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High-throughput discrimination of bacteria isolated from *Astacus astacus* and *A. leptodactylus*

N. Topić Popović[(1)], R. Sauerborn Klobučar[(1)], I. Maguire[(2)], I. Strunjak-Perović[(1),*], S. Kazazić[(3)], J. Barišić[(1)], M. Jadan[(1)], G. Klobučar[(2)], R. Čož-Rakovac[(1)]

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ABSTRACT

Key-words: *crayfish, bacteria, MALDI-TOF MS, API 20E*

Bacterial diseases and pathogens of crayfish are common, widespread, and occasionally causing serious mortalities. In order to take rapid measures for correct treatment of crayfish diseases, the turnover time and accuracy in bacterial identification is an issue. Bacteria isolated from tissues of apparently healthy *Astacus astacus* and *A. leptodactylus* were identified by the commercial phenotypic tests (API 20E) and by the matrix assisted laser induced desorption ionization connected to the time of flight mass spectrometry (MALDI-TOF MS). For Gram-negative rods, API 20E resulted in fewer species identifications than MALDI-TOF MS (5.2% versus 52.61%). The most frequently identified genus from *A. astacus* and *A. leptodactylus* was *Pseudomonas* spp.: API 20E (47.82%) and MALDI-TOF MS (52.17%). Both systems identified 60.86% of total isolates identically to the genus. *Hafnia alvei* was the only isolate for which API 20E and MALDI-TOF MS had a concordant reading to the species. MALDI-TOF MS proved to be a powerful, low-cost, rapid tool in bacterial genus identification. This is the first report of a direct comparison between the two systems for the identification of bacteria in crayfish, and also the first report on using MALDI-TOF MS for discrimination of freshwater crayfish bacterial isolates.

RÉSUMÉ

Discrimination haut-débit de bactéries isolées à partir d'*Astacus astacus* et *A. leptodactylus*

Mots-clés : *écrevisse, bactérie, MALDI-TOF MS, API 20E*

Les maladies bactériennes et les agents pathogènes des écrevisses sont communs, répandus, et de temps en temps entraînent des mortalités importantes. Afin de prendre des mesures rapides pour le traitement correct des maladies d'écrevisses, le temps nécessaire et l'exactitude dans l'identification bactérienne est une question. Des bactéries isolées dans des tissus *d'Astacus astacus* et d'*A. leptodactylus* apparemment en bonne santé ont été identifiées par les tests phénotypiques commerciaux (API 20E) et par spectromètre de masse couplant une source d'ionisation laser assistée par une matrice et un analyseur à temps de vol (MALDI-TOF MS). Pour les bâtonnets Gram-négatifs, API 20E a donné moins d'identifications d'espèces que MALDI-TOF MS (5,2 % contre 52,61 %). Le genre le plus souvent identifié à partir d'*A. astacus* et *A. leptodactylus* était *Pseudomonas spp* : API 20E (47,82 %) et MALDI- TOF MS (52,17 %). Les deux systèmes

(1) Laboratory for Ichthyopathology-Biological Materials, Ruđer Bošković Institute, Zagreb, Croatia
(2) Department of Zoology, Faculty of Science, University of Zagreb, Zagreb, Croatia
(3) Laboratory for Chemical Kinetics and Atmospheric Chemistry, Ruđer Bošković Institute, Zagreb, Croatia
* Corresponding author: strunjak@irb.hr

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ont identifié 60,86 % des isolats totaux au même genre. *Hafnia alvei* était le seul isolat dont API 20E et MALDI-TOF MS ont une lecture concordante à l'espèce. MALDI-TOF MS s'est avéré être un outil rapide, puissant, à faible coût, pour l'identification au niveau du genre bactérien. Ce travail est le premier d'une comparaison directe entre les deux systèmes pour l'identification des bactéries chez les écrevisses, et aussi le premier travail sur l'utilisation de MALDI-TOF MS pour la discrimination des isolats bactériens d'écrevisses d'eau douce.

