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INVESTIGATION OF THE RESISTANCE GENE PRESENCE IN PLASMIDS ISOLATED FROM *E. COLI* AND *SALMONELLA* STRAINS ORIGINATING FROM DOMESTIC ANIMALS BY THE METHOD OF PLASMID DNA TRANSFORMATION INTO COMPETENT CELLS

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The investigation of the presence of plasmids was performed on E. coli and Salmonella strains, originating from dogs, cats, cattle, pigs and poultry. The investigated bacterial strains were isolated from ear and skin swabs, vaginal swabs, faeces and urine, obtained from healthy and diseased animals of various age and breed categories. Up to now 45 E. coli and 35 Salmonella strains have been isolated, but the presence of plasmids was investigated in 42 strains of bacteria resistant to at least 3 or more antibiotics (23 E.coli and 4 Salmonella strains). The plasmids were isolated from 2 E. coli strains originating from dogs, 4 E. coli strains originating from cattle, 2 E.coli strains originating from pigs, 1 E. coli strain originating from a cat and 2 Salmonella strains originating from poultry. The obtained approximate size values of isolated plasmids from investigated strains, expressed in base pairs were from 2,4 kb to 17,5 kb. After the transformation process was completed and the E. coli DH 5α transformants streaking was done on LB agar with added antibiotics, the visible growth of transformed bacteria meant that the competent cells have received DNA (plasmid) with the resistance gene for the antibiotic. Lack of growth in transformants meant that the resistance gene was to be found on the chromosome.

Key words: E.coli, Salmonella, plasmid

INTRODUCTION

According to definition, bacterial resistance to antibiotics is a capability of microorganisms to resist the effects of antibiotics and continue to propagate freely in their presence (Walsh, 2003). Natural resistance to antibiotics is a genetic characteristic of all strains of a certain species of bacteria which exists independently from previous contact with a certain antibiotic. The acquired resistance in bacteria is always a consequence of genetic changes which occur after chromosome mutations or acquisition of the resistance gene. This resistance can be chromosomal and extra-chromosomal. Chromosomal resistance occurs as a consequence of spontaneous mutations in the gene locus inside the

chromosome which controls the sensitivity to the given antibiotic. Extra-chromosomal resistance occurs by horizontal transportation of the resistance gene by way of mobile genetic elements: plasmids, transposons, integrons and insertion sequences (Rowe-Magnus and Mazel, 2002). Plasmids are usually defined as stable, extra-chromosomal, covalently closed, circular DNA molecules (Hardy, 1987). They have a great significance in the adjustment and survival of bacteria during evolution in different environmental conditions. Their size can vary from 300 to 400.000 base pairs, although plasmids the size of chromosomes have also been discovered. Plasmids have been isolated from a wide range of bacterial species, both from Gram positive and Gram negative bacteria. Beside this, they have been discovered in yeasts and mould cells (Krnjaić, 2000). Phenotypic characteristics of bacteria connected with the presence of plasmids are resistance to antibiotics, disinfectants and hard metals, toxin and antibiotic production, hydrogen sulphide production, virulence factor existence, specific antigen construction, etc. Plasmids which carry resistance genes (R gene) are labeled R plasmids. All mobile genetic elements can be translocated onto other regions of DNA by mechanisms of conjugation, transformation and transduction (Freter and Brickner, 1983). Transformation is a process during which the liberated DNA from the lysed bacterium arrives to the recipient cell. The recipient cell is called “competent cell” because it possesses the conditions to pass DNA through its cellular wall into the cytoplasm. In natural conditions there is a very small number of competent cell species capable to receive foreign DNA, (e.g. *Streptococcus pneumoniae, Bacillus subtilis* and *Haemophilus influenzae),* so that the process of transformation in natural conditions is an extremely rare occurrence. For transformation in *in vitro* conditions laboratory prepared competent cells are used, most frequently strains of *E. coli* or *Salmonella,* previously treated with $CaCl_2$ with the aim of expanding pores in their cellular walls, in order to transform large segments of DNA through the newly formed apertures. Competent cells can be created also by applying electricity during the process known as "electroporation", by which the cellular wall pores’ radiuses are expanded so that DNA can pass through them. Plasmids have a great significance in genetic engineering because they are used as vectors for gene insertion inside DNA walls. Beside that, they are important for monitoring resistance gene movements among bacteria in natural conditions.

