

Article

Isolation, Identification, and Virulence Properties of *Enterobacter bugandensis* Pathogen from Big-Belly Seahorse *Hippocampus abdominalis*

Haibin Ye ^{1,†}, Chenhao Teng ^{2,3,†}, Yueqi Yang ^{2,3}, Yiyao Liu ^{2,3}, Li Li ¹, Ying Fan ¹, Youhong Wang ¹, Jing Diao ¹, Lingling Yu ^{2,3}, Chunlei Gai ^{1,*} and Haipeng Cao ^{2,3,*}

¹ Marine Science Research Institute of Shandong Province (National Oceanographic Center, Qingdao), Qingdao 266104, China; yehaibin@263.net (H.Y.); gzklili@163.com (L.L.); fy_fy123@126.com (Y.F.); 13335033571@163.com (Y.W.); doro530@sina.com (J.D.)

² National Pathogen Collection Center for Aquatic Animals, Shanghai Ocean University, Shanghai 201306, China; tengchenhhh@163.com (C.T.); m15943611800@163.com (Y.Y.); 13276450966@163.com (Y.L.); 13661690985@163.com (L.Y.)

³ Ministry of Agriculture and Rural Affairs of China Key Laboratory of Freshwater Fishery Germplasm Resources, Shanghai Ocean University, Shanghai 201306, China

* Correspondence: chunlei317@163.com (C.G.); hpcao@shou.edu.cn (H.C.)

† These authors contributed equally to this work.

Abstract

Nowadays, members of the genus *Enterobacter* have been documented as human and aquaculture pathogens. To date, no reports have described *Enterobacter bugandensis* infecting *Hippocampus abdominalis*. In the present study, an isolate of *E. bugandensis*, designated H4, was identified as a causative pathogen in cultured *H. abdominalis* following Koch's postulate, and its virulence properties were further described. The isolate's genome consisted of a single circular chromosome and harbored several virulence and resistance genes, including, but not limited to, *csgG*, *acrB*, *hcp*, *gndA*, *galF*, *rpoS*, *fur*, *rscB*, and *phoP* involved in adherence, antimicrobial activity, effector delivery systems, immune modulation, and regulation, as well as *baeR*, *blaACT-49*, *ramA*, *hns*, *ftsI*, *acrA*, *gyrA*, *fabI*, *crp*, *oqx*, *parE*, *gyrB*, *phoP*, *rpoB*, *tuf*, *ptsI*, and *fosA2* functioning against aminoglycoside, cephamycin, disinfecting agent and antiseptic, fluoroquinolone, macrolide, peptide, and other antimicrobials. Additionally, the isolate exhibited multiple resistance to cephalosporins, penicillins, and tetracyclines and demonstrated a median lethal dose (LD₅₀) of 4.47×10^5 CFU/mL in *H. abdominalis*. To our knowledge, this is the first study to describe *E. bugandensis* infecting *H. abdominalis*. These findings highlight the zoonotic potential of *E. bugandensis* and underscore the need for targeted health management in seahorse farming.

Keywords: antimicrobial resistance; virulence genes; LD₅₀; seahorse aquaculture

Key Contribution: The virulence, multiple resistance, and wide distribution of pathogenicity-related genes in *E. bugandensis* (H4) support it as a potential threat to big-belly seahorse aquaculture.



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1. Introduction

The big-belly seahorse *Hippocampus abdominalis* is one of the largest seahorse species and possesses anti-fatigue, anti-cancer, and anti-melanogenic bioactivities [1–3], which contribute to its popularity among consumers [4]. Due to the wide application of natural

products derived from this species in medicine, health products, and functional foods, its aquaculture has become increasingly significant [1]. In recent years, the big-belly seahorse has been widely cultivated in several countries, including China, New Zealand, Australia, and South Korea [5,6]. However, the global aquaculture industry of this species has been severely impacted by bacterial infections, leading to considerable economic losses [7].

Members of the genus *Enterobacter* have been associated with urinary tract infection, intestinal epithelial cell apoptosis, and neonatal sepsis in humans [8–10], as well as mortalities in grey mullet *Mugil cephalus*, striped catfish *Pangasianodon hypophthalmus*, channel catfish *Ictalurus punctatus*, silver arowana *Osteoglossum bicirrhosum*, giant freshwater prawn *Macrobrachium rosenbergii*, ornamental fish *Etroplus maculatus*, and crayfish *Procambarus clarkii* [11–18]. Recently, whole genome sequencing has been employed to identify putative virulence factors in *Enterobacter* pathogens for a better understanding of their pathogenesis [19]. For example, the genomic analysis of silver arowana-pathogenic *E. roggenkampii* has been performed to identify 730 virulence genes [18]. However, no information is available regarding the *E. bugandensis* pathogen in aquaculture.

Enteritis is a highly lethal disease that poses a serious threat to the sustainable development of seahorse aquaculture [18], resulting in a mortality of 20% to 100% in the big-belly seahorses [20]. In the present study, an isolate (H4) of *E. bugandensis* was identified as a causative pathogen of the big-belly seahorse, and its genomic characterization, antimicrobial susceptibility, pathogenicity-related genes, and virulence were further tested. To our knowledge, this is the first report of *E. bugandensis* as an enteritis agent in big-belly seahorses, and the genomic analysis revealed potential sanitary risks to aquaculture.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments and experimental protocols (Figure 1) were approved by the Institutional Animal Care and Use Ethics Committee of Shanghai Ocean University under approval no. SHOU-DW-2024-190.

