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Whole-Genome Sequence of *Aeromonas* spp. Isolated from a Dairy Farm in Central Texas

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Abstract: This study investigated the presence of *Aeromonas* spp. on a dairy farm in central Texas that employed a free-stall management system. A total of 140 samples were collected from areas of two different barns. Twenty-two presumptive *Aeromonas* isolates were cultured. Phenotypic analysis identified five *Aeromonas* spp. Twenty isolates exhibited β-lactam and one displayed tetracycline resistance. Phylogenetic analysis of the WGS data suggested only four *Aeromonas* spp. All isolates possessed at least one β-lactam resistance gene and one isolate possessed *tet*(E). No plasmids were identified from sequence alignments. Virulence genes were identified in all four *Aeromonas* spp. Mobility elements were identified in three of these, with the exception being *A. dhakensis*. Four of the transposons identified in this study have been associated with multidrug resistance in Italy, Sweden, and Singapore. There was no significant difference in the proportion of isolates from either barn. The absence of plasmids suggests mobility elements and virulence genes were localized to the chromosome. On a dairy farm of healthy cattle, these 22 *Aeromonas* isolates were considered normal environmental flora while illustrating the ubiquitous nature of *Aeromonas* spp. globally.

Keywords: *Aeromonas* spp.; dairy farm; whole genome sequencing (WGS); antimicrobial drug resistance; virulence genes; mobility elements

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1. Introduction

Aeromonas are currently comprised of 36 bacterial species and 12 subspecies. Taxonomically, the identification of the genus and species of Aeromonads has been constantly in flux due to differences in phenotypic and genotypic profiles [1,2]. *Aeromonas* are autochthonous in aquatic environments ranging from marine to freshwater, and from water treatment plants to raw sewage [3–5]. Aeromonads provide an excellent example of the importance of "One Health", where antimicrobial-resistant and pathogenic bacteria intersect the interface of environment, healthcare, and agriculture. Such interactions provide the opportunity for the dissemination of pathogenic bacteria or undesirable genetic elements to occur. There are efforts to mitigate the dissemination of antimicrobial resistance, virulence, and mobile genetic elements among human, animal, and environmental reservoirs and *Aeromonas* should be included in this effort [6,7].

It has been known for decades that Aeromonads cause disease in fish and amphibians. Aeromonads have been less frequently isolated from humans, food production animals, domesticated pets, invertebrate species, birds, ticks, insects, and soil [3,8–10]. Because they can grow at lower temperatures, *Aeromonas* spp. have been isolated from fresh groceries stored at 4 °C, such as dairy, beef, pork, poultry products, and packaged ready-to-eat meats [11–13]. The presence of Aeromonads in diverse niches results in shared microbial

communities where they could potentially cause illness in susceptible species or transfer mobile genetic elements to other bacterial genera. *A. hydrophila*, *A. caviae*, *A. veronii,* and *A. dhakensis* are the most significant species with regard to human health [1,14]. They are considered emerging pathogens and have been identified as the etiologic agent in a variety of human infections in both immunocompetent and immunocompromised humans [15–17]. Severe infections include septicemia, peritonitis, endocarditis, gastroenteritis, and wound infections [18]. *Aeromonas* spp. have also caused hemolytic–uremic syndrome and necrotizing fasciitis among humans [16,19].

Currently, *Aeromonas* infections are not common in human clinical medicine [20]. Nevertheless, the potential severity of illness is an important reason they should be correctly identified as early as possible. *Aeromonas* spp. are Gram-negative bacilli that are oxidase-, indole-, and catalase-positive and that ferment glucose [1,15]. A motile mesophilic group, typified by *A. hydrophila,* are associated with human disease and achieve optimal growth between 35 and 37 °C. A nonmotile psychrophilic group, typified by *A. salmonicida,* is associated with fish diseases and achieves optimal growth at temperatures between 22 and 25 °C [2,10].

Aeromonas spp. possess three Ambler classes of chromosomally located inducible β-lactamases: B, C, and D [21–24]. *A. hydrophila* strains confer resistance to ampicillin, other penicillins, and first- and second-generation cephalosporins [1,2,15,24,25]. FOX-type AmpC-type β-lactamases are a lineage present on the chromosome of *Aeromonas* spp. that have been mobilized to plasmids [24,26,27]. The CMY-1/MOX-family of AmpC-type β-lactamases are also present on the *Aeromonas* chromosome and have been mobilized to plasmids [28]. Chromosome-mediated mcr-3 variants in *A. veronii* have been identified in retail chicken meat in China [13]. This *A. veronii* strain displayed resistance to β-lactams and phenicol compounds and intermediate resistance to colistin [13]. Thirty-two different β-lactamase genes were identified from *Aeromonas* strains isolated from sewage influent, activated sludge, and effluent [25]. Aeromonads are also commonly resistant to heavy metals [29].

A number of incompatibility plasmids that possess antimicrobial resistance genes have been acquired by *Aeromonas* spp. These include: IncA/C, IncU, IncQ-3,4,5,7,9,11, IncFIB, IncQ-2, IncQ-3, and ColE-type. Many of these display a broad host range and are conjugative or mobilizable [29].

Pathogenesis by *Aeromonas* spp. involves multiple metabolic factors, many of which were identified as belonging to *A. hydrophila* but are now known to also belong to other *Aeromonas* spp. [30]. Regardless of species, *Aeromonas* spp. possess a number of known virulence genes: arylamidases, esterases, amylase, elastase, chitinases, lipases, peptidases, hydrolases, and others, that play roles in their pathogenesis [10,20,31–33]. Understanding the interplay between antimicrobial resistance and virulence elements is increasingly important, as many strains have acquired multiple mobile genetic elements that carry these genes among genera [16,34,35]. Acquisition of mobile genetic elements does not have to be limited to one event; strains may acquire multiple resistance or virulence genes, particularly when selection pressure exists. Multiple genetic acquisitions have resulted in multidrug-resistant bacteria in many genera that can transfer mobile genetic elements into a diversity of bacteria [29,33]. One multidrug-resistant *A. hydrophila* strain was found in a food production farm among neonatal swine with diarrhea [34,36].

With that in mind, this project was undertaken to assess the occurrence of mobility elements and virulence genes in *Aeromonas* spp. isolated from a working dairy farm with healthy adult Holstein cows. A dairy farm with two ventilation systems was chosen to determine if there was a difference in the prevalence of *Aeromonas* spp. between management systems.

