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Characterization of a hemolytic and antibiotic-resistant *Pseudomonas aeruginosa* strain S3 pathogenic to fish isolated from Mahananda River in India

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Abstract

Virulent strain *Pseudomonas aeruginosa* isolated from Mahananda River exhibited the highest hemolytic activity and virulence factors and was pathogenic to fish as clinical signs of hemorrhagic spots, loss of scales, and fin erosions were found. S3 was cytotoxic to the human liver cell line (WRL-68) in the trypan blue dye exclusion assay. Genotype characterization using whole genome analysis showed that S3 was similar to *P. aeruginosa* PAO1. The draft genome sequence had an estimated length of 62,69,783 bp, a GC content of 66.3%, and contained 5916 coding sequences. Eight genes across the genome were predicted to be related to hemolysin action. Antibiotic resistance genes such as class C and class D beta-lactamases, *fosA*, *APH*, and *catB* were detected, along with the strong presence of multiple efflux system genes. This study shows that river water is contaminated by pathogenic *P. aeruginosa* harboring an array of virulence and antibiotic resistance genes which warrants periodic monitoring to prevent disease outbreaks.

Introduction

One of the most important natural resources is water which is essential in the life of all living organisms. However, excess runoff of sewage and fertilizers into freshwater bodies provides a favorable environment for the growth and reproduction of pathogenic microorganisms [1]. It is reported that river water is usually contaminated by diverse groups of pathogenic bacteria [2]. *Pseudomonas aeruginosa* is a Gram-negative, rod-shaped and uniflagellated bacterium that can colonize a wide range of habitats including soil, water, animals, plants, and humans [3]. It is a well-known pathogen for opportunistic infections, which causes morbidness and even death in immunocompromised and cystic fibrosis-affected patients [4,5]. As a threatening fish pathogen, *P. aeruginosa* induces ulcerative syndrome and hemorrhagic septicemia leading to high mortality and significant economic losses [6]. Since water is the natural reservoir of this

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bacterium, the presence of *P. aeruginosa* is considered an indicator of contamination in water by some researchers [7,8]. This species is present in high concentrations in domestic wastewater and is a major contaminant in surface runoff. However, due to the low fecal abundance of this bacterium, agricultural leachates and barn effluents are also thought to be other important sources of *P. aeuginosa* [9]. *P. aeruginosa* displays a wide range of virulence systems which contributes to its ability to cause infection and colonization [10]. Mechanisms of virulence in these bacteria include both cell-associated factors such as lipopolysaccharides, flagella, pili, an array of extracellular secretions such as proteases, exotoxins, elastases, siderophores, extracellular polysaccharides [10,11]. Hemolysins are extracellular toxic proteins, produced by many Gram-positive and Gram-negative bacteria with lytic activities [12]. Also called cytolysins, they are responsible for forming pores in membranes of various host cells including red blood cells (RBCs), epithelial cells, and leukocytes leading to cell lysis and ultimately cell death [13,14]. The investigations on hemolysins of *P. aeruginosa* are mainly based on clinical isolates [15–17] although the hemolytic trait is reported to occur in environmental isolates as much as in clinical isolates [18]. A study reported that hemolytic *P. aeruginosa* PAO1 causes plant damage when present in the rhizosphere and disruption of the hemolysin gene lowers virulence towards poplar and barley [19].

High incidences of multidrug resistance in Gram-negative pathogenic bacteria have made the treating of bacterial diseases extremely challenging. *P. aeruginosa* readily achieves resistance to various classes of antibiotics through intrinsic and adaptive mechanisms that include low permeability of the bacterial outer membrane, biofilm formation, and involvement of robust efflux pumps, which are naturally present in bacteria to combat toxic materials in the environment [20]. In addition, acquiring resistance through horizontal gene transfer and mutational resistance has led to it joining the ranks of superbugs [21].

The current study aimed to investigate the range of virulence and antibiotic resistance in pathogenic bacteria present in river waters of North Bengal, India. Preliminary studies led to the isolation of a virulent strain *Pseudomonas aeruginosa* S3. The strain was further studied for its susceptibility to 21 common antibiotics belonging to various classes. Pathogenicity of S3 was tested in *Anabus testudineus* fishes and cytotoxicity was tested in the human liver cell line. In addition, whole genome sequencing of S3 provided information to predict genes related to virulence factors and antimicrobial resistance along with those involved in hemolytic activity.

Materials and methods

Isolation and screening of putative hemolytic strains based on hemolytic activity

The River Mahananda is a major river in North India and has a vital role in regulating the economy of the adjoining areas [22]. The river flows through the Himalayan mountains where it originates and descends into the plains in the sub-Himalayan region of Darjeeling district in West Bengal flowing through the Mahananda Wildlife Sanctuary. Then it flows through the heart of Siliguri city and meanders along several places before merging with the River Ganga. Water samples were withdrawn in sterilized vials from different locations along the Mahananda River in Siliguri.

The vials with sampled river water were kept at 4˚C and studied within 24 h of sample collection. To isolate hemolytic bacteria, serial ten-fold dilutions of the samples were made in sterile distilled water. Then, 0.1 mL of each dilution was spread plated on tryptone soya agar (TSA) medium (Himedia Laboratories, Mumbai, India) supplemented with 5% (v/v) sheep blood. The plates were incubated at 37˚C for 24h following which they were observed against the light for halo formation around the bacterial colonies. Altogether 26 colonies were picked

up based on positive halo formation from the plates and axenic cultures were maintained by streaking them on nutrient agar slants [23]. For long time preservation, the cultures were stored in glycerol stock (10% v/v glycerol) and kept at -20˚C.

Hemolytic activity

The hemolytic activity of the bacterial isolates was determined as described previously [24] with minor modifications. Briefly, sheep red blood cells were prepared by washing thrice with phosphate-buffered saline (PBS). Culture supernatant of bacterial isolates was collected by centrifuging 24 h old culture of each bacterium in tryptone soya broth(TSB). For assaying hemolysis, 245μl of culture supernatant was mixed with 5μl of washed sheep RBC and 10mM $CaCl_2$ and incubated for 1h at 37˚C. Positive control contained 1% Triton X-100 in place of culture supernatant while sterile TSB was used for negative control. After incubation, samples were centrifuged and hemoglobin release was monitored by estimating the optical density at 540nm (OD_{540}). For normalization, the negative control reading was subtracted from each sample reading. Percent hemolysis was calculated by setting the Triton X-100 control as 100% hemolysis. All the experiments were performed in triplicate and the data were represented as triplicate of mean ± SD. To find the significant differences ($P<0.05$) between the hemolytic isolates, hemolysin assay experiments were analyzed by one-way ANOVA in SPSS Software version 21.

Detection of phenotypic attributes of virulence

Production of protease was determined in skimmed milk supplemented (1.5%w/v) nutrient agar medium. This medium was inoculated by each isolate and incubated for 24h at 37˚C. Bacteria that were able to degrade casein exhibited a zone of clearance around the growth of bacteria [25]. DNase production by the isolates was observed in DNase agar medium (HiMedia laboratories, Mumbai) where the media plates were inoculated with the strains and incubated for 24h at 37˚C. Bacterial colonies surrounded by pale pink to white halos were identified as DNase positive [26]. For testing the production of lipase, the isolates were allowed to grow in Tween-80 medium at 37˚C for 24 h. The clear zone surrounding the streaked bacterial cultures due to the crystal formation indicates lipase production [27]. Amylase production by the isolates was tested in starch agar plates according to the method of Barrow and Feltham. Production of siderophore was examined by the Chromazurol S (CAS) assay [28].

Biochemical characterization

The isolate showing high hemolytic activity, multiple virulence traits, and antibiotic resistance was subjected to further investigations. The selected strain S3 was characterized through biochemical tests which included Gram staining, catalase, oxidase, indole, methyl red, Voges Proskauer, oxidation-fermentation, nitrate reduction and citrate utilization [29]. Pigment production was tested in Pseudomonas agar medium (Himedia Laboratories, India).

