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Rapid identification of *Aeromonas* species in stool samples with chromogenic media and matrix-assisted laser desorption ionization–time of flight mass spectrometry: an institutional experience

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Aim: To evaluate the routine use of chromogenic media together with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF) in gastrointestinal infections caused by *Aeromonas* spp. as rapid, practical, cost-effective and reliable methods.

Materials and methods: Between August 2007 and March 2012, a total of 13,194 stool specimens of patients were included in the study. The stool samples and the reference strains were inoculated onto Cefsulodin–Irgasan–Novobiocin agar, sheep blood agar, eosin-methylene blue agar, and Hektoen enteric agar media together with CHROMagar Salmonella Plus. All the *Salmonella* and *Aeromonas* suspected colonies were identified with an automated system (Phoenix) and MALDI–TOF.

Results: Some of the pink colonies resembling *Salmonella* were identified as *Aeromonas* spp. without any discordance (100%) between the systems. A total of 86 *Aeromonas* strains were identified: 30 *A. caviae*, 27 *A. hydrophila*, 16 *A. veronii*, 11 *A. sobria*, and 2 *A. salmonicida*. When analyzed macroscopically, those *Aeromonas* species had prominent colony appearance differences from classically detected *Salmonella* on CHROMagar Salmonella Plus media.

Conclusion: A combination of CHROMagar Salmonella Plus and MALDI–TOF will help to detect *Aeromonas* species in 24 h in a cost-effective, practical, and reliable manner.

Key words: *Aeromonas*, culture media, matrix-assisted laser desorption ionization–time of flight mass spectrometry

1. Introduction

Aeromonas species consist of ubiquitous glucose-fermenting, oxidase-positive, gram-negative rods that are widely distributed in freshwater, estuarine, and marine environments worldwide. Patients with diarrhea and harboring aeromonads often generally have had some aquatic exposure, such as an association with untreated ground water or consumption of sea food, particularly raw oysters or clams. Aeromonads grow immediately on most media used for routine stool cultures (1). In a study in Turkey, numerical taxonomy of *Aeromonas* strains isolated from different sources revealed the presence of potentially pathogenic *Aeromonas* spp., especially in food (2). Although it is an infectious agent, in Turkey *Aeromonas* spp. infections were reported as 0.37%–3.5%, while it was reported as 0%–12.2% worldwide (3).

The media most widely used for the isolation and differentiation of coliform gram-negative bacteria and other enteric pathogens from clinical specimens are MacConkey and Drigalski agars, but their ability to differentiate these types of organisms is low because of their dependence only on determination of lactose utilization (4). *Aeromonas* species in mixed coliform cultures may not always be detected but careful observers are often able to recognize mixtures of different gram-negative bacteria when they occur on a single plate (5). The combination of using both ampicillin blood agar and a modified Cefsulodin–Irgasan–Novobiocin (CIN) plate may give the highest recovery of aeromonads (1).

Rapid and reliable identification of pathogenic microorganisms, including the above-mentioned *Aeromonas*, is important for surveillance, prevention, and control of food-borne diseases. The established methods for bacterial identification in clinical microbiology are often time-consuming and laborious. Time is required for purification and the identification of pathogenic bacteria

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subspecies by biochemical typing procedures also requires long incubation times, and therefore there is a delay in final identification. These procedures require experience in interpretation and can be limited by subjectivity and low specificity. There is an increasing need for alternative procedures that allow rapid and reliable identification of microorganisms (6).

The presence of species-related disease syndromes, coupled with differences in antimicrobial susceptibilities among the species, strongly suggests that conventional identification and antimicrobial susceptibilities should be determined for clinical aeromonad isolates (7).

In our laboratory, we use chromogenic *Salmonella* media together with MacConkey agar in routine stool cultures for the isolation of *Salmonella* and *Shigella* species. By chance, we have observed that the chromogenic media we use for *Salmonella* species can also detect *Aeromonas* species, even though this feature was not mentioned in the chromogenic media prospectuses. As well as special media, there are also some techniques that can be used for the rapid identification of these microorganisms. In recent years, several reports have shown the feasibility of using matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectrometry (MS) to identify microorganisms (8,9). In whole-cell MALDI–TOF MS, characteristic "fingerprint" spectra are obtained from whole cells without biomarker prefractionation, digestion, separation, or clean-up. The procedure is very fast, requires minimal amounts of biological material (subcolony amounts), is suitable for high-throughput routine analysis, and therefore has great potential for applications in clinical microbiology or environmental monitoring. The observed protein biomarkers are typically highly expressed proteins with housekeeping functions, such as ribosomal or nucleic acid-binding proteins, which are highly conserved in bacteria; therefore, the method can be universally applied (10).

