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RESEARCH ARTICLE

ISOLATION OF *ENTEROBACTERIACEAE* FROM RAW SEAFOODS SOLD IN FISH MARKETS IN EASTERN PROVINCE OF SAUDI ARABIA.

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Abstract

Aim of this investigation is to provide the general information about prevalence of members of *Enterobacteriaceae* in raw seafoods sold in fish markets in Eastern province of Saudi Arabia. Studies were carried out to isolate and identify different bacterial species belongs to the family of *Enterobacteriaceae* in 448 seafood samples comprising Fishes (353), Prawns (49) and Cuttlefishes (46), collected during the period from January 2015 until May 2016. Bacterial isolates were characterized on specific culture media and further confirmed using API 20E Strips and identified by "api web". Out of 448 samples analysed, 304 (67.85%) samples showed positive isolation of *Enterobacteriaceae*. *Morganellamorganii* showed highest prevalence (18.52%), followed by *Proteussp* (12.27%), *Citrobactersp* (8.03%), *Enterobactersp* (6.69%), *Providenciasp* (5.58%), *Hafniaalvei* (4.46%), *Escherichia coli* (4.24%), *Klebsiellasp* (2.90%), *Rahnellaaquatilis* (2%), *Serratialiquefaciens* (1.78%) and *Pantoeaagglomerans* (1.33%). The presence of these organisms in the fresh seafood samples showed that the source of contamination may be from environment, catching, unhygienic handling and transportation. Thus it is strongly recommended that the improvements in handling and processing are needed to minimize the prevalence of pathogenic bacteria by implementing preventive measures and application of appropriate procedures for hygiene practices to monitor the quality of raw seafoods.

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Introduction:-

Fish is a valuable and nutritious food, and an essential source of high quality and cheap animal protein crucial to balance of diets in marginally food secure communities (United Nation, 2004). Production of Fish and Shellfish by aquaculture is the fastest growing sector of world food economy. World aquaculture output has been growing at 11% per annum during the past decade and has reached 33.3 million tonnes in the year 1999 (Biradar *et al*, 2001).

Unlike meat and poultry, fish are more liable to contamination with pathogenic bacteria from human reservoirs which may contaminate the water depending on the fishing and also may be further contaminated during handling, processing and packaging. While the muscle fleshes of fish, which is the main edible part is normally sterile but microorganisms can penetrate from the skin and the gut to the flesh (FAO, 1983).

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Fishery products have been recognized as a major carrier of food-borne pathogens. The contamination can occur prior to harvest, during harvest and processing operations, distribution, storage and preparation of the product. In a simplified overview, poor quality products, spoiled or decomposed are rarely responsible for food poisoning because they are usually discarded before consumption. Food poisoning in seafood products such as scombroid or histamine poisoning, normally is the result of mishandling during or after preparation. The microbial flora of seafood is related to the environment from which the fish are harvested (Wekellet *al*, 1994; Falana, 2005).

Fresh fish is a very perishable product and spoil due to microbiological activity, chemical oxidation of lipids and autolysis. However, microbial spoilage is the main mechanism affecting fresh fish quality. As the bacteria grow they utilize nutrients and produce by-products. It is well established that the accumulation of metabolic products is the primary cause of the organoleptic rejection of fresh fish. It is also known that spoilage is caused only by a fraction of the initial microbial population, known as specific spoilage organisms (SSO), which produce metabolites responsible for off-flavors and cause the organoleptic rejection of the product. The fraction of the initial micro-biota that dominate and the metabolic products that are produced are determined mostly by the temperature and atmospheric conditions during storage (Gram and Huss, 1996).

The *Enterobacteriaceae* count is considered as an index to determine the seafood quality. Some human pathogens such as *Escherichia coli*, *Klebsiellasp*, *Salmonellasp*, *Citrobactersp* and *Proteus sp*, when isolated from fish and fish products gives an identification about environmental fecal pollution of fish. These organisms have been found to survive and multiply in the gut, mucus and tissues of fish and that render fish acting as potential vector of human disease over long periods (Onyangoet *al*, 2009; Wogu and Maduakol, 2010).

