Iranian J Publ Health, Vol. 36, No.3, 2007, pp.8-15

Original Article

Assessment of *Ail* Gene Marker Amplicon for Molecular Characterization of Pathogenic *Yersinia enterocolitica* in Food Samples Collected in Iran

*MR Khorramizadeh[1], MM Soltan-Dallal[1], F Safavifar[1], F Saadat[1], S Rezaie[1], S Hashemi[1], M Taremi[2], S Ardalan[2], MR Zali[2]

[1]Dept. of Pathobiology, School of Public Health, Medical Sciences/University of Tehran, Iran
[2]RCGLD, Taleghani Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

(Received 3 Nov 2006; accepted 18 Apr 2007)

Abstract

Background: To assess the utility of the chromosomal *ail* virulence gene sequence for detection of pathogenic *Yersinia enterocolitica* in raw meet food products (beef, lamb, and chicken).

Methods: This study included 39 *Yersinia enterocolitica* positive cultures from suspicious food samples, in a working period of six months. These samples were referred to the "Food-Borne Diseases and Chronic Diarrhea Lab at Research Centre for Gastric and Liver Diseases" of the Taleghani Hospital at Shahid Beheshti University of Medical Sciences, Tehran, Iran. Isolates from 8 cultured *Y. intermedia, Y. aldovi, Y. intermedia* type O:45, *Y. kristensenii, Y. enterocolitica type* O:12/26, *Y. enterocolitica* type1/7/8, *Y. frederiksenii* type O:39, and *Y. enterocolitica* type O:8 samples were included in the study. Four non-*Yersinia* species *Salmonella typhi*, *Shigella dysenteriae*, *Shigella flexeneri*, and *Proteus mirabilis* were used for specificity testing. An established *Yersinia* type O:9 was used as positive control and for sensitivity testing. An in-house real-time PCR assay was designed in order to rapidly and specifically identifies the presence of specific *Yersinia* species.

Results: Out of 39 tested *Y. enterocolitica* samples, 6(2.3%) showed positive results for the *ail* gene PCR product, typed as O:8, and O:9, respectively. PCR products were sent for sequencing. Two sequences were registered with the National Center for Biotechnology Information (NCBI Genbank) as polymorphic *ail* gene sequences under the accession numbers of DQ157767 and DQ003329.

Conclusions: Collectively, this test is well adapted for definite confirmation of pathogenic *Y. enterocolitica* in food samples.

Keywords: Yersinia enterocolitica, *Ggenetic markers, Real- time systems, PCR, Molecular sequencing* data, *Iran*

Introduction

Yersinia entrocolitica is a ubiquitous microorganism with a wide spectrum of phenotypic variants. Some of these variants represent major causative agents for enteric infections around the world (1). Although only a few of these variants are known to cause disease in humans, traditional procedures of diagnosing *Y. entrocolitica* are time consuming and cannot distinguish conclusively between pathogenic and non-pathogenic species (2).

This ubiquitous microorganism comprises a wide spectrum of phenotypic variants, of which only a few are known to cause disease in humans. The bioserotypes most frequently associated with human yersiniosis are 4/O:3, 2/O:9, 1B/O:8, and 2/O:5,27, with bioserotype 4/O:3 representing the globally dominat variant (3).

Yersinia contamination represents a particularly significant problem in food supplies, since this bacterium needs long period to grow. Unfortunately the sporadic nature of most *Yersinia* infections and the current lack of sensitive and specific methods for detecting *Yersinia* in food have hampered the identification of infection sources (4, 5).

