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ORIGINAL ARTICLE

Comparative analysis of virulence genes, antibiotic resistance and *gyrB*-based phylogeny of motile *Aeromonas* species isolates from Nile tilapia and domestic fowl

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Significance and Impact of the Study: Many integrated fish farms depend on the application of poultry droppings/litter which served as a direct feed for the fish and also acted as pond fertilizers. The application of untreated poultry manure exerts an additional pressure on the microbial world of the fish's environment. *Aeromonas* species are one of the common bacteria that infect both fish and chicken. The aim of this study was to compare the phenotypic traits and genetic relatedness of aeromonads isolated from two diverse hosts (terrestrial and aquatic), and to investigate if untreated manure possibly enhances *Aeromonas* dissemination among cohabitant fish with special reference to virulence genes and antibiotic resistant traits.

Keywords
Aeromonas, antibiotic resistance, chicken, Nile tilapia, virulence genes.

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Abstract

The nucleotide sequence analysis of the *gyrB* gene indicated that the fish *Aeromonas* spp. isolates could be identified as *Aeromonas hydrophila* and *Aeromonas veronii biovar sobria*, whereas chicken *Aeromonas* spp. isolates identified as *Aeromonas caviae*. PCR data revealed the presence of *Lip*, *Ser*, *Aer*, *ACT* and *CAI* genes in fish *Aer. hydrophila* isolates, *ACT*, *CAI* and *Aer* genes in fish *Aer. veronii bv sobria* isolates and *Ser* and *CAI* genes in chicken *Aer. caviae* isolates. All chicken isolates showed variable resistance against all 12 tested antibiotic discs except for cefotaxime, nitrofurantoin, chloramphenicol and ciprofloxacin, only one isolate showed resistance to chloramphenicol and ciprofloxacin. Fish *Aeromonads* were sensitive to all tested antibiotic discs except amoxicillin, ampicillin–sulbactam and streptomycin.

Introduction

Aquaculture and chicken production are the most dynamic livestock sectors worldwide (Troell *et al.* 2014; Augustine and Shukla 2015). Disease outbreaks are the major constraint affecting the expansion of these sectors. From the economical point of view, the availability of natural food in earthen ponds depends on the organic fertilizers and chicken droppings/litter which constitute their main component (Prithwiraj *et al.* 2008). The application of untreated chicken manure exerts an additional pressure on the microbial load of the fish's environment. Interestingly, it has been shown that the average count of *Aeromonas* bacteria was significantly higher in pond water receiving chicken manure as fertilizers (Omojowo and Omojasola 2013).

Aeromonas spp. are considered as bacteria of a broad host range as they can infect a vast number of hosts including fish, domestic chicken, lower and higher vertebrates and human (Rey *et al.* 2009). In both freshwater and marine fish species, *Aeromonas*-associated disease conditions include Motile *Aeromonas* Septicaemia (MAS) and Epizootic Ulcerative Syndrome (EUS) (Yogananth *et al.* 2009; Viji *et al.* 2011). The clinical significance of

Aeromonas hydrophila was recorded in some of the avian species, including septicaemia in turkeys, conjunctivitis in pet parrot, salpingitis in ducks, diarrhoea and watery faeces in water chickens, diarrhoea and weight loss in canaries and cockatiels (reviewed by Setta 2004).

Aeromonads possess a wide range of virulence factors that enable them to evade the host defence system, spread and eventually killing the host. Among these factors are different toxins and enzymes, including Lipase (*Lip*), Serine protease (*Ser*), Aerolysin (*Aer*), Cytotoxic enterotoxin (*ACT*) and temperature-sensitive protease, Epr (*CAI*). These virulence-encoded genes have been widely used in determining the potential pathogenicity of *Aeromonas* species (Li *et al.* 2011; Yi *et al.* 2013). Therefore, *Aeromonas* species are considered to be emerging pathogens and their clinical significance has increased in aquaculture as well as avian and human health. *Aeromonas hydrophila, Aeromonas veronii biovar sobria* and *Aeromonas caviae* are the major aetiological agents for Aeromoniasis (Wahli *et al.* 2005), based on biochemical characterization, sequencing of housekeeping genes and DNA–DNA hybridization. Phylogenetic analyses of *Aeromonas* species based on 16S rRNA genes indicate low discriminatory power (Yanez *et al.* 2003; Küpfer *et al.* 2006). It has been reported that *gyrB* (which encodes the B-subunit of DNA gyrase, a type-II DNA topoisomerase) could be a suitable phylogenetic marker for bacterial systematics (Yi *et al.* 2013). Despite increased clinical significance, the comparison of *Aeromonas* spp. isolated from fish and chicken reared in close farms, in terms of distribution of virulence genes, antibiotic resistance and *gyrB* sequence analysis, has not been studied.

