Prevalence of trimethoprim resistance genes in *Escherichia coli* isolates of human and animal origin in Lithuania

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A total of 456 non-repetitive *Escherichia coli* isolates from human clinical specimens (urinary, *n*=134; cervix, vagina and prostate, *n*=52; blood, pus and wounds, *n*=45), healthy animals (cattle, *n*=45; poultry, *n*=20) and diseased animals (cattle, *n*=53; swine, *n*=64; poultry, *n*=43) obtained in Lithuania during the period 2005–2008 were studied for trimethoprim (TMP) resistance and the prevalence of *dfr* genes. A TMP resistance rate in the range of 18–26 % respective to the origin was found in clinical isolates, 23–40 % in isolates from diseased animals and 9–20 % in isolates from healthy animals. Of 112 TMP-resistant isolates, 103 carried at least one of the six *dfrA* genes (*dfrA1*, *dfrA5*, *dfrA8*, *dfrA12*, *dfrA14* and *dfrA17*) as determined by multiplex PCR and RFLP. The *dfrA1* and *dfrA17* genes were found most frequently in clinical isolates (17 and 19 isolates, respectively), whilst *dfrA1* and *dfrA14* genes dominated in isolates of animal origin (25 and 13 isolates, respectively). The *dfrA5*, *dfrA12* and *dfrA8* genes were detected at lower frequencies. The association with class 1/class 2 integrons was confirmed for 73–100 % of *dfr* genes found in most groups of isolates, except for the isolates from diseased swine. In this group, the majority of *dfr*-positive isolates (67 %, 8/12) carried *dfrA8* (6/12) or *dfrA14* genes (2/12) that were not associated with integrons. Non-integron location was also confirmed for the remaining *dfrA8* genes (six clinical isolates and one isolate from diseased cattle) and for *dfrA14* genes (two isolates from diseased cattle and swine each). All cassette-independent *dfrA14* genes were found to be located within the *strA* gene. This study on the prevalence and distribution of TMP resistance genes among *E. coli* isolates of human and animal origin in Lithuania demonstrates that *dfr* genes are carried most frequently as gene cassettes within class 1 and/or class 2 integrons. However, TMP resistance in some of the isolates was found to be mediated by non-integron-associated *dfrA8* and *dfrA14* genes, indicating the existence of alternative sources for the spread of resistance.

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INTRODUCTION

Trimethoprim (TMP) affects bacterial folic acid synthesis by the inhibition of dihydrofolate reductase (DHFR), which catalyses the reduction in dihydrofolate (Huovinen, 2001; Sköld, 2001). Bacteria may become resistant to TMP by several mechanisms, including the development of permeability barriers, efflux pumps, the existence of naturally insensitive target DHFR enzymes, mutational and regulation changes in target enzymes and the acquirement of drug-resistant target enzymes (Huovinen, 2001). The most common resistance mechanism is the acquirement of a TMP-insensitive DHFR variant resulting in high-level TMP resistance in various bacteria. To date, more than 30 different TMP resistance *dfr* genes are known. On the basis of the length of encoded polypeptide, they have been divided into two major types, *dfrA* and *dfrB* (White & Rawlinson, 2001).

The association of *dfr* genes with mobile genetic elements such as transposons and plasmids has often been observed (Ojo *et al.*, 2002; Sundström *et al.*, 1995). Most *dfr* genes are found to reside as gene cassettes within variable parts of

Abbreviations: CS, conserved segment; DHFR, dihydrofolate reductase; TMP, trimethoprim; UTI, urinary tract infection.

integrons, which has resulted in the rapid spread of TMP resistance in various bacteria (Blahna *et al.*, 2006). Of the five known integron classes, class 1 and class 2 integrons have been shown to harbour *dfr* gene cassettes. The *dfrA1*, *dfrA5*, *dfrA7*, *dfrA12*, *dfrA14*, *dfrA17*, *dfrA22* and *dfrA27* gene cassettes have been found to be present within variable regions of class 1 integrons (Blahna *et al.*, 2006; Grape *et al.*, 2005; Kadlec & Schwarz, 2008; Lee *et al.*, 2001; Wei *et al.*, 2009). *dfrA1* and more recently *dfrA14* have been shown to be associated with class 2 integrons (Kadlec & Schwarz, 2008; Márquez *et al.*, 2008).