2. Materials and Methods

2.1. Sample Collection

Samples were collected in June 2018 from a single free-stall dairy farm in central Texas that maintained approximately 1000 healthy Holstein cattle. Housing systems employed

two ventilation systems: (1) flow-through or (2) cross-ventilation. The flow-through or cross-ventilation housing structures were parallel to each other with roof peaks perpendicular to prevailing summer winds. Side curtain walls could be lowered to protect cows from morning or afternoon sun. Fans were present to aid ventilation. At the time of collection, barn curtains were up and air could flow throughout the entire facility. All cows were in open stanchions during hay consumption. Water misters were employed for cooling when necessary. All water troughs contained chilled circulated clean water; some sediment was present on the bottom of water troughs. Cows were free to mill around or lay down on clean sand floors. Cows were not in proximity to the two sump ponds that were in use at the time of sample collection.

A total of 140 samples were collected in sterile specimen jars from water troughs, barn aisles, and sump ponds from the flow-through and the cross-ventilated barns. All samples were aqueous in nature and were scooped into the sterile specimen jars. Ten water trough samples were collected in duplicate (n = 20) at different troughs for each barn type (Figure 1). Ten barn aisle samples were collected in triplicate (n = 30) from sites throughout the barn for each barn type. Ten sump pond samples were collected in duplicate (n = 20) from numerous sites around each of the two ponds that corresponded to each barn ventilation type. In total, 70 samples were collected representing each barn ventilation type for a total of 140 samples. The samples were stored on ice and transported back to the United States Department of Agriculture (USDA) laboratory in College Station, Texas. Upon return to the lab, 50 mL of alkaline peptone water enrichment broth was added to each jar and the jars were incubated overnight at 37 °C.

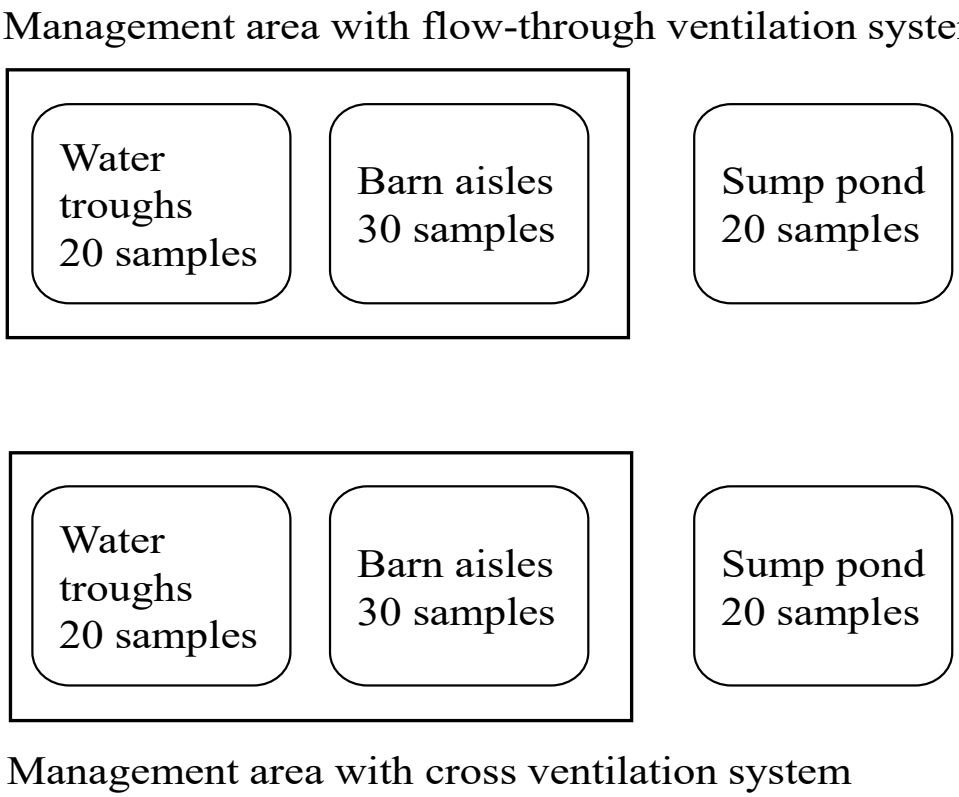

Figure 1. Location of samples on the farm.

2.2. Aeromonas Isolation and Identification

For isolation, Aeromonas medium Base (RYAN) (Oxoid™ ThermoFisher Scientific, Waltham, MA, USA) with Oxoid™ Ampicillin Selective Supplement (ThermoFisher Scientific cat. no. SR0136) at 5.0 mg/500 mL was prepared according to the manufacturer's instructions and EPA Method 1605 [37,38]. Ten milliliters of enrichment broth were filtered

through a 2.2 µm 60 mm filter. The filters were placed right-side up on the m-Aeromonas selection agar plates and incubated overnight at 35 °C. *Aeromonas* was presumptively identified by the production of acid from dextrin fermentation and bright yellow colonies that were greater than 0.5 mm. Additional microbiological testing was used to confirm the genus and speciate of the isolates. This included: catalase, oxidase, indole testing, Voges–Proskauer (VP), and hemolysis on blood agar. Biolog (Biolog, Hayward, CA, USA) phenotyping was performed using a GN2 MicroPlate™.

2.3. Statistical Testing

Pearson's chi-squared test was used to determine statistical significance for *Aeromonas* isolation associated with the flow-through and cross-vent barn locations.

2.4. Antimicrobial Susceptibility Testing

Susceptibility testing was performed on all isolates by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) methods [39,40]. A Sensititre® automated antimicrobial susceptibility system was used according to the manufacturer's instructions (Trek Diagnostic Systems, Westlake, OH, USA). The National Antibiotic Resistance Monitoring System (NARMS) panel CMV3AGNF was employed. The following antimicrobials were tested: amoxicillin/clavulanic acid (1/0.5–32/16 µg/mL), ampicillin (1–32 µg/mL), azithromycin (0.12–16 µg/mL), cefoxitin (0.5–32 µg/mL), ceftiofur (0.12–8 µg/mL), ceftriaxone (0.25–64 µg/mL), chloramphenicol (2–32 µg/mL), ciprofloxacin (0.015–4 µg/mL), gentamicin (0.25–16 µg/mL), nalidixic acid (8–32 µg/mL), streptomycin (2–64 µg/mL), sulfisoxazole (16–256 µg/mL), tetracycline (4–32 µg/mL), and trimethoprim/sulfamethoxazole (0.12/2.38–4/76 µg/mL). *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, and *Enterococcus faecalis* ATCC 29212 were used as quality control organisms. Previously, the NARMS panels CMV2AGNF and CMV3AGNF have been employed to determine susceptibility profiles for *Aeromonas* spp. [34,36,41].

