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Article

Isolation, identification and antibiogram profile of *Aeromonas hydrophila* from broiler chickens in Mymensingh Sadar, Bangladesh

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Abstract: Investigation of *Aeromonas hydrophila* was conducted to assess the microbial quality of broiler chickens from July to November 2019. A total of 60 samples from 20 broiler chickens were collected from two different locations of Mymensingh Sadar: KR market, Bangladesh Agricultural University (BAU) and Shesh mor bazar (10 birds from each location). Samples included 20 skins, 20 legs and 20 breast samples from 20 broiler chickens. PCR was done for the specific detection of each isolate and finally antimicrobial susceptibility testing was performed to check sensitivity pattern of each isolate. Alkaline peptone water was used for processing and enrichment of the samples followed by inoculation onto *Aeromonas* selective agar supplemented with ampicillin for the isolation and identification of *A. hydrophila*. Out of these 60 samples, 27 isolates were confirmed as *A. hydrophila* through biochemical tests and PCR where 55.56% isolates were recovered from Shesh mor market and other 44.4% isolates from KR market, BAU. Source-wise analysis revealed that maximum isolates of *A. hydrophila* were recovered from skin (59.26 %) followed by leg (22.22 %) and breast samples (18.52 %). PCR test revealed that all 27 isolates were found carrying *lip* gene which is specific for *A. hydrophila*. Isolates of *A. hydrophila* were found sensitive to ciprofloxacin (92%), gentamycin (66%) and chloramphenicol (50%); intermediate against erythromycin (50%), tetracycline (50%) and imipenem (50%); resistant against co-trimoxazole (84%) and ampicillin (100%). From the present study, it was found that samples were considerably contaminated with *Aeromonas hydrophila* causing risks for public health. Necessary control actions should be taken in every steps of production, processing and marketing for mitigation of this contamination.

Keywords: broiler chickens; *Aeromonas hydrophila*; molecular identification; antibiogram profile

1. Introduction

Bangladesh is an agro based country. As such poultry rearing is considered superior to the others in the agricultural sector because of a relatively short period of time to harvest. Besides, among the animal protein sources commercial poultry production ranks highest (Iyayi *et al.*, 2008). As a result rapid growth of poultry industry has been occurring around the world than other food-producing animal industries. The trade volume of poultry products has also increased parallel to the rapid growth of global poultry industry (Windhorst *et al.*, 2006). In Bangladesh, broiler meat is an important and low-cost source of animal protein that encourages the consumption of broiler meat by a large amount of consumers. The modern poultry industry can produce market

ready broiler chickens in <3 weeks through genetic selection, improved feeding and keen health management practices including usage of antibiotics as growth promoter, preventive and therapeutic agents in intensive farming systems. This irrational use of antibiotics in poultry is one of the important issues for the development of microbial resistance to antibiotics. The rise in antibiotic resistance has been reported in the past two decades in many countries including Bangladesh (Akond *et al.,* 2009). Foodborne diseases and poisoning are the widespread and great public health concerns of the modern world in both developed and developing countries. Food contaminated with pathogenic microorganisms are considered as a threat for public health which may lead to serious food poisoning outbreaks (Bagde and Tumane, 2011). Among these microorganisms *Aeromonas* spp. are also considered as a major cause of food-borne human disease in most parts of the world at present (Soultose *et al.,* 2003).