In addition, possible cross reactivity of *Yersinia* antigens and those from *Brucella* sp., *Riketsia*, *Salmonella*, *Morganella* sp., and thyroid tissue, make serologic diagnosis extremely difficult. In contrast, molecular biology methods, such as PCR, could provide both sensitivity and specificity. One of pathogenic genes of *Y. enterocolitica* that has been successfully applied as a marker for PCR diagnostic protocols is the chromosomal *ail* gene, the product of which

*Corresponding author: Tel: +98 21 66462268, Fax: +98 21 664622687, E-mail: khoramza@sina.tums.ac.ir

is involved in *Yersinia* attachment and penetration of the host cell. *Y. entrocolitica* is a ubiquitous microorganism with a wide spectrum of phenotypic variants. Some of these variants represent major causative agents for enteric infections around the world. Although only a few of these variants are known to cause disease in humans, traditional procedures of diagnosing *Y. entrocolitica* are time consuming and cannot distinguish conclusively between pathogenic and non-pathogenic species. (6-8). In this study, we investigated the presence of the *ail* gene in raw food samples contaminated with *Y. enterocolitica* in order to validate a proposed one-step PCR protocol for the rapid and specific diagnosis of pathogenic *Y. enterocolitica*. This assay was designed to use a nearly 260 bp portion of the *ail gene* as the template for PCR amplification. The resulting PCR products were subsequently sequenced and analyzed using the Basic Local Alignment Search Tool (BLAST) to check local similarity between sequences.

Materials and Methods

Thirty-nine *Yersinia* positive cultures from all suspicious food samples that have already been referred to the "Food-Borne Diseases and Chronic Diarrhoea Lab at Research Centre for Gastric and Liver Diseases" at the Taleghani Hospital, Shahid Beheshti University of Medical Sciences in Tehran, Iran. Isolates from 8 cultures of *Y. intermedia, Y. aldovi, Y. inter-media* type O: 45, *Y. kristensenii* type 0: 28, *Y. enterocolitica* type O: 12/26, *Y. enterocolitica* type 1/7/8, *Y. frederiksenii* type O: 39, *Y. enterocolitica* type O: 8, were also included in the study. Four non-*Yersinia* species, *Salmonella typhi, Shigella dysenteriae, Shigella flexeneri*, and *Proteus mirabilis*, were also included for specificity testing. An established *Yersinia* type O: 9 was used a positive control. The included specimens were isolated out of all collected samples referred to the "Food-Borne Diseases Lab" within a 6-mo working period.

The samples were incubated at 4 °C in phosphate buffered saline (PBS) for 1 mo. At weekly intervals, a suspension aliquot was cultured linearly on *Yersinia* selective agar (CIN) (Merck 1.16434) with *Yersinia* selective supplement (CIN) (Merck 1.16466.0001). The plates were examined after 24 and 48 h of incubation at 22 ºC. The colonies were inspected carefully and suspicious pin point colonies, i.e. round pink to red colonies with a clear colorless surrounding halo, were selected for further identification.

Preparation of* Yersinia *and non*-Yersinia *species Microbes were activated in TSB media and then transferred to brain heart infusion media BHI, for 20 h at 25 °C and colonies were examined for possible contamination. Characterization of the isolated colonies was performed with API 20E (bio-Me´rieux, Marcy l'Etoile, France). Identification of presumptive *Y. enterocolitica* isolates was done by biotyping with discriminatory tests (lipase, esculin, salicin, indole, xylose, and trehalose) described previously (9) and serotyped using commercial serum agglutinantion test based on heat-stable O antigens (Bio-Rad, Marnes-la-Coquette, France). A one-colony sample for each isolate was picked and used for further PCR tests.

DNA isolation Genomic DNA was isolated with the QiaAmp Tissue Kit 250 (Qiagen Inc., Leusden, The Netherlands), according to the manufacturer's instructions. Purified genomic DNA was diluted to a concentration of 10 ng/l inTE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) and stored at -20 °C.

PCR and Real Time PCR setup A primer pair flanking a sequence within the *Yersinia enterocolitica ail* gene was designed, and synthesized (Alpha DNA, Montreal, Canada) as follows: forward primer, 5'-GAA CTC GAT GAT ACC TGG GGA-3'; reverse primer, 5'-CTG CCC CGT ATG CCA TTG-3'. The conditions for PCR were optimized in a Techne Flexigene PCR machine (Techne, Chambridge, UK) and subsequently in both a Rotor-Gene RG3000 (Corbett Research, Sidney, Australia) and a STRATAGENE Mx3000P (STRATAGENE, La Jolla, USA) for analyzing the melting curves of the products acquired using SYBR Green I (Roche Diagnostics) methodology. The following amplification program was applied: after 2 min of denaturation at 95 °C, 40 cycles of three-segment amplification were accomplished with: (i) 30 sec at 95 °C for denaturation, (ii) 1 min