INTRODUCTION

There are two genera and five species from the family Astacidae inhabiting the Eurasian continent (Holdich *et al*., 2006). *Astacus astacus* (the noble crayfish) is nowadays distributed over the eastern, central, and northern parts of Europe, while *A. leptodactylus* (the narrow-clawed or Turkish crayfish) inhabits Eastern Europe and Western Asia, and is spreading naturally westwards through waterways (Holdich *et al*., 2006). In Croatia, *A. astacus* is distributed in the continental region, forming both river and lake populations, while *A. leptodactylus* is found in the rivers of eastern and central Croatia with tendency of spreading west- and southwards (Maguire and Gottstein-Matočec, 2004; Maguire *et al*., 2011). Populations of noble crayfish are considered rare; the species is designated as vulnerable and listed in the Bern Convention, EU Habitat Directive and IUCN Red List of Threatened Species (Edsman *et al*., 2010). Noble crayfish is also treated as endangered in Croatia and is protected by Croatian law (Anonymous, 2005, 2008, 2009).

Introduction of non-native American crayfish species into Europe has been responsible for the transfer of the devastating disease crayfish plague caused by *Aphanomyces astaci*, which led to mass mortalities of native crayfish species (Diéguez-Uribeondo, 2006). Due to its dramatic impact onto native European crayfish species, *A. astaci* was extensively studied from different aspects (description, characterization, diagnostics, genotypization, virulence, *etc.*) (Diéguez-Uribeondo, 2006; Makkonen, 2013). However, bacterial diseases and bacterial pathogens of crayfish have not been considered to such an extent, albeit common and widespread. Typically, bacteria isolated from crayfish include both Gram-negative and Gram-positive species, as representatives of the genera *Acinetobacter, Aeromonas, Bacillus, Citrobacter, Corynebacterium, Flavobacterium, Micrococcus, Pseudomonas, Staphylococcus* and *Vibrio* (Smith and Söderhäll, 1986; Vey, 1986; Alderman and Polglase, 1988; Edgerton *et al*., 2002; Romero and Jiménez, 2002; Quaglio *et al*., 2006a, 2006b; Jiravanichpaisal *et al*., 2009; Longshaw, 2011; Mickeniene and Šyvokiene, 2011). Bacterial infections leading to mortalities have been documented in both farmed and wild crayfish, and were also reported in asymptomatic animals (Edgerton *et al*., 2002; Quaglio *et al*., 2006a, 2006b; Cooper *et al*., 2007; Jiravanichpaisal *et al*., 2009; Johnson and Paull, 2011; Longshaw, 2011; Longshaw *et al*., 2012). Mostly, bacteria found in freshwater crayfish inhabit the ecosystem in which they live, may be found in water and sediments, and they reside on the exoskeleton, gills or in the gut. Bacteriological investigations of crayfish have predominantly been performed on their haemolymph using standard microbiological methods, and also by histopathological examinations of tissues (Colwell *et al*., 1975; Johnson, 1976; Scott and Thune, 1986; Madetoja and Jussila, 1996; Edgerton and Owens, 1999; Edgerton *et al*., 2002; Romero and Jiménez, 2002; Quaglio *et al*., 2006b; Jiravanichpaisal *et al*., 2009). When performing health status evaluations, considering correct identification and treatment of bacterial diseases and conditions, of (primarily) farmed crayfish, speed is always an issue. Rapid identification of environmental bacteria via commercial phenotypic tests allows for a wide choice of tests selection, and API 20E (Biomerieux, Marcy l'Etoile, France), an identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods developed for clinical specimens, seems to be increasingly used for the identification of aquatic pathogens (Topić Popović *et al*., 2007; Sanjuán *et al*., 2009; Bastardo *et al*., 2012; Esteve *et al*., 2012; Soto *et al*., 2012). However, due to several shortcomings of this system, such as the wrong identifications and the need

of comparison with the diagnostic schemes based on reactions in conventional phenotypic tests, more advanced methods for identification are sought after. Therefore, in addition to morphological, biochemical microbiological testing analysis, along with the molecular identification, the matrix assisted laser induced desorption ionization (MALDI) connected to the time of flight (TOF) mass spectrometry (MS) channel, is now becoming a third diagnostic pillar with strong discriminating power (Risch *et al*., 2010). Its value is in a rapid screening of the organism and the accessible protein pattern for characterization and distinction (Petersen *et al*., 2009). MALDI-TOF MS can examine the pattern of proteins detected directly from intact bacteria, giving a reproducible spectra consisting of a series of peaks corresponding to mass-to-charge ratios of ions released from bacterial proteins during laser desorption (Dupont *et al*., 2010). MALDI-TOF MS is considered a tool with potential to replace phenotypic identification of bacteria in clinical microbiology laboratories (Bizzini *et al*., 2010; Ford and Burnham, 2013; Jamal *et al*., 2013; Kok *et al.*, 2013), especially due to its time-saving benefit where the extensive time needed with culture-based methods is reduced to a few minutes.