The genus *Aeromonas* consists of two different groups of bacteria. One is non-motile psychrophilic *Aeromonas salmonicida* and the other group includes three mesophilic motile spp. *A. hydrophila, A. caviae*, and *A. sobria* (Praveen *et al.,* 2014). *Aeromonas* is an environmental microorganism that inhabits a wide range of ecosystems including aquatic environment (Wei *et al.,* 2015; Garibay *et al.,* 2006). Besides these aeromonads occur as the normal microbial flora of many aquatic and terrestrial animals including fishes, amphibians, reptiles, birds and other domestic animals (Gowda *et al.,* 2015). Along with aquatic environment, different foods, especially, fishes and other seafood, raw and cooked meat, chicken, vegetables, milk and milk products play an important role in the dissemination of aeromonads (Khajanchi *et al.,* 2010; Ghenghesh *et al.,* 2008; Fricker and Tompsett, 1989). The risk of foodborne *Aeromonas* infections has been increasing as *Aeromonas* spp. are frequently isolated from food due to their psycrotrophy and the existence of the pathogens in water and fecal materials of humans and animals (Albert *et al*., 2000). Poultry and poultry products are frequently contaminated with *Aeromonas* spp. that can be transmitted to humans through the handling of raw poultry carcasses and products, or through consumption of undercooked poultry meat (Bailey and Cosby, 2003). Contamination of poultry meat during processing, handling, marketing, and storage prior to cooking, can lead to food poisoning illness in humans (Rajakumar *et al.,* 2012; Nagar *et al.,* 2011; Ghenghesh *et al.,* 2008).

In the last few decades *Aeromonas* spp. have emerged as an important human pathogen (Praveen *et al.,* 2014). The pathogenesis of *Aeromonas* infections is multifactorial and poorly understood (Janda and Abbott, 2010). There are several evidences of their involvement in gastrointestinal and extraintestinal infection in human (Gowda *et al.,* 2015) due to the production of many putative virulence factors (Yucel and Erdogan, 2010). *Aeromonas* is considered as opportunistic pathogens in both immunocompetent and immunocompromised humans (Janda and Abbott, 2010). Besides foodborne gastroenteritis in human, some extraintestinal symptoms such as; septicemia, wound infections, meningitis, endocarditis and osteomyelitis (Stelma, 1988) with a high mortality rate in immunocompromised person have been documented. Principal virulence factors that have an effect on pathogenicity are; extracellular toxins (enterotoxin, hemolysin and protease), structural features (Pilli, S- layer, lipopolysaccharide), adhesion and invasion. *Aeromonas* spp. can grow and produce toxins in refrigerated conditions indicating that refrigeration sometimes seems to be ineffective to control the pathogens (Koca and Sarimehmetoglu, 2009). It is certain that *Aeromonas* strains may produce many different putative virulence factors such as enterotoxins, hemolysins or cytotoxins, and antibiotic resistance against different antibiotics. The ability of these bacteria to grow competitively at 5°C may be indicative of their potential as a public health hazard. Therefore, the present work is designed for isolation, identification and antibiogram profile of *Aeromonas hydrophila* from broiler chickens.

2. Materials and Methods

2.1. Sample collection and processing

This study was conducted during the period from July to November, 2019 to isolate *Aeromonas hydrophila* from different broiler samples in the laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh. A total of 60 samples from 20 broiler chickens were collected from two different locations of Mymensingh Sadar: KR market, BAU and Shesh mor bazar (10 birds from each location). The samples included 20 skins, 20 legs and 20 breast samples from 20 broiler chickens (Table 2). Collected samples were immediately brought to the laboratory maintaining proper cool chain and processed as early as possible with 1% alkaline peptone water (HiMedia).

2.2. Cultural and biochemical characterization

Isolation of *Aeromonas hydrophila* from boiler samples was performed following the procedures described by Koca and Sarimehmetoglu (2009) with some modifications. 25 g of each samples were taken, placed in sterile zipper bags and homogenized with 225 ml of 1% alkaline peptone water (HiMedia) and incubated at 30ºC for

24 hours. After incubation, enrichment fluid was streaked on *Aeromonas* selective agar (HiMedia) with ampicillin supplement and incubated at 30ºC for 24 hours. Following the incubation, dark green centered green translucent colonies were further sub-cultured until pure culture of bacteria was obtained. Presumptive *Aeromonas hydrophila* isolates were stored in 20% glycerol at –80^{0}C until further use. The isolated bacteria were identified according to their biochemical characteristics (Ahammed *et al*., 2016; Samal *et al.*, 2014).

