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Emergence of multidrug-resistant *Raoultella ornithinolytica* associated with Indian major carp

Raoultella ornithinolyitca is a Gram-negative, non-motile bacillus belonging to the family Enterobacteriaceae and has been associated with hospital-acquired clinical cases. The pathogenic potential of *R. ornithinolytica* isolates in human disease has become increasingly important[1]. The well-known factors involved in pathogenicity of *R. ornithinolytica* are its ability to adhere to human tissue, to convert histidine to histamine and to form biofilms in urinary catheters and other surgical equipment. The clinical presentations of infections include bacteraemia[2,3], enteric fever[4], diabetic foot[5], urinary tract infection, billiary tract infection, community and hospital-acquired pneumonia, pleural effusion, osteomyelitis, meningitis, cerebral abscess, pericarditis, conjunctivitis, otitis and skin infections[6]. A high rate of hospital-acquired infections has been reported in patients with immunodeficiency and those who have undergone invasive procedures, e.g. mechanical ventilation, urinary catheters and post-urethral trauma[1]. This bacterium, along with closely related species *R. planticola*, has been shown to be the causative agent of histamine toxicity from fish (also known as scombroid syndrome), but is frequently misidentified as *Klebsiella pneumoniae*. Histamine toxicity results from the expression of histidine decarboxylase, which enables the bacterium to convert histidine, and produces symptoms that include flushing, pruritus, headache and abdominal cramping[7]. Over the past decade, *R. ornithinolytica* has emerged as an infrequent, but important causative agent of human infections[8]. To the best of our knowledge, few cases of *R. ornithinolytica* human infection have been reported worldwide, linking this pathogen to bacteraemia, sepsis, soft tissue and other infections[9]. The bacterium has also been isolated from human digestive organs[1].

Indian major carp (IMC), commonly known as rohu, is one of the most preferred aquaculture fish species among the carps in India and commands a higher market price. However, with the increase in aquaculture production of rohu, occurrence of diseases proves to be significant setback for successful aquaculture by spoilage during cultivation, preservation and trading problems caused by pathogenic bacteria. The most common bacterial pathogens in IMC found in this region are *Aeromonas hydrophila*, *A. liquefaciens*, *A. sorbia*, *A. veronii*, *Edwardsiella tarda*, *Providencia vermicola*, *Acinetobacter baumanni*, *Pseudomonas fluorescence*, *Shigella* sp. and *Chondrococcus columnaris*. *A. hydrophila* accounts for the most common pathogen in IMC and has zoonotic significance[10]. The aquatic environment harbours

pathogenic organisms that can act as a hidden source of public health hazard. A report of mortality in rohu fish farm located at Jagdalpur district (lat. 19.083546N and long. 82.027617E), Bastar plateau, Chhattisgarh, India, prompted us to collect ailing *Labeo rohita* (Hamilton 1822) fishes showing external lesions and transport them to the laboratory for diagnosis.

Sheep blood agar (SBA) and MacConkey agar (MLA) were used as primary culture media for preliminary isolation according to the methods described by Quinn *et al.*[11]. Briefly, samples from kidney blood and gills were streaked on SBA and MLA and incubated at 37°C for 24 h. The purified isolate was subjected to phenotypic and biochemical test reactions. Further, the isolate was identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS, Biomeriex, France).

Apparently healthy IMC juveniles (50 ± 10 g) were obtained from a fish pond at the National Institute of Biotic Stress Management, Raipur. The fish were stocked in 1000 l cement tanks with aerated freshwater and acclimatized for 10 days before starting the experimental infection. Six groups with ten fish in each group were challenged with serial dilutions of isolate (Table 1). The bacterial suspension was made in phosphate buffered saline (PBS) (0.15 M, pH 7.4) and injected to each fish @ 0.1 ml intraperitoneally. The control group was injected with 0.1 ml of PBS intraperitoneally, and mortality pattern was observed till 10 days and the cause of mortality was reconfirmed by re-isolation from vital organs and blood.

