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MOLECULAR CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* ISOLATED FROM FOODS

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ABSTRACT

A total of 30 strains of *Listeria monocytogenes* isolated from different foods (16 of differents kinds of sausage, 14 cheese,) purchased at groceries of São Paulo City were ribotyped and analysed for the presence and expression of hemolysin gene and production of phosphatidylinositol-specific phosphalipase C - PI-PLC enzyme. The *L. monocytogenes* strains were differentiated into six ribotype classes. A total of 13 (43.3%) from these strains belong to the same ribotype (ribotype I), and was coincident to the ribotype of the standard *L. monocytogenes* prototype strain (ATCC-15313). The hemolytic activity was observed in 29 (96.7%) strains when incubated at 37ºC, but not at 4ºC. The direct colony hybridization method for hemolysin gene detection showed a positive reaction whit all the 30 *L. monocytogenes* strains, while showed negative reaction with other *Listeria* spp. The PI-PLC was produced by 27 (90%) of the strains analysed. There was no correlation between the six identified ribotypes and the virulence factors (hemolysin and PI-PLC) studied.

Key words: *Listeria monocytogenes,* ribotyping, virulence factors, food

INTRODUCTION

Several food-borne outbreaks have highlighted the importance of the *L. monocytogenes* to the public health (8, 9, 11, 18, 25). Establishing new methods to detect this pathogen in food (21) is very important because different food can be contaminated with this bacteria (11). Characterization using biochemical tests relies on expression of phenotypes that may not discriminate between species or strains within a same species (2). Alternative methods ideally should be based on the detection of *L.monocytogenes* virulence genes or gene products. One marker should be the production of a hemolysin, listeriolysin O (LLO), which is required for intracellular survival of invading bacteria in mammalian host (10). The gene encoding LLO had been named *hly*A. *L.monocytogenes* produces other hemolysins besides LLO like phosphatidylinositol-specific phospholipase C - PI-PLC (17) and phosphatidylcholine-specific phospholipase C - PC-PLC (5). Unlike LLO, which lyses host cells by forming pores in the membrane, the phospholipases disrupt host membranes by hydrolyzing membrane lipids (24).

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Methods for the characterization of *Listeria* spp. have been developed, and isoenzyme analysis shows some promise as a typing method (3). Nucleic acid hybridization has contributed significantly to the progress of bacterial classification and identification. One possible disadvantage of DNA probes is that each probe will detect only one taxonomic entity or clones harboring a given gene (14). Species specific probes for *L monocytogenes* have been developed, and used in colony hybridization assay (6, 7, 22, 23). The DNA fingerprint reveals DNA restriction fragment length polymorphisms, and has already been used in epidemiological investigations (2), but, with no success. Ribotyping, based on restriction fragment length polymorphism in the chromosomal DNA containing rRNA genes has also been used as important tool.

The purpose of this study was to type *L.monocytogenes* strains, isolated from foods in São Paulo city, by using a probe complementary to ribosomal RNA (plasmid pKK3535). Haemolytic activity was analysed in blood agar plates and the presence of the hemolysin gene detected by using the plasmid pIP5 as a probe. We also examined the enzymatic activity of phosphotidylinositol-specific phospholipase C, in order to examine the eventually relationship between the ribotypes and virulence associated genes

MATERIALS AND METHODS

Bacterial strains

A total of 30 strains of *L.monocytogenes*, serovars 1/2b and 4b, isolated from different kinds of food (16 different kinds of sausagesand 14 cheeses) purchased at groceries of São Paulo city were studied. The non-*monocytogenes Listeria* and *L. monocytogenes* type strains were from the American Type Culture Collection. The bacteria were maintained at room temperature in a solid medium (trypitic soy agar supplemented with 0.6% of yeast extract). Host strain *E.coli* (ED 8654-pKK3535), and *E.coli* DH5a -pIP5-[F^-, *end*A1 *hsd* R17 (r^-_k m^+_k) *sup*E44 thi-1l^- l^-*rec*A1 *gyr*A96 *rel*A1 (*arg* F-*lac* ZYA) U169 f80d *lac* DAM15] were maintained under selective pressure with ampicilin (100µg/ml).

