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Genotypic Identification and Evaluation of Several Selective Media for Recovery of Aeromonas spp. from Different Sources

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ABSTRACT
A total number of 250 samples were collected from Suez canal area(50) samples from Tilapia niloticus fish, (50) Mugil cephalus fish, (50) drinking water (25 tap water in addition to 25 bottled mineral water), (50) pond water and (50) childhood diarrheal samples and these samples were cultured on several selective media. The isolation rate of Aeromonas spp. from all samples using enrichment technique on starch ampicillin agar (SAA), Rimler-shotts medium (RS), Blood ampicillin agar (BAA) and MacConkey ampicillin agar (MAA) were (51.2%), (45.2%), (38.8%) and (31.6%) respectively. The incidence of Aeromonads from different sources (Tilapia niloticus fish, Mugil cephalus fish, drinking tap water, bottled mineral water, pond water and childhood diarrhea were 44 (88%), 33 (66%), 4 (16%), 0 (0%), 42 (84%) and 5 (10%) respectively. The total number of Aeromonas isolates from 250 examined samples were 260 isolates that were biochemically identified into 4 biotypes as 136 (52.31%) A. hydrophila, 81 (31.15%) A. sobria, 34 (13.08%) A. caviae and 9 (3.46%) A. schuberti. Results of antibiogram of isolated Aeromonas spp. demonstrated that all tested Aeromonas isolates were resistant to Erythromycin, Sulphamethoxazol-Trimethoprim beside Ampicillin, and while the highest degree of sensitivity towards Ciprofloxacinc,Norfloxacina, Amikacin and Gentamicin. Molecular identification of Aeromonas isolates by Polymerase chain reaction technique using 16S rRNA gene revealed that all examined Aeromonas isolates were positive, and also two virulence genes (aerolysin and hemolysin genes were identified by a specific primers and they present by a percentage of (83.3%) and (8.3%) respectively in examined Aeromonas isolates. The present study highlights the optimum recovery of Aeromonas spp. from mixed population require enrichment with an alkaline peptone water and consequence plating on more than one media and PCR technique provide rapid, sensitive and confirmatory identification of Aeromonas spp. and some virulence genes. Aeromonas spp. may use as an indicator for water quality and A. hydrophila & A. sobria are predominant, emerging and enteric pathogens.

INTRODUCTION
Aeromonas infections represent a serious problem to fresh water fish production, causing a significant economic loss to fish industry Saad et al., (2014).

Aeromonas species are facultative anaerobic Gram negative bacteria that is a member of the family Aeromonadaceae that are widespread in sea, river, fresh and ground water Hassan et al., (2012). Moreover Aeromonas species can grow at refrigerator temperatures and replicate at high salt concentration Janda and Abbott (2010). Aeromonas species cause several human diseases that vary in severity from a self-limiting gastroenteritis to potentially fatal septicemia, in addition to extra intestinal symptoms such as meningitis, endocarditis and osteomyelitis with a high mortality rate specially in immune compromised person Tsai et al., (2006). A large range of selective and differential isolation media have been evolved for the isolation of Aeromonas species from the environment, foods, and clinical samples Villari et al., (1999). Recovery of aeromonads from the contaminated samples like faeces may require usage of selective and differential media such as MacConkey media, cefsulodin irgasan novobiocin (CIN) media beside blood ampicillin agar (10 mg/L ampicillin) USEPA (2001), moreover Sarkar et al., (2012) who useda selective medium, Rimler-shotts agar for isolation of Aeromonas hydrophila from different sources like fish, pond water, river water and Starch ampicillin agar (SAA), bile salts inositol brilliant green agar (BIBG) and Aeromonas Medium (Ryan’s Medium) which were recommended Igbinosa et al., (2012). Numerous extracellular enzymes and toxins including the haemolysins, proteases, lipases, DNases, and cytotoxins that have been mentioned as virulence factors of motile Aeromonads Erdem et al., (2010) and Cai et al., (2012),however the role of each single factor regarding its pathogenesis varies John and Hatha (2014). The aim of this study was the isolation of Aeromonas on four selective media Starch ampicillin agar (SAA), Rimler-Shotts media (RS), Blood ampicillin agar (BAA) and MacConkey (MAA), evaluation of bacterial growth on different media, determination the incidence of Aeromonas spp. isolated from fish, water and childhood diarrheal samples in suz canal area, identification of isolated strains biochemically, antibiogram of such isolates and detection of some virulent genes using polymerase chain reaction PCR (aerolysin and hemolysin gene) beside 16Sr RNA gene.

**MATERIALS AND METHODS**

**Samples:**

A total of 250 samples were collected randomly from different fish farms in Suez canal area of *Tilapia niloticus & Mugil cephalus* fishes(50 samples for each), drinking tap water, bottled mineral water(25 samples for each), pond water (50 samples) and childhood diarrheal stool samples(50 samples). All samples were collected under aseptic condition and transferred immediately to microbiological lab.

