

## Research Article

# Frequency and antibiotic sensitivity of gram negative bacteria isolated from raw fish sold in Karachi, Pakistan

Maha Mansoor<sup>1</sup>, Sadaf Naeem<sup>2</sup> and Asma Naim<sup>1\*</sup>

1. Department of Microbiology, University of Karachi, Karachi, Sindh-Pakistan

2. Department of Biochemistry, University of Karachi, Karachi, Sindh-Pakistan

\*Corresponding author's email: [anaeem@uok.edu.pk](mailto:anaeem@uok.edu.pk)

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### Abstract

Fish serve as an important source of nutrition and also acts as a reservoir for different pathogenic organisms. Sewage pollution in the water bodies is the major source of pathogenic bacteria in seafood. In this study, the presence of Gram negative bacteria in raw fish samples was detected and the antibiotic susceptibility pattern of the isolates was determined. A total of 25 raw fish samples were collected at random from different local markets of Karachi in sterile zip-lock plastic bags. From these samples, a total of 73 Gram negative bacterial strains were isolated. Of these 73 Gram negative isolates, 6 were *E. coli*, 14 *K. pneumoniae*, 7 *E. aerogenes*, 4 *E. cloacae*, 5 *C. freundii*, 1 *C. koseri*, 2 *P. vulgaris*, 3 *P. mirabilis*, 7 *Salmonella* spp., 6 *Shigella* spp., 2 *Serratia* spp., 2 *Pseudomonas* spp., 7 *Yersinia* spp., 3 *Aeromonas* spp. and 4 were *Vibrio* spp. The most abundant species among all the isolated Gram negative bacteria was *Klebsiellapneumoniae* (19.17%) followed by *Enterobacter aerogenes* (9.59%), *Salmonella* spp. (9.59%) and *Yersinia* spp. (9.59%). Antibiotic susceptibility pattern of all the Gram negative isolates was determined by Kirby-Bauer disc diffusion method in which a panel of eight antibiotics was tested. The incidence of resistance was highest against Cefazolin (93.2%) and lowest resistance was observed against Gentamicin (1.4%). Of the 73 Gram negative isolates, 24 were found to be multi-drug resistant (MDR). In conclusion, the bacteriological analysis of raw fish samples showed that the quality of raw fish was poor as high bacterial load was obtained and enteric bacteria were isolated from most of the samples indicating fecal pollution. This study provides valuable information about the presence of MDR Gram negative pathogens in raw fish sold locally which could be useful in the selection of antibiotics for empirical treatment.

**Keywords:** Enteric bacteria; Fresh fish; Karachi; Multidrug resistant; Pre-enrichment

### Introduction

Fish are consumed as a major source of protein and 30 % of fish comes from aquaculture for human consumer [1]. Fish is more readily available food having low fat content and is taken into consideration because it is inexpensive or low cost source of protein in comparison with beef and goat meat so, easy to access by all social classes' individuals [2,3]. Fish are enriched with micronutrients such as Vitamin D, minerals including Iodine, Magnesium, Selenium

and Zinc than animal meat or other food (e.g. plants) and its consumption is helpful in reducing hunger or minimizing the deficiency of micronutrients thereby preventing disease in under developed countries [2]. The easy availability and high nutritional value of fresh fish is responsible for its preferable consumption by coastal people [4]. Karachi being a coastal city has a well-developed fishery industry. However, very little attention is given to the microbiological quality of fresh fish sold at

local markets. Proper storage and handling practices are not followed by local vendors mainly due to ignorance, lack of facilities and professional training.

There are many sources of contamination of seafood which are often difficult to identify [5]. The sources of contamination of fish include improper storage conditions i.e. maintaining temperature and moisture, allowing customers to deal with fish by their own, use of ice prepared from contaminated water, keeping fish on contaminated surfaces such as weighing balance and unhygienic practices [6], the intake of fecally contaminated water by fish and consumption of those fish can cause intoxication in consumers as they may be harbouring enteric pathogens [7]. All these factors can facilitate the contamination or spoilage of fish thereby resulting in foodborne illnesses.

