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Comparison of PI-PLC based assays and PCR along with *in vivo* pathogenicity tests for rapid detection of pathogenic *Listeria monocytogenes*

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Abstract

A study was carried out to compare phosphatidylinositol specific phospholipase C (PI-PLC) assay, chromogenic medium ALOA and a PCR for virulence associated genes (the *plcA*, *hlyA* and *prfA*) with mice and chick embryo inoculation for detection of pathogenic *Listeria monocytogenes* from milk and ready-to-eat (RTE) indigenous milk products. Eighteen strains of *L. monocytogenes* were isolated. A good correlation was observed among all the assays. ALOA exhibited distinct results (bluish green colonies with halo formation) within 24–48 h however; enzymatic activity was expressed on PI-PLC assay by 4 days of inoculation. About 93% hemolytic *L. monocytogenes* isolates were positive for genotypic and phenotypic expression of *plcA* gene and also proved lethal to mice and chick embryo. The detection of PI-PLC activity on ALOA with PCR targeting the *hlyA* and *plcA* genes would be reliable *in vitro* alternatives to *in vivo* assays for detecting pathogenic *L. monocytogenes*.

Keywords: Chromogenic media; *Listeria monocytogenes*; PI-PLC; Milk and milk products

1. Introduction

Listeria monocytogenes is an opportunistic intracellular pathogen that has become an important cause of human foodborne infections worldwide (Liu, 2006). It causes listeriosis in humans characterized by invasive and non-invasive illness, associated with the highest mortality (20–30%) and has a propensity to cause especially severe problems in pregnant women, neonates, the elderly, and immunosuppressed individuals (Liu, 2006; Vlaemynck, Lafarge, & Scotter, 2000). Thus, the presence of this pathogen in foods is a major concern to the food industry and public health regulators (Kathariou, 2002). Therefore rapid, specific and sensitive diagnostic tests, which can differentiate *L. monocytogenes* from other *Listeria* species, are essential for effective control of listeriosis (Liu, 2006).

Previously developed selective enrichment and plating methods (Curtis, Mitchell, King, & Griffin, 1989; Eld, Danielsson-Tham, Gunnarsson, & Tham, 1993) reduced the time needed for identification but it still required 2–3 days to get a presumptive positive result and another 2–4 days to confirm suspect colonies on selective agar through biochemical analysis (Paziak-Domanska et al., 1999). Moreover, the recommended selective agars such as Oxford, PALCAM (Gasanov, Hughes, & Hansbro, 2005) or DRIA (Dominguez-Rodriguez, Suarez-Fernadez, Fernadez-Garayzobal, & Rodriguez-Ferri, 1984) do not differentiate between pathogenic and non-pathogenic *L. monocytogenes*. Therefore, to assess the virulence properties of *L. monocytogenes*, the

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mouse pathogenicity test (Liu, 2004) and chick embryo test (Notermans, Dufrenne, Chakraborty, Steinmeyer, & Terplan, 1991) are currently in use.

Presently, a variety of chromogenic agars are commercially available which aim at shortening time for the detection of pathogenic *L. monocytogenes* (Greenwood, Willis, Doswell, Allen, & Pathak, 2005). Agar *Listeria* according to Ottaviani and Agosti (ALOA) is based on detecting phosphatidylinositol specific phospholipase C (PI-PLC) activity of *L. monocytogenes* (Greenwood et al., 2005). Phospholipases of *L. monocytogenes* are essential determinants of pathogenicity (Marquis, Doshi, & Portnoy, 1995; Smith et al., 1995) and activity of PI-PLC is expressed only by pathogenic *L. monocytogenes* (Notermans, Dufrenne, Leimeister-Wachter, Domann, & Chakraborty, 1991) and *L. ivanovii* (Leimeister-Wachter, Domann, & Chakraborty, 1991) and has been found to be a reliable marker for discrimination between pathogenic and non-pathogenic *Listeria* species (Notermans, Dufrenne, Leimeister-Wachter, et al., 1991). Recently, it has been reported that PI-PLC assay and the virulence genes (*plcA, prfA* and *hlyA* genes) targeted PCR could be utilized as a rapid and reliable methods for identifying pathogenic *L. monocytogenes* (Rawool, Malik, Shakuntala, Sahare, & Barbuddhe, 2007; Shakuntala, Malik, Barbuddhe, & Rawool, 2006). However, their results obtained by the PCR and PI-PLC assay have not been compared with chromogenic agar, ALOA, which also aids in detecting PI-PLC activity. Further, ALOA medium has proved to be a useful and significantly better assay than other media for the differentiation of *L. monocytogenes* from non-pathogenic *Listeria* species (Beumer & Hazeleger, 2003; Vlaemynck et al., 2000) but its efficacy has never been compared with *in vivo* pathogenicity tests.

