Journal of Current Veterinary Research

ISSN: 2636-4026

Journal homepage: http://www.jcvr.journals.ekb.eg

Fish diseases

Phenotypic and Molecular Characterization of *Yersinia ruckeri* and *Psychrobacter* Species Isolated from Farmed Fishes

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ABSTRACT

One of the major threats to aquaculture sector is bacterial diseases. In the current study, samples from different species of cultured fishes were collected from different fish farms at different localities in Menofiya and Kafr El-Sheikh Provinces, Egypt for the detection of causative agents of some disease. Clinically, the infected fishes were inspected for symptoms like darkness of skin and, hemorrhages in different parts of the fish's body including the base of fins, tail, and rot of fins. Identification of the retrieved bacterial pathogens using conventional biochemical and molecular methods were demonstrated. Based on the phenotypic and biochemical characterization using API20E, API 20 NE and VITEK® 2 compact, the retrieved isolates were identified as *Yersinia ruckeri* and *Psychrobacter* species. Molecular characterization was accomplished through polymerase chain reaction (PCR) and confirmed by DNA-sequencing and phylogenetic analysis. The high molecular identity and close phylogenetic relationship confirmed that the isolates were genetically homologous to *Yersinia ruckeri* and *Psychrobacter* spp.

Keywords: *Yersinia ruckeri, Psychrobacter spp., PCR, Sequencing, phylogenetic analysis.*

INTRODUCTION

Aquaculture development has become an indispensable option due to the rapidly growing population. In the past twenty years, production of fin-fish aquaculture has increased from 12.5 to 54.1 million tons (FAO 2017, 2018). Tacky water quality, high organic load and contaminated feed are considered the leading causes of bacterial diseases in aquaculture. Fishes are always very much susceptible to a wide range of pathogenic bacteria (Banerjee *et al.*, 2016). Enteric Red Mouth disease or Yersiniosis is a serious bacterial disease, sometimes can result in occurring economic havoc to Aquaculture industry. The most characterized clinical lesions are hemorrhages in different parts of the fish's body including at the base of fins, inside and around mouth and eye lesions with or without haemorrhage (Ummey *et al.*, 2020). *Psychrobacter* was described for the first time as psychrotolerant, aerobic, Gram-negative, non-motile, oxidase-positive and coccobacilli which belong to the family Gammaproteobacteria, the genus consists of 30 familiar species (Juni and Heym, 1986). Undoubtedly, dependence on traditional methods has not always been effective for the isolation of pathogens. It is obvious that, molecular technologies have upgraded the precision of bacterial identification, and it developed to be used not only in specialized laboratories but also used in that involved with routine diagnostics (Austin, 2019). Thus, aim of this study was directed to determine *Yersinia ruckeri* and *Psychrobacter* spp. recovered from naturally infected fishes collected from different regions in Menofiya and Kafr El-Sheikh Provinces, Egypt. Aside from the morphological and biochemical identification of the bacterial strains, the molecular characterization by polymerase chain reaction

(PCR) was accomplished. In addition, DNA-sequencing, and phylogenetic relationship were executed to confirm the identity of *Yersinia ruckeri* and *Psychrobacter* spp. isolates retrieved from fishes.

MATERIALS AND METHODS

Fish sample:

A total No. of 120 farmed fishes (60 *Oreochromis niloticus*, 40 *Mugil cephalus* and 20 *Clarias gariepinus*) were collected from different fish farms at different regions in (Menofiya and KafrEl-Sheikh Provinces). Fish samples were transported alive to the Department of Fish Diseases at AHRI (Animal Health Research Institute), Dokki, Giza and exposed to clinical examination and bacterial isolation.

Gross clinical and post mortem examination:

A-Naturally infected fishes have been clinically examined to investigate any clinical abnormalities acc. to the method described by Eissa, (2016) and Austin and Austin, (2016).

B-Necropsy was performed for detection of PM lesions acc. to Noga, (2010) and Austin and Austin, (2016).

Isolation and morpho- biochemical Identification:-

Samples were taken from fish's organs (gills, liver, kidney, and spleen) under full aseptic conditions. The inoculums were further streaked onto tryptic soya agar plates (TSA; Difco, Detroit, MI), blood agar (BA) and Shotts–Waltman agar (SWA) (Waltman and Shotts, 1984). Plates were incubated at 25 °C for 24–48 hours. Full identification schema preliminarily based on colonial characteristics, Gram stain and biochemical characters of the strains according to Austin and Austin, (2016). Isolates were kept at −80 °C in BHIB including 10% glycerol for further molecular identification. The biochemical characters of the identified bacterial isolates were further confirmed using API 20 E and API 20 NE Kit according to Buller, (2004) and VITEK® 2 compact (BioMérieux).

