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Enhanced Isolation of *Serpulina hyodysenteriae* by Using Sliced Agar Media†

LEROY D. OLSON

Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211

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A method has been developed for separating *Serpulina hyodysenteriae*, a large spirochete and the causative agent of swine dysentery (SD), from other fecal anaerobic bacteria in rectal and colonic swabs. This was done by cutting the blood agar in parallel cuts and streaking perpendicular to the cuts in the center of the petri dish. Migration of *S. hyodysenteriae* from the central streak was apparent by the presence of strong beta-hemolysis along the edges of the cuts. If only *S. hyodysenteriae* migrated in the cut, they migrated to the end of the cut. However, if both motile bacteria and *S. hyodysenteriae* migrated in the cut, the motile bacteria migrated to the end of the cut where they formed colonies and the *S. hyodysenteriae* located along the edges of the cut between the colonies of motile bacteria and the central streak. Although motile bacteria were present where *S. hyodysenteriae* located, the growth of the motile bacteria was partially inhibited since they rarely formed visible colonies and were low in number. The cut in the agar was thought to improve traction for the serpentine movement of the *S. hyodysenteriae* and for the flagellar movement of the motile bacteria. Use of sliced blood agar was superior to conventionally streaked blood agar in that (i) it was easier to see strong beta-hemolysis on sliced agar; (ii) frequently, a confirmatory diagnosis could be made using only one petri dish with sliced agar, thereby saving time and media; (iii) *S. hyodysenteriae* could sometimes be isolated free of other bacteria; and (iv) sliced agar was more effective in isolating *S. hyodysenteriae* from swine with chronic diarrhea and nondiarrhetic carriers of SD in which the shedding of *S. hyodysenteriae* was low.

Although much progress has been made in the in vitro propagation of *Serpulina hyodysenteriae*, the etiological agent of swine dysentery (SD) (1, 7), it frequently is difficult to separate *S. hyodysenteriae* in rectal and colonic swabs from other intestinal anaerobic bacteria when conventionally streaked and restreaked on blood agar fortified with spectinomycin (6). My observation, based on adding additional antibiotics to the blood agar (3), is that the growth of *S. hyodysenteriae* is inhibited. Isolating *S. hyodysenteriae* from other intestinal bacteria in vitro becomes more arduous when attempting to recover the organism from swine infected with *S. hyodysenteriae* but having chronic diarrhea or no diarrhea and in which the number of *S. hyodysenteriae* isolates in the feces are far fewer than in swine with acute SD diarrhea.

After observing that *S. hyodysenteriae* readily produced strong beta-hemolysis along the edges of blood agar where the agar had been accidentally cut with a streaking loop on a petri dish streaked conventionally, the idea was conceived that *S. hyodysenteriae* possibly could be isolated from the other fecal bacteria in a swab if the blood agar were sliced in parallel cuts and the plate was streaked perpendicularly to the cuts. The hypothesis was that the cut in the agar acted as a furrow improving traction for the serpentine movement of the *S. hyodysenteriae* bacteria so that they could migrate away from the other bacteria in the specimen. The objectives of this study were to (i) evaluate the use of sliced agar for promoting the migration of *S. hyodysenteriae* away from other fecal bacteria and improving the recognition of both strong and weak beta-hemolysis and (ii) make a comparison of sliced blood agar with conventional streaking for isolating *S. hyodysenteriae* from swine with acute or chronic SD diarrhea or nondiarrhetic shedders.

MATERIALS AND METHODS

Source of swine for swabs. A total of 151 rectal and colonic swab samples were collected and tested for *S. hyodysenteriae* from swine experimentally or farm exposed to SD, swine with soft stools, and nondiarrhetic swine not exposed to SD (Table 1). With each rectal or colonic sample, 3 swabs were collected: one swab for the count of large spirochetes per oil immersion field (OIF), one swab for streaking the sliced agar, and one swab for conventional streaking (5). From swine with acute SD diarrhea, 20 rectal samples were collected from 20 live swine, 16 colonic samples from 16 dead swine, 2 rectal samples of 2 swine from 2 farm outbreaks, and 20 colonic samples from 2 frozen colons. From swine with chronic SD diarrhea, 16 rectal samples were collected from two groups of four live swine each, 6 colonic samples from three dead swine, one rectal sample from one live pig of a farm outbreak, and 20 colonic samples of two frozen colons. From nondiarrhetic SD shedder swine in which the SD diarrhea had ceased, 12 rectal samples were collected from two live swine and 20 colonic samples were collected from two frozen colons. In addition, 2 rectal samples were collected from 2 live nondiarrhetic swine with soft stools but not exposed to SD, 6 colonic samples were collected from the frozen colon of a nondiarrhetic pig not exposed to SD and 10 rectal samples were collected from 10 live nondiarrhetic swine not exposed to SD. The swine with soft stools were suspected of shedding *S. innocens*, a small intestinal spirochete of swine.