**Bacteriological examination:**

- Isolation and identification of Aeromonas: A loopful was taken aseptically from internal organs, gills and skin inoculated into alkaline peptone water (APW) for enrichment then incubated at 30 °C for 24 hrs Villari et al., (2000), 25 ml of each water samples was thoroughly mixed with 225 ml of alkaline peptone water Cruickshank et al., (1980), stool samples were directly inoculated into alkaline peptone water then was inoculated aerobically at 28ºC for 24 hrs. A loopful from alkaline peptone water was subsequently streaked onto Starch ampicillin agar (SAA), Rimler-Shotts media (RS), Blood ampicillin agar (BAA), MacConkey ampicillin agar (MAA) aerobically incubated at 37ºC for 18-24 hrs. A film from typical colony of Aeromonas spp. were stained with gram stain Varnam and Evans (1991) and confirmed on the basis of the following test:Oxidase test, resistant to vibriostatic agent O/129, esculin hydrolysis, glucose fermentation in TSI, sugar fermentation and gas production, indole production and voges-proskauer test. Identification and biotyping of the isolates was carried out according to Aerokey II of Carnahan et al., (1991a).
b- Antibiotic sensitivity test for the isolated Aeromonas from fishes, water & childhood diarrheal samples was done by disc diffusion technique Ericsson and Sherris (1971).

c- Molecular typing of isolated Aeromonas was done via PCR technique: was used for the detection of 16Sr RNA gene besides 2 virulence genes (aerolysin and hemolysin genes), Sambrook et al., (1989).

RESULTS AND DISCUSSION

The present results in Table (1) and Figure (1) indicate that some selective media originally designed for isolation of Aeromonas species from different sources enrichment technique is used on several selective media such as Starch ampicillin agar, Rimler-Shotts medium, Blood ampicillin agar and MacConkey ampicillin agar was 51.2%, 45.2%, 38.8% and 31.6% respectively. These results agree with Villari et al., (1999) who stated that SAA is the most sensitive culture media and is recommended to use it in isolation of Aeromonas species. and nearly similar to results obtained by Handfield et al., (1996) in which recovery of A. hydrophila from drinking water samples on SAA was 71.4% which was higher than RS that was 50%. In addition to Thenmozhi et al., (2013) used the Starch-Ampicillin agar as a selective presumptive isolation medium for the isolation of Aeromonas isolates from the drinking water samples that grow on Starch ampicillin agar after 24 hr incubation at 37°C. These colonies were Circular, Convex, Opaque, raised, smooth and entire edges colonies, with Yellow to honey colored and amylase positive colonies (clear zone surrounding the colony).Moreover, Pin et al.,(1994) reported that Starch ampicillin agar was the most adequate media for the isolation A. hydrophila but not adequate for recovery of A. sobria. From other hand, the low selectivity of SAA for Aeromonas has been pointed out by Ribas et al., (1991).

These finding results agree with Shotts and Rimler (1973) who stated that RS medium was commonly used in fish diagnostic laboratories for cultivation of Aeromonas spp. because it contains inhibitory substances such as sodium deoxycholate, novobiocin that were added to eliminate the chance of Gram positive organisms and vibrio spp., in addition to its high sensitivity of this media which enables this media not only for the recovery of A. hydrophila from specific sources but also for the enumeration of this organism in the environment. Also, Samal et al., (2014) usedRimler-Shotts (RS) medium for isolation of Aeromonas from different freshwater diseased fish and 59 isolates grown and produced yellow, round, small to medium, convex, elevated and transparent colonies. However, these results disagreed with Robinsonet al., (1984) who considered that medium of RS was unsuitable for isolation of fecal Aeromonas spp. Also Rippey and Cabelli (1979) stated that inefficiency of RS agar as an optimum A. hydrophila recovery medium due to novobiocin contained in the medium, which suppressed the growth of sensitive environmental A. hydrophila this effect pointed out by Kaper et al., (1981) who found that A. hydrophila lysine decarboxylase positive strains from the aquatic environment were not detected in RS agar.

The present results revealed that SAA (51.2%) is better than BAA (38.8%) for isolation of Aeromonas and these results were similar to Konchel (1989) who observed a satisfactory recovery and good differential properities which make SAA with (10 µg/ml & 30 µg/ml) better than blood agar as SAA can differentiate Aeromonas from the background microflora. Also, he revealed that SAA medium was highly selective and yielded consistently higher recoveries, in addition to produce 85% Aeromonas colonies, compared with 36-40% on blood agar which means that SAA was better than BAA, Furthermore, these present results agree with Millership et al., (1983) who reported that blood agar with ampicillin was used for isolation of Aeromonas species based on beta hemolysis and oxidase test could be directly performed
on the plate, BAA was the most widely used media for isolation of Aeromonas from stool and BAA should be used in combination with another media for optimal detection of Aeromonas strains. On the other hand Andelova et al., (2006) reported that BAA is useful only for recovery of Aeromonas if screening is based on hemolysis, but approximately 10% of Aeromonas isolates would be missed because they are no hemolytic.

