IDENTIFICATION OF PLAGUE BACTERIA BY MEANS OF ANTIPLAGUE ALIZARIN SUSPENSION AGGLUTININS

A. K. Adamov, L. M. Gol'dfarb, and N. I. Kuznetsova

All-Union Scientific Research Institute "Mikrob" (Scientific Consultants, Corresponding Member AMN SSSR Professor A. Ya. Alymov and Candidate of Medical Sciences G. N. Lenskaya), Saratov
(Presented by Active Member AMN SSSR N. N. Zhukov-Verezhnikov)
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Despite the large amount of work on the problem, there is still considerable difficulty in distinguishing between plague and pseudotuberculosis bacilli in rodents.

Serological analysis, which has been widely used for distinguishing different species of bacteria, failed through lack of specificity [3-5, 9].

However, N. N. Zhukov-Verezhnikov [6], A. N. Urazova [8], and G. N. Lenskaya [7] have shown that capsular antiplague serum may be used for the differential diagnosis of the plague bacteria and the pseudotuberculosis bacilli of rodents.

Similar results were obtained by Winter and Moody [10], who used a fluorescent serum against the capsular antigens.

We here present the results of applying the agglutinizing reaction of alizarin suspension agglutinins for the identification of plague bacteria.

Antiplague alizarin suspension agglutinins were made from rabbit antiplague agglutinating sera. To immunize the rabbits we used bacteria of the vaccine strain P. pestis. They were grown in a fluid medium at 37° for 48 hours (the medium consisted of: 900 ml of Zerensen's phosphate buffer mixture at pH 7.0, diluted 1 : 20 with distilled water; peptone 10 g, glucose 2 g, Hottinger's broth 100 ml; final pH of medium 7.0; the mixture was sterilized in an autoclave at a vapor pressure of 0.5 atmospheres for 20 minutes). The bacteria from the 48 hour culture were centrifuged, the nutritive medium was decanted, and the sediment suspended in physiological saline, added so that 1 ml contained 30 billion cells. To 100 ml of the bacterial suspension was added 1 ml of 40% formalin, and the mixture was kept at 18-20° for 24 hours. The suspension of dead bacteria was injected intravenously into rabbits three times daily, as follows: first injection 15 billion, second 30 billion, and third 45 billion cells. The rabbits were exsanguinated seven days after the third injection. The serum was preserved in 1% boric acid. The antiplague agglutinating sera obtained in this way had agglutination titers against plague bacteria of 1 : 200-1 : 400; against pseudotuberculosis bacteria of rodents the titer was 1 : 100-1 : 400.

To eliminate the group agglutinins, the antiplague agglutinating sera were exposed to adsorption by the pseudotuberculosis bacteria. For this purpose we used a suspension of rodent pseudotuberculosis bacteria containing 40 billion cells per ml. The bacteria were grown for 48 hours at 37° on meat-peptone agar at a pH of 7.2. To one portion of the suspension of rodent pseudotuberculosis bacteria we added 1 ml of 40% formalin per 100 ml suspension, and to another portion an equal volume of 96% ethyl alcohol. The bacterial adsorbants were kept for 24 hours at 18-20°. Before adsorption of the antiplague agglutinating sera the cells were centrifuged until the sediment contained 80 billion cells. Before adsorption, the sera were diluted 1 : 5 with physiological saline containing 1% boric acid. To 5 ml of the agglutinating serum diluted 1 : 5 were added first 80 billion rodent pseudotuberculosis bacteria killed with formalin, then 80 billion of the same bacteria killed with alcohol. The procedure was as follows: first the sediment of rodent pseudotuberculosis bacteria killed by formalin was suspended in 5 ml of the adsorbed serum, and then in the same serum was suspended the sediment of rodent pseudotuberculosis bacteria killed with ethyl alcohol.

The mixture of the serum and bacterial cells was kept for one hour at 37°, and then centrifuged until the serum was completely transparent. The transparent serum was filtered through a Zeitz filter and an asbestos plate 1 mm thick. A second piece of filter paper was placed beneath the asbestos disk to prevent the entry of asbestos fibers.

The adsorption of the group agglutinins must be carried out so that the serum is filtered once only. Repeated filtering of agglutinating sera through asbestos filters reduces their effectiveness. Before sterilization, the asbestos plates were washed with 50-100 ml of distilled water, and before filtration of the sera with 100 ml of physiological saline containing 1% boric acid. Unwashed asbestos plates frequently contain substances which are soluble in the sera. Alizarin suspension agglutinins prepared from sera filtered through unwashed asbestos filters may cause spontaneous agglutination in physiological saline.

Sensitivity and Specificity of the Agglutination Reaction of Antiplague Alizarin Suspension Agglutinins

Species of microbe	Temperature at which the culture was grown	Number of strains tested	Number found	Minimal concentration of bacteria (millions per ml) found by means of RASA	Number of strains not revealed by RASA
P. pestis	37°	20	6	25	—
			13	10	
			1	5	
Ditto	28°	19	3	500	3
			8	250	
			2	100	
			2	50	
			1	25	
P. pseudotuberculosis rodentium	37°	19	—	—	19
Ditto	28°	19	—	—	19
V. cholerae asiaticae "inaba"	37°	1	—	—	1
Ditto "Ogava"	37°	1	—	—	1
S. paratyphi B.	37°	1	—	—	1
B. dysenteriae Flexneri	37°	1	—	—	1
B. coli	37°	1	—	—	1
Staphylococcus aureus	37°	1	—	—	1

Note. A dash indicates a negative result.

