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Comparison of the efficacy of two phenotypic identification kits and classic PCR methods to identify *Aeromonas hydrophila* isolated from fish farms

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ABSTRACT

In recent years, phenotypic identification kits have been reported to give incorrect results in identifying *Aeromonas* species, whereas molecular identification is quite reliable. In this context, *Aeromonas hydrophila* strains, isolated from fish farms (9 strains), identified by polymerase chain reaction (PCR) method, and ATCC strain bacteria were used in the present study for the determination of the usability of API 20NE and Microgen GN-ID A + B panel test kits. All strains were determined as *A. hydrophila* in molecular methods. After phenotypic identification, a 100% accuracy rate was obtained for *A. hydrophila* with API 20NE. In the Microgen GN-ID A + B, these rates were 60% for the strains used in this study. Phenotypic identification for the ATCC strain in both kits was correct. This study showed that the API 20NE test kit had high validation for the rapid and correct identification of fish pathogenic *A. hydrophila*.

Keywords: Bacterial identification, API 20NE, Microgen ID tests

Introduction

Motile aeromonads, which are generally expressed as Motile Aeromonas Complex (MAC) are Gram negative and usually capable of movement with a single polar flagellum and the disease caused by these in fish is called Motile Aeromonas Septicaemia (MAS). Also, many bacteria that cause disease of fish in this group have been reported in different studies. Important species of this genus are *Aeromonas hydrophila*, *A. caviae*, *A. sobria*, *A. veronii*, *A. schubertii*, and *A. media* (Austin & Austin, 2010). The disease caused by the bacteria belonging to this group in fish is generally called Motile *Aeromonas* Septicaemia (MAS). It is possible to see hemorrhagic septicemia in acute cases of *Aeromonas* occurring in fish, and abscesses and large ulcers in chronic cases (Austin & Austin, 2010). In this group, *A. hydrophila* has been reported in different size ranges of various fish species. It has been reported that it causes blue sac syndrome in trout alevin (Kayış et al., 2015).

Identification of the disease agent is very important in understanding the disease process. Therefore, there is continuous improvement in pathogen identification. Various phenotypic, serologic, and molecular techniques are widely used for the identification of pathogenic fish bacteria. The accuracy level of these methods and the advantages and disadvantages of between each other are a matter of discussion. There may be differences between the results of these identification methods for the Motile Aeromonas Complex. However, it can be claimed that some methods are more sensitive than others in identification. In recent years, it has been reported that phenotypic identification kits may give incorrect results in identifying *Aeromonas* species, whereas molecular identification is quite reliable (Fernández-Bravo & Figueras, 2020). Even the classical PCR method is considered to be more unsafe than the whole genome sequencing method. So, identification studies by the whole genome method for *A. hydrophila* have been reported today (Jin et al., 2020)

Some studies compare or combine the molecular method and phenotypic identification methods related to *Aeromonas* species. API tests are very common among phenotypic kits used for this purpose. Significantly, studies for identifying *Aeromonas hydrophila* by API 20NE have been reported (Dubey et al., 2021; Toobaet al., 2024). Similarly, the other test kit, the Microgen ID test, was used to identify the fish pathogens *A. hydrophila* and *A. caviae.* (Gülaydın et al., 2018). However, it is stated in many studies that these tests can give different results for the same bacteria (Santos et al., 1993). For this reason, which tests can give more accurate results for which bacteria should be carefully examined under certain conditions? In recent years, it has been desirable to present and confirm all possible identification methods for the identification of pathogenic fish bacteria. So, researchers present molecular and phenotypic identification methods together in their studies. This study aimed to identify the *Aeromonas hydrophila*, defined by molecular methods (classic PCR), with API 20NE and Microgen GN-ID A + B Panel kits. In this way, comparing both methods and using two different identification kits for commercial use in the mentioned fish pathogen bacteria, *Aeromonas hydrophila* was determined.

Material and Methods

The bacteria used in the study were obtained from the Fish Diseases Laboratory of the Fisheries Faculty of Recep Tayyip Erdogan University. Detailed information about *Aeromonas hydrophila* strains is given in Table 1. Besides, an *A. hydrophila* strain of the American Type Culture Collection (ATCC), ATCC7966, which has a whole genome analysis report, was selected, and used in this study to compare and validate the results.

