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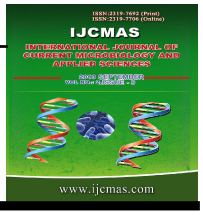

Original Research Article

Molecular diversity analysis of *Yersinia enterocolitica* isolated from marine marketed fish

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Keywords

Y. enterocolitica; multiple antibiotic resistances; haemolysin; RAPD-PCR; ERIC-PCR.

A B S T R A C T

Of 56 finfish samples collected, 11 of them were contaminated with *Y. enterocolitica*. Among the different body parts (surface, gill and intestine) of fish tested, maximum incidence has been recorded with gills with 50 %, followed by surface and intestine with 25 % and 8.3% respectively. This indicates that the emergence of pathogenic *Y.enterocolitica* in marine marketed fish. Multiple antibiotic susceptibility assay showed that all the isolates were resistant to ampicillin and sensitive to chloramphenicol and most of them showed multiple antibiotic resistance. It was also observed that 82 % of the isolates were found to be the producers of haemolysin. The lipopolysaccharide profiles of all the 11 isolate indicates existence of both rough and smooth LPSs. The molecular studies such as RAPD and ERIC PCRs clearly indicated that the wide-genetic diversity among the strains tested.

Introduction

Yersinia enterocolitica, a Gram-negative rod, belongs to family *Enterobacteriaceae* and an important food - borne and water - borne enteropathogen (Keet, 1974; Eden *et al*., 1977). Common clinical syndromes include gastroenteritis, various forms of abscesses and erythema nodosum (Bottone, 1977). Any edible raw animal food (fish, meat, pork, and poultry) may carry *Y. enterocolitica* and cause gastroenteritis among human. However, during the last two decades it was also isolated from human, animals, water, vegetables and various environments, all over the world (Hellmann and Heinrich, 1985).

Treatment of *Y. enterocolitica* infections often requires aggressive antibiotic therapy, typically involving ciprofloxacin (Margarita and Aivars, 2010). Considering the significance of food-borne illness of this bacterium, it has been taken to analyze the antimicrobial resistance and haemolysin activity among the finfish.PCR has become a potentially

powerful alternative in microbiological diagnostics due to its simplicity, rapidity, reproducibility and accuracy (Belgrader *et al*., 1999). RAPD - PCR directs random DNA sequence by using single primer (Leal *et al.,* 1999). ERIC - PCR is based on DNA sequence amplification with primer sets complementary to each end of sequences, representing the short repetitive sequence present in the genomes of *Enterobacteriaceae* (Versalovic *et al*., 1991). By using any or the combination of these molecular tools, the epidemiological investigations on *Y. enterocolitica* could be elucidated effectively. Considering the significance of food - borne illness of this bacterium, this study has been taken to analyze the diversity among the retail fish samples.

Materials and Methods

Sample collection

A total of 56 finfish samples were collected from different fish markets in Coimbatore city, Tamil Nadu. Swabbing of different body parts (surface, gill and intestine) was carried out using sterile cotton swabs and transferred to yersinia selective enrichment broth and incubated at 37 ºC for 16-24 h for enrichment.

Culture methods

Following incubation, a loopful of culture was transferred and streaked on to CIN agar (HiMedia, India), and incubated at 25 ^{o}C for 18-24 h. Suspected colonies (red bull-eye shaped colonies) were individually isolated and subcultured on nutrient agar (HiMedia, India) for further studies.

Confirmation of *Y. enterocolitica*

All the isolates were subjected to biochemical methods for identification, according to Bergey's manual of determinative bacteriology (Breed *et al*., 1944), (Table 3). Also, extraction of genomic DNA was done (Agersborg *et al*., 1977). All the samples weresubjected to 16S rRNA PCR (Neubauer *et al.,* 2000) for the confirmation of *Y. enterocolitica*.

Antimicrobial resistance

In order to check the multi - drug resistance, all the isolates were tested for their multiple antibiotic resistances against 15 different antibiotics, using Mueller-Hinton agar (HiMedia, India) by adopting standard disc diffusion method (Bauer *et al*., 1966). Following disc diffusion, all the plates were incubated at 28°C for 24 h. Zone of inhibition was observed under visual inspection.

Hemolytic activity

All the *Y. enterocolitica* strains were individually enriched in 5 ml of brain heart infusion broth (HiMedia, India) and incubated at 37 ^{o}C for 16 - 18 h. Supernatant were carefully removed after centrifugation at 10,000 g for 30 min at 4 ^{o}C. About 100 µl of supernatant were mixed with an equal volume of 2 % (v/v) suspension of sheep erythrocytes in a 96 - well 'V'- bottom microtitre plate. The mixture was incubated for 30 min at 37 ^{o}C and then for 30 min at 4 ^{o}C. An erythrocyte suspension in phosphate - buffered saline (PBS) was included in each assay as a negative control. Haemolysin production was recorded by visual inspection.