Due to increase in the cases of food intoxication or poisoning, it is a major concern now to consider presence of pathogens in food and its control [8]. Seafood or fish acts as a reservoir or vehicle for bacteria which are pathogenic for human and capable of causing infections [5,9]. Enteric bacteria present in seafood indicate fecal pollution of the aquatic environment [5]. A major source of food contamination is fecal contamination indicated by the presence of members of family Enterobacteriaceae such as *Salmonella* spp., *Proteus* spp., *Escherichia coli* and *Klebsiella* spp. To improve the quality of food and to reduce the occurrence of food borne illnesses proper screening of zoonotic bacterial pathogens in food is needed [10].

Most of the foodborne diseases in large proportion are caused by bacterial pathogens (76%) than by viruses (21%) followed by parasites (2.6%). Main pathogenic bacteria carried by seafood are *E.coli*, *Salmonella enterica*, *Listeria monocytogenes* and *Vibrio* spp. [11,12]. The contaminated raw or partially cooked food consumption poses a higher risk to the human health [13]. Common pathogenic

bacteria of family Enterobacteriaceae which can be isolated from fish and its environment are *Klebsiella* spp., *Citrobacter* spp., *Salmonella* spp., *E. coli* and *Proteus* spp. [14]. Micro-organisms carried by fish are based on their environment where they are present [15].

There is very little data available regarding the prevalence of pathogenic bacteria and the antibiotic resistance in locally available fish. Therefore, this study was aimed to detect the presence of Gram negative bacteria from raw fish samples sold at local markets in Karachi, Pakistan and to evaluate the antibiotic susceptibility pattern of the Gram negative bacteria thereby assessing the quality of fish sold locally.

## Materials and methods

### Sample collection

A total of 25 raw fish samples were randomly collected in a sterile zip lock plastic bags separately from different shops of local markets in Karachi, Pakistan. The fish species collected for this study were *Tilapia* sp., *Pangasius* sp., *Lutjanus argentimaculatus* (Red snapper), *Labeorohita* (Rohu) and *Scomberomorus guttatus* (King Mackerel/Surmai).

### Sample processing

#### Total viable count/ Total plate count

The sample was serially diluted and 0.1ml of the  $10^{-3}$  and  $10^{-4}$  dilution was transferred to Plate Count Agar (PCA) media plates separately to make lawn and plates were incubated at 37°C for 24-48 hours.

#### Pre-enrichment of sample

10 gm of fish sample was weighed and inoculated into 100 ml of Buffered Peptone Water in a flask. The flask containing sample was then incubated at 37°C for 3 hours.

#### Isolation of bacteria

##### Streaking on media plates

A loopful inoculum of pre-enriched sample was streaked on MacConkey's Agar and on Bismuth Sulfide Agar media plates with the help of sterile wire loop to obtain isolated colonies. All the media plates were incubated at 37°C for 24 hours.

### Subculture

The different types of isolated colonies were picked from different media plates and streaked on Nutrient agar media plates for the purpose of sub-culturing. All the media plates were incubated at 37°C for 24 hours.

### Purification and maintenance of culture

The isolated colonies with different morphology after sub-culturing were picked and maintained on Nutrient Agar slants. These cultures were further characterized and identified biochemically.

### Biochemical identification

Bergey's manual of Systemic Bacteriology [16] was followed to identify purified cultures. The identification scheme included Gram staining and Biochemical tests i.e. Catalase test, Oxidase test, Carbohydrate fermentation test, Indole test, MR test, VP test, Citrate utilization test, Nitrate reduction test and Motility testing.

### Antibiotic susceptibility testing

The antibiotic susceptibility pattern of all the isolated Gram negative bacteria was tested by Kirby-Bauer disc diffusion method [17] against the following antibiotics i.e. Chloramphenicol 30µg (C), Ceftriaxone 30µg (CRX), Cefazolin 30µg (KZ), Gentamicin 10µg (CN), Ofloxacin 5µg (OFX), Oxacillin 1µg (Ox), Tetracycline 30µg (TE) and Trimethoprim-Sulfamethoxazole 25µg (SXT).

