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Short Communication

Genetic Diversity among *Yersinia enterocolitica* Isolated from Chicken and Fish in and around Coimbatore City, India

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Abstract

Background: *Yersinia enterocolitica* is rapidly emerging worldwide as an enteric pathogen and has become a major cause of diarrhea even in developed countries. The aim of this study was to characterize and genetic diversity analysis among *Y. enterocolitica* strains isolated from fish and chicken sources.

Methods: From 44 strains, 55% (24 strains) found to be positive for *Y. enterocolitica* by colony morphology, biochemical tests and 16S rRNA. We investigate the diversity of *Y. enterocolitica* by hemolytic activity, antimicrobial resistance, RAPD, ERIC and REP-PCRs PCR, profiling of outermembrane proteins and lipopolysaccarides.

Results: Forty one percent of the strains were found to be the producers of haemolysin at 37 ˚C but not at 28 ˚C. All the isolates were exhibiting multi-drug resistance and found sensitive to chloramphenicol, and resistant to ciprofloxacin and amoxicillin. Eight, eleven and twelve different genotypic patterns were observed in RAPD, ERIC and REP-PCRs respectively. Five isolates have produced high molecular weight protein (HMWP) with a molecular weight of 150 – 220 kDa. Mostly LPS produce identical profiles, 22 strains have produced smooth LPS, while 2 strains have produced the rough LPS pattern.

Conclusion: Genotyping tools strongly confirm the co-existence wide genetic diversity among the strains tested. By using any or the combination of these molecular tools, epidemiological investigation on *Y. enterocolitica* could be elucidated effectively. These results showed that the REP-PCR is more informative and discriminative than other for analysis of *Y. enterocolitica* diversity.

Keywords*: Yersinia enterocolitica,* Multidrug resistance, Lipopolysaccarides

Introduction

Yersinia enterocolitica, Gram-negative rod, belongs to Enterobacteriaceae family, has been recognized as an important food and water-born pathogen (1 - 3). Any edible raw animal food (fish, meat, pork, and poultry) may carry *Y. enterocolitica* and cause diseases in humans. During the last two decades it was also isolated from animals, vegetables, various environments, water, and human, all over the world (4). The outer membrane protein (OMP) of the *Y. enterocolitica* is probably involved in the host - bacterial interactions; Because OMP is dependent on the presence of plasmid for expression (5). Protein level typing mostly determines the pathogenic species diversity.

Y. enterocolitica, heterogeneous species are divided into six biotypes and sixty serotypes (6). It is not discriminated sufficiently. PCR techniques have been used for its simplicity and reproducibility. RAPD – PCR (Random Amplification of Polymorphic DNA) directs a random DNA sequence by using a single primer (3). ERIC (Enterobacterial Repetitive Intergenic Consensus sequence) and

REP-PCR (Repetitive Extragenic Palindromic sequence) are 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 (7).

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 chicken and fish samples.

Materials and Methods

Sample collection

A total of 44 (20 chicken and 24 fish) samples were collected from different slaughter house and fish markets in Coimbatore city, Tamil Nadu during December 2010 - March 2011.

Culture methods

About 2.5 g of sample was inoculated into 125 ml *Yersinia* enrichment broth (Hi Media, India) and kept at 26 °C for 48 h. Then a loopful of culture was transferred and streaked on to CIN agar (Cefsulodin Irgasan Novobiocin agar, Hi Media, India), followed by incubating at 25 °C for 18 - 24 h, suspected colonies were individually isolated and subculture on Nutrient agar (Hi Media, India) for further studies.

Confirmation of Y. enterocolitica

All the isolates were subjected to biochemical methods for identification (Table 1), according to Bergey's Manual of Determinative Bacteriology (8). Furthermore, the genomic DNA was extracted from all the positive isolates (9) and was subjected to 16S rRNA PCR (10) to confirm the species level identification as *Y. enterocolitica*.