Each pig in sources. 1, 2, 4 to 6, and 8 to 10 (Table 1) was given orally, after withholding feed for 24 h, a total of approximately 20 g of diced infected colon (containing approximately 5×10^5 *S. hyodysenteriae* bacteria) from infected swine that had been euthanatized on the first day that diarrhea developed. The colons to be stored frozen were frozen immediately after necropsy and held at 75°C.

Slicing blood agar. Initially, individual cuts were made across the blood agar before streaking, with a single Bard-Parker knife equipped with a no. 22 sterile surgical blade. However, after observing that this method had potential for better viewing of beta-hemolysis and enhanced isolation of *S. hyodysenteriae*, five Bard-Parker knife handles equipped with no. 22 sterile surgical blades were bolted together which made five cuts with each stroke across a petri dish (Fig. 1). For this study, two strokes with a total of 10 cuts were made across each 8.5-cm-wide petri dish (Fig. 2). The width between cuts was 1 cm.

Staining smears and culturing swabs. The smear made from the first of the three swabs was stained with Victoria blue 4-R stain (4) and observed microscopically under oil immersion (×1,000) to count and average the number of spirochetes per 5 OIF (5). The second swab was streaked perpendicular to the

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TABLE 1. Comparison of streaking perpendicular to cuts on sliced blood agar with conventional streaking on unsliced blood agar of rectal and colonic swabs from swine suspected of shedding *S. hyodysenteriae*, the causative agent of SD

Type of diarrhea and source no.	Source of *S. hyodysenteriae*	No. of swine	No. of samples	Large spirochete count per OIF[a]	Streaked perpendicular to cuts in blood agar			Conventional streaking		
					No. with no hemolysis	No. with type of beta-hemolysis		No. with no hemolysis	No. with type of beta-hemolysis	
						Strong	Weak		Strong	Weak
Acute SD diarrhea										
1	Rectal swabs; live	20	20	>10	0	20	0	0	20	0
2	Colonic swabs; dead	16	16	>10	0	16	0	0	16	0
3	Rectal swabs of 2 field cases; live	2	2	>10	0	2	20	0	20	0
4	Colonic swabs of frozen colon[b]	2	20	>10	0	20	0	0	20	0
Chronic SD diarrhea										
5	Rectal swabs; live;	4	8	3–9	0	8	0	3	5	0
	2 different groups	4	8	1–2	0	8	9	4	4	0
6	Colonic swabs; dead	3	6	>10	0	6	0	1	5	0
7	Rectal swab of 1 field case; live	1	1	2	0	1	0	1	0	0
8	Colonic swabs of frozen colon[b]	2	20	3–9	0	20	0	8	12	0
Nondiarrhetic SD shedders										
9	Rectal swabs; live; recovered from diarrhea	6	12	0–2	2	10	0	11	1	0
10	Colonic swabs of frozen colon[b]; recovered from SD	2	20	0–3	4	16	0	16	4	0
Nondiarrhetic swine nonexposed to SD										
11	Rectal swabs; soft stool; live	2	2	0–2	0	0	2	1	0	1
12	Colonic swabs of frozen colon[b]	1	6	0	6	0	0	6	0	0
13	Rectal swabs; live	10	10	0	10	0	0	10	0	0

[a] 1 to 2, few; 3 to 9, moderate; >10, many.
[b] Frozen at −120°F.

cuts on the sliced blood agar. The third swab was streaked and restreaked conventionally on unsliced blood agar (5). The blood agar medium consisted of trypticase soy agar (Becton Dickins Microbiology Systems, Cockeysville, Md.) containing 5% sheep blood and 400 μg of spectinomycin per ml. Also, 10 swabs from frozen colons of swine with acute SD diarrhea and 5 swabs from frozen colons of swine with chronic SD diarrhea were streaked simultaneously on sliced SBJ blood agar (3) (SBJ is a proprietary medium from Microdiagnostics, Inc., Lombard, Ill.) and compared with sliced blood agar containing only spectinomycin. The SBJ medium contained the following antibiotics: spectinomycin, spiramycin, rifampin, vancomycin, and colistin. The plates were incubated anaerobically at 42°C in an atmosphere of 80% H_2 and 20% CO_2 as previously described (5). A pint jar one-third full of water was kept in the anaerobic jar to maintain a proper level of humidity and prevent drying of media.

In the sliced agar plates, the interpretation of the growth and type of hemolysis was made by observing the edge of the cut. If areas of beta-hemolysis developed in the conventionally streaked plates, plugs of agar were removed with a 1-ml sterile Pasteur pipette and the plates were reincubated anaerobically and reexamined 24 h later for the type of beta-hemolysis around the edge of the holes (5). A strong beta-hemolytic reaction produced by *S. hyodysenteriae* was characterized by a high intensity of brightness along the edge of the cut or around the hole and with a more discrete and defined edge where it ended, whereas a weak beta-hemolytic reaction produced by *S. innocens* was characterized by a lower intensity of brightness along the edge of the cut or around the hole and with a poorly defined boundary where it ended (5). The criteria for isolating either *S. hyodysenteriae* or *S. innocens* was spirochetal growth in an area of beta-

