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Prevalence and genetic variability of *Plesiomonas shigelloides* in temperate climate surface waters of the Pannonian Plain

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Abstract: *Plesiomonas shigelloides,* a Gram-negative bacterium and the causative agent of intestinal diseases and extraintestinal infections in humans and animals, is most frequently found in aquatic environments in tropical or subtropical areas. The present study was designed to establish the prevalence and genetic variability of *P. shigelloides* in surface waters (lakes, rivers, ponds, inlets and canals) located in a temperate climate zone, namely the Pannonian Plain of the northern part of Serbia and southern part of Hungary. The strains were isolated directly by plating samples on inositol-brilliant green-bile agar with neutral red or phenol red as indicators. Our results indicate that phenol red effectively facilitates differentiation of *P. shigelloides* from other bacteria. A number of samples were enriched using alkaline peptone water broth, peptone inositol-bile broth and tetrathionate broth. The recovery of the isolates was more successful with the first medium. Out of a total of 51 water samples collected from 28 different locations, 22 samples (43.1%) were found positive for *P. shigelloides*. Among the 37 isolated strains, 34 were from lakes (Šatrinci, Ludaš, Panonija, Krivaja, Pecs, Kapetanski rit, Pavlovci, Kovácsszénája, Dobrodol, Vranjaš, Borkovac, Hermann Ottó, Sot, Šelevrenac, Zobnatica, Palić, Orfüí, Jarkovci, Čonoplja) and 3 were from rivers (Danube, Sava). The strains were identified by phenotypic characteristic or by the VITEK2 system and confirmed by PCR using 23S rRNA species-specific oligos. The strains showed a high genetic variability, displaying a variety of RAPD profiles. Our results reveal for the first time a high prevalence of genetically diverse *P. shigelloides* populations in surface waters located in the temperate climate of central and southeastern Europe.

Key words: *Plesiomonas shigelloides*; surface waters; temperate climate; isolation; RAPD-PCR

INTRODUCTION

Plesiomonas shigelloides is a Gram-negative, rod-shaped bacterium found in aquatic environments and aquatic hosts, including fish, amphibians, shellfish, as well as in various terrestrial animals [1-5]. *P. shigelloides* is a facultatively anaerobic, motile (by means of lophotrichous flagella), catalase- and oxidase-positive bacterium, capable of producing indole from tryptophan and acid without gas, from D-glucose, m-inositol, maltose and trehalose [6]. The temperature range for *P. shigelloides* growth is between 8 and 44°C, while optimal growth occurs in the range of 37-38°C [7,8]. Due to a specific phenotype, the bacterium has been renamed several times: C27 (1947) [9], *Pseudomonas shigelloides* (1954); *Escherichia sonnei* (1956); *Pseudomonas michigani* (1959), *Aeromonas shigelloides*; *Pleisomonas shigelloides* (1960); *Fergusonia shigelloides* (1963); *Scatomonas michigani* (1964), *Vibrio shigelloides* (1971) [10,11] and *Proteus shigelloides* [12]. For a long time, it was classified as a member of the family *Vibrionaceae*, along with *Aeromonas*, as they share many phenotypic features. However, phylogenetic analyses of 5S rRNA, 16S rRNA and 23S rRNA sequences indicates that *Plesiomonas shigelloides* is more closely related to the family *Enterobacteriaceae,* especially to the genus *Proteus* [12-15]. Accordingly,

it is currently classified as the only oxidase-positive lophotrichous member of *Enterobacteriaceae*.

Most reports on the isolation of *P. shigelloides* from environmental samples are from countries situated in tropical or subtropical areas [1,16-23]. The high incidence of *P. shigelloides* in Southeast Asia has even given the adjective "Asian" to this bacterium, although it is also frequently found in freshwaters of Africa and South America [24-26]. However, there have been several successful isolations of *P. shigelloides* from environments in temperate latitudes, i.e. in the Czech Republic and the Netherlands [3,27,28], as well as cold climates, in Sweden [4,29].