In this study, the phylogenetic tree was designed to clarify the intraspecies phylogenetic relationships within *Aeromonas* spp. isolated from both moribund fish and chicken. The *gyrB* nucleotide sequences were determined from 24 *Aeromonas* strains, which were also characterized by biochemical and antibiotic resistant methods. Furthermore, the occurrence of different virulence genes (i.e. *Lip, Aer, Ser, ACT* and *CAI*) has been studied and their distribution within the two animal hosts was also investigated.

Results and discussion

Clinical examination

External examination of Nile tilapia, *Oreochromis niloticus* showed haemorrhagic patches on the dorsolateral surface of the body as well as on the ventral abdomen. The whole body showed dark discoloration with detached scales and fin rot. Internally, the diseased fish showed severe congestion of all the internal organs; liver, spleen, kidney, brain, gut and gonads with distended gall bladder as described by (Rey *et al.* 2009; Crumlish *et al.* 2010). On the other hand, the clinicopathological picture of diseased chickens showed depression, ruffled feathers, diarrhoea, impaired appetite, pericarditis, air saculitis, pneumonia, enlarged liver and spleen with occasional focal necrosis, enteritis, nephrosis and unabsorbed yolk sac.

Bacterial isolation and identification

Large flattened yellow colonies (2–3 mm in diameter) of fish *Aeromonas* spp. were cultivated on selective *Aeromonas* agar base medium. Small-sized yellow colonies (1 mm in diameter) were observed in the case of the chicken *Aeromonas* isolates. All bacterial isolates were motile, Gram-negative, short bacilli, oxidase and catalase positive, resistant to Vibrio-static reagent O/129 150 μg ml^{-1}. Further biochemical identification of these species using the API20NE confirmed both *Aer. veronii biovar sobria* and *Aer. hydrophila/caviae* (Wahli *et al.* 2005). From a total of 24 presumptive *Aeromonas* species, four isolates (16·67%) were biochemically characterized as *Aer. veronii biovar sobria* with four different API20NE profile numbers. Twenty isolates (83·33%) were identified as *Aer. hydrophila/cavaie* (11 isolates from fish and nine isolates from chicken). The profiles numbers of isolates are shown in (Table 1).

Phylogenetic analysis

Phenotypic tests are often unable to precisely identify *Aeromonas* species because of the heterogeneity that exists within the genus (Wahli *et al.* 2005). Furthermore, nucleotide sequence analysis and construction of phylogenetic tree of different protein-encoding genes have improved our understanding of bacterial population structure, as well as epidemiology (Küpfer *et al.* 2006). The 16s rRNA gene sequence is considered a proper device for the reconstruction of evolutionary history and phylogenetic relationships of bacterial genera. However, some difficulties can arise when using this technique for species identification within *Aeromonas* spp. because of its smaller discriminatory power.

Data presented in this study has shown that *gyrB* gene sequence analysis proved to be a particularly well-suited tool for phylogenetic studies of the genus *Aeromonas*. An approximately 1100 bp fragment of the *gyrB* gene of the studied strains was obtained. Comparison of the nucleotide sequences and divergence showed that all strains are deeply embedded in *Aeromonas* spp. group. Tilapia *Aeromonas* spp. isolates were identified as *Aer. hydrophila* and *Aer. veronii biovar sobria*, whereas chicken *Aeromonas* spp. isolates were confirmed to be *Aer. caviae*. Based on their sequence alignment, the intraspecies similarity for

