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INCIDENCE OF DRUG RESISTANCE AND TRANSMISSIBLE R FACTORS IN STRAINS OF E. COLI ISOLATED FROM FAECES OF HEALTHY PIGS

By

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SØGAARD, HENRY: *Incidence of drug resistance and transmissible R factors in strains of E. coli isolated from faeces of healthy pigs.* Acta vet. scand. 1973, *14*, 381—391. — Two hundred and twenty-six strains of E. coli were isolated from faeces of 107 pigs at different ages and without clinical signs of infectious diseases. The resistance of the strains to sulphonamide, tetracycline, streptomycin, chloramphenicol, ampicillin, and nalidixic acid was determined. In 74 % of the animals the predominant E. coli flora was found to be resistant to one or more of the drugs mentioned. Fifty-three % of the strains were resistant. Multiple resistance was predominant among resistant strains (67 %). R factors transmissible to a sensitive strain of E. coli K12 W3132 were demonstrated in 28 %. The proportion of resistant strains was largest in young animals (0—14 weeks) accounting for 65 % of the strains isolated, as compared to 43 % of strains from pigs and sows (6 months or more). The incidence of resistance to sulphonamide, tetracycline, and streptomycin was high, whereas most of the strains were sensitive to ampicillin and chloramphenicol. All strains were sensitive to nalidixic acid.

The incidence of resistance to antibiotics in a population of pigs to whom these drugs are not fed but applied as therapeutic agents solely seems rather high. When based on clinical findings only, the value is therefore questionable of sulphonamide, tetracycline and streptomycin treatment of infectious diseases caused by E. coli.

drug resistance; transmissible drug resistance; R factor; E. coli.

The incidence of drug resistance seems to be increasing within many species of bacteria as a result of the widespread use of antimicrobial drugs in man and domestic animals. The selection pressure exerted on microorganisms has led to increasing difficulties in treating infectious diseases.

In enterobacteriaceae transmissible or infective drug resistance is known to play an important role in the spreading of

resistance. By this mechanism R factors governing resistance to one or most frequently to several antibiotics are transmitted from an organism of one species to an organism of the same or of a different species by conjugation. In vitro transfer of R factors from multiple resistant organisms to a sensitive strain was demonstrated independently by *Ochiai et al.* (1959) and *Akiba et al.* (1960). *Kagiwada et al.* (1960) demonstrated that this transfer took place in vivo also. The main aspects of transmissible drug resistance, epidemiologically and genetically, have been reviewed by *Watanabe* (1963), *Anderson* (1968), and *Mitsuhashi* (1971).

Much work has been performed to elucidate the incidence of drug resistance and transmissible drug resistance among enterobacteriaceae in domestic animals (*Smith* 1966, *Smith & Halls* 1966, *Walton* 1966, *Bulling et al.* 1968, *Loken et al.* 1971, *Mercer et al.* 1971). Evidence has been obtained by these investigators that a large proportion of the intestinal flora in livestock is resistant to a number of antibiotics. The practice of feeding sub-therapeutic concentrations of antimicrobial drugs to animals for growth promotion purposes seems to have contributed a great deal to spreading of drug resistance (*Swann* 1969).

In Denmark it has been forbidden by law to supplement feeding stuffs with antibiotics used as therapeutic agents, and the aim of this investigation was to assess the incidence of resistance and transmissible resistance to "therapeutic" antibiotics in the intestinal E. coli flora of a population of pigs without clinical signs of infectious disease.

MATERIALS AND METHODS

Bacterial strains examined

Seventy-four strains of E. coli were isolated from faeces of 46 pigs (age about 6 months) in an abattoir. From a herd of pigs 152 strains were selected from 61 individuals. Samples were taken from 25 % of the sows, from all individuals in a single litter of 8 piglets, and from the rest of the piglets samples were collected from one individual in each of 34 litters.

Media

Conradi Drigalski agar containing 0.125 % Pril was used for the isolation of strains from faecal specimens. As selection media

in transfer experiments were used Pril-Conradi Drigalski agar supplemented with nalidixic acid (Nal) 25 μg/ml and individual antibiotics at the following concentrations: sulphonamide (Su) 100 μg/ml, tetracycline (T) 10 μg/ml, streptomycin (S) 20 μg/ml, chloramphenicol (C) 20 μg/ml, and ampicillin (A) 10 μg/ml. For sensitivity tests was used 10 % horse blood agar without peptone containing 1 % glucose. Liquid cultures were prepared in nutrient broth.

Isolation and identification of E. coli strains from faeces

Specimens of faeces were collected from rectum on cotton wool swabs which were transferred to tubes containing 0.5 ml of saline. Material from the faecal suspensions were streaked onto Pril-Conradi Drigalski plates. After overnight incubation at 35°C, pure cultures were prepared from all morphologically different types of lactose fermenting organisms growing.

