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Trimethoprim Resistance in *Escherichia coli* Strains Isolated from Patients with Urinary Tract Infection in 1989–1994

Oporność na trimetoprim wśród szczepów *Escherichia coli* izolowanych od pacjentów z zakażeniem układu moczowego w latach 1989–1994

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Abstract

Introduction. Trimethoprim (Tmp), alone or in combination with sulfamethoxazole (co-trimoxazole), is commonly used for the treatment of urinary tract infections (UTI). In Poland, Tmp has been used in monotherapy since the late nineties.
Objective. In this study the level of resistance to Tmp and the prevalence of *dfr* genes among *E. coli* strains isolated from urine in the period of 1989–1994 was investigated.
Material and Methods. Five hundred fifty-seven *E. coli* strains isolated from patients with significant bacteriuria were studied. *E. coli* C600K12 and *E. coli* J53K12 were used for the conjugational transfer of the resistance determinant of Tmp-resistant *E. coli* (donor strains) and recipients. The disc-diffusion method was performed to detect antibiotic resistance. The MICs of the antibiotics were determined by an agar dilution method. PCR was used to determine *dfr* genes encoding for dihydrofolate reductases.
Results. Of the 557 *E. coli* isolates, 15% were resistant to trimethoprim (MIC ≥ 4 mg/l). Of this group, 72% of the strains exhibited high-level resistance to Tmp (MIC > 1024 mg/l). Most of them were additionally resistant to three or more other antibiotics and resistance to doxycycline was dominant. Tmp resistance was transferred on conjugative plasmids from 55% of the donor strains to recipient *E. coli*. Co-transfer of various other resistance determinants with Tmp resistance was observed, streptomycin resistance being the most common. A gene determining Tmp resistance, *dfr*A1, was the most prevalent in the *E. coli* isolates (91%). The remaining *E. coli* strains (9%) possessed the *dfrB2* gene.
Conclusions. Most *E. coli* strains were highly resistant to Tmp (MIC > 1024 mg/l). Tmp resistance was mediated by conjugative plasmids containing *dfr* genes. Type I dihydrofolate reductase (DHFR I) was the main enzyme responsible for high-level resistance to Tmp in the studied *E. coli* strains. Despite the late introduction of Tmp (a single agent) into therapy, the ubiquitous use of co-trimoxazole has caused the development of resistance to Tmp among *E. coli* (**Adv Clin Exp Med. 2007, 16, 1, 35–42**).

Key words: *E. coli*, trimethoprim, resistance, *dfr*, DHFR.

Streszczenie

Wprowadzenie. Trimetoprim (Tmp), pojedynczo lub w połączeniu z sulfametoksazolem (kotrimoksazol), jest powszechnie stosowany w leczeniu zakażeń dróg moczowych (z.u.m.).
W Polsce trimetoprim wprowadzono do monoterapii pod koniec lat 90.
Cel pracy. Oznaczenie poziomu oporności na Tmp oraz występowania genów *dfr* wśród szczepów *E. coli* izolowanych z moczu w latach 1989–1994.
Materiały i metody. Zbadano 557 szczepów *E. coli* pochodzących od pacjentów ze znamienną bakteriurią. W badaniach nad koniugacyjnym przekazywaniem znaczników oporności wykorzystano oporne na Tmp, szczepy *E. coli* (dawcy) oraz wrażliwe na Tmp *E. coli* K12C600 i *E. coli* J53K12 (biorcy plazmidów). Oporność na antybiotyki oznaczono metodą dyfuzyjno-krążkową, a wartości MIC metodą seryjnych rozcieńczeń w podłożu stałym. Technikę PCR zastosowano do określenia typu genów *dfr* kodujących reduktazy dihydrofolianu.