MATERIALS AND METHODS

Investigated bacterial strains

The investigation of plasmid presence was performed on *E. coli* and *Salmonella* strains, originating from dogs, cats, cattle, pigs and poultry. The investigated bacterial strains were isolated from ear and skin swabs, vaginal swabs, faeces and urine, obtained from healthy and diseased animals of various age and breed categories.

Up to now 45 *E. coli* and 35 *Salmonella* strains have been isolated, and the isolation was performed from clinical material received by the Microbiology

Department laboratory from 2004 to 2005. A certain number of *E. coli* and *Salmonella* strains included in the investigation were isolates obtained from clinical materials which were sent to the Microbiology Department by various scientific and specialized veterinary institutes of Serbia.

Media and reagents

A large number of conventional bacteriological media and biochemical reactions were used in order to isolate and identify the investigated bacterial strains. Specific polyvalent diagnostic sera (produced by "Milan Jovanovic Batut" and Becton Dickinson) were utilized for *Salmonella* serological typing. Mueller Hinton agar (BioLab) and antibiogram discs (Becton Dickinson) were used to investigate antibiotic sensitivity of isolated bacterial strains, and *Salmonella* and *E. coli* strains sensitivity for ampicillin, amoxicillin with clavulic acid, cefalexin, ceftriaxone, gentamicine, chloramphenicol, tetracycline, ciprofloxacine and sulfamethoxasole+trimethoprim. Cultures of investigated 12 hours old strains, cultivated in Luria Bertani broth with tetracycline in a concentration of 15 μg/mL, or ampicillin in a concentration of 100 μg/mL, were prepared for extraction of plasmids. The solutions used for this purpose were: 5x concentrated TBE buffer, neutral phenol, 100x concentrated TE buffer, Ribonuclease A (Serva); P I solution (50mM Tris x HCl pH 8, 10mM EDTA pH 8, 100 μg/mL RNAse A); P II solution (0.2M NaOH, 1% Sodium dodecil sulphate); P III solution (3M Potassium acetate pH 5,5); P IV solution (100mM Sodium acetate pH 6); lysing buffer (2 mg lysosyme in 1 mL of water) and absolute ethyl alcohol. pH calibration in the prepared solutions was performed by using 10 N NaOH, 2 M NaOH, concentrated HCl and concentrated CH_3COOH. Agarose (Serva) was prepared for electrophoresis in a concentration of 1 % with added Ethidium Bromide (Serva) in a final concentration of 5 μg/mL. Restriction endonuclease Hae III (BsuRI) (Fermentas), and restriction R buffer (Fermentas) were used for restriction mapping, and markers DNA standard 1000 bp (Serva) and DNA standard 100 bp (Serva) were chosen for quantification of isolated DNA. For easier and more precise determination of plasmid size DNA of known size ϕX 174 (Fermentas) which was previously cut with enzyme BsuRI to 11 fragments of a precisely defined size, was also used beside the markers. Transform Aid Kit (Fermentas) was used for transformation of isolated plasmids, and competent cells were prepared during the procedure from *E. coli* DH5 α strain. The streaking of transformed cells was performed on Luria Bertani agar with added ampicillin 50 μg/mL or tetracycline12 μg/mL.