2.5. WGS and Assembly

DNA purification employed the QIAamp96 DNA QIAcube HT kit (Qiagen, Valencia, CA, USA). DNA quality and quantity were assessed by absorbance and fluorescence using a FLUOstar Omega plate reader (MG Labtech, Cary, NC, USA). DNA was sent (Novogen Corporation, INC. Sacramento, CA, USA) for library preparation and WGS on an Illumina MiSeq® sequencer (150 bp paired-end reads). Sequences were uploaded to PATRIC [42] (https://patricbrc.org/, accessed 28 July 2021) and assembly was performed on PATRIC using Unicycler version v0.4.8. The ResFinder [43], VirulenceFinder (2.0 database), PlasmidFinder (2.1 database) [42,44], and MobileElementFinder (1.0.2.0 database) databases from the Center for Genomic Epidemiology (CGE) (Danish Technical University, Lyngby, Denmark) available at (http://genomicepidemiology.org/services/, accessed 5 August 2021) were downloaded for sequence alignments. The transposon and insertion sequence finder directories were located at the following websites, respectively: (http://transposon.lstmed.ac.uk/, accessed 5 August 2021), (https://www-is.biotoul.fr, accessed 5 August 2021). The alignments were performed with MagicBlast [45]. Sequence alignments with the *Aeromonas* contigs were also conducted with *Aeromonas*-specific virulence genes selected from GenBank and the Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/VFs/, accessed 5 August 2021) [32,46]. VFDB includes *Aeromonas* spp. virulence genes that were not available from CGE databases. Average nucleotide identities (ANIs) were conducted with CJ Bioscience's online ANI calculator [47] available at (https://www.ezbiocloud.net/tools/ani, accessed 7 April 2022).

2.6. Phylogenetic Tree Construction

A phylogenetic tree was constructed using the Phylogenetic Tree Building Service on PATRIC. This service, "uses the amino acid and nucleotide sequences from defined number of the BV-BRC global Protein Families . . . which are picked randomly, to build

an alignment, and then generate a tree based on the differences within those (randomly) selected sequences (chosen by PATRIC software)." [48]. In addition to the experimental isolates (n = 22), genomes were selected from the PATRIC database for 10 different species of *Aeromonas.* If available, at least five genomes from the ten species were selected. When less than five genomes were available, all were selected. If there were more than five, the choices were based on location and date. Additionally, the reference genome from the National Center for Biotechnology Information (NCBI) was downloaded for each of the 10 *Aeromonas* species. A total of 53 additional genomes were selected: *A. hydrophila* (n = 7), including *A. hydrophila* ATCC 7966 and *A. hydrophila* CVM861, *A. media* (n = 6), *A. dhakensis* (n = 6), *A. enteropelogenes* (n = 6), *A. jandaei* (n = 5), *A. veronii* (n = 6), A. *bestiarum* (n = 3), *A. eucrenophila* (n = 2), *A. salmonicida* (n = 6), and *A. caviae* (n = 6). The phylogenetic tree was displayed using the Interactive Tree of Life (https://itol.embl.de/, accessed on) [49].

3. Results

3.1. Aeromonas Collection and Presumptive Speciation

Of the 140 samples evaluated, 22 (15.7%) isolates were recovered (A2-A23) from 20 (14.3%) separate agar plates and presumptively identified as belonging to the genus *Aeromonas*. Eighteen of the agar plates had single isolates, one agar plate had two isolates that were identified as *A. caviae* and *A. salmonicida*, and the remaining agar plate had two isolates that were identified as *A. caviae*. There were thirteen (18.6%) *Aeromonas* spp. isolated from the flow-through barn area and nine (12.9%) isolated from the cross-vent barn area. The difference in recovery between the flow-through barn area (13/70) and the cross-vent barn area (9/70) was not statistically significant (Pearson χ^2 = 1.87, p = 0.35). *Aeromonas* spp. were isolated from flow-through-associated water troughs (n = 7) and barn aisles (n = 6), but not the sump pond (Figure 2). *Aeromonas* spp. were isolated from all three cross-vent-associated sites: water troughs (n = 2), barn aisles (n = 3), and the sump pond (n = 4) (Figure 2). All isolates were catalase-, oxidase-, and indole-positive. Twenty-one of the isolates were β-hemolytic on tryptic soy agar with 5% sheep blood. Isolate A12 displayed α-hemolysis. *A. caviae* were distinguished from other *Aeromonas* spp. by a negative VP test. On the basis of the Biolog ID system, *Aeromonas* isolates were speciated as follows: *A. caviae* (n = 15), *A. bestiarum* (n = 3), *A. hydrophila* (n = 1), *A. eucrenophilia* (n = 1), *A. salmonicida* (n = 1), and *A. salmonicida subspecies pectinolytica* (n = 1) (Table 1). The Biolog ID was regarded as presumptive.

