Molecular studies on antibiotic resistant genes of *Aeromonas* species isolated from fish

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Abstract: The present study was conducted on 225 diseased fish samples, 125 Nile tilapia (*Oreochromis niloticus*) and 100 Cat fish (*Claris gariepinus*), collected from different fish markets at Kaliobia Governorate during the period from January (2016) to May (2017) for inspection of Aeromonas strains. Samples were collected from apparently pathognomic lesions in muscle, kidney, liver, intestine and spleen after clinical and postmortem examination for bacteriological examination. The results revealed that, 125 Aeromonas species were isolated from examined samples where *A. hydrophila* and *A. caviae* were the only species isolated. 114 (91.2 %) *A. hydrophila* strains, 63 (50.4%) and 51 (40.8%) were isolated from *C. gariepinus* and *O. niloticus* fishes respectively. Meanwhile, 11(8.8 %) *A. caviae* strains, 7 (5.6%) and 4 (3.2%) from *C. gariepinus* and *O. niloticus* fishes respectively. Aeromonas strains were highly resistant for ampicillin; methicillin; penicillin-G; vancomycin; oxacillin; amoxicillin, cefotaxime; oxytetracycline; erythromycin and streptomycin. Meanwhile, ciprofloxacin, enrofloxacin, gentamicin, and florphenicol were the most proper antibiotics with the highest in vitro efficiency against them. PCR results for antibiotic resistant genes in isolated Aeromonas strains showed that, they were detected in most studied strains, where, *bla*TEM gene was detected in all 10 *A. hydrophila* studied strains and in 5 out of 6 *A. caviae; tet*A (A) gene in 9 out of 10 *A.hydrophila* and in 5 out of 6 *A. caviae; sul*1gene in 8 out of 10 *A. hydrophila* and in 4 out of 6 *A. caviae*and *aad*A1gene in 7 out of 10 *A.hydrophila* and in 3 out of 6 *A. caviae*.
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Key words: Fish, bacteriological evaluation, Aeromonas species, antibiotic resistant genes

1. Introduction

Aeromon as species are responsible for wide range spectrum of diseases among fish and human (Halda-Alija and Subangi, 2004 and Ebanks *et al.,* 2005) as Motile Aeromonas Septicemia (MAS) in fish which is caused by *A.hydrophila* leading to high mortalities and high economic losses (Lu and Bi, 2007; Yu *et al.,* 2007; Dhanaraj *et al*., 2008; Abdel-Hadi *et al*., 2008 and Shayo *et al*., 2012). Moreover, motile Aeromonads due to their ubiquitous distribution are considered as bacterial indicators of freshwater environment, especially for harboring resistance genes (Schmidt *et al*., 2001). Also, they have been isolated from both Marine and freshwater environments and can cause diseases in fish under stressful conditions (Gonzalez-Serrano *et al.,* 2012).

The genus Aeromonas is a member of the family Aeromonadaceae. The genus has undergone a number of nomenclatural revisions in recent years and there are now 30 recognized species in the genus Aeromonas. The most predominant species are *A. hydrophila, A. caviae* and *A. veronii* biotype *sobria*. They are Gram-negative rods, either straight or curved facultative anaerobes, catalase-positive and most are motile by polar flagella. Gastrointestinal tract infections are the commonest source of Aeromonads followed by wound infections. In immunosuppressed individuals or those with hepatobiliary disease, aeromonads can cause otitis media, meningitis, endocarditis, peritonitis, cholecystitis, hemolytic uremic syndrome, septicemia and food poisoning (Janda and Abbott, 1998; Ko *et al*., 2000 and Guerra *et al.,*2007). Moreover, isolated *A. hydrophila* strains from patients with gastroenteritis are haemolytic (Wang *et al.*, 2003 and Wejdan *et al*., 2014). Gastroenteritis occur after intake of the pathogen via contaminated food or water (Yogananth *et al*., 2009). The consequences of horizontal gene transfer are often promoting the simultaneous spread of resistance to several unrelated classes of antibiotics, particularly if the genes for such resistance are co-located on the transmissible genetic element (Kore *et al.,* 2014).β -lactamases are enzymes produced by bacteria that inactivate β -lactam drugs by hydrolyzing the β-lactam ring of the β -lactam molecules. Most β -lactamases inactivate either penicillins or cephalosporins, but some can inactivate both classes of drugs (Stephen *et al.*, 2005). The TEM β -lactamase, conferring

