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PLASMIDS AND ANTIMICROBIAL SUSCEPTIBILITY OF *ESCHERICHIA COLI* WITH SPECIAL REFERENCE TO POULTRY ISOLATES IN SARAWAK

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SUMMARY

A total of forty six strains of *Escherichia coli* from poultry sources were examined for the occurrence of plasmid DNA in association with their susceptibility to nine antimicrobial agents. Of all the *E. coli* surveyed, 35% (16 of 46) contained plasmid DNA ranging in size from 1.5 to 64 megaDalton (mDa). Antibiotic susceptibility for the 46 poultry isolates indicated that most isolates were resistant to ampicillin, erythromycin, streptomycin, trimethoprim-sulfamethoxazole, sulfonamide and tetracycline, and no isolates were susceptible to penicillin G. More isolates containing plasmids were resistant to ampicillin, erythromycin, streptomycin, sulfonamide, trimethoprim-sulfamethoxazole and tetracycline. In addition, six selected resistant *E. coli* poultry isolates were found to transfer *enbloc* their antimicrobial resistance phenotypes with the concomitant transfer of all their respective donors plasmid species to the recipient *E. coli* K12 Nal^r, which suggested a possible relationship between plasmid carriage and resistance to the antimicrobial agents tested.

Keywords: *Escherichia coli*, antimicrobial susceptibility, plasmid DNA.

INTRODUCTION

The genes that make bacteria resistant to antimicrobial agents are usually encoded not on their chromosomes but on smaller self-replicating companion loops of DNA called plasmids (Saunders, 1984). Some plasmids bring about the conjugal transfer of their own or other genetic material, and others are non-self-transmissible (Guiney *et al.*, 1984). The accumulation of resistance genes on plasmids and their potential for transfer suggest that plasmids are major vectors in the dissemination of resistance genes through bacterial populations. This has been especially well documented by Smith and Linggood (1971) for strains of *Escherichia coli* that are pathogenic for domestic animals.

The extensive spread of resistance genes on plasmid clones was detected by selecting isolates that had highly distinctive resistance phenotypes due to their carriage either of recently emerged and still rare resistance genes or of unusual combinations of resistance genes. More research has been done on plasmids common in highly selected resistance isolates from human and animals in urban population, thus, leaving largely unsurveyed the diversity of plasmids that encode common resistance genes in unselected isolates in rural areas, which has been the basis for assuming that similar plasmids from different places are clonal and thus epidemic. This report deals with isolation of plasmids DNA in association with the antimicrobial susceptibility of selected resistant *E. coli* from poultry sources in rural Bario, Sarawak.

MATERIALS AND METHODS

Bacterial strains

Faecal specimens from poultry sources in the study area in rural Bario, Sarawak were transported in buffered glycerol saline to our laboratory for culture and isolates of *Escherichia coli*, as determined by standard laboratory procedures (Edward and Ewing, 1972) were isolated. *Escherichia coli* K12, a nalidixic acid-resistant and plasmidless strain was used as recipient for conjugation transfer experiments.

Plasmid isolation

Organisms were screened for plasmid DNA by the procedure of Birnboim and Doly (1979). Extracted plasmids were electrophoresed for 2 h at 35 mA on a 0.7% agarose gel in TBE buffer (89 mM tris base-89 mM boric acid -2.5 mM disodium EDTA) 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 plasmid was

determined by comparison with plasmids of known molecular mass from *E. coli* V517 (Macrina *et al.*, 1978).

Antibiotic susceptibility testing

Isolates were screened for resistance to ampicillin, erythromycin, gentamycin, nalidixic acid, penicillin G, streptomycin, trimethoprim-sulfamethoxazole, sulfonamide and tetracycline. Bacteria were suspended in saline to the density of a 2 McFarland standard, 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 inhibition zone size according to the specific instructions supplied for each antibiotic by the manufacturer.

Conjugation studies

Selected resistant isolates were incubated overnight at 37°C with a nalidixic acid resistant recipient of *E. coli* K12. These mating mixtures were suspended in 0.85% saline to a density of approximately 10^8 cells per mL. Plate counts were performed for estimates of donor and recipient population. Transconjugants were selected on nutrient agar plates containing 100 mg/mL of nalidixic acid and inhibiting concentration of an agent to which the donor isolate had been resistant. After incubation, transconjugants were purified and tested for resistance by the agar disc diffusion method (Bauer *et al.*, 1966).

