

Rapid Method for the Isolation of *Listeria monocytogenes* from Experimentally Infected Mice¹

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Listeria monocytogenes was successfully isolated from experimentally infected mice by placing homogenized tissues in phosphate-buffered saline (PBS), trypsin, peptone, or pepsin followed by incubation at 37 C for 24 hr. A larger number of *Listeria* isolates were recovered from the trypsin or PBS splenic homogenate suspensions incubated at 37 C for 2 hr than from the other diluent suspensions. Holding infected tissues at 4 C for at least 3 months did not increase the efficiency of *Listeria* isolation. *Listeria* L-forms were not isolated from mice injected with the bacterial form. The in vitro viability of *Listeria* L-forms suspended in PBS or PBS splenic homogenate was greatly reduced when held at 4 C.

In 1948 Gray et al. (6), studying the prevalence of listeriosis among farm animals in Michigan, found that *Listeria monocytogenes* was easily isolated from the brain in cases of ovine listeriosis. However, initial isolation attempts from the bovine brain were unsuccessful although the animals had symptoms typical of listeriosis and histopathological sections showed characteristic pathology. When the medulla of the bovine brain was held at 4 C, *L. monocytogenes* was successfully isolated after 3 weeks to 3 months of storage. Since then, many investigators using this cold-storage isolation technique have found a drastic increase in the percentage of *Listeria* isolates from infected animals (5, 14, 15), splenic explants (18), and vegetation (3, 20).

The successful isolation of *Listeria* by using the cold storage method of Gray (6) has proven to be of ecological importance but of little significance in diagnosing active cases of listeriosis. Investigators have earnestly sought a more rapid method for the isolation of *Listeria*. Olson et al. (13) compared the effectiveness of various isolation methods and observed that the highest percentage of *Listeria* isolates from sheep brains was obtained by grinding fresh tissues, inoculating the homogenate into Tryptose broth, and incubating at 37 C for 24 to 48 hr. However, Gray and Killinger (4) pointed out that Olson et al.

failed to compare their results with the cold-storage method of Gray. Other investigators have reported successful initial isolations by culturing suspected materials in thioglycolate (23), Tryptose broth plus vitamin B₁ (17), and nutrient broth plus potassium thiocyanate (22). Kramer and Jones (10) found that *Listeria* was more efficiently recovered from infected materials when the base medium contained thallose acetate and nalidixic acid rather than potassium thiocyanate, potassium tellurite, sodium azide, or various dyes. Other techniques used in the isolation of *Listeria* have been the biological method (3) and inoculation of chick embryonated eggs with suspected materials (1).

The present diagnosis of listeriosis (9) depends upon the isolation of *Listeria* by the time-consuming, cold-storage technique. The failure to isolate *Listeria* on primary culture of infected materials from animals or vegetation remains unexplained. Implications of an inhibitory substance from tissues (4, 16) or contaminating bacteria (11), a population effect (4), or L-forms (19) have not satisfactorily answered the question. The present investigation was undertaken to develop an isolation technique which would replace the cold-storage method and, in so doing, provide an explanation for its previous effectiveness.

MATERIALS AND METHODS

Cultures and maintenance. *L. monocytogenes* strain 10403, serotype 1, obtained from the late M. L. Gray, Montana State University, was used in

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these studies. The culture was maintained on Brain Heart Infusion (BHI, Difco) semisolid agar at 4 C and was examined periodically for purity during the course of this investigation.

L-forms of *L. monocytogenes* 10403 induced and maintained by the methods of Edman et al. (2) were included in this study. The L-forms were induced on penicillin gradient plates which consisted of 3.7% BHI, 0.85% Special Agar (Noble, Difco), 4% NaCl, 0.246% $MgSO_4 \cdot 7H_2O$, and 10% horse serum (Hyland Laboratories). A 0.1-ml volume of a sterile aqueous solution of 800 units of penicillin G (Squibb) per ml was placed in a 5-mm trough at one end of the agar plate. The L-forms were cultured on L-form maintenance agar which consisted of the same ingredients as the gradient induction medium plus 1.2 units of penicillin G per ml. The L-forms were induced and grown at 30 C and held at 4 C.