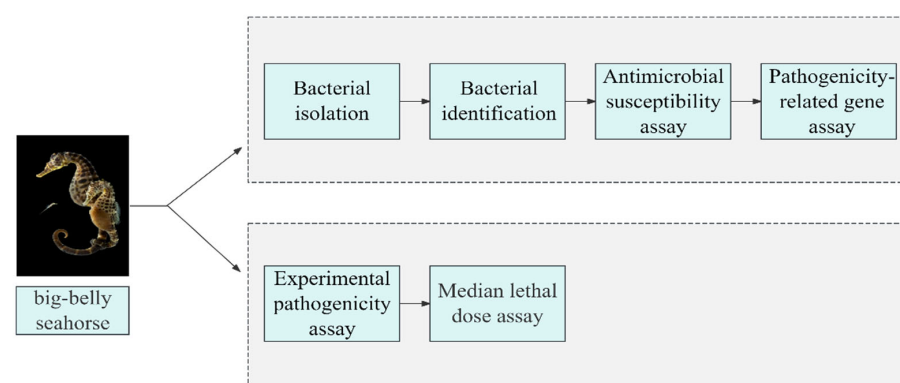


Figure 1. Illustrative workflow showcasing the analysis of bacterial pathogens from big-belly seahorses.

2.2. Bacterial Isolation

Twenty naturally diseased big-belly seahorses (8.55 ± 3.49 g in weight) suffering from enteritis were collected from an infected seahorse farm in Yantai, China, in 2023, and promptly transported to the laboratory within 1 h in sterile ice-cold containers for subsequent pathogen analysis according to Hossain et al. [21]. Diseased big-belly seahorses were externally disinfected with 70% ethanol, and liver tissues were sampled as recommended by Li et al. [22] for bacterial isolation. Bacterial isolation was performed by streaking samples onto seawater nutrient agar (NA) (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) plates using a flamed loop, as described by Wen et al. [23]. The inoculated plates were

incubated at 28 °C for 24 h, and individual colonies were purified by repeated streaking on seawater NA plates [24] after 24 h of incubation at 28 °C. In addition, the gill, muscle, and visceral tissues, such as livers and intestines, were dissected for microscopic examination of potential parasites, following the method described by Zhang et al. [25]. Ultrathin sections of the sampled tissues were also prepared and stained with uranyl acetate and lead citrate for virus observation under an electron microscope according to Zhang and Li [26].

2.3. Bacterial Identification

2.3.1. 16S rRNA Gene Sequencing Analysis

The extraction of genomic DNA from the isolate was carried out using the TIANamp bacteria DNA kit (Tiangen Biotech. Co. Ltd., Beijing, China). The 16S rRNA gene from the isolate was amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') according to Chen et al. [11]. The PCR reaction was performed in 25 µL total volume comprising 1 µL of DNA template, 1 µL of each primer (10 µM), 9.5 µL of ddH₂O, and 12.5 µL of 2× Taq Master mix (Vazyme, Nanjing, China). Amplification conditions included an initial single cycle at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 40 s, and a final single cycle at 72 °C for 10 min. The PCR product was examined by electrophoresis on a 2% (*w/v*) agarose gel stained with ethidium bromide (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and visualized by UV transillumination using a gel-imaging system (Shanghai Lingcheng Biotech. Co., Ltd., Shanghai, China). Subsequently, the resultant PCR product was purified and sequenced by Shanghai MAP Biotech. Co., Ltd., Shanghai, China. Sequence analysis was performed using the Basic Local Alignment Search Tool (BLAST) v.2.14.0 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 2 January 2024) against the GenBank database. A phylogenetic tree was constructed based on the partial sequences of 16S rRNA genes using the neighbor-joining method.

2.3.2. Genomic Sequencing Analysis

Genomic DNA was extracted from the isolate using the TIANamp bacteria DNA kit (Tiangen Biotech. Co., Ltd., Beijing, China) and was further subjected to whole genome sequencing by use of the Illumina HiSeq and PacBio platform (Shanghai Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China) according to Zhu et al. [27]. Sequence reads were assembled using SOAPdenovo2 and Canu [28,29], and the circular genome map was constructed using Circos v.0.69 [30]. The genomic features, including the number of tRNA, rRNA, sRNA, protein coding sequences (CDS), tandem repeats, prophage, genomic islands (GI), and insertion sequences (IS), were predicted using programs including tRNAscan-SE v.2.0, Barrnap v.0.8, Infernal, Glimmer v.3.02, GeneMarkS, Tandem Repeats Finder v.4.07, Phage Finder, IslandPath-DIMOB v.1.0.0, and ISEScan [31–38]. Whole genome-relatedness indices (OGRIs), including average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH), were calculated for the isolate using the BLAST v.2.15.0 algorithm (ANiB) and MUMmer alignment tool (ANIm) at JSpecies WS (<https://jspecies.ribohost.com/jspeciesws/>, accessed on 14 May 2025), as well as formula 2 of the Genome-to-Genome Distance Calculator 3.0 with the recommended BLAST+ v.2.15.0 alignment tool (<https://ggdc.dsmz.de/ggdc.php>, accessed on 14 May 2025), under the species delineation thresholds of 95% ANI and 70% dDDH [39].