Preparation and labeling of DNA probe

Plasmid pKK3535 (4) was isolated from a culture of *E.coli* (ED 8654 pKK3535), and the plasmid pIP5 (23) was isolated from a culture of *E.coli* DH5a (pIP5), grown overnight at 30°C in 2xYT broth contaning ampicilin (100µg/ml) as described by Ish-Horowicz and Burke (15). The plasmid pKK3535 was labeled using digoxigenin-dUTP (1) by nick-translation (Genius Kit, Boehring Manheim Biochemicals, Indianapolis, IN) following the manufacturer's instructions. The plasmid pIP5 was used as a probe after linearization with restriction endonuclease *Kpn*I (Promega) (23), and labeled as described above.

Ribotyping

Total DNA of *Listeria monocytogenes* strains was extracted from one liter 48h culture in 2xYT using the procedure of Sambrook *et al.* (25). DNA samples (0.5-2ug) were cleaved by restriction endonucleases *Hin*d III (Promega) according to the manufacturer's instructions, electrophoresed on 0.7% agarose gel, in Tris-acetate buffer (0.04M Tris acetate, 0.001M EDTA, pH 6.7), using 1 Kb DNA ladder (Gibco) as a marker, and stained in ethidium bromide (1.0mg/ml) for 20 min. Gels were then photographed. The DNA was transfered to nylon membrane by the capillary transfer method (27), and the fixed DNA (14) was prehybridized at 37°C in hybridization solution [25ml 20xSSC; 30ml formamide; 1ml NaCl a 10%; 0.2ml SDS a 10%; 5g blocking reagent (Genius Kit-Boehringer Mannheim); 43.8ml dd water]. The solution was replaced by 2.5ml of hybridization solution containing the labeled probe pKK3535. After 24 h incubation, the membrane was washed and positive reactions were colorimetrically visualized according to Genius Kit manufacturer's recommendations.

Hemolytic activity

The strains were stabbed on 5% horse blood agar plates (19), incubated at 37°C for 48h, and at 4°C for 30 days. The production of clear zones around the colonies indicated beta-hemolytic activity.

Colony hybridization

Bacterial colonies, from blood agar plates, were transferred to the nylon membrane, which were prepared for hibridization according to Peterkin *et al.* (23). The treated membrane, carrying DNA, was prehybridized at 68°C for 1 h in a bag containing hybridization solution. This solution was replaced by fresh hybridization solution containing the labeled pIP5 and after 24h incubation, the membrane was washed, and positive reactions were colorimetrically

visualized according to Genius Kit manufacturer's recommendations.

Detection of PI-PLC activity

PI-PLC activity were determinated as described by Notermans *et al.* (21). The plates were observed up to 5 days of incubation at 37°C, for turbid halos around colonies.

RESULTS

The 30 *L. monocytogenes* strains were differentiated in six ribotypes. The schematic representation of the ribotype patterns can be observed in Fig. 1. The type I (rI) presented 5 fragments: 2.3; 3.3; 3.6; 7.3 and 7.4Kb, the type II (rII) with 4 fragments: 2.3; 3.3; 6.9 and 7.4Kb, the type III (rIII) with 6 fragments: 2.3; 3.3; 4.0; 4.7; 7.3 and 7.4Kb, the type IV (rIV) with 4 fragments: 2.0; 2.3; 2.7 and 7.4Kb, the type V(rV) with 5 fragments: 2.3; 4.0; 4.7; 7.3 and 7.4Kb and the type VI(rVI) with 3 fragments: 2.3; 3.3 and 6.9Kb. All ribotypes presented a 2.3Kb common band, and another band of 7.4Kb was observed in five ribotypes (I, II, III, IV and V). Interestingly, a total of 13 (43.4%) of the 30 strains typed as a ribotype I were identical to the profile of the standard *L.monocytogenes* strain (ATCC-15313). The probe used was successful to differentiate *L. monocytogenes* strains from other *Listeria* spp, which lacked the 2.3Kb band. The hemolytic activity was observed in 29 (96.7%) strains when they were incubated at 37°C in blood agar, but at 4°C the listeriolysin was not produced (Table 1). The hemolytic activity using the direct colony hybridization method showed a positive reaction with all the *L.monocytogenes* strains. The phosphatidylinositol-specific phospholipase C activity was detected in 27 strains (Table 1).