Table 1: Biochemical reaction for isolated *Y. enterocolitica*

Biochemical test[a]	No. tested	No. positive	% positive
Voges –Proskauer	44	35	80
Urease	44	22	50
Sorbitol	44	24	54
Ornithine decarboxylation	44	27	61
Citrate	44	19	43
DNase	44	19	43
Raffinose	44	21	47
Esculin hydrolysis	44	4	9
Salicin fermentation	44	5	11
Lysine Iron Agar (LIA)	44	24	56

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

Antimicrobial resistance

In order to check the multi - drug resistance, all the isolates were tested for their multiple antibiotic resistances against 21 different antibiotics (Table 2), using Mueller-Hinton agar (Hi Media, India) by adopting the standard disc diffusion method (11). 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 (Hi Media, India) and incubated at 37 °C for 16 - 18 h. Supernatant were carefully removed after centrifugation at 10,000 g for 30 min at 4 °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 °C and then for 30 min at 4 °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 (Table 3).

Table 2: Antibiotic susceptibility of *Y. enterocolitica*

Antibiotic	Disc potency (µg)	No. of samples	Resistant	% Resistance
Amoxicillin	25	24	24	100
Ampicillin	10	24	11	45.83
Amikacin	30	24	19	79.16
Chloramphenicol	10	24	0	0
Ciprofloxacin	5	24	24	100
Erythromycin	15	24	23	95.83
Gentamicin	10	24	4	16.66
Imipenam	10	24	14	58.33
Kanamycin	30	24	9	37.5
Methicillin	10	24	13	54.16
Nalidixic acid	30	24	2	8.33
Novobiocin	30	24	9	37.5
Oxytetracyclin	30	24	1	4.16
Penicillin G	10 units	24	18	75
Polymyxin-B	50 units	24	2	8.33
Piperacillin	75	24	14	58.33
Rifampicin	5	24	7	29.16
Streptomycin	10	24	4	16.66
Tetracycline	30	24	7	29.16
Tobramycin	10	24	2	8.33
Trimethoprim-sul-famethoxazole	10	24	1	4.16

Table 3: Haemolytic activity of *Y. enterocolitica*

No. of strains (chicken=6 Fish=18)	Haemolysin assay (%) (Chicken=4, Fish=6)	Temperature
24	41.6%	37 °C
24	0%	28 °C

Extraction of outer membrane protein (OMP)

The *Y. enterocolitica* strains were grown in 20 ml of Nutrient broth (Hi Media, India) overnight at 28 ºC. Preparation of outer membrane protein was done (12). The samples were subjected to 12% polyacrylamide gels containing SDS (SDS-PAGE) and the gel was stained with Coomassie Brilliant Blue R-250 (13).

Extraction of lipopolysaccaride (LPS)

Cells were grown on a nutrient broth for 48 h at 25 °C. LPS from cells was obtained by the hot phenol - water method (14). 20 µl of LPS suspension was applied in each slot of 12.5% SDS - PAGE gel. The separated LPSs were visualized by silver staining (15).

Random Amplification of Polymorphic DNA (RAPD) PCR

The primer used in this study was primer 1 (5'–CCGCAGCCAA–3') and primer 2 (5'–GAGAC-GCACA–3'). Each 25 µl reaction mix contains 30 ng genomic DNA, 1 U Taq DNA polymerase, 1 X Taq DNA polymerase buffer (Chromous Bio-tech, Bangalore), 2.5 mM $MgCl_2$, 400 µMdNTPs (Helini Biomolecules, India) and 20 pmol / µl primer. RAPD - PCR conditions were maintained (6).

Enterobacterial Repetitive Intergenic Consensus sequence (ERIC) PCR

The primers ERIC 1(5'–ATGTAAGCTCCTGGGGATTCAC–3') and ERIC 2 (5'–AAGTAAGTGACTGGGGTGAGCG–3') were used. Slight modification was made in ERIC–PCR cycle, which was described earlier (16).

