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Survey of pathogenic bacteria population associated with mitten crab *Eriocheir sinensis* (H. Milne Edwards, 1853) from intertidal zones in Khor Al-Zubair canal, Basrah, Iraq

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Abstract
The present study was carried out to determine the seasonal occurrence and distribution of microbial diversity of pathogenic bacteria population present in mitten crab *Eriocheir sinensis* (H. Milne Edwards, 1853). Samples of crabs were collected during 2017, from the intertidal zones of Khor Al-Zubair canal in two seasons by hand under stones close to collection site.
A total of 24 bacterial isolates consisting five different bacterial strains such as *Pseudomonas aeruginosa, Escherichia coli, Aeromonas sp., Klebsiella pneumoniae* and *Bacillus subtilis* were isolated from gills, shells and muscle tissues of mitten crab *Eriocheir sinensis*. Microbial species were characterized based on morphological and biochemical tests.
The percentage composition of each bacterial species was recorded in different tissues of crab samples, *E. coli* (35%) and *Pseudomonas aeruginosa* (32%) are dominant among other species in crab shells and gills, respectively. *Klebsiella pneumoniae* (27%) and *Bacillus subtilis* (18%) are dominant among other species. In crab muscles, *Bacillus subtilis* (33%) and *E. coli* (28%) is dominant among other species.
Shells and gills of crab samples showed higher number of bacterial species than muscles tissues. The results of this study clearly indicate that edible crabs are contaminated with coliforms and other pathogenic microorganisms.

Keywords: Pathogenic bacteria**,** crab *Eriocheir sinensis*, Khor al-Zubair canal

Introduction
The Chinese mitten crab is a large, decapod crustacean that got its name from the light brown hairs, or setae, found on their claws that give it the appearance of wearing furry mittens (Veilleux and de Lafontaine 2007) [1]. The Chinese mitten crab *Eriocheir sinensis* (Milne-Edwards, 1853) is listed among the 100 worsummst invasive species worldwide (Lowe *et al*. 2004) [2]. Originating from China, it is currently an invasive species in many countries, including Europe and North America (Rudnick *et al*. 2000; Herborg *et al*. 2003) [3, 4]. In Europe, the first specimens were found in 1912 in the River Aller, in Northern Germany where they were presumably introduced via ballast water from ships (Gollasch 2011) [5]. As a catadromous species, the Chinese mitten crab migrates after a marine larval stage from brackish river estuaries several hundreds of kilometres upstream to reach maturity and return to the estuary to reproduce (Hymanson 1999) [6].
On the other hand, Chinese mitten crab *E. sinensis* is an economically important crustacean farmed in china with an annual production of 700,000 tons in 2012 and a culture area of more than 64,000 hectares in China. The Chinese mitten crab cultured in the Chinese provinces of Jiangsu, Anhui, Hubei, and Liaoning (Shen *et al*., 2014) [7]. With the rapid increase of the *E. sinensis* aquacultural industry, numerous bacterial diseases have recently evolved, thus resulting in huge economic losses (Shen *et al*., 2015) [8]. Many bacterial species of enteric origin can be isolated from harbours which are located around sites of human habitation, including *Bacillus cereus, Staphylococcus aureus, Vibrio parahaemolyticus, Salmonella spp., Escherichia coli, Shigella* spp., *Listeria monocytogenes*, and *Klebsiella* spp. These bacterial species are commonly isolated from waters which contain fecal materials (Badley *et al.*, 1990; Jones and Summer-Barason, 1998; Martinez-Manzanarez *et al.*, 1992) [9-11].
The crabs are in intimate contact with aquatic environment rich in pathogenic microbes and are

prone to infection by those microbes at various stages of growth, and losses due to disease can be enormous (Hudson and Lester, 1994) [12]. Microbial infections have been the major concern of aquaculturists worldwide. Various bacteria in marine and estuarine environment such as *Vibrio cholera*, and *Vibrio parahaemolyticus* and *Vibrio* species are potential human pathogens (Broza *et al*., 2007) [13].

Seafood related disease outbreaks have been reported almost throughout the world including countries like Japan, U.S, India and U.K. International Committee for Microbiological Food Safety (ICMFS) has devised permissible counts for various pathogens in different food products. Presence of these pathogens above the acceptable level is usually rejected by the importing country as unfit for human consumption, so to assess the microbiological quality of seafood in any part of the world has become significant to avoid health hazards and also economic losses (Soundarapandian and Sowmiya, 2013) [14].

