PRODUCTION AND NATURE OF *LISTERIA MONOCYTOGENES* HEMOLYSINS

AUGUSTINE N. NJOKU-OBI,[1] EDWARD M. JENKINS, JESSIE C. NJOKU-OBI, JOANNE ADAMS, AND VERDELL COVINGTON

Department of Bacteriology and Public Health, School of Veterinary Medicine, Tuskegee Institute, Alabama

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ABSTRACT

NJOKU-OBI, AUGUSTINE N. (School of Veterinary Medicine, Tuskegee Institute, Ala.), EDWARD M. JENKINS, JESSIE C. NJOKU-OBI, JOANNE ADAMS, AND VERDELL COVINGTON. Production and nature of *Listeria monocytogenes* hemolysins. J. Bacteriol. **86**:1-8. 1963.—Hemolysin produced by various strains of *Listeria monocytogenes* varied in quality and quantity, depending on medium, incubation temperature and time, and biological variations in the organisms. The hemolysin was inactivated by filtration (through Seitz, Selas, or sintered-glass filters), heat, oxygen, and formalin. Sodium thiosulfate reactivated hemolysin inactivated by filtration and oxygen. The hemolysin was protein in nature, migrating electrophoretically as a gamma-globulin, and highly antigenic in the rabbit. Although no toxicity was observed in intact mice injected with hemolysin, a possible leukocytolysis was noted with isolated mice peritoneal exudate cells. Due to the high antihemolytic activity of normal sera from various species, the possible use of an antilisteriolysin test in serological diagnosis is questioned.

Ever since Murray, Webb, and Swann (1926) first isolated and extensively described *Listeria monocytogenes*, it has been known to produce beta-hemolysin on blood agar.

Later investigators (Burn, 1934; Harvey and Faber, 1941) reported the presence of soluble, filterable hemolysins produced by this bacterium, but no attempts were made to determine conditions affecting their production, nature, or role in infection (Seeliger, 1961).

The present study sought to determine these conditions, and is one of a series of studies directed toward a better understanding of the complex factors involved in the virulence of *L. monocytogenes*.

[1] Present address: Department of Microbiology, University of Lagos Medical School, Lagos, Nigeria, West Africa.

MATERIALS AND METHODS

Strains of L. monocytogenes. A total of 112 strains were tested. Nine strains obtained from J. W. Osebold, Department of Microbiology, School of Veterinary Medicine, University of California, Davis, were used most extensively in establishing optimal conditions for hemolysin production; these were 7973, 7648, 4-52, 5348, 5214, 7647, 7644, and 1047/53. M. L. Gray, Veterinary Research Laboratory, Montana State College, Bozeman, supplied 61 strains. The remainder were laboratory strains and isolates maintained in this laboratory.

Hemolysin production. Brain Heart Infusion broth (Difco) containing an additional 0.5% glucose was used in most of the experiments. When solid medium was desired, 1.5% agar (Difco) was added. This medium gave consistently higher yields of hemolysin than Liver Infusion, Veal Infusion, or Tryptose Phosphate Broth (Difco).

For hemolysin production in broth, 0.5 ml of an 18-hr Brain Heart Infusion broth culture was inoculated into 10 ml of medium contained in screw-cap tubes (16 × 125 mm).

When solid medium was used, the same inoculum was spread out on 20 ml of solidified medium in a petri plate.

After incubation at specified temperature and time, hemolysin was obtained from broth culture by centrifuging it at 3,020 × *g* for 30 min at 8 C in a Servall RC-2 centrifuge with an SS-34 rotor. The supernatant contained the hemolysin. For solid medium, the agar culture was chopped up and extracted with normal physiological saline at 37 C for 45 min and centrifuged as above. The saline extract contained the hemolysin.

