

INVESTIGATION OF THE PRESENCE OF EXTENDED SPECTRUM BETA-LACTAMASES (ESBL) IN MULTIRESISTANT STRAINS OF *E. COLI* AND *SALMONELLA* SPECIES ORIGINATED FROM DOMESTIC ANIMALS

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Bacterial strains which possess genes to produce ESBL most often are multiresistant and also carry genes responsible for the resistance to most other antibiotics, including aminoglycosides, sulfamethoxazole+trimethoprim and fluoroquinolones. Therefore, practically the biggest contemporary clinical problem are infections of humans and animals caused by ESBL-producing strains of E. coli, Klebsiella, Enterobacter, Proteus, Serratia, Citrobacter, Salmonella and Shigella species. The investigation of the ESBL presence was completed on multiresistant E. coli and Salmonella strains originating from dogs, cats, cattle, sheep, goats, pigs and poultry. The investigated strains were isolated from ear, skin, vaginal, faecal, urine, egg and eggshell swabs, from healthy and diseased individual animals of various ages and breed categories. The sum of 112 E. coli and 45 Salmonella strains was investigated. All strains resistant to 3 or more antibiotics were categorized as multiresistant, which led to a conclusion that 35 E. coli and 6 Salmonella strains out of all investigated were multiresistant to antibiotics. The largest number of multiresistant E. coli strains was discovered in cattle – 12 in total, and the minimal number in goats and sheep, with two strains each. All multiresistant Salmonella strains belonged to the Salmonella Enteritidis species (S. enterica subsp. enterica serovar Enteritidis). The sum of multiresistant Salmonella strains compared to all investigated strains was relatively low (13.3%), but the resistance prevalence for some antibiotics in these strains was extremely high, for ampicillin and amoxicillin with clavulanic acid as high as 100%, and for tetracycline 83.3%. For the control in this investigation were used ESBL positive E. coli strains originated in human urine specimens. No presence of positive ESBL strains was established. However, when the screening investigation was performed, almost all the strains were suspect, thus a confirmatory test had to be performed for all strains.

Key words: ESBL, multiresistant, E. coli, Salmonella

INTRODUCTION

By the 1960's it was widely thought that enzymes from the beta-lactamase group were produced only by gram-positive bacteria (Livermore and Paterson, 2006). The first beta-lactamase originating from enterobacteria was discovered and purified in 1964 from the *E. coli* strain isolated from the haemoculture of a man suffering from septicaemia. It was marked as TEM-1 beta-lactamase, and in the following years TEM-1 as well as TEM-2 beta-lactamases were discovered in almost all *E. coli* strains resistant to penicillines and cephalosporins of the first generation. Soon SHV-1 beta-lactamases were discovered in strains of *Klebsiella* species resistant to beta-lactamases. After discovering OXA-lactamases it was clear that gram-negative bacteria possess several different beta-lactamase production mechanisms (Einhorn *et al.*, 2002; Katsanis *et al.*, 1994; Livermore and Paterson, 2006). By 1975, all three types of beta-lactamase were discovered in almost all strains of enterobacteria resistant to penicillines and cephalosporins, and reports on the discovery of these strains have been submitted from all continents. For this reason the pharmaceutical companies have produced and marketed cephalosporins with an expanded spectrum of action, the so-called oxyimino-cephalosporins (cefotaxime, ceftazidime, ceftriaxone) which were resistant to TEM-1, SHV-1 and OXA beta-lactamases (Thomson *et al.*, 2001; Walsh, 2003). These antibiotics were used in clinical practice, almost exclusively in treating patients suffering from intrahospital septicaemia caused by enterobacteria resistant to older generations of penicillin and cephalosporins. However, as soon as 1983, to the surprise of all experts, the first strains of *E. coli* and *Klebsiella* species resistant to this new group of antibiotics were discovered. During investigations on this form of resistance, completely new and up to then unknown enzymes belonging to the beta-lactamase group were isolated, entitled ESBL, i.e. extended spectrum beta-lactamases (due to the ability of dissolving cephalosporins and penicillins with extended spectrum of action). It is known today that the production of ESBL is coded by mutated genes responsible for the production of TEM-1, TEM-2m SHV-1 and OXA beta-lactamases (Livermore and Paterson, 2006; MacKenzie *et al.*, 2002). ESBL lactamases are created by substituting one or more aminoacids in the TEM, SHV and OXA lactamases molecules. So far over 150 new ESBL group enzymes have been discovered, which are responsible for the resistance to all cephalosporins including III generation cephalosporins, as well as to all penicillins and aztreonam (MacKenzie *et al.*, 2002; Walsh, 2003). Within the last 5 years, however, new variants of ESBL have been discovered, which do not originate from TEM, SHV and OXA lactamases, and are marked as CTX-M, PER, VEB and other beta-lactamases. The genes responsible for the production of ESBL are most frequently located on plasmids which are always larger than 100kb and by which they are easily transferred by mechanisms of conjugation and transduction to other enterobacteria in nature. Bacterial strains which possess genes to produce ESBL most often are multiresistant and also carry genes responsible for the resistance to most other antibiotics, including aminoglycosides, sulfamethoxazole+trimethoprim and fluoroquinolones. Therefore, practically the biggest contemporary clinical problem are infections of