Research methods

Isolation and identification of *E.coli* and *Salmonella* was performed by using conventional bacteriological methods. Serological typing of *Salmonella* was performed by the rapid agglutination method on disc. Disc diffusion method (Kirby Bauer) was used to investigate bacterial sensitivity to antibiotics. Plasmid extraction was performed by Birnboim and Doly method (1979), which is based on chemical lysis of bacteria after their exposure to high pH (12-12,5) – known as alkaline lysis, which causes protein, chromosomal DNA and cellular RNA

denaturation. By applying a solution of Potassium-acetate all detritus is precipitated, including chromosomal DNA. Renaturation of plasmid DNA is performed by applying absolute ethanol at low temperatures (-20°C). Although this method implies the use of Ribonuclease A, this enzyme is not necessary since cellular RNA is very unstable and disintegrates during bacterial exposure to the effects of the above mentioned chemical substances. The quality and purity of isolated plasmids were checked by the method of horizontal electrophoresis on agarose gel (1% concentration), which was prepared in 1xTBE buffer (Meyers, *et al*., 1976). Electrophoretic separation of DNA fragments was performed under 135 mA electricity, and voltage of 100 V. The results were read on transilluminator under UV light (wave length 254-366 nm). Restriction digestion of isolated plasmids was performed by restriction endonuclease BsuRI (HaeIII) on 37°C during 2-3 hours. Digested samples were applied to agarose gels of various concentrations, ranging from 0,5% to 2,2%, to obtain a clear visualization of the DNA fragments. Transformation of isolated plasmid DNA into *E. coli* DH5 α cells was performed according to manufacturer's instructions of the utilized Transform Aid Kit. Competent cells were included in the transformation process from the logarithmic growth phase. After the transformation process was completed, the transformant cells were streaked on LB agar with added antibiotic. This research investigated resistance gene presence for ampicillin and tetracycline on plasmids and their transfer by plasmids into transformed cells. After the transformed cells were streaked on LB agar with tetracycline and ampicilline, the discs were incubated for 24 hours on 37°C. As controls, untransformed *E. coli* DH5 α cells were also streaked on LB agar with added antibiotics.

RESULTS

The presence of plasmids was investigated in 42 strains of bacteria resistant to at least 3 or more antibiotics, including 23 *E. coli* strains and 4 *Salmonella* species strains. The investigated bacteria were previously cultivated on LB broth with added ampicillin and tetracycline for selective pressure and potential increase in plasmid copies number. The plasmids were isolated from 11 investigated strains: 2 *E. coli* strains originating from dogs, 4 *E. coli* strains originating from cattle, 2 *E. coli* strains originating from pigs, 1 *E. coli* strain originating from a cat and 2 *Salmonella* strains originating from poultry. Figure 1 represent the appearance of plasmid DNA after isolation from bacteria. The different visibility intensity of certain DNA fragments is a consequence of higher or lower number of plasmid copies in the cell, as well as DNA yield after isolation. The number of plasmid copies and DNA yield cannot be predicted. Also, in some columns more DNA fragments can be seen which represent different spiralization shapes of the same plasmid.

The isolated plasmids approximate size results are shown in table 1.