Repetitive Extragenic Palindromic sequence (REP) PCR

The primers REP 1 (5'-IIINCGNCGTCNGGC-3') and REP 2 (5'-NCGNCTTATCNGGCCTAC-3') (7) were used. The reaction mixtures were denaturated at 94 °C for 3 min and then subjected to 35 cycles of denaturation at 94 °C for 2 min, annealing at 50 °C for 1 min and extension at 72 °C for 5 min and with a final extension at 72 °C for 7 min.

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 and visualized using gel documentation system (UVP GelDoc – It 300 Imaging system, Cambridge, UK). A 100 bp and 1 Kb DNA ladders were used as markers.

Results

A total of 44 (20 chicken and 24 fish) samples were collected. About 55% of them (6 chicken and 18 fish) were found positive in the incidence of *Y. enterocolitica*. The greatest number of *Y. enterocolitica* were obtained after 2 days of enriched method in *Yersinia* enrichment broth incubated at 26 °C, combined with alkali treatment for 20 sec and numerous colonies of *Y. enterocolitica* without other microflora when streaked onto *Yersinia* selective cefsulodin-irgasan-novobiocin (CIN) agar (Hi Media, India) compared to MacConkey agar (Hi Media, India). CIN agar plates have mannitol in its composition. *Yersinia* sp. ferments the mannitol in the medium, producing an acidic pH which gives the colonies red color and the "bull's eye" appearance. The cultures were identified biochemically according to Bergey's Manual of Determinative Bacteriology (8). All 24 strains showed positive to LIA [Lysine Iron arginine agar (Hi Media, India)] which specific test for *Y.enterocolitica*. About 17% and 21% of the isolates were positive for esculin hydrolysis and fermentation of salicine respectively. Isolates were further subjected to 16S rRNA PCR (10) and confirmed the species as *Y. enterocolitica*. All the strains were resistant to amoxicillin, while sensitive to chloramphenicol, in general all are exhibiting multiple antibiotic resistance (MAR).

It was also observed that 45% of the isolates exhibiting 0.2 as the multiple antibiotic resistance (MAR) index value. 95.8%, 79%, 58%, 54% resistance to erythromycin, amikacin, imipenam, methicillin respectively. 41.6% (n=10) of them were the producers of haemolysin, at 37 °C, but not at 28 °C. Outer membrane protein profiles used to find the virulent strains by producing high molecular weight protein (HMWP) between 150 to 220 kDa. Five strains (S1, S2, S6, S8 and S22) produced HMWP in the range of 160 kDa (Fig. 1) which denotes pathogenic strains.

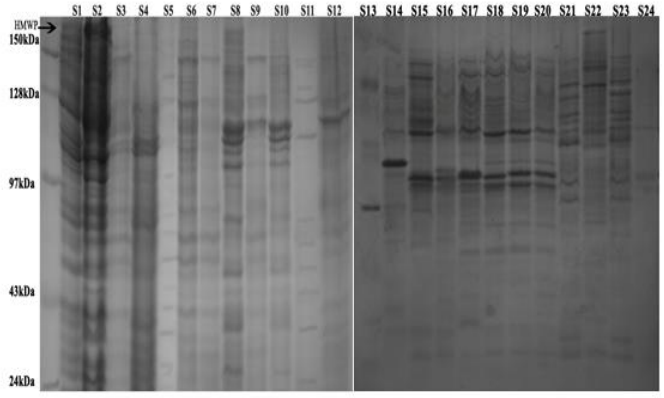

Fig. 1:SDS-PAGE analysis of Outer membrane protein of *Yersinia enterocolitica* isolated from fish and chicken. Lane 1 to 18 - Fish isolates; Lane 19 to 24 – Chicken isolates. [Arrow indicating presence of HMWP's (High Molecular Weight Proteins) between 150 to 250 kDa (S1, S2, S6, S8 and S22)]

Mostly LPS produce identical profiles, 22 strains have produced smooth LPS, while only 2 strains have produced a rough LPS pattern (Fig. 2). All the profiles and dendrogram of RAPD Fig. 3a, 3b and 4), ERIC (Fig. 5 and 6) and REP-PCR (Fig. 7

and 8) further confirm the wide genetic diversity among the strains tested. The RAPD, ERIC and REP dendrogram result (Fig. 4, 6 and 8) showed that there are 8, 11 and 12 different clusters with 0.5, 0.6 and 1 simple match similarity respectively. These shows the REP-PCR play a major role in diversity among banding patterns. This dendrogram showed no correlation between origin of isolation and fingerprint profile.