However, BAA (38.8%) was better than MAA (31.6%) for isolation of Aeromonas and these results agree with Fricker & Tompsett (1989) who examined 563 samples of various food samples to compare plating media MacConkey and Blood ampicillin agar (BAA). They showed that (BAA) gave 43.3% positive samples while MacConkey gave only 31.2%, on the other hand, Daku et al., (2004) isolated Aeromonas species from enteric samples and found that blood agar was the most sensitive media (86.5%), followed by MacConkey agar (70.3%) and this means that the isolation rate on BAA was higher than MA and the recovery rate of Aeromonas spp. On MacConkey ampicillin agar was lower, and Ifeanyichukwu et al., (2015) who used MacConkey agar and Aeromonas selective medium supplemented with ampicillin for isolation of Aeromonas species from both chlorinated and non-chlorinated water samples and yielded 60% positively. In addition to Jepessen (1995) who reported that MA was not suitable to select Aeromonas since this genus includes lactose non-fermenting besides lactose fermenting strains of the same sugar.

In the current study all the media except for Rimler-Shotts medium contain ampicillin as the selective agent and some Aeromonas spp. such as *Aeromonas trota*, are generally thought to be sensitive to ampicillin (Carnahan et al., 1991b) also *Aeromonas jandaei* which has been shown to occasionally be ampicillin susceptible (Overman and Janda 1999). In addition to Huddleston et al., (2007) who also suggested that ampicillin as a selective agent which hinder the growth of a significant portion of Aeromonas spp. and this lead to bias, misleading information and they postulate an underestimation of diversity Aeromonas spp. and its density where ampicillin was used in the isolation media.

<table>
<thead>
<tr>
<th>Media used</th>
<th>Total No. of examined samples</th>
<th>Positive samples No.</th>
<th>Positive samples %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch ampicillin agar</td>
<td>250</td>
<td>128</td>
<td>51.2</td>
</tr>
<tr>
<td>Rimler-Shotts media</td>
<td>250</td>
<td>113</td>
<td>45.2</td>
</tr>
<tr>
<td>Blood ampicillin agar</td>
<td>250</td>
<td>97</td>
<td>38.8</td>
</tr>
<tr>
<td>MacConkey ampicillin agar</td>
<td>250</td>
<td>79</td>
<td>31.6</td>
</tr>
</tbody>
</table>

Table 1: Sensitivity of solid specific media for isolation of Aeromonas species from different samples.

Fig. 1: Sensitivity of solid specific media for isolation of Aeromonas species from different samples.
The present results as shown in Table (2) demonstrated that the frequency distribution of total Aeromonas spp. isolates recovered from all samples (Tilapia niloticus, Mugil cephalus fish, drinking tap water, botteled mineral water, pond water and childhood diarrheal samples) in Suez Canal area were: 136 (52.31%) A. hydrophila, 81 (31.15%) A. sobria, 34 (13.08%) A. caviae and 9 (3.45%) A. schubertii. These results agree with (Ghenghesh et al., 2008) who stated that the most commonly isolated species from clinical samples, water and foods were A. hydrophila, A. caviae and A. veronii biovarsobria. And also Ottaviani et al., (2011) who reported that A. hydrophila and A. sobria have been frequently isolated from food and environmental samples, which supported the present findings. The mostly commonly isolated Aeromonas spp. from environmental strains (water sources) were A. hydrophila, A. sobria, A. caviae and A. schubertii, while mostly commonly isolated Aeromonas spp. associated with clinical strains (childhood diarrheal samples) were A. hydrophila and A. sobria, as shown in Table (2). These results are similar to the data reported by Kühn et al., (1997b); Ghenghesh et al., (2001) and (Razzolini et al., 2001) where A. hydrophila was the predominant species in freshwater and municipal drinking water supplies. Moreover, the present data also nearly agree with a study conducted in Turkey by Koksal et al., (2007) who reported the isolation of Aeromonas such as A. hydrophila (46%), A. sobria (34%) and A. caviae (8%) and agree with John and Hatha (2013) who stated that A. schubertii was less than 10% and was the least predominant sp. in both water and fish samples and in contrast with the data obtained in the same study which showed that the predominant species in water samples were A. sobria followed by A. caviae, and frequency distribution of different species of Aeromonas is likely to vary with geographical locations. The finding results in Table (2) and Figure (2) showed frequency distribution of Aeromonas species isolated from different sources in fish samples (Tilapia niloticus & Mugil Cephalus fishes) that were identified biochemically into the predominant species was A. hydrophila and this agree with Rathore et al., (2005) who reported that A. hydrophila was the predominant species in fish samples in India, In addition to Yadav and Kumar (2000); while in Egypt Abou El-Atta (2003) demonstrated the preponderance of A. hydrophila followed by A. sobria and A. caviae from fish. Similar finding observed by Sharma and Kumar (2011) In contrast with Yucel et al., (2005) who affirmed that among fresh water fish spp. A. caviae was the prevalent species followed by A. hydrophila and A. veronii biovar sobria in Turkey. The distribution results as shown in Table (2) revealed the isolation of Aeromonas spp. recovered from Tilapia niloticus fish samples was 107 isolates. These are biochemically identified into A. hydrophila 56 (52.33%) among other Aeromonas spp. followed by A. sobria 33 (30.84%), A. caviae 14 (13.08%) and A. schubertii 4 (3.73%). These results are similar to Maimona et al., (2015) who isolated A. hydrophila, A. sobria from tilapia fish and nearly agree with Kumar et al., (2000) who recorded isolation of A. hydrophila in fish (70.59%) followed by A. sobria (69.23 %) and A. caviae (33.33 %), but disagree with Ashiru et al., (2011) who recorded distribution of A. caviae followed by A. hydrophila and A. sobria in tilapia. On the other hand, A. schubertii is the least predominant sp. among Aeromonas spp. in present results, such result in agreement with John and Hatha (2013) who isolated A. schubertii less than (10%).