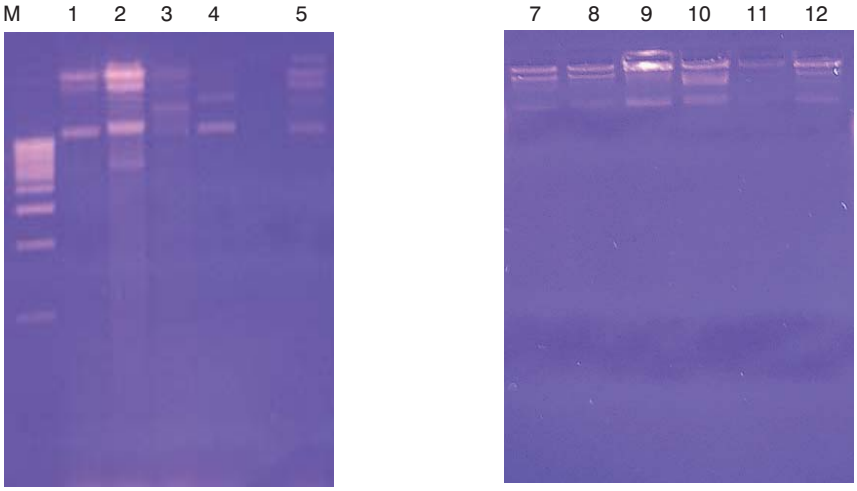

Figure 1. The appearance of plasmid DNA on 1% agarose gel after isolation

Legend: M. DNA standard (marker) furthest fragment 1000 bp, then 15000 bp, 2000 bp, 3000 bp, 4000 bp. 1. *E. coli* strain originating from dog PEC9. 2. *E. coli* strain originating from dog PEC1. 3. *E. coli* strain originating from pig SEC9. 4. *E. coli* strain originating from pig SEC4. 5. *E. coli* strain originating from cat MEC5. 7. *E. coli* strain originating from cattle GEC4. 8. *E. coli* strain originating from cattle GEC5. 9. *E. coli* strain originating from cattle GEC7. 10. *E.coli* strain originating from cattle GEC8. 11. *Salmonella* strain originating from poultry ZS5. 12 *Salmonella* strain originating from poultry ZS26

Table 1. The obtained approximate size values of isolated plasmids from *E. coli* and *Salmonella* species strains, expressed in base pairs

Origin of strain	Strain	Strain mark	Plasmid size
Dogs	*E. coli*	PEC 9	11 kb
	E. coli	PEC 1	11.5 kb
Cats	*E. coli*	MEC 5	16.5 kb
Pigs	*E. coli*	SEC 4	1.5 kb
	E. coli	SEC 9	2.5 kb
Cattle	*E. coli*	GEC 4	4.9 kb
	E. coli	GEC 5	5.5 kb
	E. coli	GEC 7	2.4 kb
	E. coli	GEC 8	9 kb
Poultry	*Salmonella*	S 5	3 kb
	Salmonella	S 26	17.3 kb

After the transformation process was completed and the *E. coli* DH 5α transformants streaking was done on LB agar with added antibiotics, the visible growth of transformed bacteria meant that the competent cells have received DNA (plasmid) with the resistance gene for the antibiotic. Lack of growth in transformants meant that the resistance gene was to be found on the chromosome.

Table 2. Explanation of resistance gene presence on isolated plasmids from clinical bacterial strains based on obtained results after performing transformation of *E. coli* DH 5 α with plasmid DNA

Origin of plasmid	Ampicillin resistance gene	Tetracycline resistance gene
E. coli SEC 4 (pig)	+	+
E. coli SEC 9 (pig)	+	–
E. coli PEC 1 (dog)	+	–
E. coli PEC 9 (dog)	+	–
E. coli MEC 5 (cat)	+	+
E. coli GEC 4 (cattle)	+	+
E. coli GEC 5 (cattle)	–	+
E. coli GEC 7 (cattlle)	+	–
E. coli GEC 8 (cattle)	–	–
Salmonella spp. S5 (poultry)	+	+
Salmonella spp. S26 (poultry)	+	–

DISCUSSION

According to new scientific literature data, plasmids isolated from wild or clinical strains of *E. coli*, as well as from other bacteria belonging to *Enterobacteriaceae*, can range in size from 1 kb to over 256 kb (2560 000 base pairs) (Sherley *et al*., 2003). All plasmids smaller than 16 kb are considered small plasmids. Plasmids ranging in size from 16 to 32 kb belong to the group of middle sized plasmids. It has been established that conjugative plasmids need to be at least 15 to 20 kb in size, because they need to contain a large number of genes to code the very process of conjugation. There are several different systems of conjugation discovered in plasmids, but they all imply the need for a large genetic apparatus, which ensures the condition that all conjugative plasmids need to be large plasmids (Alonso *et al*., 2001; Shirley *et al*, 2003). Most plasmids isolated in this research belong to the group of small plasmids, except one, sized 16,5 kb isolated from *E. coli* originating from a cat, which has been categorized as belonging to the middle sized group based on the 500 base pairs over 16 kb. Plasmids sized 17,3 kb, and isolated from *Salmonella* originating from poultry