at 62 °C for annealing, (iii) 30 sec at 72 °C for elongation and determination of (iv) fluorescence at an elevated temperature of 83 °C (23). The obtained fluorescence signals were continuously monitored to confirm amplification specificity during analyzing time. Subsequently, a melting curve program was applied with continuous fluorescence measurement. After PCR cycling amplification, Tm curve analysis was performed. The real time PCR products were cooled to 55°C and then heated to 95 °C at a rate of 0.5 °C per second. The Tm peaks of the products were calculated for triplicate assays on each sample and were based on the initial fluorescence curve (F/T) by plotting the negative derivative of fluorescence over temperature versus temperature (-dF/dT versus T).

Verification of theoretical real time PCR Sensitivity and Specificity The detection limit in the stool specimens of the assay was determined according to a method previously described, with slight modifications (2, 11). Briefly, a serial 10-fold dilution of the DNA samples in PCR-grade water was amplified with RG3000 and Mx3000P PCR. To quantify target DNA in the specimens, two microliters of a serial 10-fold dilution of DNA extracted from positive control was artificially inoculated in the in PCR-grade water and analyzed according to "Materials and Methods". The obtained data were used for the formation of a standard curve. Although real time PCR methodologies gave excellent specificity and sensitivity results, the sensitivity and specificity of the assays were also verified using conventional agarose gel electrophoresis.

Results

Detection of Pathogenic Y. enterocolitica

The presence of pathogenic *Y. enterocolitica* in raw food samples was analyzed by a one-step realtime PCR method to detect the *ail* gene amplicon. The thermal cycling and flourometric detection process was carried out in two real-time PCR machines. Fig. 1 and Fig. 2, Panel A show characteristic setup of the two machines fluorescent profiles using positive and negative controls (The primer pair was used to amplify a 260-bp fragment of the *ail* gene sequence found in pathogenic *Y. enterocolitica* strains (Fig. 3). The entire test could be completed in 3 h, including the DNA extraction step. Out of 39 *Yersinia enterocolitica* samples, 6(2.3%) showed positive results for *ail* gene PCR product, typed as O: 8, and O: 9.

Verification of theoretical real time PCR Sensitivity and Specificity PCR products could be detected when a DNA amount of 1 pg was used in the PCR reaction (Fig. 4). The average *Yersinia* genome size is 4,500 kb; therefore, 1 pg of DNA would be equivalent to approximately 200 genomes and, hence, 200 bacterial cells.

The specificity of the PCR assays was also verified by isolating genomic DNA from five non-pathogenic *Y. enterocolitica* serogroups, five other *Yersinia* spp., four different non- pathogenic *Yersinia* species, and *Y. enterocolitica* serogroup O:9 (as a positive control). Among the tested species, only DNA from pathogenic positive control *Y. enterocolitica* strains (types O: 8 and O: 9) yeilded PCR products of 260 bp (Fig. 5). No PCR products were observed when non-yersinia DNA isolates were used. The retrieval of the PCR product derived from the *ail* gene only from pathogenic *Y. enterocolitica* strains indicates the uniqueness of this virulence gene among *Yersinia* species.

