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GLOBAL WARMING: TOWARDS AN EXPERIMENTAL EVIDENCE OF THE RISE OF *VIBRIO PARAHAEMOLYTICUS*

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Abstract: *Vibrio parahaemolyticus* is a marine bacterium which is responsible for acute diarrheal illness and gastroenteritis in human beings. The present study was conducted in January-December, 2012. Marine samples from eight different stations around Cochin were intermittently collected on various seasons. Out of the 116 isolates, 88 were pathogenic strains (75.86%) having thermostable direct hemolysin (*tdh)* gene. It was found that samples from Fort Cochin, Thevara, Vypin and Polakandum were contaminated with *tdh* positive strains. The aims of this study were to elucidate the evidence of the rise of *Vibrio parahaemolyticus* by the effect of Global Warming. The isolated strains were also screened for pathogenicity. The incidence of infection can soar higher possibly due to the dilution of salt content in the oceans and rise in temperature owing to global warming. This attributes to the enhancement of optimum condition for the growth of *Vibrio parahaemolyticus* as it operates in moderate salinity range from 0.3% to 20% (optimum 2%) saline (NaCl) and moderate temperature range from 4°C to 45°C (optimum 37±1°C) and pH range from 4.8 to 11 (optimum 7.8 to 8.6). This study was supported by lab bench experiments for expounding seasonal variations largely affect the rising of *Vibrio parahaemolyticus* density. Another focus which requires attention is the emergence of multi-antibiotic resistant strains that can pose a threat to the prophylaxis and treatment of the illnesses. Considerable fraction of this pathogen delivering the virulence genes and pandemic genotype were among the samples in Cochin indicating that there is potential reservoir in Kerala and the effect of global warming might cause the rise of *Vibrio parahaemolyticus* mediated diarrhea in this region.

Key words: Global Warming, *Vibrio parahaemolyticus,* Thermostable direct hemolysin (*tdh),* Acute gastroenteritis.

INTRODUCTION

Vibrio parahaemolyticus, the marine bacterium is found in estuarine and coastal environments, (Fujino *et al*., 1951) and was first found in the United States by Barorow *et al*. (1968) in the estuarine waters of Puget Sound. It is distributed in tropical and temperate coastal waters which has a cosmopolitan distribution (De Paola *et al*., 2000). Nair (1979) carried out a study on the distribution of *Vibrio parahaemolyticus* and allied vibrios in backwaters and reported 36.8% occurrence in fishes from brackish water environments. Occurrence of *Vibrio parahaemolyticus* in the marine and brackish water of Cochin was studied by Sanjeev. S and Mahadev Iyer in 1986. The presence of *Vibrio parahaemolyticus* has been confirmed in British coastal waters and seafood. (Bartrow and Miller) and two outbreaks of infection with this organism have been recognized in Britain among persons who had eaten crab meat at a local Holiday resort (Hooper, Barrow and McNab). In airline outbreak examination of numerous colonies from primary cultures of the crab meat revealed for first time. Karunasagar *et al*. (1990) reported that *Vibrio paraha-emolyticus* was the most commonly encountered halophilic vibrio followed by V. *vulnificus* in the market samples of fish and shell fish in Karnataka state. People become infected with *V. parahaemolyticus* primarily through eating raw or undercooked seafood. The bacteria grows very quickly at room temperature so contaminated cooked seafood may also be a source. In Japan, *V. parahaemolyticus* is one of the leading causes of food

borne illness. **1.** Sporadic cases are common along the coasts of the United States but outbreaks have also been reported. **2.** Species-specific thermolabile hemolysin gene (*tlh*) for enume-rating total *V. parahaemolyticus,* and the thermostable direct hemolysin gene (*tdh*) that is associated with pathogenicity will help the molecular identifications of virulence pathogen. Human pathogenicity of the micro organism has been described to be primarily associated with thermostable direct hemolysin (*tdh*) and its presence is determined by β-hemolysis on Wagatsuma Agar. Twedt and Brown(1974) performed the detection of an enterotoxin by loop assay in Kanagawa positive *Vibrio parahaemolyticus*.