resistance to penicillin family antibiotics such as ampicillin, is encoded by the *bla* TEM gene, which is found in a group of closely related transposons that represent three of the earliest bacterial resistance transposons to be identified. TEM enzymes are important determinants of resistance in Gram-negative bacteria, and more than 180 variants derived from the TEM-1 or TEM-2 β -lactamase have been recorded (Bush and Jacoby, 2010). Aeromonas are considered one of the most important fish pathogens and can be a problem for human consumers too and fish had attained a great economic importance in Egypt, and the antimicrobial resistance among them is a serious problem, so, the present study was conducted to throw light over the Aeromonas infection in fresh water fishes, and detection of some antibiotic resistant genes of the them by using P C R.

2. Material and Methods

The present study was conducted on 225 diseased fish samples, 125 Nile tilapia (*Oreochromis niloticus*) and 100 Cat fish (*Claris gariepinus*), of various sizes were collected from different fish markets at Kaliobia Governorate during the period from January (2016) to May (2017) for inspection of Aeromonas strains. After clinical and postmortem examination of collected fish samples, 432 samples collected from 225 diseased fishes; 240samples from 125 Nile tilapia (*O. niloticus*) where the samples were collected from apparently pathognomic lesions in muscle, kidney, liver, intestine and spleen by a number of 72, 55, 68,36 and 9 respectively and 192 samples from 100 Cat fish (*Claris gariepinus*), the samples were gathered from apparently pathognomic lesions in muscle, kidney, liver, intestine and spleen by a number of 63, 41, 47,32 and 9 respectively. The surface of lesions were seared by hot spatula, then a sterilized loop ful was introduced through seared portion and inoculated onto Tryptone soya broth then incubated aerobically at 37°C for 24 hours. A loopful from incubated Tryptone soya broth was streaked onto the following media: Tryptic soya agar; MacConkey's agar plates; Aeromonas base agar; Rimler- Shotts agar (R.S.); Thiosulphate –Citrate –Bile –Sucrose (T.C.B.S) agar; Eosin methylene blue agar (EMB);, blood agar plus 10 mcg /liter ampicillin, starch agar and milk agar media. All plates were incubated for 24hours at 37ºC. The developed colonies were picked up and subculture for purification. The purified colonies were morphologically identified by Gram stain and biochemical tests (Nicky, 2004; Guadalupe *et al*., 2009; Jayavignesh *et al*., 2011and Markey *et al*., 2013).

The *In-Vitro* anti-microbial sensitivity test for isolated Aeromonas species was done on each isolated Aeromonas species strain to study its antibiotic Sensitivity according to (Koneman *et al.*, 1997).

Genotyping detection of β-lactamase ampicillin resistance gene *(bla*TEM); streptomycin resistant *(aad*A1); tetracycline resistant A *tet*A (A) and sulphonamide resistant gene (*sul*1) using conventional PCR in 16 random isolated Aeromonas spp. (10 *A. hydrophila* and 6*A. caviae*), following QIAamp® DNA Mini Kit instructions (Qiagen, Germany, GmbH), Emerald Amp GT PCR mastermix (Takara) with Code No. RR310Aand 1, 5% agarose gel electrophoreses (Sambrook *et al.,* 1989) using the Primers sequences, target genes, amplicons sizes and cycling conditions showed in Table (1).

Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94°C 5 min.	94°C 30 sec.	54°C 40 sec.	72°C 45 sec..	72°C 10 min.	Colom *et al*., 2003 Randall *et al*. 2004 Ibekwe *et al*., 2011 Randall *et al*., 2004
tetA (A)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576						
sul1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433						
aadA1	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATT	484						

Table (2): Prevalence of positive samples for Aeromonas species isolation among examined fishes

Fish type	No. of examined fish	No. of examined lesion samples	No. of Positive samples	Positive percentage %*	%**
Nile tilapia (*O. niloticus*)	125	240	55	44.0	22.9
Cat fish (*C. gariepinus*)	100	192	70	70.0	36.4
Total	225	432	125	55.6	28.9

*Percentage in relation to number of examined fish type **Percentage in relation to no. of lesion samples in each raw

3. Results

The results of bacteriological examination of examined fishes; in- vitro sensitivity tests for the isolated strains and polymerase chain reaction (PCR) were tabulated in Tables (2-5) and Figures (1-4).

