DRUG RESTANCE AND CONJUGATIVE R PLASMIDS IN *ESCHERICHIA COLI* STRAINS ISOLATED FROM CATTLE, PIGS AND CHICKENS IN INDONESIA

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ABSTRACT

Drug resistance in *Escherichi coli* strains isolated from cattle, pigs and chickens in Indonesia was investigated using six kinds of antibiotics and chemotherapeutics; oxytetracycline hydrochloride, chloramphenicol, dihydrostreptomycin, sulfadimethoxine, kanamycin and aminobenzyl penicillin. A total of 756 *E. coli* strains which consisted of 309 from cattle, 235 from pigs and 212 from chickens, resprectively, were isolated from rectal contents or fresh feces. The resistance patterns varied from single to quintuple in the cattle isolates and from single to sextuple in the pig and chicken isolates, Conjugative R plasmids were detected in 17.8% of the cattle isolates, 14.4% of the pig isolates and 42.7% of the chickens isolates, respectively. It is of interest that most of the *E. coli* strains isolated from day-old chicks before feeding has resistance, suggesting that these resistant strains were originated from hutchers which might be contaminated wirh drug-resistant bacteria. These results suggest that the high incidence of drug resistance and R plasmids in *E. coli* strains isolated from chickens is due t a reflection of the usage of antibiotics and chemotherapeutic not only for the treatment of prevention of diseases but also as growth promoters.

INTRODUCTION

Since Moore *et al* (1946) reported that a small amount of streptomycin added to the diet promoted growth of chickens. Since then many other growth-promoting agents such as antibiotics and synthetic chemotherapeutics were used as feed additives to increase the production of animal protein from livestock and poultry. However, the prolonged usage of these drugs for growth promotion, leads to a high incidence of enteric bacteria with drug resistance. The effect of the usage of antimicrobial agents at nutritional level on the emergence of resistant *E. coli* strains in the alimentary track of livestock and poultry has been investigated (Harry, 1962; Smith, 1967; Smith and Crab, 1957).

In Indonesia, several investigators performed investigation on drug resistance (Budiharta and Warudju, 1985; Mudigdo and Peranginangin, 1982; Ronohardjo *et al* 1985). Most of them determined the drug resistance of several species of bacteria using the disc method. However, in order to carry out basic studies of drug resistance, this method seems to be insufficient.

The purpose of the survey reported here was to clarify the present situation of antibiogram of *E. coli* strains isolated from cattle, pigs and chickens in Indonesia.

MATERIAL AND METHODS

Isolation of fecal *E. coli* strains from livestock and poultry as shown in Table 1, 309 *E. coli* strain from cattle, 235 from pigs and 212 from chickens were isolated in West Java and suburb of Medan in 1986. In most cases, *E. coli* strains of cattle were isolated from rectal contents, but when it was difficult to obtain rectal contents these strains were isolated from fresh fecal samples. *E. coli* strains of pigs and chickens were isolated from each of these animals. A loopful of the fecal or rectal samples were cultured on deoxycholate-hydrogen sulfide-lactose agar (DHL, Eiken Japan) agar plates. The DHL resembling *E. coli* was pick up from each DHL plate and transfered to a triple sugar iron agar (Eiken, Japan) slant. They were then subjected to the IMViC system and identified as *E. coli.*

Drugs

The following drugs were used : oxytetracycline hydrochloride (TC), chloramphenicol (CP), dihydrostreptomycin (SM), sulfadimethoxine (SA), kanamycin (KM), aminobenzyl-penicillin (APC), and nalidixic acid (NA). NA was dissolved in 1 N NaOH and SM in posphate buffer (pH 8.0). The others were dissolved in sterile distilled water at concentrayion of 4 to 16 mg per ml. These stock solutions were further diluted with distilled water before use.

Test for Drug Resistance

Heart infusion agar (Difco) plates containing serial two fold concentrations of each drug were prepared. For the test with SA, Mueller-Hinton agar (Difco) was used. Six hour cultures of the test strains in heart infusion broth (HI broth, Difco) were diluted about 100 times with sterile saline and inoculated into media with a multiple inoculator. Results were read after 18 hour of incubation at 37^{0}C.