A study of antibiotic resistance of Vibrio *parahaemolyticus* was carried out by Pradeep and Leshmananperumalsamy (1985). They reported 100% of their isolates to be resistant to penicillin due to acquisition of a penicillinase plasmid.

Combined environmental factors such as warm water and moderate salinity can increase the number of *Vibrio parahaemolyticus* by making use of double enrichment, first with Trypticase Soy Agar with salt polymixin broth and then plating on to a chromogenic agar medium. Lin, Yu and Chou (2004) studied the susceptibility of *Vibrio parahaemolyticus* to various environmental stress factors such as low temperatures and other adverse conditions.

Ottaviani *et al.* (2003) reported a biochemical method for the isolation and identification of the current *Vibrio* species using just one preparative protocol. Isolates were analyzed by Enterobacterial Repetitive Intergenic Consensus sequence (ERIC)-PCR, detection of restriction fragment length polymorphism (RFLP) in rRNA genes (ribotyping), Pulse Field Gel Electrophoresis (PFGE) and RFLP analysis of the genetic locus encoding the polar flagellum. ERIC-PCR and ribotyping will be useful for the evaluation of genetic and epidemiological relationships among *Vibrio parahaemolyticus* strains. ERIC-PCR is based on the presence of repetitive conserved consensus sequence in bacteria. ERIC- PCR is the most widely adapted PCR typing method and has been applied to the typing of new species including *Vibrio cholerae* and *Vibrio parahaemolyticus* (Marshall *et al.*, 1999).

Use of Remotely Sensed Parameters

Quantitative data on environmental levels of *V. paraheamolyticus* in fish samples and water have shown a positive correlation of *V. paraha-emolyticus* densities with water temperature, and thus an apparent seasonality with higher levels during warmer months *(*Cook *et al.*, 2002, Depaola *et al., 1990).* Salinity is also known to affect *V. parahaemolyticus* densities *(*Cook *et al.*, 2002, Depaola *et al., 1990,* Kaper *et al., 1981).* Possible links between *V. parah-aemolyticus* incidence and other environmental parameters, such as turbidity and chlorophyll, are less clear *(*Watkins *et al, 1985).* Nevertheless, if one can suitably determine the quantitative effect of environmental parameters favorable for proliferation of this bacterium and then effectively monitor these parameters, the risk of illness could be better predicted and managed. For this purpose, the use of historical data on water temperature was desirable and, on average, the model-predicted *V. parahaem-olyticus* levels based on the fixed site temperature measurements generally agreed with the levels determined by microbiological examination. However, many brackish water fishing areas are far from fixed site monitoring stations (i.e., NOAA buoys or similar monitoring stations), and such fixed site data probably do not represent the full range of temperature variations that occur within and across specific oyster-harvesting areas. This is a relevant issue to be addressed if the approach underlying to be utilized for forecasting. Furthermore, the potential for improvements in accuracy of forecasting exist if other determinant factors (e.g., salinity, turbidity, chlorophyll, etc.) substantially influence *V. paraheamolyticus* levels, are spatially variable, and can be monitored via remote sensing technology.

Remote sensing is currently being used to describe and monitor a variety of systems from local to global scales *(*Guebas, 2002; Jensen, 2000*).* For example, a study described by Lobitz *et al.*, *(*Beck *et al., 2000;* Huq *et al.*, 1995; Lobitz *et al.*, 2000) used retrospective remote sensing data to demonstrate a relationship between certain ocean parameters (sea surface temperature [SST] and sea surface salinity [SSS] and cholera (*Vibrio cholerae)* incidence in Bangladesh. In a similar fashion, remotely sensed (RS) data may prove useful to better elucidate and estimate the

quantitative effect of factors other than SST in determining *V. paraheamolyticus* densities in U.S. oysters and provide a timely, user-friendly format for risk management. In this present study, we try to elucidate the correlation between different environmental parameters and the growth of *V. paraheamolyticus* by analyzing archived RS data on water temperature and salinity with available databases under environmental concerns.

