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Characterisation of streptomycin resistance determinants in Lithuanian *Escherichia coli* isolates

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Genotypic analysis of streptomycin resistance in Lithuanian *Escherichia coli* isolates from humans, calves and pigs has been performed. *E. coli* isolates showed a high frequency (~80%) of streptomycin (Sm) resistance. Sixty nine Sm-resistant isolates from humans (n=53), calves (n = 12) and pigs (n = 4) were characterized with respect to genetic determinants encoding Sm-resistance and their diversity. Several different resistance patterns were observed. The genes *strA+str*B dominated in human isolates (47.2%), whereas the gene *aadA1* prevailed in animal isolates (37.5%); 50% of strains of animal origin contained a multiple *strA+strB+aadA* (*aadA1* or *aadA1+aadA2*) resistance pattern. The study gives the baseline information on the magnitude of the Sm resistance problem and its genetic background in contemporary Lithuanian *E. coli* of animal and human origin.

Key words: *Escherichia coli*, streptomycin resistance, *strA*, *strB*, *aadA*

INTRODUCTION

Microbial resistance to aminoglycosides has increased among *Escherichia coli* and *Salmonella enterica* isolates in Europe over the recent years [1]. A comparison of the data on the mechanisms of aminoglycoside resistance in bacteria isolated from various regions of the world over a period of almost 30 years has revealed that they became more complex over time [2]. Resistance towards Sm is the most frequent phenotype in isolates of *Escherichia coli* of both human and animal origin [3].

Four genes have been shown to be responsible for cofactor-dependent Sm modification: *aph(3")-Ib* (synonymous with *strA*) encodes the phosphoryltransferase APH(3")-Ib, *aph(6)-Id* (synonymous with *strB*) encodes the phosphoryltransferase APH(6)-Id, *ant(3")-Ia* (synonymous with *aadA*1) and *ant(3")-Ib* (synonymous with *aadA2*) encode adenyltransferases ANT(3")-Ia and Ib respectively [4]. The prevalence of Sm resistance determinants in Lithuanian *E. coli* isolates from humans and animals has not been studied to date. The aim of the present study was to determine the genetic background for Sm resistance in Lithuanian isolates.

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MATERIALS AND METHODS

Bacterial strains. Lithuanian isolates of *E. coli* from 2004 to 2005 were obtained from the pathological material of cattle (n = 16) and pigs (n = 5). Clinical *E. coli* isolates (n = 63) from humans were recovered mostly from patients with urinary tract infections (*E. coli*) in 2005. MacConkey Agar (Oxoid, United Kingdom) was used for the initial isolation of *E. coli*. Biochemical properties were determined by Microbact plates (Oxoid). Results were interpreted using Microbact 2000 (Oxoid).

Antibiotic resistance testing. The agar diffusion method according to CLSI (formerly NCCLS) guidelines [5] was applied for antimicrobial susceptibility testing. Mueller Hinton Agar (Oxoid, England) was used as the testing medium. An MSI-5 turbidometer (Latvia) was used for determination of optical turbidity. Standard discs of Streptomycin (10 μg) were used (Oxoid).

Polymerase chain reaction. Primers (Metabion, Germany) used for PCR analysis are listed in Table 1. Multiplex PCR (reaction I for amplification of *aadA1* and *strB* and reaction II for amplification of *aadA2* and *strA*) was carried out in a thermocycler (Eppendorf) for 30 cycles with denaturation at 94 °C for 30 s, annealing at 54 °C for 1 min and polymerization at 72 °C for 1 min. The PCR

Table 1. **Primers used in PCR amplification**

Gene	Primer	Sequence 5'-3'	Amplicon size, bp	Multiplex group	Source
aadA1	FANT-3	CGC CGA AGT ATC GAC TCA AC	559	I	[20]
	RANT-3	GCG GGA CAA CGT AAG CAC TA			
strB	FAPH -6	ATC GTC AAG GGA TTG AAA CCT A	510	I	[14]
	RAPH -6	GGA TCG TAG AAC ATA TTG GCG			
aadA2	FAAD2	GCT CAA TGA CCT TAT GAA GGC	379	II	This study
	RANT-3	GCG GGA CAA CGT AAG CAC TA			
strA	F1APH-3	CTT GGT GAT AAC GGC AAT TCC	547	II	[14]
	R1APH-3	CCA ATC GCA GAT AGA AGG CAA			

reaction mix (25 µl) and the primers' concentrations were as recommended by the *Taq* polymerase supplier (AB Fermentas). The DNA template for PCR (1 µl) was prepared as followed: a single colony was picked and resuspended in 200 µl of distilled water, the suspension was boiled for 5 min, and the supernatant was collected after centrifugation for 2 min. The amplification products were visualized by 2% agarose gel electrophoresis and etidium bromide staining to assess the size of the amplicons.