Contamination of seafood with *Salmonella* is a major public health concern. Salmonellosis is a worldwide problem responsible for food poisoning outbreaks but it is mainly difficult to determine whether the contamination of fishes occurred in their aqueous habitat or during their handling and marketing (Etzelet *al*, 1998). Coliforms and Fecal coliforms in fish demonstrate the level of pollution of their environment because these organisms are not the normal bacterial flora in fish. (Cohen and Shuval, 1973).

Therefore, the intention of this study is to determine the contamination level of seafood samples with *Enterobacteriaceae* collected from the Eastern province of Saudi Arabia. And to take recommendations to reduce risk associated with the consumption of seafood.

Materials and Methods:-

A total of 448 samples comprising of Fishes (353), Prawns (49) and Cuttlefishes (46) were randomly collected from retail and wholesale fish markets in Damman during the period from January 2015 until May 2016. The collected samples were individually kept in sterile polyethylene bags embedded with ice and transported rapidly to the laboratory in an insulated box.

Sample processing for Bacteriological Examination:-

Bacteriological analyses of collected seafoods were performed within 2 hours of sampling. All media used in the present study were from Oxoid, U.K. and prepared according to the standard procedures. Media were sterilized by autoclaving at 121°C at 15 psi pressure for 15 minutes, unless otherwise specified. Sterility of the media was checked by incubating at 37°C for 24 hours. The microbiological analysis was performed as per the standard methods adopted from the Online Bacteriological Analytical Manual, USFDA (BAM 8th edition Online).

Salmonella species:-

25 g of the sample was homogenized in 225 ml of Lactose Broth (LB) and incubated overnight at 37°C. After the initial pre enrichment step, samples were selective enriched in Tetrathionate Broth (TTB) at 37°C and Rappaport Vassiliadis (RV) medium at 42°C and incubated overnight. A loopful of growth from each of these media was streaked on Bismuth Sulphite Agar (BSA), Xylose Lysine Deoxycholate Agar (XLDA) and Hektoen Enteric Agar (HEA). After 24 (HEA, XLDA) – 48 (BSA) hours of incubation at 37°C, both atypical and typical colonies were picked up, purified and identified further by subsequent biochemical tests which included Gram staining, Motility, Oxidase production and then identified at the genus and species level using API 20E (Biomerieux, France) test kits. The identification was obtained by using the identification software “api web”.

Other Enterobacteriaceae:-

25 g of the sample was homogenized in 225 ml of sterile physiological saline in a filter stomacher bag and made into slurry using the BagMixer® (Interscience, France) and the diluted samples were plated on MacConkey Agar. After 24 hours of incubation at 37°C, both pink and colourless colonies were picked up, purified and identified further by subsequent biochemical tests which included Gram staining, Motility, Oxidase production and then identified at the genus and species level using API 20E (Biomérieux, France) test kits. The identification was obtained by using the identification software “api web”.

Results and Discussion:-

The *Enterobacteriaceae* are all occurring on fish products as a result of contamination from the animal/human reservoir. This contamination has normally been associated with fecal contamination or pollution of natural water or water environments, where these organisms may survive for a long time (months) or through direct contamination of products during processing (FAO).

This study was conducted to determine the incidence of *Enterobacteriaceae* in Seafoods (353 Fishes, 49 Prawn and 46 Cuttlefish) for sale in wholesale and retail outlets of fish markets in Eastern province of Saudi Arabia. The results related to the incidence and identification at the species level of *Enterobacteriaceae* from Fishes, Prawn and Cuttlefish are summarized in Table 1. The overall percentages of prevalent bacterial species are shown in Table 2.

Out of 448 samples analysed, 304 (67.85%) samples showed positive isolation of *Enterobacteriaceae*. The ranges of incidence levels of *Enterobacteriaceae* were from Fishes 233 out of 353 (66%), Prawn 44 out of 49 (89.79%) and Cuttlefish 27 out of 46 (58.69%).

