Asian J. Med. Biol. Res. 2017, 3 (1), 44-51; doi: 10.3329/ajmbr.v3i1.32035

Asian Journal of Medical and Biological Research
ISSN 2411-4472 (Print) 2412-5571 (Online)
www.ebupress.com/journal/ajmbr

Article

Morphological and biochemical characterization of two fish pathogenic bacteria: *Aeromonas salmonicida* and *Yersinia ruckeri* for rapid diagnosis of fish disease

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Received: 12 February 2017/Accepted: 14 March 2017/ Published: 30 March 2017

Abstract: *Aeromonas salmonicida* and *Yersinia ruckeri* are two common pathogenic fish bacteria responsible for furunculosis and Enteric Red Mouth disease (ERM), respectively. For the characterization of these two pathogens, a series of morphological (pigmentation, hemolyse, motility and body shape), biochemical tests (Gram staining, catalase, oxidase and API 20E strips) and a soft ionization technique (MALDI-TOF/MS) were performed in the laboratory. Pigmentation, motility, hemolysis and body shape was used as a preliminary identification of the bacteria. Both of the species were identified with the entire biochemical test without any doubt except API20E strips test. Although the API profile of *A. salmonicida* (0006104) was identified with high confidence (99.6%), but *Y. ruckeri* was misidentified as *Vibrio mimicus*. There are strong supports against *Vibrio mimicus* as it is a human pathogen, grow at a temperature more than 20^{o}C, motile and oxidase positive bacteria. The *Y. ruckeri* is a non-motile fish bacteria and oxidase negative which are consistent with the study results. Agglutination test with Bionor Mono kit was also identified the bacteria as *Y. ruckeri*. For rapid diagnosis of infectious disease, accurate identification of pathogen is very important for commercial aquaculture.

Keywords: bacteria; yersinosis; furunculosis; oxidase; agglutination

1. Introduction

Fish diseases have been identified one of the major problem in aquaculture industry, where outbreaks begin either suddenly or progress rapidly often with high mortalities. Epidemics of bacterial diseases are common in commercial aquaculture. The number of bacterial species associated with fish culture has been increasedsteadily (Austin, 2011). Several of these bacterial pathogens are members of the regular micro flora of water and/or fish. Others have been associated only with clinical diseased or covertly infected (asymptomatic) fish. *Aeromonas salmonicida and Yersinia ruckeri* are two fish pathogenic bacteria responsible for furunculosis and yersiniosis or enteric red mouth (ERM) disease, respectively. Both of the bacteria are responsible for causing disease in freshwater and marine water fishes worldwide (Welch *et al.*, 2011). Initially, *A. salmonicida* was isolated from a hatchery of brown trout (*Salmo trutta*) in Germany (Austin, 2011) and *Y. ruckeri* was from rainbow trout (*Oncorhynchus mykiss*) in the Hagerman Valley, Idaho, USA (Ross *et al.*, 1966). Presently these bacterial species are significant pathogens of cultivated salmonids in both fresh and marine water farms (Austin & Austin, 2007). Moreover, it has been documented that wide varieties of fresh and marine non-salmonids fishes were affected by these two bacterial infections (Austin, 1993; Kaku *et al.*, 1999; Austin and Austin, 2007; Austin, 2011). As fish are reared with high density in aquaculture, disease can spread rapidly with devastating consequences. The Enteric red mouth disease, if untreated, causes approximately 10 to 25% loss of immunity which results in massive economic losses in salmonid aquaculture all over the world (Meier, 1986; Welch *et al.*,

2011). To control the diseases, it is essential to identify the causative agents associated with the infections. Identification of a microbial pathogens isolate usually follows a series of morphological, biochemical, immunological, and molecular techniques. For the identification of *A. salmonicida* and *Y. ruckeri* from affected fish farm, different morphological and biochemical test are practiced in the world wide (Stevenson and Daly, 1982; Wiklund *et al.*, 1993; Gudmundsdottir, 1998; Dalsgaard and Madsen, 2000; Welch *et al.*, 2011). The accurate identification of bacteria would help to take proper steps to control the disease before massive economical loss. The general objective of the study is to rapid identification and characterization of two fish pathogenic bacteria (*A. salmonicida* and *Y. ruckeri*).