The bacterium is a concern from both human and animal health prospects. In humans, *P. shigelloides* has been mainly implicated in gastrointestinal infections, with gastroenteritis as the most common condition [21,30-33]. Although the symptoms associated with gastrointestinal illness due to *P. shigelloides* typically include diarrhea, abdominal pain, nausea, chills, fever, headaches and vomiting [34,35], *P. shigelloides* has also been isolated from persons exhibiting no apparent symptoms [36,37]. Interestingly, there are an increasing number of reports describing human gastroenteritis caused by *P. shigelloides* among immunocompetent populations [20,21,23,38,39]. Extraintestinal infections such as septicemia, meningitis, osteomyelitis, cellulitis, septic arthritis, endophthalmitis, spontaneous bacterial peritonitis and acute cholecystitis are less common and occur primarily in immunoincompetent or immunocompromised patients [21,40-43]. In animals, *P. shigelloides* is most frequently found in fish as a pathogen, causing hemorrhaging around the vent, protruded the anus and inappetence [44]. Additional symptoms in fish include emaciation, reddening of the anus with yellow exudation, petechial hemorrhages in the internal muscle wall and occasionally the accumulation of ascitic fluid in the peritoneal cavity [45,46]. Van Damme and Vandepitte [16] noted that *P. shigelloides* may be a member of the microbiota of the gastrointestinal tract in warm-water fish, possibly acting as a reservoir of infections.

Although *P. shigelloides* is primarily found in freshwaters, the most important vector for its transmission to humans seems to be food, particularly seafood [34,35,47]. De Mondino et al. [2] found a high incidence of *P. shigelloides* in the polluted salt-water environments of Rio de Janeiro City and suggested that the contamination may be due to excretion by humans and domestic animals. There are a number of case reports in the literature confirming illness caused by *P. shigelloides* following the consumption of oysters [31,34], fish [40] and crabs [48]. Other modes of transmission include contaminated drinking water [49], vegetables [50] as well as contact with amphibians and reptiles [51].

The aim of this study was to investigate for the first time the prevalence and genetic variability of *P. shigelloides* in surface waters in a region with a temperate climate, i.e. the Pannonian Plain, situated in the northern part of Serbia and the southern part of Hungary. The presence of this bacterium outside of tropical regions can be additional confirmation of the consequences of global warming; the results will indicate if the examined waters are reservoirs for *P. shigelloides* infections.

MATERIALS AND METHODS

Sampling sites and isolation

From April to August 2013, 51 water samples were collected from lakes and rivers situated in Serbia and Hungary using sterile 200-mL glass bottles (Fig. 1 and Table 1). The samples were taken 20 cm below the water surface and stored in a refrigerator at 4°C until further processing. The samples were spread directly onto inositol-brilliant green-bile agar (IBB agar or Plesiomonas differential agar (PDA) containing 1% proteose peptone (Fluka-Biochemika, Switzerland), 0.5% meat extract (Torlak, Serbia), 1% meso-inositol (Torlak, Serbia), 0.85% bile salts No. 3 (Torlak, Serbia), 0.5% NaCl, 0.0003% brilliant green (Sigma-Aldrich, USA), 1.35% agar (Torlak, Serbia) containing neutral red (0.005%) (Sigma-Aldrich, USA) or phenol red (0.005%) (Centrohem, Serbia) as pH indicator, and incubated at 37°C for 24 h. The inositol fermenting colonies (1-3 from each sample) were randomly selected, subcultured further and subjected to a variety of biochemical tests. Additionally, samples negative for *P. shigelloides* after direct plating on PDA (with the exception of four samples) were subjected to an enrichment procedure. The procedure included mixing