In this study, we isolated bacteria from various tissues of apparently healthy *Astacus astacus* and *A. leptodactylus* and compared the performances of the API 20E panels to the Bruker Biotyper MALDI-TOF MS (Bruker Daltonics, Billerica, MA) for the identification of bacterial isolates. This is the first report of a direct comparison between the two systems for the identification of bacteria in crayfish, and also the first report on using MALDI-TOF MS for the discrimination of the freshwater crayfish bacterial isolates.

MATERIALS AND METHODS

> ANIMALS, SAMPLING, AND TISSUE PROCESSING

The study was carried out in spring 2013, on 10 specimens of *Astacus astacus* (mean weight 43.63 g) and 10 specimens of *A. leptodactylus* (mean weight 55.62 g) of both sexes, all apparently healthy. All crayfish were cage-exposed in the gravel pit Jagodno in vicinity of Zagreb, Croatia. Specimens were randomly sampled, transported live to the laboratory and within few hours sacrificed by overdose of tricaine methane-sulfonate (MS-222, Sigma, St. Louis, Missouri, USA). Necropsy was performed immediately and tissues (gills, hepatopancreas, gonads, gut) were fixed in 4% neutral buffered formalin, dehydrated through a graded ethanol-xylene series and embedded in paraplast. Sagital and transverse sections (3–5 μm) were stained with hematoxylin/eosin (H&E). Microphotographs were taken with a digital camera DP 70 Olympus® connected to an Olympus® BX51 binocular microscope, and transferred to Microsoft® AnalySIS Soft Imaging System for interpretation.

Samples of scrapings of exoskeleton, mouth region, gills, stomach, hepatopancreas, and intestine were streaked onto Tryptone Soya Agar (TSA, CM0131 Oxoid Ltd, England, UK). The plates were incubated at 22 °C for 48–72 h. Representative colonies were isolated and re-streaked on fresh medium until purity was attained. Growth of colonies was ascertained by visual inspection. Pure colonies were Gram-stained and subjected to morphological, physiological and biochemical tests. The taxonomic position of the isolates was determined by API 20E panels and Bruker Biotyper MALDI-TOF MS.

> API 20E (BIOMERIEUX, MARCY L'ETOILE, FRANCE)

The API 20E tests were performed according to the manufacturer's instructions with a few alterations in order to adapt the system to the bacteria of freshwater crayfish: the incubation time was increased to 48–72 h; the incubation temperature was lowered to 22 °C; only the fermentation of sugars was allowed by sealing the cups with sterile mineral oil in the carbohydrate tests. The API 20E uses 21 standardized and miniaturized biochemical tests and a database. It consists of 21 microtubes containing dehydrated substrates. These tubes were inoculated with a bacterial suspension, which reconstituted the media. During incubation,

metabolism produced color changes that were either spontaneous or revealed by the addition of reagents. The reactions were read according to the table provided and the identification was obtained using the software provided by the manufacturer, the Apiweb. A seven-digit profile was obtained for every tested isolate. API 20E ratings were based on three parameters, including the likelihood of a match between the unknown organism's profile and the computer profile, the relative value between the likelihood of the first and the likelihood of the second choices, and the number of tests against the first choice (Brown and Leff, 1996; Topić Popović *et al*., 2007).