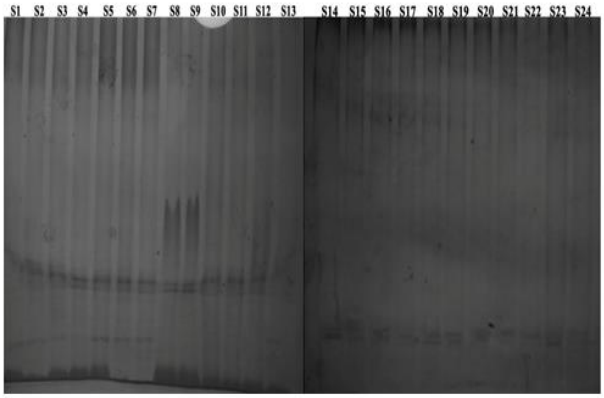

Fig. 2: SDS-PAGE analysis of lipopolysaccarides of *Yersinia enterocolitica* from fish and chicken. (Lane 1 to 18 - Fish isolates; Lane 19 to 24 – Chicken isolates)

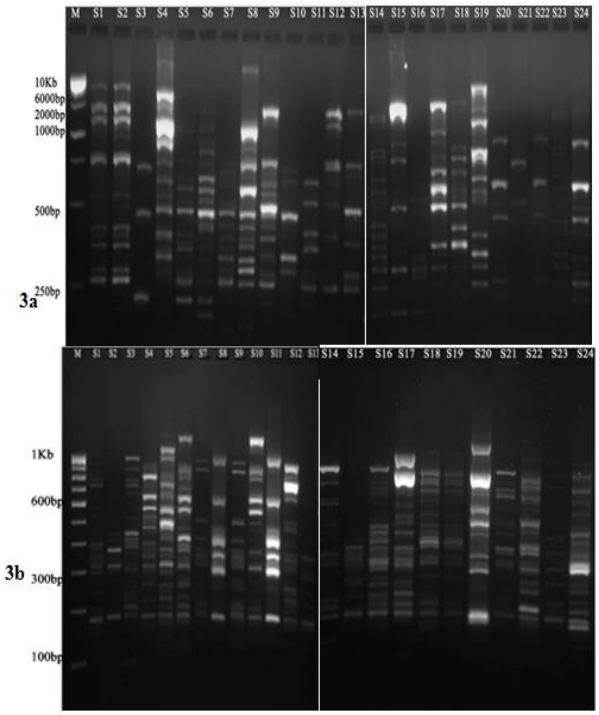

Fig. 3 (a-b): Amplification efficiency of Primer 1 and 2 for RAPD-PCR for *Y. enterocolitica* isolates from fish and chicken (Lane 1 to 18 - Fish isolates; Lane 19 to 24 – Chicken isolates)

