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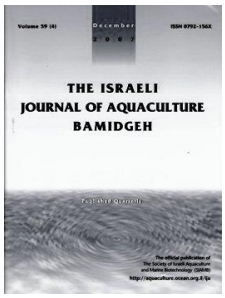


# *Acinetobacter lwoffii*: an Emerging Pathogen for Red Head Disease in Farmed Channel Catfish *Ictalurus punctatus*

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**Keywords:** red head disease; Acinetobacter iwoffii; I.ctalurus punctatus; antibiotic susceptibility

**Abstract**

Red head disease has become a significant emerging cause of economic damage in the farming of channel catfish *Ictalurus punctatus*. Only scare information is available on this disease caused by *Acinetobacter lwoffii* in channel catfish. In this study, a virulent strain, temporarily named R21, was isolated from diseased channel catfish suffering from red head disease, and identified through phylogenetic analysis and phenotypic characteristics. A phylogenetic tree was constructed to examine isolate R21 and compare it to other known isolates. In addition, isolate R21 appears to be resistant to azithromycin, ciprofloxacin, erythromycin, norfloxacin, and oxacillin, but is still susceptible to aminoglycosides, amphenicols, sulfonamides and tetracycline drugs for veterinary uses in aquaculture as revealed when screened against a range of common antibiotics. This study confirms *A. lwoffii* as an emerging pathogen for red head disease in farmed channel catfish.

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## Introduction

Channel catfish *Ictalurus punctatus* is one of the most popular cultured fish species around the world and has become an economically important aquaculture species in Brazil, China, Cuba, Mexico, Russia and the USA (Pool, 2007). Since channel catfish was introduced into China in 1984, it has been farmed on a large scale in nearly 20 provinces (Yan et al., 2013). Its annual output increased to over 450,000 tons in 2014 (Yuan & Zhao, 2015). More bacterial diseases have emerged in farmed channel catfish with increased production due to high density stocking, genetic depression, and improper fish health management (Ge et al., 2001).These have become a major economic problem in catfish aquaculture and should be given more attention to insure a sustainable catfish farming industry (Shewmaker et al., 2007).

Red head disease apparently caused by bacteria is a serious fish disease resulting in significant economic losses in fish farms. It has become the most important infectious disease in the aquaculture of European eel *Anguilla anguilla* and yellow catfish *Pelteobagrus fulvidraco* in China with a mortality of 75-100% (Hu et al., 2002; Ye, 2008). Several bacterial pathogens such as *Edwardsiella tarda* and *Edwardsiella ictarda* have been reported to cause red head disease in European eel and yellow catfish (Zhou et al., 1999; Deng et al., 2008; Zheng, 2009). Limited information is available regarding this disease in channel catfish, as well as *Acinetobacter lwoffii* as a possible causal agent for red head disease in channel catfish.

In this study, *A. lwoffii* was isolated from cage-reared channel catfish suffering from red head disease in Yuanling China during April 2016. Our aim was to characterize the phenotype, taxonomy, and antibiotic sensitivity of this strain. This study confirms *A. lwoffii* as an emerging pathogen for red head disease in farmed channel catfish.

## Materials and Methods

*Channel catfish samples.* Sixteen diseased channel catfish averaging 94.2±31.3 g and suffering from red head disease were sampled from a catfish farm in Yuanling China during April 2016. The farm has 1,000 acres of cages with catfish stocked at an initial rearing density of 50 juveniles per square meter. The water quality during the disease outbreak was pH 7.19, 0.006 mg/L total ammonia, 0.002 mg/L nitrite and 9.12 mg/L dissolved oxygen. Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory.

*Isolation of Bacteria.* Each sampled diseased catfish was externally disinfected with 75% alcohol and dissected. A 0.1 g rotting muscle sample of each catfish was streaked onto nutrient agar (NA) plates (Sinopharm Chemical Reagent Co., Ltd.). After incubation for 24h at 28$^0$C, the dominant uniform isolates were purified by streaking and re-streaking onto NA plates. Pure isolates of the dominant colonies were stored at -80$^0$C supplemented with 15% glycerol. A representative of the dominant isolates, temporarily named R21, was characterized further in the present study.