Antimicrobial susceptibility tests

To determine the antimicrobial sensitivity of *Pseudomonas aeruginosa* S3, the Kirby–Bauer disk diffusion method was used as per the standards of CLSI and EUCAST [30]. Initially, S3 was grown in nutrient broth for 24 h at 37˚C. Sterile Mueller–Hinton agar (MHA) plates were spread with S3 culture using a cotton swab and the inoculated plates were dried at room temperature. Subsequently, antimicrobial discs (Hi-media Laboratories, Mumbai, India) were placed on the inoculated plates and incubated at 37˚C for 24 h. The antimicrobial substances

used in this study were ampicillin (10 mcg), amoxicillin/clavulanic acid (10 mcg), penicillin (10 mcg), cefoperazone (75mcg), ciprofloxacin (5mcg), gentamicin (10mcg), streptomycin (25 mcg), kanamycin (30 mcg), norfloxacin (10mcg), ofloxacin (5mcg), imipenem (10mcg), cefepime (30mcg), cefixime (5mcg), cefotaxime (30mcg), cefuroxime (30 mcg), chloramphenicol (30 mcg), co-trimoxazole (15 mcg), trimethoprim (5 mcg), tetracycline (30 mcg), oxytetracycline (30 mcg) and colistin (10 mcg). Colistin susceptibility was tested by the disk elution method following CLSI guidelines. For this, specific number of colistin disks were added to 10 ml sterile cation-activated Mueller–Hinton broth (0 disk = control, 1 disk = 1 μg/mL, 2 disks = 2 μg/mL and 4 disks = 4 μg/mL) and vortexed gently for 40 min for the elution of colistin into the medium. For inoculum preparation, 3 to 5 isolated pure colonies of S3 from overnight nutrient agar plates were suspended in normal saline and added to each test-tube to attain a final inoculum density of 7.5×10^5 cfu/mL. The inoculated tubes were again vortexed at low speed and incubated overnight at 35˚C. Categorization of the strain S3 as sensitive (S) or resistant (R) towards each antimicrobial substance was done according to the suggestion mentioned in the CLSI and EUCAST interpretative guidelines for *Pseudomonas*. *Pseudomonas aeruginosa* ATCC 27853 was used as a reference strain for the antimicrobial sensitivity experiments.

Cell cytotoxicity assay

Human liver embryonic cell line WRL-68 was purchased from NCCS (National Centre for Cell Science) in Pune, Maharashtra, India, and used to check the cytotoxicity of the strain *Pseudomonas aeruginosa* S3. The strain S3 was inoculated in the Luria-Bertani (LB) broth and incubated for 24 h at 37˚C with an agitation speed of 90 rpm. Cell-free supernatant (CFS) was collected by centrifuging the 24 h culture at 10,000g for 30 mins at 4˚C followed by filtering through a 0.45 μm membrane filter. Culture filtrate of *Lactobacillus* sp., a non-pathogenic strain, was also prepared following the same procedure and included as the positive control in the experiment. WRL-68 cells were allowed to grow in Dulbeco's modified Eagle's medium (DMEM) with 10% foetal calf serum in an atmosphere containing 5% CO_2. For experimental purpose, cells were added to culture plates (60 mm) and incubated in CO_2 incubator for 24 h at 37˚C. Subsequently, the cell free supernatants (1.5 mL) were added to the cells in separate sets and incubated at 25˚C for one hour. Sterile LB medium was added to the control set. Following incubation, the cells were observed under phase-contrast inverted microscope (Olympus CK40-SLP) at 200X magnification. The percentage of viable cells of treated and control sets was determined following the trypan blue dye exclusion assay described previously [31]. Percent viability was determined as: [total number of viable cells per ml of aliquot/ total number of cells per ml of aliquot] × 100. The experiment was repeated thrice and the mean was computed. Standard error was calculated using the statistical software OriginPro R version 9.9 freely available from https://www.originlab.com.

Pathogenicity testing in fishes

Healthy small-sized fishes (25–30 g) of *A*, *testudineus* were used for testing the pathogenicity of S3 in fish. In this current study, fishes were procured from local fisheries in the Darjeeling district and acclimatized for 15 days in glass aquaria (90X35X35 cm). Each aquarium contained 8 fish and the water temperature was maintained between 25–30˚C. For pathogen injection, S3 was grown in TSB for 18 h at 37˚C under an agitation speed of 90 rpm and subsequently centrifuged to collect the cells which were resuspended in 0.85% saline solution to obtain a cell density of 1×10^7cfu/mL [32]. Benzocaine at a concentration of 25 mg/L was used to anesthetize the fishes where the fishes were kept in the solution for 1–2 min. The S3

suspension was injected into each of the fish at a concentration of 0.4 mL/25 gm body weight and maintained in a separate aquarium [30]. The fishes injected with 0.85%w/v saline solution at a similar dose were marked as a control group. The pathological lesions and death of fish were monitored and recorded every 24 h post administration for one week. The approval (IAEC approval No. IAEC/NBU/2022/39) for conducting the above experiment was obtained from Institutional Animal Ethics Committee (IAEC), Department of Zoology, University of North Bengal under the guidelines of CPCSEA, New Delhi. The fishes were anesthetized by keeping in benzocaine solution (25mg/L) for 1-2min.

Whole-genome sequencing, assembly, annotation, and comparative genome analysis

The selected strain S3 was inoculated into TSB and grown for 24 h at an incubation temperature of 37˚C. The chromosomal DNA of the bacterium was isolated using a bacterial gDNA isolation kit (Xploregen Discoveries, Bangalore, India) following the protocol obtained from the manufacturer. DNA concentration was determined using the Qubit double-stranded DNA (dsDNA) high-sensitivity (HS) assay kit (Invitrogen, USA) in a Qubit 3 Fluorometer. Total DNA was enzymatically digested to obtain the average fragment size ranging from 200–300 bp. and libraries were generated by following the NEBNext Ultra II protocol. Fragments were analyzed in Agilent 2100 bioanalyzer and sequencing was conducted with the Illumina HiSeq 4000 sequencer using the 2x150 paired end sequencing strategy to generate 15810782 reads. Read quality was assessed using FastQC and MultiQC and trimming was done using TrimGalore (version 0.6.4) to remove adapter sequences. Primary assembly of the filtered reads was done using Unicycler with default settings (version 0.4.8). MOB-suite (version 3.1.0) was used to predict plasmid sequences from the primary assemblies which showed negative results. The final finishing of the genome was done using Contiguator (https://contiguator.sourceforge.net/).

Annotation of the S3 genome was done using Rapid Annotations using Subsystems Technology (RAST) used at Pathosystems Resource Integration Center (PATRIC) and Prokka tools. The circular genome map and phylogenetic tree were created using PATRIC. Sequences for virulence factors were searched and analyzed through the Virulence Factor Database (VFDB) and Victors resource. The Comprehensive Antibiotic Resistant Database (CARD) and the NCBI National Database of Antibiotic Resistance Organism (NDARO) were used to detect antimicrobial resistance genes. In addition, hemolysin genes were predicted using the PATRIC annotation service and analyzed through BLASTP. The genomes or contigs were aligned against each other using the tool MAUVE version 20150226 for comparison [33].

Phylogenetic analysis

Using the PubMLST server [34], the identification of *Pseudomonas aeruginosa* S3 was confirmed by rMLST (ribosomal multilocus sequence typing) [35]. The PATRIC Phylogenetic Tree Building Service [36] was used to create a phylogenetic tree using amino acid and nucleotide sequences from a selected number of the BV-BRC global Protein Families (PGFams) [37]. MUSCLE was used to align the protein sequences [38] whereas Biopython was used to align the coding gene nucleotide sequences [39]. The aligned protein and nucleotides were formatted into a PHYLIP file for ease of use, and a partition file was created specifically for RaxML analysis [40]. With the help of RaxML, 100 rounds of the "Rapid" bootstrapping option [41] support values were created. The phylogenetic tree was visualized by FigTree [42].

Acquisition of the accession numbers for the nucleotide sequence

The draft genomes of S3 were submitted in the NCBI database under the accession number JAESVH000000000. The BioProject ID in GenBank is PRJNA693431. The organism was identified as *Pseudomonas aeruginosa* S3.

Results

Hemolytic activity and virulence-related traits of isolated strains

Altogether 26 bacterial strains were observed to be hemolytic among 256 strains isolated from the Mahananda River during primary screening. A quantitative estimation showed that strain S3 possessed the highest hemolytic activity (Fig 1). Further studies on virulence-related phenotypes of the isolates showed that S3 possessed the maximum number of virulence traits. It showed protease, DNase, and lipase activity and produced a siderophore in the CAS medium (S1 Table in S1 File).

Biochemical characterization and antimicrobial resistance profile of S3

The strain S3 was observed to be Gram-negative and rod in shape which produced green fluorescent pigment on Pseudomonas agar medium. The strain showed positive results in catalase, oxidase, citrate utilization and nitrate reduction tests while it showed negative results for indole, methyl red, and Voges Proskauer tests. It was found to be oxidative in the oxidation-fermentation test. These results match the phenotype of *P. aeruginosa* described in Bergey's manual [43].

