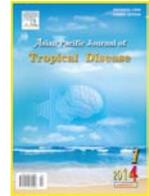




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Association of *Aeromonas salmonicida* subsp. *achromogenes* in the haemorrhagic blister of cultured carp *Cyprinus carpio* in West Bengal, India

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PEER REVIEW

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Comments

This is a valuable study as this is the first report on the disease condition caused by *A. salmonicida* subsp. *achromogenes* from India. The results of this study throw light on the spread of the pathogens and on emergence of diseases in newer regions of the world.
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ABSTRACT

Objective: To study the association of *Aeromonas salmonicida* subsp. *achromogenes* in the haemorrhagic blister of cultured exotic carp *Cyprinus carpio* in West Bengal, India, its cellular fatty acid composition and antibiotic sensitivity.

Methods: The phenotypic characterization and antibiotic sensitivity of bacterial flora of diseased fish were performed. The whole cellular fatty acid composition of *Aeromonas salmonicida* (*A. salmonicida*) was determined by MIS Sherlock automatic identification system.

Results: *Aeromonas hydrophila*, *A. salmonicida* and *Pseudomonas alcaligenes* were isolated from the haemorrhagic blister. The fatty acid methyl esters pattern confirmed the *A. salmonicida* strain as *A. salmonicida* subsp. *achromogenes*. Unsaturated fatty acid 16:1 w7c/16:1 w6c (39.09%), saturated fatty acid 16:0 (26.84%) and the mono-unsaturated fatty acids 18:1 w7c (8.89%) and 16:1 iso 1/14:0 30H (8.49%) were the most common fatty acids, which accounted for 83.31% of the total fatty acids. *A. salmonicida* subsp. *achromogenes* was highly susceptible to broad spectrum antibiotics such as chloramphenicol, ciprofloxacin, co-trimoxazole, gentamycin, nitrofurantoin and oxytetracycline.

Conclusions: The finding on the presence of *A. salmonicida* in carps necessitates molecular level study on establishing the prevalence of this bacterium in Indian aquaculture systems especially on its free-living viable but non-culturable state.

KEYWORDS

Cyprinus carpio, Haemorrhagic blister, *Aeromonas hydrophila*, *Aeromonas salmonicida* subsp. *achromogenes*, FAME analysis

1. Introduction

The Gram-negative bacterium *Aeromonas salmonicida* (*A. salmonicida*) has been known as one of the oldest fish pathogens that causes disease in a wide range of fish species[1]. *A. salmonicida* is placed within the family Aeromonadaceae, in the genus *Aeromonas* and five subspecies of *A. salmonicida* have been described, i.e., subsp. *salmonicida*, *achromogenes*, *masoucida*, *smithia*

and *pectinolytica*[1–3]. *A. salmonicida* subsp. *salmonicida* is the causative agent of classical furunculosis of salmonids and is often referred to as typical strain. Other subspecies are considered as atypical strains, causing ulcerative and systemic infections in a wide variety of fishes[1,3,4]. Atypical *A. salmonicida* strains have been isolated from a wide range of fish species in freshwater, brackishwater as well as in seawater that includes many economically important species such as salmonids, carps, goldfish, cod, eel,

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turbot, flounder, halibut and many others. The best known diseases caused by atypical *A. salmonicida* strains include goldfish ulcerative disease, carp erythrodermatitis and ulcer disease^[1,5–7]. The number, diversity and distribution of fish species that is susceptible to *A. salmonicida* enhance this bacterium's distribution worldwide. However, both typical and atypical subspecies may not be common to all geographic areas^[1]. The geographical distributions of reported isolations indicated that atypical strains mainly infect fishes of temperate regions. Some atypical strains have also been isolated from fish in Australia, Asia and in the Mediterranean^[1,5,6,8]. This paper presents the phenotypic and fatty acid methyl esters (FAME) characterization of *A. salmonicida* subsp. *achromogenes* isolated from the blister of cultured exotic carp *Cyprinus carpio* (*C. carpio*) in India.

