Primary Isolation of Brucella From Human Blood Clots

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Clotted blood has been utilized by many laboratories as a dual-purpose type of specimen for aiding the physician in his differential diagnosis of brucellosis. The serum is used for the agglutination test, and the accompanying clot is cultured for the causative organism. Unfortunately, efforts at recovering *Brucella* from clots have not always proved productive.

In the bureau of laboratories of the Indiana State Board of Health, clot examination procedures were begun in the spring of 1946 as an adjunct to the seroagglutination test. Since that time more than 41,000 specimens have been examined serologically, and more than 10,000 of the accompanying clots cultured for *Brucella*. Experimentally, in vivo methods employing guinea pigs and embryonating eggs have at times supplemented the routine in vitro enrichment broth procedure.

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In conjunction with the Indiana brucellosis project, an opportunity was provided to study the over-all technique of clot culturing. Consequently, during the period of this study (1946–50, inclusive), efforts were directed toward improvement of procedures and the development of a more productive isolation method. This paper is a report of the experimental use of several different mediums in isolating *Brucella* from clots.

Isolation Procedures

Specimens employed in this study were clots from samples of human blood submitted routinely to the Indiana State Board of Health laboratories for one or more of the febrile agglutination tests. The serum from each clot was decanted and tested for *Brucella* agglutinins with a standardized *Brucella abortus* slide test antigen. If complete agglutination occurred in the 1:80 serum dilution, or in the latter part of the study at the 1:20 level, the clot was examined by at least two of the isolation procedures. Some clots from persons exhibiting no agglutinins, or only a low titer, were included because of the special significance of such clots.

With the exception of such divergences as will be noted, the following methods were employed throughout the project.

C-V Broth Enrichment

Approximately one-half of each clot was forced through a 5-ml. syringe, without the

needle, into crystal violet tryptose broth (C–V broth), made up as follows:

Tryptose (Bacto)	3.0 gm.
Dextrose (Bacto)	1.0 gm.
NaCl	.5 gm.
Thiamine HCl (Betabion-Merck)	.5 mg.
Distilled water	100.0 ml.
Agar (Difco, granular)	.1 gm.

The ingredients were dissolved in an Arnold sterilizer and when necessary the solution adjusted to a pH of 6.6 to 6.8 with 1 N NaOH. One-tenth milliliter of crystal violet dye (1:1,000 aqueous solution) was added and mixed thoroughly. The broth was dispensed in 10-ml. amounts and autoclaved 15 minutes at 115° C.

The clot and the broth were mixed thoroughly and incubated at 36° to 37° C. under an increased CO_2 atmosphere of approximately 10 percent. The blood clot-broth mixture was incubated one week and then subcultured to crystal violet tryptose agar plates (dye concentration 1:1,000,000). Three weekly subcultures were made from the broth before discarding it as negative. This method is further discussed in a report by Damon and Albright (*1*).

Guinea Pig Injection

The remaining half of the clot was forced through the syringe into 20 ml. plain tryptose broth (Difco dehydrated product), and about once a week the accumulated specimens were transported to the department of veterinary science, Purdue University. (These clot-broth mixtures were incubated under increased CO_2 tension until the time of shipment.) At the laboratory of the department of veterinary science, a 2-ml. aliquot of each of the specimens was injected subcutaneously into the groin region of each of two guinea pigs. These pairs of animals were maintained in separate cages, and quartered in a building separate from that housing the stock supply. A month following the injections, the pigs were autopsied. Samples of heart blood were collected for agglutination tests and the spleens were cultured for *Brucella*, since previous experience had shown the spleen to be the most consistent site of isolation. Each spleen was bisected and the exposed surfaces serrated before streaking onto a plain tryptose agar plate. The inoculated plates were incubated in an atmosphere of approximately 10 percent CO_2.

Yolk Sac Inoculation

The yolk sac inoculation technique, described in detail in another paper (*2*), consisted of injecting 0.5 ml. of comminuted blood clot-tryptose broth mixture into each of four embryonating White Leghorn eggs 3 to 5 days old. The injections were made with a syringe and 20-gauge needle directly into the yolk sacs through a small opening in the large end of the eggs.

Typing of Cultures

For typing the species of *Brucella* recovered as above, both serologic and biochemical reactions were determined. Each isolated strain was checked for CO_2 growth requirements, ability to produce H_2S, and dye inhibition on basic fuchsin and thionin plates (dye concentration 1:100,000). Strain antigens were tested against known *Brucella* antiserums.

Initial Use of Enrichment Broth and Guinea Pigs

Over a period of 3 years 1,698 human blood clots were examined both by guinea pig inoculation and by C-V broth enrichment as previously described. From these specimens 90 *Brucella* strains—61 *abortus*, 21 *suis* and 8 *melitensis*—were recovered, representing a 5.3-percent isolation.

In table 1, the 90 isolations have been tabulated as to the species and the method or methods of recovery. It is to be noted that only 26 of the 90 isolations, or 28.9 percent, were obtained by both broth enrichment and guinea pig inoculation of the same specimen. In other words, 64 *Brucella* strains, or 71.1 percent, were recovered by only one or the other of the techniques. This inconsistency appeared not to involve any one species.