*Identification of the pathogen*

*Molecular identification.* The extraction of genomic DNA from isolate R21, as well as PCR amplification and sequencing of its 16S rRNA gene were performed according to our previous study (Cao et al*.,* 2010). The near complete 16S rRNA gene sequence was assembled using MegAlign, Editseq and Seqman software. A search was performed in the National Centre for Biotechnology Information (NCBI) database for sequence homology using the Basic Local Alignment Search Tool (BLAST) program. A phylogenetic tree from the near complete 16S rRNA gene sequence of the isolate and its homologous sequences was constructed using the neighbor-joining method.

*Phenotypic identification.* Isolate R21 was identified phenotypically by API 32E system recommended by Qin et al. (2014) where the isolate R21 was grown on nutrient agar (NA) plates (Sinopharm Chemical Reagent Co., Ltd.) at 28$^0$C for 24h, and the bacterial suspension was then used to inoculate the Analytical Profile Index (API 32E) test strip (Biomerieux, France) following the manufacturer's instruction. The strip was incubated at 36$^0$C and observed after 24h for checking against the API identification index and database. Previously reported information related to *A. lwoffii* (Huang et al., 1999; Dong & Cai, 2001) serves as a reference.

*Bacterial virulence assay.* Bacterial virulence was examined by experimentally infecting healthy freshwater cultured channel catfish. One hundred and fifty healthy catfish averaging 49.1±2.4 g were obtained from Baishazhou fishery Co., Ltd. in Wuhan China. Their health status was assessed according to the guidelines in our previous study (Cao et al., 2013). The catfish were acclimated in fifteen replicate aquaria (ten catfish per aquarium) supplied with 50 L of aerated filtered farming water at $18^0$C for 14 days. Prior to the bacterial virulence assay the isolate R21 was inoculated onto NA plate, incubated at $28^0$C for 24h, then washed with normal saline into a sterile tube. Its cell density was determined by counting colony forming units after a ten-fold serial dilution in sterile distilled water. Three samples of ten healthy fish were challenged by muscular injection with 0.1 mL of the isolate R21 at a concentration of $4.5 \times 10^5$ CFU/mL to $4.5 \times 10^8$ CFU/mL. Another three samples of ten healthy catfish exposed to the same experimental conditions and injected intramuscularly with 0.1 mL of normal saline remained unchallenged and served as control. The experimental catfish were kept at $18^0$C and observed daily for seven days without feeding and water change. Any dead catfish were immediately removed and sampled to confirm if mortality was caused specifically by the challenge isolate. The mean lethal dose ($LD_{50}$) value is calculated using the linear regression method as recommended by Won and Park (2008).

*Antibiotic susceptibility assay.* The antibiotic susceptibility of isolate R21 was assayed on NA plates using the Kirby-Bauer disk diffusion method as recommended by Jones et al. (2001). Nineteen fishery antibiotic discs were acquired from Hangzhou Binhe Microorganism Reagent Co., Ltd. The zones of inhibition were measured after a 24h incubation period at $28^0$C. The antibiotic susceptibility was determined according to the manufacturer's guidelines.

## Results

*Identification of the pathogenic isolate.* A dominant strain R21 was isolated from the diseased catfish and identified by molecular and phenotypic methods as *A. lwoffii.* Its near complete 16S rRNA gene sequence (1400 nucleotides) was submitted to GenBank database with the accession no. KX345374. 99% similarity was observed in the 16S rRNA gene sequence between the R21 isolate and other *A. lwoffii* isolates from the GenBank database. The phylogenetic tree confirmed that the isolate R21 is identified with *A. lwoffii* strain (Figure 1). This was again confirmed by the phenotypic features as *A. lwoffii* (Table 1) with 100% identity compared to the reference strain.

**Figure 1.** A 16S rRNA gene tree of 17 known bacteria and the R21 isolate constructed using the neighbour-joining method. The bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