Wyniki. Spośród 557 badanych szczepów *E. coli* 15% było opornych na Tmp (MIC 4 mg/L). W grupie tej 72% szczepów wykazywało wysoki poziom oporności (MIC > 1024 mg/L). Większość z nich była dodatkowo oporna na 3 lub więcej antybiotyków z wyraźną przewagą oporności na doksycyklinę. U 55% szczepów oporność na Tmp była przekazywana na plazmidach w procesie koniugacji do komórek biorcy. Wraz z opornością na Tmp przekazywane były markery oporności na inne leki, głównie streptomycynę. Gen *dfrA1* warunkujący oporność na Tmp stwierdzono u większości badanych szczepów *E. coli* (91%). Pozostałe szczepy *E. coli* (9%) zawierały gen *dfrB2*.
Wnioski. Większość badanych szczepów *E. coli* wykazywała wysoki poziom oporności na Tmp (MIC > 1024 mg/L). Za oporność odpowiadały koniugacyjne plazmidy z genami *dfr.* Reduktaza dihydrofolianu typu I (DHFR I) była głównym enzymem odpowiedzialnym za wysoką oporność na Tmp u badanych szczepów. Mimo późnego wprowadzenia Tmp (pojedynczego leku) do terapii, powszechne stosowanie w lecznictwie kotrimoksazolu spowodowało wyraźny rozwój oporności na Tmp u pałeczek *E. coli* (**Adv Clin Exp Med. 2007, 16, 1, 35–42**).

Słowa kluczowe: *E. coli*, trimetoprim, oporność, *dfr*, DHFR.

Urinary tract infection (UTI) caused by *Escherichia coli* is a frequent disease in hospitals as well as in ambulatory patients. The susceptibility patterns of antibiotics for this pathogen vary in different parts of the world [1]. Trimethoprim (Tmp), in combination with sulfonamides, has been successfully used in the treatment of UTI. Since 1972 it has also been available as a single agent in many countries [2–4]. In Poland, however, Tmp alone was introduced into clinical use at the end of the nineties. Its use in conjunction with sulametoxazole (co-trimoxazole), introduced into therapy in 1969 [5], seems to be preferable to Tmp alone.

Tmp selectively inhibits bacterial dihydrofolate reductase (DHFR), which catalyses the conversion of dihydrofolate to tetrahydrofolic acid, and this affects the biosynthesis of DNA. Several different mechanisms of Tmp resistance have been identified in bacteria, but the most common is the production of an additional plasmid-encoded Tmp-resistant DHFR [6–9]. Tmp resistance due to mutational changes in the intrinsic chromosomal DHFR confers low or intermediate levels of resistance, while a plasmid-borne DHFR usually mediates resistance to a high concentration of Tmp. *dfr* genes expressing Tmp-insensitive DHFR are localized on transferable elements, the latter being efficiently spread on plasmids, transpozons, and integron cassettes. Among Gram-negative bacteria, insensitive DHFR spreading on plasmids seems to be of the greatest clinical significance [8–11].

The epidemiology of Tmp-resistant *dfr* genes in Poland is not well known since, for many years, Tmp alone was not usually tested against the microorganisms. Sensitivity testing in Polish hospital laboratories was performed for co-trimoxazole rather than for Tmp alone. In this paper, a follow-up study of Tmp resistance among *E. coli* strains isolated from patients in the period from 1989 to 1994 and analyzed for the distribution of the *dfrA1*, *dfrA5*, *dfrA15*, *dfrA15b*, *dfrA16*, *dfrA16b*, *dfrB1*, *dfrB2*, and *dfrB3* genes encoding DHFR types I, V, XV, XVb, XVI, XVIb, IIa, IIb and IIc, respectively is described. Additionally, the resistance to other drugs 'linked' with Tmp resistance was determined.

Material and Methods

Bacterial strains

During the 5 years between 1989 and 1994, 557 consecutive *E. coli* isolates that were a factor in significant bacteriuria were collected. The strains were isolated from out- and inpatients of clinics of the Medical University in Wrocław. Repeated samples were excluded.