The present results as shown in Table (2) showed the recovery of Aeromonas spp. isolated from Mugil cephalus fish samples was 84 isolates. These are biochemically identified into A. hydrophila 40 (47.62%), A. sobria 29 (34.52%), A. caviae 13 (15.48%), A. schubertii 2 (2.38%), and this result agree with Enany et al., (2011) who stated the common bacterial pathogen
isolated from *Mugil cephalus* was *A. hydrophila*. In addition to the present result is nearly agreed to Salah El-Dien *et al.*, (2009) who recorded isolation of *Aeromonas* spp. was (30 isolates) of *A. hydrophila*, (3) *A. caviae*, (1) *A. sobria* from fresh mullet samples, but disagree with Yucel *et al.*, (2005) who affirmed that *A. veronii biovar sobria* was the most isolated Aeromonad in sea fish species (41.5%) followed by *A. sobria* (2005) who affirmed that samples, but disagree with Yucel, (1) spp. was (30 isolates) of *A. hydrophila* (30.1%) and *A. caviae* (28.3%). In general, the present results in Table (2) showed that the predominant species are 60 isolates recovered from pond water of fish that identified biochemically into *Tilapia niloticus* and *Mugil cephalus* was *A. hydrophila* and these results agreed with those recorded by Farid *et al.*, (1978) and Shalaby, (1997, 2005). The current results in Table (2) revealed that frequency distribution of *Aeromonas* species recovered from Drinking tap water samples and identified biochemically into *A. hydrophila* 3(75%) and *A. sobria* 1 (25%) and this results agree with Kühn *et al.*, (1997a) who reported that *A. hydrophila* was the major phenotype in drinking water samples in Sweden, while such results are higher than Di Bari et al., (2007) who recorded isolation of *A. hydrophila* (48.3%) from drinking water samples. The finding results in Table (2) demonstrated that isolation of *Aeromonas* species are 60 isolates recovered from pond water of fish that identified biochemically into *A. hydrophila* 34 (56.66%), *A. sobria* 16 (26.66%), *A. caviae* 7 (11.66%) and *A. schubertii* 3 (5%) and this closely agree with Abd-Elall et al., (2014) who stated that *A. hydrophila* was more frequently isolated from pond water and John and Hatha (2013) who isolated *A. hydrophila, A. sobria, A. caviae* and *A. schubertii* from water samples but vary in prevalence percentages according to variation of geographical locations, In addition the less frequently isolation of *A. schubertii* is nearly in agreement with Janda and Abbott (2010) and John and Hatha (2013) who recorded isolation of *A. schubertii* in less frequent, but disagree with Evangelista-Barreto *et al.*, (2010) who reported that high frequency and isolation of *A. caviae* in water. The current results in Table (2) showed the frequency distribution of *Aeromonas* species isolated from childhood diarrheal samples that identified biochemically into *A. hydrophila* 3 (60%) and *A. sobria* 2 (40%) are the two predominant species that isolated from stool. These results agree with Yadav and Kumar (2000) who demonstrated the same *Aeromonas* species (3 *A. sobria*, 2 *A. hydrophila*) from fecal samples of diarrheic children under five years of age, and these present finding agree with Pokhrel & Thapa (2004) who found that *A. hydrophila* was the most common species in stool then followed by *A. caviae* and *A. sobria* and nearly agree with Vasaikar *et al.*, (2002) who stated that *A. hydrophila* was the predominant species by 64.2 % of isolated *Aeromonas* from cases of gastroenteritis, then *A. sobria* 28.4 %, in addition to, Guz and Kozinska (2004) who reported that *A. hydrophila* complex and *A. sobria* complex were potential pathogens of animals and humans, characteristics of aeromonads have a public health importance, so it should be assessed, but disagree with Soltan and Moezdalian (2004) who found that *A. sobria* was the predominant species (57%) followed by *A. caviae* (36%) then *A. hydrophila* (7%) in Tehranian children presenting with diarrhea, moreover Ananthan and Alavandi (1999) who reported that the predominance of *A. caviae* in stool of children with gastroenteritis in Chennai, in addition to the frequency isolation of different species of *Aeromonas* can vary with the geographic allocations according to record of Sinha *et al.*, (2004). While the distribution of *Aeromonas* species in stool samples (childhood diarrheal sample) in present study, the predominant species of *Aeromonas* was *A. hydrophila* followed by *A. sobria* and this result was agree with Kannan *et al.*, (2010) and von Graevenitz (2007) who found *A. hydrophila* as predominant in Brazil, Thailand and India, and in contrast with previous study conducted in Europe, the United States and India, *A. caviae* was dominant followed by *A. hydrophila* and *A. veronii biovar sobria* Albert *et al.*, (2000); Borchart et al., (2003);
Shiina and Iwanaga (2004). Also it may be due to other factors like the isolation and identification methods used may be of importance Abbott et al., (2003); Janda and Abbott (2010). The four different phenol species are observed in this present study *A. hydrophila* (52.31%), *A. sobria* (31.15%), *A. caviae* (13.08%), *A. schubertii* (3.45%) that are distributed in this suez canal geographic area, and these species composition were limited to ampicillin resistant isolates and this agree with the similar finding of Oakey et al., (1996) and Ormen & Ostensvik (2001), although the similar species were reported in many previous studies but the relative isolation of these species was found to vary by John and Hatha (2013), In addition to types of *Aeromonas* spp. that isolated from fish (*A. hydrophila, A. sobria, A. caviae* and *A. schubertii*) are the same types of *Aeromonas* spp. that isolated from pond water of fish and this microbiota of pond water reflect microbiota of fish and this closely similar to Sousa and Sliva sauza (2001) who reported that *Aeromonas* in water medium was found represented in the internal fish organs, in Brazil. Furthermore, Awadallah and Abd-El All (2009) who stated that level of fish contamination with microorganisms was found to be directly proportional to their level in the overlying water, while types of *Aeromonas* spp. that isolated from drinking tap water (*A. hydrophila* and *A. sobria*) are the same types of *Aeromonas* spp. that isolated from childhood diarrheal samples and these findings may emphasize the findings of Holmberg et al., (1986) that showed acorrelation between the consumption of water and *Aeromonas* mediated diarrhea.