Identification of the isolated *Aeromonas hydrophila* was done based on detailed morphological, physiological and biochemical characterization. The isolated bacteria were sub-cultured onto TSA plates to obtain fresh 24 hours culture. Colonies grown on the TSA plates were subjected to biochemical tests. Characters such as motility, size and shape of the bacterium were recorded under morphological studies. Physiological characters included growth of each isolate at different temperature of 4°C, 5°C, 37°C and 40°C as well as growth of each isolate in nutrient broth containing different concentrations of NaCl as 0%, 1%, 2%, 3%, 3.5% and 4%. Different biochemical tests were conducted to evaluate the biochemical characteristic of the isolated bacteria such as oxidase, catalase, oxidative-fermentative test, esculin hydrolysis test, acid and gas production from sugars: glucose, lactose, sucrose, mannitol; methyl-red (MR) test, voges-Proskauer (VP) test, indole and H_2S production, decarboxylase test and citrate utilization test.

2.3. Molecular identificaion of *Aeromonas hydrophila* by PCR

Template DNA preparation was carried out by boiling method. Cultures were grown in nutrient broth at 37ºC for 24 hrs and 1 ml of the overnight culture was centrifuged at 5,000 rpm for 3 minutes using eppendorf tubes. Supernatant was carefully removed and the pellet was resuspended with 200 µl of sterile TE buffer, boiled at 100ºC for 15 minutes and immediately incubated on ice for 10 minutes. The mixture was then centrifuged at 12,000 rpm for 10 minutes and the supernatant with template DNA were then transferred into sterile tubes and stored at -80^{o}C for PCR amplification.

PCR was performed as per the method described previously by Swaminathan *et al*. (2004) with some modifications. Amplification of *lip* gene was performed to identify *Aeromonas hydrophila* with a DNA thermal cycler (Thermo cycler, ASTEC, Japan) using previously published primers. The list of primers is shown in Table 1. PCR reactions were carried out with 20 µl volume that includes 6 µl deionized water, 1 µl forward primer *(*Macrogen Inc., Korea*)*, 1 µl reverse primer *(*Macrogen Inc., Korea*)*, 2 µl DNA template and 10 µl master-mix (Promega, USA). PCR reactions were done by following conditions: initial denaturation with 1 cycle of 4 min at 94°C, followed by 40 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 69°C for 1 min, extension at 72°C for 1 min and a final extension step of 5 min at 72°C. After PCR reaction, PCR products were subjected to gel electrophoresis with 1.5% agarose gel at 100 volts for 45 minutes. Then the gel was submerged in ethidium bromide at a final concentration of 4 mg/ml for 15 minutes in a dark place followed by washing with distilled water for 5 minutes. Afterwards, the DNA was visualized under UV transilluminator (Biometry, Germany).

Table 1. Primers used for the molecular identification of *Aeromonas hydrophila*.

Primers	Sequence (5′-3′)	Amplicon size (bp)	Reference
lip-F	AACCTGGTTCCGCTCAAGCCG	760	Swaminathan *et al.* (2004)
lip-R	TTGCCTCGCCTCGGCCCAGCAGCT		

2.4. Antimicrobial susceptibility testing

Eight different antimicrobial discs: ampicillin (10 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamycin (10 µg), imipenem (10 µg) and tetracycline (30 µg) were selected for the antimicrobial susceptibility test against 12 isolated *Aeromonas hydrophila*. All the antimicrobial discs were purchased from HiMedia, India.