The obtained results were revealed that the dominant bacterial species occurred most frequently in the three types of seafood samples includes *Morganellamorganii* with 83 isolates (18.52%), followed by *Proteus* spp with 55 isolates (12.27%), *Citrobacter* spp with 36 isolates (8.03%), *Enterobacter* spp with 30 isolates (6.69%), *Providencia* spp with 25 isolates (5.58%), *Hafnia alvei* with 20 isolates (4.46%), *Escherichia coli* with 19 isolates (4.24%), *Klebsiella* spp with 13 isolates (2.90%), *Rahnella aquatilis* with 9 isolates (2%), *Serratia liquefaciens* with 8 isolates (1.78%) and the lower isolate was *Pantoea agglomerans* with 6 isolates (1.33%).

Salmonella was not detected in any of the samples analysed in this study, which was in agreement with previous studies (Popovic *et al*, 2010; Rodriguez *et al*, 2011; Figenet *et al*, 2014) in seafood products. Conversely, different workers (Shabarinath *et al*, 2007; Kumar *et al*, 2009; Bakret *et al*, 2011) reported varying incidence rates of *Salmonella* in seafood.

Morganellamorganii is widely distributed in nature, commonly found in the environment and the intestinal tracts of humans, mammals and reptiles as a part of the normal flora. Generally *Morganellamorganii* can act as an opportunistic pathogen that mainly causes wound and urinary tract infections. The drug resistance of *Morganellamorganii* is increasing in recent years, which often results in Clinical treatment failure (Hui Liu *et al*, 2016). In the present study *Morganellamorganii* showed highest prevalent (18.52%) in all the analysed seafoods. In a study conducted by SureeratPhuvasate (2008), he clearly demonstrated the ability of *Morganellamorganii* to produce Histamine in fish samples. Histamine is a biogenic amine formed through the decarboxylation of the amino acid histidine by the enzyme histidine decarboxylase. Consumption of fish containing histamine may result in illness known as Scombroid poisoning with a variety of symptoms, including rash, facial flushing, sweating, vomiting, diarrhea, head ache, burning sensation and metallic taste in the mouth.

Proteus sp. were involved in the spoilage of seafoods which can result in potential health hazard to the consumers. It can cause enteric infection in humans, summer diarrhea in infants, sinusitis, otitis as well as urinary tract infections (Bryan 1980; Varnam and Evas, 1991). The isolation of *Proteus* sp. from fish samples has been reported in previous studies by Tosunet *et al* (2016). Sanaa O. Yagoub (2009) isolated *Proteus* sp in fish with an incidence of 10.2%. In the present study, the percentages of *Proteus mirabilis* and *Proteus vulgaris* were 7.58% and 4.68% respectively. Some investigations involve *Proteus* genus as an indicator of fecal contamination in marine fish, although their use as an indicator is not very common (ILSI, 2011).

In this study, members of *Citrobacter* were identified, represented by species in the ranges of *Citrobacter freundii* (4.24%), *Citrobacter braakii* (2.67%) and *Citrobacter koserii* (1.11%). Olugbojo *et al* (2015), detected the presence of

Citrobactersp in fish samples of commercial importance collected from Nigeria. In a study conducted by Tosunet *al* (2016), *Citrobactersp* showed highest prevalence (45.56%) among all other *Enterobacteriaceae* from Horse Mackerel fish sold in public markets of Istanbul, Turkey. *Citrobactersp* are widely distributed in soil, water, food and the intestinal tract of humans and animals. They make up the normal flora of the human gut and were previously considered as a purely environmental contaminants or non-pathogenic colonizers, but known to be opportunistic pathogens, as they can cause serious infections, sepsis, food borne outbreaks of gastroenteritis, respiratory infections and urinary tract infections, especially in high-risk groups such as infants and immune compromised adults (Rusulet *al*, 1991; Dos santoset *al*, 2015).