2. Materials and methods

The exotic carp *C. carpio* of size about 720 g showing signs of haemorrhagic blister at the base of pectoral fin and cutaneous haemorrhages in a grow-out composite culture pond culturing Indian major and exotic carps in Memari (Lat 23°10'32" N; Long 88°06'24" E), West Bengal, India during the routine disease surveillance programme in November 2012 was examined following the standard methods^[9]. Inocula from the haemorrhagic blister at the base of pectoral fin of *C. carpio* were taken with the help of Hiculture transport swabs w/ Amies medium w/ charcoal (HiMedia Mumbai) and brought to the laboratory within 8 h of collection. At the laboratory, inocula from the transport medium were streaked on to *Aeromonas* isolation agar (AIA), *Pseudomonas* isolation agar (PIA) and brain heart infusion agar (BHIA, Himedia, Mumbai) and incubated at (25±2) °C for 24 h. Randomly picked five dark green colonies on AIA and also a non-pigmented translucent colony on PIA were purified by subculturing on to BHIA and characterized biochemically using Rapid HiAssorted™ biochemical test kit for Gram negative bacterial rods, Rapid Hi25™ Enterobacteriaceae identification kit and carbohydrate differentiation discs (Himedia, Mumbai). The bacterial isolates were identified following the Bergey's manual^[2,10].

The presumptive *A. salmonicida* CC32 was further characterized by FAME analysis as per Sasser^[11]. The whole cellular fatty acid composition (FAME profile) of the *A. salmonicida* was determined by MIS Sherlock automatic identification system (MIDI, Inc., Newark, USA). The FAME was extracted in accordance with the MIDI FAME™ protocol of the Microbial Identification System. The extract was analyzed by gas chromatography using a flame ionization detector after capillary column separation (Ultra 2, 25 m, 0.20 mm, 0.33 µm-phenyl methyl silicon fused silica). The individual fatty acid of the strain was identified by MIS Sherlock software (ACTIN6 method). The similarity of the FAME profile of the isolate with the species in ACTIN1 was represented

by similarity indices (IS). If the IS value was higher than 0.5, the MIS identification was used as positive and listed as additional strain features. Aerobic library (RTSBA6 6.20) was referred for the analysis. Sensitivity of the bacterial flora was tested against six antibiotics, viz., oxytetracycline (30 µg), chloramphenicol (30 µg), gentamycin (10 µg), nitrofurantoin N (300 µg), ciprofloxacin (5 µg) and co-trimoxazole (25 µg) by agar-disc diffusion assay on Mueller Hinton agar^[12].

3. Results

The cultured exotic carp *C. carpio* showed haemorrhagic blister at the base of pectoral fin (Figure 1). Cutaneous haemorrhages were seen in <1% of the cultured Indian major carps. No other external signs of infection were seen. Other fishes in the pond were apparently healthy and no mortality was recorded. Inoculation of sample on to the AIA from haemorrhagic blister yielded dark green colonies within 24 h. Only few non-pigmented colonies have grown on PIA. On the basis of the biochemical characterization (Table 1), these bacterial isolates were identified as *Aeromonas hydrophila* (*A. hydrophila*), presumptive *A. salmonicida* and *Pseudomonas alcaligenes* (*P. alcaligenes*).

Table 1

Biochemical characterization of the bacterial flora associated with haemorrhagic blister of *C. carpio*.

Biochemical tests	<i>P. alcaligenes</i> (n=1)	<i>A. hydrophila</i> (n=4)	<i>A. salmonicida</i> strain CC32 (n=1)	Standard <i>A. salmonicida</i> strain ^a
Gram reaction	–	–	–	–
Morphology	Rod	Rod	Rod	Rod
Motility	+	+	–	–
Cytochrome oxidase	+	+	+	+
O/F reaction	–/–	+/+	+/+	+/+
Gas from glucose	–	+	–	–
Voges Proskauer reaction	*	+	–	–
Indole production	*	+	+	+
H ₂ S production	–	+	–	–
Citrate utilization	+	+	+	+
ONPG	*	+	+	+
Lysine decarboxylase	–	+	–	–
Ornithine decarboxylase	–	–	–	–
Urease	–	–	–	–
Phenylalanine deamination	–	–	–	–
Nitrate reduction	+	+	+	+
Methyl red reaction	*	+	+	+
Esculin hydrolysis	*	+	(±)	–
Utilization of Glucose	–	+	+	+
Utilization of Adonitol	ND	–	–	–
Utilization of Lactose	–	–	–	–
Utilization of Arabinose	ND	+	(±)	–
Utilization of Cellobiose	*	–	–	–
Utilization of Xylose	*	–	–	–
Utilization of Rhamnose	*	–	–	–
Utilization of Melibiose	*	–	–	–
Utilization of Saccharose	*	*	+	+
Utilization of Raffinose	*	–	–	–
Utilization of Trehalose	*	+	+	+
Utilization of Melonate	*	*	+	+
Utilization of Sorbitol	ND	–	–	–

a: Standard reactions are from Bergey's manual^[2] and Austin and Austin^[4].