> BRUKER BIOTYPER MALDI-TOF (BRUKER DALTONICS, BILLERICA, MA)

Bacterial isolates (one loopful of each bacterial culture) were applied as a thin film to a 24-spot steel plate (Bruker Daltonics) in two replicates and allowed to visibly dry at room temperature (referred to as the direct colony technique). Subsequently, 2 μL of MALDI matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) was applied to the colony and dried in a fume hood. The analysis was performed in a manner that ions generated with a 337-nm nitrogen laser were captured in the positive linear mode in a mass range of 2 to 20 kDa. Positive ions were extracted with an accelerating voltage of 20 kV in linear mode. Each spectrum was the sum of the ions obtained from 200 laser shots performed in five different regions of the same well. Captured spectra were analyzed using MALDI Biotyper automation control and Bruker Biotyper 2.0 software (Bruker Daltonics). The MALDI Biotyper database contained 3 740 spectra from 319 genera and 1 946 species. For each 24-spot plate, a standard (bacterial test standard; Bruker Daltonics) was included to calibrate the instrument and validate the run. Identification criteria used were as follows: a score of 2.300 to 3.000 indicated highly probable species level identification, a score of 2.000 to 2.299 indicated secure genus identification with probable species identification, a score 1.700 to 1.999 indicated probable identification to the genus level, and a score of $<$1.700 was considered to be unreliable. The data obtained with the two replicates were added to minimize any random effect. The presence or absence of peaks was considered as fingerprints for a particular isolate. Identification of isolates corresponded to the species of the reference strain with the best match in the database.

RESULTS

The external gross signs and necropsy findings from the crayfish did not indicate to any manifest disease. Relevant bacteria were recovered from most tissues under examination. The majority of isolates were retrieved from gills (34.78%), stomach (21.74%), and mouth region (17.39%), while less from other tissues: intestine (8.7%), hepatopancreas (8.7%), and exoskeleton (8.7%). Of 23 relevant isolates, only one matched completely in both API 20E and MALDI-TOF MS readings (*Hafnia alvei*). Also, there were two unreliable identifications by MALDI-TOF MS for isolates which API 20E identified with "Good identification to the genus" as *Pseudomonas aeruginosa*. In comparison, API 20E assigned 7 isolates (30.43%) to "Unacceptable", "Doubtful", or "Low discrimination" profiles, which was expected for 3 isolates, being Gram-positive rods and identified with MALDI-TOF MS as *Bacillus cereus* with "Secure genus identification with probable species identification" (Figure 1). For Gram-negative rods, the conventional method resulted in significantly fewer species identifications than MALDI-TOF MS (5.2% versus 52.61%). Detailed comparison of identification results between API 20E and MALDI-TOF MS is presented in Table I. Overall, the most frequently identified genus from both *A. astacus* and *A. leptodactylus* was *Pseudomonas* spp.: with API 20E (47.82%) and with MALDI-TOF MS (52.17%), while both systems allocated *Pseudomonas* spp. identification for the respective isolates in 39.13% of total cases. Both systems identified 60.86% of total isolates identically to the genus. The mean time to identification with API 20E was 48 h,