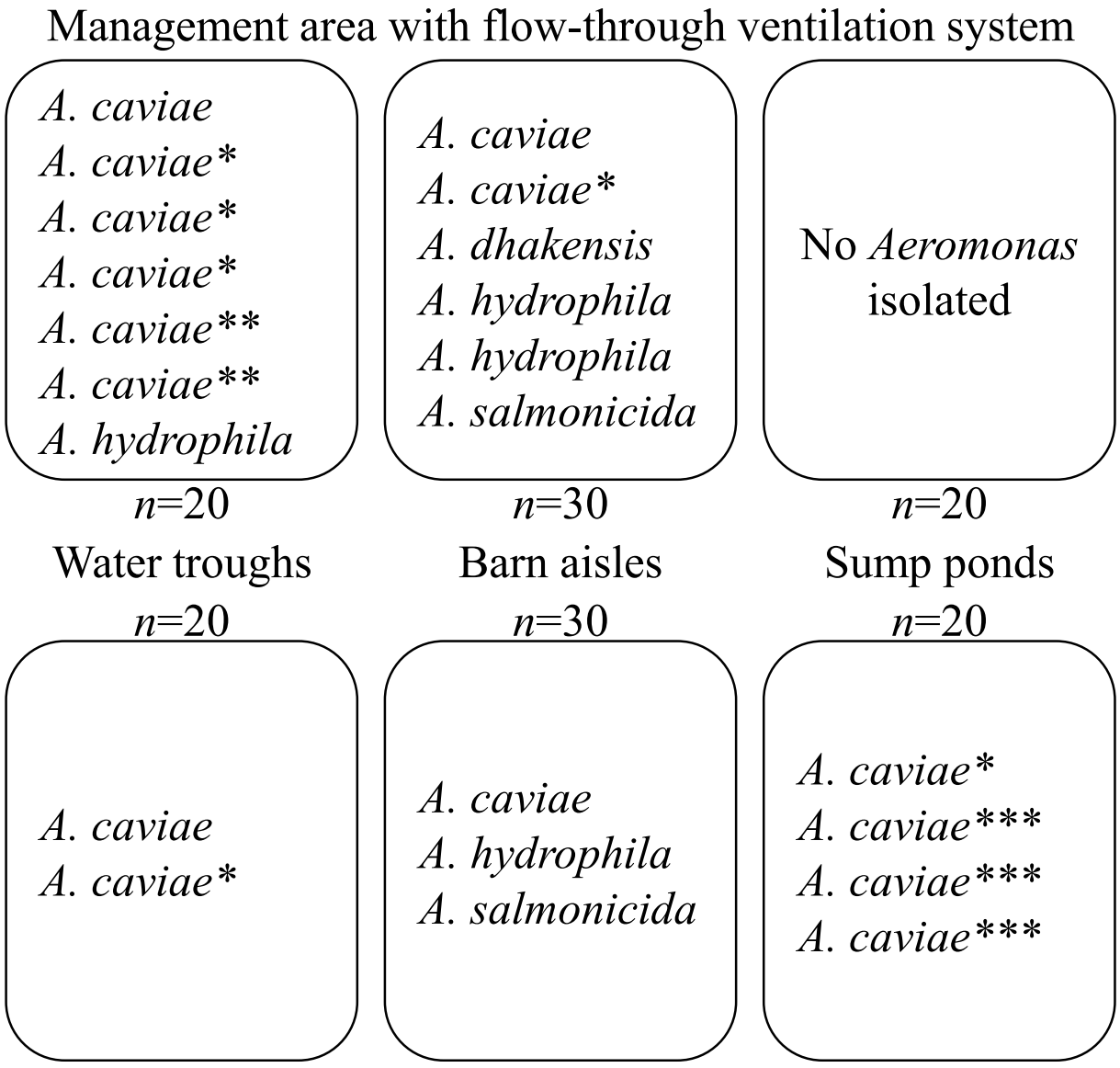

Figure 2. Location of *Aeromonas* positives on the farm.

Table 1. *Aeromonas* spp. collected from a central Texas dairy farm.

Sample ID	Farm Sample #	Site	Biolog ID	WGS ID	Resistance Profile (MIC) µg/uL	SRA Accession Number	Biosample Accession Number
Samples collected from a management area that used flow-through ventilation							
A2	AFT2	WT	*A. caviae*	*A. caviae*	Am(32)Ap(>32)	SRR15274966	SAMN20441443
A3	AFT3	WT	*A. eucrenophilia*	*A. hydrophila*	PS	SRR15274965	SAMN20441444
A4	AFT1	WT	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289137	SAMN18813779
A5	AFT5	WT	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289136	SAMN18813781
A6	AFT6	WT	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289145	SAMN18813782
A7	AFT9	WT	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289144	SAMN18813783
A8	AFT9-5	WT	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289143	SAMN18813784
A9	AFW1-3	BA	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289142	SAMN18813785
A10	AFW3-2	BA	*A. bestiarum*	*A. dhakensis*	Ap(>32)Am(>32)F(>32)T(8)Ax(2)	SRR15274964	SAMN20441445
A11	AFW4-2	BA	*A. hydrophila*	*A. hydrophila*	Ap(>32)Am(>32)F(>32)T(>8)	SRR14289141	SAMN18813786
A12	AFW5-2	BA	*A. caviae*	*A. caviae*	Ap(>32)Te(16)	SRR14289140	SAMN18813787
A21	AFW7-3	BA	*A. bestiarum*	*A. hydrophila*	Ap(>32)	SRR15274963	SAMN20441446
A23	AFW10-2	BA	*A. salmonicida*	*A. salmonicida*	Ap(>32)	SRR14289146	SAMN18813796
Samples collected from a management area that used cross-ventilation							
A18	AXT6	WT	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289149	SAMN18813793
A20	AXT9	WT	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289147	SAMN18813795
A13	AXS1-1	SP	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289139	SAMN18813788
A14	AXS1-4	SP	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289138	SAMN18813789
A15	AXS1-8-1	SP	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289135	SAMN18813790
A16	AXS1-8-2	SP	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289134	SAMN18813791
A17	AXW6-3w	BA	*A. caviae*	*A. caviae*	Ap(>32)	SRR14289133	SAMN18813792
A19	AXW6-3y	BA	*A. salmonicida* ss *pectinolytica*	*A. salmonicida*	PS	SRR14289148	SAMN18813794
A22	AXW8-2	BA	*A. bestiarum*	*A. hydrophila*	Am(>32)Ap(>32)F(32)T(>8)	SRR15274962	SAMN20441447

Farm site WT = water trough; SP = sump pond; BA = barn aisle waterway. Resistance phenotype is defined as resistance to the following antimicrobial agents: Am, amoxicillin/clavulanic acid; Ap, ampicillin; F, cefoxitin; T, ceftiofur; Ax, ceftriaxone; C, chloramphenicol; K, kanamycin; N, nalidixic acid; S, streptomycin; Su, sulfizoxazole; Te, tetracycline; Sxt, trimethoprim/sulfamethoxazole. PS = pansusceptible. Prob = probability; Sim = similarity.

3.2. WGS Phylogeny

WGS were submitted to NCBI as raw reads. The SRA and biosample accession numbers are in Table 1. The BioProject study number was PRJNA72334. The phylogenetic tree suggested the *Aeromonas* spp. were also comprised of four species, *A. caviae* (*n* = 15), *A. hydrophila* (*n* = 4), *A. salmonicida* (*n* = 2), and *A. dhakensis* (*n* = 1), but the results were different from the Biolog results (Table 1, Figure 3). WGS isolates A2, A5, A6, A9, A13, and A18 were likely the same clone that grouped together on the dendrogram (Figure 3). Overall, all the 15 *A. caviae* from the samples were close to the selected *A. caviae* on the tree.

