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Research Article

Seropositivity of Field Veterinarians for Listeric Infection by Indirect ELISAs Employing Recombinant and Wild–Type Listeriolysin O

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ABSTRACT

Listeriolysin O (LLO) being an essential virulence marker produced by all the pathogenic strains of *Listeria monocytogenes* has been reported to be an immunodominant antigen for serodiagnosis of listeric infections. The present study explores the serodiagnostic potential of recombinant listeriolysin O (rLLO) vis–a–vis wild type LLO (wLLO) employed in an indirect plate ELISA for screening sera of 221 field veterinarians from Maharashtra, India. A higher seropositivity (73.30%) for antibodies against LLO (ALLO) was observed amongst field veterinarians in wLLO–based ELISA compared to 37.56% in rLLO–based ELISA. Further, adsorption of sera with streptolysin–O (SLO) resulted in more than three–fold reduction in the seropositivity for ALLO, which was observed to be 14.93% in wLLO–based ELISA and 13.57% in rLLO–based ELISA. The rLLO–based ELISA having advantage in terms of lesser cross–reactivity and ease of production of the employed antigen, appears to be a better option for serodiagnostic purposes than wLLO–based ELISA, which is classically employed as widely accepted reliable serodiagnostic test, especially on SLO adsorbed sera. However, rLLO based–ELISA needs to be further evaluated on the sera from known clinical cases of listeriosis, especially in the high risk groups of humans, for ascertaining its efficacy as rapid and reliable serodiagnostic test for mass screening. This study seems to be the first report on comparative diagnostic potential of rLLO and wLLO in plate ELISA.

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INTRODUCTION

Listeriosis is an important bacterial infection caused by the pathogenic species of the genus *Listeria*, namely, *Listeria monocytogenes* and *L. ivanovii* (Barbuddhe et al., 2012). *L. monocytogenes* is of major concern as it accounts for about 98% of human and 85% of animal listeriosis cases (Liu, 2006). Categorized under List C of OIE, the disease in general exhibits neural, visceral and reproductive disorders particularly in various species of animals as well as humans who are immunocompromised or those that are in contact with animals (Barbuddhe and Chakraborty, 2008).

Globally, listeriosis has been reported to occur either in sporadic and epidemic form, however, there are certain Asian countries where the disease has been under reported due to lack of active surveillance systems (Tirumalai, 2013). In India, comprehensive review of reported sporadic cases of human and animal listeriosis suggested that the available epidemiological data is not sufficient to evaluate the extent of true infection in humans and animals; moreover, the disease remains mostly undiagnosed and under–reported due to unavailability of a suitable diagnostic assay (Malik et al., 2002; Barbuddhe et al., 2012). The most authentic diagnosis of listeriosis is made by isolation of the pathogen. However, it requires 2–3 days to provide presumptive positive results and additional 2–4 days for confirming suspected colonies by biochemical tests (Frece et al., 2010; Jadhav et al., 2012). On the other hand, serological tests have the advantage of large number of mass screening, and are economical, easy–to–perform and interpret. Ideally, such test must have sufficient sensitivity, specificity to detect the humoral response directed against the immunogenic component of the agent, preferably those linked to its virulence (Shoukat et al., 2013a). Many serodiagnostic assays have been developed for screening animal and human listeriosis cases either by employing the somatic (O), flagellar (H), cold–extracted or sonicated listerial antigens or outer membrane protein (OMP) of *Listeria* spp. (Chen and Chang, 1996). However, these conventional serological assays cannot be relied upon owing to their poor specificity and sensitivity (Berche et al., 1990; Chen and Chang, 1996). Moreover, these assays fail to discriminate between pathogenic and non–pathogenic *Listeria* strains. Therefore, it is important to have serodiagnostic tests based on

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virulence–linked antigens which may help in identifying true listeriosis cases. In this regard, there has been continuous search for virulence markers and/or the candidate protein antigens of *Listeria* species capable of eliciting the antibody response during listeric infection. Such virulence markers/antigens include listeriolysin–O (LLO), the *hly* gene encoded haemolysin produced by *L. monocytogenes* (Berche et al.,1990); internalins (InlA, InlB, InlC, InlC2, InlJ etc) (Das et al., 2013), the leucine rich repeat (LRR) proteins of *Listeria* spp. produced by virulence–linked family of genes (Boerlin et al., 2003); the *act*A gene encoded actin or Act A protein associated with cell–to–cell spread of the agent (Ellin Doyle, 2001), two phospholipases C namely the PI–PLC encoded by *plc*A gene and the PC–PLC encoded by *plc*B (Chaudhari et al., 2004a,b); the autolysin p60 protein (Hess et al., 1996).