RESULTS AND DISCUSSION

The increasing bacterial resistance to antimicrobials is becoming a worldwide problem. On the basis of the European Community Strategy against Antimicrobial Resistance, EU has approved and regularly updates the recommendations which prompt to collect data on antibiotic consumption and the spread of resistant bacteria, pathogenic to humans and animals, in EU member states (http://www.eurosurveillance.org/em/v09n01/0901–221.asp). The rapid economic growth of Lithuania (elevated production of food, consumption of medications, antimicrobials), changes in migrational activity, including expansion of recreation geography set the stage for the emergence of new resistant animal and human bacterial pathogens.

Systematic studies on the prevalence of antimicrobial resistance and epidemiological situation in human and veterinary medicine in Lithuania are lacking. In the few investigations on the consumption of some antibiotics used in clinical practice and the resistance level of human pathogens, conducted five to ten years ago, no data concerning streptomycin resistance of Lithuanian bacterial isolates have been presented [6, 7]. Such information as well as the data on the molecular basis of streptomycin resistance determinants would by highly valuable in evaluation of the present epidemiological situation in the country.

In this study, Sm resistance was determined in 76% of animal *E. coli* isolates and 87% of human *E. coli* isolates. The observed Sm resistance level in Lithuanian *E. coli* isolates of human and animal origin is considerably higher than that found in other European regions. Thus, according to a recent study conducted in several European countries, the Sm resistance in *E. coli* isolates from chickens was ~ 50%, from cattle – varied from zero to 15.3% and from pigs from 10.7% to 54.2% [1, 8, 9]. Streptomycin resistance of *E. coli* isolates from humans in the UK was found to be 41% [10].

In order to determine the genetic background for streptomycin resistance among Lithuanian *E. coli* isolates, all strains were analyzed for the presence of *strA*, *strB*, *aadA1* and *aadA2* genes (Figure) by multiplex PCR as described in Materials and Methods.

The *strA strB* genes dominated in isolates of human origin (n = 25, 47.2%) (Table 2). As in the original report [11], the two genes in most cases were located together. It has been suggested that both genes are needed to achieve streptomycin resistance [12].

The combination of *strA strB* genes in Lithuanian animal isolates was rare (n = 1), in contrast to German *E. coli* isolates from cattle, pigs and poultry. In this study the *strA strB* tandem was widely spread (>60%) [8]. Instead, the single gene *aadA1* was determined with a highest frequency (37.5%) in isolates of Lithuanian *E. coli* strains from animal samples.

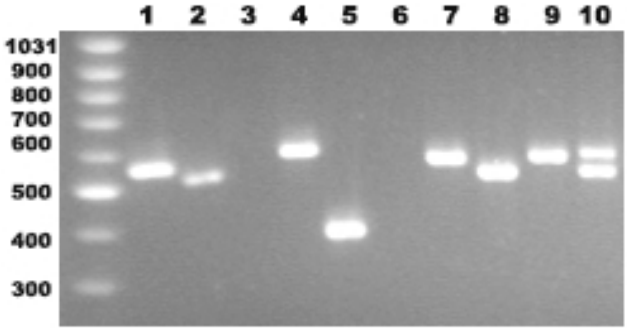

Figure. Amplicons from multiplex PCR reactions of streptomycin resistance genes from five representative *E. coli* isolates. Multiplex PCR was performed as described in Materials and Methods: reaction I for detection of *strA* and *aadA2* (even lane numbers), reaction II for *strB* and *aadA1* (uneven lane numbers). #1 isolate containing *strA* (547 bp, lane 1), *strB* (510 bp, lane2); #2, containing *aadA1* (559 bp, lane 4); #3, containing *aadA2* (379 bp, lane 5); #4, containing *strA* (547 bp, lane 7), *strB* (510 bp, lane 8); #5, containing *strA* (547 bp, lane 9), *strB* (510 bp, lane10), *aadA1* (559 bp, lane 10)

Table 2. **Presence of genes *aadA1*, *aadA2*, *strA* and *strB* in Lithuanian isolates of streptomycin-resistant *E. coli***

Gene(s)	No. of strains	
	animals	humans
aph(3")Ib (strA)	–	3 (5.6 %)
aph(6)Id (strB)	–	1 (1.9 %)
aph(3")Ib aph(6)Id (strA strB)	1 (6.25%)	25 (47.2 %)
aph(3")Ib aph(6)Id ant(3")Ia (strA strB aadA1)	3 (18.75 %)	13 (24.5 %)
ant(3")Ia (aadA1)	6 (37.5 %)	5 (9.4 %)
ant(3")Ib (aadA2)	–	2 (3.8 %)
ant(3")Ia ant(3")Ib (aadA1 aadA2)	1 (6.25 %)	2 (3.8 %)
aph(3")Ib aph(6)Id ant(3")Ia ant(3")Ib (strA strB aadA1 aadA2)	5 (31.25 %)	–
aph(3")Ib ant(3")Ia (strA aadA1)	–	1 (1.9 %)
aph(3")Ib aph(6)Id ant(3")Ib (strA strB aadA2)	–	1 (1.9 %)
Total	**16 (100 %)**	**53 (100 %)**

A quadruple gene combination of *strA strB aadA1 aadA2* was detected in five isolates from animals (31.25%). Adenyltransferases ANT(3")-Ia and Ib (encoded by *aadA1* and *aadA2*, respectively) modify spectinomycin and streptomycin resulting in resistance to an extended spectrum of aminoglycosides [13]. The gene combination of *strA strB aadA1 aadA2* was not detected in isolates of human origin.

