

Submitted: 05/10/2015

Accepted: 25/01/2016

Published: 02/03/2016

Isolation and molecular identification of *Vibrio* spp. by sequencing of 16S rDNA from seafood, meat and meat products in Libya

S.M. Azwai¹, E.A. Alfallani¹, S.K. Abolghait², A.M. Garbaj³, H.T. Naas³, A.A. Moawad⁴, F.T. Gammoudi¹, H.M. Rayes¹, I. Barbieri⁵ and I.M. Eldaghayes^{1,*}

¹Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Tripoli, P.O. Box 13662, Tripoli, Libya

²Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Suez Canal University, 41522 Ismailia, Egypt

³Department of Food Hygiene and Control, Faculty of Veterinary Medicine, University of Tripoli, P.O. Box 13662, Tripoli, Libya

⁴Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt

⁵Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via Bianchi, 9 - 25124 Brescia, Italy

Abstract

The genus *Vibrio* includes several food-borne pathogens that cause a spectrum of clinical conditions including septicemia, cholera and milder forms of gastroenteritis. Several *Vibrio* spp. are commonly associated with food-borne transmission including *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. Microbiological analysis for enumeration and isolation of *Vibrio* spp. were carried out for a total of 93 samples of seafood, meat and meat products from different geographic localities in Libya (Tripoli, Regdalin, Janzour and Tobruk). *Vibrio* spp. were detected by conventional cultural and molecular method using PCR and sequencing of 16S rDNA. Out of the 93 cultured samples only 48 (51.6%) yielded colonies on Thiosulfate Citrate Bile Salt agar (TCBS) with culture characteristics of *Vibrio* spp. More than half (n=27) of processed seafood samples (n=46) yielded colonies on TCBS, while only 44.6% of samples of meat and meat products showed colonies on TCBS. Among cultured seafood samples, the highest bacterial count was recorded in clam with a count of 3.8×10^4 CFU/g. Chicken burger samples showed the highest bacterial count with 6.5×10^4 CFU/g. Molecular analysis of the isolates obtained in this study, showed that 11 samples out of 48 (22.9%) were *Vibrio* spp. *Vibrio parahaemolyticus* was isolated from camel meat for the first time. This study is an initial step to provide a baseline for future molecular research targeting *Vibrio* spp. foodborne illnesses. This data will be used to provide information on the magnitude of such pathogens in Libyan seafood, meat and meat products.

Keywords: 16S rDNA, Libya, Meat, Seafood, *Vibrio*.

Introduction

Libya enjoys one of the longest coastlines (1800 km) on the southern Mediterranean basin; this makes seafood an important item for consumers. In general, the overall consumption of meat and meat products in Libya is increasing with the consumption of camel meat being higher than beef. This increase in meat consumption is including as well various traditional meat products that have long been known in the country and prepared by families at home or during religious feasts.

Improperly handled seafood, meat and meat products could pose a great source of infectious agents that are transmissible to humans. *Vibrio* spp. are among the infectious agents that can result in deterioration of meat or represent a potential disease source for humans. The risk of disease from ingesting pathogens found in raw meat is significantly higher than cooked meat, although both can be contaminated (Newell *et al.*, 2010). Meat can be incorrectly or insufficiently cooked, allowing disease-carrying pathogens to be ingested. In addition, meat can be contaminated during the production

process at any time, from the slicing of prepared meats to cross-contamination of food in a refrigerator. All of these situations may lead to a greater risk of disease.

From public health point of view, *Vibrio* spp. represents a greater portion of the food borne illnesses across the coast cities worldwide (Rebaudet *et al.*, 2013). This could be due to food contamination with *Vibrio* spp. shed from seafood or prevalent usage of undercooked seafood/meat or surface contamination during marine shipping of such foods. Despite the vast majority of environmental *V. parahaemolyticus* isolates are avirulent, it is leading cause of gastroenteritis linked to seafood consumption in the United States (Iwamoto *et al.*, 2010). Some *Vibrio* spp. poses a significant health threat to humans who suffer from immune disorders and liver diseases. It enters human hosts via wound infections or consumption of raw shellfish (primarily oysters), and infections frequently progresses to septicemia and death in susceptible individuals (Harwood *et al.*, 2004). The cosmopolitan distribution of *Vibrio* spp. and the lack of abattoirs for proper meat inspection, prompted us

*Corresponding Author: Dr. Ibrahim Eldaghayes. Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Tripoli, P.O. Box 13662, Tripoli, Libya. E-mail: ibrahim.eldaghayes@vetmed.edu.ly

to carry out this study. The main objective of this study was to characterize bacteria isolated from seafood, meat and meat products that may cause foodborne illnesses. We plan to use this data to help create a baseline for future research into foodborne illness in Libya.

