Role of *Atherina* Species in Transmitting some Bacterial Diseases to Human

Mohamed E. M. Mohamed, Maysa A.I. Awadallah*, Magda A. Amin, and Rasha M. M. Abou-Elez
Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt
maysavet@hotmmail.com*

Abstract: A total of 530 samples (300 from fresh water marine *Atherina*), 130 samples from water used for preparation of Atherina fish for selling, and 100 hand swabs from their handlers) were collected from randomly selected markets from 3-localities in Sharkia governorate, Egypt. All samples were examined for the presence of *Staphylococcus* species and *Enterobacteriaceae*. Moreover, the effectiveness of freezing, salting, and commercial vinegar (5% acetic acid) treatment on the survivability of *Staphylococcus* spp. and *Enterobacteriaceae* in *Atherina* fish was also evaluated. Results revealed *S. aureus* were detected in 65.7% of the surface swabs and 35.7% of muscle samples of fresh water *Atherina* fish. The prevalence of *S. aureus* in the surface swabs and muscle samples of marine *Atherina* fish were 62.2% and 25.6%, respectively. *Enterobacteriaceae* isolated from surface swabs of *Atherina* fish were; *E. coli* (5.33%), *Kl. Oxytoca* (7%), *Kl. pneumoniae* (5.7%) *Ent. cloacae* (5%), *P. vulgaris* (9%), *P. mirabilis* (6.3%), *Sh. sonnei* (1.7%), *Cit. freundii* (5%), *Cit. koseri* (6%), *Pantoea agglomerans* (38.3%), *Hafnia alvei* (1.7%), *M. morganii* (2.3%), and unidentified spp. (8.7%). The percentages of isolation of the previous species from muscle samples of *Atherina* fish were 0.7, 2.3, 1.3, 1.7, 5, 3.7, 0.7, 1.7, 2.7, 24.7, 1.3, 1.3, and 3.7, respectively. The prevalence of *S. aureus* was 53.1% in water samples used for preparation of fish for selling. *Enterobacteriaceae* isolated from water samples were *E. coli* (6.15%), *P. mirabilis* (7.7%), *P. vulgaris* (11.5%), *Ent. Cloacae* (7.7%), *Cit. freundii* (6.15%), *Cit. koseri* (6.9%), *Kl. pneumoniae* (7.7%), *Kl. oxytoca* (9.2%), *Pantoea agglomerans* (30.7%), *Hafnia alvei* (2.3%), *M. morganii* (3.1%), *Sh. sonnei* (1.5%), and unidentified species (3.8%). *S. aureus* was isolated from 73 hand swabs. *Enterobacteriaceae* isolated from hand swabs were *E. coli* (5%), *P. vulgaris* (8%), *P. mirabilis* (5%), *Kl. Pneumonia* (6%), *Kl. Oxytoca* (7%), *Ent. Cloacae* (6%), *Cit. freundii* (4%), *Cit. koseri* (5%), *Pantoea agglomerans* (36%) and unidentified species (18%). Ten representative biochemically identified *E. coli* isolates (4 from *Atherina* fish, 3 from water used for preparation of fish for selling, and 3 from hand swabs of fish handlers) were identified as O_{128} (2-strains), O_{114} (strain), and O_{136} (strain) from *Atherina* fish, O_{26} (strain), O_{111} (strain) and untyped strain from hand swabs of fish handlers. However, all isolates from water samples were O_{128}. The survivability experiment revealed that all muscle samples were negative for all bacteria species growth from the 1st week of freezing. After 1st week from freezing, all *Enterobacteriaceae* were continued to isolate (1:4 each) from the surface swabs of the 4 examined samples. On the other hand, *S. aureus* was continued to isolate at a rate of 4:4 . All *Enterobacteriaceae* except *P. mirabilis* (each with 1:4), *S. aureus* (4:4), Coagulase negative *Staphylococcus* spp. (1:4) were continued to isolate after the 2nd week from freezing. The isolated species after the 3rd week of freezing were *Kl. oxytoca*, *Pantoea agglomerans*, and un-identified species (1:4 each), and *S. aureus* (4:4). *Pantoea agglomerans*, *un-identified* species and *S. aureus* were continued to isolate after 4th week. The un-identified species (1:4) and *S. aureus* (4:4) were continued to isolate until the week 13 from freezing. *Kl. oxytoca*, *P. vulgaris*, *P. mirabilis* (1:4, each) were isolated from surface swabs of fresh water *Atherina* fish salted in NaCl solution (25%). Moreover, *Pantoea agglomerans* and *S. aureus* were isolated with ratios of (2:4) and (4:4) of the same samples, respectively. On the other hand, the bacterial spp isolated from the muscle samples of fish salted at NaCl 25% were *Kl. oxytoca*, *Pantoea agglomerans* (1:4, each). All samples salted in 50% and 75% NaCl solution were negative for the presence of *Enterobacteriaceae* from the 1st week and for the whole period of the experiment. *E. coli* was continued to isolate until the 6th hours of treatment but stop to grow after 7 hours from vinegar treatment. *S. aureus* was negative in all treated samples from the 1st hour of treatment.

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1. Introduction:

The recent food scares related to ruminant's meat (Bovine spongiform encephalopathy and Scrapie), chicken meat (Avian Influenza), the increased prices of beef and poultry meat, and the dietary changes of the consumers toward healthier food habits have resulted in a rapid rise of seafood consumption worldwide (Yasumoto, 2000).

