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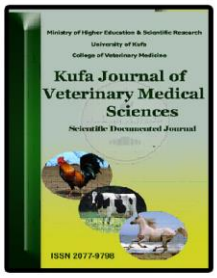
vetmed@uoKufa.edu. iq



Molecular Detection of Some *A.hydrophila* Toxins and its Antibiotics Resistance Pattern Isolated From Chicken Feces in Thi-Qar Province (Iraq)

Muslim Dhaher Musa [1] Waffa Abdulelah Ahmed[2]

1 Southern Technical University , Technical Institute[1]

2University of Baghdad , Veterinary Medicine College

Abstract

The high isolation rate of virulent *A.hydrophila* from chicken represent an important public health concern especially when these bacteria exhibit increased antimicrobial resistance to medically important antibiotics, so this study was conducted to isolate *A.hydrophila* from chicken feces and investigate the presence of some toxins genes and determine their antibiotics resistance profile. Chicken feces were collected from different regions, throughout Thi-Qar province south of Iraq, in period extended from July 2015 to January 2016. *A.hydrophila* isolates were identified by morphological, biochemical and API20E. These isolated were subjected to PCR assay for confirmation, targeting 16S RNA-23SRNA Intergenic Spacers Region and for detection of important virulence genes including hemolytic toxin Aerolysin (Aero), Heat labile enterotoxin (*Alt*) and Heat stable enterotoxin (*Ast*). Twelve *A.hydrophial* were isolated and identified to species level among 23 Aeromonas spp isolated from chicken fecal samples with overall incidence rate (52.6%). Screening for virulence genes revealed that 10/12 (83.4%) were positive for Aerolysin (*Aero*) gene and 9/12(75%) for Heat labile enterotoxin (*Alt*) gene, while none of these isolates were positive for Heat stable enterotoxin (*Ast*) gene 0/12(0%).The most prevalent genotype was (*Aer+ Alt*$^{+}$ *Ast*$^{-}$). Antibiogram against 19 antibiotics revealed that, all isolates in showed absolute susceptibility(100%) to Gentamycin, Oflaxacin, Amikacin, Norfloxacin, Imipnem Ciprofloacin. However, multidrug resistance recorded in all isolates, 2 isolates (16.7%) were resistant to eight antibiotics including , Clindamycin, Cephalothin , Vancomycin, Ticacillin-clavulnoc acid, Ceftazidime, Cefoxitin, Trimethoprim-sulfamethaxazon, Azithromycin ,with multidrug resistance index (0.42) , and 5/12 (41.7%) were resistance to seven antibiotics (58.4%) with MDRI(0.36), four isolates(33.4%) were resistant to six antibiotics with MDRI (0.31), while only one isolates 1/12(8.4%) were resistant to five antibiotic with MDRI (0.26). The present study showed that detection of pathogenic *A.hydrophila* harboring important virulence genes Aerolysin and *Alt* with resistance to many clinically important antibiotics is a good indication that chicken feces constitute important source for pathogenic *Ahydrophila* infecting human that come in contact with chicken and spread of multidrug resistance organisms in environment .

Key words : *A.hydrophila* ,virulence genes, Antibiogram profile, Chicken, Iraq.

Introduction

A.hydrophila are Gram negative rods, facultative anaerobes, oxidase and calatase positive, motile by single polar flagellum, ferment glucose with gas production [1]. They are widely distributed in nature, and have been isolated from, rivers, fresh, west water, and wells water [2, 3, 4,5,6]. Also, from food of animals origin [7,8] and from feces of different animals species including cattle [9,10], goat [11], dogs and cats [12] and chickens[13,14,15]. Feces of infected animals has been proposed as the main channel of transmission of *Aeromonas* from animals to humans that come in contact with infected animals [16]. The high occurrence of *Aeromonas* in chicken feces, suggested that chicken could be possible threat to public health [16]. *A.hydrophila* is the most frequently isolated pathogen associated with human gastroenteritis [17].It has been estimated that *Aeromonas* associated gastroenteritis cases may reached 13% in United state [18].In addition to gastroenteritis, *Aeromonas* has been implicated in meningitis, otitis, endocarditis, bacteremia and septicemia [19].

