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Spread of a Newly Found Trimethoprim Resistance Gene, *dhfrIX*, among Porcine Isolates and Human Pathogens

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A plasmid-borne gene mediating trimethoprim resistance, *dhfrIX*, newly found among porcine strains of *Escherichia coli*, was observed at a frequency of 11% among trimethoprim-resistant veterinary isolates. This rather high frequency of *dhfrIX* could be due to the extensive use of trimethoprim in veterinary practice in Sweden. After searching several hundred clinical isolates, one human *E. coli* strain was also found to harbor the *dhfrIX* gene. Thus, the *dhfrIX* gene seems to have spread from porcine bacteria to human pathogens. Furthermore, the occurrence of other genes coding for resistant dihydrofolate reductase enzymes (*dhfrI*, *dhfrII*, *dhfrV*, *dhfrVII*, and *dhfrVIII*) among the porcine isolates was investigated. In addition, association of *dhfr* genes with the integraselike open reading frames of transposons Tn*7* and Tn*21* was studied. In colony hybridization experiments, both *dhfrI* and *dhfrII* were found associated with these integrase genes. The most common combination was *dhfrI* and *int*-Tn*7*, indicating a high prevalence of Tn*7*.

Resistance to trimethoprim, usually plasmid borne, is rather common in clinical human isolates. The resistance is mediated by plasmid genes expressing drug-resistant variations of the target enzyme, dihydrofolate reductase (DHFR) (20). Several different types of genes coding for resistant DHFRs are known. The resistant enzyme variations mediate different levels of resistance (9). The most commonly found gene coding for resistant DHFR is *dhfrI* borne on Tn*7* (1, 4, 7, 19), which was first observed in the R plasmid R483 (1, 20). It has recently been established that *dhfrI* can also be located in an element named integron (23), which can be found in Tn*21*-like transposons (28). The integron is recombinationally active and carries several other antibiotic resistance genes, among them *dhfrII*, *dhfrV*, and *dhfrVII* (25, 26).

The newly characterized *dhfrIX* gene (11) has so far been observed only among porcine isolates of *Escherichia coli* in Sweden, where overall trimethoprim resistance frequency was about 16% in 1991. The rather high frequency of trimethoprim resistance and the appearance of a new resistance gene among porcine *E. coli* could be regarded as a consequence of the extensive use of trimethoprim (in combination with sulfonamides) in veterinary practice, including in the treatment of pig diarrhea. The *dhfrIX* gene was originally found in isolates from porcine *E. coli* collected in 1982 from several farms spread over the southern part of Sweden and sometimes from animals not treated with trimethoprim. The *dhfrIX* gene was found to be borne on conjugative plasmids mediating resistance to a drug level of about 250 mg/liter. In this study, we wanted to investigate the epidemiology of *dhfrIX* and other trimethoprim resistance genes among porcine isolates and the possible spread of *dhfrIX* among human pathogens. To investigate the prevalence of *dhfrIX* among veterinary isolates, 279 trimethoprim-resistant porcine isolates of *E. coli* were studied by colony hybridization. Thirty-one of these showed hybridization to the probe for *dhfrIX*. The ability of the *dhfrIX* gene to spread could also reflect a risk of its moving into human pathogens. This was investigated in a collection of more than 400 human trimethoprim-resistant enterobacterial strains, among which one *dhfrIX*-positive isolate was actually found.