However, as demands for and production of seafood increased, it becomes important to pay more

attention to seafood safety and seafood related diseases (Gillespie *et al.*, 2001). Center of Disease Control (CDC) and U.S. Department of Agriculture recorded that seafood is 25 times more likely than poultry to cause illness in man (FAO, 2001).

Atherina species are small fishes in coastal areas belonging to family *Atherinidae* which feeds on small crustaceans and fish larvae (Maugé, 1990). They are considered an important source of animal protein especially for poor people in Egypt.

Nowadays, water pollution with domestic waste water is most common in River Nile and its tributaries; the greatest volume of wastes discharged into the water course is sewage. Such pollution reduces the water quality and had been reported as a precursor to fish infection with microorganisms that can infect both consumers and handlers (Omer *et al.*, 2004) either through ingestion of fish meal or through direct contact through abraded skin (Czachor, 1992). Moreover, *Atherina* species are liable for contamination after being harvested either from infected fish handlers or contaminated utensils and equipment during transportation, distribution and food preparation.

Fish may serve as carrier for several zoonotic bacteria as *E. coli*, *Salmonella* sp., *Aeromonas* sp., *Pseudomonas* sp., *Proteus* spp., *Shigella* spp., *Staphylococcus aureus*, *Erysipelothrix* which are incriminated in food poisoning, skin disorders, allergic condition as well as other infection (Janssen, 1970).

Due to the perishable nature of fish, various fish preservation techniques have been developed through history. Both land and marine fish suffer from wide seasonal variation in catch volume. To maintain a stable fish supply during times of scarity and to maintain such fish with low microbial load, drying, salting, fermentation, smoking, addition of antimicrobials and freezing were widely practiced (Al-Jedan *et al.*, 1999). Sodium chloride, trisodium phosphate, lactic acid, and acetic acid have been used as food preservatives are generally recognized as safe (Branen *et al.*, 1990). On the other hand, icing of fish was found to cause a decrease in the aerobic, mesophilic, and psychrotrophic bacteria after 5-days of storage (Lalitha and Surendran, 2006).

2. Materials and methods:

A total of 530 samples (300 from fresh water marine *Atherina*, 130 samples from water used for their preparation for selling, and 100 hand swabs from their handlers) were collected from randomly selected markets from 3-localities in Sharkia governorate, Egypt. All samples were examined for the presence of *Staphylococcus* species and *Enterobacteriaceae*. Moreover, the effectiveness of freezing, salting, and commercial vinegar (5% acetic acid) treatment on the survivability of *Staphylococcus* spp. and *Enterobacteriaceae* in fresh water *Atherina* fish was also evaluated.

I. Occurrence of *Staphylococcus* spp. and *Enterobacteriaceae* in *Atherina* fish, water used for their preparation for selling, and hand swabs from their handlers.

A. Sampling:

1.1.1. *Atherina species*:

Fish samples were identified as marine or fresh water *Atherina* fish according to their characteristic morphology (Maugé, 1990) then placed in polyethylene bags, ice packed and transferred to the laboratory within short time. Surface swabs and muscle samples from each fish were collected according to (Sanaa, 2009) as the followings:

1. Surface swabs:

A sterile swab was rolled over the surface of each fish to be examined and immersed in a tube containing buffered peptone water (BPW).

2. Muscle samples:

The surface of fish was sterilized with a red hot scalpel; a part from the muscle underlying the sterilized surface was then picked up by the mean of sterile spatula and placed into sterile tube containing buffered peptone water.

3. Water used for preparation of *Atherina* fish for selling:

One hundred and thirty water samples that used for preparation of *Atherina* fish for selling were collected from the same localities from which *Atherina* fish were collected. All samples were collected under complete aseptic conditions in sterile colourless glass bottles provided with stopper. The samples were dispatched on ice and transferred to the laboratories with a minimum time of delay. Twenty-five ml of each water sample were added to 225 ml BPW and incubated at 37ºC for 18-24 hours.

4. Hand swabs from fish handlers:

Sterile swabs were moistened in sterile BPW, rolled all over the hand and then immersed into test tubes containing BPW. The tubes were labeled with respect to name, age, sex, and date of collection. The tubes were put on ice, packed and transferred to the laboratory with in a minimum time of delay.

B. Bacteriological examination according to (Cruickshank *et al.*, 1975):

1. Isolation and identification of *Staphylococcus* species*:*

It was done according to (Sonnerwirth and Jarett, 1980). Characteristic yellow colonies with yellow zones and small red colonies with no colour change in surrounding medium from each plate of mannitol salt agar (Oxoid, CM $_{85}$) were purified on nutrient agar (Oxoid, CM_3) slants and incubated at 37°C for 18-24 hours for further identification including:

1.1. Microscopical examination:

Films were prepared from the pure cultures and stained by Gram's stain technique and examined under ordinary microscope (10x and 40 x) to verify the presence of characteristic features of the organisms and to confirm the specificity of the colonies.

1.2. Biochemical identification (Catalase test and Tube coagulase test)

2. Isolation and identification of *Enterobacteriaceae*:

It was done according to Koneman et al., (1997). Characteristic pink colonies and pale colonies on MaCconky agar (Oxoid, CM7) plates were purified on nutrient agar (Oxoid, CM_3) slants and incubated at 37°C for 18-24 hours for further identification including:

2.1. Microscopical examination:

2.2. Detection of motility:

It was detected by stabbing the suspected microorganisms into semi-solid agar tubes containing 0.4% agar and incubated at 37°C for 3 days. Spreading of the growth from the stab line was considered positive.