Materials and Methods

Collection of samples

A total of 93 samples (Table 1) of seafood, meat and meat products that includes 21 of shrimps; 5 of clam; 20 of fish; 34 samples of raw meat (10 beef, 9 camel meat, 6 mutton and 9 chickens) and 13 samples of meat products (2 beef sausages, 5 beef burgers, 5 chicken burgers and 1 kebab) were randomly collected from different geographic localities in Libya [Tripoli, Regdalin (120 km west of Tripoli), Janzour (30 km west of Tripoli) and Tobruk (1400 km east of Tripoli)]. Each sample was 250 g in weight. The Samples were packed in sterile plastic bags and stored in an insulated box containing crushed ice. The samples were transferred as quickly as possible to Food Hygiene and Control Laboratory at the Faculty of Veterinary Medicine, University of Tripoli. All samples were subjected to *Vibrio* spp. microbiological enumeration and isolation.

Samples processing

Preparation of samples, decimal dilutions, culturing and enumeration techniques of bacteria were performed according to the methods described previously (Downes

and Ito, 2001). Briefly, 25 g from each sample was aseptically transferred into a sterile polyethylene stomacher bag and blended with 225 ml of sterile alkaline peptone water (Catalogue #610098, LIOFILCHEM, Italy) in a stomacher homogenizer (Stomacher 400, Seaward medicals, UK.) at 230 rpm for 60 s. Serial dilutions were made using sterile 0.1% peptone water.

Isolation, cultural characteristics and enumeration of *Vibrio* spp.

Determination of the *Vibrio* spp. count was performed using *Vibrio* spp. selective Thiosulfate Citrate Bile Salt agar plates (TCBS: catalogue #611010, LIOFILCHEM, Italy). TCBS agar plates were inoculated by spreading 0.1 ml of the serial dilutions and incubated at 37°C for 48 h. TCBS plates were examined for the presence of either yellow, round, 2-3 mm diameter colonies (suspect: *V. cholera*, *V. fluvialis* or *V. alginolyticus*) or green, round, 2-3 mm diameter colonies (suspect: *V. parahemolyticus* or *V. vulnificus*). Countable plates are those containing 25 to 250 colonies (Kaysner and DePaola, 2001).

Purification of *Vibrio* spp.

For purification, single colony from each grown type of *Vibrio* suspect colonies was streaked onto another TCBS agar and incubated overnight at 37°C. This process has been performed until obtaining pure consistent colonies.

Table 1. Total number, enumeration and molecular identification of suspected *Vibrio* spp. in processed samples.

Type of sample	No. of samples	No. of suspected <i>Vibrio</i> spp. growth on TCBS (%)	No. of positive <i>Vibrio</i> spp. by 16S r DNA sequencing	Average CFU/g
Shrimp	21	11 (52.3)	1	6.25×10 ²
Clam	5	4 (80)	1	3.8×10 ⁴
Sardine	8	7 (87.5)	3	3.7×10 ³
Mackerel	4	3 (75)	3	3.8×10 ³
Annular sea bream	2	1 (50)	1	4.5×10 ³
Amberjack	1	None	None	-
Common dentex	1	1 (100)	1	3.7×10 ³
Shark	1	None	None	-
Dusky grouper	2	None	None	-
Sea needle	1	None	None	-
Beef	10	5 (50)	None	2.9×10 ³
Camel meat	9	3 (33.33)	1	5.3×10 ²
Chicken	9	5 (55.55)	None	7.2×10 ³
Mutton	6	None	None	-
Beef burger	5	4 (80)	None	1.2×10 ⁴
Chicken burger	5	4 (80)	None	6.5×10 ⁴
Beef kebab	1	None	None	-
Beef sausage	2	None	None	-
Total	93	48 (51.6)	11	

Identification of *Vibrio* spp. by PCR and sequencing of 16S rDNA

DNA extraction of *Vibrio* isolates

The procedure of DNA extraction of *Vibrio* isolates was done using the GF-1 bacterial DNA extraction kit (Cat# GF-BA-100, Vivantis, Malaysia). Briefly, a single colony of pure isolate was picked up from TCBS agar and inoculated into 5 ml nutrient broth then incubated at 37°C. A total volume of 1-3 ml of bacterial culture was centrifuged at 10000 rpm for 2 min then supernatant was discarded. The pellet was then re-suspended by adding 100 µl of buffer R1 (Cat. # GF-BA-100, Vivantis, Malaysia).