Table 1. Fish hosts of the bacterial isolates used in this study and their acceptance numbers in the National Centre for Biotechnology Information (NCBI).

Code	Host/Samples	Tissue	Acceptance Number
D7	*Capoeta banarescui*	Spleen	MT730008
D13	*Capoeta ekmekciae*	Spleen	MT730009
D17	*Squalius orientalis*	Spleen	MT730010
D22	*Squalius orientalis*	Spleen	MT730011
Y1	*Salmo* sp.	Egg	MT730013
Y21	Hatchery	Water	MT730014
Y28	Hatchery	Water	MT730015
K31	*Alburnus derjugini*	Kidney	MK548537
Y33	*Oncorhyncus mykiss*	Kidney	MT730016

For the molecular identification of the *Aeromonas* species, their genomic DNA was obtained by boiling method (Kayiş et al., 2015). The primers specific to the 16S rRNA region of eubacteria (27 Fwd 5'-AGA GTT TGA TCC TGG CTC AG-3', 1492 Rev 5'-GTT TAC CTT GTT ACG ACT T-3') were used. Then PCR reaction was carried out using bacterial genomic DNA and the given primers (Model Px2 ThermoHybrid; Thermo Electron Inc., Waltham, MA, USA). The 1465-bp amplified products were purified with a NucleoSpin PCR

purification kit (Macherey-Nagel) and sent for sequencing by double-sided reading (ABI PRISM 310 genetic analyzer, Applied Biosystems). Accession numbers of bacteria in the National Center for Biotechnology Information (NCBI) are given in Table 1. The API 20NE test kit (BioMerieux, France) and Microgen ID A+B (Microgen, UK) were used for the phenotypic identification of the bacterial strains. The tests and their differences included in both test kits are given in Table 2.

Pure bacterial cultures were inoculated into the kits as specified in the instructions of the test kits. API 20NE kits were incubated at 29 ±1ºC and 22 ±1ºC, while Microgen GN-ID A + B was incubated at 34 ±1ºC for 24 hours. Many researchers have stated that API tests need some modifications in them to use in fish pathogens (Popovic et al., 2014). The most important of these changes is perhaps the incubation temperature. Therefore, a low-temperature trial (22 ±1ºC) was also conducted, which is more suitable for fish pathogens. At the end of the incubation, different reagents determined for both kits were added to the tests. The codes of bacteria were formed according to the colour changes mentioned in the instructions, and the results were interpreted via the APIWEB and Microgen ID software systems for identification.

Kovac's reagent, VPI-VPII, Nitrate A and Nitrate B, and TDA reagent were added to the tests for Microgen, and Mineral oil, Nit1, and Nit2, Zn, and James solutions were added for API 20 NE. As a result of the reagents applied, the codes obtained according to the colour changes were uploaded to the licensed APIWEB and Microgen ID software systems, and the bacteria were identified.

Table 2. Comparison of the test contents of the kits used in this study.

API20 NE		**Microgen GN-ID A+B**		
NO_3	Potassium nitrate	OX	Oxidase	
TRP	L-tryptophan	MOT	Motility	
GLU	D-glucose (fermentation)	NIT	Nitrate	
ADH	L-Arginine	LYS	Lysine	
URE	Urease	ORN	Ornithine	
ESC	Esculin ferric citrate	H_2S	H_2S	
GEL	Gelatine	GLU	Glucose	GN-A
PNG	4-Nitrophenyl-β-D- glucopyranoside	MAN	Mannitol	
GLU	D-Glucose (assimilation)	XLY	Xylose	
ARA	L-Arabinose	ONPG	o-nitrophenyl-beta-D-galactoside	
MNE	D-Mannose	IND	Indole	
MAN	D-Mannitol	URE	Urease	
NAC	N-Acetyl glucosamine	VP	Voges Proskauer	
MAL	D-Maltose	CIT	Citrate	
GNT	Potassium gluconate	TDA	Triptofan	
CAP	Capric acid	GEL	Gelatine	
AD	Adipic acid	MAN	Malonate	
MLT	Malic acid	INO	Inositol	
CIT	Trisodium citrate	SOR	Sorbitol	
PAC	Phenylacetic acid	RHM	Rhamnose	
		SUC	Sucrose	GN-B
		LAC	Lactose	
		ARA	Arabinose	
		ADO	Adonitol	
		RAF	Raffinose	
		SAL	Salicin	
		ARG	Arginine	

