JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2016. Vol. **10**(3), p. 1835-1846

Isolation and Identification of Bacterial Flora from Catfish *(Clarias gariepinus)* with Antimicrobial Susceptibility and Herbal Sensitivity

Soheir S. Abd El-salam[1], F.M. Ghaly[2], Dina M. Baraka[1], Shahira H. Mahmoud[3] and Abeer A. El-makhzangy[3]

[1]Department of Botany, Faculty of Science, Benha University, Egypt
[2]Department of Botany, Faculty of Science, Zagazig University, Egypt.
[3]Animal Health Research Institute, Zagazig University, Egypt.

https://doi.org/10.22207/JPAM.10.3.18

(Received: 12 May 2016; accepted: 22 June 2016)

This study was carried out for isolation and identification phenotypically morphological and biochemical characteristics of bacteria infected *Clarias gariepinus* and genotypically by Random Amplified Polymorphic DNA (RAPD) also, its susceptibility to different antibiotics and some natural plant extract. A total 289 strains of bacteria isolates , the number of bacterial isolates differed according to the organ of isolation . The highest rate of isolation was obtained from liver (67 isolates) followed by skin (62), intestinal contents (55), kidney (47) and spleen (46) while the lowest number of isolated bacteria were recovered from gills (12). The most commonly isolated bacteria were *Aeromonas Sorbia* (46.7%), *A.caviae* (19%), *A.jamdaei* (8.6%), *A.veronii* (8.6%), *Ps.aeruginosa* (2.4%), *Ps.fluorescens* (2%), *Staph.aureus* (2.7%), *Staph.epidermidis* (1%), *Vibrio alginolitcus* (4.5%) and *V.anguillarum* (4.15%). All tested isolates were sensitive to Ciprofloxacin followed by Cefuroxime then Gentamycin, the lowest (MIC) and (MBC) were recorded for Ciprofloxacin and alcoholic Clove extract among the plant species tested, Cinnamon, Hibiscus, Nutmeg, Thymus and Rosemary against all bacterial isolates.

Keywords: Antibiotic susceptibility, Herbal sensitivity, *Aeromonas spp*., Phenotypic, Genotypic, Catfish.

There is an increasing demand for fish and fish products around the world[1]. Fish is a low fat food, a great source of protein, vitamins and minerals; so it is an important food source for human especially , freshwater fish as Catfish (*Clarias gariepinus*) in high economic and nutritional value[2,3].

Bacteria is the major group of water microbes are responsible for heavy mortalities and which invade the tissue fish host[4] and can contaminate the surface gills and intestinal tract of fresh shell frish and other sea foods[5].

* To whom all correspondence should be addressed.
Tel.: +2/01067140872;
E-mail: Dr.Soheir_Saad@yahoo.com

Fish are susceptible to a wide variety of bacterial pathogens, the majority of bacterial infections are caused by Gram-negative (*Aeromonas spp.*, *Pseudomonas spp*. and *Vibrio spp.*) which cause most diseases in tropical fish . Several workers have conducted investigations on these bacteria[6,7].

The genus *Aeromonas* comprises of several species of gram negative, rod shaped, motile and non-motile, oxidase and catalase positive[8]. Owing to varied nature of these bacteria, they have been difficult to classify, making the taxonomy of the genus[9]. There is therefore a need for a system for identification, RAPD fingerprinting is rapid, sensitive and making the detection and identification relatively easy[10] found that the motile

Aeromonads, isolated from the intestines of farm raised fresh water fish, had been classified to species level on the morphological and physiological bases .

Uses of antibiotic to cure bacterial infection and prevent fish mortality in aquaculture is becoming limited as pathogen developed resistance to drugs[11], many studies showed that natural plants and their extracts have multi-antimicrobial properties[12]. So treatments of bacterial disease with various herbs have been safely used such as cinnamon, clove, garlic, thyme, mint and vanilla[13-14] determined that association of antibiotics and plant showed synergistic antibacterial activity against antibiotic-resistant bacteria.

The aim of the present study was to isolate and identify the isolated from the affected catfish (*Clarias* fish) based on the biochemical properties and to screen their phenotypic and genotypic traits which might be helpful for identification of this species in the diagnostic laboratories and determination the sensitivity of bacterial isolates to antibiotic and some natural plant extract.

MATERIALS AND METHODS

Collection of fish

Total number of two hundred *Clarias gariepinus* were collected from different localities in Sharkia governorate fish were transferred alive to the laboratory and subjected to clinical, postmortem and bacteriological examinations. Forty of them were apparently healthy and chosen as a control; while hundred and sixty showing clinical abnormalities and lesions.

Isolation of bacteria

Isolation of bacteria was carried out aseptically from collected samples of the affected areas of skin, gills and the internal organs (kidney, liver, spleen and intestine) were cultivated on different media for the isolation of bacteria as tryptic soya broth at 25°C and at 37°C for (18 - 24)hours, then poured onto tryptic soya agar, blood agar, Rimler-Shoots agar, Thiosulfate Citrate Bile-salt Sucrose agar (TCBS), and incubate at the same time and temperature . Purification of the isolated bacterial strains was performed according to[15].

Identification of bacterial isolates

All the bacterial isolates were preliminary differentiated according to their morphological characters, physiological and biochemical rests were performed to identify *Aeromonas spp., Pseudomonas spp.* and Gram-positive cocci according to[16,15] and so *Vibrio spp.*[17,18]. The identification was performed by Gram staining, catalase activity, oxidase test, oxidation-fermentation (OF) test, methyl red test, Voges Proskaver (VP) test, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, indole, urease and hydrogensulfide (H_2S), production test, gelatin degradation, acid production and nitrate reduction[15].