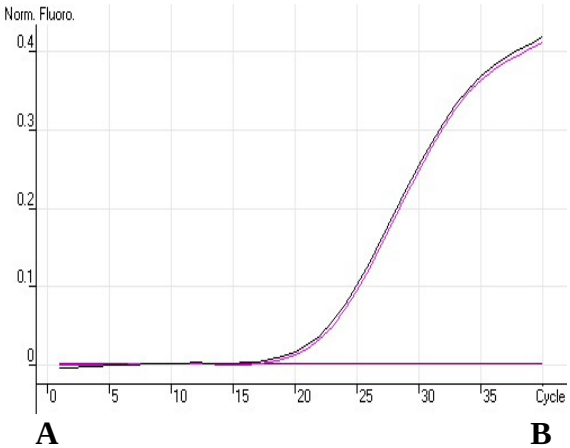
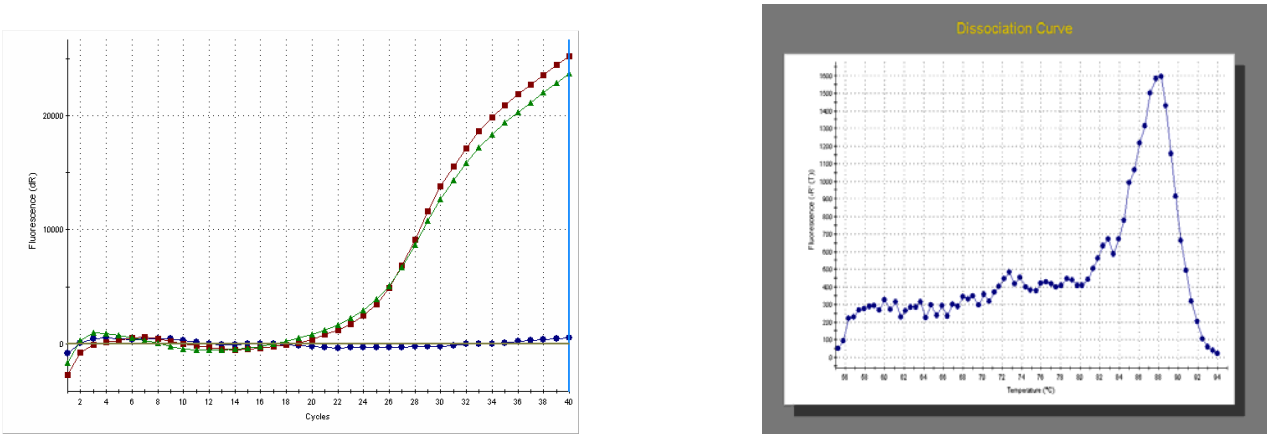


Fig. 1: Amplification setup plot of *ail* gene using RG3000

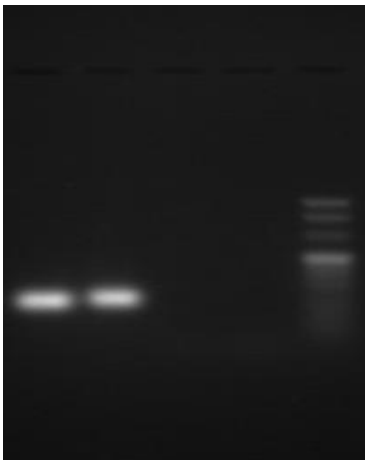
As shown above, two positive PCR products are emitting rising fluorescence. A: a non pathogenic bacterial DNA and water which were used as negative controls, emitting no fluorescence. "X" axis depicts cycle number and "Y" axis represents fluorescence detected by the real time machine and reported in "arbitrary units".



A. **B.**

Fig. 2: Amplification setup plot of *ail* gene using Mx3000P

As shown above, two positive PCR products are emitting rising fluorescence. A. non pathogenic bacterial DNA and water which were used as negative controls, emitting no fluorescence. "X" axis depicts cycle number and "Y" axis represents fluorescence detected by the real time machine and reported in "arbitrary units". B. representative depiction of dissociation (melt) curve analysis of one positive PCR product, showing a dissociation peak temperature of 88 ºC.