humans and animals caused by ESBL-producing strains of *E. coli*, *Klebsiella*, *Enterobacter*, *Proteus*, *Serratia*, *Citrobacter*, *Salmonella* and *Shigella* species (Baraniak *et al.*, 2002; Barker, 1999; Einhorn *et al.*, 2002). In gram-positive bacteria ESBL enzymes have not yet been discovered, and they are also not recorded in gram-negative bacteria which do not belong to the *Enterobacteriaceae* family. Enzymes from the ESBL group do not work on 7- α -methoxy cephalosporine, the so-called cephamycine (cephoxitin and cefotetan), as well as carbanepems which remain virtually the only medicine of choice to treat infections caused by ESBL-producing strains since ESBL strains resistant to imipenem, meropenem and ertapenem have not yet been discovered (Thomson *et al.*, 2001). Colistin also works on ESBL-producing strains, although this antibiotic shows a strong toxic effect on the host organism, as well as do nitrofurantoin and fosfomicin which can, due to their specific pharmacokinetics, only be used for treatment of urinary infections (MacKenzie *et al.*, 2002; Walsh, 2003). The strains which produce ESBL show varying sensitivities to beta-lactamase inhibitors (clavulanic acid, sulbactam, tazobactam). It is interesting that enzymes belonging to the ESBL group do not work on cefepime, which belongs to IV generation cephalosporins. However, during therapy with cefepime in patients suffering from infections caused by ESBL strains, other resistance mechanisms quickly develop, thus making the use of cefepime not advisable unless in combination with aminoglycosides (MacKenzie *et al.*, 2002; Thomson *et al.*, 2001). Strains of *Enterobacter*, *Citrobacter*, *Morganella* and *Serratia* species have been discovered which are resistant also to oxyimino-cephalosporins, but this is an inducible resistance and relates to the development of chromosomal mutations responsible for the production of *AmpC* beta-lactamases which do not belong to the ESBL lactamases (Weinbren and Borthwick, 2005). The ESBL strains are geographically widely spread and have been also discovered in animals. There are no precise epidemiological data concerning the incidence of ESBL strains due to non-existence of standard methods for their detection (CLSI, 2006; Isenberg, 2004). The biggest danger to patient's lives represents the sensitivity phenomenon of ESBL-producing strains to numerous beta-lactam antibiotics in standard disk diffusion investigation, while in conditions *in vivo*, the therapeutic effect of these antibiotics does not take place. That is why the appearance of the ESBL enzymes is also called "a hidden resistance mechanism". The most important risk factors for creation of ESBL-producing strains is the prolonged use of beta-lactam antibiotics, especially in hospitals. Considering the fact that the III generation cephalosporins are also used in treating animals, ESBL positive strains can easily appear on farms as well as in pets. The most concern is caused by the appearance of ESBL positive *Salmonella* strains. Up to 1999, as much as 3,4% of ESBL positive *Salmonella* strains originating from humans and animals were discovered in the EU (Katsanis *et al.*, 1994; Baraniak *et al.*, 2002; Einhorn *et al.*, 2002; MacKenzie *et al.*, 2002). Despite the fact that ESBL enzymes were discovered 25 years ago, this issue is addressed by a relatively small number of laboratories. In Serbia, of all registered veterinary laboratories, only the bacteriological laboratory of the Microbiology department at the Faculty of

veterinary medicine, Belgrade, deals with routine investigation of ESBL presence in animals.