Pathogenicity of *Aeromonas* is said to be multifactorial, involving structural components(flagella, capsule, fambirae, S-layer, Lipopolysacchrides and outer membrane proteins) siderophores, quorum-sensing mechanisms, secretion systems, extracellular enzymes, and exotoxins[20]. Exotoxins are the most important virulence factors which are divided into two main categories: hemolytic toxins (hemolysin and Aerolysin),and enterotoxins which divided into two main groups; enterotonic enterotoxin (Heat labile enterotonic enterotoxin and heat stable enterotonic enterotoxin) and enterotoxic enterotoxin including heat stable enterotoxic enterotoxin [21,22].The risks to human health from various *Aeromonas hydrophila* strains may differ depending on their carriage oftoxin coding genes,[23]. An interesting approach for the direct detection of pathogenic *A. hydrophila* isolates is the use of virulence determinants as genetic markers. It has been suggested that variations in the distribution of potential virulence genes amongst *A.hydrophila*, might contribute to their degree of pathogenicity [24].Therefore, the detection of virulence genes in *Aeromonas* is essential in determining potential pathogenicity of these organism and subsequent possible targets for prevention of infection. In veterinary medicine, antibiotics are used for therapeutic and for growth promoter to whole flock rather than individual animal [25], thus this technique in Antibiotic usage, especially in poultry, raise the antibiotics selection pressure in poultry intestine as a result their intestinal flora contain relatively high percentage of resistant bacteria. Resistant bacteria from chicken intestine may contaminate the chicken meat as result this may pose a risk to human health by direct contact with these animals or by ingestion of poultry meat [26]. Resistance to commonly used antibiotics is relevant among pathogenic *Aeromonas spp*. in which, in addition to their classical resistance to β-lactamase antibiotics, multiple antibiotic resistance has been reported [27]. According to our best knowledge no previous study undertaken the prevalence, or distribution of toxins genes and antibiotics resistance profile of *A.hydrophila* from chicken feces in Iraq, regarding these facts this study aimed for detection of some toxins genes and examine the antimicrobial resistance profile of *A.hydrophila* isolated from chicken feces.

Materials and methods

- **Samples collection**

One hundred and twenty five chicken fecal samples were collected at random months in period extended from July 2015 to February 2016, from different locations throughout Thi-Qar province south of Iraq. The fecal samples were collected in sterile screw caped container, placed in ice and transported to laboratory within period less than two hours .

- **Isolation procedure**

Method described by Ghenghesh *et al*.,(2008) [28] was adapted for isolation of *Aeromonas spp*. from chicken feces as follow: five grams of chicken fecal samples was inoculated in 50 ml alkaline peptone water broth (APW pH=8.4) and incubated 18-24 hours at 37C ° for enrichment . A loopful of enrichment media was streaked onto MacConkey agar and Ampicillin Blood Agar (ABA30) containing 5% blood and 30 mg/L ampicillin, all plates were then incubated at 37C° for 24 hours.

- **Identification**

Colonies that appeared as large flattened colorless with non lactose fermentation on MacCkony agar and white to grayish with β-hemolytic zone on ABA30 agar were selected and sub cultured on ABA30 agar to obtain pure colonies, the colonies were subjected to following biochemical tests; oxidase, resistance to O/129(2,4 diamino-6, 7-diisopropylpteridine), glucose fermentation (KIA), motility (wet mount), Gram stain and salt tolerance test. Colonies showed positive reaction for Oxidase, resistance to O/129, glucose fermentation with gas, motile, Gram negative rods and unable to grow in nutrient broth containing 6.5% NaCl were presumptively identified as *Aeromonas spp*. The presumptively identified colonies were confirmed as *Aeromonas spp.* by using PCR technique with primer targeting Intergenic Spacer Regions (ISR) specific for genus *Aeromonas* [29].The primer sequence and amplification reaction conditions were presented in tables (1and 2) . PCR reaction was performed in 50µl PCR tube containing 5 µl PCR premix (Bioneer/ Korea), 10µl of template DNA, 1µl forward and reveres primer at final concentration (20 picomoles/µl) and the remaining volume was completed with nuclease free water .

Species identification was accomplished according to AerokeyII biochemical scheme[30] and *A.hydrophila* isolates were further confirmed by API20E.