Titration of hemolysin. Hemolytic activity was titrated by twofold dilutions in 0.5 ml of

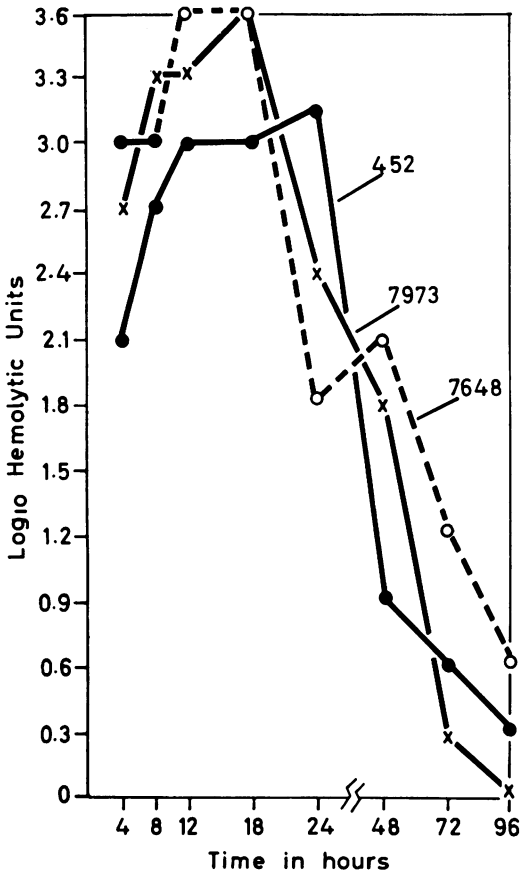

FIG. 1. *Effect of incubation time at 37 C on hemolysin production in Brain Heart Infusion Agar.*

Streptolysin O Buffer Solution (Cappel Laboratory, West Chester, Pa.). An equal volume of a 1% suspension of sheep red blood cells (Cappel) in Streptolysin O Buffer Solution was added to the tubes of diluted hemolysin. A reading was made after 2 hr of incubation in a water bath (37 C), and the final reading was made after overnight storage at 10 C.

Titers were expressed usually as minimal hemolytic units (MHU), defined as the reciprocal of the highest dilution of hemolysin showing lysis. When expressed as complete hemolytic units (CHU), the highest dilution showing complete hemolysis was taken as the end point.

Titration of antihemolytic activities of various biological fluids. Sera from various normal animals and man were diluted twofold in 0.5 ml of Streptolysin O Buffer Solution. Hemolysins were diluted to contain one CHU in 0.4 ml, and were added in this amount to each serum dilution. The mixtures were incubated at 37 C for 30 min, and then 0.1 ml of a 5% suspension of sheep red blood cells was added to each tube. Hemolysis was read after 2 hr in a water bath (37 C) and after overnight storage at 10 C.

For titrating antihemolytic activity of purulent and peritoneal exudates, the same procedure was used except that the hemolysin was diluted to contain four CHU in 0.4 ml.

Production of antihemolysin sera. Partially purified hemolysin was employed. The method of Herbert and Todd (1941) was used to partially purify the hemolysin after the hemolytic fraction had been isolated from 18-hr Brain Heart Infusion broth culture supernatant with 66% saturated ammonium sulfate. This increased activity 150 times.

The partially purified hemolysin was diluted with normal physiological saline to contain 0.1 μg per 0.5 ml. New Zealand White rabbits were immunized by injecting intravenously a total of 18 μg of hemolysin over a 3-week period. At 10 days after the last injection the animals were bled intracardially for serum.

RESULTS AND DISCUSSION

Rate of hemolysin production. Incubation time and temperature affected the rate of hemolysin production. With the plate culture method, appreciable hemolysin could be detected after only 4 hr of incubation at 37 C (Fig. 1). Although there was considerable strain difference in time required for development of maximal titer, the average was 16 hr. After 24 hr, titers decreased, sometimes rather rapidly, and were almost completely absent at 96 hr.

Broth cultures showed an essentially identical trend (Fig. 2). However, titers were much lower than with plate cultures.

Average time for maximal hemolysin production was 64 hr at 20 C, and 11 hr at 42 C . Plate cultures at 4 C for 7 days yielded appreciable hemolysin titers (Table 1). Increased hemolysin production in broth cultures incubated at 4 C for up to 4 weeks (Girard et al., 1962) could not be confirmed. It appeared that hemolysin was produced primarily during the period of active bacterial growth (Fig. 2).

Effect of oxidation on hemolysin activity. Since the hemolysin titer decreased rapidly after 24 to 48 hr, studies were made to determine whether

oxidation was responsible for the deterioration, as was reported for pneumococcus, streptococcus, and staphylococcus. When the hemolysin was reduced by sodium thiosulfate (Todd and Hewitt, 1932), it was still present but inactive (Fig. 3).