Extraction of lipopolysaccaride (LPS)

Cells were grown on nutrient broth for 48 h at 25 °C. LPS from cells was obtained by the hot phenol - water method (Rezania *et al.,* 2011). About 20 µl of LPS

suspension was applied in each slot of 12.5 % SDS - PAGE gel. The separated LPSs were visualized by silver staining (Tsai and Frasch, 1982).

RAPD – PCR

Two primers have been used in this study, primer-1 (5'–CCGCAGCCAA –3') and primer-II (5'–GAGACGCACA –3'). Each 25 µl reaction mix contains 30 ng genomic DNA, 1 U Taq DNA polymerase, 1 X Taq DNA polymerase buffer (Chromous Biotech, Bangalore), 2.5 mM $MgCl_2$, 400 µM dNTPs (Helini Biomolecules, Chennai) and 20 pmol / µl primer. The RAPD - PCR conditions were adopted according to Leal *et al*., (1999).

ERIC – PCR

The primers ERIC-1 (5'–ATGTAAGCTCCTGGGGATTCAC–3') and ERIC – 2 (5'–AGTAAGTGAC TGGGGTGAGCG–3') were used. Each 25 µl reaction mix contains 40 ng genomic DNA, 2.5 U Taq DNA polymerase, 1 X Taq DNA polymerase buffer, 3 mM$MgCl_2$, 400 µM dNTPs and 20 pmol / µl of each primer. Slight modification was made in ERIC–PCR cycle, which was described by Szczuka and Kaznowski (2004).

Visualization of PCR products

Amplification was performed using a thermal cycler (MJ Research, Model PTC 100 Watertown, Mass., USA). All the PCR products were resolved by agarose gel electrophoresis with 1.5 % (w/v) concentration with 1 X Tris borate EDTA buffer and the gel were stained with 1 µl / ml of ethidium bromide and visualized using gel documentation system (UVP GelDoc – It 300 Imaging system, Cambridge, UK). A 100 bp DNA ladders were used as markers.

Results and Discussion

Y. enterocolitica has emerged as one of the significant causes of food - borne gastroenteritis in humans in developed countries for the past 20 years (Wojciech *et al*., 2004). An infection transmitted through consumption of contaminated food is a significant source of human morbidity. Of 56 finfish samples collected, 20 % (n=11) of them were contaminated with *Y. enterocolitica* (Table 1). It was also observed that none of the finfish sample collected from Quilon was positive for this bacterium. This significant level of incidence of this bacterium is being reported for the first time in South India. In previous study, it was reported that 1 % of the *molluscus and 3 % shellfish* samples were contaminated with *Y. enterocolitica* (Ripabelli *et al.*, 2004). In a study, from 15 fish samples collected, 5 (33.3 %) isolates found contaminated by this bacterium (Khareb *et al.*, 1996). The higher degree of incidence of *Y. enterocolitica*in this present work reveals serious issues of food borne contamination with respect to the public health point of view. Maximum incidence of *Y. enterocolitica* has been recorded from gill samples, with 50 %, followed by body surface and intestine with 25% and 8.3 % respectively (Table 2). This might be due to the poor hygienic conditions of the water bodies and improper handling and storing practices. The transmission of this bacterium from contaminated fish to a fresh fish is quite normal during poor hygienic conditions. Such kind of contamination results in high-degree of contamination in any type of food-borne infections.

Production of haemolysin is said to be one of the important pathological characters of *Y. enterocolitica*. The ability of

Table.1 Station-wise incidence of *Y. enterocolitica* in marketed fish

Station	No. of Samples	No. of Positive samples	% incidence
Quilon	7	-	-
Coimbatore	49	11	22
TOTAL	**56**	**11**	**20**

Table.2 Fish-wise incidence of *Y. enterocolitica* in marketed fish

Location	Name of fishes	Surface	Gut	Intestine	Total
Quilon	_	_	_	_	_
Coimbatore	Para	_	+	_	10
	PlicofollisDussumier	+	_	_	
	SardinellaGibbosa	+	_	_	
	Vaasanthi	_	+	_	
	Jilebi	_	+	_	
	Lizard	+	_	_	
	Lizard	_	_	+	
	StolethorusTriostegus	_	+	_	
	SardinellaGibbosa	_	+	_	
	Stolethorus Triostegus	_	+	_	
	RestrelligerKanaguria	_	+	_	1
% positive		25%	50%	8.3%	11