Escherichia coli is the most frequent Gram-negative bacterium isolated from blood cultures in clinical settings, as well as the most frequent cause of community and hospital-acquired urinary tract infections (UTIs) (Wiles *et al.*, 2008; Yamamoto, 2007). TMP, most commonly in combination with sulfonamides (sulfamethoxazole), is the antimicrobial of choice for the treatment of UTIs and respiratory and gastrointestinal tract infections (Masters *et al.*, 2003). Resistance to TMP among clinical *E. coli* isolates varies greatly, ranging from 10 to 70 % in different parts of the world (Kahlmeter & ECO.SENS, 2003; Lee *et al.*, 2001). The presence of *dfr* gene cassettes within integrons is a potential source of horizontal spread of TMP resistance among bacteria present in different environments, including livestock, where TMP is used for antimicrobial therapy and prophylaxis of food-producing animals (Mathew *et al.*, 2007; Prescott, 2008).

Systematic data on antibiotic consumption and the prevalence of antibiotic resistance at the national level in Lithuania have been lacking until recently. However, several studies have revealed the extensive use of antimicrobials for clinical therapy and in livestock, the high prevalence of self-medication in the country and an increase in antibiotic resistance of some important human pathogens (Beržanskytė *et al.*, 2006; EARSS Management Team, 2008; Grigoryan *et al.*, 2006; Špakauskas *et al.*, 2004). During the period 1994–2002, *E. coli* was isolated as a major infection agent of childhood UTIs in two of the largest children's hospitals in Lithuania, with the prevalence of TMP resistance ranging from 17.5 to 35.5 % (Kaltenis *et al.*, 2003; Pundzienė *et al.*, 2003). Despite these observations, the prevalence of TMP-resistant bacterial isolates from different environments in Lithuania is not clear, and the molecular basis of resistance has not been investigated.

In this study, we analysed TMP resistance in *E. coli* isolates of human and animal origin in Lithuania obtained during the period 2005–2008, the distribution of *dfr* genes and their association with class 1/class 2 integrons and other genetic structures.

METHODS

Bacterial isolates. A total of 231 and 225 *E. coli* isolates of human and animal origin, respectively, were isolated during the period 2005–2008. The bacterial isolates of human origin were collected randomly from diverse units of nine hospitals located in different regions of Lithuania and were recovered from patients with various infections from urine (n=134), cervix, vagina and prostate (n=52), and blood, pus and wounds (n=45). A single isolate per patient was selected before antibiotic treatment.

The bacterial isolates of animal origin were obtained from 23 farms (eight cattle, ten swine and five poultry) from different regions of the country. *E. coli* isolates from healthy animals (cattle, n=45; poultry, n=20) and animals with various disease conditions (cattle, n=53; swine, n=64; poultry, n=43) were selected for further testing. All isolates from healthy animals were obtained from faeces. The isolates from diseased animals were obtained from faeces or from internal organs (poultry). Diarrhoea in all animal species as well as chronic respiratory disease in poultry were the main manifestations of disease. Only one randomly selected isolate per healthy animal (in the case of diseased animals, only one typable or non-typable isolate) from one herd was used for further testing. Samples were obtained before treatment with antimicrobial drugs.

E. coli strains were initially isolated on MacConkey agar (Oxoid). Biochemical properties were determined using Microbact plates and results were interpreted using Microbact 2000 (Oxoid).

Antimicrobial susceptibility testing. Antibiotic susceptibility testing was performed by the disc diffusion (Kirby–Bauer) method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2006). The isolates were interpreted as susceptible or resistant according to the inhibition zone diameter using CLSI recommendations. Intermediate susceptible strains were considered as non-susceptible. Discs were obtained from Oxoid.

The antimicrobials used for the susceptibility testing of *E. coli* clinical isolates were as follows (μg per disc): ampicillin (10 μg), ampicillin–sulbactam (10/10 μg), gentamicin (120 μg), TMP–sulfamethoxazole (1.25/23.75 μg), ciprofloxacin (5 μg), cefuroxime (30 μg), cefotaxime (30 μg), piperacillin–tazobactam (100/10 μg), norfloxacin (10 μg) and nitrofurantoin (300 μg).

