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Molecular characterization and pathogenicity of *Klebsiella pneumoniae* species complex associated with mass mortality of *Oncorhynchus mykiss* in the Ladakh trans-himalayas

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Bacterial diseases pose a significant threat to the entire aquaculture system, both in cold and warm-water environments. The Himalayan region has been widely utilised for trout farming. However, this region is presently under tremendous stress due to the outbreak of several diseases, which are responsible for a lot of mortality and have resulted in significant economic loss. In the present study, *Klebsiella pneumoniae* belonging to the *K. pneumoniae* species complex was isolated from diseased rainbow trout *Oncorhynchus mykiss* cultured in the trans-Himalayan region of Ladakh. Infected fish were identified based on exhibited lethargy, tail fin erosion, abdominal distension, abnormal swimming, and intraperitoneal haemorrhage. The pathogen was identified through morphological, biochemical, and molecular analyses, including partial 16 S rRNA gene sequencing. The phylogenetic analysis confirmed the close genetic relationship of the SLB4 isolate to *K. pneumoniae* (NR_036794.1), as supported by strong bootstrap values, though we acknowledge that 16 S rRNA sequencing has limitations in discriminating among members of the *K. pneumoniae* species complex. Antimicrobial susceptibility testing was also carried out, revealing multidrug resistance to ceftazidime, ampicillin, rifampicin, ceftriaxone, trimethoprim-sulfamethoxazole, cefixime, and cefepime, but sensitivity to several aminoglycosides, carbapenems, fluoroquinolones, and other agents. Histopathological examination of infected muscle, liver and kidney tissues revealed severe lesions, including melano-macrophage centres, cytoplasmic vacuolation, focal necrosis, and structural disruption of renal tubules and glomeruli. The present findings confirm a member of the *K. pneumoniae* species complex as a pathogenic agent in *O. mykiss*, underscore its capacity to cause systemic organ damage, and highlight its potential role in disseminating multidrug resistance in aquaculture environments. In addition, based on the above findings, active surveillance and antibiotic stewardship have been recommended to mitigate the impact of these infections in cultured rainbow trout.

Keywords Trans-Himalayas, Bacterial disease, *Klebsiella pneumoniae* species complex, Multidrug-resistance, *Oncorhynchus mykiss*

The trans-Himalayan higher altitude region holds a significant position for its rich biodiversity, encompassing a wide range of terrestrial and aquatic ecosystems. Within this region, the cold desert of Ladakh supports only a limited number of freshwater bodies, including streams and rivers fed primarily by glacial meltwater from the

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Himalayas. Among all the water bodies, the Suru River, which flows through Kargil, is a major contributor to the livelihoods of the sizeable population. Originating in the Zanskar range of the northern Himalayas, the Suru River also serves as a critical hydrological and ecological lifeline to the entire region. It exhibits marked seasonal changes appearing highly turbid during the summer months and developing a frozen surface layer in winter. Fisheries are considered an essential source of livelihood and nutrition for mountain communities. The ichthyofauna of the region is mainly dominated by trout groups, with *Oncorhynchus mykiss* being one of the most widely cultivated. Owing to its rapid growth rate and superior flesh quality, this species has become a significant focus of modern aquaculture practices. Current aquaculture systems in the area often involve pond management strategies that include the use of inorganic fertilisers and chemicals to boost productivity and maintain favourable rearing conditions. However, significant achievements have been made in the production of rainbow trout cultured under cold-climate conditions, the entire Himalayan region faces significant threats and losses due to disease outbreaks in the farming system to increased stocking densities and the use of artificial feeds to accelerate the growth of cultured fish[30]. The close confinement and high stocking densities inherent in these systems increase the likelihood of disease outbreaks compared to free-living fish in natural environments. Adverse alterations in the physical, chemical, biotic, and abiotic conditions of freshwater habitats, when persistent or left unaddressed, can rapidly trigger infectious diseases in fish populations[3]. Consequently, fish farms and hatcheries frequently experience substantial losses of rainbow trout due to the widespread occurrence of pathogenic bacteria[12,41,51]. *Klebsiella spp*. are pathogenic bacteria that can infect a wide range of hosts, including fish. These rod-shaped, non-motile, Gram-negative, oxidase-negative organisms belong to the genus *Klebsiella* and naturally occur as part of the microflora in diverse environments such as water bodies and soil wastes [24,35]. Taxonomically, they fall under the family Enterobacteriaceae and encompass several species, including *Klebsiella pneumoniae*, *K.terrigena*, *K. mobilis*, *K. oxytoca*, *K. variicola*, *K.singaporensis*, *K. planticola*, *K. granulomatosis*, *K. trevisanii*,and *K. ornithinolytica*[16,17]. Recent genomic studies have refined our understanding of the *Klebsiella* genus, revealing that *K. pneumoniae* comprises a species complex (KpSC) that includes *K. pneumoniae sensu stricto*, *K. variicola*, *K. quasipneumoniae* (with subspecies), and several more recently described taxa[54,55] PMID: 32055025). *Klebsiella pneumoniae* (*sensu lato*) is a versatile pathogen implicated in a wide spectrum of infections across multiple hosts. In humans, it causes urinary tract infections, wound infections, pneumonia, and bacteremia[22,28]. In canines, it has been associated with septicemia and severe enteritis[43] whereas in *Zalophus californianus* (California sea lions), it has been linked to suppurative pneumonia and pleuritis[25]. In aquatic organisms, members of the *K. pneumoniae* species complex cause diverse pathological manifestations. Several studies in the past have reported the effects of *Klebsiella pneumoniae* (*sensu lato*) in fish species such as *Cyprinus carpio*, infections present as haemorrhages and red spots on the body[1,35]. Skin discoloration, ulcers, and exophthalmia in *Nemipterus japonicus*[14], *Oreochromis niloticus*[48], and *Amphiprion nigripes*[21] which are characterised by skin haemorrhages and systemic effects. The pathogenicity of members of the *K. pneumoniae* species complex is mediated by an array of virulence genes. These include allS (activator of the allantoin regulon), uge (uridine diphosphate galacturonate 4-epimerase), ureA (part of the urease operon), magA (microviscosity-associated gene A), mrkD (type 3 fimbriae adhesins), kfuBC (iron acquisition system), rmpA (regulator of mucoid phenotype), wabG (outer core lipopolysaccharide synthesis), and fimH (type 1 fimbriae adhesins). These factors enhance bacterial survival by facilitating host tissue invasion, immune evasion, and the establishment of infection[9,18,20,27]. Despite its significance in other hosts, reports of *K. pneumoniae* species complex infections in fish remain scarce. The present investigation represents the first documented isolation of a member of the *K. pneumoniae* species complex from diseased *O. mykiss* cultured in the trans-Himalayan region of Ladakh, India. This study aimed to identify the causative agent of a bacterial disease outbreak in *O. mykiss* through a comprehensive approach integrating morphological and biochemical analyses, 16 S rRNA gene sequencing, and pathogenicity assessment, while acknowledging that precise species-level identification within the complex would require whole-genome sequencing.