2. Materials and Methods

Isolation of fish pathogenic bacteria wascarried out in the laboratory of Technical University of Denmark (DTU). A number of morphological and standard biochemical tests were used for the identification of fish bacteria *A. salmonicida* and *Y. ruckeri*. The tests are described below:

2.1. Culture and dilution of bacteria

Prior to the laboratory analysis, the collected pathological fish samples were cultured in a nutritive marine agar plate at 20° C for 48 hours. While *Y. ruckeri* grows with colorless colonies in the medium, the *A. salmonicida* form brownish colonies in the culture medium (Hanninen and Hirvela-Koski, 1997; Gudmundsdottir, 1998). To see the hemolysis, subculture of the sample were done in a fresh growth medium containing agar, meat and 5% calf blood. The cultured samples were diluted in physiological saline solution (PS) (0.9% [wt/vol] NaCl) (Schmidt *et al.*, 2000) in order to carry out the confirmation test for *A. salmonicida* and *Y. ruckeri.*

2.2. Motility, shape and hemolysis test

Motility pattern and shape of the cultured bacterial samples were performed using an oil immersion microscope. Hemolytic characteristics were observed using blood agar plate in aerobic condition.

2.3. Gram staining

As a primary aid a rapid non-staining potassium hydroxide (KOH) method was used for the identification of the Gram reactions (Buck, 1982). To perform the test, 3% aqueous KOH solution was used. Formation of gel and string within 5-60 seconds with the KOH was recorded gram negative bacteria while no strings with loop identify the Gram positive bacteria.

2.4. Cytochrome oxidase and catalase test

Cytochrome oxidase and catalase are two enzyme-based tests played a crucial part in bacterial identification. Commercially prepared paper disk that contains substrate 1% tetramethyl-p-phenylenediamine dihydrochloride was used for oxidase test. When the enzyme present in bacteria, it oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product. In case of absence of the enzyme, the reagent remains reduced and is colorless. For the catalase test of bacteria, commercially available 3% Hydrogen peroxide (H_2O_2) was used (Versalovic *et al.*, 2011). Catalase positive reactions of the bacteria are evident by immediate formation of gas bubble formation with the H_2O_2. In case of no formation of bubbles was recorded after 20 to 30 seconds, therefore, it was considered as a negative catalase test.

2.5. Agglutination

For the identification of *Y. ruckeri*, Agglutination test was performed for the one sample that did not perform any color in agar culture. For rapid agglutination test of *Y. ruckeri*, a Bionor Mono kit was used, which was developed by a Norwegian diagnostic company (BIONOR AS). These kits use latex particles coated with specific polyvalent sheep antisera against numerous bacterial pathogens such as *Y. ruckeri* (Mono-Y) (Romalde *et al.*, 1995).The Mono-Yr test reagent contains of monodisperse particle coated with antibodies and it forms a granular particle agglutination pattern when mixed with the sample consists *Y. ruckeri*. A clear agglutination within 30 S after mixing the test reagent with the bacteria was considered as positive results and in case of no agglutination the test was considered negative. To obtain maximum reliability, the kit consists a control reagent was used, containing of mono-dispersed particles coated with non-specific protein. *Y. ruckeri* does not give an ordinary agglutination with the control reagent.

2.6. Analytical Profile Index (API) strips test

For rapid diagnosis of fish pathogen, an Analytical Profile Index (API) strips test was performed. The commercial miniaturized API-20E system (BioMerieux) was used for this biochemical test. Suspension of inoculate bacteria was prepared with sterile saline water. After incubation at 20^{o}C for 48 hours, the color change of the strips were compared with the standard API Reading Scale (color chart) and the results (positive or negative) were recorded on a sheet triplets by black triangles for further scoring. The oxidase was performed separately and was recorded on the API test result sheet, which constitutes the 21st test.