- **Molecular Detection Of Toxins Genes**

Presto™ Mini gDNA Bacteria Kit (Genaid, Thailand) has been employed to extract genomic DNA from 24 hours *A.hydrophila* broth culture. Polymerase chain reaction (PCR) was used to detect hemolytic toxin, Aerolysin (*AeroA*) gene and enterotoxins heat labile toxin (*Alt*) gene and Heat stable toxin gene(*Ast*) using specific oligonucleotid primers presented in table (1), these primers are specific for *A.hydrophila* according to previous studies. The PCR reaction was done in 50 µl PCR tube containing ,5µl PCR premix (Bioneer/ Korea) , 5µl template DNA, 1µl of forward and reverse primer at final concentration (10 picomole/ µl), and the remaining volume was completed with nuclease free water,the amplification conditions for each gene was presented in table (2). Presence of amplified product was confirmed by agarose gel electrophoresis using 1.5% garose in TBE X-1, applying voltage 100 for 1 hour and examined under ultraviolet light after staining with ethidium bromide. After electrophoresis, photographs were taken by digital camera.

Table (1) . Sequences and product size of primers used for detection of *Aeromonas spp* and toxins coding genes of *A.hydrophila*

Gene	Cycles	Initial denaturation		Denaturation		Annealing		Extension		Final extension	
		Temperature C □	Duration Seconds	Temperature C □	Duration Seconds	Temperature C □	Duration Seconds	Temperature C □	Duration Seconds	Temperature	Duration Seconds
ISR	25	94	120	94	60	58	60	72	60	72	600
Aero	35	95	300	92	30	52	30	72	120	72	60
Alt	30	94	180	92	30	63	30	72	60	72	120
Ast	30	94	180	92	30	66	30	72	60	72	120

Gene name	Primers sequences 5′ ⟶ 3′	Product size	Purpose of use	Reference
ISR	F - GGAAACTTCTTGGCGAAAAC R - GGTTCTTTTCGCCTTTCCCT	550	Detection of Aeromonas spp	Osman *et al* .,2012
Aero	F- GCC TGA GCG AGA AGG T R-CAG TCC CAC CCA CTT C	416	Detection of Aerolysin specific for *A.hydrophila*	Wong *et al* .,1998
Alt	F-CCATCCCCAGCCTTTACGCCAT R-TTTCACCGAGGTGACGCCGT	338	Detection of heat labile enterotonic enterotoxin	Martínez *et al* 2009
Ast	F-ATG CAC GCA CGT ACC GCC AT R-ATC CGG TCG T C G CTC TTG GT	260	Detection of heat stable enterotonic enterotoxin	Martínez *etal*.,2009

Table (2). PCR Amplification conditions of genes of Aeromonas

Antibiotic sensitivity test

Antibiotics sensitivity test was carried out according to kirby-Bauer method using Mueller- Hinton agar . Antibiotics resistance profile of *A.hydrophila* isolates was tested against 20 antibiotics these are : Cholamphincol (30), Oflaxacine (5), Amikacin(30), Clindamycin(2), Naldxic acid (30), Streptomycin (10), Cephalothin (30), Norfloxacin(10), Vancomycin(30, Erythromycin (15), Deoxycyclin (30), Azithromycin(15), Aztreonam(30), Cefteriaxon(30), Trimethoprim-sulfamethaxazon, Imipenem(10), Ticacillin-clavulnoc acid (75/10), ciprofloxacin(5) , Ceftazidime (5),and Cefoxitin(10).

The diameter of inhibition zone for individual antimicrobial agent was interpreted in terms of sensitive, intermediate and resistant categories by comparison with the standard inhibition zone according to Clinical and Laboratory Standards Institute (CLSI , M45-A2 (CLSI 2006) .

Results

Out of 125 chicken fecal samples, 23 (18.4%) isolates were isolated and identified as *Aeromonas spp*.the results of biochemical tests used for initial identification of *Aeromonas spp.* were presented in table (3). Molecular identification revealed that all 23(100%) isolates were *Aeromonas spp*. based on ampilifcation of intergenic spacer regions (ISR) which yield product size (550bp) that is specific for *Aeromonas spp*. figure(1) indicating an excellent correlation between biochemical tests used for presumptive identification and molecular method (PCR) using highly conserved region of 16S-RNA 23SRNA for genus identification .

Speciation of 23 *Aeromonas* isolates by using Aeroky II biochemical scheme revealed that these isolates were belong to four species with *A.hydrophila* 12 (52.17) was the most predominant followed by *A.sobriae* 6(26.08), *A.caviae* 4(17.39), and *A.veronii* 1(4.34). The twelve *A.hydrophila* isolates were further confirmed by API20E , figure (2).