Materials and methods

Sampling

Naturally infected *Oncorhynchus mykiss* specimens were obtained from the Government Trout Culture Farm located in Damsna, Suru Valley (34°10′33″N; 75°56′10″E), Kargil, Ladakh, India, for the isolation of pathogenic bacteria (Fig. 1).

The specimens were collected after observing clinical signs, such as irregular swimming patterns and distended abdomens in juveniles. Adult moribund fish exhibited pronounced haemorrhages near the caudal and anal fin regions, along with severe erosion of the tail fin. Upon detailed examination, the pathological manifestations were not uniform among all affected individuals. To facilitate handling, fish were euthanised prior to sampling using an overdose of tricaine methane sulfonate using MS-222 (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. E10521). Fish were immersed in buffered MS- 222 solution (250–300 mg L^{-1}) until complete loss of opercular movement and absence of reflex response were observed. Before dissection, specimens were externally disinfected with alcohol to minimize contamination. Swab samples were aseptically collected from skin lesions, liver, and kidney tissues and directly inoculated onto Nutrient Agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India; Cat. No. M001), MacConkey Agar (HiMedia; Cat. No. M081), and Blood Agar Base (HiMedia; Cat. No. M073) supplemented with 5% sterile defibrinated sheep blood. Following incubation at 37 °C for 24–48 h, dominant colonies were subcultured to obtain pure cultures, with the confirmed isolate (designated SLB4) maintained in tryptic soy broth and preserved in glycerol at – 20 °C for long-term storage.

Biochemical profiling of the isolated bacterial strain

Initial characterisation of the isolated strain involved Gram staining, followed by a comprehensive set of biochemical assays. These included tests for catalase activity, esculin hydrolysis, malonate utilization, and carbohydrate fermentation using xylose, arabinose, adonitol, rhamnose, raffinose, cellobiose, lactose, melibiose,

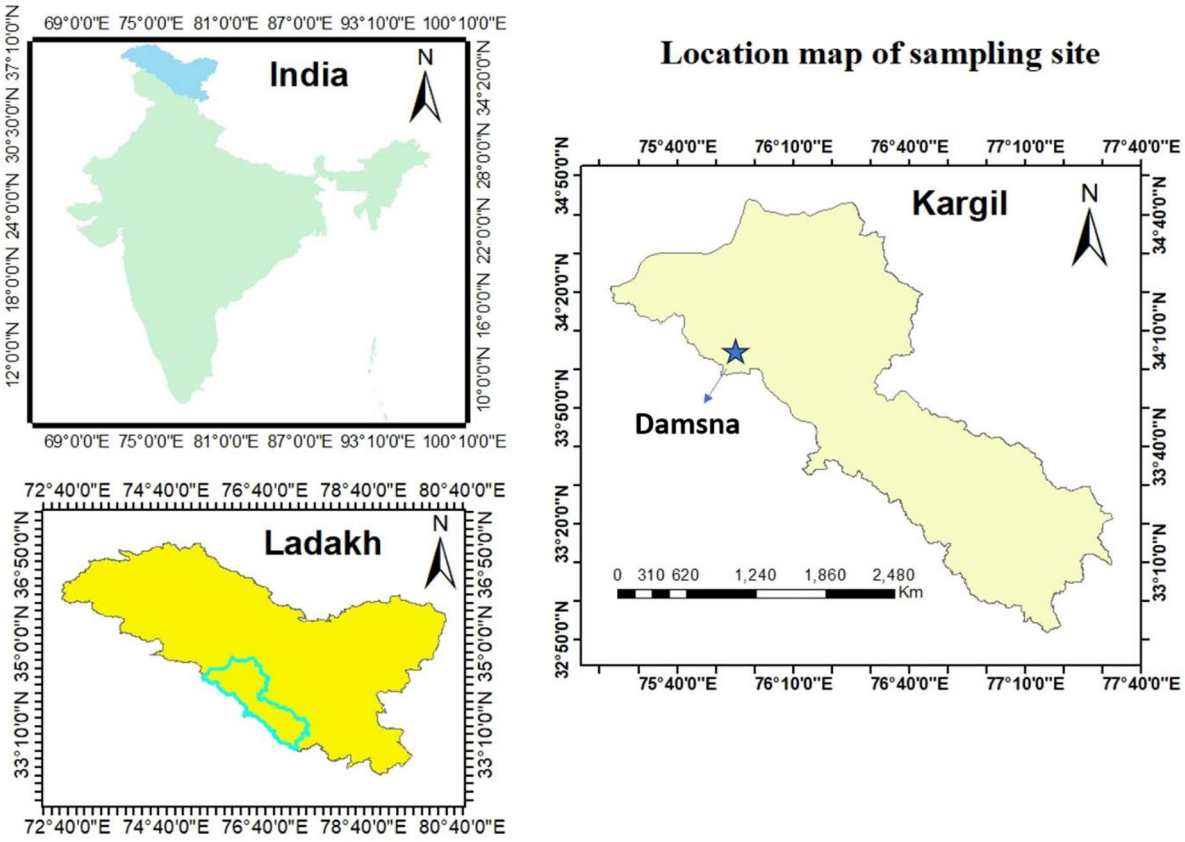

Fig. 1. Location map of sampling site Damsna in district Kargil (Ladakh).