The antimicrobials used for the susceptibility testing of *E. coli* isolates of animal origin were as follows (μg per disc): ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), nalidixic acid (30 μg), tetracycline (30 μg), TMP–sulfamethoxazole (1.25/23.75 μg), ciprofloxacin (5 μg), cephalotin (30 μg), florfenicol (30 μg) and ceftiofur (30 μg).

E. coli ATCC 25922 was used as the reference strain.

Preparation of DNA templates. All bacterial DNA templates used in PCR were prepared by boiling bacterial colonies suspended in 100 μl sterile water. The boiled samples were centrifuged briefly and the supernatants used as DNA templates.

Detection of *dfr* genes by multiplex PCR and RFLP. In this study, the modified method of Navia *et al.* (2003) was used, together with newly designed primers for multiplex PCR, targeting all *dfr* genes published to date. The *dfr* gene sequences were downloaded from GenBank, compared and grouped by sequence similarity. The primers were designed for analysis of similar genes of groups A1 (*dfrA1*, *dfrA15*, *dfrA15b*, *dfrA16*, *dfrA16b* and *dfrA28*), A5 (*dfrA5*, *dfrA14*, *dfrA25* and *dfrA27*), A7 (*dfrA7* and *dfrA17*), A12 (*dfrA12*, *dfrA13*, *dfrA21* and *dfrA22*) and B (*dfrB1*, *dfrB2*, *dfrB3*, *dfrB4*, *dfrB5* and *dfrB6*), and for individual screening of the *dfrA3*, *dfrA3b*, *dfrA6*, *dfrA8*, *dfrA9*, *dfrA10*, *dfrA19*, *dfrA20*, *dfrA23*, *dfrA24* and *dfrA26* genes (Table 1).

The PCR mix (25 μl) included 2 μl template DNA, 1× PCR buffer, 1 U *Taq* DNA polymerase (AB Fermentas), 200 μM each dNTP, 1 μM each primer (Table 1) and 2.5 mM $MgCl_2$. DNA amplification

Table 1. Primers for amplification of the *dfr* genes and lengths of amplicon restriction fragments for RFLP

