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Comparison of microplate, agar drop and well diffusion plate methods for evaluating hemolytic activity of *Listeria monocytogenes*

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Hemolytic activity is an important characteristic for the differentiation of *Listeria monocytogenes* from apathogenic *Listeria* species within of conventional laboratory practices. We compared the efficacy of the agar well diffusion method with respect to two previously described methods such as the agar drop and microplate methods in quantifying hemolysis of *L. monocytogenes* cultures. The hemolytic activities of 13 strains of *L. monocytogenes* were tested. Two culture media (Mueller Hinton blood agar and Mueller Hinton blood agar supplemented with 0.2% activated charcoal and 1 mmol/L $CaCl_2$) were evaluated, using the agar drop and well diffusion methods as plating procedure. The agar well diffusion method was the best plating procedure for detecting the hemolysis of all strains studied after 24 and 48 h of incubation ($p < 0.01$). In addition, this plating procedure showed a greater sensitivity compared to microplate method at a read time of 6 h, giving positive reactions with all strains at an inoculum of 10^8 cfu/ml. The supplementation of charcoal on blood agar had a positive effect only when the plates were incubated after 48 h ($p < 0.01$). The results indicate that the agar well diffusion method can detect and quantify *L. monocytogenes* hemolytic activity faster and with higher sensitivity than the other two methods here studied.

Key words: *Listeria monocytogenes*, hemolytic activity, microplate, agar drop and well diffusion methods.

INTRODUCTION

Listeria monocytogenes is a Gram positive rod and a notable food-borne human pathogen causing an elevated percentage of fatalities. The conventional laboratory methods for its identification frequently involve primary isolation followed by subsequent biochemical characterization. The hemolytic activity caused by a pore-forming toxin called listeriolysin O (LLO) differentiates *L. monocytogenes* from a closely related nonpathogenic species, *L. innocua*; thus, substantial efforts have been made to establish new methods and to enhance its classical identification (Capita et al., 2001).

Recent advances in nucleic acid and immunoassay based methods offer alternative sophisticated systems for the detection of *L. monocytogenes* virulence factors, but they are unavailable for low complexity microbiological Laboratories (Churchill et al., 2006). At present, phenoltype tests employing saccharides (rhamnose, xylose), evaluation of the hemolytic activity (Gasanov et al., 2005), as well as evidence of phosphatidylinositol-specific phospholipase C activity on ALOA type chromogenic media (Leclercq, 2004) are currently used for *L. monocytogenes* identification. In the case of the LLO detection, the evidence of hemolysis on blood agar may display some difficulties, such as variable intensity of hemolysis or false-positive reactions of nonpathogenic *Listeria* species according to the type of agar base used (Capita et al., 2001). The influence of environmental conditions and a number of other factors on the hemolytic phenoltype of *L. monocytogenes* on agar plates has been described, including blood sources, plating procedure, potassium telurite, iron and selenium ions (Fernández-Garayzábal et al., 1992; Fisher and Martin, 1999; Capita et al., 2001). Thus, an agar drop plating assay on blood agar supplemented with activated charcoal was reported

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by Lemes-Marques and Yano (2004) as a new approach for hemolytic activity detection.

Furthermore, the agar well diffusion method has been previously used as a plate assay procedure for determining this activity from culture supernatants of *L. monocytogenes* (Fernández-Garayzábal et al., 1992) . Even though both methods were efficient, they required 48 h of incubation. To reduce the analysis time, a simple test using microplate had already been proposed (Domínguez Rodríguez et al., 1986), which is still frequently cited as a rapid method for hemolysis semi quantification of *Listeria* strains.