Table.3 Biochemical reaction for isolated *Y.enterocolitica*

Biochemical test[a]	No. tested	No. positive	% positive
Voges –proskauer	56	45	80
Urease	56	34	60
Sorbitol	56	40	71
Ornithine decarboxylation	56	24	48
Citrate	56	15	28
DNase	56	12	21
Raffinose	56	11	19
Esculin hydrolysis	56	13	23
Salicin fermentation	56	23	41
Lysine Iron Agar (LIA)	56	11	19

[a]All biochemical tests completed at 36°C unless otherwise noted

haemolysis is claimed to be one of the virulence factors among pathogenic microorganisms. It was observed that 82 % (n=10) of the strains were positive for haemolysin production. Pedersen (2009) reported that ability of lysis of human erythrocytes by *Y. enterocolitica* haemolysin differs individual to individual. He also reported that the *Y. enterocolitica* serotypes O8 and O9 did not produce any haemolytic substance. Similarly, Tsubokura *et al.,* (1979) reported that none of their isolates were positive for haemolysin production. These strains might be belongs to O8 and O9 serovars. The production of haemolysin is effectively influenced by temperature. It has been reported that *Y. enterocolitica* could produce haemolysin at 28 ^{o}C and 37 ^{o}C as well (Phillips *et al.,* 2000). They also reported that the haemolysin produced at 37 ^{o}C is comparatively effective than at 28 ^{o}C. In the present study, 10 of 11 isolates were positive for haemolysin production at 37 ^{o}C, but not 28 ^{o}C (Table 4). While, Franzinet al (1984) reported that of 131 *Yersinia* sp. 74 were positive for haemolytic activity at 28 ^{o}C.

Antimicrobial resistance among food-borne pathogens and therapeutical intervention has always been an important issue in public health. In the present study, 7 strains (64 %) were resistance to tetracycline. While, of 50 *Y. ruckeri* of salmon fish isolates, only 2 showed resistance to tetracycline (Grandis and Stevenson, 1985). In our study, all the isolates were found resistance to amoxicillin and sensitive to chloramphenicol. Stock and Wiedemann, (1999) and Okwari *et al.,* (2007) also reported that all the milk isolates of *Y. enterocolitica* exhibiting resistance to amoxicillin. In another study, Subha *et al.,* (2009) reported all the raw milk isolates of *Y. enterocolitica* were sensitive to chloramphenicol. In contrast, they have recorded that 63 % of the strains were resistant to amoxicillin. In the present study, it was also observed that 81 %, 64 %, 55% and 45 % isolates showed resistance methicillin, riframpcin, erythromycin and novobiocin respectively (Table 5). In general, the antimicrobial resistance pattern for *Y. enterocolitica*, reported by several researchers having significant difference, world-wide. There is no identical profile for a set of antibiotics has been reported by any researcher. This strongly supports the significant impact of geographical location, local selective pressure and other factors in the determination of antibiotic resistance among the *Y. enterocolitica* isolates.

Lipopolysaccharide is an essential component of the outer membrane of most Gram-negative bacteria and causative agent of severe septic shock in humans (Bos *et al.,* 2004). Several investigators worked on the characterization of LPSs produced by *Y. enterocolitica* (Boyce *et al.,* 1979; Francis *et al.,* 1980; Hoffman *et al.,* 1980; Zhang *et al.,* 1997). In the present investigation, majority of the isolates have produced smear like profiles for the LPS. Out of 11 strains used, only one is exhibiting ladder like pattern for LPS (Figure 1).

The diversity of 11 strains in RAPD-PCR was analyzed using two primers(primer-I and primer-II), which showed four and five different banding pattern for primer-I and primer-II respectively (Figures2 and 3). Similarly in a study conducted by Odinot *et al.,* (1995) with 48 *Y. enterocolitica* isolates of clinical origin, 13 different profile groups have been reported. While, Leal *et al.,* (1999), used

the

Table.4 Haemolytic activity of *Y. enterocoltica* isolated from marketed fish

No. of strains	Haemolysin assay (%) (n= 11)	Temperature
11	82%	37°C
11	0 %	28°C

Table.5 Antibiotic susceptibility of *Y.enterocolitica*

Antibiotic	Disc potency (µg)	No. of samples	Resistant	% Resistance
Amoxicillin	25	11	11	100
Ampicillin	10	11	8	73
Amoxyclav	30	11	7	64
Chloramphenicol	10	11	0	0
Erythromycin	15	11	6	55
Gentamicin	10	11	2	18
Kanamycin	30	11	4	36
Methicillin	10	11	9	81
Nalidixic acid	30	11	6	55
Norfloxacin	30	11	4	36
Novobiocin	30	11	5	45
Polymyxin-B	50 units	11	6	55
Rifampicin	5	11	7	64
Streptomycin	10	11	5	45
Tetracycline	30	11	7	64