The recipient strains used in conjugation experiments were *E. coli* C600K12 (Tmp^S, Na^R, lac^-, tre^-, leu^-, the^-) and *E. coli* J53K12 *(Tmp^S, Na^S, Rf^R, lac^+, pro^-, met^-), obtained from the Institute of Immunology and Experimental Therapy PAN (IIET), Poland (Na-nalidixic acid, lac-lactose, tre-trehalose, leu-leucine, the-thiamine, Rf-rifampicin, pro-proline, met-methionine, S-sensitive, R-resistant, (–) negative, (+) positive.). For control plasmids, the *E. coli* NCTC 50535, 50536, and 50515 strains were used. Bacteria were grown in Nutrient Broth/Agar or in Brain Heart Infusion broth (BHI) (Biomed, Poland) and identified to the species level by biochemical tests (ID 32E, bioMerieux, France). Iso-Sensitest medium (Oxoid, UK) was used for susceptibility testing (supplemented with lysed horse blood 5% v/v) when the MIC for Tmp was determined [12], Luria-Bertani (LB) medium (Difco, USA) ws used for isolation of plasmids and Falkow minimal medium for the conjugation experiments [16].

Plasmids

The plasmids used as a source of positive control DNA for PCR were pFE872 for *dfrA1* gene [13] and pWZ820 for *dfrB2* gene [14].

Susceptibility Testing

The disc-diffusion technique was applied using commercial discs with the following antimicrobial agents: trimethoprim, doxycycline, spectinomycin, amikacin, gentamicin, streptomycin, kanamycin, ampicillin, piperacillin, carbenicillin, ceftazidime, cefuroxime, cefotaxime, and chloramphenikol [15]. The minimal inhibitory concentration (MIC) was determined by an agar dilution method [12]. The concentration of the antibiotics ranged from 4 to 1024 mg/l. High-level resistance to Tmp was defined as MIC > 1024 mg/l.

Resistance Transfer Experiments

In conjugation studies, selected *E. coli* strains which were highly resistant to Tmp were used as donor strains. They were mated with the recipient *E. coli* C600K12 strain. The resulted Tmp-resistant transconjugants were then mated with a second type of recipient, *E. coli* J53K12 (confirmation of transfer of resistance determinants). All mating experiments were performed as follows: overnight cultures of donor and recipient strains diluted 10 times with BHI broth were incubated at 37°C and 28°C. After the incubation period, the donor and recipient strains were mixed in the ratio 1:1 in the same broth and incubated for 24 h at 37°C and 28°C. From each overnight culture, 0.1 ml was spread on the selective Falkow agar supplemented with growth factors for the recipient and antibiotics from the spectrum resistance determined by the plasmid carried by the donor. After the incubation period, the number of colonies was counted. The colonies were purified three times on an appropriate selective medium. The frequency of transfer of Tmp resistance was expressed as the number of transconjugant cells in relation to the number of donor cells [16].

DNA Isolation

Bacterial strains were incubated for 20 h at 37°C with constant agitation (196 r.p.m.) in LB medium (1% yeast extract, 1% bactotryptone, and 0.5% sodium chloride) containing 500 µg/ml of Tmp. Plasmid DNA was extracted from the 3-ml culture with a Plasmid Mini kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions.

Molecular Typing

PCR was performed in a volume of 25 µl containing a 1-µl DNA sample, 2.5 µl of PCR buffer and 2.5 U of Taq polymerase (Biotools Labs, Madrid, Spain), 0.2 mM of each dNTP and 50 pmol of both forward and reverse primers. The reactions were carried out in a DNA Engine PTC-200 (JM Research, USA) thermocycler, comprising 30 cycles of 1 min at 94°C, 1 min at 65°C, 1 min at 72°C, and a final extension of 5 min at 72°C.