RESULTS

Plasmid analysis

Forty six strains of *Escherichia coli* from chickens in Bario, a remote area in Sarawak were examined for the occurrence of plasmid DNA in association with the antibiotic susceptibility of the bacterial host. Of all the *E. coli* isolates surveyed, 35% (16 of 46) contained plasmid DNA, and all isolates were screened at least twice (Table 1). DNA bands were discerned in photographs of electrophoresis gels of plasmids extract of the 16 *E. coli* poultry isolates. Photographs of the gel (Figure 1) illustrates the general diversity of these plasmids detected among the 16 *E. coli* isolates as manifested in plasmids of differing sizes and differing staining intensities, the later varying in part presumably with plasmid copy number. Amidst this diversity, however the poultry isolates can be seen to have yielded plasmids with similar molecular weight. For instance, a 51 mDa plasmid was found in 56% (9 of 16) of the *E. coli* isolates; similarly, 88% (14 of 16) had common plasmids in the range of 1.5 to 6 mDa. This suggested a common source for the dissemination of *E. coli* among chickens in the study area, and it was suspected that the high plasmid occurring rate of 35% (16 of 46) obtained was due to repeat isolation of organisms from different birds of the same flock. The *E. coli* plasmids ranged in size from 1.5 to 64 mDa (Table 1).

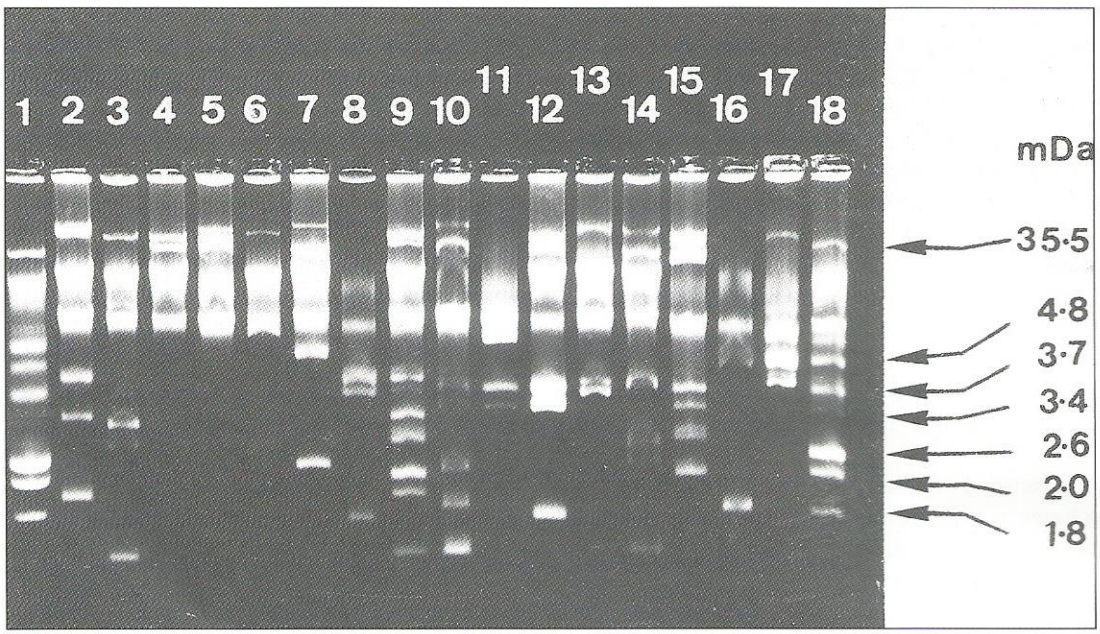

Figure 1. Agarose gel (0.7%) electrophoresis of plasmid DNA from *Escherichia coli* isolates. Lanes 1 and 18, V517 standard containing 35.5, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8 mDa size reference plasmids; 2, EC401; 3, EC402; 4, EC403; 5, EC404; 6, EC405; 7, EC406; 8, EC407; 9, EC408; 10, EC409; 11, EC410; 12, EC411; 13, EC412; 14, EC413; 15, EC414; 16, EC415; and 17, EC416.

Table 1. Plasmid profiles and antimicrobial susceptibility among chicken isolates of *Escherichia coli* with plasmid DNA.

