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Research Paper

Detection of Biofilm Producing *Vibrio parahaemolyticus* from Anchovy Fish (*Stolephorus indicus*) Sold in Puducherry

C. Vijayan, V. Ajaykumar*, A. Bhattacharya and S. Leonal Rabins

Department of Veterinary Public Health and Epidemiology
Rajiv Gandhi Institute of Veterinary Education and Research, Kurumbapet, Puducherry

ABSTRACT

Food borne illnesses have major social and economic impacts. The WHO estimates that worldwide food borne and waterborne diarrheal diseases together kill about 2.2 million people annually and in India around 6 per cent of the population. *Vibrio parahaemolyticus* has recently been recognized as one of the most important food borne pathogens as the leading causal agent of human acute gastro enteritis and also it has the biofilm forming capacity. This study was conducted to detect the biofilms producing *Vibrio parahaemolyticus* from Anchovy fish (*Stolephorus indicus*) sold in Puducherry and was isolated as per the standard procedure. A total of 50 samples were screened for presence of *Vibrio parahaemolyticus*. Out of 50 samples, only 20 samples (40 %) were positive for *Vibrio parahaemolyticus*. This isolates were further screened for detection of biofilm production ability by using Modified Congo Red Agar (CRA). Out of 20 isolates, only 15 isolates (75%) of *Vibrio parahaemolyticus* were having biofilm producing ability. This can be a major concern as microorganisms growing in a biofilm can be associated with chronic and recurrent human infections. Such organisms are highly resistant to cleaning, antimicrobial agents and reduce the efficiency of chemicals on sanitization of food processing equipments in food industry.

Keywords : *Anchovy fish, Biofilm, Vibrio parahaemolyticus, Foodborne illness*

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INTRODUCTION

The World Health Organization defines food borne illness as 'diseases', usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food. The WHO (2015) estimates that worldwide food borne and waterborne diarrheal diseases together kill about 2.2 million people annually and in India around 6 per cent of the population. The major causative agents of these illnesses involve viruses, bacteria, parasites, toxins, metals, and prions. In particular, microorganisms, especially the bacteria, have become an important group of causative agents as most of morbidity and mortality from food borne illnesses are related to them (Nyenje *et al*. 2012).

Vibrio parahaemolyticus is one such organism whose infection may not cause high mortality but considerable morbidity. This bacterium was first identified as a cause of food-borne illness during the fall of 1950 within the southern suburbs of Osaka, Japan, where an outbreak of acute gastro enteritis following the consumption of semidried juvenile sardines sickened 272 and killed 20 individuals (Fujino *et al*. 1953). *Vibrio parahaemolyticus* has recently been recognized as one of the most important food borne pathogens as the leading causal agent of human acute gastro enteritis, primarily following the consumption of raw, under cooked or mishandled seafood and marine products (Su and Liu 2007; Pal and Das 2010; Roman *et al*. 2012).

Vibrio parahaemolyticus is a halophilic, Gram-negative rod-shaped bacterium. The optimal growth conditions of V. *parahaemolyticus* are 35-37°C, pH 7.5-8.0 and approximately 0.5 M NaCl (Joseph *et al*. 1982). The colony morphology of *V. parahaemolyticus* is variable. Multiple colony morpho-types can occur in colony descendants from a single isolate. Moreover, the colony types can switch reversibly from translucent (TR) to opaque (OP). The switching mechanism is believed to be a response to specific environmental conditions (McCarter 1999). *Vibrio parahaemolyticus* are highly competent in biofilm formation although the biofilm structures are developed differently in TR and OP strains.

The mean incubation period for *V. parahaemolyticus* infection is 1-5h (range: 4–96h). The illness is self-limiting with moderate severity, lasting an average of 3 days in immunocompetent patients. Because of its self-limiting nature, most cases of infection by *V. parahaemolyticus* can be treated by oral rehydration alone (CDC 2013).

