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RAPID DETECTION OF *listeria monocytogenes* IN FOOD BY POLYMERASE CHAIN REACTION

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Abstract – The standard conventional methods for the detection of *Listeria monocytogenes* in foods require high time 7 to 10 days to give ready results. To dissolve this problem we have evaluate a short method using Polymerase Chain Reaction (PCR) to analyze food samples. In parallel with this study, a comparison was made between PCR amplification from templates directly prepared from food and the official standard ISO procedure 11290-1. In this study we have used a Half Frazer broth as an enrichment medium; there were positive results of PCR detection of *L. monocytogenes* in different food sample analyzed (milk, cheese and meat) with approximately 1.5 10^1 Colony Forming Units /25g in less than 36 h. This PCR procedure has proved to be rapid and sensitive method suitable for the routine analysis; firstly, because this assay required just a short pre-enrichment step before PCR. Secondly, this procedure is very simple and time-saving; it could take less than one working day to obtain results if initial microbiological load was very important.

Key words: Cheese, *Listeria monocytogenes*, meats, milk, Polymerase Chain Reaction.

INTRODUCTION

Bacteria of the genus *Listeria* are ubiquitous in the environment and *L. monocytogenes* has been recognized as the most important food born pathogens (25, 34). Physiological characteristics make this species difficult to control in food and capable of causing severe infections like septicaemia, encephalitis, and meningitides especially in immunocompromised individuals but also new born infant and the elderly (7). The ability of *L. monocytogenes* to survive and grow over a wide range of temperatures (1, 31), in low pH (29), and in the presence of high salt concentrations which has been largely built on the virulent strains of this species (19), makes it a potential hazard in milk, dairy products and meats. The incidence of *L. monocytogenes* in raw milk and cheese is well documented (21, 33); because they often implicated as the source in several outbreaks (3, 4, 5, 22). The reported ability of *L. monocytogenes* to grow and proliferate on many of processed meat products at low temperatures (11), suggests that consumption of these products could play a role in the spread of human listeriosis. Studies have also found a presence of *Listeria* in meat and meat products (6, 32). Identification of *L. monocytogenes* with standard conventional methods are laborious and time consuming and require up to 7 days according to ISO 11290-1 (14) to produce results. However, much effort has been expended to facilitate the

Abbreviations: **bp,** base pairs; **CFU,** Colony Forming Unit; **ISO,** International Organization for Standardization; **PCR,** Polymerase Chain Reaction; **UV,** ultraviolet

rapid detection of this microorganism in foods. The first studies to determine the sensitivity of PCR (Polymerase Chain Reaction) detection carried out on artificially contaminated food have been developed by Furrer et al. and Wang et al. (10, 36). Recently molecular methods have been extensively applied for the rapid detection of *L. monocytogenes*; using primer sets derived from the listeriolysin O gene (35), the Dth 18 gene (37), and the iap gene (24). Other faster techniques have been employed which might allow the specific detections of *L. monocytogenes* in foods, as dealing with a pre-enrichment of food samples prior to PCR detection of *L. monocytogenes* (26, 28, 35), or separating bacteria using a monoclonal antibody (9, 27). But in some cases certain suffer from the presence of inhibitors factors found in food samples which can alter PCR results (30, 37). In our laboratory, a directly detection of *L. monocytogenes* from food has been developed as a rapid procedure to shorten the time of detection of this pathogenic bacteria. To realize this work, three selected food items representing typical samples (milk, cheese and meat) were used.

MATERIALS AND METHODS

Reference strain

Listeria CIP 7839 strain was used for artificial contamination of ground meat, milk and cheese. The strain was incubated at 37°C for 24 h in Luria Bertani (LB) broth for preparing initial suspension.