Table (3) . Biochemical test used for presumptive identification of *Aeromonas spp*

Test	Typical reaction	No .of positive isolates
Gram stain	G ve- bacilli occur singly	23 (100%)
Oxidase	Positive	23(100%)
Glucose fermentation	Positive	23(100%)
Resistance to O/129	Resistant	23(100%)
String test	Negative	23(100%)
Growth at 6.5% NaCl	Negative	23(100%)
Growth on ABA30	Positive	19 (82.6%)
Hemolysis on blood agar	Positive	21 (91.3%)

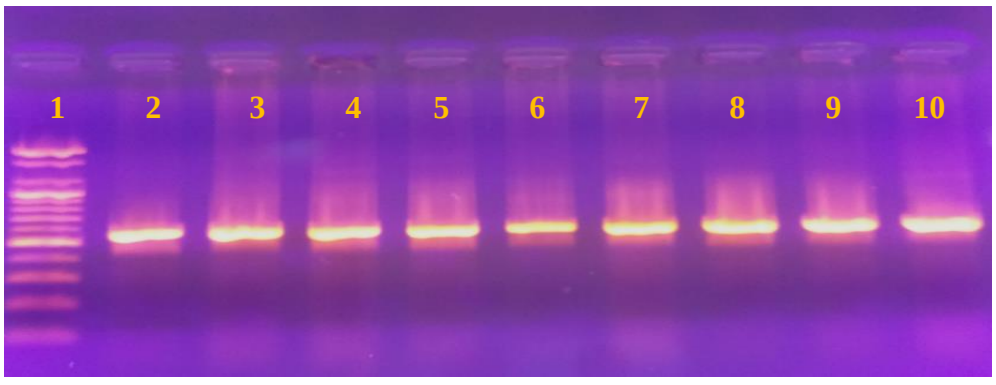


Figure (1). Agaros gel electrophoresis (1.5%) of amplified ISR (550 bp) after 1 hour at 100 V, stained with ethidum bromide and visualized by a UV transilluminator. **Lane 1**: 2Kbp ladder, **Lanes 2-10** : positive *Aeromonas spp*

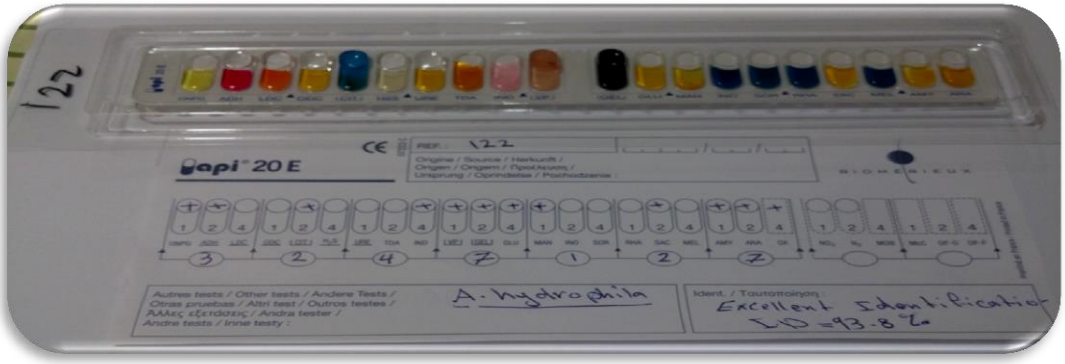


Figure (2). Reaction on *A.hydrophila* in Api20E, shows excellent identification

Detection of *A.hydrophila* Toxin Coding Ggenes

Molecular characterization of all 12 isolates of *A.hydrophila* revealed that all isolates were harboring at least one toxin coding gene in that, Aerolysin gene was detected in 10/12(83.4%), and heat labile enterotoxin (*Alt*) was detected in 9/12(75%),while heat stable enterotoxin gene was not detectedat all 0/12 (0%), figures (3 and 4). According to different combinations of the detected genes, three genotypes were detected, the most predominant genotypes 7/12(58.4%) was ($Aero^{+}$ Alt^{+} Ast^{-}) , followed by the genotype ($Aero^{+}$ Alt^{-} Ast^{-}) in which only Aerolysin was detected 3/12(25%) and only two strains (16.7%) were shown to be positive for Alt gene only(Aer^{-} Alt^{+} Ast^{-}), table (4).