No information has been found about the suitability of the agar well diffusion method to support an enhanced response compared with the agar drop plate and microplate methods. In this work, the use of the agar well diffusion method to quantify the hemolysis produced by *L. monocytogenes* strains is proposed. The efficacy of this method with that of the microplate method is also compared in order to obtain a new, simple and rapid test to evaluate its hemolytic activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The strains used in this study consisted of 13 *L. monocytogenes* strains which included 4 strains obtained from the *Listeria* Collection of the Pasteur Institute, Paris, France (CLIP 74902, CLIP 74903, CLIP 74904, CLIP 74910) and 9 strains isolated in our laboratory from different sources (five from clinical samples: SL-A, SL-B, SL-C, SL-MF2, SL-4425; two from food SL-I, SL-K; and two from animals sources: SL-D, SL-E). Five *L. innocua* strains (CLIP 74915, CLIP 74916 plus three freshly isolated strains from food samples, SL-G, SL-H, SL-56) were used as negative control. Each isolated strain was identified by Gram staining, catalase reaction, motility at 25°C and using Api Listeria kit (bioMérieux, Marcy l'Étoile, France) (Donnelly, 1999). They were further confirmed and serotyped by Institute Pasteur, Paris, France (Seeliger and Höhne, 1979; Doumith et al., 2004). Bacteria were kept in our laboratory in Luria-Bertani broth containing 20% glycerol at -70°C. The strains were subcultured on brain heart infusion agar and incubated at 37°C for 24 h before use.

Hemolytic activity on blood agar

The hemolytic activity of the *Listeria* strains was screened on Mueller Hinton agar containing 5% horse blood (MHBA) and on MHBA supplemented with 0.2% activated charcoal and 1 mmol/L $CaCl_2$ (MHBAAC + Ca^{+2}) (Lemes-Marques and Yano, 2004). Two plating procedures were used:

Agar drop plate method

Inocula were prepared by diluting scraped cell mass in 0.9% saline solution (SS) and adjusted to a turbidity corresponding to a 0.5 of McFarland standard turbidity scale (approximately 1.5 x 10^8 cfu/ml). Five microlitres of each suspension were spotted on both culture media. All plates were incubated at 37°C and read at 24 and 48 h after inoculation. The hemolytic activity was expressed as a ratio of the clear halo diameter surrounding the colony to the diameter of the colony itself. When a *Listeria* strain showed no activity, the value considered was zero. When a distinctive translucent halo was displayed only under the colony, this ratio was equal to one.

Agar well diffusion plate method

Cell suspensions in SS to a final concentration of 10^8 to 10^{10} cfu/ml were prepared from *Listeria* subcultures. Six millimeters - diameter wells were punched into MHBA and MHBAAC + Ca^{+2}. A 20 µl aliquot of each suspension was dispensed into the wells. All plates were incubated at 37°C and read on hours 6, 24 and 48 after inoculation. The hemolytic activity was expressed as a ratio of the clear halo diameter to the diameter of the cell growth around the well perimeter. When a *Listeria* strain showed no activity, the value considered was zero. When the positive reaction was displayed under the cell growth around the well perimeter, this ratio was equal to one.

Every experiment using both plating procedures was carried out in parallel, and the results represented the average of at least three independent experiments done in duplicate.

Hemolytic activity by the microplate method

The hemolytic activity of all *Listeria* strains was determined by using a modified procedure originally developed by Domínguez Rodríguez et al. (1986). Briefly, cell suspensions from 10^8 to 10^{10} cfu/ml in SS were prepared from all strains. Serial twofold dilutions were made by mixing 100 µl of each suspension with SS in microtitre plates with U-form wells. Fifty µl of a 3% suspension of washed horse red blood cell (to which 10 ml of a 10% gelatin solution with 0.43% sodium azide had been added per liter) was added to each dilution. The microplates were incubated at 37°C for 6 h. The hemolytic activity titre was expressed as complete hemolysis units (CHU: the reciprocal of the highest dilution at which 100% hemolysis took place) . The results represented the average of at least three independent experiments done in duplicate.

Enumeration techniques

The number of cfu/ml was determined by serial dilution of samples taken from each *Listeria* cell suspension using SS for the dilutions and a non- selective medium, tryptic soy agar (TSA), to estimate the number of colonies. The plates were incubated for 48 h at 37°C. All the samples were replicated two-fold.

