ANTIMICROBIAL RESISTANCE AND CONJUGATIVE R PLASMIDS IN *ESCHERICHIA COLI* STRAINS ISOLATED FROM ANIMALS IN PENINSULAR MALAYSIA

CHONG-LEK KOH and CHOY-HAR KOK

Department of Genetics and Cellular Biology, University of Malaya, Kuala Lumpur 22-11, Malaysia.

INTRODUCTION

In the livestock industry, antibiotics and synthetic chemotherapeutics have been used in large quantities not only as therapeutic drugs for disease treatment of infected animals but also as feed additives for growth promotion and disease prophylaxis of healthy animals. However, prolonged veterinary use of these agents has resulted in high incidence of antibiotic resistant bacteria in many countries throughout the world (Dulaney and Laskin, 1971; Krcmery *et al.*, 1972; Falkow, 1975; Broda, 1979). In most of these cases, R plasmids, which are extrachromosomal genetic elements coding the resistance markers, have been found to be responsible for the drug resistance.

Hitherto, no study has been conducted in Malaysia to associate transferable antibiotic resistance traits in bacteria of animal origin with conjugative R plasmids. Hence, the present study was undertaken to (1) examine the antibiograms of some *Escherichia coli* strains isolated from animals in Peninsular Malaysia, (2) determine the frequencies and patterns of transfer of antibiotic resistance in these isolates and (3) correlate the presence of conjugative R plasmids with the transferable resistance in these strains.

MATERIALS AND METHODS

Bacterial strains : Fifteen independent *E. coli* strains, isolated from avian, bovine and porcine sources, (Table 1) were obtained from the Veterinary Diagnostic Laboratory, Petaling Jaya, Selangor, Malaysia. Plasmidless *E. coli* K12 (ERL 14R525, F^- $1ac^+$ Nal^r, from Dr. E.J. Threlfall, London, England), sensitive to all antibiotics tested except nalidixic acid, was used as the recipient in mating experiments.

Media: LB and M9 media were prepared as described previously (Koh *et al.*, 1983). Mueller-Hinton agar (BBL) and Isosensitest agar (Oxoid) were prepared as described by the manufacturers.

Antibiotic sensitivity test : Each *E. coli* isolate was tested for susceptibility with ten different antimicrobial agents. Strains not inhibited by 40 μg/ml of ampicillin (Ac), carbenicillin (Cb) or streptomycin (Sm); 10 μg/ml of gentamicin (Gm) or tetracycline (Tc); 25 μg/ml of chlorampenicol (Cm); 20 μg/ml of kanamycin (Km); 100 μg/ml of nalidixic acid (Nx); 16 μg/ml of trimethoprim (Tp); or cotrimoxazole (Ct) containing 16 μg/ml of Tp and 80 μg/ml of sulphamethoxazole were regarded as resistant. Mueller-Hinton agar was used to incorporate all drugs except Ct and Tp, which were incorporated in Isosensitest agar. *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as sensitive controls.

Mating procedure : Each antibiotic resistant strain was mated with *E. coli* K12 to verify if the resistance traits were transferable, and quantitative bacterial matings were performed by a plate mating method as described pre-

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Table 1

Characteristics of veterinary *E. coli* strains isolated in Peninsular Malaysia.

Strain	Animal origin	Antibiogram									
		Ac	Cb	Cm	Ct	Gm	Km	Nx	Sm	Tc	Tp
KE-1	Bovine	–	–	+	+	–	–	–	+	+	+
KE-2	Bovine	–	–	–	–	–	–	–	–	–	–
KE-3	Avian	–	–	–	–	–	–	–	–	+	–
KE-4	Bovine	–	–	–	–	–	–	–	–	+	–
KE-5	Bovine	–	–	–	–	–	–	–	–	–	–
KE-6	Bovine	–	–	–	–	–	–	–	–	–	–
KE-7	Bovine	–	–	–	–	–	–	–	–	+	–
KE-8	Bovine	+	+	+	–	–	+	–	+	+	–
KE-9	Bovine	+	+	+	–	–	–	–	+	+	–
KE-10	Bovine	–	–	–	–	–	–	–	–	–	–
KE-11	Bovine	–	–	–	–	–	–	–	–	–	–
KE-12	Bovine	–	–	–	–	–	–	–	–	–	–
KE-13	Bovine	+	+	–	–	–	+	–	+	+	–
KE-14	Bovine	–	–	+	–	–	–	–	+	+	–
KE-15	Porcine	–	–	–	–	–	–	–	–	–	–