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MATERIALS AND METHODS

Bacterial strains. A collection of 279 trimethoprim-resistant *E. coli* strains of porcine origin from many farms in different parts of Sweden and isolated at the National Veterinary Institute in the years 1984 through 1989 was studied. All strains varied in serotypes and showed various degrees of trimethoprim resistance, which, however, always corresponded to a MIC of >8 mg/liter. Forty-eight trimethoprim-sensitive strains of porcine *E. coli* (collected in 1987 through 1989) were used as controls. In a second study, 434 human trimethoprim-resistant enterobacterial strains were studied. A part of these were strains collected in 1989 to 1991 from patients with urinary tract infections (UTI), 97 were from Academic Hospital in Uppsala (Carl Påhlson), 54 were from Danderyd Hospital in Stockholm (Bengt Wretlind), 27 were from Huddinge Hospital in Stockholm, and 44 were from Karolinska Hospital in Stockholm (Signe Ringertz), which also provided 26 trimethoprim-resistant strains collected in Addis Ababa, Ethiopia, in 1986 (17). Forty-six strains were from Finland (Turku and Helsinki) (Elina Heikkilä), and 14 were fecal *E. coli* strains from day care centers in Houston, Tex. (Barbara Murray). Also, 46 *Shigella* strains were obtained from Bangkok, Thailand (Panida Jayanetra), and 80 other strains of the family *Enterobacteriaceae* were obtained from Lagos, Nigeria (Adebayo Lamikanra).

Materials and media. Bacteria were grown in Luria-Bertani medium (14) or in Iso-Sensitest medium (Oxoid, Basingstoke, United Kingdom). Trimethoprim lactate was a gift from Wellcome Research Laboratories, Beckenham, United Kingdom. Radioactively labeled deoxynucleotides [α-^{32}P] dCTP and [γ-^{32}P]dATP were from DuPont Co., Boston, Mass. Restriction endonucleases and T4 polynucleotide kinase were bought from Boehringer GmbH, Mannheim, Germany, and agarose (DNA grade) was from BioRad Laboratories, Richmond, Calif. Oligonucleotide probes were

TABLE 1. Probes for colony hybridization

Probe target	Fragment (plasmid)	Reference
dhfrIX	0.34-kb *Eco*RV-*Hin*dIII (pCJO01-1) and 5'-AAAACAGTACCACCCACCCAGAACACT (oligonucleotide)[a]	11
dhfrI	0.5-kb *Bam*HI-*Kpn*I (pLKO627)	28
dhfrII	0.28-kb *Eco*RI-*Sal*I (pLKO601)	6
dhfrV	0.5-kb *Bam*HI-*Kpn*I (pLKO22)	25
dhfrVII	5'-AGTGTCGAGGAAAGGAATTTCAAGCTC (oligonucleotide)[b]	26
int-Tn*7*	1.7-kb *Hpa*I-*Ava*I (pRSS021)	22
int-Tn*21*	1.3-kb *Bam*HI-*Kpn*I (pLKO26)	28

[a] Synthetic oligonucleotide includes bases 196 to 222 of the structural gene of *dhfrIX*.

[b] Synthetic oligonucleotide includes bases 203 to 229 of the structural gene of *dhfrVII* (cf. GenBank/EMBL accession no. X58425).

synthesized by Scandinavian Gene Synthesis AB, Köping, Sweden.

MIC determinations. MICs of trimethoprim were determined by the agar dilution method (3).

DNA probes and labeling procedures. The gene-specific probes are described in Table 1. The *dhfrIX* gene was represented by the 0.34-kb *Eco*RV-*Hin*dIII fragment, which includes 72 bases upstream of the start codon of *dhfrIX* (11). As specific probes for the integraselike open reading frames of transposons Tn*7* and Tn*21* (15, 25), the probes *int*-Tn*7* and *int*-Tn*21* were used (Table 1). The fragment probes were labeled with [α-^{32}P]dCTP by using an Oligolabeling Kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Labeled DNA was purified on Sephadex G-50 columns (Pharmacia LKB). Oligonucleotide probes were 5' labeled with [γ-^{32}P]dATP and T4 polynucleotide kinase (18). Labeled DNA was purified on Sephadex G-25 DNA columns.

Colony hybridization. Preparation of filters for colony hybridization was as described earlier (16). Prehybridization and hybridization were done at 42°C in a slowly rotating cylinder. For colony hybridization, strains C600 and JM83 without and with the vector plasmids pBR322 and pUC19 were used as negative controls. Positive controls for the different DNA fragment probes are given in Table 1. For oligonucleotide probes, the positive controls also included pCJO01-1 (Table 1), pLKO221 (26), and pLMO226 (24).

For DNA fragment probe hybridization, filters were washed at 68°C for 30 min, twice in 1 liter of 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0)–0.2% sodium dodecyl sulfate (SDS) and twice in 1 liter of 1× SSC–0.2% SDS.