RAPD-PCR Analysis

DNA Extraction

The protocol developed by[19] with modifications was used for isolation of DNA from broth culture of bacteria at log phase. The modified protocol without the use of proteinase K has given good result, yielding quality DNA of approximately 20 µg from a 2 ml bacterial culture .

Primers

A panel of 9 numbers of decamer random primers from M/S Operon Technologies, was used for PCR amplification of bacterial DNA template. Two out of 9 primers, viz., OPX-03, OPV-19 were selected for final screening as they only generated several reproducible amplicons that could be resolved as distinct bands by agarose gel electrophoresis .

PCR amplification and resolution of RAPD markers

PCR reactions has been optimized for important parameters such as annealing temperature, concentration of $Mgcl_2$, template DNA, *Taq* DNA polymerase, dNTP's and primers. The PCR reaction components consists of 200 mm dNTP, 7.5 pico moles of primer, 2 units of *Taq* DNA polymerase enzyme, assay buffer with working concentration of 1.5 mM $Mgcl_2$, 20-30 ng template DNA in an assay volume of 25 ml. These concentrations were determined by a series of preliminary standardizing experiments .

Thermal cycling was performed with Perkin-Elmer thermocycler (GeneAmp PCR System 2400). Each of the 35 PCR cycles standardized for this work consisted of denaturation of DNA at 92°C for one minute, primer annealing at 37°C for one minute and primer extension at 72°C for one minute. All PCR samples were subjected to an initial two

minutes denaturation for 94°C and post amplification extension at 72°C for ten minutes. PCR products were stored at -20°C until electrophoresis was performed.

The PCR amplification products were resolved by carrying out electrophoresis using a 1.5% agarose gel, stained with ethidium bromide. The marker used was ë DNA cut with HindIII/ EcoRI. The DNA bands were visualized and documented using a Vilber Lourmat Get documentation system .

Data analysis

The data of RAPD analysis were scored computer as (1) and (0) for the presence and absence of bands, respectively. Similarity coefficients among the studied *Aeromonas* isolates as implemented in the computer program SPSS version 10[20].

Antibiotic Sensitivity test

Antibiotic susceptibility of isolates was determined using the disc diffusion technique[21,22]. The antibiotics and the concentration ranges tested were: Ampicillin (AM 10µg), Clindamycin (DA 2µg), Ciprofloxacin (CIP 5µg), Erythromycin (E 15µg), Gentamycin (GN 10µg), Norofloxacin (NOR 10µg), Spectinomycin (SPT 100µg) and Cefuroxime (CRO 30µg).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) method

Is the lowest concentration of an antimicrobial agent (MIC) that will inhibit the visible growth of a microorganism after over-night incubation [23]. A two-fold serial dilutions of the antibiotics were prepared in Muller-Hinton broth (MHB) and to determine (MBC),100µl aliquot from the tube showing (MIC) was placed on (MBC) agar plate antibiotic free, after incubation and examine the plates at which 99.9% killing of bacteria concentration; which prevented visible growth .

Antimicrobial evaluation of medicinal plants

The disc diffusion method was used as described by[24]. A total of six selected herb extracts was used in this study to examine their sensitivity to experimental isolates. Medicinal plants were collected from local market in Sharkia governorate. Cinnamon (*Cinnamomum verum*), Clove (*Syzygium aromaticum*), Thymus (*Thymus vulgaris)*, Nutmeg (*Myristica fragrans)*, Rosemary (*Rosmarinus officinalis*) and Hibiscus (*Hibiscus sabdariffa*) plants were finely grinded to powder, soaked in 100ml hot sterile water and allowed to stand for (72) hours[25], each plant material extracted in Soxhlet apparatus with 150 ml of 95% ethanol at 60°C for (4) hours[26]. 15µl of the herb extract was added on top o the disc of 6mm diameters, the plate was incubated at 37°C for (24) hour , the antimicrobial activity was expressed as the mean of inhibition diameter (mm) produced by plant extracts.

Determination of MIC and MBC of ethanolic Clove extract against isolated bacteria

The micro dilution method was used to determine the MIC of Clove extract. A two-fold serial dilution of ethanolic extract of Clove were prepared in sterile Muller-Hinton broth. Each dilution was seeded with 100 µl of standard bacterial inoculum, then inoculate the lowest concentration of extract; showing no visible growth[27].

RESULTS AND DISCUSSIONS

Clarias gariepinus is freshwater fish, a popular delicacy relished throughout tropical Africa. As we know, fishery products are of great importance for human nutrition worldwide and provide clear health benefits[3]. Fish are susceptible to a wide variety of bacterial pathogens causes large mortality in the aquaculture .

Isolation of bacteria isolates

In the present study, bacteriological examination of 166 diseased *Clarias gariepinus* revealed the isolation of (289) different bacterial isolates in Table (1), isolate (1) was found to

Table 1. Showed percentage (%) of bacterial isolates from naturally diseased *Clarias garpinus*

Bacterial isolates	Total	Percentage %
Isolate (1)	135	46.7%
Isolate (2)	55	19.03%
Isolate (3)	25	8.6%
Isolate (4)	25	8.6%
Isolate (5)	6	2.07%
Isolate (6)	7	2.4%
Isolate (7)	8	2.7%
Isolate (8)	3	1.03%
Isolate (9)	13	4.5%
Isolate (10)	12	4.15%
Total	289	

represent the most predominate isolates from clinically diseased *Clarias gariepinus* in an incidence of 46.7% , other bacteria were isolated in much lower numbers as 1.03% of isolate[8] .

Identification of isolated bacteria

All the bacteria isolates were preliminary differentiated according to their morphological , physiological and biochemical properties .The results of this study, *Aeromonas spp.* were the most predominant bacterial species isolated from diseased fish in an incidence 83%, these results agreement with those recorded by[28,10,29,30].