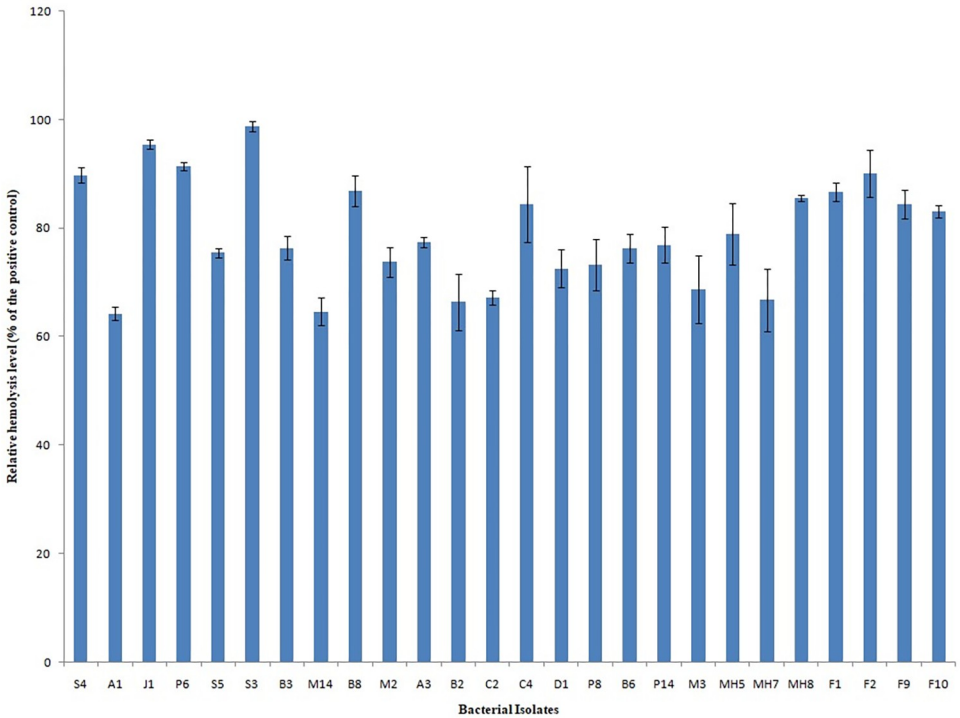

Fig 1. Hemolytic activity of the bacterial isolates. Data are presented as triplicate of mean ± SD. One-way ANOVA suggests a significant difference ($p<0.05$) in hemolysis (%) by the isolates.

https://doi.org/10.1371/journal.pone.0300134.g001

Table 1. Antimicrobial sensitivity testing of *P. aeruginosa* S3.

Class	Antimicrobial substance	Disk content (mcg)	Interpretation
Aminoglycosides	Gentamicin	10	S
	Streptomycin	25	R
	Kanamycin	30	R
β-lactam	Penicillin Ampicillin Amoxycillin/Clavulanic acid	10 10 10	R R R
Carbapenems	Imipenem	10	R
Fluoroquinolones	Ciprofloxacin	5	S
	Ofloxacin	5	S
	Norfloxacin	10	R
Cephalosporin	Cefepime	30	R
	Cefixime	5	R
	Cefotaxime	30	R
	Cefoperazone	75	R
	Cefuroxime	30	R
Phenicols	Chloramphenicol	30	R
Folate pathway inhibitors	Co-trimoxazole	15	R
	Trimethoprim	5	R
Tetracyclines	Tetracycline	30	R
	Oxytetracycline	30	R
Polymyxins	Colistin	10	R

https://doi.org/10.1371/journal.pone.0300134.t001

The antibiotic resistance profile of *P. aeruginosa* S3 is listed in Table 1, which reveals that among the 21 antimicrobial substances tested, strain S3 was resistant to 19 of them. It showed resistance to all tested beta-lactam class of antibiotics including penicillins, all five cephalosporins, and carbapenem. S3 was also resistant to chloramphenicol, colistin, tetracyclines, and folate pathway inhibitors. It showed resistance to the aminoglycosides streptomycin and kanamycin but was susceptible to gentamycin. Similarly, it was found to be susceptible to fluoroquinolones, ciprofloxacin, and ofloxacin but was resistant to norfloxacin.

Pathogenicity in fish

The pathogenicity of S3 was tested by injecting it into *A. testudineus* fishes intramuscularly. Almost 90% mortality was recorded within the first 24 h and by 36 h, all injected fishes were dead. The fishes developed clinical signs like hemorrhagic spots on the body surface especially on the head and ventral aspect of the abdomen, loss of scales, fin erosions, and exophthalmia (Fig 2).

Cell cytotoxicity assay

The CFS of S3 showed cytotoxicity towards human liver cell line WRL-68. The trypan blue dye exclusion assay revealed a considerable decrease in cell viability when WRL-68 cells were exposed to the CFS of S3 compared to the controls (Fig 3). The viability of the S3-treated WRL-68 cell was observed to be 3.1% which was significantly lower compared to the cell treated with *Lactobacillus* (69%).

Genetic features and phylogeny of S3

Genomic characteristics and annotated gene information of strain S3 are briefly presented in Table 2. The draft genome length of strain S3 was 62,13,354 bp with a GC composition of

Fig 2. Pathogenicity testing of *Pseudomonas aeruginosa* S3 in *Anabaena testudineus*.

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66.32% and harbored 5916 protein-coding sequences. The circular viewer application of PATRIC was used to construct the circular whole genome map of S3 and shown in Fig 4. Prospective genes obtained from the draft genome of S3 were analyzed and annotated using the subsystem approach of the RAST server (Fig 5). Subsystem refers to an assemblage of functionally related protein families, collectively referring to a precise biological process or structural complexes [44]. It had been observed that in strain S3, membrane transport function was controlled by 166 genes, biotic and abiotic stress response was regulated by 104 genes, virulence, defense, and disease-related function was controlled by 62 genes, and whereas metabolism and acquirement of iron were taken care of by 52 genes. Careful analysis of the genome of S3 also revealed the involvement of four genes associated with prophages, transposable elements, and plasmids. Listeria Pathogenicity Island LIPI-1 extended was found to be present under this subsystem. The subsystem's description obtained at the SEED viewer revealed the presence of *prf A*, *plcA*, *plcB*, *mpl*, and *actA* in LIPI-1 extended.

A Phylogenetic tree using the core genes of the whole genome sequence of S3 was constructed using the PATRIC database and presented in Fig 6. The microbial taxonomy of S3 was confirmed as *Pseudomonas aeruginosa* at 100% identity based on the variation of 56 genes

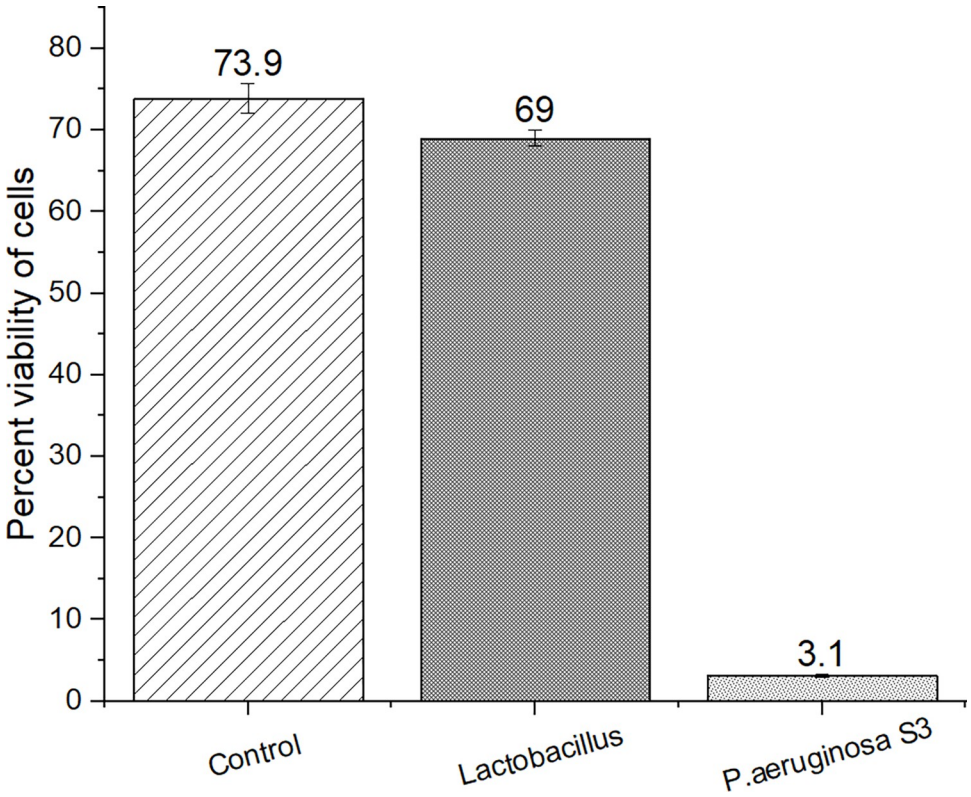

Fig 3. Cytotoxic effect of *Pseudomonas aeruginosa* S3 cell-free culture supernatant fluid on human liver cell line (WRL-68).

https://doi.org/10.1371/journal.pone.0300134.g003

Table 2. Genomic characteristics and annotation information of the chromosome of *Pseudomonas aeruginosa* S3 based on PATRIC.

