Research article

DIFFERENTIATION BETWEEN *PSEUDOMONAS* AND *STENOTROPHOMONAS* SPECIES ISOLATED FROM FISH USING MOLECULAR AND MALDI-TOF METHOD

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For the purpose of precise antibiotic susceptibility testing it is necessary to clearly distinguish *Pseudomonas* and *Stenotrophomonas* genera, considering acquired resistance of *Pseudomonas* species, as well as the intrinsic resistance of *Stenotrophomonas* species. This is why in the identification of the 51 isolates originated from fish, the following methods were used: standard PCR, 16S rRNA gene sequencing, and MALDI-TOF.

The results of the standard PCR test, 16S rRNA gene sequencing and MALDI-TOF analysis confirmed 35 strains to belong to the *Pseudomonas* genus. Standard PCR test and VITEK MS device confirmed that 10 strains belong to *Stenotrophomonas maltophilia* species.

Three strains were positive in both standard PCR tests for *Pseudomonas* and *Stenotrpohomonas*. 16S rRNA gene sequencing identified these 3 strains to be 99% *Pseudomonas* sp. and 99% *Stenotrophomonas* sp. VITEK MS first identified these three strains as 99% *Stenotrophomonas*, and in the repeated identification it identified them as 99% *Pseudomonas*. MALDI TOF/TOF 4800 Plus device identified these strains as *Stenotrophomonas*.

Three strains were negative in both standard PCR tests for *Pseudomonas* and *Stenotrpohomonas*. 16S rRNA gene sequencing identified these 3 strains to be 99% *Pseudomonas* sp. and 99% *Stenotrophomonas* sp. VITEK MS first identified these three strains as 99% *Stenotrophomonas*, and in the repeated identification it identified them as 99% *Pseudomonas*. MALDI TOF/TOF 4800 Plus device identified these strains as *Stenotrophomonas*.

Although modern test methods that have very high specificity (PCR, 16S rRNA gene sequencing, MALDI TOF) were used in this study, precise differentiation between

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Pseudomonas and *Stenotrophomonas* species for 6 isolates could not be reached using the above mentioned methods.

Key words: fish, *Pseudomonas*, *Stenotrophomonas*, PCR, 16S rRNA, MALDI-TOF

INTRODUCTION

Antibiotics susceptibility testing in bacteria isolated from fish is significantly more complex compared to the same investigations for bacteria isolated from humans and domestic animals. Fish are colonized with bacteria that are intrinsically resistant to a large number of antibiotics (*Stenotrophomonas*) and therefore do not have great significance in assaying resistance mechanisms. It is not known what significance or how great the significance is when it comes to the spread of antibiotic resistance genes in the *Flavobacterium, Brevundimonas, Plesiomonas, Chryseobacterium, Psychrobacter, Shewanella, Rahnel, Raoultella and Chromobacter*species, which are an integral part of the microbiome of the skin and guts of the fish. Many of the listed species are genetically related and once belonged to the same genus, so this is the reason why it is difficult to differentiate them, particularly using conventional methods. Most bacteria species originating from fish, require lower temperature (about 22°C-25°C) for their growth, compared to the recommended incubation temperature for disk diffusion and microdilution methods or for E- test (35ºC -37ºC, CLSI, EUCAST) [1], which is why it is not known whether the results obtained are valid if the antibiotic susceptibility testing is performed at temperatures lower than recommended. In 2011 EUCAST (*European Committee on Antimicrobial Susceptibility Testing*) issued a document [2] which serves as the basic guide to differentiate the intrinsic resistance from an acquired resistance in various bacteria species. In this document, only a small number of bacteria species that can be found in fish are listed, these are members of the *Pseudomonas*, *Stenotrophomonas*, *Yersinia* and *Enterobacter* genera. For *Aeromonas* only information on the intrinsic resistance was given in the document that was jointly published by EUCAST and the French association of microbiologists [3]. For almost all other bacteria species characteristic for fish, including *Vibrio*, there is no information on which antibiotics they are intrinsically resistant to, and which resistance may be considered acquired.