MATERIALS AND METHODS

In situ data: Archived data of southern Arabian sea from January 2012 to December 2012 were obtained from the NOAA/NESDIS, the available data were synchronized and interpreted by Ms. Suhatha Rahima, Research Scholar, Department of Applied Geology, Gandhigram Rural Institute, Tamil Nadu. The following variables were used in this study: Total colony plate count of *V. paraheamolyticus* per gram of fish sample (geometric mean of two replicate samples), water temperature (SST), and salinity (SSS). The present study compared these *V. paraheamolyticus* density and environmental data with archived RS data. Thus, only in situ observations recorded at times for which RS data were available were used. This occurred for 55 of 78 in situ observations.

RS data: SST derived from NOAA's Advanced Very High Resolution Radiometer (AVHRR) (O'Reilly *et al., 2000)* determined from Sea-Viewing Wide Field-of-View Sensor data, are all characterized by a pixel-to-pixel ground sample distance of 1.1 km. The Aqua (EOS PM) satellite was used to generate Fig. 2 and 3. These estimates of RS data were provided by the Department of Applied Geology and Geo informatics, Gandhigram Rural Institute, Tamil Nadu, in processed form via an automated processing system. The automated processing system contains programs for sensor calibration, atmospheric correction, geometric registration, and product algorithms.

Statistical analyses: Effects of different environmental parameters like SST and SSS on total *V. parahaemolyticus* densities were analyzed by regression analysis. All statistical analyses were conducted using the SPSS (SPSS Inc., Chicago, Ill) from the Department of Applied Research, Gandhigram Rural Institute, Tamil Nadu.

Bacterial isolation and identification

Microbiological analysis: For the present study, fish samples collected from Fort Cochin, Thevara, Vypin and Polakandum landing sites over a year period (Jan – Dec 2012). In each landing site, three samples were collected at different time period. for the isolation of the organism were maintained aseptically in sterile conditions. The samples include crabs, clams, prawns, squid etc. The samples were then transported to the Microbiology Laboratory in sterile polythene bags for the isolation and further Microbial, Biochemical and Molecular investigations. The samples were then sorted out; weighed 25 g of gills, skin and muscles altogether, and macerated using a homogenizer. Each sample was streaked on thiosulphate citrate bile salt agar and tryptone soy agar supplemented with 3 % NaCl (TCBS-3 and TSA-3 respectively). All plates were incubated at 25°C for 24 to 48 h. Isolates were characterized by morphological tests and API 20E strips (BioMerieux). Similar analysis was made of the water from collection site in which the fish were held as well as the food supply. Further characterization of the isolates was carried out by additional biochemical tests.

Haemolytic activities*:* Haemolysis of human blood was tested on TSA-3 supplemented with 1 % freshly obtained erythrocytes (Amaro *et al.,* 1992). Plates were incubated at 25 or 37'C for testing haemolysis of human erythrocytes. The Kanagawa test was performed using Wagatsuma agar (Nishibushi *et al*., 1989) supplemented with human 5 % erythrocytes. Haemolysis and Kanagawa plates were streaked in duplicate, plates were spot inoculated with 10 ìl of overnight cultures on basal medium (BM) and BM supplemented with 5 mM taurocholic acid (Sigma) as described by Osawa and Yamai (1996). This test is used to detect the presence of the virulent (tdh) strain of *Vibrio parahaemolyticus*. A clear zone (β-haemolysis) in the Wagatsuma agar indicates the presence of virulent strain (having *tdh* gene).