Results and Discussion

The identification of bacteria in the NCBI database as a result of the molecular identification is given in Table 1. The results showed that all bacteria were *Aeromonas hydrophila* in both incubation temperatures. According to the API 20NE test results of bacteria in the Apiweb system, all strains, including the ATCC, were confirmed as *A. hydrophila* (Table 3). In the results of all Microgen ID test kits strains, 6 out of 10 different bacteria could be identified as *A. hydrophila* (Table 4). In addition, the ATCC strain was identified as *A. hydrophila*.

According to the data obtained from the study, reference strain *A. hydrophila* (ATCC7966) and D17, D22, Y28 Y21, and Y33 strains were defined as *A. hydrophila* in all three identification methods (molecular, API 20NE, and Microgen ID A + B). API 20NE codes of only three bacteria (D7, D22, and Y21) were identified as the same as the ATCC strain. On the other hand, in the Microgen ID system, none of the bacteria codes could be identified the same as the ATCC strain. In the API system, six tests (TRP, ARA, MNE, MAN, NAG, MAN, and CIT) differed with ATCC strains. All other tests were similar to the ATCC strain. The test with the most variability in the API system was determined as citrate. (Table 3 and Figure 1). On the other hand, only 11 tests were observed, similar to ATCC strains in the Microgen ID system. The most variable tests in the Microgen ID systems were VP, gelatine, mannitol, and hydrogen sulphide (Table 4 and Figure 2).

Molecular methods have been used frequently in identifying fish-origin bacteria for the last two decades (Altinok & Kurt, 2003). However, traditional phenotypic methods are still commonly used for bacteria. Many studies indicate that phenotypic identification methods for bacteria have some problems. Such as, some bacteria can be misidentified due to incubation temperature values and aquatic system differences (Popovic et al., 2004). For these reasons, the scientific authorities recommend the application of molecular techniques in the identification of bacteria. This question is an important detail that researchers ask; How is the compatibility of both methods? The presented study is a narrow answer to the accuracy of this approach. So, in the present study, *Aeromonas hydrophila* strains were identified with the classical PCR technique, and the two different test kits and the results were compared. According to the PCR technique, all bacteria were identified as *Aeromonas hydrophila*. Molecular identification was not performed on the ATCC strains used in the study. On the other hand, all strains were identified as *A. hydrophila* in the same bacterial group according to the API 20NE test. In contrast, six strains were defined as *A. hydrophila* according to Microgen tests. This study demonstrated that the API 20NE kit successfully identified *A. hydrophila*. On the other hand, it was determined that the Microgen ID system was more unsuccessful in identifying *A. hydrophila*. For *A. hydrophila*, all tests except citrate showed slight variation between the reference strain and isolates. In this sense, it can be said that the API 20NE test kit is quite successful in identifying *A. hydrophila.*

Table 3. Evaluation of the results of both test kits for *Aeromonas hydrophila*

Code	API 20NE	(%)	Bacteria	Microgen ID	(%)	Bacteria
K31	7576455	91.2	*Aeromonas hydrophila*	746622001	99.5	*Aeromonas sobria*
D7	7577755	99.3	*Aeromonas hydrophila*	644424000	95.9	*Burkholderia cepacia*
D13	5573754	99.8	*Aeromonas hydrophila*	706424123	99.7	*Vibrio fluvialis*
D17	7577754	99.9	*Aeromonas hydrophila*	777664123	98.2	*Aeromonas hydrophila*
D22	7577755	99.3	*Aeromonas hydrophila*	744660523	96.8	*Aeromonas hydrophila*
Y1	7574454	99.4	*Aeromonas hydrophila*	706424001	82.6	*Aeromonas sobria*
Y28	7577754	99.9	*Aeromonas hydrophila*	717624003	99.9	*Aeromonas hydrophila*
Y21	7577755	99.3	*Aeromonas hydrophila*	707624023	98.2	*Aeromonas hydrophila*
Y33	7574455	99.2	*Aeromonas hydrophila*	716624023	99.7	*Aeromonas hydrophila*
ATCC	7577755	99.3	*Aeromonas hydrophila*	754660101	98.7	*Aeromonas hydrophila*