The objective of the present study was to evaluate the methods for rapid detection of pathogenic *L. monocytogenes* strains isolated from milk and ready-to-eat (RTE) indigenous milk products employing PI-PLC assay, ALOA and a PCR for the *plcA* gene encoding PI-PLC, *hlyA* gene encoding listeriolysin O and a regulatory gene *prfA* vis-à-vis their correlation with *in vivo* pathogenicity tests.

2. Materials and methods

2.1. Bacteria

The standard strains of *L. monocytogenes* 4b (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), *Rhodococcus equi* (MTCC 1135), *Streptococcus faecalis* (MTCC 439), *Bacillus cereus* (MTCC 1272), *Escherichia coli* (MTCC 443), *Aeromonas hydrophila* (MTCC 646) used in the study were obtained from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India.

The other reference strains of *Listeria* namely, *Listeria monocytogenes* 4b (NCTC 11994), *L. monocytogenes* 1/2a (NCTC 7973), *L. monocytogenes* 1/2b (NCTC 10887), *L. ivanovii* (NCTC 11846), *L. innocua* (NCTC 11288), *L. seeligeri* (NCTC 11856), *L. grayi* (NCTC 10812), *L. welshimeri* (NCTC 11857) were kindly provided by Dr. S.V.S. Malik, Indian Veterinary Research Institute, Izatnagar, India. The strains of *Salmonella* (1117) and *Vibrio cholerae* (0139) were procured from the Division of Veterinary Public Health, Indian Veterinary Research Institute, India. The strains were tested for their purity besides morphological and biochemical characteristics (described in Section 2.4). The pathogenic potential of reference strains of *Listeria* spp. was tested by *in vitro* as well as *in vivo* pathogenicity tests.

2.2. Samples

A total of 1098 samples comprising of 471 bovine raw milk (374 from buffaloes and 97 from cattle) (collected from unorganized sectors) and 627 ready-to-eat (RTE) indigenous milk products (collected from markets) were analyzed for isolation of pathogenic *Listeria* spp. All the samples were collected aseptically and transported on ice to the laboratory, stored at 4 °C and processed within 24 h of collection.

2.3. Enrichment and isolation of Listeria spp.

Isolation of *Listeria* from the milk and milk products was attempted as per the US Department of Agriculture (USDA) method described by McClain and Lee (1988) after making suitable modifications. Approximately 10 ml of milk/10 g milk product was directly inoculated into 90 ml of University of Vermont-1 (UVM-1) and incubated overnight at 30 °C. The enriched UVM-1 inoculum (0.1 ml) was then transferred to UVM-2 medium added with ceftazidime pentahydrate (Glaxo, India) at the rate of 37.6 mg/l (referred as modified UVM-2, MUVM-2) and again incubated overnight at 30 °C.

For plating, Dominguez-Rodriguez isolation agar (DRIA) (Dominguez-Rodriguez et al., 1984) was used. The inoculum from enriched MUVM-2 was streaked on DRIA and the inoculated plates were incubated at 37 °C for 48 h. The greenish-yellow glistening, iridescent and pointed colonies of about 0.5 mm diameter surrounded by a diffuse black zone of aesculin hydrolysis were suspected to be listeriae. The presumed colonies of *Listeria* (at least 5/plate) were further confirmed. These typical colonies were further characterized.

2.4. Confirmation of the isolates

Morphologically typical colonies were verified by Gram's staining, catalase reaction, tumbling motility at 20–25 °C, methyl red-Voges Proskauer (MR-VP) reactions, CAMP test with *S. aureus* and *R. equi*, nitrate reduction, and fermentation of sugars (rhamnose, xylose, and α-methyl-D-mannopyranoside) and hemolysis on 5% sheep blood agar (SBA). The DL-alanine β-napthylamide