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Original article ■

Bacterial Etiology of Diarrheal Syndrome

Biljana Miljković-Selimović[1,2], Tatjana Babić[1,3], Predrag Stojanović[1,2]

[1]*National Referent Laboratory for Campylobacter and Helicobacter*
[2]*Institute for Microbiology and Immunology, Faculty of Medicine in Niš, Serbia*
[3]*Center for Microbiology, Public Health Institute Niš, Serbia*

SUMMARY

Bacteria which cause enterocolitis belong to the genera *Salmonella*, *Shigella*, *Vibrio*, to the thermophilic campylobacters, species of *Yesrsinia enterocolitica* (*Y. enterocolitica*) and *Clostridium difficile* (*C. difficile*), and to the group of diarrhoeagenic *Escherichia coli*. The aim of this paper was to determine the frequency of isolation of these microorganisms as well as their resistance to antibiotics.

Stool samples of the patients with acute diarrhea were investigated in one-year period. Bacteria were isolated and identified using standard microbiological procedures. Sensitivity testing of *Salmonella*, *Shigella* and *Yersinia* was preformed against the panel of 12 antibiotics, while *Campylobactera jejuni/coli* against the panel of 7 antibiotics.

Among enteric pathogens, *Salmonella* was the predominant bacterium, with *Salmonella* Enteritidis (*S.* Enteritidis) on the first position. *S.* Enteritidis was resistant to one ore more antimicrobial drugs in 35.6%. Resistance to streptomycin was the most frequently reported - it occured in 21.8% of strains. Resistance to one or more antibiotics in *Y. enterocolitica* O3 was detected in 87.5% of strains. This bacterium expressed resistance against ampicillin in 87.5% as well. All *Shigella* spp. isolates were resistant against three or more drugs. Resistance to fluoroquinolones occured in 60% of campylobacter strains.

In the examined samples, the predominant bacterium was *Salmonella*. In *S.* Enteritidis strains, a notable resistance occurred against streptomycine. *Y. enterocolitica* O3 expressed relatively high level of resistance, mostly against ampicillin. Multiple resistance occurred in the genus *Shigella.* In *Campylobacter* strains, resistance to fluoroquinolones (nalidixic acid and ciprofloxacine) was the most frequent.

Key words: **bacterial pathogens, *Salmonella* spp., diarrhea**

Corresponding author:

Biljana Miljković -Selimović •
tel. 064/433 22 38•
e-mail: biljams@eunet.rs •

INTRODUCTION

Bacterial infections of digestive tract are considered to be the major cause of morbidity and mortality, especially in children, elderly and immunosuppressed patients, both in industrialized and developing countries. These bacteria belong to genera of *Salmonella*, *Shigella*, *Vibrio*, to the thermophilic campylobacters, species of *Yersinia enterocolitica* (*Y. enterocolitica*) and *Clostridium difficile* (*C. difficile*), as well as to the group of diarrhoeagenic *Escherichia coli* (*E. coli*)(1).

In the last two decades, *S.* Enteritidis was the most frequently isolated *Salmonella* serotype, especially in Europe, where it participates with up to 85% of salmonelloses cases (2). Besides permanent significance of *Salmonella enterica* subsp. *enterica*, in the last few years, the importance of *Campylobacter* spp. and *Clostridium difficile* have been emphasized. The roles of *Bacteroides fragilis* and some new "patotypes" of diarrhoeagenic *Escherichia coli* in the etiology of diarrhea have been described, too.

AIMS

The aim of the paper was to determine the frequency of isolation of these microorganisms, as well as their resistance to a panel of antibiotics, in outpatients and hospitalized patients with diarrhea, whose stool was examined at the Public Health Institute Niš.

MATERIAL AND METHODS

The investigation was conducted during one-year period, from January 1, 2008 to December 12, 2008.

For bacterial isolation of the genera *Salmonella* and *Shigella,* the stool was cultured on McConkey agar, Salmonella - Shigella (SS) agar (Biomedics, bioMérieux, Madrid, Spain), and selenite F-broth which was subcultured on SS - agar after 24 hours of incubation. Nutrient media were incubated at 37°C under aerobic conditions. Identification was performed on the basis of biochemical activities and antigen structure detection.