2.7. Interpretation of API strips test

The results of the API strip tests were obtained with the numerical profile. On the triplets result sheet, the tests were marked into three groups with a given value of 1, 2 and 4 for each group. The score was added only for the positive tests in each triplet. The possible highest and lowest score for a triplet are 7 and 0, respectively. By adding together the values for the positive reactions within each group, a 7 digit profile number was obtained for the 20 tests of the API 20E strips. The 7-digits numerical profiles were put into a computerized database (apiweb V5.0) for the identification of organisms.

2.8. Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS)

The MALDI-TOF is a rapid, inexpensive and accurate bacterial identification technique compared to conventional phenotypic and molecular techniques (Biswas and Rolain, 2013). This test was performed for the identification of fish pathogenic bacteria. Small colonies of cultured bacteria were placed in a cell of a stainless steel target plate (UV-absorbing MALDI-TOF sample plate). Three cells were used for the each bacterial sample and the cells were marked as A1, A2, A3 for the *A. salmonicida* and A4, A5, A6 for the *Y. ruckeri*. One drop of matrix solution (alpha-cyano-4-hydroxycinnamic acid (HCCA) which absorbs laser energy) was added into the bacterial colonies of the cells and kept it for a few minutes to dry. The prepared plate was then put on a fixed, pulsed laser beam (Bruker Biotyper MALDI Automation Control software) for analyzing the samples. The results were recorded electronically in a database.

3. Results

Primarily, *A. salmonicida* and *Y. ruckeri*were characterized on the basis of colony morphology and biochemical test results (Gram stain, cell morphology, motility, haemolyse, cytochrome oxidase, and catalase activity) (Table 1). Then, API 20E strips (Biomerieux), agglutination (BioNor Mono Yr) and MALDI-TOF/MS test were performed for the confirmation of the experimental bacteria.

3.1. Morphology

After 48 hours of incubation, single brown pigmented colony of *A. salmonicida* and colorless single colony for *Y. ruckeri* were observed in new culture medium. *A. salmonicida* cultured sample in agar medium showed brown pigment. The same bacteria were observed non-motile and coccoid rod shaped. Some of the cells were attached each other and several bacterial cells formed chain. Further, *Y. ruckeri* cultured sample didn't show any color, they are non-motilile and rod shaped cells when observed under oil immersion microscope. Unlike *A. salmonicida*, it did not display any chain formation under microscope. Although from the blood agar medium culture *A. salmonicida* was identified as hemolysis positive, *Y. ruckeri* showed negative reaction.

3.2. Gram staining, oxidase and catalase test

Both of the cultured bacteria showed viscous, slime string with the Gram staining method (3% KOH) (Table 1). It was indicated that the bacteria (*A. salmonicida* and *Y. ruckeri*) were gram negative (Buck, 1982). *A. salmonicida* showed positive reaction with oxidase test as dark purple color was found within 10 seconds. *Y. ruckeri* didn't show any color, indicates that it is negative with oxidase test. Both of the species have shown catalase positive test with 3% H_2O_2.

3.3. Agglutination test

The non-pigmented cultured bacterial sample was showed granular particles when mixed with the Mono-Yr test reagent (Figure 1a). There was no ordinary agglutination found when mixed with the control reagent (Figure 1b). The observed results indicate that the cultured colonies contained *Y. ruckeri* species.

Table 1. Morphological and biochemical characteristics of fish bacteria *A. salmonicida* and *Y. ruckeri.*

Characteristics	*A. salmonicida*	*Y. ruckeri*
Motility	Non-motile	Non-motile
Shape	Coccoid rod	Rod
Gram staining (3% KOH)	Gram negative (-)	Gram negative (-)
Haemolyse	+	-
Pigmentation	+	-
Catalase	+	+
Oxidase (Cytochrome)	+	-
Agglutination		+

+ and – indicate positive and negative reaction, respectively

Table 2. Scores obtained from the API 20E strips test for the study of fish bacteria (*A. salmonicida* and *Y. ruckeri*).