A combination of genes *strA strB* and *aadA1* was detected at similar frequency in isolates of animal and human origin instead (18.75 and 24.5%, respectively). In total, 50% of Sm-resistant *E. coli* isolates of animal origin carry the combination of *strA strB aadA* (*aadA1* or *aadA1* + *aadA2*). In similar studies, the genes *strA strB aadA* were determined at a frequency of 2% in *E. coli* (Germany), 6.6% in *E. coli* (Norway) and 13% in *Salmonella* (Denmark) in Sm-resistant isolates of animal origin [8, 9, 14].

Such a diversity of the streptomycin resistance determinants could result from a distinct transferable genetic source. The *strA* and *strB* genes are usually associated with the Tn5393-like transposons that reside on large conjugative plasmids and with the small nonconjugative plasmid RSF1010 that carries this tandem [15]. In particular, *aadA1* usually was the most prevalent gene detected in class 1 integrons – DNA elements which may contain transferable antimicrobial resistance genes [8, 9].

A recent study has shown that the genes encoding resistance to streptomycin can greatly influence the distribution of streptomycin MICs (minimum inhibitory concentration) among *E. coli* [9]. The study further indicated that the *strA strB* genes mediate substantially higher MICs than the *aadA* gene cassettes (the authors did not discriminate between *aadA1* and *aadA2*). Strains harboring both the *strA strB* and *aadA* gene cassettes had higher MICs than strains harboring only the *strA strB* [9].

It is widely supposed that reduction or cessation in the use of particular antibiotics will diminish the prevalence of resistance. There is evidence that this may be the case in some instances: a restriction in macrolide prescription in Finland, for example, was followed by a reduction in erythromycin resistance among group A streptococci [17]. In contrast, however, the persistence of resistance to sulphonamides in *E. coli* in the UK in the period 1999–2004 was demonstrated [10]. Most studies showed a strong association of sulphonamide resistance with resistance to streptomycin. This is perhaps to be expected, given that the *sul2* gene (coding the resistance to sulphonamides) is often found adjacent to the resistance genes *strAB* [18], while *sul1* (coding for resistance to sulphonamides) is often associated with *aadA* gene cassettes in type 1 integrons [8, 16]. In our study eight different type 1 integrons were detected in *E. coli* isolates of clinical and animal origin, and their DNA sequence has been determined. In six cases *sulI* was associated with *aadA1* or *aadA* [19].

Streptomycin, like the sulphonamides, has a limited clinical use presently and it is not obvious why resistance to either class of drugs should have persisted. The possible explanations for the persistence of sulphonamide and streptomycin resistance include the properties of the mobile elements on which the determinants are carried, and the potential selection pressures other than in human medical use. The non-human use of antimicrobial agents in agriculture may also be important. Streptomycin was used extensively in veterinary medicine for a very long time. It is also among the antimicrobials most frequently used for the treatment of cattle, pigs, dogs and other animals.

Our results show that Sm resistance in Lithuanian *E. coli* isolates from humans and animals is high and has a complex genetic pattern. The obtained information will be used in further studies of the molecular mechanisms of Sm resistance. It also highlights the need of the implementation of programmes of aprudent usage of antimicrobials both in human and animal treatment in the country.

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GENETINIŲ DETERMINANČIŲ, LEMIANČIŲ LIETUVOJE PAPLITUSIŲ ŽMOGUI IR GYVŪNAMS PATOGENIŠKŲ *Escherichia coli* ATSPARUMĄ STREPTOMICINUI, TYRIMAS

S a n t r a u k a

80% patogeninių *Escherichia coli* padermių, išskirtų iš veterinarinių ir klinikinių pavyzdžių, buvo būdingas atparumas streptomicinui. 69 padermėse, išskirtose iš žmonių (n = 53), galvijų (n = 12) ir kiaulių (n = 4), buvo nustatytos atsparumą streptomicinui koduojančios genetinės determinantės. Padermėse, išskirtose iš klinikinių pavyzdžių, vyrauja *strA+strB* (47,2%) genai, padermėse iš veterinarinių pavyzdžių – pavienis *aadA1* genas (37,5%) ir daugybinės determinantės *strA + strB + aadA* (*aadA1* arba *aadA1 + aadA2*) (50%). Šie tyrimai leidžia įvertinti Lietuvoje paplitusių žmogui ir gyvūnams patogeniškų *E. coli* būdingą atsparumo streptomicinui mastą ir pateikia pirminę informaciją apie šiuo metu vyraujančių padermių atsparumo genetines determinantes.