Stenotrophomonas is an opportunistic pathogen that is intrinsically more resistant to a large number of antibiotics, including the aminoglycosides, cephalosporins and carbapenems. In contrast, *Pseudomonas* species are naturally sensitive to most antibiotics to which *Stenotrophomonas* species are intrinsically resistant. Finding resistance to carbapenems, aminoglycosides and some cephalosporins (cefepime, ceftazidime and cefotaxime) in *Pseudomonas* species isolated from fish has a great epidemiological significance. As *Stenotrophomonas maltophilia* species once belonged to the *Pseudomonas* genus, there are significant difficulties when trying to identify and clearly distinct between the *Pseudomonas* and *Stenotrpohomonas* genera. Results from the conventional systems, such as API 20 NE (bioMerieux, France) and BBL Crystal Enteric / Nonfermenter ID kit (Becton Dickinson, USA) are not considered

authoritative in the scientific research field because, even if they are categorized as "excellent" "very good" or "good identification", these systems exhibit a high level of errors in differentiating between *Pseudomonas* and *Stenotrophomonas* species [4]. The most common cause of confusion with the classical identification is discrepancy in the oxidase test in *Stenotrophomonas* species that are oxidase negative, but atypical, positive oxidase test occurs in as many as 20% of the strains [5]. In addition, some isolates of *Pseudomonas* species can be oxidase negative. Besides the oxidase test, susceptibility testing to sulfamethoxasole/trimethoprim and intrinsic resistance test to imipenem as typical characteristics of *Stenotrophomonas* species is being proposed as the main conventional test for distinguishing *Stenotrophomonas* from *Pseudomonas* species. However, due to the widespread of acquired resistance of *Stenotrophomona s*species to sulfamethoxasole/trimethoprim, and *Pseudomonas* species to imipenem, proposed phenotypic tests have also become unreliable. This is why in the recent years exclusively molecular methods [6],and MALDI-TOF [7] are being proposed in order to get precise and clear identification of bacteria isolated from fish. In this research, differentiation of *Pseudomonas* and *Stenotrophomonas* species isolated from fish was carried out using standard PCR methods, the 16S rRNA gene sequencing, MALDI-TOF, as well as some conventional methods.

MATERIAL AND METHODS

Tested strains of bacteria

In this study strains of bacteria were isolated from swabs taken from the gills and skin of healthy, live carp, originating from different ponds in Serbia. The strains were isolated in order to study their sensitivity to antibiotics. For the assay, only Gram-negative species were selected, as to test their resistance to carbapenems, aminoglycosides and III and IV generation cephalosporins. Out of 200 isolated strains, in the preliminary, conventional test, 35 strains were suspected to belong to the *Pseudomonas* genus, 10 to belong to the *Stenotrophomonas* genus, and 6 strains could not be categorized, actually they were oxidase positive and resistant to sulfamethoxazole/trimethoprim or showed multi-resistance to aminoglycosides, carbapenems and cephalosporins, so it was necessary to rule out the suspicion that they belong to the *Stenotrophomonas* genus.

Isolation and identification using conventional methods

Culturing of swabs was performed on UTI agar (Urogenital tract infections chromogenic agar, HI Media) from where pure cultures were subcultivated and maintained on Columbia blood agar supplemented with 5% sheep blood (bioMerieux, France). The plates were incubated at a 25°C. All isolated strains were assayed in the oxidase test using oxidase reagent dropper (Becton Dickinson, USA). The diagnostic susceptibility test to sulfamethoxasole/trimethoprim and imipenem was performed using disk diffusion method on Mueller Hinton agar (Becton Dickinson, USA) with discs manufactured by Becton Dickinson, as well as by using E test strips of the

same antibiotic (bioMerieux), in order to determine the MIC value, also on Mueller Hinton agar. Reading and interpretation of the results were carried out according to the EUCAST recommendations (2016).