No.	Oligo Name	Sequence (5`à 3`)	Tm (°C)	GC- Content
1	16 S Forward	GGATGAGCCCGCGGCCTA	57	72.22%
2	16 S Reverse	CGGTGTGTACAAGGCCCGG	58	68.42%

Table 1. Primer sequences and melting temperatures (Tm) used for the PCR amplification of the 16 S rRNA gene in *Klebsiella pneumoniae* isolate SLB4.

trehalose, glucose, and sucrose. Additional assessments comprised ornithine utilization, oxidase activity, phenylalanine deamination, urease activity, ONPG, citrate utilization, nitrate reduction, lysine utilization, Voges–Proskauer (VP) reaction, hydrogen sulfide (H_2S) production, methyl red, and indole production. The assays were conducted using the Biochemical Identification Kit (HiMedia Laboratories Pvt. Ltd., Mumbai, India; Cat. No. KB003). For further confirmation, biochemical profiling was performed using the BD Phoenix M50 (USA) automated bacterial identification system.

DNA extraction and 16 S rRNA gene amplification

Genomic DNA from the bacterial isolate was extracted using the standard protocol. The integrity and quality of the extracted DNA were assessed via electrophoresis on a 1% agarose gel stained with ethidium bromide. The 16 S rRNA gene was amplified using gene-specific primers in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, USA). Details of the primers and their corresponding annealing temperatures are provided in Table 1. The PCR amplicons were subsequently analyzed on a 2% agarose gel stained with ethidium bromide to confirm successful amplification.

DNA Sequencing and phylogenetic characterization of the bacterial isolate

The PCR-amplified products were bidirectionally sequenced using the ABI 3130 Genetic Analyzer (Applied Biosystems, USA) to obtain high-accuracy sequence data. The 16 S rRNA gene sequence of the SLB4 isolate was compared with sequences available in GenBank using the NCBI–BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). A multiple sequence alignment of SLB4 with reference Klebsiella species retrieved from the NCBI GenBank database was performed using MEGA 11[49]. Phylogenetic relationships were inferred via the

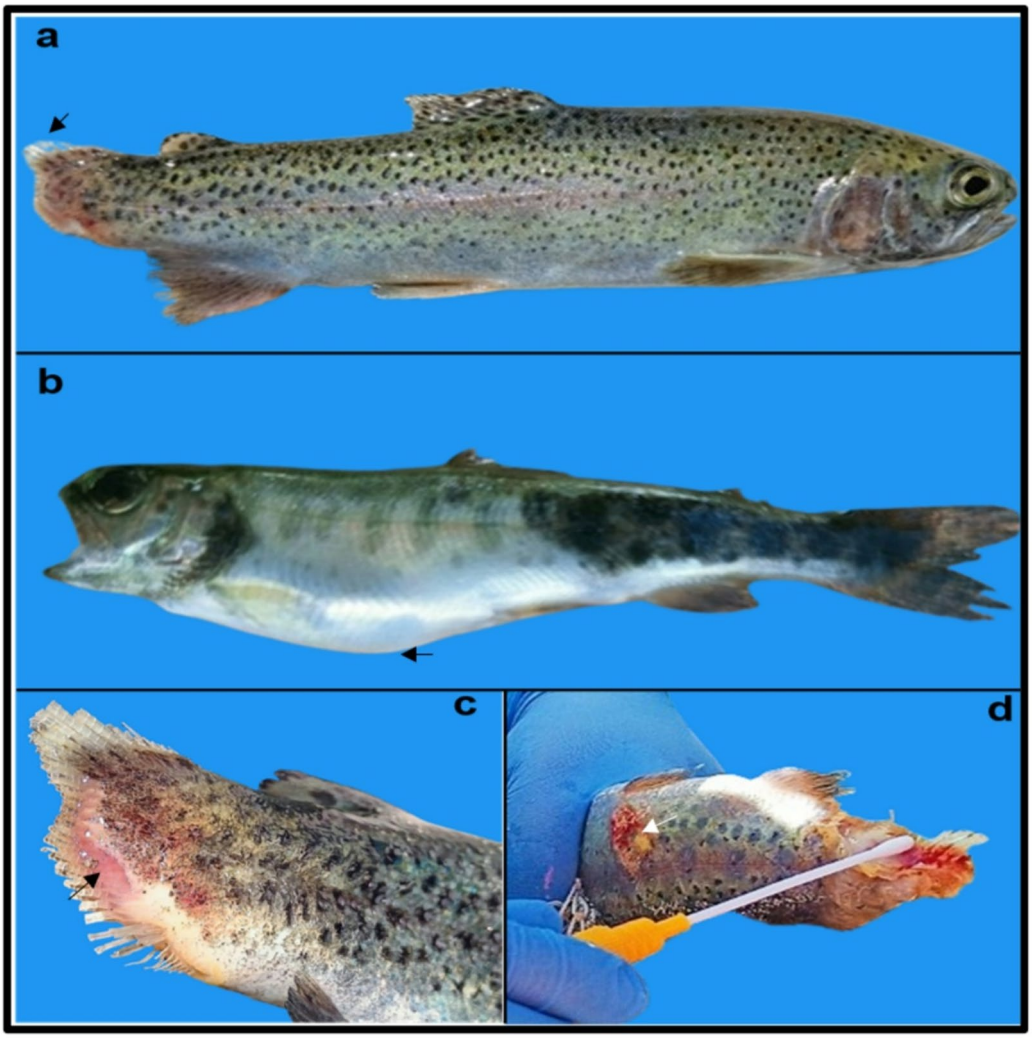

Fig. 2. External pathological signs in *Oncorhynchus mykiss* (rainbow trout) naturally infected with isolate SLB4 (GenBank accession no. PQ676432), isolated and characterized in this study. Manifestations include: (a) adult trout with complete necrosis of the tail fin, (b) juvenile trout showing abdominal distension, (c) close-up of necrotic tail fin with hemorrhages at the caudal region, and (d) ulcers with prominent hemorrhages on the body surface.

neighbour-joining method, with *Salmonella enterica* serving as the outgroup. The reliability of the phylogenetic tree topology was evaluated through bootstrap analysis with 1000 replicates in MEGA 11.