Table 2: Distribution of different *Aeromonas* spp. isolates from (*Tilapia niloticus & Mugil cephalus* fishes, Drinking Tap, Botteled mineral water, Pond water and Childhood diarrheal stool samples):

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of isolates</th>
<th>Distribution of <em>Aeromonas</em> isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>A. hydrophila</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Tilapia fish</td>
<td>107</td>
<td>56</td>
</tr>
<tr>
<td>Mugil fish</td>
<td>84</td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td>191</td>
<td>96</td>
</tr>
<tr>
<td>Tap water</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Botteled mineral water</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Pond water</td>
<td>60</td>
<td>34</td>
</tr>
<tr>
<td>Childhood Diarrhea</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>260</td>
<td>136</td>
</tr>
</tbody>
</table>
Fig. 2: Frequency distribution of total Aeromonas isolates from all samples (Tilapia niloticus fish, Mugil cephalus fish, drinking tap water, drinking bottled mineral water, pond water of fish and Childhood diarrheal samples).

The antibiotic resistance patterns against 10 antimicrobial agents were established for the 48 strains from Aeromonas species isolated from the fish, water and childhood diarrheal stool samples and demonstrated in Table (3) and Figure (3). The present results revealed that all strains of A. hydrophila, A. sobria, A. caviae and A. schubertii are highly resistant to antibiotics like Ampicillin and Erythromycin as well as Sulphamethaxazole–trimethoprim. Such results were in concordance with Sreedharan et al. (2012) and Carnahan et al. (1991a). On the other hand, the present results showed that A. hydrophila is sensitive to Gentamicin and Ciprofloxacin by a percentage (100%), Norfloxacin by a percentage (90%), Amikacin by a percentage (60%), Doxycycline and Cefotaxime by a percentage (20%), but resistant to Ampicillin and Erythromycin antibiotics as well as Sulphamethaxazol-trimetoprim and Rifampicin and such results agree with John and Hatha (2013) who stated the sensitivity of Aeromonas spp. to Gentamicin and Ciprofloxacin (100%), and also agree with Enany et al., (2011) who recorded that A. hydrophila had been resistant to Erythromycin, and nearly agree with Samal et al., (2014) who stated that Norfloxacin was sensitive by (84.6%), while disagree with Awan et al., (2009) that showed that Cefotaxime (90.9%), Amikacin (100%) the more sensitive. The present study revealed that A. sobria is sensitive to Amikacin by a percentage (100%), Ciprofloxacin, Cefotaxime by a percentage (66.6%), Norfloxacin (58.33%) Rifampicin (41.66%), Doxycycline (33.3%), Gentamicin (16.66%), while is resist to Ampicillin, Erythromycin, Sulphamethoxazol-trimetoprim and this agree with Henadek (2002) that stated that A. sobria was sensitive to Doxycycline (33%), and agree with Awan et al., (2009) who reported A. sobria was sensitive to Amikacin (100%) but disagree with John and Hatha (2013) who showed A. sobria is sensitive to Ciprofloxacin and Gentamicin by a percentage (100%). The present study

showed that *A. caviae* showed sensitivity toward Amikacin, Gentamicin, Norfloxacin (100%), Ciprofloxacin (87.5%), Cefotaxime (50%), Doxycycline (37.5%), while was resist to Ampicillin, Erythromycin, Sulphamethoxazol - trimethoprim and Rifampicin, and this agree with Awan et al., (2009) who reported *A. caviae* was sensitive to Gentamicin (100%) and nearly similar with Amikacin (96.2%), Ciprofloxacin (88%), and in contrast with them when they stated that *A. caviae* was sensitive to Cefotaxime (96%), Sulphamethoxazol-trimethoprim was sensitive by (46.2%), Erythromycin sensitive by (18.2%). In addition *A. schuberti* was sensitive to Doxycycline (100%), Cefotaxime (100%), Amikacin (100%), Norfloxacin (100%), Gentamicin (100%), Ciprofloxacin (100%) beside Rifampicin (37.5%), while was resist to antibiotics as Ampicillin and Erythromycin as well as Sulphamethoxazol-trimethoprim and this was similar to John and Hatha (2013) with that showed *A. schuberti* was sensitive to Ciprofloxacin and Gentamicin (100%), and Awan et al., (2009) who reported *A.schubertii* was sensitive to Ciprofloxacin, Cefotaxime and Amikacin (100%), but disagree with the data obtained in the same study which revealed *A. schuberti* was sensitive to Sulphamethoxazol-trimethoprim (50%).