Tests	Active ingredients	*A. salmonicida*	No. of code	Total code	*Y. ruckeri*	No. of codes	Total code
ONPG	2-nitrophenyl-$^{\beta}$D-galactopyranoside	-	0	0	+	1	5
ADH	L-arginine	-	0		-	0	
LDC	L-lysine	-	0		+	4	
ODC	L-ornithine	-	0	0	+	1	3
CIT	Trisodium citrate	-	2		+	2	
H2S	Sodium thiosulfate	-	0		-	0	
URE	Urea	-	0	0	-	0	0
TDA	L-tryptophane	-	0		-	0	
IND	L-tryptophane	-	0		-	0	
VP	Sodium pyruvate	-	0	6	-	0	6
GEL	Gelatin	+	2		+	2	
GLU	D-glucose	+	4		+	4	
MAN	D-manitol	+	1	1	+	1	1
INO	Inositol	-	0		-	0	
SOR	D-sorbitol	-	0		-	0	
RHA	L-rhamnose	-	0	0	-	0	0
SAC	D-sucrose	-	0		-	0	
MEL	D-melibiose	-	0		-	0	
AMY	Amygdalin	-	0	4	-	0	0
ARA	L-arabinose	-	0		-	0	
OX	Tetramethyl-p-phenylenediamine	+	4		-	0	

+ and - indicate positive and negative reaction, respectively

Table 3. Identification of fish bacteria using MALDI-TOF/MS Biotyper.

Cells No.	Analytic name	Organisms best match	Score value	Organisms (2nd Best Match)	Score value
A1	(+++), B	*A. salmonicida*	2.546	*A. salmonicida*	2.545
A2	(+++), B	*A. salmonicida*	2.572	*A. salmonicida*	2.552
A3	(+++), B	*A. salmonicida*	2.616	*A. salmonicida*	2.587
A4	(-), C	No peaks found	<0	No peaks found	<0
A5	(+++), B	*Y. ruckeri*	2.689	*Y. ruckeri*	2.637
A6	(+++), B	*Y. ruckeri*	2.545	*Y. ruckeri*	2.505

(+++), Highly probable species identification, (-), Not reliable identification
B, Genus consistency and condition of species consistency are not fulfilled
C, No consistency: Neither species nor genus consistency

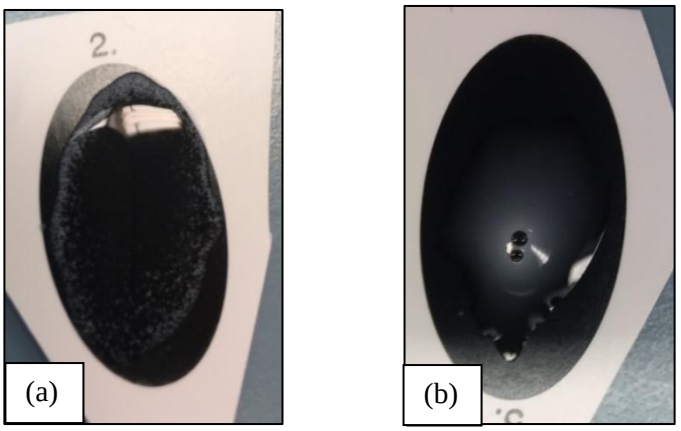

Figure 1. Identification of *Y. ruckeri* using agglutination test (a) Granular particles with Mono-Yr test reagent (b) No particles with control reagent.

3.4. API strip tests

The results of the API tests were recorded in Table 2. The estimated 7 digit codes for the pigmented (*A. salmonicida*) and non-pigmented (*Y. ruckeri*) bacterial strains were 0006104 and 5306100, respectively. The computer database (apiweb V5.0) significantly identified the *A. salmonicida* (ID of 99.6%. and T value 0.97). Although the non-pigmented bacterial sample was recoded as *Y. ruckeri* from morphological and other biochemical tests, the apiweb misidentified this sample as a *Vibrio mimicus* with low confidence (ID 83.9% and T value 0.38).

3.5. MALDI-TOF/MS test

In total, 6 cells of the MALDI-TOF plate were studied for two bacterial samples with MALDI-Biotyper system. Among the studied cells of MALDI-TOF plate, the recorded Biotyper score were more than 2.5 from the five cells, indicating highly probable to secure species identification (Table 3). No reliable identification was recorded from one cell (A4) of the non-pigmented bacterial colony (*Y. ruckeri*).