(±): Weak reaction; ND: No data available; *: Not done.



Figure 1. Haemorrhagic blister at the base of pectoral fin of *C. carpio*.

The chromatogram of presumptive *A. salmonicida* CC32 showing the fatty acid peaks is presented in Figure 2. Analysis of the FAME profile of *A. salmonicida* CC32 and the chromatogram yielded a total of 13 different fatty acids. The FAME profile similarity index of 0.769 in the aerobic library (RTSBA6 6.20) identified the strain as *A. salmonicida* subsp. *achromogenes*. Unsaturated fatty acid 16:1 w7c/16:1 w6c was the predominant fatty acid (39.09%). The saturated fatty acid 16:0 (26.84%) and the mono-unsaturated fatty acids 18:1 w7c (8.89%) and 16:1 iso I/14:0 3OH (8.49%) were the second, third and fourth most common fatty acids, which accounted for 83.31% of the total fatty acids (Table 2). The *A. salmonicida* subsp. *achromogenes* strain was highly sensitive to chloramphenicol, ciprofloxacin, co-trimoxazole, gentamycin, nitrofurantoin and oxytetracycline. All strains were sensitive to chloramphenicol and ciprofloxacin. *A.*

hydrophila and *P. alcaligenes* strains were resistant to co-trimoxazole and exhibited varying degrees of sensitivity to other antibiotics (Table 3).

Table 2

FAME profile of *A. salmonicida* subsp. *achromogenes* CC32 from haemorrhagic blister of *C. carpio*.

Peak name	Percentage (%)
12:00	8.42
12:0 3OH	0.25
14:00	6.32
15:0 anteiso	0.26
16:1 w5c	0.19
16:00	26.84
16:0 3OH	0.27
18:00	0.35
19:0 iso	0.23
12:0 aldehyde? and/or unknown 10.9525	8.49
16:1 w7c/16:1 w6c and/or 16:1 w6c/16:1 w7c	39.09
18:1 w7c and/or 18:1 w6c	8.89
16:0 10-methyl and/or 17:1 iso w9c	0.41

Table 3

Antibiotic sensitivity of bacterial flora isolated from haemorrhagic blister of *C. carpio*.

Antibiotics	<i>A. salmonicida</i>	<i>A.</i>	<i>A.</i>	<i>A.</i>	<i>A.</i>	<i>P.</i>
	subsp. <i>achromogenes</i> CC32	<i>hydrophila</i> CC31	<i>hydrophila</i> CC33	<i>hydrophila</i> CC34	<i>hydrophila</i> CC35	<i>alcaligenes</i> CC36
Chloramphenicol (30 µg)	S	S	S	S	S	S
Ciprofloxacin (5 µg)	S	S	S	S	S	S
Co-trimoxazole (25 µg)	S	R	R	R	R	R
Gentamycin (10 µg)	S	S	S	S	I	S
Nitrofurantoin N (300 µg)	S	S	S	I	S	I
Oxytetracycline (30 µg)	S	S	S	S	I	R

S: Sensitive; I: Intermediate; R: Resistant.

4. Discussion

The original epizootic spread of atypical strains of *A. salmonicida* was in the temperate regions such as Canada,