Genome Features	Chromosome
Genome length (bp)	62,69,783
Protein-coding genes	5916
GC content (%)	66.32298
The number of tRNA	55
The number of rRNA	3
Contigs	46
Contig L50	1
Contig N50	61,73,608
Coarse Consistency(%)	99.9
Fine Consistency(%)	99.7
CheckM Completeness	99
CheckM Contamination	0.6
Hypothetical proteins	1112
Proteins with functional assignments	4804
Proteins with Subsystem assignments	2073
Proteins with EC number assignments	1288
Proteins with GO assignments	1095
Proteins with Pathway assignments	971

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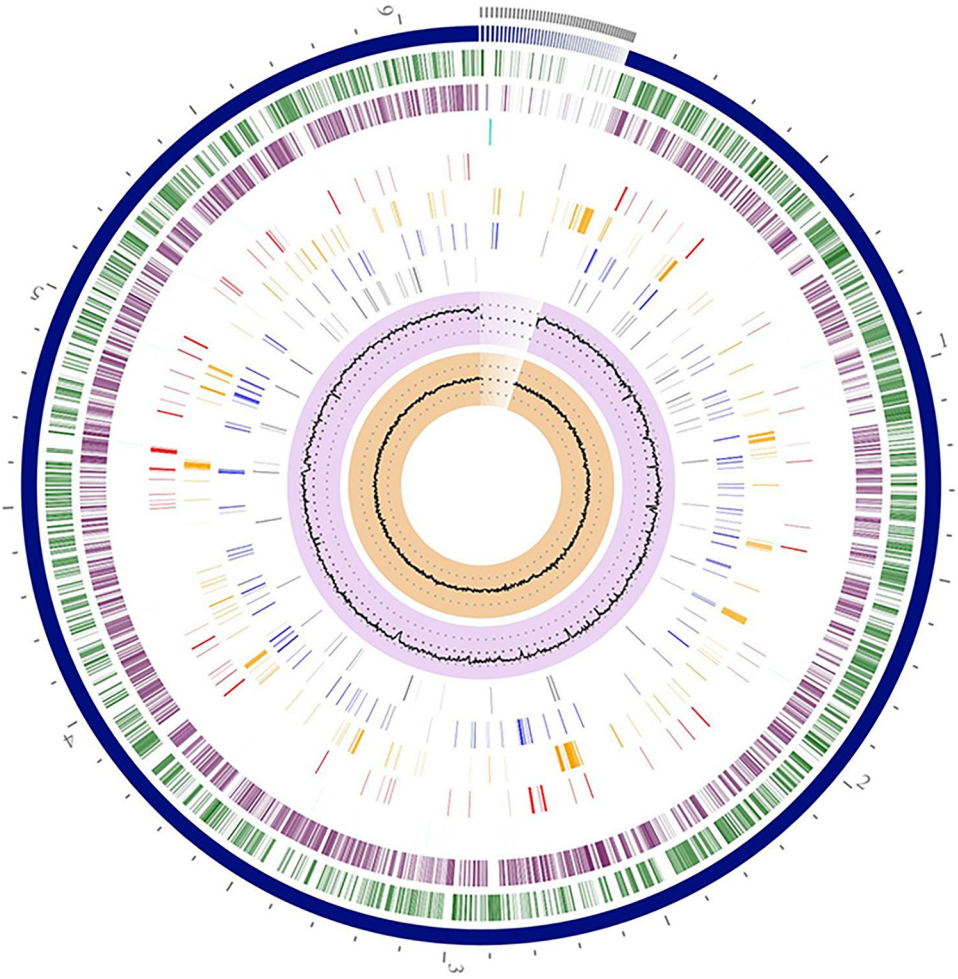

Fig 4. The circular genome visualization of *P. aeruginosa* S3 was created using the PATRIC circular viewer with the layout starting from the outermost layer contigs and moving towards the center, with forward and reverse coding sequences, non-CDS features, AMR genes, VF Genes, transporters, and drug targets. The two inner tracks are GC content and GC skew.

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encoding ribosomal protein subunits. Pairwise alignment of S3 with whole genomes of *P. aeruginosa* PAO1 and *P. balearica* DSM 6083 was done using progressive MAUVE and S3 showed the highest similarity with *P. aeruginosa* PAO1 as shown in Fig 7. The sequence type was found as 549 using the MLST database which also matched with *P. aeruginosa* PAO1.

Prediction of genes encoding virulence factors, antibiotic resistance, and hemolysin action

Several genes were predicted as virulence factors in the draft genome of *P. aeruginosa* S3 according to the Victors (89 genes) (Table 3) and VFDB (239 genes) database (S2 Table in S1 File). CARD and NDARO databases were used to find genes responsible for antibiotic resistance of S3 as shown in Table 4. *P. aeruginosa* S3 was found to contain many multidrug efflux systems and also showed the presence of antibiotic resistance genes of fosfomycin, chloramphenicol, and beta-lactam antibiotics. Eight genes across the genome were predicted through the PATRIC database to be related to hemolysin action displayed by S3 (Table 5). A BLASTP-based study revealed that S3 carried two hemolysin genes PapA and ChoE which were

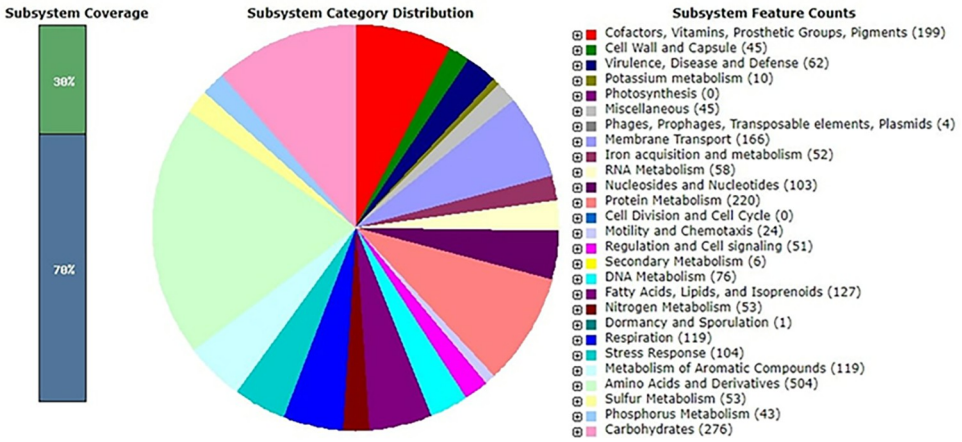

Fig 5. An annotated draft whole genome of *Pseudomonas aeruginosa* S3 from the RAST server was analyzed to identify subsystem categories. The pie chart revealed the number of genes related to individual subsystems. Subsystem coverage is presented in the bar graph in the left. The ratio of coding sequences annotated in the SEED subsystem (30%) and outside of the SEED subsystem (70%) is indicated.

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annotated as Phospholipase/lecithinase/hemolysin. In addition, apart from the known *plcH* gene, a predicted homolog of the hemolysin III family protein and another putative hemolysin belonging to the GNAT family were present. Rest three genes encoded activation or secretion proteins of hemolysin.

Discussion

The presence of pathogens in river water causes the spread of several diseases, ultimately with serious consequences for human health [45]. In this present research, a pathogenic strain of *P. aeruginosa* S3 resistant to multiple antibiotics was isolated from the Mahananda river. Hemolytic activity, a key indicator of virulence in several bacteria in the aquatic environment [46]

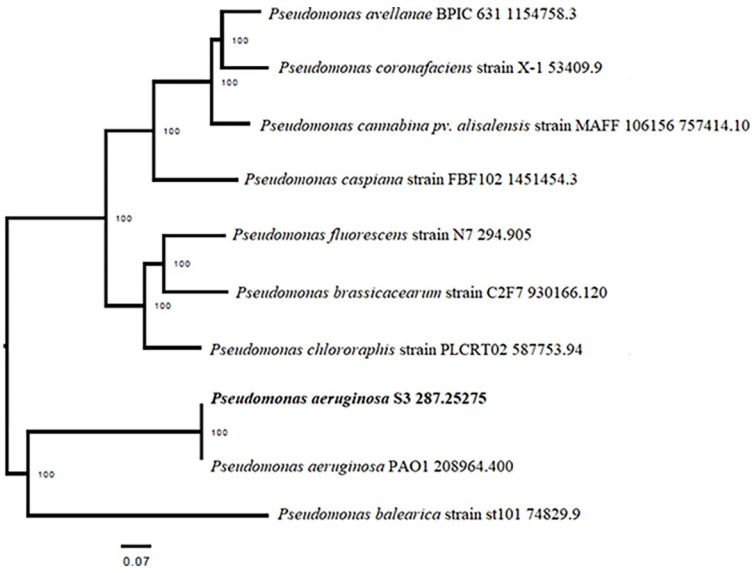

Fig 6. Phylogenetic analysis based on core genes of *Pseudomonas aeruginosa* S3 using PATRIC.