Figure.1-SDS-PAGE analysis of lipopolysaccaride of *Yersinia enterocolitica* from fish

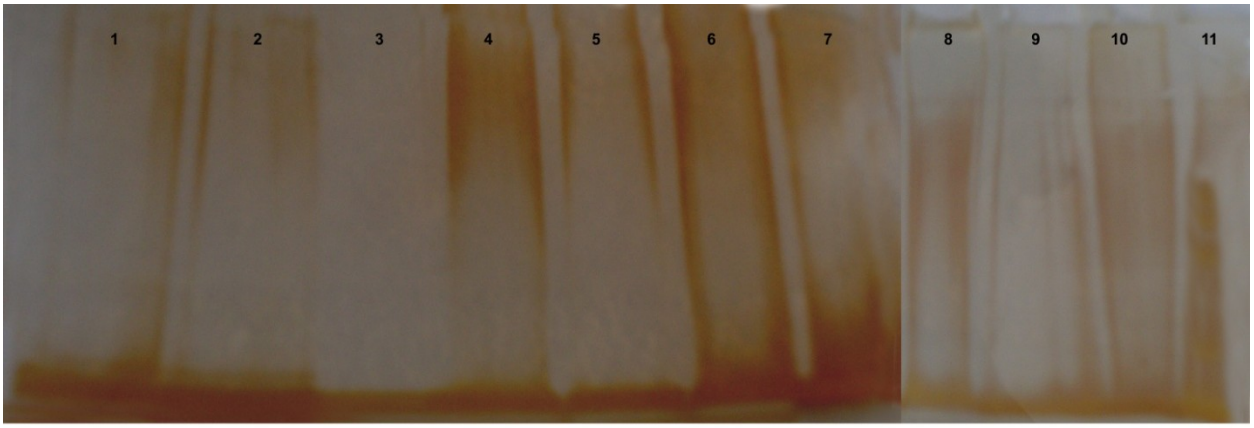


Figure.2 Primer I- RAPD-PCR fingerprints of *Y. enterocolitica* isolates from fish

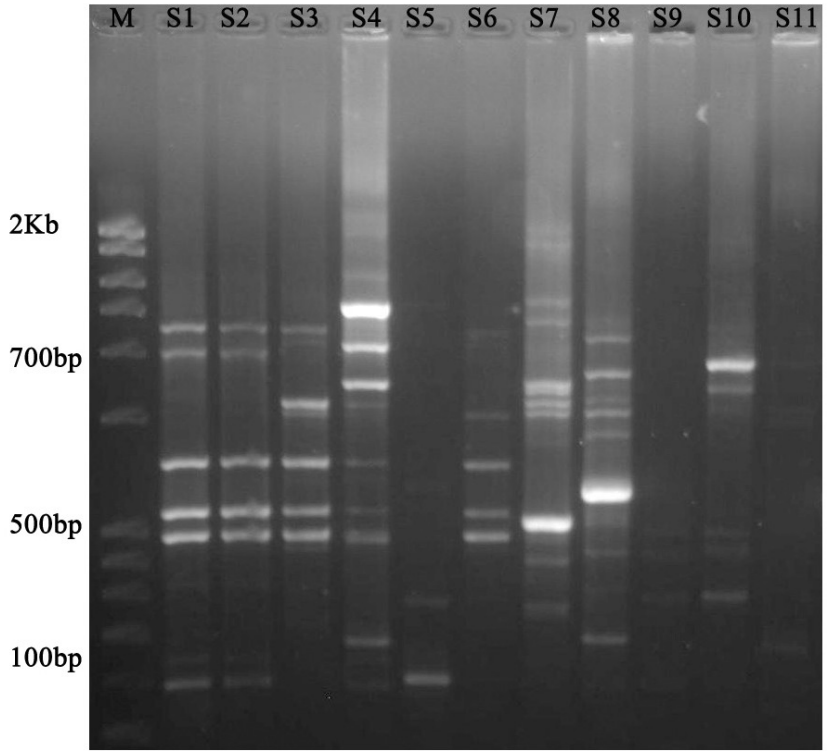


Figure.3 Primer II- RAPD-PCR fingerprints of *Y. enterocolitica* isolates from fish