2.3.3. Phenotypic Identification

Phenotypic identification of the isolate was conducted in technical triplicate using the API 20E strip (BioMerieux, Marcy l'Etoile, France) according to the manufacturer's

instructions [40]. The phenotypic traits of *E. bugandensis* type strain EB-247, previously described by Doijad et al. [41], were used as references.

2.4. Antimicrobial Susceptibility Assay

The antimicrobial susceptibility of the isolate was examined in technical triplicate using the Kirby–Bauer disc diffusion method [42]. Briefly, the Mueller–Hinton (MH) agar plates were inoculated with the isolate (100 µL) at 1.0×10^7 CFU/mL. Antimicrobial discs containing ampicillin, piperacillin, cefazolin, cefuroxim, ceftazidime, cotrimoxazole, gentamicin, kanamycin, doxycycline, minocycline, tetracycline, and ciprofloxacin (Hangzhou Binhe Microorganism Reagent Co., Ltd., Hangzhou, China) were then aseptically placed onto the surface of inoculated MH agar plates. Following incubation at 28 °C for 18 h, antimicrobial inhibition zone diameters (mm) were measured and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) antibiotic susceptibility testing standards [43,44].

2.5. Pathogenicity-Related Gene Assay

The pathogenicity-related genes in the isolate were identified by aligning its genome (see Section 2.3.2) against the virulence factors database (VFDB, <http://www.mgc.ac.cn/VFs/>, accessed on 20 May 2025) and comprehensive antibiotic resistance database (CARD, <http://arpcard.mcmaster.ca>, accessed on 20 May 2025) [38,45], at E-values $\leq 1 \times e^{-5}$, identity $\geq 80\%$ and coverage $\geq 70\%$ as recommended by Liu et al. [46] and Ministry of Agriculture and Rural Affairs of China [47].

2.6. Experimental Seahorses

Healthy big-belly seahorses (3.76 ± 0.17 g in weight), sourced from a seahorse farm in Weihai, China, and free of any bacterial pathogens through an examination of culturing blood and liver smears from a few sampled seahorses on sea water NA plates according to Biswas et al. [48], were acclimated in aerated seawater (18 °C, pH7.8, and salinity of 22 ppt, monitored directly using temperature meter (CWY122; Zhejiang LECAN Precise Tools Co., Ltd., Huzhou, China), pH meter (pH-100A; Shanghai Lichen Instrument Technology Ltd., Shanghai, China) and salinity meter (EC-30; INESA Scientific Instrument Co., Ltd., Shanghai, China)) under a photoperiod of 14 h:10 h (light–dark) for 14 days. During the acclimation period, healthy seahorses were fed twice daily at 8:00 and 15:00 with *Artemia* [24].

2.7. Experimental Pathogenicity Assay

Prior to the bacterial challenge, the suspension of the isolate was prepared as described in our previous study [49] and enumerated as 1.0×10^8 colony-forming units (CFU)/mL by counting CFU on seawater NA plates from a series of ten-fold dilutions in sterile normal saline [50]. Bacterial challenge was conducted in biological replicates for four isolates from the diseased seahorses according to Xie et al. [51] in aquaria (38 cm $\times$ 52 cm $\times$ 48 cm) containing 50 L aerated seawater at 18 °C via intraperitoneal infection [52]. Healthy big-belly seahorses of uniform size [53] were randomly divided into control and treatment groups (three replicate aquaria per group, ten seahorses per aquarium, thirty seahorses per group). The seahorses in the treatment group were intraperitoneally injected with 0.1 mL of the isolate suspension at 1.0×10^8 CFU/mL according to Li et al. [54], while the control group of seahorses received intraperitoneal injection with 0.1 mL of sterile normal saline. During the bacterial challenge, no water exchange or feeding was conducted. All seahorses in both control and treatment groups were observed daily for seven continuous days according to Li et al. [54] to record pathological signs and calculate mortality rates. The challenge strain was re-isolated from artificially infected seahorses and identified following

16S rRNA gene sequencing and phenotypic methods described by Cao et al. [15] and Wen et al. [23] to confirm the causative agent.

2.8. Median Lethal Dose Assay

Median lethal dose (LD₅₀) of the isolate was assayed in biological replicates in aquaria (38 cm × 52 cm × 48 cm) containing 50 L aerated seawater at 18 °C via intraperitoneal infection, as recommended by Wang et al. [52]. Prior to this assay, the isolate suspensions were freshly prepared and enumerated as 2.0×10^5 , 2.0×10^6 , 2.0×10^7 , and 2.0×10^8 CFU/mL according to our previous study [49]. A total of 150 healthy big-belly seahorses of uniform size [53] were randomly divided into one control group and four treatment groups (three replicate aquaria per group, ten seahorses per aquarium). The seahorses in the treatment groups were intraperitoneally injected with 0.1 mL of the isolate suspensions at concentrations of 2.0×10^5 , 2.0×10^6 , 2.0×10^7 , and 2.0×10^8 CFU/mL, respectively, and the seahorses in the control group received intraperitoneal injection with 0.1 mL of sterile normal saline. During the experiment, no water exchange or feeding was carried out. All seahorses in both control and treatment groups were observed daily for ten continuous days to record mortality and calculate survival rates. The 10-day intraperitoneal LD₅₀ value was calculated using the probit method [55] to assess the virulence of the isolate. Additionally, re-isolation and identification of the isolate from experimentally infected seahorses were performed following the procedure described by Cao et al. [15] and Wen et al. [23] to confirm the causative agent.