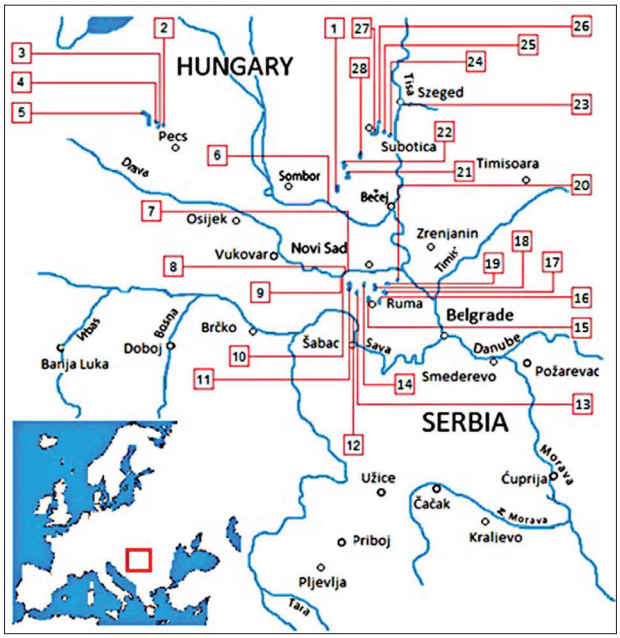

Fig. 1. Sampling sites in northern Serbia and southern Hungary. 1 – Lake Panonija (Serbia); 2 – Lake Orfűi (Hungary); 3 – Lake Pecs (Hungary); 4 – Lake Hermann Ottó (Hungary); 5 – Lake Kovácsszénája (Hungary); 6 – Canal Danube-Tisa-Danube (Serbia); 7 – River Danube (Serbia); 8 – Inlet Bager (Danube river) (Serbia); 9 – Lake Tikvara (Serbia); 10 – Pond Cvrcina bara (Serbia); 11 – Lake Sot (Serbia); 12 – River Sava (Serbia); 13 – Lake Vranjaš (Serbia); 14 – Lake Bešenovo (Serbia); 15 – Lake Pavlovci (Serbia); 16 – Lake Borkovac (Serbia); 17 – Lake Dobrodol (Serbia); 18 – Lake Šatrinci (Serbia); 19 – Lake Šelevrenac (Serbia); 20 – Lake Jarkovci (Serbia); 21 – Lake Krivaja (Serbia); 22 – Lake Čonoplja (Serbia); 23 – River Tisza (Hungary); 24 – Lake Kapetanski rit (Serbia); 25 – Lake Ludaš (Serbia); 26 – Lake Krvavo (Serbia); 27 – Lake Palić (Serbia); 28 – Lake Zobnatica (Serbia).

equal volumes (25 mL) of water samples with a double-strengthened tetrathionate broth without iodine (TTB; 0.25% casein peptone (Torlak, Serbia), 0.25% meat peptone (Torlak, Serbia), 0.1% bile salts (Torlak, Serbia), 1% $CaCO_3$, 3% $Na_2S_2O_3$, pH 8.4), alkaline peptone water (APW; 1% peptone (Torlak, Serbia), 1% NaCl, pH 8.4) [52] or peptone inositol bile broth (PIBB; 1% inositol, 0.823% Na_2HPO_4, 0.5% NaCl, 0.5% peptone, 0.15% bile salts No. 3, 0.12% NaH_2PO_4, pH 7.6) [17].

Bacterial cultures

The reference type strain *P. shigelloides* ATCC14029 served as a positive control for preliminary identification tests and the PCR identification method based on 23S rRNA, while the reference type strain *Aeromonas hydrophila* ATCC7966 was used as a negative control for the PCR. For examination of usability of IBB with two different pH indicators, beside *P. shigelloides* and *A. hydrophila*, two bacterial species were also used: *K. pneumoniae* ATCC 31488 and *P. mirabilis* ATCC 35659. In addition, *P. shigelloides* ATCC51903 was used in the random amplification of polymorphic DNA (RAPD) analysis. The strains were grown on Luria-Bertani (LB) agar and LB broth under aerobic conditions at 37°C for 24 h.