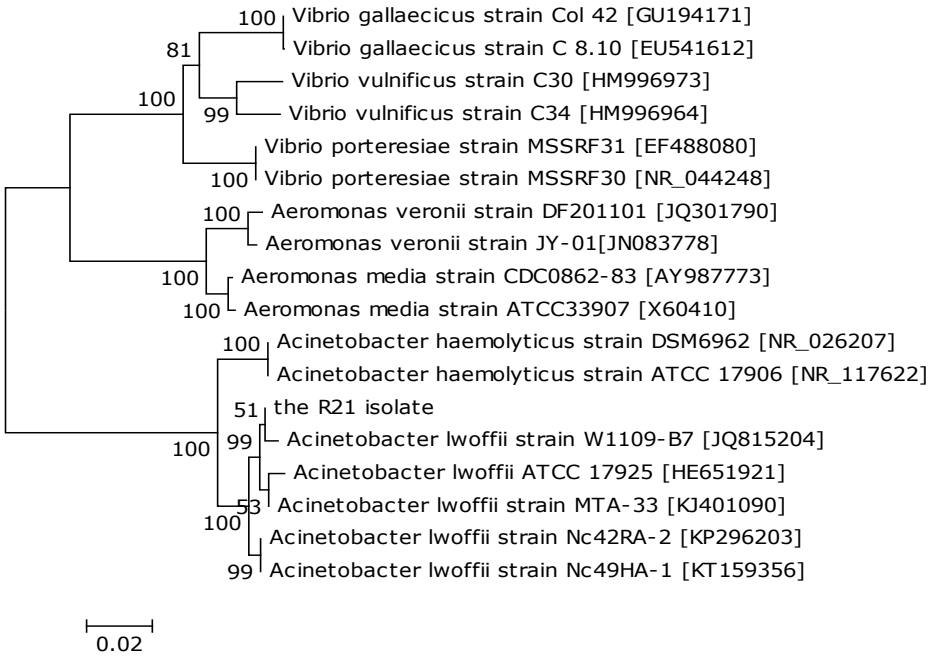


**Table 1.** Phenotypic characterization of isolate R21.

| *Tests* | *Reaction* | |
|---|---|---|
| | *R21* | *A. lwoffii*[a] |
| Arginine dihydrolase | $R^-$ | $R^-$ |
| Lysine decarboxylase | $R^-$ | $R^-$ |
| Lipase | $R^-$ | $R^-$ |
| L-aspartate aminase | $R^-$ | $R^-$ |
| N-acetyl-β-glucosaminidase | $R^-$ | $R^-$ |
| α-galactosidase | $R^-$ | $R^-$ |
| α-glucosaccharase | $R^-$ | $R^-$ |
| α-maltosidase | $R^-$ | $R^-$ |
| β-galactosidase | $R^-$ | $R^-$ |
| β-glucosaccharase | $R^-$ | $R^-$ |
| β-glucuronidase | $R^-$ | $R^-$ |
| Urease | $R^-$ | $R^-$ |
| Orinithine decarboxylase | $R^-$ | $R^-$ |
| Indole production | $R^-$ | $R^-$ |
| Malonate utilization | $R^-$ | $R^-$ |
| Acid production from | | |
| Adonitol | $R^-$ | $R^-$ |
| Galacturonic acid | $R^-$ | $R^-$ |
| Inositol | $R^-$ | $R^-$ |
| L-arabinose | $R^-$ | $R^-$ |
| L-arabitol | $R^-$ | $R^-$ |
| L-rhamnose | $R^-$ | $R^-$ |
| D-arabitol | $R^-$ | $R^-$ |
| D-cellobiose | $R^-$ | $R^-$ |
| D-glucose | $R^-$ | $R^-$ |
| D-maltose | $R^-$ | $R^-$ |
| D-mannitol | $R^-$ | $R^-$ |
| D-sorbitol | $R^-$ | $R^-$ |
| D-sucrose | $R^-$ | $R^-$ |
| D-trehalose | $R^-$ | $R^-$ |
| 5-ketone-potassium gluconate | $R^-$ | $R^-$ |
| Palatinose | $R^-$ | $R^-$ |
| Sodium pyruvate | $R^-$ | $R^-$ |