To prepare normal alizarin suspension globulins used to control the agglutinating reaction of the alizarin suspension agglutinins (RASA), normal (nonimmune) rabbit blood sera were used. Normal rabbit sera were adsorbed by rodent pseudotuberculosis bacteria in the same way as were the antiplague agglutinating sera, by means of a standard number of bacterial cells. To 5 ml of normal rabbit sera diluted 1 : 5 in physiological saline and 1% boric acid were added 80 billion rodent pseudotuberculosis bacteria killed in formalin.

Antiplague alizarin suspension agglutinins were prepared from the adsorbed antiplague agglutinating sera (of titer 1 : 200-1 : 400) prepared as described above. The synthesis of the antiplague alizarin suspension agglutinins and of the normal suspension globulins was carried out by a slightly modified version of A. K. Adamov's method [2].

To 10 ml of alizarin suspension prepared from twice-distilled water were added 2 ml of adsorbed agglutinating serum diluted 1 : 5 in physiological saline containing 1% boric acid. The mixture of the alizarin suspension with agglutinating serum was kept for two hours at 37°, and then placed for 18 hours in a refrigerator at 8°. After 18 hours in the cold the mixture of the agglutinating serum and the alizarin suspension was centrifuged, and to the sediment was added 10 ml of normal rabbit serum diluted 1 : 25 with physiological saline containing 0.5% boric acid. A similar method was used to prepare normal alizarin suspension globulins from normal rabbit sera. For preservation, to the suspension agglutinins and globulins we added 0.1-0.05 mg/ml of merthiolate. The alizarin suspension antibodies and the normal suspension globulins retained their activity for a year.

The antiplague alizarin suspension agglutinins and normal alizarin suspension globulins prepared by the method described above were tested for sensitivity and specificity with 20 strains of plague bacteria, 19 strains of rodent pseudotuberculosis bacteria, and 6 strains of other bacterial species. The experiments were carried out by the following method. From the 2 hour agar cultures, by means of an optical standard, a suspension was prepared of bacteria in physiological saline containing 500 million cells per ml: by dilution, suspensions containing 250, 100, 50, 25, 12.5, 10.5, and 2.5 million cells per ml were prepared.

RASA tests with the bacterial suspensions were carried out by A. K. Adamov's method [1]. The results of the reactions were assessed in 5-10 minutes.

We tested the cultures of rodent plague and pseudotuberculosis bacteria grown at 28 and 37°. Cultures of both species were grown at 37°. The result of the experiments with the suspensions of pure bacterial cultures are shown in the table.

It can be seen that agglutination reaction with antiplague alizarin suspension agglutinins takes place at a density of 5-25 million plague bacteria per ml grown at 37°. The RASA with plague bacterial suspensions grown at 28° occurs at a content of 25-500 million per ml or more of these bacteria. Then of the 19 strains of plague bacteria grown at 28°, three caused no agglutination reaction at a density of 500 million per ml.

Differences in the sensitivity of the antiplague alizarin suspension agglutinins shown by carrying out the agglutination reaction with plague cultures grown at 28° and 37° appear to depend on the amount of specific capsular antigen in the bacterial cells.

Antiplague suspension agglutinins did not react with rodent pseudotuberculosis bacteria, pathogenic intestinal bacteria, B. coli, or staphylococci.

Besides carrying out the RASA, we also tested 20 strains of plague bacilli and 19 cultures of the causative agent of rodent pseudotuberculosis in an agglutination reaction with adsorbed antiplague serum from which the antiplague suspension agglutinins were prepared. Of the bacteria tested, two strains of rodent pseudotuberculosis were agglutinated by the adsorbed antiplague serum, two strains of bacteria of the same species and three cultures of plague bacilli gave a nonspecific agglutination in physiological saline. In the RASA reaction with the antiplague suspension agglutinins, there was no nonspecific reaction.

Following the results given above, we developed the following method for the identification of plague bacilli.

The culture to be studied was grown on a meat-peptone agar slope at 37° for 1-2 days (according to the rate of growth). From the agar culture we prepared a suspension in physiological saline containing 500 million bacterial cells per ml (the concentration of cells was determined in terms of an optical standard). By dilution, suspensions containing 250 and 100 million bacteria per ml were prepared. With these suspensions we carried out the agglutination reaction with the antiplague alizarin suspension agglutinin, and with normal alizarin suspension globulin. Also, as a control of the specificity of both alizarin suspensions, they were tested with physiological saline for spontaneous agglutination. When there was no agglutination of the control suspension agglutinins or normal suspension globulins, the occurrence of a marked agglutination in the drops of antiplague suspension agglutinins mixed with bacterial suspensions of different concentrations indicated that the culture studied was one of plague bacteria.

The method of identification of plague bacteria by means of antiplague suspension alizarin agglutinins is specific and simple, and may be used in mobile laboratories.

SUMMARY

This paper deals with the results of using the agglomeration reaction of antiplague alizarin suspension for the identification of plague bacteria. We showed that antiplague alizarin suspension agglutinins could be used to differentiate plague bacteria from the causative agent of rodent pseudotuberculosis and from other bacterial species.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.