### Results

Total aerobic count was determined and a very high bacterial load was observed on Plate Count Agar from all 25 raw fish samples which was interpreted as too numerous to count (TNTC). A Total of 73 Gram negative bacterial strains were isolated. Among 73 Gram negative bacterial strains, 64 belonged to different genera of the family Enterobacteriaceae (i.e. *Escherichia*, *Klebsiella*, *Enterobacter*, *Salmonella*, *Shigella*, *Citrobacter*, *Serratia*, *Proteus* and *Yersinia*), while 9 belonged to non-Enterobacteriaceae genera (i.e. *Pseudomonas*, *Aeromonas* and *Vibrio*). The most frequently isolated Gram negative

bacteria was found to be *Klebsiella pneumoniae* (19.17%) followed by *Enterobacter aerogenes*, *Salmonella* spp., and *Yersinia* spp. (9.6% each) (Table 1).

Antibiotic susceptibility pattern of all the Gram negative isolates was determined against Ofloxacin (5µg), Gentamycin (10µg), Trimethoprim-Sulfamethoxazole or Co-trimoxazole (25µg), Chloramphenicol (30µg), Cefazolin (30µg), Ceftriaxone (30µg), Tetracycline (30µg) and Oxacillin (1µg). The resistance pattern of the isolated Gram negative bacteria against different antibiotics is given in (Table 2). The highest number of organisms were resistant to Cefazolin (93.15%) followed by Oxacillin (89.04%) and Ceftriaxone (30%) while maximum number of organisms were susceptible to Gentamicin (98.63%) followed by Ofloxacin (93.15%) and Chloramphenicol (93.15%).

Of the 73 Gram negative isolates obtained from fish, 24 were resistant to multiple drugs, however none of the isolates of genus *Proteus* showed multidrug resistance. Highest frequency of multidrug resistance was observed among *Yersinia* spp. and *Salmonella* spp. followed by *Serratia marcescens*, *Pseudomonas*, *Escherichia coli*, *Enterobacter aerogenes* and *Citrobacter freundii* (Table 3).

The resistance of bacteria with respect to the number of antibiotics is given in (Figure 1). The highest number of isolates were resistant to a combination of two antibiotics and none of the isolates were resistant to all the antibiotics tested. Few strains were sensitive to all the eight antibiotics tested and organisms were found to be tolerant to a maximum of six antibiotics simultaneously. The occurrence of multi-drug resistance (MDR) was also noted among the different species of Gram negative bacteria isolated. *Salmonella* (57%) and *Yersinia* (57%) species exhibited the highest rate of MDR strains, followed by *E. coli* (50%) (Table 4).

**Table 1. Frequency of gram negative bacteria isolated from fish samples**

Gram negative bacteria	Frequency	
	Number (#)	Percentage (%)
<i>Escherichia coli</i>	6	8.22
<i>Enterobacter aerogenes</i>	7	9.59
<i>Enterobacter cloacae</i>	4	5.48
<i>Citrobacterfreundii</i>	5	6.85
<i>Citrobacterkoseri</i>	1	1.37
<i>Klebsiella pneumoniae</i>	14	19.17
<i>Proteus vulgaris</i>	2	2.74
<i>Proteus mirabilis</i>	3	4.11
<i>Salmonella</i> spp.	7	9.59
<i>Shigella</i> spp.	6	8.22
<i>Serratia</i> spp.	2	2.74
<i>Yersinia</i> spp.	7	9.59
<i>Pseudomonas</i> spp.	2	2.74
<i>Aeromonas</i> spp.	3	4.11
<i>Vibrio</i> spp.	4	5.49
<b>Total</b>	<b>73</b>	<b>100</b>