A. eucrenophila (*n* = 1) and *A. bestiarum* (*n* = 3) appeared to be closely related to *A. hydrophila* in this study (Figure 3). This suggested that the *A. eucrenophila* isolate and the three *A. bestiarum* isolates presumptively identified by the Biolog ID system were misclassified. Consequently, ANIs were determined for all isolates against reference genomes downloaded from NCBI. The isolate presumptively identified as *A. eucrenophila*, A3, had an ANI of 85.2% to the reference genome *A. eucrenophila* CECT 4224 (RefSeq GCF_000819865.1), an ANI of 96.46% to the reference genome *A. hydrophila* OnP3.1 (RefSeq GCF_017310215.1), and an ANI of 96.34% to the traditional laboratory strain *A. hydrophila* subsp. *hydrophila* ATCC 7966 (RefSeq GCF_000014805.1). The three presumptive *A. bestiarum*, A10, A21, and A22, had ANIs of 87.97%, 88.44%, and 88.53%, respectively, to the reference genome *A. bestiarum* GA97-22 (RefSeq GCF_002906925.1), ANIs of 93.08%, 96.96%, and 97.13% to *A. hydrophila* OnP3.1, and ANIs of 93.08%, 96.86%, and 97.03% to *A. hydrophila* 7966. Sample A10, which had an ANI to *A. hydrophila* OnP3.1 below 95%, was subsequently compared to the reference genome *A. dhakensis* 71431 (RefSeq GCF_020405345.1) and had an ANI of 97.26%. Thus, isolates A3, A21, and A22 were reclassified as *A. hydrophila* and isolate A10 was reclassified as *A. dhakensis*.

The presumptive *A. hydrophila*, A11, *A. salmonicida*, A19, and *A. salmonicida*, A23, had ANIs to their respective reference genomes, *A. hydrophila* OnP3.1 (RefSeq GCF_017310215.1) and *A. salmonicida* SRW-OG1 (RefSeq GCF_012931585.1), of 97.01%, 97.40%, and 97.36%. All 15 *A. caviae* isolates had ANIs to the reference genome, *A. caviae* WP8-S18-ESBL-04 (RefSeq GCF_014169735.1), that ranged from 97.85% to 98.09%.

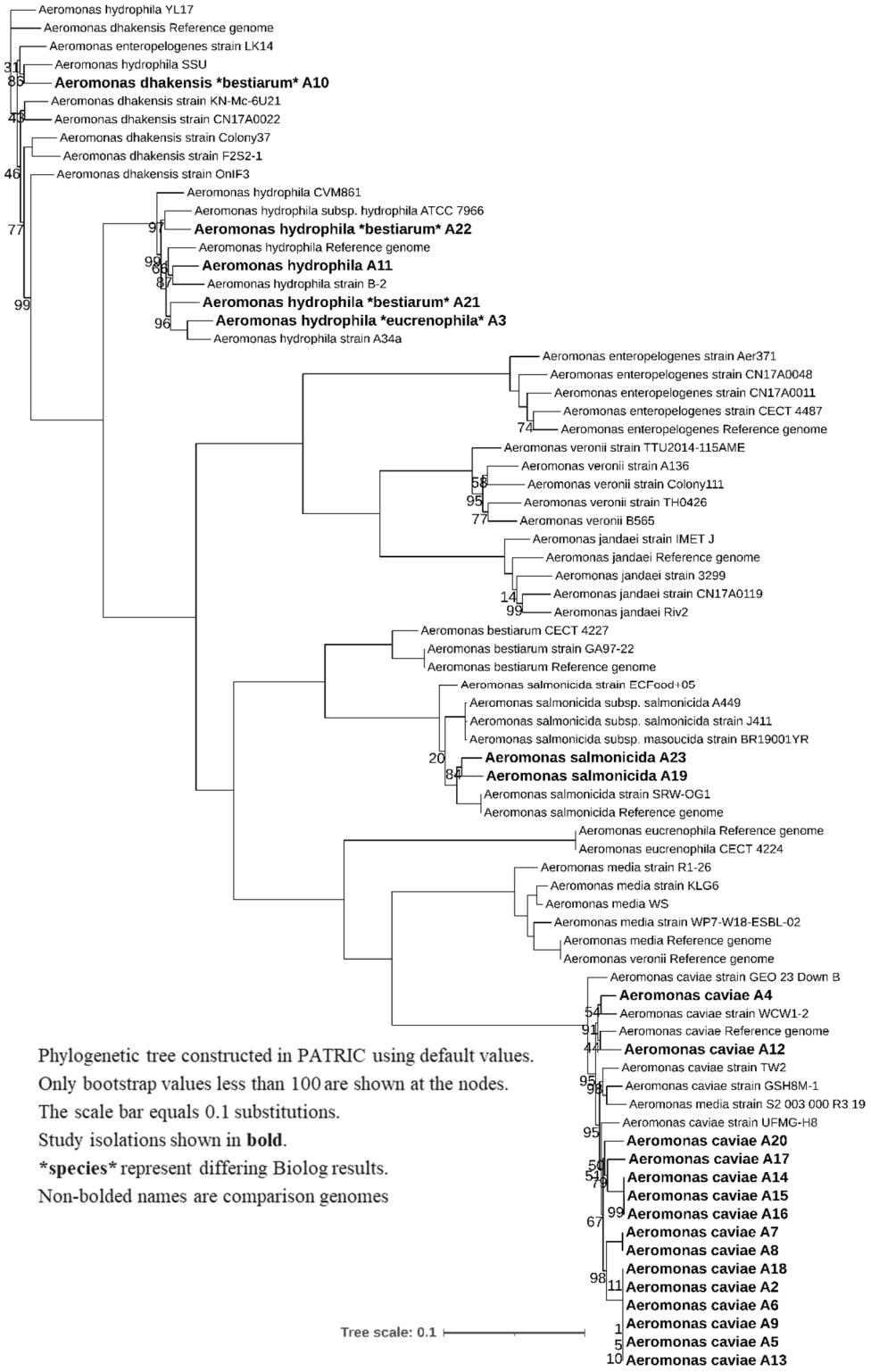

Figure 3. Phylogenetic tree.