The present study provides an account of microbial communities isolated from shells, gills and muscles of crab samples and also the density of bacterial population from different tissues of crabs collected from the intertidal zones in the banks of Khor Al-Zubair canal, Basrah, Iraq.

Materials and Methods

The mitten crab *Eriocheir sinensis* were collected from under the stones by hands in the intertidal zones of Khor Al-Zubair canal, Basrah, Iraq. Figure (1). Were brought to the laboratory and maintained in plastic tubs.

This study involved 60 mitten crabs (weight 12.8-45.5 g, length of carapace 22.7-58.6 mm, width of carapace 28.8-65.4 mm) obtained in four seasons during 2017. They were transported to microbiological laboratory of marine science center for analysis.

The medium was prepared by dissolving nutrient agar (NA) (Merck) in distilled water and was autoclaved.

The crab tissues such as gills, shells (carapace) and muscles were isolated by serial dilution technique using nutrient agar medium (Richmond, 1997) [15]. The shells, gills and muscles tissues of crab samples were dissected under a sterile condition and homogenized tissue was used for bacterial isolation tests. Each homogenized tissue sample was serially diluted and spread on nutrient agar medium separately, with one plate maintained as a control without sample. The plates were incubated at 37 °C for 24 to 48 h for observation of the bacterial colonies. Confirmation test were also performed using MacConkey agar, EMB (Eosin-Methylene blue agar) and Pseudomonas agar (APHA, 1998) [16].

To estimate bacterial numbers, the inoculated plates were incubated at 37 °C for two days and duplicates were prepared for each dilution. Following incubation, the total number of colony forming unit (CFU) was determined and representative colonies were subcultured for identification. Bacterial numbers were calculated as the average of each set of duplicates and expressed as CFU/ml of the homogenate. Bacteria were isolated by a random collection of colonies from the agar plates. The colonies were purified by repeatedly sub culturing them on agar.

Phenotypic characteristics, Gram staining, and biochemical tests were determined for all isolates according to (Holt *et al*., 1994) [17]. The isolated bacterial species were identified by the following procedures. The morphological and biochemical characteristics of the individual colony was recorded. The individual colony was transferred to nutrient agar. The isolates were subjected to different biochemical tests, for example, Gram staining, motility test, indole test, methyl red test, Voges Proskauer test, catalase test, nitrate test and carbohydrate fermentation test as described by (Buchanan and Gibbons, 1985) [18].

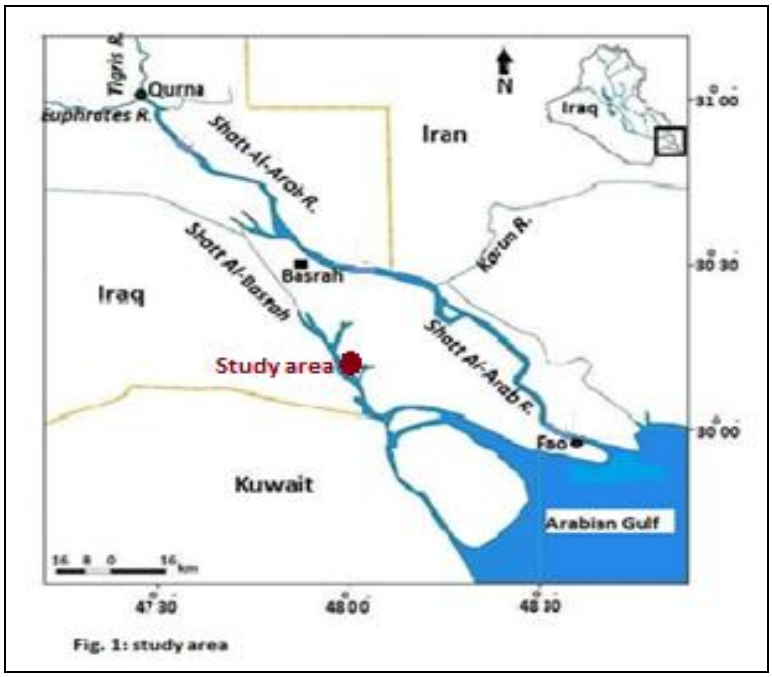

Fig 1: Map of study area at Khor Al-Zubair canal

Results

Specimens of mitten crab *Eriocheir sinensis* (H. Milne Edwards, 1853) were collected from the intertidal zones in the banks of Khor Al-Zubair, Basrah, Iraq. A total of 24 bacterial isolates were obtained from 60 crabs representing five species. *Pseudomnas aeroginosa* 21(35%) Followed by *E. coli* 17(28%), *Aeromonas sp.* 12(20%), *Klebsiella pneumoniae* 8(13%) and *Bacillus subtilis* 8(13%): as shown in

Table (1).