2.3. Biochemical identification: (Oxidase test, Indole test, Methyl red test, Voges proskauer test, Citrate utilization test, Urea hydrolysis test, Sugar fermentation test, H_2S production, and Decarboxylation of arginine, ornithine and lysine).

2.4. Serological identification

Ten representative isolates of the bacteriologically and biochemically identified colonies of *E. coli* from *Atherinae* fish (No. = 4), water used for their preparation for selling (No. =3), and hand swabs of their handlers (No. = 3) were subjected to serological identification at Animal Health Research Institute, Dokki, Giza, and the Central laboratories of the Ministry of Health and Population, Cairo, Egypt. Serodiagnosis was done by slide agglutination technique (Sojka, 1965) using set 1:O polyantisera and related mono antisera (Denka Seiken Co).

II. Effectiveness of freezing, salting (NaCl), and commercial vinegar (5% acetic acid) treatment on the survivability of *Enterobacteriaceae* and *Staphylococcus* species in *Atherina* fish:-

1. Freezing

Ten *Atherina* fish were randomly taken from a total quantity of one kilogram of fresh water *Atherina* and examined for the presence of both pathogens before freezing process. The remaining *Atherina* fish were divided into 13 parts. Each part was placed into sterile polyethylene bag. Each bag was tightly sealed and kept in deep freezer at -20°C. Four *Atherina* fish from the first bag was thawed and their surface swabs and muscle samples were examined for the presence of *Enterobacteriaceae* and *Staphylococcus* species after one week from freezing. This step was repeated with the remaining bags until 13-week.

2. Salting (NaCl- treatment):

Ten-representative fresh water *Atherina* samples randomly taken from a total quantity of one kilogram of fresh water *Atherina* were examined for the presence of *Enterobacteriaceae* and *Staphylococcus* species before salting treatment. The remaining quantity of *Atherina* fish was divided into 3-groups. The first group was immersed in NaCl solution at a concentration of 25% (wt/vol). The 2nd and 3rd groups were immersed in the same solution with concentrations of 50%, and 75%, respectively. From each groups 4-representative *Atherina* fish samples were picked up, washed with sterile water to remove the NaCl residues then surface swabs and muscle sample of each fish were examined for the presence of *Enterobacteriaceae* and *Staphylococcus* species. This process was repeated at an interval of 1-week for 6 weeks (Ez El-Din, 1999).

3. Commercial vinegar (Acetic acid 5%) treatment:

Ten-representative fresh water *Atherina* samples randomly taken from a total quantity of one kilogram of fresh water *Atherina* were examined for the presence of *Enterobacteriaceae* and *Staphylococcus* species before their treatment with commercial vinegar solution containing 5% acetic acid. The remaining quantity of *Atherina* fish was immersed in vinegar. Four fish samples were picked up and washed with sterile water to remove the vinegar residues. Surface swabs and muscle samples of fish were examined for the survival of *Enterobacteriaceae* and *Staphylococcus* at an interval of 1-hour and for 24 hours (Bin-Jasass, 2008).

3. Results and Discussion:

Atherina spp and other fish species are subjected to many risks of contamination which result from sewage pollution of their aquatic environment or from unhygienic handling during

harvesting, transportation and processing (Ibrahim *et al*., 2009). *S. aureus*, the main cause of food intoxication in man due to consumption of the preformed toxins in various food stuffs, and members of the family *Enterobacteriaceae* are the predominant bacterial pathogens transmitted from *Atherina* spp to man. They are involved in enteritis, urinary and respiratory tract infection as well as pyogenic infection (Novothy *et al*., 2004).

Results in Table (1) clarifiy that the overall prevalence of *S. aureus* infection in surface swabs versus muscle samples of all examined *Atherina* spp. were 64.7% Vs 32.7%.The comparable prevalence of coagulase negative *Staphylococcus* spp in the same samples were 40% Vs 22%. The percentage of *S. aureus* infection in muscle samples of *Atherina* fish is nearly similar to that recorded by Mugula and Lyimon (1992). Lower percentages were previously reported by Wienke *et al*. (1994) in fish and shellfish flesh; Normanno *et al*. (2005) in fish flesh, and Simon and Sanjeev (2007) in flesh of fishery products.

Regarding the occurrence of *Staphylococcus* spp in fresh water *Atherina*, it is clear from table (1) that *S. aureus* was isolated from surface swabs and muscle samples of fresh water *Atherina* with the percentages of 65.7 and 35.7, respectively. The recorded infection rate of *Staphylococcus* spp in the muscle samples of fresh water *Atherina* is in agreement with those reported by Noha and Ghada (2006) in sardine fish and Metawea and Abdel-Ghaffar (2007) in *Tilapia nilotica.* On the other hand, higher infection rate with *S. aureus* (43% and 53%) was recorded by Omaima and El-Kewaley (2008) and Ibrahim *et al.* (2009). In contrary, lower infection rates of 4.35% and 3.93% of *S. aureus* in *Tilapia nilotica* and *Labea niloticus*, respectively were previously recorded by Abo-El-Alla and Bastawrows (1999).

