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Comparative performance of TCBS and TSA for the enumeration of *trh*+ *Vibrio parahaemolyticus* by direct colony hybridization

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ABSTRACT

Vibrio parahaemolyticus is one of the important foodborne pathogens is of public health concern due to the emergence of pandemic strains causing disease outbreaks worldwide. We evaluated the DNA based colony hybridization technique for the detection and enumeration of total and pathogenic *V. parahaemolyticus* from the bivalve shellfish, clam using non-radioactive, enzyme-labeled probe targeting the *tlh* and *trh* genes, respectively. The digoxigenin (DIG) labeled probes designed in this study showed 100% specificity by dot blot assay. Colony hybridization using DIG probes was performed using both non-selective, trypticase soy agar (TSA) and the selective medium, thiosulfate citrate bile salts sucrose (TCBS) agar. Of 32 clam samples analyzed, 71.88% had > 10,000 *V. parahaemolyticus* cells/g in TSA whereas it was 18.75% in case of TCBS. All the samples showed the presence of total *V. parahaemolyticus* in TSA and 97% in the case of TCBS. Interestingly, results of the *trh*$^{+}$ *V. parahaemolyticus* samples were quite high while using TCBS plates (62.5%) as compared to TSA (43.75%). However, the cell numbers obtained from TSA were higher than from TCBS. Several yellow colonies on TCBS turned out to be *V. parahaemolyticus* using colony hybridization, which was further confirmed by PCR and sucrose utilization test. Colony hybridization using DIG-labeled probe was found to be highly sensitive and could differentiate and enumerate pathogenic and non-pathogenic strains of *V. parahaemolyticus*. Since traditional methods are not only labor-intensive and time-consuming but also less sensitive, colony hybridization using DIG-labeled probes would be a useful alternative for the enumeration of *V. parahaemolyticus* in naturally contaminated seafood.

1. Introduction

Surveillance of foodborne diseases is crucial for public health worldwide. *Vibrio parahaemolyticus*, a Gram-negative halophilic bacterium, widely distributed in the marine environment is responsible for gastroenteritis associated with the consumption of raw, undercooked or contaminated seafood (Raghunath, 2015). Epidemiological data show *V. parahaemolyticus* to be distributed worldwide and associated with foodborne illness in counties like USA, Spain, Italy, Norway, and several other Asian countries (Caburlotto et al., 2008; Scallan et al., 2011; Ottaviani et al., 2013). The pathogenicity of *V. parahaemolyticus* is mainly attributed to the presence of two key virulence factors *viz.*, thermostable direct hemolysin (TDH) encoded by the *tdh* gene, and TDH- related hemolysin (TRH) encoded by the *trh* gene (Raghunath, 2015). Clinical isolates are found to be carrying any of the virulence factors whereas most of the environmental isolates lack the virulence genes (Ronholm et al., 2016). Therefore, it is essential to detect and enumerate *V. parahaemolyticus* from the seafood samples to confirm the safety of seafood.

Commonly used conventional, culture-based techniques like total plate count (TPC), and most probable number (MPN) offers less sensitivity and septicity, time-consuming and laborious. On the contrary, molecular techniques such as PCR, qPCR and their variants have been employed to overcome the these drawbacks of conventional methods (Givens et al., 2014; Banerjee and Farber, 2017; Zhu et al., 2018; Federici et al., 2018; Liu et al., 2018; Yang et al., 2018). PCR based detection of bacteria is increasingly used due to its rapidity, sensitivity, and specificity (Federici et al., 2018). However, due to the presence of various inhibitors in the samples, the results of PCR based assays may be misleading the actual result. Colony hybridization, a molecular

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method, involves the DNA-DNA hybridization using specific probes and is a useful method for the accurate enumeration of *V. parahaemolyticus* (Nishibuchi et al., 1985; McCarthy et al., 1999). However, it is not extensively used for enumeration in seafood industries because of the inadequate validation.

The use of radioisotopic probes in hybridization is hazardous and not recommended in a standard laboratory set up. Hence, non- radioactive enzyme-labeled probes are frequently used in hybridization assays (McCarthy et al., 1999; Deepanjali et al., 2005). In this study, we evaluated colony hybridization using digoxigenin (DIG)-labeled probes targeting the *tlh* and *trh* gene. Colony hybridization has been developed using various types of probes (Lee et al., 1995; Nordstrom et al., 2006; Raghunath et al., 2007; Suffredini et al., 2014; Cole et al., 2015) to target organisms that come upon non-selective media. In this study, we performed colony hybridization using the culture from both non-selective as well as selective media followed by detection and enumeration of *V. parahaemolyticus* in clam samples harvested from the natural bed.

