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Identification of Aeromonas species in trout in Tunceli province by MALDI-TOF MS method

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ABSTRACT

The genus Aeromonas has undergone a number of taxonomic and nomenclature revisions over the past 20 years, and new (sub)species and biogroups are continuously described. The aim of this study was to determine the prevalence of Aeromonas species in freshwater fish farms and factors affecting their prevalence in Tunceli province. In addition, it was aimed to compare the different methods such as MALDI-TOF MS, 16S rDNA sequencing and housekeeping gene sequencing (gyrB), method for species class identification of Aeromonas. In our study, tissue samples taken from the intestine, kidney, spleen and liver of 200 juvenile and adult rainbow trout randomly selected from the farms in Mazgirt, Çemişgezek, Ovacık, Pertek districts of Tunceli province in March-May and November-January were used. In addition, 64 water samples taken from net cages and concrete pools of the same facilities were used. Five species of Aeromonas were more prevalent (MP) in fish farms, A. sobria (47.52%) was the highest, followed by A. veronii (26.95%), A. media (12.06%) and A. hydrophila (7.80%). The less prevalent species were A. salmonicida (5.67%). In our study, the accuracy of MALDI-TOF MS results of 141 isolates identified as Aeromonas species was found to be 100% (141 isolates) at genus level and 92.2% (130 isolates) at species level. In the phylogenetic studies of the genus Aeromonas, it was concluded that 16S rDNA and gyrB gene sequencing methods are reliable methods at the point where no distinction can be made at the species level.

Keywords: *Aeromonas species, rainbow trout, 16S rDNA, MALDI-TOF MS, gyrB*

INTRODUCTION

Most of the species belonging to the genus Aeromonas have been isolated from pathogenic bacteria that are frequently encountered in aquatic production enterprises in the world and in Turkey. Aeromonas genus is rod-shaped bacteria belonging to the Aeromonadaceae family; being gram negative, cytochrome oxidase positive, facultatively anaerobic and catalase positive, they convert nitrate to nitrite, show fermentative reaction to O/F glucose test, are sporeless and resistant to Vibriostat (O/129) test. Since bacteria belonging to the genus Aeromonas, whose natural habitats are marine and freshwater, are common in waters, the causative agent is found in the intestinal microbiota and body surfaces of crustaceans and amphibians, and freshwater and marine fish. Some species of this genus cause infections in humans, fish and other aquatic animals. A. hydrophila, A. sobria, A. caviae are motile Aeromonas species that are important fish pathogens (1,2,3).

Although there are 25 accepted species in the genus Aeromonas, 14 of these species were described in the last edition of Bergey's Manual (4). Bacteria of the genus Aeromonas are divided into two groups according to their growth conditions and biochemical characteristics. The psychrophilic group consists of bacteria that reproduce between 22 and 25 °C and do not move. A non-motile species, A. salmonicida is characterized by being pathogenic for fish and reptiles. Bacteria belonging to the mesophilic group such as A. hydrophila, A. caviae and A. veronii are reported to cause infections and other diseases more frequently in humans, although they are seen in sea creatures (5).

Rainbow trout is the most produced trout species in Turkey, both in fresh waters and in the seas (6). Bacterial species belonging to the genus Aeromonas, which is an extremely important pathogen for human health, are important because they are frequently isolated from fisheries and pose a danger to public health. Diseases in intensive fish farming can cause great economic problems due to the difficult and expensive treatment and cause the business economy to be adversely affected. For this reason, early diagnosis of fish diseases, effective treatment and taking necessary control measures are of great importance in aquaculture. Identification of Aeromonas species found in fish is required before examining their effects on human and animal health. This work describes the development of a matrix-assisted laser desorption/ ionization–time-of-flight mass spectrometry (MALDI-TOF MS) method database for rapid identification of clinical and environmental Aeromonas isolates.

MATERIALS AND METHODS

After the applications were made to the Ethics Committee of Fırat University Faculty of Medicine and the necessary approval was obtained, the commercial kits and chemicals used in molecular analyzes were met by the Munzur University Scientific Research Projects Coordination Unit. (Project number: YLMUB021-02).