Concerning the occurrence of *Staphylococcus* spp in marine *Atherina* fish, results illustrated in table (1) show that *S. aureus* was isolated with percentages of 62.2 and 25.6, respectively. Mohamed *et al.*, (2001) and Omaima and El-Kewaley (2008) isolated the same pathogen from flesh of shellfish (*Crabs* and *Shrimps*) and *Mugile cephalus* flesh with the percentages of 25 and 32, respectively. On the other hand, Papadopoulou *et al*. (2007) recorded higher infection rate of 80% in the flesh of marine fish.

It was clear from table (1) that the prevalence of *S. aureus* is high either in fresh water or marine *Atherina* spp. This may due to higher prevalence of *S. aureus* in human being either on their skin or in their nose that create a great chance for contamination of *Atherina* species up on improper handling through persons who are not observing the basic rules of personal hygiene. This substantiates the conclusion of Papadopoulou *et al*. (2007) that the origin of *S. aureus* is the handlers rather than fish.

Coliforms are commonly inhabitant in the intestinal tract of animals and human and its presence in fish may be attributed to absence of acceptable hygienic measures during harvesting or to sewage contamination of the aquatic environment of fish (Mona *et al*., 2003). The flesh of healthy fish is considered bacteriologically sterile (National Academy of Science, 1985). The bacterial isolates from gills and skin could be mainly accounted for filtering ability of the gills or the slime layer of the skin and particularly as a result of active multiplication and adaptation. Such bacteria may spread to the vascular system and invade the fish flesh, which are not in direct contact with the external aquatic environment (Shewan, 1971).

Table (1): Occurrence of *Staphylococcus* spp. in surface swabs and muscle samples of *Atherina* fish.

Isolated microorganisms	*Atherina* spp								Total number (300)			
	Fresh water *Atherina* spp (210)				Marine *Atherina* spp (90)							
	SU.S		Ms		SU.S		Ms		SU.S		Ms	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
S. aureus	138	65.7	75	35.7	56	62.2	23	25.6	194	64.7	98	32.7
Coagulase negative *Staphylococcus* spp	82	39.1	55	26.2	38	42.2	11	12.2	120	40	66	22

SU.S: Surface swabs of fish MS: Muscle samples of fish No.: Number of positive

The results of Table (2) verify that the isolated *Enterobacteriaceae* were *E. coli, Kl. oxytoca, Kl. pneumoniae, Ent. cloacae, P. vulgaris, P. mirabilis, Sh. sonnei, Cit. freundii, Cit. koseri, Pantoea agglomerans, Hafnia alvei, M. morganii* and unidentified microorganisms. The respective overall prevalence of those pathogens in surface swabs versus muscle samples of the all examined *Atherina* spp were 5.33% Vs 0.7%, 7% Vs 2.3%, 5.7% Vs 1.3%, 5% Vs 1.7%, 9% Vs 5%, 6.3% Vs 3.7%, 1.7% Vs 0.7%, 5% Vs 1.7%, 6% Vs 2.2%, 38.3% Vs 24.7%, 1.7% Vs 1.3%, 2.3% Vs 1.3%, and 8.7% Vs

3.7%, respectively. Nearly similar results were obtained by Abo El-Alla and Bastawrows (1999), who isolated *Kl. pneumoniae* (0.58%), *Kl. oxytoca* (1.73%), *Cit. freundii* (10.14%), *P. vulgaris* (1.73%), and *P. mirabilis* (8.41%) from *Tilapia nilotica*. They also isolated these pathogens with respective percentages of 0.56, 2.81, 7.87, 5.16 and 6.74 from *Labeo niloticus.* However, all samples they examined were free from *E.coli*.

On the other hands, Maysa and Abd Elall (2009) isolated the same pathogens from *Tilapia nilotica* and *Clarias lazera*, their results were *E. coli* (30%), *Ent. cloaca* (20%), *Ent. agglomerans* (8.33%), *Ent. aerogens* (3.33%), *Cit. freundii* (13.33%), *Kl. pneumoniae* (16.67%), *Kl. oxytoca* (8.33%), *P. vulgaris* (21.67%), and *P. mirabilis* (8.33%). Moreover, Sifuna *et al.,* (2008) found that all the examined fish samples (100%) were positive for presence of *E. coli*. They concluded that the pollution of river's and lakes' water by products of man continues to create public health problem especially food borne diseases caused by members of family *Enterobacteriaceae*.

Results of (Table 2) also verify that *Enterobacteriaceae* isolated from the surface swabs versus muscle samples of fresh water *Atherina* fish were *E.coli* 5.71% Vs 0.95%, *Kl. Oxytoca,* 8.1% Vs 2.4%, *Kl. pneumoniae* 6.7% Vs 1.4%, *Ent. cloacae* 6.2% Vs 1.9%, *P. vulgaris* 9.5% Vs 5.7%, *P. mirabilis* 6.7% Vs 4.3%, *Sh. sonnei* 1.9% Vs 0.95%, *Cit. freundii* 5.71% Vs 1.9%, *Cit. koseri* 6.2% Vs 2.9%, *Pantoea agglomerans* 38.1% Vs 26.2%, *Hafnia alvei* 1.9% Vs 1.4%, *M. morganii* 2.4% Vs 1.4%, and unidentified microorganisms 6.7% Vs 4.3%.

