Isolation And Identification of *Vibrio parahemolyticus* Recovered From Some Shellfish Marketed In Zagazig City

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ABSTRACT

The present investigation was undertaken to study the occurrence of *V. parahaemolyticus* in shellfish and to assess the genomic diversity within *V. parahaemolyticus* strains isolate using ERIC-PCR technique. A total of 130 samples of shellfish were collected from fish markets, Zagazig city included white shrimps (40); blue crabs (40) and mussels (50). *V. parahaemolyticus* were isolated and identified using standard bacteriological methods. ERIC-PCR was carried out for a total of 9 *V. parahaemolyticus* clones, representing white shrimp, blue crab and mussels (3 each). *V. parahaemolyticus* were isolated with the percentages of 17.5(7/40), 12.5(5/40) and 16.0(8/50), respectively. The overall percentage of the examined shellfish was 15.4(20/130). Overall, the ERIC-PCR results showed 6 separate clusters at similarity level 0.53. Isolates No 4, 2, 5, 3 grouped together (group I). Isolates No 1, 8, 9, 7 and 6 each clustered in their own groups (II, III, IV, V and VI, respectively). In addition, there is sub-clustering with group I at similarity level 0.66. The amplified bands were analyzed by dendrogram using Dice correlation coefficient. The phylogenetic analysis of tested strains exhibits high amount of heterogeneity. It could be concluded from the present study that *V. parahaemolyticus* are circulating among the marketed shellfish in Zagazig city. Variable *V. parahaemolyticus* clones indicate different sources of contamination and infections under the research conditions.

INTRODUCTION

Vibrio parahaemolyticus is a gram-negative, halophilic bacterium that occurs in the marine environment naturally and isolated from seafood samples frequently (**1**). *V. parahemolyticus* were always associated with gastrointestinal and extra-intestinal troubles related to the consumption of insufficiently cooked seafood or deep contact with contaminated seawater *(2, 3, 4)*. *V. parahaemolyticus* is often isolated from seawater, sediment and a variety of seafood including shrimp, crab, oyster and clam due to its halophilic characteristics *(1)*. Previous studies determined the occurrence of *V. parahaemolyticus* in shellfish in different geographic areas over the world: in shrimp, in Croatia *(5)*; in India *(6)*; in Egypt *(7)*. Whereas in mussels, in Italy *(8)*; in Turkey *(9)*; in Spain *(10)*. El-Sahn et al *(11)* found that sea invertebrate samples collected in the summer months contained higher levels of *V. parahaemolyticus* than winter samples. Deficiencies in data available to conduct the present risk assessment of *V. parahaemolyticus* in marketed shell fish in Egypt were found. So, the present investigation was undertaken to study the occurrence of *V. parahaemolyticus* in shell fish and to assess the genomic diversity within *V. parahaemolyticus* strains isolate using ERIC-PCR technique.

MATERIAL AND METHODS

A total of 130 samples of shellfish were aseptically collected from different

localities in fish markets, Zagazig city, Egypt during May and June, 2013.The examined samples included white shrimps (40); blue crabs (40) and mussels (50) were collected in sterile polyethylene bags placed in an ice box. The collected samples were transferred to the Laboratory of Diagnosis, Academic Veterinary Hospital, Zagazig University with minimum of delay.

Enrichment procedures

Five grams of individual shellfish flesh were incised using a sterile scalpel after removal of the carapace. These 5 gm flesh samples were homogenized in 45 ml of 3% NaCl containing 1% alkaline peptone water (APW, pH: 8.6) using a sterile blender. The shellfish homogenates were incubated at 37°C for 18 hrs ***(5 , 12)***. While, water samples were enriched by adding 100 ml of each sample aseptically to equal volume of 1% alkaline peptone water containing 3% NaCl then were incubated at 37°C for 18 hrs ***(13)***.