Molecular Identification, DNA sequencing, and phylogenetic relationships:

The three different isolates were submitted to identification by16SrRNA. The isolates were inoculated into brain heart infusion broth (BHIB) and incubated for 24 hrs. DNA was purified using PathoGene-spin™ DNA Extraction Kit acc. to the manufacturer's instructions. PCR was accomplished using oligonucleotide primer for general gram negative 16srRNA gene. The PCR protocol was accomplished as following an initial denaturation at 95°C / 5 min, followed by 35 cycles 94°C / 2 min; 35 cycles of 94°C /30 s, 55°C / 30 s, and 72°C /30 s; 72°C / 5 min. QIAquick PCR Product extraction kit has been used to extract PCR products (Qiagen, Valencia). Sequencing was done using Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) and then it was purified using Centrisep spin column. DNA sequencing was performed by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990), originally was conducted to confirm sequence identity to GenBank accessions. The phylogenetic tree was created using neighbor-joining method in MEGA 7 (Tamura *et al.*, 2013).

RESULTS

Gross clinical and PM examination of Fishes:

Clinical examination of different fish species including (*Oreochromis niloticus*, *Mugil cephalus* and *Clarias gariepinus*) revealed darkness of skin, hemorrhages in different parts of the fish's body including the base of fins, tail, and rot of fins, abdominal distension, ulcers and sloughing of fish scales. While, the post-mortem examination of examined fishes revealed septicemia represented by gills congestion and viscera, enlargement of different internal organs and some cases exhibited that liver paleness and intestine .

Bacterial examinations

Morphological, culture characters, and Biochemical identification

Colonies suspected of *Yersinia ruckeri* appeared small round, white opaque colonies of approximately 1–2 mm in diameter on TSA media after 48 hrs of incubation. Gram staining of these colonies indicated the presence of Gram negative, short bacilli. Isolates were weakly motile. On blood agar (BA) medium produced white and creamy colored colonies, while on Shotts–Waltman agar (SWA) appeared small yellow colonies with opaque halo. All isolated *Yersinia ruckeri* were Catalase positive and they gave negative results toward Oxidase and H2S production.

Colonies suspected of *Psychrobacter* spp. on (TSAS) (TSA in addition to 1.5% NaCl) appeared creamy-white to opaque, low convex and favors growth at 20°C to 15°C for 48 hrs.

Gram staining of these colonies gives Gram-negative, non-motile coccobacilli. All isolated *Psychrobacter* Spp. were Oxidase and Catalase positive and they gave negative results toward urea hydrolysis, nitrate reduction and H2S production. The data existed in (Table 1) illustrated full identification schema of *Yersinia ruckeri* and two *Psychrobacter* Spp. using API 20E system.

Results of VITEK® 2 compact system

Three strains were identified using Vitek®2 compact system. Three isolates were identified as *Yersinia ruckeri* and 2 isolates were *Psychrobacter* spp., with a probability of 99%.

Molecular identification, DNA Sequencing and Phylogenetic relationship:

QIAquick PCR Product extraction kit has been used to extract PCR products. DNA sequencing was performed by Applied Biosystems3130 genetic analyzer, a BLAST® analysis (Basic Local Alignment Search Tool), initially was conducted to confirm sequence identity to GenBank accessions. From the sequence of 16SrRNA gene, the isolates sequence was *Yersinia ruckeri* and 2 isolates were *Psychrobacter* spp., and were submitted to Genbank data base under accession no. MW494455, MW494438 and MW494452 respectively, (Figure 1, 2 and 3).

Table (1): illustrated the full identification schema of *Yersinia ruckeri* and two *Psychrobacter* spp. using API 20E system.

	Yersinia ruckeri	*Psychrobacter* spp.1	*Psychrobacter* spp.2
Gram stain	-ve	-ve	-ve
Motility	Motile	Non motile	Non motile
API 20E reactions			
(ONPG)ß-galactosidase	+ve	-ve	-ve
(ADH)Arginine dihydrolase	+ve	-ve	
(LDC) Lysine decarboxylase	+ve	-ve	-ve
(ODC)Ornithine decarboxylase	+ve	-ve	-ve
(CIT) Citrate utilization	-ve	-ve	-ve
(H2S) H2S production	-ve	-ve	-ve
(URE) Urea hydrolysis	-ve	-ve	-ve
(TDA) Tryptophan deamination	-ve	-ve	-ve
(IND) Indol production	-ve	-ve	-ve
(VP) Acetoin production	+ve	-ve	
(GEL) Gelatin hydrolysis	+ve	-ve	-ve
(GLU) Glucose fermentation	+ve	+ve	-ve
(MAN) Mannitol	+ve	+ve	-ve
(INO) Inositol	-ve	-ve	-ve
(SOR) Sorbitol	-ve	-ve	-ve
(RHA) Rhamnose	-ve	-ve	-ve
(SAC) Sucrose	-ve	-ve	+ve
(MEL) Melibiose	-ve	-ve	-ve
(AMY) Amygdalin	-ve	-ve	-ve
(ARA) Arabinose	-ve	-ve	+ve
(Oxidase) Cytochrome oxidase	-ve	+ve	+ve