On the other hand, the respective occurrence of the aforementioned pathogens in surface swabs versus muscle samples of marine *Atherina* were 4.4% Vs 0%, 4.4% Vs 2.2%, 3.3% Vs 1.1%, 2.2% Vs 1.1%, 7.8% Vs 3.3 %, 5.6% Vs 2.2%, 1.1% Vs 0%, 3.3% Vs 1.1%, 5.6% Vs 2.2%, 38.89% Vs 21.1%, 1.1% Vs 1.1%, 2.2% Vs 1.1% and 13.3% Vs 2.2%. Higher percentages of the isolated pathogens were previously reported by Papadopoulou *et al.* (2007). The highest infection rate may be attributed to unsatisfactory sanitation during handling and processing.

From Table (2) it could be easily concluded that the presence of *Enterobacteriaceae* in fish may be related to fecal pollution of surface water or aquatic environment of fish or to improper handling. From zoonotic point of view, it constitutes a public health hazard.

Table (2): Occurrence of *Enterobacteriacae* in surface swabs and muscle samples of *Atherina* fish.

Isolated microorganisms	*Atherina* spp. A								Total number (300)			
	Fresh water *Atherina* spp. (210)				Marine *Atherina* spp. (90)							
	SU.S		MS		SU.S		MS		SU.S		MS	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
E. coli	12	5.71	2	0.95	4	4.4	0	0	16	5. 33	2	0.7
Kl. Oxytoca	17	8.1	5	2.4	4	4.4	2	2.2	21	7	7	2.3
Kl.pneamonie	14	6.7	3	1.4	3	3.3	1	1.1	17	5.7	4	1.3
Ent. Cloaca	13	6.2	4	1.9	2	2.2	1	1.1	15	5	5	1.7
P. vulgaris	20	9.5	12	5.7	7	7.8	3	3.3	27	9	15	5
P.mirabilis	14	6.7	9	4.3	5	5.6	2	2.2	19	6.3	11	3.7
Sh. Sonnei	4	1.9	2	0.95	1	1.1	0	0	5	1.7	2	0.7
Cit. freundii	12	5.71	4	1.9	3	3.3	1	1.1	15	5	5	1.7
Cit. koseri	13	6.2	6	2.9	5	5.6	2	2.2	18	6	8	2.2
Pantoea agglomerans	80	38.1	55	26.2	35	38.89	19	21.1	115	38.3	74	24.7
Hafnia alvei	4	1.9	3	1.4	1	1.1	1	1.1	5	1.7	4	1.3
M. morganii	5	2.4	3	1.4	2	2.2	1	1.1	7	2.3	4	1.3
Unidentified microorganisms	14	6.7	9	4.3	12	13.3	2	2.2	26	8.7	11	3.7

SU.S: Surface swabs of fish. MS: muscle samples of fish No.: Number of positive.

Table (3) shows that *S. aureus* and coagulase negative *Staphylococcus* spp were isolated from 69 (53.1%) and 55 (42.3%) of the examined water samples, respectively. Lower percentages of 20, and 10 were previously recorded by Mohamed *et*

al. (2003), and Metawea and Abd El-Ghaffar (2007), respectively.

The highest percentages of *S. aureus* and coagulase negative *Staphylococcus* spp. may be attributed to contamination of the hands of *Atherina* fish handlers which in turn contaminates *Atherina* fish and water used for their preparation for selling. It was clarified by Le-Loir *et al.* (2003), that *S. aureus* is a main cause of gastroenteritis resulting from the consumption of contaminated food. Information from many episodes of Staphylococcal gastroenteritis outbreaks indicates that *S. aureus* was isolated from the implicated food and from the hands and nose of food handlers (Docarmo *et al.*, 2004). On the other hand, coagulase negative *Staphylococcus* spp. was reported to produce enterotoxin and affect the public health (Udo *et al.*, 1999).

Regarding the occurrence of *Staphylococcus* spp. in hand swabs of *Atherina* fish handlers; table (3) clarifies that S*taph. aureus* was isolated from 73% of hand swabs. Nearly similar results were previously reported by Lues and Tonder (2007) and Simon and Sanjeev (2007). Lower frequencies were also cited by Mohamed *et al.* (2001), Shojaei *et al.* (2006), Dhorod *et al.* (2009) and Ibrahim *et al.* (2009).

On the other hand, high percentage of 95 was recorded by Aycicek *et al.* (2004). The variation between the obtained results and the previous studies may be due to geographical distribution, the extent of applying the personal hygiene and the time of sampling.

Table (3) shows also that the percentage of caogulase negative *Staphylococcus* spp. in hand swabs was 50. Higher percentage of 70 was obtained by Aycicek *et al.* (2004). However, lower result was previously recorded by Mohamed *et al.* (2001).

The higher percentage of *S. aureus* (73) and caogulase-negative *Staphylococcus* spp. (50) from the hand swabs of *Atherina* fish handlers can be explained by the fact that these organisms are found as a permanent flora on the skin. It was previously reported that approximately 35-40% of the healthy adults carry *S. aureus* asymptomatically either on their skin or mucosa (Ulrich, 1965). So, it could be easily concluded that the fish handlers are still a potential hazard for Staphylococcal food contamination.

Table (3): Occurrence of *Staphylococcus* spp in hand swabs of *Atherina* fish handlers (n=100) and water used for preparation of *Atherina* spp for selling (n=130).