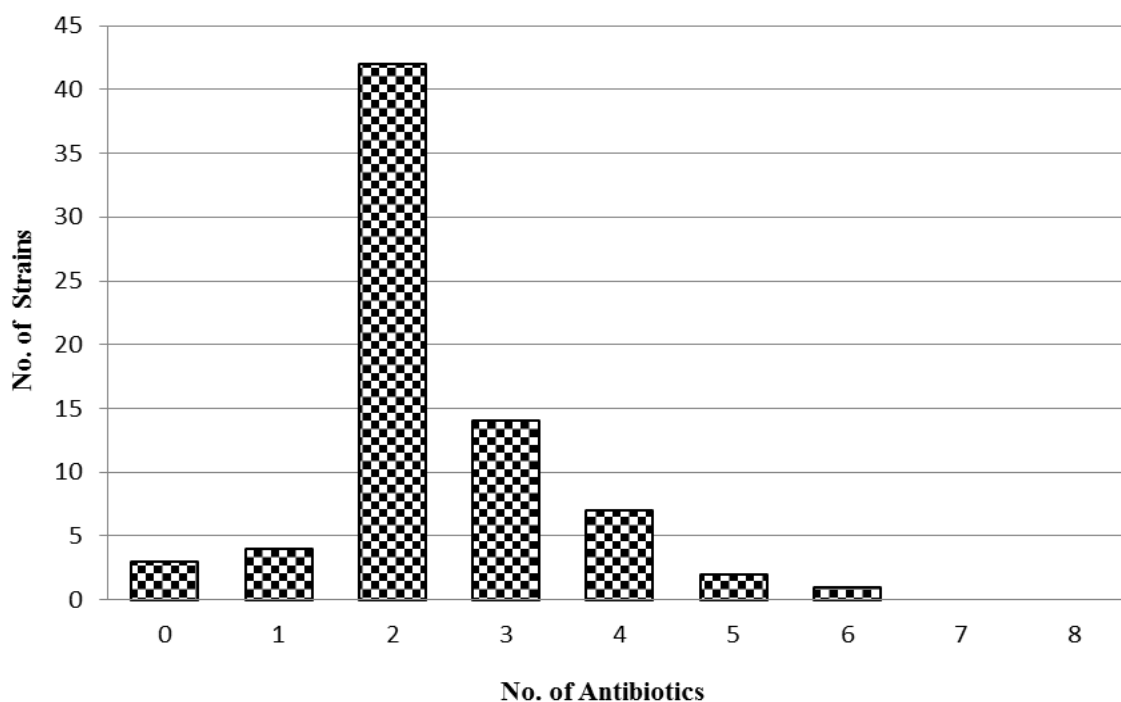
**Table 2. Antibiotic susceptibility pattern of gram negative bacteria (n=73)**

Antibiotics	Frequency of isolates								
	Resistant			Intermediate			Susceptible		
	No.	%	Average zone of inhibition (mm) $\pm$ SD	No.	%Age	Average zone of inhibition (mm) $\pm$ SD	No.	%Age	Average zone of inhibition (mm) $\pm$ SD
Chloramphenicol (30 $\mu$ g)	02	2.74	5.5 $\pm$ 6.40	3	4.11	14 $\pm$ 1.73	68	93.15	26.13 $\pm$ 5.31
Cefazolin (30 $\mu$ g)	68	93.15	4.02 $\pm$ 6.54	02	2.74	23 $\pm$ 1.41	03	4.11	29.33 $\pm$ 4.93
Trimethoprim-sulfamethoxazole (25 $\mu$ g)	05	6.85	0	01	1.37	14 $\pm$ 0	57	78.08	26.51 $\pm$ 4.85
Gentamicin (10 $\mu$ g)	00	0.00	0	01	1.37	14 $\pm$ 0	72	98.63	19.15 $\pm$ 3.44
Ofloxacin (5 $\mu$ g)	03	4.12	11 $\pm$ 1.73	02	2.74	14 $\pm$ 0	68	93.15	28.39 $\pm$ 6.65
Oxacillin (1 $\mu$ g)	65	89.04	9 $\pm$ 0	01	1.36	12 $\pm$ 0	07	9.58	20.42 $\pm$ 6.94
Ceftriaxone (30 $\mu$ g)	19	26.02	17.35 $\pm$ 5.64	14	19.18	23.94 $\pm$ 0.680	40	54.79	28.69 $\pm$ 3.67
Tetracycline (30 $\mu$ g)	04	5.48	5.25 $\pm$ 6.07	05	6.84	12.6 $\pm$ 0.894	64	87.67	23 $\pm$ 6.10