Identification of environmental isolates

P. shigelloides identification was performed by subjecting inositol fermenting colonies to several preliminary tests: Gram staining, oxidase, catalase, indole production, gelatin hydrolysis, citrate utilization and carbohydrate fermentation tests (glucose, maltose, sorbitol, sucrose and arabinose). The identification of some strains was confirmed by the VITEK2 system (BioMérieux, France). All strains identified by phenotype as *P. shigelloides* were additionally confirmed by the PCR method. Genomic DNA was isolated from LB broth overnight cultures using the GeneJet Genomic DNA purification kit (Thermo Scientific, Lithuania). The concentration and purity of the isolated DNA was determined using a BioSpec-nano spectrophotometer (Shimadzu Corporation, Japan). The PCR was conducted using PS23FW3 primer (5′-CTC CGA ATA CCG TAG AGT GCT ATC C-3′) and PS23RV3 primer (5′-CTC CCC TAG CCC AAT AAC ACC TAA A-3′), designed for a specific amplification of a region of the *P. shigelloides* 23S rRNA [53]. PCR reactions were performed as described [53] using a thermocycler (Biometra, TProfessional, Germany). The PCR products were analyzed on 2% agarose gel, stained with ethidium bromide and visualized under UV light using a BioDoc Analyze (Biometra, Germany). All PCR reactions were conducted in duplicate. The reference strains of *Plesiomonas shigelloides* ATCC51903 and *Aeromonas hydrophila* ATCC7966 served as a positive and negative control, respectively.

RAPD fingerprinting

An initial comparison of strains was conducted using a set of 16 decamer oligos (OPA 02, OPA 07, OPA 08, OPB 06, OPB 11. OPB 12, OPB13, OPC 04, OPE 09,

Table 1. Sample origin, method of isolation and isolated strains of *P. shigelloides* from surface waters

Sample origin	Sampling site	Mark on the map	GPS coordinates	No. of samples per site (n=54) (No. of directly plated/ enriched samples)	Media* (media from which strains were isolated are underlined)	Strain isolated (n=37)
Lakes	Šatrinci, Serbia	18	46°04'5"N, 19°55'0"E	2 (1/1)	PDA/APW+PDA	2SHD
(n=43)	Ludaš, Serbia	25	46°50'0"N, 19°49'0"E	2 (2/0)	PDA	1LD, 2LD
	Panonija, Serbia	1	45°44'1"N, 19°31'4"E	2 (2/0)	PDA	6PAND, 4PAND
	Krivaja, Serbia	21	45°50'1"N, 19°29'5"E	2 (2/0)	PDA	1KD, 8KD
	Pecs, Hungary	3	46°09'3"N, 18°08'2"E	2 (2/0)	PDA	1PECD, 4PECD
	Kapetanski rit, Serbia	24	46°02'2"N, 19°56'3"E	2 (2/0)	PDA	2KRD, 5KRD, 6KRD
	Pavlovci, Serbia	15	45°04'2"N, 19°47'5"E	3 (2/1)	PDA/APW+PDA	2PD
	Kovácsszénája, Hungary	5	46°10'4"N, 18°06'5"E	2 (2/0)	PDA	1KOVD, 4KOVD
	Dobrodol, Serbia	17	45°02'3"N, 19°56'4"E	2 (1/1)	PDA/APW+PDA	2DD, 5DD
	Vranjaš, Serbia	13	45°05'4"N, 19°35'5"E	1 (1/0)	PDA	2VD, 8VD
	Borkovac, Serbia	16	45°02'3"N, 19°49'1"E	2 (1/1)	PDA/APW+PDA	2BD, 4BD
	Hermann Ottó, Hungary	4	46°10'3"N, 18°07'5"E	2 (2/0)	PDA	6HERD, 9HERD
	Sot, Serbia	11	45°09'3"N, 19°20'2"E	2 (2/0)	PDA	7SD
	Šelevrenac, Serbia	19	45°04'3"N, 19°59'5"E	2 (2/0)	PDA	3SHELD
	Zobnatica, Serbia	28	45°50'2"N, 19°37'5"E	2 (2/0)	PDA	1ZD, 3ZD, 5ZD
	Palić, Serbia	27	46°05'1"N, 19°45'3"E	2 (2/0)	PDA	1PAD
	Orfűi, Hungary	2	46°08'4"N, 18°09'2"E	2 (2/0)	PDA	2ORFD, 14ORFD
	Jarkovci, Serbia	20	45°02'5"N, 20°01'3"E	2 (2/0)	PDA	2JD, 5JD
	Čonoplja, Serbia	22	45°51'1"N, 19°17'5"E	2 (2/0)	PDA	4CHD
	Bešenovo, Serbia	14	45°06'2"N, 19°42'4"E	2 (2/0)	PDA	-
	Krvavo, Serbia	26	46°05'4"N, 19°46'1"E	2 (2/0)	PDA	-
	Tikvara, Serbia	9	45°14'2"N, 19°22'6"E	1 (1/1)	PDA/(APW/TTB/PIBB)+PDA	-
Rivers	Danbe, Serbia	7	45°14'2"N, 19°26'5"E	1 (1/1)	PDA/(APW/TTB/PIBB)+PDA	DBPM1
(n=3)	Tisza, Hungary	23	46°14'6"N, 20°09'2"E	1 (1/1)	PDA/(APW/TTB/PIBB)+PDA	-
	Sava, Serbia	12	44°54'4"N, 19°45'3"E	1 (1/1)	PDA/APW+PDA	D737A2
Ponds (2)	Cvrcina bara, Serbia	10	45°14'3"N, 19°23'0"E	2 (2/2)	PDA/(APW/TTB/PIBB)+PDA	-
Inlet (1)	Bager (Danube river), Serbia	8	45°14'2"N, 19°24'4"E	1 (1/1)	PDA/(APW/TTB/PIBB)+PDA	DBPM2
Canals (2)	Danube-Tisa-Danube, Serbia	6	45°17'2"N, 19°47'2"E	2 (2/2)	PDA/(APW/TTB/PIBB)+PDA	-