The primers IAF (5'-GTGAAACTATCAC-TAATGG-3'), IAR (5'-TTAACCCTTTTGC CAGATTT-3'), IIF (5'-GATCGCCTGCGCAA-GAAATC-3'), and IIR (5'-AAGCGCAGCC ACAGGATAAAT 3'), described previously by Navia et al. [17], were used**.** The pair of primers IAF/IAR results in an expected PCR product of 474 bp for the *dfrA1*, *dfrA5*, *dfrA15*, *dfrA15b*, *dfrA16*, and *dfrA16b* genes, while the primers IIF/IIR yield a band of 141 bp for the *dfrB1*, *dfrB2*, and *dfrB3* genes [17]. For the final *dfr* genes identification, the amplicons were digested by the restriction enzyme *Tas*I (*Tsp*509I, Fermentas, Lithuania) and the resulting RFLP (Restriction Fragments Length Polymorphism) pattern was compared with data published by Navia et al. [17].

Whole PCR products and restriction fragments were visualized by electrophoresis on 1.3% or 2% agarose (Basica LE, Prona, Spain) gels with ethidium bromide (0.25 µg/ml) using standard procedures [18].

Results

Trimethoprim Resistant Isolates

The results showed that from 557 *E. coli* strains isolated during the period of study, 15% (86) were resistant to Tmp. Among them, 72% (62) exhibited high-level resistance (MIC > 1024 mg/l). The remaining strains showed resistance to Tmp with MIC of 4–1024 mg/l (Table 1).

Resistance to Other Drugs

All (86) strains resistant to Tmp (MIC ≥ 4 mg/l) were also tested for their resistance to other antibiotics and chemotherapeutics. The variety of resistance patterns for *E. coli* Tmp^R (Tmp-resistant) isolates is shown in Table 1. Resistance to doxycycline (93%) was the most common. Over 70% of Tmp^R strains were found to carry resistance to ampicillin. More strains presented resistance to carbenicillin (69%), piperacillin (54%), and chloramphenicol (48%) than to kanamycin (13%) and gentamicin (12%). Resistance to streptomycin and spectinomycin was noted in 71% and 53% of the strains, respectively. Less frequent was resistance to cefuroxime (8%) and ceftazidime (6%). Only 2% of the Tmp^R strains showed resistance to cefotaxime and 1% to amikacin.

Tabela 1. Wzory oporności na antybiotyki 86 szczepów *E. coli* (Tmp^R)

Table 1. Antibiotic resistance patterns in the 86 *E. coli* (Tmp^R) isolates

Number of strains (Liczba szczepów)	MIC of Tmp (mg/l)	Resistance pattern (Wzór oporności)
2	8	Tmp
10	8	Tmp, Dk
3	> 1024	Tmp, Dk
1	8	Tmp, Dk, Sp
1	> 1024	Tmp, Dk, Sp
1	8	Tmp, Dk, Ge
1	8	Tmp, Dk, Pip
1	8	Tmp, Dk, S
1	> 1024	Tmp, S, Sp
1	> 1024	Tmp, Dk, S, Sp
1	8	Tmp, Pip, Am, Cb
1	> 1024	Tmp, Dk, Sp, Am
2	> 1024	Tmp, S, Pip, Am, Cb
1	> 1024	Tmp, Dk, Am, Cb, C
2	> 1024	Tmp, Dk, S, Sp, Am
1	> 1024	Tmp, Dk, S, Pip, Am
1	8	Tmp, Dk, Pip, Am, Cb
1	8	Tmp, Dk, S, Pip, Am, Cb
1	256	Tmp, Dk, S, Pip, Am, Cb
3	> 1024	Tmp, Dk, S, Pip, Am, Cb
1	8	Tmp, Dk, S, Am, Cb, C
1	> 1024	Tmp, Dk, S, Am, Cb, C
1	128	Tmp, Dk, S, Am, Cb, Cxm
2	> 1024	Tmp, Dk, Sp, Am, Cb, C
2	> 1024	Tmp, Dk, S, Sp, Km, C
1	> 1024	Tmp, Dk, Pip, Am, Cb, C
1	8	Tmp, Dk, S, Sp, Pip, Am, Cb
8	> 1024	Tmp, Dk, S, Sp, Pip, Am, Cb
1	8	Tmp, Dk, S, Sp, Am, Cb, C
2	> 1024	Tmp, Dk, S, Sp, Am, Cb, C
2	> 1024	Tmp, Dk, S, Pip, Am, Cb, Ge
5	> 1024	Tmp, Dk, S, Pip, Am, Cb, C
1	> 1024	Tmp, Dk, S, Sp, Am, Cb, C, Cxm
8	> 1024	Tmp, Dk, S, Sp, Pip, Am, Cb, C
3	> 1024	Tmp, Dk, S, Sp, Am, Cb, C, Km
2	> 1024	Tmp, Dk, S, Sp, Pip, Am, Cb, Ge
1	> 1024	Tmp, Dk, S, Pip, Am, Cb, Km, Ge
1	> 1024	Tmp, Dk, S, Sp, Pip, Am, Cb, C, Cxm
2	> 1024	Tmp, Dk, S, Sp, Pip, An, Cb, C, Km
1	> 1024	Tmp, Dk, S, Sp, Pip, Am, Cb, C, Km, Cxm
1	> 1024	Tmp, Dk, S, Sp, Pip, Am, Cb, Ge, Cxm, Caz
1	> 1024	Tmp, Dk, S, Sp, Pip, Am, Cb, Ge, Km, An
1	> 1024	Tmp, Dk, S, Sp, Pip, Am, Cb, Ge, Km, Cxm, Caz, Ctx,
1	> 1024	Tmp, Dk, S, Sp, Pip, Am, Cb, C, Ge, Km, Cxm, Caz, Ctx

