

**RESISTANT STRAINS OF *ESCHERICHIA COLI* IN THE ENVIRONMENT  
OF THE MARTIN REGION COMPARED TO RESISTANT STRAINS IN SLOVAKIA  
REZISTENTNÉ KMENE *ESCHERICHIA COLI* V ŽIVOTNOM PROSTREDÍ  
V REGIÓNE MARTIN V POROVNANÍ S REZISTENTNÝMI KMEŇMI NA SLOVENSKU**

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**ABSTRACT**

**Introduction:** This paper concerns testing for microorganisms with antimicrobial resistance (AMR) in the urban environment, focusing on the samples of microorganisms that can transiently colonise the human gut microbiome from children's sandpits.

**Aim:** The aim was to diagnose resistant *Escherichia coli* strains from sandpits in the Martin region in 2018. The findings were compared with other data on resistance in *Escherichia coli* in Slovakia gathered between 2017 and 2021.

**Methods:** Saline solutions were prepared from the sandpit samples, which were then cultivated on 3 different agars (Endo agar, deoxycholate-citrate agar and HiChrome agar for *Escherichia coli*). Analysis with MALDI-TOF (matrix assisted laser desorption/ionization - time of flight) mass spectrometry confirmed the presence of various bacterial species in the samples including *Escherichia coli*. The antimicrobial susceptibility of the *E. coli* strains was determined using a disc diffusion method and a quantitative method establishing a minimum inhibitory concentration.

**Results:** A total of 32 different bacterial species were identified in the sand samples, of which the most numerous species was *Escherichia coli*. Three of the strains of *E. coli* isolated from the environment were resistant to multiple antibiotics (aminopenicillins, fluoroquinolones, tetracyclines) at time when other research found only a slight decrease in resistance to four types of antibiotics in Slovakia (aminopenicillins, fluoroquinolones, third generation cephalosporins and aminoglycosides). A high percentage of resistance (50 – 70 %) persists to the present at least in the case of aminopenicillins.

**Conclusion:** The reported findings of the presence of resistant *E. coli* strains in an urban environment indicate that it would be beneficial to include playgrounds in monitoring and to conduct regular testing to track the changes in resistance.

**Key words:** Antimicrobial resistance. *Escherichia coli*. Environment.

**ABSTRAKT**

**Úvod:** V našom príspevku sme sa zamerali na prítomnosť mikroorganizmov v urbánom prostredí. Skúmali sme výskyt antimikrobiálne rezistentných mikroorganizmov, ktoré môžu transientne kolonizovať mikrobióm detí na pieskoviskách.

**Cieľ:** Naším cieľom bolo diagnostikovať rezistentné kmene *Escherichia coli* z pieskovísk v regióne Martin z roku 2018. Naše výsledky sme porovnali s údajmi o rezistencii kmeňov *Escherichia coli* na Slovensku v rokoch 2017 – 2021.

**Metódy:** Zo získaných vzoriek piesku sme pripravili soľné roztoky, ktoré sme kultivovali na 3 rôznych agaroch (Endo agar, deoxycholát-citrát agar a HiChrome agar pre *Escherichia coli*).

Metódou hmotnostnej spektrometrie s maticou asistovanej laserovej desorpcie/ionizácie s analyzátorom doby letu sme potvrdili vo vzorkách prítomnosť rôznych druhov baktérií vrátane *Escherichia coli*. Antimikrobiálnu citlivosť kmeňov *Escherichia coli* sme stanovili difúznou diskovou metódou a kvantitatívnu metódou stanovenia minimálnej inhibičnej koncentrácie.

**Výsledky:** Vo vzorkách piesku sme detekovali 32 rôznych druhov baktérií, z ktorých najpočetnejšie bol zastúpený druh *Escherichia coli*. Vyizolovali sme 3 kmene *Escherichia coli* rezistentné na rôzne antibiotiká (aminopenicilíny, fluorochinolóny, tetracyklíny) z prostredia, a to v období, kedy bol na Slovensku zaznamenaný len mierny pokles rezistencie pre 4 druhy antibiotík (aminopenicilíny, fluorochinolóny, cefalosporíny 3. generácie a aminoglykozidy). Vysoké percento rezistencie (50 – 70 %) minimálne u aminopenicilínov pretrváva do súčasnosti.

**Záver:** Na základe našich zistení o prítomnosti rezistentných kmeňov *Escherichia coli* v urbánom prostredí, by bol prospešný ich monitoring v tomto prostredí (detské ihriská) spolu s trendom výskytu rezistencie.

