JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1982, p. 503–507
0095-1137/82/030503-05$02.00/0

Vol. 15, No. 3

Routine Test for In Vitro Differentiation of Pathogenic and Apathogenic *Listeria monocytogenes* Strains

BORIS SKALKA,* JIŘÍ SMOLA, AND KAMILA ELISCHEROVÁ

Department of Epizootiology and Microbiology, School of Veterinary Medicine, 612 42 Brno, and Research Institute for Preventive Medicine, 809 58 Bratislava, Czechoslovakia

Received 9 July 1981/Accepted 5 October 1981

The exosubstance of *Rhodococcus equi* in a prepurified form strongly enhanced the hemolytic effect of certain strains of *Listeria monocytogenes*. The strains which produced positive synergic hemolysis with this exosubstance were also pathogenic for guinea pigs and white mice. The other strains, which remained nonhemolytic in the presence of the *R. equi* exosubstance, were apathogenic for those animals. A routine test was devised for the in vitro determination of the pathogenicity of *L. monocytogenes* strains.

Listeria monocytogenes, well known for its antigenic and pathogenic heterogeneity, is now regarded as a single species (18) even though there are reasons for distinguishing two or even three separate species (13, 20). Apart from the existing species, also called the "classical" pathogen (9, 10, 16), the recognition of another previously described (11) pathogen, *Listeria bulgarica*, has been proposed (12). It is especially recommended that the apathogenic strains be recognized as the species *Listeria innocua* (16). However, the apathogenicity of this species is not absolute (15). Besides pathogenicity and antigen structure, there is a further common characteristic of the proposed species: hemolytic activity. The colonies of *L. monocytogenes* are generally said to be surrounded by a zone of hemolysis when cultivated on agar with ovine erythrocytes. Only the strains of serotype 5 (*L. bulgarica*) have colonies surrounded by a zone of total hemolysis and a peripheral zone of partial hemolysis. An exosubstance producing partial hemolysis is similar to staphylococcal beta toxin (3). Phospholipase C production has been established in an *L. monocytogenes* strain (14), and the production of phospholipase D has also been described (19). Strains of serotype 6 (*L. innocua*) are nonhemolytic (16). Hemolysis of some classic pathogenic *L. monocytogenes* strains is, however, not particularly striking, and it is recommended that staphylococcal beta toxin be used to increase hemolytic activity (4, 7, 9, 10, 14). A still stronger hemolytic effect was described in the zone of activity of the exosubstance of *Rhodococcus* (*Corynebacterium*) *equi* (5, 8, 12). This exosubstance, more recently designated *equi*-factor (1, 2), does not hemolyze alone (5, 8). The subject of this study is the establishment of a routine test for the precise differentiation of hemolytic and nonhemolytic *L. monocytogenes* strains and the correlation of hemolytic activity to pathogenicity.

MATERIALS AND METHODS

Media. The media used were nutrient agar CM 3, brain heart infusion CM 225, brain heart infusion agar CM 375, and Columbia agar base CM 331, all from Oxoid Ltd. For hemolytic tests, 5% washed ovine erythrocytes were added to the agar base. Nalidixic acid and acriflavin were added to some media as previously described (6).

Bacterial strains. The strains used were *R.* (*Corynebacterium*) *equi* NCTC 1621, *Staphylococcus aureus* CCM 6188, previously known as K-126 (17), and the following *L. monocytogenes* strains from the Czechoslovak Collection of Microorganisms (CCM) and from the laboratory of one of the authors of this paper: (i) 64 strains of serotype 1/2 without determination of H antigens; (ii) 4 strains of serotype 1/2a, including CCM 5576; (iii) 1 strain of serotype 1/2b (CCM 5879); (iv) 2 strains of serotype 1/2c, 1 of which was CCM 5577; (v) 1 strain each of serotypes 3a (CCM 5578), 3b (CCM 5880), 3c (CCM 5881), and 4a (CCM 5779); (vi) 8 strains of serotype 4b, 1 strain of serotype 4c, and 1 strain of serotype 4d (CCM 5883); (vii) 6 strains of serotype 5, including CCM 5884; (viii) 46 strains of serotype 6, including CCM 5885, without determination of H antigens, 21 strains of serotype 6a, and 2 strains of serotype 6b; and (ix) 1 strain of serotype 7 (CCM 5886). The hemolytic properties of the listeria strains used were assessed on ovine blood agar.

