Recreational pools filled with mineralized thermal water are potential reservoirs of pathogenic *Vibrio* spp.

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ABSTRACT
Vibrio spp. are Gram-negative rod-shaped bacteria commonly present in marine, estuarine and natural freshwater environments. A few members of this genus are associated with human diseases. Here we present the study of *Vibrio* spp. isolations from 20 artificial recreational pools in Slovakia. Water samples were collected from artificial pools filled with mineralized thermal water in eight recreational areas in Slovakia in 2019 and 2020. Ninety six out of 176 samples were positive for *Vibrio* spp. Totally 118 different strains of *Vibrio* spp. were isolated, from which 77 belonged to potentially pathogenic species – *V. cholerae* (34 isolates), *V. vulnificus* (4 isolates), *V. furnissii* (3 isolates), *V. fluvialis* (25 isolates), *V. alginolyticus* (10 isolates) and *V. mimicus* (1 isolate). To our knowledge this is the first study demonstrating the presence of pathogenic or potentially pathogenic *Vibrio* spp. in artificial pools filled with thermal mineralized waters even disinfected with chlorine compounds.

KEYWORDS
vibrio – mineralized water – recreational pool – biofilm

SÚHRN
Sojka M., Umrian M., Kaniková M., Petrovičová K.: Rekreačné bazény plnené termálnou mineralizovanou vodou sú potenciálnymi rezervoármi patogénnych *Vibrio* spp.

Vibrio spp. sú gramnegatívne tyčinkovité baktérie, prirodzene sa vyskytujúce v morskej, brakickej i sladkej vode. Niektoré druhy môžu spôsobovať ochorenia ľudí. V publikácii prezentujeme štúdiu výskytu *Vibrio* spp. v 20 umelých rekreačných bazénoch na Slovensku. Vzorky vôd boli odoberané z umelých bazénov, plnených mineralizovanou termálnou vodou, v deviatich rekreačných oblastiach na Slovensku, v rokoch 2019 a 2020. *Vibrio* spp. bolo izolované v 96 zo 176 vzoriek vôd. Spolu bolo izolovaných 118 rôznych kmeňov vibrií, z ktorých 77 patrilo k niektorému z potenciálne patogénnych druhov – *V. cholerae* (34 izolátov), *V. vulnificus* (4 izoláty), *V. furnissii* (3 izoláty), *V. fluvialis* (25 izolátov), *V. alginolyticus* (10 izolátov) and *V. mimicus* (1 izolát). Táto štúdia je, podľa našich informácií, prvou vo svete, dokumentujúcou prítomnosť patogénnych alebo potenciálne patogénnych *Vibrio* spp. v umelých bazénoch s chlórovou dezinfekciou, plnených termálnou mineralizovanou vodou.

KĽÚČOVÉ SLOVÁ
vibrio – mineralizovaná voda – rekreačný bazén – biofilm

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INTRODUCTION

Vibrionaceae family and especially *Vibrio* spp. are a group of common, Gram-negative, rod-shaped, bacteria. They are the natural constituents of freshwater, estuarine and marine environments [1]. Vibrios are typically halophilic and most of them are unable to grow without Na^+ supplementation or Na^+ cations serve at least as a factor supporting the growth of these bacteria [2].

Only a few species of *Vibrionaceae* family are associated with human infections. Cases of *Vibrio* spp. infections have a marked seasonal distribution, with most cases occurring during warmer months [1]. *Vibrio* spp. infections are usually initiated from exposure to contaminated water or consumption of raw or undercooked contaminated seafood. They cause a variety of symptoms in humans, depending on the *Vibrio* species (or serotype) and portal of entry. Cholera is caused exclusively by *V. cholerae* serogroup O1 or O139, producing cholera toxin. Other vibrio infection manifestations are – cholera-like syndrome, gastroenteritis, ear, eye, wound infections and rarely sepsis [1, 3]. The most common and most important pathogenic vibrio species are *V. cholerae*, *V. mimicus, V. parahaemolyticus*, *V. vulnificus, V. alginolyticus, V. fluvialis* and *V. furnissii* [1].