In our study, tissue samples taken from the intestines, kidneys, spleens and livers of 200 juvenile or adult rainbow trout randomly selected from the facilities in Mazgirt, Çemişgezek, Ovacık, Pertek districts of Tunceli province in March-May and November-January were used. In addition, 64 water samples obtained from mesh cages and concrete pools of the same facilities were used. Fish samples were examined for ecto and endoparasites before the bacterial study. In the Fırat University Fisheries and Experimental Animals Research Center (SÜDAM) unit, the body surface of the fish was wiped with 70% ethyl alcohol after anesthetizing with 50 ppm benzocaine, and the abdominal cavity was opened under sterile conditions. The skin was cut with a sterile scalpel and samples were taken from the liver, spleen, kidney and intestines with sterile forceps. It was placed in sterile bags with the help of sterile plastic loops and delivered to İnönü University Faculty of Medicine, Department of Basic Medical Sciences, Medical Microbiology laboratory under cold chain. After adding 225 ml of 0.1% Alkaline Peptone Water to each of the liver, spleen, kidney and intestinal tissue samples in sterile bags, they were homogenized for 2 minutes and incubated at 28 °C for 18-24 hours. After incubation, a loopful of enrichment liquid

was taken and planted on Aeromonas Agar by scratching method, and petri plates were incubated at 28 °C for 18 to 24 hours (7). Colonies with an opaque dark green center growing on Aeromonas agar were accepted as a suspicious colony for Aeromonas. Isolates suspected to be Aeromonas species were purified. Bacteria grown for purification were added to the solid medium as a single colony. The purification process was completed by repeating the process twice. Approximately three to four loops of pure culture were added to MRS broth with 30% glycerol in 1.5 ml eppendorf tubes and vortexed. Stock cultures were stored at -80 °C for later use (7). Isolated Aeromonas species were identified with the MALDI-TOF MS instrument. The matrix solution was mixed with 2 mg of HCCA (α-cyano-4-Hydroxycinnamic Acid) matrix and 150 µl of organic solvent, and 1 ml of organic solvent was taken and added to a sterile eppendorf tube and mixed. Cultures grown on Aeromonas Selective Agar for up to 24 hours were loaded directly into the distribution system. After the logarithmic step, bacterial colonies suspected of Aeromonas status were pseudo-eliminated from petri dishes and metal plate with a single formed ring-end loop. For calibration, the standard bacterial strain E. coli ATCC 8739 (American Type Culture Collection Manassas, USA) was transferred to designated wells on the target slide. For quality control purposes, positive controls (E. coli strains) were analyzed in each assay. The plates were dried, placed in the device and started to be read (8).

Spectra peak images were obtained by using linear positive ion model with 20 hz laser pulse frequency of structures weighing 2.000-20.000 da. Again, the proximity and distance of the strains were calculated using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm using "Pearson Correlation" (0.8% tolerance) (8). From the results using the MALDI-TOF MS method, A. hydrophila sequence analysis of 16S rDNA and gyrB gene regions was performed for isolates identified as A.punctata. Template DNA was prepared for sequence analysis of 16S rDNA and gyrB gene regions. For this, several bacterial colonies were taken and suspended in 750 µl of TE and vortexed. The supernatant was removed by centrifugation at 12000 rpm for 2 minutes. Washing was then repeated twice and the pellet was suspended in 250 µl of TE. It was incubated at 95 °C for 20 minutes and DNA was exposed by lysing the bacteria. The supernatant formed by centrifugation at 12000 rpm for 2 minutes was transferred to a clean tube and template DNA was prepared. In the study, gyrB housekeeping gene and 16S rDNA gene regions were amplified as PCR primers. The primers used in the 16S rDNA and gyrB sequence analysis in the study are given in Table 1(9,10,11,12).

TABLE 1: Primers used in 16S rDNA and gyrB sequence analysis (9,10,11,12)

Name of Gene	Primer sequences	Size
16SrDNA	p8FPL 5'-AGTTTGATCCTGGCTCAG-3' p806R 5'-GACTACCAGGGTATCTAAT-3'	~834bp
gyrB	gyrB3F (F) 5'-TCCGGCGGTCTGCACGGCGT–3' gyrB14R (R) 5'-TTGTCCGGGTTGTACTCGTC–3'	~1100

Nucleotide sequences obtained by 16S rDNA and gyrB sequence analysis were visually checked for electropherograms. Sequences obtained by sequence analysis in both directions were matched. Validated sequences were compared with the gene bank database using the BLAST program on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BALST/) (9,10,11,12).

The analysis of the findings obtained in our study was performed using the descriptive statistics method.

RESULTS

It was determined that there were suspicious colonies of Aeromonas spp on Aeromonas selective agar in 141 cultures isolated from the media by conventional method. In the distribution of Aeromonas species identified by MALDI-TOF MS method according to their localization in intestinal, kidney, spleen and liver tissues, it was determined that A. sobria species was the most common bacterial species and spread most in the intestine among organs. Table 2 shows that A. media is mostly localized in the intestine, spleen and liver tissue, but not in the kidney.