Table 4. Similarities of the strain for ATCC strain in API 20NE tests.

Tests	**Bacteria**									
	K31	D7	D13	D17	D22	Y1	Y28	Y21	Y33	ATCC
NO_3	+	+	+	+	+	+	+	+	+	+
TRP	+	+	-	+	+	+	+	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+
ADH	+	+	+	+	+	+	+	+	+	+
URE	-	-	-	-	-	-	-	-	-	-
ESC	+	+	+	+	+	+	+	+	+	+
GEL	+	+	+	+	+	+	+	+	+	+
PNG	+	+	+	+	+	+	+	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+
ARA	-	+	+	+	+	-	+	+	-	+
MNE	+	+	+	+	+	-	+	+	-	+
MAN	+	+	-	+	+	+	+	+	+	+
NAC	-	+	+	+	+	-	+	+	-	+
MAL	-	+	+	+	+	-	+	+	-	+
GNT	+	+	+	+	+	+	+	+	+	+
CAP	+	+	+	+	+	+	+	+	+	+
ADI	-	-	-	-	-	-	-	-	-	-
MLT	+	+	+	+	+	+	+	+	+	+
CIT	+	+	-	-	+	-	-	+	+	+
PAC	-	-	-	-	-	-	-	-	-	-
OX	+	+	+	+	+	+	+	+	+	+

Table 5. Similarities of the strain for ATCC strain in Microgen ID tests.

Tests	**Bacteria**									
	K31	D7	D13	D17	D22	Y1	Y28	Y21	Y33	ATCC
OX	+	+	+	+	+	+	+	+	+	+
MOT	+	+	+	+	+	+	+	+	+	+
NIT	+	+	+	-	+	+	+	+	+	+
LYS	+	+	-	+	+	-	-	-	-	+
ORN	-	-	-	+	-	-	-	-	-	-
H_2S	-	-	-	+	-	-	-	-	+	+
GLU	+	+	+	+	+	+	+	+	+	+
MAN	+	-	+	+	-	+	+	+	+	-
XLY	-	-	-	+	-	-	+	+	-	-
ONPG	+	+	+	+	+	+	+	+	+	+
IND	+	-	-	+	+	-	+	+	+	+
URE	-	-	-	-	-	-	-	-	-	-
VP	-	-	-	+	+	-	-	-	-	+
CIT	+	+	+	+	+	-	+	+	+	+
TDA	-	-	-	-	-	-	-	-	-	-
GEL	-	+	+	+	-	+	+	+	+	-
MAL	+	-	-	-	-	-	-	-	-	-
INO	-	-	-	-	-	-	-	-	-	-
SOR	-	-	-	-	+	-	-	-	-	-
RHM	-	-	-	-	-	-	-	-	-	-
SUC	-	-	+	+	+	-	-	-	-	+
LAC	-	-	-	-	-	-	-	-	-	-
ARA	-	-	+	+	+	-	-	+	+	-
ADO	-	-	-	-	-	-	-	-	-	-
RAF	-	-	-	-	-	-	-	-	-	-
SAL	-	-	+	+	+	-	+	+	+	-
ARG	+	-	+	+	+	+	+	+	+	+