In Table (2), the biochemical properties as the growth at 5° C and on 6% NaCl, acid production from sucrose, salicin and arabinose were used to differentiate between the four species of *Aeromonas* as described[31,32,33,15,22]. all *Aeromonas* (group 1, 2, 3 and 4) isolates gave negative result with Inositol, Arginie dehydrolase and give positive result with Maltose and Mannitol. Group 1,3 and 4 gave negative result with Arabinose while group 2 gives positive result. Group 1,2 and 3 gave negative result with salicin, while group 4 gave positive result. Sucrose fermentation test gave positive result with group 1, 2 and 4 while group 3 gave negative result, that similar to those recorded by[30,33,15,29]. so according to[17] the four groups are *A.sobria, A.caviae, A.jandaei and A.veronii,* respectively .

In Table (3), *Vibrio spp.* were gram negative motile , oxidase and catalase positive grown on TCBS gave yellow colonies and in oxidation fermentation test gave positive result , no growth appeared at 0% NaCl but positive at 2,

Table 2. Morphological and biochemical characters of suspected *Aeromonas spp.* Isolates from naturally infected *Clarias garpinus*

Biochemical tests	*Aeromonas spp.*			
	Group 1	Group 2	Group 3	Group 4
Gram stain	-ve	-ve	-ve	-ve
Shape	Short rod	Short rod	Short rod	Short rod
Motility	+	+	+	+
Cytochrome oxidase	+	+	+	+
O/F	F	F	F	F
Growth at 5°C	+	-	-	-
Growth on 0% Nacl	+	+	+	+
Growth on 3.5% Nacl	+	+	+	+
Growth on 6% Nacl	+	-	-	-
Catalase test	+	+	+	+
H_2S (TSI)	+	-	-	-
Indol	+	+	+	+
Starch hydrolysis	+	+	-	-
Methyl red	+	+	-	-
Vogus Proskauer	+	-	+	+
Citrate	+	+	+	+
Gelatin Liquefaction	+	+	-	-
Hemolysis	β	-	γ	-
Acid production from :				
Arabinose	-	+	-	-
Salicin	-	-	-	+
Inositol	-	-	-	-
Maltose	+	+	+	+
Tween 80	+	+	+	+
Glucose	+	-	+	+
Ornithen decarboxylase	+	-	+	+
Lysine decarboxylase	+	-	+	+
Arginine dehydrolase	-	+	-	-

5, 7% NaCl and Indole , citrate utilization positive but arginine hydrolysis negative . They ferment carbohydrates with production of acid but no gas. Strain 1 had comma shape rods under oil immersion lens and growth 10% NaCl while Strain 2 was short thick bacilli , no growth at 10%NaCl .[34,35,36,37] as[17], strain 1 & 2 are most likely to be *Vibrio alginolticus* and *Vibrio anguillarum,* respectively .

The result in Table (4) showed that the biochemical properties for differentiation between *Pseudomonas spp.* were oxidation fermentation test, growth at 37 C , arginin dehydrolysis , production of acid from glucose , Arabinose, mannitol and sucrose . These results were in agreement with[38,39,40,41,33,15]. Isolate 1 grows well onto blood agar, nutrient agar, Tryptic soya agar (TSA) media, on R-S medium produce green color, smooth colonies also Isolate 2 grows well onto nutrient, TSA agar and R-S media but producing large irregular spreading colonies. This exhibit that isolate 1 is *Pseudomonas fluorescens* and isolate 2 is *Pseudomonas aerginosa* .

Table 3. Morphological and biochemical character of suspected *Vibrio spp.* Isolated from naturally infected *Clarias garpinus*

Biochemical tests	*Vibrio spp.*	
	Strain 1	Strain 2
Gram stain	-ve	-ve
Shape	Curved rod (comma) shape	Short rods
Motility	+	+
Cytochrome oxidase	+	+
O/F	F	F
Catalase	+	+
Growth on TCBS	+ Yellow colonies	+ Yellow colonies
Methyl red	+	+
Vogus Proskauer	+	+
Arginine dehydrogenase	-	-
Indole	+	+
Citrate Utilization	+	+
Growth on 0% Nacl	-	-
Growth on 2% Nacl	+	+
Growth on 5% Nacl	+	+
Growth on 7% Nacl	+	+
Growth on 10% Nacl	+	-
Marg reaction	Evitage N	Evitage N

Table 4. Morphological and biochemical characters of suspected *Pseudomonas spp.* Isolates from naturally infected *Clarias garpinus*

Biochemical tests	*Pseudomonas spp.*	
	Isolate 1	Isolate 2
Gram stain	-ve	-ve
Shape	Long curved rods	Long curved rods
Motility	+	+
Cytochrome oxidase	+	+
O/F	o	o/-
Growth at 5°C	+	+
Growth on 0% Nacl	+	+
Arginine dehydrogenase	v	+
Catalase	+	+
H_2S (TSI)	-	-
Indole	-	-
Lysine decarboxylase	-	-
Gelatin degradation	+	v
Ornithen decarboxylase	-	-
Urease test	v	v
Acid production from :		
Glucose	+	+
Sucrose	-	-
Salicin	-	-
Lactose	v	-
Maltose	+	+
Inositol	+	-
Fructose	-	-
Nitrate reduction	-	-
Esculin hydrolysis	-	-

Table 5. Morphological and biochemical character of suspected *Staphylococcus spp.* Isolated from naturally infected *Clarias garpinus*

Biochemical tests	*Staphylococcus spp*	
	Strain 1	Strain 2
Gram stain	+ve	+ve
Shape	Large yellow colony on rich media	Small white colony
Growth temperature	15-45ºC	37ºC
Catalase	+	+
Coagulase	+	-
Cytochrom oxidase	-	-
D-mannitol fermentation	+	+
Hemolysis on blood agar	Hemolysis β	Non hemolysis