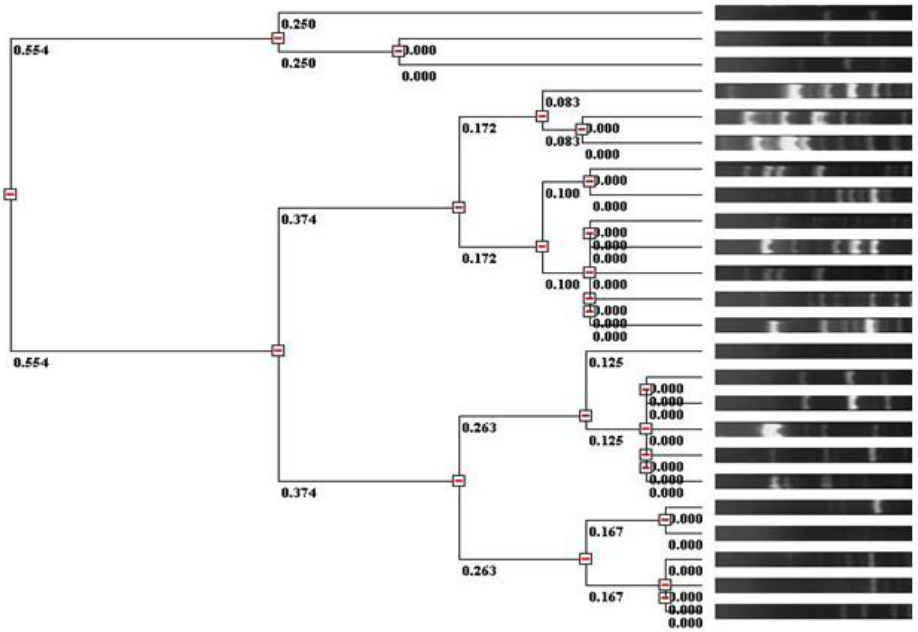

Fig. 4: Combined dendrogram for RAPD-PCR (amplified by two different primers) for *Y. enterocolitica* using simple-match similarity matrix clustered by the unweighted pair-group with arithmetic mean

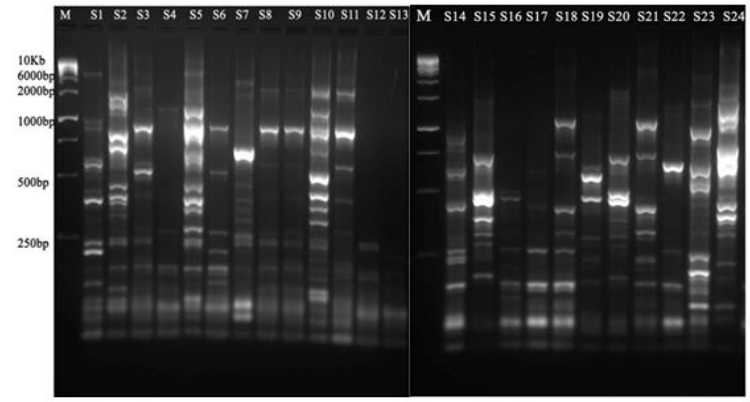

Fig. 5: ERIC-PCR fingerprints of *Y. enterocolitica* isolates from fish and chicken. (Lane 1 to 18 - Fish isolates; Lane 19 to 24 – Chicken isolates)

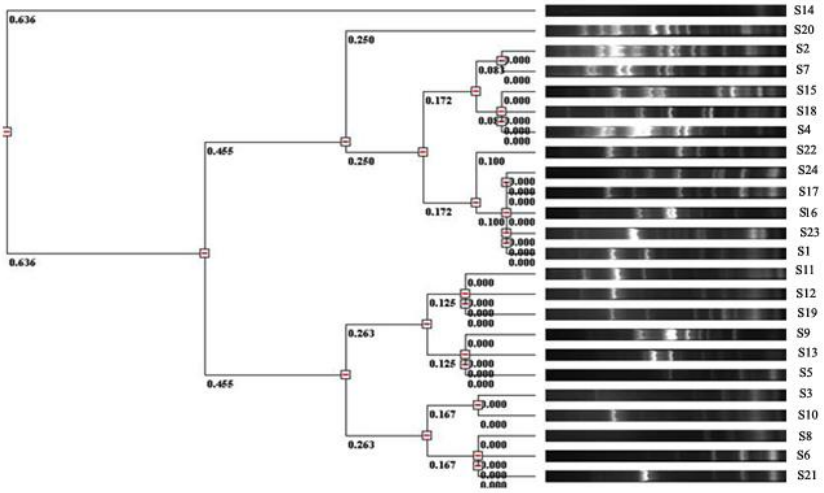

Fig. 6: Dendrogram for ERIC-PCR of *Y. enterocolitica* using simple- match similarity matrix clustered by the un-weighted pair-group with arithmetic mean