MATERIALS AND METHODS

Investigated bacterial strains

The investigation of the ESBL presence was completed on multiresistant *E. coli* and *Salmonella* strains originating from dogs, cats, cattle, sheep, goats, pigs and poultry. The investigated strains were isolated from ear, skin, vaginal, faecal, urine, egg and eggshell swabs, from healthy and diseased individual animals of various age and breed categories.

The isolation was made from the clinical material delivered to the Institute for veterinary science and freshwater fish at the Biotechnical Institute in Podgorica, and to the Specialistic veterinary microbiological laboratory in Podgorica in the period 2004-2005. A certain number of strains for *Salmonella* and *E. coli* species included in the research were isolated from clinical materials sent from various epizootiological areas to the Microbiology Department, Faculty of veterinary medicine, Belgrade.

Media and reagents

A large number of conventional nutrient media and biochemical reactions were used in order to isolate and identify the investigated bacterial strains. For serological typisation of *Salmonella* specific polyvalent diagnostical sera (produced by National health protection Institute "Milan Jovanovic Batut" and Becton Dickinson) were used. In order to detect multiresistance in investigated *E. coli* and *Salmonella* strains, Mueller Hinton agar (BioLab) and antibiogram disks of ampicillin, amoxicillin with clavulanic acid, tetracycline, chloramphenicol, sulfamethoxazole+trimethoprim, enrofloxacin and ceftriaxone (Becton Dickinson) were used in the research. In order to detect positive ESBL strains Mueller Hinton agar (BioLab) and antibiogram disks of amoxicillin with clavulanic acid (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefotaxime with clavulanic acid (30/10 µg) and ceftazidime with clavulanic acid (30/10 µg) were used.

Research methods

Isolation and identification of *E. coli* and *Salmonella* were completed using conventional bacteriological methods. Serological typisation of *Salmonella* was done using rapid slide agglutination method. To investigate bacterial sensitivity to antibiotics disk diffusion method by Kirby Bauer was used, and the density of inoculated suspensions of all investigated bacterial strains was approximately $1-2 \times 10^8$ bacteria/mL. The plates were incubated for 24h at 37°C. The choice of antibiotics used in this research was based on recommendations by CLSI (Clinical Laboratory Standards Institute, USA, 2006).

Since there is no standardized method for detection of ESBL positive bacterial strains, recommendations by CSLI from 2006, were used. The density of streaked inoculums of all investigated strains was approximately $1-2 \times 10^8$ bacteria/mL. A screening test was first performed ie. disk diffusion test with some

of the above mentioned antibiotics or with all of them: cefpodoxime (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), aztreonam (30 µg). Depending on the obtained zones of growth inhibition, the presence of ESBL enzymes were either suspected, or the strain was discarded as ESBL negative. Two confirmatory tests were performed on strains suspected of being ESBL positive. The first was disk diffusion test with disks of ceftazidime, cefotaxime, ceftriaxone and aztreonam set around the disk of amoxicillin with clavulanic acid, in such a way that the distance between the disk's centres was 2 to 3 cm. The second test was also a disk diffusion test performed with disks of ceftazidime, cefotaxime with clavulanic acid, and ceftazidime with clavulanic acid. The results were read after 24h of incubation at 37°C.