This observation suggested that incubation in an atmosphere of CO_2 might enhance hemolysin production; however, the reverse was found. Cultures incubated in air yielded higher titers than those incubated under 5% CO_2, and many strains failed to produce detectable hemolysin in this environment.

Effect of hydrocolloids on hemolysin production. Addition of some common hydrocolloids to the broth cultures potentiated the hemolytic titer and prevented deterioration of the hemolysin (Table 2). Carrageenan was least effective. Its highly charged SO_4^{--} groups caused considerable precipitation of ingredients in the medium.

The nature of this potentiation and prevention of deterioration by hydrocolloids is not known. It was reported for staphylococcus hemolysin by Burnet (1930) and confirmed by Seiffert (1935). Although unconfirmed, hydrocolloids may absorb inhibitory substances in the medium, allowing greater production and release of the hemolysin or loosely binding hemolysin, or both, preventing its inactivation by oxidizing agents. The effect of silica gel was similar to that of hydrocolloids.

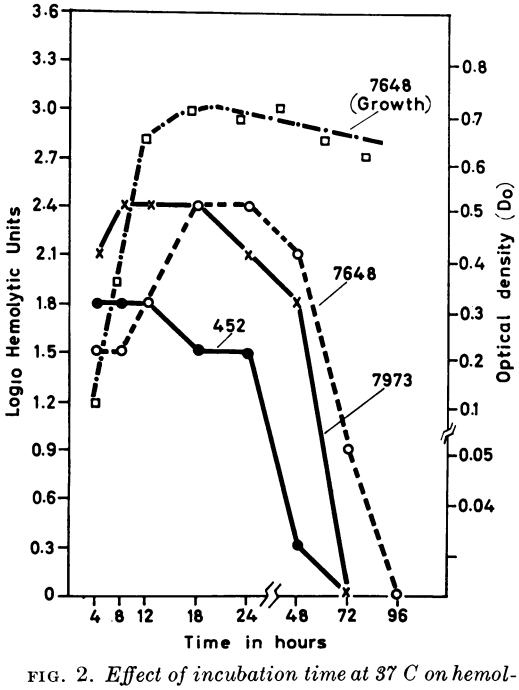

FIG. 2. *Effect of incubation time at 37 C on hemolysin production in Brain Heart Infusion broth.*

TABLE 1. *Hemolysin production on Brain Heart Infusion Agar at various temperatures**

Strain	Incubation temp						
	4 C†	20 C		37 C		42 C	
	Titer	Titer	Time	Titer	Time	Titer	Time
			hr		*hr*		*hr*
7973	32	2,048	72	2,048	18	2,048	10
7648	128	2,048	72	4,096	18	4,096	12
452	16	512	48	1,024	12	512	10

* The titer given is the highest titer, and the time is the time the highest titer was obtained.

† At 4 C, incubation was for 7 days only.

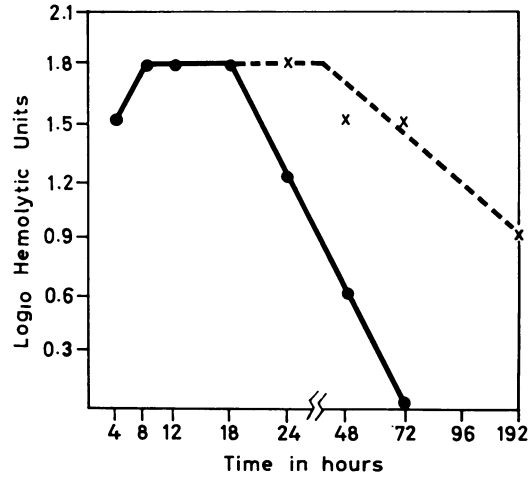

FIG. 3. *Activation of denatured hemolysin by $Na_2S_2O_4$. Symbols: ●—●—● = detectable hemolysin; ×--×--× = total hemolysin (reduced).*

The higher hemolysin titers obtained by the agar plate method might be related to this action of the hydrocolloids. However, it does not explain the rapid deterioration of hemolysin which occurs with this method after 24 hr of incubation, as shown earlier. It is possible that the degree of agar solidification might be an important factor in preventing deterioration.

Influence of initial pH of agar medium. No significant difference was observed with pH values from 5 to 8. At pH 9, most strains grew scantily and hemolysin production was poor. The hemolytic extracts were uniformly in the range of pH 5.5.