Staphylococci spp. were gram positive , facultative anaerobes that grow by aerobic respiration or by fermentation Table (5) . The bacteria were catalase - positive and oxidase - negative, strain 1 formed a fairly large yellow colony on rich medium , hemolytic on blood agar and produce the coagulase enzyme . Strain 2 had a relatively small white colony , non hemolytic , lack coagulase enzyme, from the previous result strain 1 identified as *S.aureus* & strain 2 identified as *S.epidermids*.[33,42,43] which revealed that 73.5% of the examined fish were infected with various gram positive bacteria, namely *Streptococcus spp., Staphylococcus spp.* and *Clostridium perfringens.*

Table (6) revealed that the most common of bacterial isolates from organ and tissues of clinical diseased *Clarias gariepinus* were *Aeromonas sobria* (135 isolates) 40 of them from skin , 20 from liver and intestinal content , 25 from (kidney and spleen) while gills showed the lowest rate of isolation 5 isolates , also the highest rate of isolation was obtained from liver (67 isolates) followed by skin (62 isolates) ,intestinal contents (55) , kidney (47 isolates) and spleen (46 isolates) and finally gills (12 isolates). Our results in agreement with those reported by[44,45] which demonstrated that the motile *Aeromonas* dominated the skin & gills[46] found that the highest

Table 6. Distribution of bacterial isolates from organs and tissues of naturally infected *Clarias garpinus*

Bacterial isolates	Liver	Gills	Kidney	Spleen	Intestinal contents	Skin	Total
Aeromonas sobria	20	5	25	25	20	40	135
Aeromonas caviae	10	5	11	9	15	5	55
Aeromonas jandaei	7	1	2	5	5	5	25
Aeromonas veronii	7	1	5	5	5	2	25
Pseudomonas fluorescens	4	-	-	-	-	2	6
Pseudomonas aeruginosa	2	-	-	-	2	3	7
Staphylococcus aureus	5	-	-	-	3	-	8
Staphylococcus epidermidis	2	-	-	-	1	-	3
Vibrio alginolitcus	5	-	2	1	2	3	13
Vibrio anguillarum	5	-	2	1	2	2	12
Total	67	12	47	46	55	62	289

Table 7. Code & sequence of the two DNA random primer that revealed RAPD products in the isolates as well as number & types of amplified DNA bands generated by these primers

Primer code	Sequences	Monomorphic bands	Unique polymorphic bands	Polymorphic bands	Total
OPX-03	5'-TGG CGC AGT G -3'	-	8	20	28
OPV-19	5'- GGG TGT GCA A -3'	-	7	12	19

rate of isolation was obtained from the intestine content (68 isolates) followed by the liver (43 isolates), kidney (16 isolates) , while the lowest number of isolated bacteria were recovered from skin , spleen and gall bladder (12,11 and 9 isolates).

Fig (1a and b) illustrates the amplification products of four *Aeromonas* isolates. Nine 10-*mer* random primers were tested the present study to identify and discriminate among these isolates. But only primers (OPX-03 and OPV19) succeeded to get amplified and reproducible fragments. As shown in figure (1a and b) each land (PCR-DNA product of each isolate) appeared to be different. The molecular size of the PCR products generated by these primers are ranged from 2176 to 260 bp. Forty-seven polymorphic bands were generated by the two primers. A total of 15 polymorphic bands were scored as unique ones. The primer OPX-03 was found to be the more potent in generating 11 unique bands while the latter primer produced 7 unique

Table 8. Molecular size in bp of the amplified polymorphic (unique) DNA bands generated by 2 DNA random primers used to identify the four *Aeromonas* isolates Monomorphic: the band appeared in all isolates

Isolates	OPX-03	OPV-19
A.caviae	980	1800-1700-615
A.jandaei	1280	680
A.sobria	1033-830-750-570	780
A.veronii	-	820-570

bands.

The primer OPX-03 revealed clear variations in RAPD products between the studied bacterial isolates. Figure (1a) illustrates the DNA polymorphism obtained with this primer. A total of 28 reproducible bands were generated by the primer OPX-03, which not necessary generated by all isolates. All the generated bands were scored as polymorphic ones (100% polymorphism). Eight of these bands were identified as a unique bands (Table 7) the first and second unique bands were

Table 9. Minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC) for three antibiotics against bacteria isolated from naturally infected *Clarias garpinus*

Isolated Bacteria	Ciprofloxacin (CIP)		Cefuroxime (CRO)		Gentamycin (CN)	
	MIC (0.39-100µ/L)	MBC	MIC (0.39-100µ/L)	MBC	MIC (0.39-100µ/L)	MBC
Aeromonas sobria	1.5	1.5	50	100	25	25
Aeromonas caviae	1.5	1.5	50	100	3.1	6.25
Aeromonas jandaei	0.39	0.39	1.5	1.5	6.25	6.25
Aeromonas veronii	1.5	1.5	50	100	6.25	12.5
Pseudomonas fluorescens	25	25	6.25	6.25	50	50
Pseudomonas aeruginosa	25	25	3.1	3.1	50	50
Staphylococcus aureus	6.25	6.25	12.5	12.5	1.5	1.5
Staphylococcusepidermids	3.1	3.1	6.25	6.25	1.5	1.5
Vibrio alginoliticus	3.1	3.1	6.25	6.25	12.5	12.5
Vibrio anguillarum	3.1	3.1	6.25	6.25	12.5	12.5

Table 10. Minimum inhibitory concentration (MIC) & Minimum bactericidal concentration (MBC) of ethanolic clove extract against bacteria isolated from naturally infected *Clarias garpinus*

Ethanolic clove extract	Isolated bacteria									
	1	2	3	4	5	6	7	8	9	10
MIC (mg/ml)(0.19-100mg/ml)	25	3.1	6.25	6.25	12.5	6.25	3.1	6.25	1.5	1.5
MBC	25	3.1	6.25	6.25	12.5	6.25	3.1	6.25	1.5	1.5

detected at about 980 and 1820 bp in *A.caviae and A.jandaei*, respectively. The third, forth, fifth and sixth unique bands were recognized at about 1033, 830, 750 and 570 bp in *Aeromonas sobria*. The remainder unique DNA bands were detected at about 570 and 820 bp in *A.veronii* (Table 8).