RESULTS

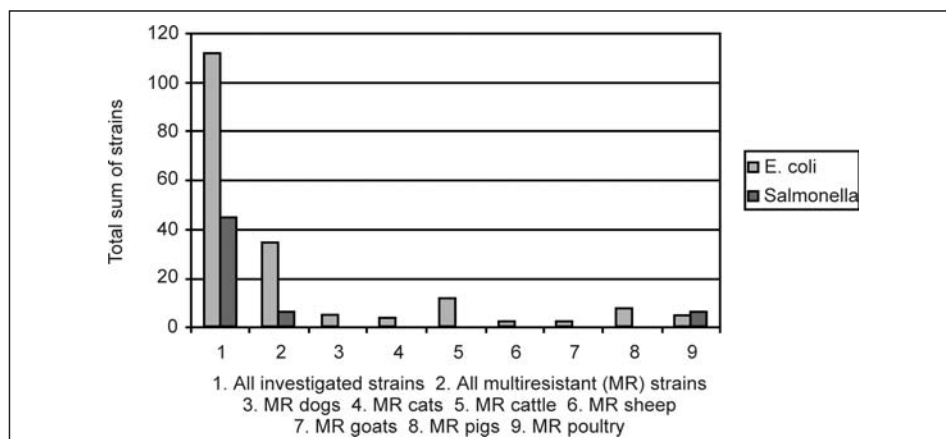
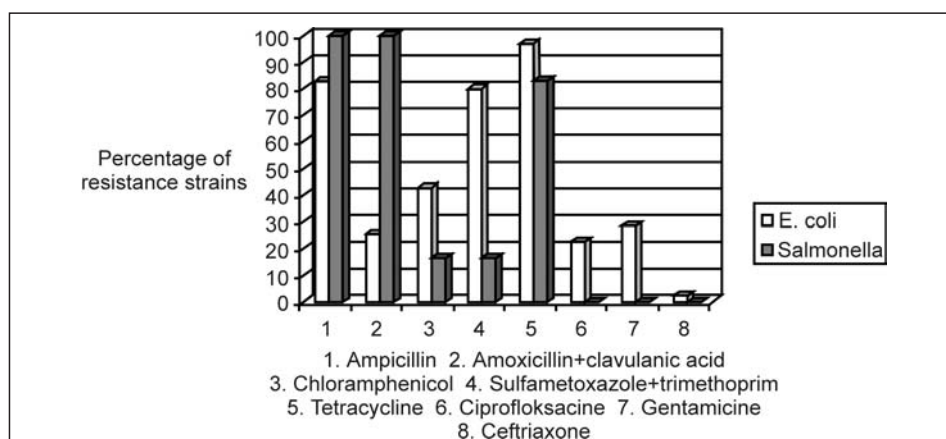
The sum of 112 *E. coli* and 45 *Salmonella* strains was investigated. All strains resistant to 3 or more antibiotics were categorized as multiresistant, which led to a conclusion that 35 *E. coli* and 6 *Salmonella* strains out of all investigated were multiresistant to antibiotics. The largest number of multiresistant *E. coli* strains (MR *E. coli*) was discovered in cattle – 12 total, and the minimal number in goats and sheep, with two strains each. Only one dog-originated *E. coli* strain was resistant to all eight used antibiotics in the disk diffusion test. All other MR strains were resistant to 3-6 antibiotics. Almost all investigated MR *E. coli*, 34 in all, were sensitive to ceftriaxone. In MR *E. coli* strains originating in cats, pigs and poultry no resistance to amoxicillin with clavulanic acid was discovered. No resistance was also discovered to chloramphenicol, ciprofloxacin or gentamicin in MR *E. coli* strains originating in sheep and goats. The biggest resistance prevalence in MR *E. coli* was discovered for tetracycline (97.1%), and for ampicillin (82.8%). Very high resistance prevalence was determined for sulfamethoxazole+trimethoprim (80%), and chloramphenicol (42.8%), while for all other investigated antibiotics the resistance prevalence was about 30%. The results of the multiresistance presence investigation in *E. coli* strains are shown in tables 1 and 2 and on figures (1-4).

Table 1. Resistance profiles of multiresistant *Salmonella* strains

Number of antibiotics	Antibiotics to which resistance was shown	Number of strains
4	ampicillin, amoxicillin with clavulanic acid, tetracycline, chloramphenicol,	1
3	ampicillin, amoxicillin with clavulanic acid, tetracycline,	4
3	ampicillin, amoxicillin with clavulanic acid, sulfamethoxazole + trimethoprim	1

Table 2. Screening test for ESBL positive strains

Antimicrobial agent	Disk potency (μg)	Zone diameter (mm) for positive screen
Cefpodoxime	10	plain 17
Ceftazidime	30	plain 22
Cefotaxime	30	plain 27
Ceftriaxone	30	plain 25
Aztreonam	30	plain 27

Figure 1. The number of isolated multiresistant *E. coli* and *Salmonella* strains compared to the sum of investigated strains originating in domestic animalsFigure 2. Resistance prevalence for investigated antibiotics in multiresistant *Salmonella* strains