In vitro L-form survival. *Listeria* L-forms were harvested from maintenance medium, washed once in 4% NaCl, and resuspended in the same diluent. A 0.1-ml volume of the L-form suspension was pipetted into separate tubes which contained 5 ml of 4% NaCl, 0.067 M phosphate-buffered saline (PBS) at pH 7.2, or PBS with splenic homogenate. The homogenate was prepared by aseptically excising spleens from uninoculated mice and homogenizing the spleens in PBS with a chilled tuberculin syringe (B-D plastipak). All tubes were incubated at 4 C and sampled periodically. Samples removed from the tubes were diluted in 4% NaCl or PBS and plated on L-form maintenance agar. The plates were incubated at 30 C for 5 days before determining the per cent survival. Samples also were plated on McBride agar (Difco) and incubated at 37 C for 4 days to ascertain the presence of L-form revertants.

Animal Infectivity. Two inbred white mouse strains, Washington State University (WSU) and Swiss-Webster (SW), were maintained for experimentation. The two colonies were housed and bred in physically separate quarters. *L. monocytogenes* was grown in 10 ml of BHI broth at 37 C for 16 hr. The cells were harvested and washed twice in 0.5% peptone (Difco) and resuspended in the same diluent. Mice were injected intraperitoneally (ip) with 0.2 ml of the desired bacterial concentration by using a tuberculin syringe and a 25-gauge hypodermic needle (B-D Yale, disposable). In general, 1-month-old mice, weighing 16 to 20 g, were used throughout the investigation. All experimental mice, infected and controls, were held in animal quarters physically separate from the stock mouse colonies. Animals were provided with feed and water ad libitum.

Necropsy and pathology. A postmortem examination was performed on all infected and control mice immediately after deaths caused by an acute case of listeriosis or by sacrificing with an overdose of chloroform. The mice were disinfected externally, and the peritoneal cavity was surgically opened exposing the viscera. The liver and spleen were examined for the presence of lesions, which appeared as greyish-white discrete circumscribed necrotic foci. A portion of the liver and upper section of the small intestine was

excised; the spleen, left kidney, and gall bladder were completely removed.

Isolation and identification. Agar smears of the excised tissues were made on McBride agar by making multiple incisions in the organs followed by smearing these specimens over the agar surface. The plates were incubated at 37 C for 72 hr. Preliminary identification of bacterial colonies was based on the Henry reflected-light technique (7) in which *Listeria* colonies appear blue to blue-green. Confirmed identification was based on the Gram stain, motility at room temperature, and fermentation reactions in glucose, sucrose, salicin, lactose, D-xylose, inulin, maltose, and trehalose.

Tissues collected during necropsy were discarded if the tissue smears on McBride agar were positive for *Listeria*. Otherwise, the liver and spleen were minced separately and each tissue was placed into Kahn tubes which contained 1 ml of either PBS, 0.5% peptone, 0.25% trypsin (Difco certified, 1:250) suspended in PBS at pH 7.2, or 2% pepsin (National Biochemicals Corp.) suspended in PBS at pH 7.2. All tubes were incubated at 37 C for 24 hr with periodic mixing of the tissue suspensions. A sample from each tissue suspension was plated on McBride agar and incubated at 37 C for 72 hr. Also, samples of the splenic tissue suspensions were plated on L-form maintenance agar immediately after the tissues were minced. These plates were incubated at 30 C for 3 weeks with periodic microscopic observations. All tissue suspensions were held at 4 C and sampled at intervals over a 3-month, or longer, holding period.

Quantitative recovery. Spleens of injected mice were aseptically excised, washed in PBS, and homogenized in 1 ml of PBS with a tuberculin syringe. The splenic homogenate was divided into equal parts (0.2 ml) and placed into 0.8 ml of one of the following diluents: PBS, 0.25% trypsin, 2% pepsin, 0.5% peptone, or distilled water. A 0.1-ml volume of the PBS tissue suspension was plated immediately on McBride agar in duplicate and incubated at 37 C for 72 hr. The remaining PBS tissue suspension along with the trypsin, pepsin, and peptone tissue suspensions were incubated at 37 C for 2 hr followed by plating 0.1 ml of each suspension on McBride agar in duplicate and incubating at 37 C for 72 hr. The water-tissue suspension was placed at -20 C for 4 hr, thawed at room temperature, refrozen, and again thawed at room temperature. A 0.1-ml volume of the final thawed tissue suspension was plated on McBride agar in duplicate and incubated at 37 C for 72 hr.