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Although cholera is rare in developed countries, vibriosis cases are becoming increasingly frequent. Several authors link this with global warming and subsequent changes in vibrio ecology in marine and estuarine environment and non-cholera vibrios are nowadays considered as emerging pathogens [4, 5, 6].

There is extensive research done worldwide in vibrio ecology in marine and estuarine water environment and in natural fresh waters [1–7]. Very little is known about presence of these bacteria in artificial swimming or recreational pools. No study regarding the occurrence of vibrios in artificial swimming pools or study about risk of exposure for humans to pathogenic *Vibrio* spp. during recreational activities in artificial swimming pools has been published until now. As vibrios are generally characterized as halophilic bacteria, in this study we focused on the possible occurrence of different pathogenic vibrios in artificial swimming pools and recreational pools filled with chlorinated mineralized thermal water. We also focused on ability to form a biofilm, which might be one of the strategies for survival in this harsh environment.

MATERIALS AND METHODS

Total 176 water samples were collected from 20 artificial pools in eight different recreational areas (eight different operators) in Slovakia. The pools were filled with waters with different chemical composition and declared as mineralized waters by the operators. Declared total mineralization varied from 247 mg/L to 18724 mg/L and declared Na^+ content varied form 21,2 mg/L to 3100 mg/L (table 1).

Water samples were transported to the laboratory in sterile glass bottles in thermally insulated bags without cooling to prevent temperature shocks which may affect the recovery of vibrios. The samples were processed within 12 hours after collection. Filtration combined with enrichment in alkaline peptone water was used. Briefly, 500 mL of water sample was filtered through the nitrocellulose membrane filter (0,45 µm pore size, Millipore) and filter was pre-incubated for 8 hours in alkaline peptone water (Oxoid) in 37 °C and subsequently inoculated onto TCBS agar (Oxoid) and Chromatic Vibrio agar (Liofilchem) and incubated in 37 °C for 24 hours. Suspected colonies were subcultured on blood agar (Oxoid) and presumptively identified as *Vibrio* sp. using combination of colonial charasteristics, oxidase activity, string test, resistance to O129 vibriostatic agent (150 µg), typical appearance on TSI agar (HiMedia) and MIU combined medium (HiMedia). Vibrios were finally identified using 35 classical biochemical and physiological characteristics in media enriched with 1% of NaCl. A set of dichotomous keys according to Noguerola and Blanch [8] was used for presumptive identification of particular strain in the first step. The final identification of the strain was confirmed by comparing the bacterial strain biochemical and physiological profile with the consensus matrix of the particular species [2, 8]. *Vibrio cholerae* isolates were serogrouped by a slide agglutination with O1 polyvalent and O1 monovalent antisera (Denka Seiken) and possible production of cholera toxin was examined using reverse passive latex agglutination (VET-RPLA toxin detection kit, Oxoid).

To determine the ability of adhering to artificial surfaces and to produce biofilm, the microplate method according to Stepanovic was employed [9]. Briefly, the strains were grown for 24 hours in 37°C in tryptic soy broth (Oxoid) in 96-well polystyrene microplate allowing them to adhere to plate walls and form biofilm. Subsequently the medium with planktonic bacteria was aspirated and wells were washed with saline. Formed bacterial biofilms were fixed with methanol and stained with crystal violet. The biofilms were then destained with 96 % ethanol and optical density (OD) at 595 nm was measured. All the strains were tested in triplicates. The cut off OD (OD_C) was defined as three standard deviations above the mean of the negative control and ability to produce biofilm was classified as strong if OD of tested strain (OD_S) was higher than 4x OD_C, moderate if OD_S was between 2x OD_C and 4x OD_C and weak if OD_S was below 2x OD_C.