From the foregoing results, it was evident that the two techniques for isolation were quite inconsistent in yielding duplicate recovery of *Brucella* strains. Although actually more recoveries were obtained from the C-V broth enrichment of the clots, the use of the guinea pigs added considerably to the total isolations.

As previously noted, that portion of the clot received by the guinea pigs was first inoculated into plain tryptose broth and held at 37° C. under increased CO_2 tension for several days

Table 1. Recovery of Brucella from C-V broth inoculated with clotted blood and from guinea pigs inoculated with clotted blood enriched in plain tryptose broth

Species	Guinea pig+ broth−	Guinea pig− broth+	Guinea pig+ broth+	Total recoveries from	
				Guinea pig	Broth
Br. suis	8	8	5	13	13
Br. abortus	16	28	17	33	45
Br. melitensis	2	2	4	6	6
Total	26	38	26	52	64
Percent isolations	28. 9	42. 2	28. 9	57. 8	71. 1
	Grand total=90 strains				

prior to injection. It seemed plausible that this preliminary enrichment of the inoculums might have been a determining factor in the incongruity of the results. The question then arose as to how efficient a medium of isolation the guinea pig would be if the clotted blood material were inoculated directly, following the same technique as before but excluding the initial enrichment of the clot.

The following experiment was then conducted in order to determine, if possible, the value of the guinea pig as an isolation medium when unenriched clotted blood was injected. Furthermore, since by this time an egg yolk sac inoculation technique was being used experimentally with some success, it seemed desirable to expand the study so as to compare this method with the guinea pig and C-V tryptose enrichment broth procedures.

Experimental Methods

The type of specimen was the same as previously employed, except that all clots from serums showing complete agglutination with the slide antigen at the 1:20 dilution level were included.

One-half of each clot was inoculated into C–V tryptose broth for enrichment and subcultured periodically as previously described. The other half was expelled through a syringe into a bottle containing 5 to 7 ml. of plain tryptose broth and small glass beads. The bottle was then shaken 3 minutes to further disintegrate the clot. This was the inoculum for the guinea pigs and for the embryonating eggs whenever used. Duplicate guinea pigs were inoculated subcutaneously with 1.0 to 1.5 ml. of this mixture at the Indiana State Board of Health laboratories. Each pair was placed in a separate cage and the animals were transported to the veterinary research laboratory, Purdue University, where they were kept for 1 month before they were sacrificed for the examination. For a part of the experiment, 0.5 ml. of the same inoculum was injected concurrently into each of four embryonating White Leghorn eggs 3 to 5 days old, as already described.

Results

Over a period of 1½ years, 276 blood clots were examined for the presence of *Brucella* organisms. A total of 181 specimens was injected into both C-V tryptose broth and into guinea pigs; and 95 were examined by inoculating C-V tryptose broth, guinea pigs, and embryonating eggs as previously described.

Ten *Brucella* strains were isolated from the group of 181 specimens inoculated into guinea pigs and C-V broth. Two of the strains were recovered from the guinea pigs, whereas portions of the same specimens, following enrichment in the C-V broth, yielded 9 strains. One strain was isolated from both mediums. All recoveries were identified as *Br. abortus.*

From the 95 specimens examined by inoculation into the C-V broth, guinea pigs, and embryonating eggs, a total of 13 *Brucella* strains was recovered. The species and methods of isolation are tabulated in table 2. Twelve of the 13 strains were isolated via the embryo yolk sac technique. The guinea pigs yielded 1 strain

Table 2. Methods of recovery of Brucella (13 strains) from blood clots examined by injection into guinea pigs, C-V broth, and embryonating eggs

Species	Number of recoveries from		
	C-V broth	Guinea pig	Eggs
Br. suis	2	1	3
Br. abortus	4	0	8
Br. melitensis	0	0	1
Total	6	1	12

and the C-V broth 6 strains. The 1 strain which was not recovered from the eggs by the method employed was a *Br. suis* and was isolated from both the guinea pigs and the C-V broth.

The results of the entire study as reported in this paper are summarized in table 3, with a division of the data into three categories. The first group comprises the initial attempt at isolating *Brucella*, during which time that portion of the clot to be injected into guinea pigs received a preliminary broth enrichment. The second group is the experimental group, in which the guinea pigs were injected without prior enrichment of the specimens and concurrently with the inoculation of the C-V broth. The third group of data includes only those clots which were examined by injection into all three mediums—embryonating eggs, C-V tryptose broth, and guinea pigs.

The use of the three mediums as indicated in the third group increased the total percentage of isolations from 5.3 to 13.6 percent. This increase was primarily due to the inclusion of the egg embryo technique. Statistically, there was no significant difference found in the percentage of isolations yielded by the C-V broth alone in any of the three categories. Likewise, in the first group, the difference in the percentage of isolations yielded by the C-V broth and by the guinea pigs was not found to be significant; however, in the second series, the opposite was true. The guinea pig technique was significantly inferior to the use of C-V broth in the isolation of *Brucella* from the blood clots.