Table (3): Prevalence of Aeromonas species isolated from examined fishes

Fish type	No. of examined lesion samples	positive samples for Aeromonas species					
		A. hydrophila		*A. caviae*		Total	
		No.	%*	No.	%*	No.	%*
Nile tilapia (*O. niloticus*)	240	51	40.8	4	3.2	55	44.0
Cat fish (*C.gariepinus*)	192	63	50.4	7	5.6	70	56.0
Total	432	114	91.2	11	8.8	125	100.0

*Percentage in relation to number of Aeromonas species isolated (125)

Table (4): In-Vitro anti-microbial sensitivity test for isolated *A.hydrophila* strains

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		
		No.	%	No.	%	No.	%	AA
Amoxicillin	25µg	0	0.0	11	9.6	103	90.4	R
Ampicillin	10µg	0	0.0	12	10.5	102	89.5	R
Cefotaxime	30µg	3	2.6	9	7.9	102	89.5	R
Ciprofloxacin	5 µg	96	84.2	10	8.8	8	7.0	S
Enrofloxacin	5 µg	95	83.3	11	9.7	8	7.0	S
Florphenicol	30 µg	91	79.8	13	11.4	10	8.8	S
Gentamicin	10 µg	92	80.7	15	13.2	7	6.1	S
Methicillin	5 µg	0	0.0	10	8.8	104	91.2	R
Oxacillin	1µg	0	0.0	15	13.2	99	86.8	R
Oxytetracycline	30 µg	3	2.6	15	13.2	96	84.2	R
Penicillin-G	10 u	0	0.0	9	7.9	105	92.1	R
Streptomycin	10 µg	4	3.5	15	13.2	95	83.3	R
Trimethoprim/ Sulphamethoxazol	(1.25/23.75) mcg	12	10.5	35	30.7	67	58.8	R
Vancomycin	30 µg	0	0.0	9	7.9	105	92.1	R

No.: Number of isolates AA: Antibiogram activity %: Percentage in relation to total number of isolates (114)

Table (5): *In-Vitro* anti-microbial sensitivity test for isolated *A.caviae* strains

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		
		No.	%	No.	%	No.	%	AA
Amoxicillin	25µg	0	0.0	3	27.3	8	72.7	R
Ampicillin	10µg	0	0.0	3	27.3	8	72.7	R
Cefotaxime	30µg	1	9.1	2	18.2	8	72.7	R
Ciprofloxacin	5 µg	9	81.8	1	9.1	1	9.1	S
Enrofloxacin	5 µg	9	81.8	2	18.2	0	0.0	S
Florphenicol	30 µg	6	54.5	3	27.3	2	18.2	S
Gentamicin	10 µg	8	72.7	2	18.2	1	9.1	S
Methicillin	5 µg	0	0.0	1	9.1	10	90.9	R
Oxacillin	1µg	0	0.0	1	9.1	10	90.9	R
Oxytetracycline	30 µg	1	9.1	2	18.2	8	72.7	R
Penicillin-G	10 u	0	0.0	1	9.1	10	90.9	R
Streptomycin	10 µg	1	9.1	3	27.3	7	63.6	R
Trimethoprim/ Sulphamethoxazol	(1.25/23.75) mcg	2	18.2	3	27.3	6	54.5	R
Vancomycin	30 µg	0	0.0	1	9.1	10	90.9	R

No.: Number of isolates AA: Antibiogram activity %: Percentage in relation to total number of isolates (11)

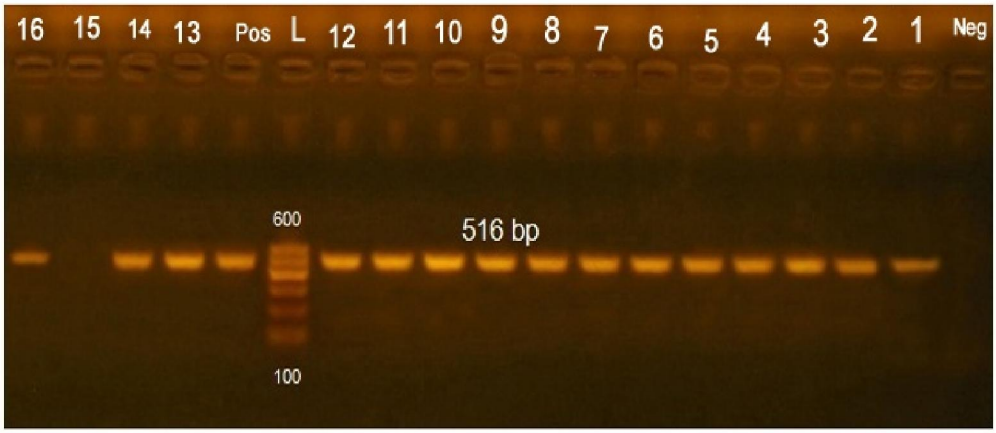

Fig. (1): β-lactamase ampicillin resistance (*bla*TEM) gene.
Lane L: 100-600 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control (at 516 bp.).
Lane 1-10: *A.hydrophila* (Positive). Lane 11- 14 & 16: *A. caviae* (Positive). Lane 15: *A. caviae* (Negative)