For oligonucleotide probe hybridization, filters were washed twice in 1 liter of 2× SSC–0.1% SDS at room temperature for 15 min and twice in 1 liter of 0.5× SSC–0.1% SDS at 42°C for 15 min.

RESULTS

Occurrence of *dhfrIX* and other *dhfr* genes among porcine isolates. DNA probes for different types of *dhfr* genes, including *dhfrIX* (cf. Materials and Methods and Table 1), were investigated among the 279 trimethoprim-resistant strains from pigs. As a control, 48 trimethoprim-sensitive porcine *E. coli* strains were examined with the probe for *dhfrIX*. No positive hybridizations were observed among these sensitive strains.

The type I *dhfr* gene was, as expected, most common (Table 2). Of the 279 strains, 166 harbored the *dhfrI* gene, and in 102 of these (61%), *dhfrI* was most likely borne on Tn*7* as judged from the concomitant *int*-Tn*7* hybridization. The combination of *dhfrI* and *int*-Tn*21* hybridizations was observed in 12 strains (4.3%). Twenty-five strains carrying *dhfrI* hybridized to *int*-Tn*7* and *int*-Tn*21* in combination. This is most likely explained by a Tn*7* location of *dhfrI* and a parallel occurrence of an integron structure carrying *int*-Tn*21*. Six strains hybridized to the probes for *dhfrII* and *int*-Tn*21*. This group could represent strains in which *dhfrII* is inserted as a genetic cassette in a Tn*21*-like structure (25). None of the porcine strains harbored the genes coding for DHFRV, DHFRVII, or DHFRVIII. In 18 strains, hybridizations were observed with the probe for *int*-Tn*7* but not with the gene-specific probe for *dhfrI*. One group of 24 strains hybridized only to the *int*-Tn*21* probe, and 5 isolates showed hybridization to the probes for both of the integraselike open reading frames of transposons Tn*7* and Tn*21* but not to any of the *dhfr* gene probes.

TABLE 2. Number of veterinary strains hybridizing to probes used

No. of strains	Hybridization to gene-specific probe for[a]:				
	dhfrIX	*dhfrI*	*dhfrII*	*int*-Tn*7*	*int*-Tn*21*
28	+	−	−	−	−
1	+	−	−	+	−
2	+	−	−	−	+
27	−	+	−	−	−
102	−	+	−	+	−
12	−	+	−	−	+
25	−	+	−	+	+
6	−	−	+	−	+
18	−	−	−	+	−
24	−	−	−	−	+
5	−	−	−	+	+
29	−	−	−	−	−
279 (total)	31	166	6	151	74

[a] For descriptions of probes used, see Table 1. +, hybridization to probe; −, no hybridization. None of the studied strains hybridized to gene-specific probes for *dhfrV*, *dhfrVII*, or *dhfrVIII* (24).

Thirty-one of the isolates (11%) showed hybridization to the *dhfrIX* probe. Among these, one also hybridized to the *int*-Tn*7* probe, and two hybridized to both the *dhfrIX* probe and the *int*-Tn*21* probe. The *dhfrIX* gene, which does not resemble a cassette, and the integraselike open reading frames of Tn*7* and Tn*21* are probably on different locations in these cases. In our previous study, one of the original wild-type isolates, which harbored *dhfrIX*, was hybridizing to the probes for both *dhfrIX* and *int*-Tn*21*, but after transfer of *dhfrIX* into a recipient strain, the transconjugant did not show any hybridization to the *int*-Tn*21* probe (11). The 31 isolates which hybridized to the probe for *dhfrIX* were collected from 1984 through 1989 in geographically separate areas.

Finally, 29 strains did not hybridize to any of the probes used in this study. These strains thus seem to carry still other trimethoprim resistance genes.

