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Studies on the Prevalence of *E.Coli* and *Salmonella* in Mullet Fish from Different Sources

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ABSTRACT

A total of 120 samples of mullet fish were collected from three different sources; marine water (Port Said), fresh water (Kafr ElZayate) and farms (Kafr Elshiekh) (40 samples from each). The samples were examined for the presence of *E. coli* and *Salmonella*. Thirty-Seven *E. coli* isolates (21 from gills, and 16 from muscles) and fifty-six *Salmonella* isolates (31 from gills, and 25 from muscles) were identified by culture, biochemical analysis. The isolates were studied for their antimicrobial susceptibility using 12 Antibiotics that are mainly used in the veterinary field. *E. coli* isolates were highly sensitive to Amikacin (100%) and ciprofloxacin (97.3%) while resistant to Streptomycin and Doxycycline. *Salmonella* isolates were sensitive to ciprofloxacin (100%) and resistant to Nalidexic acid and Streptomycin. High multiple antibiotic resistance (MAR) index was detected in *E. coli* and *Salmonella* isolates from fresh water fish. By using PCR, the *aad*A2 gene responsible for resistance to Streptomycin was detected in the examined isolates.

1. INTRODUCTION

Enterobacteriaceae is a common water-borne bacterium, which may be present in tissues of apparently normal fish (Newaj et al., 2008). In most instances, disease occurs as result of complex interactions between pathogens, fish and environmental stress, which affect susceptibility of the host to disease (Song et al., 2010). Whenever fish are exposed to environmental stress or injury, it causes serious outbreaks of disease with variable mortalities (Sekar, et al., 2008). The isolation and prevalence of Escherichia coli (*E. coli*) can be used as indicator of water contamination and the surrounding environment of fish (ELsaidy et al., 2015). Further, fish meat spoil more quickly than other foods, particularly about 30% of landed fish are lost through microbial activity alone (Ghaly et al., 2010).

E. coli and *Salmonella* are regarded as the principle diseases causing mortalities, economic losses, condemnation and public health hazard (Fatma et al., 2012). Antimicrobial therapy is an important tool in reducing both the incidence and mortality associated with *E. coli* infection in fish but not the best solution due to bacterium`s acquired resistance to antibiotics (Schroeder et al., 2002). Infections with antibiotic resistant bacteria lead to difficult therapeutic options for infection treatment (Murugan et al., 2011).

The aim of this study is the isolation and biochemical identification of E. coli and Salmonella isolated from fish from different sources. Moreover, evaluation of antibiotic sensitivity for the isolated bacteria and identify the *aad*A2 gene responsible for resistance to Streptomycin to by using PCR.

2. MATERIAL AND METHOD:

2.1. Sample collection:

A total of 120 samples of mullet fish from marine water source (Port said), fresh water source (Kafr El.Zayate) and farm source (Kafr Elsheikh) were collected. Forty samples from each source were collected in separate plastic bag and kept in icebox then transferred directly to the laboratory (Rocha et al., 2014).

Table (1): Oligonucleotide primers sequences for amplification of streptomycin resistance gene of *E. coli*.

Primer	Sequence	Amplified product	Reference
*aad*A2	TGTTGGTTACTGTGGCCGTA	622 bp	Walker et al. (2001)
	GATCTCGCCTTTCACAAAGC		

2.2.1. Isolation and identification of *E. coli* and *Salmonella*

2.2.1. Fish swab samples were taken from gills and muscles then inoculated onto nutrient broth and tetrathionate broth and incubated at 37ºC for 24h (2 h). After that the colonies were sub-cultured on MacConkey agar medium and Eosin Methylene blue medium (EMB) and incubated at 37ºC for 24h (Gupta et al., 2013). Colonies suspected to be *E. coli* were subjected to Gram staining and biochemical identification (Alexander et al., 2010). While in case of colonies suspected to be Salmonella were subjected to Selenite F. broth and incubated at 37ºC for 24h and sub-cultured on Salmonella-Shigella agar medium (SS) or Xylose Lysine Deoxycholate Agar medium (XLD) then incubated at 37ºC for 24h. The colonies were subjected to Gram staining and biochemical identification (Alexander et al. 2010).