The strains so isolated were identified by the following tests: growth in semi-solid agar, Voges-Proskauer reaction, indole production, fermentation of glucose and malonate, gelatin liquefaction, H_2S production (*Lautrop* 1956), and lysin and ornithin decarboxylase determination as described by *Møller* (1955).

Two hundred and twenty-six strains with reactions typical for E. coli were included in the investigation described.

Tablets for sensitivity tests

Neo-sensitabs®, Rosco, containing: sulphamethizole 4 mg, tetracycline 400 μg, streptomycin 1 mg, chloramphenicol 400 μg, ampicillin 100 μg, and nalidixic acid 1 mg.

Test for sensitivity to antibiotics

One loopful of the cultures was spread evenly on 10 % horse blood agar, and Neo-sensitabs® were placed immediately after inoculation. Incubation overnight at 35°C. Strains giving inhibition zone diameters corresponding to the following or higher minimal inhibitory concentrations (MIC) were regarded as resistant (μg/ml): Su 15, T 4, S 16, A 4, C 16, and Nal 12.

Recipient strain in transfer experiments

As prospective recipient strain in resistance transfer experiments was used E. coli K12 W 3132, obtained from I. Ørskov,

International Escherichia Centre, Copenhagen. This strain is F^-, requires methionine for growth, and is sensitive to sulphonamides, tetracycline, streptomycin, chloramphenicol, ampicillin, and nalidixic acid. A nalidixic acid resistant mutant of the strain was selected by spreading 0.1 ml volumes of a 24 hrs. broth culture onto Pril-Conradi Drigalski plates containing nalidixic acid 25 μg/ml. MIC for the selected mutant strain as determined by two-fold tube dilution test was 256 μg/ml.

Transfer of resistance

Each strain resistant to one or more of the drugs mentioned (potential donors) was grown overnight at 35°C in nutrient broth. A broth culture of E. coli W 3132, resistant to nalidixic acid, was prepared in a similar way (prospective recipient).

The potential donor cultures (0.02 ml) and recipient strain (0.1 ml) were transferred to 10 ml of nutrient broth, and the mating cultures were incubated overnight at 35°C; 0.1 ml were spread onto a series of Pril-Conradi Drigalski plates supplemented with nalidixic acid 25 μg/ml and each separate drug to which the potential donor strains were resistant. The mating cultures were then diluted in saline 10^{-2} and 10^{-4} and 0.1 ml of the dilutions were plated onto the same selection media. Finally the cultures were centrifuged and 0.03 ml of the deposit was spread as well.

The potential donor cultures and the prospective recipient culture (0.1 ml of each) were spread separately onto each selection medium in order to determine the number of spontaneously occurring mutants.

All selection plates were incubated overnight at 35°C and the number of growing colonies was counted after 24 and 48 hrs. Colonies appearing from the mating cultures were considered to be resistant recombinants of E. coli W 3132 when growing in a number at least 10 times higher than from the separate cultures on the control plates. Two colonies from each selection medium were picked and the resistance patterns transferred were determined.

RESULTS

The incidence of drug resistance and transmissible drug resistance in E. coli isolated from faeces of apparently healthy pigs at different ages is illustrated in Table 1.

Table 1. Incidence of drug resistance and transmissible drug resistance in faeces of pigs of different age groups.

Age groups	Number examined	Number with resistant E. coli	Number of strains isolated	Number of strains resistant	Number of resistant strains with transmissible R factors
piglets (0—14 weeks)	42	37(88 %)	103	67(65 %)	23(34 %)
pigs (6 months)	46	30(65 %)	74	34(46 %)	7(21 %)
sows (> 12 months)	19	12(63 %)	49	19(39 %)	3(16 %)
Total	107	79(74 %)	226	120(53 %)	33(28 %)

Test of significance for differences between age groups:

Proportion of individuals with resistant E. coli, piglets versus pigs + sows: $\chi^2 = 7.30$, $f = 1$, $0.01 > P > 0.001$

Proportion of resistant E. coli, piglets versus pigs + sows: $\chi^2 = 10.86$, $f = 1$, $P < 0.001$

Proportion of resistant strains with transmissible R factors, piglets versus pigs + sows: $\chi^2 = 3.60$, $f = 1$, $0.1 > P > 0.05$

From 74 % of the animals examined, one or more strains chosen as described from non-selective plates were resistant to one or more of the following drugs: sulphonamide, tetracycline, streptomycin, chloramphenicol, and ampicillin. All strains tested were sensitive to nalidixic acid. Among the strains isolated 53 % were resistant, and 28 % of the resistant strains contained R factors transmissible to E. coli W 3132.