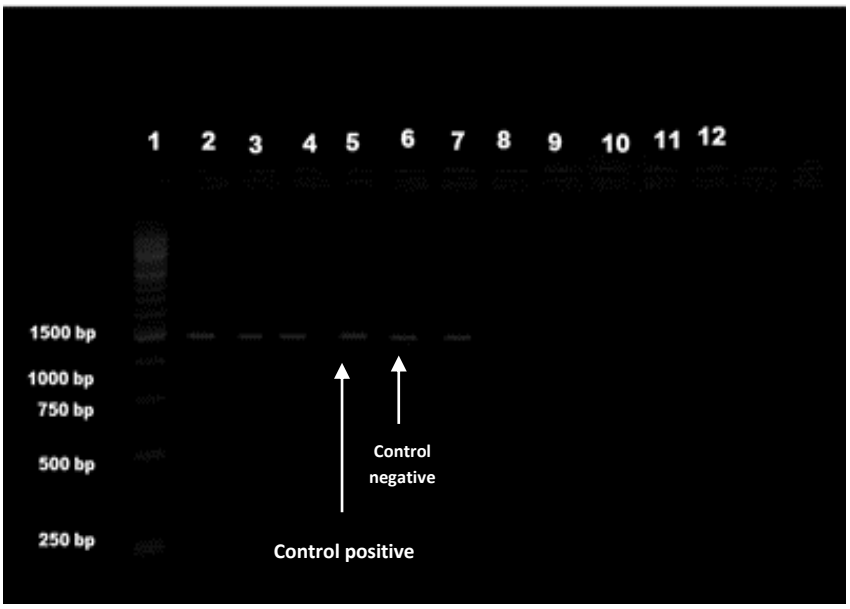

Figure (1): Agrose gel electrophoresis of 16SrRNA gene
Lane (1):1000 bp DNA Ladder
Lanes (2-4): positive isolates for 16srRNA gene.
Lanes (5): Control positive *Escherichia coli* (ATCC; 25922)
Lanes (6): Control negative *Staphylococcus aureus subsp. Aureus (*ATCC; 6538)