Isolation and identification procedures

The enriched shellfish homogenates were inoculated on Thiosulphate Citrate bile salts sucrose agar media (TCBS, Hi Media, India) using an inoculating loop and kept at 37°C for 18 hrs ***(14 , 9)***.

The isolated colonies were subjected to Gram staining and growing at various salt concentrations by transferring colonies into tubes containing peptone water at 0%, 3%, 6% and 10% NaCl, and these tubes were incubated at 37°C for 24 hrs ***(15)***. Suspected colonies on TCBS plates were selected to be streaked onto the surface of Trypticase Soya agar slants (TSA; Oxoid, UK) supplemented with 2% NaCL, then incubated at 37°C for 24 hrs ***(16)***. The further identification of *Vibrio* species was done using morphological, physiological and different biochemical tests ***(17 , 18)***.

T he *V. parahaemolyticus* isolates were then grown in Luria-Bertani broth overnight at 37°C with shaking at 220 rpm in an orbital shaker (Lab-line Incubator-shaker).

DNA extraction

PCI (phenol-chloroform-isoamyl) based method was selected for preparation of template DNA. The method used was the mini preparation method ***(19)***. The cell suspension of *V.parahaemolyticus* isolates was transferred to 1.5 ml microfuge tube and was centrifuged at 10,000 rpm for 2 minutes in a Eppendorf centrifuge (Model 5415C) to get the cell pellet. The pellet was resuspended in 700 µl GET (Glucose-EDTA-TrisHCl) buffer (17 mg/ ml) and vortexed to mix. Ten µl 25% SDS (Sodium Dodecyl Sulphate) plus 5 µl Proteinase K (25 mg/ ml) (as an additional to rupture the cells) were added to the tube and mixed gently. The tube was incubated in water bath at 60°C for 20 minutes to lyse the cell or until the solution mixture become clear. Then, 500 µl PCI mix (phenol-chloroformisoamylalcohol) solutions were added and mixed gently. The tube was centrifuged at 12000 rpm for 1 minute. 200 µl of the upper aqueous layer (clear layer) was carefully transferred into a new sterile eppendorf tube. After that, 200 µl KAc (potassium acetate) and 400 µl cold isopropanol (100%) were added followed by incubation at -20°C for 5-10 minutes. The tube was centrifuged at 12000 rpm for 7 minutes. The supernatant was discarded and the pellet was washed with 500 µl of cold 70% ethanol. After centrifugation at 12000 rpm for 5 minutes, the ethanol solution was discarded; the pellet was air-dried and dissolved in 30 µl sterile distilled water.

ERIC PCR

Two specific primers were used to correlate to ERIC sequence ***(20)***; ERIC 1 (R): 5' ATGTAAGCTCCTGGGGATTCA- 3' and ERIC 2 (F): 5' AAGTAAGTGACTGGGGTGAGC- 3'. The PCR technique was carried out in 0.5 ml microfuge tubes. The total volume consisting of reaction mixtures was 25.0 µl. Consisting of 17.5 µl sterile distilled water, 2.5 µl 10x PCR buffer with MgCl2, 0.5 µl 25 mM deoxyribonucleotide phosphate, 1.0 µl of each primer, 0.5 of 0.5 units *Taq* DNA polymerase and 2 µl template DNA. The cycling conditions were as follows; predenaturation at 95°C for 7 minutes, denaturation at 90°C for 30 seconds,

annealing at 58°C for 1 minute, and extension at 65°C for 8 minutes, with a final extension at 68°C for 16 minutes at the end of 30 cycles. Agarose gel electrophoresis