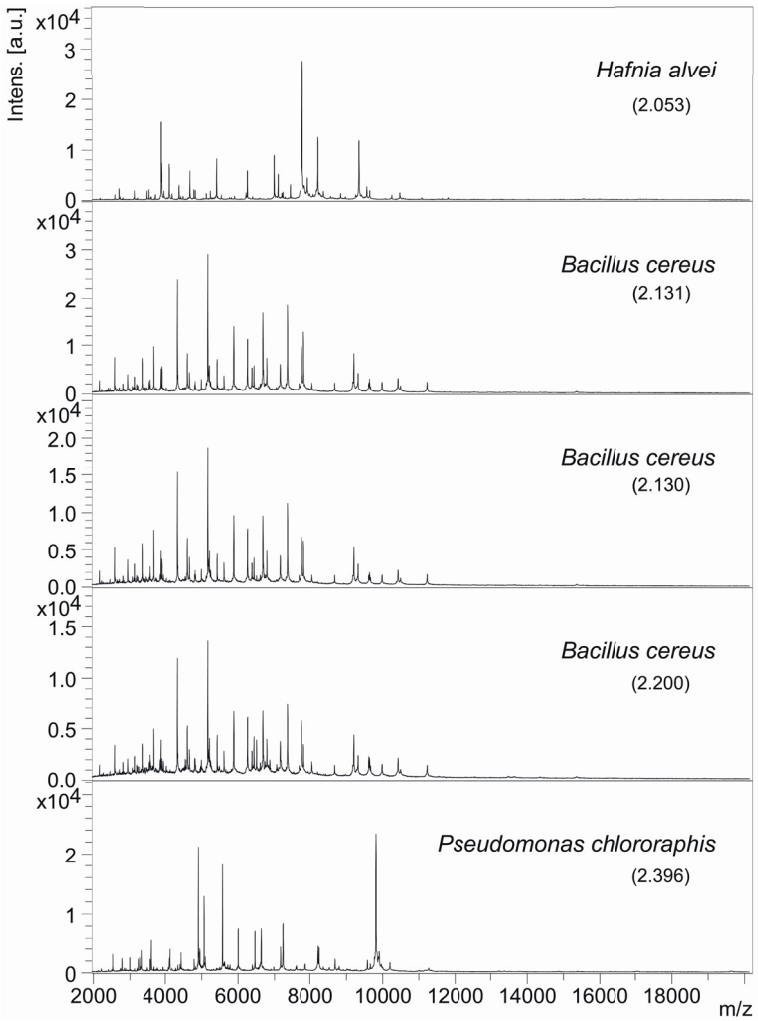

Figure 1
MALDI-TOF MS spectral profiles of bacterial isolates: Hafnia alvei isolated from hepatopancreas of A. leptodactylus with "Highly probable species identification"; 3 isolates of Bacillus cereus from intestine, hepatopancreas and gills of A. leptodactylus, all within the category "Highly probable species identification"; Pseudomonas chlororaphis isolated from gills and mouth region of A. astacus, both isolates here presented as the identical spectral profile with "Highly probable species identification".

whereas MALDI-TOF MS needed less than 10 minutes per bacterial isolate. Histopathological findings of the hepatopancreas (Figure 2) indicated to its vacuolization as well as nodular formations in haemal sinus. Tissue sections of gill lamellae (Figure 3) showed epithelial wall lifting and presence of dead cells.

DISCUSSION

Bacteria isolated from tissues of apparently healthy *Astacus astacus* and *A. leptodactylus* were identified by the API 20E panels and the MALDI-TOF MS, and the two systems were compared for their usefulness for identification of bacteria in crayfish. The most prevalent genus identified by both API 20E and MALDI-TOF MS was *Pseudomonas*. Indeed, *Pseudomonas* spp. is one of the most frequently isolated Gram-negative bacteria from crayfish (Scott and Thune, 1986; Edgerton *et al*., 2002; Mickeniene and Šyvokiene, 2011). Neither *A. astacus* nor *A. leptodactylus* under this survey demonstrated any of the previously described signs of *Pseudomonas*-related bacterial septicemia (Edgerton *et al*., 2002)

Table I
Comparison of identification results between API 20E and MALDI-TOF MS for isolates from Astacus astacus and A. leptodactylus.

API 20E (result)*	MALDI-TOF MS (score)**	Comment
***Astacus astacus* isolates**		
Pseudomonas fluorescens/putida **(Excellent identification to the genus)**	*Pseudomonas koreensis* (1.913)	Bacterial colonies (cream in color) lacked fluorescing properties. *Ps. koreensis* is an unlikely isolate in central Croatia.
Aeromonas hydrophila **group 2 (Very good identification to the genus)**	*Aeromonas eucrenophila* (2.165)	*A. eucrenophila* is found in fresh waters and has been isolated from fish and crayfish.
Pseudomonas aeruginosa **(Very good identification to the genus)**	*Pseudomonas koreensis* (2.067)	Cream-colored bacterial colonies with yellow diffusing pigment. *Ps. koreensis* is an unlikely isolate in central Croatia.
Pseudomonas aeruginosa **(Very good identification to the genus)**	*Pseudomonas cedrina* (1.914)	Matching identification to the genus. *Ps. cedrina* belongs to the *Ps. fluorescens* group.
Pseudomonas aeruginosa **(Good identification to the genus)**	Not reliable identification (1.683)	Not reliable id. after multiple measurements.
Pseudomonas aeruginosa **(Good identification to the genus)**	Not reliable identification (1.683)	As above.
Pseudomonas aeruginosa **(Good identification)**	*Pseudomonas chlororaphis* (2.396)	Orange-colored colonies possibly associated with *Ps. chlororaphis* subsp. *auranthiaca* or *aureofaciens.*
Pseudomonas aeruginosa **(Good identification)**	*Pseudomonas chlororaphis* (2.396)	As above.
Shewanella putrefaciens **(Good identification)**	*Shewanella baltica* (1.766)	*Sh. putrefaciens* has been isolated from marine environments, *Sh. baltica* is found mainly in waters of the Baltic Sea.
Brucella **spp. (Low discrimination)**	*Pseudomonas thivervalensis* (2.045)	*Ps. thivervalensis* is a soil bacterium.
Ochrobactrum anthropi **(Low discrimination)**	*Pseudomonas frederiksbergensis* (2.272)	Secure genus identification (*Pseudomonas*) with less probable species identification.
Pantoea **spp. (Low discrimination)**	*Arthrobacter aurescens* (2.180)	Gram-positive rods, yellow-colored colonies. Arthrobacteria are commonly found in soil.
Aeromonas hydrophila **group 1 (Unacceptable profile)**	*Pseudomonas proteolytica* (2.080)	Secure genus identification (*Pseudomonas*). *Ps. proteolytica* is a psychrophilic bacterium not likely to be found in central Croatia.