have also been categorized as middle sized. Based on available data, these two plasmids can be supposed to belong to conjugative plasmids. The presence of plasmids in *Salmonella* species is mostly investigated in order to detect the virulence gene on plasmid DNA (Ang-Kucuk *et al.,* 2000). Bacteria from this genus rarely carry plasmids, which explains the low prevalence of resistance to antibiotics in these bacteria. The existence of plasmids also does not imply the presence of the resistance gene, which has been confirmed by detecting large plasmids in Salmonella strains sensitive to all investigated antibiotics (Martinez-Martinez *et al*., 1998). In our research a plasmid has been isolated from ZS 26 *Salmonella* strain, which is most probably responsible for the resistance to amoxicillin with clavulic acid, beside the resistance gene to ampicillin which has been proved to be present on the isolated plasmid. The mentioned strain was also resistant to tetracycline. Since all the bacterial strains from which plasmid isolation was performed were proven to be resistant to ampicillin and tetracycline by previous disc diffusion method, the absence of resistance gene on isolated plasmids meant that the given genes were localized on chromosomes of investigated strains. The only result that cannot be clearly explained concerns a plasmid originating from *E. coli* GEC 8. The size of this plasmid was 9 kb, but after transformation to competent cells, the growth on LB agar with ampicillin and tetracycline failed to happen. This can be explained in several ways. One of the explanations is that the process of transformation was not successful, which is unlikely considering the very clear results for other transformed cells. It is also possible that a complete degradation of this plasmid occurred during manipulation in the transformation process, but this possibility is also not likely, since the factor which could have possibly degraded this DNA had to have degraded all the other plasmids. Finally, resistance genes for both antibiotics can be localized on chromosome *E. coli* GEC 8 strain, due to which the transformants on LB agar with added antibiotics failed to grow.

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ISPITIVANJE PRISUSTVA GENA REZISTENCIJE NA PLAZMIDIMA IZOLOVANIM IZ SOJEVA *E. COLI* I *SALMONELLA* VRSTA POREKLOM OD DOMAĆIH ŽIVOTINJA METODOM TRANSFORMACIJE PLAZMIDSKE DNK U KOMPETENTNE ĆELIJE

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SADRŽAJ

Ispitivanje prisustva plazmida vršeno je na sojevima *E. coli i Salmonella* vrsta poreklom od pasa, mačaka, goveda, svinja i živine. Ispitivani sojevi bakterija izolovani su iz briseva ušiju, kože, vaginalnih briseva, fecesa i urina i to kako od zdravih tako i od bolesnih jedinki različitih starosnih kategorija i rasa. Ukupno je izolovano 45 sojeva *E. coli* i 35 sojeva *Salmonella* vrsta a prisustvo plazmida ispitano je kod 42 soja bakterija rezistentnih na najmanje 3 i više antibiotika. Plazmidi su izolovani iz 11 ispitanih sojeva i to iz 2 soja *E. coli* poreklom od pasa, 4 soja *E. coli* poreklom od goveda, 2 soja *E. coli* poreklom od svinja, 1 soja *E. coli* poreklom od mačke i kod 2 soja *Salmonella* vrsta poreklom od živine. Nakon izvršene transformacije i zasejavanja transformanata *E. coli* DH 5 α na LB agar sa dodatkom antibiotika, vidljivi porast transformisanih bakterija značio je da su kompetentne ćelije primile DNK (plazmid) sa genom rezistencije na taj antibiotik. Izostanak porasta transformanata značio je da se gen rezistencije nalazio na hromozomu.