ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1976, p. 6-10

Vol. 9, No. 1
Printed in U.S.A.

Antibiotic Resistance and Hly Plasmids in Serotypes of *Escherichia coli* Associated with Porcine Enteric Disease

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Received for publication 3 September 1975

A total of 359 hemolytic *Escherichia coli* strains, representing eight pig pathogenic serotypes and isolated from pigs with enteric disease, was tested for transferable resistance to eight antibiotics. The co-transfer of plasmids controlling hemolysin production (Hly) with antibiotic resistance plasmids (R-factors) was evaluated. Transferable resistance to tetracycline, streptomycin, and/or sulfonamides was found in 47% of the total number of strains and 80% of the resistant ones. Chloramphenicol resistance was seldom seen. Co-transfer of Hly with R-factors occurred in 22.5% of the strains, generally to a degree excluding a genetic linkage. Stable coexistence of two R-factors in a cell was indicated by transfer patterns in 29 of the strains.

Resistance to various antibiotics in *Escherichia coli* is very often controlled by plasmids—R-factors (22). They are generally conjugative, but nonconjugative R-factors exist (2). Production of α-hemolysin may also be plasmid borne (15), but the genetic information can be found on the chromosome as well. Plasmids controlling α-hemolysin production are named Hly factors. Like R-factors they can be either conjugative or nonconjugative (17).

R-factors present major problems in the treatment of acute diseases with an *E. coli* etiology, such as neonatal and postweaning diarrhea in pigs. The hemolytic phenotype is also common among strains isolated from such cases, especially from postweaning diarrhea (4, 18). Therefore, production of hemolysin was earlier believed to indicate pathogenicity, but according to more recent investigations (17) it does not seem to play any decisive role in the etiology of *E. coli* diarrhea.

As *E. coli* strains from diseased pigs often show both the antibiotic resistant and the hemolytic phenotype, an investigation with a dual purpose was started, first, to evaluate the incidence of conjugative R-factors in pathogenic serotypes and, second, to examine whether the degree of coinheritance of Hly with various R-factors would suggest a genetic linkage.

MATERIALS AND METHODS

Strains. The material consisted of 359 hemolytic *E. coli* strains isolated from pigs less than 12 weeks old, the carcasses of which were autopsied at the State Veterinary Serumlaboratory. Hemolytic colonies cultured from the jejunal contents of the pigs were serotyped after biochemical identification as *E. coli*. The strains represented the following eight O:K types: O149:K91, O147:K89, O141:K85ab, O141:K85ac, O139:K82, O138:K81, O45:K?, and O8:K87. They were not tested for the presence of the plasmid-determined K88 antigen. The strains were not in contact with antibiotics during isolation; determination of resistance patterns took place in this department less than 4 weeks after isolation and serotyping. Strains carrying resistance markers were tested for donor ability in conjugation experiments with a K-12F$^-$ *lac*$^-$ *nal*^{r} *Hly*$^-$ recipient strain. A rifampin-resistant mutant (*rif*^{r}) of *E. coli* K-12 HfrH was used as a recipient when markers subsequently were transferred from K-12 F$^-$. The antibiotic resistance genes of both K-12 strains were chromosomally located. All wild-type donor strains fermented lactose. The nomenclature used is that recommended by Demerec et al. (5).

Media. Penassay broth (Difco) was used for conjugation experiments, and McConkey agar (Oxoid) containing the appropriate antibiotics was applied in selection plates. Resistance patterns before and after conjugation were determined on DST agar (Oxoid) by means of Multodisk (Oxoid, code 1744 E) containing the following antibiotics: ampicillin, streptomycin, compound sulfonamides, tetracycline, chloramphenicol, furazolidone, neomycin, and nalidixic acid. Production of hemolysin was investigated on Penassay agar (PA) medium with 5% calf blood. The PA medium was composed of Penassay broth with the addition of 1.5% agar (Difco). Strains were kept in stock culture agar (Difco).

Antibiotics. All antibiotics applied in selection plates were dissolved in sterile distilled water, except chloramphenicol where a sterile borate buffer was used as solvent (sodium borate, 3.4 g; boric acid, 14.1 g; water to 1,000 ml). The concentrations of the antibiotics in the selection plates were as follows: sodium nalidixate, 20 μg/ml; dihydrostreptomycin sulfate, 12.5 μg/ml; tetracycline, 12.5 or 10 μg/ml; rifampin, 250 μg/ml; and chloramphenicol, 12.5 μg/ml. All antibiotics were of reagent grade purity. Because of difficulties with contra selection of the recipient when the donor was found to be sulfonamide resistant, sulfonamides were not included in the selection plates. Transfer of sulfonamide resistance (Su) was only recorded when Su was coinherited with streptomycin (S) or tetracycline resistance (T). This means that Su was recorded merely as an unselected marker.

