. coli* were obtained from water samples at ten stations along Gombak River on the outskirts of Kuala Lumpur metropolis. All samples were collected in accordance with recommended procedures (Anon., 1981). Poultry strains of *E. coli* were obtained from carcasses wash of fresh poultry meat supplied to a residential college of a local university. Water drippings from at least four carcass were obtained for each sample of water from the carcass wash. Several crystals of sodium thiosulphate were added to each sample to neutralise any residual chlorine.

Identification of bacteria

The *E. coli* strains were purified by streaking on standard plate count agar and identified using API 20E kits (Analytab Products, Inc.).

Antimicrobial susceptibility testing

Isolates were screened for resistance to ampicillin (10 µg/mL), chloramphenicol (30 µg/mL), erythromycin (30 µg/mL), gentamicin (10 µg/mL), kanamycin (30 µg/mL), nalidixic acid (30 µg/mL), streptomycin (10 µg/mL), trimethoprim (2.5 µg/mL) and tetracycline (30 µg/mL), using commercial discs (BBL Senci-Disc, Becton Dickinson). Bacteria were suspended in saline to give a density of 2 McFarland standards, diluted 1:20, and streaked, by the method of Bauer *et al.* (1966) on Mueller Hinton agar. Plates were incubated for 24 h at 37°C. Characterisation of strains as sensitive or resistant was based on the size of inhibition zone around each disc.

Plasmid isolation

DNA isolation procedure and gel electrophoresis were performed a minimum of three times for each isolate. Plasmid DNA from *E. coli* was isolated by the procedure of Birnboim and Doly (1979). Extracted plasmid DNAs were electrophoresed for 2 h at 35 mA on a 0.7% agarose gel in TBE buffer as described by Meyers *et al.* (1976). After the gels were stained with ethidium bromide (1.5 mg/L for 30 min), they were photographed under UV illumination. The approximate molecular mass of each plasmid was calculated by using, as reference, plasmids from *E. coli* V517 (Macrina *et al.*, 1979).

Conjugal transfer study

The ability of poultry R-plasmid to transfer in sterile river water was tested in laboratory simulations. The recipient and donor strains (Table 3) were randomly selected among the aquatic and poultry isolates. To permit selection against the donor in conjugation experiments, nalidixic acid-resistant derivative of the plasmidless recipient strains (EA101 and EA106) was generated by subculture in increasing concentrations of nalidixic acid in plate count broth. Nalidixic acid was then used to select against donors when recipients were enumerated in the mating mix. A laboratory strain of *E. coli* K12 C600 F^- Lac^- Na^r was used as control. River water was sterilised by filtering through 0.22 mm pore membrane filters: no viable organisms were recovered when the sterilised water was streaked onto agar plates. The standard mating conditions were as followed: Separate 5 mL tubes of LB broth (Oxoid) were inoculated with overnight cultures of donor and recipient cells and incubated at 37°C with shaking to mid-log phase. Mating was done in fresh medium to a final cell density of about 10^8 cfu/mL, and with donor:recipient ratio of about 10, at 37°C without shaking for 3 h. Investigators often use 10^8 cfu/mL cell density in genetic studies to enhance

To assess plasmid transfer in sterile river water (SRW), donor and recipient cells were grown to mid-log phase as described above, pelleted by centrifugation (5000 rev/min for 10 min) and finally suspended to the original volume in SRW. Matings were performed in the same proportion as described above. Conjugal transfer was conducted at 25°C without shaking for 18 h. A temperature of 25°C, considered to be closest to natural environment temperature, has been proposed by many workers as the optimal temperature to obtain maximal transfer frequencies (Travers and Oddie, 1986; Rochelle *et al.*, 1989; Sandt and Herson, 1991). After incubation, serial dilutions of mating mixtures were spread on MacConkey agar plates supplemented with both nalidixic acid (50 µg/mL) and a single appropriate antibiotic to which the potential donor was resistant and to which the recipient strain was sensitive. Just before plating for the recovery of tranconjugants, nalidixic acid was added to the mating mixture at the final concentration of 50 µg/mL to inhibit gene transfer by affecting the DNA metabolism of the donor strains (Fernandez-Astorga *et al.*, 1992). Control, donor, or recipient alone samples were also plated on the same selective medium. Fifty colonies grown on MacConkey agar plates after a 24 h period at 25°C (experimental conditions) or 37°C (optimal conditions) were recovered and tested for their antimicrobial resistance and for the presence of plasmid band(s). The transfer frequency for each mating pair was calculated as the number of transconjugants per initial number of donor cells in the conjugation mixtures (Shaw and Cabelli, 1980).