3.3. Antimicrobial Resistance

No plasmids were identified when the isolates were compared to the Plasmid Finder database. Nevertheless, as expected, all isolates possessed at least one beta-lactam resistance gene when analyzed with ResFinder (Table 2). Phenotypically, 20 isolates displayed resistance to ampicillin. Resistance genes included: *amp*H_1_AJ276031 (n = 3); *bla*MOX-6_1_GQ152601 (n = 12); *bla*MOX-6_1_GQ1526012 (n = 9); *cph*A1_4_AY261376 (n = 1); *cph*A5_4_AY22051 (n = 1); *imi*H_1_AJ548797 (n = 1); *amp*H_2_HQ586946 (n = 4); *cph*A1_AY261379 (n = 1). One isolate carried the *tet*(E)_2_L06940 gene (Table 2).

Table 2. Percent alignment of genes identified in *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Aeromonas caviae* isolates from a central Texas dairy farm to antimicrobial resistance genes.

Gene	A3—*A. hydrophila*	A10—*A. dhakensis*	A11—*A. hydrophila*	A21—*A. hydrophila*	A22—*A. hydrophila*	A19—*A. salmonicida*	A23—*A. salmonicida*	A2—*A. caviae*	A5—*A. caviae*	A6—*A. caviae*	A9—*A. caviae*	A13—*A. caviae*	A18—*A. caviae*	A7—*A. caviae*	A8—*A. caviae*	A4—*A. caviae*	A12—*A. caviae*	A14—*A. caviae*	A15—*A. caviae*	A16—*A. caviae*	A17—*A. caviae*	A20—*A. caviae*
*amp*H_1	97.7	95.3	97.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*amp*H_2	97.7	95.3	-	97.1	98.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
bla_{MOX}-6	-	-	-	-	-	-	-	96.6	96.6	96.6	96.6	96.6	96.6	97.6	97.6	97.2	97.2	-	-	-	97.3	97.6
bla_{MOX}-7	-	-	-	-	-	-	-	96.6	96.6	96.6	96.6	96.6	96.6	-	-	-	-	97.5	97.5	97.5	-	-
*cph*A1	-	95.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*cph*A1	-	-	-	-	-	-	96.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*cph*A5	-	-	-	-	-	95.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*imi*H	-	-	-	96.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tet(E)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	99.9	-	-	-	-	-

When categorized by species, *A. hydrophila* isolates A3, A10, and A11 all possessed *amp*H_1 AJ276031. Isolates A3 and A10 also possessed *amp*H_2 HQ589797, along with A21 and A22. *A. hydrophila* isolates A10, A11, and A22 displayed the same antibiogram: amoxicillin/clavulanic acid, ampicillin, cefoxitin, and ceftiofur. *A. hydrophila* A3 was phenotypically pansusceptible (Table 1). A10 had intermediate resistance to ceftriaxone and possessed a third gene, *cph*A1. *A. hydrophila* A21 was the only isolate that possessed *imi*H and was only resistant to ampicillin (Table 1). *A. salmonicida* A19 and A23 possessed *cph*A5 and *cph*A1, respectively (Table 2). *A. salmonicida* A19 was phenotypically pansusceptible (Table 1). All *A. caviae* displayed phenotypic resistance to ampicillin except for *A. caviae* A12, which also displayed resistance to tetracycline (Table 1). Genotypically, *A. caviae* isolates A2, A5, A6, A9, A13, and A18 all carried the bla_{MOX-6} and bla_{MOX-7} genes (Table 2). *A. caviae* isolates A4, A7, A8, A17, and A20 all possessed bla_{MOX-6} alone. Isolate A12 carried the bla_{MOX-6} and *tet*(E) genes (Table 2). Isolates A14, A15, and A16 possessed bla_{MOX}-7 alone.

3.4. Mobility Elements

Thirty mobility elements (Table 3) were identified; none were integrons. *A. hydrophila* isolates A11, A21, and A22 possessed a single *A. hydrophila*-associated insertion element, ISAhy1. *A. salmonicida* A19 possessed ICEEcoUMN026-1, IS5, four ISAs elements known to be associated with *A. salmonicida*, and transposons Tn*6234* and Tn*6240*. *A. salmonicida* isolates A23 possessed six ISAs elements (Table 3).

Table 3. Percent alignment of genes identified in *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Aeromonas caviae* isolates from a central Texas dairy farm to genes in the MobileElementFinder database.

Gene	A3—A. hydrophila	A10—A. dhakensis	A11—A. hydrophila	A21—A. hydrophila	A22—A. hydrophila	A19—A. salmonicida	A23—A. salmonicida	A2—A. caviae	A5—A. caviae	A6—A. caviae	A9—A. caviae	A13—A. caviae	A18—A. caviae	A7—A. caviae	A8—A. caviae	A4—A. caviae	A12—A. caviae	A14—A. caviae	A15—A. caviae	A16—A. caviae	A17—A. caviae	A20—A. caviae
ICEEcoUMN026-1	-	-	-	-	-	99.3	-	-	-	-	-	-	-	99.2	99.2	99.6	99.2	-	-	99.3	-	-
IS5	-	-	-	-	-	99.3	-	-	-	-	-	-	-	99.2	99.2	99.7	99.4	-	-	-	-	-
ISAeme15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0	99.7	-	-	-	-	-
ISAeme16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	99.8	99.8	-	-	-	-	-
ISAeme19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	99.5	99.6	-	-	-	-	-
ISAeme21	-	-	-	-	-	-	-	96.6	96.6	96.6	96.6	96.6	96.6	-	-	-	-	-	-	-	-	-
ISAeme3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	96.8	-	-	-	-
ISAhy1	-	-	95.5	96.1	97.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ISAhy3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	99.0	-	-	-	-
ISAs1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	99.3	-	-	-	-	-	-
ISAs15	-	-	-	-	-	-	99.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ISAs16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	99.3	-	-	-	-	-	-
ISAs17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	95.6	-	99.6	-	-	-	-
ISAs18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	95.3	-	-	-	-	-	-
ISAs19	-	-	-	-	-	-	-	-	-	-	95.3	96.1	-	-	-	97.8	-	-	-	-	-	-
ISAs20	-	-	-	-	-	-	-	-	-	-	-	-	-	98.2	98.2	-	99.6	-	-	-	-	-
ISAs22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0	-	-	-	-	-	-
ISAs23	-	-	-	-	-	98.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ISAs24	-	-	-	-	-	99.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ISAs26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	99.6	100.0	99.7	99.4	99.3	-	-
ISAs31	-	-	-	-	-	97.2	97.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ISAs32	-	-	-	-	-	-	96.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ISAs34	-	-	-	-	-	98.1	98.2	-	-	-	-	-	-	99.7	99.7	-	-	-	99.0	99.0	-	-
ISAs5	-	-	-	-	-	-	95.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ISAs7	-	-	-	-	-	-	95.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ISAve3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	96.6	96.5	96.5	96.5	-	-
ISEc28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0	100.0	-	-	-	-	-
MITEAeme1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0	-	-	-	-	-	-
Tn6180	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0	100.0	-	-	-	-	-
Tn6234	-	-	-	-	-	98.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tn6240	-	-	-	-	-	98.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tn6279	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100.0	100.0	-	-	-	-	-