*PDA– Plesiomonas differential agar; APW – Alkaline Peptone Water; TTB – Tetrathionate Broth without Iodine; PIBB – Peptone Inositol Bile Broth

OPM 10, OPM 18, OPN 01, OPN 02, KO 1, K 15, NO 11) in order to identify suitable primers for the RAPD fingerprinting. Based on the performance, five primers were selected: OPA 07, OPB 11, OPM 10, OPN 02 and K 01. The RAPD PCR reaction was performed in a 20-μL volume containing 2.5 μL 10x Dream Taq Green Buffer with 20 mM $MgCl_2$, 1 μL DreamTaq polymerase (Thermo Scientific, Lithuania), 0.5 μL 10 mM dNTPs, 25 ng of corresponding primer and about 100 ng of template DNA. The amplification was carried out in a thermocycler (Biometra, TProfessional, Germany) with the following temperature program: one cycle of initial denaturation at 94°C for 5 min followed by 40 cycles, including denaturation at 94°C for 2 min, annealing at 36°C for 1 min, extension at 72°C for 2 min, and a final extension step at 72°C for 10 min, followed by cooling to 4°C. The obtained DNA fragments were separated by electrophoresis on a 2% agarose gel containing ethidium bromide along with 1 kb DNA Ladder (Thermo Scientific, Lithuania) and visualized under UV light using a BioDoc Analyze Systems (Biometra, Germany). All PCR reactions were conducted in duplicates.

The bands were scored in a binary mode with 1 indicating its presence and 0 its absence. A pairwise genetic similarity matrix was calculated from binary data using Jaccard's coefficient. The similarity matrix was subjected to cluster analysis by unweighted paired group method using arithmetic average (UPGMA). Bootstrap analysis, with 100 repetition value, was used to assess the tree topology robustness. These analyses were carried out using D-UPGMA [54, 55] and TreeGraph 2 [56].

RESULTS

Out of 51 water samples collected from different water sources, 22 samples (40.7%) were positive for *P. shigelloides*. The list of isolated strains describing the inoculation procedure and media used are shown in Table 1. Out of the 37 positive isolates, 34 were from lakes and 3 from rivers, with no positive samples from ponds and canal waters. According to preliminary physiological, biochemical and morphological tests, 37 colonies obtained from various sampling sites or using different procedures were chosen for molecular identification. All the *P. shigelloides* suspected isolates subjected to PCR amplification with primers specific for *P. shigelloides* 23S rRNA generated a product of expected molecular size 284 bp (Fig. 2). Of the 37 positive isolates, 34 were from lakes and 3 were from rivers, with no positive samples from ponds and canal waters.