The re-suspended cells were centrifuged at 10000 rpm for 5 min then the supernatant was decanted completely. The protein of the pellet was denaturated by re-suspension in 180 µl of Buffer R2 (Cat. # GF-BA-100, Vivantis, Malaysia) and 20µl of proteinase K, then incubated at 65°C for 20 min with shaking every 5 min. Homogenization was achieved by adding 400 µl of Buffer BG (Cat. # GF-BA-100, Vivantis, Malaysia) and mix by inverting tube and incubation at 65°C for 10 min. 200 µl of absolute ethanol was added with immediate mixing to prevent precipitation of DNA due to high ethanol concentration. The sample was transferred (maximum volume 650 µl) into the column and centrifuged at 10000 rpm for 1 min. The flow was discarded and the column was washed by 750 µl of wash buffer (Cat. # GF-BA-100, Vivantis, Malaysia) by centrifugation at 10000 rpm for 1 min. The flow was discarded the DNA was eluted in 50 µl of elution buffer, which left for 5 min at room temperature and then centrifuged at 1500 rpm 1 min.

Amplification of 16S rDNA

Partial 16S rDNA was amplified using the universal oligonucleotides primers Forward S-D-Bact-0341-b-S-17 and Reverse S-D-Bact-0785-a-A-21 adopted from (Herlemann *et al.*, 2011). Briefly, 0.2 µg of genomic DNA was added to 25 µl Maximo Dry PCR Master Mix (Cat. # S295, GeneON, UK). The mixture was then amplified in a DNA Thermal Cycler (TECHNE TC-512) using the following program: one denaturation step at 94 °C for 5 min; 35 cycles of denaturation, 92 °C for 30 s, annealing temperature for 30 s at 55°C, extension at 68 °C for 60 s; and a final extension at 72 °C for 10 min.

The PCR products were electrophoresed in 2% agarose gel (Cat. # 604-005, GeneON, UK) incorporated with nucleic acid gel stain – 10000X (Gel RED, Cat. # S420, GeneON, UK) at voltage 100 volt for one hour (SCIE-PLAS, UK). The sizes of the amplified fragments were determined by comparison with the GelPilot 100 bp increment Ladder (Qiagen, Cat. No. 239035, Melbourne Australia) a ready-to-use 6 fragments (100–600 bp) DNA marker. The gel was

photographed with gel-documentation system micro DOC with UV-trans-illuminator (CSLUVTS312, Cleaver Scientific, UK).

DNA sequencing and analysis

The amplified 16S rDNA PCR fragment (464 bp) was excised from the gel and the DNA was extracted from the gel using GF-1 Ambi Clean kit (Cat. # GF-GC-100, Vivantis, Malaysia). Briefly, the net weight of gel slice was determined and 1 volume of Buffer DB was added to 1 volume of gel (A gel slice of mass 0.1g will have a volume of 100 µl). Then the gel was incubated at 50°C until gel has melted completely. The sample was transferred into a column assembled in a clean collection tube. Centrifuge at 10000 rpm for 1 min. The flow was discarded and the column was washed with 750 µl buffer and centrifuged at 10000 rpm for 1 min. The flow was discarded and the column was dried by centrifugation at 10000 rpm for 1 min to remove residual ethanol. DNA was then eluted by adding 30 µl of elution buffer and mixture was left for 2 min.

The purified 16S rDNA amplicons underwent cycle sequencing by Big Dye® Terminator v1.1 kit (AB Applied Biosystems) and sequence reactions were separated on a four capillary ABI PRISM® 3130 Genetic Analyzer at IZSLER (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Bianchi, 9 - 25124 Brescia, Italy). Sequences were assembled and edited using SeqMan module within Lasergene package, (DNASTar Inc., Madison, WI, USA) The obtained consensus sequences were subjected to BLAST search both at NCBI (<http://www.ncbi.nlm.nih.gov/pubmed>) and at 16S bacterial cultures Blast Server for the identification of prokaryotes (<http://bioinfo.unice.fr/blast/>).

Results

Isolation, cultural characteristics and enumeration of *Vibrio* spp.