Antibiotic susceptibility of the isolates was determined using the disc diffusion or Kirby–Bauer method (Bauer *et al.*, 1966). Stock cultures of the bacterial strains were grown on TSA for 24 h at 37^{o}C. Then colonies of each of the isolate were adjusted to 0.5 McFarland's turbidity standard (equivalent to 1x10^8 colony forming unit/ml) in sterile phosphate buffered saline (PBS) and the bacterial suspension was spread onto Mueller–Hinton agar (Oxoid). Antibiotic-impregnated discs were kept on the solid medium and the plates were incubated at 37^{o}C for 24 h. Zone of inhibition formed around the discs was measured and antibiotic sensitivity was assayed from the length of the diameter of the zones (in mm). The zone radius was actually scaled from the centre of the antibiotic disc to the end of the clear zone where bacteria could be seen growing. Tested bacterial strains were

classified into three categories: sensitive, intermediate, and resistant depending on the diameters of inhibition zones and standards supplied by HiMedia Laboratories and comparing with other related references (Table 4).

3. Results and Discussion

3.1. Occurrence of *Aeromonas hydrophila*

In this study, a total of 60 samples were assessed for isolation and identification of *Aeromonas hydrophila* from poulty sources. From these 60 samples, 30 samples (10 skin, 10 leg and 10 breast samples) were collected from KR market, Mymensingh Sadar and another 30 samples (10 skin, 10 leg and 10 breast samples) were collected from Shesh mor bazar, Mymensingh Sadar. Out of 60 samples, a total of 37 isolates showed positive growth on *Aeromonas* Selective Agar plates (HiMedia, India) and produced greenish with dark green centre, round, small to medium, convex and translucent colonies. In this study, out of 60 different poultry samples, 27 (45%) samples were positive for *Aeromonas hydrophila* based on biochemical and molecular test (Table 2). The above result is quite similar with the results reported by Dallal *et al.* (2012), Koca and Sarimehmetoglu (2009) and Ternstrom and Molin (1987) who had found 41%, 53.75% and 53.3% positive *Aeromonas hydrophila* respectively from different poultry sources. Singh (1997) reported that all of ground turkey meat samples of his study were contaminated with *Aeromonas* spp. where 56% isolates were identified as *A. hydrophila* which is also nearly similar to present study. Higher and lower isolation rate of *Aeromonas* spp. from poultry sources compared to our findings have also been documented in previous study. Yucel and Citak (2003) detected motile aeromonads in 87% of poultry meat samples with predominance of *Aeromonas hydrophila* and *Aeromonas sobria* strains. Some other studies on chicken samples performed by Yucel and Erdem (2004), Sarımehmetoglu and Kuplulu (2001) and Hanninen (1993) had found a higher contamination levels of 86.95%, 82.9% and 93% with *Aeromonas* spp. respectively compared to present findings. On the other hand lower recovery rate of *Aeromonas hydrophila* had also been reported by Nagar *et al.* (2011), Sharma *et al.* (2009) and Chang *et al.* (2008). Variations in the percentage of *Aeromonas hydrophila* may be due to the differences in the geographical distribution, origin of the samples, sampling period, methodology of analysis, number of samples for analysis and poor hygienic practices during handling and processing (Nagar *et al.*, 2011; Sharma *et al.*, 2009; Koca and Sarimehmetoglu, 2009).

Significant economic losses are being experienced in commercial poultry sector worldwide due to diseases caused by bacterial agents (Barnes *et al*., 2003). Many previous studies revealed that *Aeromonas hydrophila* to be found more frequently in meat and meat products (Dallal *et al.*, 2012; Osman *et al.*, 2012; Sharma and Kumar, 2011; Hanninen, 1993). It is probable that the *A. hydrophila* infection of chicken occurred horizontally via the oral route through drinking water from contaminated sources and unhygienic feeds containing contaminated fish meals or similar products (Dashe *et al.*, 2013) as fish is considered to be the reservoir of *A. hydrophila* (Sharma *et al.*, 2009). Whereas contamination of poultry meat with *A. hydrophila* is attributed to the washing of carcasses with contaminated water along with insufficent sanitary measures during their handling and processing (cutting and mincing) **(**Stratev and Odeyemi, 2016; Rajakumar *et al.*, 2012; Stratev *et al.*, 2012).