Table 1. Biochemical characteristics of *Raoultella ornithinolytica* isolate of fish origin

Biochemical tests	Results
Oxidase	Negative
Catalase	Positive
O/F test	Facultative anaerobe
Motility	Non-motile
Indole production	Negative
H_2S production	Positive
Citrate utilization	Positive
Nitrate reduction	Positive
Urease	Negative
Phenylalanine deamination	Positive
Histidine breakdown	Positive
Lysine utilization	Negative
Ornithine utilization	Positive
Gas from glucose	Positive
Dextrose	Positive
Adonitol	Positive
Lactose	Negative
Arabinose	Negative
Sorbitol	Negative

The isolate was subjected to antibiotic sensitivity testing using disc diffusion method on Mueller–Hinton agar plates as described by Bauer *et al.*[12]. Antimicrobial susceptibility testing was performed according to Clinical and Laboratory Standards Institute, 2017 (ref. 13). The antibiotic discs and EZY MIC strips were obtained from Himedia, Mumbai. The isolate was tested against most commonly used antibiotics and the results were interpreted according to the chart furnished by the manufacturer. Minimal inhibitory concentrations (MICs) were determined against mecillinam, cefotaxim, gentamicin, amikacin, kanamycin, streptomycin, rifampicin and erythromycin by Epsilometer test (E-test). Confirmation for the presence of antibiotic-resistant genes (carbapenemase), extended spectrum-lactamase (^{bla}*TEM*), tetracycline resistant genes (^{bla}*tetW*), fluoroquinolone resistant genes (^{bla}*QnrA*) and class-2 Integerase gene (^{bla}*Int-2*) was carried out employing conventional PCR using previously reported primers[14,15]. Briefly, DNA was extracted from the 18-h-old broth culture using DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's protocol and stored at –20°C till further use. PCR amplification of the genes *QnrA*, *TEM*, *Int-2* and *tetW* using universal primer sets was carried out with DreamTaq Green PCR master mix (ThermoFisher Scientific, USA). The PCR products were further gel-purified using Zymoclean Gel DNA Recovery kit (Zymo Research, USA) according to the manufacturer's instruction and purified products were sequenced commercially (Eurofins India Pvt Ltd, Bengaluru). The sequence data were subjected to BLAST, NCBI homology analysis.

R. ornithinolytica was isolated as a polymicrobial culture with *Escherichia coli* and *Aeromonas veronii*. It was the second most dominant colony of 1 mm diameter size with smooth edges, non-haemolytic on SBA and pale non-lactose-fermenting colonies on MLA after 24 h of incubation. The organism was Gram-negative, non-motile, facultative anaerobic bacillus. It was found to be positive for catalase, phenylalanine deamination, Simmon's citrate, nitrate and H_2S production; while negative for cytochrome oxidase and urease. The organism could ferment glucose and adonitol. Until now, biochemical characterization of the genus *Raoultella* has scarcely been described. Unlike earlier studies, *R. ornithinolytica* in this study yielded non-fermenting colonies on MLA after 24 h of incubation. According to Bhatt *et al.*[16], *R. ornithinolytica* was found to be positive for simon citrate and ornithine decarboxylation but unlike to their findings our isolate could not produce indole. The isolate was further identified as *R. ornithinolytica* (with confidence level 99.9%) by MALDI TOF-MS analysis. Due to the rapidity and sensitivity of MALDI-TOF MS, it has considerable potential for application in large-scale screening for isolates. At present, the extent of diversity within pathogens is a crucial knowledge gap that is slowing down the biotechnological exploitation of this pathogen as well as our capacity to further elucidate its ecological roles. To the best of our knowledge, there are no earlier reports on the isolation and characterization of *R. ornithinolytica* from fishes or aquatic ecosystem from India. Cases of *R. ornithinolytica* may be misidentified as *Klebsiella* species in clinical laboratories using conventional phenotypic identification and therefore, the organisms may be under-recognized as a human pathogen[1]. *R. ornithinolytica* appears to be a Gram-negative aquatic commensal with the ability to adhere to human tissues and form biofilms in urinary catheters[5]. The isolate was found to be positive for histidine breakdown and has been presumed to be the causative agent of histamine toxicity, also known as histamine fish poisoning caused by eating spoiled fish[7,17]. *R. ornithinolytica* has been reported to be the dominant histamine-producing bacteria in fish.