To account for mutation in the donor and/or recipient, mutation frequencies were determined by spreading the donor and the recipient separately on appropriate selective MacConkey agar plates. To account for the possible role of transformation or viral transduction in mediating antibiotic resistance transfer, the recipient strain was mated with cell-free supernatant fluid from the donor culture in the same proportion; this was then spread on the same selective agar plates for selection of transconjugants. No transconjugants were obtained in any of the culture media.

RESULTS

Plasmid DNA isolation

Plasmid DNA was extracted from a total of 25 aquatic and 30 poultry isolates of *E. coli*. The plasmid DNA from each strain was separated by electrophoresis on agarose gels, and the plasmid profiles were compared. Approximately 76% aquatic and 73% poultry strains contained plasmid bands ranging in size from 2.3 kb to 102 kb. Interestingly, small plasmid bands (<15 kb) occurred mainly within the plasmid-containing isolates from both aquatic and poultry sources. Using plasmid band number and size as a basis, we could not separate the 25 aquatic and 30

Table 1. Strains of *E. coli* used in this study.

E. coli strain	Resistance patterns[a]	Plasmids (kb)
Aquatic isolates:		
EA101	Sm	–[b]
EA102	ErSm	49, 5.2
EA103	ErSm	68, 4.7, 4.9
EA104	ErSm	67, 5.2
EA105	ErSm	59, 5.2, 2.5
EA106	ErSm	–
EA107	ErSm	7.6, 3.8
EA108	ErSm	46
EA109	ErSm	4.3,3.7
EA110	ErSm	81, 55, 5.8, 3. 7,3. 3, 2.7, 2.3
EA111	SmKm	61, 6.5, 5.6, 3.5, 3.2
EA112	ErTe	5, 3.5
EA113	ErSmTe	4.9, 2.3
EA114	ErSmTe	55, 4.9
EA115	ErSmTe	90
EA116	ErSmTe	7.3, 5, 2.3
EA117	ApSmTe	90, 5.6
EA118	ApErT	7, 5.9
EA119	ErGmSmKm	–
EA120	ApErCmKm	4.4, 2.3
EA121	ApErSmT	–
EA122	ApErSmT	68
EA123	ApErSmT	–
EA124	ApErGmSmT	–
EA125	ApErSmTKm	3.3
Poultry isolates:		
EP201	ApCmSmTTe	68
EP202	ApErSmTTe	4.9, 3.2
EP203	ApKmSmTTe	82, 6.1, 4.7, 2.7
EP204	ApKmSmTTe	–
EP205	ApErKmSmTe	87, 61
EP206	ApErKmSmT	–
EP207	ApErKmSmTe	–
EP208	ApErKmSmTTe	–
EP209	ApCmErKmSmT	84, 61, 7.5
EP210	ApGmNaSmTTe	90, 12.5, 5.3
EP211	ApErKmSmTTe	81, 6.4, 5
EP212	ApErKmSmTTe	81, 8, 2.7
EP213	ApErKmNaSmTTe	102, 81, 6.4, 5
EP214	ApCmErKmSmTTe	75, 32, 4.9
EP215	ApCmErNaSmTTe	81, 4.9
EP216	ApErGmKmSmTTe	10, 8, 6.1, 5.2
EP217	ApCmErKmSmTTe	90
EP218	ApCmErKmSmTTe	82, 61, 6.5, 4.7, 4.3, 3.3, 3, 2.6
EP219	ApCmErKmSmTTe	6.2, 5.2
EP220	ApCmGmKmSmTTe	82, 11, 6.5, 4.7, 4.3, 3.3, 3, 2.6
EP221	ApErGmKmSmTTe	–
EP222	ApErGmKmSmTTe	13.4
EP223	ApCmErKmSmTTe	67, 61
EP224	ApErKmNaSmTTe	87, 4, 2.4
EP225	ApErGmKmSmTTe	100, 94, 79, 6.4, 4.6, 2.9
EP226	ApErGmKmSmTTe	–
EP227	ApCmErKmSmTTe	–
EP228	ApErGmKmSmTTe	7, 5.6, 4.7, 3.8
EP229	ApErGmKmSmTTe	13.2, 5.6, 2.9, 2.7
EP230	ApCmErGmKmSmTTe	–