The results from culture, enumeration and molecular identification of suspected *Vibrio* spp. in processed samples are shown in Table 1. Out of the 93 cultured samples, only 48 (51.6%) yielded colonies on TCBS with culture characteristics suggestive of *Vibrio* spp. (Fig. 1; Fig. 2a and 2b). More than half (27) of processed seafood samples (46) yielded colonies on TCBS, while only 21 out of 47 (44.6%) cultures of meat and meat products samples resulted in colonies on TCBS. No bacterial growth was revealed from the cultured samples of amberjack, shark, dusky grouper and sea needle, in addition to those from mutton, beef kebab and beef sausage (Table 1).

The highest bacterial count was recorded in a clam with a count of 3.8×10^4 CFU/g from the seafood samples. The highest bacterial count in meat products was from chicken burger samples with 6.5×10^4 CFU/g (Table 1 and Fig. 3).

Identification of *Vibrio* spp. by amplification and sequencing of 16S rDNA

All suspected isolates on TCBS were further analyzed molecularly by extraction of their DNA followed by sequencing of a portion of their 16S rDNA. Sequence



Fig. 1. TCBS plate showing the characteristic *Vibrio* spp. colonies (Green and yellow) during enumeration procedures.

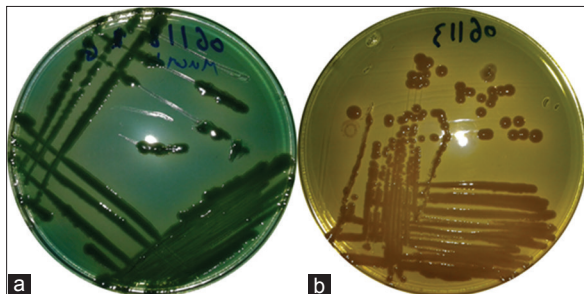


Fig. 2. Showing the pure consistent colonies of *Vibrio parahemolyticus* (a) and *Vibrio alginolyticus* colonies (b).

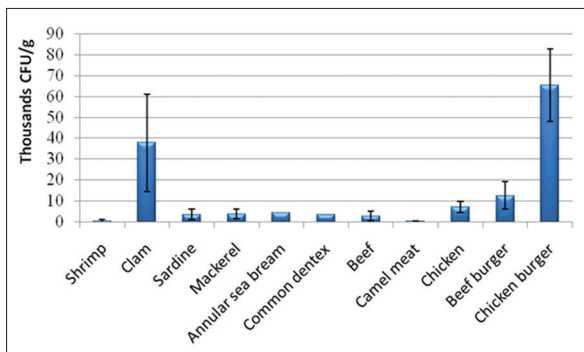


Fig. 3. Mean counts of *Vibrio* spp. in seafood, meat, and meat products samples. Error bar represents SD.

analysis showed that only 11 (22.9%) of these isolates were *Vibrio* spp. (Fig. 4 and Table 2).

Four out of the 11 *Vibrio* isolates were found to be *Photobacterium damsela* subsp. *Damsela* using the PCR-16S rDNA technique with 99 % nucleotide identity (Table 2). Moreover, the molecular test has revealed the presence of *Vibrio parahemolyticus* in a camel meat sample, which is the first report of the isolation of *Vibrio* from camel meat.

Discussion

Our results represent the first report of isolation and identification of *Vibrio* spp. by sequencing of 16S rDNA from seafood, meat and meat products samples in Libya. Only 11 samples out of 48 suspected *Vibrio* isolates (22.9%) in this study were identified to be *Vibrio* spp. by 16S rDNA sequencing. *V. parahemolyticus* and *V. alginolyticus* were the most frequently detected making up 27% of the isolates and the least frequently species was *V. owensii* 9%. A previous study, which examined the occurrence of *Vibrio* in mussels harvested from Adriatic Sea, found 48.4% of samples were positive for *Vibrio* spp., among which *V. alginolyticus* was most frequently found (32.2%) while *V. parahaemolyticus* was the least frequent (1.6%) (Ripabelli *et al.*, 1999). Another study determined the incidence of food borne pathogens in some European fish (France, Britain, Greece and Portugal) found *V. parahaemolyticus* was recorded in 35% of samples from Portugal and 14% from Greece but not in those from Britain or France (Davies *et al.*, 2001).