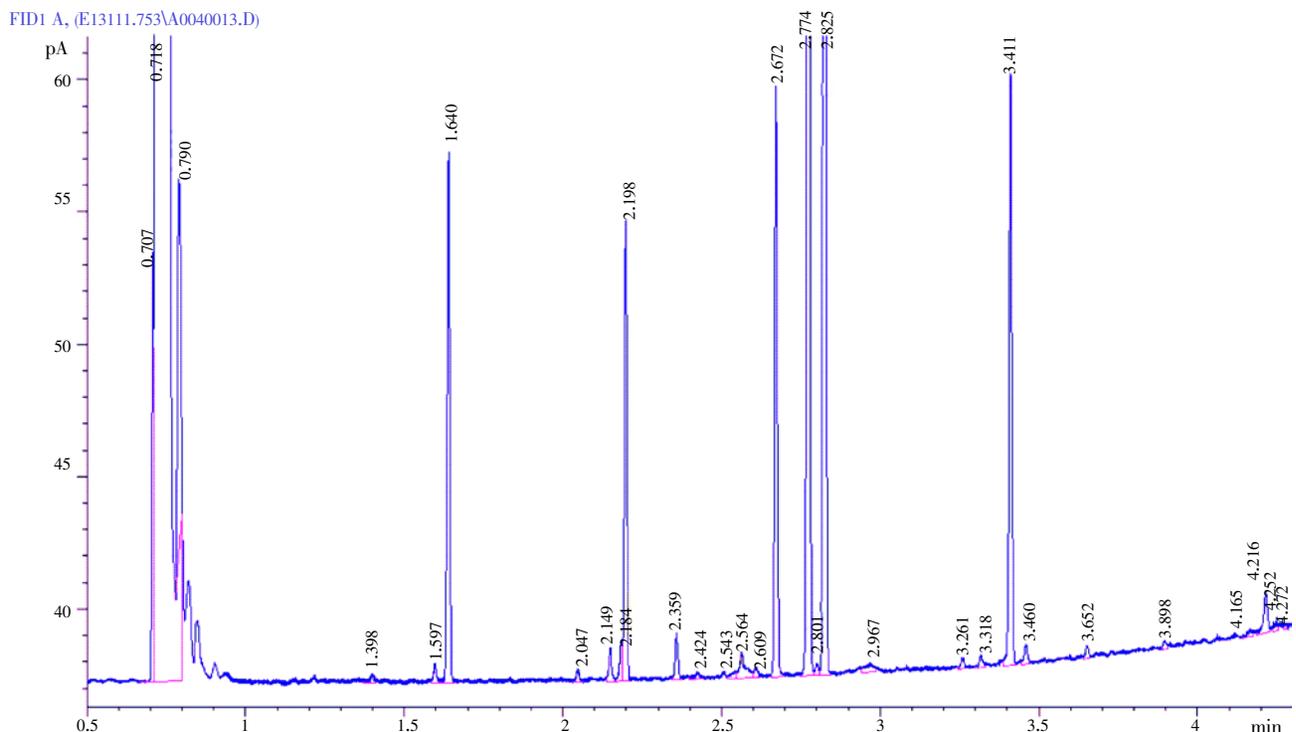


Figure 2. Chromatogram of *A. salmonicida* subsp. *achromogenes* showing the fatty acid peaks through gas chromatography.

USA, Japan, Europe including the Nordic countries, but nowadays their distribution is nearly global, including Australia^[1,8,13], Republic of Korea^[8], South Africa^[14], from fish exported from Singapore where it was formerly believed to be absent^[13] and Bangladesh^[15]. The epizootiology as well as the general homogeneity of *A. salmonicida* strains suggested that this bacterium has been spread principally through introductions and trade^[13]. The observations of the present study revealed mixed bacterial infection and association of *A. hydrophila*, *A. salmonicida* and *P. alcaligenes* in the haemorrhagic blister of *C. carpio*. Except for the weak reactions for esculin hydrolysis and arabinose utilisation in rapid HiAssorted biochemical test kit for Gram negative bacterial rods (Table 1), the *A. salmonicida* CC32 was biochemically consistent with criteria described for *A. salmonicida* subsp. *achromogenes*^[2,4].