Table 2. Number (%) of isolated *Aeromonas hydrophila* from different types of broiler samples collected from two different locations of Mymensingh Sadar.

Sample sources	KR Market, Mymensingh Sadar			Shesh mor, Mymensingh Sadar		
	No. of sample tested	**No. (%) of culture positive isolates**	**No. (%) of positive *A. hydrophila***	**No. of sample tested**	**No. (%) of culture positive isolates**	**No. (%) of positive *A. hydrophila***
Skin	10	9 (90)	7 (70)	10	9 (90)	9 (90)
Leg	10	6 (60)	3 (30)	10	6 (60)	3 (30)
Breast	10	3 (30)	2 (20)	10	4 (40)	3 (30)
Total	**30**	**18 (60)**	**12 (40)**	**30**	**19 (63.33)**	**15 (50)**

In this present study, out of 27 positive isolates of *Aeromonas hydrophila*, 55.56% isolates were recovered from shesh mor market (Figure 1). The reasons behind higher recovery of *A. hydrophila* from shesh mor market may be due to the use of a funnel like device for the bleeding of each birds after slaughtering that may act as a vehicle for transmission of pathogens. However, this device was not observed in KR market. Among three different samples of a bird, skin was found most contaminated part followed by leg and breast (Figure 2). This was happened because skin is the most outer part of a bird and always gets exposed to the faecal contamination. *Aeromonas* spp. are very sensitive to pH below 5.5 and 7.2 is considered as the optimum pH for growth (Kirov,

1993). Koca and Sarimehmetoglu (2009) investigated and documented that average pH value of turkey leg samples was 6.0, on the other hand, 5.7 for breast samples. This finding also supports our present study and clarifies the higher susceptibility of leg samples to *A. hydrophila* over the breast samples.

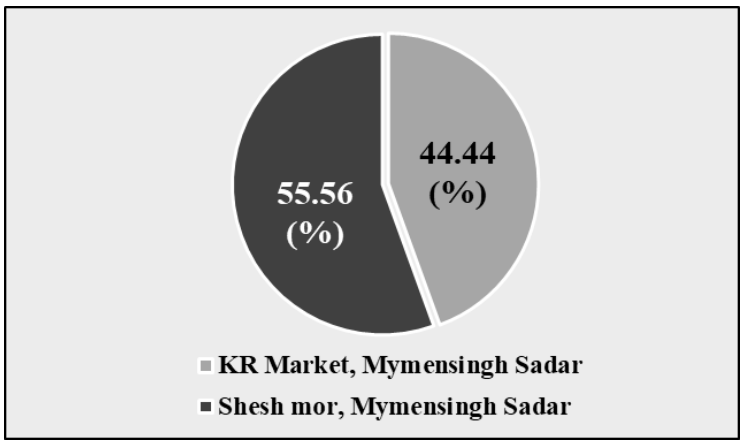

Figure 1. Location-wise percentage of *A. hydrophila* from 27 positive isolates.

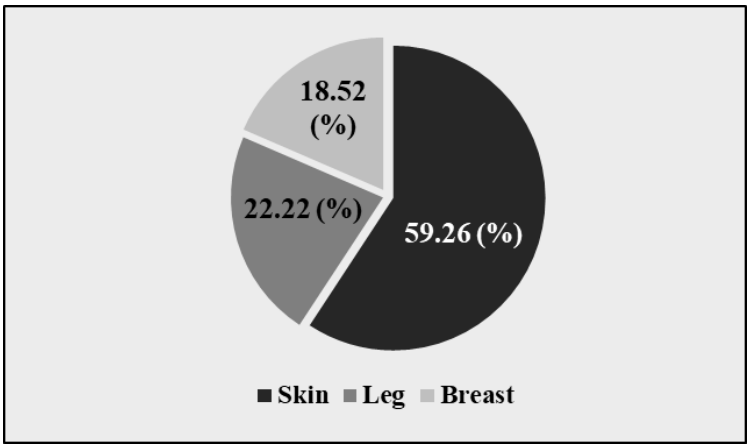

Figure 2. Sample-wise percentage of *A. hydrophila* from 27 positive isolates.