Tmp – trimetoprim, Dk – doksycyklina, Sp – spektynomycyna, Am – ampicylina, Cb – karbenicylina, C – chloramfenikol, S – streptomycyna, Ge – gentamycyna, An – amikacyna, Km – kanamycyna, Pip – piperacylina, Cxm – cefuroksym, Caz – ceftazydym, Ctx – cefotaksym.

Tmp – trimethoprim, Dk – doksycycline, Sp – spectinomycin, Am – ampicillin, Cb – carbenicillin, C – chloramphenicol, S – streptomycin, Ge – gentamicin, An – amikacin, Km – kanamycin, Pip – piperacillin, Cxm – cefuroxime, Caz – ceftazidime, Ctx – cefotaxime.

Transfer of Trimethoprim Resistance

For conjugational crosses, 55 highly Tmp^R strains were selected as donors. Seven isolates resistant to one of the selection markers were rejected. In the case of 30 (55%) donor strains, resistance to Tmp was successfully transferred to recipients. No further transfer of resistance determinants was observed, even when conjugation

was repeated at 28°C. These strains required mobilization the X^+ factor (*E. coli* C20, F^+ (IIET, PAN)). Three of these strains transferred Tmp resistance via mobilization. All transconjugants obtained were highly resistant to Tmp (MIC > 1024 mg/l). The frequency of conjugational transfer varied from 2.4×10^{-6} to 4.4×10^{-1} transconjugants per donor. In most cases the transfer frequency amounted to $10^{-4} – 10^{-3}$.

Linkage of Trimethoprim Resistance with Resistance to Other Antibiotics

For all transconjugants highly resistant to Tmp, resistance profiles (MIC) for other drugs were determined. More than 80% of transconjugants showed a linkage of Tmp and streptomycin resistance. However, co-transfer with spectinomycin resistance was shown only in 32% of the transconjugants. Determinants expressing resistance to ampicillin, doxycycline, carbenicillin, and piperacillin were found in (rates for transconjugants obtained from matings with recipients a) *E. coli* C600K12 and b) *E. coli* J53K12) [a]87–[b]79%, 84–76%, and 71–68% of the transconjugants, respectively. Lower co-transfer with Tmp was demonstrated for chloramphenicol (21–18%), gentamycin (21–16%), and kanamycin (11–13%). Resistance to 3 cephalosporines was co-transferred to less than 6% of the transconjugants. None of the Tmp^R transconjugants exhibited resistance to amikacin.