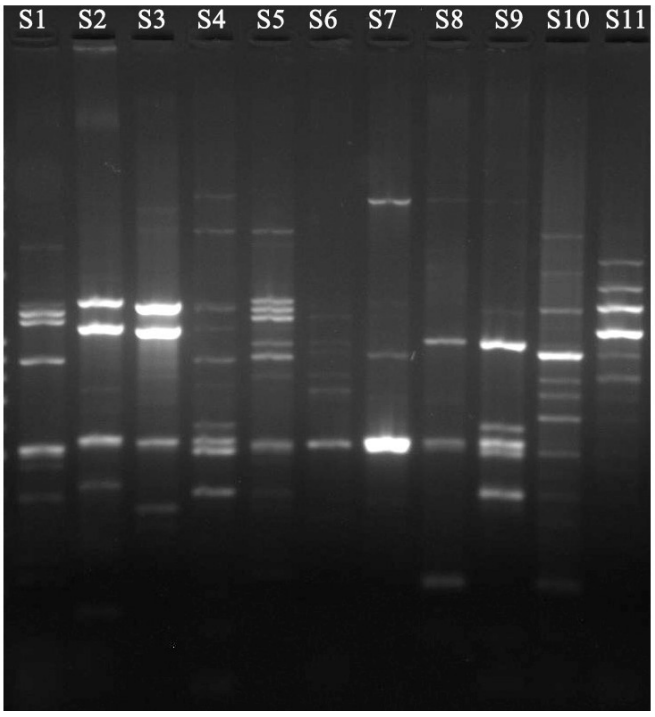


Figure.4-Dendrogram for RAPD-PCR of *Y. enterocolitica* isolates from fish

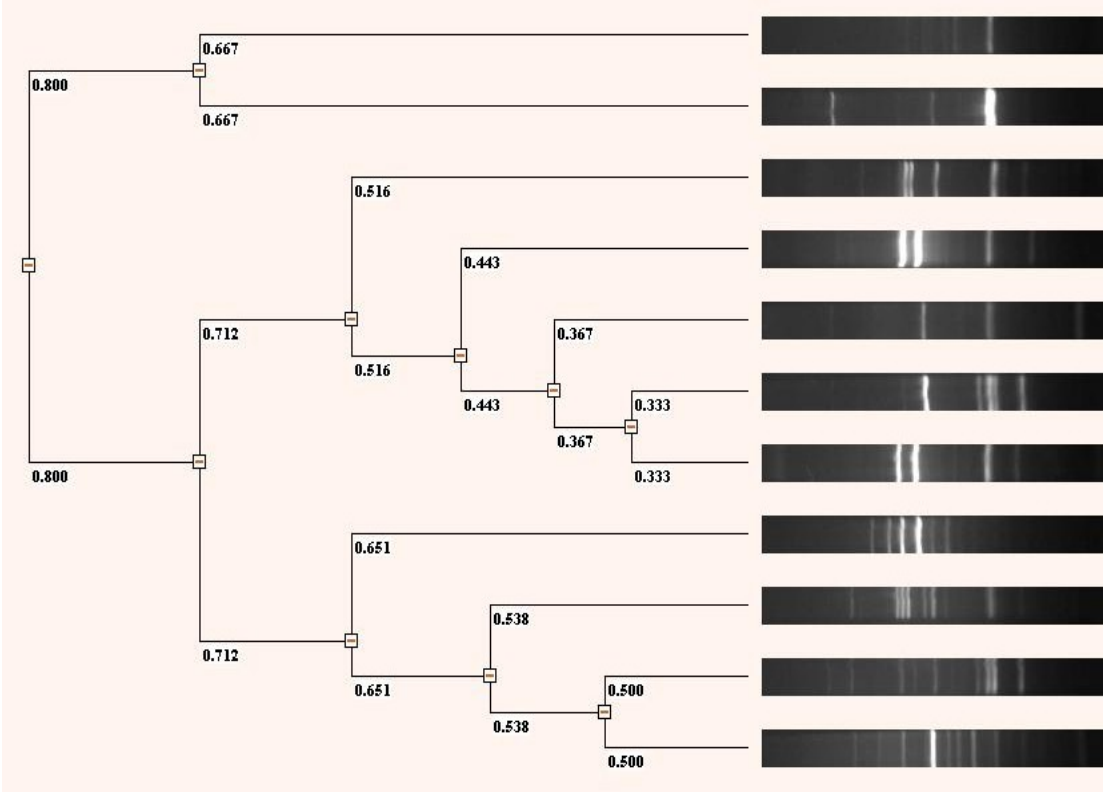


Figure.5 ERIC-PCR fingerprints of *Y. enterocolitica* isolates from fish

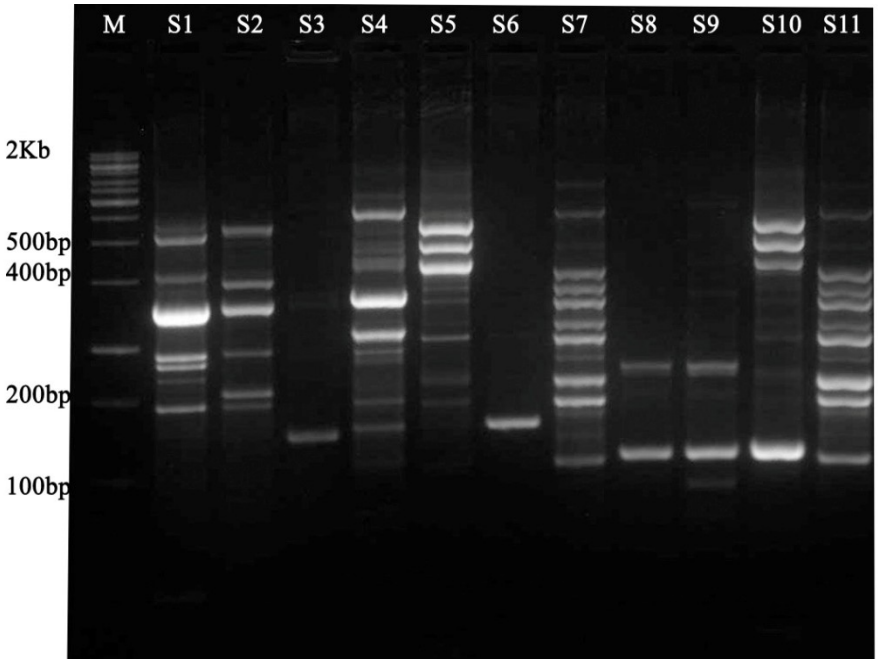