+ = resistant; – = susceptible

viously (Koh *et al.*, 1983). Transconjugants from each mating were selected on different LB agar plates, containing Nx (100 μg/ml) and one of the antibiotics to which the donor strain was resistant. The concentrations of antibiotics incorporated into the selective media were 20 μg/ml for Km, 40 μg/ml for Sm and 10 μg/ml for Tc.

Analysis of transconjugants : Single transconjugant colonies from each mating growing on the selective media were purified and toothpicked onto different LB agar plates, each containing one of the following antibiotics to which the donor strain was resistant: Ac (40 μg/ml), Cb (40 μg/ml), Cm (25 μg/ml), Km (20 μg/ml), Sm (40 μg/ml) or Tc (10 ug/ml). To screen for the acquisition of Ct or Tp resistance, transconjugant colonies were toothpicked onto M9 minimal plates supplemented with glucose and Ct (containing 16 μg/ml Tp and 80 μg/ml sulphamethoxazole). The donor and recipient cells were always inoculated on the same antibiotic plates as the transconjugants to act as controls.

Transfer frequencies are expressed as the number of transconjugants per ml of the mating mixture divided by the number of donor cells per ml of the same mating mixture.

Isolation of plasmid and agarose gel electrophoresis : The rapid extraction method of Kado and Liu (1981) was used to isolate plasmid DNA from the *E. coli* donors and transconjugants. Horizontal agarose gel electrophoresis for the detection of plasmid DNA was performed according to Meyers *et al.*, (1976). At the end of the electrophoretic run, gels were stained with ethidium bromide (5 μg/ml) and visualized and photographed on a 302 nm UV transilluminator

(Model TM 36, UV Products, Inc.), using a Polaroid MP-4 Land camera system fitted with a yellow filter and Polaroid type 665 black-and-white Land films.

Antibiotics : Antibiotics added to agar or broth were from the following sources : ampicillin sodium (Penbritin; Beecham Research Laboratories, England), carbenicillin sodium (Pyopen; Beecham), chloramphenicol (Sigma Chemical Co., U.S.A.), cotrimoxazole (Bactrim; SA F. Hoffmann-La Roche & Co. Ltd., U.S.A.), gentamicin sulphate (Sigma), kanamycin sulphate (Sigma), nalidixic acid (Sigma), streptomycin sulphate (Sigma) and tetracycline HCl (Sigma).

RESULTS

Antibiotic resistance phenotypes : Table 1 shows that seven of the fifteen *E. coli* isolates were susceptible to all the ten antibiotics tested, just like the sensitive controls. Among the resistant isolates, three were mono-resistant and five were multi-resistant, i.e., resistant to three or more antimicrobial agents. All of them were resistant to Tc and susceptible to Gm and Nx. Most of them showed various combinations of resistance to Ac, Cb, Cm, Km, Sm and Tc.

Transfer of antibiotic resistance by conjugation to *E. coli* K12 : All the mono- and multiresistant *E. coli* isolates were examined for transferability of resistances. Three strains, KE-1, KE-8 and KE-13, were able to transfer Km, Sm and Tc resistance traits to the *E. coli* recipient, and the transfer frequencies ranged from 4.5×10^{-8} to 6.8×10^{-7} (Table 2).

Coinheritance of unselected resistance traits: Table 2 shows that KE-1, KE-8 and KE-13 proved capable of simultaneously transferring all or part of their resistance traits. In mating involving KE-1 donor, all transconjugants inherited the complete antibiotic resistance pattern, CmCtKmTcTp, regardless of whether selection was made on Km or Tc. This indicates that all the five resistance determinants were acquired as a single linkage group, suggesting the presence of a conjugative R plasmid.

In contrast, segregated transfer was observed among transconjugants derived from mating with KE-8 donor. Selection for Tc

Table 2

Characteristics of antibiotic resistance transfer from animal *E. coli* strains to *E. coli* K12.