Table 1 API20NE profiles and *gyrB* Accession numbers

No.	Isolate	Source	*Aeromonas* spp	API-20NE profiles	*gyrB* Accession no.
1	Fay1209	Nile tilapia	*Aeromonas sobria*	7176754	LC012334
2	Fay1208	Nile tilapia	*Aer. sobria*	7176755	LC012335
3	Fay1207	Nile tilapia	*Aer. sobria*	7176715	LC012336
4	Fay1206	Nile tilapia	*Aer. sobria*	7176655	LC012337
5	Fay1205	Nile tilapia	*Aeromonas hydrophila*	7177754	LC012338
6	Fay1204	Nile tilapia	*Aer. hydrophila*	7456754	LC012339
7	Fay1203	Nile tilapia	*Aer. hydrophila*	7456754	LC012340
8	Fay1202	Nile tilapia	*Aer. hydrophila*	7456754	LC012341
9	Fay1201	Nile tilapia	*Aer. hydrophila*	7177754	LC012342
10	Fay1200	Nile tilapia	*Aer. hydrophila*	7467744	LC012343
11	Fay1101	Nile tilapia	*Aer. hydrophila*	7576755	LC012344
12	Fay1102	Nile tilapia	*Aer. hydrophila*	7576755	LC012345
13	Fay1103	Nile tilapia	*Aer. hydrophila*	7576755	LC012346
14	Fay1104	Nile tilapia	*Aer. hydrophila*	7467754	LC012347
15	Fay1105	Nile tilapia	*Aer. hydrophila*	7467744	LC012348
16	Chi2201	Chicken	*Aeromonas caviae*	7567354	LC012325
17	Chi2202	Chicken	*Aer. caviae*	7567354	LC012326
18	Chi2203	Chicken	*Aer. caviae*	7567354	LC012327
19	Chi2204	Chicken	*Aer. caviae*	7567744	LC012328
20	Chi2205	Chicken	*Aer. caviae*	7567744	LC012329
21	Chi2206	Chicken	*Aer. caviae*	7567354	LC012330
22	Chi2207	Chicken	*Aer. caviae*	7567755	LC012331
23	Chi2208	Chicken	*Aer. caviae*	7567755	LC012332
24	Chi2209	Chicken	*Aer. caviae*	7567354	LC012333

tilapia *Aer. hydrophila* isolates (n = 11) was 99–100% with nucleotide difference ranged from 2 to 10. The *gyrB* gene sequence analysis of tilapia *Aer. veronii biovar sobria* revealed that all isolates were identical. The intraspecies similarity for chicken *Aer. caviae* isolates (n = 9) was 99–100% with nucleotide difference ranged from 0 to 9.

The derived neighbour-joining tree method based on Kimura 2-parameter model revealed strong nodal support for two major lineages. The first clade included tilapia *Aer. hydrophila* isolates grouped with chicken *Aer. caviae* isolates to form two subclades. The first subclade grouped tilapia *Aer. hydrophila* isolates of this study together with other fish and human *Aer. hydrophila* isolates, forming a distinct phylogenetic subclade with a bootstrap value of 99%. The second subclade included chicken *Aer. caviae* isolates that grouped with other *Aer. caviae* and was strongly supported by a high bootstrap value of 100% to form a monophyletic group (Fig. 1). Interestingly, in this study, the phylogenetic analysis of *Aer. hydrophila* and *Aer. caviae* isolated from fish and chickens, respectively, has clearly shown a high relatedness to *Aer. hydrophila* and *Aer. caviae* of human origin, pointing out the zoonotic significance of these bacteria. The second major clade included *Aer. veronii bv sobria* of fish that grouped with other *Aer. sobria, Aer. veronii* and *Aer. veronii bv sobria* and was strongly supported by a high bootstrap value of 100%.

According to available literature, commercial phenotyping diagnostic systems used routinely in microbiology laboratories are not exactly correct for the identification of *Aeromonas* spp. (Chuang *et al.* 2011). The accuracy of API-20NE system was more reliable for *Aer. hydrophila* and *Aer. veronii biovar sobria*, but not for *Aer. caviae*. In contrast, *gyrB* sequence combined with phylogenetic analysis could easily differentiate *Aer. hydrophila* from *Aer. caviae* and *Aer. veronii bv sobria*. Additional tests, like Voges–Proskauer reaction and Ornithine decarboxylase production are essential for confirmation of *Aeromonas* species identified by the API-20NE system, although their accuracy of identification is still not comparable with the molecular method.