Multiplex group	PCR primer sequence (5′→3′, forward/reverse)	Reference	Amplicon size (bp)	Resistance gene (GenBank accession no.)	Restricted amplicon products (bp) (enzyme)
I	GTGAAACTATCACTAATGG/ACCCTTTTGCCAGATTTG	Navia *et al.* (2003)/This study	471*	*dfrA1* (X00926)	236, 163, 42, 30 (*Tas*I); 471 (*Pvu*I)
				dfrA15 (Z83311)	356, 62, 30, 23 (*Tas*I); 274, 197 (*Alu*I)
				dfrA15b (AF156486)	356, 62, 30, 23 (*Tas*I); 197, 143, 131 (*Alu*I)
				dfrA16 (AF077008)	216, 101, 94, 41, 19 (*Tas*I); 250, 190, 31 (*Tru*I1)
				dfrA16b (AJ131405)	216, 101, 94, 41, 19 (*Tas*I); 190, 155, 95, 31 (*Tru*I1)
				dfrA28 (FN263373)	236, 162, 40, 30 (*Tas*I); 320, 148 (*Pvu*I)
	TTGGGAAGGACAACGCACTT/ACCATTTCGGCCAGATCAAC	Chen *et al.* (2004)	382	*dfrA8* (U09273)	
	GGTGAGCARAAGATYTTTCGC/TGGGAAGAAGGCGTCACCCTC	This study/Navia *et al.* (2003)	309	*dfrA12* (Z21672)	121, 99, 89 (*Alu*I)
				dfrA13 (Z50802)	188, 121 (*Alu*I); 309 (*Msp*I)
				dfrA21 (AY552589)	188, 121 (*Alu*I); 212, 97 (*Msp*I)
				dfrA22 (AJ628423)	201, 108 (*Alu*I)
II	GCBAAAGGDGARCAGCT/TTTMCCAYATTTGATAGC	This study	394	*dfrA5* (X12868)	344, 35, 15 (*Alu*I)
				dfrA14 (Z50804)	204, 175, 15 (*Alu*I)
				dfrA25 (DQ267940)	379, 15 (*Alu*I)
				dfrA27 (FM957879)	208, 118, 53, 15 (*Alu*I)
	AAAATTTCATTGATTTCTGCA/TTAGCCTTTTTTCCAAATCT	This study/Navia *et al.* (2003)	471	*dfrA7* (X58425)	268, 203 (*Alu*I)
				dfrA17 (AF169041)	216, 203, 52 (*Alu*I)
	TTTATTGTGGTAAGCAATAC/GTATACATCTGCATCAAAAC	This study	201	*dfrA3b* (AY162283)	
III	ACCTGCCGATCTGCGTCAT/TCGCAGGCATAGCTGTTCTT	Chen *et al.* (2004)	387	*dfrA3* (J03306)	
	ACCAGAGCATTCGGTAATCA/TTGGATCACCTACCCATAGA	Chen *et al.* (2004)	445	*dfrA10* (M69220)	
	CACAGTCTATCGCCTTAATC/ATAGACCACAAAGCTAAACG	This study	233	*dfrA26* (AM403715)	
IV	GTTTCCGAGAATGGAGTAAT/GGTACGTGTAATCAATATTTG	Chen *et al.* (2004)	429	*dfrA6* (Z86002)	
	TCACCAAGAAGTCAGAGATT/TAAAACCAGATTCGACTTTC	This study	311	*dfrA24* (AJ972619)	
	AGAATTCCCTTCTCTTTGAT/ATGCCAACAGTTGAGATTAT	This study	218	*dfrA23* (AJ746361)	
V	GATCACGTRCGCAAGAARTC/GACTCGACVGCRTASCCTTC	This study	95	*dfrB1* (U36276)	
				dfrB2 (J01773)	
				dfrB3 (X72585)	
				dfrB4 (AJ429132)	
				dfrB5 (AY943084)	
				dfrB6 (DQ274503)	
	TGAACCAGAAGATTTAAAACAC/AATGGTCGGGACCTCAGAT	This study/Chen *et al.* (2004)	384	*dfrA9* (X57730)	
	AGTCGCTGTGGATTCTAAGT/CAATGTGAAAATTGTTCTGG	This study	455	*dfrA19* (AM234698)	
	ATGATTTGCTTTGGCACTTA/CCACCAATAATGAAGCATGT	This study	250	*dfrA20* (AJ605332)	

*Amplicon size is 468 bp for the *dfrA28* gene.

was carried out in a Mastercycler (Eppendorf) using the following conditions: 2 min initial denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at various temperatures for 30 s [46 °C (for multiplex group I), 44 °C (II), 52 °C (III and IV) and 56 °C (V)] and extension at 72 °C for 30 s. PCR products were fractionated by gel electrophoresis (2 % agarose in 1 × TAE buffer with 0.5 μg ethidium bromide ml^{-1}) and visualized using a UV transilluminator. The length of amplicons was determined by comparing them with a 100 bp ladder (AB Fermentas).

The appropriate restriction enzymes were selected for RFLP analysis of each homology group of all known *dfr* genes to distinguish similar genes within the group (Table 1). The restriction mixture (20 μl) included: 10 μl PCR restriction mixture (~0.1–0.5 μg DNA), 1 × recommended buffer for restriction enzyme and 10 U restriction enzyme (*Alu*I, *Msp*I, *Pvu*I, *Tru*I1 or *Tas*I; AB Fermentas). Restriction products were fractionated by gel electrophoresis (3 % agarose in 1 × TAE buffer with 0.5 μg ethidium bromide ml^{-1}) and the length of the restriction products was determined as above.

Determination of *dfr* gene localization. The localization of *dfr* genes within integrons was determined as follows. Isolates containing *dfr* genes were subjected to PCR to amplify the junction between the 5′ conserved segment (5′CS) of the class 1/class 2 integrons and the appropriate *dfr* gene. Forward primers used in this study were FINT-1 (5′-GGCATCCAAGCAGCAAG-3′) for the class 1 integron and FINT-2 (5′-CCGGACGGCATGCACGATTTG-3′) for the class 2 integron. The reverse primers complementary to DNA sequences of the *dfr* genes are listed in Table 1. Annealing temperatures used were 49 and 51 °C for the class 1 and class 2 integrons, respectively. The length of amplicons varied depending on the position of the *dfr* gene cassette within the variable region of the integrons.