Y. enterocolitica was isolated by cultivation on cefsulodin-irgasan-novobiocin agar (CIN) (Biomedics, bioMérieux, Madrid, Spain). Nutrient media were incubated at 25°C over the 48-hour period. Identification of these bacteria was conducted by the investigation of biochemical activities and agglutination in the specific hyperimmune sera.

E. coli was isolated using McConkey agar (Biomedics, bioMérieux Madrid, Spain), identified by biochemical array, and determination of the group was performed by agglutination with specific hyperimmune sera on the glass plate.

Investigation of drug susceptibility was performed by Kirby-Bauer disc diffusion method against ampicillin (10μg), amoxicillin/clavulanic acid (20+10 μg), ceftriaxone (30μg), cefotaxime (30μg), kanamycin (30 μg), gentamicin (10μg), trimethoprim/sulfamethoxazole (1.25+23.75μg), streptomycin (10μg), chloramphenicol (30μg), tetracycline (30μg) ciprofloxacine (5μg), and nalidixic acid (30μg) using tablets (Neo-Sensitabs, Rosco Diagnostica A/S, Taastrup, Denmark). CLSI interpretive criteria (3) were used.

Bacteria of the genus Campylobacter were isolated on Blaser's solid medium (Biomedics, bioMérieux, Madrid, Spain) after 48 hours of incubation at 37°C under microaerophilic conditions. Identification was performed on the basis of colony appearance on microscopic slide (Gram-negative "gull wings" shaped rods), by positive catalase and oxidase tests, and by hippurate hydrolysis test. Investigation of the strain drug susceptibility was performed by disc-diffusion method against erythromycin (15μg), gentamicin (10μg), tetracycline (30μg), ciprofloxacin (5μg), nalidixic acid (30 μg), cephalothin (30μg), and chloramphenicol (30μg) using tablets (Neo-Sensitabs, Rosco Diagnostica A/S, Taastrup, Denmark). The results were interpreted by CLSI interpretive criteria (3): the standard for staphylococcus was used for erythromycin, while standards for Enterobacteriaceae family were used for the rest of the drugs. Strains with intermediate sensitivity were considered as resistant ones.

C. difficile was isolated on cycloserin-cephoxitin-fructose agar - CCFA (Biomedics, bioMérieux, Madrid, Spain) after 48 hours incubation at 37°C, under anaerobic conditions. Identification was performed on the basis of the colony growth on the selective medium, its colony appearance and microscopic appearance in Gram-stained preparation. A/B toxins production was measured by ELISA test (Ridascreen, R-Biopharm Ag, Darmstadt, Germany).

RESULTS

In this period, there were 7.376 stool samples for examination: 1.086 clinic samples and 6.290 outpatient ones. Positive findings were detected in 12.4% of the clinic samples and only in 3.4% of the outpatient samples.

Taking into consideration all the investigated samples, the genus *Salmonella* dominated over other isolates with 59% (Table 1).

Additionally, in primo-isolates, both from clinical and outpatient samples, it was ascertained that microorganisms of the genus *Salmonella* were also predominant (51.4%), and the most frequent serotype was *Salmonella* Enteritidis (*S.* Enteritidis) (41.7%) (Graph 1).

The presence of other Salmonella serotypes *i.e. S.* Typhimurium (3.7%), *S.* Infantis, *S.* Bovismorbificans, *S.* Paratyphi B (Table 2) was proven, too.