Prepurified bacterial exosubstances. Beta toxin was obtained from the above-mentioned *S. aureus* strain by the cellophane-on-agar technique and subsequent precipitation with acetone as described previously (17). The determination of activity units (AU) was performed by making use of a tube test with ovine erythrocytes and the hot-cold effect. To obtain the exosubstance of *R. equi* (ERE), the same method used for staphylococcal beta toxin was used. The assay for the activity of ERE was based on synergistic hemolysis with staphylococcal beta toxin. To the tubes containing a series of ERE dilutions we added a suspension of ovine erythrocytes that had been exposed for

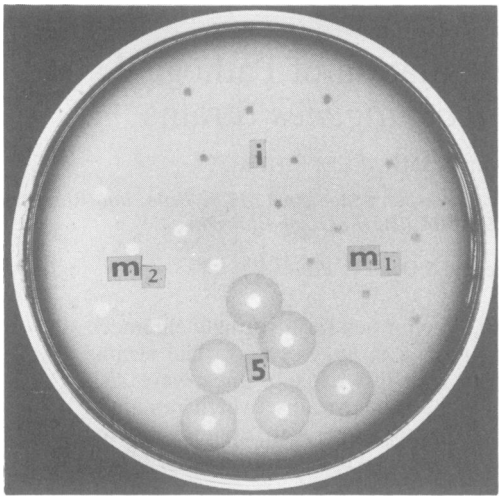

FIG. 1. Quadrants on ovine blood agar with colonies of the following strains: *i*, *L. monocytogenes* serotype 6 (*L. innocua*); m_1 and m_2, classical pathogenic *L. monocytogenes* strains with different hemolytic activities; and 5, a strain of serotype 5 (*L. bulgarica*).

20 min at room temperature to staphylococcal beta toxin. The volume of the mixture in each tube was 1 ml, and the concentration of the erythrocytes corresponded to 1%; the amount of staphylococcal beta toxin was 5 AU/ml. An AU was the final concentration of ERE which gave complete hemolysis within 1 h at room temperature.

RESULTS

The hemolytic activity of all listeria strains was examined on ovine blood agar. Punctiform cultivation was used since it gives results that can be more easily evaluated than those obtained from cultivation in streaks. The strains of serotype 5 grew in colonies surrounded by a narrow zone of total hemolysis and a broad peripheral zone of partial hemolysis, resembling the effect of staphylococcal beta toxin. A second group of strains grew in colonies surrounded by a narrow ring of total hemolysis, whereas the rest of the strains failed to hemolyze (Fig. 1).

On the basis of these results, the pathogenicity of all listeria strains on the eyes of guinea pigs and in mice inoculated intraperitoneally was investigated. The strains represented by *i* on Fig. 1, belonging to serotype 6, were apathogenic for both kinds of laboratory animals. The strains of other serotypes were pathogenic and caused death in white mice and conjunctivokeratitis in the eyes of guinea pigs. With regard to hemolytic activity, the pathogenic strains formed three groups: the serotype 5 group, with characteristic bizonic hemolysis, represented by 5 on the plates; the group represented by m_2, which produced a narrow zone of total hemolysis around its colonies; and the nonhemolytic group represented by m_1.

A further series of experiments was used to test the synergistic hemolysis of listeria strains with strains of *S. aureus* and *R. equi* (Fig. 2). The strains represented by *i* on Fig. 2 were nonhemolytic in the staphylococcal beta toxin zone and in the ERE zone. Strains represented by m_1 produced only slight hemolysis in the staphylococcal beta toxin zone and a negative or slightly positive hemolytic effect in the ERE zone. The listeria strains represented by m_2 expressed synergistic hemolysis in the zones of both bacterial indicator strains, especially in the ERE zone. Hemolysis of strains of serotype 5 was not affected in the *S. aureus* beta toxin zone but was intensively enhanced in the ERE zone.