Molecular Analysis

a) Random amplified polymorphic DNA (RAPD) amplification: Prior to amplification, genomic DNA of *V. parahaemolyticus* was

extracted using Sigma Aldrich® Genomic DNA purification Kit (Sigma®, USA) for RAPD-PCR amplification: Amplification reactions were performed in 25 µl volume containing 3.5 mM $MgCl_2$, 0.5 µl dNTPS, 0.2 µ M primer, 0.5 µl Units of *Taq* polymerase, 2 µl genomic DNA. Amplifications were carried out in the thermal cycler for 35 cycles of 1 min at 94UC, 1 min at 36°C and 2 min at 72 ÚC. A final elongation step at 72 UC for 5 min was included. Amplification products were fractionated by electrophoresis through 1.5% Agarose gel and detected by staining with Ethidium bromide (EtBr). DNA ladder was used as DNA size marker. During the primer screening procedure ten random primers having the 50% G+C contents gene sequence (10-mer) were screened and three primers, the P 11-(5-AGG GGT CTT G-3),P 21-(5-AAT CGG GCT G-3) and P 31-(5-GGG AAC GTG T-3) were selected for further study as they provide reproducible and discriminatory patterns. Amplifications were carried out in a thermal cycler, GeneAMP PCR System 2400 (PE Applied Biosystems, USA).

***b) ORF8, tdh* and *trh* ASSAYS:** The presence of *tdh, ORF8* and *trh* in the strains was determined by PCR with a set of primers for *tdh*, 5_-GGTACTAAATGGCTGACATC-3_(sense) and 5_-CCACTACCACTCTCATATGC-3_ (antisense), and another set of primers for *trh*, 5_-GGCTCAAAATGGTTAAGCG-3_ (sense) and 5_-CATTTCCG-CTCTCATATGC-3 (antisense),and another set of primer for ORF8, 5-GTTCGCATACAGTTGAGG-3-(sense) and 5-AAGTACAGCAGGAGTGAG-3 (antisense), by the protocols established by Tada.

***c) tlh* and *toxR*-TARGETED PCR:** PCR was performed as described by Matsumoto et al. with the genomic DNAs of the strains in order to detect the *toxR* sequence of the samples with primers 5_-TAATGAGGTAGAAACA-3 and 5_-ACGTAACGGGCCTACA-3 and for *tlh,* primers5-AAAGCGGATTATGCAGAAGCACTG-3 and 5-GCTACTTTCTAGCATTTTCTCTGC-3 .PCR amplification was then performed in a DNA thermal cycler (GeneAMP PCR System 2400, PE Applied Biosystems, USA) with the genomic DNAs of all strains by the methodology described by Tada et al. The PCR products were electrophoresed in a 1.5% agarose gel, and after EtBr staining, the specifically amplified fragments were visualized by UV illumination.

d) ERIC- PCR (Enterobacterial Repetitive Intergenic Consensus sequences): Two specific primers were used to correlate to ERIC sequence (Versalovic *et al*., 1991; Son *et al*., 1998) ERIC 1 (R): 5'- ATGTAAGCTCCTGGGGATTCA-3' and ERIC 2 (F): 5' AAGTAAGTGACTGGG GTGAGC- 3'. The PCR technique was carried out in 0.5 ml microfuge tubes. The total volume consisting of reaction mixtures was 20ìl. Consisting of 12.5ìl sterile distilled water, 2.5 ìl 10x PCR buffer, MgCl2-1.8 µl, 0.5 ìl 25 mM deoxyribonucleotide phosphate, 1.0 ìl of each primer, 0.5 of 0.5 units *Taq* DNA polymerase and 2 ìl template DNA. The cycling conditions were as follows; pre-denaturation 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.

RESULTS

SSA and SST derived from NOAA/NESDIS data based on the four sampling stations near Central Institute of Fisheries and Technology, Wellington Island, Cochin, correlated well. Generally, SST was slightly higher than bottom water temperature, although this pattern was reversed on rare occasion. Overall, the estimated annual average temperatures based on these data were in close agreement (23.05°C for AVHRR versus 22.94°C for in situ). Fig 1 presents the summary statistics for the in situ and RS environmental parameters, as well as actual and predicted mean log total *V. parahaemolyticus* density, based on the FDA risk assessment model *(31)* The following model was used to derive these predictions: The observed *V. parahaemolyticus* densities were slightly higher than were either set of predictions, and the observed log *V. parahaemolyticus* densities were more variable. Greater variability of observed densities is expected because these model predictions are of mean log *V. parahaemolyticus* per gram, based on water temperature (SST) and salinity (SSS). The predictions include the effect of salinity (SSS) account for the negative variability exhibited in the growth. But the prediction based on the sea surface temperature (SST) account the positively correlated change in Total colony count. A positive residual corresponds to an observed level greater than that predicted based on water temperature and salinity. Despite some differences between remotely sensed temperature and salinity