Table 1. Characteristics of water samples collected from the artificial pools filled with mineralized waters in Slovakia in 2019 and 2020

	Declared Na^+ concentration (mg/L)	Number of examined pools	Number of collected samples	*Vibrio* spp. positive samples No. (%)
Operator 1	3100	4	53	53 (100)
Operator 2	3100	1	17	17 (100)
Operator 3	764	2	15	3 (33)
Operator 4	391	2	12	4 (33)
Operator 5	388	5	10	10 (100)
Operator 6	197	2	22	5 (23)
Operator 7	172	3	13	3 (23)
Operator 8	21	4	34	1 (3)

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RESULTS AND DISCUSSION

Ninety-six (54,5%) out of 176 mineralized water samples taken from artificial pools in eight different recreational areas in Slovakia in years 2019 and 2020 were positive for *Vibrio* spp. by means of classical culture technique of vibrio isolation (see table 1). Only pools in which presence of mineralized thermal water was declared by the operator were examined in each of the eight enrolled recreational areas, as it is known that vibrios are stimulated by Na^+ or require this as a growth factor. Only with few exceptions, vibrios grow preferentially in waters with salinity below 2,5% NaCl and with temperature more than 18 °C [5]. This is probably the reason why they exhibit a strong seasonality in natural environments, being most abundant in summer months. This seasonality was not present in vibrios isolated in this study from artificial pools, these were isolated from the studied water independently on the season.

From 23 pools monitored in this study, 20 were positive for *Vibrio* spp. All three pools which were negative for *Vibrio* spp. were operated by the same operator in one recreational area (operator 8). Although these were declared to be filled with mineralized thermal water, Na^+ concentration was only 21 mg/L. As *Vibrio* spp. are generally halophilic microorganisms and Na^+ enhances their growth [2], this could be the explanation for our findings in those pools. Only one sample from this particular recreational area was positive for *Vibrio* sp.; four different *Vibrio* spp. strains were isolated from this sample but no one was considered as potentially pathogenic species (Figure 1). Although from this data the Na^+ concentration in water seems to be one of the factors responsible for the presence of pathogenic and potentially pathogenic vibrios in pools, further studies are needed to evaluate this speculation.

Totally 118 different strains of *Vibrio* spp. were isolated (see Figure 1), from which 77 belonged to potentially pathogenic species – *V. cholerae* (34 isolates), *V. vulnificus* (4 isolates), *V. furnissii* (3 isolates), *V. fluvialis* (25 isolates), *V. alginolyticus* (10 isolates) and *V. mimicus* (1 isolate). Forty-one isolates were classified as *Vibrio* sp., characterized as gram-negative, vibrio-shaped, oxidase-positive, glucose-fermenting organisms, resistant to O129 vibriostatic agent, but not identified as potentially pathogenic species by means of other biochemical and physiological properties.

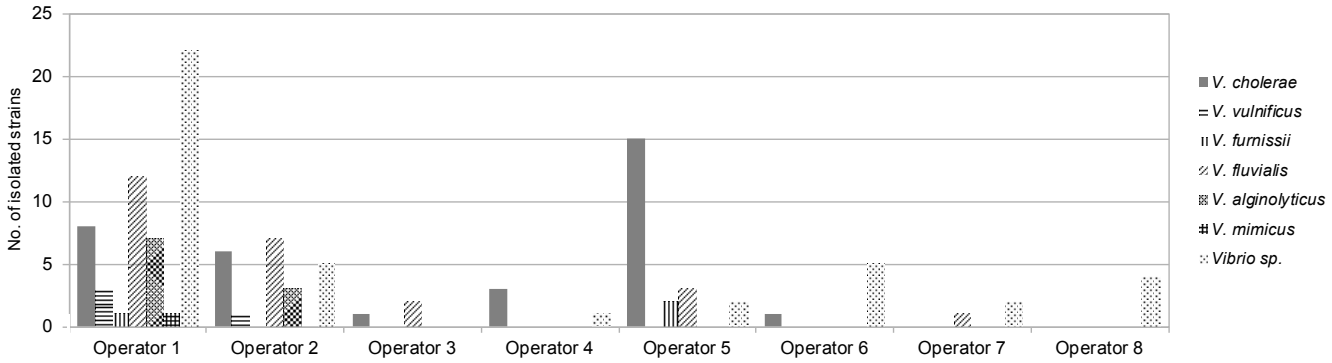

Figure 1. *Vibrio* spp. isolated from artificial pools filled with mineralized waters in Slovakia in 2019 and 2020.