Antibiotic susceptibility test

The antibiotic susceptibility profile of the bacterial isolate was assessed using the BD Phoenix M50 automated system. The antimicrobial agents tested included amoxicillin–clavulanate, ampicillin, amikacin, tetracycline, cefepime, cefixime, trimethoprim–sulfamethoxazole, gentamicin, ciprofloxacin, imipenem, meropenem, colistin, streptomycin, rifampicin, nitrofurantoin, nalidixic acid, piperacillin–tazobactam, ofloxacin, ceftazidime, ceftriaxone, cefoperazone–sulbactam, erythromycin, ertapenem, and levofloxacin.

Results

Clinical signs and gross pathology

Gross examination of diseased rainbow trout revealed marked external and internal pathological alterations indicative of a severe systemic infection. Externally, affected fish exhibited extensive haemorrhages, particularly around the caudal and anal fin regions, along with progressive erosion of the tail fin (Fig. 2). The severity of fin damage varied among individuals; approximately 75% of the affected fish showed complete erosion of the caudal fin with exposure of the underlying musculature, whereas the remaining 25% exhibited partial fin rot characterized by fraying of fin margins and erosion of up to half of the fin tissue. In several cases, abdominal distension and loss of normal body coloration were also observed. On internal examination, the visceral organs appeared congested and pale, with occasional enlargement of the liver and spleen. The musculature adjacent to

the eroded fin regions showed signs of necrosis and tissue degradation, suggesting progressive bacterial invasion and tissue destruction. Petechial haemorrhages were also noted in some specimens, supporting the presence of systemic septicaemia. Overall, the gross pathological findings were consistent with a severe Gram-negative bacterial infection associated with extensive tissue damage, vascular compromise, and systemic involvement.

Histopathology

For reference, healthy rainbow trout tissues typically exhibit well-organised hepatic parenchyma with polygonal hepatocytes containing centrally placed nuclei and uniform cytoplasm; renal tissue with intact glomeruli and well-defined renal tubules lined by cuboidal epithelial cells; and skeletal muscle with orderly arranged myofibers showing distinct striations and minimal interstitial spaces. Histopathological examination of infected *O. mykiss* revealed severe, multi-organ pathological alterations compared to normal tissue architecture (Fig. 3). In the liver, marked hepatocellular degeneration was evident, characterized by cytoplasmic vacuolation, scattered necrotic hepatocytes, and disruption of cellular integrity. Prominent melano-macrophage centres appeared as dense aggregates of pigment-laden immune cells, indicating chronic inflammatory response and active phagocytosis of cellular debris and pathogens. These degenerative and necrotic changes suggest metabolic disturbance, toxin-mediated injury, and hypoxic stress associated with systemic infection. Kidney tissue exhibited pronounced pathological lesions, including glomerular degeneration with disruption of capillary tuft integrity and tubular degeneration marked by cytoplasmic vacuolation, epithelial cell swelling, and focal necrosis. Diffuse infiltration of inflammatory cells and the presence of melano-macrophage centres further indicated a strong systemic immune response and impaired renal filtration and excretory functions. Similarly, muscle tissue showed marked degenerative changes, including myofiber disruption, loss of normal striations, irregular fiber outlines, and variable eosinophilia, indicating myopathic damage likely associated with bacterial invasion and toxin-mediated injury. Inter-fiber edema and myofibrillar separation were observed as widened interstitial spaces between