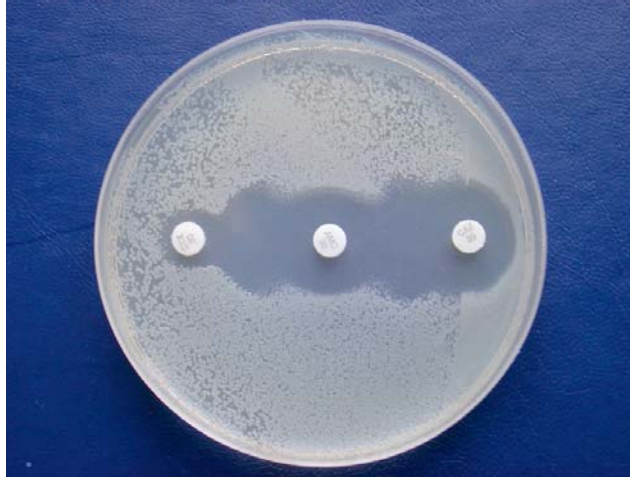


Figure 3. The first confirmatory ESBL test (ESBL positive *E. coli* strain, originated in a human specimen)

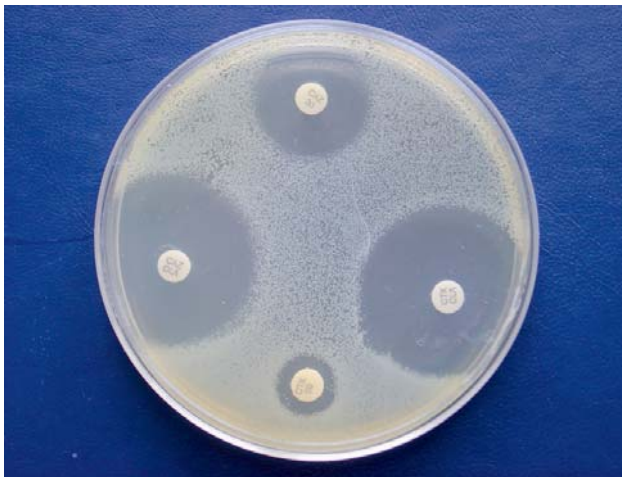


Figure 4. The second confirmatory ESBL test (ESBL positive *E. coli* strain originated in a human specimen)

All multiresistant *Salmonella* strains were of the *Salmonella enteritidis* species (*S. Enterica* subsp. *Enterica* serovar. Enteritidis). The sum of multiresistant *Salmonella* strains compared to all investigated strains was relatively low (13.3%), but the resistance prevalence for some antibiotics in these strains was extremely high, for ampicillin and amoxicillin with clavulanic acid as high as 100%, and for

tetracycline 83.3%. The resistance profiles in these *Salmonella* strains are shown in Table 1. Although one of the tasks of this investigation was to detect the resistance to fluoroquinolones in *Salmonella* strains, since this form of resistance causes the greatest concern of the expert public, no *Salmonella* strains resistant to ciprofloxacin were discovered, despite the fact that fluoroquinolones are widely used in poultry in Serbia and Montenegro for therapeutic and prophylactic purposes. One *Salmonella* Enteritidis strain was discovered to be resistant to chloramphenicol which can be explained only by the resistance gene transfer or by assuming that chloramphenicol is used for poultry, despite the legal ban.

As far as ESBL presence in the investigated bacterial strains is concerned, no presence of positive ESBL strains was established. However, when the screening investigation was performed, almost all the strains were suspect, so a confirmatory test had to be performed for all strains.

DISCUSSION

Based on CLSI recommendations, when performing the screening test for the presence of ESBL enzymes in enterobacterial strains it is recommended to use 1 or all mentioned antibiotics from Table 2 (CLSI, 2006; Isenberg, 2004). Table 2 shows growth inhibition zone diameters based on which the strains are declared suspect to ESBL or ESBL negative.