Conjugation. Broth cultures of donor and recipient strains were grown overnight at 38 C. They were subsequently transferred to 10-ml amounts of fresh broth in a donor-recipient ratio of 1:5 and grown in a shaking water bath for 3 to 4 h. A sample from each tube was spread on McConkey plates containing sodium nalidixate or rifampin and one of the antibiotics to which the donor was found resistant. Both the donor and the recipient strain were tested for ability to grow on the selection plate used for the mating mixture to exclude mistakes. Whenever the donor was resistant to more than one of the above mentioned antibiotics, each marker was selected separately to see if transfer occurred independently of the selecting antibiotic.

Transfer of resistance patterns to *E. coli* K-12 F^- was verified by examining three single colonies from each selection plate. When discrepancies were found between the resistance patterns of the three transconjugants, 100 to 200 single colonies from the respective selection plates were replicated (10) on PA plates containing the appropriate antibiotics. This procedure was not used for Su markers since the possible presence of sulfonamide inhibitors in the media might blur the results. When resistance markers were not transferred in the first conjugation experiment, the cross was repeated on a cellophane membrane (3). This method allowed detection of transfer at frequencies lower than those detectable by the broth conjugation method (11, 12).

Test for coinheritance of Hly with R factors. From each selection plate 50 to 100 single colonies were point inoculated on PA containing 5% calf blood and incubated overnight at 38 C before reading.

RESULTS

In the present material each of the serotypes O149:K91, O138:K81, and O8:K87 was represented by approximately 80 strains, whereas O147:K89, O139:K82, and O45:K? each comprised less than 20 strains. From Table 1, it appears that resistance (except in types O139 and O45) was found in 53 to 74% of the strains and in the majority of the cases was governed by R-factors. It is possible that even greater percentages of R-factor-carrying strains (columns 4 and 5, Table 1) would have been found if attempts of plasmid mobilization had been made. The only criterion for plasmid-borne characters applied in the present work, however, was transmission to *E. coli* K-12 F^-.

The distribution of the observed transmissible resistance patterns among serotypes is listed in Table 2. Most of the patterns involved combinations of tetracycline, streptomycin, and sulfonamide resistance, although monoresistance to tetracycline also occurred in several strains. Chloramphenicol resistance was rarely observed, did not occur separately, and was always transmissible. None of the strains proved resistant to ampicillin, neomycin, or furazolidone.

The co-transfer of Hly with various R-factors appears in Table 2, columns 3 and 4. Only 38 out of the 169 R-factor-harboring strains (22.5%) showed transmission of Hly as an unselected marker (this is biased upwards by 11 O149 strains carrying the SSuC markers, because they came from the same herd). The frequency of co-transfer within strains was often low; in most matings less than 20% of the examined

TABLE 1. *The incidence of transferable and nontransferable antibiotic resistance in enteropathogenic E. coli serotypes*

O:K type	No. of strains	Resistant strains no.	Resistant strains/total strains (%)	R^+ strains no.	R^+ strains/resistant strains (%)	R^+ strains/total strains (%)
O149:K91	80	59	74	52	88	65
O147:K89	7	5	71	4	80	57
O141:K85ab	54	33	61	19	58	35
O141:K85ac	32	17	53	9	53	28
O139:K82	8	0		0		
O138:K81	79	42	53	37	88	47
O45:K?	19	3	16	2	67	11
O8:K87	80	52	65	46	88	58

TABLE 2. *R-factors and Hly plasmids demonstrated in E. coli of the following antigenic types*

O:K type	Resistance pattern[a]	No. of strains	Co-inh of Hly with R (no. of strains)	Co-inh of Hly with R Hly colonies/100 R+ colonies
O149:K91	SSuT	11	NF	NF[c]
	SSuC	11	11[b]	10–15
	SSu	16	NF	NF
	ST	6	NF	NF
	T	8	1	1
O147:K89	SSuT	2	NF	NF
	ST	2	NF	NF
O141:K85ab	SSuT	9	4	2–8
	SSu	4	NF	NF
	ST	3	1	97[d]
	S	1	NF	NF
	T	2	2	11–26
O141:K85ac	SSuT	1	NF	NF
	SSuC	2	NF	NF
	SSu	3	NF	NF
	ST	1	NF	NF
	T	2	NF	NF
O138:K81	SSuT	2	1	36
	SSu	6	4	60 (1) 100 (3)[d]
	ST	2	NF	NF
	T	27	9	1–8
O45:K?	SSuT	1	NF	NF
	T	1	NF	NF
O8:K:87	SSuTC	2	NF	NF
	SSuT	15	6	33–59
	SSu	17	NF	NF
	ST	7	2	83–88
	T	3	1	50
	S	2	NF	NF