The diseased fish under experimental infection grossly showed ocular opacity and haemorrhages in skin externally. On necropsy there were haemorrhagic enteritis, congestion in heart, liver and kidneys. The lamellae of gills were fused and hyperaemic. The isolate was reconfirmed by morphology, biochemical reactions and MALDI TOF-MS analysis as *R. ornithinolytica*. The organism has been reported as an opportunistic pathogen and recovered as polymicrobial

Table 2. Antibiotic sensitivity pattern of *Raoultella ornithinolytica* isolate of fish origin

Antibiotic discs/symbol	Disc diffusion method		Minimum inhibitory concentrations	
	Zone of inhibition (mm)	Interpretation	Minimum inhibitory concentrations (μg/ml)	Interpretation
Amikacin/Ak	11	R	3.5	S
Ampicillin/AMP	0	R	64	R
Amoxyclav/AMC	0	R	Not performed	
Cefixim/CFM	22	S	Not performed	
Cefotaxim/CTX	25	I	0.094	R
Cefotaxim/Clavulanic acid/CEC	24	I	12/0.32	R
Ceftriaxone/CTR	20	I	Not performed	
Ciprofloxacin/CIP	22	I	3	I
Colistin/CL	0	R	No inhibition	R
Co-trimoxazole/COT	0	R	Not performed	
Doxycycline/DO	0	R	Not performed	
Gentamicin/G	13	I	1	S
Kanamycin/K	12	R	1.5	S
Levofloxacin/LE	15	I	Not performed	
Ofloxacin/OF	13	I	Not performed	
Pefloxacin/PF	18	R	Not performed	
Oxytetracycline/O	0	R	Not performed	
Tetracycline/TE	0	R	Not performed	
Streptomycin/S	12	I	8	–
Trimethoprim/TR	0	R	Not performed	
Rifampicin/RIF	0	R	No inhibition	R
Mecillinam/MEC	0	R	No inhibition	R
Erythromycin/E	0	R	No inhibition	R

*As per Clinical and Laboratory Standards Institute, 2017. Performance Standards for Antimicrobial Susceptibility Testing, Wayne, PA, USA.

culture, which suggests its dependence on co-flora to cause the disease[9].

The isolation of *R. ornithinolytica* isolate with reduced susceptibility to antibiotics was relatively high in this study and in previously reported cases. Frequent hospital outbreaks of infection caused by the selection of multidrug-resistant *R. ornithinolytica* strains have been reported worldwide. The isolate in the present study was highly resistant to ampicillin, amoxicillin, clavulanic acid, tetracycline, doxycycline, co-trimoxazole, rifampicin, kanamycin, trimethoprim, oxytetracycline, colistin and pefloxacin, which confirmed its multidrug-resistant nature. Intermediate resistance was shown towards cefotaxim, cetriaxone, ciprofloxacin, levofloxacin, gentamicin, streptomycin and cephotaxim, whereas the isolate was found to be sensitive to cefixim (Table 2). However, MIC values revealed *R. ornithinolytica* to be sensitive to aminoglycoside antibiotics, while resistant to ampicillin, cefotaxim, cefotaxim + clavulanic acid, colistin, mecillinam, erythromycin and rifampicin, and intermediate resistant to ciprofloxacin (Table 2).