Table (4). Distribution of toxin coding genes of *A.hydrophila* isolated from chicken fecal samples

Isolate cod	Aerolysin	Heat labile entrotoxin	Heat stable enterotoxin	Genotype
AH1	+	+	-	$Aero^{+}$ Alt^{+} Ast^{-}
AH2	+	-	-	$Aero^{+}$ Alt^{-} Ast^{-}
AH3	+	+	-	$Aero^{+}$ Alt^{+} Ast^{-}
AH4	-	+	-	$Aero^{-}$ Alt^{+} Ast^{-}
AH5	+	+	-	$Aero^{+}$ Alt^{+} Ast^{-}
AH6	+	+	-	$Aero^{+}$ Alt^{+} Ast^{-}
AH7	+	+	-	$Aero^{+}$ Alt^{+} Ast^{-}
AH8	+	+	-	$Aero^{+}$ Alt^{+} Ast^{-}
AH9	+	-	-	$Aero^{+}$ Alt^{-} Ast^{-}
AH10	-	+	-	$Aero^{-}$ Alt^{+} Ast^{-}
AH11	+	+	-	$Aero^{+}$ Alt^{+} Ast^{-}
AH12	+	-	-	$Aero^{+}$ Alt^{-} Ast^{-}
TOTAL	10 (83.4%)	9(75%)	0(0%)	

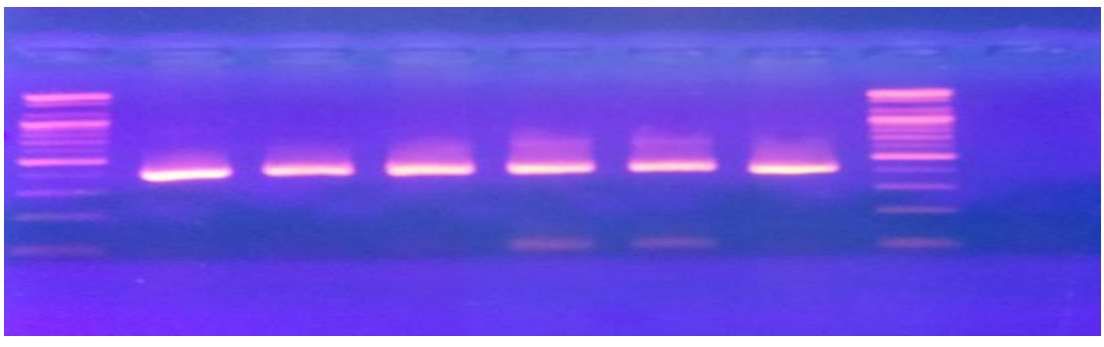


Figure (3).Agarose gel electrophoresis (1.5%) of amplified *Aerolysin* gene (416 bp) of *A. hydrophila* for 1 hour at 100V , stained with ethidum bromide and visualized by a UV transilluminator. Lanes 1,8: Molecular weight marker using 2000 bp ladder ,Lane 9 :negative control , Lane 2,3,4,5,6,7, PCR amplification products of *A. hydrophila* Aerolysin (*Aero*) gene

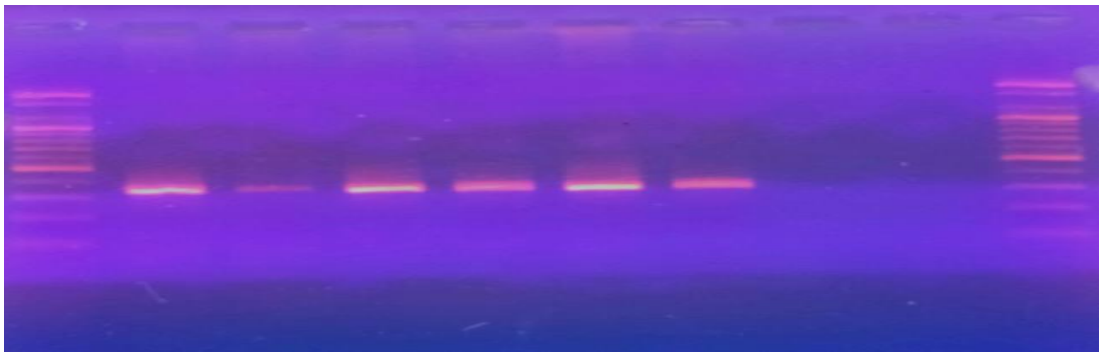


Figure (4). Agarose gel electrophoresis (1.5%) of amplified *Alt* gene (338 bp) of *A. hydrophila* for 1 hour at 100V , stained with ethidum bromide and visualized on a UV transilluminator. Lanes 1,10: molecular weight marker using 2000 bp ladder ,Lanes 8,9 :negative control , Lanes 2,3,4,5,6,7, PCR amplification products of *A. hydrophila alt* gene