Donor	Antibiogram	Selective donor antibiotic	Transfer frequency	No. of transconjugants examined	No. and resistance phenotype of transconjugants
KE-1	CmCtKmTcTp	Km	6.8×10^{-7}	30	30 CmCtKmTcTp
		Tc	3.0×10^{-7}	30	30 CmCtKmTcTp
KE-8	AcCbCmKmSmTc	Km	4.5×10^{-8}	30	30 CmKmTc
		Tc	4.7×10^{-8}	30	30 AcCbCmKmSmTc
KE-13	AcCbKmSmTc	Km	7.2×10^{-8}	30	30 AcCbKmSm
		Sm	9.3×10^{-8}	30	30 AcCbKmSm
		Tc	0		

resistance yielded one class of transconjugants showing acquired resistance to all the six antibiotics; whereas, selection with Km gave transconjugants all showing resistance to Cm, Km and Tc. This shows that Cm, Km and Tc resistance traits were cotransferred as a single linkage group, and Ac, Cb and Sm resistance traits were coinherited independently as another linkage group.

All transconjugants from mating with KE-13 donor acquired resistance to all antibiotics to which KE-13 was resistant except Tc, irrespective of whether initial selection was for Km or Sm resistance. This indicates that the four transferable resistance traits, Ac, Cb, Km and Sm, were linked. Tc resistance in KE-13 was not transferable by conjugation.

Analysis of plasmid profiles : The results of the mating experiments showing *en bloc* transfer of unselected resistance traits suggest that the donor *E. coli* strains harboured one or more conjugative R plasmids.

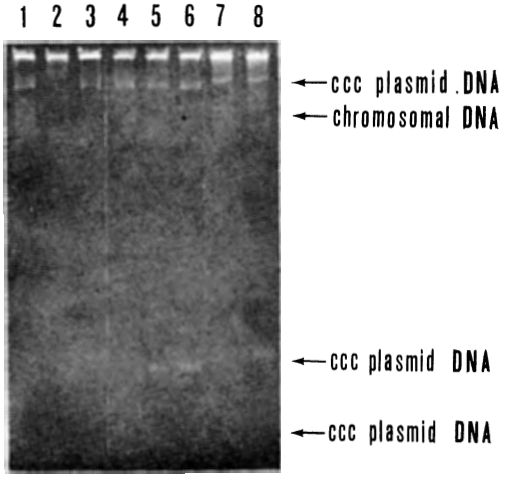

Fig. 1—Agarose (0.5%) gel electrophoresis of DNA extracted from *E. coli* donors, recipient and transconjugants. Lane 1, KE-1; lane 2, *E. coli* K12 recipient; lane 3, transconjugant from KE-1 mating; lane 4, CmKmTc resistant transconjugant from KE-8 mating; lane 5, KE-8; lane 6, AcCbCmKmSmTc resistant transconjugant from KE-8 mating; lane 7, transconjugant from KE-13 mating; lane 8, KE-13.

As shown in Fig. 1, a distinct slow-migrating covalently closed circular (ccc) DNA band was detected in both the KE-1 donor (Lane 1) and the *E. coli* transconjugant (Lane 3), but was absent in the *E. coli* K12 recipient (Lane 2). This band shows the presence of a high molecular weight (M.W.) plasmid DNA (M.W. greater than 41×10^6) in both the donor and its transconjugant.

Fig. 1 also shows two types of plasmid patterns for the two classes of transconjugants derived from KE-8 mating. Transconjugants which were resistant to Ac, Cb, Cm, Km, Sm and Tc harboured two, a large (M.W. greater than 41×10^6) and a small (M.W. between $2.6 - 3.4 \times 10^6$), ccc plasmid species (Lane 6). However, transconjugants which were resistant to Cm, Km and Tc harboured only the large plasmid (Lane 4). KE-8 donor was shown to carry three different ccc plasmid species (Lane 5).

All transconjugants from KE-13 mating possessed only a large plasmid (M.W. greater than 41×10^6) (Lane 7, Fig. 1), which corresponded to one of the three plasmid species in KE-13 (Lane 8, Fig. 1).