Standard PCR

DNA isolation from investigated strains was carried out using the boiling method. In short: 1-2 colonies of overnight culture were resuspended in 1 mL PBS buffer (Dulbecco's, Serva) in Eppendorf tubes of 1.5 mL volume. After vortexing, the suspension was centrifuged at 13,200 rpm for 5 minutes at RT. The supernatant was discarded, and the pellet was resuspended in 100 µl of TE buffer (Thermo Scientific). Subsequently, the tubes with TE buffer were soaked in boiling water for 10 minutes, incubated on ice for 1 minute, and the remaining content that contains the genomic DNA was resuspended and transferred to 900 µl of TE buffer. The isolated DNA was stored at -20°C. In order to precisely determine if it belongs to the *Pseudomonas* genus the PCR method that was previously described [8] was used. Primers (Invitrogen) had the following sequences: PA-GS-F 5'-GACGGGTGAGTAATGCCTA-3 and PA-GS-R 5'CACTGGTGTTCCTTCCTATA – 3', and the size of the amplified product was 618 bp. The PCR mixture in an amount of 25µl consisted of 2 mM $MgCl_2$, 1 x Hot Start PCR Buffer (200 mM Tirs-HCl pH 8,3; 200 mM KCl; 50 mM $(NH_4)2SO4$; Thermo Scientific), 0.4mM deoxynucleoside triphosphates each (Thermo Scientific), 0.4 µM of each primer, 1 U Maxima Hot Start Taq Polymerase (Thermo Scientific) and 2 µl of DNA. Conditions of PCR reactions took place according to the following scheme: initial denaturation of 95°C for 4 minutes, 25 cycles of denaturation at 95°C for 30 seconds, annealing 54°C 30 seconds, extension 72°C for 1 minute and a final extension 72°C for 5 minutes. As a positive control *P. aeruginosa* ATCC 27853 strain was used, and as a negative control *S. maltophilia* ATCC 13637.

For precise determination of the investigation strains of the *Stenotrophomonas* genus the method previously described [9] was used. Sequences of the applied primers (Invitrogen) were as follows: F 5'-GCTGGATTGGTTCTAGGAAAACGC-3'and R 5'-ACGCAGTCACTCCTTGCG -3', and the size of the amplified product was 278 bp. The PCR mixture in an amount of 25µl was composed of 1 mM $MgCl_2$, 1 x Hot Start PCR Buffer (200 mM Tirs-HCl pH 8,3; 200 mM KCl; 50 mM $(NH4)_2SO_4$;Thermo Scientific), 0.2 mM deoxynucleoside triphosphates each (Thermo Scientific), 0.2µM of each primer, 2 U Maxima Hot Start Taq Polymerase (Thermo Scientific) and 2µl of DNK. Conditions of the PCR reaction took place according to the following scheme: initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 45 seconds, 55°C annealing for 30 sec, extension 72°C for 45 seconds and a final extension at 72°C for 10 minutes.

Isolation and characterization of bacterial isolates by 16SrRNA gene sequencing

Genomic DNA from isolated strains was extracted (Kapa Express Extract Kit, KapaBiosystems, Wilmington, MA, USA) and 16S ribosomal RNA (16S rRNA) gene was amplified by polymerase chain reaction (PCR) using universal bacterial primers 27f and 1492r [10]. Obtained PCR products were sequenced using Applied Biosystems 3130 Genetic Analyzer (Foster City, CA, USA) and sequences were analyzed and assembled by SeqMan Pro software (DNASTAR Inc., Madison, WI, USA). The BLASTN program (NCBI, http://www.ncbi.nlm.nih.gov) [11] was used for sequences similarity search.