The *Enterobactersp* can be found in soil, water, sewage, intestinal tract of man and animals (Banwart, 1989). In the present study, several species of *Enterobacter* has been isolated which includes *Enterobacter cloacae* (2.67%), *Enterobacter aerogenes* (2.23%), *Enterobacteramnigenus* (1.11%) and *Enterobactersakazakii* (0.66%). In a study conducted by Mai F. Elsheriefet *al* (2014), he isolated *Enterobactersp* along with other members of *Enterobacteriaceae* from the farmed and retailed fishes of Tilapia and Mugilcephalus. *Enterobactersp* can act as opportunistic pathogens that rarely cause primary diseases in humans. They are described as agents associated with respiratory infections, skin, urinary tract, bones, joints, central nervous system and gastro intestinal tract infections (Dos Santos *et al*, 2015).

In this study, members of *Providencia* were isolated, identified and represented by species in the percentages of *Providenciastuartii* (2.67%), *Providenciaertgeri* (1.56%) and *Providenciaalcalifaciens* (1.33%). Sanaa O. Yagoub (2009), isolated *Providencia* app along with other members of *Enterobacteriaceae* including, *Esherichia coli*, *Enterobactersp*, *Klebsiellasp*, *Citrobactersp*, *Salmonellasp*, *Shigella* sp, *Serratiasp* and *Proteussp* from raw fish sold in fish market in Khartoum state of Sudan. *Providenciasp* are generally considered to be commensals in the gastrointestinal tract of humans, warm blooded animals, often also in soil and sewage water. *Providenciaertgeri*, *Providenciastuartii* and *Providenciaalcalifaciens* has been isolated from samples of human feces, either as part of the human intestinal microbiota as well as a cause of gastric disorder, such as traveler's diarrhea. In 2001, *Providenciaalcalifaciens* was reported in a major outbreak of food borne illness among children in japan. They can act as opportunistic pathogen involved in urinary tract infections, keratitis, dacryocystitis, conjunctivitis and endophthalmitis. The bacteria *Providenciastuartii* is reported as more resistant species of the family *Enterobacteriaceae*. The emerging of resistance in *Providenciasp* is a clinical concern as it may lead to increased treatment cost and delays in treatment, which can be fatal to the patient (Dost Santos *et al*, 2015).

Hafniaalvei, a member of the family *Enterobacteriaceae* and the only species of the genus *Hafnia*described, is part of the human gastrointestinal flora, but can be found in environmental habitats such as surface water and food. *Hafniaalvei* is normally considered as a colonizing organism and rarely appears to be a pathogen. Recent findings suggest that it is a rare but may contribute to opportunistic infections in humans apart from diarrhea; it can cause extra intestinal infections such as septicaemia, liver abscess, peritonitis and pneumonia (Podschunet *al*, 2001; Dos Santos *et al.*, 2015). In the present investigation, the prevalence range of *Hafniaalvei* is around 4.46% in seafoods, followed by *Escherichia coli* with the percentage of 4.24% and members of *Klebsiellasp*, represented by its species in the ranges of *Klebsiellaterrigena* (1.56%) and *Klebsiellapneumoniae* (1.33%). Isolation of *Escherichia coli* and *Klebsiellasp* from the seafood samples analysed in this study was in agreement with the previous studies done by different workers (Yagoub, 2009; Mai F. Elsheriefet *al*, 2014; Romero Jarero Jorge, 2016; Tosunet *al*, 2016) who reported varying incidence rates of such organisms in seafoods.

Infections associated with *Escherichia coli* through the ingestion of seafoods are directly related to fecal contamination. *Escherichia coli* is normally present in the intestinal flora of humans and warm-blooded animals which may transfer to the foods due to the poor hygienic conditions, cross contamination or contaminated water (Huss *et al*, 2004; Renata Albuquerque costa, 2013). *Klebsiellasp* can also act as an opportunistic pathogen that cause wide range of infections including foodborne outbreaks of gastroenteritis, urinary tract infections, pneumonia, bacteremia, neonatal sepsis and wound infections (Rahkiaet *al*, 1998; Podschunet *al*, 1998).