2.2.2Antimicrobial susceptibility testing (disc diffusion)

The Antimicrobial susceptibility testing by using disc diffusion method was carried out according to the Clinical Laboratory standards Institute (CLSI, 2012). The following antibiotics were used: Penicillin (P; 10µg), Amoxicillin clavaunic acid (Amc ;25µg), Cefotaxime (CTX; 30µg), Cefaclor (CEC; 30µg), Ciprofloxacin (CIP; 5µg), Ofloxacin (OFX; 5µg), Amikacin (AK; 30µg), Ceftriaxone (CRO; 30µg), Sulfamethoxazole-trimethoprim (SXT; 25µg), Cefidime (CAZ; 30µg), Doxycycline (Do; 30µg), Streptomycin (S; 25µg), Ampicillin (AM; 10µg), Nalidixic acid (NA; 30µg) (Rocha et al., 2014).

2.2.3. Polymerase chain reaction (PCR) for detection of Streptomycin Resistance Gene in *E. coli* isolates:

DNA was extracted from 10 pure cultured *E. coli* by phenol-chloroform method according to Sambrook et al., 1989 using Emerald Amp GT PCR master mix kit (Takara). The PCR mixture was prepared by adding 12.5 µl Emerald Amp GT PCR master mix, 4.5 µl PCR grade water, 1 µl Forward primer (20 pmol), 1 µl Reverse primer (20 pmol), 6 µl Template DNA to a total volume of 25 µl. The primers used are listed in Table (1).

3. RESULTS

3.1. Incidence of pathogenic *E. coli* and *Salmonella* isolates in examined fish samples:

A total of 120 samples were collected from three different sources (120) from gills and (120) from muscles; 40 from each source. Thirty-seven samples were *E. coli* positive (21 from gills, and 16 from muscles) and fifty-six samples were *Salmonella* positive (31 from gills and 25 from muscles) (Table 2). The *E. coli* isolates gave the characteristic greenish-black metallic sheen and pink colonies on Eosin Methylene blue media(EMB) and MacConkey agar, respectively. While, the salmonella isolates gave characteristic black and yellowish Ash colonies on Xylose Lysine Deoxycholate Agar media (XLD), Salmonella-Shigella agar media (SS)and MacConkey agar, respectively. Moreover, both *E. coli* and salmonella revealed Gram-negative rods under microscopic examination. *E. coli* isolates were indole, Methyl red, sugar fermentation and gas production positive, Citrate utilization, urease and H2S production negative while *Salmonella* isolates were H_2S production positive.

3.2. Antimicrobial susceptibility

Antimicrobial susceptibility was observed in all *E. coli* isolates from different samples: from gills; n = 21 and muscles; n = 16. Antimicrobial susceptibility was also observed in all *Salmonella* isolates from different samples: from gills; n= 31 and muscle; n=25 as shown in Table (3). High multiple antibiotic resistance (MAR) index was detected from *E. coli* isolates (Table 4) and *Salmonella* isolates (Table 5).

Table (2): Incidence of *E. coli* and *Salmonella* isolated from Mullet fish from different sources

Source	Gills			Muscles			Total +ve *E. coli* No. (%)	Total +ve *Salm* No. (%)
	No. of samples	*E. coli* +ve No. (%)	*Salm* +ve No. (%)	No. of samples	*E. coli* +ve No. (%)	*Salm* +ve No. (%)		
Fresh water	40	11 (27.5)	13 (32.5)	40	9 (22.5)	10 (25)	20 (25)	23 (28.75
Farm	40	6 (15)	10 (25)	40	5 (12.5)	8 (20)	11 (13.75)	8 (22.5)
Marine fish	40	4 (10)	8 (20)	40	2 (5)	7 (17.5)	6 (7.5)	15 (18.75)
Total	120	21 (17.5)	31 (25.8)	120	16 (13.3)	25 (20.8)	37 (15.4)	56 (23.3)

Salm; *Salmonella*

Table (3): Antimicrobial resistance of *E. coli* and *Salmonella* isolated from Mullet fish from different sources

Antibiotics	Resistance No. (%)	
	E. coli	*Salmonella*
Cefaclor (CRO)	8/37 (21.6%)	ND
Pencillin (P)	23/37 (62%)	46/56 (82.14%)
Ciftrixione (CEC)	9/37 (24%)	ND
Amikacin (AK)	0/37 (0%)	52/56 (92.9 %)
Amoxicillin\ clavulinic acid (AMC)	31/37 (83.8%)	15/56 (26.8 %)
Cefotaxime (CTX)	32/37 (86.5%)	22/56 (39.3%)
Ceftazdime (CAZ)	30/37 (81%)	ND
Ciprofloxacin (CIP)	1/37 (2.7%)	0/56 (0%)
Ofloxacin (OFX)	2/37 (5.4%)	3/56 (5.4%)
sulfamethoxazol Trimethoprim (SXT)	27/37 (73%)	2/56 (3.6 %)
Doxycycline (DO)	35/37 (94.6%)	ND
Streptomycin (S)	37/37 (100%)	56/56 (100%)
Ampicillin (AM)	ND	4/56 (7.14 %)
Nalidixic acid (NA)	ND	54/56 (96.4%)