The percentage composition of each bacterial species was recorded in different tissues of crab samples (Table 2). *E. coli* (35%) and *Pseudomonas aeruginosa* (32%) were dominated among other species in crab shells and gills, respectively, *Klebsiella pneumoniae* (27%) and *Bacillus subtilis* (18%) are dominant among other species. In crab muscles, *Bacillus subtilis* (33%) and *E. coli* (28%) is dominant among other species.

The crab tissues such as shell, gills and muscles showed that mean value of CFU were found maximum during summer season and were found minimum during winter season. *Pseudomonas aeruginosa* and *E. coli* found in higher density in gill and shell tissues. *Aeromonas sp.* was found in higher density in muscle tissues. *Klebsiella pneumoniae* in shell tissues was found in higher density, *Bacillus subtilis* was found in higher density in muscle tissues. The seasonal counts in summer and winter seasons of *pseudomonas aeruginosa* in crab gill tissues ranged from 2.61- 7.29 $x10^5$ CFU/g, *Bacillus subtilis* counts ranged from 1.19 - 3.23 $x10^5$ CFU/g in muscles, *Klebsiella pneumoniae* counts ranged from 2.94-4.47 $x10^5$ CFU/g in muscles, *E. coli* counts ranged from 4.53-10.21 $x10^5$ CFU/g in shells during summer and winter seasons, respectively. (Table 3).

All bacterial isolates including pathogens antagonistic isolates were identified using biochemical and morphological tests. A total of 5 bacterial species were isolated and were predominant by gram-negative bacteria. Of these, *Pseudomonas aeruginosa, E. coli, Aeromonas sp., Klebsiella pneumoniae and Bacillus subtilis* were found common in shells, gills and muscles of crab tissues Table (4).

Table 1: Bacteria isolated from Gills, Shells and Muscles of specimens mitten crab *Eriocheir sinensis*

Bacteria species	Gills	Shells	Muscles	No. of crabs	% of crabs
Bacillus subtilis	+	+	+	8	13
Escherichia coli	+	+	-	17	28
Aeromonas sp.	-	+	-	12	20
Klebsiella pneumonia	+	+	-	10	16
Pseudomonas aeruginosa	+	+	+	21	35

+: presence of bacteria species -: Absence of bacteria species.

Table 2: the percentage composition of bacterial population during 2017 isolated from Gills, Shells and Muscles of mitten crab *Eriocheir sinensis*

Bacteria species	Gills %	Shells %	Muscles %
Bacillus subtilis	15	18	33
Escherichia coli	30	35	28
Aeromonas sp.	0	12	16
Klebsiella pneumonia	18	27	12
Pseudomonas aeruginosa	32	15	11

Table 3: Seasonal variation of bacterial species in mean value of CFU/g in two seasons during 2017 isolated from Gills, Shells and Muscles of mitten crab *Eriocheir sinensis*

Bacteria species	**Gills** CFU/g		**Shells** CFU/g		**Muscles** CFU/g	
	Summer	**winter**	**summer**	**Winter**	**summer**	**Winter**
Bacillus subtilis	$4.56x10^3$	$2.84x10^3$	$2.38x10^4$	$1.26x10^3$	$3.23x10^5$	$1.19x10^5$
Escherichia coli	$7.77x10^5$	$4.84x10^5$	$10.21x10^5$	$4.53x10^5$	$1.27x10^5$	$0.34x10^5$
Aeromonas sp.	0	0	$2.03x10^5$	$1.00x10^5$	$3.68x10^5$	$1.93x10^5$
Klebsiella pneumonia	$7.29x10^5$	$2.06x10^5$	$2.16x10^5$	$2.72x10^5$	$4.47x10^5$	$2.94x10^5$
Pseudomonas aeruginosa	$4.56x10^5$	$2.61x10^5$	$3.92x10^5$	$1.15x10^5$	$2.60x10^5$	$1.10x10^5$

Table 4: Morphological and Biochemical characteristics of bacteria species isolated from specimens mitten crab *Eriocheir sinensis*