In this investigation, ceftazidime, cefotaxime and ceftriaxone were used for the screening test. Inhibition zones for ceftazidime and cefotaxime in all cases were bigger than the listed diameters, i.e. bigger than 22 mm and 27 mm. However, in almost all MR *E. coli* strains the growth inhibition zones for ceftriaxone were smaller than 25 mm, based on which all strains had to be declared suspect to ESBL. It is interesting to note that the inhibition zone diameter for ceftriaxone prescribed by the disk's manufacturer for the "sensitive" category is 21 mm and more. In our investigation, inhibition zones for most of the strains were between 21 mm and 25 mm, so that, according to the manufacturer's (Becton Dickinson) manual, which is also in accordance with the CLSI recommendations, all those strains could be categorized as sensitive to ceftriaxone, while according to CLSI recommendations these strains ought to have been categorized as ESBL suspect, so that in reports they would all be categorized as resistant to all beta-lactams, including ceftriaxone (Katsanis *et al.*, 1994; Isenberg, 2004; Weinbren and Borthwick, 2005; CLSI, 2006; Livermore and Paterson, 2006).

Besides that, the appearance of multiresistance in enterobacterial strains is often connected with the presence of ESBL gene, which makes all MR *E. coli* and *Salmonella* strains ESBL suspect (Katsanis *et al.*, 1994; Baraniak *et al.*, 2002).

For each strain two confirmatory tests were performed. The first test was done with ceftazidime, cefotaxime and amoxicillin with clavulanic acid disks in the center of the slide. All disks were placed at a distance of 2 to 3 cm (Isenberg, 2004; Weinbren and Borthwick, 2005; CLSI, 2006). In cases of positive ESBL testing, a spreading of the inhibition zone appears on the side of amoxicillin with clavulanic acid (Figure 3).

The second confirmatory test was, as was already mentioned, performed with ceftazidime, cefotaxime, cefotaxime with clavulanic acid and ceftazidime with clavulanic acid. In cases where growth inhibition zones are bigger than 5mm or more, around disks with ceftazidime with clavulanic acid or cefotaxime with clavulanic acid when compared with growth inhibition zones around cefotaxime and ceftazidime, the strain is declared ESBL positive (Isenberg, 2004) (Figure 4).

According to data available from literature, prevalence of ESBL positive *E. coli* and *Salmonella* strains in animals is still very low, ie. these strains are still rarely discovered in animals (Barker, 1999; Baraniak *et al.*, 2002). Statistically, in human medicine ESBL are most frequently discovered in strains of *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Enterobacter* species isolated from humans previously treated with cephalosporins with extended spectrum of action over a longer period of time (Livermore and Paterson, 2006). It is in this sense, of great importance to investigate animal-originated strains from animals which have been treated with III generation cephalosporins. For example, strains of *E. coli*, *Klebsiella*, *Enterobacter* and *Proteus* species isolated from cat and dog urine are interesting, since ceftriaxone is often used for treating these animals in our country and throughout the world. Strains of *E. coli* and *Klebsiella* species isolated from the milk of cows suffering from mastitis are also interesting, since it is known that there has been a medical preparation of ceftriaxone for intramammary use commercially available in Serbia and Montenegro. However, although the pathogenicity of these bacterial strains in animals has been proved, it is debatable what significance *Klebsiella* and *Enterobacter* species in veterinary medicine can really have and especially in small animals practice. For example, during 2005 and 2006 as much as 2500 various samples originating in dogs and cats have been microbiologically tested in the Microbiology Department bacteriological laboratory, but in only 5% of urine samples *Enterobacter aerogenes* was isolated, and *Klebsiella pneumoniae* was isolated from only 3% of urine samples, and 1.3% of skin swab samples (Krnjačić *et al.*, 2005). As opposed to that, *Proteus* species as potential carriers of ESBL are commonplace in all sorts of animal samples, and ESBL test is performed on these strains almost every day. All the mentioned strains have proven to be ESBL negative, although they were investigated by the recommendations of the then active NCCLS which highly vary from the current CLSI recommendations.

Unfortunately samples originating in large animals are seldom delivered to the mentioned laboratory, which makes it impossible to gain a better insight on the presence of *Klebsiella*, *Enterobacter* and *Citrobacter* species in cows and horses. Also, when animal and especially poultry feces samples are microbiologically tested, the tests are mostly performed to isolate the most significant intestinal pathogens such as *Salmonella*, *Campylobacter* and *Clostridium* species, while the potential ESBL carriers such as *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter*, and even *E. coli* (unless it produces hemolysis) are rejected as species which are physiologically present in the intestines. The routine isolation of mentioned species from feces samples would immensely heighten the cost of such tests. Therefore the ESBL investigations are practically performed only in cases where the mentioned species have been isolated as primary pathogenic causative

agents, and such cases are statistically still very rare (except in *E. coli* and *Proteus* species).