TABLE 2. *Effect of hydrocolloids on titer and deterioration in broth at 37 C*

Hydrocolloid added	Time of incubation (hr)					
	Strain 452			Strain 7648		
	18	72	120	18	72	120
Furcellaran (0.1%)	64	64	32	1,024	512	256
Carrageenan (0.1%)	0	8	4	32	8	8
Na carboxy-methylcellulose (cellulose gum; 0.3%)	128	256	64	1,024	2,048	256
Sodium alginate (0.3%)	256	512	128	2,048	1,024	128
None	32	2	0	256	16	0

TABLE 3. *Effect of repeated subculture on hemolysin production**

Strain	Time retested (months)†			
	0	2	4	6
7648	2,048	2,048	4,096	2,048
7973	4,096	2,048	512	128
452	512	1,024	128	32
354	128	64	16	4

* Results are expressed as minimal hemolytic units.

† Each strain was subcultured eight times (once a week) between the 2-month intervals.

Lytic action on other erythrocytes. The action of the hemolysin was essentially identical on guinea pig, cow, sheep, horse, and human red blood cells. Slightly higher titers were obtained with rabbit red blood cells.

Biological variation. As has been described for *Staphylococcus* (Bigger, 1933) and for *Pneumococcus* (Cowan, 1934), *Listeria* strains could lose either completely or partially their ability to produce hemolysins. Although an increase was not observed in the present study, this might possibly occur under proper conditions, as yet undetermined.

Some strains retested at intervals of 2 months over a 6-month period gave similar results, whereas others varied considerably (Table 3). A similar variability was noted when colonies were picked from a single strain, and each colony tested for hemolysin production (Table 4). This extreme variation within a single strain is disturbing and difficult to control but is not unexpected.

Survey of hemolysin production of 112 strains. From the data presented in Fig. 4, some conclusions could be derived. Under the conditions of these studies, more strains (7.2%) produced hemolysins in the range of 1,024 to 4,096 MHU by the agar plate method than in broth (2.0%). Whereas about 9.0% failed to produce any detectable hemolysin on solid medium, 11% failed in broth. The majority of the strains, about 62.3% on solid medium and 56% in broth, produced hemolysins with titers ranging from 16 to 128 MHU.

It was also observed that the ability to produce soluble hemolysin in broth is not concomitant with hemolysis on blood agar plate.

Of the eight strains producing high-titered hemolysins on solid medium, six were of decreased virulence. It has been reported that all newly isolated strains of *L. monocytogenes* are hemolytic. Although it was not possible to test this, it was possible to simulate the condition by serial passage in mice. Cultures isolated from artificially infected moribund or dead mice were tested for soluble hemolysin production. As shown in Table 5, ability to produce high-titered hemolysin decreased with continued passage. Although the number of strains tested was small, the consistency of the results indicated that, in some cases, increased virulence might decrease soluble hemolysin production.

Qualitative differences in hemolytic action. The hemolytic action pattern exhibited by the 112 strains seemed to follow three modes, when in-

TABLE 4. *Variation in hemolysin production within strains**

Strain	Isolates									
	1	2	3	4	5	6	7	8	9	10
7648	2,048	2,048	2,048	1,024	4,096	1,024	2,048	4,096	4,096	2,048
7973	32	1,024	128	16	2,048	2,048	4,096	512	1,024	16
452	128	32	512	512	0	1,024	0	0	0	128
354	0	0	8	0	64	512	0	128	8	64

* Results are expressed as minimal hemolytic units.

cubated at temperature and time shown in Table 6. The hemolysin was obtained from plate cultures.

Pattern I. Only 4% of the strains produced hemolysin which acted rapidly at room temperature, almost attaining the end point within 1 hr. The hemolysis was sparkling and not enhanced greatly by incubation at 37 C or overnight incubation at 10 C.