Detection of virulence genes

There are several reports suggesting that motile *Aeromonas* isolates could carry either one or multiple virulence factors that may play an important role in the development of disease, either in avian species or in fishes. In this study, PCR screening of five virulence genes (*Lip, Aer, Ser, ACT* and *CAI*) was performed. All of the five virulence genes were present in 45·45% (n = 5/11) of fish *Aer. hydrophila* strains, and two genes 100% (n = 11/11) from all chicken *Aer. caviae* isolates. Three virulence genes (n = 3/4, 75%) were detected in *Aer. veronii biovar sobria* of fish (*ACT*, *CAI* and *Aer*) (Table 2).

The prevalence of virulence genes of fish *Aeromonas* isolates were distributed as follows: *ACT* the most frequent virulence gene, was detected in 15 strains, *Aer* was detected in 12 isolates of *Aer. hydrophila* and *sobria*. *Lip* was detected in all *Aer. hydrophila* fish isolates and has not been detected in *Aer. veronii biovar sobria*. It has been shown that *ACT* and *Aer* exhibit both haemolytic and cytolytic properties (Niamah 2012) whereas Lipase alter the structure of the cytoplasmic membrane of the host tissue cells and help the colonization of *Aer. hydrophila* on the host tissues, causing their necrosis (Oliveira *et al.* 2012), as well as digest the cellular components of the host tissue cells (Zhang *et al.* 2000). *Ser* gene was detected in six *Aer. hydrophila* isolates and absent in *Aer. veronii biovar sobria. CAI* was detected in seven *Aeromonas* strains; which has an important role in the invasiveness and establishment of *Aer. hydrophila* infections by overcoming initial host defences and by providing nutrients for microbial cell proliferation (Rivero *et al.* 1990). *ACT* and *Aer* genes were present in all *Aer. veronii biovar sobria* isolates.

On the other hand, both *Ser* and *CAI* genes were expressed in all *Aer. caviae* isolated from poultry. Accord-