2. Materials and methods

2.1. Bacterial cultures

V. parahaemolyticus and other cultures comprising non-Vibrios and Vibrios other than *V. parahaemolyticus* were retrieved from the stock culture in glycerol broth stored at −80 °C (Table 1). The cultures were inoculated for recovery into tryptone soy broth (TSB) and incubated in a shaker water bath at 35 °C ± 2 for 24 h. Fresh *V. parahaemolyticus* cultures were streaked onto the selective media, thiosulfate citrate bile salts sucrose (TCBS) agar and other cultures on the non-selective media, trypticase soy agar (TSA) to obtain single colony for further study.

Table 1
Bacterial strains used for specificity determination.

SL. No	Culture code	Place of isolation/Source	PCR Assay[a]		DNA blotting[b]	
V. parahaemolyticus						
1	HP1	Kumta	*tlh*	*trh*	*tlh*	*trh*
2	SF1	Kumta	+	−	+	−
3	CLSH2804 (1)	Thokottu	+	−	+	−
4	SRST2804(1)	Thokottu	+	−	+	−
5	SMT1/2806	Deralakatte	+	−	+	−
6	SHH1/2806	Deralakatte	+	−	+	−
7	SM/171016/1	Sasihithlu	+	−	+	−
8	OM/171016/1	Sasihithlu	+	−	+	−
9	SHP/171016/1	Sasihithlu	+	−	+	−
10	SHT3/2806	Sasihithlu	+	+	+	+
11	181	LSC	+	−	+	−
12	VP13	LSC	+	−	+	−
13	TRH10	LSC	+	+	+	+
14	VP4	LSC	+	−	+	−
15	AQ4037	LSC	+	+	+	+
16	81TDH2	LSC	+	+	+	+
17	3SST7	LSC	+	+	+	+
18	24STTDH1	LSC	+	+	+	+
19	VP49	LSC	+	−	+	−
20	TRHAP5	LSC	+	−	+	−
21	31A6	LSC	+	+	+	+
22	12ETRHST4	LSC	+	−	+	−
23	88TRH2VP21	LSC	+	+	+	+
24	17AP8	LSC	+	−	+	−
25	859	LSC	+	−	+	−
26	VP20	LSC	+	−	+	−
27	VP38	LSC	+	−	+	−
28	VP30	LSC	+	−	+	−
29	VP32	LSC	+	−	+	−
30	86	Bapatla	+	−	+	−
31	87	Bapatla	+	−	+	−
32	88	Bapatla	+	−	+	−
33	89	Bapatla	+	−	+	−
34	91	Bapatla	+	−	+	−
35	93	Bapatla	+	−	+	−
Other vibrio's						
36	*V. cholerae*	LSC	−	−	−	−
37	*V. campbellii*	LSC	−	−	−	−
38	*V. vulnificus*	LSC	−	−	−	−
39	*V. alginolyticus*	LSC	−	−	−	−
40	*V. harveyi*	LSC	−	−	−	−
Other non-vibrio bacteria						
41	*Edwardsiella tarda*	LSC	−	−	−	−
42	*Aeromonas hydrophila*	LSC	−	−	−	−
43	*Salmonella* Weltevreden	LSC	−	−	−	−
44	*Salmonella* Newport	LSC	−	−	−	−
45	*Salmonella* Typhi	LSC	−	−	−	−
46	*Salmonella* Arizona	LSC	−	−	−	−
47	*Pseudomonas aeruginosa*	LSC	−	−	−	−
48	*Acinetobacter* sp.	LSC	−	−	−	−
49	*Klebsiella pneumonia*	LSC	−	−	−	−
50	*Escherichia coli*	LSC	−	−	−	−
51	*Listeria* sp.	LSC	−	−	−	−
52	*Bacillus* sp.	LSC	−	−	−	−
53	*Micrococcus* sp.	LSC	−	−	−	−
54	*Staphylococcus epidermidis*	LSC	−	−	−	−
55	*Staphylococcus aureus*	LSC	−	−	−	−

LSC: Laboratory stock culture of Nitte University Centre for Science Education and Research, Mangalore, India.
(+): Positive reaction; (−); Negative reaction.