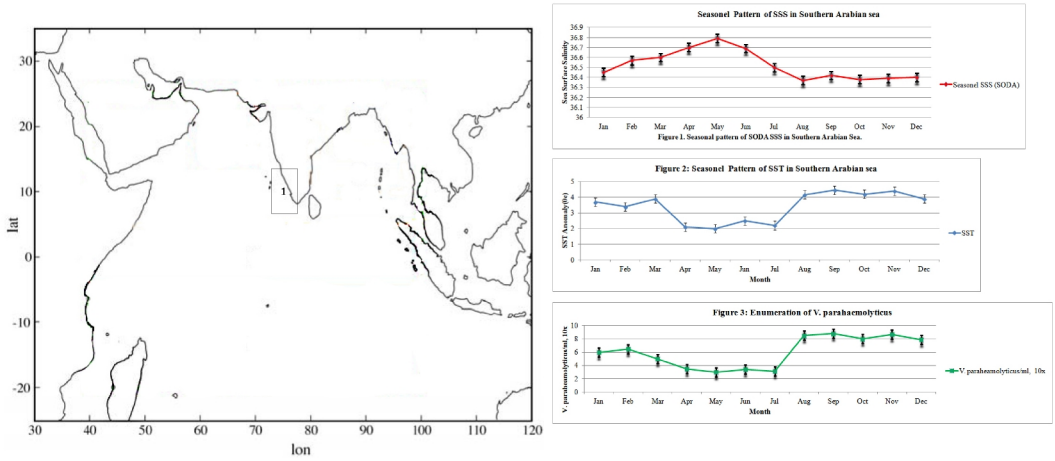

Fig. 1. Enumeration of *V. paraheamolyticus* growth (cells/ml,10x), under the effect of Remotely sensed and *in vitro* SST and SSA