Staphylococcus species isolate	hand swabs of Atherina fish handlers (n=100)		Water used for preparation of Atherina spp for selling (n=130)	
	No. of +ve	%	No. of +ve	%
S. aureus	73	73	69	53.1
Coagulase negative Staphylococcus spp	50	50	55	42.3

The results in Table (4). The table shows that the isolated *Enterobacteriaceae* from water samples were *E. coli* 8 (6.15%), *P. vulgaris* 15 (11.5%), *P. mirabilis* 10 (7.7%), *Ent. cloacae* 10 (7.7%), *Cit. freundii* 8 (6.15%), *Cit. koseri* 9 (6.9%), *Kl. pneumoniae* 10 (7.7%), *Kl. oxytoca* 12 (9.2%), *Pantoea agglomerans* 40 (30.7%), *Hafnia alvei* 3 (2.3%), *M. morganii* 4 (3.1%), and *Sh.sonnie* 2 (1.5%). Nearly similar results were cited by Zaki (1989), Metawea and Abd El-Ghaffar (2007), and Maysa and Abd El-All (2009).

The variation in the percentages of the isolation rates of *Enterobacteriaceae* between this study and others may be attributed to the type of water examined. In this study, the water samples examined were tape water in which *Atherina* spp were immersed for preparation for selling, however, water samples examined in other studies were surface water from rivers or seas into which large quantities of sewage and animal wastes may be dumped.

It could be easily concluded from table (4) that water might play an important role as a reservoir and a source of human infection with *Enterobacteriaceae*. The presence of such pathogens in water may be a result of fecal contamination of water by dirty hands of *Atherina* fish handlers, who are not observing the basic rules of personal hygiene especially after using toilet. Also it throw light on the role of *Atherina* in transmitting pathogenic bacteria to man.

Table (4) also points to the isolation of *E. coli, P. vulgaris, P. mirabilis, Kl. pneumoniae, Kl .oxyotica, Ent. cloacae, Cit. freundii, Cit. koseri, Pantoea agglomerans and unidentified* spp. with the percentages of 5, 8, 5, 6, 7, 6, 4, 5, 36, and 18, respectively. These results agree with Maysa and Abd-Elall (2009) and Shojaei *et al.* (2006).

From the results illustrated in table (4), one can easily concluded that the isolation of *E .coli, Ent. cloacae, P. vulgaris, P .mirabilis, Kl. pneumoniae, Kl. oxytoca, Cit. freundii, Cit. koseri and Pantoea agglomerans* from hands of *Atherina* fish handlers were due to faecal to hand spread with no application of the basic rules of personal hygien. This was previously verified by Ampofo and Clerk (2002), who reported that the isolation of *E. coli, Kl .pneumoniae, P. mirabilis and P.vulgaris* were common in individuals of communities of sewage fed

ponds. De Wit and Rombouts (1992) concluded that the presence of *Enterobacteriaceae* on hands is not a good indicator of personal and toilet hygiene.

From the aforementioned results, it is evident that *Enterobacteriaceae* are potentially present in water and are not known as classical fish pathogens. The oxygen depletion and higher water temperature render fishes to be easily infected with those bacteria (Badran *et al.,* 1994). Moreover, fish might act as a carrier of human pathogens such as *Staphylococcus* species and members of *Enterobacteriaceae* in water environments polluted by human sewage or diseased animal. Therefore, good water quality is the key to improve the production and hygiene of fish as a food (Omaima and El-Kewaley, 2008).

Table (4): Occurrence of *Enterobacteriacae* in hand swabs of *Atherina* fish handlers (n=100) and water used for preparation of *Atherina* spp for selling (n=130).

Isolated microorganisms	Hand swabs of Atherina fish handlers (n=100)		Water used for preparation of Atherina spp for selling (n=130)	
	No. of +ve	%	No. of +ve	%
E. coli	5	5	8	6.15
P. vulgaris	8	8	15	11.5
P. mirabilis	5	5	10	7.7
Ent. Cloacae	6	6	10	7.7
Cit. freundii	4	4	8	6.15
Cit. koseri	5	5	9	6.9
Kl. Pneumoniae	6	6	10	7.7
Kl. Oxytoca	7	7	12	9.2
Pantoea agglomerans	36	36	40	30.7
Hafnia alvei	0	0	3	2.3
M. morganii	0	0	4	3.1
Sh. Sonnie	0	0	2	1.5
Unidentified microorganism	18	18	5	3.8

Results in Table (5) reveal that O_{128} (2 strains), O_{114} (1 strain), and O_{136} (1 strain) were isolated from *Atherina*. However, O_{26}, O_{111}, and untyped strain (1 strain, each) were identified in hand swabs of *Atherina* fish handlers. While, the 3- *E. coli* isolates from water samples used for preparation of *Atherina* fish for selling were O_{128}.

It was clear from table (5) that the most predominant serogroup of *E. coli* was O_{128} which isolated from *Atherina* spp. and water samples. Hefnawy *et al.* (1989) isolated enteroopathogenic *E. coli* from *Tilapia nilotica* and their serogrouping were O_{55}, O_{86a}, O_{128a}, O_{128b} (one strain, each) and O_{119} (2 strains). On the other hand, Metawea and Abdel Ghaffar (2007) identified *E. coli* O_{55}: K_{59}, O_{124}: K_{72}, O_{111}: K_{55}, and O_{128}: K_{67} from fish samples and their aquatic environment. They concluded that the presence of the same serotypes in both water and fish samples indicates that water may act as a dangerous source of these pathogens to fish and consequently act as a vehicle of human infection, constituting therefore public health problems.