2.9. Statistical Analysis

All values are expressed as mean ± standard deviation (SD). Antimicrobial inhibition zone diameters and survival rates were subjected to normality testing and analyzed using one-way analysis of variance (ANOVA) with Dunnett's post hoc test in SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Bacterial Isolation

Four isolates, designated H1, H2, H3, and H4, were recovered from the liver of naturally diseased seahorses, with no parasites or viruses detected. Phylogenetic analysis of their 16S rRNA genes (GenBank accession nos PP724349, PP725560, PP724360, and PP133232) revealed that these isolates belonged to the genera *Bacillus* and *Enterobacter* (Figure S1 and Figure 2). Among these, only isolate H4 was virulent to big-belly seahorses (Table S1) and thus was further described.

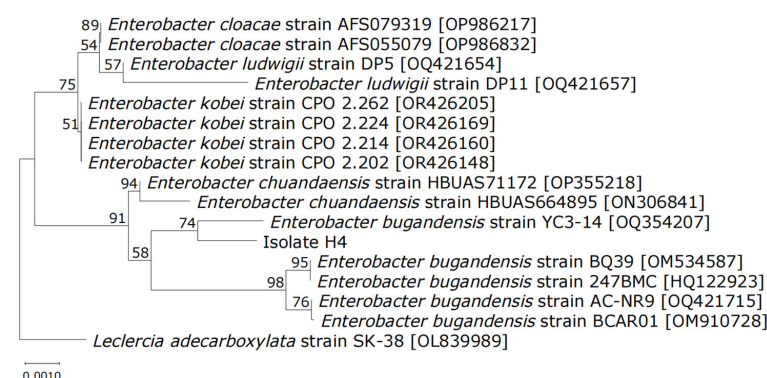


Figure 2. The 16S rRNA phylogenetic tree of isolate H4 and 16 known bacteria constructed based on the partial sequences of 16S rRNA genes using the neighbor-joining method. The bootstrap values (%) are shown beside the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.

3.2. Bacterial Identification

The partial 16S rRNA gene and whole-genome sequences of isolate H4 were deposited at the NCBI BioSample and GenBank databases under accession nos. PP133232, SAMN39216373, and CP142002, respectively. Sequence analysis showed that isolate H4 shared 99% similarity with available *E. bugandensis* strains available in the GenBank database. The phylogenetic analysis (Figure 2) further confirmed isolate H4 as an *E. bugandensis* strain.

The complete genome of isolate H4 (Figure S2) consisted of a single circular chromosome with a genome size of 4,821,486 bp and GC content of 55.91%. A total of 83 tRNAs, 25 rRNAs, 181 sRNAs, 4438 CDS, 104 repeats, 4 prophages, 12 GI, and 1 IS were predicted in the genome of isolate H4 (Table 1). Furthermore, the ANIb, ANIm, and dDDH values between isolate H4 and *E. bugandensis* type strain EB-247 were calculated as 98.47%, 98.76%, and 95.48%, exceeding the species delineation thresholds of ANI (95%) and dDDH (70%). In contrast, the ANI and dDDH values between isolate H4 and other *Enterobacter* species were below these thresholds (Figure 3), further supporting isolate H4 as *E. bugandensis*.

Table 1. The genomic features of isolate H4.

Feature	Genome
Genome size (bp)	4,821,486
Plasmid	0
Chromosome	1
GC content (%)	55.91
Number of CDS	4438
Gene average length (bp)	971.21
Number of tRNAs	83
Number of 5S rRNAs	9
Number of 16S rRNAs	8
Number of 23S rRNAs	8
sRNAs	181
Repeated regions (%)	0.29
Number of repeats	104
Number of GIs	12
Number of prophages	4
Number of IS	1

GC, guanine–cytosine; CDS, coding sequences; GIs, genomic islands; IS, insertion sequence.

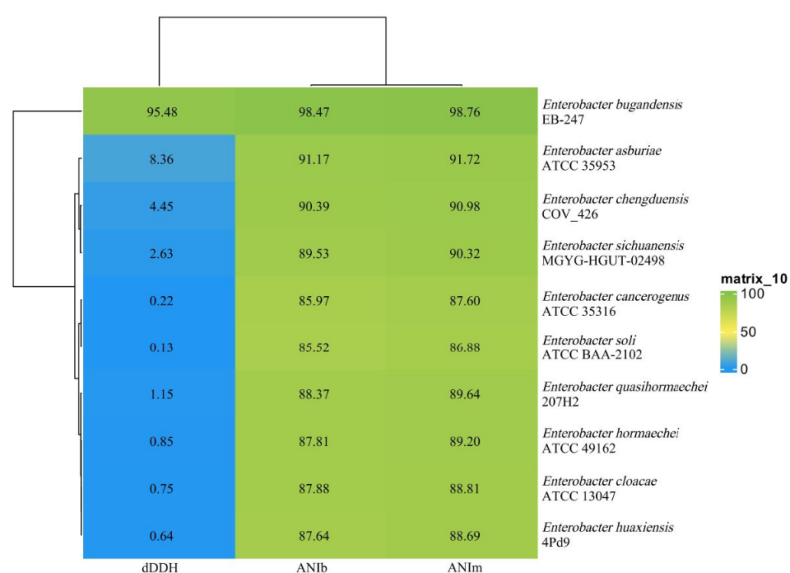


Figure 3. Heat map analysis of the genetic similarity between strain H4 based on the whole genome sequence and other *Enterobacter* species (ANIb, the average nucleotide identity with the alignment algorithm BLAST+ v.2.15.0 (National Center for Biotechnology Information, Bethesda, USA); ANIm, the average nucleotide identity with the alignment algorithm MUMmert; dDDH, the digital DNA–DNA hybridization values with the Genome-to-Genome Distance Calculator 3.0).