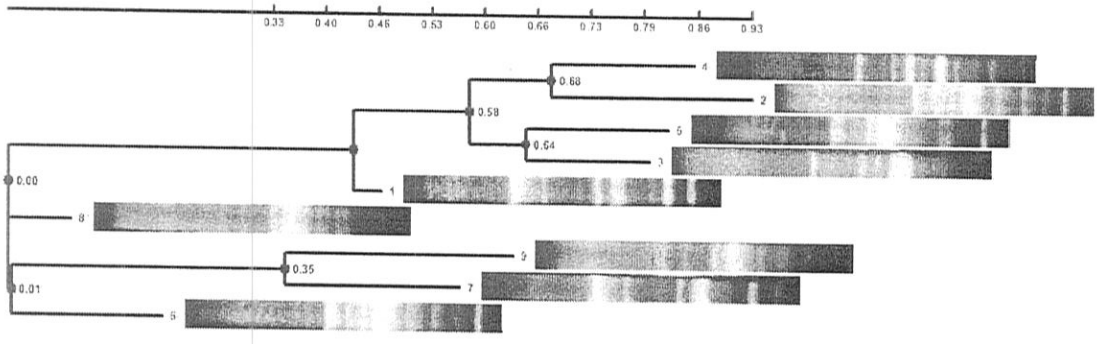
After the PCR assay, the PCR products were run on 1.2% agarose gel (Sigma) in 1x Tris-Borate- EDTA (TBE). About 15 – 20 µl PCR products were loaded into sample wells and voltage at 100 volt was used for 1 hour. The gel was stained with ethidium bromide (0.5µg/ml) solution for 1 min and de-stained in water for 30 min. The gel was visualized under UV transilluminator and photographed. Similarity matrix was built for *V. parahaemolyticus* isolates using Dice correlation coefficient. Cluster analysis was performed and dendrogram was constructed using the data matrix of 9 strains based on unweighted pair group method (UPGMA) using Gel Compar II software version 4.0 (Applied-Maths, St-Martens-Latem, Belgium).

RESULTS

Table 1. Occurrence of *V. parahaemolyticus* in some shellfish marketed in Zagazig city

Fish species	No. of examined samples	Positive	
		No	%
White shrimp	40	7	17.5
Blue crab	40	5	12.5
Mussels	50	8	16.0
Total	130	20	15.4

Fig. 1. ERIC-PCR analysis of 9 *V. parahaemolyticus* isolates. A dendrogram (left) and the horizontal gel like images (right) are the reproduced ERIC-PCR banding patterns for each isolate. Isolates No 1-3 represent shrimp; No 4-6 represent Crab and No 7-9 represent mussels.



Overall, the ERIC-PCR system showed 6 separate clusters at similarity level 0.53. Isolates No 4, 2, 5, 3 grouped together (group I). Isolates No 1, 8, 9, 7 and 6 each clustered in their own groups (groups II, III, IV, V, VI, respectively). In addition, there is sub-clustering with group I at similarity level 0.66 as shown in figure 1.

DISCUSSION

V. parahaemolyticus are major food-borne bacteria causing gastrointestinal disorders. Detection of current status of *V. parahaemolyticus* in marketed shellfish may help to establish an effective prevention and control measures, to reduce the risk of *V. parahaemolyticus* infection and to ensure the shellfish bio-safety. Table 1 shows the

occurrence of *V. parahaemolyticus* in some shellfish marketed in Zagazig city. *V. parahaemolyticus* were isolated from white shrimp, blue crab and mussels with the percentages of 17.5(7/40), 12.5(5/40) and 16.0(8/50), respectively. The overall percentage of the examined shellfish was 15.4(20/130). A previous study reported higher results (27.6%) of *V. parahaemolyticus* in shrimps from Abu-Kir, Egypt *(7)*. Lower results were found in shrimp (2.6%) and blue crabs (0.0%) *(21)*. Similar results (12.2%) were found in India *(6)*. The difference of occurrence of *V. parahaemolyticus* in shrimp may be due to variation of salinity level and the sample sizes of studies *(22)*. *V. parahaemolyticus* was isolated from mussels in Italy with the frequency of 2.7% *(8)* and 1.6% *(23)*. Higher results were recorded in mussels in Italy with the frequency of 32.6% *(12)*. Similar results (11.2%) were recorded in Spain *(10)*. Higher reports of *V. parahaemolyticus* may be associated with the growing of bivalve mollusks in uncontrolled waters subjected to contamination and their peculiar characteristic of filtering large amounts of water *(8)*. The obtained results may represent a public health hazard for the consumers, not only through ingestion of contaminated shellfish but also from cross contamination. Further studies are needed to count *V. parahaemolyticus* in the marketed shellfish to correlate the biological hazard and infective dose of shellfish.