Statistical analysis

The results were submitted to paired Student's t - test for means comparison using the Statgraphics plus 3.1 software. Bacterial cell counts were converted to a log cfu/ml. A linear regression and a correlation coefficient between hemolytic activity for all *L. monocytogenes* strains and log of bacterial counts were calculated using the same software.

RESULTS

Table 1 shows the hemolytic activity of all *L. Monocytogenes* strains in MHBA and MHBAAC + Ca^{+2} media using two plating procedures: agar drop and agar well diffusion plate methods. The results varied according to the strain studied, the supplementation or not of activated charcoal on blood agar, the plate assay procedure and the incuba-tion time employed.

Table 1. Comparison of the agar drop and well diffusion plate methods for the hemolytic activity evaluation of *L. monocytogenes* strains on MHBA and MHBAAC + Ca^{+2}.

Plating method	Medium	Incubation time (h)	Hemolytic activity of *L. monocytogenes* strains:												
			SL-A	SL-B	SL-C	SL-D	SL-E	SL-I	SL-K	SL-MF2	SL-4425	CLIP 74902	CLIP 74903	CLIP 74904	CLIP 74910
Drop	MHBA	24	1.17 ± 0.08	1.28 ± 0.05	1.22 ± 0.10	1.28 ± 0.11	1.22 ± 0.06	1.17 ± 0.09	1.28 ± 0.08	1.17 ± 0.04	1.19 ± 0.10	1.25 ± 0.09	1.15 ± 0.01	1.14 ± 0.06	1.13 ± 0.02
		48	1.24 ± 0.08	1.26 ± 0.09	1.28 ± 0.11	1.27 ± 0.12	1.21 ± 0.05	1.23 ± 0.08	1.28 ± 0.10	1.18 ± 0.03	1.14 ± 0.00	1.52 ± 0.06	1.35 ± 0.10	1.18 ± 0.05	1.35 ± 0.07
	MHBAAC + Ca^{+2}	24	1.15 ± 0.01	1.12 ± 0.08	1.16 ± 0.03	1.12 ± 0.08	1.11 ± 0.07	1.12 ± 0.08	1.14 ± 0.05	1.04 ± 0.07	1.04 ± 0.08	1.08 ± 0.10	1.04 ± 0.08	1.00 ± 0.00	1.00 ± 0.00
		48	1.23 ± 0.11	1.19 ± 0.11	1.24 ± 0.06	1.24 ± 0.10	1.18 ± 0.06	1.23 ± 0.12	1.26 ± 0.09	1.24 ± 0.13	1.19 ± 0.10	1.43 ± 0.11	1.20 ± 0.11	1.00 ± 0.00	1.20 ± 0.07
Well-diffusion	MHBA	24	1.41 ± 0.12	1.42 ± 0.11	1.43 ± 0.08	1.52 ± 0.14	1.44 ± 0.09	1.40 ± 0.08	1.37 ± 0.07	1.31 ± 0.13	1.37 ± 0.11	1.43 ± 0.05	1.41 ± 0.13	1.43 ± 0.08	1.46 ± 0.07
		48	1.57 ± 0.09	1.62 ± 0.14	1.60 ± 0.08	1.63 ± 0.14	1.62 ± 0.16	1.52 ± 0.06	1.51 ± 0.07	1.58 ± 0.16	1.50 ± 0.11	1.67 ± 0.15	1.44 ± 0.07	1.55 ± 0.10	1.67 ± 0.14
	MHBAAC + Ca^{+2}	24	1.56 ± 0.09	1.54 ± 0.11	1.53 ± 0.07	1.53 ± 0.07	1.43 ± 0.07	1.49 ± 0.03	1.46 ± 0.09	1.43 ± 0.08	1.42 ± 0.13	1.42 ± 0.09	1.45 ± 0.09	1.43 ± 0.07	1.46 ± 0.07
		48	1.79 ± 0.09	1.93 ± 0.15	1.68 ± 0.16	1.84 ± 0.14	1.63 ± 0.13	1.57 ± 0.09	1.88 ± 0.17	1.92 ± 0.19	1.75 ± 0.17	1.64 ± 0.14	1.75 ± 0.17	1.60 ± 0.09	1.66 ± 0.16

