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Evaluation of two different laboratory methods for the identification of *Aeromonas* spp. in stool sample of patients with diarrhea

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Background: *Aeromonas* spp. can cause diarrhea and various infections in humans. Access to rapid techniques with a high sensitivity and specificity is strongly needed for the identification of *Aeromonas* species. The aim of this study was to evaluate two different methods including API 20E bacterial identification tests and the molecular detection using PCR primers specific for 16s-rRNA and 23S-rRNA genes sequences for identification of *Aeromonas* spp. in stool samples from patients with diarrhea.
Materials and Methods: One hundred stool samples from diarrheal patients were collected. All isolates were subjected toAPI 20 E strip tests and PCR using specific primers for identification of *Aeromonas* spp.
Results: The API 20E analysis identified 2 (2.2%) isolates as *Aeromonas* spp. Molecular identification by aero-23S-rRNA gene confirmed the same 2 isolates as identified by the API 20E strips.
Conclusion: Both API 20E system and PCR method using Aero 23S-rRNA primer were found to be accurate in identification of *Aeromonas* spp. isolates with highconfidence.

Keywords: API bacterial identification, *Aeromonas* spp., PCR

1. Background

Aeromonas is a gram negative and facultative anaerobic rod that belongs to Enterobacteriaceae family. This organismis found in aquatic environment and foodsespecially in fish, meat and vegetables (1) and therefore, *Aeromonashy drophila* has been placed on the United States Environmental Protection Agency (EPA) Contaminant Candidate List as awaterborne pathogen (2). *Aeromonas* spp. can cause diarrhea and various infections such as gasterointestinal infections, meningitis, bacteremia and lung infections in humans (3). The exact role of *Aeromonas* spp. in causation of diarrhea in humans is not completely understood and maybe due totheir heterogeneous populationamong which only some certain species are pathogenic (4). *Aeromonas* spp. have many different virulence factors such as cytotoxins, aerolysins, enterotoxins, hemag-glutinins, hemolysins and these species are capable of adhering and invadingtissue culture cell lines however, the correlation between these factors and the diarrheagenic ability of *Aeromonas* spp. has not been clearly recognized (5). The isolation rate of *Aeromonas* spp. from human's feces is various depending on the geographic regions and the use of various isolation methods and different study populations make comparison of these rates difficult, however,it has been demonstrated that isolation of *Aeromonas* spp. is seasonally associated (6). Significant isolation of *Aeromonas* spp. from food, water or human's feces is problematic and this complexity poses a major dilemma and threat to public health. In the past, conventional biochemical testshave been used for the identification of *Aeromonas* spp. however,they are time consuming and their sensitivity is not so much to guarantee the accurate identification of isolates (7). New rapid tests and molecular techniques such as API strips (API-bioMérieux, Inc., La Balme les Grottes, France), Polymerase Chain Reaction (PCR) and outer membrane proteins based immunoassays (8, 9), DNA/RNA probes (10, 11) and flow cytometry are now used for identification of *Aeromonas* spp. from food, aquatic environment and clinical samples (12, 13). Access to rapid techniques with a high sensitivity and specificity is strongly needed for the identification of*Aeromonas* species.

2. Objectives

The aim of this study was to evaluate two different methods including API 20E strip testsand the molecular detection using PCR primers specific for 16s-rRNA and 23S-rRNA genes sequences for the identification of *Aeromonas* spp. in stool samples from patients with diarrhea.

3. Materials and Methods

3.1. Isolation of strains

One hundred stool samples from diarrheal patients that were referred to 3 hospitals in Tehran were collected and transferred to our laboratory in Carry-Blair medium and suspended in 2 ml sterile normal saline. For the isolation of *Aeromonas* species the bacterial suspension was cultured on both Blood Agar (B.A) plates with 20 µg ml^{-1} ampicillin (incubated at 37°C) and CIN (cefsulodin-Irgasan-novobiocin) medium (incubated at room temperature for 48h). The colonies with positive hemolysis on B.A and bull's eye shape on CIN medium were selected and subjected to oxidase test. Colonies with positive oxidase test were considered as suspected colonies for further identification tests (14). *Aeromonas* ATCC7965 was used as positive control and a subset of other enteric bacteria including *Vibrio cholerae* ATCC14035, *Campylobacter jejuni* ATCC29428, *Shigella sonnei* ATCC 9290, *Shigella flexneri* ATCC 12022**,** *Escherichia coli* ATCC25922, *Enterobacter aerogenes* ATCC 13048 were used as negative controls**.**

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3.2. API 20 E for identification of Aeromonas spp.