Phenotypically, isolate H4 showed 100% identity to *E. bugandensis* (Table 2). It was positive for arginine dihydrolase, ornithine decarboxylase, β -galactosidase, citrate utilization, and fermentation of glucose, sucrose, mannitol, rhamnose, melibiose, arabinose, inositol, and sorbitol. Conversely, it was negative for lysine decarboxylase, urease, phenylalanine deaminase, production of acetoin, indole, and H_2S , gelatin hydrolysis, and amygdalin fermentation. These phenotypic characteristics confirmed isolate H4 as *E. bugandensis*.

Table 2. Phenotypic characterization of isolate H4.

Characterization	Isolate H4	<i>E. bugandensis</i> Strain EB-247 ^a
Voges–Proskauer	-	-
Arginine dihydrolase	+	+
Lysine decarboxylase	-	-
Ornithine decarboxylase	+	+
β -galactosidase	+	+
Urease	-	-
Phenylalanine deaminase	-	ND
Indole production	-	-
H_2S production	-	-
Gelatin hydrolysis	-	-
Citrate utilization	+	+
Glucose fermentation	+	+
Sucrose fermentation	+	+
Mannitol fermentation	+	+
Rhamnose fermentation	+	+
Melibiose fermentation	+	+
Arabinose fermentation	+	+
Inositol fermentation	+	+
Sorbitol fermentation	+	+
Amygdalin fermentation	-	-

+, positive reaction; -, negative reaction; ND, not described; ^a, data previously reported [41].

3.3. Antimicrobial Resistance Assessment

Antimicrobial susceptibility testing revealed that ciprofloxacin produced the largest inhibition zone (30.43 mm), which was significantly larger ($p < 0.05$) than that for cotrimoxazole (21.80 mm), kanamycin (19.43 mm), gentamicin (16.20 mm), and tetracycline (9.43 mm). No inhibition zones were observed for cefazolin, cefuroxime, ceftazidime, ampicillin, piperacillin, doxycycline, and minocycline (Table 3). These findings revealed that isolate H4 exhibited multiple resistance to ampicillin, cefazolin, cefuroxime, ceftazidime, doxycycline, minocycline, piperacillin, and tetracycline, but remained susceptible to cotrimoxazole, gentamicin, kanamycin, and ciprofloxacin. This suggests that isolate H4 is multi-resistant to cephalosporins, penicillins, and tetracyclines.

Table 3. Susceptibility of isolate H4 to antimicrobials.

Antimicrobial Category	Antimicrobial Agent	Content (μ g/disc)	Zone Diameter Breakpoints (mm)			Inhibition Zone Diameter (mm)	Susceptibility
			S	I	R		
Aminoglycosides	Gentamicin	10	≥ 15	13–14	≤ 12	16.20 ± 0.26^d	S
	Kanamycin	30	≥ 18	14–17	≤ 13	19.43 ± 0.42^c	S
Cephalosporins	Cefazolin	30	≥ 23	20–22	≤ 19	0 ± 0^f	R
	Cefuroxime	30	≥ 18	15–17	≤ 14	0 ± 0^f	R
	Ceftazidime	30	≥ 21	18–20	≤ 17	0 ± 0^f	R

Table 3. Cont.

Antimicrobial Category	Antimicrobial Agent	Content (µg/disc)	Zone Diameter Breakpoints (mm)			Inhibition Zone Diameter (mm)	Susceptibility
			S	I	R		
Penicillins	Ampicillin	10	≥17	14–16	≤13	0 ± 0 ^f	R
	Piperacillin	100	≥21	25–29	≤17	0 ± 0 ^f	R
Quinolones	Ciprofloxacin	5	≥21	22–25	≤15	30.43 ± 0.21 ^a	S
Sulfonamides	Cotrimoxazole *	23.75/1.25	≥16	11–15	≤10	21.80 ± 0.52 ^b	S
Tetracyclines	Doxycycline *	30	≥14	11–13	≤10	0 ± 0 ^f	R
	Minocycline	30	≥16	13–15	≤12	0 ± 0 ^f	R
	Tetracycline *	30	≥15	12–14	≤11	9.43 ± 0.42 ^e	R

Data are presented as mean ± SD. S, susceptible; R, resistant; *, antimicrobials in aquaculture use. Data with different superscript letters indicate a significant difference ($p < 0.05$) following one-way ANOVA. The antimicrobial susceptibility was examined in technical triplicate and analyzed against the antibiotic susceptibility testing standards [43,44].