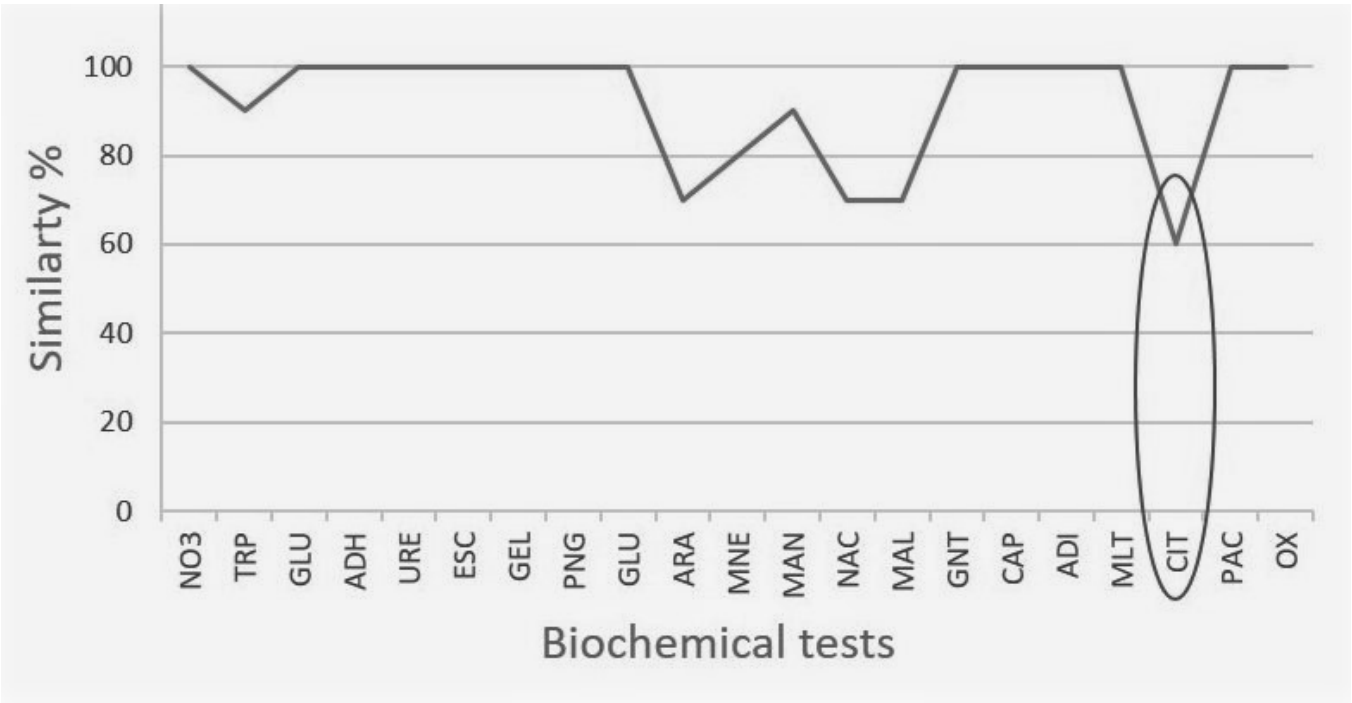

Figure 1. Similarities of the bacteria in API 20 NE tests for ATCC strain.