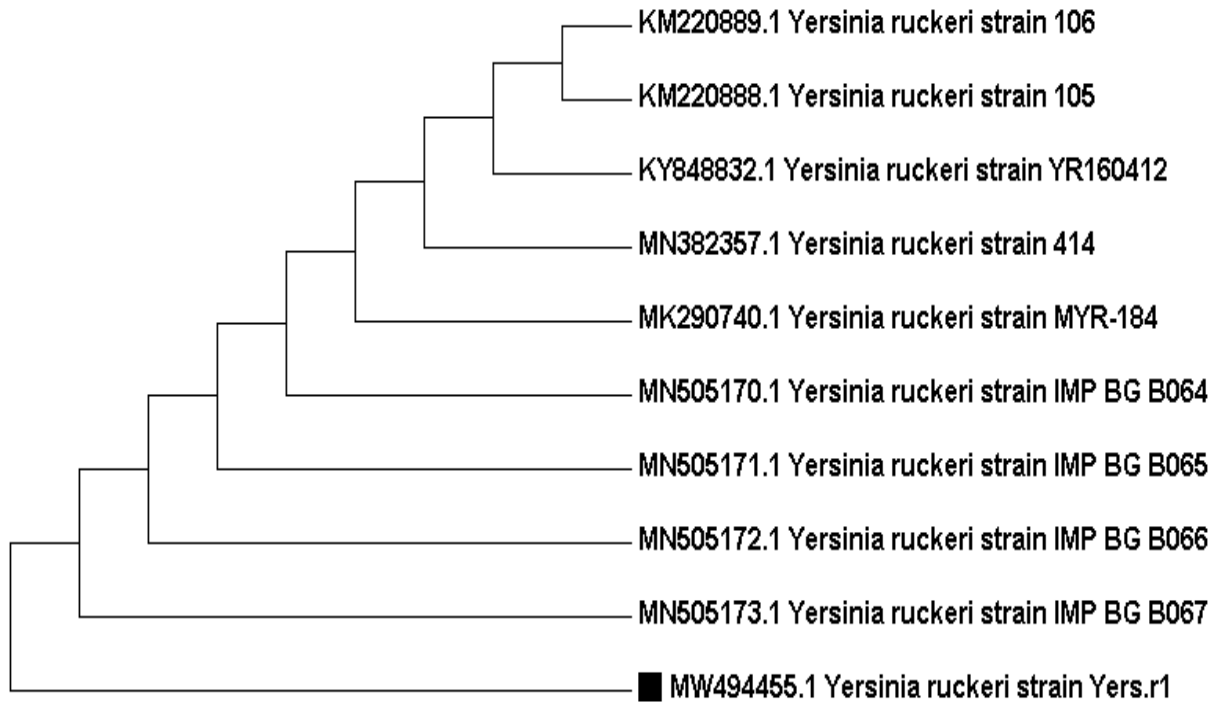

Fig. (2): Phylogenetic tree of *Yersinia ruckeri* strain by general gram negative 16SrRNA gene demonstrating the relationships of *Yersinia ruckeri* with related species. The neighbor-joining method has been used to create phylogenetic tree.

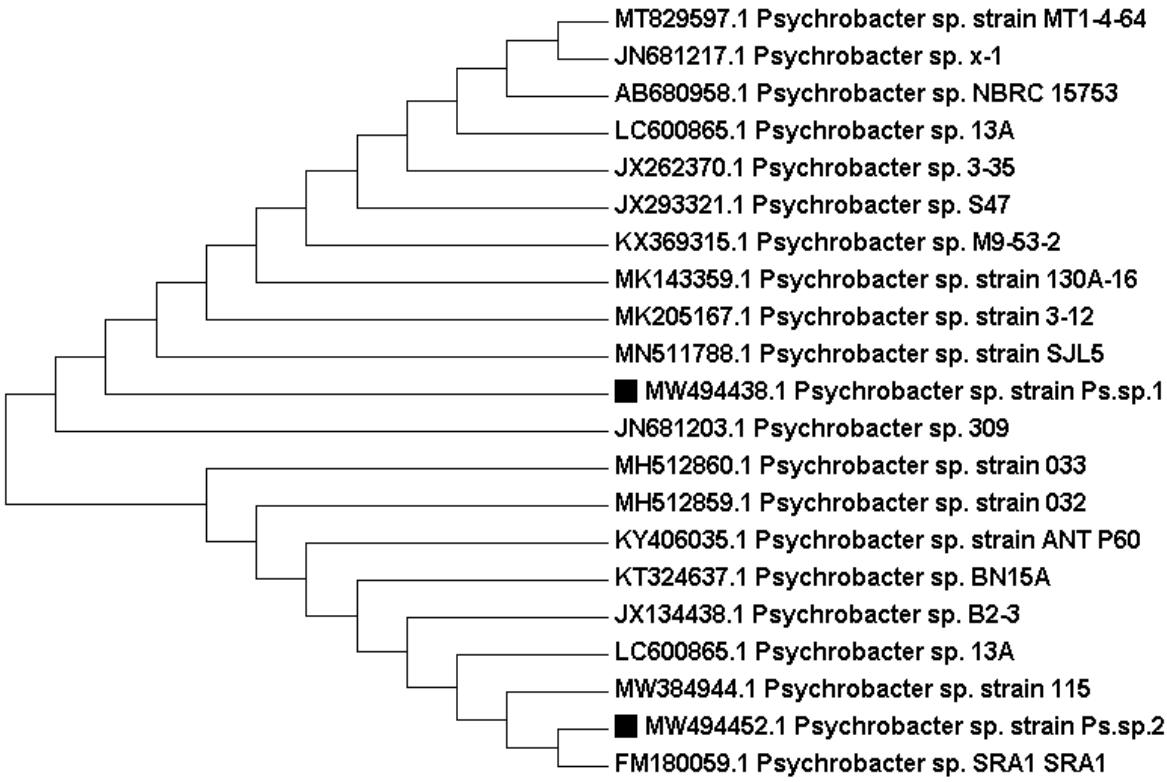

Fig. (3): Phylogenetic tree of two *Psychrobacter* spp. strains by general gram negative 16SrRNA gene demonstrating the relationships of two *Psychrobacter* spp. with related species. The neighbor-joining method has been used to create phylogenetic tree.