Artificial contamination of food samples

Decimal dilutions of 24 h culture of *L. monocytogenes* were made in LB; from $1.5.10^1$ to $1.5\ 10^7$ CFU (Colony Forming Units). The exact inoculum level was controlled by plating the innocula diluted in trypticase soya agar plate with 0,6% yeast extract (TSAYE; Biokar Diagnostics) and the colonies were enumerated after 24 h incubation at 37°C. Then, twenty five g of ground meat and cheese, and 25 ml of milk (previously tested free *L. monocytogenes* and analysed for pH, and quantity of fate and calcium) were separately homogenized using a stomacher for 1 min in 225 ml of half Frazer enrichment broth (Biokar Diagnostics BK115HA). Samples were artificially contaminated with *L. monocytogenes* by directly adding 1 ml of serial dilutions and incubated at 30°C during time (2 h to 24 h and 48 h).

DNA extraction and cells lysate

PCR templates were prepared from 0 h, 2 h to 24 h pre-enrichment broths. Aliquots (1ml) taken from different enrichment broth during time were centrifuged (14.000 g for 2 min), the supernatant decanted, and the pellet resuspended in 250 µl of distilled water. Boiled in a bather bath 10 min at 100°C and centrifuged 14.000g for 1 min. 1µl of supernatant was used as template in the PCR reaction (16).

PCR analysis

A reaction mixture of 50 µl contained 5 µl of PCR buffer 10 x:Tris-HCl 100 mmol/l , KCl 500 mmol/l, Triton x-100 1 % (Promega M 1661), 3 mmol/l $MgCl_2$ (Promega A 351B), 150 µmol /l of each dNTP, 1 µmol /l of the primer (hly1: 5'-CGGAGGTTCCGCAAAAGATG-3' and hly2: 5'-CCTCCAGAGTGATCGATGTT-3'), 0,2 µmol tween 20%, 1,5 U Taq DNA plymerase and 1 µl of cells lysate. (Positive and negative control samples were included in each PCR). Amplification started with an initial denaturation step at 94°C for 5 min, followed by 30 cycles (94 °C for 1 min, 56 °C for 45 s, and 72°C for 45 s). Final extension was performed at 72° for 10 min (15).

Detection of amplification product

A portion of 10 µl of PCR product was analysed by electrophoresis in 1.2% agarose gel. 2 µl of molecular weight marker were used (φ x 174 diggers by Hae III). The gels were stained with ethidium bromide (0.5µg/ml), visualised in UV light and photographed.

Microbiological analysis

The official standard ISO procedure 11290-1 (14) was conducted in parallel with the rapid detection procedure (PCR). A 0.1 ml portion of each pre-cultured was streaked on Palcam (Bio.Rad, 64754) and Oxford (AES Laboratory AEB151993N) agar plates with supplement. Plates were incubated at 37°C for 24 to 48 h. At the same time we transferred 0,1ml of pre-enrichment culture to enrichment Frazer broth and incubated at 37 °C for 24 h. Then, 0.1 ml portion from this secondary enrichment were re-streaked on Palcam and Oxford agar and incubated at 37°C for 24 h. Colonies thought to be *Listeria* were tested according to Bacteriological Analytical Manuel (13) by microbiological, biochemical tests and ß Hemolysis test onto sheep Blood Agar (BA) 5%. Cultures were identified with API *Listeria* test (API *Listeria*, Bio Mérieux. Marcy l'etoile, FR).

RESULTS

Detection of L. monocytogenes by cultural method

In parallel with the detection procedure of *L. monocytogenes* by PCR, a comparison was made with the official standard method according to ISO 11290 -1 (14) in order to determine whether the type of technique was rapid and sensitive to produce results, results were shown in table1. However, after 12 h and 24 h of incubation in Half Frazer broth, the results are negative of three food samples (no growth of suspect colonies neither onto Palcam or Oxford agar plates. To achieve results of 1.510^1 CFU/25g and 1.510^2 CFU/25g in (milk and cheese) and in ground meat respectively; a second enrichment in Frazer broth has been used. Five suspect colonies of *L. monocytogenes* selected on agar plates were characterized by several biochemical tests and confirmed by hemolysis test and identified with API *Listeria*.