The integration of *dfrA14* within the *strA* gene was determined by amplification of junction regions. The primer F1APH-3 (5′-CTTGGTGATAACGGCAATTCC-3′) and the *dfrA14* reverse primer (Table 1) were used for amplification of the junction region located upstream of *dfrA14*. The primer R1APH-3 (5′-CCAATCGCAGAT-AGAAGGCAA-3′) and the *dfrA14* forward primer (Table 1) were used for amplification of the junction region located downstream of *dfrA14*.

Conjugation experiments. Conjugation experiments were performed by mating *E. coli* harbouring the *dfrA14* gene as a donor strain with rifampicin-resistant *E. coli* HK225 as recipient strain at a donor : recipient ratio of 1 : 5 as described by Kadlec & Schwarz (2008). Transconjugants were selected on Luria–Bertani agar plates containing rifampicin (100 mg l^{-1}) and TMP (10 mg l^{-1}).

Statistical methods. Differences in the prevalence of antibiotic resistance between different groups were assessed by a χ^2 or Fisher's exact test. A P value of <0.05 was considered to be statistically significant. Statistical analyses were performed using GraphPad Prism software.

RESULTS AND DISCUSSION

TMP-resistant *E. coli* isolates of human and animal origin

A total of 456 *E. coli* isolates of human and animal origin obtained during the period 2005–2008 in Lithuania were tested for antibiotic susceptibility by the disc diffusion method. Of these, 112 (25 %) were found to be TMP resistant. Table 2 shows the frequency of resistance to TMP among the different groups of isolates. Overall, 23 % of human clinical isolates were TMP resistant. Statistical analysis showed that there was no significant difference in the frequency of TMP-resistant human clinical isolates according to the origin. The resistance rate for clinical *E. coli* isolates was comparable to that found in other European countries: Germany (22.5 %), Ireland (22.1 %), Portugal (26.7 %) and Spain (25.1 %), as observed in the ECO.SENS study (Kahlmeter & ECO.SENS, 2003). According to the same study, the proportion of TMP-resistant *E. coli* was found to be lowest in Nordic countries and Austria (5.5–9.5 %). The frequency of TMP resistance among animal isolates was 23, 33 and 40 % for diseased swine, poultry and cattle, respectively. Within the healthy animals, 9 % of isolates from poultry and 20 % of isolates from cattle were TMP resistant (Table 2). The observed TMP resistance rate among isolates recovered from

Table 2. TMP resistance and distribution of the *dfr* genes in *E. coli* isolates of human and animal origin

Origin of isolates (*n*)	No. (%) of TMP-resistant isolates	No. (%) of isolates with *dfr* genes							No. (%) of *dfr*-negative isolates
		All	*dfrA1*	*dfrA5*	*dfrA8*	*dfrA12*	*dfrA14*	*dfrA17*	
Human clinical specimens									
Urine (134)	35 (26)	34* (100)	13* (38)	3 (9)	3 (9)	1 (3)	1 (3)	14* (41)	1 (3)
Cervix, vagina, prostate (52)	11 (21)	11 (100)	3 (27)	1 (9)	3 (27)	–	2 (18)	2 (18)	–
Blood, pus, wounds (45)	8 (18)	8 (100)	1 (13)	1 (123)	–	1 (13)	2 (25)	3 (38)	–
Healthy animals									
Cattle (45)	4 (9)	3 (75)	2 (67)	–	–	–	1 (33)	–	1 (25)
Poultry (20)	4 (20)	4 (100)	1 (25)	–	–	2 (50)	1 (25)	–	–
Diseased animals									
Cattle (53)	21 (40)	21 (100)	13 (62)	–	1 (5)	–	6 (29)	1 (5)	–
Swine (64)	15 (23)	12 (100)	3 (25)	–	6 (50)	1 (8)	2 (17)	–	3 (20)
Poultry (43)	14 (33)	10 (100)	6 (60)	1 (10)	–	–	3 (30)	–	4 (29)

