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Research Note

Molecular Serogrouping of *Listeria monocytogenes* from Brazil Using PCR

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ABSTRACT

We assessed the serotype distribution of *Listeria monocytogenes* isolates from clinical, beef, and environment samples using two PCR-based protocols for serogrouping. A panel of 134 isolates (22 clinical samples, 79 samples of beef cuts, and 33 samples from the beef processing environment) were subjected to conventional serology and identified as serotypes 1/2a ($n = 12$), 1/2b ($n = 21$), 1/2c ($n = 71$), and 4b ($n = 30$). Isolates from clinical samples were predominantly serotype 4b, and the most prevalent serotype among the beef cut and environment samples was 1/2c. The protocol described by M. Doumith, C. Buchrieser, P. Glaser, C. Jacquet, and P. Martin (*J. Clin. Microbiol.* 42:3819–3822, 2004) produced contradictory results for seven 1/2a isolates, which were positive for *lmo1118* and had the profile IIc (serotypes 1/2c and 3c). Fifteen serotype 4b isolates amplified the target *lmo0737,* with the atypical profile IVb variant 1. The results obtained with the protocol described by M. K. Borucki and D. R. Call (*J. Clin. Microbiol.* 41:5537–5540, 2003) were in full agreement with those of the conventional serology. We recommend using this multiplex PCR approach by adding one pair of the reported primers to the panel to reduce total effort by one PCR while maintaining specificity. We present additional recommendations to improve the efficiency and reproducibility of this serogrouping assay.

Listeria monocytogenes is a gram-positive foodborne pathogen that causes infections in animals and humans. This pathogen is widespread in the environment, survives under a wide range of conditions, and is found in many different food products *(8)*. Infections occur through the consumption of contaminated foods, and ready-to-eat foods are often described as products with a high risk of transmission *(25, 26)*. Despite the low morbidity, infection with this intracellular bacterium results in 20 to 30% mortality and 95% hospitalization. Neonates, pregnant women, the elderly, and immunosuppressed individuals are particularly at risk *(10, 13)*.

A variety of methods have been used to differentiate *L. monocytogenes* strains and predict their virulence potential *(16)*. The classical serotyping method previously described by Seeliger and Höhne *(22)* is routinely used to identify potentially pathogenic strains. This method is based on variation in the somatic (O) and flagellar (H) antigens, allowing the identification of 13 different serotypes; among them, serotypes 1/2a, 1/2b, and 4b are responsible for more than 95% of listeriosis cases *(25)*. This classical method, however, is expensive, labor intensive, and time-consuming and demands high-quality antisera *(2, 6)*.

Several molecular assays have been developed to identify the main *L. monocytogenes* serogroups associated with listeriosis *(2, 6, 12, 24, 27, 30)*. Some of these methods have been used extensively because they are relatively simple to perform and relatively rapid, and once the infrastructure is in place, assay expense is limited. The multiplex PCR assay described by Doumith et al. *(6)* is a popular protocol for presumptive differentiation of *L. monocytogenes* strains, grouping them into four serogroups *(7, 12, 23, 27–29)*. Borucki and Call *(2)* developed a similar assay *(2, 5, 24),* but it has been used less frequently.

Herein we assess the serotype distribution of *L. monocytogenes* isolates from clinical, beef cut, and environment samples from Brazil and evaluate the performance of these two multiplex PCR protocols relative to the serotyping "gold standard."

MATERIALS AND METHODS

Microorganisms. A panel of 134 isolates identified as *L. monocytogenes* was used in this study; all isolates were obtained from the *Listeria* Culture Collection of Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Brazil) and isolated by Camargo et al. *(3)*. Isolates were collected between 1978 and 2012 from three sources: clinical samples ($n = 22$), beef cuts ($n = 79$), and beef processing environments ($n = 33$). Isolates were stored at –20°C in Trypticase soya broth (TSB; Oxoid Ltd., Basingstoke, UK) supplemented with 20% glycerol (vol/vol).