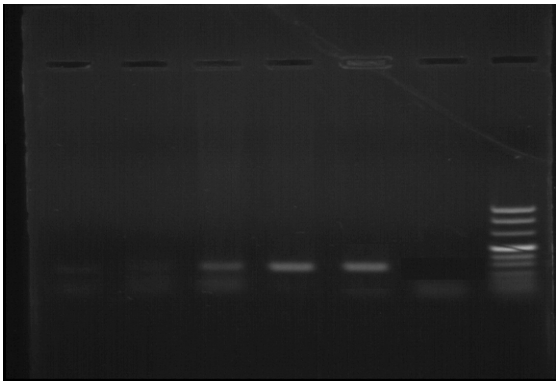


260 bp

1 2 3 4 5

Fig. 3: PCR Amplification of *Yersinia enterocolitica* "*ail*" Gene

Example of agarose gel electrophoresis of PCR products which have already been amplified by RG3000 and Mx3000P realtime machines. Each lane was loaded with 20 ul of a 25 ul PCR mixture.
Lanes: 1 & 2- *Y. enterocolitica* PCR products as "positive controls" obtained from "RG 3000" and "Mx3000P" real time machines, respectively. 3-Non yersinial PCR product used as "negative control".
4- Water used as "non-template negative control".
5- DNA Size Marker (Roche No. VIII) molecular sizes in base pairs (Roche Diagnostics, Almere, The Netherlands).



260 bp

10fg 100fg 1pg 10pg 100pg NTC SM

Fig. 4: Verification of real time PCR sensitivity

Chromosomal DNA from *Y. enterocolitica* serotype O: 9 was isolated and PCR was performed according to "Materials and Methods" using 100 pg to 10 fg of serially diluted chromosomal DNA as a template. SM: DNA Size Marker (Roche No. VIII)

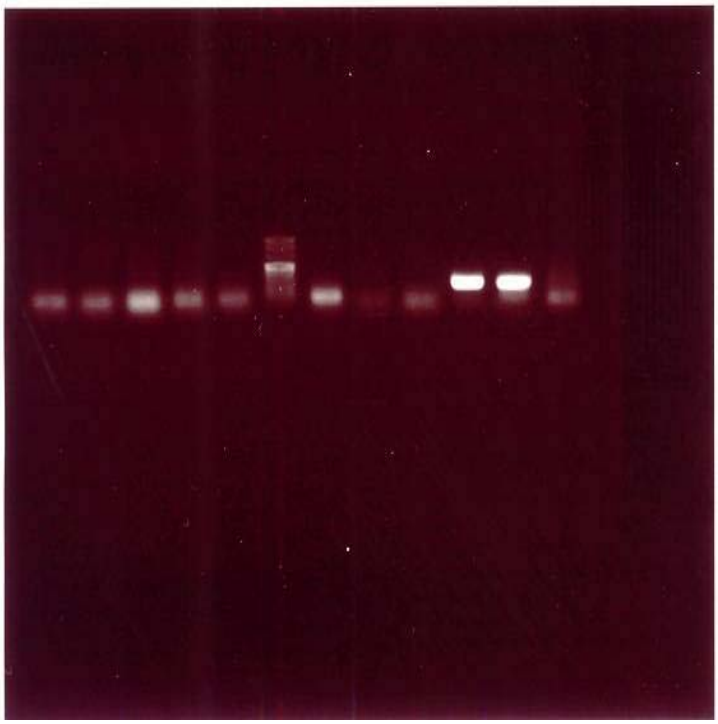


Fig. 5: Verification of real time PCR specificity using non-pathogenic and pathogenic yersinial DNA isolates

From left to right (np stands for non-pathogenic):
1. *Y. intermedia* (np)
2. *Y. aldovi* (np)
3. *Y. intermedia* O:45(np)
4. *Y. kristensenii* O:28 (np)
5. *Y. enterocolitica* type O:12/26 (np)
6. Roche DNA size marker VIII
7. *Y. enterocolitica* type 1/7/8 (np)
8. *Y. frederiksenii* type O:39(np)
9. *Y. enterocolitica* type O:12/26 (np)
10. *Y. enterocolitica* type O:8
11. *Y. enterocolitica* type O:9 (positive control)
12. Non-template negative control

Discussion

The objective of the present study was to assess the presence of *ail* gene in raw food samples contaminated with *Y. enterocolitica*. A sensitive and specific single-reaction PCR assay for the detection of pathogenic *Y. enterocolitica* strains was used as a diagnostic tool for the rapid typing of pure *Yersinial* cultures. Although the majority of *Y. enterocolitica* isolates recovered from asymptomatic carriers, food, and environmental samples are nonpathogenic, it is important to identify those isolates that are pathogenically significant (12-15). Several phenotypic tests can be used to differentiate between pathogenic and nonpathogenic isolates. However, these tests are time-consuming and not always reliable (16). In contrast, molecular diagnostic tests involving PCR, real time PCR, and DNA colony hybridization assays have been used to rapidly identify and characterize pathogenic *Y. enterocolitica* isolates with high specificity (17, 18).