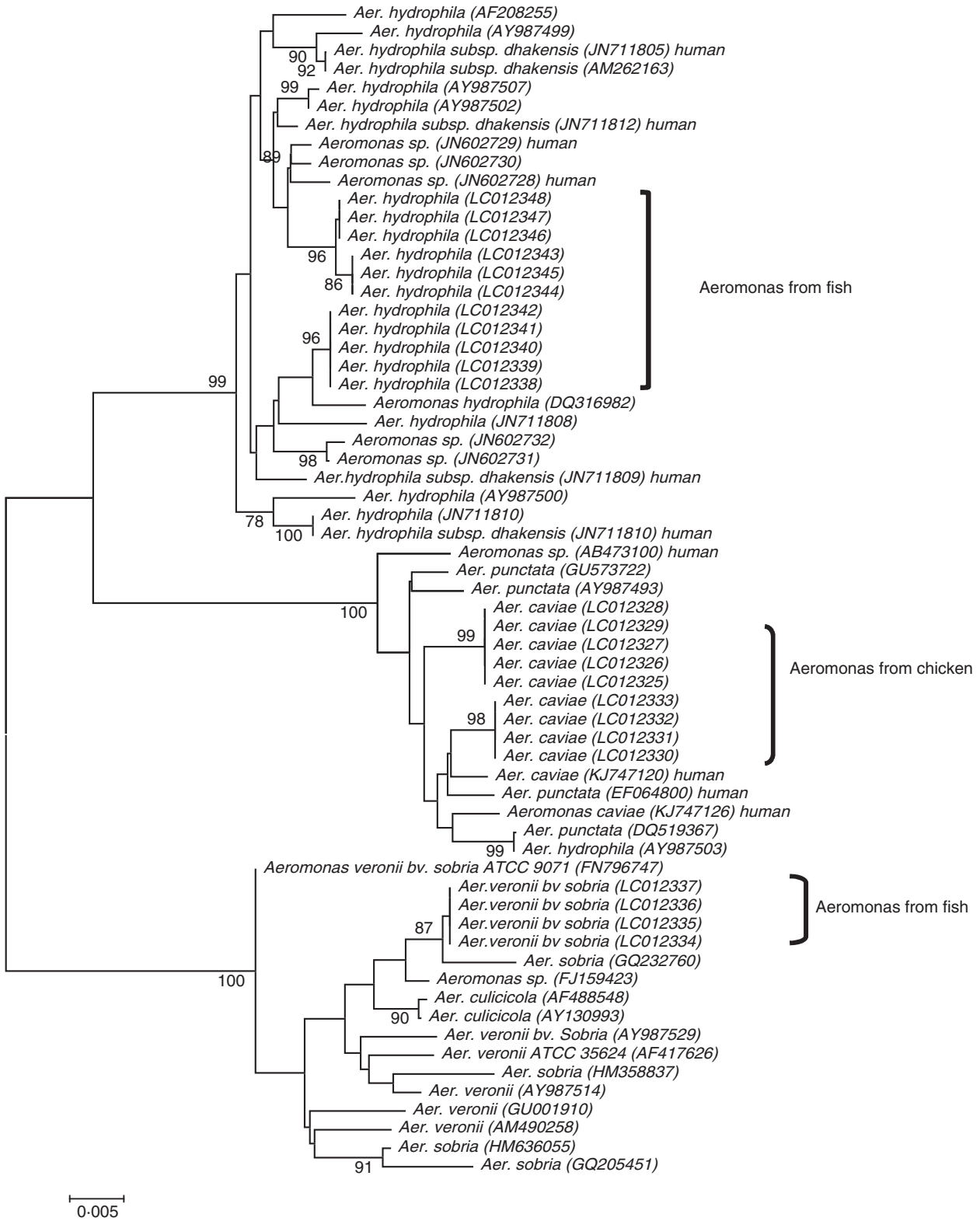

Figure 1 Phylogenetic tree generated based on the comparative analysis of the *gyrB* gene sequences, showing the relationship among the Nile tilapia and chicken strains of *Aeromonas* in this study and related isolates of *Aeromonas* of human and other terrestrial mammal.

Table 2 Genetic detection of five virulence genes in *Aeromonas* species isolated from Nile tilapia and chicken

	Fish		
Virulence genes	*Aeromonas hydrophila*	*Aeromonas veronii biovar sobria*	*Chicken Aeromonas caviae*
Lipase (*Lip*)	(11/11) 100%	(0/4) 0%	(0/9) 0%
Aerolysin (*Aer*)	(8/11) 73%	(4/4) 100%	(0/9) 0%
Serine protease (*Ser*)	(6/11) 55%	(0/4) 0%	(9/9) 100%
Cytotoxic enterotoxin (*ACT*)	(11/11) 100%	(4/4) 100%	(0/9) 0%
Temperature sensitive protease (*CAI*)	(5/11) 46%	(3/4) 75%	(9/9) 100%

ing to the available literature, this is the first description of these genes in Aeromonas of avian origin. The clinical significance of these findings in poultry is still not well-understood. Nonetheless, it has been shown that these genes are required for host cell invasion and pathogenesis of *Aer. hydrophila in vitro* (Rivero *et al.* 1990). This finding could also suggest that Aeromonas, under certain conditions, could be a primary pathogen in poultry in addition to its involvement as a complicating factor to other infectious agents affecting poultry. This finding concurs with the results of Chuang *et al.* (2011), who reported that *Aer. caviae* has poor ability to produce cytotoxic eneterotoxins and therefore is less virulent than *Aer. hydrophila* or *Aer. veronii biovar sobria*.

Antimicrobial susceptibility

Fish *Aeromonas* were sensitive to cefotaxime, chloramphnicol, nitrofurantoin and ciprofloxacin as shown in Table 3. It has been shown previously that they were completely resistant to Amoxicillin with high levels of resistance to ampicillin–sulbactam and streptomycin (74%). Variable responses were observed towards, nalidixic acid, gentamycin, trimethoprim–sulfamethoxazole and oxytetracycline; the sensitivity levels were 74, 67, 67 and 53%, respectively, these results were coherent with Akinbowale *et al.* (2006); Daood (2012); Zanella *et al.* (2012). Regarding the chicken *Aeromonas*, this study has shown that all chicken Aeromonas were highly sensitive to cefotaxime followed by chloramphicol, nitrofurantoin and ciprofloxacin. Antibiogram profiling and biofilm formation by Aeromonas recovered from chicken faecal samples have been studied, where Aeromonas showed the highest susceptibility to ciprofloxacin, gentamicin and tetracyclines (Igbinosa *et al.* 2013). In this study, the increased resistance of avian Aeromonas to antibacterial agents could be influenced by several factors, including the type of Aeromonas itself, the type of organ sampled, the type of water used for drinking different chicken flocks, in addition to the improper use of antibiotics in commercial chicken farms. The resistance levels towards the other antibiotics used have been documented in Table 3. A similar observation has been reported by Chuang *et al.* (1997), Ko *et al.* (2011) and Ko *et al.* (2003).