One of the major challenges in the food industry is the ability of food borne pathogens to form biofilm on the food contact surfaces. Bioflim forming capacity of organisms on various

*Corresponding author E-mail address: ajaykins@gmail.com

food processing equipments can lead to complete tolerance against various chemical disinfection methods in food processing industries. Bioflim is defined as assemblage of microbial cells that are irreversibly associated with a surface and enclosed in a matrix primarily of polysaccharides. Biofilm mode of bacterial growth exhibits a distinct phenotype with respect to altered gene transcription and growth rate as well as increased resistance to chemical and physical treatment (Costerton *et al*. 1995). Biofilm producing *V. parahemokyticus* can remain in the surfaces like cutting board and knives which can act as a source of contamination. This study was conducted to detect the biofilms producing *Vibrio parahaemolyuticus* from Anchovy fish (*Stolephorus indicus*) sold in Puducherry.

MATERIALS AND METHODS

The study was carried out on the sea fish sample Anchovy fish. A total of 50 fish samples were collected from different fish vendors and fish markets in Puducherry. The fish samples were collected aseptically in polythene bags and it was kept in the ice box. The samples were immediately transported to laboratory for further analysis.

***Processing of samples*:** The collected samples were processed in the Biosafety level–II laboratory. Fish's head, tails and guts were removed and then put into stomacher for homogenization. The samples remaining after the tests and negative samples were discarded as per standard methods including proper decontamination.

Isolation of *Vibrio parahaemolyticus:* *Vibrio parahaemolyticus* was isolated and identified as described in the *Bacteriological Analytical Manual* of the Food and Drug Administration (Elliot *et al.* 1995). The 50 grams of processed fish sample was placed in 450 ml of alkaline peptone water to obtain a 10^{-1} dilution and then it was incubated for 24 hours at 37^0C. A loop full of enrichment broth was streaked on to thiosulfate citrate bile salts sucrose agar plates and were incubated at 37°C for 24 hours. After the incubation, *V. parahaemolyticus* was observed as blue-green coloured colonies. These colonies (round, 1 to 2 mm in diameter, humid, shiny, sucrose fermenting) were selected for biochemical tests.

Characterization and identification of isolates: The suspected colonies of *V. parahaemolyticus* were subjected to various tests and confirmed based on the biochemical characteristics. The individual colonies from TCBS agar were transferred to TSB and incubated at 37^0C for 24 hours. Primary Identification Tests like Gram's staining, Catalase test (Slide test), Oxidase test and motility test were performed. Secondary identification tests like Indole production, methyl red (MR) reaction, Voges Proskauer (VP) reaction, citrate utilization test, urease activity, gelatin hydrolysis/ liquefactions and carbohydrate utilization test were preceded as per the standard procedures.

Biofilm production assay for vibrio parahaemolyticus: Biofilm production in terms of slime production by isolates was determined by cultivation on Congo Red Agar (CRA) plates (Freeman *et al*. 1989 and Dadawala *et al*. 2010). A loop full of isolate was streaked onto CRA plates. The plates were incubated at 37°C for 24 hours followed by storage at room temperature for 48 hours. The production of rough black colonies by bacterial cultures indicated the ability for bioflim production.

RESULTS AND DISCUSSION

Isolation and identification of V. parahaemolyticus: Out of 50 Anchovy fish sample only 23 samples produced typical colony morphology of *V. parahaemolytics* in TCBS agar. These 23 isolates were further subjected to various biochemical and carbohydrate utilization tests. Out of 23 isolates only 20 (40 per cent) isolates given the typical results for *V. parahemolyticus*. Remaining 3 isolates were identified as *Vibrio vulnificus*. Characterization and identification of *V. parahaemolyticus* was done as per the results shown in the Table 1 and 2.