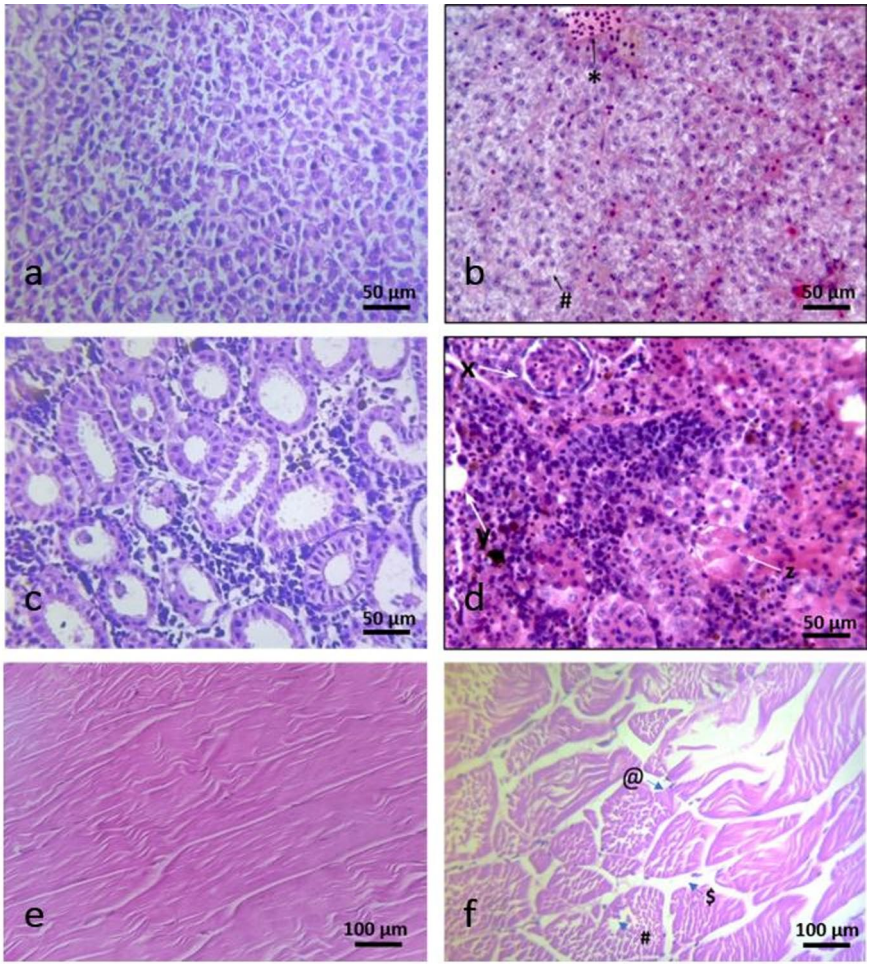

Fig. 3. Histopathological alterations in liver, kidney, and muscle of healthy control and infected *Oncorhynchus mykiss* (H&E). Control liver (A) shows normal hepatocytes, while infected liver (B) displays vacuolation, necrosis (#), and melano-macrophage centres (*). Control kidney (C) has intact tubules and glomeruli; infected kidney (D) shows glomerular degeneration (X), inflammatory infiltration (Y), and tubular necrosis (Z). Control muscle (E) exhibits orderly myofibers with striations; infected muscle (F) shows myofiber degeneration (#), inter-fiber edema ($), and structural disruption (@). Scale bars: 50 µm (liver, kidney); 100 µm (muscle).

muscle bundles, along with vacuolar degeneration within the sarcoplasm, suggesting metabolic injury and early cellular degeneration.

Cultural and morphological characteristics

Following incubation at 37 °C for 24–48 h, distinct colony morphologies were observed across different media. On nutrient agar, the majority of colonies appeared smooth and shiny, characteristic of Klebsiella species. MacConkey agar produces pink mucoid colonies indicated active lactose fermentation, a key biochemical characteristic of members of the *K. pneumoniae* species complex within the Enterobacteriaceae family. On blood agar, large, moist colonies developed without haemolysis, reflecting the non-hemolytic nature typical of this group (Fig. 4). The consistent cultural and morphological traits across different media provided strong preliminary evidence for the pathogen's identification, which was later validated by biochemical and molecular analyses.

Phenotypic and biochemical characterization

Biochemical characterization identified the SLB4 isolate as a Gram-negative rod. The strain tested positive for malonate, urease, glucose, arabinose, melibiose, oxidase, cellobiose, ONPG, nitrate reduction, lysine, Voges-Proskauer (VP), adonitol, esculin hydrolysis, rhamnose, raffinose, lactose, sorbitol, saccharose, mannitol, sucrose, and trehalose. Negative reactions were recorded for ornithine decarboxylation, hydrogen sulfide (H_2S) production, citrate utilization, phenylalanine deamination, indole, and methyl red (summarized in Table 2; detailed BD Phoenix results in Supplementary Table S1). These biochemical features are consistent with member in the *K. pneumoniae* species complex.

Molecular identification and phylogenetic analysis

The 16 S rRNA gene of the bacterial isolate SLB4 was successfully amplified using PCR, yielding an amplicon of approximately ~ 1.4 kb. The amplification was confirmed by agarose gel electrophoresis (Supplementary Figure S1). The PCR product was subsequently sequenced and deposited in GenBank under accession number PQ676432. BLAST analysis of the 16 S rRNA gene sequence revealed ≥ 99–100% similarity with multiple *Klebsiella pneumoniae* reference strains available in GenBank. Phylogenetic analysis (Fig. 5) showed that isolate SLB4 clustered with *K. pneumoniae* reference strains (e.g., NR_036794.1) with strong bootstrap support (≥ 99%). However, it should be noted that the 16 S rRNA gene is highly conserved among members of the *K. pneumoniae* species complex, and strong bootstrap values primarily reflect sequence similarity rather than definitive taxonomic discrimination at the species level. Therefore, while our analyses confirm that SLB4 belongs to the *K. pneumoniae* species complex, precise species-level identification whether *K. pneumoniae*, *K. variicola*, *K. quasipneumoniae*, or a closely related taxon would require higher-resolution molecular approaches such as whole-genome sequencing.

Antibiogram study

Antimicrobial susceptibility testing of *Klebsiella pneumoniae* strain SLB4, isolated from diseased *O. mykiss*, revealed a distinct multidrug-resistance (MDR) profile (Table 3). The isolate exhibited resistance to 7 of 24 antibiotics (29.2%), yielding a Multiple Antibiotic Resistance (MAR) index of 0.29, indicating prior exposure to antibiotic-contaminated environments. The highest resistance frequencies were observed against third- and fourth-generation cephalosporins (ceftazidime, ceftriaxone, cefixime, and cefepime) and β-lactam agents, including ampicillin and rifampicin. In contrast, complete sensitivity (100%) was observed toward carbapenems (imipenem, meropenem, ertapenem), aminoglycosides (amikacin, gentamicin, streptomycin), and fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin).