DISCUSSION AND CONCLUSION

Epidemiological tracking of food-borne pathogens requires methods of analysis that allow discrimination between phenotypically indistinguishable strains within a species (2).

Grimont and Grimont (14) analyzed the DNA from 41 different bacterial species, and demonstrated that patterns of hybridized fragments could be used to identify biochemically indistinguishable strains. Baloga and Harlandar (2) studied 28 strains of *L.monocytogenes* isolated from food implicated in food-borne illness and from patients with listeriosis. The authors determinated the fingerprints, ribotypes, and the resulting subtypes were compared with multiloccus enzyme electrophoresis classification schemes. They observed that the serotypes were distributed among several distinct fingerprints and ET categories.

The mechanisms of pathogenicity of *L. monocytogenes* have been studied, and usually is associated with hemolysin production (12). There have been a number of reports suggesting the possibility of the temperature regulation of virulence genes expression in *L. monocytogenes*, but the results have been conflicting (13, 17). The hemolytic activity of *Listeria* spp., and the level of listeriolysin produced may be dependent on enrichment procedures, selective media, temperature and virulence of the bacteria (8, 21).

In our study, hemolytic activity was detected in 29 *L. monocytogenes* strains when incubated at 37°C, but, this activity was not observed when incubated at 4°C. Ours data agree with the Girard *et al.* (13) and Leimeister-Wachter *et al.* (17) results, which

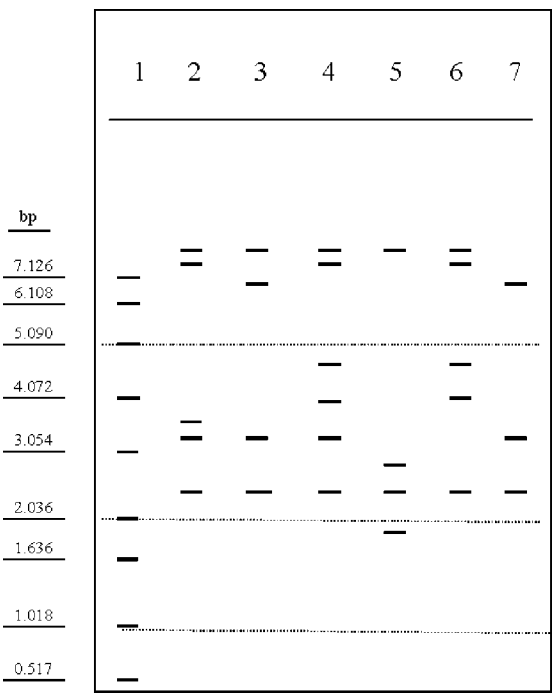

Figure 1: Schematic representation of six *L. monocytogenes* ribotype patterns.
Lane l: 1Kb DNA ladder (Gibco-BRL); Lane 2: ribotype I; Lane 3: ribotype II; Lane 4: ribotype III; Lane 5: ribotype IV; Lane 6: ribotype V; Lane 7: ribotype VI.

observed higher levels of the hemolysin production in different temperatures (between 20 to 37ºC), when compared to 4ºC.

Leimeister-Wachter *et al.* (17) found that low temperature have no significant effect on the *L. monocytogenes* virulence factors production. A likely explanation for the absence of hemolytic activity in one strain could be an alteration in the environment or in the regulatory gene (17, 20).