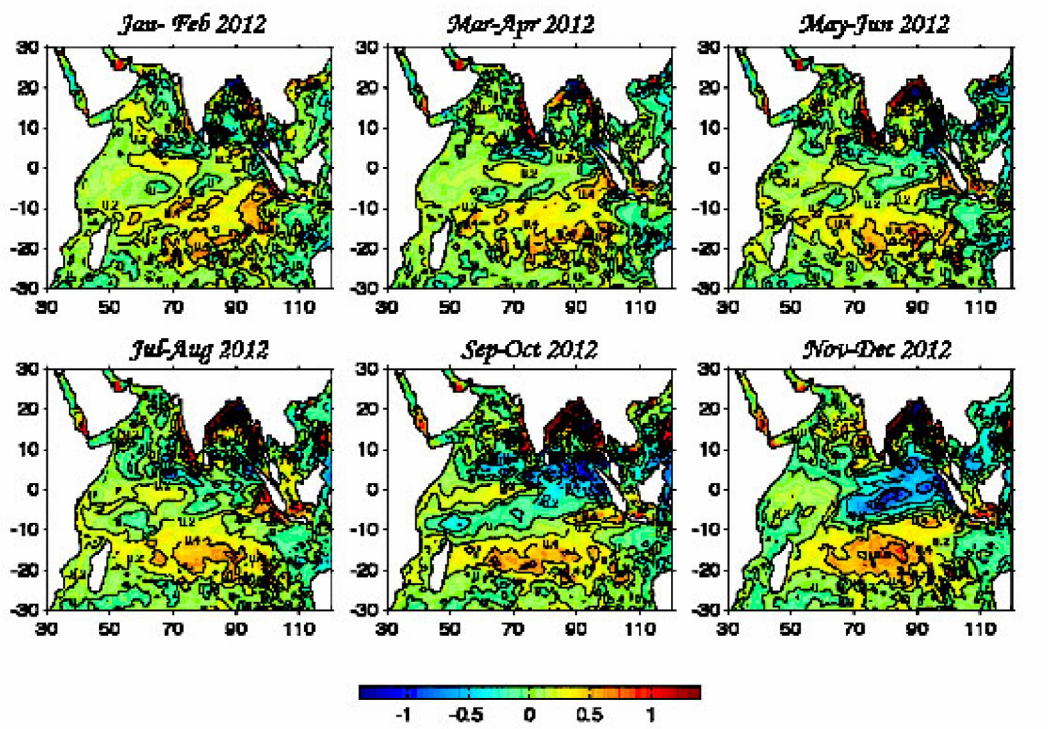

Fig. 2. Monthly variation of Sea Surface Salinity (SSS) anomalies from Jan to December 2012

measurements, the spatial variations in water temperature across a given region or estuary on a given day appears to be of equal or greater magnitude it is negatively correlated with salinity. For example, RS SST and SSS data for September to October 2012 are shown in Figure 2&3. Here, one can see a variation of between 22 and 25°C.

Microbiological analysis

Pure cultures were obtained from liver and ascitic fluid of diseased fish. Colonies were 3 to 4 mm and opaque on TSA-3 and green on TCBS. The strains were Gram-negative straight rods, motile, oxidase- and catalase-positive, sensitive

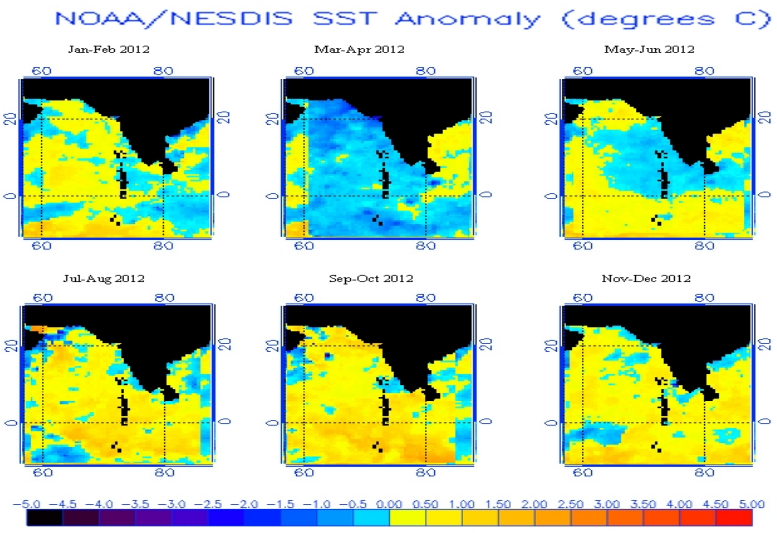

Fig. 3. Monthly variation of Sea Surface Temperature (SST) anomalies from Jan to December 2012.

to the vibriostatic o/129 at 150 pM and fermentative. Isolates were first characterized by API 20E (BioMerieux) strips, which gave profiles after reading the strips, seven digits 7037645 obtained, it is typical of *Vibrio parahaemolyticus*. On the basis of these results and those listed in Table 1, the isolates were identified as V. *parahaemolyticus*. V. *parahaemolyticus* strain were also recovered from the water from the sampling sites and the skin of the fish.