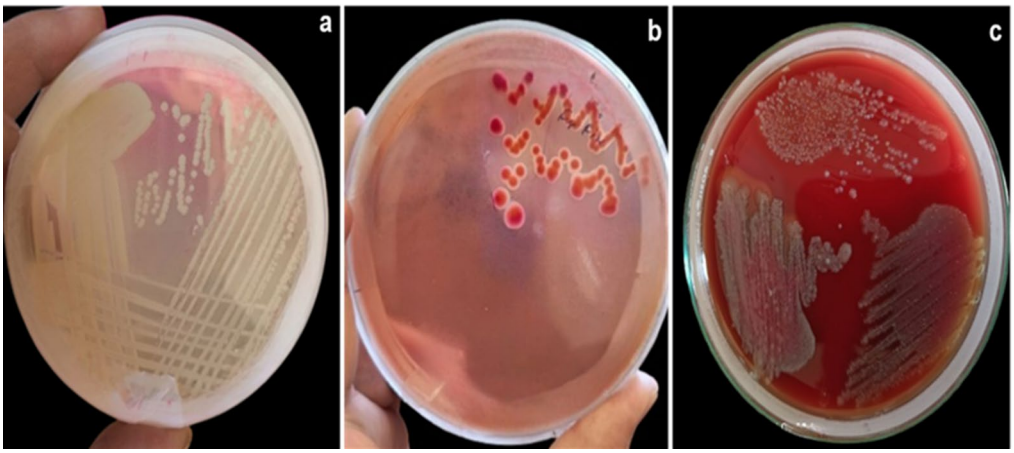

Fig. 4. Colony morphology of the isolate after 24–48 h at 37 °C: (a) smooth, shiny colonies on nutrient agar, (b) pink, mucoid colonies on MacConkey agar (lactose fermentation), and (c) large, moist, non-hemolytic colonies on blood agar.

Test Category	Parameter	SLB4 Result	Reference *K. pneumoniae*
Morphology	Gram stain	-	-
	Shape	Rod-shaped	Rod-shaped
	Motility	Non-motile	Non-motile
Basic Enzyme Tests	Catalase	+	+
	Oxidase	+	+
	Urease	+	+
	Phenylalanine deamination	-	-
Sugar Fermentation	Glucose	+	+
	Lactose	+	+
	Sucrose	+	+
	Mannitol	+	+
	Sorbitol	+	+
	Trehalose	+	+
	Raffinose	+	+
	Saccharose	+	+
	Melibiose	+	+
	Cellobiose	+	+
	Rhamnose	+	+
	Adonitol	+	+
	Xylose	+	+
	Arabinose	+	+
	Esculin hydrolysis	+	+
Other Biochemical Reactions	Malonate utilization	+	+
	ONPG (β-galactosidase)	+	+
	Lysine utilization	+	+
	Ornithine utilization	-	-
	Citrate utilization	-	-
	Hydrogen sulfide (H_2S) production	-	-
	Indole production	-	-
	Methyl red	-	-
	Voges–Proskauer (VP)	+	+
	Nitrate reduction	+	+

Table 2. Morphological and biochemical properties of isolate SLB4 compared with reference strain data. *(Reference strain characteristics from Bergey's Manual, Frederiksen 2015).*

Discussion

Bacterial diseases continue to pose significant threats to sustainable aquaculture production worldwide, particularly both intensive and semi-intensive farming systems where fish are exposed to complex interactions of environmental, nutritional, and husbandry-related stressors[4,13]. Opportunistic pathogens exploit these stress-induced immunosuppressive states, especially in cold-water salmonids that are inherently sensitive to fluctuations in water temperature, dissolved oxygen, and stocking density. In recent years, an increasing number of emerging and non-classical pathogens have been reported in aquaculture systems. These includes *Providencia*, *Achromobacter*, *Raoultella*, and *Klebsiella* species[31,40]. Among these, bacteria belonging to the *Klebsiella pneumoniae* species complex, traditionally considered as a human and terrestrial animal pathogen, has received growing attention for its ability to colonize in aquatic environments reseulted disease in fish[5,47]. The present study documents the natural occurrence of an infection caused by a member of the *K. pneumoniae* species complex in *O. mykiss* collected from the world's highest altitude rainbow trout culture farm in Ladakh, India. In the first phase of the study, clinical signs observed in diseased trout, including lethargy, frayed fins, abdominal distension, haemorrhages, and erratic swimming, were consistent with systemic Gram-negative bacterial infections and closely resembled those documented in other infections caused by members of the *K. pneumoniae* species complex in fish species such as *Oreochromis niloticus*[2] and *Amphiprion nigripes*[37]. These clinical manifestations reflect septicaemia, extensive tissue damage, and host immune responses triggered by endotoxins and virulence determinants commonly associated with this group, including lipopolysaccharide (LPS), siderophores, fimbriae, and capsule polysaccharides[36]. The observed endothelial injury and haemorrhagic lesions further support the occurrence of systemic infection, and such vascular damage is likely multifactorial, involving (i) direct cytotoxic effects of bacterial exotoxins (e.g., haemolysins and cytotoxins) on endothelial cells[36], (ii) an exaggerated host inflammatory response leading to neutrophil-mediated damage and vasculitis [54]. It should be noted, however, that while the clinical signs, bacterial isolation, and histopathological findings strongly suggest that the isolated strain is the causative agent, experimental challenge studies fulfilling Koch's