Results of antibiotics resistance pattern was presented in table (5). All *A.hydrophila* isolates 12 (100%showed resistance to Clindamycin, Cephalothin, Vancomycin, Ticacillin-clavulnoc acid, and Ceftazidime.
Seven isolates (58.4%) were resistant to cefoxitin, and five isolates (41.7%) were resistant to Cloramphenicol while resistance to Trimethoprime-Sulfamethoxazol and Aztreonam was (25%)

All isolates showed multi-antibiotic resistance with varying MAR index ranging from 0.25 to 0.4, most of isolates, 5(41.7%) were resistant to seven antibiotics with MAR index (0.35), 4(33.4%) were resistant to six antibiotics with MAR index (0.3), only two isolates (16.7%) were resistant to eight antibiotics, MAR index(0.4), and only one isolates (8.4%) was resistant to five antibiotics, MAR index (0.25) .

On the other hand, all *A.hydrophila* isolates in this study were sensitive to Gentamycin, Oflaxacin, Amikacin, Norfloxacin, Imipnem , Ciprofloacin, and naldexic acid, the streptomycin showed high activity on *A.hydrophila* isolates as 9 (75%) were susceptible while the remaining three isolates were intermediate susceptibility , also Deoxycyclein was potent against A.hydrophila isolates in that (83.4%) of isolates were susceptible.

Table (5) Antibiotics Resistance Pattern of *A.hydrophila* Isolated From Chicken feces

Antibiotic Disc	AH 1	AH 2	AH 3	AH 4	AH 5	AH 6	AH 7	AH 8	AH 9	AH 10	AH1 1	AH 12	Resistane %
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	S	0
Cholamphenicol	S	S	R	S	R	R	I	R	R	S	S	I	41.7
Ofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	0
Amikacin	S	S	S	S	S	S	S	S	S	S	S	S	0
Clindamycin	R	R	R	R	R	R	R	R	R	R	R	R	100
Nalidixic acid	S	S	S	I	S	S	S	S	S	S	S	S	0
Streptomycin	I	S	I	S	I	S	S	S	S	S	S	S	0
Cephalothin	R	R	R	R	R	R	R	R	R	R	R	R	100
Norfloxacin	S	S	S	S	S	S	S	S	S	S	S	S	0
Vancomycin	R	R	R	R	R	R	R	R	R	R	R	R	100
Erythromycin	S	S	S	S	S	S	S	S	S	S	S	S	0
Doxycycline	S	S	S	S	I	S	S	S	S	S	S	S	0
Aztreonam	R	R	I	I	I	I	I	I	R	I	I	R	25
Ceftriaxone	S	S	S	S	S	S	S	S	S	S	S	S	0
Trimethoprim-Sulfamethoxazole	R	S	S	S	R	R	I	I	R	I	I	I	25
Imipenem	S	S	S	S	S	S	S	S	S	S	S	S	0
Ticarcillin-clavulanic acid	R	R	R	R	R	R	R	R	R	R	R	R	100
Ciprofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	0
Ceftazidime	R	R	R	R	R	R	R	R	R	R	R	R	100
Cefoxitin	S	R	R	S	R	S	R	S	S	R	R	R	58.4
MAR index	0.35	0.35	0.35	0.25	0.4	0.35	0.3	0.3	0.4	0.3	0.3	0.35	

Discussion

In current study, *Aeromonas spp*. isolated from chicken feces with an incidence rate (18.4%), a similar results reported by [16,31,13],however, the results of this study are higher than those reported by Kumar *et al*.(2000)[33] who found (16.7%) and Mahmoud and Tanios,(2008)[14] who found *Aeromonas* recovered from chicken at percentage (9%), this could be attributed to the difference in geographical region. Researchers from various parts of the world reported isolation of *Aeromonas* from chicken with varying isolation percentage ranging from as low as 6% as reported by [32], moderate recovery percentage (24%) was reported by [34], and high recovery rate (55%) was reported by [35]. Identification of *Aeromonas spp*. using set of biochemical tests including; Oxidase, glucose fermentation (KIA), resistance to O/129, String test and salt tolerance test, approved to be suitable for initial identification of *Aeromonas spp*, as a excellent correlation was found between biochemical identification and molecular identification by PCR technique using genus specific primer designated by Kong *et al*.,(1999)[26],who found amplification of ISR fragment provide a very specific and rapid screening