Table 1. Number of *Escherichia coli* strains isolated from feces of animal

	Animals	No. of strains	Place of isolation
Cattle	Dairy cattle	218	Tangerang, Sukabumi, Bogor*, Lembang, Cisarua
	Beef cattle	77	Sukabumi, Bogor*
	Buffalo	14	Bogor*
		309	
Pig		235	Tangerang, Cisarua, Cimahi, Medan
Chicken	Layer	66	Bogor, Tangerang
	Breeder layer	34	Medan
	Brolier	35	Bogor, Medan
	Breeder broiler	77	Ciomas, Medan
		212	
Total		756	

*Slaughter house

Transfer of Drug Resistance

The recipient strain used was *E. coli* ML 1410 (a nalidixic acid-resistant, and methionine-requiring F derivative of *E. coli* K-12). Donor and recipient strains were cultured in HI broth for 6 hr at 37°C. One ml of the donor culture and 0.1 ml of the recipient culture were mixed and conjugated for 18 hrs at 37°C and then spread on DHL agar plates containing (25 µg of TC, CP, SM, KM and APC per ml). After 18 hrs incubation at 37°C, the plates were inspected for transconjugants. One colony per donor was purified and tested for their resistance patterns to TC, CP, SM, SA, KM or APC to which the donor strain was resistant. If no transcojugant colonies were grown, another incubation for mating culture was done at 25°C for 18 hrs. Transconjugant colonies grown on each DHL agar plate were purified and tested in such a manner as described above. They are considered to be strains carrying thermosensitive R plasmids.

RESULTS

Incidence of Strains Resistant to Individual Drugs

The frequency of isolation of *E. coli* strains resistant to each of the six drugs is shown in Table 2. Resistance in the isolates from chickens, pigs and cattle was demonstrated most frequently to TC, followed by SM, SA, APC, KM, and CP. *E. coli* strains resistant to each drug were always isolated most frequently drom chickens regartless the kind of drugs used, and resistant strains from pigs were observed moderately. In cattle, resistant strains were very few and there were no strains resistant to CP and KM.

Table 2. Frequencies of drug resistance to each drug in *Eschirichia coli* strains isolated from animals

Animal	No. of	Percentage of strains resistant to					
	strains	TC	CP	SM	SA	KM	APC
Cattle	309	6.1	0	8.4	12.0	0	0.6
Pig	235	29.8	5.1	25.1	17.4	2.6	6.0
Chicken	212	74.5	10.4	69.8	65.6	11.3	23.1

Incidence of Drug Resistance and Conjugative R plasmids

As shown in Table 3, the incidence of resistance was the lowest in *E. coli* strains isolated from cattle and among three breeds of cattle, the incidence varied from 17.4 to 0%. Incidence of resistance in *E. coli* strains isolated from dairy cattle was the highest and there was no resistant strain isolates from the buffalos. Totally, out of the 309 strains only 45 (14.6%) were resistant. Conjugantive R plasmids were found in 8 of these resistant strains and one of the showed thermosensitive transfer.

In *E. coli* strains isolated from various types of chickens, the incidences of resistance were extremely high except for *E. coli* strains from layer chickens, Totally, 185 (87.3%) out of 212 strains were resistant. The incidence of *E. coli* strains carrying R plasmids was also relatively high (79 strains, 42.7%) compared to those isolated from pigs and cattles although the incidence varied from 84.4% to 25.0%, and 22 of them showed thermosensitive transfer. Incidence is not only resistant strains but also R plasmid carrying strains was always the lowest in *E. coli* strains isolated from layer chickens among four types of chickens.

Table 3. Frequencies of drug resistance in *Escherichia coli* strains isolated from feces of animals

Animal	No. of strains isolated	No. of resistant (%) strains	No. of strains carrying (%) R plasmid
Dairy cattle	218	38 (17.4%)	5 (1)* (15.8%)
Beef cattle	77	7 (9.1%)	2 (28.6%)
Buffalo	14	0 (0%)	0 (0.0%)
	309	97 (41.6%)	7 (1) (17.8%)
Pig	235	97 (41.3%)	14 (17.4%)
Layer	66	44 (66.7%)	11 (25.0%)
Breeder layer	34	32 (94.1%)	19 (8) (84.4%)
Broiler	35	34 (97.1%)	13 (3) (47.1%)
Breeder broiler	77	75 (97.4%)	14 (11) (33.3%)
	212	185 (87.4%)	57 (22) (42.7%)

* No. of strains showing thermosensitive transfer is indicated in parenthesis.