High frequency of trimethoprim resistance is a consequence of extensive use of trimethoprim. Trimethoprim in combination with a sulfonamide was introduced into veterinary practice in Sweden in 1974, and in that year, no trimethoprim-resistant strains could be found among porcine *E. coli*. As early as 1982, however, 10% of *E. coli* isolates from pigs with diarrhea were resistant to trimethoprim (5). The rather

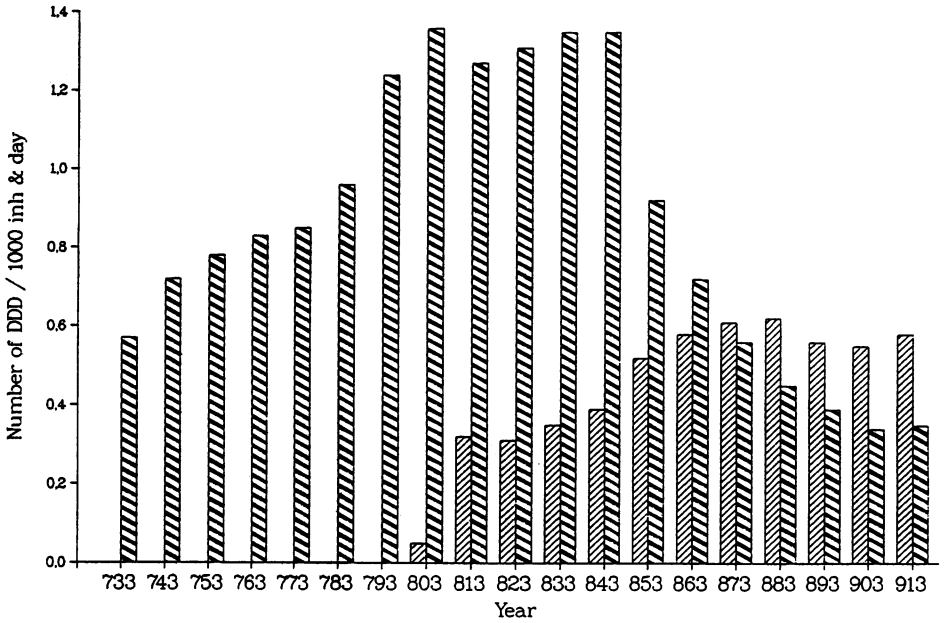

FIG. 1. Sales of trimethoprim (▨) and trimethoprim-sulfonamides (▩) in DDDs per 1,000 inhabitants (inh) per day. Values were reported for the month of March of each year.

high frequency of trimethoprim resistance among those isolates is most probably a consequence of the extensive use of trimethoprim-sulfonamides in the treatment of piglets with diarrhea. The use of trimethoprim (in combination with a sulfonamide) mainly for the treatment of UTI in humans has decreased since 1980 (Fig. 1). This decrease was caused by the side effects of the sulfonamide component. The utilization of trimethoprim as a single drug has not compensated for this decrease (Fig. 1). Furthermore, Fig. 1 shows that the utilization of trimethoprim-sulfonamide increased from 0.56 defined daily doses (DDDs) per 1,000 inhabitants per day in 1973 to 1.35 in 1980 but that it has since decreased to a level of 0.35 DDDs per 1,000 inhabitants per day in 1991. Trimethoprim was used as a single drug for 0.05 DDDs per 1,000 inhabitants per day in 1980, and use increased until 1988. Use has stayed at an almost constant level since then. The utilization of trimethoprim alone amounted to 0.58 DDDs per 1,000 inhabitants per day in 1991. These data are based on sales of trimethoprim and trimethoprim-sulfonamides in DDDs per 1,000 inhabitants per day in Sweden and were obtained from the Swedis Development Center in Uppsala. The use of sulfonamides in single-drug therapy in animals was affected in a similar way (Table 3). The use of trimethoprim in combination with sulfonamides in veterinary practice has increased every year since 1980 (Table 3) in spite of the fact that the incidence of neonatal piglet diarrhea has decreased because of the extensive use of highly efficient vaccines against this disease (21). Apparently, this decrease in disease has not been accompanied by the expected decrease in the use of trimethoprim. In consequence, records from the National Veterinary Institute show that the frequency of trimethoprim-resistant strains among porcine *E. coli* remained at about the same level in 1989 (16%) as in 1982 (10%). All values in Table 3 are from the Swedish Drug Company (Apoteksbolaget), which is a government-owned distributor of all prescription drugs.