Identification using MALDI-TOF method

Identification of strains using MALDI-TOF method (Matrix-assisted laser desorption/ionization time of-flight mass spectrometry) was performed on VITEK MS device (bioMerieux Industry, France). To prepare strains VITEK MS-CHCA matrix, (bioMerieux) was used, and to read the result database VITEK MS Knowledge Base V2.0 - Industry Use was used. To calibrate the device, *E. coli* ATCC 8739 calibration strain was used. A small number of strains was also investigated on MALDI TOF/TOF 4800 Plus (AB SCIEX, USA), besides being assayed on VITEK MS device. The bacteria were identified by MALDI MS/MS biotypization protocol described at http://rapidcell.proteinreader.com/maldi-msms-biotypization-protocol/[12].

RESULTS

Out of the 51 strains investigated in conventional tests, 35 were oxidase positive, most were resistant to sulfamethoxasole/trimethoprim with obtained MIC values > 32 μg/mL and were suspected to belong to the *Pseudomonas* genus. A total of 10 strains were negative in oxidase test and were sensitive to sulfamethoxasole/trimethoprim with obtained MIC values <4 μg/mL, and they were suspected to belong to the *Stenotrophomonas* genus. A total of 6 strains were oxidase positive with a delayed (slow) oxidase reaction, and in the readings to sulfamethoxazole/trimethoprim sensitivity after 18[h], strains were clearly sensitive, with MIC values of <4 μg/mL and when re-read after 6 more hours incubation, they were clearly resistant, with MIC values> 32 μg/mL. This part of the test was repeated several times, and identical results were always obtained - uneven sensitivity in just 6[h] difference in the length of incubation. Identical results were obtained while repeating the above mentioned tests using the disk diffusion method, where the zone around the sulfamethoxazole/trimethoprim disk in readings after 18[h] of incubation was > 16 mm, and in re-reading after 2-6[h], the area was 0 mm. These strains were resistant to carbapenems, III and IV generation of cephalosporins, and aminoglycosides.

Based on the results of standard PCR test, 16S rRNA gene sequencing and MALDI-TOF analysis, 35 strains that were suspected of belonging to *Pseudomonas*, were confirmed to belong to that genus. In the standard PCR assay, these strains gave positive results in the form of the presence of the desired PCR product the size of 618 bp (**Figure 1**). Out of 35 strains, 3 stains were identified as *P. aeruginosa* with 99% accuracy, in both assays performed: 16S rRNA gene sequencing analysis and using VITEK MS device. In the 16S rRNA gene sequencing, two more strains were identified as *P. fluorescens* with 99% accuracy, and VITEK MS identified one out of the two strains as *P. fluorescens* (99% accuracy), while the other was not clearly identified up to the species, but it was confirmed to belong to the *Pseudomonas* genus. The remaining 30 strains were identified as *Pseudomonas* spp. with 99% accuracy with both 16S rRNA gene sequencing and using VITEK MS device.
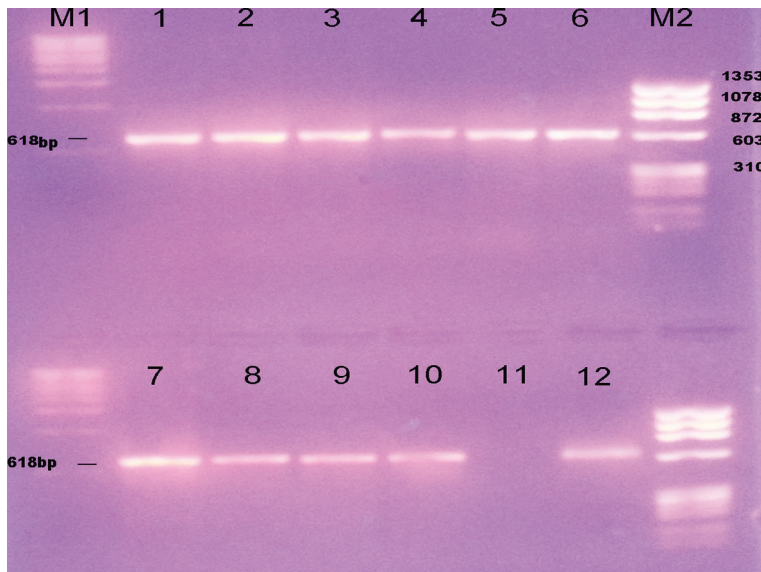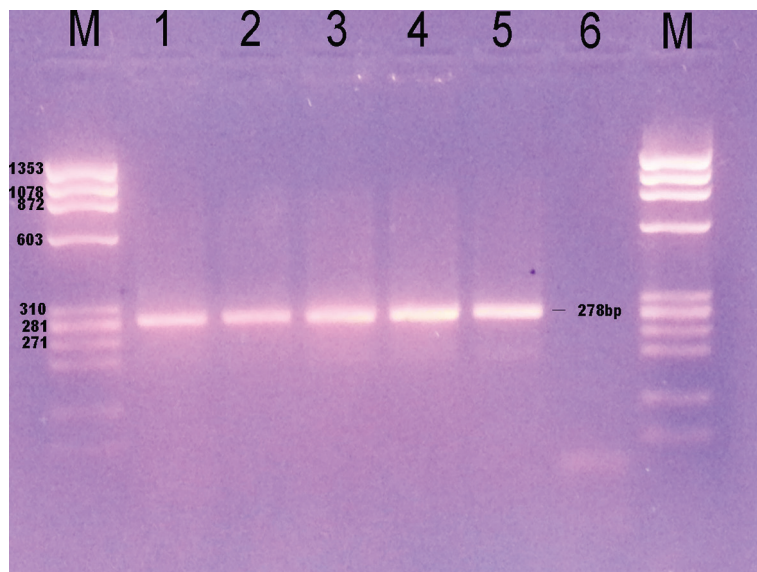