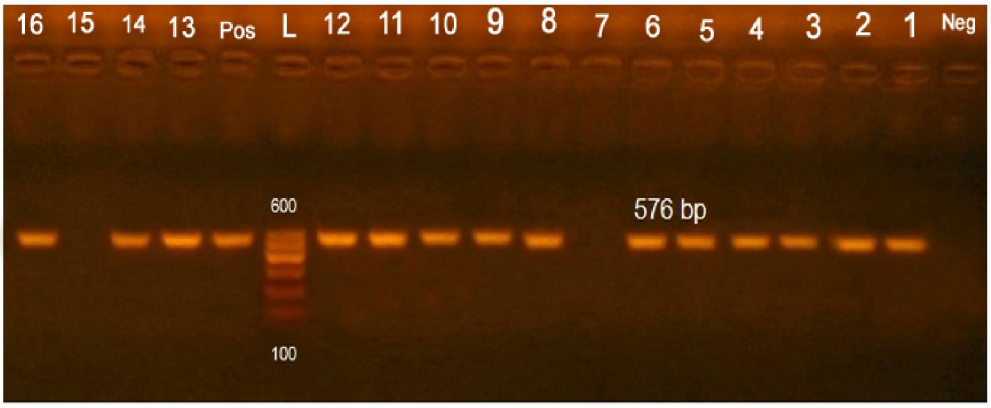

Fig. (2): Tetracycline resistant A (*tetA*) gene.
Lane L: 100-600 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control (at 576 bp.).
Lane 1-6, 8, 9 & 10: *A.hydrophila* (Positive). Lane 7: *A.hydrophila* (Negative). Lane 11- 14 & 16: *A. caviae* (Positive). Lane 15: *A. caviae* (Negative)

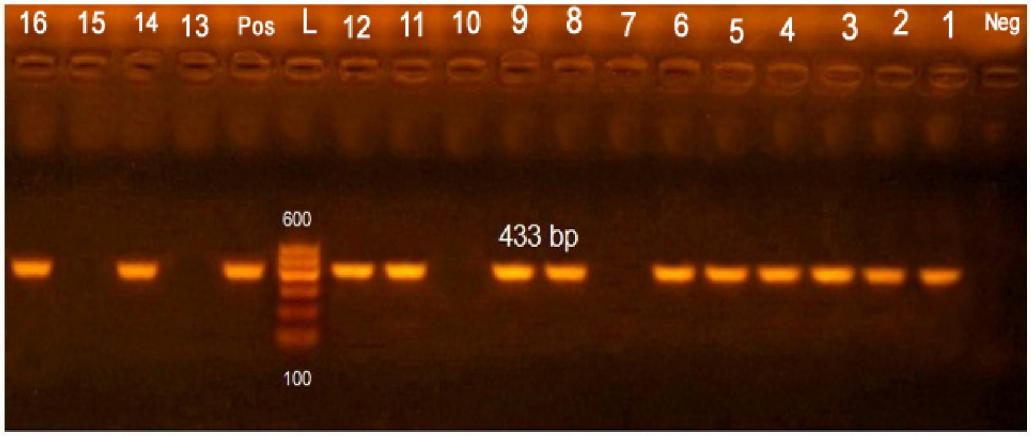

Fig. (3): Sulphonamide resistant (*sul*1) gene.
Lane L: 100-600 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control (at 433 bp.). Lane 1- 6, 8 & 9: *A.hydrophila* (Positive). Lane 7 & 10: *A.hydrophila* (Negative). Lane 11, 12, 14 & 16: *A. caviae* (Positive). Lane 13 & 15: *A. caviae* (Negative).