3.2. Morphological and biochemical characterization

The isolated *A. hydrophila* from were further identified based on the morphological, physiological, conventional, and biochemical characteristics. Morphologically the isolated colonies showed greenish with dark green centre, round, small to medium, convex and translucent colonies on *Aeromonas* Selective Agar plates (HiMedia, India). Microscopically *A. hydrophila* was a Gram negative short plump rod, motile by polar flagella with swarming movement, positive for oxidase and catalase test similar to the characteristics reported by Monir *et al.* (2017), Samal *et al.* (2014) and Noga (2000). The isolates were found to produce acid and gas from different sugar media such as glucose, lactose, sucrose, dextrose, maltose, mannitol, whereas did not ferment inositol, sorbitol, rhamnose etc. Moreover, they utilized citrate for growth and produced acetoin, produced indole, reduced nitrate, showed positive reaction towards Voges proskauer (VP) test, gelatinase test, arginine decarboxylase test; esculin hydrolysis test, alkyl sulfatase test, acetate utilization test; showed negative reaction towards methyl red (MR) test, lysine decarboxylase test, urease test (Monir *et al.*, 2017; Ahammed *et al.*, 2016; Samal *et al.*, 2014; Jayavignesh *et al.*, 2011; Mostafa and Ahamed, 2008). Consequently, the isolates showed positive growth at 37°C with the optimum at 24°C but no growth was found at 4°C and 40°C. Furthermore, *A. hydrophila* strains grow in nutrient broth with 0-2% NaCl, however, no growth was noted in 2-4% NaCl media (Table 3).

Table 3. Results of biochemical characteristics of the isolated bacteria.

Characters	Characterization by Ahammed *et al.* (2016)	Characterization by Mostafa and Ahamed (2008)	Present result
Gram stain	-	-	-
Shape	Rod	Rod	Rod
Motility	+	+	+
Oxidase	+	+	+
Catalase	+	+	
Glucose	+	+	+
Lactose	+	+	+
Sucrose	+	+	+
Maltose	+	+	+
Mannitol	+	+	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Esculin hydrolysis	+	+	+
Methyl-red test	-	-	-
Voges-Proskaur	+	+	+
Indole	+	+	+
H_2S production	+	+	-
Arginine decarboxylation	+	+	+
Lysine decarboxylation	-	-	-
Citrate utilization	+	+	+
Growth at: 4°C	-	-	-
5°C	+	+	+
37°C	+	+	+
40°C	-	-	-

3.3. Molecular identificaction of *Aeromonas hydrophila* by PCR

DNA extracted from all culture positive samples were used in the PCR assay for specific identification of *Aeromonas hydrophila*. PCR primers targeting *lip* gene in the isolated genomic DNA of *Aeromonas hydrophila* amplified 760 bp that confirmed the identity of *Aeromonas hydrophila* (Figure 3). PCR product of 760 bp was obtained in 27 isolates of *A. hydrophila* out of 37 culture positive samples (Table 2).

lip gene codes for a thermostable extra cellular lipase of *A. hydrophila* and the PCR primers containing *lip* gene are designed for the specific detection of *A. hydrophila* (Swaminathan *et al.*, 2004). Cascon *et al*. (1996) screened 50 strains of bacteria including *Aeromonas* spp., through amplification of *lip* gene. A DNA fragment of approximately 760 bp was amplified only in the strains of *A. hydrophila*. Swaminathan *et al.* (2004) identified nine isolates of *A. hydrophila* from fish and water samples by amplification of the *lip* gene through known primer sequences at a modified annealing temperature.