Serotyping. All *L. monocytogenes* isolates were subject to conventional serology using polyclonal somatic and flagellar

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TABLE 1. *Serotypes of the 134* L. monocytogenes *isolates used in the present study according to their sources*

Source	Sample type	No. (%) of isolates by serotype				Total no.
		1/2a	1/2b	1/2c	4b	
Clinical samples[a]		5 (22.7)	2 (9.1)		15 (68.2)	22
Beef cuts		6 (7.6)	19 (24.1)	40 (50.6)	14 (17.7)	79
Beef processing environment	Carcass	1 (14.3)		5 (71.4)	1 (14.3)	7
	Equipment			11 (100)		11
	Employee hands			15 (100)		15
Total		12	21	71	30	134

[a] One isolate was from a clinical sample of sheep brain, and other isolates were from human clinical samples.

antisera produced by the Laboratory of Bacterial Zoonoses (FIOCRUZ) as previously described by Seeliger and Höhne *(22)*.

Molecular analysis. Colonies from each isolate were transferred to TSB and incubated at 35°C for 24 h, and the resulting cultures were subjected to DNA extraction and purification using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI). The DNA was then subjected to a multiplex PCR assay as previously described by Doumith et al. *(6)*. The expected PCR products were *lmo0737* (691 bp), *lmo1118* (906 bp), ORF2110 (597 bp), ORF2819 (471 bp), and *prs* (370 bp). The extracted DNA was also subjected to the Borucki and Call *(2)* PCR assay. Expected PCR products were obtained with primers D1 (214 bp), D2 (140 bp), FlaA (538 bp), and GLT (483 bp).

In addition, a modified PCR protocol was developed based on the Borucki and Call *(2)* assay. In this case, extracted DNA was subjected to a multiplex PCR composed of 12.5 μl of GoTaq Green Master Mix (Promega), 2 μl of DNA, 40 μM concentrations each primer (D1 F and R, D2 F and R, and GLT F and R), and ultrapure PCR water (Promega) to a final volume of 25 μl. Amplification conditions were the same as those described by Borucki and Call *(2)* for the D1, D2, and GLT primers. A separate PCR was conducted with the FlaA primers *(2)*.

L. monocytogenes strains Scott A (serotype 4b), ATCC 7644 (serotype 1/2c), and ATCC 15313 (serotype 1/2a) were included as positive controls for all assays. Atypical results obtained with the Doumith et al. method were confirmed independently by the Laboratory of Bacterial Zoonoses (FIOCRUZ) using the same protocols, except that *L. monocytogenes* reference strains CDC F4555 (serotype 4b) and CDC F6254 (serotype 1/2c) were used as positive controls for these reactions.

RESULTS AND DISCUSSION

Based on conventional serology, the majority of clinical isolates were serotypes 4b (68.2%) and 1/2a (22.7%) (Table 1). For beef cut isolates, serotype 1/2c was detected most frequently (50.6%), but other serotypes were also detected: 1/2b (24%), 4b (17.7%), and 1/2a (7.6%). According to Orsi et al. *(20),* serotypes from lineage I (1/2b, 3b, 3c, and 4b) can be isolated from various sources but are overrepresented among human isolates, whereas serotypes from lineage II (1/2a, 1/2c, and 3a) are overrepresented among foods and environmental sources. Cartwright et al. *(4)* reported that 60.7% of the clinical isolates in the United States were serotype 4b and 33.1% were serotype 1/2a. Martín et al. *(17)* reported a high prevalence of serotype 1/2c (38.4%) in meat products, whereas serotype 1/2a prevailed on food contact surfaces (45.5%). According to Ochiai et al. *(19),* serotype 1/2c is most commonly isolated from pork meat in Japan, and Moreno et al. *(18)* reported a high prevalence of serotypes 1/2c and 4b in the pork processing environment and in pork meat in São Paulo state, Brazil. The handling of beef and pork in the same establishment may explain the high level of environmental contamination by serotype 1/2c among beef products.

The Doumith et al. *(6)* protocol correctly classified serotype 1/2b, 1/2c, and 4b isolates into their corresponding

TABLE 2. *Summary of* Listeria monocytogenes *PCR results after amplification using the protocols of Doumith et al. and Borucki and Call*[a]

Serotype	*n*	Doumith et al. *(6)*							Borucki and Call *(2)*					
		lmo1118	*lmo0737*	ORF2110	ORF2819	*prs*	Profile (serotypes)	*n*	D1	D2	*flaA*	*glt*	Division (serotypes)	*n*
1/2a	12	−	+	−	−	+	IIa (1/2a, 3a)	5	−	+	+	−	II (1/2a, 3a)	12
		+	+	−	−	+	IIc (1/2c, 3c)[b]	7						
1/2b	21	−	−	−	+	+	IIb (1/2b, 3b, 7)	21	+	−	−	+	I (1/2b, 3b)	21
1/2c	71	+	+	−	−	+	IIc (1/2c, 3c)	71	−	+	−	−	II (1/2c, 3c)	71
4b	30	−	−	+	+	+	IVb (4b, 4d, 4e)	15	+	−	−	−	I (4b, 4d)	30
		−	+	+	+	+	IVb-v1 (4b)[c]	15						