In the present study members of *Enterobacteriaceae* such as *Rahnellaaquatilis*, *Serratialiquefaciens* and *Pantoeaagglomerans* were isolated in the ranges of 2%, 1.78% and 1.33% respectively from the analysed seafoods. All the above organisms can act as opportunistic pathogen in humans. *Rahnellaaquatilis* is typically associated with fresh water, since been isolated from blood, surgical wounds, urine, sputum, bronchial washings and stool, usually in immune compromised patients or in young children (KaleyTash, 2004). *Serratialiquefaciens* also an opportunistic

pathogen which is capable of colonizing a wide variety of surfaces in water, soil, the digestive tracts of rodents, plants, insects, fish and humans (Grimont and Grimont, 1978). *Pantoea agglomerans* inhabits plants, soil, and water, reported as both commensal and pathogen of animals and humans. They rarely cause disease in healthy individuals, such as septic arthritis, outbreak secondary to contaminated parental nutrition, occupational respiratory infections and skin allergy (Maria Carla Liberto *et al.*, 2009).

Most of the *Enterobacteriaceae* members isolated and identified in this study were comes under the group of Coliforms and Fecal Coliforms. Austin and Austin (1985) have demonstrated the presence of *Escherichia coli*, *Enterobacter* sp, *Citrobacter* sp, *Salmonella* sp, *Proteus* sp, in fish that lives in contaminated marine water by domestic waste water. Priyanka Rani Majumdar *et al.* (2014), stated that the untreated and the improper way of sewage disposal system in the water body is one of the main sources for microbial water contamination which results in the accumulation of these bacterial pathogenic species in the commercial edible fishes. Moreover, unhygienic handling by infected people or healthy carriers, during the capture, transport and processing may also act as a source of contamination by pathogenic organisms in seafoods.

Table 1:-List of Bacterial species isolated from Fishes, Prawn and Cuttlefish.

FISHES	PRAWN	CUTTLEFISH
<i>Enterobacter cloacae</i>	<i>Enterobacter aerogenes</i>	<i>Proteus mirabilis</i>
<i>Enterobacter aerogenes</i>	<i>Enterobacter amnigenus</i>	<i>Proteus vulgaris</i>
<i>Enterobacter amnigenus</i>	<i>Enterobacter cloacae</i>	<i>Providencia stuartii</i>
<i>Enterobacter sakazakii</i>	<i>Enterobacter sakazakii</i>	<i>Providencia rettgeri</i>
<i>Citrobacter freundii</i>	<i>Citrobacter freundii</i>	<i>Morganella morganii</i>
<i>Citrobacter braakii</i>	<i>Citrobacter braakii</i>	<i>Hafnia alvei</i>
<i>Citrobacter koserii</i>	<i>Citrobacter koserii</i>	<i>Citrobacter braakii</i>
<i>Providencia stuartii</i>	<i>Proteus vulgaris</i>	<i>Escherichia coli</i>
<i>Providencia alcalifaciens</i>	<i>Proteus mirabilis</i>	<i>Klebsiella pneumoniae</i>
<i>Providencia rettgeri</i>	<i>Morganella morganii</i>	<i>Rahnella aquatilis</i>
<i>Proteus mirabilis</i>	<i>Escherichia coli</i>	
<i>Proteus vulgaris</i>	<i>Hafnia alvei</i>	
<i>Klebsiella terrigena</i>	<i>Rahnella aquatilis</i>	
<i>Klebsiella pneumoniae</i>	<i>Serratia liquefaciens</i>	
<i>Morganella morganii</i>	<i>Pantoea agglomerans</i>	
<i>Escherichia coli</i>		
<i>Hafnia alvei</i>		
<i>Rahnella aquatilis</i>		
<i>Serratia liquefaciens</i>		
<i>Pantoea agglomerans</i>		

Table2:- Prevalence of Bacterial species among Fishes, Prawn and Cuttlefish.