**Kľúčové slová:** Antimikrobiálna rezistencia. *Escherichia coli*. Prostredie.

**INTRODUCTION**

Antimicrobial resistance (AMR) is a condition in which a microorganism becomes resistant to antibiotics that were previously effective against it. The European Union (EU) estimates that approximately 33 000 people die each year from infections caused by resistant microorganisms. The EU's approach to AMR is based on the "One Health" concept, recognising the connections between human and animal health as well as environmental factors [1]. The pathogen in which AMR was most frequently reported in European countries in 2021 was *Escherichia coli* (*E. coli*), according to European Antimicrobial Resistance Surveillance Network (EARS-Net). Between 2017 and 2021, the highest level of resistance in *E. coli* samples from humans was against aminopenicillins (overall 55.98 %). This was followed by *E. coli* resistance to fluoroquinolones (24.92 %) and

cephalosporins (15.12 %). The antibiotics with the lowest percentage of resistance were the aminoglycosides (10.78 %) [2]. Antimicrobial resistant *E. coli* have also been found in the environment (in soil samples), where the prevalence of isolates with resistance is 11.7% [3]. *E. coli* bacteria could be used as an indicator of environmental AMR caused by anthropogenic factors. The National References Laboratories could easily implement culture-base methods to detect AMR in environmental samples, using standardised protocols for selective cultivation of AMR *E. coli* from human and animal samples [4]. Similar resistant genes of *E. coli* microorganisms have been detected in animal (hog) faecal samples and environmental (soil) samples in their vicinity. These findings suggested that the AMR genes could be transmitted horizontally from the animal faeces to surrounding environments [5]. Birds living in urban areas should also be considered as potential disseminators of resistant *E. coli* strains [6].

One goal of our study was to compare the presence of resistant *E. coli* strains from environmental samples in the vicinity of Martin in 2018 with resistant *E. coli* strains isolated from human samples collected all over Slovakia during the period 2017–2021. The article also reviews antimicrobial policy in Slovakia in relation to the presence of resistant *E. coli* strains from 2017 to the present.

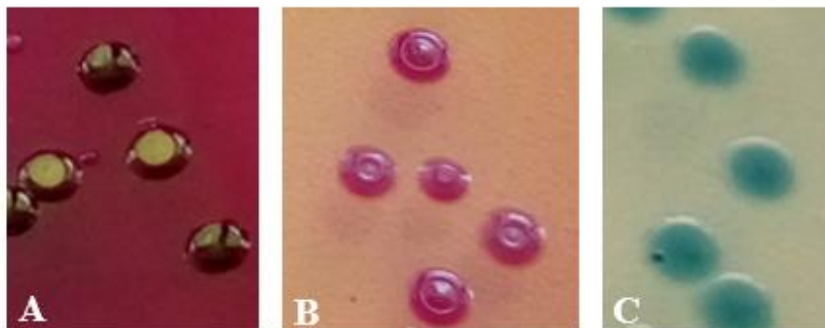
## METHODS

AMR data for 4 types of antibiotics (aminopenicillins, fluoroquinolones, third generation cephalosporins and aminoglycosides) in Slovakia in the last 5 years were taken from the Surveillance Atlas of Infectious Diseases [7], which is administered by the European Centre for Disease Prevention and Control (ECDC).

The environmental samples were collected from 35 sandpits in 2018. Sand samples were taken at 5 places in each sandpit. The samples were processed immediately after sampling. A part of each sand sample in the amount 10 g was suspended in 90 ml of saline solution for one hour. At the same time 3 g of each sand sample was taken and cultivated in 5 ml of nutrient broth for 24 hours at 37 °C. After one hour of incubation, 500 µl of each saline suspension was inoculated to ENDO agar, deoxy-cholate citrate agar (DC agar) and chromogenic coliform agar (HiChrome agar) for *E. coli* after one hour of incubation. The samples were incubated for 24 hours at 37 °C. The cultivated microorganisms were diagnosed using colony morphology. Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) was used to verify the colony morphology findings for every microorganism, focusing especially on *E. coli* strains. *E. coli* strains were tested using disk diffusion method for antimicrobial susceptibility to third generation cephalosporins, fluoroquinolones and aminoglycosides. Resistant strains were also tested with dilution minimal inhibition concentration (MIC) assays to verify the initial findings of antimicrobial susceptibility.