Table 3: Antibiogram of random isolated Aeromonas species

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Discount</th>
<th>A. hydrophila n=20</th>
<th>A. sobria n=12</th>
<th>A. caviae n=8</th>
<th>A. schuberti n=8</th>
<th>Total N=48</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>S</td>
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<tr>
<td>Ampicillin (AMP)</td>
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<td>20</td>
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<td>12</td>
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<td>0</td>
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<td>8</td>
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<tr>
<td>Erythromycin (E)</td>
<td>15</td>
<td>20</td>
<td>100</td>
<td>12</td>
<td>100</td>
<td>8</td>
</tr>
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<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Sulphamethoxazol-</td>
<td>25</td>
<td>20</td>
<td>100</td>
<td>12</td>
<td>100</td>
<td>8</td>
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<tr>
<td>Trimethoprim(SXT)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>7</td>
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<td>0</td>
<td>5</td>
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<td>80</td>
<td>8</td>
<td>66.6</td>
<td>5</td>
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<tr>
<td></td>
<td>S</td>
<td>4</td>
<td>20</td>
<td>4</td>
<td>33.3</td>
<td>3</td>
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<tr>
<td>Cefotaxime (CTX)</td>
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<td>80</td>
<td>4</td>
<td>33.3</td>
<td>4</td>
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<tr>
<td></td>
<td>S</td>
<td>4</td>
<td>20</td>
<td>8</td>
<td>66.6</td>
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<td>Gentamicin (CN)</td>
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<td></td>
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<td>12</td>
<td>60</td>
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<td>100</td>
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<td>Norfloxacin (NOR)</td>
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<td></td>
<td>S</td>
<td>20</td>
<td>100</td>
<td>8</td>
<td>66.6</td>
<td>7</td>
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</tbody>
</table>

S = Sensitive  R = Resistant

**Fig. 3:** Antibiogram of isolated Aeromonas species.
Conventional PCR using 16SrRNA gene for 12 tested Aeromonas strains which were identified biochemically as 5 strains of *A. hydrophila* and 3 strains of *A. sobria*, 2 *A. caviae*, 2 *A. schubertii*, the present results revealed that all examined strains were positive for 16SrRNA gene as shown in Table (4) Figs. (4 &5) and Photo (1). These results were nearly similar with Martinez-Murcia (1999) and Wang *et al.*, (2003) who used 16SrRNA gene for identification of the tested strains of Aeromonas which give the same results that all isolated strains were positive for this gene presence.

PCR assay was developed with specific primers for detection of different Aeromonas spp. virulence genes (Aerolysin and Hemolysin). The current results showed that Aerolysin gene was detected in 10 strains out of 12 (83.3%), Table (4), photo (2) and Figs. (4 &5) and this result is closely similar to Abd-ElAll *et al.*, (2014), Ottaviani *et al.*, (2011) and Singh *et al.*, (2008) who reported that total aerolysin gene detection in Aeromonas spp. in fish and pond water samples was (80%), (83.7%), (85%) respectively. They also nearly agree with Ormen and Ostensvik (2001) who used a PCR assay to detect the aer A gene in Aeromonas spp. environmental water isolates in Norway and reported that 79% were positive.

**Table 4: Frequency distribution of 16SrRNA, Aerolysin and Hemolysin genes of isolated Aeromonas spp.:**

<table>
<thead>
<tr>
<th>Aeromonas strains</th>
<th>16SrRNA gene</th>
<th>Aerolysin Gene</th>
<th>Haemolysin gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td><em>A. hydrophila</em></td>
<td>5</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td><em>n</em>= 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. sobria</em></td>
<td>3</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td><em>n</em>= 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. caviae</em></td>
<td>2</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td><em>n</em>= 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. schubertii</em></td>
<td>2</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td><em>n</em>= 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>12</td>
<td>100</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 4: Percentage of positive isolates for 16SrRNA gene, Aerolysin gene and Hemolysin gene.
The current study revealed very low percentage of hemolysin gene (1 out of 12 strains) (8.3%) which belong to A. schubertii, while the remaining strains were not harbouring the hemolysin gene. This finding was observed also by Abdullah et al., (2003). On other hand, Yucel and Citak (2003) who reported that A. hydrophila and A. sobria were been stronger producer of hemolysin but A. caviae was non hemolytic.

In the current study some Aeromonas strains lacked both aer A and hlyA genes and this was observed before in earlier studies done by (Santos et al., 1999 and Herrera et al., 2006) who stated that aerolysin-like gene was activable under certain conditions and can be detected in apparently non haemolytic strains.