For *P. shigelloides* isolation from surface water, PDA was used with or without previous enrichment on APW, TTB and PIBB. PDA with neutral red or phenol red was successfully used for this purpose resulting in the isolation of 28 strains (75.7%) by spreading 0.5 mL of samples directly onto the agar plates. The application of these two indicators revealed that phenol red seems to be a more suitable medium as it facilitates *P. shigelloides* differentiation from other bacteria by producing a more obvious medium color change (Fig. 3). Of the 13 samples subjected to the enrichment procedure, the presence of the bacterium was detected in 7 samples with 9 strains (24.3%) iso-

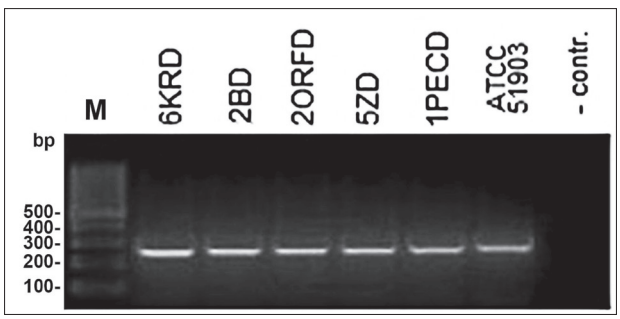

Fig. 2. PCR identification of selected *P. shigelloides* strains. M – 100 bp marker; strain designations are indicated above the electrophoregram; the strain *P. shigelloides* ATCC 51903 was a positive control, and *Aeromonas hydrophila* ATCC 7966 was a negative control (-).

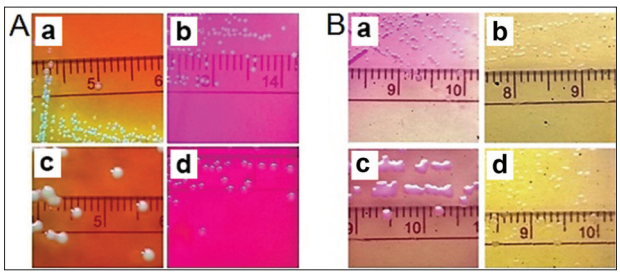

Fig. 3. Colony growth of different bacteria on Plesiomonas differential agar (PDA). **A** – PDA containing the indicator phenol red; only colonies of *P. shigelloides* ATCC14029 (a) turned the medium to a yellow color while *Aeromonas hydrophila* ATCC7966 (b), *Klebsiella pneumoniae* ATCC31488 (c), and *Proteus mirabilis* ATCC35659 (d) did not change the color of the medium (orange or red); **B** – PDA containing the indicator neutral red; this indicator did not allow for differentiation between *P. shigelloides* ATCC14029 (a) and *K. pneumoniae* ATCC31488 (c), and *A. hydrophila* ATCC7966 (b), and *P. mirabilis* ATCC35659 (d) did not change the color of the medium.

lated. No positive samples were detected after enrichment with TTB and PIBB due to a massive presence of competing microorganisms.

The RAPD analyses of 37 strains with five different primers resulted in 649 amplified fragments. The number of detected loci varied between primers from 0 to 14 (OPB11). The fragment size varied, ranging from about 150 bp to about 4.3 kb. The total number of polymorphic fragments by strain was between 8 for strain 2PD and 35 for strain 2BD. The UPGMA dendrogram based on Jaccard's genetic similarity coefficients showed unusual subclustering among different strains (Fig. 4). According to the analysis, most isolates of *P. shigelloides,* including the two reference strains ATCC51903 and ATCC14029, were classified into group A, whereas the strain 1PECD isolated from Lake Pecs (Hungary) forms a unique group B. The strains from group A are clustered into two subgroups, A1 and A2, and both were further divided into several subclades with low bootstrap values. Subgroup A1 contains ATCC14029 along with 14 other strains, all from different lakes, except the isolates 2ORFD and 14ORFD from Lake Orfűi (Hungary), and 3ZD and 1ZD from Lake Zobnatica (Serbia). Although these isolates were from the same samples, different RAPD patterns were obtained. Group A2 consists of 22 strains, including ATCC51903. The isolates 5KRD, 6KRD and 2KRD from the Lake Kapetanski Rit (Serbia) were represented by different RAPD patterns along with the isolates 4PAND and 6PAND from the Lake Panonija (Serbia).