O_{136} is one of the enteroinvasive *E. coli* (EIEC) that invades and multiplies with in human colonic epithelial cells. Human is its natural reservoir. Person to person transmission through fecal-oral route and ingestion of contaminated food and water that have been contaminated through dirty hands of fish or food handlers who are not observing the basic rules of personal hygiene are the principal modes of its transmission (Meng *et al.,* 2001).

The isolation of O_{136} in *Atherina* fish may arise from pollution of aquatic environment with sewage or from dirty hands of fish handlers. This result substantiates the role of water and fish handlers in transmission of pathogens to fish which in turn transmit these pathogens to other susceptible people through ingestion of improperly cooked fish or through direct contact.

Microorganisms differ in their response to freezing, some survive virtually unharmed, some resist freezing but are susceptible to damage during frozen storage (Hossain *et al.,* 2008). The damage caused to microorganism by freezing was due to sudden drop in temperature, ice crystals formation, and increase of solutes concentration (Lund, 2000).

Table (5): Serogrouping of *E. coli* isolates from *Atherina* spp., water used for preparation of *Atherina* spp. for selling and their handlers.

E. coli serotypes	Source			Total
	Atherina species	water used for preparation of *Atherina* species for selling	Hand swabs of *Atherina* fish handlers	
O26	0	0	1	1
O111	0	0	1	1
O114	1	0	0	1
O128	2	3	0	5
O136	1	0	0	1
Untyped	0	0	1	1
Total	4	3	3	10

Table (6) shows that after the 1st week from freezing, all *Enterobacteriacea* detected before freezing were continued to isolate with a ratio of (1:4, each) from the surface swabs of the four examined *Atherina*. On the other hand, *S. aureus* and coagulase negative *Staphylococcus* spp were continued to recover from surface swabs with ratios of (4:4), and (2:4), respectively.

All *Enterobacteriaceae* except *P. mirabilis* (each with a ratio of 1:4), *S. aureus* (4:4), and coagulase negative *Staphylococcus* spp (1:4) were continued to isolate from surface swabs after the 2nd week from freezing. On the other hand, the isolated species after the 3rd week of freezing were *Kl. Oxytoca* and *Pantoea agglomerans* (1:4, each), and *S. aureus* (4:4). Meanwhile, after the 4th week of freezing, the isolated species were *Pantoea agglomerans (1:4)* and *S. aureus* (4:4). *S. aureus* (4:4) was found positive until the 13th week from freezing. In contrary, all muscle samples were negative since the 1st week of freezing.

These results agree with Chattopadhyay (1999), Lund (2000), and Hossain *et al.* (2008), who stated that gram negative bacteria are more sensitive to freezing than gram positive bacteria. Thushani *et al.* (2003) also concluded that *S. aureus* survived during storage at -24°C over a period of 8 weeks. The resistance of *S. aureus* to freezing may be related to the presence of mucoprotein complex and diaminopimelic acid in the cell walls of gram positive cells. The mucoprotein complex and diaminopimelic acid was found to protect the membrane protein against denaturation (Singhal and Pushpa, 1999). From hygienic point of view, freezing at -20°C for suitable time prevent transmission of some pathogenic bacteria to man through eating *Atherina* spp.

Salting of fish is an ancient method used by the Pharos for preservation of fish. The effect of salting on the microorganisms arise from the changes of the osmotic pressure which cause lowering the moisture content of the microbial cell, leading to its shrinkage and death. Moreover, salting cause denaturation of the protein content of the microbial cell, this considered as an important factor in suppression of the microbial growth (Ez El-Din, 1999).

Table (6): The effect of freezing on the survival of *Staphylococcus* spp. and *Enterobacteriaceae* in fresh water *Atherina* fish.

Isolated Bacteria	Before treatment *				After freezing**												
	SU.S		MS		1w	2w	3w	4w	5w	6 w	7 w	8 w	9 w	10 w	11 w	12 w	13 w
	No.	%	No.	%	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.
Kl. oxyotica	2	20	1	10	1	1	1	0	0	0	0	0	0	0	0	0	0
P. vulgaris	1	10	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
P. mirabilis	1	10	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Pantoea agglomerans	5	50	3	30	1	1	1	1	0	0	0	0	0	0	0	0	0
Cit.freundii	1	10	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
Cit. koseri	1	10	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
S. aureus	6	60	4	40	4	4	4	4	4	4	4	4	4	4	4	4	4
Coagulase negative Staphylococci	4	40	3	30	2	1	0	0	0	0	0	0	0	0	0	0	0

No. : Number of positive. *Ten *Atherina* fish were examined before treatment

** Surface swabs and muscle samples of 4 frozen *Atherina* fish was examined/week.

-Muscles of all fish examined after the 1st week from freezing treatments were found to be negative.

The results of salting experiment were shown in table (7). The illustrated results verify that the ratios of the isolation of the bacterial species from surface swabs after salting treatment was lower than before treatment. It is also clear from table (7) that *Kl. Oxytoca, P. Vulgaris, P. Mirabilis* (1:4, each) were isolated from surface swabs of fresh water *Atherina* salted in 25% NaCl solution. Moreover, *Pantoea agglomerans* and *S. aureus* were isolated from the same samples with ratios of (2:4) and (4:4), respectively. On the other hand, only *Kl. oxytoca* and *Pantoea agglomerans* were isolated from the muscle samples of *Atherina* fish salted in 25% NaCl solution with a ratio of (1:4, each). Meanwhile, *S. aureus* was isolated from the same samples at a ratio of (2:4).