No known mobility elements were identified in *A. hydrophila* A3, *A. dhakensis* A10, *A. caviae* A17, or *A. caviae* A20. *A. caviae* isolates A2, A5, A6, A9, A13, and A18 possessed ISAeme21. In addition to ISAeme21, A9 and A13 also possessed ISAs19 (Table 3). Isolates A7 and A8 were identical to each other and aligned to four mobility elements. *A. caviae* isolates A4 and A12 had considerably more mobility elements than the other *A. caviae*; they carried sixteen and eleven mobility elements, respectively, including transposons Tn*6180*, Tn*6279*. *A. caviae* A4 possessed MITEAeme1 (Table 3).

3.5. Aeromonas Virulence Genes

Alignments to the VFDB from the 22 isolates in this study identified 52 *Aeromonas* virulence genes (Table 4). *A. caviae* isolates A2, A3, A6, A9, A13, A18, and A20 possessed two virulence genes, *syc*X and *tpp*E, whereas A7, A8, and A12 only had *sysX*. There were no alignments to any virulence genes in *A. caviae* isolates A14, A15, A16, and A17. A4 aligned to *mshE*. Forty virulence genes were identified from the flow-through and 36 from the cross-vent barn isolates, respectively. Isolates A2, A5, A6, A9, A13, and A18 possessed two virulence genes: *exe*E general secretory pathway protein E and *vasK/icmF* Type VI secretion system protein.

Table 4. Percent alignment of genes identified in *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Aeromonas caviae* isolates from a central Texas dairy farm to genes in the Virulence Factor database.

VFDB Identifier	A3—*A. hydrophila*	A10—*A. dhakensis*	A11—*A. hydrophila*	A21—*A. hydrophila*	A22—*A. hydrophila*	A19—*A. salmonicida*	A23—*A. salmonicida*	A2—*A. caviae*	A5—*A. caviae*	A6—*A. caviae*	A9—*A. caviae*	A13—*A. caviae*	A18—*A. caviae*	A7—*A. caviae*	A8—*A. caviae*	A4—*A. caviae*	A12—*A. caviae*	A14—*A. caviae*	A15—*A. caviae*	A16—*A. caviae*	A17—*A. caviae*	A20—*A. caviae*
YP_856360	-	-	96.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_008043135	95.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_858602	-	-	96.8	96.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_008045131	97.4	95.7	-	98.7	97.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_008043134	-	-	-	97.9	96.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145297253	-	-	-	-	-	97.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145299407	-	-	-	-	-	96.2	96.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_855102	96.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_857337	-	-	96.8	96.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
111143381	-	98.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145297589	-	-	-	-	-	97.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145300643	-	-	-	-	-	98.9	95.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_855103	-	-	-	-	-	-	-	95.1	95.1	95.1	95.1	95.1	95.1	95.3	95.3	-	95.0	-	-	-	-	95.3
YP_856379	97.0	95.9	97.4	96.5	97.4	-	-	96.3	96.3	96.3	96.3	96.3	96.3	-	-	-	-	-	-	-	-	97.0
145298366	-	-	-	-	-	-	98.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145299432	-	-	-	-	-	-	97.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_857806	-	-	-	-	98.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_857151	-	-	-	-	98.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145300802	-	-	-	-	-	97.8	97.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145297730	-	-	-	-	-	97.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
507519905	-	-	-	-	97.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_858122	-	-	-	97.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_856293	-	-	-	-	99.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145299468	-	-	-	-	-	96.0	96.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_857189	95.7	-	97.9	97.7	97.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_008041840	-	-	97.9	97.3	98.0	-	-	-	-	-	-	-	-	-	-	97.8	-	-	-	-	-	-
145297762	-	-	-	-	-	96.7	96.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145297254	-	-	-	-	-	-	95.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_008043465	97.8	-	99.0	99.0	98.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145299828	-	-	-	-	-	98.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_856993	-	-	-	96.2	95.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145300215	-	-	-	-	-	98.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145300516	-	-	-	-	-	97.5	97.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_855918	-	-	-	98.8	98.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_008042625	98.0	-	97.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_001140282	-	-	-	-	-	97.1	97.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145300595	-	-	-	-	-	98.7	99.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_858308	-	-	99.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_855659	-	96.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145300501	-	-	-	-	-	98.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_857681	96.5	-	97.0	97.2	97.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145298152	-	-	-	-	-	97.5	97.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_857247	-	-	98.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145298624	-	-	-	-	-	-	98.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145297880	-	-	-	-	-	98.1	98.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
507523204	97.8	96.0	97.5	97.2	97.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_855347	97.4	-	-	97.8	99.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145298519	-	-	-	-	-	97.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145299774	-	-	-	-	-	98.7	98.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
145300766	-	-	-	-	-	98.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YP_858604	-	96.5	-	-	95.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
507520337	-	-	97.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Three cross-vent isolates, A18, A20, and A13, possessed the *Aeromonas* virulence factor SSU cytonic enterotoxin (ast), GenBank number AF419157.

Alignments to 10 *Aeromonas*-specific virulence genes downloaded from NCBI identified 10 genes, shown in Table 5. Three of fifteen *A. caviae* isolates showed alignment to just

A. hydrophila (AF419157) SSU enterotoxin (ast). These were A14, A15, and A16. Neither of the *A. salmonicida* showed alignments.