A score of 2.5 indicates highly acceptable species identification; assuming that there is a minimum of 5% score variation between the best match and different genera or species with closely related spectra. A high confidence score (>2.5) for the *A. salmonicida* were recorded from the first three cells of the stainless matrix plate (Table 3). While no result was observed from the cell A4; a high confidence score (>2.5) were recorded for *Y. ruckeri* from the last two cells (A5 and A6) of the MALDI-TOF sample plate (Table 3).

4. Discussion

The fish pathogenic bacteria *A. salmonicida* produce brown pigment and *Y. ruckeri* didn't form any color on nutritive agar growth medium which was used as a primary property for the identification of these species (Griffin *et al.*, 1953; Altmann *et al.*, 1992). Non-motile stains of *A. salmonicida* were identified from the studied sample. With respect to the motility and shape, *A. salmonicida* has described as non-motile subspecies and coccoid rod shape bacteria (Gudmundsdottir, 1998; Austin and Austin, 2007). Typically, *Y. ruckeri* do not produce any pigmentation on nutritive growth medium. While most of the infection related to the *Y. ruckeri*were associated with motile subspecies (Austin and Austin, 2007; Welch *et al.*, 2011), serotype O1 was identified as a non-motile bacteria (Austin *et al.*, 2003; Evenhuis *et al.*, 2009). Non-motile *Y. ruckeri*, serovar type-I was described from rainbow trout (*Oncorhynchus mykiss)* in Spain (Fouz *et al.*, 1990) and from hatchery-reared brown trout (*Salmo trutta)* in USA (Arias *et al.*, 2007). The *Y. ruckeri* was also documented as non-motile, rod shaped phenotype from Europe and USA (Evenhuis *et al.*, 2009; Tinsley *et al.*, 2011; Verner-Jeffreys *et al.*, 2011; Welch *et al.*, 2011).

Both of the experimental fish pathogen *(A. salmonicida and Y. ruckeri*) were Gram negative when staining was performed using Gram staining method (Buck, 1982). Several studies have suggested that the *A. salmonicida* (Pedersen *et al.*, 1994; Gudmundsdottir, 1998; Schmidt *et al.*, 2000) and *Y. ruckeri* are gram negative fish bacteria (Tinsley *et al.*, 2011; Verner-Jeffreys *et al.*, 2011; Welch *et al.*, 2011) which are reliable with the present results.

The oxidase test was used to identify the presence of respiratory enzymes, cytochrome oxidase in the bacterial isolates. Positive oxidase test was observed for *A. salmonicida* (Hanninen and Hirvela-Koski, 1997; Gudmundsdottir, 1998; Dalsgaard and Madsen, 2000). However, there is also negative cytochrome oxidase

reaction recorded from another stain of *A. salmonicida* (Chapman *et al.*, 1991). Like oxidase test, *A. salmonicida* give positive reaction with catalase test. A Positive oxidase and catalase test of *A. salmonicida* were recoded from turbot fish farm in Denmark (Pedersen *et al.*, 1994; Dalsgaard and Madsen, 2000). The studied *A. salmonicida* was observed as cytochrome oxidase positive like other research.In contrast, negative oxidase test for *Y. ruckeri* was recorded from the rainbow trout farm in Spain (Fouz *et al.*, 1990). The serotype O1 of the *Y. ruckeri* was observed as a catalase positive and oxidase negative from a fish farm in USA (Arias *et al.*, 2007). Moreover, *Y. ruckeri* was found positively reacting with biochemical test on Agglutinin (Verner-Jeffreys *et al.*, 2011). The Mono-Yr kit was able to identify the classical serotypes O1, O2 and O3 (most common serotypes) of *Y. ruckeri* that showed positive results with agglutination test, but the kit was unable to detect *Y. ruckeri* strains of serotypes O5 and O6 (Romalde *et al.*, 1995). The studied *Y. ruckeri* stain was O1 which showed positive reaction with agglutination test.