In additional assays, *S. aureus* and *R. equi* were replaced by staphylococcal beta toxin and ERE. Both exosubstances were adjusted to a concentration of 2,000 AU/ml and were streaked on the surface of blood agars. The listeria strains were cultivated in streaks perpendicular to those of the exosubstances (Fig. 3). The strains represented by *i* grew without hemolytic effect. The pathogenic listeria strains without hemolytic activity of their own, represented by m_1, were slightly hemolytic in the staphylococcal beta toxin zone and gave a substantial hemolytic reaction in the ERE zone. The strains represented by m_2 had slightly enhanced hemolysis in the staphylococcal beta toxin zone and strongly expressed synergistic hemolysis in the ERE zone. The hemolytic activity of serotype 5 strains was not affected by staphylococcal beta

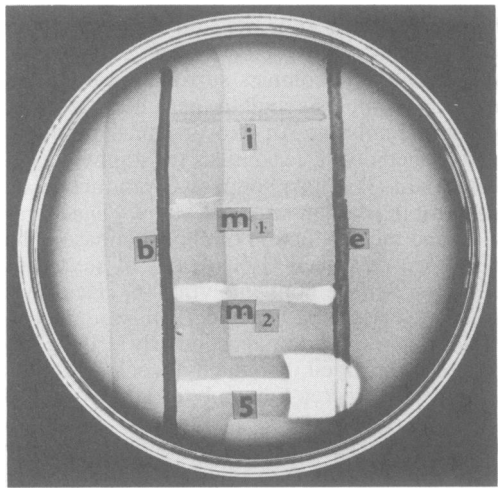

FIG. 2. Same *L. monocytogenes* strains as described in the legend to Fig. 1 on ovine blood agar. Their streaks lead perpendicularly to the beta toxin-producing *S. aureus* strain (*b*) and the *R. equi* strain (*e*).

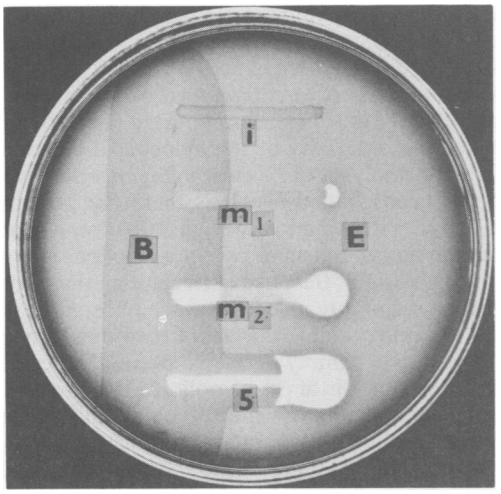

FIG. 3. Same listeria strains as described in the legend to Fig. 1 and 2 on ovine blood agar. The streaks lead perpendicularly to prepurified staphylococcal beta toxin (*B*) and prepurified ERE (*E*). The hemolytic effects of the interactions are pronounced.

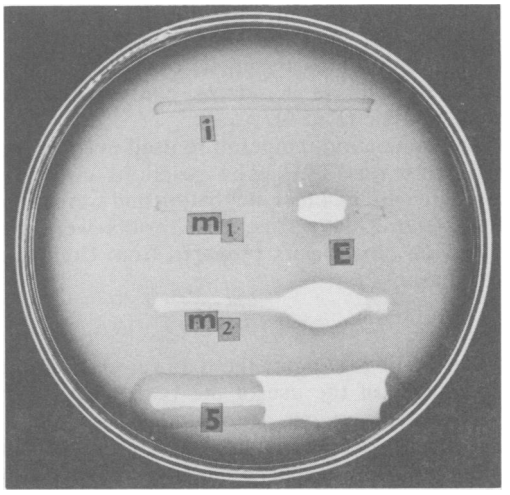

FIG. 4. Same listeria strains as described in the legends to Fig. 1, 2, and 3 cultivated across a streak of prepurified ERE (*E*). The hemolytic interactions are evident.

toxin but was strongly enhanced in the ERE zone.