Using conventional cultivation on TCBS, Jakšić *et al.* (2002) determined the occurrence of *Vibrio* spp. in fish and shrimps harvested from Adriatic Sea. They were able to isolate *Vibrio* spp. from 19.65% of samples. The most frequently found were *V. parahemolyticus* (9.4%), *V. vulnificus* (6.8%) and *V. alginolyticus* (3.4%). Das *et al.* (2009) examined the occurrence of *Vibrio parahaemolyticus* in samples of finfish and *Penaeus monodon* from wholesale fish markets in Kolkata, India, by standard culture technique. The bacterium was isolated from 45.8% of shellfish and 16.7% of finfish samples. Xu *et al.* (2014) have investigated

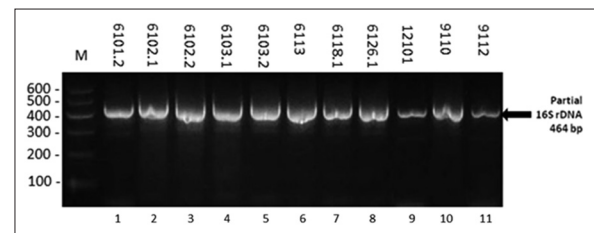


Fig. 4. Amplification of partial 16S rDNA (464 bp) of isolated *Vibrio* strains using the universal oligonucleotides primers (FOR S-D-Bact-0341-b-S-17 and REV S-D-Bact-0785-a-A-21). Lane (M) contains the 6 fragments (100–600 bp) DNA marker. Lanes 1-11 contains isolated *Vibrio* spp.

Table 2. Identification of suspected *Vibrio* spp. by PCR and sequencing of 16S rDNA.

Blast NCBI search results	Nucleotide identity %	Isolates code	Type of sample	Sampling area
<i>Vibrio owensii</i>	99	6101.2	Chub mackerel (<i>Scomber japonicas</i>)	Tripoli
<i>Vibrio harveyi/Vibrio alginolyticus</i>	99	6102.2	Chub mackerel (<i>Scomber japonicas</i>)	Tripoli
<i>Vibrio harveyi/Vibrio alginolyticus</i>	99	6113	Clam shellfish	Tripoli
<i>Vibrio harveyi/Vibrio alginolyticus</i>	99	12101	Shrimp	Tripoli
<i>Vibrio parahaemolyticus</i>	99	6118.1	Camel meat	Regdalin
<i>Vibrio parahaemolyticus</i>	99	9110	Common dentex	Tobruk
<i>Vibrio parahaemolyticus</i>	99	9112	Annular sea bream	Tobruk
<i>Photobacterium damsela</i> subsp. <i>Damsela</i> ; 99% <i>Vibrio</i>	99	6102.1	Chub mackerel (<i>Scomber japonicas</i>)	Tripoli
<i>Photobacterium damsela</i> subsp. <i>Damsela</i> ; 99% <i>Vibrio</i> sp.	99	6103.1	Sardine	Tripoli
<i>Photobacterium damsela</i> subsp. <i>Damsela</i> ; 99% <i>Vibrio</i> sp.	99	6103.2	Sardine	Tripoli
<i>Photobacterium damsela</i> subsp. <i>damsela</i> ; <i>Vibrio olivaceus</i>	99	6126.1	Sardine	Tripoli

the prevalence, pathogenicity, and serotypes of *V. parahaemolyticus* in shrimp from Chinese retail markets. *V. parahaemolyticus* was detected in (37.7%) of samples by the most probable number method.

V. owensii, which has been isolated from cultured crustaceans in Australia and recognized as a novel *Vibrio* spp. (Cano-Gómez *et al.*, 2010) was among the suspected isolates in our study from samples of mackerel, a case which have not been reported earlier. From the results obtained in the present study, *Photobacterium damsela* subsp. *damsela* (formerly *V. damsela*) was the most frequently bacterium isolated (36%) from the processed samples. This microorganism has been recognized as a pathogen for a wide variety of aquatic animals, such as crustaceans, molluscs, fish and cetaceans. In addition, this bacterial pathogen has been reported to cause diseases in humans and, for this reason, it may be considered as an agent of zoonoses (Austin, 2010). According to Bergey's Manual of Systematic Bacteriology (Thyssen and Oliver, 2005), *P. damsela* subsp. *damsela* belongs to the genus *Photobacterium* included in the family *Vibrionaceae*, displaying morphological characteristics typical of members of the family.