Among all the virulence associated proteins, LLO which is produced by only virulent strains of *L. monocytogenes*, has been extensively used as an antigen in development of Enzyme– linked immunosorbent assay (ELISA) for serodiagnosis of listeric infection in sheep (Low et al., 1992; Lhopital et al., 1993; Barbuddhe et al., 2000; Shoukat et al., 2013b), goats (Miettinen et al., 1990; Miettinen and Husu, 1991; Bhanu Rekha et al., 2006), buffaloes (Chaudhari et al., 2001) and cattle (Thakur, 2000) as well as in humans (Berche et al., 1990; Kaur et al., 2006). However, the cross–reactivity of antibodies against LLO (ALLO) with those produced against streptolysin O (SLO), a haemolysin produced by *Streptococcus* spp. remains a major limitation of this assay, which calls for adsorption of test sera with SLO prior to its testing by this assay (Berche et al., 1990; Kaur et al., 2006; Shivaramu, 2008; Shoukat et al., 2013a). Therefore, recombinant forms of LLO (rLLO) have been explored as an alternative to wild type LLO (wLLO) as a diagnostic antigen in Western blot assays (OIE, 2008).

In this context, the present study was undertaken to compare diagnostic potential of recombinant listeriolysin O (rLLO) with wild type LLO (wLLO) in indirect ELISA employed for screening the human serum samples of field veterinarians. The developed assays were also evaluated after adsorption of the test sera with SLO.

MATERIALS AND METHODS

Sample Collection

A total of 221 blood samples were collected aseptically from field veterinarians of Maharashtra State, India during September, 2013–March, 2014. All the samples were transported to the laboratory under chilled conditions. The sera from all the blood samples were separated and stored at –20 ºC for its further use in the developed assays. Before collection of the samples, informed consent was taken from all the persons.

Purification of Wild Type LLO (wLLO)

The wLLO was extracted and purified from the cell free supernatant of 18h–old *Listeria monocytogenes* (MTCC 1143, IMTECH, Chandigarh, India) growth in brain–heart infusion (BHI) broth (Himedia, India) at 37 ºC by ion–exchange chromatography technique as described by Lhopital et al. (1993).The eluted fractions having wLLO were pooled together and the protein concentration was estimated using BCA™ Protein Assay kit (Pierce, USA, Catalog No. 23225). Purity of the wLLO was checked by SDS–PAGE confirming it to be a homogenous 58.0 kDa protein. The pooled wLLO was stored at –80ºC til further use.

Production of Recombinant LLO (rLLO)

A precise directional cloning of *hly* gene of *L. monocytogenes* was performed using Expresso T7 Cloning and Expression System (Lucigen, USA, Catalog No. 49001–1). Primers were designed as per the manufacture's kit protocol (Table 1). The expression of the 58 kDa target protein was confirmed by Western blot analysis (Figure 1) using anti–histidine antibodies (Abcam, USA, Catalog No. ab6442) and Goat anti–Rabbit IgG Fc (HRP) secondary antibodies (Abcam, USA, Catalog No. ab97200). The rLLO was purified using Ni–NTA Fast Start Kit (Qiagen, USA, Catalog No. 30600). Finally, the protein concentration of rLLO was measured using BCA™ Protein Assay kit (Pierce, USA, Catalog No. 23225) and stored at –80ºC until further use.