[a] Performed with *tlh* (Bej et al., 1999) and *trh* (Tada et al. 1992) primers.

[b] Performed with *tlh* and *trh* probes (Table 2).

2.2. Extraction of genomic DNA

Genomic DNA was extracted from all the cultures grown in the respective media according to the protocol of Ausubel et al. (1995) with minor modifications. The extracted DNA pellet was washed with 70% ethanol, dried under vacuum (Savant ISS110 SpeedVac concentrator, Thermo Scientific, USA) and finally re-suspended in 1 × TE buffer (10 mMTris-Cl; 1 mM EDTA; pH 8.0). DNA concentration and purity was measured in a spectrophotometer (BioSpectrometer® basic, Eppendorf, USA).

2.3. Preparation of the tlh & trh probe

The *tlh* probe was prepared by random primed labeling method. For this, genomic DNA of the standard culture *V. parahaemolyticus* AQ3047 was used. The *tlh* gene fragment was amplified by PCR (Bej et al., 1999) using ~200 ng of extracted DNA in a thermocycler (S1000™, Bio-Rad, USA). The product was purified using a PCR purification kit (Qiagen, USA). A 100 ng of purified the *tlh* gene fragment denatured by heat was labeled with DIG-High Prime (Roche, Germany) and used as *tlh* probe. For the preparation of the *trh* probe, a set of *trh* gene sequences of *V. parahaemolyticus* were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) in FASTA format. These sequences were used to locate a specific and conserved region using multiple sequence alignment programme, MultAlin (multalin.toulouse.inra.fr/multalin/). Primer designing tool Primer3Plus (www.bioinformatics.nl/primer3plus) was used to design the probe sequence. Of several sets of probes generated, the best sequence with optimum GC content and melting temperature was selected and labeled with a DIG at 5′ end of oligos (M/s- Eurofins Scientific, India) (Table 2).

2.4. Specificity test of the tlh & trh probe using DNA blot assay

Initially, both the probes were standardized for optimum annealing

Table 2
Details of the oligo sequence used for the preparation of probes in this study.

Gene	Oligo sequence (5′-3′)	DIG labeling	Reference
tlh[a]	F-AAAGCGGATTATGCAGAAGCACTG R-GCTACTTTCTAGCATTTTCTCTGC	Random priming using PCR assay	This study
trh	CACGACTTCAGGCTCAAAATGGTTAAGCGCCTAT	5′- end labeling	This study

[a] PCR performed with *tlh* (Bej et al., 1999) primers.

temperature. Annealing temperatures ranging from 40 to 50 °C were used to perform hybridization. Overnight hybridization was carried out with denatured *tlh* probe (25 ng/ml) as recommended by the manufacturer (DIG-High Prime DNA Labeling kit I, Roche, Germany). For the *trh* probe, various concentrations (20 pmol/µl, 10 pmol/µl and 5 pmol/µl) were used to optimize the hybridization reaction. Probe concentration of 5 pmol/µl was found to be optimum and selected for further study. To perform DNA blotting, the extracted genomic DNA was denatured by incubating at 95 °C for 10 min, and two microliters spotted on a positively charged nylon membrane (Ambion™, Bright Star, Thermofisher, USA) followed by U·V crosslinking (Cross linker CL-1, Herolab, Germany). The membrane was then incubated for hybridization with the probe in the presence of DIG Hyb buffer (supplied with the kit). After which, the membrane was subjected to stringent washing with SSC buffer containing 0.1% SDS and incubated in 1 × blocking solution (supplied with the kit) at room temperature. The membrane was incubated in antibody solution (1:5000) (supplied with the kit) at room temperature, washed using washing buffer, and the result visualized as blue dots on the membrane following the addition of a freshly prepared colored substrate solution (NBT/BCIP, supplied with the kit).

2.5. Evaluation of colony hybridization using the tlh & trh probe

Colony hybridization was performed using Whatman 541 membrane (Merck, Germany). Agar plates showing ~30–300 colonies after overnight incubation were selected. Colonies were spotted on membrane followed by the lysis of cells using lysis buffer (0.5 M NaOH; 1.5 M NaCl) and the addition of neutralization buffer (2 M $C_2H_7NO_2$). The membrane was then subjected to UV- crosslinking (Herolab, Germany) at 2.5 J/cm^2 for three minutes. Colony hybridization was performed using the specific *tlh* & *trh* probe (as described previously). After the addition of the color substrate, blue or dark brown dots that appeared on the membrane were enumerated and expressed as colony forming units (CFU)/g.