Drug Resistance Patterns of Original Strains and Conjugative R plasmids

Resistance patterns of 38 *E. coli* strains isolated from dairy cattle are shown in Table 4. Nine different resistance

patterns were found. Double and single resistant strains were found frequently (20 and 12 out of 38 strains, respectively). SM SA-resistant strains were relatively frequent. Sextuple and quintuple resistant strains were not found. Conjugative R plasmids which were found in the *E. coli* showed four different resistance patterns. Transferred resistance patterns were double and single resistance. Resistance patterns of seven *E. coli* strains from beef cattle were very simple (data not shown).

Table 4. Resistance patterns and transferred resistance patterns of *Escherichia coli* strains isolated from feces of dairy cattle

Original strains		R plasmids	
Resistance pattern	No. of strains	Resistance pattern	No. of strains
TC CP SA APC	1		
TC SM SA	4	SM SA	(1)*
		TC SA	1
TC SM APC	1		
TC SM	1	SM	1
TC SA	5	TC SA	3
SM SA	14		
TC	3		
SM	2		
SA	7		
Total	38		5(1)(15.8%)

* No. of strains showing thermosensitive is indicated in parenthesis.

Resistance patterns of 75 *E. coli* strains isolated from pigs in West Java (Tangerang, Cisarua, Cimahi) are shown in Table 5. Sixteen different resistance patterns were found. Although single and double resistant strains were found frequently (34 and 23 out of 75 strains, respectively). Sextuple and quintuple resistant strains which were not evident in *E. coli* strains isolated from cattle could be isolated. Conjugative R plasmids showed six different resistance patterns and they varied from single to sextuple resistance. Resistance patterns of 22 strains isolated from pigs at the suburb of Medan were almost the same as those above mentioned.

In *E. coli* strains isolated from breeder broiler and broiler chickens, the resistance patterns are shown in Table 6. Twenty-two different resistance patterns were found. Triple and quadruple resistant strains were found frequenty

Table 5. Resistance patterns and transferred resistance patterns of *Escherichia coli* isolated from pigs in West Java

Original strains		R plasmids	
Resistance pattern	No. of strains	Resistance pattern	No. of strains
TC CP SM SA KM APC	3	TC CP SM SA KM APC	2
TC CP SM SA KM	1	CP SM SA KM	1
TC CP SM SA	1		
TC SM SA APC	3		
TC SM SA	8	TC	1
TC SM APC	2	TC SM APC	1
TC CP	2		
TC SM	14	TC SM	1
		SM	1
TC SA	3		
TC APC	1		
CP APC	1		
SM SA	2		
TC	25	TC	2
SM	5	SM	1
SA	2		
APC	2		
Total	75		10 (13.5%)

(39 and 31 out of 109 strains, respectively) and TC SM SA-resistant, TC SM SA APC-resistant strains were relatively frequent. Conjugative R plasmids showed 16 different resistance patterns. Transferred resistance patterns varied from single to sextuple resistance.

In *E. coli* strains isolated from layer chickens, the resistance patterns are shown in Table 7. Ten different resistance patterns were found. Single and triple resistant strains were found frequenty (12 and 13 out of 44 strains, respectively) and TC-resistant, TC SM SA-resistant strains were relatively frequent. Conjugative R plasmids showed 6 different resistance patterns. Transfered resistance patterns varied from single to pentaple resistance.

Fluctuation of Frequency of Drug Resistance in *E. coli* Strains Isolated from Chickens According to the Age

Table 8 showed the fluctuation of the frequency of drug resistance according to the age. Almost all strains isolated from 4 types of chickens except for layer chickens were resistant throughout the period. Even *E. coli* strains isolated from one day old breeder and broiler chickens before feeding had already resistance, which persisted at high frequencies throughout the period observed. On the other hand, in *E. coli* strains from layer chickens, the frequencies of resistance gradually decreased according to the age.