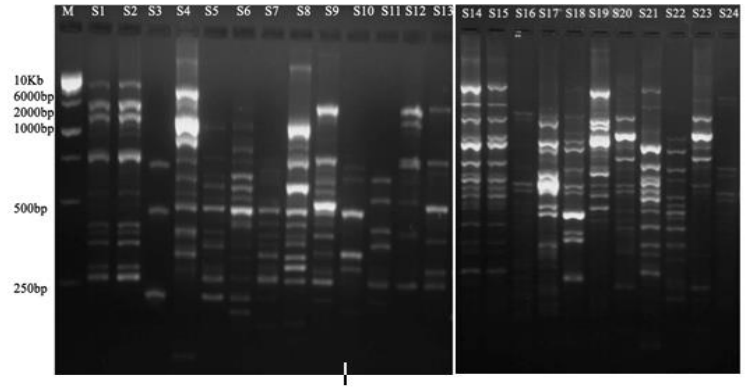

Fig. 7: REP-PCR fingerprints of *Y. enterocolitica* isolates from fish and chicken. (Lane 1 to 18 - Fish isolates; Lane 19 to 24 – Chicken isolates)

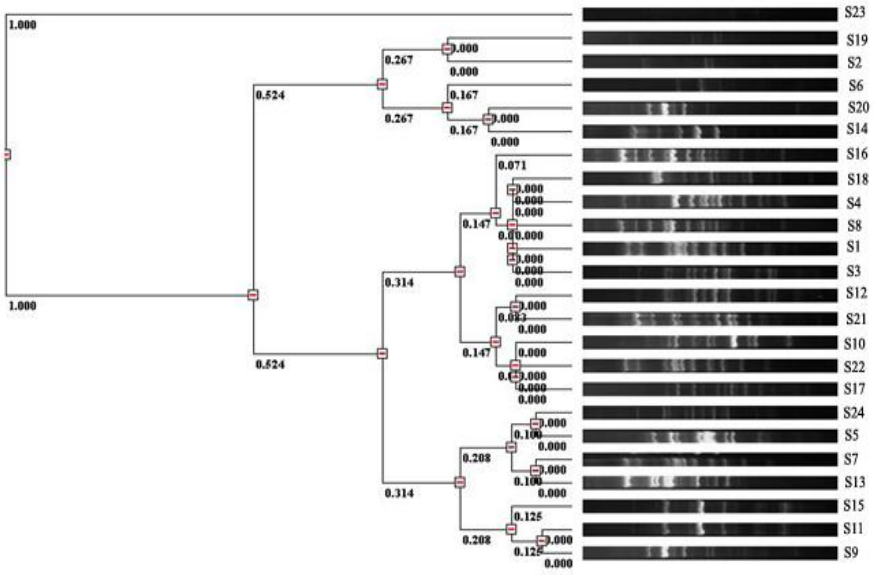

Fig. 8: Dendrogram for REP-PCR of *Y. enterocolitica* using simple- match similarity matrix clustered by the unweighted pair-group with arithmetic mean

Discussion

A wide genetic diversity among *Y. enterocolitica* isolates has been observed in the present study. *Y. enterocolitica* emerged as one of the important causes of food - borne gastroenteritis in human in developed countries for the last 20 years (17). An infection transmitted through consumption of contaminated food is a significant source of human morbidity. A previous study of 720 chicken samples collected in Western Iran, 132 (18.33%) of them were positive for *Y. enterocolitica* (18). Another report demonstrated that of 70 samples, 4.3% of the chicken carcasses have been found positive for the incidence of *Y. enterocolitica* (19). In the present investigation, 25% (n=6) of the chicken samples were contaminated with *Y. enterocolitica*, which is significantly a higher level than the previous reports. This might be due to the method involved in maintenance and preservation of chicken samples in retail outlets. The higher degree of incidence reveals the poor hygienic practices in retail outlets.