Characterization of Tmp^R Plasmids

Most of the donor strains (91%) were shown to harbor plasmids ranging in size from 1 to 60 kb. Although in three strains plasmids were not visualized, the transfer of resistance to Tmp and other drugs was successful. In 70% of the isolates, large plasmids (50–60 kb) were detected. This plasmid was transferred to 42% of the transconjugants in the first conjugation mating and to 40% in the second. The most commonly observed resistance patterns which correlated with a transfer of the above plasmid were Tmp-Dk-S, Tmp-Pip-Am-Cb, and Tmp-Dk-S-Pip-Am-Cb. The remaining transconjugants possessed 1–5 small plasmids in the size range of 1–7 kb. In several multiresistant transconjugants, no plasmids were visualized.

Genes Conferring Tmp Resistance

The presence of *dfr* genes was confirmed by PCR in *E. coli* clinical isolates and in transconjugants obtained from conjugational crossing with the second recipient, *E. coli* K12J53 (Figures 1–3). Genes detected in all transconjugants correlated well with those detected in the donor strains. Most (30/33) of the strains harbored the gene *dfrA1* and only 2/33 strains possessed the gene *dfrB1*. The genes *dfrA5*, *dfrA15*, *dfrA15b*, *dfrA16*, *dfrA16b*, *dfrB2*, or *dfrB3* were not found in the studied strains. One strain expressed Tmp resistance encoded by an unidentified gene.

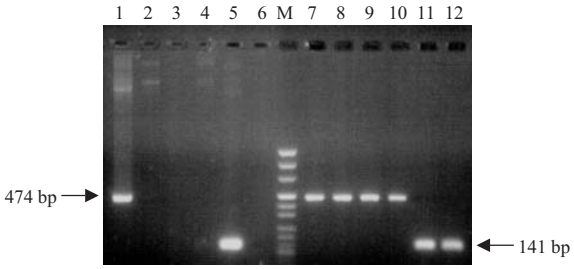

Fig. 1. Specific amplification of selected fragments of *dfr* genes. Electrophoretic separation of amplicons obtained with the following primer pairs and DNA: lane (1) primers IAF/IAR and control plasmid pFE872 (*dfrA1*); (2) IAF/IAR and control plasmid pWZ820 (*dfrB2*); (3) negative control for PCR with IAF/IAR primers; (4) primers IIF/IIR and control plasmid pFE872 (*dfrA1*); (5) IIAF/IIAR and control plasmid pWZ820 (*dfrB2*); (6) negative control for PCR with IIAF/IIAR primers; (7) IAF/IAR and DNA from clinical isolate 383; (8) IAF/IAR and DNA from clinical isolate 917; (9) IAF/IAR and DNA from transconjugant J383/1/1; (10) IAF/IAR and DNA from transconjugant J917/1/29; (11) IIF/IIR and DNA from clinical isolate 907; (12) IIAF/IIAR and DNA from transconjugant J907/1/9. M: molecular weight marker (pUCMix8, Fermentas). The arrows indicate the expected amplicon size of 474 bp and 141 bp [17]

Ryc. 1. Specyficzna amplifikacja wybranych fragmentów genów *dfr*. Elektroforeza amplikonów uzyskanych z użyciem następujących par starterów i matrycowych DNA: ścieżka (1) – startery IAF/IAR i plazmid kontrolny pFE872 (*dfrA1*); (2) – IAF/IAR i plazmid kontrolny pWZ820 (*dfrB2*); (3) – kontrola negatywna reakcji PCR ze starterami IAF/IAR; (4) – startery IIF/IIR i plazmid kontrolny pFE872 (*dfrA1*); (5) – IIAF/IIAR i plazmid pWZ820 (*dfrB2*); (6) – ujemna kontrola reakcji ze starterami IIAF/IIAR; (7) – IAF/IAR i DNA ze szczepu klinicznego nr 383; (8) – IAF/IAR i DNA ze szczepu klinicznego nr 917; (9) – IAF/IAR i DNA z transkoniuganta nr J383/1/1; (10) – IAF/IAR i DNA z transkoniuganta nr J917/1/29; (11) – IIF/IIR i DNA ze szczepu klinicznego nr 907; (12) – IIAF/IIAR i DNA z transkoniuganta nr J907/1/9; M – marker masy (pUCMix8, Fermentas). Strzałkami zaznaczono wielkość spodziewanych amplikonów 474 bp i 141 bp [17]