Appearance and possible spread of *dhfrIX* among human isolates of trimethoprim-resistant enterobacteria. From a general point of view regarding the use of antibiotics, it is of interest to ask whether the type IX *dhfr* gene newly found among porcine isolates will occur and spread among human pathogens. A total of 434 trimethoprim-resistant human isolates were studied by colony hybridization. Of these, 222 were UTI isolates from four Swedish hospitals, while the rest were from Finland, Nigeria, Ethiopia, Texas, and Thailand (see Materials and Methods; Table 4). Among the 434 human isolates, only one gave a clearly positive hybridization signal with the oligonucleotide probe for *dhfrIX* (Table 1). Thus, one human isolate seems to harbor the *dhfrIX* gene (Table 4). This *E. coli* isolate was from a sample analyzed at a bacteriological laboratory in Uppsala in 1991 and was from a patient with UTI who had no connection to animal farms.

DISCUSSION

Trimethoprim is a clinically useful antibacterial agent because of its selective inhibition of bacterial DHFRs. Structural differences make the human enzyme practically insensitive to the antifolate action of the drug. Bacterial resistance to trimethoprim is mediated by foreign, plasmid-borne genes which express drug-resistant variations of DHFRs. For, more than a dozen of these resistant enzymes, the

TABLE 3. Use of trimethoprim and sulfonamides in animals in Sweden

Yr	Kg of active substance in animals[a]	
	Tp	Su
1980	134	6,600
1982	142	4,931
1984	186	4,325
1986	197	3,093
1987	208	2,932
1989	264	2,198
1990	285	

[a] Tp, trimethoprim; Su, sulfonamide.

TABLE 4. Colony hybridization to oligonucleotide probe for *dhfrIX* in human isolates

Organism	Origin	Collection yr	No. of isolates	
			Total	Hybridizing to probe for *dhfrIX*
Enterobacteriaceae	Sweden	1989–1991	222	1
E. coli	Finland	1986–1987	46	0
Enterobacteriaceae	Nigeria	1990	80	0
E. coli	Ethiopia	1986	26	0
E. coli	Texas	1986	14	0
Shigella spp.	Thailand	1983–1988	46	0
Total			434	1

amino acid sequences are now known. Most of them show some similarity to each other and to the chromosomal enzyme of, for example, *E. coli* as well as to the human enzyme (11, 13). The origins of the plasmid-encoded enzymes are unknown, but it is reasonable to assume that they were once picked up from environmental organisms naturally resistant to trimethoprim. Some of these resistant enzyme genes have been incorporated into genetic structures like transposon Tn*7* (1, 7) and the integron (28), which have effected their efficient spread under the selection pressure of a ubiquitous use of the drug. The emergence of *dhfrV* could illustrate the plausibility of this argument. This trimethoprim resistance trait seems to have first appeared locally in Sri Lanka, Southeast Asia (29), where it is the dominating type of trimethoprim resistance. Presumably, it has since spread around the world, and a few isolates carrying *dhfrV* have been found in Europe (25, 31). The *dhfrV* gene is borne on an integron, which could effect its efficient dissemination (25). The *dhfrIX* gene described here and in a previous paper (11) seems to be an even more clear-cut and more extended illustration of dissemination from a gene reservoir. The *dhfrIX* gene seems originally to have appeared in strains of *E. coli* among fecal bacteria in pigs reared in the southern part of Sweden and then spread under the heavy selective pressure of frequent therapeutical use of trimethoprim in veterinary practice. As shown in this paper, it is now widely spread among porcine isolates of *E. coli*, even those from pig herds with no history of trimethoprim treatment. The *dhfrIX* gene could have appeared in one animal in one herd and then spread by the extensive trade and transport of piglets between farms in Sweden. This spread is not the dissemination of one single bacterial clone, since the strains carrying *dhfrIX* belonged to several different serotypes (data not shown). In an earlier study (11), *dhfrIX* was found on two different conjugative plasmids, which formed efficient vehicles for the spread of resistance under trimethoprim selection pressure.

