Spread of a Newly Found Trimethoprim Resistance Gene, \textit{dhfrIX}, among Porcine Isolates and Human Pathogens

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Received 9 April 1992/Accepted 28 September 1992

A plasmid-borne gene mediating trimethoprim resistance, \textit{dhfrIX}, newly found among porcine strains of \textit{Escherichia coli}, was observed at a frequency of 11% among trimethoprim-resistant veterinary isolates. This rather high frequency of \textit{dhfrIX} could be due to the extensive use of trimethoprim in veterinary practice in Sweden. After searching several hundred clinical isolates, one human \textit{E. coli} strain was also found to harbor the \textit{dhfrIX} gene. Thus, the \textit{dhfrIX} gene seems to have spread from porcine bacteria to human pathogens. Furthermore, the occurrence of other genes coding for resistant dihydrofolate reductase enzymes (\textit{dhfrI}, \textit{dhfrII}, \textit{dhfrIV}, \textit{dhfrVII}, and \textit{dhfrVIII}) among the porcine isolates was investigated. In addition, association of \textit{dhfr} genes with the integrase-like open reading frames of transposons \textit{Tn7} and \textit{Tn21} was studied. In colony hybridization experiments, both \textit{dhfrI} and \textit{dhfrII} were found associated with these integrase genes. The most common combination was \textit{dhfrI} and \textit{int-Tn7}, indicating a high prevalence of \textit{Tn7}.

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 \textit{dhfrI} borne on \textit{Tn7} (1, 4, 7, 19), which was first observed in the R plasmid R483 (1, 20). It has recently been established that \textit{dhfrI} can also be located in an element named integron (23), which can be found in \textit{Tn21}-like transposons (28). The integron is recombinationally active and carries several other antibiotic resistance genes, among them \textit{dhfrII}, \textit{dhfrV}, and \textit{dhfrVII} (25, 26).

The newly characterized \textit{dhfrIX} gene (11) has so far been observed only among porcine isolates of \textit{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 \textit{E. coli} could be regarded as a consequence of the extensive use of trimethoprim (in combination with sulphonamides) in veterinary practice, including in the treatment of pig diarrhea. The \textit{dhfrIX} gene was originally found in isolates from porcine \textit{E. coli} collected in 1982 from several farms spread over the southern part of Sweden and sometimes from animals not treated with trimethoprim. The \textit{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 \textit{dhfrIX} and other trimethoprim resistance genes among porcine isolates and the possible spread of \textit{dhfrIX} among human pathogens. To investigate the prevalence of \textit{dhfrIX} among veterinary isolates, 279 trimethoprim-resistant porcine isolates of \textit{E. coli} were studied by colony hybridization. Thirty-one of these showed hybridization to the probe for \textit{dhfrIX}. The ability of the \textit{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 \textit{dhfrIX}-positive isolate was actually found.

MATERIALS AND METHODS

\textbf{Bacterial strains}. A collection of 279 trimethoprim-resistant \textit{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 \textgreater{}8 mg/liter. Forty-eight trimethoprim-sensitive strains of porcine \textit{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 strains were collected in 1989 to 1991 from patients with urinary tract infections (UTI), 97 were from Academic Hospital in Uppsala (Carl Pålsson), 54 were from Danderyd Hospital in Stockholm (Bengt Wretlind), 27 were from Huddinge Hospital in Stockholm, and 44 were from Karolinska Hospital in Stockholm (S jean 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 Heikkinä), and 14 were fecal \textit{E. coli} strains from day care centers in Houston, Tex. (Barbara Murray). Also, 46 \textit{Shigella} strains were obtained from Bangkok, Thailand (Panida Jayanetra), and 80 other strains of the family \textit{Enterobacteriaceae} were obtained from Lagos, Nigeria (Adebayo Lamikikin).

\textbf{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 \([\alpha-32P]dCTP\) and \([\gamma-32P]dATP\) 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

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MICS of trimethoprim were determined by the agar dilution method (3).

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 EcoRV-HindIII fragment, which includes 72 bases upstream of the start codon of dhfrIX (11). As specific probes for the integrase-like open reading frames of transposons Tn7 and Tn21 (15, 25), the probes int-Tn7 and int-Tn21 were used (Table 1). The fragment probes were labeled with [α-32P]dCTP by using an Oligolabeling Kit (Pharmac LKB Biotechnology AB, Uppsala, Sweden). Labeled DNA was purified on Sepahed G-50 columns (Pharmac LKB). Oligonucleotide probes were 5' labeled with [γ-32P]dATP and T4 polynucleotide kinase (18). Labeled DNA was purified on Sepahed 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 pGJ001-1 (Table 1), pLKO221 (26), and pLMO224 (24).