Isolate no.	Antimicrobial resistance[a]	Plasmid size (mDa)	Frequencies of transfer[b,c]
EC401	PG	52, 3.7, 3, 1.9	ND
EC402	ApEmGmPGSmSSxtTe	51, 2.8, 2.5, 1.5	1×10^{-3}
EC403	ApEmPGSmSSxtTe	51, 42	ND
EC404	EmGmPGSmS	64, 51, 42	2.3×10^{-4}
EC405	ApNalPGSmSSxtTe	51, 7.4, 2.4	ND
EC406	ApPGSmSSxtTe	64, 35, 4.5	3.1×10^{-3}
EC407	ApEmPGSmSSxtTe	51, 3.6, 3.4, 1.7	ND
EC408	PGSmS	51, 42, 3.6, 3.4, 2.6, 2.2, 2, 1.5	1.8×10^{-4}
EC409	ApGmPGSmSSxtTe	64, 42, 3.6, 3.2, 2.2, 1.9, 1.5	5.2×10^{-6}
EC410	ApEmGmPGSmSSxtTe	9.5, 5.8, 3.6, 3.2	ND
EC411	ApEmPGS	52, 38, 3.4, 3.1, 1.8	ND
EC412	EmPGSmS	51, 3.4	ND
EC413	ApEmPGSmSSxtTe	51, 35, 3.4, 2.5, 2.3, 1.8, 1.5	ND
EC414	ApEmNalPGSmSSxtTe	51, 42, 28, 3.4, 3.1, 2.6, 2.2	ND
EC415	ApEmPGSmSTe	64, 4.2, 1.8	3.7×10^{-4}
EC416	ApEmPGSmSSxtTe	51, 5.7, 3.8, 3.4	ND

a Tested for Ap = ampicillin, Em = erythromycin, Gm = gentamycin, Nal = nalidixic acid, PG = penicillin G, S = sulfonamide, Sm = streptomycin, Sxt = trimethoprim-sulfamethoxazole and Te = tetracycline.

b Frequencies are expressed as the number of transconjugants per input donor cells.

c Transconjugants obtained showed *enbloc* transfer of donors antimicrobial resistance phenotypes and plasmid species.

ND = Not determined.

Antibiotic susceptibility

The 46 *E. coli* isolates with and without plasmids were tested for their susceptibility to nine antibiotics commonly used as feed supplements and therapeutics in animal husbandry industry. Table 1 presents the results of antibiotic susceptibility of 16 of these plasmid-containing organisms, and for a comparison, the susceptibility of 30 isolates without plasmid DNA (Table 2). All isolates listed in Table 2 were compared to correlate antibiotic sensitivity with the presence or absence of plasmid DNA in the host organism. In general, most isolates were resistant to ampicillin, erythromycin, streptomycin, trimethoprim-sulfamethoxazole, sulfonamides and tetracycline, and no isolates were susceptible to penicillin G; whereas only three and seven isolates were resistant to nalidixic acid and gentamycin, respectively (Table 2). When the resistance of isolates carrying plasmids were compared with that of isolates without plasmids, the results were similar for resistance towards gentamycin, penicillin G and nalidixic acid; however more isolates containing plasmids were resistant to ampicillin, erythromycin, streptomycin trimethoprim-sulfamethoxazole, sulfonamide and tetracycline (Table 2).

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

Antibiotic tested	Total no. (%) of strains[a] resistant	No. of *E. coli* chicken isolates resistant[b]	
		P+(16)	P-(30)
Ampicillin	26 (56%)	12 (75%)	14 (47%)
Erythromycin	19 (41%)	11 (68%)	8 (27%)
Gentamycin	7 (15%)	4 (25%)	3 (10%)
Nalidixic acid	3 (6%)	2 (12%)	1 (3%)
Penicillin G	46 (100%)	16 (100%)	30 (100%)
Sulfonamide	39 (67%)	15 (93%)	24 (80%)
Streptomycin	29 (63%)	14 (87%)	15 (50%)
Trimethoprim-sulfamethoxazole	23 (50%)	10 (62%)	13 (43%)
Tetracycline	20 (43%)	11 (68%)	9 (30%)

a The total number of strains tested were 46.

Conjugal transfer studies

In mating experiments, all six selected resistant *E. coli* poultry isolates, EC402, EC404, EC406, EC408, EC409 and EC415 transferred *enbloc* their antimicrobial agent resistance phenotypes to the recipient *E. coli* K12 Nal^r, yielding transconjugants containing their respective donors plasmid species. The frequencies of conjugal transfer ranged from 1×10^{-3} to 5.2×10^{-6} transconjugants per donor cell.

DISCUSSION

Although the origin of the many genes coding for antibiotic resistance remains speculative, it is well known that genetic variation in bacteria has been rapid, especially in their resistance to antibiotics (Saunders, 1984); strains resistant to many antibiotics have been selected where antibiotic use is intensive and multiple (Falkow, 1975). For example, it is estimated that nearly half the antibiotics sold in the United States are given to food-producing animals (O'Brien *et al.*, 1982). Consequently, the spread of antibiotic-resistant *Salmonella* spp. to humans for instance, by way of contaminated meat is becoming increasingly more evident (Holmberg *et al.*, 1984). Animal feed supplementation with antibiotics is known to produce antibiotic-resistant bacteria in the normal enteric flora of the animals (Ahart *et al.*, 1978); however, where antibiotic use has been restricted, the incidence of strains resistant to the agent has declined (Lacey, 1984). There is little doubt that the veterinary and medical use of antibiotics plays a crucial role in selecting resistance.