RESULTS

In vitro L-form survival. The survival of *Listeria* L-forms in 4% NaCl, PBS, and PBS with splenic homogenate at 4 C was determined (Fig. 1). In splenic homogenate, viable L-forms decreased 300-fold in 3 days. However, L-forms suspended in PBS declined 100-fold in 3 days, whereas less than a 10-fold drop in titer was observed in 4% NaCl over the same time period. The recovery of L-forms was always lower when

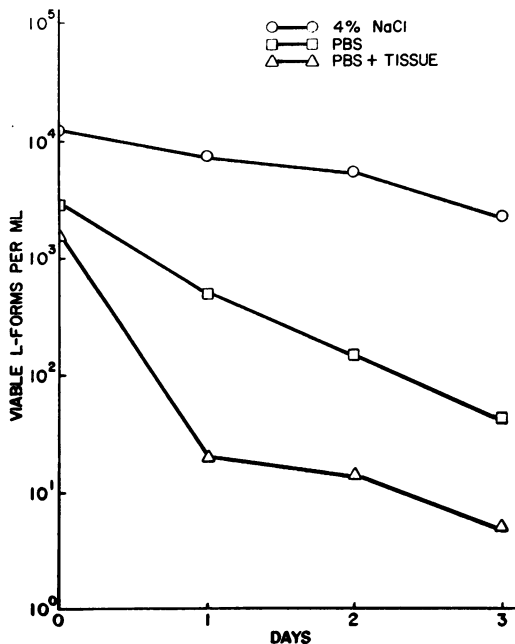


FIG. 1. Survival of L-forms of *Listeria monocytogenes* in 4% NaCl, PBS, and PBS plus splenic homogenate at 4 C.

dilutions of the original sample were made in PBS rather than 4% NaCl. Bacterial revertants were not recovered from the L-form suspensions during the 3-day holding period at 4 C.

Pathology. Mice injected with concentrations of *Listeria* ranging from 10^8 to 10^8 viable cells were observed for the presence of lesions in the liver and spleen. Mice which died from listeriosis prior to day 3 postinjection did not show any evidence of lesions. Lesions appeared in the liver and spleen of approximately 50% of the mice which died from listeriosis on day 3 through day 9 postinjection. Lesions appeared more frequently in the spleen. Mice that died from listeriosis after day 9 generally developed hind-quarter paralysis without evidence of lesions in the liver or spleen.

Isolation. *L. monocytogenes* was isolated from all mice that died from listeriosis regardless of the absence or presence of lesions in the liver or spleen. Isolation of the causative agent was made directly from the liver, spleen, kidney, and small intestine by using the tissue-smear culture method.

Mice injected ip with 1.9×10^8 virulent *Listeria* were sampled daily over a 16-day period. Table 1 illustrates the recovery of *L. monocytogenes* from the liver, spleen, kidney, and small intestine of infected mice. *Listeria* was isolated by

the tissue-smear culture method from most of the organs sampled through day 5 postinjection. The liver-smear cultures were generally positive for *Listeria* through day 7 postinjection, whereas *Listeria* isolates were obtained sporadically from the other organs after the 5th day by using the tissue-smear culture method. The number of liver samples showing positive *Listeria* isolates was increased by placing pieces of the liver in trypsin, peptone, or pepsin diluents. Nine liver samples, which were negative with the tissue-smear culture method, were positive when cultured with the diluent technique. Homogenization of the spleen increased the number of *Listeria*-positive samples. Twenty splenic samples which were negative when cultured by the smear culture method yielded *Listeria* isolates when cultured after the tissue was homogenized. Three spleens which were negative for *Listeria* by both the tissue-smear culture method and homogenization gave positive isolates when the splenic homogenate was placed in trypsin, peptone, or pepsin prior to culturing. More tissue samples were positive for *Listeria* when trypsin was the diluent rather than peptone or pepsin. *L. monocytogenes* was isolated from the liver or spleen of all mice sacrificed through day 9 postinjection and at least 50% of the mice sacrificed through day 11.