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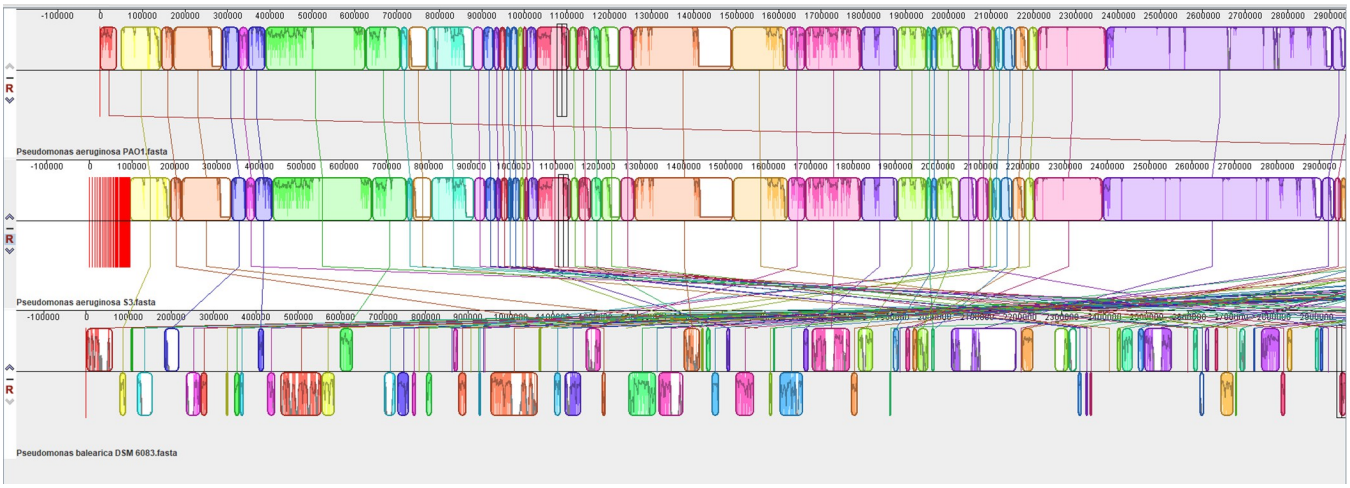

Fig 7. Genome comparison for *Pseudomonas* strains using Mauve. Pairwise alignment of *P. aeruginosa* S3 with other whole genomes of *P. aeruginosa* PAO1 and *P. balearica* DSM 6083.

https://doi.org/10.1371/journal.pone.0300134.g007

was utilized during the initial screening. Among 26 hemolytic isolates, S3 possessed the highest hemolytic activity and exhibited multiple virulence traits which led to its selection for further studies to genotypically and phenotypically characterize this strain to understand the threat it poses to aquatic organisms and human health.

Virulence factors of *P. aeruginosa* exhibit a wide range of mechanisms controlled by diverse signaling systems and multiple complex and interconnected regulatory circuits, giving this pathogen extraordinary versatility in disease manifestation [10]. Gene prediction in the RAST server showed the existence of Listeria Pathogenicity Island LIPI-1 extended in the S3 genome. All genes of the *L. monocytogenes* LIPI-1 were present in LIPI-1 extended of S3 except *hly* which encodes listeriolysin O [47]. LIPI-1 has been reported to occur in other genera such as in *Bacillus* sp. 87 isolated from food [48]. It was also found in *P. aeruginosa* MZ4A isolated from clinical waste [49]. The presence of LIPI-1 in *P. aeruginosa* S3 might increase its virulence but further studies are required to confirm this possibility.

P. aeruginosa is considered one of the significant recurrent emerging pathogens isolated from fishes such as *Oreochromis niloticus*, *Clarias gariepinus* [6,50], *O. mossambicus* [51] and *Labeo bata* [52]. S3 displayed high mortality in fish injection studies with clinical observations agreeing with some previous findings [6,50]. However, as reported by Haque et al. (2021) [52], deep necrotic skin ulcers were not found. In addition, S3 showed cytotoxic activity toward human liver cell lines WRL-68. Previous studies reported *P. aeruginosa* clinical isolates to exhibit cytotoxic properties in human liver cell lines [53] but such studies involving environmental isolates are rare.

Among the various virulence factors found in S3, the two-component system regulatory network comprising signal-sensing histidine kinases and response regulators that play a vital function in *P. aeruginosa* virulence and environmental adaptation [54] were found. *P. aeruginosa* S3 carried the *PA1157* gene which is a two-component response regulator that regulates the response to changes in different environmental conditions. It is a part of the OmpR family which controls porin expression [55]. In addition, the transcriptional regulator gene *algR*, (alginate biosynthesis) which is considered essential for pathogenesis [56] was also detected along with *algB* (response regulator) and *algZ* (sensor histidine kinase). Moreover, the Chp/FimS/AlgR network involved in biofilm formation and virulence was found [57]. In addition, genes encoding the virulence regulator MvfR (PqsR) and the quorum sensing regulator LasR

Table 3. Prediction of genes related to virulence factors of *Pseudomonas aeruginosa* S3 according to Victors database.

Gene	Product	Source ID	Source Organism
nuoD	NADH-ubiquinone oxidoreductase chain C (EC 1.6.5.3) / NADH-ubiquinone oxidoreductase chain D (EC 1.6.5.3)	15597835	*P.aeruginosa* PAO1
PA3001	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13)	15598197	*P.aeruginosa* PAO1
phzS	FAD-dependent monooxygenase PhzS	15599413	*P.aeruginosa*
PA14_37260	Outer membrane low permeability porin, OprD family = > OccK3/OpdO pyroglutamate, cefotaxime uptake	116050053	*P.aeruginosa* UCBPP-PA14
exoT	hypothetical protein	15595242	*P.aeruginosa* PAO1
PA0151	Ferrichrome-iron receptor @ Iron siderophore receptor protein	15595349	*P.aeruginosa* PAO1
pyrF	Orotidine 5'-phosphate decarboxylase (EC 4.1.1.23)	15598072	*P.aeruginosa* PAO1
algW	Outer membrane stress sensor protease DegS	15599642	*P.aeruginosa* PAO1
prpL	Lysyl endopeptidase (EC 3.4.21.50)	116052217	*P.aeruginosa* UCBPP-PA14
exoY	Adenylate cyclase ExoY (EC 4.6.1.1)	15597387	*P.aeruginosa* PAO1
PA5327	Oxidoreductase, FAD-binding	15600520	*P.aeruginosa*
exoS	putative exoenzyme T	15599036	*P.aeruginosa* PAO1
fur	Ferric uptake regulation protein FUR	15599958	*P.aeruginosa* PAO1
wspF	Chemotaxis response regulator protein-glutamate methylesteraseCheB (EC 3.1.1.61)	116051700	*P.aeruginosa* UCBPP-PA14
htpG	Chaperone protein HtpG	15596793	*P.aeruginosa* PAO1
plcH	Phospholipase C (EC 3.1.4.3) = >hemolyticPlcH	116048767	*P.aeruginosa* UCBPP-PA14
dsbA	Periplasmic thiol:disulfide interchange protein DsbA	116053639	*P.aeruginosa* UCBPP-PA14
PA1157	Two-component transcriptional response regulator, OmpR family	15596354	*P.aeruginosa* PAO1
estA	Phospholipase/lecithinase/hemolysin	15600305	*P.aeruginosa* PAO1
thrC	Threonine synthase (EC 4.2.3.1)	15598930	*P.aeruginosa* PAO1
pscJ	Type III secretion bridge between inner and outermembrane lipoprotein (YscJ, HrcJ, EscJ, PscJ)	116049673	*P.aeruginosa* UCBPP-PA14
aprF	Type I secretion outer membrane protein, TolC family @ ABC-type protease exporter, outer membrane component PrtF/AprF	15596445	*P.aeruginosa* PAO1
PA3756	L,D-transpeptidase	15598951	*P.aeruginosa* PAO1
PA14_03530	Transcriptional regulator	116053999	*P.aeruginosa* UCBPP-PA14
hfq	RNA-binding protein Hfq	15600137	*P.aeruginosa* PAO1
purH	IMP cyclohydrolase (EC 3.5.4.10) / Phosphoribosylaminoimidazolecarboxamideformyltransferase (EC 2.1.2.3)	15600047	*P.aeruginosa* PAO1
pgk	Phosphoglycerate kinase (EC 2.7.2.3)	15595749	*P.aeruginosa*
PA0158	Multidrug efflux system, inner membrane proton/drug antiporter (RND type) = >TriC of TriABC-OpmH system	15595356	*P.aeruginosa* PAO1
PA4489	UPF0192 protein YfaS	15599685	*P.aeruginosa*
relA	Inactive (p)ppGpp 3'-pyrophosphohydrolase domain / GTP pyrophosphokinase (EC 2.7.6.5), (p)ppGpp synthetase I	15596131	*P.aeruginosa* PAO1
wbpX	Mannosyltransferase	15600642	*P.aeruginosa* PAO1
nqrB	Na(+)-translocating NADH-quinone reductase subunit B (EC 1.6.5.8)	15598194	*P.aeruginosa* PAO1
purL	Phosphoribosylformylglycinamidine synthase, synthetase subunit (EC 6.3.5.3) / Phosphoribosylformylglycinamidine synthase, glutamine amidotransferase subunit (EC 6.3.5.3)	15598958	*P.aeruginosa* PAO1
PA14_37650	hypothetical protein	116050024	*P.aeruginosa* UCBPP-PA14
lasR	N-(3-oxododecanoyl)-L-homoserine lactone-binding transcriptional activator @ Acyl-homoserine lactone-binding transcriptional activator, LuxR family @ Transcriptional regulator LasR	116049375	*P.aeruginosa* UCBPP-PA14
katA	Catalase KatE (EC 1.11.1.6)	116052272	*P.aeruginosa* UCBPP-PA14
narK2	Nitrate/nitrite transporter NarK/U	15599071	*P.aeruginosa* PAO1
PA3826	FIG018175: Predicted transmembrane protein	15599021	*P.aeruginosa*