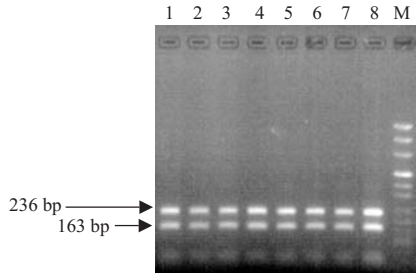

Fig. 2. Electrophoresis of amplikons obtained with primers IAF/IAR and digested by *TasI* enzyme. Lanes 1–8: clinical isolates number 654, 716, 893, 917, 16, 294, 975, and 28, respectively. M: molecular weight marker (pUCMix8, Fermentas). The arrows indicate the 236 bp and 163 bp restriction fragments typical for the *dfrAI* gene [17]

Ryc. 2. Elektroforeza amplikonów uzyskanych parą starterów IAF/IAR i trawionych enzymem restrykcyjnym *Tas*I. Ścieżki 1–8 odpowiednio szczepy kliniczne numer 654, 716, 893, 917, 16, 294, 975 i 28. M – marker masy (pUCMix8, Fermentas). Strzałkami zaznaczono fragmenty restrykcyjne o wielkości 236 bp i 163 bp charakterystyczne dla genu *dfrA1* [17]

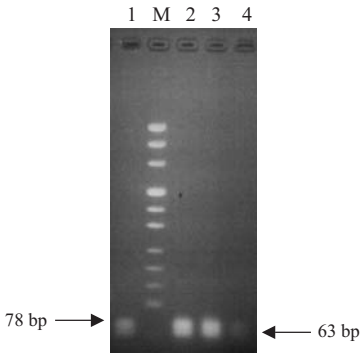

Fig. 3. Electrophoresis of amplicons obtained with primers IIAF/IIAR and digested by *Tas*I enzyme. Lanes 1–4: clinical isolates and their transconjugants 907 and J907/1/9, 223, and J223/1/5, respectively. M – molecular weight marker (pUCMix8, Fermentas). The arrows indicate the 78 bp and 63 bp restriction fragments typical for the *dfrB1* gene [17]

Ryc. 3. Elektroforeza amplikonów uzyskanych parą starterów IIAF/IIAR i trawionych enzymem restrykcyjnym *Tas*I. Ścieżki 1–4 odpowiednio szczepy kliniczne i ich transkoniuganty 907 i J907/1/9 oraz 223 i J223/1/5. M – marker masy (pUCMix8, Fermentas). Strzałkami zaznaczono fragmenty restrykcyjne o wielkości 78 bp i 63 bp charakterystyczne dla genu *dfrB1* [17]

Discussion

Trimethoprim-resistant bacteria have been isolated worldwide since the combination of trimethoprim and sulfonamides was introduced for clinical treatment. Resistance to this chemotherapeutic in Gram-negative bacteria and a variety of mechanisms responsible for it have been widely reported [19–25]. In Poland, Tmp alone has been registered for clinical use since 1998. Co-trimoxazole, however, has been extensively used for the treatment of various infections, including UTI, during the last 30 years.