TABLE 2: Distribution of Aeromonas Species Detected from Tissue Samples of Rainbow Trout Taken from Rainbow Trout Breeding Facilities in Pertek, Çemişgezek, Ovacık and Mazgirt Districts of Tunceli Province

Sample Type	Number of Positive Samples (Aeromonas spp.)	Bacteria species									
		A.hydrophila		A. sobria		A.veronii		A.salmonicida		A. media	
		n	%	n	%	n	%	n	%	n	%
Bowel	58	3	5.17	29	50.00	12	20.69	4	6.90	10	17.24
Kidney	24	1	4.17	12	50.00	10	41.67	1	4.17	0	0
Spleen	32	2	6.25	13	40.63	9	28.13	2	6.25	6	18.75
Liver	27	5	18.52	13	48.15	7	25.93	1	3.70	1	3.70
Toplam	141	11	7.80	67	47.52	38	26.95	8	5.67	17	12.06

In addition, in our study, it was determined that Escherichia coli 2, Lelliottia amnigena 1 and 1 Raoultella planticola bacteria reproduced from 64 water samples taken from concrete pools and net cages of rainbow trout breeding facilities.

In our study, 16S rDNA sequencing and gyrB sequencing analysis were performed in order to make a complete distinction between the two species from the samples that yielded A.hydophila/ A.punctata (caviae) as a result of MALDI TOF-MS analysis. When the results of conventional method, MALDI-TOF MS method, 16S rDNA sequence analysis and gyrB sequence analysis of Aeromonas bacteria were compared; Aeromonas spp by conventional method 11 suspected isolates were identified as A.hydophila/A.punctata (caviae) by the MALDI-TOF MS method, and according to the results of the sequence analysis performed on the 16S rDNA and gyrB gene regions of 11 suspected isolates, 11 suspicious isolates were determined and to be Aeromonas hydrophila species Table 3 shown in

TABLE 3: Typing Results of Aeromonas Species Bacteria by Conventional and Molecular Methots, MALDI-TOF MS.

No	NCBI Registration Number	Conventional Method	MALDI-TOF MS	16S rDNA	gyrB
1	AY987739.1	Aeromonas spp.	A.hydrophila/A.punctata(caviae)	A.hydrophila	A.hydrophila
2	AY987732.1	Aeromonas spp.	A.hydrophila/A.punctata(caviae)	A.hydrophila	A.hydrophila
3	AY987736.1	Aeromonas spp.	A.hydrophila/A.punctata(caviae)	A.hydrophila	A.hydrophila

4	AY987736.1	Aeromonas spp.	A.hydrophila/A.punctata(caviae)	A.hydrophila	A.hydrophila
5	AY987736.1	Aeromonas spp.	A.hydrophila/A.punctata(caviae)	A.hydrophila	A.hydrophila
6	AY987736.1	Aeromonas spp.	A.hydrophila/A.punctata(caviae)	A.hydrophila	A.hydrophila
7	AY987736.1	Aeromonas spp.	A.hydrophila/A.punctata(caviae)	A.hydrophila	A.hydrophila
8	AY987736.1	Aeromonas spp.	A.hydrophila/A.punctata(caviae)	A.hydrophila	A.hydrophila
9	AY987736.1	Aeromonas spp.	A.hydrophila/A.punctata(caviae)	A.hydrophila	A.hydrophila
10	AY987736.1	Aeromonas spp.	A.hydrophila/A.punctata(caviae)	A.hydrophila	A.hydrophila
11	AY987736.1	Aeromonas spp.	A.hydrophila/A.punctata(caviae)	A.hydrophila	A.hydrophila

DISCUSSION

Considering the international studies investigating the presence of bacterial species in the organs of fish, their spread in the organs; Kapetanovic et al. investigated the bacterial flora of the fry in rainbow trout hatcheries and reported that the bacteria belonging to Aeromonas and Pseudomonas genera were the most common species in the ponds (13). John reported that bacterial agents are responsible as primary agents in infections with high mortality, and that some microorganisms found in the normal flora of the host may occur as secondary infections in cases where the host's immune system is suppressed (2). In their study, Fowoyo and Achimugu investigated the liver, kidney, skin and gut organs of freshwater catfish using both phenotypic and genotypic methods. They reported that they detected A. hydrophila in the liver, kidney, skin and intestines of fish and that the incidence of A. hydrophila was common in the intestines of all fish (14). Kumar et al. reported that the prevalence of Aeromonas species in freshwater fish farms is generally affected by spatial and temporal variables, host species of farmed fish, and on-farm biosecurity measures (15).