Table 1: Characterization and identification of *V. parahaemolyticus*

S. No.	Identification tests	Characterisation/Reactions
1	Gram's staining	Gram negative & curved rods/rods
2	Catalase	Positive
3	Oxidase	Positive
4	Motility	Positive
5	Indole production	Positive
6	Methyl red	Negative
7	Voges Proskauer	Negative
8	Citrate utilization	Negative
9	Triple Sugar Iron agar reaction	Negative for both H_2S and gas production
10	Urease test	Positive
11	Sodium Chloride tolerance (0, 3, 6, 8 and10 per cent)	Positive at 3, 6, 8, 10 per cent concentration
12	Lysine decarboxylase test	Positive

Table 2: The carbohydrate utilization by *Vibrio parahemolyticus*

S. No.	Sugars	Acid from carbohydrate
1	D-Glucose	Positive
2	Lactose	Negative
3	Sucrose	Negative
4	Sorbitol	Negative
5	D-Mannitol	Positive
6	Inositol	Negative
7	Maltose	Positive
8	Cellobiose	Negative
9	Arabinose	Positive

Differentiation between Vibrio parahaemolyticus and Vibrio vulnificus: *Vibrio parahaemolyticus* and *Vibrio vulnificus* are phenotypically similar, and difficult to differentiate. The differentiation between the *Vibrio parahaemolyticus* and *Vibrio vulnificus* were done as per the characteristics in the Table 3.

Table 3: Differentiation between *Vibrio parahaemolyticus* and *Vibrio vulnificus*

S. No.	Characteristics	*Vibrio parahaemolyticus*	*Vibrio vulnificus*
1	TCBS agar	Bluish green	Bluish green
2	Oxidase	Positive	Positive
3	Growth in 0, 3, 6, 8 and 10 per cent NaCl	Growth noticed in 3,6,8 and 10 per cent NaCl	Growth noticed only in 3 and 6 per NaCl
4	Acid from Sucrose	Negative	Negative
5	Acid from Lactose	Negative	Positive
6	Acid from Arabinose	Positive	Negative
7	Acid from D-mannitol	Positive	Variable

Different superscripts in a column differ significantly (P<0.05)

Study conducted by Adebayo-Tayo *et al*. (2011) from the Itu creeks reported that among the species isolated, *Vibrio cholerae* was most predominant (30.4%). This was closely followed by *Vibrio mimicus* (27.8%), *Vibrio parahaemolyticus* (21.5%), *Vibrio fluvialis* (17.7%) and *Vibrio vulnificus* (2.5%). In the present study, less number of *V. parahemolyticus* was isolated but no *Vibrio cholerae* suspected isolates were found.

Das *et al*. (2009) examined the occurrence of *V. parahaemolyticus* in shell fish and finfish from wholesale fish markets in Kolkata. The bacterium was isolated from 45.8% of shellfish and 16.7% of finfish samples. These results are in tune with the results of the present study.

Xu *et al*. (2014) have investigated the prevalence, pathogenicity, and serotypes of *V. parahaemolyticus* in shrimp from Chinese retail markets. *V. parahaemolyticus* was detected in 37.7% samples by the most probable number method. Jaksic *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%). The percent of isolates of *V. parahemolyticus* obtained in lower than this present study.

Biofilm production assay: In biofilm production assay of *V. parahaemolyticus* by using the modified Congo Red Agar (mCRA), out of 20 isolates of *V. parahaemolyticus* only 15 (75 per cent) isolates were having the ability of biofilm production. The results, developed in this study, showed that *Vibrio*, food-borne pathogen, is able to produce biofilm on abiotic surface. Similar type of results was observed in a study by Elexson *et al*. (2014) in Malaysia. They isolated a total 36 strains of *V. parahaemolyticus* from seafoods and screened for the *in vitro* biofilm formation in the wells of commercially available microtiter plates. The biofilm production was measured at room temperature and at the end it had 61.1% of weak biofilm producers, 13.89% of moderate biofilm producers and 25% of strong biofilm producers.

CONCLUSION

Results of the present study show that seafood available in the region is grossly contaminated with *V. parahaemolyticus*. As

the seafood in Puducherry is used to make exotic dishes which involve minimal cooking, the presence of this organism can cause public health hazard. This study also reveals about 75 per cent of the isolates have the ability to produce biofilm. This is the matter of concern as this organism can resistant to the normal cleaning practices. This may results in contamination of food in the kitchen. This result clearly indicates the need for proper handling and processing of seafood. It is also important that in kitchen, whenever seafood is prepared, care should be taken to avoid cross contamination as the study reveals isolates of *V. parahaemolyticus* can produce biofilm. Further studies are required to understand to behavior of *V. parahemolyticus* in biofilm.

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