Results of these studies show that 15% of *E. coli* isolated from UTI in the Wrocław area were resistant to Tmp. These data are comparable to those seen in 1998 in Rovaniemi (14%), Turku (16%), and Helsinki (19%) in Finland [20]. The first screening work on Tmp resistance in our geographical region was conducted by Złotorzycka et al. on *E. coli* isolated from urine and fecal samples [data not published]. The report demonstrated heightened resistance from 0% in 1977–1979 to 1% in 1980–1981 and 1.2% in 1983. Compared with these data, in the years 1989–1994 a 15-fold increase of Tmp resistance in *E. coli* strains is observed. These findings therefore indicate a slow development of trimethoprim resistance to the year 1994. This incidence is much lower than that seen in India [26], Chile [27], and Taiwan [28], where by the end of the eighties more than 40% of isolates were resistant to Tmp. This variable resistance may reflect the consumption and control of drugs in particular countries. Our results also show a high proportion (72%) of high-level resistance to this agent in the studied strains. Similarly, Brumfitt et al. [29] reported an increase in highly Tmp resistant *E. coli* isolates from 22% in 1973 to 91% in 1981. Heikkilä et al. [20] reported high-level Tmp resistance in more than 90% of strains in Finland and Tsakris et al. [22] of 89% in Greece at the beginning of the nineties. This increasing tendency may suggest plasmid/transpozon-mediated Tmp resistance dissemination.

Sixty percent (33/55) of the strains studied could have transferred Tmp resistance determinants via conjugation, indicating the presence of R-plasmids. Similar proportions (more than half of the strains) are reported by Tsakris et al. [30] and Mayer [31]. Lin-Li Chang reported 66% of transferable high-level Tmp resistance in 1987, but at the same time emphasized a decreasing tendency to 53.7% in 1989, suggesting an influence of non-transferable plasmids or transpozons [28].

In bacteria, genetic changes concerning resistance to antimicrobial agents have been rapid. Intensive, multiple, and sometimes prolonged application of drugs usually leads to the selection of multiresistant strains. Such strains may carry linked determinants of antibiotic resistance [32]. The present authors observed that along with resistance to Tmp, many other determinants were co-transferred. This result probably reflects the wide spectrum of antibiotics used in Poland. The most

frequent in Tmp-resistant transconjugants was the linkage with resistance to streptomycin, ampicillin, doksycycline, and piperacyllin. This may follow (except streptomycin) their role in the treatment of urinary tract infections. Amyes indicated that Tmp-resistant bacteria often had linked plasmid genes conferring both trimethoprim and ampicillin resistance [33]. Thus, ampicillin might play the role of a powerful selector for plasmid *dfr* genes in bacterial populations. Resistance to gentamicin and kanamycin was co-transferred less frequently than for streptomycin and spectinomycin. None of the transconjugants was resistant to amikacin, which may reflect their low usage compared with other aminoglycosides during the study period. Similar rates were observed for chloramphenicol and cephalosporines.

In the most of the trimethoprim-resistant transconjugants, variable plasmids were present. They differed in their molecular mass and antibiotic resistance patterns. However, additional studies are needed to determine their relationship.

Here, the prevalence of *dfr* genes in *E. coli* urinary isolates was investigated. These data showed that *dfrA1* was the most prevalent gene, while *dfrB2* was detected in only a few strains. The presence of *dfrA5*, *dfrA15*, *dfrA15b*, *dfrA16*, *dfrA16b*, and *dfrB2* or *dfrB3* genes in the studied strains was not observed. Similar results, i.e. the prevalence of type I DHFR (*dfrA1*) in *E. coli*, have been reported in other parts of the world [22, 3–36]. The present authors believe that this is the first report on the distribution of trimethoprim resistance genes in Gram-negative rods in Poland. The results suggest that the plasmid-borne *dfrA1* gene has spread efficiently among *E. coli* from this geographical region, probably under the selective pressure of the ubiquitous use of Tmp in combination with sulfamethoxazole. In this study it was not determined whether *dfrA1* gene was a component of Tn7 or was integrated in gene cassettes. The high percentage of transconjugants resistant to both trimethoprim and streptomycin may suggest, however, the presence of transpozon Tn7 as a mediator of Tmp resistance in the studied strains. Further studies will be conducted for the exact calculation of trimethoprim resistance gene dissemination in hospital settings in Poland.

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