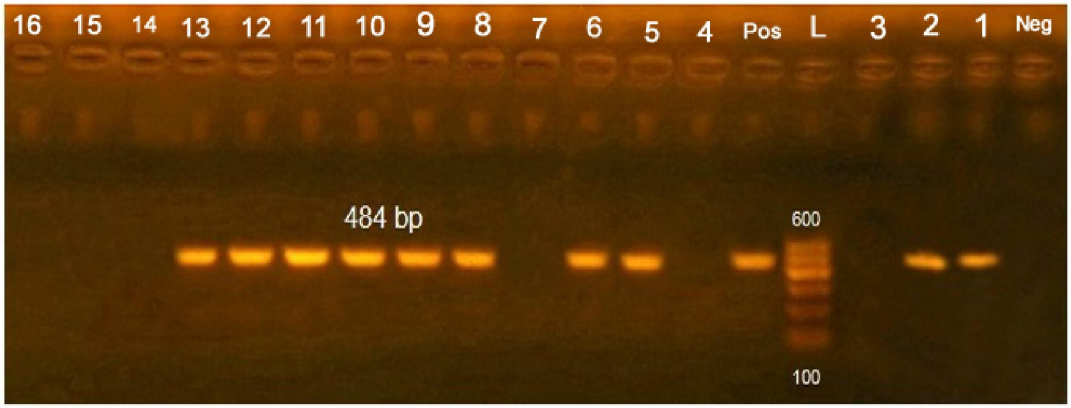

Fig. (4):Streptomycine resistant (*aad*A1) gene.
Lane L: 100-600 bp. DNA Ladder.
Neg.: Negative control. Pos.: Positive control (at 484 bp.).
Lane 1, 2, 5, 6, 8 & 9: *A.hydrophila* (Positive). Lane3,4 & 7: *A.hydrophila* (Negative).
Lane 11, 12 & 13: *A. caviae* (Positive). Lane 14, 15 & 16: *A. caviae* (Negative).

4. Discussion

Aeromonas species are widely spread microorganisms that responsible for wide range spectrum of diseases among fish, as Motile Aeromonas Septicemia (MAS) leading to high mortalities and high economic losses (Abdel-Hadi *et al*., 2008 and Shayo *et al*., 20 12), beside their role in gastrointestinal and extra intestinal infections in humans (Subashkumar *et al*.,2006 and Parker and Shaw, 2011).

The prevalence of Aeromonas septicemia with Aeromonas species isolation (Table, 2) revealed that, 125(55.6%) out of 225 examined fish represented as 55 positive samples (44.0%) from125 *O. niloticus* and 70 (70.0) from 100 *C. gariepinus* examined fish samples were positive for Aeromonas species isolation. These results came in accordance with that obtained by El- Dien *et al.* (2010); Yucel and Balo (2011) and Ibrahim-Lamis (2015). The results of bacteriological examination (Table, 3) revealed that, 125 Aeromonas species were isolated from examined samples where *A. hydrophila* and *A. caviae* were the only species isolated. Similar results were recorded by Stratev *et al*. (2012). A total of 114 (91.2 %) *A.hydrophila* strains, 63 (50.4%) and 51 (40.8%) were isolated from *C. gariepinus*and *O. niloticus* fishes respectively. Meanwhile, 11(8.8 %) *A.caviae* strains, 7 (5.6%) and 4 (3.2%) from *C. gariepinus* and *O. niloticus* fishes respectively. These results agree with those of Abu- Leila (2005); Mohamed *et al.* (2006); Mahdy (2007); Ibrahim-Lamis (2015) and Sayed (2017). Meanwhile, disagreed with others who recorded lower incidence, El- Dien *et al.* (2010) and Noor El- Deen *et al.* (2014).

The results of *in- vitro* sensitivity tests for the isolated *A. hydrophila* (Table, 4) revealed that, the isolated *A. hydrophila* were highly resistant for penicillin-G and vancomycin followed by methicillin; amoxicillin; ampicillin; cefotaxime; oxacillin; oxytetracycline; streptomycin and trimethoprim/ sulphamethoxazol. Meanwhile, they were highly sensitive to ciprofloxacin followed by enrofloxacin; gentamycin and florphenicol. Moreover, (Table, 5) revealed that, the isolated *A. caviae* were highly resistant for methicillin; oxacillin; penicillin-G and vancomycin followed by amoxicillin; ampicillin; cefotaxime; oxytetracycline; streptomycin and trimethoprim/ sulphamethoxazol. Meanwhile, they were highly sensitive to ciprofloxacin andenrofloxacin followed by gentamycin and florphenicol. The rise in incidence of Multiple antibiotic resistance (MAR) bacteria has been attributed to the indiscriminate use of antimicrobials in animal culture and in medicine (Del Castillo *et al*., 2013) especially in increased resistance to β-lactam antibiotics in the genus Aeromonas may be attributed to the presence of β-lactamases genes (Ndi and Barton, 2011). Nearly similar results were recorded by Kaskhedikar and Chhabra (2010); Jayavignesh *et al.* (2011); Igbinosa and Okoh (2012); Khairul *et al*. (2013); Kore *et al.* (2014); Ibrahim-Lamis (2015); Ali *et al.* (2016) and Didugu *et al.* (2016). These results are of serious concern as these drugs, especially β-lactam antibiotics, are still considered the most recommended for the treatment of bacterial infections in fish, animals and human; however their efficiency has greatly deteriorated due to the production of β-lactamases by resistant bacterial strains.