Figure 1. Agarose gel electrophoresis with PCR products of standard PCR for *Pseudomonas* genus identification: M1- DNA Standard 1 KBp DNA ladder (SERVA); lanes 1-5 strains identified as *Pseudomonas* spp with PCR products size 618-bp; lane 6 positive control *P. aeruginosa* ATCC 27853; M2-ΦX174 DNA/BsuRI (HaeIII) Marker, 9 (Fermentas); lanes 7-10 strains identified as *Pseudomonas* spp; lane 11 negative control *S. maltophilia* ATCC 13637; lane 12 positive control *P. aeruginosa* ATCC 27853

In a standard PCR assay, 10 strains which were suspected of belonging to the *Stenotrophomonas* genus gave a positive PCR product of 278 bp in size, which is characteristic of this genus (**Figure 2**). These strains were not analyzed by 16S RNA gene sequencing. Identification on the VITEK MS device, with 99.9% accuracy confirmed that these 10 strains belong to *Stenotrophomonas maltophilia* species (**Table 1**).

Out of the 6 remaining strains for which there was doubt to what species and genus they belonged, three strains yielded positive results in PCR reactions for both assayed genera. These six disputable isolates were further identified by 16S rRNA gene sequencing (1300 bp) (**Table 2**). 16S rRNA gene sequences of isolates shared 99%

-100% of the 16S rRNA gene sequence identity with their closest matching entries in GenBank.

Figure 2. Agarose gel electrophoresis with PCR products of standard PCR for *Stenotrophomonas* genus identification: M-ΦX174 DNA/BsuRI (HaeIII) Marker, 9 (Fermentas); lanes 1-4 strains identified as *Stenotrophomonas* with PCR products size 278-bp; lane 5 positive control *S. maltophilia* ATCC 13637; lane 6 negative control *P.aeruginosa* ATCC 27853

Table 1. Results of the identification of investigated strains using standard PCR and MALDI TOF

| Isolate	Standard PCR *Pseudomonas* spp.	Standard PCR *Stenotrophomonas* spp.	VITEK MS
Pseudomonas spp. 35 isolates	35/35	0/35	35/35
Stenotrophomonas spp. 10 isolates	0/10	10/10	10/10