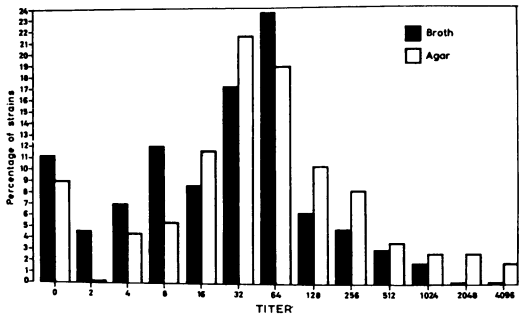

FIG. 4. *Hemolysin production by 112 strains of Listeria monocytogenes in Brain Heart Infusion medium (18 hr at 37 C).*

TABLE 5. *Effect on hemolysin production of serial passage in mice*

Strain	Passage*			
	0	1st	2nd	3rd
452	64	32	8	0
354	32	32	2	0
1122-3†	256	1,024	64	16
7973	256	512	64	8

* Mice were injected intraperitoneally with living culture and killed when moribund. The spleen or liver was seared, an inoculating needle introduced into the tissue, and inoculated in Brain Heart Infusion broth. After 18 hr at 37 C, culture was checked for purity and tested for soluble hemolysin.

† Supplied by M. L. Gray.

TABLE 6. *Qualitative differences in hemolytic action, as shown by hemolysis rate patterns**

Time and temp of incubation	Pattern		
	I	II	III
1 hr at 20 to 25 C....	128	TR†	0
1 hr at 37 C.....	256	64	8
Overnight at 4 C....	512	64	64
Approximate percentage of strains.....	4	75	11

* Results shown for each pattern are for a typical strain. Strains shown are 7973 for pattern I, 452 for pattern II, and 5348 for pattern III.

† Slight hemolysis.

TABLE 7. *Effect of filtration (Seitz) on titer**

Strain	Before filtration	After filtration	Filtered + $Na_2S_2O_4$
7648	2,048	64	512
1122-3	2,048	256	1,024
1R200,000	512	8	128
5348	64	TR	32
452	64	TR	32

* Results are expressed as minimal hemolytic units. TR = slight hemolysis.

Pattern II. About 75% of the strains produced hemolysin which showed some lysis at 20 C within 1 hr. Unlike pattern I, hemolysis increased greatly with incubation at 37 C for 2 hr.

Pattern III. In 11% of the cultures, little or no lysis occurred at 20 C, but at 37 C there was evidence of lysis characterized by discoloration of the red blood cells. This discoloration cleared and the hemolysis became sparkling after incution at 10 C. In view of the recent reports of beta-conditioning factors elaborated by some strains of *L. monocytogenes* (Gray, 1961; Fraser, 1962), it is speculated that beta-lysins similar to those elaborated by *Staphylococcus* are produced by some *Listeria* strains.

At present, it is not possible to determine whether these patterns represent differences in the nature of the hemolysin of *L. monocytogenes* or of differing degrees of inactivation of the hemolysin. Studies are planned to explore these possibilities.

Effect of filtration on the stability of hemolysin. Filtration through Seitz, Selas, or sintered-glass filters inactivated the hemolysin. The extent of inactivation was dependent on the initial concentration of hemolysin and appeared to result from oxidation of the hemolysin (Table 7). Sometimes the hemolysins were reactivated to their initial potency by addition of sodium thiosulfate.

The use of a Millipore filter under positive pressure obviated inactivation.

Effect of heat. Thermal inactivation was dependent on concentration and time and temperature of incubation (Table 8). One CHU could be inactivated in 30 min at 56 C. This heat lability was one indication of the protein nature of the

TABLE 8. *Heat lability of hemolysin**

Expt	Hemolytic units	Temp (C)							
		52		56		60		65	
		10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min
1	2,048	+	+	+	+	+	+	+	TR
2	1,024	+	+	+	+	+	+	+	−
3	256	+	+	+	+	+	TR	−	−
4	1	+	+	+	−	−	−	−	−
5	1	+	+	TR	−	−	−	−	−
6	1	+	+	+	−	−	−	−	−

* Symbols: + = 50% or more of 1% sheep red blood cells lysed; TR = slight hemolysis; − = no hemolysis.

hemolysin. Thermally inactivated hemolysin could not be reactivated with sodium thiosulfate.

Other factors affecting the stability of hemolysin. A concentration of 0.25% formalin inactivated the hemolysin in 3 to 5 days at 37 C.

Bubbling oxygen through the hemolysin for 4 hr produced varying degrees of inactivation, depending on the initial concentration of the hemolysin, suggesting that reduced sulfide groups might be activity centers of the hemolysins. These hemolysins were reactivated by sodium thiosulfate.