[a] Streptomycin resistance; C, chloramphenicol resistance; Su, sulfonamide resistance; and T, tetracycline resistance.

[b] From the same herd.

[c] NF, No co-transfer found.

[d] Only 20 to 35 colonies were available for testing after each conjugation because of low transfer frequencies.

transconjugants carried Hly. In a few cases, however, co-transfer of Hly took place at frequencies from 83 to 100%. The high co-transfer frequency was often found in conjugation experiments where the cellophane membrane method was applied, because the transfer frequency of the prospective R-factor was too low to be detected by the broth conjugation method.

The plasmids from two of the three strains which co-transferred Hly 100% with the R-factor were transmitted from K-12 F$^-$ *nal*^{r} to HfrH *rif*^{r} to see whether Hly and the SSu resistance markers would occur separately in the new recipient. All out of 30 single colonies in one strain and 21 out of 22 in the other strain were hemolytic after selection for streptomycin. These results suggest that Hly and SSu are located to the same plasmid, but conclusive evidence cannot be obtained until transduction experiments have been performed.

Unselected resistance markers were generally co-transferred 100% with each other, suggesting that the markers were on the same plasmid, but in 29 strains transfer patterns indicated the presence of more than one R-factor.

DISCUSSION

In spite of the ban on therapeutic antibiotics in feedstuffs, effective in Denmark by 1 March 1971, the incidence of antibiotic resistance in pig pathogenic *E. coli* types is rather high. It was found to vary from 74% in the O149 strains to 16 and 0% in the O139 and O45 strains. These results are of the same magnitude as those reported from Ireland (19), but the latter in-

vestigation did not include any statement on transferable resistance. R-factors were carried by 47% of the total number of strains in the present material or 80% of the resistant ones. The incidence of R-factors in resistant strains is thus twice as high in our material as the one found by Larsen and Larsen (9) and Jørgensen (Ph.D. thesis, Royal Veterinary and Agricultural Univ., Copenhagen, 1974) in healthy Danish pigs of the same age (40%). Presumably our results reflect the situation in "problem farms" only, i.e., farms with repeated outbreaks of disease and frequent treatment with antibiotics.

The variation in R-factor incidence between serotypes seen in Table 1 appears to be more or less random, reflecting their varying occurrence among diseased pigs (4) and the following unequal exposure to antibiotics.

Exact information about administration of antibiotics could seldom be obtained, but when available it was in agreement with at least one of the resistance markers in the strains. Apart from a few cases of chloramphenicol resistance, only T, S, and Su determinants were found. This is in accordance with findings in healthy Danish pigs (9, 21; Jørgensen, Ph.D. thesis). The same markers are found on R-factors in strains from healthy and diseased pigs throughout the world (1, 6, 15), but frequently accompanied by ampicillin and chloramphenicol resistance (7, 8).

Although R-factors were demonstrated in most of the resistant strains in this investigation, co-transfer of antibiotic resistance and α-hemolysin production was not frequent. The hemolytic character was seldom transferred from our strains, either because the hemolysin production in most strains was not coded for by a plasmid or because Hly transfer can be detected only when occurring at a high rate, due to lack of a sensitive selection technic (16). A third possibility is that hemolysin production was controlled by nontransmissible plasmids that could not be mobilized by the resistance transfer factor.

Co-transfer of R- and Hly-plasmids seems to be a rare phenomenon. Smith and Linggood (16) transferred R-factors from 30 enteropathogenic, α-hemolytic pig strains without finding one single hemolytic transconjugant. Similar results were obtained by Smith and Gyles (14), who failed to show linkage between Hly and two R-factors from naturally occurring strains. In a Polish investigation (20), co-transfer was reported of T and Hly and a determinant for colicin production (col) in 6 of 12 strains. Hly and col and T and col were also jointly transferred, but Hly and T were not. It was concluded that the resistance marker and Hly were transferred as two independent events.