**Table 3. Antibiotic resistance profile of different species of gram negative isolates from fish samples**

Gram negative isolates	No.	Antibiotic resistance (%)							
		C	KZ	SXT	CN	OFX	Ox	CRX	TE
<i>Escherichia coli</i>	6	-	6(100)	1(17)	-	1 (17)	6 (100)	3 (50)	-
<i>Enterobacter aerogenes</i>	7	1 (14)	7 (100)	1 (14)	-	-	7 (100)	3 (43)	1 (14)
<i>Enterobacter cloacae</i>	4	-	3 (75)	-	-	-	4 (100)	-	1 (25)
<i>Citrobacterfreundii</i>	5	-	3 (60)	1 (20)	-	-	2 (40)	3 (60)	-
<i>Citrobacterkoseri</i>	1	-	1 (100)	-	-	-	1 (100)	-	-
<i>Klebsiella pneumoniae</i>	14	-	14 (100)	2 (14)	-	1 (7)	14 (100)	1 (7)	-
<i>Proteus vulgaris</i>	2	-	2 (100)	-	-	-	2 (100)	-	-
<i>Proteus mirabilis</i>	3	-	3 (100)	-	-	-	3 (100)	-	-
<i>Salmonella spp.</i>	7	1 (14)	6 (86)	2 (29)	-	-	6 (86)	3 (43)	-
<i>Shigella spp.</i>	6	-	5 (83)	-	-	-	4 (67)	1 (17)	1 (17)
<i>Serratia spp.</i>	2	-	2 (100)	-	-	-	-	1 (50)	-
<i>Yersinia spp.</i>	7	-	7 (100)	-	-	-	7 (100)	3 (43)	1 (14)
<i>Pseudomonas spp.</i>	2	1 (50)	2 (100)	-	-	-	2 (100)	1 (50)	-
<i>Aeromonas spp.</i>	3	1 (33)	3 (100)	-	-	1 (33)	-	2 (67)	1 (33)
<i>Vibrio spp.</i>	4	-	4(100)	-	1(25)	-	4(100)	1(25)	-
<b>Total</b>	<b>73</b>	<b>4 (5.5)</b>	<b>68(93.2)</b>	<b>6 (8.2)</b>	<b>1 (1.4)</b>	<b>3 (4)</b>	<b>62 (85)</b>	<b>22 (30)</b>	<b>5(6.8)</b>

C=Chloramphenicol, KZ=Cefazolin, SXT= Trimethoprim-sulfamethoxazole, CN=Gentamicin, OFX= Ofloxacin, Ox= Oxacillin, CRX= Ceftriaxone and TE= Tetracycline



**Figure 1. Multidrug resistant gram negative bacteria with respect to the number of antibiotics**

**Table 4. Frequency of multidrug resistance among different species of gram negative fish isolates**

Gram negative bacteria	Total number of isolates	Multiple antibiotic resistant organisms	
		Number (#)	Percentage (%)
<i>Escherichia coli</i>	6	3	50
<i>Enterobacter aerogenes</i>	7	2	28.57
<i>Enterobacter cloacae</i>	4	1	25
<i>Citrobacterfreundii</i>	5	2	40
<i>Citrobacterkoseri</i>	1	-	-
<i>Klebsiella pneumoniae</i>	14	3	21.43
<i>Proteus vulgaris</i>	2	-	-
<i>Proteus mirabilis</i>	3	-	-
<i>Salmonella</i> spp.	7	4	57.14
<i>Shigella</i> spp.	6	1	16.66
<i>Serratia</i> spp.	2	1	50
<i>Yersinia</i> spp.	7	4	57.14
<i>Pseudomonas</i> spp.	2	1	50
<i>Aeromonas</i> spp.	3	1	33.33
<i>Vibrio</i> spp.	4	1	25
<b>Total</b>	<b>73</b>	<b>24</b>	<b>100</b>
<b>Percentage</b>	<b>100</b>	<b>32.87</b>	