API 20 E strips (API-bioMérieux, Inc., La Balme les Grottes, France) consisted of 27 essential biochemical tests for the identification of Enterobacteriacae was used. All of the suspected isolates (88) were subjected to API 20 E strip system according to manufacturer instruction. After inoculation, each strip was placed in incubation box (tray and lid) provided by the manufacturer with 5 ml distilled water in wells of incubation box to provide a humid atmosphere.

3.3. Molecular identification of Aeromonas species

The whole genome of all suspected isolates (88) was extracted by boiling method. Two pairs of primers were used for molecular identification of *Aeromonas* spp. including 16S-rRNA gene and 23S-rRNA gene reported to specifically identify the *Aeromonas* spp. (Table 1). PCR was performed in a reaction mixture with total volume of 25 µl, containing 2.5 µl 10x Taq polymerase buffer, 0.3 µl dNTPs (10 mmol l^{-1}), 1 U Taq DNA polymerase, 0.6 µl $MgCl_2$ (50 mmol l^{-1}) and 0.3 mol l^{-1} from each primer. PCR was done as follows: Initial denaturation step at 94°C for 5 min followed by 30 cycles consisting of denaturation (94°C for 1 min), annealing (54°C for 1 min, separately was set for each primer pair), and extension (72°C for 1 min), followed by a final extension step at 72°C for 5 min. The genomes of other standard enteric bacteria were used as negative control ineach PCR assays.

3.4. Sequencing of amplified fragment and BLAST software analysis

PCR products were purified using QIAquick Gel Extraction Kit (Qiagen), and direct sequencing of the amplified fragments was performed using ABI 3730X capillary sequencer (Genfanavaran, Macrogen, Seoul, Korea). The amplified Aero 23S-rRNA fragments were analyzed with previously deposited sequences in the GenBank using BLAST software.

3.5. Effectiveness of different methods

Considering 23S-rRNA gene PCR-sequencing as gold standard, the effectiveness of API strips was evaluated via following formula:sensitivity of API strips = [(number of isolates determined as positive by both API and PCR-sequencing)/(total number of isolates determined as positive by 23S-rRNA gene PCR-sequencing)] ×100], specificity of API strips = [(number of isolates determined as negative by API and 23S-rRNA gene PCR-sequencing)/(total number of isolates determined by 23S-rRNA gene PCR-sequencing)] ×100], sensitivity of 16S-rRNAgene PCR-sequencing method = [(number of isolates determined as positive by both 16S-rRNA gene PCR-sequencing and 23S-rRNA gene PCR-sequencing)/(total number of isolates determined as positive by 23S-rRNA gene PCR-sequencing)] ×100], specificity of 16S-rRNA gene PCR-sequencing = [(number of isolates determined as negative by both 16S-rRNA gene PCR-sequencing tests and 23S-rRNA gene PCR-sequencing)/(total number of isolates determined as negative by 23S-rRNA gene PCR-sequencing)] ×100] (17).

4. Results

4.1. Preliminary biochemical tests and colony morphology

Eighty eight out of 100 stool samples were identified as suspected *Aeromonas* spp. due to colony appearance on B.A plates supplemented with Ampicillin and CIN medium. Twelve were excluded from further investigation because their culture morphology did not matchwith*Aeromonas* spp. characteristics.

4.2. Identification of isolates by API 20 E strips

The API 20E analysis identified 2(2.3%) isolatesas *Aeromonas* spp. and 86remainingisolateswere identifiedas follows: 29 (33%) *E.coli*, 20 (22.7%) *Enterobacter* spp., 15 (17%) *Rotella* spp., 8(9.1%) *Klebsiella* spp., 6 (6.8%) *Bacillus* spp. and 8 (9.1%) *Proteus* spp. (Table 2).