Table I
Continued.

API 20E (result)*	MALDI-TOF MS (score)**	Comment
***Astacus leptodactylus* isolates**		
Hafnia alvei **(Excellent identification)**	*Hafnia alvei* (2.053)	Previously isolated from freshwater crayfish (Longshaw *et al*., 2012). Opportunistic pathogen of freshwater fish (Austin and Austin, 1999).
Pseudomonas aeruginosa **(Excellent identification to the genus)**	*Pseudomonas koreensis* (2.084)	Matching identification to the genus. *Ps. koreensis* is an unlikely isolate in central Croatia.
Pseudomonas aeruginosa **(Excellent identification to the genus)**	*Pseudomonas koreensis* (1.967)	As above. *Pseudomonas aeruginosa* has zoonotic potential (Austin and Austin, 1999).
Aeromonas hydrophila **group 1 (Very good identification to the genus)**	*Aeromonas bestiarum* (2.193)	*A. bestiarum* and *A. hydrophila* stand in the same phenogroup, described as the *A. hydrophila* complex (Martino *et al*., 2011).
Pseudomonas fluorescens/putida **(Good identification to the genus)**	*Pseudomonas kilonensis* (1.915)	Matching identification to the genus.
Shewanella putrefaciens **(Good identification)**	*Shewanella baltica* (1.947)	*Sh. putrefaciens* has been isolated from marine environments, *Sh. baltica* is found mainly in waters of the Baltic Sea.
Pseudomonas aeruginosa **(Doubtful profile)**	*Pseudomonas kilonensis* (1.823)	Matching identification to the genus.
Burkholderia cepacia **(Low discrimination)**	*Bacillus cereus* (2.131)	Gram-positive rods.
Burkholderia cepacia **(Low discrimination)**	*Bacillus cereus* (2.130)	Gram-positive rods.
Burkholderia cepacia **(Low discrimination)**	*Bacillus cereus* (2.200)	Gram-positive rods.

* API 20E result: excellent identification (% id ⩾ 99.9 and T index ⩾ 0.75); very good identification (% id ⩾ 99.0 and T index ⩾ 0.50); good identification (% id ⩾ 99.9 and T index ⩾ 0.25); acceptable identification (% id ⩾ 80.0 and T index ⩾ 0). Identification to the taxon: one single taxon has been selected; identification to the genus level: 2, 3 or 4 taxa belonging to the same genus have been selected; Low discrimination: 2, 3 or 4 taxa belonging to different genera have been selected; The identification is “not reliable” if the sum of the % id proposed is less than 80.0; The profile is doubtful if a taxon having several tests against the identification is present among those proposed; the profile is unacceptable if the number of choices proposed is 0, all the gross frequencies being less than the threshold value.
** MALDI-TOF MS score: 2.300 to 3.000: highly probable species identification; 2.000 to 2.299: secure genus identification with probable species identification; 1.700 to 1.999: probable identification to the genus level; <1.700: not reliable identification.