2.6. Direct detection and enumeration of total and pathogenic V. parahaemolyticus

Sample collection was carried out from February 2018 to May 2018. The clam samples (n = 32) were collected twice a week from the local markets of three different southwest coastal zones *viz* Kasaragod (Z1), Mangaluru (Z2) and Udupi zone (Z3). Each zone is separated by about 60 km. The collected clam samples were brought to the laboratory in an ice box and processed within two hours. A 10 g clam meat was homogenized in 90 ml phosphate buffered saline (PBS), and tenfold serial dilution (10^1 to 10^5) prepared. A100 µl of homogenate was spread on to the selective (TCBS) and non-selective agar plates (TSA) in triplicate (Table S1). Colony hybridization was performed as described previously. For further confirmation, five each of green and yellow colonies from TCBS and five colonies from TSA were randomly selected and subjected to crude DNA extraction (Dileep et al., 2003) for PCR assay using the *tlh* (Bej et al., 1999) and the *trh* gene (Tada et al., 1992). Additionally, ten representative yellow colonies (reacted with the *tlh*/*trh* probe) appeared on the TCBS were subjected to sucrose utilization test using phenol red broth (Biosca et al., 1996). Cell counts obtained for total, and pathogenic *V. parahaemolyticus* on TSA and TCBS were compared and analyzed.

2.7. Statistical analysis

The significance of differences between the two media was calculated using student *t-test* with a probability of $p < 0.05$. Statistical assays were carried out using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Graphs were generated using Prism version 5.0 Software (Graph Pad, Inc., La Jolla, USA).

3. Results

3.1. Specificity of the tlh & trh probes

In dot blot assay, 45 °C was found to be the optimum annealing temperature for both the *tlh* and *trh* probe. All 35 *V. parahaemolyticus* isolates included in this study, generated strong signals for the *tlh* probe and eight *trh*$^+$ isolates gave a strong signal for *trh* probe. However, no signal generation as color dot was observed on nylon membrane for non–*V. parahaemolyticus*. This showed that both the DIG-labeled probes prepared were 100% specific (Fig. S1) and could be used for the specific detection and enumeration of *V. parahaemolyticus* in shellfish samples.

3.2. Enumeration of V. parahaemolyticus in naturally contaminated clam

In colony hybridization, positive results were obtained as blue dots on the Whatman membrane, and negative results were seen as no color or light yellow spots (Figs. 1 and 2). Clam samples showed variation in the cell count obtained using the *tlh* probe that ranged from 10^2 to 10^6 CFU/g and with the *trh* probe, the counts were 10^2 to 10^5 CFU/g on both on TSA and TCBS. Of 32 clam samples, 23 (71.88%) samples had 10,000 or more cells/g and 9 (28%) showed < 10,000 cells/g on TSA media. However, in case of TCBS, 26 (81.25%) samples showed < 10,000 cells/g and only 6 (18.75%) samples had > 10,000 cells/g (Table S2). Twenty-two samples (68.75%) harbored *V. parahaemolyticus* carrying the virulence gene *trh*. Samples collected from Kasaragod (Z1) contained more *trh*$^+$ *V. parahaemolyticus* (90% and 50% on TCBS and TSA plates respectively) compared to the other zones. Samples from Udupi (Z3) showed *trh*$^+$ *V. parahaemolyticus* but with a maximum count of 7×10^5 CFU/g (Table S3). On the other hand, all the samples from three zones showed a positive reaction with the *tlh* probe, confirming the presence of total *V. parahaemolyticus* in clam samples.

All the samples were positive for the *tlh* probe in TSA and TCBS, except one sample in case of TCBS. Interestingly, along with green colonies, several yellow colonies on TCBS showed a positive reaction with the *tlh* probe which was further confirmed by PCR assay and sucrose utilization test. In addition, few of yellow colonies also showed positive reaction with the *trh* probe (data not shown). Of 10 representative yellow colonies considered, eight colonies were able to utilize the sucrose and showed color change (red to yellow) (data not shown). This further confirms the presence of sucrose utilizing *V. parahaemolyticus.* In the case of hybridization using the *trh* probe, results on TCBS plates were better than TSA plates, showing 20 (62.5%) and 14 (43.75%) samples positive respectively. However, the cell number was found to be more in TSA (maximum log10 = 5.5 CFU/g)