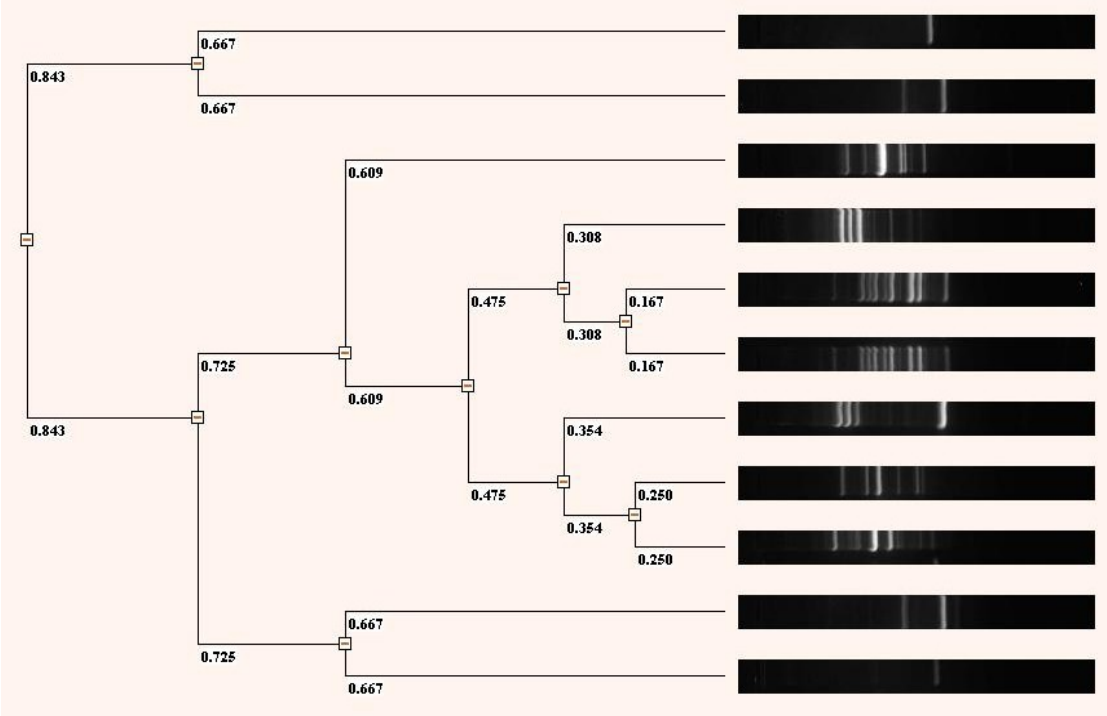


Figure.6 Dendrogram for ERIC-PCR of *Y. enterocolitica* isolates from fish



same primers and reported five different genotypic profiles for 20 *Y. enterocolitica* isolated from human and swine sources.