In the third group of data only 95 specimens are included as being examined by all three methods. Although this number is not sufficient for a final statistical evaluation, it is obvious that a difference in the abilities of the three mediums to isolate *Brucella* does exist. Of the 13 recoveries from this group, only 1 is accredited to the guinea pig and less than half to the C-V broth. On the other hand, the embryonating eggs yielded all but 1 of these strains.

Of the guinea pigs injected, only the ones yielding an isolation had developed a titer. The remaining guinea pigs showed no evidence of agglutinins.

Discussion

The guinea pig, as employed in this study, appeared of little value in the primary isolation of *Brucella* from clotted blood material when the specimens were injected directly, without a preliminary enrichment. However, when the

Table 3. Summary of results of using different techniques for isolating Brucella from blood clots

Group	Number specimens	Treatment of clot	Total strains recovered		Results of examinations		
			Number	Percent	Strains isolated	Strains missed	Percent isolations
1	1,698	Incubated in tryptose broth, then injected into guinea pigs	90	5.3	52	38	3.1
		Incubated in C-V broth			64	26	3.8
2	181	Injected directly into guinea pigs	10	5.5	2	8	1.1
		Incubated in C-V broth			9	1	5.0
3	95	Injected directly into guinea pigs			1	12	1.1
		Incubated in C-V broth	13	13.6	6	7	6.3
		Inoculated into embryo yolk sacs			12	1	12.6

blood inoculums were incubated in tryptose enrichment broth for several days prior to injection, different results were obtained. In the former instance *Brucella* was recovered from only 1.1 percent of the specimens; under the latter conditions the organism was recovered from 3.1 percent. It is believed that the number of organisms in the inoculums, rather than increased resistance of the guinea pig stock to *Brucella* infection, was a principal factor in the differing results obtained during these two phases of the investigation. This guinea pig stock was also being currently employed in other experiments, and there was never an indication that the animals might have suddenly developed an immunity to *Brucella* infection.

Of the in vivo methods, which involved the injection of both unenriched and enriched clots into guinea pigs, and of unenriched clots into embryonating eggs, the latter procedure gave the highest percentage of recoveries from the clots examined and has to date appeared superior to the in vitro C-V tryptose enrichment broth technique.

From the 1,974 specimens included in this study, 113 (5.7 percent) recoveries were made. This proportion is much higher than that reported by West and Borman (*3*) when clots from all specimens submitted for agglutination tests were cultured, including those from negative serums. It is lower than the total percentage of recoveries made in the laboratories of the Georgia Department of Public Health (*4*) when special blood culture outfits were used for collecting specimens from persons exhibiting agglutination titers of at least 1:320 in order to supplement clot culturing. However, based on the limited number of clots injected into embryonating eggs, this medium yielded as high a percentage of isolations as the Georgia laboratories obtained with their special supplementary specimens.

The culturing of all clots routinely, without regard to titers of the accompanying serums or symptomatology of patients, has not proved very profitable in this laboratory. From 7,906 unselected clots, only 0.3 percent isolations were obtained, with only two recoveries from specimens with negative agglutination titers (*1*). In conclusion, clot examinations in the bureau of laboratories of the Indiana State Board of Health have been quite profitable when clots were selected from specimens with an agglutination titer of at least 1:40, and especially when these clots were injected into embryonating eggs.

Summary

During 4½ years, three basic techniques for the isolation of *Brucella* from human blood clots—employing guinea pigs, a crystal violet tryptose enrichment broth, and embryonating chick eggs—were studied.

Altogether, 1,974 specimens were examined and 113 *Brucella* strains were recovered: 79 *Br. abortus*, 9 *Br. melitensis*, and 25 *Br. suis*.

It was found that the incubation of portions of the blood clots in tryptose broth for several days before injecting them into guinea pigs apparently accounted for most of the isolations obtained from guinea pigs. Injection of clotted blood directly into these animals yielded very few isolations. Of the three methods of isolation, the guinea pig proved the least efficient and the egg inoculation technique yielded the largest percentage of recoveries.

REFERENCES

(*1*) Damon, S. R., and Albright, K.: The isolation of *Brucella* from blood clots. *In* Brucellosis, a symposium under the joint auspices of National Institutes of Health of U. S. Public Health Service, Federal Security Agency; U. S. Department of Agriculture; and National Research Council. Washington, D. C., American Association for the Advancement of Science (1950).

(*2*) Gay, Kathleen, and Damon, S. R.: A yolk sac technique for the routine isolation of *Brucella*. Pub. Health Rep. **66**: 1204–1211 (1951).

(*3*) West, D. Evelyn, and Borman, Earle K.: The culturing of blood clots for *Brucella* organisms. J. Infect. Diseases. **77**: 187–192 (1945).

(*4*) Brim, Alice, Morris, Janie, and Sunkes, E. J.: Methods of isolation and incidence of *Brucella* types found in Georgia. J. Lab. and Clin. Med. **35**: 483–487 (1950).