The entire group of animals was divided into 3 groups according to age, referred to as piglets, pigs, and sows. There was no difference as to the incidence of drug resistant E. coli between pigs and sows, whereas a comparison between these two groups together and the group of piglets showed differences regarding as well the proportion of individuals with resistant strains ($\chi^2 = 7.30$, $f = 1$, $0.01 > P > 0.001$) as the proportion of strains carrying resistance ($\chi^2 = 10.86$, $f = 1$, $P < 0.001$).

From 34 % of resistant strains isolated from piglets R factors could be demonstrated. This was the case among 19 % of resis-

tant strains in pigs and sows as a whole. The difference, however, is not statistically significant ($\chi^2 = 3.60$, $f = 1$, $0.1 > P > 0.05$).

The proportion of individuals within the different age groups carrying E. coli strains resistant to each of the antibiotics separately is shown in Table 2. The incidence of strains with resist-

Table 2. Incidence of resistance to individual antibiotics in E. coli in faeces of pigs of different age groups.

Antibiotics	Piglets with resistant E. coli	Pigs with resistant E. coli	Sows with resistant E. coli	Total
Su	22(52 %)	13(28 %)	9(47 %)	44(41 %)
T	28(67 %)	23(50 %)	6(32 %)	57(53 %)
S	34(81 %)	16(35 %)	11(58 %)	61(57 %)
C	2(5 %)	1(2 %)	1(5 %)	4(4 %)
A	0	5(11 %)	1(5 %)	6(6 %)
Nal	0	0	0	0

ance to streptomycin was remarkably high in the group of piglets as compared with pigs and sows put together ($P < 0.001$). Similar differences were in evidence regarding resistance to sulphonamide and tetracycline, but not so well-marked (Su: $0.05 > P > 0.02$, T: $0.02 > P > 0.01$).

Fourteen different resistance patterns were found (Table 3).

Table 3. Drug resistance patterns of resistant E. coli strains from pigs, and R factors demonstrated in the resistant strains.

Resistance patterns	Number of strains	R factors demonstrated (numbers transferred in brackets)	Frequency of R factor transfer
Su T S	31	Su T S (17) T S (1)	$4.0 \times 10^{-2} - 1.3 \times 10^{-7}$
Su S	25	0	
T	21	T (2)	$5.8 \times 10^{-5} - 7.2 \times 10^{-8}$
T S	18	T S (5)	$2.4 \times 10^{-2} - 1.3 \times 10^{-7}$
S	13	S (4)	$6.0 \times 10^{-7} - 6.9 \times 10^{-8}$
Su T S C	3	Su T S C (3)	$1.0 \times 10^{-2} - 8.1 \times 10^{-3}$
A	2	0	
Su	1	0	
Su A	1	0	
Su T A	1	0	
Su T C A	1	0	
Su T S A	1	0	
T S A	1	T S (1)	2.4×10^{-2}
C	1	0	

Thirty-eight strains (33 %) were singly resistant. Of these strains transmissible R factors could be demonstrated in 6 (15 %), whereas this was the case in 27 of 82 multiple resistant strains. The resistance pattern found most frequently was Su T S. Twenty-five strains carried resistance to sulphonamide and streptomycin together. In no instance R factors could be demonstrated in these strains. The proportion of strains with resistance to tetracycline only which could transfer their resistance was remarkably small (2/21).

The frequency of transfer per introduced donor cell of the various R factors is indicated in Table 3. R factors carrying two or more resistance determinants were transferred at a frequency somewhat higher than what was found in those strains containing R factors with one resistance determinant only.

DISCUSSION

Several surveys have elucidated the correlation between the level of antibiotic pressure and the incidence of resistant E. coli strains in the intestinal flora of domestic animals. These surveys have dealt mainly with problems arising from continuous feeding to animals of antimicrobial drugs with the purpose of obtaining a growth promoting effect (*Walton* 1966, *Smith* 1966, *Smith & Halls* 1966, *Loken et al.* 1971, and *Mercer et al.* 1971).

A British Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine in a report (*Swann* 1969) concluded that the administration of antibiotics especially at sub-therapeutic levels to livestock presents certain hazards to human and animal health by considerably increasing the proportion of enteric bacteria resistant to one or more drugs. Following the recommendations of this report, the Danish Ministry of Agriculture decided to restrict from the 1st of March 1971 the range of antimicrobial drugs allowed as feed additives to such substances which were not applied as therapeutic agents in man and livestock.