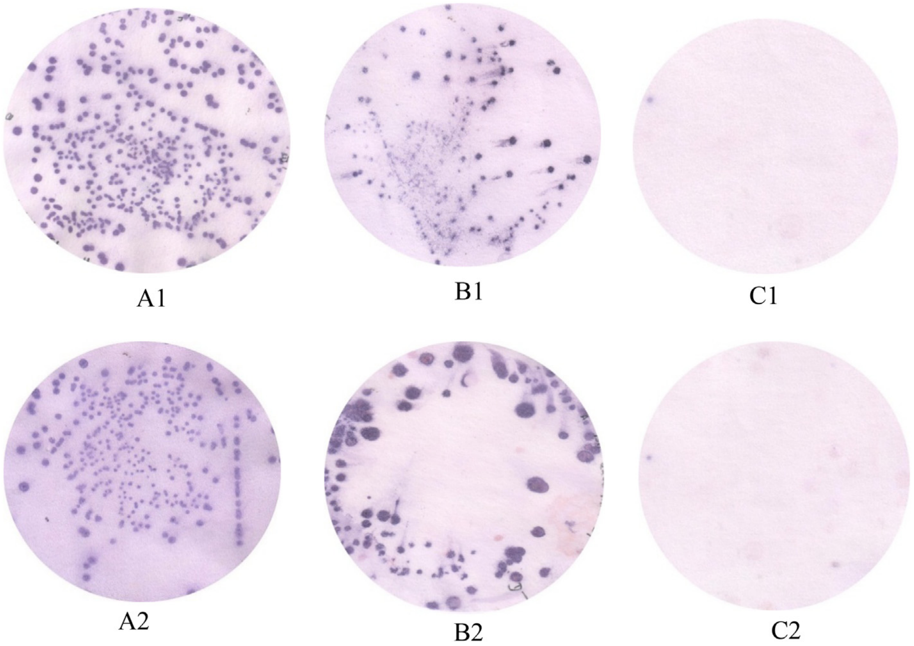

Fig. 1. Representative image of colony hybridization for the detection of *V. parahaemolyticus* using the *tlh* gene. A1 & A2: Positive control for the *tlh* gene on TSA and TCBS plates respectively. B1 &C1: Sample showing a positive result and negative results on TSA. B2 & C2: Sample showing a positive result and negative results on TCBS.

than in TCBS (maximum log10 = 4.7 CFU/g) in all the samples, except one.

Cell counts obtained in TSA, and TCBS plates for the *tlh* probe showed that 25% of samples had no log difference with least cell count of 3.6 and 3.5 CFU/g respectively. However, 30%, 18% and 3% of samples showed one, two and three log difference in the cell count respectively. For the *trh*, cell counts obtained in TSA and TCBS plates revealed 18% of the samples had no log difference, 12% of samples with one, 6% of samples with two, 21% of samples with three and 6% of samples with five log difference (Fig. 3). Statistical analysis performed indicated that there was a significant difference in the cell count obtained between TSA and TCBS using the *tlh* ($p < 0.05$) and the *trh* probe ($p < 0.05$) (Table S4).

4. Discussion

Enumeration of *V. parahaemolyticus* by DNA-based colony hybridization has been performed by different scientists who have used non-radioactive enzyme-labeled probe like alkaline phosphatase (Yamamoto et al., 1992; Nordstrom et al., 2006; Raghunath et al., 2007; Karunasagar et al., 2009; Cole et al., 2015). They were reported to be specific and significant for enumeration of the organism. However, the unavailability and cost-effectiveness of probe preparation is a significant drawback. In this study, we developed and evaluated a DIG-labeled probe for the specific enumeration of *V. parahaemolyticus* in the clam. An added advantage of the DIG-labeled probe as compared to AP-labeled probe is the reusability and the long shelf-life (Lee et al., 1995)