*The *dfrA1* gene and *dfrA17* gene were both found in one isolate.

diseased cattle and swine was comparable to that reported for some countries in the Baltic region such as Finland and Sweden (Hendriksen *et al.*, 2008a, b; Kahlmeter & ECO.SENS, 2003). However, a comparison of our findings with geographical areas closer to Lithuania was not possible due to a lack of epidemiological data from neighbouring countries. *E. coli* from diseased cattle showed a significantly higher TMP resistance rate compared with that for indicator bacteria recovered from healthy cattle (40 vs 9 %, $P < 0.05$). Similar findings were reported in a complex study on antibiotic resistance among bacterial pathogens and indicator bacteria in animals in Europe, where the prevalence of antibiotic-resistant *E. coli* strains from diseased pigs was higher than that from healthy pigs (Hendriksen *et al.*, 2008b). The observed difference in resistance rate is thought to be due partially to the sampling methodology, when isolates recovered from diseased animals generally represent cases of severe disease or treatment failure, possibly resulting in the emergence of resistant pathogens during previous (unsuccessful) therapy.

Identification of *dfr* genes

We combined multiplex PCR and RFLP methods to search for the 33 currently known *dfr* genes in selected TMP-resistant *E. coli* isolates (Table 1) (Agersø *et al.*, 2006; Grape *et al.*, 2007b; Wei *et al.*, 2009). By using this approach, we were able to determine the prevalence and distribution of integron-borne *dfr* genes and those not carried within known mobile elements in a large collection of isolates of human and animal origin. Of the 112 TMP-resistant *E. coli* isolates, 103 harboured one of the following genes: *dfrA1*, *dfrA5*, *dfrA8*, *dfrA12*, *dfrA14* and *dfrA17*. The distribution of *dfrA* gene variants among different groups of isolates is presented in Table 2.

The *dfrA17* and *dfrA1* genes were detected most frequently. One clinical isolate from urine harboured a combination of *dfrA1* and *dfrA17* genes (Table 2). The *dfrA1* gene prevailed in TMP-resistant isolates of animal origin, followed by the *dfrA14* gene, whereas the *dfrA1* and *dfrA17* genes dominated in clinical isolates. Only a single isolate of animal origin (diseased cattle) harboured *dfrA17*. The latter observation indicates the difference in acquired *dfrA17*-mediated TMP resistance between *E. coli* strains of human and animal origin. Our results obtained for clinical isolates are consistent with recent European–Canadian and Swedish studies, in which a prevalence of *dfrA1* and *dfrA17* genes in *E. coli* isolates from UTIs was observed (Blahna *et al.*, 2006; Grape *et al.*, 2007a).

Unexpectedly, we observed that the *dfrA14* gene was among the most prevalent *dfr* genes in TMP-resistant *E. coli* animal isolates, in contrast to recent reports on the rare incidence of class 1 integron-associated *dfrA14* in TMP-resistant *E. coli* strains of animal origin in Germany and Portugal (Kadlec & Schwarz, 2008; Machado *et al.*, 2008).

The remaining *dfr* genes identified in this study (*dfrA5*, *dfrA8* and *dfrA12*) were less prevalent and showed different distribution patterns within the groups of isolates (Table 2). Nine TMP-resistant isolates (eight isolates of animal origin) were negative for the *dfr* genes tested.

Association of *dfr* genes with class 1 and class 2 integrons

To investigate the association of *dfr* genes with integrons, we amplified the junction of the 5′CS of the class 1/class 2 integrons and *dfr* genes that were identified in this study and are known to be located within the variable regions of integrons (*dfrA1*, *dfrA5*, *dfrA12*, *dfrA14*, *dfrA17*; Blahna *et al.*, 2006; Grape *et al.*, 2005; Kadlec & Schwarz, 2008). The PCR generally yielded amplicons of 590 and 640 bp, indicating that the *dfr* genes were situated in the first gene cassettes within the variable parts of the class 1 and 2 integrons, respectively. In one case (an isolate from diseased poultry), however, the junction of the class 1 integron and the *dfrA1* gene was amplified with a length of 1940 bp, suggesting that the *dfr* gene cassette was located further downstream within the variable region of the integron.