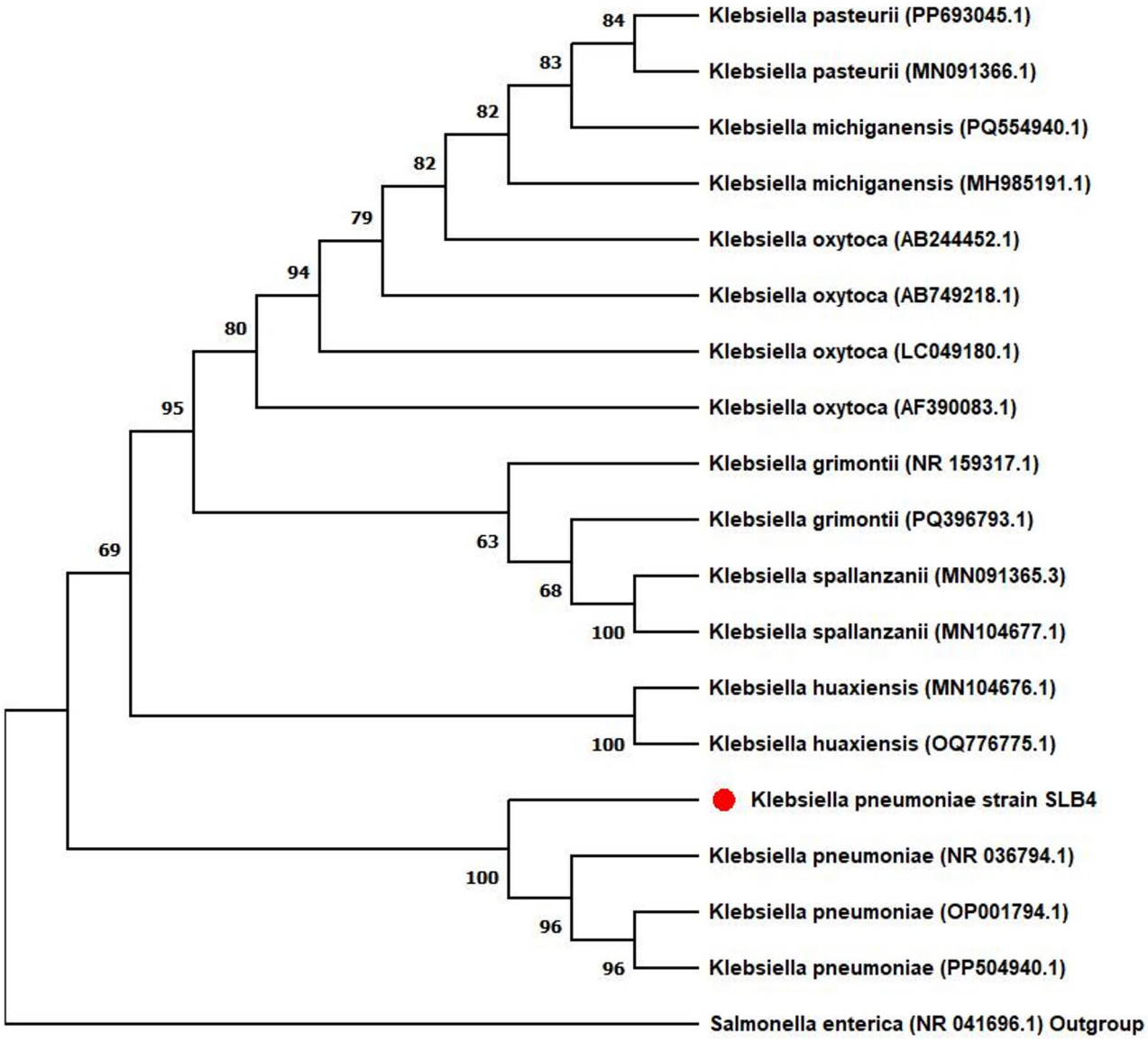

Fig. 5. Phylogenetic tree based on 16 S rRNA gene sequences showing the relationship of isolate SLB4 with closely related *Klebsiella* reference strains retrieved from GenBank was constructed using MEGA 11 software. Bootstrap values (> 50%) are indicated at the branch nodes. *Salmonella enterica* was used as the outgroup.

postulates would be required to definitively establish causality. The naturally occurring infection documented in the present study is particularly significant, as it demonstrates that members of the *K. pneumoniae* species complex can induce disease in trout under farm conditions without any experimental challenge, suggesting that the bacterium persisted in the culture environment long enough to establish infection. Possible environmental reservoirs include sediment, inflowing stream water, contaminated feed, human activity, and the resident fish microbiota, as previously reported in environmental studies of *K. pneumoniae*[29,39]. The biochemical characteristics of the isolate carried out in the present study further supported its identification as a member of the *K. pneumoniae* species complex, showing typical features such as lactose fermentation and indole negativity, consistent with classical descriptions[23]. Phenotypic identification remains highly valuable, especially in remote aquaculture settings, because it provides rapid confirmation before molecular results are obtained. The molecular identification through partial 16 S rRNA gene sequencing carried out in the present study provided strong corroboration, with the isolate (SLB4) showing ≥ 99–100% sequence similarity to reference *K. pneumoniae* strains. However, we acknowledge that the 16 S rRNA gene is highly conserved among members of the *K. pneumoniae* species complex and may not always discriminate *K. pneumoniae sensu stricto* from closely related species such as *K. variicola* or *K. quasipneumoniae*[7,44,55]. Therefore, while our phylogenetic analysis confirms that SLB4 belongs to the *K. pneumoniae* species complex, definitive species-level classification would require higher-resolution approaches such as whole-genome sequencing, which would also enable detailed characterization of virulence genes, antimicrobial resistance determinants, and surface antigen (wzi) typing as described by[8] and [56] (PMID: 24088853). Histopathological observations in infected trout further confirmed the pathogenicity of

Class	Antibiotic	MIC (µg/mL)	Result
Aminoglycosides	Amikacin	≤16	S
	Gentamicin	≤4	S
	Streptomycin	≤25	S
Carbapenems	Imipenem	≤0.25	S
	Meropenem	≤0.5	S
	Ertapenem	≤0.25	S
Cephalosporins	Cefixime	>4	R
	Ceftazidime	32	R
	Ceftriaxone	>4	R
	Cefepime	>16	R
	Cefoperazone-Sulbactam	≤0.5/8	S
β-lactams	Ampicillin	>32	R
	Amoxicillin-Clavulanate	≤8/4	S
	Piperacillin-Tazobactam	≤4/4	S
Polymyxins	Colistin	≤0.5	S
Sulfonamides	Trimethoprim-Sulfamethoxazole	>8/152	R
Nitrofurans	Nitrofurantoin	≤32	S
Fluoroquinolones	Ciprofloxacin	≤0.5	S
	Levofloxacin	≤1	S
	Ofloxacin	≤2	S
	Nalidixic acid	≤30	R
Tetracyclines	Tetracycline	≤10	S
Macrolides	Erythromycin	≤10	S
Rifamycins	Rifampicin	>5	S

Table 3. Antimicrobial susceptibility profile of isolate SLB4 recovered from *Oncorhynchus mykiss*. Abbreviations: MIC = Minimum Inhibitory Concentration; S = Sensitive; R = Resistant.