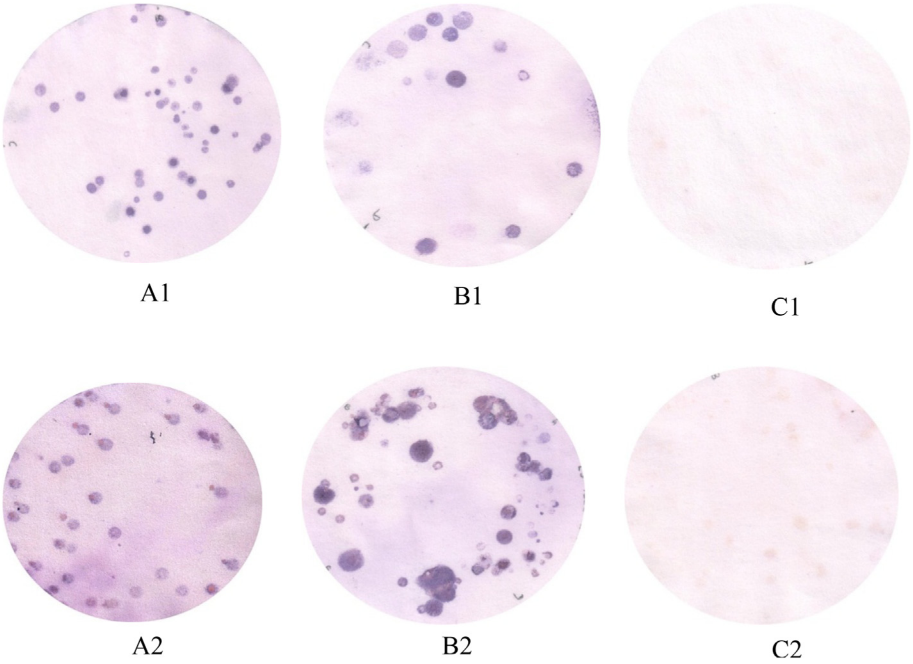

Fig. 2. Representative image of colony hybridization for the detection of pathogenic *V. parahaemolyticus* using the *trh* gene. A1 &A2: Positive control for the *trh* gene on TSA and TCBS plates respectively. B1 & C1: Sample showing a positive result and negative results on TSA. B2 & C2: Sample showing a positive result and negative results on TCBS.

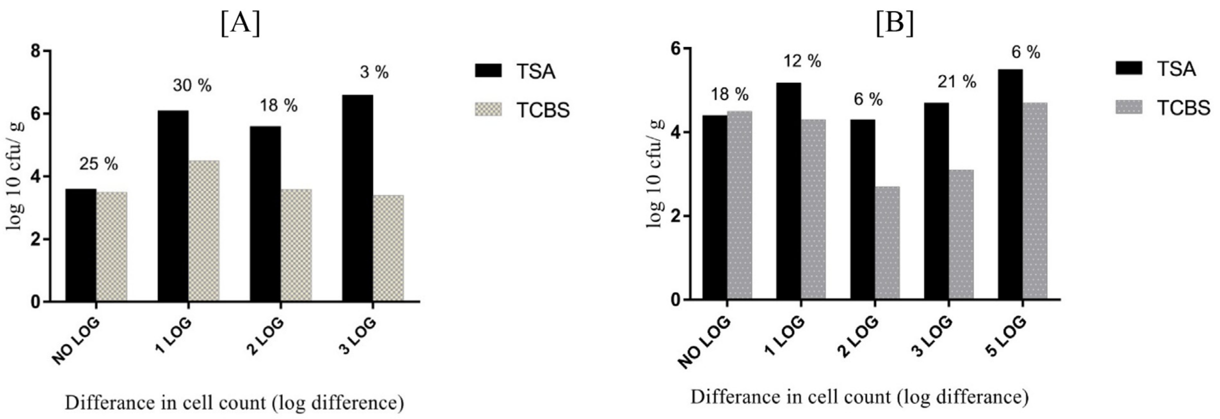

Fig. 3. Variation in colony counts using colony hybridization on TSA and TCBS media. A: Difference in colony count between TSA and TCBS using the *tlh* probe. B: Difference in colony count between TSA and TCBS using the *trh* probe.

which is vital for routine enumeration. The *tlh* probe prepared by random primed labeling using DIG to enumerate total *V. parahaemolyticus.* To our best knowledge, this is the first report where this type of probe is being used to detect and enumerate *V. parahaemolyticus.*Durisin et al. (1998) developed a randomly labeled DIG probe for the detection of *Yersinia enterocolitica*. McCarthy et al. (1999) and Suffredini et al. (2014) did develop a DIG-labeled probe using end labeling method targeting the *toxR*, *trh* and gene for the enumeration of total and pathogenic strains of *V. parahaemolyticus*.