The association with class 1/class 2 integrons was confirmed for 73–100 % of the *dfr* genes found in most groups of isolates, except for the isolates from diseased swine, where only four out of 12 identified *dfr* genes (33 %) were present on integrons (Table 3). All *dfrA1*, *dfrA5*, *dfrA12* and *dfrA17* genes identified in this study were found as cassettes within integrons. One human clinical isolate recovered from urine carried *dfrA1* in a gene cassette on class 1 and class 2 integrons. Another isolate of this group contained the *dfrA1* and *dfrA17* genes in cassettes on class 2 and class 1 integrons, respectively.

Analysis of the *dfrA14* genes showed their diverse location in isolates obtained from different sources. Thus, all *dfrA14* genes in clinical isolates were found on either a class 1 or a class 2 integron, whereas only seven out of 13 *dfrA14* genes (54 %) in isolates of animal origin were integron-associated, all of them with class 1, as determined by amplification of the junction between the 5′CS and the *dfrA14* gene (Table 3). To our knowledge, this is the first study showing the presence and the frequency of class 1 integron-associated *dfrA14* in clinical *E. coli* isolates. Notably, the incidence of *dfrA14* in class 2 integrons appears to be rare, as only one report on the presence of such a structure in a class 2 integron carried by an *E. coli* isolate from the urinary tract has been published (Márquez *et al.*, 2008).

Further PCR analysis of the full-length variable regions of *dfrA14*-positive class 1 integrons using primers annealing to the 5′CS and 3′CS yielded no products with the exception of two isolates (diseased cattle), suggesting the absence of the 3′CS in the majority of isolates (data not shown). A similar finding has been reported previously (Sunde, 2005) and was supported by the recent determination of the nucleotide sequence of a defective *dfrA14*-

Table 3. Distribution of *dfr* gene cassettes associated with class 1 and class 2 integrons in *E. coli* isolates of human and animal origin

Origin of isolates carrying *dfr* genes (*n*)	No. of isolates carrying *dfr* gene cassettes within integrons								No. of isolates carrying *dfr* gene not associated with integrons	
	All (%)	Class 1					Class 2			
		dfrA1	*dfrA5*	*dfrA12*	*dfrA14*	*dfrA17*	*dfrA1*	*dfrA14*	*dfrA8*	*dfrA14*
Human clinical specimens										
Urine (34)	29 (85)	6*	3	1	1	14†	8*†	–	3	–
Cervix, vagina, prostate (11)	8 (73)	2	1	–	1	2	1	1	3	–
Blood, pus, wounds (8)	8 (100)	1	1	1	–	3	–	2	–	–
Healthy animals										
Cattle (3)	3 (100)	2	–	–	1	–	–	–	–	–
Poultry (4)	4 (100)	–	–	2	1	–	1	–	–	–
Diseased animals										
Cattle (21)	18 (86)	10	–	–	4	1	3	–	1	2
Swine (12)	4 (33)	1	–	1	–	–	2	–	6	2
Poultry (10)	8 (80)	5	1	–	1	–	1	–	–	2

**dfrA1* gene cassette within class 1 and class 2 integrons in one urinary isolate.
†*dfrA1* gene cassette within class 2 and *dfrA17* gene cassette within class 1 integron in one urinary isolate.

containing class 1 integron carried by the plasmid pK245 in a multidrug-resistant *Klebsiella pneumoniae* isolate (Chen *et al.*, 2006).

DNA sequencing of the variable regions of integrons confirmed the location of the *dfr* genes observed in this study. The *dfrA1*, *dfrA17* and *dfrA12* gene cassettes were found to be present within the same type of class 1 or class 2 integron in *E. coli* isolates of human and animal origin, suggesting a horizontal spread of certain integron types among *E. coli* present in different ecological niches in Lithuania (J. Povilonis, V. Šeputienė, M. Ružauskas, R. Šiugždinienė, M. Virgailis, P. Žlabys, A. Pavilonis and E. Sužiedėlienė, unpublished data).