Table 1: Primers used for cloning *hly* gene of *L. monocytogenes*

Oligo	Sequence 5' – 3'	Product size
hly F	CATCATCACCACCATCACAA GATAATGCTAGTTTTCATTA CG	1.6kb
hly R	GTGGCGGCCGCTCTATTAT TCGATTGGATTATCTACACT	

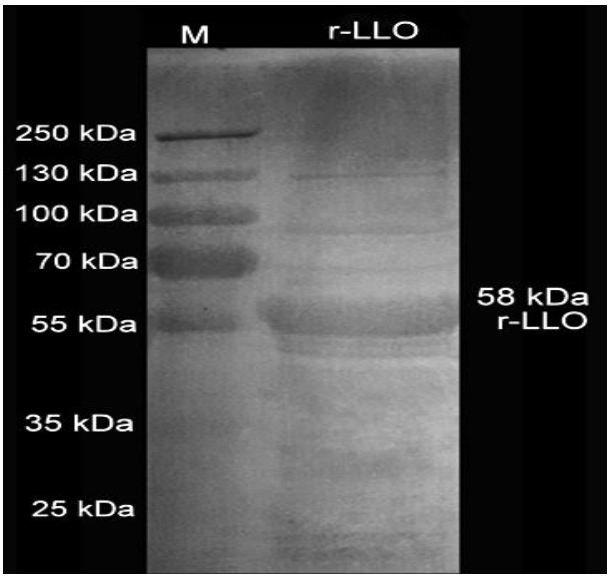

Figure 1: Confirmation of expressed r–LLO by Western Blotting. Lane1– Marker (PageRuler™ Plus prestained Protein Ladder, Thermo scientific, USA, Catalog No. 26619), Lane 2– recombinant LLO

Indirect ELISA

The indirect plate ELISA was performed as described by Low et al. (1992). The ELISA was standardized by using checker–board titration method. A serum sample at a dilution of 1:200 with a positive to negative (P/N) ratio of more than 2 was considered as positive for listeriosis in a standardized ELISA by employing either wLLO (1µg/well) and rLLO (0.125µg/well) as an antigen and rabbit anti–human HRPO conjugate (1:2000, Sigma–Aldrich, India,

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Product No. A8792). These standardized indirect ELISA tests were then employed for screening ALLO before and after adsorption of the test sera with SLO (Sigma Aldrich, USA, Product No. S5265) as per the protocol described by Berche et al. (1990). All the test sera were evaluated three times independently by both the ELISAs.

Statistics

The data obtained from the present study was analyzed using paired student't' test.

RESULTS

Detection of ALLO Antibodies by wLLO

Screening of sera from field veterinarians by indirect ELISA employing wLLO revealed seropositivity for ALLO in 73.30% (162/221) cases (Table 2). On retesting of the sera showing positivity against wLLO following their adsorption with SLO, the seropositivity was considerably reduced to the level of 14.93% (33/221) (Table 2).

Detection of ALLO by rLLO–based ELISA

Screening of sera samples by rLLO–based ELISA revealed a positivity of 37.56% (83/221) for antibodies against rLLO. On retesting of the positive sera following their adsorption with SLO, the seropositivity was reduced to 13.57% (30/221) (Table 2).

Table 2: Comparison of seropositivity for antibodies against wild type LLO and recombinant LLO

Antigen	Total sera screened	Before SLO adsorption Positive (%)	After SLO adsorption Positive (%)
w–LLO	221	162 (73.30)	33 (14.93)
r–LLO	221	83 (37.56)	30 (13.57)

Comparison of Seropositivity for Listeric Infection by wLLO and rLLO–based ELISAs

On overall basis, a high seropositivity (73.30%) was observed among the field veterinarians in wLLO–based indirect ELISA. However, subsequent to adsorption with SLO, the seropositivity reduced significantly ($p<0.05$) to 14.93% (Table 2). On comparison of these observations with that of rLLO–based ELISA, significantly lower seropositivity ($p<0.05$) was observed in case of unadsorbed sera i.e., 37.56% and SLO adsorbed sera, i.e., 13.57 % (Table 2).