The agar well diffusion method was the best plating procedure for detecting the hemolysis of the studied *L. monocytogenes* strains. When comparing with the agar drop plate method, it was observed that the degrees of hemolysis were greater in all assayed conditions ($p < 0.01$) (Table 1). Using the agar well diffusion method, no significant differences were obtained when the strains were inoculated on MHBA and MHBAAC + Ca^{+2} media after 24 h of incubation ($p > 0.05$). The hemolytic activity was stronger in the presence of activated charcoal only after 48 h of incubation ($p < 0.01$).

When the agar drop plate method was used, all strains showed an easily distinguishable positive reaction with values above one in both MHBA and MHBAAC + Ca^{+2} media, except for CLIP 74904 and 74910 strains on MHBAAC + Ca^{+2} medium after 24 h of incubation. The former maintained weak hemolysis even after 48 h of incubation. The supplementation of charcoal showed no great impact on the hemolytic capacity of the assayed strains after 48 h of incubation, thus showing no significant difference ($p > 0.05$). The zones of hemolysis were greater on MHBA than on MHBAAC + Ca^{+2} media after 24 h of incubation ($p < 0.01$).

As expected, none of the five tested *L. innocua* strains had any positive activity for the studied virulence factor.

The usefulness of the agar well diffusion method was then compared with the microplate method. As summarized in Table 2, there was a clear trend in the data; the microplate method was less sensitive than the agar well diffusion method for the studied strains. In all cases, except for the CLIP 74902 strain, cell concentrations higher than 10^8 cfu/ml were required to visualize the complete hemolysis. Three strains required a very high cellular concentration (10^{10} cfu/ml) to detect the hemolytic activity.

In contrast, all strains showed an easily visualized positive reaction with a cellular concentration of 10^8 cfu/ml when the agar well diffusion method was used, with the exception of two of them on MHBAAC + Ca^{+2} medium (SL-4425 and CLIP 74910 strains).There was no significant difference when activated charcoal was added to MHBA medium ($p > 0.05$). In most cases, there was not a good linear correlation between the log bacterial counts and the hemolytic activity. The evidence of complete hemolysis at all cell concentrations of *L. monocytogenes* CLIP 74902 assayed by the microplate method allowed to ascertain the interrelation of both variables. Likewise, the same correlation was made with the data from agar well diffusion method (Figure 1). In this condition, the

Table 2. Comparison of the micro plate method and the agar well diffusion plate method for the hemolytic activity evaluation of *L . monocytogenes* strains after 6 h of incubation.

Strains	cfu/ml	Hemolytic activity by:		
		Microplate method	Agar well diffusion method	
		CHU	MHBA	MHBAAC + Ca^{+2}
SL-A	2.16×10^8	0	1.20 (0.03)	1.23 (0.05)
	4.00×10^8	2	1.20 (0.05)	1.26 (0.10)
SL-B	4.30×10^8	0	1.23 (0.03)	1.23 (0.03)
	1.12×10^8	2	1.24 (0.01)	1.26 (0.10)
SL-C	2.30×10^8	0	1.22 (0.04)	1.26 (0.02)
	1.50×10^{10}	1	1.24 (0.02)	1.34 (0.03)
SL-D	3.95×10^8	0	1.20 (0.04)	1.39 (0.02)
	5.00×10^9	1	1.23 (0.03)	1.30 (0.09)
SL-E	2.40×10^8	0	1.21 (0.02)	1.37 (0.08)
	3.55×10^9	1	1.19 (0.07)	1.27 (0.06)
SL-I	5.10×10^8	0	1.39 (0.09)	1.40 (0.08)
	8.60×10^9	3	1.36 (0.02)	1.47 (0.11)
SL-K	1.40×10^8	0	1.24 (0.01)	1.18 (0.05)
	4.70×10^9	1	1.22 (0.06)	1.24 (0.04)
SL-MF2	2.10×10^8	0	1.26 (0.01)	1.25 (0.05)
	5.50×10^9	4	1.21 (0.05)	1.29 (0.01)
SL-4425	3.07×10^8	0	1.16 (0.05)	1.11 (0.09)
	1.59×10^{10}	1	1.22 (0.05)	1.29 (0.04)
CLIP 74902	3.10×10^8	1	1.17 (0.07)	1.17 (0.05)
	3.20×10^9	4	1.34 (0.05)	1.33 (0.06)
CLIP 74903	1.90×10^8	0	1.22 (0.01)	1.34 (0.01)
	2.04×10^9	1	1.21 (0.02)	1.38 (0.01)
CLIP 74904	7.90×10^8	0	1.20 (0.05)	1.23 (0.03)
	2.50×10^9	4.67	1.47 (0.03)	1.29 (0.06)
CLIP 74910	1.20×10^8	0	1.21 (0.07)	1.08 (0.03)
	1.30×10^9	2	1.20 (0.02)	1.23 (0.02)