The most recent developments in methodologies for the detection of *L. monocytogenes* have involved gene probes. A commercial hybridization assay based on 16S rRNA sequences (Gene Trak) was developed for the detection of *Listeria* spp. in dairy products and environmental samples (16). Some researchers developed probes specifically for *L. monocytogenes* (7, 23). Those probes are used to detect the listeriolysin gene (*hly*A), and are used in colony

Table 1. Ribotype (*Hin*dIII), hemolytic activity, presence of *hly*A gene and phosphatidylinositol-specific phosfolipase C (PI-PLC) production of *Listeria* spp, and *L. monocytogenes* isolated from food in São Paulo-BR.

Strains	Ribotype	Hemolytic activity 4°C	37°C	(Gene *hly*A)	PI-PLC
L. gray		-	-	-	-
L. innocua		-	-	-	-
L. ivanovii		+	+	-	-
L. monocytogenes	I	-	+	+	+
L. murrayi		-	-	-	-
L. seeligeri		-	+	-	-
L. welshimeri		-	-	-	-
1	I	-	+	+	+
2	II	-	+	+	+
3	II	-	+	+	+
4	VI	-	+	+	+
5	VI	-	+	+	+
6	I	-	+	+	+
7	I	-	+	+	+
8	I	-	+	+	-
9	V	-	+	+	+
10	II	-	+	+	+
11	V	-	+	+	+
12	I	-	+	+	+
13	I	-	+	+	-
14	I	-	+	+	+
15	I	-	+	+	+
16	II	-	+	+	+
17	I	-	+	+	+
18	V	-	+	+	+
19	II	-	+	+	+
20	II	-	+	+	-
21	III	-	+	+	+
22	III	-	+	+	+
23	IV	-	+	+	+
24	I	-	+	+	+
25	I	-	+	+	+
26	I	-	-	+	+
27	III	-	+	+	+
28	I	-	+	+	+
29	IV	-	+	+	+
30	IV	-	+	+	+

(+) positive reaction (-) negative reaction

hybridization assays. In our study, the direct colony hybridization method gave positive reaction with all the 30 *L. monocytogenes* strains analysed, while showed a negative reaction with other *Listeria* spp, as expected.

The *pfr*A gene is a positively acting factor that transcriptionally activates the expression of the *pic, hly*A and other genes (17). In this study, the PI-PLC activity was expressed only by the majority of the strains. Probably, the negative PI-PLC activity in three tested strains should be explained by the absence of or mutation on the *pic* or *prf*A genes as suggested by Notermans *et al.* (21)

Our data permit us to conclude that there were no correlation between the six identified ribotypes and some *L. monocytogenes* virulence factors (hemolysin and PI-PLC).

RESUMO

Caracterização molecular de *Listeria monocytogenes* isolada de alimentos

Foram estudadas 30 cepas de *Listeria monocytogenes* isoladas a partir de diferentes alimentos (16 diferentes tipos de linguiça, 14 de queijo), adquiridos em supermercados da cidade de São Paulo. As cepas foram classificadas através da ribotipagem e analisadas quanto à presença e expressão do gene da hemolisina e à produção da enzima fosfolipase C fosfatidilinositol-específica PI-PLC. As cepas de *L. monocytogenes* foram diferenciadas em 6 ribotipos. As cepas do tipo I possuiam o mesmo perfil da amostra padrão de *L.monocytogenes* (ATCC 15313), sendo 13 (43,3%) das cepas estudadas correspondentes a ele. A atividade hemolítica foi observada em 29 (96,7%) das cepas, quando incubadas a 37°C em agar sangue, mas não a 4°C. O método da hibridização direta de colônias, utilizando sonda para hemolisina, revelou resultado positivo para todas as cepas. A PI-PLC foi produzida por 90% das amostras analisadas. Deste modo, foi possível concluir que não há relação entre os seis ribotipos de *L. monocytogenes* identificados nesse estudo e os fatores de virulência estudados (hemolisina e PI-PLC).

Palavras-chave: *Listeria monocytogenes*, ribotipagem, fatores de virulência, alimentos.

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