technique for identification of *Aeromonas spp*. Osman *et al* .,(2012)[24], found primer complementary to 16S rRNA and 23S rRNA genes was successful way to detect *Aeromonas spp*. from food of animals origin. In current study, speciation of 23 *Aeromonas* isolates revealed that *A.hydrophila* was predominant (52.17%), similar finding obtained by Akan and Dieker ,(1996)[31] and Akan *et al.* (1998)[36] as they found that *A.hydrophila* was the predominant species in chicken feces.

Results of current study, showed that all *A.hydrophila* strains were positive to one or two toxins coding genes. Generally, *Aero* gene coding for aerolysin has been detected in most of strains 10/12(83.4%). Detection of this gene, aerolysin(*Aero*) has been proposed as an excellent indicator of pathogenicity [37,38]. Findings of this study are in line with Ftima and Mohamed,(2012)[13] who found all *A.hydrophila* isolates from diseased chicken feces were harboring aerolysin gene, while only (42%) from apparently healthy chicken.

Interestingly, in current study all A.*hydrophila* strain were phenotypically β-hemolytic even those strains which were PCR negative for aerolysin (*Aero*$^{-}$ *Alt*$^{+}$ *Ast*$^{-}$, and *Aero*$^{-}$ *Alt*$^{+}$ *Ast*$^{-}$),in this regard finding of this study are in agreement with [39], this could be attributed to presence of other hemolytic toxin. Indeed, two types of β-hemolytic toxins, hemolysin (*hlyA*) and aerolysin (*Aero*) are found in most *Aeromonas* species[21]. Also, it has been reported that *Aeromonas* can produce two types of lipase enzymes; lipase A1 and Phospholipase C, and both have hemolytic activity [40].

On the other hand, result of this study found that most isolates 9/12(75%), were harboring heat labile enterotoxin (*Alt*) gene. The *Alt* detection percentage in this study are in concordance with range (64%-97%) which published by several investigators (41, 42,18, 43,44). An important evidence that poultry provide source of *A.hydrophila* infection in human is conducted by[19], who found that the high prevalence of *Aeromonas* in poultry farmers suffering from gastroenteritis was related to frequent contact with poultry. Also, Albert *et al* .,(2000)[45] found that *A.hydrophila* strains from cases of sever gastroenteritis of children as sole pathogen were positive for *Alt* gene. Since most of strains(58.4%) detected in this study were carrying genotype (*Aero*$^{+}$ *Alt*$^{+}$ *Ast*$^{-}$), so it is safely to assume the A.*hydrophila* isolated from chicken feces are constitute public health risk.

In this study, all *A.hydrophila* strains were displayed multi-antibiotics resistance, this results are similar to those obtained by [46,47,48]. In this study, *A.hydrophila* were displayed resistance (100%) to β-lactam antibiotics including; Cephalosporins, Ticarcillin-Clavulanic acid and lesser extent to Aztreonam (25%). This can be explained by the ability of these bacteria to express inducible β-lactimases which can hydrolyze variety of β-lactam antibiotics [49]. It has been reported that *A.hydrophila* simultaneously producing the class A, B, C, and D β-lactamases [50]. For Cephalosporins, *A.hydrophila* showed to have varying resistance pattern, in that all strains (100%) were resistance to Cephalothin, Ceftazidime and (58%) of strains were resistant to Cefoxitin. The resistance to Cephalothin in current study was expected since it is characteristic feature that is useful in differentiation of *A.hydrophila* from *A.veronii* [30]. Resistance to third generation cephalosporins have been documented in local studies [51,52]. An explanation of third generation resistance observed in this study could be attributed to Class

C β-lactamase is primarily Cephalosporinase which is chromosomally mediated in *A.hydrophila*. An exposure to β-lactam antibiotics or mutation in gene that inhibit the expression of Class C β-lactamase, lead to expression of this enzyme which confer resistant to extended spectrum cephalosporins[53]. In poultry, antibiotics are generally administered to flocks rather than individual chicken for therapeutic and growth promoter, which is inevitably lead to antibiotics resistance, since Aeromonas can retain resistance upon exposure to low concentration of antibiotics **[54]**.

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