The generated RAPD profile by the primer OPV-19 shows a total of 19 bands, which not necessary identified in all isolates. No monomorphic bands (common bands) were detected by OPV-19 primer among the studied isolates (Figure 1b). The profile comprised 7 polymorphic unique bands and 12 non-unique ones. Three unique bands at apparent molecular size of 1800, 1700 and 615 bp were identified in the *A.caviae*. The two unique bands at about 680 and 780 bp were identified on *A.jandaei* and *A.sobria*, respectively. Also, the two bands at about 820 and 570 bp were observed in the *A.veronii* .

The average similarity index between *Aeromonas* isolates was calculated as Nei's original measures of genetic identity and genetic

distance, considering all the amplicons resulting from all the primers. The coefficient of genetic identities ranged from 0.427 to 0.848, most of them were of high magnitude . This result confirmed by[47] used RAPD markers to analyze Avine *E.coli* isolates from the USA (predominately, Gorgia) It is concluded that RAPD provides a rapid and powerful tool to study the epidemiology of avain *E.coli*. As several authors have suggested[48,49,50,51] molecular typing has great discriminating power and could be used in epidemiological studies of *Aeromonas* species .

Fig (2) illustrated that , *Aeromonas spp.* high susceptible to Ciprofloxacin , Ceftriaxone . Moderate susceptible to Gentamycin , Norofloxacin but resistance to Ampicillin , Erythromycin , Spectomycin , this result agree with[52].While , *Pseudomonas spp* were found to be

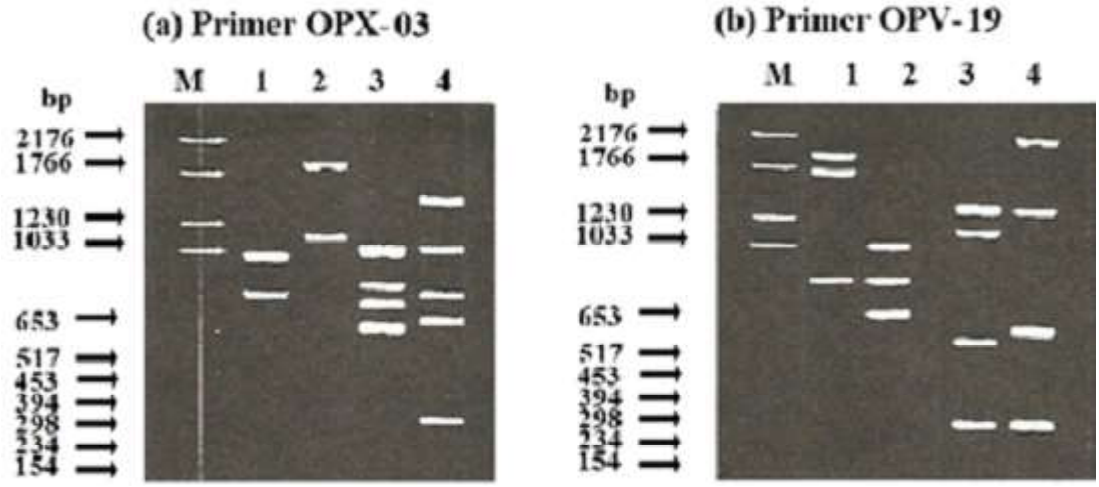

Fig. 1. RAPD fingerprints of the four bacterial isolated using two random primers. Lanes 1-4 are *Aeromonas sobria, A. caviae, A. jandae* and *A. veronii,* respectively Lane M is the standard DNA marker

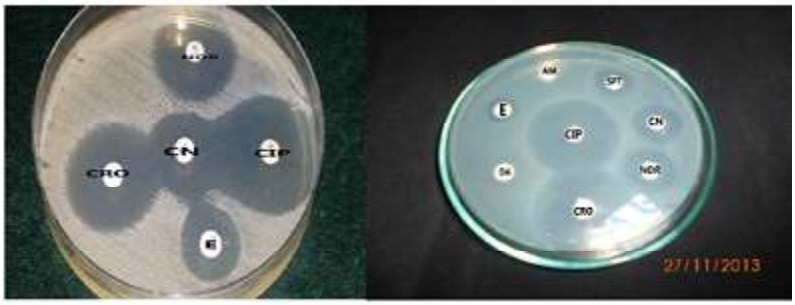

Fig. 2. Antibacterial sensitivity of bacteria isolated from naturally infected *Clarias garpinus* to different antibiotics

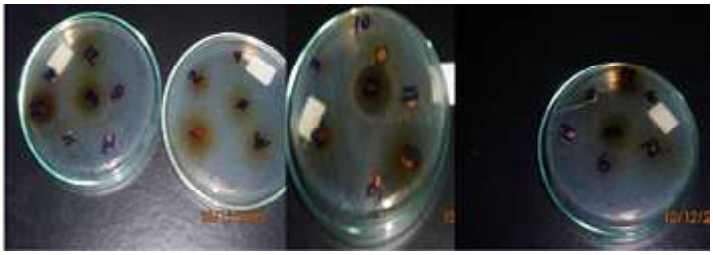

Fig 3. Antibacterial activity of different plant extracts against bacteria isolated from naturally infected *Clarias garpinus*

susceptible to Ceftriaxone and moderate susceptible to Ciprofloxacin but resistance to Ampicillin , Erythromycin , Spectomycin and Gentamycin these results nearly similar to the recorded by[53]. *Vibrio spp* were highly sensitive to Ciprofloxacin , intermediated to Norofloxacin and Ceftriaxone and resistance to remaining antibiotic used . This result in agreement with those reported by[54,37]. *Staphylococcus spp.* susceptible to Ciprofloxacin Ceftriaxone , Gentamycin , Norofloxacin , also resistance to Erythromycin , Ampicillin. Our result incorporate with those reported by[55].