The fatty acid profile results of Huys *et al.*^[16] revealed 44 different fatty acids and two alcohols in the members of genotypically characterized *Aeromonas* spp. other than *A. salmonicida*. In this study, the FAME analysis of *A. salmonicida* yielded 13 different fatty acids. The FAME profile of the isolate with similarity index of 0.769 in the aerobic library (RTSBA6 6.20) confirmed the strain as *A. salmonicida* subsp. *achromogenes* (Table 2, Figure 2). About 83.3% of the total fatty acids of *A. salmonicida* subsp. *achromogenes* comprised of unsaturated fatty acid (16:1 w7c/16:1 w6c), saturated fatty acid (16:0) and mono-unsaturated fatty acids (18:1 w7c and 16:1 iso I/14:0 3OH), which corroborate the findings of Bektas *et al.*^[17] in *A. salmonicida* isolated from rainbow trout, *Oncorhynchus mykiss*. They identified 16:1 w7c/15 iso 2OH (46.12%) as the dominant fatty acid, together with other fatty acids such as 18:1 w7c (8.33%), 14:0 3OH/16:1 iso 1 (8.83%) and 16:0 (23.75%). All of them accounted for about 87% of the total fatty acids. The *A. salmonicida* subsp. *achromogenes* strain failed to grow on BHIA up on repeated subculture and with increasing ambient temperature (>30 °C) towards the summer months. The ideal water temperature for *A. salmonicida* to survive is within the range of 12.8–21.1 °C^[18]. It is also reported that the strains of the non-motile psychrophilic species *A. salmonicida* grow weakly when they are cultivated at 28 °C^[2,16]. Reports on the prevalence of *A. salmonicida* in the Southeast Asia are scarce^[1] and *A. salmonicida* is sensitive to environmental conditions^[4,19]. Since this bacterium is quickly outcompeted in growth by most commonly occurring aquatic bacteria, it is difficult to isolate it from mixed microbial populations^[1,20]. Despite the controversy as to whether or not the organism is capable of a free-living existence in the natural environment, it is now clear that *A. salmonicida* has the ability to persist in the aquatic environment for protracted periods^[4,21] by entering a viable but non-culturable (VBNC) state. Austin *et al.*^[19] in their experiments reported that factors like temperature at about 15–22 °C and nutritional changes are responsible for reactivating the *A. salmonicida* cells. Perhaps the pond water temperature of $\leq 18^{\circ}\text{C}$ observed in the present study

during the sampling period might have favoured the growth of the atypical strain, *A. salmonicida* subsp. *achromogenes* from its VBNC state. Presumably due to this reason, it might have not been reported earlier in the Indian subcontinent.

The antibiotic sensitivity of the *A. salmonicida* subsp. *achromogenes* of the present study suggested that the bacterium is highly susceptible to broad spectrum antibiotics of human therapeutic importance compared to other bacterial strains (Table 3), which corroborate the observations of Karatas *et al.*^[7]. Aminov and Mackie^[22] reported that bacterial population eliminate antibiotic resistance genes in order to reduce the additional metabolic cost imposed by the carriage of antibiotic resistance and to overcome environmental conditions. It can be assumed that the antibiotic susceptible *A. salmonicida* subsp. *achromogenes* of the present study would have behaved in a similar way due to the unfavourable growth conditions in the composite fish culture pond.

The fishes in the composite culture pond were apparently healthy and no mortality was recorded. As the atypical strain, *A. salmonicida* subsp. *achromogenes* is known to cause ulcer disease and carp erythrodermatitis, involvement of this species in haemorrhagic septicaemia and ulceration of immunologically suppressed Indian major and exotic carps during winter season could not be ruled out. This warrants molecular biology based study on establishing the prevalence of this bacterium especially on its free-living VBNC state in aquaculture systems in the cooler regions of India in order to tackle future disease outbreaks due to exotic species introduction and current trade practices.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Aquaculture involves raising of commercially important fishes and other aquatic organisms. Disease is the major problem that causes production and economic losses in aquaculture worldwide. Identification of the pathogens causing diseases in cultured fishes and evolving effective treatment methods would help in the sustainability of aquaculture.

Research frontiers

The present study reports the occurrence of *A. salmonicida* subsp. *achromogenes* for the first time from India. The study stresses the need to monitor the movement of live fishes as this pathogen was once confined to cold countries.

Related reports

Holt *et al.* 1999 have reported the atypical form of disease caused in fishes by *A. salmonicida* subsp. *achromogenes* often characterized by dermal ulcerations with or without subsequent septicemia.

Although conventional methods like growth on specific media with supplements have been suggested and followed for the identification of *A. salmonicida* isolates (Cipriano and Bertolini, 1988), confirmation of the *A. salmonicida* by FAME, which is more accurate, is followed in this study.

Innovations & breakthroughs

Prevalence of *A. salmonicida* subsp. *achromogenes* has not been reported in cultured fishes from India. This report would help the researchers and diagnosticians working on fishes to widen their diagnostic approach to look for many emerging diseases.

Applications

It may be significant to know the prevalence of atypical strains of *A. salmonicida* as it is often overlooked during fish disease diagnosis and treatment. Hence diagnostic protocols followed for fish diseases should include the procedures to look for such pathogens which were not prevalent before in our country.

Peer review

This is a valuable study as this is the first report on the disease condition caused by *A. salmonicida* subsp. *achromogenes* from India. The results of this study throw light on the spread of the pathogens and on emergence of diseases in newer regions of the world.

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