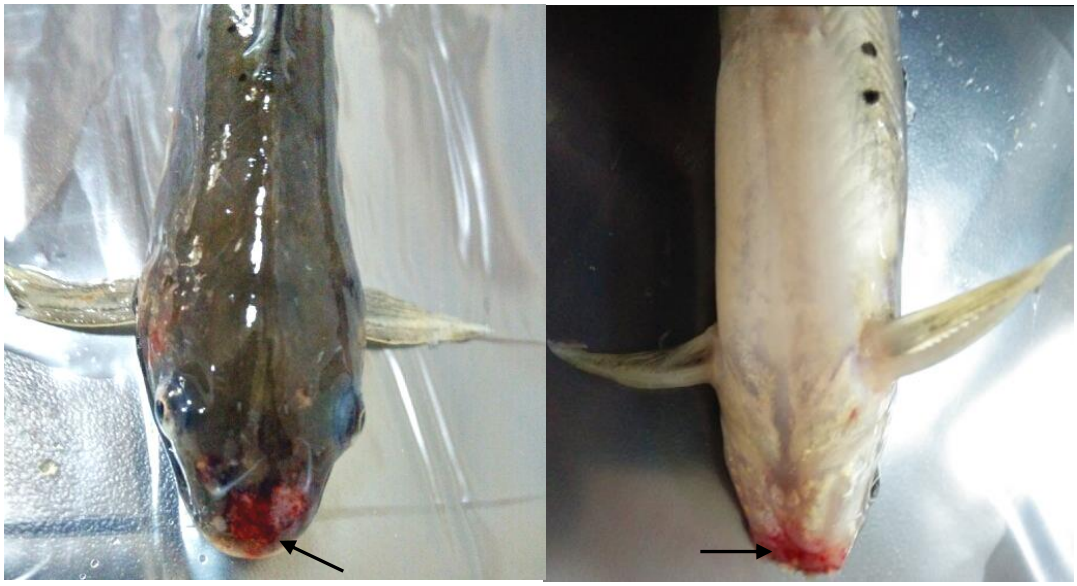
$R^+$: positive reaction; $R^-$: negative reaction. [a]The reference strain's data are in accordance with those previously reported (Huang et al., 1999; Dong & Cai, 2001).

Isolate R21 exhibits potential pathogenicity in an experimental challenge. A mortality of 13.3%-100% of the experimental catfish died after the challenge with isolate R21 at a concentration of $4.5 \times 10^5$ CFU/mL to $4.5 \times 10^8$ CFU/mL (Table 2) with a $LD_{50}$ value of $4.0 \times 10^6$ CFU/mL. All showed signs of red head disease described by Song et al. (2001), similar to those seen in the originally diseased catfish (Figure 2). The re-isolated bacteria from experimentally dead catfish were identified phenotypically and molecularly as isolate R21. No clinical signs or mortality were noted in the control catfish.

**Table 2.** Cumulative mortality of experimental channel catfish infected by isolate R21.

| *Group* | *Concentration (CFU/mL)* | *No. of fish* | *Cumulative mortality (%)* | *Average cumulative mortality (%)* |
|---|---|---|---|---|
| Control | 0 | 10 | 0 | 0 |
| | | 10 | 0 | |
| | | 10 | 0 | |
| Treated 1 | $4.5 \times 10^5$ | 10 | 10 | 13.3 |
| | | 10 | 20 | |
| | | 10 | 10 | |
| Treated 2 | $4.5 \times 10^6$ | 10 | 60 | 56.7 |
| | | 10 | 50 | |
| | | 10 | 60 | |
| Treated 3 | $4.5 \times 10^7$ | 10 | 90 | 86.7 |
| | | 10 | 90 | |
| | | 10 | 80 | |
| Treated 4 | $4.5 \times 10^8$ | 10 | 100 | 100 |
| | | 10 | 100 | |
| | | 10 | 100 | |

**Figure 2.** Pathological symptoms of the farmed channel catfish suffering from red head disease. A: arrow indicates hemorrhage and ulceration in the snout; B: arrow indicates hemorrhage in the lower jaw.



*Antibiotic susceptibility.* The antibiotic susceptibility of isolate R21 is shown in Table 3. The data indicate that isolate R21 is sensitive to amikacin, amoxicillin, cefotaxime, chloromycetin, clindamycin, doxycycline, florfenicol, gentamycin, neomycin, rifampin, streptomycin, sulfamethoxazole, tobramycin, intermediately susceptible to levofloxacin, and resistant to five other tested antibiotics. This suggests that in general, isolate R21 has not developed resistances to aminoglycosides, amphenicols, sulfonamides, and tetracycline antibiotics.