In view of these results, a cross-streak technique was used for demonstrating the synergism produced by ERE and the pathogenic strains of *L. monocytogenes*. With this technique, an increased intensity of positive reactions of the pathogenic strains was obtained, whereas the apathogenic strains of serotype 6 remained nonhemolytic (Table 1, Fig. 4).

The results of cultivation on ovine blood agar containing 5 AU of ERE per ml are shown in Fig. 5. The colonies of serotype 5 and those represented by m_2 were surrounded by a broad zone of total hemolysis; the colonies represented by m_1 were surrounded only by a narrow zone. The colonies of serotype 6 grew without hemolysis.

The final assay was made on medium the same as that described above but supplemented with acriflavin and nalidixic acid, which negatively influenced the hemolytic effect of the listeria strains. The hemolysis least affected was that of serotype 5 strains. There was a marked decrease

TABLE 1. Results of hemolytic interaction with ERE and determination of pathogenicity of *L. monocytogenes* strains

L. monocytogenes serotype	No. of strains tested	Hemolytic reaction with ERE (+/−)	Pathogenicity for:	
			Guinea pig eyes (+/−)	Mice injected intraperitoneally (+/−)
1/2	64	64/0	64/0	64/0
1/2a	4	4/0	4/0	4/0
1/2b	1	1/0	1/0	1/0
1/2c	2	2/0	2/0	2/0
3a	1	1/0	1/0	1/0
3b	1	1/0	1/0	1/0
3c	1	1/0	1/0	1/0
4a	1	1/0	1/0	1/0
4b	8	8/0	8/0	8/0
4c	1	1/0	1/0	1/0
4d	1	1/0	1/0	1/0
5	6	6/0	6/0	6/0
6	46	0/46	0/46	0/46
6a	21	0/21	0/21	0/21
6b	2	0/2	0/2	0/2
7	1	1/0	1/0	1/0

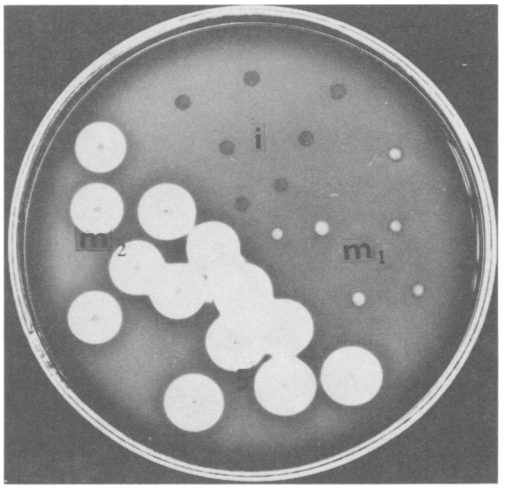

FIG. 5. Colonies of *Listeria* strains on ovine blood agar containing 5 AU of ERE per ml. Strains 5 and m_2 are surrounded by a broad zone of total hemolysis. Strain m_1 has only a narrow hemolytic zone, and strain *i* is nonhemolytic.

in the hemolytic manifestation of strains represented by m_2, and the hemolytic effect of the strains represented by m_1 was completely suppressed. Serotype 6 strains continued to be nonhemolytic (Fig. 6).

The incubation temperature used in these experiments was 37°C. The reactions could be evaluated after 24 h of incubation and were quite clear after 2 days. The best results were obtained on blood agars prepared from Columbia agar base.

