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The Detection of *Yersinia enterocolitica* in Biological Samples by RT-PCR Technique

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Abstract. The research has been performed in order to optimize Yersinia enterocolitica diagnosis from blood samples and other biological samples from different human clinical cases. Following this objective, the general technique of collecting and processing the samples was adapted and optimized, raising the rapidity of diagnosis. There have been artificially contaminated different types of samples with Yersinia enterocolitica stems, checking the efficiency and accuracy of optimized method. In finally was developed a methodology of Yersinia identification from naturally contaminated biological samples. As a target-gene there has been used the chromosome gene *ail*, this being present in the pathogenic strains of Y. enterocolitica. The sample was marked at the 5' end with a report dyeing solution and at the 3' end with an extinction one. The reading was performed at the RT-PCR, with a work protocol consisting of 2 stages, including 45 cycles. This qualitative method demonstrated some specific results with high sensitivity, positive *ail* gene detection rate in Y. enterocolitica in 267 blood samples and other type of biological products, being of 82 % using PCR and 56 % using culture methods.

Key-words: RT-PCR, blood, *Yersinia enterocolitica*, ail gene

INTRODUCTION

There have been published several research articles on this theme, and the studies in this direction developed very fast. In the last decade a great number of researchers put in the highlight the fact that the bacterial species of Yersinia genus are frequently involved in human food poisonings, manifested through acute diarrhea syndrome (3, 10, 13, 15). The available culture methods for Y. enterocolitica detection from food were time-eaters and only sometimes would have given satisfying results. The traditional methods used to detect Yersinia enterocolitica in food products depend on the toilsome and time-consuming culture methods, taking up to 4 weeks. The bulk of isolated samples from food products and from the samples collected from the environment are not pathogenic. The difficulties are associated with the isolation of Yersinia enterocolitica pathogenic strain, from a very low number of pathogenic strains in samples and the high number of microorganisms from the specific flora, especially in food products and the samples collected from the environment. Using the DNA detection methods, like PCR, this pathogenic agent can be quickly and accurately detected. Although clinically Yersinia diseases do not show any specific complications and generally are treated with efficiency, the epidemiological risk is important, consisting of outbreaks with hundreds of cases (3, 8, 11, 12). Initially, Yersinia pestis was the one considered having the most important epidemiological risk (1, 3). Later on, several research groups demonstrated that a higher number of Yersinia species were involved in food poisoning outbreaks (1, 4, 7, 8), some of them being able to determine different diseases and on other animal species also (11). Nowadays, although there have been discovered and registered a larger number of Yersinia species, the main pathogens for humans are: Yersinia pestis, Yersinia

pseudotuberculosis and Yersinia enterocolitica. Taking into account the fact that the contamination sources, for human beings, are represented generally by the food products with no thermal processing or the ones that include short thermal treatments, at a maximum of 70^{o}C, we considered as important the performing of several investigations which would reveal the optimal conservation and development temperatures of Yersinia enterocolitica strains, the survival period of this bacterial species at low temperature values (in refrigerated and frozen products) or at high temperature values (in thermal processed food products).

MATERIALS AND METHODS

The bacterial strains subjected to analysis were isolated from different blood samples or from faeces samples collected from human subjects suffering of acute diarrhea syndrome, identified through biochemical comparative tests and marked by a code. In the first stage there have been obtained young cultures (maximum 24 hours) in liquid media (alkaline saline peptonated water, glucose saline broth, Mehlman and Aulisio sorbitole-phosphate biliar salts broth, irgasan-tircarcyline-potassium chloride broth produced by De Zuter and co.) or solid ones (Salmonella-Shigella agar, Hektoen Enteric agar, several lactose media, as MacConkey or Drigalski). Yersinia strains were introduced in CIN (cefsulodine-irgasan-novobiocin) agar dishes, incubated at 30^{o}C and other strains in blood agar dishes, incubated at 37^{o}C for 18-20 hours. The DNA extraction procedure used the chelation properties of Chelex resin. A 100 µl quantity from the enrichment media left over the night was centrifuged at 13,000 x rpm for 10 minutes. The supernatant was discarded and the rest was resuspended in a 50 µl quantity of buffer, incubated afterwards at 56^{o}C for 30 minutes and at 100^{o}C for 10 minutes. After the centrifugation, the supernatant was carefully transferred in another flask and a 2 µl quantity from this supernatant was used as a pattern.

The RT-PCR test developed in order to detect the positive ail strains of Yersinia enterocolitica used primers as 5' - ACT CGA TGA TAA CTG GGG GAG-3' şi 5'-CCC CCA GTA ATC CAT AAA GG-3' for *ail* gene elaborated by Nakajima, with an amplification target on a 170 bp fragment. The hydrolysis sample 5' was a fluorogenic internally labeled sample with 6-carboxifluorescein at the 5' end and interrupted with 6-carboxi-tetramethyl-rodamine at 3' end. The fluorescence was detected using an optical detection system. The data referring to the fluorescence were obtained during the annealing/prolongation stage of each PCR cycle. The software introduces in the graph the relative fluorescence units and the number of PCR cycle in work. The initial step of each cycle, used to determine the abundance of a certain nucleic acid sample, is the number of amplification cycles necessary for the accumulated fluorescence in order to obtain a significantly higher value in comparison with the background one. A higher Ct value of 40 indicated a negative result.