(*Continued*)

Table 3. (Continued)

Gene	Product	Source ID	Source Organism
PA4115	Pyrimidine/purine nucleotide 5'-monophosphate nucleosidase PpnN (EC 3.2.2.4) (EC 3.2.2.10)	15599310	*P.aeruginosa*
modA	Molybdenum ABC transporter, substrate-binding protein ModA	15597060	*P.aeruginosa* PAO1
clpV1	T6SS AAA+ chaperone ClpV (TssH)	15595288	*P.aeruginosa* PAO1
pvdS	Sigma factor PvdS, controlingpyoverdin biosynthesis	15597622	*P.aeruginosa* PAO1
PA3498	Flavodoxin reductases (ferredoxin-NADPH reductases) family 1; Vanillate O-demethylase oxidoreductase (EC 1.14.13.-)	15598694	*P.aeruginosa* PAO1
PA0082	T6SS component TssA (ImpA)	15595280	*P.aeruginosa* PAO1
PA4564	Conserved uncharacterized protein CreA	15599760	*P.aeruginosa*
purD	Phosphoribosylamine—glycine ligase (EC 6.3.4.13)	15600048	*P.aeruginosa* PAO1
cca	CCA tRNA nucleotidyltransferase (EC 2.7.7.72)	15595781	*P.aeruginosa* PAO1
mdoG	Glucans biosynthesis protein G precursor	15600271	*P.aeruginosa* PAO1
fabF1	3-oxoacyl-[acyl-carrier-protein] synthase, KASII (EC 2.3.1.179)	116050967	*P.aeruginosa* UCBPP-PA14
flhB	Flagellar biosynthesis protein FlhB	116049394	*P.aeruginosa* UCBPP-PA14
wzz	hypothetical protein	15598356	*P.aeruginosa* PAO1
PA4692	Protein-methionine-sulfoxide reductase catalytic subunit MsrP	15599886	*P.aeruginosa*
PA14_04850	hypothetical protein	116054099	*P.aeruginosa* UCBPP-PA14
galU	UTP—glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	15597219	*P.aeruginosa* PAO1
pilI	type IV pili signal transduction protein PilI	116054141	*P.aeruginosa* UCBPP-PA14
fliC	Flagellin protein FlaB	15596289	*P.aeruginosa* PAO1
phzM	Phenazine-specific methyltransferase PhzM	15599404	*P.aeruginosa*
PA14_38610	Short-chain fatty acids transporter	116049953	*P.aeruginosa* UCBPP-PA14
PA2895	hypothetical protein	15598091	*P.aeruginosa* PAO1
pepA	Cytosol aminopeptidase PepA (EC 3.4.11.1)	15599026	*P.aeruginosa*
pilF	Type IV pilus biogenesis protein PilF	116051830	*P.aeruginosa*
PA0041	Putative large exoprotein involved in heme utilization or adhesion of ShlA/HecA/FhaA family	15595239	*P.aeruginosa* PAO1
pgm	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (EC 5.4.2.12)	15600324	*P.aeruginosa*
mvfR	Multiple virulence factor regulator MvfR/PqsR	116048931	*P.aeruginosa* UCBPP-PA14
PA4887	Uncharacterized MFS-type transporter	15600080	*P.aeruginosa*
PA1009	Glycine cleavage system transcriptional antiactivatorGcvR	15596206	*P.aeruginosa* PAO1
algU	RNA polymerase sigma factor RpoE	15595959	*P.aeruginosa* PAO1
PA2972	Maf-like protein YceF	15598168	*P.aeruginosa* PAO1
PA5312	Aldehyde dehydrogenase (EC 1.2.1.3)	15600505	*P.aeruginosa*
PA14_03120	Transcriptional regulator, MarR family	116053966	*P.aeruginosa* UCBPP-PA14
pilD	Leader peptidase (Prepilin peptidase) (EC 3.4.23.43) / N-methyltransferase (EC 2.1.1.-)	15599724	*P.aeruginosa* PAO1
PA4491	Uncharacterized protein YfaA	15599687	*P.aeruginosa*
PA5441	hypothetical protein	15600634	*P.aeruginosa*
PA5437	Transcriptional regulator PA5437, LysR family	15600630	*P.aeruginosa*
PA0073	ABC transporter, ATP-binding protein	15595271	*P.aeruginosa* PAO1
rhlB	RhlB, TDP-rhamnosyltransferase 1 (EC 2.4.1.-)	15598674	*P.aeruginosa* PAO1
metE	5-methyltetrahydropteroyltriglutamate—homocysteine methyltransferase (EC 2.1.1.14)	15597123	*P.aeruginosa* PAO1
gacA	BarA-associated response regulator UvrY (= GacA = SirA)	116050573	*P.aeruginosa* UCBPP-PA14

(*Continued*)

Table 3. (Continued)

Gene	Product	Source ID	Source Organism
pilY1	Type IV fimbrial biogenesis protein PilY1	15599750	*P.aeruginosa* PAO1
PA3286	beta-ketodecanoyl-[acyl-carrier-protein] synthase (EC 2.3.1.207)	15598482	*P.aeruginosa* PAO1
toxA	hypothetical protein	15596345	*P.aeruginosa* PAO1
PA3173	Putative short-chain dehydrogenase	15598369	*P.aeruginosa* PAO1
mucC	Sigma factor RpoE regulatory protein RseC	15595962	*P.aeruginosa* PAO1
PA14_41070	FIG005121: SAM-dependent methyltransferase (EC 2.1.1.-)	116049764	*P.aeruginosa* UCBPP-PA14
algR	Alginate biosynthesis two-component system response regulator AlgR	15600454	*P.aeruginosa* PAO1
PA4488	Uncharacterized protein YfaQ	15599684	*P.aeruginosa*
mucA	Sigma factor RpoE negative regulatory protein RseA	15595960	*P.aeruginosa* PAO1

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which controls the expression of many virulence factors [58] were detected. *PA1157*, *algB*, *algR and algZ* were found to match with *P. aeruginosa* PAO1 genes while MvfR and LasR genes matched with *P. aeruginosa* PA14. However, genes encoding RhlR, another major quorum-sensing regulator, was not found. Nevertheless, the presence of other virulence regulatory genes in *P. aeruginosa* S3 appears to be sufficient to express its pathogenic phenotype. Production of siderophores is reported to be essential in the pathogenesis of *P. aeruginosa* [59,60]. *P aeruginosa* S3, which exhibited siderophore production in the phenotype expression experiment was found to carry thirteen *pvd* cluster genes associated with pyoverdin synthesis and its regulation and secretion; and another nine genes involved in pyochelin synthesis and secretion. We found the presence of several other important virulence-associated genes similar to PAO1 that included *Las A* (protease precursor) and the pseudomonas elastase gene *Las B* (extracellular zinc protease) which render the ability to invade tissues and thus cause damage to host cells [61,62].