DISCUSSION

Our findings support the data referring to the advantages of the use of the hemolytic synergism of *L. monocytogenes* and *R. equi* (5, 8) over the analogous cooperative hemolysis of the beta toxin of *S. aureus* (4, 9, 10). The possibility of distinguishing *L. monocytogenes* from *Erysipelothrix rhusiopathiae* by means of interaction with *R. equi* (5) is extended here to include the feasibility of differentiation within the species *L. monocytogenes* or a simple method for differentiating newly proposed species of the genus *Listeria* (12, 16). The use of prepurified exosubstance seems to be superior to the use of the bacterial strain since the synergistic hemolysis is more pronounced and the results can be standardized by adjusting for various AU. Since only prepurified ERE was used in our study, we should choose a designation different from *equi*-factor, the latter being recommended for the purified product (1, 2). This factor was characterized as phospholipase C (2) and was undoubtedly the main component of the prepurified substance, which we called ERE. The fact that some strains of *L. monocytogenes* produce phospholipase C has been reported (14), and the functional similarity of staphylococcal beta toxin and hemolysin of *L. monocytogenes* serotype 5 has been studied (3). It is therefore possible that the phenomenon we describe represents hemolytic synergism of two phospholipase Cs of different origin. Because of the finding that some listeria strains are able to produce phospholipase D (19), it is possible that more than one form of cooperative hemolysis takes place. Nevertheless, the analysis of the hemolytically synergistic phenomenon was not the subject of this paper. We intended to elaborate a routine test for in vitro determination of *L. monocytogenes* pathogenicity. A correlation was found between the pathogenicity of a listeria strain and its hemolytic behavior in the zone of activity of ERE. The routine test developed is suitable for use on recently isolated strains as well as for the identification of laboratory strains and may well prove itself in the differentiation of *L. monocytogenes* strains.

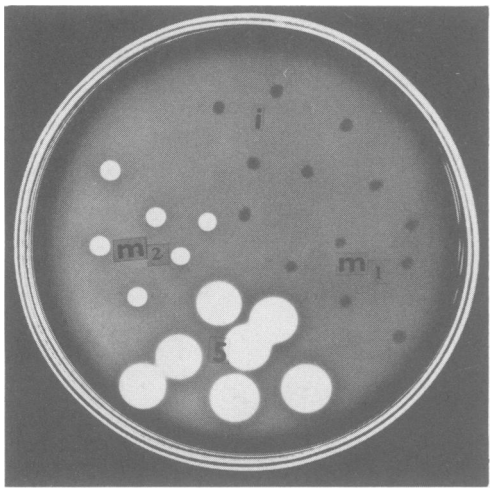

FIG. 6. Growth on medium the same as that described in the legend to Fig. 5 but supplemented with acriflavin and nalidixic acid. Strains 5 and m_2 show hemolysis, although weaker than in medium without inhibitors. The hemolysis of strain m_1 is suppressed, and strain *i* is nonhemolytic.

ACKNOWLEDGMENTS

We thank H. P. R. Seeliger of the Listeria Reference Laboratory Würzburg for the serotyping of some of the strains, the Czechoslovak Collection of Microorganisms for providing the strains, and A. Horňáčková for excellent technical assistance.