3.4. Pathogenicity-Related Genes

Genomic analysis revealed 51 virulence genes and 63 resistance genes in isolate H4 through alignment with the VFDB and CARD databases (Tables S2 and S3). Notably, nine virulence genes, encoding curli (*csgG*), acriflavine resistance protein B (*acrB*), hemolysin-coregulated protein (*hcp*), 6-phosphogluconate dehydrogenase (*gndA*), GalU regulator (*galF*), RNA polymerase sigma factor (*rpoS*), ferric uptake regulator (*fur*), transcriptional regulator (*rscB*), and PhoP two-component regulatory system (*phoP*), possessed >91% sequence identity to those associated with adherence, antimicrobial activity, effector delivery systems, immune modulation, and regulation. Additionally, 17 resistance genes, encoding response regulator of the two-component regulatory system BaeSR (*baeR*), beta-lactamase (*blaACT-49*), transcriptional regulator RamA (*ramA*), histone-like nucleoid structuring protein (*hns*), penicillin-binding protein 3 (*ftsI*), membrane fusion protein AcrA (*acrA*), gyrase subunit (*gyrA*), enoyl-acyl carrier protein reductase (*fabI*), C-reaction protein (*crp*), open reading frame B (*oqx*B), DNA topoisomerase IV subunit ParE (*parE*), DNA gyrase subunit B (*gyrB*), response regulator PhoP (*phoP*), RNA polymerase β-subunit (*rpoB*), elongation factor Tu (*tuf*), phosphoenolpyruvate–protein phosphotransferase system I (*ptsI*), and fosfomycin glutathione transferase FosA2 (*fosA2*), yielded >95% sequence identity to those functioning against aminoglycoside, cephamycin, disinfecting agent and antiseptic, fluoroquinolone, macrolide, peptide, and other antimicrobials. Furthermore, the presence of *hcp*, *phoP*, *hns*, *ftsI*, *blaACT-49*, *ramA*, *crp*, *acrA*, and *oqx*B genes in isolate H4 provided a genetic basis for the manifestation of typical enteritis signs of intestinal hyperemia, as well as resistance against ampicillin, piperacillin, cefazolin, cefuroxime, ceftazidime, doxycycline, tetracycline, and minocycline. These virulence and resistance genes could probably contribute to the pathogenic potential of isolate H4 in seahorses.

3.5. Experimental Pathogenicity

Isolate H4 demonstrated pathogenicity in big-belly seahorses with a mortality of 100% at 1.0×10^8 CFU/mL (Table S1). Experimentally infected seahorses exhibited the typical enteritis symptom of intestinal hyperemia, similar to that observed in naturally infected individuals (Figure 4). The same strain (isolate H4), showing the same molecular and phenotypic characteristics as shown in Figure 2 and Table 2, was successfully re-isolated from the experimentally infected seahorses. In contrast, no mortality or observable pathological signs were recorded in the control individuals. These findings, following Koch's postulate, revealed that isolate H4 was the causative agent of enteritis in big-belly seahorses.



Figure 4. Gross signs of enteritis-infected big-belly seahorses. (A). Naturally affected seahorse. The arrow shows intestinal hyperemia. (B). Experimental seahorse challenged with isolate H4. The arrow shows hyperemia in the intestinal wall.

3.6. Virulence Assessment

The survival rates of the big-belly seahorses acutely declined following challenge with isolate H4 at concentrations of 2.0×10^5 , 2.0×10^6 , 2.0×10^7 , and 2.0×10^8 CFU/mL and reached a plateau after 8 days. Finally, the 10-day mortality rates were calculated respectively as 46.67%, 76.67%, 96.67%, and 100.00% in the experimental big-belly seahorses infected with isolate H4 at concentrations of 2.0×10^5 , 2.0×10^6 , 2.0×10^7 , and 2.0×10^8 CFU/mL, significantly higher ($p < 0.05$) than the control (Figure 5). The same strain (isolate H4) was successfully re-isolated from all the experimentally dead seahorses, as confirmed by molecular and phenotypic analyses. No visible pathological changes or mortality were recorded in the control big-belly seahorses. These findings indicated that isolate H4 was dose-dependently virulent to big-belly seahorses, with an LD_{50} of 4.47×10^5 CFU/mL calculated using log-probit calculation based on the linear correlation Equation (1) between survival probability (y) and challenge concentrations ($\lg x$) (Figure S3).

$$y = -1.635x + 14.245 \quad (1)$$

Wherein, when x is $\lg LD_{50}$, y is 5.0.