the isolate. The liver displayed marked necrosis, cytoplasmic vacuolation, sinusoidal dilation, and prominent melano-macrophage centers, all of which indicate severe hepatocellular damage and immune activation. Similar hepatic alterations have also been documented in the past in infections caused by members of the *K. pneumoniae* species complex in clownfish[37] and in other gram-negative bacterial infections, including *Edwardsiella tarda* in *Clarias gariepinus*[32] and *Providencia vermicola* in *Labeo rohita*[45]. The kidney lesions characterized by tubular degeneration, glomerular necrosis, hemorrhages, and vacuolation reflect impaired renal filtration and are hallmark features of systemic bacterial septicemia in fish[42]. As the kidney is a primary hematopoietic and excretory organ in teleosts, the observed degeneration strongly indicates systemic spread of the pathogen and significant physiological disruption. In addition to hepatic and renal injury, the muscle tissue also showed clear pathological alterations indicative of bacterial myopathy. Affected sections exhibited myofiber degeneration, inter-fiber edema, and prominent myofibrillar separation, together with early vacuolar degeneration changes consistent with the destructive effects of bacterial toxins and inflammatory mediators on skeletal muscle. Comparable muscle fiber degeneration has been described in bacterial infections of salmonids and catfish, where systemic septicemia results in tissue hypoxia, metabolic stress, and structural breakdown of myofibers[33,42]. Such muscular involvement suggests compromised locomotor performance and overall weakening of infected fish, further exacerbating disease progression and mortality. The antibiotic susceptibility results provide an alarming yet globally consistent picture of multidrug-resistant (MDR) strains within the *K. pneumoniae* species complex. The SLB4 isolate exhibited resistance to ampicillin, cefotaxime, ceftriaxone, cefepime, rifampicin, and trimethoprim-sulfamethoxazole, while retaining susceptibility to aminoglycosides, carbapenems, fluoroquinolones, and colistin. Such patterns are widely reported in environmental and clinical isolates and are often associated with extended-spectrum β-lactamase (ESBL) genes such as blaCTX-M, blaTEM, and blaSHV, as well as emerging carbapenemase genes like blaKPC and blaNDM[10,34,54] . Aquatic environments have increasingly been recognized as reservoirs for MDR strains, facilitated by selective pressure from antibiotic use in aquaculture, introduction of resistant bacteria from agricultural runoff, and horizontal gene transfer mediated by plasmids, integrons, and transposons[5,15]. The susceptibility of SLB4 to carbapenems and colistin is unsurprising, as these antibiotics are rarely used in aquaculture, but it raises concerns about potential future acquisition of carbapenemase genes a scenario that has been documented in marine and freshwater environments globally[26,46] .

The natural detection of an MDR member of the *K. pneumoniae* species complex in a high-altitude, cold-water aquaculture system such as Ladakh is particularly noteworthy. Cold temperatures generally inhibit bacterial proliferation; however, members of this group are known for their metabolic versatility and capacity to form robust biofilms, enabling long-term persistence in low-nutrient and stressful environments[38]. The cold-water aquaculture units in Ladakh often depend on stream-fed systems where water sources may be subjected to human activity, livestock access, and environmental contamination, potentially introducing and maintaining

these bacterial populations. Moreover, hypoxic conditions, high stocking density, and fluctuations in water chemistry in such systems may predispose trout to infection, as suggested for other bacterial pathogens in extreme environments[6,52].

From a one-health perspective, the presence of MDR members of the *K. pneumoniae* species complex in fish farms poses a multifaceted risk. Aquaculture settings can serve as convergence points for bacteria from human, animal, and environmental sources, facilitating gene exchange and dissemination of pathogens[19]. The possibility of resistant bacteria or resistance genes transferring from aquaculture systems to human-associated microbiomes cannot be ignored, particularly when water bodies are shared for both domestic and agricultural purposes[53]. The detection of natural infections in trout in the present study highlights the urgent need to implement integrated surveillance systems combining fish health monitoring, environmental microbial assessment, and prudent antibiotic stewardship. While certain preventive measures such as improved water sanitation, biosecurity protocols, vaccination research, and alternative therapeutics like probiotics and phage therapy should be considered to reduce dependence on antibiotics and limit resistance spread[11,50]. Future investigations employing whole-genome sequencing, virulence gene screening (including rmpA, aerobactin, and other siderophores), and wzi typing will be instrumental in elucidating the genetic basis of pathogenicity and resistance in this isolate.

Conclusion

The present study provides the first detailed account of a naturally occurring infection caused by a member of the *K. pneumoniae* species complex in cultured rainbow trout, *O. mykiss* collected from the high altitude trans-Himalayan belt of Ladakh, confirmed through phenotypic, molecular, and phylogenetic analyses. While our findings strongly implicate the isolated strain as the causative agent based on clinical signs, bacterial isolation, and histopathological damage. Unlike previous studies based on experimental infections, our findings demonstrate that members of the *K. pneumoniae* species complex can infect fish under natural aquaculture conditions, leading to characteristic clinical signs and pronounced histopathological damage. The multidrug-resistant profile of the isolate underscores its potential role both as pathogen and a reservoir of antimicrobial resistance genes in aquatic environments. These observations have far-reaching implications for fish health, aquaculture sustainability, and public health. Therefore, strengthening farm-level biosecurity, adopting prudent antibiotic stewardship, and implementing comprehensive one health surveillance strategies are critical steps toward mitigating the emergence and spread of multidrug-resistant pathogens in aquaculture systems.

Data availability

The sequence data generated during the current study are available in the NCBI GenBank database. The accession number assigned to the sequence is PQ676432.

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Author contributions

Riyaz Ahmad: Conceptualization, validation, visualization, original draft preparation, and review.Imtiaz Ahmed: Visualisation, validation, supervision, and manuscript revision.Firdous A. Khanday: Co-Supervision.Mohammad Abul Farah: Validation. Francesco Fazio: Manuscript editing.

Declarations

Competing interests
The authors declare no competing interests.

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