DISCUSSION

Egyptian aquaculture industry has evinced a distinguished expansion; even so, it has faced some threats related to disease outbreaks. In Egypt, Oreochromis niloticus is the most popular cultured fish, it is susceptible to a variety of bacterial diseases when exposed to stress factors (Elsheshtawy *et al.,* 2019). Enteric Red mouth disease is considered a serious bacterial disease, affected mainly freshwater fishes. It was first isolated from Oncorhynchus mykiss in the early 1950's from a trout farm at Idaho, USA (Rucker, 1966). It is now found across Europe, Europe, South Africa, Middle East and China, in North- and South America (Shaowu *et al.,* 2013). *Psychrobacter* spp. has been isolated from the kidney of the moribund fish as the predominant bacterial type sampled in one Atlantic salmon site (Salmo salar) and a rainbow trout (Oncorhynchus mykiss Walbaum). The organism was isolated in three further farm sites, followed by skin lesions or gills of dying salmon. Whereas psychobactor species have been often identified as the main isolate of the kidney of compromised individuals in fish gill and intestinal flora, bacterial recovery suggests that it has the ability to develop opportunistic salmonid infections (McCarthy *et al.,* 2013). Clinical examination of different fish species revealed darkness of skin, hemorrhages in different parts of the fish's body including the base of fins, tail, and rot of fins, abdominal distension, ulcers and sloughing of fish scales. While, the post-mortem examination of examined fishes revealed septicemia represented by gills congestion and viscera, enlargement of different internal organs and some cases exhibited that liver paleness and intestine. The observed clinical signs and PM lesions were similar to that reported by (McCarthy *et al.,* 2013; Kumar *et al.,* 2015; Mohammed, 2017; Ummey *et al.,* 2020). The exhibition of virulence factors and formation of biofilms are the major factors for the establishment of disease in aquaculture animals by the bacterial pathogens (Osman *et al.,* 2020). Isolation and identification of isolated strains based on the colonial characters on selective media and the results of the biochemical reactions. Colonies of *Yersinia ruckeri* appeared small round, white opaque colonies of approximately 1–2 mm in diameter on TSA media after 48 hrs of incubation. Isolates were Gram negative weakly motile short bacilli. On blood agar (BA) medium produced white and creamy colored colonies, while on Shotts–Waltman agar (SWA) appeared small yellow colonies with opaque halo. All isolated *Yersinia*

ruckeri were Catalase positive and they gave negative results toward Oxidase and H2S production, these outcomes are in accordance with that described previously (Eissa *et al.*, 2008; Şeker *et al.*, 2011; Abdel-latief *et al.*, 2014; Zorriehzahra *et al.*, 2017). Colonies of *Psychrobacter* spp. on TSAS (TSA in addition to 1.5% NaCl) appeared creamy-white to opaque, low convex and favors growth at 20°C to 15°C for 48 hrs. Gram staining of these colonies gives Gram-negative, non-motile coccobacilli. All isolated *Psychrobacter* spp. were Oxidase and Catalase positive and they gave negative results toward urea hydrolysis, nitrate reduction and H2S production. These results are matching to that confirmed previously (Romanenko *et al.*, 2002; McCarthy *et al.*, 2013). API 20 E, API 20 NE and VITEK 2 compact system were used to confirm the results of biochemical reactions. In aquaculture systems, precise bacterial identification and molecular characterization have a great importance to control the likelihood of risks occurring, especially bacteria that are able to cause diseases in cultured fishes and human (Netzer *et al.*, 2021). The isolated *Yersinia ruckeri* in the current study had high nucleotide identity with other isolates MN505173, MN505172, MN505171, MN505170, MK290740.1, MN382357, KY848832, KM220889 and KM220888 which were isolated from channel catfish, sturgeon, Rainbow trout, Micropterus salmoides and Oncorhynchus mykiss. The isolate *Psychrobacter* spp. MW494438 had high nucleotide identity with, MN511788, MK205167, MK143359, KX369315, JX293321, JX262370, LC600865, AB680958, MT829597, JN681217which were all isolated from waste water while isolate *Psychrobacter* spp. MW494452 was nucleotide identity with FM180059, FM180059, MH512860, MH512859, KY406035, KT324637, JX134438, LC600865, MW384944 which were all isolated from waste water also.

CONCLUSION

Yersinia ruckeri and *Psychrobacter* spp. are responsible for the occurrence of diseases in freshwater fishes which consequently result in huge economic losses in aquaculture industry. So, DNA-Sequencing is considered one of the most trustworthy diagnostic techniques in *Yersinia ruckeri* and *Psychrobacter* spp identification.

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