Identification of Bacteria. / **Reaction agent**	***Bacillus subtilis***	***Escherichia coli***	***Aeromonas salmonicida***	***Klebsiella pneumoniae***	***Pseudomonas aeruginosa***
Gram staning Shape	G^{-ve} cocci	G^{-ve} bacilli	G^{-ve} bacilli	G^{-ve} bacilli	G^{-ve} bacilli
Motility	M	M	M	M	M
Indole test	+	-	-	-	-
Methyl red test	+	-	-	-	-
Voges proskuer test	-	-	-	-	-
Citrate utilization test	-	+	+	-	+
Urease test	-	+	+	+	+
TSI test		A/A+G	A/A	A/A+G	K/K
H2S	-	-	-	+	-
Gas	-	+	+	+	+
Nitrate reduction test	+	-	-	+	-
Catalase test	+	+	+	-	+
Oxidase test	+	+	+	-	+
Carbohydrate test	+	+	+	+	+
Glucose	+	+	+	-	+
Maltose	-	-	-	-	-
Sucrose	-	-	-	-	-

Note: - Negative, + Positive, A/A -Gulcose & lactose and /or sucrose fermentation, K/K -no fermentation, A/A+G- Gulcose & lactose and /or sucrose fermentation+ gas produced.

Discussion

Environmental loading of fecal byproducts from humans and their associated animals is significant and can affect the quality of water and food resources in coastal ecosystems (Fayer, 2004; Kim *et al*., 2004) [19 20]. In addition, infected crabs can represent a significant public health problem. The Higher microbial counts in some samples may be attributable to handling during harvest or processing. The total bacteria

count on fish rarely indicate the quality of the fish but it gives an indication of the risk of spoilage induced since each of these organisms had different ways of effecting health conditions of consumers of such contamination fish (Krantz *et al*., 1969) [21].

The bacterial populations during different seasons were also fluctuated widely depending on physico-chemical parameters of the environment (Mahalakshmi *et al*., 2012) [22]. Thampuran *et al*., (2005) [23] also reported that the microbial quality of the *Tilapia* indicated that all tissue samples except muscle were contaminated with fecal coliform where *Escherichia coli* is the most common contaminant and is often encountered in high numbers. The isolation of these groups of organisms indicted faecal and environmental pollution and these supported the findings of Yagoub *et al*., (2004) [24].

The isolation of *Pseudomonas* sp. from the collected crab samples is of highly importance because this bacterium plays a considerable role as potential pathogenic bacteria for human and as an indicator of food quality as spoilage organism. This is in accordance with previously mentioned by Jeyasekaran *et al*., (2005) [25] and Koutsoumanis and Nychas (2000) [26] who identified pseudomonas as a good spoilage index. Although *Pseudomonas sp.* is not referred to as the cause of food borne illnesses they are closely associated to food deterioration (Tryfinopoulo, 2002) [27].

In the present study bacteria isolated could be pathogenic and involved in disease transmission to human, previous studies on pathogenic bacteria in crabs include: (Faghri, 1984) [28] who found pathogenic bacterial in several species of crabs such as tanner crab *Chionoectes opilio*, Dungeness crab *Cancer magaster*, king crab *Paralithodes camtschatia* and Rock crab *Cancer irroratus*, this could serve as accumulation sites for human pathogens particularly in crabs collected from contaminated area. (Hauxhurst *et al*., 1980; Hauxhurst *et al*., 1981) [29, 30]. indicated that crab tissues contained higher number of bacteria than their surrounding environments. Most of the bacterial species found in the present study were comparable to bacteria found in cultivated mitten crab *Eriocheir sinensis* Farmed in Lake Tai(Chen *et al*. 2015) [31], and China oyster in Setia Wetland (Najiah *et al*., 2008) [32].

The higher presence of *E. coli* and *P. aeruguinosa* in the present study further suggested that fecal contamination occurred in Khor Al-Zubair canal.

Conclusion

On the basis of the results of this study the bacteria were isolated from muscles, shells and gills of crab samples. The isolated bacterial species were predominately fecal coliforms associated with the tissues of crabs indicate possible fecal contamination that may have occurred through sewage effluents and others like; *E. coli*, *P. aeruguinosa* etc. are also found. The sampling site were contaminated by human activities. The occurrence of high counts of pathogens in sea food may cause food poisoning; especially in individuals who consume this sea food raw, or lightly or insufficiently cooked. Before sewage disposal into the sea, it should be treated otherwise it may influence the organic load directly and bacterial load indirectly.

Authors' contributions

All authors in this paper have contributed equally toward the publication of this paper.

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