One of the chromosomal genes required for *Y. enterocolitica* virulence is the attachment invasion locus (*ail*) gene (19). The *ail* gene product has been shown to be involved in attachment, invasion, and serum resistance (20). In addition, *ail* has been shown to associate only with disease-causing strains of *Y. enterocolitica* and not with avirulent biotype 1A strains

(21, 22). Several PCR assays have been developed to detect pathogenic *Y. enterocolitica* in clinical, food, and environmental samples. Some of these methods use primers targeting plasmid genes; however, these are often unreliable due to plasmid loss during subculture and storage (23). PCR methods targeting chromosomal virulence genes have also been created for natural samples. The *ail* gene, located in the chromosome of pathogenic *Y. enterocolitica* strains, is the most frequently used target (24-27).

In a recent study on the prevalence of pathogenic *Y. enterocolitica* in pig herds all isolates contained the *ail* gene (28). In contrast, another survey that analyzed virulence factors and associated markers in a strain of *Y. enterocolitica* isolated from human diarrheic feces reported negative results for virulence determinant genes *vir F* and *ail*. The clinical isolates indicated in this report, however, were *Y. enterocolitica* of the biotype 1A, which is considered avirulent. This biotype could be the etiological agent for illness through other mechanisms of virulence that differ from those characterized in species of enteropathogenic *Yersinia* (11). In another study, Thoerner (2003) used a PCR-based assay for the detection of plasmid- and chromosome-borne virulence genes in *Y. enterocolitica* and *Y. pseudotuberculosis* to investigate the distribution of these genes in isolates from various sources. Most biotypes resulted in (nearly) homogeneous genotypes for the chromosomal virulence genes (*ystA*, *ystB*, and *ail*); however, plasmid-borne genes (*yadA* and *virF*) were detected with variable efficiency, due to heterogeneity within the bacterial population for the presence of the virulence plasmid. Of the virulence genes, only *ystB* was present in biotype 1A; however, within this biotype, pathogenic and apathogenic isolates could not be distinguished based on the detection of virulence genes (29).

Our one-step *ail* gene PCR method shows excellent correspondence with fluorogenic PCR assays. The primer set was able to detect pathogenic *Y. enterocolitica* in pure culture at a limit of 1 pg of chromosomal DNA, demonstrating that conventional PCR can be as sensitive as fluorogenic PCR (11, 28). The detection limit of our PCR seems to be comparable to the other reports (2). In addition to the high sensitivity, PCR-based assays are also suggested to provide a more rapid means to accurately identify pathogenic *Y. enterocolitica* than present standard methods, such as biotyping combined with serotyping (4). Use of our proposed one-step PCR methodology would significantly reduce the amount of time required to identify pathogenic *Y. enterocolitica* strains and can be used directly after primary selective culture of this pathogen, with the biotyping and serotyping steps omitted, if necessary. The 2.3% positivity of our *ail* gene PCR results reflects the source of the analyzed food samples, which were non-pork origins. *Y. enterocolitica,* a foodborne pathogen, is mainly transmitted through ingestion of contaminated pork, milk, or water.

PCR products were sent for sequenceing to confirm the data. The designed primers in this study as well as the sequenced PCR products were subjected to a stand-alone Basic Local Alignment Search Tool (BLAST) system in which the predicted PCR product and/or primers can be compared against the genome of interest or a similar genome to find related genes or estimate primer quality (30-32). The sequenced PCR products matched mostly with types O: 8, or O: 9. Two isolates with more than 3% different sequence profile were registered as polymorphic species in the National Center for Biotechnology Information (NCBI Genbank http://www.ncbi.nlm.nih.gov/) with accession numbers of DQ157767 and DQ003329, respectively.