Virulence detection of *Vibrio parahaemolyticus*

All the isolates were tested in Wagatsuma agar. Out of them, 88 isolates produced â-hemolysis by Kanagawa Reaction. For PCR based identification, *toxR* and *tlh* genes were used, which are species-specific for *Vibrio parahaemolyticus* and all isolates produced specific amplicons except one isolate (MLT V-1). PCR performed for both *tdh* and *trh*. The *tdh* virulence factor was detected in 88 samples, whereas *trh* factor was not highlighted at all. The fish samples collected from different locations during the season January – December were used for the study. Fort Cochin, Vypin , Polakandum market and Thevara market were found to be infected with virulent pandemic strain of Vibrio haemolyticus specifically Crab,

Table 1. Morphological and Biochemical Test Results

Sl.No	Test/Reaction	*Vibrio paraheamolyticus.*
1	Growth on TCBS agar	Green /Bluish green colonies
2	Gram reaction & Motility	Gram negative roads , Motile
3	Indole	Positive
4	Klinger Iron Agar	Positive
5	Oxidase	Positive
6	Catalase	Positive
7	Nitrate Reduction	positive
8	Methyl Red	Positive
9	Voges-Proskaur	Negative

Octopus & Squid (Fort Cochin); Mackerel & Silver belly (Polakandum); Crab, Mackerel and Pearl Spot(Varapuzha); and Mullet (Thevara).

RAPD amplification and analysis

The RAPD PCR yields various amplification products resulting in a pattern of bands normally from 5-11 bands and the molecular size ranges between 250 bp and 5 kbp. Primer P11 produced 9 band patterns. Band pattern 'I' was prominent which was shown by 47 strains. Primer P21 produced 9 band patterns. Here band pattern 'b' was found to be prominent

which was exhibited by 33 strains. Primer P31 produced 11 band patterns. The band pattern obtained here featured two types of prominent band patterns - '1' & '7' which were shown by 21 strains and 22 strains respectively.

ERIC- TYPING

36 banding patterns were obtained normally 5 – 12 bands with molecular size ranging from 250 bp to 4 kbp numbered from '1' to'36' in the figure: 10. Band pattern '21' found to be prominent which was shown by 29 isolates. Out of 11 tdh positive isolates, 3 followed this banding pattern. Out of the remaining 8 isolates, 2 exhibited band pattern '3', 1 exhibited band pattern '11', 2 showed the band pattern '30', 2 exhibited the band pattern '32' and 1 showing the band pattern '25'.

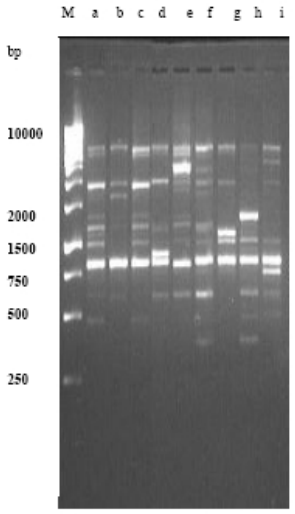

Fig. 4. RAPD pattern by random primer P21 All representative variants of single RAPD pattern are included. Each specific pattern designated by a letter (a-i) and 1 Kb molecular markers are given on the left.

SUMMARY AND CONCLUSIONS

This study scrutinized the value of remote sensing data for prediction of *V. parahaemolyticus* levels under environmental concerns. RS SST and SSS data were found to relate to in situ temperature and salinity data with relatively high correlation, and observed systematic differences were small and consistent with expectations *(23)*. Thus, predictions based on RS water temperature appear feasible now and provide much greater coverage than available with sporadically located weather buoys. Additionally, the imagery provides a user friendly format for the interpretation and use of risk assessment outputs for forecasting the effect of global warming and risk management. Approaches that may be used to mitigate the effect of global warming by remote sensing data collected over multiple previous days and/or statistically interpolating, both spatially and temporally *(6, 16)*. Use of additional sources of