(Standard deviations in parentheses)

CLIP 74902 strain showed a good correlation between log bacterial counts and hemolytic activity on both agar culture media, while the interrelation was poor when the microplate method was used.

DISCUSSION

According to Ermolaeva et al. (1999) the presence of activated charcoal in the culture medium induces a greater production of virulence factors of *L. Monocyto-genes* . It has been suggested that charcoal supplemen-tation may affect the adsorption of a diffusible autorepres-sor substance of the transcriptional regular PrfA, with a consequent induction of this virulence gene activator (Ermolaeva et al., 2004). The hemolytic activity of *L. monocytogenes* strains in MHBAAC + Ca^{+2} medium using the agar drop plate method has been previously evalua-ted by Lemes-Marques and Yano (2004) with satisfactory results compared to those from MHBA medium after 48 h of incubation. The data presented here suggest that this was not the case. The presence of activated charcoal did not show a clear advantage of the culture medium when this plating method was used after 24 - 48 h of incubation (Table 1).

The streak, red cell blood top layer and drop plate methods are the most extensively used for detection of listerial virulence factors with *L. monocytogenes* cultures (Ermolaeva et al., 2003; Blanco et al., 1989; Capita et al., 2001; Notermans et al., 1991). The agar well diffusion method is less frequently used and the available data report the employment of culture supernatants for successful LLO detection (Fernández Garayzábal et al., 1992), although the procedure is time-consuming and labor intensive. In this study, the use of the agar well diffusion method with pure *L. monocytogenes* cultures showed the tendency observed by Lemes-Marques and Yano (2004), enhancing the response obtained with the

