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New Methodology for the Isolation of *Listeria* Microorganisms from Heavily Contaminated Environments

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A new methodology was devised for the isolation of microorganisms of *Listeria* genus from heavily contaminated materials, by which 88% of tested sheep were identified as carriers. There were 286 strains isolated by our methodology versus 19 by the classical system.

Listeria microorganisms are widely distributed in nature. Their isolation is not unduly difficult, except from heavily polluted material because of overgrowth by other microbial species. This is particularly arduous when using conventional techniques and media to isolate these microorganisms from feces or from certain chronic infections, etc. (4–6, 8, 9). These problems may blur the diagnosis of listeriosis. Also, traditional study methodology has tended to describe isolates of *Listeria monocytogenes* rather than the other *Listeria* species potentially present.

In the present study, 100 samples of feces obtained from a herd of sheep were studied. The classic methodology was employed and compared with methodologies involving modifications in culture media and design of isolation techniques. Samples (1 g) were taken aseptically from the rectum ampoule of adult, crossbred, meat-providing, grazing animals of both sexes over 1 year old, with no previous history of listeriosis or exposure to any kind of antibiotic treatment in the year previous to the sampling.

Classic methodology. A 1-g sample of feces was diluted in 15 ml of peptone-rhamnose broth and kept at 4°C for 48 h before being transferred to Despierres medium (3). It was enriched at the same temperature. The isolations were made on day 15; isolates were subcultured periodically for up to 6 months on Beerens nalidixic acid-blood agar (1) and on Ralovich tripaflavin (acriflavin)-nalidixic acid-serum agar (10, 11) and then incubated for 24 h at 37°C. The subsequent selection of colonies for the biochemical study was made on the basis of the procedure described below.

Modified methodology. Table 1 shows the composition of the different media used in the collection, enrichment, and isolation stages. The media containing esculin were prepared by the method of Buttiaux et al. (2), although, as a general rule, the mineral salts were sterilized separately. Enrichment medium no. 3 was distributed into flasks with Craigie tubes.

The feces (1 g) were diluted in 20 ml of media A and B. During sampling and transportation in the above-mentioned media, the temperature was kept at 4°C. Once in the laboratory, the feces were first homogenized in both collection media (A and B). Four milliliters of medium A was transferred to 20 ml of medium C. The three collection media were incubated at 4°C for 24 h, after which 2 ml of medium A was transferred to enrichment media 1 and 2; in the case of medium no. 3, this addition was fixed by the capacity of the Craigie tube. The samples were enriched at 22°C and then isolated after 48 h when the indicator turned from trypan blue to colorless and ammonia ferric citrate turned to black; this was repeated every 7 or 15 days in negative cases in which no growth was observed. Under no circumstances during this stage were the collected media rejected, but they were still kept at 4°C to be used for new enrichments and isolations in the event of absence of positive results. Isolations were made in media I, II, and III after incubation at 22°C for a period of 2 to 5 days, starting from both collection and enrichment media. Once the isolation of the *Listeria* strains had been achieved, the same medium was used for subsequent subcultures, since undesirable contaminations from handling the culture are avoided. In such cases, it is advisable to decrease the concentration of acriflavine to 50% (6 mg), thereby increasing the growth rate of the various strains, without an evident loss of selectivity. The isolation plates were studied with a stereoscopic magnifying glass at a magnification of 15- to 50-fold with incident light. Colonies suspected of belonging to the *Listeria* genus were grown in blood agar and identified according to the usual procedure. The pathogenicity for mice and production of hemolysin were determined before cultivation in blood agar.

Tables 2 and 3 show the results of the isolations made on each of the collection and enrichment media, as well as the number of strains of each species recovered by the various isolation media (by the traditional and modified methodologies). It is noteworthy that by the end of the experiment a total of 286 strains from 116 samples corresponding to 88 animals were isolated by the modified method. Of these, 60 were found to be carriers of the species *L. grayi*, 4 of *L. monocytogenes*, 8 of *L. grayi* and *L. murrayi*, 8 of *L. grayi* and *L. monocytogenes*, 4 of *L. monocytogenes*, 4 of *L. monocytogenes* and *L. denitrificans*, and 4 of *L. grayi*, *L. murrayi*, and *L. monocytogenes*, whereas 12 were found to be negative for all four species. In contrast, by the traditional method, only 11 samples corresponding to nine animals were found to be positive, from which a total of 19 strains were isolated.