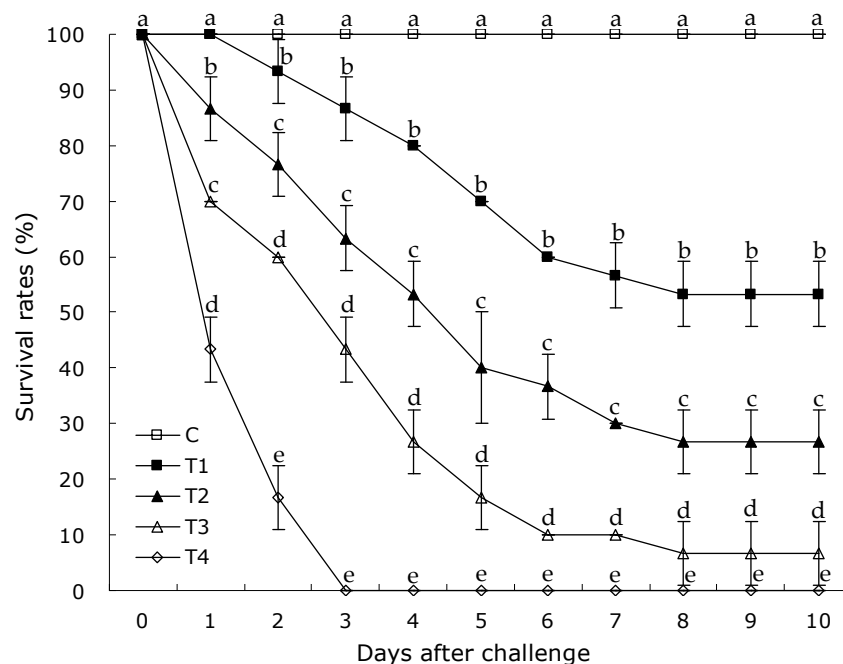


Figure 5. Survivals of experimental big-belly seahorses infected by isolate H4 at different cell densities for 10 days (C, 0 CFU/mL; T1, 2.0×10^5 CFU/mL; T2, 2.0×10^6 CFU/mL; T3, 2.0×10^7 CFU/mL; T4, 2.0×10^8 CFU/mL). Data are means of three replicates. The error bars indicate SD. Data with different letters are significantly different ($p < 0.05$) following one-way ANOVA.

4. Discussion

Nowadays, several bacterial pathogens have been implicated in mortality in cultured seahorses, including *V. alginolyticus*, *V. fortis*, *V. rotiferianus*, *V. harveyi*, *V. tubiashii*, *V. vulnificus*, *V. parahaemolyticus*, *E. tarda*, *Pseudoalteromonas spongiae*, *Pseudomonas* sp., *Photobacterium* sp., and *Mycobacterium chelonae* [22–25,52,54,56–62]. Although strains of *E. bugandensis* have previously been isolated from water environments [63], there are currently no reports of *E. bugandensis* infection in the big-belly seahorse. In the present study, a pathogenic isolate H4 of *E. bugandensis* was successfully isolated from enteritis-infected big-belly seahorses and further characterized with respect to its genomic features, virulence, antimicrobial susceptibility, and pathogenicity-related genes, thereby indicating that *E. bugandensis* could pose a threat to seahorse aquaculture. However, there are still several limitations of this study, such as a limited sample size and missing gene functional analysis that should be overcome in the future to better understand the pathogenesis of *E. bugandensis*.

Given that 16S rRNA gene sequencing analysis alone is insufficient for acute differentiation of *Enterobacter* species, whole genome sequencing and biochemical characterization analyses are proposed for precise species identification [64,65]. In the present study, whole genome-based analysis showed > 98% ANI identity and >95% dDDH similarity to the type strain EB-247 of *E. bugandensis*, taxonomically suggesting isolate H4 as *E. bugandensis* based on the species delineation thresholds of 95% ANI and 70% dDDH [66]. Moreover, the biochemical characteristics of isolate H4 were highly consistent with those of the type strain EB-247 of *E. bugandensis*, further confirming it as *E. bugandensis*. However, it should be noted that *E. bugandensis* strains are often misidentified phenotypically as *E. ludwigii* using Vitek®2 Compact and BioLog systems due to the lack of comprehensive phenotypic data in the reference database [65]. Therefore, there is an urgent need to collect extensive phenotypic profiles to improve the accuracy of identifying this species.

Recently, the genomes of several pathogenic *E. bugandensis* isolates from humans have been characterized through whole genome sequencing. For example, the genome of *E. bugandensis* strain EB-247, isolated from a sepsis-infected neonate, consists of a single chro-

mosome (4,717,613 bp) and contains 4355 CDS, 16 GI, and 72 virulence genes [10]. Similarly, the genome of *E. bugandensis* CMCC(B) 45301, obtained from China National Center for Medical Culture Collections, is composed of a chromosome (4,631,472 bp) and a plasmid (81,691 bp) and possesses a total of 4499 CDS [67]. However, no information is currently available regarding the genomic features of *E. bugandensis* pathogens in aquaculture. In our study, the genomic characterization of the seahorse-pathogenic *E. bugandensis* was first reported and differed from virulent strains EB-247 and CMCC(B) 45301 of *E. bugandensis* in genomic features such as the genome size, number of CDS, GI, and virulence genes, revealing substantial genomic diversity among pathogenic *E. bugandensis* isolates. This is probably attributed to different environmental and nutritional stresses faced by these isolates [68]. Therefore, it is necessary to conduct more studies on the genomic and virulence profiles of *E. bugandensis* in different environments worldwide [10].

The emergence and spread of antimicrobial resistance among pathogenic *E. bugandensis* strains have raised increasing concerns due to their strong adaptive capability and high-level multidrug resistance [69]. For example, *E. bugandensis* strain EB-247 has developed resistance to aminoglycosides, cephalosporins, penicillins, quinolones, sulfonamides, and tetracyclines [70]. Similarly, *E. bugandensis* strains IF2SW-B1 and IF2SW-P2, isolated from the International Space Station environmental surfaces, have exhibited resistance to aminoglycosides, cephalosporins, penicillins, and quinolones [71]. In the present study, isolate H4 also showed resistance to cephalosporins, penicillins, and tetracyclines, supporting the hypothesis that *E. bugandensis* possesses intrinsic resistance against cephalosporins and penicillins [70,71]. This is probably due to the presence of resistance genes such as *hns*, *ftsI*, *blaACT-49*, *ramA*, *crp*, *acrA*, and *oqxB* genes in isolate H4, which confer resistance against penicillins, cephalosporins, and tetracyclines [72–77].