[a] Isolates were previously identified to serotype based on conventional serological tests *(22)*.
[b] Doumith et al. (6) and Kérouanton et al. *(12)*.
[c] Leclercq et al. *(14)*.

serogroups with 100% accuracy (Table 2). Accuracy was problematic for 1/2a isolates; only 42% were correctly serogrouped and the remainder were misclassified (Table 2). An additional refinement to the Doumith et al. assay, as described by Leclercq et al. *(14),* did not distinguish the 4b isolates (serogroup IVb) as being different from an additional IVb variant 1 (IVb-v1) grouping. The isolates that had atypical results (7 serotype 1/2a isolates and 15 serotype 4b isolates) were subjected to independent PCR testing at the Laboratory of Bacterial Zoonoses (FIOCRUZ), and all results were confirmed by using the two tested protocols. The Borucki and Call *(2)* protocol correctly classified all isolates by the proposed divisions and serogroups (Table 2). The modified Borucki and Call assay produced results identical to those of the original assay (data not shown).

Serogrouping as proposed by Doumith et al. differentiates *L. monocytogenes* into four molecular serogroups: IIa corresponded to serotypes 1/2a and 3a; IIc to serotypes 1/2c and 3c; IIb to serotypes 1/2b, 3b, and 7; and IVb to serotypes 4b, 4d, and 4e. This protocol is the one most commonly used for serogrouping *L. monocytogenes* isolates obtained from food and clinical samples. However, some strains of serotype 4b can have an atypical amplification profile, IVb-v1 *(9, 14)*, which was found for 15 isolates in the present study (Table 2). Other researchers have described strains with this atypical IVb-v1 profile from food processing plants, foods, and clinical samples *(9, 14, 15, 21)*. Lee et al. *(15)* found that IVb-v1 strains had a specific gene cassette comprising *lmo0734* to *lmo0739* (6.3 kb), which are common to 1/2a and 3a strains and 1/2c and 3c strains, suggesting horizontal gene transfer to serotype 4b (lineage I), generating the atypical profile IVb-v1.

We identified seven strains of serotype 1/2a with positive results for *lmo1118,* consistent with the inclusion of these strains in profile IIc (related to serotypes 1/2c and 3c) (Table 2). Similar results were observed by Doumith et al. *(6)* for the EGDe strain and by Kérouanton et al. *(12)* for isolates from food processing sources and foods. Bécavin et al. *(1)* compared the genomes of widely used serotype 1/2a strains 10403S, EGD, and EGD-e and found that the pathogenic EGDe is more closely genetically related to serotype 1/2c strains. Consequently, many potentially pathogenic strains of serotype 1/2a may have been incorrectly classified as serotype 1/2c, which is a pathogenic serotype but is not common among clinical isolates. This discordance can be resolved after an additional PCR assay based on the amplification of *flaA* in these atypical strains *(12)*.

The serogrouping method proposed by Borucki and Call *(2)* categorizes *L. monocytogenes* isolates into groups similar to those proposed by Doumith et al. *(6)* but takes into consideration the major phylogenetic divisions within this species. Division I includes serotypes 1/2b, 3b, 4b, 4d, and 4e, and division II includes serotypes 1/2a, 1/2c, 3a, and 3c. A third division (III) includes serotypes 4a and 4c (which are less commonly found in clinical and food samples) and can be identified by adding MAMA-C primers to the protocol *(11)*. We did not include these primers in our study because division III isolates are not usually identified in human and animal clinical listeriosis cases *(20)*. Additional PCR assays are required for complete identification of possible serotypes in each division *(2, 24)*.

Our recommended changes to the Borucki and Call protocol provided results identical to those obtained with the original protocol. However, the original protocol required three PCRs (one multiplex and two simplex) for complete serogrouping, whereas our recommended modification requires only two PCRs (one multiplex and one simplex). With this improvement in efficiency and the 100% correspondence with the results of conventional serotyping, this improved protocol may serve practitioners' needs better than the protocol described by Doumith et al. *(6)*.

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