It has been a longstanding debate whether the use of antibacterial agents in animals will lead to resistance genes eventually spreading into human pathogens and to difficulties in the treatment of infectious disease as a sequel. The same kinds of antibiotics are used in both humans and animals, and the environments are not separated. Exchange of drug-resistant enterobacteria between animals and humans is known to occur and to cause disease (8), but it is not well understood how specificity to animal hosts contributes to the biologic containment of, for example, *E. coli*. Campbell and Mee (2) have shown that the same *dhfr* gene is carried by identical plasmids in both human bacterial strains and porcine isolates, but it was not possible to determine in which direction the transfer had occurred. In a study performed in a farm environment, where the spread of an *E. coli* strain with markers could be followed, Marshall et al. (12) showed that an *E. coli* strain harboring a transferable plasmid rapidly spread among different animal species and to humans even when there was no treatment with antibiotics. Similarly, plasmid-borne streptothricin resistance genes were demonstrated to move from farm animals to humans (10). Streptothricin was used for 2 years for growth promotion in industrial pig farms in a relatively large geographic area of eastern Germany. Streptothricin was not used for any other purpose. Plasmids coding for streptothricin resistance were found in fecal bacteria from pigs which had been fed streptothricin but also in *E. coli* isolated from humans in direct or indirect contact with the animals and even from people in the community, who had had no contact at all with the farms.

The case of *dhfrIX* seems to be a similar phenomenon just emerging. The transfer of this trimethoprim resistance trait, which is ubiquitous among porcine isolates, seems to be on the verge of transferring into human bacterial strains. Of the 434 trimethoprim-resistant human isolates investigated, only one, an *E. coli* strain, showed the presence of *dhfrIX*. It originated from an elderly patient with UTI living in a city in the middle part of Sweden who had had no farm contacts. The patient was given several trimethoprim courses of therapy over 10 years.

Trimethoprim has been used extensively in Sweden, mostly for the treatment of UTI. Since all prescription drugs in Sweden are distributed by one state-owned company, it is possible to obtain sales figures representing the total utilization of every drug. Figures for trimethoprim utilization are shown in Fig. 1, where it can be seen that in the years 1980 through 1984, more than 1.25 DDDs were utilized per day per 1,000 inhabitants. This means that statistically, almost 4.5% of the Swedish population was exposed to trimethoprim in those years, provided that the average period of treatment was 10 days. This ought to represent a sizable selection pressure for the spread of trimethoprim resistance.

The distribution of other trimethoprim resistance traits besides *dhfrIX* in the studied collection of porcine isolates seems to reflect what has been observed earlier among human isolates, among which the most common gene for trimethoprim resistance is *dhfrI* (7). This gene has mostly been found on transposon Tn*7*, which seems to have functioned as a very efficient vehicle for the dissemination of this resistance trait. Transposon Tn*7* occurs on plasmids like R483 (1) but can also insert itself into the *E. coli* chromosome. This location seems in fact to be dominating in clinical contexts, since Heikkilä et al. (7) have shown that 98% of their *dhfrI*-carrying *E. coli* isolates harbored that gene in the chromosome. The *dhfrI* gene has, furthermore, recently been observed also to occur at a specific insertion site in a recombinationally active genetic structure (28), an integron (23). This integron was earlier shown to contain several other resistance genes (*dhfrII*, *dhfrV*, etc.) at the same GTTA locus (25). The 12 *dhfrI*-carrying strains hybridizing to the probe for *int*-Tn*21* but not to that for *int*-Tn*7* (Table 2) could be examples of this *dhfrI* location. The 18 strains in Table 2 which hybridized to the probe for *int*-Tn*7* but not to the probe for *dhfrI*, on the other hand, could represent transposons similar to Tn*1825* and Tn*1826*, which in turn were shown to be very similar to Tn*7* in mediating streptothricin (nourseothricin) resistance and in carrying the same

spectinomycin resistance gene (*aadA1*) as Tn*7* but which lack *dhfrI* (27, 30).

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