Table 2. Quantitative comparison of bacterial strains isolated by conventional method and API 20E system

Organisms	Identification by API 20E system. No. (%)
Aeromonas spp.	2 (2.3%)
Proteus spp.	8 (9.1%)
E. coli	29 (33%)
Salmonella spp.	0 (0%)
Bacillus spp.	6 (6.8%)
Edwardosiella spp.	0 (0%)
Enterobacter spp.	20 (22.7%)
Rotella spp.	15 (17%)
Klebsiella spp.	8 (9.1%)
Total	88

4.3. Molecular characterization of suspected Aeromonas spp. isolates

PCR amplification of 16S-rRNA gene showed a single band of expected size (599bp) in 79 (90%) of 88 suspected *Aeromonas* spp. isolates and even in other species of enteric bacteria thatwere used as negative controls in this study (Figure 1A).Moreover, molecular identification by aero-23S-rRNAgene targeted primers generated an amplification band of 550bp in the same 2 isolates as identified by the API 20E strips (figure 1B). Analysis of amplified Aero 23s-rRNA sequences with the previously deposited sequences in the GenBank using BLAST software confirmed the identity of *Aeromonas* spp. isolates.

Considering aero-23s-rRNA gene sequencing as gold standard, the sensitivity and specificity of API strips was calculated as 100% and 100%, respectively while, the sensitivity and specificity of PCR with 16S-rRNA gene primers was calculated as 100% and 10.5%, respectively.

Table 1. Primers used in this study.

Target	Forward and Reverse	Primer sequences 5'-3'	Amplicon size	References
Aeromonas spp. (IGS region of 23S-rRNA)	aero-23S-rRNA-F aero-23S-rRNA-R	GGAAACTTCTTGGCGAAAAC GGTTCTTTTCGCCTTTCCCT	550 bp	15
Aeromonas spp. (*16s-rRNA* gene)	16S-rRNA-F 16S-rRNA-R	TCATGGCTCAGATTGAACGCT CGGGGCTTTCACATCTAACTTATC	599 bp	16

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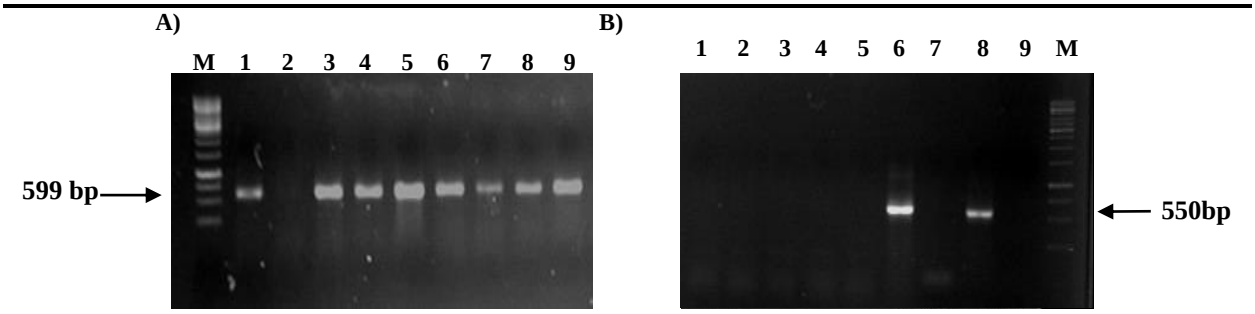

Figure 1. A) PCR amplification of 16S-rRNA gene among suspected isolates. M: 1kb DNA size marker; lane 1: positive control *Aeromonas* ATCC7965; lane 2: negative control; lane 3: *Vibrio cholerae* ATCC14035; lane 4: *Campylobacter jejuni* ATCC29428; lane 5: *Shigellasonnei* ATCC 9290; lane 6: *Shigella flexneri* ATCC 12022**;** lane 7: *Escherichia coli* ATCC25922; lane 8: *Enterobacter aerogenes* ATCC 13048; lane 9: suspected *Aeromonas* isolate.
B) PCR amplification of aero-23S-rRNA gene among suspected isolates. Lane 1: *Vibrio cholerae* ATCC14035; lane 2: *Campylobacter jejuni* ATCC29428; lane 3: *Shigella sonnei* ATCC9290; lane 4: *Shigella flexneri* ATCC12022; lane 5: *Escherichia coli*ATCC25922; lane 6: suspected *Aeromonas* isolate; lane 7: *Enterobacter aerogenes* ATCC13048; lane 8: positive control *Aeromonas* ATCC7965; lane 9: negative control; M: 1kb DNA size marker.