### Discussion

The prevalence of antibiotic resistant bacteria in fish is a public health concern. In the present study, total aerobic count was performed to assess the hygienic quality of fish. The results represented that the fish were of poor quality and unsafe to consume without proper washing and cooking as high bacterial load was observed on Plate Count Agar in form of uncountable colonies which was interpreted as too numerous to count (TNTC). Ghosh and Mandal [18] also reported a high viable bacterial count ( $25.1 \pm 0.28 \times 10^6$  CFUg<sup>-1</sup>) in gills of fish. In another study, a very high total viable count (TVC) was observed in fresh fish in comparison with frozen fish. The main reason for low bacterial load in frozen fish is the storage temperature that facilitates the inactivation of bacterial enzymes ceasing their cellular activity and division [19].

In this study, Gram negative bacteria identified as *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Proteus mirabilis*, *Proteus vulgaris*, *Salmonella* spp., *Shigella* spp., *Citrobacter freundii*, *Pseudomonas*

spp., *Serratia marcescens*, *Yersinia* spp., *Aeromonas* spp. and *Vibrio* spp. were isolated from fish samples. These opportunistic and potential pathogenic organisms have also been reported in other studies from different parts of the world [20-24]. The members of the family Enterobacteriaceae are universally considered as a marker of hygienic quality of food and water [25]. In this study, the most frequently isolated Gram negative bacteria was found to be *Klebsiella pneumoniae* (19.17%) which could be due to the unhygienic processing of fish and fecal contamination, as *Klebsiella* are not only found in the mucosal lining of mammals where they colonize and contribute towards the source of fecal contamination of food but are also common in the environment i.e. water, sewage, soil and plants [26].

Antibiotic susceptibility pattern of the Gram negative bacteria isolated from raw fish was also determined against eight commonly used antibiotics representing different classes of antibiotics. Most of the isolates were found to be resistant to Cefazolin (93.2%) and Oxacillin (85%)

while Gentamicin and Ofloxacin were the most effective antibiotics against these isolates.

In this study, 24 bacterial strains out of 73 were found to be multidrug resistant. Organisms exhibiting resistance to 3 or more antibiotics were considered as multidrug resistant (MDR). High frequency of MDR bacteria in fish have also been reported by Matyar *et al.* [27] from Turkey, Schmidt *et al.* [28] from Denmark and Chelossi *et al.* [29] from Western Mediterranean fish farms. The prevalence of antibiotic resistant pathogenic bacteria in fish in the current study concurs with the findings of Agoba *et al.* [30] and Barat *et al.* [31] who have also reported a high rate of antibiotic resistance among Gram negative bacteria. The emergence of antimicrobial resistance which can be due to different mechanisms like organism is naturally resistant, horizontal transfer of resistance genes from one organism to another, unnecessary addition of antibiotics in animal feed to increase their productivity and also the overuse of antimicrobial agents result in the spread of antibiotic resistance among pathogenic bacteria. Otherwise these antimicrobial agents are used for the prevention of diseases and in the maintenance of health in its appropriate use [32].

Cefazolin is the first generation Cephalosporin which inhibits cell wall synthesis by binding Penicillin binding proteins to stops peptidoglycan synthesis. In this study, 93.15% resistance was observed. In a previous study, 15-48% resistance was reported against Cefazolin from fish farm samples [33]. In case of Oxacillin, 89.04% resistance was observed which is higher than 15.8% resistance reported by Schmidt *et al.* [28]. Ceftriaxone, a third generation Cephalosporin, works by inhibiting bacterial cell wall synthesis after binding to penicillin-binding proteins (PBPs) or transpeptidases preventing the cross linking of peptidoglycan. In this study, 30% of the isolates were resistant to this antibiotic.