Table 1. Results of microbiological analysis

Samples	contamination level CFU /25g	Growth *L. monocytogenes* longer time (h)		
		12h	24h	48h
Ground meat	$1.5\ 10^3$	-	-	+
	$1.5\ 10^4$	-	-	+
	$1.5\ 10^5$	-	-	+
	$1.5\ 10^6$	-	-	+
	$1.5\ 10^7$	-	-	+
Milk	$1.5\ 10^1$	-	-	-
	$1.5\ 10^2$	-	-	+
	$1,5\ 10^3$	-	-	+
	$1,5\ 10^4$	-	-	+
	$1.5\ 10^5$	-	-	+
	$1.5\ 10^6$	-	-	+
	$1.5\ 10^7$	-	-	+
Cheese	$1.5\ 10^1$	-	-	-
	$1.5\ 10^2$	-	-	+
	$1.5\ 10^3$	-	-	+
	$1.5\ 10^4$	-	-	+
	$1.5\ 10^5$	-	-	+
	$1.5\ 10^6$	-	-	+
	$1.5\ 10^7$	-	-	+

- : No growth
+ : Growth of *L. monocytogenes*

PCR detection of L. monocytogenes in artificially contaminated food samples

In the present study, we have detected *L. monocytogenes* in three food samples by PCR with pre-enrichment step after different incubation times at 2, 24 h and 48 h (table 3). Results of amplification of the listeriolysin O gene are shown in Fig 1. These samples were previously tested free *L. monocytogenes* and analysed for pH, and quantity of fate and calcium that can affect sensitivity of PCR detection. Results are shown in table 2.

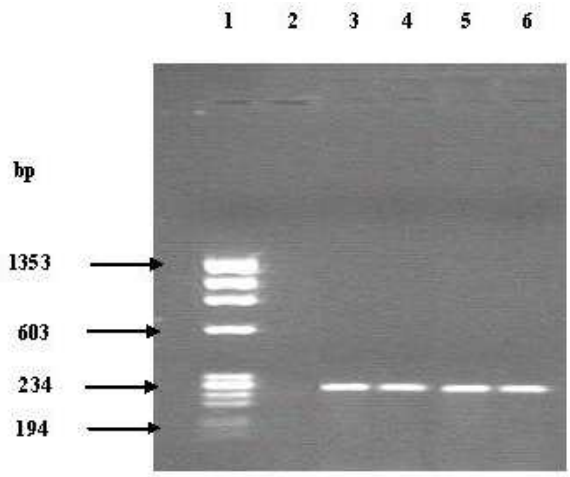

Figure 1. Detection of *Listeria monocytogenes* directly from food by PCR

Lanes1: molecular size marker φ x 174 diggers by Hae III, Lane 2: negative control, Lane 3: reference strain of *Listeria monocytogenes* (positive control), Lanes 4: ground meat sample artificially contaminated with *Listeria monocytogenes* after 6h of incubation, Lane 5: milk sample artificially contaminated with *Listeria monocytogenes* after 12h of incubation, Lane 6: cheese sample artificially contaminated with *Listeria monocytogenes* after 12h of incubation.