In this present study the aim was to evaluate the usefulness of CHROMagar Salmonella Plus medium and MALDI–TOF identification in the rapid, practical, correct, and cost-effective detection of *Aeromonas* species (6).

2. Materials and methods

The practical use of CHROMagar Salmonella Plus medium together with MALDI–TOF analysis is tested in isolation of *Aeromonas* species. For comparison an automated system (Phoenix, BD, Franklin Lakes, NJ, USA) is also used in identification of both bacteria.

The stool specimens of 13,194 patients collected from 16 hospitals of our institution were included in this study. The symptoms of the patients from whom those bacteria species were isolated included abdominal pain, nausea, vomiting, and diarrhea. Those patients had a generally mild type of disease. The analysis was performed in our central laboratory between August 2007 and March 2012. For comparison with commercial systems, *Aeromonas* suspected strains and reference strains *Aeromonas hydrophilia* ATCC 7966 and *Aeromonas caviae* ATCC 7966 were also inoculated onto CIN agar, sheep blood agar, eosin-methylene blue agar, and Hektoen enteric agar media together with CHROMagar Salmonella Plus (BD). In CHROMagar Salmonella Plus media, *Aeromonas* suspected pink or mauve and/or diffuse pigmented colonies were identified in an automated system (Phoenix, BD).

Salmonella species had pink and bigger colonies than *Aeromonas* colonies. After identification of *Aeromonas* species, those strains were inoculated onto other media and the supporting data were achieved by observing hemolysis on blood agar and the growth of oxidase-positive *Aeromonas* colonies on CIN agar.

All of the *Aeromonas* strains were also identified with MALDI–TOF and their concordances were evaluated. In the MALDI–TOF mass spectrometry method the isolate of the suspected colony is dropped on a metal target, and a matrix solution is added onto this drop and dried in the air. After the target is placed in the device with the adjusted laser shots, the matrix absorbs the light and the molecules of the isolate become ionized. The matrix solution contains alpha-cyano-4-hydroxycinnamic acid, trifluoroacetic acid, and acetonitrile. After the laser shots, the molecules are ionized and fly in the flight tube according to their molecular weights. The digitalized data accumulate in order to form a time of flight (TOF) mass spectrum and are detected via a detector. In our study we used an Autoflex II Smartbeam MALDI–TOF MS (Bruker Daltonics, Bremen, Germany) device.

The peaks of the microorganisms' protein molecules were identified with a Biotyper (Bruker Daltonics). The antibiotic susceptibility testing of *Aeromonas* strains was done using the Phoenix automated system (BD).

3. Results

Using CHROMagar Salmonella Plus medium, pink colonies were the point of interest. The pink or mauve colonies on the medium led the laboratory staff to mostly think of *Salmonella* colonies. This was almost always true, but when they were processed in the automated system and MALDI–TOF, some of them were detected to be *Aeromonas* species. When analyzed carefully those colonies had prominent differences from classically detected *Salmonella* colonies. Those little differences enabled the laboratory technician and physician to suspect and isolate *Aeromonas* species. The *Aeromonas* pink pigment diffused into the medium, creating a wide, pink cloudy appearance around the colonies. However, *Salmonella* species do not have as

wide a pigment diffusion as *Aeromonas* species colonies. They were pink colonies with smooth surroundings. The difference in colony appearance between *Salmonella* and *Aeromonas* are shown in Figures 1 and 2, respectively.

The pink pigmentation was most noticeable after 24 h of incubation at 37 °C on the inoculated chromogenic *Salmonella* medium. The appearances of the microorganisms' colonies were therefore evaluated according to their growths after 24 h incubation at 37 °C.