ACKNOWLEDGMENTS

Our sincere thanks are due to A. Dam, The State Veterinary Serumlaboratory, Copenhagen, who kindly supplied the bacterial strains.

This work was supported by grant no. 513-2616 from the National Research Council (Statens jordbrugs-og veterinaer-videnskabelige Forskningsråd).

LITERATURE CITED

1. Aden, D. P., N. D. Reed, N. R. Underdahl, and C. A. Mebus. 1969. Transferable drug resistance among *Enterobacteriaceae* isolated from cases of neonatal diarrhea in calves and piglets. Appl. Microbiol. 18: 961-964.
2. Anderson, E. S., and M. J. Lewis. 1965. Characterization of a transfer factor associated with drug resistance in *Salmonella typhimurium*. Nature (London) 208:843-849.
3. Chabbert, Y. A., and J. C. Patte. 1959. Cellophane transfer. Application to the study of activity of combinations of antibiotics. Appl. Microbiol. 8:193-199.
4. Dam, A., and B. Knox. 1974. Haemolytic *Escherichia coli* associated with enteritis and enterotoxaemia in pigs in Denmark. Nord. Veterinaermed. 26:219-225.
5. Demerec, M., E. A. Adelberg, A. J. Clark, and P. E. Hartman. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics 54:61-76.
6. Grant, A. J., and J. Pittard. 1974. Incompatibility reactions of R plasmids isolated from *Escherichia coli* of animal origin. J. Bacteriol. 120:185-190.
7. Guinée, P. A. M. 1971. Bacterial drug resistance in animals. Ann. N.Y. Acad. Sci. 182:40-51.
8. Kashiwazaki, M., S. Namioka, and N. Terakado. 1972. Distribution of transferable drug-resistance factors in fecal *Escherichia coli* from healthy pigs. Natl. Inst. Anim. Health Q. 12:17-22.
9. Larsen, H. E., and J. L. Larsen. 1972. Occurrence and distribution of antibiotic resistant *Escherichia coli* in feces from cattle and swine. Nord. Veterinaermed. 24:651-659.
10. Lederberg, J., and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63:399-406.
11. Le Minor, L., C. Coynault, and G. Pessoa. 1974. Plasmid determinants of the atypical lactose positive character in *S. typhimurium* and *S. oranienburg* isolates from epidemic in Brasil 1971-1973. Ann. Inst. Pasteur Paris 125:261-285.
12. Scavizzi, M. R. 1973. New incompatibility groups of plasmids. Interest in O111:B4 *Escherichia coli* outbreaks observed in pediatrics department. Ann. Inst. Pasteur Paris 124:153-167.
13. Smith, H. W. 1968. Antimicrobial drugs in animal feeds. Nature (London) 218:728-731.
14. Smith, H. W., and C. L. Gyles. 1970. The relationship between different transmissible plasmids introduced by F into the same strain of *Escherichia coli K12*. J. Gen. Microbiol. 62:277-285.
15. Smith, H. W., and S. Halls. 1967. The transmissible nature of the genetic factor in *Escherichia coli* that controls haemolysin production. J. Gen. Microbiol. 47:153-161.
16. Smith, H. W., and M. A. Linggood. 1970. Transfer factors in *Escherichia coli* with particular regard to their incidence in enteropathogenic strains. J. Gen. Microbiol. 62:287-299.

17. Smith, H. W., and M. A. Linggood. 1971. Observations of the pathogenic properties of the K88, Hly and Ent plasmids of *Escherichia coli* with particular reference to porcine diarrhoea. J. Med. Microbiol. 4:467–485.
18. Svendsen, J. 1974. Enteric *Escherichia coli* diseases in weaned pigs. Nord. Veterinaermed. 26:226–233.
19. Sweeney, E. J. 1973. Typing of swine-enterotoxaemia *Escherichia coli* according to behavior with antibiotics: research application. Irish Vet. J. 27:57–60.
20. Swiderski, M., and T. M. Lachowicz. 1974. Occurrence and transfer of antibiotic resistance, colicinogeny and haemolysin production in *Escherichia coli* strains isolated from cases of colibacillosis. Acta Microbiol. Pol. Ser. A 6:155–162.
21. Sögaard, H. 1973. Incidence of drug resistance and transmissible R-factors in strains of *E. coli* isolated from faeces of healthy pigs. Acta Vet. Scand. 14: 381–391.
22. Watanabe, T. 1963. Infective heredity of multiple drug resistance in bacteria. Bacteriol. Rev. 27:87–115.