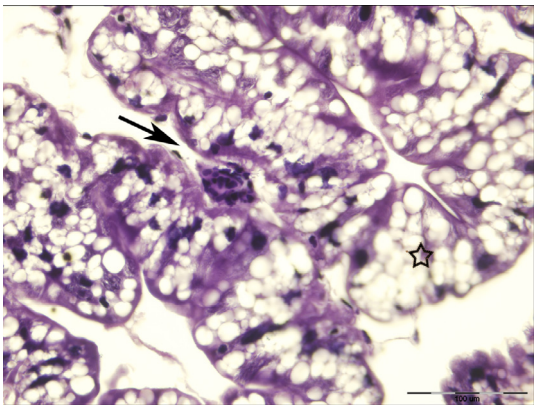

Figure 2
Histopathological sections of A. astacus hepatopancreas showing nodular formations in haemal sinus, consisting of agglomerated phagocytic cells surrounding or ingesting bacterial cells (arrow). Also present is thinning of the cell membrane leading to major vacuolization of hepatopancreas (asterisk). H&E, Scale bar 100 μm.

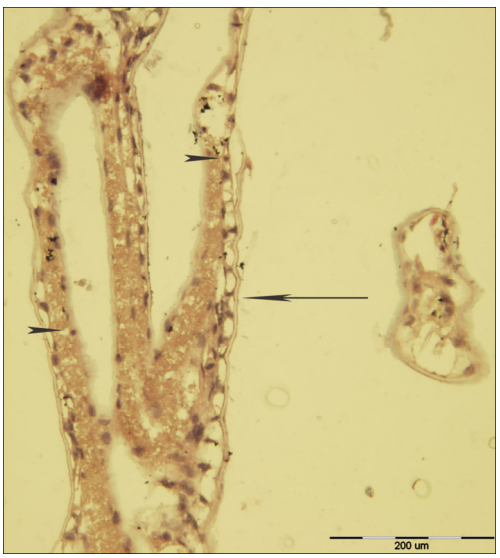

Figure 3
Tissue sections of A. leptodactylus gill lamellae showing epithelial wall lifting (arrow) and presence of dead cells with pyknotic nuclei in the haemal canal (arrow tips). H&E, Scale bar 200 μm.

in form of the presence of gross clinical signs (lethargy, reduced response to stimuli, postural abnormalities). Except for several nodular formations in haemal sinus of hepatopancreas (agglomerated phagocytic cells surrounding or ingesting bacterial cells), asymptomatic septicemic cases were not diagnosed through histopathological examination (absence of lesions or granulomas). Like other bacteria ubiquitous in the freshwater environment (Longshaw, 2011), Pseudomonads can be isolated from apparently healthy crayfish, and are considered to have the potential to cause problems under stress or culture conditions. Interestingly, although both identification systems identified almost half of the isolates as Pseudomonads (MALDI-TOF MS 52.17%, API 20E 47.82%), only 39.13% of isolates had concordant identification to the genus level with both MALDI-TOF MS and API 20E. MALDI-TOF MS scored highly for *Pseudomonas* spp. in 13% of isolates which API platform could not discriminate. Since *Ps. aeruginosa* is easily identified as the species by MALDI-TOF MS (van Veen *et al*., 2010), and MALDI-TOF MS did not identify *Ps. aeruginosa* in this work, 39% of isolates recognized as *Ps. aeruginosa* with the API 20E (with more or less discrimination) can be dismissed as such and attributed only to the genus.

It has been demonstrated that classical phenotypic methods can frequently misidentify non-fermenting bacteria (*Pseudomonas* included), and for this class of bacteria molecular tools such as 16S rRNA gene sequencing provide reliable results, but less accurate at the species level (Campos Braga *et al*., 2013). Therefore, a reference database for MALDI-TOF MS based on the identification of non-fermenters was established (Mellmann *et al*., 2009; Campos Braga *et al*., 2013). MALDI-TOF MS was shown to identify correctly to the species level a number of Pseudomonads, and outperform 16S rRNA sequencing at identifying members of both *Pseudomonas* and *Bacillus* genera (Böhme *et al*., 2013). In this work it identified *B. cereus* "Securely to the genus and probably to the species" with a high score.