S. No	Bacterial Isolates	Bacteria l Genus	Overall No. (%) of Prevalence in Samples			No. (%) of Prevalence for Total 448 Samples	No. (%) of Prevalence of Bacterial Genus
			Fishes (353)	Prawn (49)	Cuttlefish (46)		
01.	<i>Morganellamorganii</i>	<i>Morganellaspp</i>	63 (17.84%)	14 (28.57%)	06 (13.04%)	83 (18.52%)	83 (18.52%)
02.	<i>Proteus mirabilis</i>	<i>Proteus spp</i>	28 (07.93%)	02 (04.08%)	04 (08.69%)	34 (07.58%)	55 (12.27%)
03.	<i>Proteus vulgaris</i>		14 (03.96%)	03 (06.12%)	04 (08.69%)	21 (04.68%)	
04.	<i>Citrobacterfreundii</i>	<i>Citrobacterspp</i>	16 (04.53%)	03 (06.12%)	---	19 (04.24%)	36 (08.03%)
05.	<i>Citrobacterbraakii</i>		07 (01.98%)	03 (06.12%)	02 (04.34%)	12 (02.67%)	
06.	<i>Citrobacterkoserii</i>		04 (01.13%)	01 (02.04%)	---	05 (01.11%)	
07.	<i>Enterobacter cloacae</i>	<i>Enterobacterspp</i>	11 (03.11%)	01 (02.04%)	---	12 (02.67%)	30 (06.69%)
10.	<i>Enterobacter aerogenes</i>		06 (01.69%)	04 (08.16%)	---	10 (02.23%)	
08.	<i>Enterobacteramnigenus</i>		03 (00.84%)	02 (04.08%)	---	05 (01.11%)	
09.	<i>Enterobactersakazakii</i>		02 (00.56%)	01 (02.04%)	---	03 (00.66%)	
11.	<i>Providenciastuartii</i>	<i>Providenciaspp</i>	09 (02.54%)	---	03 (06.52%)	12 (02.67%)	25 (05.58%)
13.	<i>Providenciarettgeri</i>		06 (01.69%)	---	01 (02.17%)	07 (01.56%)	
12.	<i>Providenciaalcalifaciens</i>		06 (01.69%)	---	---	06 (01.33%)	
14.	<i>Hafniaalvei</i>	<i>Hafniaspp</i>	13 (03.68%)	03 (06.12%)	04 (08.69%)	20 (04.46%)	20 (04.46%)
15.	<i>Escherichia coli</i>	<i>Escherichiaspp</i>	14 (03.96%)	04 (08.16%)	01 (02.17%)	19 (04.24%)	19 (04.24%)
16.	<i>Klebsiellaterrigena</i>	<i>Klebsiellasp</i>	07 (01.98%)	---	---	07 (01.56%)	13 (02.90%)
17.	<i>Klebsiellapneumoniae</i>		05 (01.41%)	---	01 (02.17%)	06 (01.33%)	
18.	<i>Rahnellaaquatilis</i>	<i>Rahnellaspp</i>	07 (01.98%)	01 (02.04%)	01 (02.17%)	09 (02.00%)	09 (02.00%)
19.	<i>Serratialiquefaciens</i>	<i>Serratiaspp</i>	07 (01.98%)	01 (02.04%)	---	08 (01.78%)	08 (01.78%)
20.	<i>Pantoeaagglomerans</i>	<i>Pantoeaspp</i>	05 (01.41%)	01 (02.04%)	---	06 (01.33%)	06 (01.33%)
TOTAL NUMBER OF ISOLATES			233 (66.00%)	44 (89.79%)	27 (58.69%)	304 (67.85%)	304 (67.85%)

Conclusion:-

The monitoring and control of seafood quality is one of the main goals in the seafood industries. The findings of this study revealed that the raw seafoods sold in fish markets in the eastern province of Saudi Arabia could be a source of food borne bacterial pathogens. The presence of such contaminating bacteria could be attributed to cross contamination from the environment, source and unhygienic handling by the sellers. Hence, the findings of this study may be considered as an additional knowledge to enhance proper controlling of the storage and shelf life of seafoods by implementing preventive measures such as HACCP and application of Sanitation Standard Operating

Procedures (SSOP) in fish markets to monitor the quality of seafoods by minimizing the prevalence of the pathogens.

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