Table 1 . *Total number of isolated bacteria*

Microorganism	Outpatients' samples	Hospital samples	Total number of isolates	Percentage
S. Enteritidis	138	28	166	47,3
C. difficile	11	84	95	27,1
Other salmonellas	25	16	41	11,7
***Campylobacter* spp.**	19	4	23	6,6
***Y. enterocolitica* O3**	19	1	20	0,6
***Shigella* spp.**	4	2	6	1,7
Total	216	135	351	100

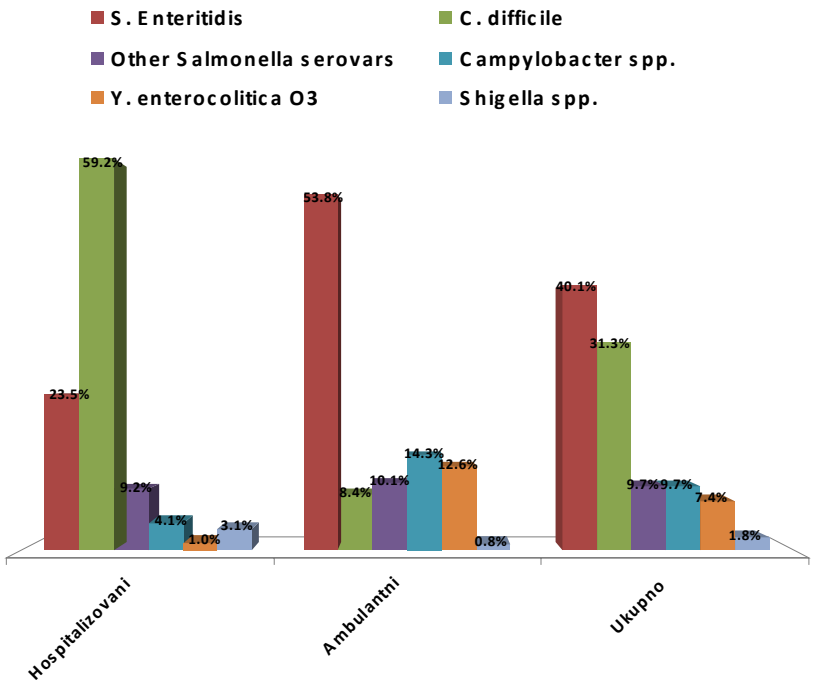

Figure 1. *Primo-isolates of bacterial enteric pathogens in hospitalized and outclinic patients*

The most often isolated microorganisms in hospitalized patients were *C. difficile* (59.2%), followed by *Salmonella* spp. (32.7%), *Campylobacter jejuni/coli* (4. 1%), *Shigella* spp. (3.1%), and *Y. enterocolitica* (1%) (Figure 1).

In outpatients' samples, *S.* Enteritidis prevailed (53.8%), while *Campylobacter jejuni/coli*, *Y. enterocolitica* O3, *C. difficile* and *Shigella flexneri* 2a were found in lower percentage: 14.3%, 12.6%, 8.4% and 0,84%, respectively (Figure 1).

S. Enteritidis strains were resistant to one or more antimicrobial drugs in 35.6%. Resistance to streptomycin was more often (21.8%) compared to tetracycline (10.3%), ampicillin (5.7%), trimethoprim / sulfamethoxazole (4.6%), nalidixic acid (3.4%), and amoxicillin/clavulanic acid (1.1%) (Figures 2, 3). Resistance to ceftriaxone, cefotaxime, kanamycin, gentamicin, chloramphenicol, and ciprofloxacin were not observed in any of S. Enteritidis isolates.

Table 2. *Serovars of isolated Salmonella enterica subsp. enterica*

Serovar	Number	Percentage
***S.* Enteritdis**	87	80,6
***S.* Typhimurium**	4	3,7
***S.* Infantis**	2	2,8
***S.* Bovismorbificans**	3	2,8
***S.* Paratyphi B**	2	1,9
***S.* Senftenberg**	3	1,9
***S.* Mbandaka**	2	1,9
***S.* Ohio**	1	0,9
***S.* Hadar**	1	0,9
***S.* Bispebjerg**	1	0,9
***S.* Thompson**	1	0,9
***S.* Bareilly**	1	0,9
Total	108	100