Rapid visual identification and selection of the suspected colonies was possible with reference to the collection media; minimum media were chosen and no inhibitor was added. The isolation of *Listeria* spp. was almost exclusively based on their ability to grow at 4°C (7). The isolation media tested by other authors, such as nalidixic acid-blood agar (1) and tripaflavin-nalidixic acid-serum agar (10, 11), provided the basis for our modifications. In this respect, first the serum in the tripaflavin-nalidixic acid-serum agar was substituted by an equal amount of sheep blood, which favored the selection of colonies, making the contrasts clearer. It was necessary to

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TABLE 1. Composition of the collection, enrichment, and isolation media employed in the modified method[a]

Components[a]	Collection medium			Enrichment medium			Isolation medium		
	A	B	C	1	2	3	I	II	III
Peptone	5 g	10 g	5 g	5 g	5 g		5 g	5 g	3 g
Neopeptone	5 g		5 g	5 g	5 g		5 g	5 g	5 g
Proteose peptone						5 g			3 g
Tryptone						5 g			
Lab-Lemco powder				10 g	10 g	5 g	7 g	10 g	
Yeast extract				5 g		5 g		5 g	
Glucose				5 g			1 g	5 g	
Rhamnose		2 g			2 g				
Trehalose dihydrate	3 g								
Esculin			1 g			1 g			1 g
NaCl	10 g	10 g	8 g	50 g	50 g	20 g	5 g	40 g	5 g
Disodium phosphate 2-hydrate	12 g	12 g	12 g	53.22 g	53.22 g	24 g	11.83 g	1.83 g	12 g
Potassium phosphate monobasic	1 g	3 g	2 g	1.35 g	1.35 g	1.35 g	1.35 g	1.35 g	
Potassium phosphate dibasic		1.5 g							
Ammonia-ferric citrate			1 g			1 g			1 g
Nalidixic acid				50 mg	50 mg	30 mg	40 mg	40 mg	40 mg
Polymixin B				$8 \cdot 10^5$ IU				$3 \cdot 10^4$ IU	
Acriflavin HCl							12 mg	18.7 mg	12 mg
Trypan blue				80 mg	80 mg	40 mg			
Agar			1.5 g			3 g	15 g	15 g	15 g
Defibrinated sheep blood							50 ml	50 ml	50 ml

[a] All media contained 1,000 ml of distilled water, had a pH of 7.2 to 7.5, and were autoclaved at 116°C for 20 min.
[b] Components were purchased from the following: peptone, neopeptone, proteose peptone, tryptone, and yeast extract, Difco Laboratories; Lab-Lemco powder, Oxoid Ltd.; glucose and esculin, E. Merck AG; rhamnose, trehalose dihydrate, nalidixic acid, acriflavin HCl, and trypan blue, Serva.

TABLE 2. Strains isolated from the collection and enrichment media

Species	No. of strains isolated from:							
	Collection medium				Enrichment medium			
	Modified			Traditional[a]	Modified			Traditional[b]
	A	B	C		1	2	3	
L. monocytogenes	44	15	4	6	4	3	5	3
L. grayi	108	29	36	2	4	0	11	0
L. murrayi	12	1	2	0	0	1	2	0
L. denitrificans	0	0	5	3	0	0	0	

[a] Peptone water and rhamnose.
[b] Peptone water, rhamnose, nalidixic acid, polymixin B, and methylene blue.

TABLE 3. Strains obtained on the isolation media

Species	No. of strains isolated by:				
	Modified			Traditional	
	I	II	III	NABA[a]	TNBA[b]
L. monocytogenes	2	5	68	4	5
L. grayi	0	0	188	2	0
L. murrayi	2	0	16	0	0
L. denitrificans	1	1	3	4	4

[a] Nalidixic acid-blood agar.
[b] Tripaflavin-nalidixic acid-blood agar.

change to epi-illumination rather than transillumination. Second, the concentration of acriflavin was lowered to 12 mg/liter, since it was found that growth of most *L. murrayi* and *L. grayi* was inhibited. In these cases it is advisable to use the agar nutritive medium with nalidixic acid and polymixin B or other selective media. Esculin was added to some as the only usable carbohydrate as well as ammonia-ferric citrate as an indicator of hydrolysis (3). The incubation temperature of the isolation media should be approximately 22°C, because higher temperatures can promote an evident loss of the iridescent appearance of the *Listeria* colonies. Furthermore these high temperatures favor the growth of a greater number of undesirable microorganisms, making the task of selection more difficult. From the results set out in Tables 2 and 3, the efficiency of each of the media in the three stages of our study (collection, enrichment, and isolation) was greatly superior to that of traditional methods for the species *L. monocytogenes*, *L. grayi*, and *L. murrayi*. In contrast, for *L. denitrificans*, the traditional method obtained a slightly superior yield to the modified method, despite the similar efficiency of collection medium C.

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