The current results revealed that aerolysin gene is 100% in A. caviae (2/2) and A. schubertii (2/2) were positive for aerolysin gene, while in only 80% of A. hydrophila (4/5) and 60% of A. sobria (2/3). This result nearly agree with Abd-El All et al., (2014) and Umesh et al.,(2011) who detected Aerolysin gene in (100%) of A. hydrophila recovered from fish samples, furthermore Abdullah et al., (2003) detected aerolysin gene in all the isolates, and nearly similar to Herrera et al., (2006) who mentioned that 8/9 of A. hydrophila were positive for aerolysin gene but differ with another study which reported a result of 2/4 of A. caviae and 2/2 of A. sobria that were positive for aerolysin gene. In contrast with Heuzenroeder et al., (1999) who made a survey of clinical and environmental isolates of the Aeromonas spp. and stated that aerA-like sequences were found in 78%, 97% and 41% respectively, in A. hydrophila, A. sobria and A. caviae isolates, Moreover Pollard et al., (1990) and Lior and Johnson (1991) showed that the aerA gene was detected only in hemolytic, cytotoxic and enterotoxigenic strains of A. hydrophila but not in A. sobria and A. caviae.

The current results revealed that Aerolysin gene was 66.6 % positive in A. sobria and this result is similar to Yousr et al., (2007) who detected that the same percentage of aerolysin gene of A. sobria, but disagree with the percentage of A. hydrophila and A. caviae where the aerolysin gene were (52.6%) and (44.7%) respectively.

The frequency and distribution of the aerolysin gene in the Aeromonas strains in this study was nearly similar with an earlier
PCR survey by Husslein et al., (1991) who detected the aer A gene in all strains belonging to *A. hydrophila* and *A. sobria* species, so the aerolysin gene seems to be as ubiquitous like the Aeromonas spp.

In the current result, the clinical strains possessless hemolytic activity and this observation is also reported by Altwegg (1985) who stated that although, it is very likely that clinical isolates possess less number of virulence gene, it should kept in mind that Aeromonads were recognized as opportunistic microorganism that may be present in diarrheal stool as commensals rather than as primary pathogens.

Another observation, which is that one of the isolated *A. sobria* strain was lacking both aerolysin & hemolysin genes and developed multi drug resistance and another isolated *A. hydrophila* strain was lacking hemolysin genes and developed also multi drug resistance and such results may strongly force the point of view that pathogenicity and virulence of Aeromonas spp. are multifactorial and complex Janda and Abbott (1998); Chopra et al., (2000), and this agrees with Shome et al., (1999) who mentioned that the production of enzymes or toxins is not reflective of biological virulence and they neither satisfy the strain to be virulant nor avirulant in spite of; this appear to enhance the process of disease in-vivo. The whole process of pathogenesis is a complex interaction between the host, agent and environmental determinants.

**Photo (1):** illustrated the positive for amplification of (685 bp) fragment of 16SrRNA gene from extracted DNA of 12 Aeromonas spp. from fish, water and human stool samples.

**Photo (2):** illustrated (326 bp) fragment of (aerA) gene where (10) amplification Aeromonas strains were positive for aerolysin gene.

**Photo (3):** illustrated (1500bp) fragment of hemolysin gene from extracted DNA of *A. schubertii* isolated from water.

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Photo 1: Electrophoretic pattern of 16SrRNA gene amplification of 12 Aeromonas spp. isolated from different sources. **Lanes 1-12:** showed 16SrRNA gene of 685bp from various Aeromonas spp. of different sources positive of Aeromonas spp from water: *A. hydrophila* (Lane 1), *A. schubertii* (Lane 2), *A. sobria* (Lane 3) and *A. caviae* (Lane 4); from fish: *A. hydrophila* (Lane 5,6,10), *A. caviae* (Lane 7), *A. sobria* (Lane 8), *A. schubertii* (Lane 9); from stool: *A. sobria* (Lane 11) & *A. hydrophila* (Lane 12).

* Lane (L) for ladder (100 bp DNA ladder).
* Pos. = +ve control
* Neg. = -ve control
Genotypic identification and evaluation of several selective media for recovery of Aeromonas spp.

Photo 3: Electrophoretic pattern of Hemolysin gene amplification of 12 Aeromonas spp. isolated from different sources.

Lanes 1-12: showed Hemolysin gene of 1500 bp from various Aeromonas spp. of different sources positive of Aeromonas spp from water: A. hydrophila (Lane 1), A. schubertii (Lane 2), A. sobria (Lane 3) and A. caviae (Lane 4); from fish: A. hydrophila (Lane 5, 6, 10), A. caviae (Lane 7), A. sobria (Lane 8), A. schubertii (Lane 9); from stool: A. sobria (Lane 11) & A. hydrophila (Lane 12).

Pos. = +ve control
Neg. = -ve control

Lane (L) for ladder (100 bp DNA ladder).