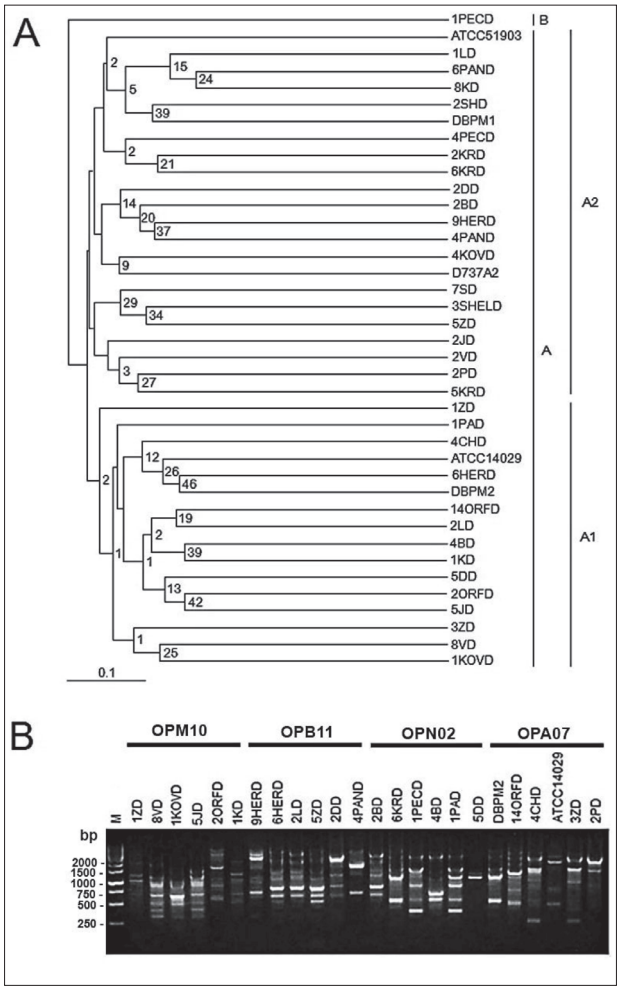

Fig. 4. Genetic variability of 37 *P. shigelloides* strains isolated from surface waters of Pannonian Plain. **A** – dendrogram of *P. shigelloides* genetic variability is constructed by the UPGMA method based on the presence/absence of RAPD-PCR fragments amplified using five different primers (OPA 07, OPB 11, OPM 10, OPN 02 and K 01). Bootstrap values based on 100 replications are shown at the nodes. **B** – An example of obtained RAPD-PCR profiles using OPM10, OPB11, OPN02 and OPA07 oligos. *P. shigelloides* strain designations are indicated above the electrophoregram; 1 kbp marker (M).

DISCUSSION

Numerous studies have shown a high incidence of *P. shigelloides* in tropical and subtropical regions. In studies conducted in Brazil [2], Bangladesh [17], Japan [1] and Nigeria [24], the prevalence of the bacterium in pond and river-water samples ranged from 7.4% to 25%. Depending on the study, PDA, taurocholate-tellurite-gelatin agar (TTGA), SS agar, DHL agar and xylose lysine deoxycholate (XLD) agar were used. Moreover, the isolation procedures involved filtration or centrifugation of water samples, with or without using enrichment broths such as APW, gram negative broth and peptone broth. In the mentioned studies, relatively high sample volumes were processed, varying from 50 to 500 mL (in most instances it was 100 mL).