DISCUSSION

A disquieting feature of the present study is the relatively high incidence (53%) of antibioic resistant isolates among the *E. coli* strains purified from animals in Peninsular Malaysia. Among them, 62.5% were resistant to three or more antibiotics, i.e., multiresistant. A variety of antibiotic resistance patterns was observed and they involved various combinations of Ac, Cb, Cm, Km, Sm and Tc. All of them were resistant to Tc and sensitive to Gm and Nx. This finding is not unexpected because similar observations have been reported in other countries by Smith (1966, 1968), Fein *et al.*, (1974), Hartley *et al.*, (1975), Marsik *et al.*, (1975) and Kanai (1983). The high incidence of Tc resistance

was reported to be directly related to the widespread incorporation of tetracyclines in animal feeds. On the other hand, both Gm and Nx are rarely used in veterinary medicine. In Malaysia, similar situations prevail. Chlortetracycline, oxytetracycline, bacitracin, colistin, spiramycin, sulphonamides and trimethoprim are permitted feed additives for calves, swine and poultry. Ampicillin, streptomycin and neomycin are used extensively for individual antibacterial treatment in veterinary practice.

Three of the eight resistant strains, KE-1, KE-8 and KE-13, transferred all or part of their resistance traits to an *E. coli* recipient by conjugation. The transfer frequencies and the cotransfer of antibiotic resistance during conjugation indicated that all the three *E. coli* donors harboured self-transmissible R plasmids. The presence of R plasmids encoding the transferable resistance traits in all the three donors and their respective transconjugants was confirmed by analysing their plasmid profiles after agarose gel electrophoresis. In KE-1, the five resistance traits were encoded by a large conjugative R plasmid (M.W. greater than 41×10^6). In KE-8, the five resistance traits were mediated by two plasmids. The Cm, Km and Tc resistance traits were encoded by a large R plasmid (M.W. greater than 41×10^6), and the Ac, Cb and Sm resistance traits were borne on a smaller R plasmid (M.W. between $2.6 - 3.4 \times 10^6$). In KE-13, four of the five resistance traits were located on a large self-transmissible R plasmid (M.W. greater than 41×10^6).

The discovery that drug resistant *E. coli* strains, including some carrying infectious R plasmids, were present in animals in Peninsular Malaysia is of concern to public health, animal husbandry and therapeutic treatments. The use of antibiotics in animals has been known to exert a selection pressure to maintain a pool of resistant enterobacteria in the animals, which then constitute a reservoir of antibiotic resistance (Swann Committee, 1969). These bacteria may be transmitted to human contacts (Fein *et al.*, 1974; Hartley *et al.*, 1975; Marsik *et al.*, 1975; Levy *et al.*, 1976a, b; Linton *et al.*, 1977; Saida *et al.*, 1981; O'Brien *et al.*, 1982) and some of these may transfer their resistance traits to initially sensitive enteric pathogens within the bowels of their human and animal hosts (Walton, 1966; Smith, 1970; Farrar *et al.*, 1972; Anderson *et al.*, 1973; Anderson, 1975; Smith, 1977).

Our present findings emphasize the need in Malaysia to investigate the impact of veterinary antibiotic use on the emergence and persistence of antibiotic resistant bacteria, to study the extent of human infection by antibiotic resistant bacteria of animal origin, and to review the policy of application of antibiotics for growth-promoting and for prophylactic and therapeutic purposes in the livestock industry. The objective of these studies will eventually be to formulate a safe and effective antibiotic policy in line with that recommended by the Swann Committee (1969) in the United Kingdom.

SUMMARY

Fifteen independent *E. coli* strains of avian, bovine and porcine origin in Peninsular Malaysia were tested for antibiotic resistance and conjugative R plasmids. Eight (53%) isolates were found to be antibiotic resistant. Among them, 37.5% were mono-resistant and 62.5% were resistant to three or more antibiotics, i.e., multi-resistant. All of them were resistant to Tc and sensitive to Gm and Nx.

Three of the eight antibiotic resistant strains were able to transfer all or part of their resistance to an *E. coli* K12 recipient by conjugation. The transfer frequencies of Km, Sm and Tc resistance of the three donors varied between 4.5×10^{-8} to 6.8×10^{-7}. Analysis of the plasmid profiles of all the

three donors and their respective transconjugants after agarose gel electrophoresis provided conclusive evidence that the transferable resistance traits were plasmid-mediated.

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