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Ciprofloxacin against *Aeromonas spp* was recorded in Table (9) range from 0.39-1.5micro /ml , *Pseudomonas spp*. MIC and MBC was 25 micro / ml , *Staphylococcus spp*. gave (6.25 and 3.1 micro /ml) for *Staph. aureus* and *Staph. epidermidis* respectively , *Vibrio spp.* recorded 3.1 micro/ml[56,57], in an other hand[58] reported that MIC of Ciprofloxacin was (1 microgram/ml) for 90% of clinical isolates tested, on another hand[59] revealed that the Ciprofloxacin MICs 0.5 to 1.0 ug/ml species for pathogenic bacteria such as *Pseudomonas* , *Staphylococci* and susceptible *S.Pneumonia* , in spite of this broad range in pathogen sensitivities .

Fig (3) showed that alcoholic clove (*Syzygium aromaticum*) extract has the highest inhibitory activity in comparison with other plant extracts , also ethanolic cinnamon (*Cinnamomum verum*) extract showed good inhibitory activity against bacterial isolates while alcoholic extracts of Nutmeg (*Myristica fragrans*) and Hibiscus (*Hibiscus sabdariffa*) have intermediated inhibitory activity against bacterial isolates , but alcoholic extracts of Thymus (*Thymus vulgrais*) and Rosemary (*Rosmarinus officinalis*) have low inhibitory activity, this accordance with[60].

Table (10) showed that minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethanolic clove extract against isolated bacteria recorded 25 micro/L *A.sobria , A.caviae* and *Staph.aureus* resulted 3.1 micro /L while *A.jandaei , A.veronii , Ps.aeruginosa* and *Staph.epidermids , Ps. Fluorescens* gave 12.5 micro /L MIC and MBC . In the case of *Vibrio spp.* recorded 1.5 micro/L[61,62,63].

REFERENCES

1. Feldhusen, F., " The role of seafood in bacterial food borne diseases ". *Microbe Infection,* 2000; **2**: 1651-1660 .
2. Gabriel, U. U.; Akinrotiml, O. A.; Bekibele, D. O.; Onunkwo, D. N. and Anyanwa, F. E., " Locally produced fish feed potentials for aquaculture development in Subsaharan Africa ". *African Journal of Agriculture Research* 2007; **2**(7): 287-295 .
3. Darlington, L. G. and Stone, T. W.,: " Antioxidants and fatty acids in the amelioration of rheumatoid arthritis and related disorders ". *Brazilian J. of nutrition.* 2001; **85**: 251-269 .
4. Roberts, R. J., " *Fish pathology* " (2nd ed.). Bailliere Tindall, London. 1989; **469**: 295-310 .
5. Mortia, K.; Watanabe, N.; Kurata, S. and Kanamori, M. " Beta-lactam resistance of motile *Aeromonas* isolates from clinical and environmental sources ". *Antimicrobial Agents Chemotherapy*, 1994; **38**: 353-355 .
6. Ducenci, S. K. and Candan, A., " Isolation of *Aeromonas* strains from intestinal for a of Atlantic salmon (Salmo solar L.; 1758). *Turk. J. Vet. Anim. Sci*., 2003; **27**: 1071-1075 .
7. Kar, N. and Ghosh, K, : " Enzyme producing bacterial in the gastrointestinal tracts of *Labe rohita* (Hamilton) and *Channa punctata* (Bloch)". *Turk. J. Fish Aquat. Sci*., 2008; **8**: 115-120 .
8. Cantas L., Sorby, J.R., Alestrom, P. and Sorum, H., " Culturable gut microbiota diversity in zebrafish". *Zebrafish* 2012; **9**(1): 26-37 .
9. Carnaham, A. M. and Altwegg, M. Taxonomy, In Austin, B., Altwegg, M., Gosling, P. J. And Joseph, S. W. (Ed.) the genus Aeromonas. John Wiley & Sons Ltd., Chichester, England, 1995; 1-38 .
10. Hatha, M.; Vivekanandhan, A. A.; Julie Joice, G. and Christol., " Antibiotic resistance pattern of motile *Aeromonads* from farm raised fresh water fish". *Int. J. Food Microbial* 2005; **98**: 131-134 .
11. Gomez-Gil, B.; Rogue, A. and J. F. Tumbull. " the use and selection of aquatic organisms in aquaculture ". 2000; **191**: 259-270 .
12. Boklari, F. M., " Antifungal activity of some medicinal plants used in Jeddah Saudi Arabia ". *Mycopathologia*, 2009; **7** (1) : 51-57 .
13. Tassou, C.; Koutsoumanis, K. and Nychas, G. J. E. " Inhibition of Salmonella enteritis's and *Staphylococcus aureus* in nutrient broth by mint essential oil ". *Food Research International,* 2000; **33**: 273-280 .