Table 5. Percent alignment of genes identified in *Aeromonas hydrophila* isolates from a central Texas dairy farm to *Aeromonas*-specific virulence genes selected from GenBank.

Identifier	Gene Description	A3—*A. hydrophila*	A10—*A. dhakensis*	A11—*A. hydrophila*	A21—*A. hydrophila*	A22—*A. hydrophila*
AB237183	*A. hydrophila ahlip*11 gene for lipase	-	97.4	-	-	-
AF419157	*A. hydrophila* isolate SSU enterotoxin (*ast*)	-	-	96.1	96.5	-
DQ650654	*A. hydrophila* strain AH-1 LafK (*lafK*)	97.6	-	98.3	98.9	98.8
GQ856318	*A. hydrophila* strain BSK-10 phospholipid-cholesterol acyltransferase (GCAT)	-	97.0	-	-	98.7
JN215210	*A. hydrophila* strain FSP 384/10 flagellin (*fla)*	-	-	95.5	-	-
U81555	*A. hydrophila* hemolysin (*hlyA*)	96.7	95.5	96.5	96.4	96.6

4. Discussion

This study reports four *Aeromonas* spp. on a dairy farm of 1000 healthy Holstein cattle in Texas. *Aeromonas* clones were disseminated across the physical area of the working farm where the samples were collected.

WGS and phylogenetic analysis of the *Aeromonas* genomes separated taxonomic clusters into four species, *A. hydrophila*, *A. caviae*, *A. salmonicida*, and *A. dhakensis*, while the Biolog system identified slightly different results based on biochemical profiles. The Biolog profiles did not include *A. dhakensis*. This illustrates the importance of Whole Genome Sequencing to confirm biochemical identifications.

A. caviae A14, A15, and A16 were isolated from the cross-vent-associated sump pond and are likely clones with genetic differences only in mobility elements. Evaluation of all genetic traits indicated A2, A5, A6, A9, A13, and A18 were *A. caviae* clones. They all possessed the $bla_{\text{MOX-6}}$ and $bla_{\text{MOX-7}}$ beta-lactamase genes, the mobility element ISAeme21, and virulence genes *sycX* and *tppE*. *A. caviae* A2, A3, A6, and A18 were genotypically identical and differed from A9 and A13 by the possession of ISAs19 in the latter two. This clone was distributed across different areas of the dairy farm where sample collection was allowed (Figure 2). This illustrates that the type of ventilation management was not a factor in bacterial distribution and suggests that *Aeromonas* spp. are a ubiquitous environmental resident that could be found anywhere on a farm this large regardless of these two management types.

In addition to elucidating phylogenetic relationships among the farm isolates, genomic features could be compared to *A. hydrophila* CVM861 and other *Aeromonas* isolates that have been sequenced. WGS of the 22 dairy isolates gives insight into a pool of mobile elements (e.g., plasmids, transposons, integrons, and Integrative Conjugative Elements (ICEs), Miniature Inverted–Repeat Transposable Elements (MITEs), and (IS) insertion sequences) that may lead to the dissemination of virulence and antimicrobial resistance genes. The first published whole genome sequence of an *Aeromona*s strain was *A. hydrophila* ATCC 7966. This strain had no transposase or insertion sequences, which was considered unusual for a 47 Mb genome [50]. In our study, *A. hydrophila* A3, *A. dhakensis* A10, and *A. caviae* A17 and A20 had no mobility elements. *A. hydrophila* A3 and *A. dhakensis* A10 possessed the same two resistance genes, *amp*H_1 and *amp*H_2. However, A3 was phenotypically pansusceptible, suggesting the beta-lactamases were not expressed or capable of conferring resistance.

A. caviae A4 and A12 each had the same two transposons: Tn*6180* was initially detected in *Acinetobacter baumannii* in Singapore [51] and Tn*6279* was initially detected in *Acinetobacter baumannii* in Sweden [52]. *A. hydrophila* CVM861 harbors plasmid IncQ1_1_M28829, which likely carries the Tn*21* transposon that is adjacent to the class I integron and macrolide resistance gene operon. In comparison, none of the dairy isolates had alignments to any plasmids. In this report, genotypic analysis showed that all *A. caviae* harbored one or two β-lactamase genes above the 95% identity cut-off. *A. caviae* A12 possessed only one β-lactamase gene, but also had a *tet*(E) gene. The *A. hydrophila* isolates had one to three β-lactamase genes above the 95% identity cut-off. *A. salmonicida* A19 had the *cphA*5 gene and *A. salmonicida* A23 had the *cphA*1 gene.

Phenotypically, all *A. caviae* were resistant to ampicillin. In addition, *A. caviae* A12 was also resistant to tetracycline. This was consistent with the genotypic analysis. The *A. hydrophila* isolates were all resistant to one or more β-lactams, which was also consistent with the genotypic analysis. *A. salmonicida* A23 was resistant to ampicillin and also had a β-lactamase gene. *A. salmonicida* A19, however, was phenotypically pansusceptible despite having a β-lactamase gene.

Aeromonas spp. secrete a wide variety of extracellular enzymes that are considered virulence factors involved in pathogenesis. These genes encode for proteins that include hemolysins, aerolysins, enterotoxins, cytotoxins, and others. *Aeromonas* spp. can regulate gene expression by quorum sensing, which is also coordinated with biofilm formation [15,53]. Several of the *A. caviae*, A2, A5, A6, A7, A8, A9, A13, and A18, possess the *exeE* gene, necessary for extracellular export and normal outer membrane assembly [54]. A previous study identified *Aeromonas* isolates from cattle that exhibited resistance to carbapenem [55]. This study did not screen for resistance to carbapenem, but several relevant genes (*cph*A1_4_AY261376, *cph*A5_4_AY22051, and *cph*A1_AY261379) were identified.

The different ventilation systems did not appear to influence the recovery of *Aeromonas* isolates. Even though there were different ventilation systems present the cattle were in relatively close proximity to each other. Water runoff, biting flies, and a number of environmental factors could have easily transmitted bacteria between the herds. Although we did encounter some antibiotic resistance among the isolates, none displayed multidrug resistance. The mobility elements and virulence genes suggested they were localized to the chromosome and were unlikely to be horizontally transferred to other bacterial genera. Thus, on a dairy farm of 1000 healthy cattle, the existence of these 22 *Aeromonas* isolates could be considered as normal environmental flora.

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Data Availability Statement: Sequence reads available at NCBI using accession numbers available in Table 1.

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