Table (4): MAR Index of *E. coli* isolated from mullet fish from different sources

MAR	Number of isolate		Total
	Gills	Muscles	
0.25	1	0	1
0.33	0	1	1
0.4	3	3	6
0.5	8	5	13
0.58	3	5	8
0.66	6	2	8

Table (5): MAR Index of *Salmonella* isolated from mullet fish from different sources

MAR	Number of isolate		Total
	Gills	Muscles	
0.3	3	1	4
0.4	15	12	27
0.5	10	8	18
0.6	2	4	6
0.7	1	0	1

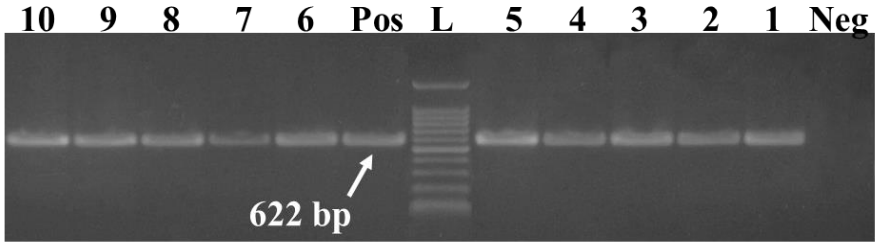

Figure 1. Agarose gel electrophoresis of amplified DNA showing the specificity of the single reactions for the detection of the *aad*A2 gene. Pos; positive control, Neg; negative control, L; 100 bp DNA ladder. 1-10; *E. coli* isolates.

3.2. Detection of Streptomycin (*aad*A2) gene in *E. coli* isolates

All *E. coli* isolates (Table 2) were 100% resistant to streptomycin as shown in Table (3). Therefore, the *aad*A2 gene responsible for resistance of *E. coli* to streptomycin was detected in 10 isolates. ALL isolates were positive to the *aad*A2 gene as shown in Fig.1.

4. DISCUSSION

Enterobacteriaceae comprises many species that have been reported to be of health hazard for the consumer. They are also economically important as they may cause spoilage deterioration of fish meat and fish meat product (ICMSF, 1980 & National Academy of Science, 1985).

Bacterial diseases in fish generally do not develop simply as the result of exposing a host to an infectious agent. In most instances, disease occurs as the result of complex interactions between pathogen, fish and environmental stress, which affect the susceptibility of the host to disease (Wedekind et al., 2010).

In the present study, *E.coli* and *Salmonella* were isolated from gills and muscles of apparently healthy mullet fish from fresh water, farm and marine sources. The overall incidence of *E.coli* and *Salmonella* in mullet fish as in Table (2) was 15.4 % and 23.3 %, respectively. The percent of isolation varied according to the source of the fish for both *E.coli* and *Salmonella*: 25% and 28.75 % from fresh water, respectively, 13.75 % and 22.5 from farm fish, respectively, and 7.5% and 18.75 % from marine water, respectively. The total incidence of *E.coli* and *Salmonella* isolation from the gills of all samples were 17.5% and 25.8 %, respectively. And from the muscles of all samples were 13.3% and 20.8 %, respectively (Table 2). This result nearby with the results reported by(Azza et al.,2012) for *E.coli*, who recorded 14.3% in gills but in muscles 0% while lower than the results of Arafat et al., (2013) who recorded the detection of *Salmonella* by 33.3% in the gills and 46.7% in the muscle with a total percent 40% of all samples.

The results showed that high bacterial load was observed in both gills and muscles with the highest level isolated from fresh water fish. was collected from Kafr El-Zayat region, which is characterized by the presence of high level of contamination because of factories sewage. This agrees with (Austin B & Austin D 1999) who reported that most of the microorganisms in fish tissue are thought to result from the surface, gills, or intestinal contamination, where microorganisms are adsorbed on the surfaces of the fish and found in their intestinal contents and muscle. Therefore, the initial microbial flora on the caught fish is dependent upon the contamination of the water, bottom sediment from the area of catch and the food entering the digestive tract. Further, the total results of *E. coli* recorded in this study is similar to that of Shaltout et al. (2009) who evaluated the total microbial count including fungi, yeasts and found it to be high in Kafr El-Zayat region (Total Bacterial Counts) indicating fecal and/or industrial pollution. While in farm fish results in this study show 15% &12.5% in gills and muscles for E.coli , and 25% &20% in gills and muscles for salmonella this result is differ totally to Elham ., (2017) who reported that E.coli in farm fish gills &muscle 25%,22.5% respectively ,and for salmonella 7.5%&5% in gills and muscle respectively.also in marine fish of this study result show 7.5% & 18.75% for *E.coli* and *Salmonella* respectively that near in result of(El sheriff et., al 2014) who record 8% &12% for *E.coli* and *Salmonella* respectively