Aeromonads produce threes β-lactamase classes, which predetermines their resistance to a broad spectrum of β-lactam antibiotics (Chen *et al*., 2012). They are considered universally resistant to penicillins

(penicillin, ampicillin, carbenicillin and ticarcillin). That is why, ampicillin is included as a supplement to some selective culture media for the isolation of aeromonads from contaminated samples (Awan *et al*., 2009 and Daood, 2012). Moreover, the presence of β-lactamase gene in Aeromonas has been reported in several studies also in Gram-negative bacteria which primarily mediated by β-lactamases leading to hydrolyzing the β-lactam ring and inactivate the antibiotic. Many different β-lactamases have been described; however, TEM-, SHV- OXA-, CMY- and CTX-M- β-lactamases are the most dominant in Gram-negative bacteria (Bradford, 2001). The results of PCR for amplification of *bla*TEM gene in *A.hydrophila and A. caviae* strains (Fig., 1) showed that, the *bla*TEM gene was amplified in all 10 *A.hydrophila* studied strains and in 5 out of 6 *A. caviae* studied strains giving product of 516 bp. Similar results were obtained by Verner-Jeffreys *et al.* (2009); Ramalivhana *et al*. (2010); Shah *et al*., 2012; Ye *et al*., 2013; Ibrahim-Lamis (2015) and Okolie (2015). However, the results were not incoordinance with (Ndi and Barton, 2011) who failed to detect *bla*TEM virulent gene in these strains although there was a phenotype β- lactam resistance. The genetics of tetracycline resistance in Aeromonads has been investigated previously (Gon˜i-Urriza *et al*., 2000 and Schmidt *et al*., 2001). Among various *tet* genes, five classes of genetically distinguishable tetracycline resistance determinants (*tet* A to *tet* E) have been described in Aeromonas spp. And the most predominant ones are *tet*A and *tet* E (Nawaz *et al.,* 2006 and Balassiano *et al.,* 2007). Moreover, most of these determinants are tetracycline inducible and provide resistance to other tetracycline analogs, such as oxytetracycline (Schmidt *et al*., 2001). Meanwhile, the results of PCR for amplification of *tet*A *(*A*)* gene in *A.hydrophila and A. caviae* strains (Fig., 2) showed that, the *tet*A *(*A*)* gene was amplified in 9 out of 10 *A.hydrophila* studied strains and in 5 out of 6 *A. caviae* studied strains giving product of 576 bp. These results were agreed with those of Verner-Jeffreys *et al.* (2009); Ndi and Barton (2011); Ibrahim-Lamis (2015). The results were not incoordinance with (Igbinosa and Okoh, 2012) who failed to detect *tet* virulent gene in these strains. Also, the results of PCR for amplification of *sul*1gene in *A.hydrophila and A. caviae* strains (Fig., 3) showed that, the *sul*1gene was amplified in 8 out of 10 *A.hydrophila* studied strains and in 4 out of 6 *A. caviae* studied strains giving product of 433 bp. These results were agreed with those of Verner-Jeffreys *et al.* (2009); Nawaz *et al*. (2010); Ndi and Barton (2011); Igbinosa and Okoh (2012); kore *et al*. (2014) and Okolie (2015). Moreover, the results of PCR for amplification of *aad*A1gene in *A.hydrophila and A. caviae* strains (Fig., 4) showed that, the *aad* A1gene was amplified in 7 out of 10 *A.hydrophila* studied strains and in 3 out of 6 *A. caviae* studied strains giving product of 484 bp. These results were agreed with those of Verner-Jeffreys*et al.* (2009); Ndi and Barton (2011) and Okolie (2015).

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