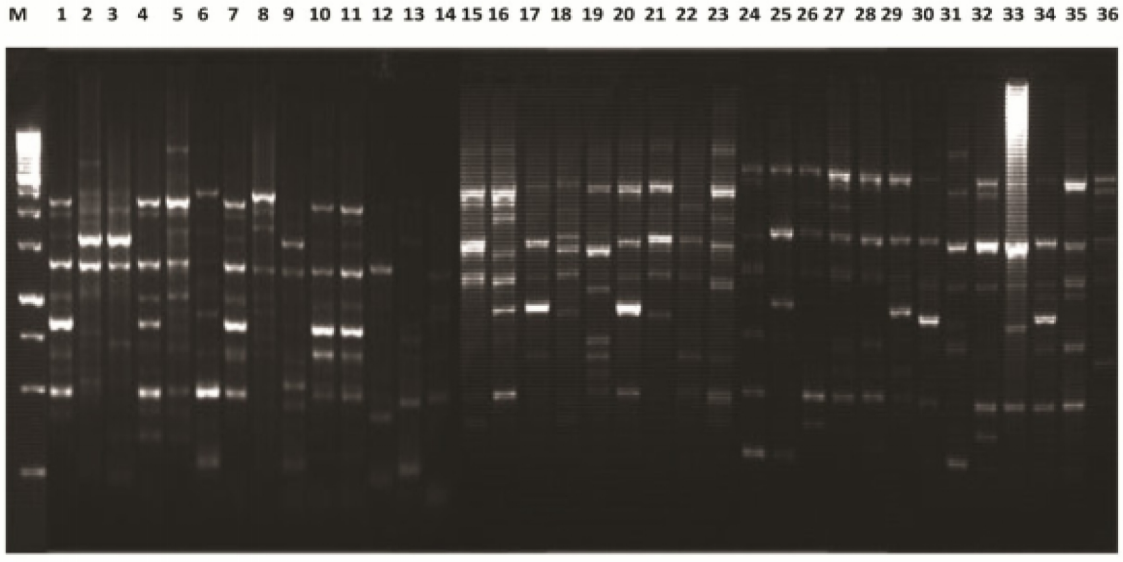

Fig. 5. ERIC pattern of genomicDNA of *Vibrio paraheamolyticus*. All representative variants of single RAPD patterns are included. Each specific pattern is designated by a number (1-36) and 1 Kb molecular size marker(M) are given on the left.

data, including buoys and vessels, may also help overcome the remote sensing data gaps. The present study was conducted in January-December 2012. Marine samples from eight different stations around Cochin were collected. Of the 116 isolates, 88 (76%) were pathogenic strains having *tdh* gene. It was found that samples from Fort Cochin, Thevara, Vypin and Polakandum were infected with *tdh* positive strains. But they were found to be deficient in ORF8 and *trh* genes. Squid, Octopus and Crab from Fort Cochin emerged to be the carriers of the *tdh* positive virulent strain of *Vibrio paraheamolyticus*. Samples from Varapuzha emerged as positive for the presence of virulent strains of Vibrios parahaemolyticus. Factors such as salinity and air temperature that affect *V. parahaemolyticus* growth level. The RS data–based predictions shown in Figure 1 include effects of salinity, Temperature and enumeration of *V. paraheamolyticus* plate count. The tremendous hike of SST and SSS will provide optimum growth parameters for this pathogenic strain. This Data source for salinity with spatial resolution equal to or better than that of water temperature, RS water temperature– based predictions are either contingent on specified salinity levels, or such predictions can be averaged over the likely distribution of salinity in southern Arabian sea sampling area, considered independent of location. Alternatively, a range of salinity-specific predictions may be of value in the absence of such information.

Implication of studies on *Vibrio parahaemolyticus* is the effect of global warming to the pathogenicity of the organism. The incidence of *Vibrio parahaemolyticus* infection can soar higher possibly due to the dilution of salt content in the oceans and rise in temperature owing to global warming. This may be attributed to the enhancement of optimum conditions of growth of *Vibrio parahaemolyticus* as it operates in moderate salinity and moderate temperature. This prediction is supported by the fact that seasonal variations largely affect the booming of *Vibrio parahaemolyticus*.

With precisely specified molecular analysis and expected prediction models, RS data can be used for the study of environmental impacts and risk assessment, Both to investigate past outbreaks and virulence patterns and to provide a basis for forecasting and rapid, near real-time dissemination of pertinent information for risk management.

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