Identification profiles of fish bacteria in this study were obtained using two commercial miniaturized systems used for Enterobacteria identification: API 20E and MALDI-TOF/MS. The API-20E system is used extensively in aquaculture for the rapid diagnosis of bacterial fish disease. Depending on the species, the API-20E strips give negative and positive reactions with different active reagent (i.e. glucose, urea, sucrose etc.). The API profile for the *A. salmonicida* was 0006104 which gave 99.6% confidence for the identification of this species by the API database (apiweb V5.0). McCasland and True (2001) had documented that *A. salmonicida* strains confirmed serologically by FAT and by the standard biochemical tests gave 2006104 and 0006104 API profile and were correctly identified as *A. salmonicida.*

According to the API database (apiweb V5.0), the *Y. ruckeri* strains were misidentified as *Vibrio mimicus* with low confidence (ID 83.9%) against the test mode of 100% oxidase test and 99% IND (L-tryptophane). The *V. mimicus* are human pathogen responsible for gastroenteritis and diarrhea and growth temperature range from 21 to 35^{o}C which is a motile and oxidative positive bacteria (Chowdhury *et al.*, 1989; Campos *et al.*, 1996). In contrast, our cultured bacteria was a fish bacteria, incubated at 20^{o}C, non-motile and oxidative negative which are in favor of the fish bacterial species *Y. ruckeri*. Similarly, misidentification of the *Y. ruckeri* with API-20E kits was recorded from a Spanish rainbow trout farm (Fouz *et al.*, 1990). Austin *et al*. (2003) were identified different species (*Hafnia alvei)* when examined reference strains of *Y. ruckeri* in API 20E test (Austin *et al.*, 2003). Therefore, it has been suggested that the addition of new API 20E profile in the API database generated by *Y. ruckeri* strains would improve the rapid diagnosis of ERM (Yancey *et al.*, 1989; Austin *et al.*, 2003).

The identification and characterization of microorganisms by MALDI-TOF MS is based on the detection of mass signals from biomarkers. The mass signals are specific at genus, species or sub-group level for each microorganism. Although, it is widely used for the identification of human pathogen, it is also able to identify fish bacteria i.e. *A. hydrophila, A. salmonicida, Flavobacterium columnare, Y. ruckeri, Vibrio* sp. (Dumpala *et al.*, 2010; Benagli *et al.*, 2012; Wang *et al.*, 2013; Jansson *et al.*, 2015). The MALDI-TOF results showed up to species consistency with a higher confidence score (>2.5) and provided identification of the strains *A. salmonicida (ATCC33658) and Y. ruckeri (ATCC0305222-1)* species in the present study. The results suggest that the proteometric based MALDI-TOF MS-based identification represents a powerful device for quick and correct classification and characterization of *A. salmonicida* and *Y. ruckeri* species.

5. Conclusions

The *A. salmonicida* and *Y. ruckeri* are the common bacteria for fish disease worldwide. It affects more in farm fish than wild fish and causes a huge loss of commercial fish production. In this respects, rapid and accurate diagnosis of the causative agent of fish disease is essential to take proper steps for the treatment of the infections and trace-back of disease outbreaks associated with microbial infections. It is difficult to set correct identification of a microbial species using one/two biochemical test. Therefore, combination of a series of rapid biochemical as well as PCR tests is important for diagnosis of pathogens. In this study, *A. salmonicida* was confirmed by the entire applied test. While API 20E strips test misidentified *Y. ruckeri*. The others test such as Gram staining, agglutination, catalase, oxidase and MALDI-TOF provides accurate results for the identification of the bacteria. For the rapid identification of furunculosis and ERM disease, the above mentioned test would be helpful for aquaculture operation. Additionally, molecular identification is needed about the host specificity of the stains.

Acknowledgements

The author sincerely thanks toInger Dalsgaard for support this special arrangement with different tests. Special thanks to Lisbeth Schade Hansen for her cordial cooperation during the laboratory work and giving me necessary information to carry out the study.

Conflict of interest
None to declare.

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