Table 6. Resistance patterns and transferred resistance patterns of *Escherichia coli* isolated from breeder and broiler chickens

Original strains		R plasmids	
Resistance pattern	No. of strains	Resistance pattern	No. of strains
TC CP SM SA KM APC	2	TC CP SM SA KM APC	2
TC CP SM SA APC	7	TC SM APC	1
		TC CP SA	1
		TC SA	(1)*
		TC	1
TC CP SM SA	4		
TC CP SA APC	1		
TC SM SA KM	2	TC SA	1
TC SM SA APC	19	TC SM SA APC	1(1)
		SM SA	(1)
		SA APC	1
		SM	2
TC SM KM APC	4	TC SM KM APC	3
CP SM SA APC	1		
TC CP SM	1	TC CP SM	1
TC SM SA	33	TC SM SA	2(6)
		TC SM	1
		TC SA	(1)
		SM SA	2(1)
		SM	3(2)
TC SM KM	2	SM	1
TC SM APC	1	TC SM	1
SM SA APC	1		
SM KM APC	1		
TC CP	1		
TC SM	3	TC SM	1
		TC	(1)
TC SA	10		
SM SA	3	SM SA	2
TC	5		
SM	2		
SA	3		
APC	1		
Total	109		27(14)(37.6%)

* *No. of strains showing thermosensitive transfer is indicated in parenthesis*

Table 7 : Resistance patterns and transferred resistance patterns of *Escherichia coli* strains isolated from layer chickens

Original strains		R plasmids	
Resistance pattern	No. of strains	Resistance patten	No. of strains
TC CP SM SA KM APC	1	TC CP SM SA KM	1
TC CP SM SA APC	1	SM SA	1
TC SM SA APC	2	TC SM APC	1
TC CP SA	1		
TC SM SA	13	TC SM SA	2
		SM SA	1
SM SA KM	2	SM SA KM	2
TC SM	6	TC SM	1
		SM	
TC SA	2		
SM SA	4		
TC	12		
Total	44		11(25.5%)

Table 8 : Frequencies drug resistance in *Escherichia coli* strains isolated from different kinds of chicken

Kinds of chicken	Age	No. of strains	No. of resistance strains (%)
Breeder broiler	1 day	8	8
	1 month	11	11
	2months	8	8
	8 months	11	11
	Total	38	38(100)
Broiler	14 days	9	8
	24 days	4	4
	36 days	6	6
	Total	19	18(94.74)
Breeder layer	1 day	9	8
	2 months	10	10
	7 months	8	8
	14 months	7	6
	Total	34	32(94.12)
Layer	8 months	10	10
	12 months	10	10
	14 months	9	7
	18 months	8	6
	19 months	10	7
	20 months	9	4
	Total	56	44(7857)

DISCUSSION

There has been several reports about drug resistance of *E. coli* strains isolated from livestock in Indonesia. Mudigdo and Peranginangin (1982) reported that most of the *E. coli* strains isolated from diarrhea piglets were resistant to TC and that the administration of TC in rations was correlated with the resistance of *E. coli* against TC in piglets. Other investigators (Budiharta and Warudju, 1985; Ronohardjo *et al,* 1985) also carried out tests for drug resistance of *E. coli,* Staphylococcus, Salmonella and so on and demonstrated resistant bacteria among them. However, these results were obtained by the use of a disc method and most of them did chemotherapeutics in determining drug resistance. Although studies using disc are important and practical, other basic studies are needed in performing surveys on drug resistance up to date. For example, MICs of individual drug are required to understand the level of drug resistance to bacteria to be tested and detection of conjugative R plasmids is also necessary for the epidemiological purpose because conjugative R plasmids can be transferred to other pathogenic bacteria (Mitsuhashi *et al,* 1967).

It is emphasized that this was probably the first report about investigations of R plasmid detection in *E. coli* strains isolated from livestock and poultry in Indonesia. *E. coli* strains from rectal contents or fresh feces as representatives of Enterobacteriaceae drug resistance as well as the detection of R plasmids were performed.