CONCLUSION AND RECOMMENDATIONS

It could be concluded from the present study that the isolation of Aeromonas species from mixed population such as fishes, waters and childhood diarrhea, require enrichment in alkaline peptone water and consecutive plating on more than one media such as Starch ampicillin media and Rimler-Shotts media to avoid the missing of some Aeromonas spp. As the isolation of Aeromonas species is laborious process and biochemical identification lack specificity, so PCR technique provide rapid and sensitive method for confirmatory identification of Aeromonas species and detection of some virulence genes.

Aeromonas species seem to be prefer fresh water than brackish water and marine water, so freshwater fish (Tilapia niloticus) showed heavier contamination than Mugil cephalus. Also the Aeromonas spp. isolated from drinking tap water and childhood diarrhea This data suggesting that the bacterial population of Aeromonas on fish and water may reflect the level of human infection. This study show that Aeromanas not only primary fish pathogen but also potentiate the evidence that Aeromonas is water born and an emerging pathogen for human. The four phenotypes species that recovered from
Suez Canal area were *A. hydrophila*, *A. sobria*, *A. caviae* and *A. schubertii*. So routinely examination for Aeromonas spp. in Clinical laboratory of hospitals is necessary specially for *Aeromonas hydrophila* and *Aeromonas sobria* that are the predominant enteric and emerging species in Suez canal area Aeromonas species not only seems to be ubiquitous in habitats, but also Aerolysin gene and multiple resistances to antibiotics are ubiquitous. In current study Aeromonas spp. developed multiple drug resistant to Erythromycin, Sulphamethoxazol-trimethoprim, Rifampicin, Doxycycline and Cefotaxime beside the classical resistant to Ampicillin, Higher frequency of multi-drug resistance was observed for *Aeromonas sobria* than *Aeromonas hydrophila* this may be attributed to the fact that *Aeromonas sobria* is more virulent than *Aeromonas hydrophila*. so The legal restrictions is highly recommended in using antibiotics for controlling of Aeromonads infections in fishes, water and human, and recommended using of Ciprofloxacin and Norofloxacin as first line treatment followed by Gentamicin and Amikacin as 2nd line of treatment in control fish infection while in human, Amikacin or Gentamicin can used as first line treatment followed either by Norofloxacin or Ciprofloxacin as 2nd line of treatment.

Hemolytic activity could be the landmark for genus Aeromonas and in the present study Aerolynsin gene is broad spread in the isolated strains of Aeromonas and Hemolytic activity of Aeromonas spp. not enhance the disease process in fish and human and not serve species specific marker so this study may enforced that pathogenicity and virulence of Aeromonas spp. are multifactorial and complex so the advance investigation of other factors rather than haemolysin genes is required to understand the pathogenicity of Aeromonas.

Regular examination of pond water and their input supplies should make for prohibition their contamination with Aeromonas from sewage pollution of pond water of fish. And improving water quality may improve fish health condition In addition to apply sanitary and hygienic measurements to control biofilm formation as it may play an important role in contamination of drinking water to avoid risk of human infections.

**REFERENCES**


Genotypic identification and evaluation of several selective media for recovery of Aeromonas spp.


PAGE analysis.” Open Journal of Medical Microbiology, 2, 37-40.


ARABIC SUMMARY

التصنيف الجيني وقيمة العينات من أنواع الأتربة المختارة للكشف عن الأتربات الورمية من مصادر مختلفة

محمد السيد عاطفي1، أماني محمود شبيل2، حسنين صالح3، رباح السيد أبو الخير إبراهيم3

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2. رئيس بحوث المجتمعية بحث صحة البيئات والأغذية. جامعة قلقان النمسا.
3. مدرسة الباكلوريا الإكلينيكية كليلة الطب – جامعة قلقان النمسا.

تم تجميع 250 عينة شفطية (50 عن كون من الأسماك، 50 عن كون من الأسماك، 50 عن كون من الأسماك بالإضافة إلى تجميع 50 عن كون من الأسماك من الأحياء الساحلية). ورص دعو الفحص الإكلينيكية للكشف عن هذا العيب. وذلك استخدام التفاعلات الفيزيولوجية والكيميائية والكيميائية اعتمادا على كمية المراد تحليلها. يشمل ذلك استخدام التفاعلات بحرية متصلة لميكروب من الأتربات الورمية من الأحياء الساحلية.

وتلك الإجراءات التي تشمل: 
- عزل وميز ميكروب الأتربات الورمية من مصادر مختلفة.
- دراسة مشاكل السائل المناعي في كل من الأسماك، واللبس، وال يوجد.
- إجراء اختبار الحفاظ الدائم للكشف عن ميكروب مثير.$$G$$ عزل وتحقيق نتائج.
- دراسة جديدة لتحديد بعض الجوانب الجينية للكشف عن ميكروب و يتم توجيه في كل من العينات المختلطة من الأسماك، والميكروبات، واللبس، والأخلاقيات.

وقد كشفت الدراسة أن عزل ميكروب الأتربات الورمية من المصادر المختلفة على مصادر متنوعة، (ABA): (RS)، (SAA)، (MAA)، و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. 

ولقد أظهرت النتائج أن نسبة الأسماك ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، والميكروبات، واللبس، والأخلاقيات. و (51.2%) و (55%) و (60%) و (63%) و (60%) و (65%) بنسب تم رصد ميكروب الأتربات الورمية من الأسماك، الم