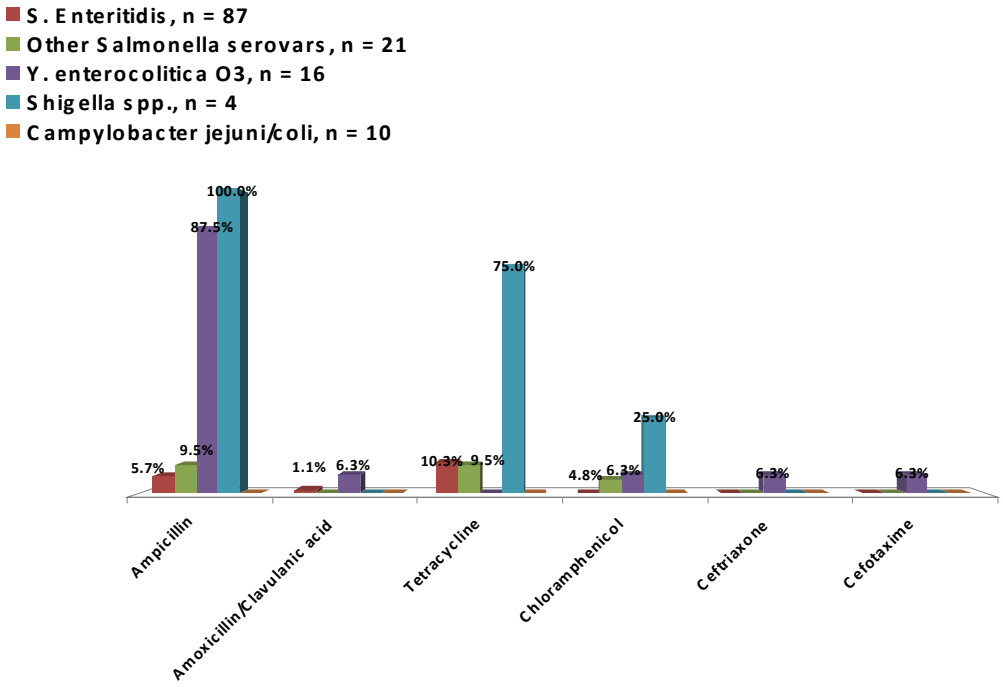

Figure 2. *Resistance to Ampicillin, Amoxicillin/Clavulanic acid, Tetracycline, Chloramphenicol, Ceftriaxone, Cefotaxime*

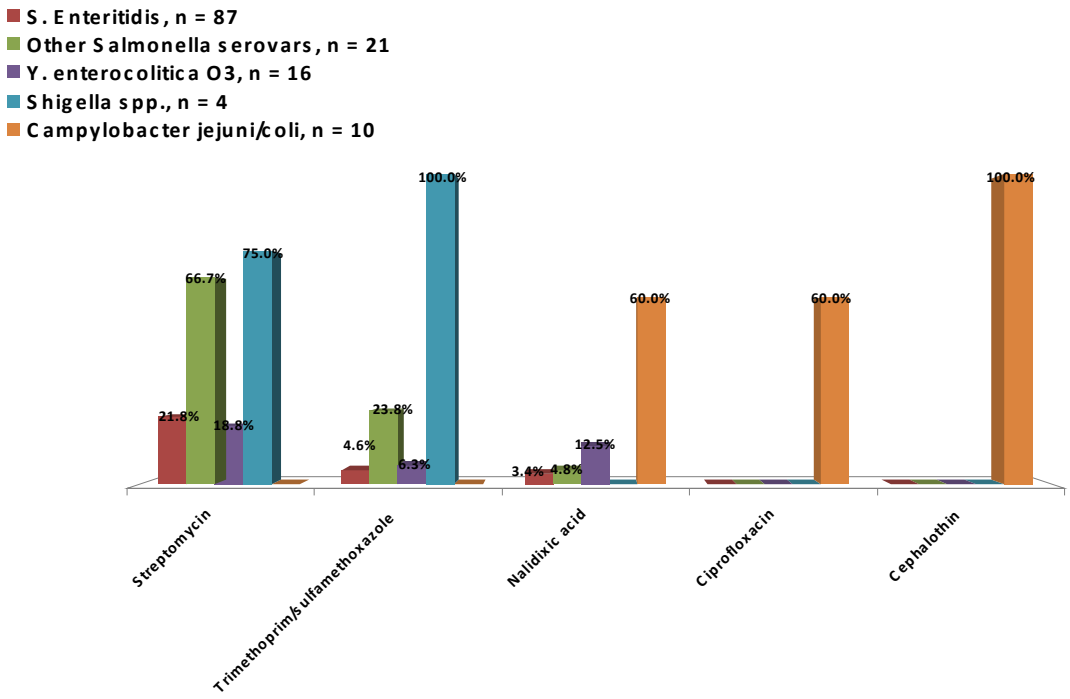

Figure 3. *Resistance to Cephalothin, Streptomycin, Nalidixic acid, Ciprofloxacin, Trimethoprim/Sulfamethoxazole*