14. Nascimento, G. G. F.; Locatelli, J.; Freitas, P. C. and Silva, G. L.,: " Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria ". *Braz. J. Microbiol*. 2000; 3194. ISSN 1517-8382 .
15. Austin, B. and Austin, D. A., " Bacterial fish pathogens . Disease of farmed and wild fish " . Praxis publishing, U. K; 2007; 581 pp .
16. Carter, G. R., " Isolation and identification of bacteria from clinical specimens. In: Carter GR, Cole JR. (Eds.) Diagnostic procedures in Veterinary Bacteriology and Mycology. 5th edition, Academic press Inc., San Diego, California 1990.
17. Bergeys Manual of Systematic Bacteriology., Williams and Wilkins Baltimore, London, 1982; 1.
18. Finegold, S. M. and Martin, W. J., " Bailey and Scott's Diagnostic Microbiology. 6th ed. The C. V. Mosby. Co., St., Lowis, Toranto, London, 1982 .
19. Murray, M. G. and Thompson, W. F., " Isolation of genomic DNA from bacteria ". *Nucleic acid research* 1980; **8**: 4321-4325 .
20. Dice, L. R. : " Measures of the amount of ecologic association between species ". *Ecol.* 1945; **26**: 297-302 .
21. Muniruzzaman, M. and Chowdhug, M. B. R., : " Sensitivity of fish pathogenic bacteria to various medicinal herbs ". *Bangladesh. J. Vet. Med.,* 2004; **2**: 75-82 .
22. Rahman, M. M. and Hossain, M. N., " Antibiotic and herbal sensitivity of some *Aeromonas spp.* collected from diseased crap fish. *Progress. Agric,* 2010; **21**(1&2): 117-129 .
23. Thongson, C.; Davidson, P. M.; Mahkarrchanakul, W. and Weiss, J., " Antimicrobial activity of ultrasound-associated solvent extracted species ". *Lett. Appl. Microbiol.* 2004; **39**: 401-406 .
24. Nostro, A.; Germano, M. P.; Angelo, V.; Marino, A. and Cannatelli, M. A.: " Extraction methods and bioautography for evaluation of medicinal plants antimicrobial activity ". *Letti. Microbiol* 2000; **30**(1): 379-384 .
25. Indu, M. N.; Hatha, A. A. M.; Abirosh, C.; Harsha, U. and Vivekanandan, G., " Antimicrobial activity of some of the south-indian spices against serotypes of *E. coli, Salmonella, Listeria monocytogenes* and *Aeromonas hydrophilia* " *Braz. J. Microbiol.* 2006; **37**(2) : 153-158 .
26. Soxhlet, F., " Polytechnisches ". *J. Dingler*, 1879; 232, 461 .
27. Irkin, R. and Korukluoglu, M. " Control of *Aspergillus niger* with garlic , onion and leek extracts ". *Afr. J. Biotechnol;* 2007; **6**: 384-387 .
28. Ahmed, M. M. El-Ashram " On *Aeromonas hydrophilia* infection among cultured *Tilapias* : a biological , histopathological and management studies ". *Egypt. J. Aquat. Biol. & Fish*., 2002; 6(3): 181-202.
29. Wael, G. M., " A trial to detect pathogenic behavior of members of genus *Aeromonas* isolated from fish by using DNA manipulation ". Ph. D. V. Sci. A thesis (Bacteriology) Faculty of Vet. Medicine, Zagazig University 2011.
30. Somayah, M. Awad., " Studies on prevailing bacteria diseases in crap ". Ph. D. V. Sci. A thesis (Fish Disease and Management). Faculty of Vet. Medicine Suez Canal University, 2011.
31. Rahman, M.; Patricia, C. N.; Kuin, I.; Huys, G.; Swingm J. and Mollby, R., " Identification and characterization of pathogenic *Aeromonas veronii biovar sobria* associated with Epizotic ulcerative syndrome in fish in Bangladesh ". *Applied and Environmental Microbiology*, 2002; **68**: 650-655 .
32. Martin-Carnahan, A. and Joseph, S. W.,: " Genus 1: *Aeromonas* stainer 1943, 213. In: Brenner, D. J.; Krieg, N. R. and Staley, J. T. (eds), Bergey's Manual of Systematic Bacteriology, 2nd ed. Vol. 2 the proteobacteria, part B, the gammaproteobacteria. New York, Springer-Verlag, 2005; 557-578 .
33. Austin, B. and Austin, D. A. " Bacterial fish pathogens in disease in farmed and wild fish Ellis Horwood Ltd. Publisher, Chichester, England,1993 .
34. Baumann, P.; Furniss, A. L. and Lee, J. V., " Genus I Vibrio Pacini 1854, 411. In N. R. Kreig and J. G Holt (ed.), Bergey's manual of systematic bacteriology, 1984; **1**: 518-538 .
35. Megahed, A. A.: " Studies on some gram negative bacteria of fish ". M. S. C. Thesis (Microbiology) Fac. of Vet. Med, Suez Canal University, 2000.
36. Eleonor, V. A.; Tendencia, L. and Dureza, A.., " Isolation of *Vibrio* species from *Penaeus monodon* (fabricious) with red disease syndrome. Aquaculture, 1997; **154**: 107-114 .
37. Taghrid, M. N. A., : " Polymerase chain reaction for diagnosis of some fish bacterial pathogens ". M. V. Sci. A thesis (Bacteriology) Faculty of Vet. Medicine, Zagazig University, 2011.
38. Badran, A. F., " Studies on *Pseudomonas* septicemia outbreak among intensively cultured fresh water fishes with special reference to its control ". *Zag. Vet. J*., 1993; **21**(4): 737-746 .
39. Aly, S. E. " Pathological studies on some fish in Suez Canal area ". Ph. D. Thesis, Pathology *Dept. Faculty of Vet. Med., Suez, Canal*

University, 1994.