E. bugandensis is an important human pathogen associated with several diseases, such as septicemia and neonatal sepsis, and is generally positive for a variety of virulence genes [10,41]. For example, *E. bugandensis* isolate EB-247 carries the virulent *vgrG* gene as well as a series of adhesion-encoding genes that are implicated in pathogenesis [10,78]. In our study, in addition to the essential virulence *vgrG* gene involved in the pathogenesis of human diseases [78], *E. bugandensis* isolate H4 was found to harbor other potential virulence genes, such as *csgD*, *rpoS*, *gndA*, *rpoS*, *fur*, *rscB*, and *hcp* associated with bacterial adhesion, disease development, biofilm formation, virulence factor expression regulation, resistance enhancement to host environmental stresses, and colonization to host [79–85]. These findings further underscore the zoonotic potential of *E. bugandensis*. Given that genomic islands-facilitated horizontal gene transfer (HGT) can promote the exchange of virulence genes and thereby increase bacterial pathogenicity [86,87], there is an urgent need for robust surveillance systems to mitigate the impact of HGT-mediated virulence gene exchange on human and animal health [88].

Resistance genes play a crucial role in determining the antimicrobial susceptibility of *E. bugandensis* [71]. For example, the *TolC* gene has been shown to enhance the resistance of *E. bugandensis* to a range of antimicrobials, including cefuroxime, cefoperazone, amikacin, streptomycin, minocycline, doxycycline, levofloxacin, florfenicol, trimethoprim-sulfamethoxazole, azithromycin, lincomycin, piperacillin, tetracycline, and clindamycin [67]. In this study, the presence of the *TolC* gene in isolate H4 provided a genetic explanation for its multiple resistance to minocycline, doxycycline, piperacillin, and tetracycline. Furthermore, *E. bugandensis* isolate H4 also carried a variety of resistance genes, such as *baeR*, *rpoB*, and *ftsI* associated with bacterial susceptibility to tetracyclines, rifamycins, and β -lactams [89–91]. The presence of these resistance genes undoubtedly increases bacterial virulence and treatment difficulty [86]. Although cotrimoxazole exhibited strong inhibitory activity against isolate H4 and might be considered for the treatment of *E.*

bugandensis infections, its excessive use could accelerate the emergence and dissemination of drug-resistant strains and negatively impact the aquaculture environment [88]. Recent studies have demonstrated that the application of *Enterococcus faecium* in aquaculture systems significantly enhances the expression of intestinal immune-related genes and improves disease resistance against bacterial infection in big-belly seahorses [92]. Therefore, the use of *E. faecium* may represent a promising alternative to cotrimoxazole for the prevention and control of *E. bugandensis*-induced enteritis in big-belly seahorses.

The LD₅₀ value obtained from a challenge test is widely recognized as a precise indicator for assessing bacterial virulence [93]. Generally, bacterial isolates are categorized as highly virulent, virulent, and avirulent based on LD₅₀ values of below 10^{4.5–5.5}, 10^{5.5–7.0}, and above 10^{7.0} CFU/mL [94]. In this study, isolate H4 exhibited an LD₅₀ of 4.47 × 10⁵ CFU/mL in big-belly seahorses, indicating it as a highly virulent strain [94]. This agrees with Pati et al. [10] that *E. bugandensis* is a highly virulent pathogen. Previous studies have shown that the presence of the *phoP* gene in Gram-negative bacterial pathogens, along with the type VI secretion system (T6SS) induced by *hcp* gene expression, can contribute to intestinal inflammation [95,96]. Therefore, the enteritis sign of intestinal hyperemia observed in our study could probably be associated with the presence of *phoP* and *hcp* genes in isolate H4. In addition to the virulence factors of *E. bugandensis*, attention should also be directed toward other contributing factors to the outbreak of enteritis in seahorses, such as the use of natural diets contaminated with bacterial pathogens [97].

5. Conclusions

The results of this study demonstrated *E. bugandensis* as a causative pathogen of enteritis in *H. abdominalis* and provided insights into its antimicrobial resistance and pathogenicity. These findings revealed that the *E. bugandensis* pathogen was multi-resistant and highly virulent and carried numerous pathogenicity-related genes contributing to the pathogenic potential. These virulence properties could highlight the zoonotic potential of *E. bugandensis* and reveal its potential sanitary risks to aquaculture. Future studies are needed for epidemiological surveillance and targeted management of *E. bugandensis*-induced enteritis in seahorse farming.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/fishes10080411/s1>. Table S1: Cumulative mortality of experimental seahorses infected by bacterial isolates. Table S2: Potential virulence genes in the genome of isolate H4 through alignment with the VFDB database. Table S3: Potential resistance genes in the genome of isolate H4 through alignment with the CARD database. Figure S1: The 16S rRNA phylogenetic tree of isolates H1, H2, H3, and 18 known bacteria constructed using the neighbor-joining method. Figure S2: Circular complete genome map of the chromosome from isolate H4. Figure S3: The linear correlation between survival probability and challenge concentrations.

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