Other *Salmonella* serotypes were resistant to one or more antimicrobial drugs in 71.4%. Resistance to streptomycin appeared even in 66.7% of the observed strains, while it was in lower degree reported against trimethoprim / sulfamethoxazole (23.8%), ampicillin (9.5%), tetracycline (9.5%), nalidixic acid (3.4%), and chloramphenicol (4.8%) (Figures 2, 3). Resistance to amoxicillin / clavulanic acid, ceftriaxone, cefotaxime, kanamycin, gentamicin, ciprofloxacin was not observed in any of the isolates.

For *Y. enterocolitica,* O3 resistance to one or more antibiotics was detected in 87.5% of strains. The highest level of resistance was against ampicillin, also in 87.5% (Figure 2), followed by resistance to streptomycin (18.8%), and nalidixic acid (12.5%) (Figure 3). Resistance to amoxicillin/clavulanic acid, chloramphenicol, trimethoprim/sulfamethoxazole, ceftriaxone, and cefotaxime was at the same level with 6.25% (Figures 2, 3). Resistance to tetracycline, kanamycin, gentamicin, and ciprofloxacin was not confirmed.

All *Shigella* spp. isolates expressed resistance against three or more drugs including ampicillin and tetracycline (Figures 2, 3). Strains resistant to amoxicillin/clavulanic acid, ceftriaxone, cefotaxime, kanamycin, gentamicin, chloramphenicol, and ciprofloxacin were not observed.

Inherited resistance of *Campylobacter jejuni/coli* to cephalothin was expressed in all strains, while 60% of the examined strains were resistant to quinolones: nalidixic acid and ciprofloxacin (Figure 3). Resistance to erythromycin, gentamicin, tetracycline and chloramphenicol was not confirmed.

DISCUSSION

The survey results confirmed the domination of *Salmonella* spp. with *S.* Enteritidis as a predominant bacterium in the examined samples.

It has been shown that diverse geographic areas are characterized by the domination of different causes of enterocolitis. In some parts of Asia (Orissa, India), the most prevalent causes are *V. cholera* (17.3%) and diarrhoeagenic *E. coli* (13.2%), while *Shigella* spp. (4.5%) and *Salmonella* spp. (0.7%) are present in a lower degree (4). In Hong Kong, the most isolated microorganism is *Vibrio parahaemolyticus* (42.3%) (5). However, in the European Union (EU), *Campylobacter* spp. and *Salmonella* spp. are dominant (6), whereas in the United States of America, *Salmonella* spp. and *Campylobacter* spp. are the most reported (7).

As this investigation also confirmed, *S.* Enteritidis is the most frequent isolated serotype of the genus *Salmonella*, especially in Europe, with 85% of strains. On the second place is *S.* Typhimurium, while *S.* Hadar, *S.* Virchow, and *S.* Infantis shift their position from the third to the fifth place, periodically (2). In 2002 and 2003, in Patra, Greece, the most isolated serotype among *Salmonella* was also *S.* Enteritidis (68%) followed by *S.* Typhimurium (19%), *S.* Newport (7%), *S.* Infantis (4%), and *S.* Paratyphi A (8).

Additionally, the examination of stool specimens of patients with diarrhea in Crete, Greece, confirmed *Salmonella enterica* as the most often isolated bacterium, followed by *Campylobacter* spp, enteropathogenic *Escherichia coli*, *Yersinia enterocolitica*, *Shigella* spp.,

and *Aeromonas hydrophila*. *Clostridium difficile* was confirmed in 14.4% of the examined samples (9).