[a] Tested for ampicillin (Ap), chloramphenicol (Cm), erythromycin (Er), gentamicin (Gm), kanamycin (Km), nalidixic acid (Na), streptomycin (Sm), trimethoprim (T) and tetracycline (Te).
[b] Presence of plasmid DNA band(s) not detected.

group. Table 1 illustrates the general diversity of the plasmid bands detected among the 25 aquatic and 30 poultry *E. coli* isolates as manifested in plasmid bands of differing sizes. Amidst this diversity, however, the *E. coli* isolates can be seen to have yielded common plasmid bands in the range of 2.3 kb to <15 kb.

Antimicrobial susceptibility

The 25 aquatic and 30 poultry *E. coli* isolates with and without plasmids were tested for their susceptibility to nine antibiotics. Table 1 presents the results of antibiotic susceptibility of all the *E. coli* isolates used in this study. Table 2 present the results of antibiotic susceptibility of the plasmid-containing organisms, and for comparison, the susceptibility of isolates without plasmid DNA. All isolates listed in Table 1 were compared to correlate the antibiotic sensitivity with the presence or absence of plasmid DNA in the host organism (Table 2).

Transfer studies

Table 3 shows the variation in antimicrobial and plasmid transfer frequency according to the mating medium and temperature. Transfer frequency was lower when the donor cells isolated from poultry sources were grown and mated with the recipient cell in the same sterilised river water (SRW) compared with LB medium. All four selected resistant *E. coli* poultry

Table 2. Antibiotic susceptibility of *E. coli* with and without plasmid.

Antibiotic tested	Total no.(%) of strains resistant[a]		No. of *E. coli* isolates resistant[b]			
	A 25	P 30	P(+) A (19)	P(+) P (22)	P(-) A (6)	P(-) P (8)
Ampicillin	8(32%)	30(100%)	5(26%)	22(100%)	3(50%)	8(100%)
Chloramphenicol	1 (4%)	11(37%)	1 (5%)	9(41%)	c	2(25%)
Erythromycin	22(88%)	25(83%)	17(90%)	18(82%)	5(83%)	7(88%)
Gentamicin	2 (8%)	10(33%)	-	7(32%)	2(33%)	3(38%)
Kanamycin	4(16%)	26(87%)	3(16%)	18(82%)	1(17%)	8(100%)
Nalidixic acid	-	4(13%)	-	4(18%)	-	-
Streptomycin	22(88%)	30(100%)	16(84%)	22(100%)	6(100%)	8(100%)
Trimethoprim	6(24%)	28(93%)	3(16%)	28(93%)	3(50%)	7(88%)
Tetracycline	6(24%)	28(93%)	6(32%)	21(96%)	-	7(88%)

a Total number of strains tested (Aquatic, A=20; and Poultry, P=30).
b P+, with plasmid; P-, without plasmid. Number in parenthesis are total number of isolates.
c None detected.

Table 3. Transfer frequencies for *E. coli* donors from poultry sources (EP) crossed with recipients from aquatic sources (EA) and a laboratory recipient (K12).

Donor strain	Plasmid size (kb)	Resistance pattern	Recipient strain[a,b]	37°C		25°C
				LB	SRW	SRW
EP201	68	ApCmSmTTe	EA101	4.0×10^{-4}	5.3×10^{-4}	2.1×10^{-6}
EP205	87, 61	ApErKmSmTe	EA101	4.5×10^{-5}	6.7×10^{-4}	1.9×10^{-6}
EP210	90, 12.5, 5.3	ApGmNaSmTTe	EA101	2.7×10^{-4}	4.4×10^{-4}	3.6×10^{-7}
EP211	81, 6.4, 5	ApErKmSmTTe	EA101	3.8×10^{-4}	3.3×10^{-3}	2.5×10^{-6}
EP201	68	ApCmSmTTe	EA106	3.2×10^{-3}	4.7×10^{-4}	3.8×10^{-5}
EP205	87, 61	ApErKmSmTe	EA106	1.8×10^{-4}	7.1×10^{-4}	2.4×10^{-7}
EP210	90, 12.5, 5.3	ApGmNaSmTTe	EA106	5.4×10^{-4}	8.1×10^{-4}	2.5×10^{-6}
EP211	81, 6.4, 5	ApErKmSmTTe	EA106	3.7×10^{-4}	5.8×10^{-4}	2.5×10^{-6}
EP201	68	ApCmSmTTe	K12	4.1×10^{-3}	5.8×10^{-4}	3.7×10^{-7}
EP205	87, 61	ApErKmSmTe	K12	3.9×10^{-3}	5.2×10^{-4}	2.4×10^{-7}
EP210	90, 12.5, 5.3	ApGmNaSmTTe	K12	5.1×10^{-3}	6.6×10^{-4}	1.8×10^{-6}
EP211	81, 6.4, 5	ApErKmSmTTe	K12	5.5×10^{-3}	7.3×10^{-3}	1.5×10^{-7}