In our study, when tissue samples taken from intestine, kidney, spleen and liver of rainbow trout were examined; A. sobria and A. veronii are the most isolated bacteria, respectively. The reason why these species are isolated as the most common bacterial species is that the bacterial flora of freshwater fish reflects the microflora of the aquatic environment in which they live. It is related to the fact that the bacterial flora of the same species may also vary among fish living in different fisheries, as well as the differences in genus and species. It is seen that our findings are in parallel with the studies reported by other researchers.

A. sobria has been identified as the predominant bacterial species in the intestine, kidney, liver and spleen. Since the transmission route is the outer skin of the fish, the aquaculture environment, the quality of the water and feed used in the facilities, and its widespread presence in the waters have been taken into account. Since the bacterial load in fish will cause fish diseases, facilities that produce in concrete pools, especially in Mazgirt district, are risky in this respect. It was thought that the water used in the pools of the facility was not suitable for aquaculture, since the dam and hydroelectric power plant operating on the Peri Stream, where the facilities were established, changed the ecological and hydrological characteristics of the basin.It was concluded that the mixing of the hot water of the spa with the water coming to the facility affects the prevalence of Aeromonas. It was concluded that due to the high number of bacteria detected in the fish taken from the facilities on the Peri Stream in Mazgirt district, the fish could get sick under adverse stress conditions. The isolation of A. salmonicida from all the fish organs we studied was associated with the presence of suitable breeding conditions with the temperature of the water used in the facilities below 37 ºC.

When the microbiological and molecular analyzes of the samples taken from the waters used by the rainbow trout breeding facilities are examined; Lam et al. reported that Raoultella planticola bacteria grew in the blood culture of a 56-year-old patient, which was taken twice (16). Yuk et al. reported that Lelliottia bacteria species are generally found in soil and foods, L. nimipressuralis species are mostly found in plants, L. amnigena species are detected as a food contamination marker, and L. nimipressuralis species are detected in human blood tissue (17). Çarbaş et al. reported that they detected E. coli in pond water samples as a result of their research in terms of total aerobic mesophilic bacteria, Enterobacteriaceae, Pseudomonas, coliform group bacteria and Escherichia coli by taking aquaculture water samples from 6 different farms raising rainbow trout (18). The data we obtained as a result of our study show parallelism with the results of other researchers' studies. The presence of E. coli bacteria in the water, which was detected in the facility where aquaculture is carried out by the concrete pool method in Ovacık district, indicates that the water is contaminated with animal or human feces.

When the identification methods of bacterial species belonging to the genus Aeromonas are examined, in international studies; Laupland et al. reported that in the diagnosis of bacterial infections, classical microbiological diagnostic methods and molecular methods based on 16S rDNA sequence analysis are accepted as the gold standard and maintain their importance in order to identify the bacteria at the genus and species level (19). Elbehiry et al. stated that the identification of Aeromonas with traditional methods is difficult, some species are phenotypically very similar, time consuming, and results are problematic due to variability in results. It has been reported that various molecular methods such as MALDI-TOF MS and rRNA sequence analysis have been developed. (20). Guo et al. reported in a study that MALDI-TOF MS could not distinguish between A. hydrophila and A. caviae species in the genus Aeromonas and produced uncertain species identification results, and that the updates made in the databases of the device could solve the species-related problem. They reported that this problem was temporarily resolved by identifying the strains as A. hydrophila/caviae, and the solution was satisfactory in routine practice, since precise identification does not normally affect clinical management (8). Justesen et al. reported that in their study on species identification of anaerobic bacteria with MALDI TOF, they found the correct species detection rate to be 67.2% (21). Veen et al., in their study in the microbiology laboratory, reported that they detected 92% accurate detection rate of both bacteria and yeast species using the MALDI-TOF method, and the correct detection rate of classical biochemical tests as 83% (22).