For these strains PCR reaction was repeated 3 times, and each time, all three strains were double positive, ie. the PCR products contained both- 618 bp *Pseudomonas* fragment, and 278 bp *Stenotrophomonas* fragment. Positive and negative controls in all replications were valid (**Figure 3**). In the 16S rRNA gene sequencing each of the three strains was identified as 99% *Pseudomonas* sp. and 99% *Stenotrophomonas* sp., respectively. In other words same as in the PCR method, these strains were categorized with 99% accuracy as members of both genera. These three strains VITEK MS in the first identification identified as 99% *Stenotrophomonas* and in the repeated identification it identified them as 99% *Pseudomonas*. The MALDI TOF/TOF 4800 Plus device identified these strains as *Stenotrophomonas*.

Table 2. Results of standard PCR, 16S rRNA gene sequencing and MALDI TOF for 6 strains that could not be clearly identified by applied methods

Isolate	Standard PCR Pseudomonas spp.	Standard PCR Stenotrophomonas spp.	VITEK MS	MALDI TOF/ TOF 4800Plus	16s RNA identification-The best GenBank match Classification	Accession number	nt	% Identity
1	–	–	P.fluorescens 34.2% P.veroni 34% P.oleovorans 31.7% S.maltophilia 99.9%	S.maltophilia	P.hibiscicola strain AT 106 S.maltophilia strain ISMMS2 P.geniculata strain IR66	KT387346 CP011305 KJ396843	1352	99%
2	–	–	P.fluorescens 99.9% S.maltophilia 99.9%	S.maltophilia	P.hibiscicola strain AT 106 S.maltophilia strain ISMMS2	KT387346 CP011305	1344	99%
3	–	–	P.fluorescens 99.9% S.maltophilia 99.9%	S.maltophilia	S.maltophilia strain RAT3A	KT248840	1351	99%
4	+	+	P.fluorescens 99.9% S.maltophilia 99.9%	S.maltophilia	P.geniculata strain R6-798 S.maltophilia strain ISMMS2	JQ659864 CP011305	1347	99%
5	+	+	P.fluorescens 99.9% S.maltophilia 99.9%	ND	P.geniculata strain IR81 S.maltophilia strain ISMMS2	KJ396858 CP011305	1341	100%
6	+	+	P.fluorescens 99.9%	ND	P.geniculata strain IR81 S.maltophilia strain ISMMS2	KJ396858 CP011305	1346	99%

Note: ND – not done
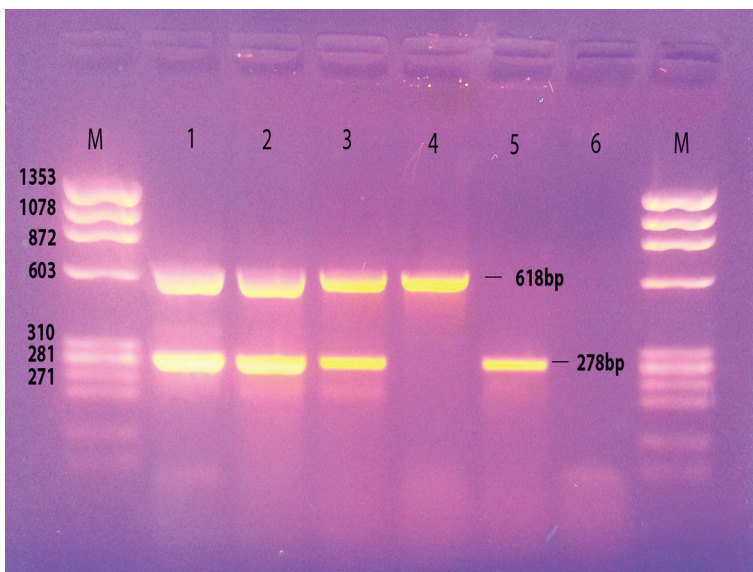
Figure 3. Agarose gel electrophoresis with PCR products obtained in multiplex PCR for *Pseudomonas* and *Stenotrophomonas* (for 3 unidentified strains): lane M-ΦX174 DNA/BsuRI (HaeIII) Marker, 9 (Fermentas); lanes 1-3 strains positive on both genera with 618 and 278 bp PCR products; lane 4 *P.aeruginosa* ATCC 27853; lane 5 *S. maltophilia* ATCC 13637; lane 6 negative control for PCR.