Table (7) shows also that all samples salted in 50% and 75% NaCl solution were negative for the presence of *Enterobacteriaceae* from the 1st week and for the whole period of the experiment. Moreover, all muscle samples were negative at all dilutions except the dilution of 25%. On the other hand, *S. aureus* was isolated from surface swabs of *Atherina* fish at a rate of (2:4) after 2 and 3 weeks and at a rate of (1:4) after four and five weeks.

The obtained results agree with Ez El-Din (1999), who reported that salting appeared to have a little effect on the growth of aerobic, mesophilic bacteria, *Streptococcus feacalis, S. aureus*, and mold but completely suppressed coliforms growth. Slonczewski *et al.* (2008) also found that *S. aureus* resists salting for long period. He concluded that lower water activity (0.85) of *S. aureus* enable to grow in up to 25% NaCl solution. It is easily concluded from table (7) that salting has a great effect on *Enterobacteriaceae* and other gram negative bacteria and a little effect on *S. aureus*. This may throw light on the cause of food intoxication after consumption of salted fish meal.

Table (7): The effect of salting on the survival of *Staphylococcus* spp. and *Enterobacteriaceae* in fresh water *Atherina* fish.

Bacteria Isolated	Before treatment *				After salting**												
					1w				2w			3w			4w	5w	6w
					25%		50%	75%	25%	50%	75%	25%	50%	75%	25%	25%	25%
	SU.S	MS	SU.S	MS	SU.S	MS	SU.S	SU.S	SU.S	SU.S	SU.S	SU.S	SU.S	SU.S	SU.S	SU.S	SU.S
	No.	%	No.	%	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.
Kl. Oxyotica	2	20	1	10	1	1	0	0	0	0	0	0	0	0	0	0	0
P. vulgaris	1	10	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
P.mirabilis	1	10	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Pantoea agglomerans	5	50	3	30	2	1	0	0	0	0	0	0	0	0	0	0	0
Cit. Freundii	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cit. Koseri	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus	6	60	4	40	4	2	0	0	2	0	0	2	0	0	1	1	0
Coagulase negative *Staphylococci*	4	40	3	30	0	0	0	0	0	0	0	0	0	0	0	0	0

No. : Number of positive. *Ten *Atherina* fish were examined before treatment

** Surface swabs and muscle samples of 4 frozen *Atherina* fish was examined/week.

- Muscles of all *Atherina* fish salted 50%, 75% NaCl were found to be negative from the 1st week of salting.

The results of commercial vinegar treatment on the survivability of *Enterobacteriaceae* and *Staphylococcuc* spp in Atherina fish are shown in table (8). It is clear that *Pantoea agglomerans* and *Kl. oxytoca* continued to isolate from surface swabs of treated *Atherina* untill 3 hrs after vinegar treatment. On the other hand, *E. coli* continued to isolate with a ratio of (1:4) from the treated *Atherina* until 6 hrs from vinegar treatment. In contrary all treated *Atherina* fish were negative for *S. aureus* since the 1st hour of treatment. These results are in agreement with Entani *et al.* (1998), who reported that the growth of some food borne pathogenic bacteria was inhibited with 0.1% concentration of acetic acid in the used vinegar. From zoonotic point of view the obtained results illustrated in table (8) revealed that the commercial vinegar is effective for reducing bacterial population in fish due to its content of acetic acid. It was reported by Rhee *et al.* (2003) that the antimicrobial effect of acetic acid on the inactivation of food borne pathogenic bacteria stored at 22°C was more effective than at 5°C. Heat and acetic acid treatments act synergistically to inhibit the growth of food borne pathogens (Shin *et al.*, 2006).

Table (8): The effect of commercial vinegar (5% acetic acid) on the survival of *Staphylococcus* spp. and *Enterobacteriaceae* in fresh water *Atherina* spp. at room temperature

Isolated Bacteria	Before treatment *				After treatment**											
					1 h		2 h		3 h		4 h		5 h		6 h	
	SU.S		MS		SU.S	MS	SU.S	MS	SU.S	MS	SU.S	MS	SU.S	MS	SU.S	MS
	No.	%	No.	%	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.
E. coli	1	10	1	10	1	1	1	1	1	1	1	1	1	1	1	1
P. vulgaris	2	20	1	10	1	1	1	1	0	0	0	0	0	0	0	0
P. mirabilis	1	10	1	10	1	0	0	0	0	0	0	0	0	0	0	0
Pantoea agglomerans	5	50	3	30	2	2	2	1	2	0	0	0	0	0	0	0
Kl. Pneumoniae	1	10	1	10	1	0	0	0	0	0	0	0	0	0	0	0
Kl. Oxytoca	1	10	1	10	1	1	1	0	1	0	0	0	0	0	0	0
S. aureus	6	60	5	50	0	0	0	0	0	0	0	0	0	0	0	0
Coagulase negative *Staphylococcus* spp	4	40	3	30	0	0	0	0	0	0	0	0	0	0	0	0

No. Number of positive *Ten *Atherina* fish were examined before treatment
**Surface swabs and muscle samples of 4 frozen *Atherina* fish was examined/week.
All treated fish samples examined after 7 h were negative.

Corresponding author
Maysa A.I. Awadallah
Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt
maysavet@hotmmail.com*

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