The gall bladder of mice injected ip with 1.2×10^8 *Listeria* was sampled on days 14, 18, 22, 26, and 32 postinjection. *L. monocytogenes* was not isolated from the gall bladders of 10 mice sacrificed per time period. However, *Listeria* was isolated from the splenic homogenate by using the diluent technique through day 10 postinjection. The splenic homogenate was examined for the presence of L-forms on days 10, 14, 18, 22, and 32 postinjection. *Listeria* L-forms were not isolated from the 10 mice sacrificed per time period.

Quantitative recovery. A comparison of the quantitative recovery of *Listeria* from the splenic homogenate suspended in PBS, trypsin, peptone, pepsin, and distilled water was made for the purpose of determining the best diluent for isolating *Listeria*. Before making these determinations, the effect of various diluents on splenic cell destruction was examined. Splenic homogenate suspended in PBS showed very little cellular destruction when examined microscopically at 0 hr and after 2 hr of incubation at 37 C. Splenic homogenate suspended in pepsin exhibited slight cellular destruction after 2 hr at 37 C. Approximately 50% cellular destruction of the splenic tissue was observed when the homogenate was suspended in peptone for 2 hr at 37 C or when placed in distilled water followed

TABLE 1. Recovery of *Listeria monocytogenes* from mice injected with 1.9×10^8 *Listeria* cells

Time post-injection (day)	No. of mice	Liver				Spleen ^a					Kidney smear	Intestine smear
		Smear	Trypsin	Peptone	Pepsin	Smear	PBS	Trypsin	Peptone	Pepsin		
1	3	+ ^b	ND ^c	ND	ND	+	+	ND	ND	ND	+	+
	1	+	ND	ND	ND	- ^d	+	ND	ND	ND	-	+
2	3	+	ND	ND	ND	+	+	ND	ND	ND	+	+
	1	+	ND	ND	ND	-	+	ND	ND	ND	-	-
3	1	+	ND	ND	ND	+	+	ND	ND	ND	+	+
	2	+	ND	ND	ND	+	+	ND	ND	ND	-	+
	1	+	ND	ND	ND	+	+	ND	ND	ND	+	-
4	3	+	ND	ND	ND	+	+	ND	ND	ND	+	+
	1	+	ND	ND	ND	+	+	ND	ND	ND	-	-
5	2	+	ND	ND	ND	+	+	ND	ND	ND	+	+
	1	+	ND	ND	ND	+	+	ND	ND	ND	+	-
	1	-	+	+	+	-	-	-	-	-	-	-
6	1	+	ND	ND	ND	-	+	ND	ND	ND	-	+
	3	+	ND	ND	ND	-	+	ND	ND	ND	-	-
7	1	+	ND	ND	ND	+	+	ND	ND	ND	+	+
	1	+	ND	ND	ND	-	+	ND	ND	ND	-	-
	1	-	+	+	+	-	+	ND	ND	ND	-	-
	1	-	-	-	-	-	+	ND	ND	ND	-	-
8	1	-	+	-	-	-	+	ND	ND	ND	-	+
	1	-	+	-	-	-	+	ND	ND	ND	-	-
	1	+	ND	ND	ND	-	-	-	-	-	-	+
	1	-	-	-	-	-	+	ND	ND	ND	-	-
9	1	-	-	-	-	+	+	ND	ND	ND	-	-
	1	-	+	+	+	+	+	ND	ND	ND	-	+
	2	-	-	-	-	-	+	ND	ND	ND	-	-
10	1	-	+	+	+	-	+	ND	ND	ND	-	+
	2	-	-	-	-	-	+	ND	ND	ND	-	-
	1	-	-	-	-	-	-	-	-	-	-	-
11	1	+	ND	ND	ND	-	-	+	+	+	-	+
	1	-	-	-	-	-	-	+	+	+	-	+
	2	-	-	-	-	-	-	-	-	-	-	-
12	4	-	-	-	-	-	-	-	-	-	-	-
13	1	-	-	-	-	-	+	ND	ND	ND	-	+
	3	-	-	-	-	-	-	-	-	-	-	-
14	1	-	+	-	-	-	+	ND	ND	ND	-	-
	3	-	-	-	-	-	-	-	-	-	-	-
15	1	-	-	-	+	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	+	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-
16	1	-	+	+	+	-	+	ND	ND	ND	-	-
	3	-	-	-	-	-	-	-	-	-	-	-

^a Spleen was homogenized in PBS and sampled immediately before placing the homogenate in the other diluents.