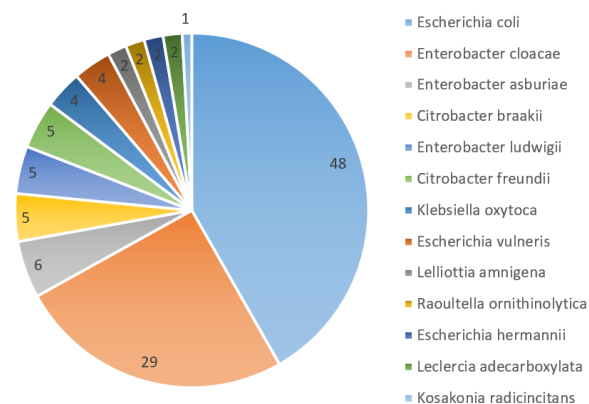
## RESULTS

On ENDO agar, tested *E. coli* strains produced round-shaped red colonies with a greenish metallic sheen. On DC agar, *E. coli* strains grew in pink coloured colonies with well-defined edges. Finally, colonies of *E. coli* on HiChrome agar had a bluish green colouration (Figure 1). The colony morphology of the tested strains isolated from sand samples was in accordance with the defined colony morphology of *E. coli*.



**Figure 1** Colony morphology of *E. coli* strains. A: ENDO agar, B: DC agar, C: HiChrome agar.

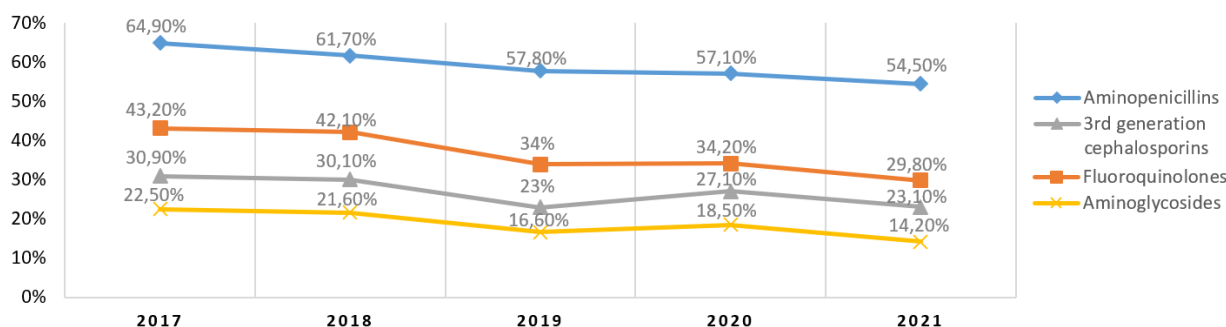
A total of 175 sand samples were examined, from which 149 bacteria colonies were successfully isolated and identified by MALDI-TOF MS. Based on microbial taxonomy, 32 bacterial species were identified of which 13 belonged to the family Enterobacteriaceae (Fig. 2). The most prevalent strain was *E. coli* (48/149, i.e., 32.2 % of total microorganisms). The second most prevalent strain was *Enterobacter cloacae* (29/149, i.e., 19.46 % of total microorganisms). The strains *Enterobacter asburiae* was identified 6 times (i.e., 4 % of total microorganisms). *Citrobacter braakii*, *Enterobacter ludwigii*, *Citrobacter freundii* were each detected 5 times (i.e. 3.35 % of overall number of microorganisms). The strains *Klebsiella oxytoca* and *Escherichia vulneris* were isolated 4 times (i.e., 2.68 % of total microorganisms) (Fig. 2).



**Figure 2** 13 different species of Family Enterobacteriaceae isolated from sand samples in Martin region in Slovakia

The research findings indicate that the resistance of *E. coli* strains has decreased since 2017. The percentage range of resistance was different for each antibiotic group. The highest percentage was for resistance to aminopenicillins (50 – 70 %), followed by fluoroquinolones (25 – 50 %). Resistance to third generation cephalosporins was detected in 25 – 50 % of isolates during the years 2017 and 2018. The percentage decreased to under 25 % in 2021. The last group of antibiotics, aminoglycosides, had a lower percentage range (10 – 25 %) during the years 2017 – 2021 (Fig. 3).

Three strains of *E. coli* resistant at least to one antibiotic were detected in the environmental samples. Strains exhibited resistance to aminopenicillin (ampicillin), second generation quinolones (ciprofloxacin), tetracycline and two antibiotic combinations (trimethoprim + sulfonamide and ampicillin + sulbactam). *E. coli* strain 1 was the most resistant strain of the three, with resistance to four antibiotics. Strain 3 had almost the same antimicrobial resistance as strain 1, with resistance to three antimicrobial drugs. Strain 2 showed the highest antimicrobial susceptibility of the three strains (Tab. 1).



**Figure 3** Percentage of resistant *E. coli* isolates between years 2017-2021 for 4 different antibiotics in Slovakia [7].