There is a certain absence of publications reporting the isolation of *P. shigelloides* in freshwater environments in cold and temperate climates. Among the few publications, a successful isolation has been reported from the continental climate rivers and lake water samples in Slovakia [53], as well as from samples taken in a subpolar region of Sweden [4,29]. In the analysis of cold climate freshwater samples from two lakes and two different sites of a river in Sweden, Krovacek et al. [4] determined the presence of

P. shigelloides after APW enrichment in all examined samples. In the study, 400 mL from each water sample was mixed with 40 mL of 10x concentrated APW, which was a significantly higher sample volume than we used. The same media and method were used in the survey conducted by Gonzáles-Rey et al. [53] who revealed the presence of *P. shigelloides* in one of the six lakes in the northern Sweden. Taking into account previous reports and the results of the present study, APW appears to be the enrichment medium of choice for *P. shigelloides* isolation.

When comparing our results with all the results published by other authors mentioned above, it is obvious that we have detected a higher percentage of positive samples, although many strains were isolated directly from samples, without an enrichment procedure. Differences in the performance of *P. shigelloides* isolation in the abovementioned studies can be the result of various media application. Several studies have shown that *P. shigelloides* grows on a variety of media, including the MacConkey [31,43,57-59], SS agar [1,31,60], modified salmonella-shigella agar with inositol substitution for lactose [60], deoxycholate citrate (DC) agar [59], XLD agar [57], Hektoen enteric (HE) agar [57,58], MSDS agar [61], Endo agar [4] and PDA agar [62].

Aside from the used media, another explanation for the successful isolation of *P. shigelloides* directly from samples in our study could be global warming. The detected abundance of *P. shigelloides* in geographical regions with temperate climates such as the Czech Republic [3,28], the Netherlands [27] and now Serbia and Hungary, is not the only fact supporting this. Namely, most of the previous studies were conducted during the second half of the last century when global warming occurred, and there is a significant temporal distance in comparison to our study.

The conducted RAPD-PCR analysis revealed a notable genetic variability among all 37 isolates as none of the isolates had the same RAPD pattern. Accordingly, even the strains isolated from the same sampling sites were not clonally identical. The dendrogram did not reveal geographical relations among strains since isolates from spatially unrelated samples were grouped in the same cluster. The results of our RAPD analysis are in accordance with the findings of Gu et al. [63] who used the RAPD technique to determine genetic variability among 26 isolates from fresh water (10 isolates), fish (6 isolates) and human clinical isolates (10 isolates) and found a high genetic variability among most of the isolates while none of the isolates had the same composite RAPD profile. Similarly, among 24 strains belonging to nine O:H serovars isolated from humans, animals and the environment, 22 different RAPD-PCR patterns were found [64]. The results of high genetic variability of *P. shigelloides* are not very surprising since the species has been proven to have high levels of nucleotide diversity (the nucleotide difference between strains is about 1.49%) [65]. This is the result of frequent homologous recombination in housekeeping genes, which affects *P. shigelloides* alleles and nucleotides 7 and 77 times more frequently than mutations, respectively [65]. Moreover, *P. shigelloides* stands out among members of the *Enterobacteriaceae,* as recombination in other species is much less frequent [66-68].

CONCLUSIONS

To our knowledge, this is the first published report on the isolation of *P. shigelloides* from surface waters of the Pannonian Plain in Serbia (15 lakes and 2 rivers) and Hungary (4 lakes). Since we have identified a relatively high percentage of samples positive for *P. shigelloides*, as well as a high genetic variability indicating a potential for an elevated evolution producing more virulent strains, it is important to emphasize that surface waters in temperate climates could potentially act as a reservoir of the bacterium. The results indicate that the bacterium is widely distributed and as such should not be considered as a tropical or subtropical species, and its obvious presence in a temperate climate could be interpreted as a sort of a microbiological consequence of global warming. More importantly, since *P. shigelloides* frequently causes an acute illness with symptoms common to gastrointestinal infections by *E. coli*, *Salmonella*, *Shigella* and other Enterobacteriaceae, it can easily lead to a diagnostic error with possible medical implications. Additional studies are needed to determine the actual importance of *P. shigelloides* as an etiological agent of waterborne gastrointestinal diseases and the possible microbiological risk to public health in temperate climates.

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