DISCUSSION

Longer 24 h incubation of 3 various food samples artificially contaminated (ground meat, milk and cheese) allowed PCR detection in different contamination level from 1.5 10^1 to 1.5 10^7 CFU/25g. But if we decrease incubation time, *L. monocytogenes* could be directly detected from milk and in cheese in a course of maximum 12 h with 1.510^6 CFU/25g and 1.510^7 CFU/25g respectively. In ground meat, 6 h was required to detect 1.510^7CFU/25g. As shown in table 2, the quantity of the calcium in milk and cheese is higher than that reported in meat; it may be the only reason why in this later sample the time incubation is decreasing for 6 h. Similarly, Bickley et al. (2) have reported that the inhibition of PCR was not attributed to the fat content of the milk, but calcium ions were, however, identified as a major source of PCR inhibition. Indeed, compared with previous other approaches assayed for the detection of *L. monocytogenes*, Wernars et al. (37) had added a purification step for reducing inhibition of DNA amplification, and 10^3 CFU/0.5g of cheese have been visualised. Using alcohol precipitation of DNA in the presence of Nal, Makino et al. (23) showed that *L. monocytogenes* was detected at 10^3 CFU/0.5g of soft cheese and minced meat. Regarding; Herman et al. (12), these authors have developed a direct detection method for *L. monocytogenes* in raw milk on the basis of chemical extraction of the milk components. This method involves very efficient concentration, purification, and lysis of *L. monocytogenes* cells present in raw milk, and the detection limit is situated between 10 and 5 CFU in 25 ml raw milk. The sensitivity of this technique is enhanced by application of a two-step PCR amplification procedure with two nested pairs of primers specific for *L. monocytogenes*. While, in a study done by Furrer et al. (10) the direct detection of *L. monocytogenes* was determined to 10 bacteria /10 ml milk in artificially contaminated pasteurized milk. Compared to our finding, this procedure had taken less than two working days, it based on in-vitro amplification of haemolysin gene fragment system. Almost same time (55 h) was required in a study done by Fluit et al. (9) to detect 1 CFU of *L. monocytogenes* /g of cheese after a second enrichment in Frazer Broth, by using a magnetic immuno-PCR assay which uses beads coated with specific monoclonal antibodies to concentrate *Listeria* cells out the enrichment culture prior to lysis. Recently, Kim and Cho (17) have developed a PCR-enzyme-linked immunosorbent assay (PCR-ELISA) for the rapid detection of *L. monocytogenes* which could be achieved within only 5 h. The detection limit of PCR-ELISA for *L. monocytogenes* was determined to be as low as 10 cells per PCR reaction. As we noted, the results of these studies mentioned above are not all comparable, since different procedures were used to eliminate factors that inhibit the PCR. Majority of these methodologies were fast, high specific and sensitive. Unfortunately, some approaches among them required expensive equipment and could not been available in all laboratories. Thereby, our procedure is very simple and had several advantages based on its possibility to

Table 2. pH, fat and calcium in analyzed food samples

Food items	pH	Fat (g) /100g and/or ml of sample	Calcium (mg)/100g and/or ml
Ground meat	6.0	11.5	10
Milk	6.92	3	113
cheese	5.55	33.5	600

Table 3. PCR detection of *L. monocytogenes* in artificially contaminated food samples

Contamination level CFU/g	Results of PCR detection								
	ground meat			milk			cheese		
	24h	12h	6h	24h	12h	6h	24h	12h	6h
$1.5\ 10^1$	+	-	-	+	-	-	+	-	-
$1.5\ 10^2$	+	-	-	+	-	-	+	-	-
$1.5\ 10^3$	+	-	-	+	-	-	+	-	-
$1.5\ 10^4$	+	-	-	+	-	-	+	-	-
$1.5\ 10^5$	+	-	-	+	-	-	+	-	-
$1.5\ 10^6$	+	+	-	+	+	-	+	-	-
$1.5\ 10^7$	+	+	+	+	+	-	+	+	-

obtain results in less than 36 h. With one short step of enrichment in half Frazer broth, it doesn't need pure DNA or any other treatment prior to PCR. Moreover, less than one working day was required to produce results if initial microbiological load was very important. In conclusion, because of *L. monocytogenes* is a common foodborne pathogen that has the capacity to cause severe clinical illness in vulnerable human population groups. The availability of rapid and specific laboratory tests to identify this bacterium is essential for preventing an otherwise easily treated malaise from developing into a life-threatening disease (20). However, in order to ensuring food quality and to meet the needs for the industrialists in fast analysis; the replacement of Standard method EN ISO 11290-1 (14) and the conventional procedures for isolation of nucleic acids from *L. monocytogenes* (8, 18) by our rapid procedure using PCR is very convenient and useful for research in routine of *L. monocytogenes* in food.

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