MALDI-TOF MS exceeded API 20E in species identification of Gram-negative rods. In this work, *Hafnia alvei* was the only isolate for which both API 20E and MALDI-TOF MS had a concordant reading to the species level. Interestingly, although API 20E gave "Excellent identification" for the profile 5305112 of the strip, that very profile was previously described for the reference culture of *Yersinia ruckeri* (Austin *et al*., 2003; Topić Popović *et al*., 2007). MALDI-TOF MS however, identified it "Securely to the genus and probably to the species" and therefore confirmed the API result. *H. alvei* has previously been isolated from freshwater crayfish (Longshaw *et al*., 2012), although its disease-causing properties in crayfish have not been described.

The disparity and problems in *Aeromonas* spp. identification (*A. hydrophila* group 1 (API 20E) versus *A. bestiarum* (MALDI-TOF MS)) can be attributed to close relatedness of the two species, which according to Martino *et al*. (2011) belong to the same phenogroup, described as the *A. hydrophila* complex. The current taxonomic database of the MALDI-TOF MS Biotyper system recognizes species that are currently of different taxonomic status and have not been updated in the Apiweb system (Kierzkowska *et al*., 2013). The genus *Aeromonas* comprises 21 validly proposed species, and some of them are phenotypically very similar. MALDI-TOF MS can however provide their good separation at the genospecies level comparable with the phylogenetic tree obtained by *gyrB* gene sequencing; it categorized in clusters well differentiated *A. bestiarum* and *A. hydrophila* (Benagli *et al.*, 2012). Genus-level accuracy of clinical and environmental *Aeromonas* isolates identified by MALDI-TOF MS in the work of Lamy *et al*. (2011) was 100%, while species-level accuracy reached 90.6%, making this system one of the most accurate and rapid methods for phenotypic identification of Aeromonads, albeit with the need of improvements in its database composition, taxonomy and discriminatory power (Lamy *et al.*, 2011).

When comparing the performance of MALDI-TOF MS with conventional and API systems for clinical isolates of human material, the percentage of correct identifications is significantly higher than in this work, mainly due to the customized databases (Bizzini *et al*., 2010; Dupont *et al*., 2010; Risch *et al*., 2010; Martiny *et al*., 2011; Saffert *et al*., 2011; El-Bouri *et al.*, 2012; Nagy *et al*., 2012; Campos Braga *et al*., 2013; Kierzkowska *et al*., 2013). For example, 97.2% of isolates had identical genus identification by both methods, while 79.9% yielded exactly the same species identification in the work of van Veen *et al*. (2010), and conventional methods also resulted in fewer species identification (83.1% versus 92% MALDI-TOF MS). The databases of the both identification systems used in this work (MALDI-TOF MS and API 20E) are not comprehensive for environmental isolates, and therefore most discordant results were due to the systematic database-related taxonomical differences. Obviously, the quality and reliability of the identification by MALDI-TOF MS depends on the quality and amount of reference spectra present in the database (Seng *et al*., 2009; Calderaro *et al.*, 2013).

The disparities on the species level between the two systems which identically identified the isolates on the genus level are not necessarily of the major concern if one looks solely into rapid screening of crayfish bacterial flora with the purpose to get insight into the health status of apparently healthy animals. However, diagnostics of bacterial diseases in crayfish require a completely different approach, and necessitate precision. Generally, diagnostic methods based on phenotypic analysis are less frequently used, and molecular methods dominate over traditional techniques, the golden standard being the 16S rRNA gene sequencing. Nevertheless, the high cost of this assessment makes this technique impossible

to use in routine microbiology diagnostics (Kierzkowska *et al*., 2013) and the next step is the mass spectrometry-assisted identification. MALDI-TOF MS has demonstrated to be a competent bacterial typing tool that extends phenotypic and genotypic approaches, allowing a more ample classification of bacterial strains (Böhme *et al*., 2013). It seems to be a powerful, low-cost, rapid proteomic tool in bacterial genus (and frequently species) identification from freshwater crayfish, however we do suggest combining it with classical microbiological methods, despite their drawbacks such as time-consuming reactions and sometimes subjective morphological observations requiring experience, at least until we benefit from the MALDI-TOF MS database extension. That done, rapid and accurate identification of crayfish pathogens with MALDI-TOF MS will significantly improve the bacterial disease recognition, immediate therapy approach, and enhance the outcomes of farmed crayfish populations, with a single direct colony testing.

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