^b +, *Listeria* was isolated from the sample(s).

^c Not done.

^d -, *Listeria* was not isolated from the sample(s).

by two freeze-thaw treatments. Complete cellular destruction was observed when the splenic homogenate was suspended in trypsin for 2 hr at 37 C.

The comparative effects of the various diluents on the quantitative recovery of *L. monocytogenes* from the spleen of experimentally infected mice are illustrated in Table 2. The largest number of

Listeria isolates was obtained more often from the trypsin or PBS splenic suspensions incubated for 2 hr at 37 C than from the peptone, pepsin, distilled water or zero time PBS tissue suspensions.

The pH of all splenic homogenate suspensions was determined with hydron paper (Micro Essential Laboratory) immediately after the

TABLE 2. Comparison of the effect of various diluents on the recovery of *Listeria monocytogenes* from the spleen of mice injected with 1.2×10^8 *Listeria* cells

Time post-injection (day)	Mouse	Colony counts from splenic homogenate					Water F-T ^a
		Trypsin 2 hr, 37 C	Peptone 2 hr, 37 C	Pepsin 2 hr, 37 C	PBS		
					0 hr	2 hr, 37 C	
6	A	5	5	0	0	ND ^b	0
6	B	3500	1280	3100	570	ND	230
6	C	15900	6550	10150	3300	ND	935
6	D	9850	1315	3500	655	ND	600
6	E	19100	20600	20000	3950	ND	1005
6	F	650	515	490	35	ND	25
6	G	10	5	0	0	ND	5
6	H	2105	795	1450	965	4690	175
8	I	100	15	60	20	65	5
8	J	125	55	55	15	160	10
8	K	4175	1825	1980	1110	5150	350
8	L	160	45	110	55	150	5
10	M	0	0	0	10	5	0
10	N	385	295	175	100	445	10

^a Freeze-thaw.

^b Not done.

addition of the homogenate to the diluents and after the 2-hr incubation period at 37 C. A neutral pH was found in all of the samples tested.

Cold storage recovery. The effect of 4 C on the recovery of *L. monocytogenes* from tissues of experimentally infected mice was determined. The liver, spleen, and kidney of mice injected ip with 2×10^8 *Listeria* were minced separately and placed in trypsin, peptone, or PBS. Also, the spleens of mice injected with 1.2×10^8 *Listeria* were homogenized and placed in trypsin, peptone, pepsin, or PBS. All tissue diluent samples, which were cultured after 0, 2, and 24 hr of incubation at 37 C, were held at 4 C and sampled over a 3-month, or longer, holding period. *L. monocytogenes* was isolated from only two tissue samples out of 419 specimens which were shown negative prior to holding at 4 C. These positive samples came from a splenic homogenate after 1 month of cold storage in PBS and from another splenic homogenate in PBS after 1.5 months at 4 C. However, *L. monocytogenes* was previously isolated from other tissues of these mice. All tissue samples which were shown positive after 0, 2, or 24 hr of incubation at 37 C remained positive throughout the cold-storage holding period.

The spleens of mice injected ip with 1.2×10^8 *Listeria* were homogenized and placed in PBS. The splenic homogenate was examined

for the presence of L-forms immediately after homogenization and after 1 month of cold storage. *Listeria* L-forms were not isolated from 50 splenic samples before or after cold storage.

DISCUSSION

According to Gray and Killinger (4), ip injections of virulent *Listeria* in mice result in a septicemia with possible development of necrotic lesions in the liver, spleen, lungs, adrenal glands, tonsils, and intestinal tract. The lesions are characterized histologically by focal necrosis with infiltration of mononuclear cells and, at times, neutrophils which characterizes a suppurative inflammation. Louria et al. (12) injected mice in the tail vein with 10^6 to 10^7 virulent *Listeria* and observed, after 48 hr, focal abscesses in the liver and spleen that were subsequently replaced by epithelial cells resulting in granuloma formation. Similar pathology was observed in the lungs and kidneys of mice 7 days postinjection. Inhorn et al. (8) described the splenic involvement in a human suffering from listeriosis. Histologically, the lesions in the spleen were characterized as focal granulomas with central caseation necrosis.