Looking at the sequence analysis methods for the identification of bacterial species belonging to the genus Aeromonas; studies with higher standardization using conserved gene regions such as gyrB and rpoD developed in recent years seem to limit the use of 16S rRNA PCR analyzes. (23,24,25,26,27). In a study, Yanez et al. investigated the phylogenetic relationships of all known species of the genus Aeromonas using the sequence of gyrB, a gene encoding the B-subunit of DNA gyrase. They identified 53 strains. They stated that the results supported the recognition of the Aeromonadaceae family as distinct from Plesiomonas shigelloides and other enteric bacteria, and that this phylogenetic marker revealed species groups consistent with the taxonomic organization of all Aeromonas species described so far. In particular, the gyrB results were consistent with the 16S rDNA analysis; They suggest that gyrB is a useful target for the simultaneous identification of species and strains due to the sequence diversity found at the intraspecies level. In the results of their study, they reported that the gyrB gene is an excellent molecular chronometer for phylogenetic studies of the genus Aeromonas (10). In their study, Shin et al. analyzed 65 Aeromonas isolates to compare the accuracy and feasibility of different methods for identifying Aeromonas at the species level. As a result, while the traditional biochemical method and 16S rRNA sequencing identified Aeromonas very accurately at the genus level, they stated that the identification at the species level was not satisfactory. They reported that housekeeping gene sequencing by phylogenetic

analysis is the most accurate method to identify Aeromonas at the species level. (28). In a study by Mursalim and colleagues to determine their antimicrobial susceptibility to Aeromonas distribution and diversity, they identified the biochemical properties of a total of 86 isolates of the Aeromonas species, derived from diseased freshwater fish from 13 farms in Thailand. They used MALDI-TOF MS, PZR experiments and gyrB gene sequence analysis. They reported that the MALDI-TOF MS result showed 100% accuracy in breed level identification (86 isolates) and 88.4% accuracy in species level identification (76 isolates) (29). The data obtained from our study parallels the work of researchers who reported that the MALDI-TOF MS method sometimes fails to differentiate at the species level.

As a result of the evaluation, as a result of the MALDI TOF-MS analysis, it was accepted that the species identification of 130 isolates with accuracy values between 89.7% and 99.9% gave correct results; 11 suspicious isolates were detected as A.hydophila/ A.punctata (caviae) with accuracy values between 51.0% and 99.9%. 16S rDNA gene sequence analysis of 11 suspected isolates was performed and it was determined that the nucleotide sequences of 11 suspected isolates belonged to Aeromonas hydrophila strain. In order to confirm the species identification of 11 suspect isolates with 16S rDNA gene sequence analysis, gyrB sequence analysis was performed and it was determined that 11 suspicious isolates belonged to Aeromonas hydrophila strain. The results of the two sequencing methods were found to be compatible with each other. As a result of molecular analysis, a total of 141 isolates of Aeromonas species were identified using MALDI-TOF MS, 16S rDNA and gyrB gene sequence analysis. It was seen that MALDI-TOF MS result showed 100% (141 isolates) accuracy in genus level identification and 92.2% (130 isolates) accuracy in species level identification, and gyrB results were consistent with 16S rDNA analysis. The gyrB results were consistent with the 16S rDNA analysis results. It showed a higher capacity to differentiate between species. We consider the current analysis useful to elucidate reported discrepancies between different DNA-DNA hybridization sets, making gyrB a useful target for simultaneous identification of species and strains due to the sequence diversity found at the intraspecies level. The gyrB gene is an excellent molecular chronometer for phylogenetic studies of the genus Aeromonas. In our study, it was determined that the gyrB results were compatible with the 16S rDNA analysis and the gyrB gene sequence analysis had a higher capacity to differentiate between species. Therefore, we suggest using gyrB gene sequence analysis as a molecular method in phylogenetic studies.

In the literature review; It is seen that many studies have been carried out on Aeromonas bacterial species in rainbow trout in our country so far. Most of the studies on Aeromonas have been carried out for the detection of the disease agent. In this study, the identification of Aeromonas bacterial species in rainbow trout in Tunceli province in Turkey by MALDI-TOF MS method and the identification of suspected isolates by 16S rDNA and gyrB gene sequence analyzes at the point where this method could not differentiate in identification at the species level was carried out for the first time in this study.

CONCLUSIONS

In our study, the accuracy of MALDI-TOF MS results of 141 isolates identified as Aeromonas species was found to be % 100 (141 isolates) at genus level and % 92,2 (130 isolates) at species level. It is recommended to use the MALDI-TOF MS method because it gives accurate and fast results in molecular analysis.

However, it has been concluded that 16S rDNA and gyrB gene sequencing methods are more reliable methods at the point where no distinction can be made in identification at the species level due to the higher capacity to distinguish between species in phylogenetic studies of the genus Aeromonas.

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