Of 20 fish samples collected, 90% (n=18) of them were contaminated with *Y. enterocolitica*. This high percentage of contamination is being reported for the first time in South - India. In a previous study, it was reported that 1% of the Molluscs *and 3%* shellfish samples were contaminated with *Y. enterocolitica* (20). In a study, from 15 fish samples collected, 5 (33.3%) isolates found contaminated with this bacterium (21). 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.

The ability of haemolysis is claimed to be one of the virulence factors among pathogenic microorganisms. In a study conducted in Italy, of 131 *Yersinia* sp., 74 were positive for haemolytic activity at the 28 °C (22). *Y. enterocolitica* could produce haemolysin at 28 °C and 37 °C as well (23). Here 24 isolates of *Y. enterocolitica*, 41.6% (n=10) of them were found to possess haemolytic activity at 37 °C, not at 28 °C.

Antimicrobial resistance among food-borne pathogens and therapeutic intervention has always been an important issue in public health. This study observed that all the *Y. enterocolitica* was sensitive to chloramphenicol and resistance towards amoxicillin and ciprofloxacin. *Y. enterocolitica* isolated from milk were found resistant to amoxicillin (24, 25) and sensitive to chloramphenicol (26).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.

Some of these outer membrane proteins are encoded by plasmid and play significant role in the pathogenicity of these bacteria (27). In another study the presence of both HMWP1 and HMWP2 are present in pathogenic strains of *Y. enterocolitica* (28).

In our study also, it was reported that five strains have produced a similar kind of protein, which confirms the presence of high pathogenic strains among our isolates (Fig. 1).

"The lipopolysaccharides have been reported as one of the essential components of outer membranes of Gram negative bacteria, which responsible for severe septic shock in human" (29). Several investigators worked on the characterization of LPSs produced by *Y. enterocolitica* (30-34). The isolates could be rough mutants that arose from an original smooth isolate during *in vivo* passage (35).

The diversity of 24 strains in RAPD-PCR was analyzed using two (primer 1 and primer 2) primers, which combined dendogram shows eight different banding pattern (Fig. 3 and 4). Similarly in a study conducted with 48 *Y. enterocolitica* isolates of clinical origin, they were able to group them into 13 different groups (36). In another study, it was analyzed with the same primer, the genetic diversity of 20 *Y. enterocolitica* isolated from human and swine sources and found five different genotypic profiles among them (6).

In the present investigation, profiles of both ERIC and REP-PCRs clearly revealed the coexistence of genetically diverse *Y. enterocolitica,* which was confirmed by highly reproducible, identical profiles (Fig. 5 and 7). ERIC-PCR tool to study the diversity among differentiated 106 isolates of *Y. enterocolitica* and reported that gave 11 different profiles (37). In contrast, the *Y. enterocolitica* isolates were having limited number of genetic diversity in ERIC and REP-PCRs (38). Comparing the discriminating efficiency between ERIC and REP-PCRs for *Y. enterocolitica,* it was reported that ERIC-PCR was better than REP-PCR (39). In our study, both the tools gave better results, particularly REP-PCR is much better than ERIC-PCR, due to the higher number of amplified products present in the profiles. This was further confirmed by the dendrogram (Fig. 4, 6 and 8) also.

To the best of our knowledge, this is the first report, involving three different PCR tools (RAPD, ERIC and REP-PCRs) along with outer membrane protein and lipopolysaccarides profiling in studying the genetic diversity among the isolates of *Y. enterocolitica*. Interestingly, all the five tools have proved their ability in differentiating the isolates of *Y. enterocolitica* in this study.

Conclusion

We strongly recommend using these typing techniques in the epidemiological investigations with special reference to *Y. enterocolitica* also.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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