The results of *E. coli* antibiotic sensitivity showed that Amikacin, Ciprofloxacin and Ofloxacin were the most effective antimicrobials with resistance of 0%, 2.7% and 5.4%, respectively. The resistance was observed in all isolates from both gills and muscles. These

results agreed with zahraei.t and farashi.s, (2006) who reported low antimicrobial resistance of *E.coli* to Amikacin, ciprofloxacin, ofloxacin with 0%, 2.2% and 2.3%, respectively. The *E.coli* isolates were highly resistant to sulfamethoxazole-Trimethoprim, Amoxicillin, Cefotaxime, Ceftazidime, Doxycycline and Streptomycin, which were less effective with 83.8%, 86.5%, 81%, 94.6%, 100%, respectively in comparison to results reported by Lambie et.al., (2000) who recorded that *E.coli* isolates were highly resistant to Amoxicillin with 100%. On the other hand, Giruov, (1985) recorded the sensitivity of *E.coli* to Amoxicillin with 83.8 %. Streptomycin results showed that 100% of *E.coli* isolates were resistant agreed with Machado et.al., (2008) and Theresa et.al., (2009) who recorded that *E.coli* isolates were resistant to sterptomycin with 93% and resistant to sulfamethoxazole Trimethoprim with 67%.

The results of Multiple antibiotics resistance (MAR) showed that MAR were detected in high percent in gills and muscles of samples from fresh water than other two sources. These results were agreed with Rosha et al., (2014) and Jiao et al., (2007) who reported lower MAR index than in our study. However, MAR index reported by Sakr et al., (2015) was nearly similar to our results with referring to water pollution and its effect on antibiotic resistance.

Results of antibiotic sensitivity to salmonella:

The results of antimicrobial agents showed that the most effective agents with low resistance to salmonella were Ampicillin ,Ciprofloxacin, Sulfamethoxazol Trimethoprim and Ofloxacin with 7.4% ,0%,3.6% and 5.4% respectively. These results were agreed with (Akter et al. 2007) and Luciana et al. 2011).

On the other hand, ciprofloxacin Sulphamethoxazole/ Trimeoprim and ofloxacin showed antibiotic resistance with 20%,0% and 8%, respectively which disagreed with results that recorded by El-Jakee et al. (2010) who recorded that those antimicrobial agents resistancy to salmonella were 100%,100% and 98%, respectively.

Our results showed resistance to penicillin,Amikacin ,streptomycin, nalidixic acid were less effective with percent 82.14%, 92.9% , 100%,96.4%. This result is different to results recorded by (De Oliveira et al. (2006) whose results were resistant to sulfamethoxazole/trimethoprim or sulfazotrim and only one was resistant to chloramphenicol. But similar in result in the predominant resistance to nalidixic acid (21.5%), and streptomycin (11.4%). Higher Mark index were detected from Salmonella isolates in gills as show in table (5)

Detection of gene aadA2 in *E. coli* strain which one of genes that responsible for resistance of E. coli to streptomycin

Recording the result of RCR for the detection of aadA2 genes among 10 *E.coli* isolated strains , all isolates contain aadA2 encoding genes, this gene was one of genes that responsible for resistance of *E.coli* to streptomycin , this result similar toAshraf. M. Ahmed et al.,(2004):who recorded gene aadA2 confers resistance to streptomycin in *E.coli* strains isolated from travelling patients but Marianne Sunde et al.,(2005): reported that The distribution of streptomycin in *E. coli* can be greatly influenced by the genes encoding resistance to streptomycin. The strA-strB genes are probably involved in conferring high-level resistance to streptomycin, whereas the case for the aadA gene cassettes shown in low level resistance to streptomycin. Also most isolates of *E. coli* exhibited resistance to antibiotics used in many animal products, mainly streptomycin, sulphonamides, integrons were commonly identified and corresponding to a few integron types that have been mostly found among Enterobacteriaceae from food animals some of them globally desiminated as type I1 (aadA1), type II1 (dfrA1-aadA1),typeIII1 (dfrA12-orfF-aadA2) Antunes et al.,(2006), Bass et al.,(1999), Box et al.,(2005).

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