**Table 3.** Susceptibility of isolate R21 to antibiotics.

| *Antibiotics* | *Content (μg/disc)* | *Inhibition zone diameter (mm)* |
|---|---|---|
| Amikacin | 30 | $21.93\pm0.15^{S}$ |
| Amoxicillin | 20 | $27.07\pm0.21^{S}$ |
| Azithromycin | 15 | $9.94\pm0.11^{R}$ |
| Cefotaxime | 30 | $29.69\pm0.15^{S}$ |
| Chloromycetin | 300 | $22.98\pm0.91^{S}$ |
| Ciprofloxacin | 5 | $9.91\pm0.54^{R}$ |
| Clindamycin | 2 | $20.97\pm1.31^{S}$ |
| Doxycycline* | 30 | $30.03\pm1.41^{S}$ |
| Erythromycin | 15 | $9.04\pm0.04^{R}$ |
| Florfenicol* | 75 | $28.95\pm1.23^{S}$ |
| Gentamycin | 10 | $24.24\pm1.10^{S}$ |
| Levofloxacin | 5 | $14.02\pm1.41^{I}$ |
| Neomycin* | 30 | $22.09\pm1.46^{S}$ |
| Norfloxacin | 10 | $0\pm0^{R}$ |
| Oxacillin | 1 | $0\pm0^{R}$ |
| Rifampin | 5 | $22.87\pm0.27^{S}$ |
| Streptomycin | 10 | $21.49\pm0.74^{S}$ |
| Sulfamethoxazole* | 300 | $26.84\pm0.40^{S}$ |
| Tobramycin | 10 | $20.12\pm0.32^{S}$ |

Data are presented as the mean ± standard deviation; $^{S}$Susceptible; $^{I}$Intermediately susceptible; $^{R}$Resistant. *Antibiotics for aquaculture use.

## Discussion

The association of *A. lwoffii* for erecting body disease in striped catfish *Clarias fuscus* (Li et al., 2001), ascites disease in bullfrog *Rana catesbeiana* (Yu et al., 2013), skin hemorrhages and enteritis in common carp *Cyprinus carpio* (L.) (Kozińska et al., 2014) has been documented in aquaculture. However, there is limited information on *A. lwoffii*

as a causal agent for red head disease in cultured catfish. In this study, we characterized the phenotype, taxonomic position, and antibiotic susceptibility of *A. lwoffii* R21. This demonstrates the emergence of *A. lwoffii* as a causative agent for red head disease in farmed channel catfish.

Various virulence factors are involved in the pathogenicity of *Acinetobacter*, including the adherence to epithelial cells, production of extracellular enzymes and toxins, as well as the ability to protect against phagocytosis (Braun, 2008). Diseases caused by *A. lwoffii* are usually associated with the production of these virulent factors. In the present study, the death of healthy channel catfish was caused experimentally by the R21 isolate with a $LD_{50}$ value of $4.0\times10^6$ CFU/mL. This further demonstrates the potential pathogenicity of *A. lwoffii* to farmed channel catfish. Apart from the virulence of the R21 isolate, there might be other secondary factors that induce red head disease in channel catfish, such as high density stocking, misuse of contaminated feed, incomplete disinfection of farming water and improper fish health management; these should also be raised as concerns.

Intensive fish farming has resulted in the massive use of antibacterial agents for treatment of fish bacterial diseases (Smith et al., 1994; Hu et al., 2015). Antibiotic susceptibility in *Acinetobacter* species has become a major concern because of their rapid development of resistance to a wide range of antimicrobials. Five *A. lwoffii* isolates from Chilean salmon farm were susceptible to gentamycin, kanamycin and resistant to tetracycline, oxytetracycline (Doughari et al., 2011; Miranda & Zemelman 2002). Two *A. lwoffii* isolates from diseased *Cyprinus carpio* (L.) showed susceptibility to gentamycin, enrofloxacin, flumequine, norfloxacin, and developed resistance to amoxicillin and cephalotin (Kozińska et al. 2014). In our study, the susceptibility of the isolate obtained is the same as that confirmed by Miranda & Zemelman (2002) and Kozińska et al. (2014) in gentamycin. R21 isolate has also exhibited sensitivity to aminoglycosides, amphenicols, sulfonamides, and tetracycline antibiotics used in fish farming regions, suggesting that the outbreak of this disease may not be the result of antibiotics abuse.

In conclusion, the present study identified *A. lwoffii* isolate as a causal agent for red head disease in cultured channel catfish. The pathogenicity of the R21 isolate supports this infection as an emerging threat in catfish farming. Better management, lower densities, and treatment with efficient antibiotics are suggested to avoid future outbreaks of this disease.

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