a All the recipients were sensitive to tetracycline, resistant to nalidixic acid and plasmid-free. Transconjugants were selected and enumerated on MacConkey agar plates double-supplemented with appropriate concentration of a single antibiotic to which the

isolates transferred *en bloc* their antimicrobial resistance phenotypes to the respective recipients, yielding transconjugants exhibiting plasmid bands with similar electrophoretic mobilities to their respective donors. All resistance acquisition occurred by conjugation since no recombinants were demonstrated among the susceptible recipient strains placed in the supernatants as transduction-transformation controls.

DISCUSSION

The results obtained provided information on the incidence of distribution of antimicrobial resistance and plasmid profiles among *E. coli* from poultry and aquatic sources. Many of the *E. coli* isolated from both sources exhibited resistance towards ampicillin, erythromycin, kanamycin, streptomycin, trimethoprim and tetracycline, although resistances to chloramphenicol, gentamicin and nalidixic acid were less common. These results concur with the data among human and animal *E. coli* isolates in Peninsular Malaysia (Koh and Kok 1984; Son *et al.*, 1995). Multiple resistances (resistant to 5 or more antibiotic) were prevalent among the poultry isolates (Table 1), in general agreement with the findings reported elsewhere (Grabow *et al.*, 1973; Fontaine and Hoadly, 1976) who showed that bacteria with high numbers of multiple resistances have been isolated from environments with the greatest potential for significant contamination by antimicrobial agents, e.g., poultry farms where antimicrobial prophylaxis has provided the selective pressure necessary to maintain R plasmids in the etiological agents (Jukes, 1972; Sato *et al.*, 1975). However, resistant bacteria have also been isolated from apparently nonselective environments, including estuaries, deep ocean water and sediment, and drinking water (McNicol *et al.*, 1980; Hada and Sizemore, 1981; Armstrong *et al.*, 1981), which generally agreed with the resistance patterns seen in this study for river water isolates.

The total number of plasmids present in any given bacterial population can affect the results of the epidemiologic analysis. A small total number can compromise our ability to discriminate among strains. In contrast, the more plasmids an organism contains, the more specific is the plasmid profile as a marker for a single strain, although it may sometimes complicate the interpretation of the results when large, diverse populations are studied (Tacket *et al.*, 1984). Occasionally, isolates that are part of an outbreak may exhibit different, but related plasmid profiles pattern. In the present study, the use of plasmid size and number did not allowed us to classify all the *E. coli* into any specific groups or profiles. We observed that *E. coli* from both sources is made up of a heterogenous group of strains exhibiting different plasmid profiles. Without the plasmid studies one could have thought that all these multiple-resistant *E. coli* strains were plasmid analysis helped to recognise the presence of more than a single clone as the carrier of the resistance characteristics. These laboratory observations, based on differences in plasmid content and antibiotic susceptibility, suggest how complex the epidemiology of *E. coli* may be in this geographical area. The antibiotic resistances among bacteria with and without plasmids was compared and it was found that a high frequency of ampicillin, erythromycin, streptomycin and tetracycline resistance was observed among isolates with plasmids. However, there appear to be no concordance between the plasmid profiles and antibiotic resistance patterns.