In conclusion, our results revealed noticeable differences in terms of distribution of virulence genes, antibiotic resistance and *gyrB* sequence analysis between *Aeromonas* spp. isolates from tilapia and chicken. The biochemical, molecular and phylogentic analysis demonestrated that *Aer. hydrophila* and *Aer. veronii biovar sobria* are the predominant species among *O. niloticus*. The phenotypic identification of chicken *Aeromonas* strains demonstrated homogeneity with *Aer. hydrophila*, whereas the genetic identification confirmed its belonging to *Aer. caviae* with bootstrap value 100% and high relevance to human Aeromonas. Fish isolates of *Aeromonas* spp. harboured the five

Table 3 Patterns of Antibiogram phenotype of *Aeromonas* isolates from fish and chicken

	Fish isolates (15 isolates)						Chicken isolates (9 isolates)					
	S		I		R		S		I		R	
Antimicrobial agents	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Amoxycillin (30 μg)	0	0	0	0	15	100	0	0	0	0	9	100
Ampicillin–sulbactam (20 μg)	3	20	1	6	11	73	0	0	0	0	9	100
Cefotaxime (30 μg)	15	100	0	0	0	0	9	100	0	0	0	0
Chloramphenicol (30 μg)	15	100	0	0	0	0	7	78	1	11	1	11
Nitrofurantoin (300 μg)	15	100	0	0	0	0	7	78	2	22	0	0
Ciprofloxacin (5 μg)	13	87	2	13	0	0	6	67	2	22	1	11
Nalidixic acid (30 μg)	11	74	2	13	2	13	0	0	2	22	7	78
Gentamicin (10 μg)	10	67	3	20	2	13	1	11	1	11	7	78
Oxytetracycline (30 μg)	8	54	1	6	6	40	3	33	0	0	6	67
Streptomycin (10 μg)	0	0	4	27	11	73	0	0	0	0	9	100
Trimethoprim–sulfamethoxazole (25 μg)	10	67	3	20	2	13	3	33	1	11	5	56

virulence genes assayed and showed sensitivity to different antibiotic discs, in contrast with the results obtained from chicken isolates of *Aeromonas* spp.

Our findings confirmed the existence of multispecies aetiology of aeromoniasis; a disease common to fish and chicken; caused by motile aeromonads in fish and chicken. In addition, fish and chicken *Aeromonas* spp. carried some virulence and resistant traits that may be responsible for motile *Aeromonas* spp. virulence and pathogenesis. This will be of benefit for public health monitoring; especially for farmers and livestock handlers. Therefore, fish and chicken *Aeromonas* spp. isolates should not be disregarded as putative infectious disease agents in humans and mammals.

Material and methods

Samples

A total of 24 strains of *Aeromonas* species were isolated; 15 isolates from moribund *O. niloticus* at a private fish farm, Fayoum governorate, Egypt (Table 1). This farm used untreated chicken droppings/litter as direct fish feeding and fertilizers. Recently, this farm had reported disease outbreaks to General Authority for Fish Resources Development, GAFD. On the other hand, nine isolates have been recovered from diseased chicken in the same territory at the same time. Specimens of fish/chicken were rapidly transported on ice to our laboratories for clinical and bacteriological examination. All institutional and national guidelines for the care and use of animals (fisheries and chicken) were followed.

Bacterial isolation and identification

The strains were isolated from the internal organs (kidney and spleen) of individual diseased fish and chicken. *Aeromonas* selective agar base (Havelaar) (Biolife Italiana, Milano, Italy) supplemented with Ampicillin was used to culture *Aeromonas* spp. The suspected *Aeromonas* isolates were identified by oxidase and catalase tests and were further confirmed by the sensitivity to Vibrio-static reagent O/129 (150 μg ml^{-1}; Sigma, St. Louis, MO). The isolates were then identified to the species level using API20NE biochemical identification strips (bioMérieux, Marcy L'Etoile, France). The pure stock isolates of *Aeromonas* spp. were stored in Brain heart infusion (BHI) broth with 15% (vol/vol) glycerol (LB; Difco, Spark, MD, USA) at −80°C.