The remaining three strains were negative in the standard PCR, actually they did not belong to either *Pseudomonas* or *Stenotrophomonas* genera. PCR method was repeated 3 times for these strains as well, and each time all three strains were negative for the requested PCR products. However, in the 16S rRNA gene sequencing each of the three strains was identified as 99% *Pseudomonas* sp. and 99% *Stenotrophomonas* sp., actually, based on 16S rRNA gene sequencing these strains were identified as belonging to both genera. VITEK MS device identified these three strains as 99% *Stenotrophomonas* in one identification, and as 99% *Pseudomonas* in the other. MALDI TOF/TOF 4800 Plus device identified these strains as both *Stenotrophomonas*.

DISCUSSION

Given the fact that resistance in bacteria isolated from fish has been drawing great attention in scientific and professional circles in the recent years, there is growing literature about this research topic being published in reputable scientific journals. However, the validity of these results may be questioned from various aspects. One of the papers [13] showed the results of an extremely worrying resistance in bacteria isolated from sharks located in the Atlantic Ocean. Isolated strains mentioned in this paper were resistant to a great number of antibiotics that are used exclusively in the human medical practice, for example, piperacillin, ceftazidime and cefotaxime.

However, what is arguable is that all isolates in this paper that were tested to antibiotics sensitivity were not precisely identified and were presented only as Gram-negative bacilli. In this aspect, it is possible that the results shown in this paper were of intrinsic resistance to listed antibiotics. On the other hand, although intrinsically resistant to carbapenems, aminoglycosides, penicillins and cephalosporins, *Stenotrophomonas* can carry plasmids with genes for TEM [14] and CTX-M [15] beta-lactamases that have extremely great importance in the resistance of bacteria isolated from people. This is why it is important to accurately identify the *Stenotrophomonas* and for the research on the resistance found in these species to be redirected to plasmids and the aforementioned beta-lactamases. In the papers written by Spilker and Gallo [8.9] it was claimed that designed primers and proposed PCR cycle conditions guarantee to obtain highly specific results with 100% accuracy. In our study, the 3 strains showed positive findings and the required PCR products for both genera, which indicates a relatively high non-specificity of this method and this occurred in 5.88% of the strains. Using standard PCR method it was not determined if 3 of the strains belonged to either of the genus so it could also be rated as an error or non-specificity of the method, because 16S rRNA gene sequencing and MALDI TOF methods identified these strains as *Pseudomonas* and/or *Stenotrophomonas*. These results emphasize the important point that effective identification by the 16S rRNA gene sequencing is not necessarily a sufficient criterion to guarantee species identity. So, if these 6 strains all generated nonspecific results, or an error in the standard PCR, it means that standard PCR non-specificity in this research has appeared in 11.76% of the strains. However, the 6 debatable strains could not be accurately identified to the end and therefore the findings obtained in a standard PCR cannot be clearly interpreted for these 6 strains. Thus, although 16S rRNA gene sequences can be used routinely to distinguish and establish relationships between genera and well-resolved species, very recently diverged species may not be recognizable.

MALDI TOF is considered one of the most accurate methods in the identification of bacterial species. However, it is known that in the analysis of the detected sequence of proteins, which database is used plays an important part. In this sense, a vague finding does not mean that the MALDI TOF is a nonspecific method, but the database has not been fully updated. In this study MALDI TOF device intended for the identification of bacteria isolated from a variety of waters: from wells, rivers, seas, etc. was used, so the database for both devices was comprehensively updated for types of bacteria found in waters, and therefore in fish. However, in this field of study research has probably not yet determined or clearly defined all the protein sequences of all species of bacteria that can be found in fish because the databases obviously recognize joint sequences for *Pseudomonas* and *Stenotrophomonas*.