The incidence of plasmids conferring resistance to a particular antibiotic(s) and/or to certain other harmful agents, in aquatic bacterial isolates has been the subject of a number of studies (Burton *et al.*, 1982; Frederickson *et al.*, 1988; Jobling *et al.*, 1988). Many plasmids have been found to be transmissible to a large number of bacterial species, even when a recipient of a plasmid may be genetically and physiologically distant from the donor (Arturo *et al.*, 1993). This phenomenon increases the capacity for plasmid dissemination in aquatic systems within the natural microbial populations. The various reports on the capacity of genetic exchange between bacteria under laboratory conditions (Trevors and Oddie, 1986; Sandt and Herson, 1991) may not always be directly applicable to genetic transfer under simulated environmental conditions. The results obtained in this study demonstrated the ability of natural plasmid conjugal transfer among *E. coli* isolates from poultry to aquatic sources to occur in sterilised river water, compared with that assessed in nutrient medium (LB) under similar conditions in the laboratory. However, our results showed that transfer frequencies in SRW at 25°C are generally several orders of magnitude below (2 to 3 logs lower) than that observed in LB medium (Table 3). The decrease in transfer frequencies in river water could be explained by a stress due to abiotic environmental factors affecting gene transfer, as demonstrated by Khalil and Gealt (1987), Fernandez-Astorga *et al.* (1992), and/or nonavailability of nutrients reported by Trevors and Oddie (1986) and Sandt and Herson (1991). These authors found a close link between transfer and availability of nutrients.

Isolation of *E. coli* containing R plasmid and the demonstration of the capacity of the aquatic isolates to act as recipients for R plasmid transfer under both laboratory and simulated environmental conditions is an important step toward understanding the impact of wastes on autochthonous aquatic microorganisms. Although the frequencies of transfer were low (10^{-5} to 10^{-7}) when compared with those in controlled laboratory matings (10^{-3} to 10^{-4}), bacteria in aquatic habitats present in sufficiently high numbers and in close enough proximity to one another can effect significant transfer and retransfer of R plasmids and of other transferrable extrachromosomal genetic elements,

An accidental intake of river water, during swimming for example, would mean that a person would ingest *E. coli* containing R plasmid and could result in either a transient or permanent colonisation of the digestive tract. In consequence, they have a proportionately increased chance of passing their genetic information to the indigenous microbial population. Isolation of *E. coli* containing R plasmid demonstrates that the problem of multi-resistant bacteria exist in Malaysian waters and poultry sources. The reduced effectiveness of antibiotics may be attributed to factors such as the misuse of antibiotics as feed additives or for prophylaxis purposes, but is surely also due to the contribution of water as a mean of spreading bacteria containing R plasmids, as evident by the results obtained in this study.

ACKNOWLEDGEMENTS

The authors wish to acknowledge B. Dora for her assistance in the preparation of this paper.

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RINGKASAN

PEMINDAHAN PLASMID DAN KETAHANAN ANTIMIKROB DARIPADA ESCHERICHIA COLI *STRAIN AYAM-ITIK KEPADA STRAIN AKUA*

Analisis DNA plasmid dan kerentanan antibiotik telah diguna untuk mengkaji strain Escherichia coli *yang dipencil daripada sumber ayam-itik dan akua. Sembilan belas daripada 25 dan 22 daripada 30, masing-masing daripada strain akua dan ayam itik telah didapati mengandungi jalur DNA plasmid dengan julat saiznya di antara 2.3 hingga 102 kilobes (kb). Ketahanan terhadap lima atau lebih agen antimikrob yang diuji paling banyak terdapat di kalangan strain ayam-itik. Bagaimanapun, tiada satupun rentan terhadap kesemua agen antimikrob diuji. Lebih banyak pencilan yang mengandungi plasmid tahan ampisilin, eritromisin, streptomisin dan tetrasiklin. Potensi pemindahan plasmid semulajadi di antara strain ayam-itik dan akua telah dikaji. Empat pencilan ayam-itik multitahan antibiotik (EP201, EP205, EP210 dan EP211) yang mempunyai plasmid besar bakal mengkonjugat telah dikawankan dengan dua pencilan akua (EA101 dan EA106) dan satu strain makmal* E. coli *K12 C600 Na^r dalam air sungai tersteril pada 37°C dan 25°C, dan dalam medium LB pada 37°C. Pengkonjugatan berlaku dalam semua sistem yang diuji dengan pemindahan* en bloc *jalur-jalur ketahanan antibiotik dan DNA plasmid penderma. Dalam pemindahan in vitro yang dilakukan pada 25°C dalam air sungai steril, nilai kekerapan pemindahan menurun sekadar 2 hingga 3 log daripada keadaan optimum pada 37°C.*