Shellfish are known to be the reservoirs of *V. parahaemolyticus* infections (Su and Liu, 2007; Yu et al., 2013). Our goal was to enumerate the *V. parahaemolyticus* including the pathogenic strains that are associated with clam. The results show colony hybridization to efficiently quantify the number of *V. parahaemolyticus* cells present in the clam. As per the guidelines of FDA, > 10,000 *V. parahaemolyticus* cells/g of meat is not recommended for consumption with a total absence of pathogenic strains (DHHS/FDA, 1995). In this study, 71.88% of the samples had > 10,000 *V. parahaemolyticus* cells/g using the *tlh* probe, and 68.75% of them were found to be pathogenic (*trh*+). This demonstrates the sensitivity and accuracy of colony hybridization using a specific DIG-labeled probe and also throws light on the poor microbiological quality of shellfish in the market in this area.

In some of the earlier studies, it was reported that only a small proportion of the environmental strains of *V. parahaemolyticus* were pathogenic (Okuda et al., 1997; Matsumoto et al., 2000; Dileep et al., 2003). Though the incidence of pathogenic *V. parahaemolyticus* in seafood is usually low; there can be variations based on the geographic area (FAO/WHO, 2011). In this study, an unusually high number of samples with trh^{+} *V. parahaemolyticus* were identified using colony hybridization. Studies based on PCR assay carried out to date, suggests the presence of 1–10% pathogenic strains in an environmental sample (Depaola et al., 2000; IFT, 2004; Paranjpye et al., 2012). The low prevalence reported could be due to the lack of the enrichment process, low amount of template DNA, inhibitory agents present in the sample to perform PCR. Di Pinto et al. (2008) suggested enriching the samples for PCR particularly for the identification of virulent strains of *V. parahaemolyticus.* Quantification of *V. parahaemolyticus* were also performed using qPCR assay which failed to enumerate accurately due to the presence of contamination, and the pre-enrichment process was required for the assay (Cai et al., 2006; Robert-Pillot et al., 2010; Suffredini et al., 2014). Conly hybridization could efficiently enumerate both the pathogenic and non-pathogenic strains of *V. parahaemolyticus* in seafood samples. In addition, the method is relatively simple and does not require any sophisticated instrument perform.

In our study, confirmation of reaction obtained by colony hybridization was done by performing PCR assay. In direct PCR with samples, several trh^{+} samples were found to be negative (17 out of 22 trh^{+} samples or 22.7% positive). However, they were confirmed as positive after the samples were enriched overnight and used for PCR assay.

In our study, several yellow colonies on TCBS plates were positive for the *tlh* and *trh* using colony hybridization followed by PCR and sucrose utilization test. A similar observation was also reported earlier (Hara-kudo et al., 2001; Di Pinto et al., 2011). 90% of clam samples collected from Kasaragod (Z1) region were found to positive for the trh^{+} *V. parahaemolyticus* (90%). Thislikely due to the handling of clams in that particular region (New et al., 2016).

We also compared the colony hybridization using both non-selective (TSA) and selective (TCBS) agar plates. Our result analysis revealed that TSA could provide better detection and enumeration of total *V. parahaemolyticus* as compared to TCBS. This was expected and could mainly be due to the selectivity associated (presence of inhibitors) with of TCBS agar in which metabolically damaged cell may not be able to grow (Raghunath et al., 2007). Interestingly, TCBS plates were able to detect more trh^{+} *V. parahaemolyticus* in clam samples as compared to TSA. This could be due to the presence of bile salts in TCBS, which might favor the growth of pathogenic strains. Raghunath et al. (2009) showed that enrichment broth containing bile salts gave better recovery of pathogenic *V. parahaemolyticus* from seafood samples compared to alkaline peptone water alone. Pace et al. (1997) reported that bile might be involved in the release of *V. parahaemolyticus* from dormancy and also trigger the virulence in them. However, the cell count in TCBS was relatively lower compared to that in TSA plates.

5. Conclusion

Development of an efficient technique for the detection and enumeration of *V. parahaemolyticus* becomes very important, which could facilitate surveillance for this pathogen. The results obtained in this study suggests that colony hybridization using DIG-labeled probe would be useful for sensitive and specific detection and enumeration of *V. parahaemolyticus* and could also be used to differentiate pathogenic and non-pathogenic strains. The comparison of two growth media suggested that selective media, TCBS could be a better choice over TSA for the detection of pathogenic *V. parahaemolyticus* (trh^{+}) in clam samples. However, non-selective medium TSA could be considered for enumeration of total *V. parahaemolyticus*.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2018.12.020.

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