The results for the reference strains (*Aeromonas caviae* ATCC 15468, *Aeromonas hydrophilia* ATCC 7966) were similar to those of the patient sample inoculations of these bacteria. There were no false positives at all using our criteria for differentiating *Aeromonas*. *Aeromonas* suspected colonies were found to be different colors, such as mauve or blue, compared to the classic pink of *Aeromonas* types such as *Aeromonas caviae*. Some of them were mauve-blue pigmented colonies with a halo around them of the same color. Some of them, such as *Aeromonas hydrophilia*, had mucoid colonies diffusing into the media, although they had pink halos with no prominent edges around the colonies.

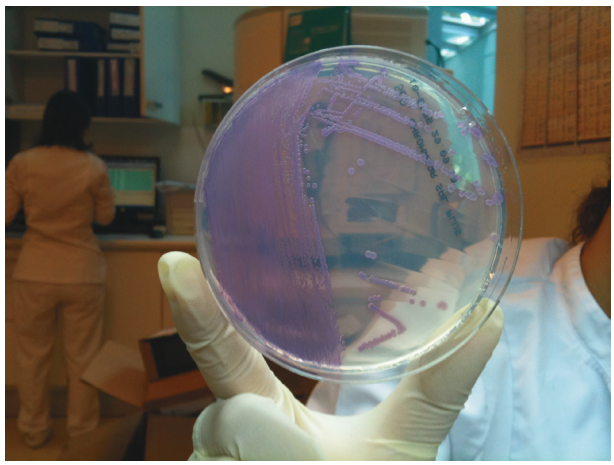

Figure 1. *Salmonella* species in CHROMagar Salmonella Plus.

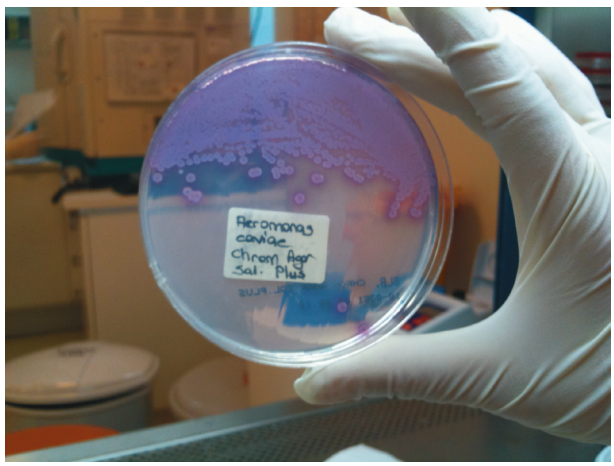

Figure 2. *Aeromonas* species in CHROMagar Salmonella Plus.

We detected 86 *Aeromonas* spp. These included 30 *A. caviae*, 27 *A. hydrophila*, 16 *A. veronii*, 11 *A. sobria*, and 2 *A. salmonicida*. Typically, more than 300 peaks, mainly between 2000 and 25,000 Da, were detected, and for *Aeromonas* subspecies-specific protein peaks were detectable (Figure 3).

For both of the organisms, while there were no difference at species identification level between MALDI–TOF and the Phoenix automated system, some differences were detected at subspecies level. *Aeromonas* subtyping with MALDI–TOF was completely compatible with the Phoenix automated system.

4. Discussion

Aeromonas can be the agent of infections and septicemia. It is a rarely detected pathogen of traveler's diarrhea. Although most cases of *Aeromonas*-associated gastroenteritis are self-limited, antimicrobial therapy is often indicated (11). The MacConkey plate has been the most widely used medium in the isolation and differentiation of coliform organisms and enteric pathogens. Definitive identification of the oxidase-positive, glucose-fermenting, gram-negative rods of aeromonads is accomplished with a small number of biochemical tests and antimicrobial markers.

In routine laboratory workflow, *Aeromonas* detection is not always so successful or practical. Tests for identification of *Aeromonas* species from routine media are time-consuming. Special media such as CIN agar is not always used in routine stool cultures in every laboratory. Therefore, some of the *Aeromonas* species may not be detected.

Recently, MALDI–TOF MS has been used more often in the diagnosis of bacteria and fungus. It has been preferred because it is reliable, rapid, and cost-effective (12,13).