40. El-Attar, A. A. and Mostafa, M., " Some studies on tail and fin rot disease among cultured *Tilapia* fishes ". *Assiut Vet. Med. J.* 1996; **35**(70): 155-161 .
41. Azza, M. Abd-El-Rahman " Studies on bacterial diseases among cultured *tilapias* ". Thesis (Fish Disease and Management) Faculty Vet. Med., Suez Canal University, 1996.
42. Todar, K. " *Staphylococcus auerus* ". University of Wisconsin, Madison, Department of Bacteriology, 2005.
43. Alshimaa, A. H. M., " Preliminary studies on gram positive bacterial infection in some fresh water fishes " . M. V. Sci. A thesis (fish disease and management) Faculty of Vet. Medicine , Zagazig University, 2007.
44. Maniati, M.; Petinaki, E. and Maniatis, K. A. N., " Antimicrobial susceptibility of *Aeromonas spp., Vibrio spp.* and *Plesiomonas, Shigelloides* isolated in the Philippines and Thailand ". International Journal of Antimicrobial Agents, 2005.
45. Sreedharan, K.; Philip, R. and Singh, S. B., " Virulence potential and antibiotic susceptibility patterns of motile *Aeromonad* associated with fresh water ornamental fish culture systems: A possible threat to public health ". *Brazilian Journal of Microbiology*: 2012; 754-765 .
46. Abd El-Aziz, A. M., " Study the effect of some different factors on some types of bacteria and fungi isolated from armout catfish . " Ph. D. V. M. Sci. A thesis (Bacteriology , Immunology and Mycology) faculty of Vet. Medicine , Moshtohor , Zagazig University (Benha branch) 1996.
47. Maurer, J. J.; M. D. Lee; C. Lobsinger; T. Brown; M. Maier and S. G. Thayer., " Molecular typing of avain *Escherichia coli* isolates by random amplifion of polymorphic DNA". *Avain Diseases.* **42**(3): 431-451 .
48. Davin-Regli, A., Bollet, C., Chamorey, E., Colonna D'istria , V. and Cremieux, A., " A cluster of cases of infections due to *Aeromonas hydrophilia* reavealed by combined RAPD and ERIC-PCR". *Journal of Medical Microbiology* 1998 ;**47**: 499-504 .
49. Solar, L., Figueras, M. J. Chacon, M. R., Guaroo, J. and Martinez-Murcia, A. J., Comparsion of three molecular methods for typing *Aeromonas popoffii* isolates". Antonie van Leeuwenhoek 2003; **83**(4): 341-349 .
50. Szczuka, E. And Kaznowki, A." Typing of clinical and environmental *Aeromonas spp.* Strains by Random Amplified Polymorphic DNA PCR, Repetitive Extragenic Palindromic PCR, and Enterobacterial Repetitive Intergenic Consensus Sequence PCR". *Journal of Microbiology* 2004; **42**(1): 220-228 .
51. Subaskumar, R.' Thayumanavan, T., Thilagavathy, C., Vivekanandhan, C., Savithamani, K. and Lakshmanaperumalsamy, P., " Typing of hemolytic and antibiotic resistant *Aeromonas hydrophilia* isolated from raw milk of Coimbatore, South India*." International Journal of Dairy Sciences* 2006; **1**(1): 70-83 .
52. Sarker, M. A.; Buddin, M. N.; Sarker, M. G. A. and Chowdhury, M. B. R., " Pathogenicity of *Aeromonas sobria* to Thai Silver Barb (Barbodes Gonionotus Bleeker) and its sensitivity to some antibiotic agents ". *Bangladesh. T. Fish. Res.,* 2000; **4** (2): 185-170.
53. Wolska, K.; Jakubczak, A.; Anusz, Z. and Bukowiski, K., " Susceptibility of *Pseudomonas aeruginosa* strains to antibiotics and chemotherapy ". *Med. Ycyna Watery Naryjna.* 1999; **55**(12): 812-817 .
54. Akinbowale, O. L. ; Peng, H. and Barton, M. D., : " Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia *" J. Applied Microbiology,* 2006; **100**(5) : 1103-1113 .
55. Pu, S.; Wang, F. and Ge, B., " Characterization of toxin genes and antimicrobial susceptibility of *Staphylococcus aureus* isolates from Louisiana retail meats ". *Foodborne Pathog. Dis*.; 2011; **8** (2): 299-306 .
56. Elliot, L. Rank.; Iris B. Smith, and Maryfarn, L., Bacteremia caused by *Vibrio hollisae J. Clinical Microbiology*, 1988; 375-376 .
57. Qadri, F.; Alam, M. S.; Nishibuchi, M.; Rahman, T.; Alam, N. H. and Chisti, J., " Adaptive and inflammatory immune responses in patients infected strains of *Vibrio haemolyticus*. *J. Infect. Dis*. 1993; **187**: 1085-1096 .
58. Ko, W. C.; Chiang, S. R.; Lee, H. C.; Tang, H. J.; Wang, Y. Y. and Chuang, Y. C., " In vitro activities of fluoroquinolones against *Aeromonas hydrophilia* ". *Antimicrob Agents Chemother* 2003; **47**: 2217-2222 .
59. Neu, H. C.," Bacterial resistance to fluoroquinolones ". *Rev. Infect. Dis*. 1998; **10**: S57-S63 .
60. Ibtisam, M. A. " Antimicrobial activity of Ethanolic extracts from some medicinal plants ". *Australian Journal of Basic and Applied Sciences*. 2011; **5**(11). 678-683 .
61. Ahmed, A. Tayel. and Wael, F. El-Tras: " Possibility of fighting food borne bacteria by Egyptian Folk medicinal herbs and spices extracts ". *J. Egypt Public Health Assoc*. 2009; **84** (1&2) : 21-32 .

62. Gupta, C.; Garg, A. P.; Uniyal, R. C. and Gupta, S., " Comparison of antimicrobial activities of clove oil and its extract on some food borne microbs. *The International Journal of Microbiology*, 2009; 7 .
63. Maha, M. I.; Essam, T. M.; Mohamed, A. F. and Fathia, E. M., " Screening for the antimicrobial activities of alcoholic and aqueous extracts of some common species in Egypt ". *Int. J. Micro. Resch.* 2012; **3**(3): 200-207.