By comparing the resistance frequencies in *E. coli* strains isolated from cattle, pigs and chickens, it became clear that most *E. coli* strain from chickens and about half of *E. coli* strains from pigs are drug-resistant and it was also demonstrated that most of the *E. coli* strains isolated from cattle are drug-sensitive and it was noted that resistant strains could not be isolated from the buffalos.

Compared these finding to the results obtained in Japan which is referred to as one of the countries where antibiotics are widely used (Nakamura, 1984), the resistance frequency in *E. coli* strains isolated from chickens is almost the same in both countries (Kanai, 1978). However, resistance frequencies in *E. coli* strains isolated from pigs and calves are much higher in Japan than in Indonesia because the resistance frequencies in *E. coli* isolated from these animals were extremely high in Japan (80 to 90%) (Nakamura *et al,* 1978; 1979). This difference of resistance frequencies is thought to be due to difference usage of antibiotics and synthetic chemotherapeutics because the effect of the usage of antimicrobial drugs on the emergence of drug-resistant strains of bacteria in animals have already been demonstrated (Edward, 1962; Harry, 1962; Smith, 1967; Smith abd Crabb, 1957). As a matter of cource, a significant amount of antibiotics and synthetics chemotherapeutics are used not only in the treatment and preventions of diseases but also as growth promoters in Japan and even the net weight of antimicrobial products used for livestock and poultry has already been published (Nakamura, 1984). On the other hand, the present situation about the usage of antimicrobial drugs is not clear in Indonesia. However, it is obvious that a considerable amount of antibiotics and synthetics chemotherapeutic used for broiler as not only for the therapeutics or preventive purpose of diseases control but also as growth promoting agents because most of the *E. coli* strains isolated from chickens are drug-resistant. In the case of layer chickens, it is thought that antibiotics and synthetic chemotherapeutics are not given after their egg production started because resistant *E. coli* strains decrease after their egg production.

A small amount of antimicrobial agents may be used in pigs as growth promoters in addition to treatment and provention of diseases. As for the cattle, it is thought that the antimicrobial agents are given only for the treatment of diseases because most of *E. coli* strains isolated from them are drug-sensitive.

Conjugative R plasmids could be detected at frequencies of 42.7%, 14.4% ad 1.8% in resistant strains isolated from chickens, pigs and cattle, respectively, in Indonesia. In Japan, conjugative R plasmids and over 50% of the pig and calf isolates carried conjugative R plasmid (Kanai and Igarashi, 1980; Nakamura, 1978; 1979). Therefore the isolation frequency of conjugative R plasmid in *E. coli* strains of chickens is almost the same in both countries. However, in *E. coli* strains from other species, the frequencies were much higher in Japan than in Indonesia. These results may also reflect the difference of antibiotic usage in both countries as mentioned above. Thermosensitive R plasmids could be isolated in *E. coli* strains from chickens (22 strains) and from a cattle. Because thermosensitive transfer may more likely occur outside the animal body, special attention had to be paid to their incidence in disposed feces and sewage (Smith *et al,* 1978).

The present situation of drug resistance in *E. coli* strains isolated from livestock and poultry was reported by Mudigdo and Peranginangin (1982) as well as Budiharta and Warudju (1985). This paper is the report of drug resistance in *E. coli* strains isolated from livestock and poultry in Indonesia regarding to detection of conjugative R plasmids, these data migh contribute to provide a certain index to the drug resistance surveys that will be conducted in the future.

It is of interest that most of the *E. coli* strains isolated from day-old chicks before feedng had resistance and that these resistant strains persisted at high frequencies for a certain period. In Japan, it had already been demostrated that day-old chicks before feeding had drug-resistant *E. coli* strains and that these resistant strains originated from the hutchers which were contaminated with drug-resistant bacteria, proved by the serological study of these bacteria (Nakamura, 1981). It is thought that the contamination with resistant *E. coli* strains spread to the hutchers up to the present in Indonesia the same as in Japan.