The results of this study allow us to tentatively conclude that the PCR assay methods described in this report offer a useful tool for the rapid, sensitive, and specific detection of pathogenic *Y. enterocolitica*. In addition, this assay can be performed in any laboratory equipped with either conventional or fluorumetric PCR facilities, even without prior biotyping and serotyping.

Acknowledgements

This study was supported by a grant from RCGLD, Taleghani Hospital, Shahid Beheshti, University of Medical sciences, Tehran, Iran.

References

1. Neubauer H, Sprague LD, Scholz H, Hensel A (2001). Diagnosis of *Yersinia enterocolitica* infections: a review on classical identification techniques and new molecular biological methods. *Berl Munch Tierarztl Wochenschr*, 114: 1-7.
2. Wannet WJ, Reessink M, Brunings HA, Maas HM (2001). Detection of pathogenic *Yersinia enterocolitica* by a rapid and sensitive duplex PCR assay. *J Clin Microbiol*, 40: 739.
3. Thisted Lambertz S. Danielsson-Tham ML (2005). Identification and Characterization of Pathogenic *Yersinia enterocolitica* Isolates by PCR and Pulsed-Field Gel Electrophoresis. *Applied and Environmental Microbiology*, 71: 3674-81.
4. Fredriksson-Ahomaa M, Korkeala H (2003). Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: a methodological problem. *Clin Microbiol Rev*, 16: 220-29.
5. Gray JT, WaKabongo M, Campos FE, Diallo AA, Tyndal C, Tucker, CA (2001). Recognition of *Yersinia enterocolitica* multiple strain infection in twin infants using PCR-based DNA fingerprinting. *J Appl Microbiol*, 90: 358–64.
6. Gierczynski R, Jagielski M, Rastawicki W (2001). The presence of the ail gene in clinical strains of *Yersinia enterocolitica* isolated from stools in Poland and characteristics of gene variant. *Acta Microbiol Pol*, 50: 19-25.
7. Kot B, Trafny EA (2004). The application of PCR to the identification of selected virulence markers of Yersinia genus. *Pol J Vet Sci*, 7: 27-31.
8. Lee TS, Lee SW, Seok WS, Yoo MY, Yoon JW, Park BK et al. (2004). Prevalence, antibiotic susceptibility, and virulence factors of *Yersinia enterocolitica* and related species from ready-to-eat vegetables available in Korea. *J Food Prot*, 67: 61123-7.
9. Wauters G, Kandolo K, Janssens M (1987). Revised biogrouping scheme of *Yersinia enterocolitica*. *Contrib Microbiol Immunol*, 9: 14–21.
10. Pfaffl MW (2001). Development and validation of externally standardized quantitative insulin like growth factor-1 (IGF-1) RT-PCR using Light Cycler SYBR® Green I technology. In *Rapid Cycle Real-time PCR: Methods and Applications*. Eds, Meuer S, Wittwer C, Nakagawara K, Springer. Heidelberg, pp. 21-34.
11. Paz M, Muzio H, Teves S, Santini P (2004). Analysis of a *Yersinia enterocolitica* isolated from human diarrheic feces in Argentina. *Rev Argent Microbiol*, 36: 164-69.
12. Bottone EJ (1999). *Yersinia enterocolitica*: overview and epidemiologic correlates. *Microbes Infect*, 1: 323-23.
13. De Boer E (1995). Isolation of *Yersinia enterocolitica* from foods. *Contrib Microbiol Immunol*, 13: 71-3.
14. Kapperud G (1991). *Yersinia enterocolitica* in food hygiene. *Int J Food Microbiol*, 12: 53-66.
15. Sacco C, Ciapini A, Santomauro F, Dei, R, Donato R (2005). Evaluation of the *Yersinia enterocolitica* pathogenicity through some phenotypic and genotypic characters: CRMOX agar positivity and presence of the ail gene. *Ann Ig*, 17(6): 501-7.
16. Kwaga J, Iversen JO (1992). Laboratory investigation of virulence among strains of *Yersinia enterocolitica* and related species isolated from pigs and pork products. *Can J Microbiol*, 38: 92-7.
17. Buchrieser C, Weagant SD, Kaspar CW (1994). Molecular characterization of *Yersinia enterocolitica* by pulsed-field gel electrophoresis and hybridization of DNA fragments to ail and pYV probes. *Appl Environ Microbiol*, 60: 4371-79.
18. Favier GI, Escudero ME, de Guzman AM. (2005). Genotypic and phenotypic characteristics of *Yersinia enterocolitica* isolated from the surface of chicken egg-