The incidence of drug resistance among E. coli in healthy pigs as described in this paper can therefore be ascribed to the impact on bacterial drug resistance of the use of antibiotics in therapy and prevention of infectious diseases. *Mercer et al.* demonstrated that 80 % of E. coli strains isolated from pigs and calves fed antimicrobial drugs carried resistance to one or more drugs, whereas the proportion in herds not receiving antibiotics

was only 16 %. The present survey has revealed that under Danish conditions 53 % of E. coli isolated from healthy pigs not fed antibiotics with therapeutical applications were resistant to such drugs.

This proportion seems quite noteworthy and indicates that in a population of animals not continuously exposed to low level concentrations of antibiotics, the therapeutic use of these drugs will exert a selection pressure high enough to change the predominant flora of E. coli to being resistant in 74 % of the population.

The findings presented in this paper clearly indicate that the benefits of application per os of sulphonamides, tetracyclines, and streptomycin in pigs are rather doubtful, if based on clinical findings only.

Sixty-seven % of the resistant strains were resistant to more than one drug, in 5 cases 4 resistance determinants were involved in the resistance patterns. The prevalence of multiple resistance in the main proportion of resistant E. coli makes an efficient treatment of colibacillosis, one of the most important diseases causing morbidity and mortality in newborn and weanling pigs, an utmost difficult task.

The incidence of resistance in E. coli is higher in piglets (0—14 weeks) than in pigs of 6 months or more. *Ringarp* (1965) refers to a group of pigs in which necropsy was done. Eighty-four of the animals had died at the time of weaning or shortly after. *Wittig* (1961) reported that of 1105 pigs of various age groups 42 % died from colienterotoxinaemia, as compared to 68 % of pigs which died at the time of weaning. The prevalence of resistance among E. coli in the group of young pigs as described above may well be assigned to the higher morbidity caused by E. coli generally found within this group, leading to a larger consumption of antimicrobial drugs.

Information about the consumption of antibiotics in the group of piglets and in the group of sows was available, but could not be obtained for the group of pigs. During a period of 15 months previous to the start of this investigation in the herd of sows and piglets, the following antibiotics were prescribed by the veterinary surgeon: tylosine, sulphonamide, nitrofurantoin, streptomycin, and chloramphenicol. Tetracycline was not applied at all. The average consumption of the prescribed drugs per year was calculated and is illustrated in Table 4.

Table 4. Average consumption per year of antibiotics in a herd of sows and piglets.

Antibiotic	mg per individual
tylosine	1200
sulphonamide	750
nitrofurantoin	105
streptomycin	95
chloramphenicol	85

In 28 % of the resistant strains R factors transmissible to E. coli K12 W 3132 could be demonstrated. This does not necessarily mean that R factors were not present in 72 %. It is well known that R factors may be segregated and accordingly loose their transmissibility (*Anderson* 1968). *Hashimoto & Mitsuhashi* (1971) have found that R factors which have lost tetracycline resistance are often non-transmissible. This might be the explanation why 25 strains carrying resistance determinants to sulphonamide and streptomycin did not transfer resistance. The use of more than one recipient strain might have increased the possibility to demonstrate R factors. E. coli W 3132, however, was shown to be a competent recipient of some R factors, since the frequency of transfer in some experiments was higher than 10^{-2}.

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SAMMENDRAG

Forekomst af antibiotikaresistens og overførbare R faktorer i E. coli stammer isoleret fra faeces af raske svin.

Tohundredeseksogtyve E. coli stammer er isoleret fra faeces af 107 svin i forskellige aldersgrupper og uden kliniske symptomer på infektionssygdomme. Stammernes følsomhed over for sulfonamid, tetracyklin, streptomycin, chloramphenicol, ampicillin og nalidixan er undersøgt. Hos 74 % af dyrene var den overvejende del af E. coli

floraen resistent over for et eller flere af de nævnte antibiotika. Treoghalvtreds % af stammerne var resistente. De fleste stammer udviste resistens over for flere antibiotika (67 %). Otteogtyve % af de resistente stammer indeholdt R faktorer, der kunne overføres til en følsom stamme, E. coli K12 W 3132. Hos grise i aldersgruppen 0—14 uger var 65 % af de isolerede stammer resistente, i modsætning til gruppen af baconsvin og søer, hvor 43 % af stammerne udviste resistens. Hyppigheden af resistens over for sulfonamid, tetracyclin og streptomycin var stor, hvorimod de fleste stammer var følsomme for ampicillin og chloramphenicol. Samtlige stammer var følsomme for nalidixan.

Hyppigheden, hvormed der forekommer tarmbakterier, der er resistente over for en række antibiotika, der ikke finder anvendelse som fodertilskud, synes under danske forhold at være ganske betydelig. På denne baggrund forekommer anvendelse af sulfonamid, tetracyklin og streptomycin til behandling af E. coli infektioner hos svin at være problematisk, såfremt behandlingen indledes uden forudgående bakteriologisk undersøgelse.

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