However, de Almeida *et al.* [34] reported a single isolate resistant to Ceftriaxone and Verner-Jeffreys *et al.* [35] reported all the isolates susceptible to it, which are contrasting to current findings. In this study, 9.6% isolates were resistant to trimethoprim-sulfamethoxazole, a bactericidal drug which works by inhibiting folic acid synthesis. This finding is consistent with the 0-15% resistance reported by Newaj-Fyzul *et al.* [36] but lower than 23.4% resistance reported by Brahmi *et al.* [37] in bacteria including *E. coli*, *K. pneumoniae* and *Enterobacter* isolates.

Tetracycline is a bacteriostatic drug and inhibits bacterial protein synthesis. In this study, only 6.8% resistance was observed to tetracycline among the isolated Gram negative bacteria which is much lower than the 52% reported by Newaj-Fyzul *et al.* [36]. In this study, only 5.5% isolates were resistant to Chloramphenicol. Verner-Jeffreys *et al.* [35] reported a high resistance (56%) to chlormaphenicol in their fish isolates. Ofloxacin, a broad spectrum second generation fluoroquinolone drug acts by inhibiting DNA gyrase or topoisomerase IV to inhibit bacterial cell division. In this study, 4.1% resistance was observed by the isolated Gram negative bacteria. Similar findings of low resistance to ofloxacin was also reported in a previous study where out of 94 clinical isolates from fish, 78% to 80% of isolates (members of family Enterobacteriaceae and Staphylococcus) were susceptible to Ofloxacin [32]. Gentamicin, a member of aminoglycoside drug whose mode of action is to inhibit the protein synthesis by binding with 30s ribosomal subunit was found to be the most effective antibiotic. In this study, only a single isolate belonging to *Vibrio* spp. was resistant to Gentamicin. Similar findings were reported by Kathleen *et al.* [38] where 1.1% isolates were also resistant to gentamicin. In another study, 50% to 90% of bacterial isolates among which most were Gram negative and member of family

Enterobacteriaceae showed susceptibility to Gentamicin [32].

The differences in the antibiotic resistance pattern of these isolates from other researches could be due to the fact that fish sold in the local market are the catch from natural marine waters while most of the reported studies are based on the samples obtained from aquacultures and also bacterial flora differs among various geographical regions.

The quality of fish can be maintained or food products can be saved not only from pathogenic bacterial species but also from other micro-organisms by following the guidelines provided by Centre for Disease Control and Prevention (CDC), The International Commission on Microbiological Specifications for Foods (ICMSF) and Hazard Analysis Critical Control Point (HACCP). Fish should be microbiologically examined for the presence of pathogens especially enteric pathogens (belonging to family Enterobacteriaceae), hygienic practices should be followed during collection, processing, transportation and distribution of food. Fish processing environment, equipment and storage conditions such as appropriate cold temperature and humidity should also be maintained and fish should be properly cooked before it is consumed in order to prevent foodborne illnesses or food poisoning.

### Conclusion

In this study a number of Gram negative food-borne pathogens were isolated from raw fish samples including *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., *Salmonella* spp., *Shigella* spp., *Serratia* spp., *Pseudomonas* spp., *Yersinia* spp., *Aeromonas* spp. and *Vibrio* spp. Resistance against different antibiotics was also observed by most of the isolates and some isolates also showed multidrug resistance. So, it is important to assess the quality of fish before it is being consumed and to consider the emergence of resistance among Gram negative bacteria by the

observed isolates because they are a threat to public health worldwide and difficult to treat. Efforts should be directed to improve the quality of fish sold at local markets and proper storage facilities should be provided to the vendors/merchants.

### Authors' contributions

Conceived and designed the experiments: A Naim, Performed the experiments: M Mansoor, A Naim & S Naeem, Analyzed the data: A Naim & M Mansoor, Contributed materials/ analysis/ tools: A Naim & S Naeem, Wrote the paper: A Naim & M Mansoor.

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