C. difficile rarely causes community - acquired infective diarrhea and occurs more frequently in hospital settings (10), as this investigation also showed.

In comparison to other *Salmonella* serotypes, lower rates of drug resistance were determined for *S.* Enteritidis. The most expressed resistance was against streptomycin (21.8%), followed by resistance to tetracycline (10.3%), ampicillin (5.7%), trimethoprim / sulfamethoxazole (4.6%), nalidixic acid (3.4%), and amoxicillin/clavulanic acid (1.1%).

Resistance of other *Salmonella enterica* subsp. *enterica* to streptomycin was evidenced in 66.7% of isolates, followed by trimethoprim/sulfamethoxazole in 23.8%, ampicillin in 9.5%, tetracycline in 9.5%, and in the cases of nalidixic acid and chloramphenicol in 4.8% of the examined strains.

The investigation of *Salmonella* susceptibility in Crete, Greece revealed that resistance rate to ampicillin was 31.5%, and to trimethoprim/sulfamethoxazole 4.4% (9). In some other research, it was confirmed that 57 of 65 examined isolates of *S.* Enteritidis were susceptible to all the applied antibiotics. Resistance to amoxicillin and amoxicillin/clavulanic acid was exhibited in two strains, four strains were resistant only to tetracycline, and one strain expressed resistance both to streptomycin and tetracycline, while one strain was multiple-resistant (8). In the examination of other salmonella serotypes in Patra, Greece, the multiple-resistance to antibiotics was confirmed in case of *S.* Typhimurium (8). This serotype, isolated in China, expresses significant resistance. In 2002, only three isolates expressed susceptibility to all the applied antibiotics, while 82% of the examined isolates were resistant to at least eight antibiotics. Resistance to nalidixic acid was at the same rate, while resistance to ciprofloxacin was in 70% of tested strains (11).

In this research, *Y. enterocolitica* O3 expresses relatively high resistance rate, especially to ampicillin (87.5%) and streptomycin (18.75%). Resistance to nalidixic acid was observed in 12.5% of isolates, whereas resistance to ciprofloxacin was not confirmed.

Drug susceptibility testing of 151 strains of *Yersinia enterocolitica* isolated from humans, animals and environment, in Germany, confirmed that 99% of total isolates were resistant to amoxicillin (12). In Spain, the spreading of outbreak-related strains resistant to nalidixic acid has been evidenced (13). One of the earlier studies had suggested a high level of susceptibility of the examined strains to aminoglycosides: gentamicin, netilmicin, tobramycin, neomycin, and amikacin (14). This study also confirmed gentamicin susceptibility of the tested strains.

In this study, it was observed that bacteria of the genus *Shigella* have high resistance rate to ampicillin (100%) and trimethoprim/sulfamethoxazole (100%). Additionally, high rate of multiple resistance was determined (100%), too. However, the resistance to quinolones was not confirmed.

The investigation of *Shigella* drug susceptibility isolated in Crete, Greece revealed that 58.3% of isolates were resistant to ampicillin, while 30.5% of tested *Shigella* strains were resistant to trimethoprim/sulfamethoxazole (9). The investigation of shigellosis in Spanish travelers that mostly visited India revealed that 12 of 200 tested isolates were resisted to quinolones (15). The survey of *Shigella* isolated in Bangalore, India, conducted for the period 2002 to 2007, demonstrated that the tested strains were resistant to ampicillin (55.2%), cotrimoxazole (81.3%), chloramphenicol (43.3%), nalidixic acid (61.9%), and ciprofloxacin (20.9%), although there were not strains resistant to ceftriaxone (16), which was also confirmed in our study.

Bacteria of the genus *Campylobacter* express high resistance rate to quinolones in even 60% of tested strains. Strains resistant to erythromycin, gentamicin, tetracycline, and chloramphenicol were not confirmed in this investigation. The investigation of strains of the thermophilic campylobacters isolated in Niš in 2002 and 2003 revealed resistance to ciprofloxacin in 30% of the examined strains (17).