5. Discussion

The API system is one of thewell documentedmethods thatare based on conventional biochemical tests with much higheraccuracy (18). Oxidase test is one of the principal tests in the initial characterization of gram-negative bacteria. The cytochrome system is found in aerobic, or microaerophilic, and facultatively anaerobic organisms. Thus, the oxidase test is important in identifying organisms that either lack the enzyme or are obligate anaerobes. The test is most helpful in screening colonies suspected of being one of the Entero-bacteriaceae member (all negative) from colonies suspected of belonging to other genera such as *Aeromonas*, *Pseudomonas*, *Neisseria*, *Campylobacter*, and *Pasteurella* (all positive) (19). This test may produce false positive results during the procedure that uses nickel, steel, and other wire loops. Oxidase reagent will turn purple over time due to oxygen in the air and if it is not stored in a good condition, the false positive result will frequently occur in laboratories.

According to the result of this study, among doubtful isolates only 2.3% of isolates were identified as *Aeromonas* spp. by aero-23S-rRNA gene sequencing and API 20E.

Many studies have been performed to evaluate the API system for the identification of different genus of bacteria and have compared this method by other reports (18, 20-22). In a study by Devenish and Barnum (1980), the accuracy of API 20E to identify Enterobacteace isolated from clinical specimens of animal origins was reported as 97.9 % and 235 out of 240 isolates were correctly identified by this method (23).

Malloy and colleagues (1983), compared API 20E system with two automated systems, AutoMicrobic system (Vitek Systems, Inc., Hazelwood, Mo.) and the MS-2 (Abbott Diagnostics, Dallas, Tex) for the direct identification of Enterobacteriaceae isolated from blood cultures and have reported the correlation of 90% between API 20E and two other systems (21).

The results of the present study validate the accuracy of API 20E system for the identification of *Aeromonas* spp. and their results are comparable with molecular identification. PCR method has been mostly used for the phylogenetic positioning or for virulence characterization of *Aeromonas* spp. (12,16, 24-26). In a study by Arora and colleagues (2006) duplex-PCR using primers targeted for 16s rRNA gene and aerolysin gene was performed for the detection of *Aeromonas* spp. in foods of animal origin. The primer set used in this study for 16s rRNA was supposed to be genus specific for the detection of all species of *Aeromonas* however, almost all isolates of different genera (*Vibrio cholerae*, *Campylobacter jejuni*, *Shigella sonnei*, *Shigella flexneri*, *Escherichia coli*, *Enterobacter aerogenes*) also produced positive results which is probably because of similarities within the 16s-rRNA gene sequences of different genera of enteric bacteria (27). Moreover, the Aero23S-rRNA primer designed by Osman and colleagues (2011) was proved with the present study to be specific enough to ensure accurate detection of *Aeromonas* spp. (15). The target sequence of this primerset is located within intergenic spacer (IGS) region of 23s-rRNA in *Aeromonas* spp. Analysis of our suspected *Aeromonas*isolates with the later primer set revealed that only2 isolates out of 100 harbored this gene and identified as *Aeromonas* spp. This finding is well correlated with the results of API 20E system.

6. Conclusion

In conclusion, both API 20E system and PCR method using Aero 23S-rRNA primer were found to be accurate enoughforthe identification of *Aeromona*s spp. isolated from stool samples of patients with diarrhea, however,molecular technique based on 16S-rRNA sequences showed high level of disparity which make the interpretation of results more problematic and emphasizes on the need to more extensively re-assess and validate the accuracy of this method.

Conflict of Interests

The authors declare they have no conflict of interests.

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Authors' Contributions

Mina Boustanshenas design the study and wrote the manuscript, Majid Akbari performed the isolation and biochemical characterization of the strains, Niloofar Rezaie performed all molecular producers.

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