Occurrence of *Vibrio* spp. in meat and meat products is not widely reported. However, Zakhariyev *et al.* (1976) has investigated *V. parahemolyticus* in pork sausage. Gardner (1980) have associated *V. costicola* with the spoilage of cured meats. Garcia-Lopez *et al.* (1998) had indicated the association of *Vibrio* spp. among other Gram-negative bacteria associated with spoilage of meat and meat products. Similarly, Doulgeraki *et al.* (2012) had concluded that *V. parahemolyticus* are among those organisms which are responsible

for spoilage of raw meat during storage at different conditions. An interesting finding in this study was the isolation of *V. parahemolyticus* from camel meat, which has never been reported previously. On the other hand, none of the bacteria isolated from all the processed samples (beef, chicken, mutton, beef burger, chicken burger, beef kebab and beef sausage) belonged to *Vibrio* spp. Further, the mixed selling of chicken meats, beef and camel meat together with seafood products at the retail markets could have allowed the cross contamination from contaminated seafood/water directly to meat (Herrera *et al.*, 2006; Nyachuba, 2010). The irresponsible and unhygienic act of washing chicken, beef and camel meats with sewage contaminated water/seawater could explain the reported *Vibrio* incidences (especially *V. cholera* and *V. parahemolyticus*) among these types of fresh meats (Maheshwari *et al.*, 2011). This may also explain the isolation of *V. parahemolyticus* from camel meat in this study.

No *Vibrio* detection from mutton meat samples. This may be related to the low pH (acidic), associated with high conjugated linolenic and free fatty acids intermingling the mutton meat. This assumption ideally coincides with the relative bacterial inhibitory effect of sheep meat (mutton) fat reported by several studies (Reineccius, 1979; Sofos, 1994; Garcia-Lopez *et al.*, 1998). However, lack of detection of *Vibrio* spp. in beef kebab and beef sausage samples may be attributed to the excessive dressing in spice, lemon/vinegar, garlic and onions that possess inhibitory effects on *Vibrio* growth (Beuchat, 1976).

In Libya, there is a lack of data concerning actual causes of food borne infections in general. So it is difficult to

find a link between *Vibrio* spp. isolations and any of the reported food poisoning outbreaks in the country. However, correlations between *V. parahaemolyticus* and food borne infections have been described in Taiwan (Pan *et al.*, 1997), USA (Fyfe *et al.*, 1997), France (Geneste *et al.*, 2000), Mexico (Velazquez-Roman *et al.*, 2012) and China (Ma *et al.*, 2014).

One of the major risks involves the consumption of raw or undercooked seafood, meat and meat products that may be contaminated by food borne pathogens present in the marine/retail markets (Genigeorgis, 1985; Jay *et al.*, 2005). Such risks are further increased if the food is mishandled during handling, slaughter, transportation, and processing where pathogens could multiply exponentially under favorable conditions (Oliver and Kaper, 1997).

In contrast to most other food borne pathogens, *Vibrio* spp. utilize aquatic habitats as their natural niche (Oliver and Kaper, 1997; Reidl and Klose, 2002). As a result, *Vibrio* spp. are commonly associated with polluted water, seafood, and other aquatic animals as the main source of contamination (Sutherland and Varnam, 2002). Food borne infections with *Vibrio* spp. are common in coastal cities where retail markets are close to the sea basin (Rebaudet *et al.*, 2013). The close vicinity of seafood, meat and meat products retail markets as well as processing facilities to the sea basin amplifies the potentials of *Vibrio* spp. contamination to such foods (Jackson *et al.*, 1997; Feldhusen, 2000; Sofos, 2008).

Finally, it is empirical to mention that the identity of the retrieved *Vibrio parahaemolyticus* and *V. harveyi* were presumptively identified using morphological characteristics extracted from morphological cultural characteristics on the selective TCBS agar media. All isolates matched the standard morphological criteria previously established (Alsina and Blanch, 1994; Perilla *et al.*, 2003; Austin and Austin, 2012). Molecular confirmation of the retrieved *Vibrio* isolates was done using partial amplification of 16S rDNA using the universal oligo-nucleotides primers (FOR S-D-Bact-0341-b-S-17 and REV S-D-Bact-0785-a-A-21) where the specific 464 bp amplicon has been documented coinciding with that reported by (Herlemann *et al.*, 2011) for the same *Vibrio* spp. using the same specific primers and 16S rDNA PCR protocol.

Acknowledgements

This study was part of a project titled “Genetic authentication of bacterial isolates from meat and milk products in Libya and establishing the Food-borne Libyan-type Bacterial Collection (FLBC)” that was supported by a grant provided by the Libyan Authority for Research, Science and Technology (LARST). Authors are grateful to Veronica Papini, a technician in Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna, Brescia, Italy, who performed the sequencing of the Partial 16S rDNA.

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