**Table 1** Isolated resistant strains of *E. coli* in sand samples in Slovakia

ATB Resistant <i>E. coli</i>	Trimethoprim +sulfonamide	Ampicillin	Ampicillin +sulbactam	Ciprofloxacin	Tetracycline
Strain 1	R (>4)	R (>32)	S	R (>4)	R (>16)
Strain 2	S	S	S	S	R (>16)
Strain 3	S	R (>32)	R 16	R (>4)	S

**Abbreviations:** ATB = antibiotic, R = resistant, S = susceptible

## DISCUSSION

The samples taken from children's playground sandpits in and around Martin, Slovakia, are evidence that strains of *E. coli* resistant to ampicillin, trimetoprim+sulfonamid, ciprofloxacin and tetracycline are present in the urban environment. *E. coli* isolated strains from stool samples should be tested for AMR to 4 antibiotics: ampicillin, ciprofloxacin, trimethoprim + sulfonamide, tetracycline according to outpatient data [8]. Isolates of *E. coli* from non-urban environments (forest and potting soil mix) showed resistance only to sulfisoxazole (81 %). Isolates from the urban environment (garden landscapes, residential yards, and indoor potted plants) showed resistance to sulfisoxazole (78.05 %), ampicillin (7.32 %) and cefoxitin (4.88 %) [9]. Resistance in *E. coli* strains isolated in groundwater affected mainly aminoglycoside, penicillin, and tetracycline antibiotics [10]. This resistance pattern was confirmed in a study where the most prevalent AMR genes were for  $\beta$ -lactam group, then tetracyclines, aminoglycosides and last sulphonamides [6]. Multiple AMRs are associated with integrons and mobile genetic elements in the bacterial genome [11]. Drivers of AMR include antimicrobials, biocides (surfactants and disinfectants), plant-derived compounds, xenobiotics (toluene, hexane, and octanol) and heavy metals. These drivers are transmitted in the environment by aquaculture systems, sewage sludge, spreading of animal manure as well as industrial and municipal wastewater [12]. The main point of antimicrobial transmission is sewage outflows from hospitals and the general populace which are mixed in wastewater treatment plants. Treatment of AMR requires a holistic approach based on "One Health" principles in which human health, animal health and environment are considered jointly [11]. Multi-sectoral and One Health collaboration in Slovakia are on a good level according to the Surveillance report from the year 2020. Additionally, the national surveillance system for AMR in humans is on a very good level [13]. Implementation of AMR environmental monitoring could be of benefit as it would resistance monitoring on an ongoing basis. *E. coli* strains could be used as indicators of resistance phenotypes in the environment. The advantage of monitoring this microorganism is that it can be isolated from animal and human samples, thus making it possible to compare strains between

species [4].

Some steps have been taken on the national level in Slovakia to improve the handling of AMR. The first one was the National Action Plan on Antimicrobial Resistance in the Slovak Republic for the period 2019-2020, the next one is the Standard Diagnostic and Therapeutic Procedure for the Implementation of Antimicrobial Policy in Institutional Healthcare Settings, which was approved by the Minister of Health of the Slovak Republic on 1 April 2020 [14,15]. The last two are the Slovak National Antimicrobial Resistance Surveillance System and the Slovak Antimicrobial Resistance Veterinary Database [16]. This database includes 4 projects supported by the Slovak Research and Development Agency relating to AMR. The results of the projects are described in articles, but the database does not yet contain official guidance on AMR in animal or environmental samples.

The European Food Safety Authority studied transmission of zoonotic bacteria between poultry, pigs and cattle from other farms or slaughterhouses [17]. The prevalence of complete susceptibility in *E. coli* strains from food-producing animals was around 20 % in Slovakia in 2018. There have been only a few reports about AMR of indicator *E. coli* strains in animals in Slovakia since 2018. Indicator *E. coli* strains in pigs had an extremely high level of resistance for tetracycline (74.1 %) in 2019. The resistance of indicator *E. coli* strains in broilers reached extremely high levels for ampicillin (82.1 %) and ciprofloxacin (96.4 %) in 2020 [18]. It has been shown that higher antibiotic consumption in food-producing animals has consequences for the full susceptibility of *E. coli* strains in these animals [19]. Use of antibiotics in Slovakia in both the community and hospital sectors has shown a decreasing trend since 2017 [20].

## CONCLUSION

We isolated 3 resistant *E. coli* strains from sandpits in 2018. The number of resistant strains is not especially significant because just the presence of resistant strains indicates the potential for their transient colonisation of the human gut biome, especially in children. Our findings on AMR to aminopenicillins and fluoroquinolones in *E. coli* strains in the environmental samples correlates with data on the most common AMR *E. coli* from human samples. We assume that there is a potential transmission vector for AMR strains between these

types of samples.

We did not investigate antimicrobial resistant genes in isolated *E. coli* strains. Another recommendation would be to identify microorganisms from environmental samples more frequently to have an overview of changes in AMR in the urban environment.

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