In the present investigation, profiles of ERIC - PCRs clearly revealed the co-existence of genetically diverse *Y. enterocolitica*, with seven differentprofile groups (Figure 5). Juliana *et al.*, (2006) also reported that ERIC-PCR is a powerful tool in the discrimination of genetic diversity among the *Y. enterocolitica*. They worked on 106 strains of *Y. enterocolitica* isolated from human, animal and food observed 11 different profiles among them.

In contrast, Pooja and Virdi, (2004) recorded that ERIC profiles of *Y. enterocolitica* were having limited number of genetic diversity. Wojciech *et al*., (2004) reported that ERIC PCR is much better than REP PCR in the discriminating capacity in genetic diversity analysis with reference to *Y. enterocolitica*. In our study, seven different patterns observed. This was further confirmed by the dendrogram (Figures4 and 6) also. Interestingly, these three tools have proved their efficiency in species diversity analysis among *Y. enterocolitica* used in this study. In conclusion, we strongly recommend using these typing techniques in the epidemiological investigations with special reference to *Y. enterocolitica.*

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References

Agersborg, A., R. Dahl and Martines, I. 1997. Sample preparation and DNA extraction procedures for polymerase chain reaction identification of *Listeria monocytogenes* in seafoods. Int. J. Food Microbiol. 35: 275-280.

Bauer, A.M., W.M.M. Kirby, J.C. Sherris and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45: 493-496.

Belgrader, P., W. Benett, D.Hadley, J. Richards, P. Stratton, R. Mariella, and Milanovich, F. 1991. PCR detection of bacteria in seven minutes. Science. 284: 449–450.

Bos, M.P., B. Tefsen, J. Geurtsen and Tommassen, J. 2004. Identification of an outer membrane protein required for the transport of lipopolysaccharide to the bacterial cell surface. Proc. Natl. Acad. Sci. USA. 101(25): 9417-22.

Bottone, E.J., 1997. *Yersinia enterocolitica*: The charisma continues. Clin. Microbiol. Rev. 10: 257-276.

Boyce, J.M., E.J. Evans Jr, D.G. Evans and DuPont, H.L. 1979. Production of heat-stable, methanol-soluble enterotoxin by *Yersinia enterocolitica*. Infect. Immun. 25:532-537.

Eden, K.V., M.L. Rosenberg, M. Stoopler, B. Wood, A.K. Highsmith, P. Skaliy, J. Wells and Feeley, J.C. 1977. Waterborne gastroenteritis at a ski resort associated with the isolation of *Yersinia enterocolitica*. Public. Health Rep. 92: 245-250.

Francis, D.W., P.L. Spaulding and L-Vitit, J. 1980. Enterotoxin production and thermal resistance of *Yersinia enterocolitica* in milk. Appl. Environ. Microbiol. 40: 174-176.

Franzin, L., P. Fantino and Valerio, V. 1984. Isolation of *Yersinia enterocolitica* and *Yersinia* like Organisms from Raw Milk in Italy. Curr. Microbiol. 10: 357-360.

Grandis S.A., and Stevenson, R. M. W. 1985. Antimicrobial susceptibility patterns and R plasmid-mediated resistance of the fish pathogen *Yersinia ruckeri.* Antimicrob. AgentsChemother. 27(6): 938-942.

Hellmann, E., and Heinrich, G. 1985. Growth rates of two virulence plasmids carrying *Yersinia enterocolitica* after contamination of heated milk, raw minced pork and vegetables. Zbl. Bakt. Mik. Hyg. *B.* 182: 1-16.

Hoffman, J., B. Lindberg and Brubaker, R.R. 1980. Structural studies of the O-specific side-chains of the lipopolysaccharide from *Yersinia enterocolitica* Ye 128. Carbohydr. Res. 78: 212-214.

Jann, K., and Jann, B. 1984. Structure and biosynthesis of O-antigens. In E.T. Rietschel (*ed.*), Handbook of endotoxin. Chemistry of endotoxin. Elsevier Science Publishers, Amsterdam, 1:138-186.

Juliana, P.F., P. Deise, P.S. Andre, A.C. Malaspina and Marcelo, B. 2006. Molecular typing and virulence markers of *Yersinia enterocolitica* strains from human, animal and food origins isolated between 1968 and 2000 in Brazil. J. Med. Microbiol. 55: 1539–1548.

Keet, E.E., 1974. *Yersinia enterocolitica* septicemia. N. Y. State J. Med. 74: 2226-2230.

Khare, S.S., A.S Kamat, T.R. Doctor and Nair, P.M. 1996. Incidence of *Yersinia enterocolitica* and related species in some fish, meat and meat products in India. J. Sci. Food. Agric. 72(2): 187–195.