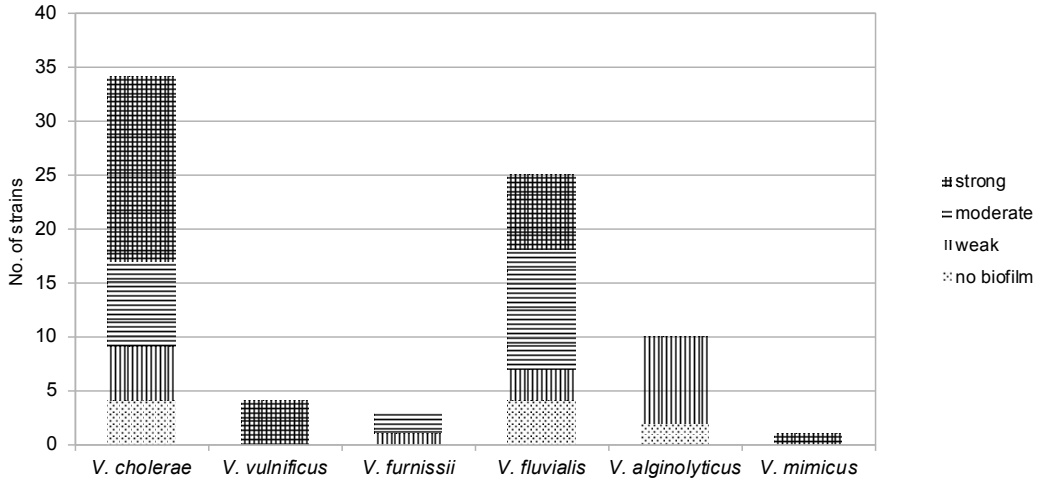

Figure 2. Biofilm formation of potentially pathogenic *Vibrio* spp. isolated from artificial pools filled with mineralized waters in Slovakia in 2019 and 2020

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Out of 34 *V. cholerae* strains, 32 were identified clearly as non-O1, non-O139, two isolates agglutinated spontaneously and thus were non-agglutinable, even after boiling. All 34 *V. cholerae* strains were tested for cholera toxin production using reverse passive latex agglutination with negative results.

Vibrio sp. are ubiquitous in natural aquatic ecosystems. While many vibrios are free living, a small group can form pathogenic or symbiotic interactions with eukaryotic hosts. The potentially pathogenic vibrios alternate between growth within their hosts and prolonged survival in aquatic habitats. Adaptation of vibrios to changing parameters of the aquatic ecosystem as well as to those of their respective hosts is critical to their survival and colonization success. One key factor for environmental survival and transmission is the ability to form biofilms (reviewed in [10]). The ability to form biofilm structures was also demonstrated in 67 (87 %) out of 77 tested potentially pathogenic vibrios isolated during this study (Figure 2).

CONCLUSIONS

Six different *Vibrio* species which are considered as pathogenic or potentially pathogenic were isolated from artificial recreational pools in Slovakia in 2019 and 2020, including emerging pathogens *V. fluvialis*, *V. furnissii* and *V. vulnificus*. Biofilm formation ability was shown in majority of tested strains. We can estimate that this could be one of the factors enabling them to survive challenging conditions in chlorinated recreational pools. To our knowledge this is the first study demonstrating the presence of pathogenic or potentially pathogenic *Vibrio* spp. in artificial pools filled with thermal mineralized waters even disinfected with chlorine compounds. From this data we may conclude that such pools may serve as reservoirs of these bacteria. Further studies are needed to quantify the risk potential of such recreational activities for acquiring infection with *Vibrio* sp.

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