Confirmation for the presence of carbapenemase and extended spectrum-lactamase genes was carried out by PCR amplification and sequencing of the amplicons. PCR screening revealed the presence of ^{bla}*QnrA* (516 bp), ^{bla}*TEM* (481 bp), ^{bla}*Int-2* (403 bp) and ^{bla}*tetW* (168 bp) genes. The amplicons were further confirmed by sequencing of the PCR products and subsequent homology analysis using NCBI BLAST showed 90–98% identity with the available sequences in the public database. The isolate was negative for other metallo-beta-lactamase (MBL) genes. *R. ornithinolytica* produces at least two different chromosomally encoded class A β-lactamases and accordingly they remain resistant to amicillin[18–21]. Resistance of *R. ornithinolytica* to ampicillin has also been reported in 187 isolates from hospital-acquired human infections in China[1] and other cases of human infections[21]. The combination of amino- and carboxypenicillin with clavulanic acid makes *R. ornithinolytica* environmental isolates susceptible to these molecules[20]. The isolate reported in this study was found resistant to amoxicillin–clavulanic acid combination. The isolate was resistant to fluoroquinolones. Similar to our findings, two isolates of *R. ornithinolytica* were recovered from the blood specimen of a 4-year-old child with acute encephalitis and from the sputum specimen of a 43-year-old male with pneumonia in China. Both the strains were resistant to beta lactams, but remained resistant to fluoroquinolones[23]. *R. ornithinolytica* was isolated from sport-related injury in a 13-year-old boy in China and found to harbour ^{bla}*TEM* gene[6]. NDM producing *R. ornithinolytica* isolates have been described in different clinical cases from hospital-acquired infections in India and China[9,16,23]. Borba[24] isolated *R. ornithinolytica* from an environmental sample in Rock Creek Park, Kensington, Maryland, USA, which was found to harbour 12 antibiotic-resistant genes from 7 different categories. To the best of our knowledge, there are no previous reports of the detection of *QnrA*, *Int-2* and *TetW* genes in *R. ornithinolytica*. Multidrug-resistance in Enterobacteriaceae, including resistance to quinolones, is currently among the major antibiotic resistance problems worldwide[25].

According to Okamura and Feist[26], the emerging disease is the one which appeared in the population for the first time or which existed previously, but is now rapidly increasing and spreading to other geographic areas. *R. ornithinolytica* has the potential to be regarded as an emerging opportunistic pathogen. These bacteria

are known to transmit antibiotic resistance genes in the food chain and environment. Therefore, the emergence of this pathogen may cause public health hazards. Further studies to explore detailed genetic features of this isolate are under way.

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Nesting sites of birds and spiders in the semi-arid zone of Rajasthan, India

A plant when present outside its native range is termed as non-native/exotic. Some of these non-native species can outcompete the native species, becoming invasive owing to either phenotypic plasticity, absence of natural predators and pathogens, or the presence of efficient seed dispersal and allelopathic mechanisms[1,2]. Successful eradication of such invasive species is almost impossible and extremely expensive, posing a significant threat to the native biodiversity and community[3,4]. *Prosopis juliflora*, a native plant of Central America, northern South America and the Caribbean islands has invaded several regions throughout the world, including India[5]. According to IUCN 2009 ranking, *P. juliflora* is among the top 100 invasive alien species of the world invading land at a rapid rate[6]. The estimated invasion rate in Ethiopia, and Gujarat, India was 3.48 km^2/year and ~6.19 km^2/year respectively[7,8]. The increased invasion rate was attributed to its high adaptability, germination and dispersal rate[8]. Very few animals graze on the foliage of *P. juliflora* because of its unpalatable leaves and long spines. *P. juliflora* is slowly replacing grassland habitats in Great Rann of Kutch, Gujarat, negatively impacting the livestock population in these areas[7]. *P. juliflora* invaded region has altered soil chemistry[9] and microbiota[10], and reduced the watertable[11] that can further affect the native plant diversity. Within the invaded region *P. juliflora* has impacted indigenous biodiversity and plant communities changing their composition and adversely affecting endangered plant species like *Commiphora wightii* in Jamnagar district, Gujarat[7,12]. *P. juliflora* negatively impacts biodiversity due to its chemical and morphological characteristics[13–17]. In a competition assay, *P. juliflora* outperformed *P. cineraria* in terms of germination, growth rate and drought tolerance[18]. The allelochemicals secreted