According to several studies, the rates of resistance to TMP among clinical bacterial isolates are increasing despite the decreased use of TMP or co-TMP in clinical settings (Kärpänoja *et al.*, 2008; Sörberg *et al.*, 2002). Thus, the proportion of TMP-resistant *E. coli* urinary isolates in Europe rose from 0 to 5 % before 1990, and to 9–26 % in 2003 (Kahlmeter & ECO.SENS, 2003). The persistence of and even increase in TMP resistance among bacterial pathogens is thought to be facilitated by the common presence of *dfr* gene cassettes within integrons and their spread among bacteria present in different environments (e.g. livestock), where specific antibiotic pressure supporting the selection of such genetic structures could still be high (Blahna *et al.*, 2006; Cocchi *et al.*, 2007).

The trends in TMP consumption and resistance of bacterial pathogens or indicator bacteria were not investigated in Lithuania; however, the high prevalence of integron-associated *dfr* genes in TMP-resistant *E. coli* isolates of different origins and TMP resistance rates, similar to those in other European countries, show that a pool of transferable resistance determinants has already been established and disseminated.

Identification of *dfr* genes not associated with integrons

We analysed further the location of *dfrA14* genes for which the association with integrons was not confirmed. For this purpose, we attempted to amplify the junction region between the *dfrA14* and *strA* genes, which has been reported to be the integration site for *dfrA14* in a resistance plasmid carried by uropathogenic *E. coli* (Ojo *et al.*, 2002). The PCR using DNA from the six isolates of animal origin (two isolates each from diseased cattle, swine and poultry) yielded amplicons of ~780 bp (downstream of the *dfrA14* gene) and ~760 bp (upstream of the *dfrA14* gene), confirming disruption of the *strA* gene by the insertion of a 570 bp DNA fragment containing the *dfrA14* gene. The gene arrangement Δ*strAdfrA14*Δ*strA* in these isolates closely resembled one residing within a small transferable plasmid, pSTOJO1 (6.8 kb), isolated from uropathogenic *E. coli* in Nigeria, which has been shown to lack integron structures (Ojo *et al.*, 2002). We confirmed the conjugal transfer of the Δ*strAdfrA14*Δ*strA* arrangement at a frequency of 5×10^{-4} c.f.u. per recipient cell. Previous studies have shown that plasmids carrying *sul2–strA* genes are widespread among Gram-negative bacteria and are capable of accepting other resistance genes, such as *catA3* and *dfrA14*, to form new resistance gene clusters in a response to antibiotic selective pressure (Kehrenberg *et al.*, 2003).

There is a group of *dfr* genes that has been shown not to be associated with integrons and is disseminated by other mechanisms such as being part of the common region (*dfrA3b*, *dfrA9*, *dfrA10*, *dfrA19*, *dfrA20*, *dfrA23* and *dfrA26*), unknown genetic structures (*dfrA3*, *dfrA24*) or transposons (*dfrA8*) (Sundström *et al.*, 1995; Toleman *et al.*, 2006). In this study, the *dfrA8* gene was identified in half of the *dfr*-positive isolates recovered from diseased swine (6/12), as well as in human clinical isolates obtained from urine (three isolates) and cervix (three isolates). Of all the *dfr* genes observed in this study, the *dfrA8* gene was found to be neither class 1 nor class 2 integron-associated. This finding is consistent with the observations that *dfrA8* does not appear to be a recombination substrate for integrases but has been assembled into the plasmid-borne transposon Tn*5091* (Sundström *et al.*, 1995). Only fragmented studies on the prevalence of *dfrA8* among *E. coli* isolates in Europe and in other countries have been conducted, showing considerable variation (Pallecchi *et al.*, 2007; Sundström *et al.*, 1995).

In summary, this study showed that integron-associated *dfr* genes are in most part responsible for the TMP resistance of *E. coli* isolates of human and animal origin in Lithuania. However, the resistance of some isolates is mediated by cassette-independent *dfrA8* and also by *dfrA14* present in a non-integron structure, responsible for resistance spread through a conjugative plasmid. For this reason, integron-associated DHFRs seem not to be unique and single players contributing to the current TMP resistance.

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