In the present study, *Listeria* was isolated from the liver, spleen, kidney, and small intestine of all experimentally infected mice which died from listeriosis irrespective of the time of death postinjection and the presence or absence of lesions in the liver or spleen. In mice injected ip with virulent *Listeria*, the organism persisted in the peritoneal cavity for 8 days and in the spleen for 10 consecutive days followed by sporadic recovery from the spleen during the next 6 days (21). The degree of difficulty in isolating *Listeria* was directly proportional to the amount of time which elapsed postinjection. The immunological response of the host apparently governed the in vivo persistence of *Listeria*. If the listeric infection proceeded unchecked by the host defenses, a generalized infection ensued to cause death of the host. *L. monocytogenes* was always isolated by agar smear cultures of tissues from mice which died from listeriosis. If the immunological response prevents death of the host but allows the causative agent to become deep seated in a granuloma, isolation of *Listeria* is impeded. Even homogenization of the organ may not liberate *Listeria* from the granuloma. However, if the tissue is allowed to undergo autolysis, *Listeria* can often be isolated. The cold-storage method of Gray (6) for isolating *Listeria* from suspected infected tissues certainly encompasses the effects of autolytic enzymes on the tissues. Pittman and

Cherry (16) postulated that the success in isolating *Listeria* from tissues held at 4 C was possibly due to the release of *Listeria* from slowly autolysing tissue cells. However, if the suspected tissues are incubated at 37 C, cellular autolysis would most likely proceed at a faster rate than at 4 C. The present investigation demonstrates that if tissues obtained from experimentally infected mice prior to day 17 postinjection were minced and placed in PBS, trypsin, peptone, or pepsin followed by incubation at 37 C for 24 hr, *L. monocytogenes* was isolated from a high percentage of the sampled tissues.

The quantitative recovery study showed that more *Listeria* isolates were obtained from the trypsin and PBS tissue suspensions incubated at 37 C for 2 hr than from the other tissue diluent suspensions. The type of diluent and treatment was apparently important for maximum *Listeria* recovery from the infected splenic homogenate. Since PBS did not cause any cellular lysis of the splenic homogenate after 2 hr at 37 C and the trypsin tissue suspension showed complete cellular destruction, the results would indicate that cellular lysis was not a prerequisite for isolating *Listeria*. However, a sufficient amount of autolysis must have occurred in the PBS tissue suspensions to break down the integrity of the remaining structural components, since more *Listeria* were recovered from the PBS tissue suspensions after 2 hr at 37 C than recovered from the PBS tissue suspensions immediately after homogenization. The increase in the number of recovered *Listeria* was apparently not due to multiplication alone, since the increase was greater than would be expected from *Listeria* reproduction. Also, if replication were to account for the increase in the number of *Listeria* isolates, one would expect a comparable increase in isolates from the peptone tissue suspensions.

Further evidence to substantiate the efficiency of the diluent technique in isolating *Listeria* was obtained by culturing all negative tissue samples during a 3-month or longer holding period at 4 C. Gray et al. (5, 6), Osebold and Inouye (14), and Osebold et al. (15) have demonstrated a substantial increase in the number of *Listeria* isolates from suspected tissues after 3 weeks to 3 months of holding at 4 C. The present investigation demonstrated positive isolates from less than 0.5% of all refrigerated tissues which were shown to be negative by the tissue-smear, homogenization, and diluent techniques. Thus, cold storage did not enhance the isolation of *Listeria* from tissues of experimentally infected mice. Also, the results appear to indicate that the mechanism involved in cold-storage isolation was similar to that obtained by incubation of

suspected tissues in diluents at 37 C, that is, tissue autolysis. The advantage of the diluent technique is of paramount importance to the clinician, since the time needed for isolation of *Listeria* could be reduced from 3 months to 2 days.

Localization of *Listeria* in the gall bladder was not observed in the experimentally infected mice. Implications of an inhibitory substance in the spleen has been previously disproven (21). The role of the *Listeria* L-form as an explanation for the failure to isolate the bacterial form was not evident. The probability of isolating *Listeria* L-forms from refrigerated tissue samples was poor, since the *in vitro* studies indicated a high mortality rate of *Listeria* L-forms.

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