In the present study, genotypic diversity of *V. parahaemolyticus* strains was done by ERIC-PCR technique. Fig. 1 represents the patterns obtained with ERIC2 primer tested with *V. parahaemolyticus* clones isolated from marketed shell fish *in* Zagazig city. ERIC-PCR technique was done to 9 strains representing shrimp, crab and mussels (3 each) and showed a reproducible patterns consisting of several amplicons. The amplified bands were analyzed by dendrogram using Dice correlation coefficient. The phylogenetic analysis of tested strains exhibits high amount of heterogeneity. Similar genetic heterogeneity results were found in Tunisia *(24)* for *Vibrio* species originated from a fish farm using ERIC-PCR technique. It could be concluded from the present study that *V. parahaemolyticus* are circulating among the marketed shellfish in Zagazig city. Variable *V. parahaemolyticus* clones indicate different sources of contamination and infections under the research conditions.

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الملخص العربى

عزل وتصنيف الواويات شبه المحللة المستفردة من بعض الأسماك القشرية المسوقة بمدينة الزقازيق

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أجريت الدراسة الحالية لتحديد تواجد الواويات شبه المحللة فى الاسماك القشرية وكذلك لتقييم التنوع الجينومى بنسخ الواويات شبه المحللة المستفردة باستخدام تقنية تفاعل البلمرة المتسلسل ERIC . تم تجميع عدد كلى ١٣٠ عينة من الأسماك القشرية من سوق السمك بمدينة الزقازيق مشتملة على عدد ٤٠ عينة جمبرى وعدد ٤٠ كابوريا وعدد ٥٠ من أم الخلول. تم عزل وتعريف الواويات شبه المحللة باستخدام الطرق البكتريولوجية القياسية. وقد تم عمل تفاعل البلمرة المتسلسل لعدد ٩ نسخ من الواويات شبه المحلله ممثلة للجمبرى والكابوريا وأم الخلول (٣ لكل نوع منهم). وقد عُزلت الواويات شبه المحللة بنسب مئوية ١٧،٥(٤٠/٧) ، ١٢،٥(٤٠/٥) و ١٦،٠(٥٠/٨)، على التوالى. وكان إجمالى تواجد الواويات شبه المحللة فى العينات التى تم فحصها ١٥،٤ % (١٣٠/٢٠). وأسفرت نتائج تفاعل البلمرة المتسلسل عن تواجد ٦ انماط جينية مختلفة عند مستوى تشابه ٠،٥٣. ووقعت المستفردات رقم ٤ ، ٢، ٥ ، ٣ ضمن النمط الجينى الأول. بينما المستفردات رقم ١، ٨، ٩، ٧، ٦ وقع كل منهم فى نمط جينى خاص به (الثانى ، الثالث ، الرابع ، الخامس ، السادس ،على التوالى). بالإضافة إلى ذلك وجدت أنماط جينية تحتية عند مستوى تشابه ٠،٦٦. وقد حللت الحزم المتضاعفة بطريقة التفرع الشجرى باستخدام معامل ارتباط ديس. وقد أسفر تحليل التباين الجينى عن اختلاف شديد بين العترات. ونستنتج من الدراسة الحالية أن الواويات شبه المحللة متواجدة فى الاسماك القشرية المسوقة بمدينة الزقازيق. وتنوع الانماط الجينية للواويات شبه المحللة يشيرالى تعدد مصادر التلوث والعدوى تحت ظروف الدراسة.