DNA extraction

All isolates were aerobically grown on Brain Heart Infusion (BHI) agar, and then incubated at 25°C for 24 h. Genomic DNA was extracted from cultivated strains using prepMan Ultra reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

PCR identification and Partial sequences of *gyrB* gene

Internal fragment of the *gyrB* gene was amplified using set of primers designed by Hu *et al.* (2012) (Table 4). Generally, the PCR reaction mixture was subjected on a thermal cycler to the following programme; a denaturation at 95°C for 4 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 90 s, and a final extension at 72°C for 10 min. The amplified fragment of *gyrB* gene of 24 *Aeromonas* spp. isolates was sequenced using Sanger DNA sequencer, Applied Biosystem in two directions.

The nucleotide sequences were analysed by using BIO EDIT ver. 7.0 (Hall 1999), and the phylogenetic analysis was then carried out by the neighbour-joining method using MEGA ver. 5 (Tamura *et al.* 2011). The options used

Table 4 Nucleotide sequences used in this study

Genes	Primers sequences	Size/bp	References
gyrB	F 5′-TCCGGCGGTCTGCACGGCGT-3′ R 5′-TTGTCCGGGTTGTACTCGTC-3′	1100	Hu *et al.* (2012)
Aerolysin (*Aero*)	F 5′-GAGCGAGAAGGTGACCACCAAGAAC-3′ R 5′-TTCCAGTCCCACCACTTCACTTCAC-3′	417	Nam and Joh (2007)
Serine protease (*Ser*)	F 5′-ACGGAGTGCGTTCTTCCTACTCCAG-3′ R 5′-CCGTTCATCACACCGTTGTAGTCG-3′	211	
Lipase (*Lip*)	F 5′-GACCCCCTACCTGAACCTGAGCTAC-3′ R 5′-AGTGACCCAGGAAGTGCACCTTGAG-3′	155	
Cytotoxic enterotoxin (*Act*)	F 5′-GAGAAGGTGACCACCAAGAACA-3′ R 5′-AACTGACATCGGCCTTGAACTC-3′	232	Hu *et al.* (2012)
Temperature-sensitive protease, Epr (*CAI*)	F 5′-GCTCGACGCCCAGCTCACC-3′ R 5′-GGCTCACCGCATTGGATTCG-3′	387	

were: bootstrap (1000 replicates); gaps/missing data: pairwise deletion; codon positions: 1st +2nd +3rd + noncoding; substitution model: Kimura two-step algorithm; substitutions to include: transitions and transversions; pattern among lineages: same (homogeneous); rates among sites: uniform rates. Although more sophisticated tree-building methods are available, we assumed that this approach was sufficient to resolve relationships at branch terminals.

Nucleotide sequence accession numbers

The complete nucleotide sequence of *gyrB* locus of fish and chicken *Aeromonas* spp. strains were submitted to the DNA Data Bank of Japan and the accession numbers are presented in Table 1.

Detection of virulence genes

All *Aeromonas* strains were subjected to PCR assays to detect the five virulence genes Lipase (*Lip*), Serine protease (*Ser*), Aerolysin (*Aer*), Cytotoxic enterotoxin (*ACT*) and temperature-sensitive protease, Epr (*CAI*); using the same primers sequences and PCR conditions described by Nam and Joh (2007), Hu *et al.* (2012). The nucleotide sequences of the primers used in this study are documented in (Table 4).

Antimicrobial susceptibility

Susceptibility to antimicrobials was evaluated by disc diffusion method (Igbinosa *et al.* 2013) on Muller–Hinton agar (Difco Laboratories) using commercial antibiotic discs: amoxicillin (30 μg), ampicillin–sulbactam (20 μg), cefotaxime (30 μg), chloramphenicol (30 μg), Nitrofurantoin (300 μg),ciprofloxacin (5 μg), gentamicin (10 μg), nalidixicacid (30 μg), oxytetracycline (30 μg), streptomycin (10 μg) and trimethoprim–sulfamethoxazole (25 μg). The inhibition zones were interpreted using published standards of the Clinical Laboratory Standard Institute Guidelines, CLSI (Wikler *et al.* 2009) and the isolates reported as susceptible, intermediate or resistant against the antimicrobial agents tested.

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Conflict of Interest

No conflict of interest declared.

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