Leal, T.A., C. Nilma, M. Alzira and Almeida, P. 1999. RAPD-PCR Typing of *Yersinia enterocolitica* (Enterobacteriaceae) O:3serotype strains isolated from pigs and humans. *Genet. Mol. Biol.* 22(3): 315-319.

Margarita, T., and Aivars, B. 2010. Prevalence and antimicrobial resistance of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in slaughter pigs in Latvia. J. Food Prot. 57(3): 1335–1338.

Neubauer, H., A. Hensel, S. Aleksic and Meyer, H. 2000. Identification of *Yersinia enterocolitica* within the genus *Yersinia*. System. Appl. Microbiol. 23:58-62.

Odinot, P.T., J.F.G. Meis, P.J.J.C. Van Den Hurk, J.A.A. Hoogkamp and Melchers, W.J.G. 1995. PCR-based characterization of *Yersinia enterocolitica*: comparison

with biotyping and serotyping. Epidemiol. Infect. 115: 269-277.

Okwari, A.E.J., S.E. Agina, A.O. Olabode, M.A.K. Fadera, J. Ibu and Odugbo, M. 2005. Faecal carriage of *Yersinia* species in pig sheep and poultry on display for sale in Vom and Bukuru areas of Jos South Local Government Area (LGA). Plateau state, Nigeria. Nig. J. Micriol. 19: 444-451.

Pedersen, K.B., 2009. Studies on a haemolytic substance in *Yersinia enterocolitica*. Acta. Pathol. Microbiol. Scand. B. 89 (1-6): 2011 – 214.

Phillips, A.D., S. Navabpour, S. Hicks, G. Dougan, T. Wallis and Frankel, G. 2000.EnterohaemorrhagicEscherichia coli O157: H7 target Peyer's patches in humans and cause attaching lesions in both human and bovine intestine. Gut 47: 377–381.

Pooja, S., and Virdi, J.S. 2004. Repetitive elements sequence (REP/ERIC)-PCR based genotyping of clinical and environmental strains of *Yersinia enterocolitica* biotype 1A reveal existence of limited number of clonal groups. FEMS Microbiol. Lett. 240:193–201.

Rezania, S., N. Amirmozaffari, B. Tabarraei, M. Jeddi-Tehrani, O. Zarei, R. Alizadeh, F. MasjedianZarnani, A.H. 2011. Extraction, Purification and Characterization of Lipopolysaccharide from *Escherichia coli* and *Salmonella typhi*. Avicenna J. Med. Biotechnol. 3: 3-9.

Ripabelli, G., M.L. Sammarco, I. Fanelli and Grasso, G.M. 2004. Detection of *Salmonella, Listeria* spp., *Vibrio* spp., and *Yersinia enterocolitica* in frozen seafood and comparison with enumeration for faecal indicators: implication for public health. Ann. Ig. 16(4): 531-539.

Stock, I., and Wiedemann, B. 1999. An invitro study of the antimicrobial susceptibility of *Yersinia enterocolitica* and the definition of a database. J.Antimicrob. Chemother. 43: 37-45.

Subha, B., D. Ramakrishnan and Suganthi, V. 2009. Antimicrobial resistance pattern of selected *Yersinia enterocolitica* isolated from raw cow milk and pork samples of Namakkal District, Tamilnadu, South India. Global J. Environ. Res. 3(3): 169 – 177.

Szczuka, E., and Kaznowski, A. 2004. Typing of clinical and environmental *Aeromonas*sp. strains by random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR and enterobacterial repetitive intergenic consensus sequence PCR. J. Clin. Microbiol. 42: 220-228.

Tsai, C.M., and Frasch, C.E. 1982. Staining of lipopolysaccharide in SDS polyacrylamide gels using silver staining method. Anal. Biochem. 119:115–119.

Tsubokura, M., K. Otsuki, I. Shimohira and Yamamoto, H.1979. Production of indirect hemolysin by *Yersinia enterocolitica* and its properties. Infect. Immun. 939-942.

Versalovic, J., T. Koeuth and Lupski, J.R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19: 6823-6831.

Wojciech, L., Z. Staroniewicz, A. Jakubczak and Ugorski, M. 2004. Typing of *Yersinia enterocolitica* isolates by ITS profiling, REP and ERIC PCR. J. Vet. Med. 51: 238-244.

Zhang, L., J. Radziejewska-Lebrecht, D. Krajewska-pietrasik, P. Toivanen and Skurnik, M. 1997. Molecular and chemical characterization of the lipopolysaccharide O-antigen and its role in the virulence of *Yersinia enterocolitica* serotype O: 8. Mol. Microbiol. 23: 63-76.