In this study, most of the *E. coli* isolates exhibit resistance towards ampicillin, erythromycin, penicillin G, streptomycin, trimethoprim-sulfamethoxazole, sulfonamides and tetracycline, although resistance to nalidixic acid and gentamycin was less common. Similar studies have shown that there is a high frequency of resistance to ampicillin, streptomycin and tetracycline among human and animal *E. coli* isolates in Peninsular Malaysia (Koh and Kok, 1984; Cheong *et al.*, 1990); likewise the results obtained in this study showed that a high percentage of the *E. coli* isolates from chickens in rural Bario in Sarawak to be resistant to ampicillin, streptomycin and tetracycline. In addition, all the *E. coli* isolates were resistant to penicillin G.

The antibiotic resistance among bacteria with and without plasmids was compared and it was found that a higher frequency of ampicillin, erythromycin, streptomycin, sulfonamides, trimethoprim-sulfamethoxazole and tetracycline was observed among isolates with plasmids than among isolates without plasmids. However, there did not appear to be a consistent relationship between a particular plasmid and resistance to an antibiotic. Although there is no firm evidence of plasmids associated with resistance to antibiotics, the high frequency of plasmid DNA observed among the poultry isolates may result from overall exposure of the organism to antibiotics present in animal feeds. Under normal conditions the bacterial cell has no need for plasmids and they tend to be lost, but when a plasmid function becomes essential for survival, as for example when antibiotics are used, cells containing the appropriate plasmid will be selected at the expense of those that do not (Lacey, 1984). However, these new genetic loci are transient and highly unstable and may be lost as rapidly as they were gained.

The overall impression from this study is that the plasmid content of the 16 resistant *E. coli* isolates is highly diverse, exhibiting various electrophoresis patterns (Figure 1). All of these isolates, moreover, were of one genus, *Escherichia coli*, and from one host species, chickens, raised in presumably similar environment by farmers in one region. Plasmid content of unselected bacteria sampled from more varied sources, therefore, might be even more diverse (Selander and Levin, 1980). Such a high level of diversity makes it highly probable that identical or nearly identical plasmids found in widely separated isolates are clonal.

Attempt to demonstrate transfer of resistance from six selected resistant *E. coli* strains by conjugation was successful (Table 1). *Enbloc* transfer of the donors resistance phenotypes was observed in their respective transconjugants, with the concomitant transfer of all the plasmid species of the respective donor strains. Although there did not appear to be a constant relationship between a particular plasmid and resistance to an antibiotic, there is potential for spread of resistance, as evidence by the findings in this study on the six strains of *E. coli* containing transferable antimicrobial resistance and plasmids DNA. Thus, there is no doubt that the close association between *E. coli* and human enteric disease makes it a potential agent for dissemination of antibiotic resistance genes among human bacterial flora. In this report, the results obtained emphasised the important role that *E. coli* isolates and their associated plasmid would have to offer for tracing the spread of antibiotic resistance through animal and human population, and thus plasmid can be considered as additional procedure in national and statewide control programmes.

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RINGKASAN

KERENTANAN PLASMID DAN ANTIMIKROB ESCHERICHIA COLI *DENGAN MERUJUK KHUSUS KEPADA PENCILAN AYAM ITIK DI SARAWAK*

Sejumlah empat puluh enam strain Escherichia coli daripada sumber ayam itik telah diperiksa untuk menentu wujudnya DNA plasmid berkaitan dengan kerentannya terhadap sembilan agen antimikrob. Daripada semua E. coli *yang ditinjau, 35% (16 daripada 46) mengandungi DNA plasmid yang julat saiznya di antara 1.5 dan 64 megaDalton (mDa). Kerentanan antibiotik untuk 46 pencilan ayam itik menunjukkan yang kebanyakannya tahan ampisilin, eritromisin, streptomisin, trimetoprim-sulfametoksazol, sulfonamida dan tetrasikilina, dan tiada pencilan yang rentan terhadap penisilin G. Lebih banyak pencilan yang mengandungi plasmid rentan terhadap ampisilin, eritromisin, streptomisin, trimetoprim-sulfametoksazol, sulfonamida dan tetrasikilina. Juga, enam daripada pencilan* E. coli *ayam itik tahan pilihan didapati memindah secara* en bloc *fenotip ketahanan antimikrobnya, diiringi pemindahan kesemua spesies plasmid yang ada pada penderma masing-masing kepada E. coli penerima K12 Nal^r, dan ini menyarankan mungkin ada perkaitan di antara pembawaan plasmid dan ketahanan terhadap agen antimikrob yang diuji.*