Several genes encoding multiple secretion systems that are associated with host colonization and virulence were detected, e.g., genes associated with the type III secretion system which are grouped in five operons comprising structural and regulatory genes *viz*. *pscNOPQRSTU*, *popNpcr1234DR*, *pcrGVHpopBD*, *exsCEBA*, and *exsDpscBCDEFGHIJKL* [63]. Besides, genes of several effectors associated with the type III secretion system such as *exoS*, *exoY*, and *exoT* were found. ExoS supports cell invasion and intracellular persistence through its ADP-ribosyltransferase activity [64]. However, *exoU*, considered the most virulent of the T3SS effectors and the main driver of the cytotoxic phenotype was not found in *P. aeruginosa* S3. Isolation of virulent *P. aeruginosa* strains lacking one or more such effectors is not unusual and PAO1, known to be hemolytic and cytotoxic [19], also lacks this gene. Moreover, the presence of *exoS* and *exoU* is almost exclusive [63]. In the context that some high-risk strains carry either *exoU* or *exoS*; authors have suggested that environmental strains are more likely to carry *exoS* which is more prevalent while strains of specific clinical origin mostly carry *exoU* [65]. Despite not carrying *exoU*, the cytotoxic phenotype showed by *P. aeruginosa* S3 nevertheless implies that it is a health risk that demands attention. Genes associated with the type IV secretion system which matched with *P. aeruginosa* PAO1 included *pilA* (the major abundant pilin subunit PilA), *fimUpilVWXY1Y2E* operon (several less abundant, fiber-associated pilin-like proteins and surface sensor), the *pilMNOPQ* operon (proteins necessary for assembly and twitching motility), and PilD, the prepilin leader peptidase needed for processing the major and minor pilins [57,66,67] were also found. This system is involved in host tissue adherence and colonization and promoting surface-associated twitching motility [67]. In addition, S3, like PAO1, carried the virulence locus HSI-1 of the type VI secretory system which structurally resembles

Table 4. Prediction of antimicrobial resistance genes of *Pseudomonas aeruginosa* S3 based on CARD, and NDARO database.

Gene	Product	Source ID	Source Organism
oprN	Multidrug efflux system, outer membrane factor lipoprotein = >OprN of MexEF-OprN system	NP_251185.1	*P.aeruginosa* PAO1
opmE	Multidrug efflux system, outer membrane factor lipoprotein = >OpmE of MexPQ-OpmE system	BAE06009.1	*P.aeruginosa*
mexL	Transcriptional regulator, AcrR family	NP_252368.1	*P.aeruginosa* PAO1
PmrB	Sensory histidine kinase QseC	AEX49906.1	*P.aeruginosa*
mexM	RND efflux system, membrane fusion protein	BAE06005.1	*P.aeruginosa*
oprD	Outer membrane low permeability porin, OprD family = > OccD1/OprD basic amino acids, carbapenem uptake	NP_249649.1	*P.aeruginosa* PAO1
gyrA	DNA pase subunit A (EC 5.99.1.3)	NP_251858.1	*P.aeruginosa* PAO1
amrB	Multidrug efflux system, inner membrane proton/drug antiporter (RND type) = >MexY of MexXY/AxyXY	NP_250708.1	*P.aeruginosa* PAO1
mexE	Multidrug efflux system, membrane fusion component = >MexE of MexEF-OprN system	NP_251183.1	*P.aeruginosa* PAO1
mexH	Multidrug efflux system, membrane fusion component = >MexH of MexHI-OpmD system	NP_252895.1	*P.aeruginosa* PAO1
mexZ	Transcriptional repressor of mexXY operon, MexZ	NP_250710.1	*P.aeruginosa* PAO1
parC	DNA topoisomerase IV subunit A (EC 5.99.1.3)	BAA37152.1	*P.aeruginosa*
oprM	Multidrug efflux system, outer membrane factor lipoprotein = >OprM of MexAB-OprM	NP_249118.1	*P.aeruginosa* PAO1
TriC	Multidrug efflux system, inner membrane proton/drug antiporter (RND type) = >TriC of TriABC-OpmH system	NP_248848.1	*P.aeruginosa* PAO1
oprJ	Multidrug efflux system, outer membrane factor lipoprotein = >OprJ of MexCD-OprJ system	AAB41958.1	*P.aeruginosa*
TriB	Multidrug efflux system, membrane fusion component = >TriB of TriABC-OpmH system	NP_248847.1	*P.aeruginosa* PAO1
phoQ	Sensor histidine kinase PhoQ (EC 2.7.13.3)	NP_249871.1	*P.aeruginosa* PAO1
nalC	Transcriptional regulator, AcrR family	NP_252410.1	*P.aeruginosa* PAO1
mexD	Multidrug efflux system, inner membrane proton/drug antiporter (RND type) = >MexD of MexCD-OprJ system	NP_253288.1	*P.aeruginosa* PAO1
mexA	Multidrug efflux system, membrane fusion component = >MexA of MexAB-OprM	NP_249116.1	*P.aeruginosa* PAO1
OpmH	Outer membrane channel TolC (OpmH)	NP_253661.1	*P.aeruginosa* PAO1
mexK	Multidrug efflux system, inner membrane proton/drug antiporter (RND type) = >MexK of MexJK-OprM/OpmH system	AAG07064.1	*P.aeruginosa* PAO1
FosA	Fosfomycin resistance protein FosA	NP_249820.1	*P.aeruginosa* PAO1
mexW	Multidrug efflux system, inner membrane proton/drug antiporter (RND type) = >MexW of MexVW-OprM system	AAG07763.1	*P.aeruginosa* PAO1
mexF	Multidrug efflux system, inner membrane proton/drug antiporter (RND type) = >MexF of MexEF-OprN system	NP_251184.1	*P.aeruginosa* PAO1
PmrA	Two-component system response regulator QseB	NP_253464.1	*P.aeruginosa* PAO1
mexN	Multidrug efflux system MdtABC-TolC, inner-membrane proton/drug antiporter MdtB-like	BAE06006.1	*P.aeruginosa*
TriA	Multidrug efflux system, membrane fusion component = >TriA of TriABC-OpmH system	NP_248846.1	*P.aeruginosa* PAO1
PDC-1	Class C beta-lactamase (EC 3.5.2.6) = > PDC family	ACQ82807.1	*P.aeruginosa* PAO1

(*Continued*)

Table 4. (Continued)

Gene	Product	Source ID	Source Organism
mexV	Multidrug efflux system, membrane fusion component = >MexV of MexVW-OprM system	AAG07762.1	*P.aeruginosa* PAO1
mexC	Multidrug efflux system, membrane fusion component = >MexC of MexCD-OprJ system	AAB41956.1	*P.aeruginosa*
mexS	Putative oxidoreductase	ADT64081.1	*P.aeruginosa* PAK
mexI	Multidrug efflux system, inner membrane proton/drug antiporter (RND type) = >MexI of MexHI-OpmD system	NP_252896.1	*P.aeruginosa* PAO1
arnA	UDP-4-amino-4-deoxy-L-arabinose formyltransferase (EC 2.1.2.13) / UDP-glucuronic acid oxidase (UDP-4-keto-hexauronic acid decarboxylating) (EC 1.1.1.305)	NP_252244	*P.aeruginosa* PAO1
mexR	Multidrug resistance operon repressor MexR, MarR family	NP_249115.1	*P.aeruginosa* PAO1
parE	DNA topoisomerase IV subunit B (EC 5.99.1.3)	NP_253654.1	*P.aeruginosa* PAO1
mexP	Multidrug efflux system, membrane fusion component = >MexP of MexPQ-OpmE system	BAE06007.1	*P.aeruginosa*
mexB	Multidrug efflux system, inner membrane proton/drug antiporter (RND type) = >MexB of MexAB-OprM	AAA74437.1	*P.aeruginosa*
nfxB	Transcriptional regulator NfxB	NP_253290.1	*P.aeruginosa* PAO1
amrA	Multidrug efflux system, membrane fusion component = >MexX of ofMexXY/AxyXY	NP_250709.1	*P.aeruginosa* PAO1
catB7	Chloramphenicol O-acetyltransferase (EC 2.3.1.28) = >CatB family	NP_249397.1	*P.aeruginosa* PAO1
mexJ	Multidrug efflux system, membrane fusion component = >MexJ of MexJK-OprM/OpmH system	NP_252367.1	*P.aeruginosa* PAO1
opmD	Multidrug efflux system, outer membrane factor lipoprotein = >OpmD of MexHI-OpmD system	NP_252897.1	*P.aeruginosa* PAO1
nalD	Transcriptional regulator, AcrR family	NP_252264.1	*P.aeruginosa* PAO1
APH(3')-IIb	Aminoglycoside 3'-phosphotransferase (EC 2.7.1.95) = > APH(3')-II/APH(3')-XV	CAA62365.1	*P.aeruginosa*
mexG	hypothetical protein	NP_252894.1	*P.aeruginosa* PAO1
OXA-50	Class D beta-lactamase (EC 3.5.2.6) = > OXA-50 family, oxacillin-hydrolyzing	AAQ76277.1	*P.aeruginosa*
mexQ	Multidrug efflux system, inner membrane proton/drug antiporter (RND type) = >MexQ of MexPQ-OpmE system	BAE06008.1	*P.aeruginosa*
phoP	Transcriptional regulatory protein PhoP	NP_249870.1	*P.aeruginosa* PAO1
emrE	small multidrug resistance family (SMR) protein	NP_253677.1	*P.aeruginosa* PAO1

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the contractile tails of bacteriophages [68]. Hemolysin-coregulated protein (Hcp) and valine–glycine repeat protein G (VgrG) act as major structural components and effectors of this system which are associated with key functions in virulence, biofilm formation and competition with microorganisms in the environment [57,69]. The presence of these versatile secretary machinery might play a role in the pathogenicity of *P. aeruginosa* S3.