That is why one of the aims of this paper, besides to highlight to everyone who works in veterinary clinical microbiology the significance and danger of ESBL strains, is also an attempt at awakening the interest for investigating the presence of ESBL strains especially in large animals, in other microbiological laboratories in Serbia and Montenegro.

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**ISPITIVANJE PRISUSTVA BETA-LAKTAMAZA PROŠIRENOG SPEKTRA
DELOVANJA (ESBL) KOD MULTIREZISTENTNIH SOJEVA *E. COLI* I *SALMONELLA*
VRSTA POREKLOM OD DOMAĆIH ŽIVOTINJA**

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SADRŽAJ

Sojevi bakterija koji poseduju gene za proizvodnju beta-laktamaza proširenog spektra delovanja (ESBL) najčešće ispoljavaju multirezistenciju i istovremeno su nosioci gena odgovornih za rezistenciju na većinu drugih antibiotika, uključujući i aminoglikozide, sulfametoksazol+trimetoprim i fluorohinolone. Zbog navedenih razloga, najveći problem danas u kliničkoj praksi predstavljaju infekcije ljudi i životinja izazvane ESBL produkujućim sojevima *E. coli*, *Klebsiella*, *Enterobacter*, *Proteus*, *Serratia*, *Citrobacter*, *Salmonella* i *Shigella* vrsta. U ovim ispitivanjima materijali za izolovanje sojeva bakterija predstavljali su brisevi ušiju, kože, vaginalni brisevi, feces i urin, jaja i ljuske od jaja. Primenom klasičnih i komercijalnih testova mikrobiološke dijagnostike izolovani su i identifikovani sojevi *E. coli* i *Salmonella* vrsta. Navedenim sojevima je ispitivana i osetljivost na određeni broj antibiotika i hemioterapeutika primenom metoda propisanih od strane CLSI, USA iz 2006. Samo oni sojevi *E. coli* i *Salmonella* poreklom od pasa, mačaka, goveda, ovaca, koza, svinja i živine kod kojih je ustanovljena rezistencija na 3 i više antibiotika kategorizovani su kao multirezistentni i dalje su ispitivani na prisustvo beta-laktamaza proširenog spektra delovanja. Ovim ispitivanjem obuhvaćeno je ukupno 112 sojeva *E. coli* i 45 sojeva *Salmonella*. Takođe su ispitivanjem bila obuhvaćena i dva ESBL pozitivna soja *E. coli* koji su izolovani u našoj laboratoriji iz urina ljudi, a služili su nam kao kontrola. Od 112 ispitivanih sojeva *E. coli*, kod 35 je utvrđena multirezistencija na određeni broj antibiotika, dok je od 45 sojeva *Salmonella* koji su ispitivani samo kod 6 sojeva ustanovljena multirezistencija. Najveći broj multirezistentnih sojeva *E. coli* (MR *E. coli*) otkriven je kod goveda, ukupno 12, a najmanje kod koza i ovaca, po dva soja. Svi multirezistentni sojevi *Salmonella* pripadali su vrsti *Salmonella enteritidis* (*S. enterica* subsp. *enterica* serovar. *enteritidis*). Ukupan broj multirezistentnih sojeva *Salmonella* u odnosu na broj sojeva koji su ispitivani bio je relativno nizak (13,3 %). Međutim, izuzetno visoka prevalencija rezistencije kod ispitivanih sojeva utvrđena je na ampicilin i amoksicilin sa klavulanskom kiselinom (100 %), a na tetraciklin 83,3%. Primenom "screening" ispitivanja, gotovo svi sojevi uključeni u ispitivanje bili su sumnjivi na prisustvo ESBL i zbog toga je u ispitivanje bio uključen i potvrdni test. Tek po završenim ispitivanjima, mogao se izvesti zaključak da među izolovanim sojevima *E. coli* i *Salmonella* poreklom od životinja nisu otkriveni sojevi koji poseduju β - laktamaze proširenog spektra delovanja.