Böhme et al. used MALDI–TOF MS successfully in the identification of the main 26 species of seafood spoilage and pathogenic gram-negative bacteria, including *Aeromonas hydrophila*, *Acinetobacter baumannii*, *Pseudomonas* spp., and *Enterobacter* spp. among others. Thus, the proteomic approach has been demonstrated to be a competent tool for species identification (14). Recently, the extraction process accuracy of MALDI–TOF MS was evaluated in identifying aeromonads. In this study, genus-level accuracy was detected as 100% in comparison with rpoB gene sequencing, which makes this system one of the most accurate and rapid methods for phenotypic identification (15).

In our laboratory workflow we use chromogenic *Salmonella* medium for the detection of *Salmonella* species.

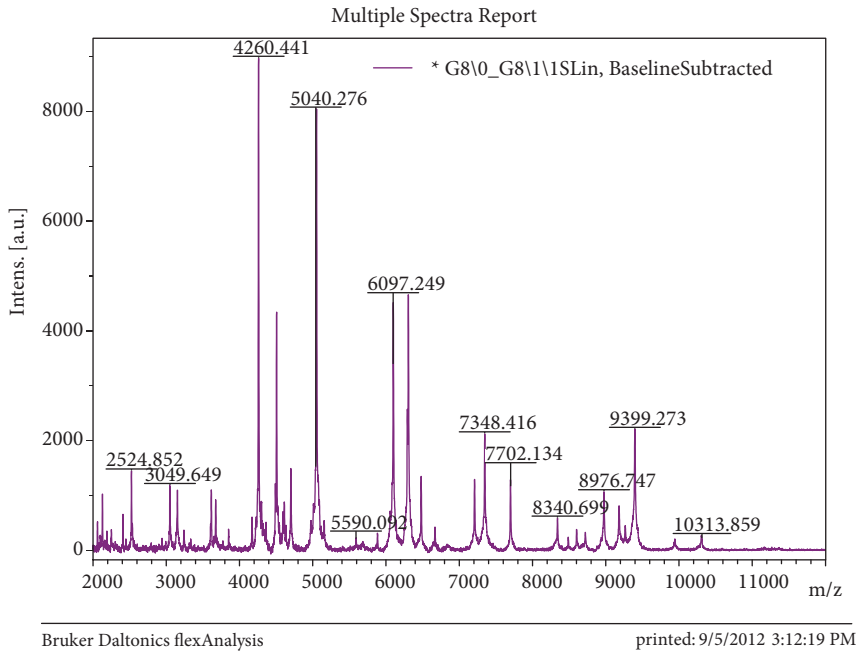

Figure 3. Fingerprint image of *Aeromonas veronii* in MALDI-TOF MS.

It is a time saving, cost-effective, and specific medium for *Salmonella* species. On this medium the typical appearances of *Salmonella* (including *S. typhi*, *S.* Paratyphi A, and lactose-positive *Salmonella*) are mauve. When we examined our routine stool cultures we noticed that some of those pink or mauve colonies that were suspected to be *Salmonella* were something other than *Salmonella* (16). They had the small difference of having diffuse pigmentation throughout the chromogenic *Salmonella* agar plate. We identified these organisms, using an automated system, as *Aeromonas* species. For comparison we also inoculated those microorganisms onto other conventional media used for the identification of *Aeromonas* species, used a reference strain to see the reactions on all of the media, and performed biochemical tests.

As a result, we have observed and isolated 86 *Aeromonas* species in our central laboratory in a 5-year period. With a MALDI–TOF MS device it is much easier and more rapid to detect *Aeromonas* species.

We did not perform any process other than this routine, and we are now also detecting *Aeromonas* species with this system, without any effort or other special media. We do not require any other biochemical reactions and thus can work without any delay. It is practical to observe *Aeromonas* species on routinely used chromogenic *Salmonella* medium along with *Salmonella* species without using any other medium or method. This may enable the identification of *Aeromonas* species in stool samples without any extra cost, time, or effort.

We strongly suggest this medium for the detection of *Aeromonas* species, including subspecies detection, while performing routine *Salmonella* species detection.

It is cost-effective, necessitating only chromogenic media and observation, and provides accurate identification in 24 h, or in other words 1 or 2 days earlier. Reliable and practical diagnosis can be achieved by the help of the MALDI–TOF MS method.

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