Antibiotic resistance machinery in bacteria develops through a spectrum of genomic changes [70]. Genomic studies revealed the presence of two crucial beta-lactamases, class C beta-lactamase *PDC-1* and class D beta-lactamase *OXA-50*, which occur naturally in *P. aeruginosa* [71]. Therefore, *P. aeruginosa* is, in general, resistant to the beta-lactam class of antibiotics [72,73] but, higher susceptibility is often found towards cephalosporins and imipenem in both clinical [74] and environmental [75] isolates. Notably, our strain showed complete resistance towards all beta-lactam class of antibiotics. The expression of class C beta-lactamase PDC

Table 5. Hemolysin genes prediction of *Pseudomonas aeruginosa* S3 based on PATRIC database.

Hemolysin-related gene annotations (PATRIC)	Identity of the Gene (based on NCBI BLASTP)	Percentage identity(%)	Source organism
Hemolysin activation/secretion protein associated with VreARIsignalling system	ShlB/FhaC/HecB	100	*Pseudomonas* sp.
Putative hemolysin	GNAT family N(alpha)-acetyltransferase	100	*Pseudomonas aeruginosa*
Hemolysin activation/secretion protein	ShlB/FhaC/HecB	100	*Pseudomonas* sp.
Hemolysin activation/secretion protein	two-partner secretion system transporter CdrB	100	*Pseudomonas* sp.
FIG01964566: Predicted membrane protein, hemolysin III homolog	Hemolysin III family protein	99.51	*Pseudomonas aeruginosa*
Phospholipase/lecithinase/hemolysin	cholinesterase/ ChoE	100	*Pseudomonas* sp.
Phospholipase/lecithinase/hemolysin	PapA	100	*Pseudomonas aeruginosa* PAO1
Phospholipase C, hemolytic PlcH	phospholipase C, phosphocholine-specific	100	*Pseudomonas* sp.

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(Pseudomonas-derived cephalosporinase) is considered the main resistance mechanism in *P. aeruginosa* against the beta-lactam class of antibiotics [76]. On the other hand, the most common type of carbapenemases is the Class D β-lactamases, also known as oxacillinases (OXA) which hydrolyses imipenem [71]. The presence of both *PDC-1* and *OXA-50* in the draft genome of S3 might play a role in the resistance shown by S3 towards all beta-lactam type of antibiotics. However, other mechanisms might also be involved, including those of RND (resistance-nodulation-cell division) family efflux pumps. Apart from the intrinsic mechanisms, several efflux systems have been characterized in *P. aeruginosa* which contribute more actively to resistance by extrusion of antibiotics from the cell [77]. Genes encoding ten of these multidrug efflux systems belonging to the RND family which are mexAB-OprM, mexC-D-OprJ, mexEF-OprN, mexGHI-OpmD, mexJK-OprM, mexMN-OprM, mexPQ-OmpE, mexVW-OprM, mexXY-OprM and TriABC-OmpH were found in S3. In addition, the gene encoding efflux pump of the SMR (small multidrug resistance) family, namely *emrE* was also detected [78]. All of these genes matched with PAO1. While the expression of most of these is tightly regulated, overexpression resulting from antibiotic exposure occurs due to mutations in regulatory proteins [77]. Resistance to tetracyclines, folate pathway inhibitors and norfloxacin in S3 observed in this study might be due to overexpression of some of the efflux systems or mutations in the topoisomerase genes *gyrA/gyrB* and *parA/parB* [79,80]. However, further studies are required to explain these resistant phenotypes. Antibacterial colistin (polymyxin E) has recently received increasing attention as a last-resort treatment option against multidrug-resistant Gram-negative bacteria [81]. Reported data suggests that adaptive resistance to polymyxins in Gram-negative bacteria occurs due to modifications of outer membrane lipopolysaccharide (LPS) mediated by *pmrHFIJKLME* operon which is under the regulation of the two two-component systems, PhoPQ and PmrAB [82]. Equally important is resistance mediated by the *mexAB-oprM* operon, which is involved in nonspecific antimicrobial efflux [83]. The *pmrHFIJKLME* operon was not detected in S3 but the *mexAB-oprM* operon and the genes coding for regulatory components PmrAB and PhoPQ were found. Colistin resistance in S3 may be due to other mechanisms preliminarily confirmed to occur in colistin-resistant *P. aeruginosa* [84,85]. Hence, further studies on molecular mechanisms involved in colistin resistance in S3 should be of interest. Aminoglycoside resistance in *P. aeruginosa* is mainly due to the structural modification by three types of enzymes, aminoglycoside phosphotransferase (APH), aminoglycoside acetyltransferase (AAC), and aminoglycoside nucleotidyl transferase (ANT). APH in *P. aeruginosa* transfers a phosphoryl group to the 3′-hydroxyl of aminoglycosides, making antibiotics such as streptomycin and kanamycin inactive [86]. The *APH* gene was

found in S3 and matched with PAO1, but the other two genes were absent which is consistent with the finding that S3 displayed resistance to kanamycin and streptomycin but not to gentamycin. The chloramphenicol resistance gene *catB7* encoding chloramphenicol O-acetyltransferase was detected which corroborates with chloramphenicol resistance observed in the disc diffusion test but identification of the exact mode of resistance, especially in the presence of the robust efflux system which can use chloramphenicol as substrate requires further investigations. Additionally, *fosA* encoding the fosfomycin resistance protein, widely distributed among several Gram-negative bacteria including PAO1 [87] was found.

Five genes were predicted as putative hemolysins in *P. aeruginosa* S3. It included the *plcH* gene that encodes hemolytic phospholipase C [4]. This gene, found to be upregulated in biofilm-forming cells compared to planktonic cells [88], was also reported to be present in hemolytic *P. aeruginosa* strains isolated from environmental sources [89]. The capability of *P. aeruginosa* to form biofilms is associated with its ability to induce long-lasting chronic lung infections and antibiotic resistance [90]. Phospholipase C contributes to the breakdown of lipids and lecithin and facilitates its establishment in the respiratory tract. Moreover, it helps in its survival within the tissue by suppressing the respiratory burst response of neutrophils [91]. Another gene predicted as hemolysin was cholinesterase (ChoE) which is reported to act together with PlcH and PchP (acid phosphatase) in *P. aeruginosa* to obtain choline and inorganic phosphate as nutrients for bacterial metabolism [92]. However, PchP was not detected in the draft genome of S3 thus suggesting the occurrence of multiple modes of action. It was observed that *P. aeruginosa* cholinesterase (PA4291 gene) hydrolyses acetylcholine and is regarded as a pathogenic factor that supports corneal infection [93]. Clinical changes in the fish eyes induced by S3 were also observed in the current study but understanding the extent of involvement of this gene here requires further studies. Another predicted gene was identified as the hemolysin III family protein. Well-characterized in *B. cereus*, hemolysin III family proteins are regarded as pore-forming toxins [94]. But, the role of this gene in pathogenicity in *P. aeruginosa* remains to be studied. The fourth putative hemolysin was a GNAT family protein known as a large group of enzymes that acylates various substrates using acyl-coenzyme A [95]. They execute a wide range of cellular functions, including the activation of pore-forming toxins by a distinct group of this family known as toxin-activating acyltransferase [96]. Shin and Choe [97] characterized the PA4534 protein from *P. aeruginosa*, a GNAT protein, and found it closely related to N-terminal acetyltransferase. The fifth gene predicted to encode a putative hemolysin in S3 was identified as the PapA gene which codes for type P pilin in *E. coli* and is involved in uropathogenicity [98]. All of these genes matched with several distinct strains of *Pseudomonas*, including PAO1. The participation of all or some of these proteins might be instrumental in the strong hemolytic activity observed in *P. aeruginosa* S3 and further research on understanding their contribution towards hemolysin action should be of interest.

Conclusion

This study reported the genotypic and phenotypic characterization of *P. aeruginosa* S3, a pathogenic and antibiotic-resistant strain isolated from the Mahananda River. The draft genome of S3 provided better insights into the virulent and antibiotic-resistant phenotype. The presence of eight genes related to hemolysin action was detected. In addition, several genes involved in antibiotic efflux mechanisms were found. To our knowledge, this is the first report characterizing pathogenic and antibiotic-resistant *P. aeruginosa* from this river though, additional research is required to comprehend how the virulent and antibiotic resistance genes function fully. *P. aeruginosa* is a common recurrent pathogen frequently isolated from clinical settings. However, the isolation of a virulent and antibiotic-resistant strain from river water raises

public health concerns and gives a warning about the appropriate use of antibiotics. Therefore, periodic monitoring of *P. aeruginosa* in the river water is needed as it indicates poor water quality that could lead to disease outbreaks.

Supporting information

S1 File.
(DOCX)

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