For DNA fragment probe hybridization, filters were washed at 68°C for 30 min, twice in 1 liter of 2x SSC (1x 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 1x SSC-0.2% SDS.

 For oligonucleotide probe hybridization, filters were washed twice in 1 liter of 2x SSC-0.1% SDS at room temperature for 15 min and twice in 1 liter of 0.5x 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 dhfrIX gene, and in 102 of these (61%), dhfrIX was most likely borne on Tn7 as judged from the concomitant int-Tn7 hybridization. The combination of dhfrIX and int-Tn21 hybridizations was observed in 12 strains (4.5%). Twenty-five strains carrying dhfrIX hybridized to int-Tn7 and int-Tn21 in combination. This is most likely explained by a Tn7 location of dhfrIX and a parallel occurrence of an integron structure carrying int-Tn21. Six strains hybridized to the probes for dhfrII and int-Tn21. This group could represent strains in which dhfrII is inserted as a genetic cassette in a Tn21-like structure (25).

Thirty-one of the isolates (11%) showed hybridization to the dhfrIX probe. Among these, one also hybridized to the int-Tn7 probe, and two hybridized to both the dhfrIX probe and the int-Tn21 probe. The dhfrIX gene, which does not resemble a cassette, and the integrase-like open reading frames of Tn7 and Tn21 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-Tn21, but after transfer of dhfrIX into a recipient strain, the transconjugant did not show any hybridization to the int-Tn21 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 diarrrhea were resistant to trimethoprim (5). The rather...
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

![Graph showing sales of trimethoprim and trimethoprim-sulfonamides](image)

**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.

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

| Yr | 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 | |

*Tp, trimethoprim; Su, sulfonamide.
TABLE 4. Colony hybridization to oligonucleotide probe for

\( \text{dhfrI}X \) in human isolates

| Organism          | Origin | Collection yr | No. of isolates |
|-------------------|--------|---------------|----------------|
| Enterobacteriaceae| Sweden | 1989–1991     | 222            |
| E. coli           | Finland| 1986–1987     | 46             |
| Enterobacteriaceae| Nigeria| 1990          | 80             |
| E. coli           | Ethiopia| 1986          | 26             |
| E. coli           | Texas  | 1986          | 14             |
| Shigella spp.     | Thailand| 1983–1988    | 46             |
| Total             |        |               | 434            |

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 \( \text{dhfrI}X \) 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 \( \text{dhfrI}X \). 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 \( \text{dhfrI}X \) 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 \( \text{dhfrI} \) (7). This gene has mostly been found on transposon Tn7, which seems to have functioned as a very efficient vehicle for the dissemination of this resistance trait. Transposon Tn7 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 Heikillé et al. (7) have shown that 98% of their \( \text{dhfrI} \)-carrying \( E. \) coli isolates harbored that gene in the chromosome. The \( \text{dhfrI} \) 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 (\( \text{dhfrII}, \text{dhfrV} \), etc.) at the same GTTA locus (25). The 12 \( \text{dhfrI} \)-carrying strains hybridizing to the probe for \( \text{int-Tn21} \) but not to that for \( \text{int-Tn7} \) (Table 2) could be examples of this \( \text{dhfrI} \) location. The 18 strains in Table 2 which hybridized to the probe for \( \text{int-Tn7} \) but not to the probe for \( \text{dhfrI} \), on the other hand, could represent transposons similar to Tn1825 and Tn1826, which in turn were shown to be very similar to Tn7 in mediating streptothricin (nourseothricin) resistance and in carrying the same

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spectinomycin resistance gene (aadA1) as Tn7 but which lack dhfrI (27, 30).

ACKNOWLEDGMENTS

We thank Verena Rehbinder for skillful technical assistance. This work was supported by a grant from the Swedish Council for Forestry and Agricultural Research to Anders Franklin and Catarina Jansson, a grant from the Swedish Medical Research Council to Ola Skōld, and a fellowship from the I. F. Foundation for Pharmaceutical Research for Catarina Jansson.

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