In Holland, it has been observed that resistance to tetracycline varies from 7% up to 15%. However, an increase of strains resistant to ofloxacin from 11% in 1994 to 29% in 1997 was demonstrated (18). High resistant rate to quinolones (44.5% to norfloxacin, and 40.5% to ciprofloxacin) were found with 44.5% among Campylobacter isolates in Crete, Greece. The same study revealed that 14.9% of tested isolates were resistant to erythromycin (9), which we did not find.

CONCLUSION

The most common cause of bacterial enterocolitis is *Salmonella enterica* subsp *enterica*. In the stool samples of hospitalized patients at the University Clinical Center Niš the most often isolate was *C. difficile*. In outpatients, the most frequently isolated bacteria were of the genus *Salmonella*. As for the salmonella isolates, the predominant serotype was *S.* Enteritidis. Resistance of *S.* Enteritidis was found in lower percentage than other salmonella serotypes, though with the highest rate against streptomycin (21.8%). *Y. enterocolitica* O3 exhibits a relatively high resistance rate especially to ampicillin (87.5%). Bacteria of the genus *Shigella* expressed multiple resistance. Bacteria of the genus *Campylobacter* exhibits significant resistance to quinolones.

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BAKTERIJSKI UZROČNICI DIJAREJNOG SINDROMA

Biljana Miljković-Selimović[1,2], Tatjana Babić[1,3], Predrag Stojanović[1,2]

[1]Nacionalna Referentna laboratorija za *Campylobacter* i *Helicobacter*
[2]Institut za mikrobiologiju i imunologiju, Medicinski fakultet u Nišu, Srbija
[3]Centar za mikrobiologiju, Institut za javno zdravlje Niš, Srbija

Sažetak

Bakterijski izazivači enterokolitisa pripadaju rodovima *Salmonella*, *Shigella*, *Vibrio*, termofilnim kampilobakterima, vrstama *Yesrsinia enterocolitica* (*Y. enterocolitica*) i *Clostridium difficile* (*C. difficile*), kao i grupi dijarejagenih *Escherichia coli*. Cilj rada bio je utvrđivanje učestalosti ovih mikroorganizama u stolici osoba sa dijarejom kao i određivanje njihove rezistencije na antibiotike.

Ispitivani su uzorci stolice bolesnika sa akutnom dijarejom tokom jednogodišnjeg perioda. Bakterije su izolovane i identifikovane korišćenjem standardnih mikrobioloških procedura. Ispitivanje osetljivosti *Salmonella*, *Shigella* i *Yersinia enterocolitica* vršeno je prema panelu od 12 antibiotika, a ispitivanje osetljivosti *Campylobactera jejuni*/*coli* prema panelu od 7 antibiotika, disk-difuzionom metodom.

Bakterije iz roda *Salmonella* dokazane su 51,4% primoizolata, dok je među njima najčešća *Salmonella* Enteritidis (*S.* Enteritidis). Sojevi *S*. Enteritidis bili su rezistentni na jedan ili više antibiotika u 35,6%. Najčešće je bila ispoljena rezistencija prema streptomicinu (21,8%). Rezistencija na jedan ili više antibiotika kod sojeva *Y. enterocolitica* O3 dokazana je u 87,5%. Najčešće je bila prisutna rezistencija prema ampicilinu, takođe, u 87,5%. Svi izolati *Shigella* spp. bili su rezistentni prema tri ili više antibiotika. Kod 60% ispitanih sojeva kampilobaktera dokazana je rezistencija prema fluorokvinolonima: nalidiksinskoj kiselini i ciprofloksacinu.

U ispitivanom materijalu dominirao je nalaz *Salmonella* spp. Kod *S*. Enteritidis, zapažena je izražena rezistencija prema streptomicinu. *Y. enterocolitica* O3 je ispoljila dosta visok nivo rezistencije, uglavnom prema ampicilinu. Multipna rezistencija zapažena je u rodu *Shigella.* Kod vrsta *Campylobacter*, najčešća je bila rezistencija prema fluorokvinolonima.

Ključne reči: **bakterijski patogeni, *Salmonella* spp., dijareja**