DISCUSSION

L. monocytogenes is a zoonotic agent and its presence in all the critical stages of the food production and distribution chain, including the epidemiological surveillance of livestock plays a decisive role in the prevention of food–borne listeriosis in humans (Swaminathan and Gerner–Smidt, 2007). Thus, a sensitive and specific test to identify *L. monocytogenes* infected animals is of great importance in carrying out epidemiological surveys to develop appropriate control strategies. LLO is an important virulence marker of *L. monocytogenes* and a known dominant antigen target of anti–listerial immunity (Bouwer et al., 1992) which induces T–cell recognition during the course of an acute listeric infection (Berche et al., 1987). Antibodies to LLO (ALLO) have been detected soon after the clinical onset of disease in man (Morandi et al., 1981; Berche et al., 1990; Aljicevic et al., 2006) and their detectable levels have been found to persist for several months. Even the low dose of experimental infection in lambs has been reported to elicit a detectable ALLO response just like the high dose of infection (Lhopital et al., 1993). Therefore, LLO, which is produced *in vivo* during the process of intracellular multiplication of pathogenic *Listeria* spp., seems to be a good virulence–associated marker in clinical infection. Moreover, LLO has been reported to avoid the necessity of using multiple serotype antigens in immunoassays (Low and Donachie, 1997). However, LLO has been reported to share common antigenic domains with other haemolysins namely pneumolysin from *Streptococcus pneumoniae*, perfringolysin O from *Clostridium perfringens*, cereolysin O from *Bacillus cereus*, alveolysin from *Bacillus alvei* (Geoffroy et al., 1987).

In order to avoid the level of cross–reactivity in seropositivity observed against native or wild type of LLO (wLLO) we evaluated the serodiagnostic potential of rLLO as an antigen in indirect ELISA and compared with that of wLLO in terms of reduction caused, if any, in the cross–reactivity so as to have a more reliable serodiagnosis of listeric infection in field veterinarians. As expected, in wLLO–based ELISA a very high seropositivity (73.30%) for listeric infection was observed in field veterinarian which significantly reduced to 14.93% following sera adsorption with SLO. In rLLO–based ELISA, a lower seropositivity was observed (37.56%) which further reduced to 13.57% following adsorption of test sera with SLO.

The results of the present study are in agreement with earlier studies wherein a very high seropositivity for ALLO was observed in case of spontaneous abortions (48%) and in abattoir personnel (49.2%) when tested against purified LLO employed as antigen in indirect plate ELISA (Kaur et al., 2006; Barbuddhe et al, 1999). The observations are also comparable with earlier reports on seropositivity for listeric infection in case of animals ranging from 77% (Osebold and Aalund, 1968), 37% (Nass and Ortel, 1977), 53% (Husu, 1990; Lida et al., 1991), 53.33% (Morandi et al., 1981) as well as in case of human subjects 60% (Larsen and Jones, 1972) and 60% in women in reproductive age and some with spontaneous abortions (Aljicevic et al., 2006).

LLO is antigenically related to a number of cytolysins, including SLO from *Streptococcus pyogenes*, pneumolysin from *Streptococcus pneumoniae* and perfringolysin from *Clostridium perfringens*. Adsorption of the test sera with SLO has been found to cause 3–fold reduction in ALLO titers on account of eliminating the marked cross–reactivity in human clinical case of encephalitis (Berche et al., 1990) and abortions (Kaur et al., 2006) as well as animal listeriosis cases in cattle (Shivaramu, 2008) and sheep (Shoukat et al., 2013a).

Summarily, the rLLO–based ELISA turned out to be a superior serodiagnostic assay for human listeriosis than the conventional wLLO–based ELISA as the former assay showed less cross–reactivity. The exact reason for less cross–reactivity of rLLO in comparison to wLLO observed in the ELISA is not known, however, the differences in the antigenicity of both the antigens might be a probable cause. On comparison of seropositivity data, the rLLO–based ELISA employed on SLO adsorbed sera exhibited significantly superior serodiagnostic efficacy in terms of less cross–reactivity than that observed with rLLO–based

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ELISA employed on unadsorbed sera. Moreover, the rLLO employed as a diagnostic antigen in ELISA was having additional advantages, as it was easy to produce with assured consistent quality in each batch and cost effective in terms of sample analysis compared to the wLLO, which was tedious, time–consuming, cumbersome and costly to produce besides having the likelihood of batch–to–batch variations in the quality and quantity produced, owing to its vulnerability to proteolytic enzymes during its *in vitro* production and purification.

In conclusion, the rLLO–based ELISA employed on SLO adsorbed human sera can be used for reliable serodiagnosis of listeriosis in humans, especially in high risk groups. However, the assay needs to be validated on clinically confirmed listeriosis cases, with and without isolation of the pathogen from such subjects, before making final recommendation for its utilization as the diagnostic tool of choice for reliable serodiagnosis of listeriosis in human population.

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