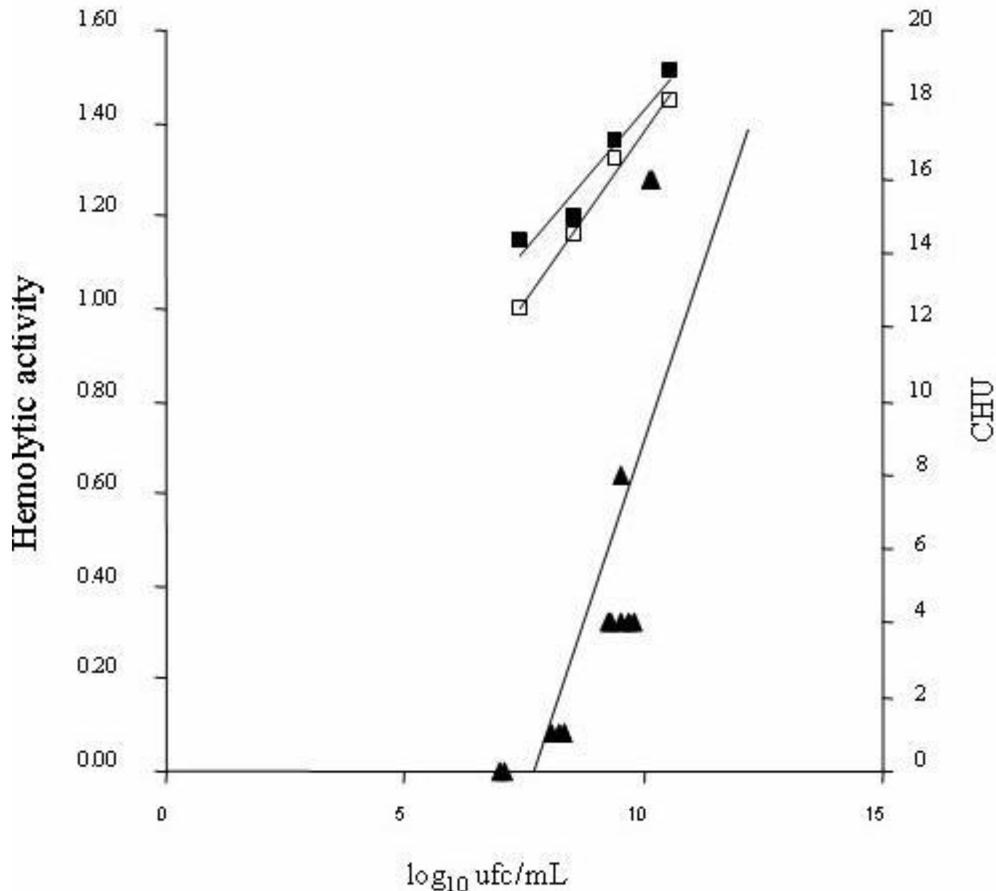

Figure 1. Relationship between hemolytic activity and log bacterial counts of *L. monocytogenes* CLIP 74902 for microplate method ( ) and agar well diffusion method on MHBA ( ) and MHBAAC+ Ca^{+2} ( ). All assays were incubated at 37 ºC for 6 h. Linear regression equations: ( ) y = 3.8448 x – 29.643, R^2 = 0.585; ( ) y = 0.1218 x + 0.2089, R^2 = 0.9504; ( ) y = 0.1464 x - 0.084, R^2 = 0.9928.

drop plate method in all assayed conditions (Table 1). The proposed method allows the use of greater inoculation volumes with a more homogeneous radial diffusion, increasing the contact area between bacteria and erythrocytes.

The increase in the test sensitivity reduced the read time, obtaining positive hemolytic reactions in both agar media after 6 hours of incubation. This incubation time made it possible to compare the agar well diffusion method with the microplate method (Table 2).

Ripio et al. (1996) reported that the size of the hemolysis halo on blood agar correlated with secreted LLO titres (CHU) from *L. monocytogenes* culture supernatant when weak and strong hemolytic strains were compared. However, a detailed analysis of those data from hyperhemolytic strains shows poor correlation between the two methods (R^2 = 0.58). In this study, the results are consisent with the latter tendency (R^2 = 0.53).

It is known that an autorepressor substance is released by *L. monocytogenes* during early exponential phase in broth cultures, approximately after 6 h of growth (Ermaloeva et al., 2004). But, the growth on solid media of this bacterium can be even slower due to O_2 availability and other factors (Wu et al., 2000). These conditions may have contributed to find no significant differences when the agar well diffusion method was used in both media after 6 - 24 h of incubation.

The agar well diffusion plate method gave good responses using *L. monocytogenes* concentrations in the order of 10^8 ufc/ml. These results increased the sensitivity achieved with the microplate method, making it a suitable test since this cell concentration can be easily obtained by a 0.5 McFarland suspension.

In addition, the good linear correlation found between the cellular concentration and the size of the hemolysis halo using the agar well diffusion method for *L. Monocytogenes* CLIP 74902 shows the strength of this approach for quantitative estimations of hemolytic activity.

In summary, the described agar well diffusion method provides an accurate, rapid, simple and sensitive tool to detect and quantify hemolytic activity of *L. monocytogenes* strains without equipment requirements. Hence,

this assay might be useful in microbiology laboratories in which hemolysis tests are routinely carried out.

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