On the other hand, while performing this survey the team asked to the owner of a certain pig farm to gain some information relating to the animal health. The owner explained that the diarrhea of the piglet had been occuring for a long time. Judging from the clinical signs and the owners explanation, the team suspected that the diarrhea was caused by pathogenic bacteria. Several isolates of *Salmonella typhimurium* were isolated not only from these piglets but also from their dam. It was surprising that these isolates of *S. typhimurium* showed quintuple resistance (TC CP SM SA APC-resistance) (Data not shown) althougth conjugative R plasmids could not be detected. Their dam excreted multiple resistant *Salmonella* as a carrier and piglets which were very sensitive to this pathogen suffered from diarrhea. It is difficult to cure the disease caused by such multiple resistant Salmonella strain.

In order to avoid such cases and reduce the amounts of resistant bacteria including *E. coli* strains carrying R plasmids and other pathogenic strains, it is needed to prevent the excess usage of antimicrobial agents.

Antibiotics and synthetic chemotherapeutics shall be used to minimize the beneficial effect in the future.

REFERENCES

Budiharta, S. and B. Warudju. 1985. Mastitis di Daerah Istimewa Yogyakarta II. Isolasi bakteri penyebab dan resistensinya terhadap beberapa antibiotika. *Hemera Zoa.* **72**:58-68.

Edwads, S.J. 1963. Effect of antibiotics on the growth rate and intestinal flora *(Escherichia coli)* of calves. *Journal of Comparative Pathology.* **72:**420-432.

Harry, E.G. 1962. The ability of low concentration of chemotherapeutic substances to induce resisten in *E. coli. Research Veterinary Science* **3:**85-93.

Kanai, H. and T. Igarashi. 1980. Drug resistance and R Plasmids in *Escherichia coli* isolated frm one flock of growing brioler at the age of one day and 28 days old. *Japanese Poultry Science.* **17:**193-198.

Mitsuhashi, S. H. Hashimoto and K. Suzuki. 1967. Drug resistance of enteric bacteria. XIII. Distribution of R factors in *Escherichia coli* strains isolated from livestock. *Journal of Bacteriology.* **94**:116-1169.

Moore, P.R.A., Evenson, T. D. Lucky, E. Mckoy, C.A. Elveiijem and E. B. Harta. 1946. Use of sulfasxidine, straptohricin, and streptomycin in nutritional studies with chick. *Journal Biological Chemistry.* **165:** 437-441.

Mudigdo, R and Peranginangin, A. 1982. Kejadian resistensi *Escherichia coli* terhadap tetracycline pada babi di daerah Medan dan sekitarnya. *Annual Report on animal disease investigation in Indonesia during the periode of 1976–1981*: 53-60.

Nakamura, M. 1984. Recent advance on the antibiotics used for feed supplements and feed additives. *Japanese Journal of Zootecnical Science.* **55**:291-298.

Nakamura, M., K. Omae and T. Koeda. 1978. Drug resistance and R plasmids distribution in fecal *Escherichia coli* strains isolated from calves and pigs in Japan. *Annual Report of National Veterinary Assay Laboratory.* **15**:21-27.

Nakamura, M., K. Omae, H. Yoshimura and T. Koeda. 1979. Drug resistence and R plasmid distribution in fecal *Escherichia coli* strains isolad from calves and pigs in 1977. *Annual Report of National Veterinay Assay Laboratory.* **16**:31-37.

Ronohardjo, P., S. Poernomo and L.H. Prasetyo. 1985. Pengujian in vitro imequyl terhadap berbagai kuman patogen gram negatif isolat lapangan unggas. *Buletin Penyakit Hewan.* **17;**301-303

Smith, H. W. 1967. The effect of the use of antibacterial drugs, particulary an food addvitive on the emergency of drug Resistant strains of bacteria in Animals. *New Zealand Veterinary Journal.* **15**:153-166.

Smith, H.W. 1967. and W.E. Carbb. 1957. The effect of the continous administration of diets containing low levels of tetracyclines on the incidence of drug-resistant *Bacterium coli* in the faeces of pigs and chickens: The sensitivity of the coli to other chemotherapeutic agents. *Vet. Rec.* **69:** 24-30.

Smith, H. W., Z. Parsell and P. Green. 1978. Thermosensitive antibiotic resistance plasmids in enterobacteria. *Journal of General Microbiology.* **109;**37-47.