shells obtained in Argentina. *J Food Prot,* 68: 1812-15.
19. Miller VL, Farmer III JJ, Hill WE, Falkow S (1989). The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. *Infect Immun,* 57: 121-31.
20. Bottone E (1997). *Yersinia enterocolitica*: the charisma continues. *Clin Microbiol Rev,* 10: 257–76.
21. Harnett N, Lin YP, Krishnan C (1996). Detection of pathogenic *Yersinia enterocolitica* using the multiplex polymerase chain reaction. *Epidemiol Infect,* 117: 59-67.
22. Lambertz ST, Ballagi-Pordany A, Nilsson A, Norberg P (1996). Danielsson-Tham M-L. A comparison between a PCR method and a conventional culture method for detecting pathogenic *Yersinia enterocolitica* in food. *J Appl Bacteriol,* 81: 303-8.
23. Blais BW, Philippe LM (1995). Comparative analysis of *yadA* and *ail* polymerase chain reaction methods for virulent *Yersinia enterocolitica. Food Control,* 6: 211-14.
24. Bhaduri, S (2002). Comparison of multiplex PCR, PCR-ELISA and fluorogenic 5' nuclease PCR assays for detection of plasmid-bearing virulent *Yersinia enterocolitica* in swine feces. *Mol Cell Probes,* 16: 191-96.
25. Kong RY, Lee SK, Law TW, Law SH, Wu RS (2002). Rapid detection of six types of bacterial pathogens in marine waters by multiplex PCR. *Water Res,* 36: 2802-12.
26. Ramesh A, Padmapriya BP, Chrashekar A, Varadaraj MC (2002). Application of a convenient DNA extraction method and multiplex PCR for the direct detection of Staphylococcus aureus and *Yersinia enterocolitica* in milk samples. *Mol Cell Probes,* 16: 307-14.
27. Heusipp G, Schmidt MA, Miller VL (2003). Identification of rpoE and nadB as host responsive elements of Yersinia enterocolitica. *FEMS Microbiol Lett,* 226: 291-98.
28. Gurtler M, Alter T, Kasimir, S, Linnebur M, Fehlhaber K (2005). Prevalence of *Yersinia enterocolitica* in fattening pigs. *J Food Prot,* 68: 850-54.
29. Thoerner P, Bin Kingombe CI, Bögli-Stuber K, Bissig-Choisat B, Wassenaar TM, Frey J, Jemmi1 T (2003). PCR Detection of Virulence Genes in *Yersinia enterocolitica* and Yersinia pseudotuberculosis and Investigation of Virulence Gene Distribution. *Appl Env Microbiol,* 69: 1810-16.
30. Wang X, Zhou D, Qin L, Dai E, Zhang J, Han Y, Guo Z, Song Y, Du Z, Wang J, Wang J, Yang R (2006). Genomic comparison of Yersinia pestis and Yersinia pseudotuberculosis by combination of suppression subtractive hybridization and DNA microarray. *Arch Microbiol,* 186: 151-19.
31. Ng KW, Lawson J, Garner HR (2004). PathoGene: a pathogen coding sequence discovery and analysis resource. *Biotechniques,* 218: 220-22.
32. Pallen MJ, Beatson SA, Bailey CM (2005). Bioinformatics analysis of the locus for enterocyte effacement provides novel insights into type-III secretion. *BMC Microbiol,* 5: 9.