In this study there has been used MasterMix 2X (diagnosis kit with premixed solutions) and the optimal primers, the sample concentrations beinb of 200 nM and 80 nM respectively. The reading was performed on a LightCycler thermo-cycler 2.0 Roche. A 2µl quantity was added over 23 µl of primary mix. The normalizing optimal temperature of 54oC was determined using the degree characteristic included in the thermo-cycler software. The specificity studies were realized using 32 bacterial strains. The Yersinia spp. pure cultures were maintained in BHI broth at 30oC and for other bacterial species at 37oC, for 16-18 hours. The bacterial DNA was extracted from a 1µl quantity of an enrichment media left over night, which was transferred in a 100 µl sterile distilled water and successively boiled for 10 minutes in order to realize the bacteria lysis. A 2 µl quantity from this bacterial suspension was subjected to thermal treatment through boiling and used as a pattern for PCR technique.

The sensitivity studies that used pure cultures of Y. enterocolitica, serotype 4/O:3 were performed in order to identify the detection limit for 5'-nuclease test. The culture was inoculated in 10 ml BHI medium, incubated at 30oC for 18 hours, afterwards being obtained the decimal serial dilutions in 3 copies and numbered as duplicate using the CIN agar dishes and incubated at 30oC for 18-20 hours. The DNA was isolated from 100 µl from each dilution. For the inoculation studies, 4 quantities of whole milk 10 m each, were tested in order to verify the absence of Y. enterocolitica strains, mixed with 90 ml TSB broth in sterile mix bags, then inoculated in 5 different levels. For each experiment, a non-inoculated sample was used as control, being obtained positive results in this case (the absence of Y. enterocolitica). The mixing bags were incubated over night (16-18 hours) at 25^{o}C. The enrichment media samples left over night were studied through PCR technique and through culture methods. The DNA was extracted from a 100 µl from the enrichment medium left over night. The culture method included the direct introduction of CIN selective agar in the dishes before the enrichment stage and 2 enrichment stage, the enrichment stage on TSB broth over night and the selective enrichment media with irgasan-tircarcyline-potassium chloride, before introducing the culture in CIN agar dishes.

RESULTS AND DISCUSSIONS

The sample-based RT-PCR test in order to detect Y. enterocolitica *ail*-positive strains is a specific one for all the strains with pathogenic potential. Y. enterocolitica strains which belong to pathogenic serotype showed a positive reaction with Ct values of 25-26, meanwhile the non-pathogenic Y. enterocolitica strains. Yersinia spp., including Y. pseudotuberculosis and other bacterial species showed negative results. The specificity of used primers in this study was tested by Nakajima and co. (12). They obtained similar results for 14 pathogenic strains of Y. enterocolitica, 13 non-pathogenic strains of Y. enterocolitica, 31 for Y. pseudotuberculosis, 11 for Y. frederksenii, 17 for Y. intermedia and other 12 bacterial strains were tested through conventional PCR technique. The lowest detection limit of this PCR test performed in order to identify ail gene of Y. enterocolitica in pure cultures was 103-104 CFU/ml, because the DNA was extracted from a 100 µl pure culture quantity and a 2 µl quantity used as a pattern. Nevertheless, the detection limit of PCR reaction was 1-10 CFU for a PCR test. These data are correlated with the latest research on the same topic.

The former enrichment stage is normally necessary to raise the sensitivity when studying the naturally contaminated samples. Moreover, the enrichment stage recedes the risk of obtaining some false positive results when using PCR technique, due to the presence of dead cells. The detection rates of *ail*-positive Y. enterocolitica strains, especially on naturally contaminated milk were very low when performing the two working techniques. A reason for the high number of negative results can be the low number of pathogenic strains existent in these samples. The 2 µl pattern used in this study revealed low values. An alternative considering the raising of the sensitivity could be using a pattern of 5 µl, this being afterwards added to a quantity of 45 µl of primary mix. Another reason for obtaining negative results could be the high number of microorganisms which form the environmental microbial flora. The non-selective enrichment stage used in this research permits the development of all bacteria, this leading to a recession of the sensitivity of this test, if the environmental microbial flora is present in a high concentration. It is demonstrated that a high external DNA concentration could represent the cause of a negative effect on the amplification efficiency. This could be due to different interactions between the bacterial DNA and PCR reagents. In order to overcome this problem, it can be used a stage of selective enrichment, which doesn't

allow the inhibition of the necessary factors for PCR technique. The enrichment stage demonstrated that it exists the possibility of inhibiting agents removal from the sample and the prevention of false positive results due to dead cells' DNA.

CONCLUSIONS

The bio-statistically interpretation allows the formulation of the next conclusions:

- The prevalence of Yersinia enterocolitica species in naturally contaminated blood samples and other kind of biological samples was evidently higher than when using RT-PCR technique in comparison with the culture methods (classical techniques).
- There has been imagined a work protocol for detection of Yersinia enterocolitica species presence in whole biological (especially blood) samples, a protocol which could be extended for the detection of this species and from other types of biological products and from different types of food products.
- The molecular detection method developed during our research proved its efficiency in detecting also the blood samples in which the bacterial cells were found destroyed, their presence being impossible to detect through classical bacteriological techniques. This makes possible the optimal diagnosis of clinical cases, in order to rapidly institute an optimal therapeutically conduct.

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