CONCLUSIONS

Although for this research modern test methods that have very high specificity for this field of study (PCR, 16s rRNA gene sequencing, MALDI TOF) were used, it is

obvious that these methods have not been sufficiently perfected yet so that precise results could be obtained in 100% of cases; in other words, precise differentiation of *Pseudomonas* and *Stenotrophomonas* species could not be obtained using the listed methods. For the standard PCR it is necessary to find the gene sequences in *Pseudomonas* and *Stenotrophomonas* that do not have similarities, and to design a new, more specific primer pairs based on which these genera could be precisely distinguished. Although the 16S ribosomal RNA (rRNA) gene sequencing is a well-established method for studying phylogeny and taxonomy of samples from complex microbiomes or environments that are difficult or impossible to study, there are certain cases that this methodology is best to combine with the biochemical or other identification approaches to yield the definite answer about the identity of a particular bacterial isolate. In MALDI TOF method, it is necessary to also determine the precise protein sequences that are unique to the *Pseudomonas* and to the *Stenotrophomonas* species.

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Authors' contributions

AK, AJ, MD and MD carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. ČS, BA and CM carried out the MALDI-DOF. AK, AJ and MD participated in the design of the study and performed the statistical analysis. AK, AJ, MD, MD, ČS, BA and CM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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DIFERENCIJACIJA *PSEUDOMONAS* I *STENOTROPHOMONAS* VRSTA IZOLOVANIH IZ RIBA PRIMENOM MOLEKULARNIH METODA I MALDI-TOF METODE

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S obzirom na značaj stečene rezistencije *Pseudomonas* vrsta, kao i na intrinzičnu rezistenciju *Stenotrophomonas* vrsta, a u cilju preciznog ispitivanja osetljivosti na antibiotike, neophodna je jasna diferencijacija pripadnika ovih rodova bakterija. U tom cilju su u identifikaciji 51 izolata poreklom od riba korišćene metode: standardni PCR, 16S rRNA sekvenciranje gena, MALDI-TOF.

Rezultati standardnog PCR testa, 16S rRNA sekvenciranja gena i MALDI-TOF analize su za 35 sojeva potvrdili pripadnost rodu *Pseudomonas*. Standardnim PCR testom i primenom aparata VITEK MS utvrđeno je da 10 sojeva pripada vrsti *Stenotrophomonas maltophilia*.

U 16S rRNA sekvenciranju gena 3 soja koja su bila pozitivna u oba standardna PCR testa identifikovana su kao 99% *Pseudomonas* sp. i 99% *Stenotrophomonas* sp. VITEK MS je ova tri soja u prvoj identifikaciji identifikovao kao 99% *Stenotrophomonas,* a u ponovljenoj identifikaciji kao 99% *Pseudomonas*. Ti sojevi su na aparatu MALDI TOF/TOF 4800 Plus bili identifikovani kao *Stenotrophomonas*.

U 16S rRNA sekvenciranju gena tri soja koja su bila negativna u oba standardna PCR testa su identifikovana kao 99% *Pseudomonas* sp. i 99% *Stenotrophomonas* sp. Aparat VITEK MS je ova tri soja identifikovao u jednoj identifikaciji kao 99% *Stenotrophomonas,* a u drugoj identifikaciji 99% *kao Pseudomonas*. Ti sojevi su na aparatu MALDI TOF/TOF 4800 Plus bili identifikovani kao *Stenotrophomonas*.

Iako su u ovom istraživanju korišćene savremene metode ispitivanja koje imaju vrlo visoku specifičnost (PCR, 16s rRNK sequencing, MALDI TOF) precizna diferencijacija *Pseudomonas* i *Stenotrophomonas* vrsta nije mogla biti postignuta.