LITERATURE CITED

1. **Barksdale, L., R. Linder, I. T. Sulea, and M. Pollice.** 1981. Phospholipase D activity of *Corynebacterium pseudotuberculosis* (*Corynebacterium ovis*) and *Corynebacterium ulcerans*, a distinctive marker within the genus *Corynebacterium*. J. Clin. Microbiol. **13**:335–343.
2. **Bernheimer, A. W., R. Linder, and L. S. Avigad.** 1980. Stepwise degradation of membrane sphingomyelin by corynebacterial phospholipases. Infect. Immun. **29**:123–131.
3. **Brzin, B.** 1977. *Listeria monocytogenes* serovar 5 in the CAMP test, p. 133–135. *In* I. Ivanov (ed.), Proceedings of the seventh international symposium on the problems of listeriosis, 1977. National Agroindustrial Union Center for Scientific Informations, Sofia, Bulgaria.
4. **Brzin, B., and H. P. R. Seeliger.** 1975. A brief note on the CAMP phenomenon in *Listeria*, p. 34–37. *In* M. Woodbine (ed.), Problems of listeriosis. Leicester University Press, Leicester, Great Britain.
5. **Buxton, A., and G. Fraser.** 1977. Animal microbiology, vol. 1. Blackwell Scientific Publications, Ltd., Oxford, Great Britain.
6. **Elischerová, K.** 1975. *Listeria monocytogenes* findings in raw materials of animal origin. Čs. Epidemiol. **24**:47–53. (In Czechoslovakian.)
7. **Fraser, G.** 1962. The effect of staphylococcal toxins on ruminant erythrocytes treated with diffusible substances formed by corynebacteria. Vet. Rec. **74**:753–760.
8. **Fraser, G.** 1964. The effect on animal erythrocytes of diffusible substances produced by bacteria. J. Pathol. Bacteriol. **88**:43–53.
9. **Groves, R. D., and H. J. Welshimer.** 1977. Separation of pathogenic from apathogenic *Listeria monocytogenes* by three in vitro reactions. J. Clin. Microbiol. **5**:559–563.

10. **Groves, R. D., and H. J. Welshimer.** 1977. Separation of pathogenic from apathogenic *Listeria monocytogenes* (U.S.A. strains) by three in vitro reactions, p. 43–52. *In* I. Ivanov (ed.), Proceedings of the seventh international symposium on the problems of listeriosis, 1977. National Agroindustrial Union Center for Scientific Informations, Sofia, Bulgaria.
11. **Ivanov, I.** 1962. Untersuchungen über die Listeriose der Schafe in Bulgarien. Monatsh. Veterinaermed. **17**:729–736.
12. **Ivanov, I.** 1975. Establishment of non-motile strains of *Listeria monocytogenes* type 5, p. 18–26. *In* M. Woodbine (ed.), Problems of listeriosis. Leicester University Press, Leicester, Great Britain.
13. **Ivanov, I. (ed.)** 1979. Proceedings of the seventh international symposium on the problems of listeriosis, 1977. National Agroindustrial Union Center for Scientific Informations, Sofia, Bulgaria.
14. **Leighton, I., D. R. Threlfall, and C. L. Oakley.** 1975. Phospholipase C activity in culture filtrates from *Listeria monocytogenes* Boldy, p. 239–241. *In* M. Woodbine (ed.), Problems of listeriosis. Leicester University Press, Leicester, Great Britain.
15. **Patočka, F., E. Menčíková, H. P. R. Seeliger, and A. Jirásek.** 1979. Neurotropic activity of a strain of *Listeria innocua* in suckling mice. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **243**:490–498.
16. **Seeliger, H. P. R., and M. Schoofs.** 1977. Serological analysis of non-hemolyzing Listeria-strains belonging to a species different from *Listeria monocytogenes*, p. 24–28. *In* I. Ivanov (ed.), Proceedings of the seventh international symposium on the problems of listeriosis, 1977. National Agroindustrial Union Center for Scientific Informations, Sofia, Bulgaria.
17. **Skalka, B., J. Smola, and J. Pillich.** 1979. Diagnostical availability of the hemolytically active exosubstance of *Corynebacterium pseudotuberculosis* for isolation and identification of *Streptococcus agalactiae* and its comparison with the beta-toxin of *Staphylococcus aureus*. Zentralbl. Veterinaer med. Reihe B **26**:679–687.
18. **Skerman, V. B. D., V. McGovan, and P. H. A. Sneath (ed.).** 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. **30**:225–420.
19. **Souček, A., F. Patočka, and A. Součková.** 1969. Production of extracellular toxin by *Listeria monocytogenes* in vitro. J. Hyg. Epidemiol. Microbiol. Immunol. **13**:271–272.
20. **Woodbine, M. (ed.).** 1975. Problems of listeriosis. Leicester University Press, Leicester, Great Britain.