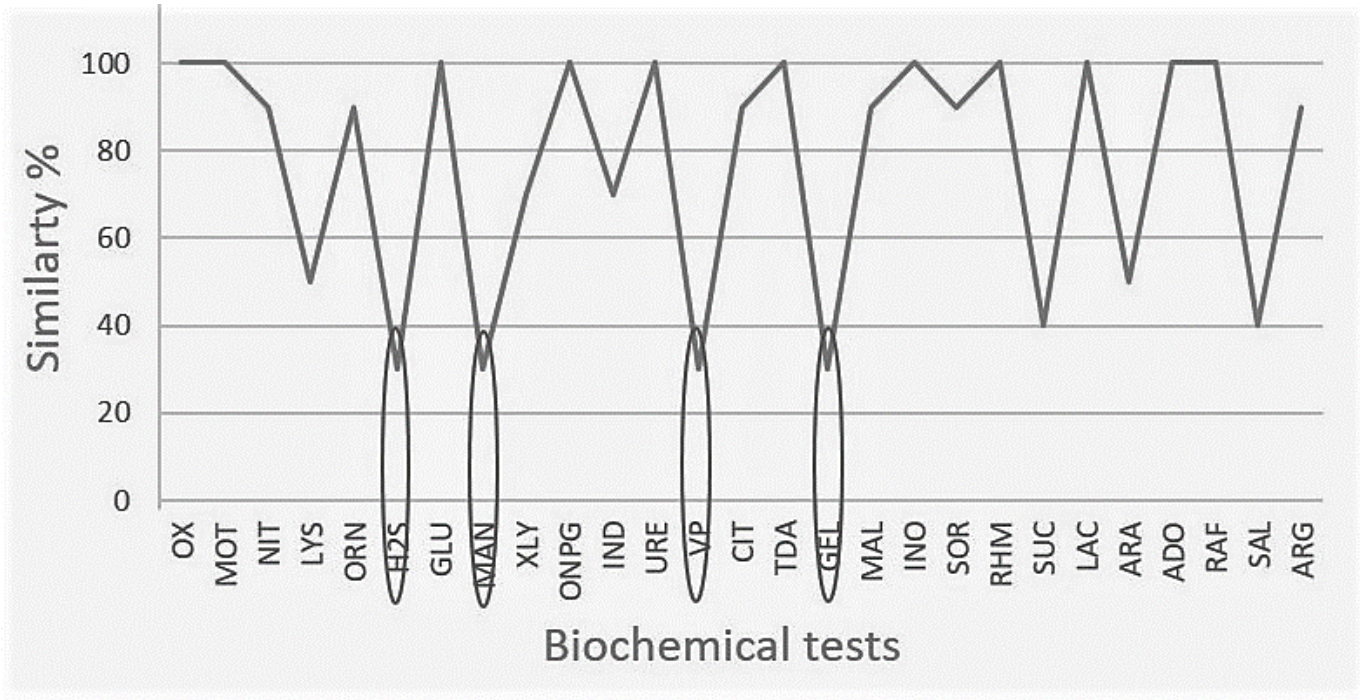

Figure 2. Similarities of the bacteria for ATCC strain in Microgen ID.

Microgen ID test kits are not as widely used as API tests in identifying pathogenic bacteria. However, it has been reported to be used in some studies. In a study conducted with bacteria isolated from sea turtle eggs, comparing API and Microgen ID tests with molecular identification methods contains quite detailed information. This study states that Microgen ID tests give the best result in identifying bacteria with Gram-negative rods (Awong-Taylor et al., 2007). Microgen ID kits were used to identify bacteria obtained from pearl mullet (*Chalcalburnus tarichi*) samples, and *A. hydrophila*, and *A. caviae* were identified in the mentioned study (Gülaydın et al., 2018). In the present study, it was observed that the Microgen ID test successfully identification of *A. hydrophila* isolates at 60% rates. The reasons for this situation (not completely successful in all isolates) should be investigated, and studies should be done to increase the reliability of the test. In particular, the incubation temperatures of the tests may have caused these false results. Successful identification of the reference strain ATCC isolate in the Microgen test indicates that the reliability of the test will increase after minor improvements. The kit database should be improved by adding more data on different strains of the same species. Additionally, different incubation temperatures can be studied.

Bacterial fish pathogens are known to prefer low incubation temperatures. However, the recommended incubation temperatures of commercial identification kits are relatively higher. Literature information indicates that these temperature preferences are a problem, especially in API tests. The present study reveals that the temperature difference does not differ in identifying *Aeromonas hydrophila* with these commercial kits.

Conclusion

The identification kits used in this study are generally designed for bacteria that are human pathogens. Therefore, it may give misleading results in the identification of pathogenic fish bacteria. However, with the studies to be done, the most accurate results can be achieved. With this study, it is understood that the API 20 NE test kit, which is frequently used, gives quite accurate results for *A. hydrophila*. Both kits were found as the test kit more suitable for identification with molecular methods and ATCC strains. Therefore, the use of these test kits can be recommended for the mentioned bacteria in the same conditions as the present study. However, the most correct approach is to study using both methods.

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: Ethics committee approval is not required for this study.

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