The hemolysin could be stored in the lyophilized state for 6 months without significant loss of activity. Similar stability was obtained when hemolysin was stored at −20 C in nearly filled screw-cap tubes. However, if the tubes were only half filled and stored at 4 C, the hemolysin deteriorated rapidly with only half activity left after 2 to 3 weeks.

Nature of the hemolysin. The hemolysin is definitely protein in nature. The fraction obtained with ammonium sulfate between 66 and 33% saturation was most active.

About 90% of the activity was found in a euglobulin fraction, which precipitated when salts had been dialyzed out against distilled water. The other 10% was found in a fraction that remained in solution.

The hemolysin was partially purified by adsorption and elution from calcium phosphate gel (Herbert and Todd, 1941). This increased the activity nearly 150 times on a volume-to-volume basis.

Paper electrophoresis resolved two fractions. One had a relative mobility of gamma-globulins; the other migrated more rapidly (even more so then the albumin fraction of rabbit serum used as a reference). The gamma-globulin fraction contained 90 to 95% of the activity.

Preliminary studies indicated that electrophoresis with starch gel (Robinson, Thatcher, and Gagnon, 1958) could be used to isolate larger quantities of purified hemolysin. It is now being used to obtain hemolysin for further studies on biochemical and biophysical properties.

Qualitatively, the isolated hemolysin reacted very strongly in the biuret test. The Molisch test was very weak. Before hydrolysis, the ninhydrin test was weak, but became very strong after hydrolysis.

The hemolysin produced specific antibodies in the rabbit. The antisera were highly active in the tube precipitation test, forming visible precipitates with as little as 0.1 μg of partially purified hemolysin. These antisera were also antihemolytic in very high dilutions. In one instance, an antihemolysin serum diluted 10^5 completely inhibited five CHU of test hemolysin, whereas the preimmunization serum was antihemolytic at a dilution of only 1:50.

Biological effects. No toxicity was noted when mice were injected intravenously with up to 3 mg of crude hemolysin, or when 15 mg were injected intraperitoneally. Also, there was no detectable skin reaction in the rabbit. This might be a reflection of the antihemolytic activity of normal sera or peritoneal and purulent exudates (Table 9).

Peritoneal exudates were induced with glycogen and with live *Listeria* cells, and harvested 24 hr after exposure. After centrifugation at 3,020 $\times$ *g* in a Servall RC-2 centrifuge with SS-34 rotor at 8 C, the supernatants were tested for antihemolytic activity.

Attempts to demonstrate the presence of hemolysins produced in vivo in peritoneal exudates induced with living *Listeria* cells were unsuccessful.

Preliminary results with glycogen-induced peritoneal exudate cells, which consisted predominantly of polymorphonuclear leukocytes, indicate that the hemolysin exerts a possible toxicity of a lytic nature. For this study, leukocytes were washed three times with cold Hank's solution and suspended in the same solution in

TABLE 9. *Antihemolytic activity of biological fluids*

Fluid	Complete hemolytic units	Strain			
		7648	5348	452	7973
Sera					
Guinea pig	1	>512*	256	>512	256
Rabbit	1	128	128	>512	64
Cow	1	>512	512	>512	>512
Sheep	1	128	256	256	32
Human	1	>512	512	>512	>512
Pus					
Dog	4	512	256	512	1,024
Peritoneal exudate					
Glycogen	4	128	128	256	128
Listeria	4	>8,192	>8,192	>8,192	>8,192

* Highest dilution inhibiting lysis.

silicone-coated screw-cap tubes. To a 2-ml sample, an equal volume of purified hemolysin in phosphate-buffered saline (pH 6.5) containing 1,000 CHU/ml was added and incubated in a water bath (37 C). As control, an equal volume of buffered saline was added in place of hemolysin. Total cell counts were made at the beginning and at 30-min intervals for 2 hr. During this period, cell populations exposed to hemolysin showed a reduction of approximately 90%, contrasting significantly with 15% in the control. A more detailed study of this is in progress. It has been proposed that a cytolytic factor is operative in interactions of sheep peritoneal exudate cells and *L. monocytogenes* in vitro (Njoku-Obi and Osebold, 1962). It is possible that this factor might be elaborated by the bacterium within the phagocyte to provoke the extensive lysis of normal sheep exudate cells observed in that study.

The rather high antihemolytic property of normal sera from various animal species suggests that the antilisteriolysin test has little value for serological diagnosis of listeriosis.

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