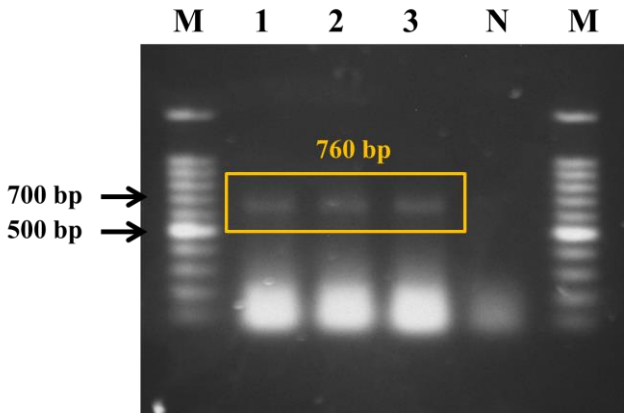

Figure 3. *lip* gene-based PCR of *Aeromonas hydrophila* showing positive band at 760 bp. Lane M: 100 bp DNA ladder; lane N: negative control; lanes 1-3: positive samples of *Aeromonas hydrophila*.

3.4. Antimicrobial susceptibility testing

Out of 27 isolated *Aeromonas hydrophila*, randomly 12 isolates (6 from each location) were tested against eight commercially available and widely used antibiotics in poultry industry of Bangladesh namely ampicillin, ciprofloxacin, gentamicin, chloramphenicol, erythromycin, co-trimoxazole, imipenem and tetracycline. The isolates of *Aeromonas hydrophila* showed varying levels of susceptibility/resistance to the different antimicrobial agents (Table 4).

All *Aeromonas hydrophila* strains were found resistant to ampicillin and few isolates showed higher resistance to co-trimoxazole (84%), tetracycline (34%) and erythromycin (34%). Higher resistance to *β*-lactamases like ampicillin had also been reported by some other researchers (Samal *et al.*, 2014; Nagar *et al.*, 2011; Vaseeharan *et al.*, 2005; Radu *et al.*, 2003). The high resistance is due to the production of inducible chromosomal *β*-lactamases (Janda and Abbott, 2010). In this present study, most of the isolates were found sensitive to ciprofloxacin (92%), gentamycin (66%), chloramphenicol (50%) and Imipenem (34%) as reported by Monir *et al.* (2017), Nagar *et al.* (2011), Ashiru *et al.* (2011), Akinbowale *et al.* (2007), Palu *et al.* (2006) and Ottaviani *et al.* (2006). The sensitivity and resistance pattern of *Aeromonas* may vary due to different isolation sources, environmental conditions, and variable use of drug from place to place (Nagar *et al.*, 2011).

Table 4. Antibiogram profile of the isolated *Aeromonas hydrophilla* (n=12).

Antibiotics	No. (%)		
	Sensitive	Intermediate	Resistant
Ciprofloxacin (5 µg)	11 (92)	1 (8)	0 (0)
Gentamicin (10 µg)	8 (66)	4 (34)	0 (0)
Tetracycline (30 µg)	2 (16)	6 (50)	4 (34)
Chloramphenicol (30 µg)	6 (50)	4 (34)	2 (16)
Erythromycin (15 µg)	2 (16)	6 (50)	4 (34)
Co-trimoxazole (25 µg)	0 (0)	2 (16)	10 (84)
Ampicillin (10 µg)	0 (0)	0 (0)	12 (100)
Imipenem (10 µg)	4 (34)	6 (50)	2 (16)

4. Conclusions

In conclusion, the findings of the present study indicate the involvement of *A. hydrophila* in apparently healthy broiler chickens. In addition, *Aeromonas hydrophila* isolates were found resistance to a variety of commercially available antibiotics due to indiscriminate and irrational use in poultry sector. Hence, intensive and continuous monitoring of potentially pathogenic *Aeromonas* spp. along with their antibiogram profile from the poultry value chain in Bangladesh are highly recommended to assess the human health risk.

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Conflict of interest

None to declare.

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