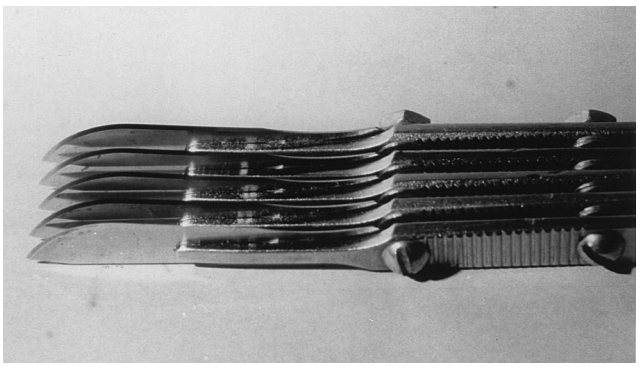

FIG. 1. Five Bard-Parker surgical knives bolted together for making cuts.

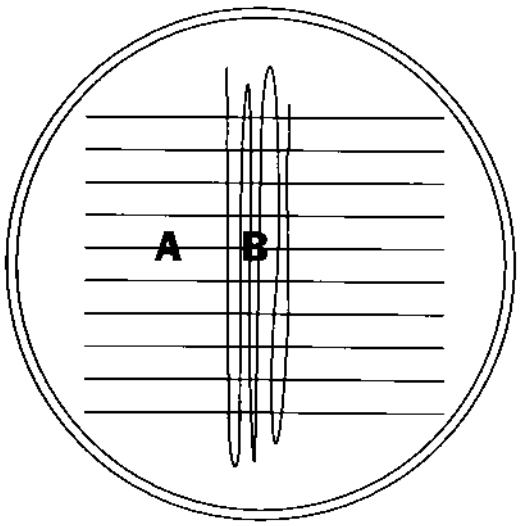

FIG. 2. Pattern for making cuts (A) in sliced blood agar and central streaking (B).

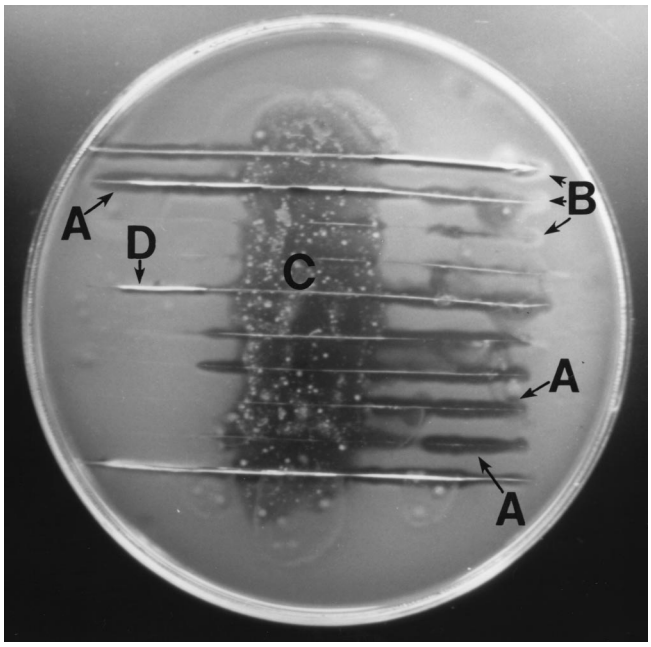

FIG. 3. Sliced blood agar plate streaked with swab from frozen colon of a pig with acute SD diarrhea and incubated for 48 h. Notice that the edges of 13 cuts have strong beta-hemolysis (A). Colonies of motile bacteria at end of cuts (B). Central streak (C). Widening of cut because of agar shrinkage (D).

hemolysis which was confirmed by staining a scraping with Victoria blue 4-R and observing the spirochetes microscopically. However, the *S. hyodysenteriae* and the *S. innocens* were not necessarily in purified form.

RESULTS

The principal feature observed in the sliced blood agar after streaking rectal or colonic swabs carrying *S. hyodysenteriae* in the center of the plate and perpendicular to the slices was a strong beta-hemolysis along both sides of one or more cuts in the agar (Fig. 3). This hemolysis usually was most evident from swabs of swine with acute SD diarrhea which had a higher number of *S. hyodysenteriae* and fewer motile anaerobic bacteria than infected swine with chronic SD diarrhea or no diarrhea and involved from 3 to all 20 cuts after 24 to 48 h of incubation (Fig. 3). If *S. hyodysenteriae* migrated in a cut from the central streak and motile bacteria were not present, the *S. hyodysenteriae* usually migrated to the end of the cut where their presence was evident as an area of strong beta-hemolysis. If only motile bacteria migrated in a cut, they usually migrated to the end of the cut where they were evident as visible colonies. If both *S. hyodysenteriae* and motile bacteria migrated in the same cut, the motile bacteria usually migrated to the end of the cut where they were evident as colonies and the *S. hyodysenteriae* located and produced strong beta-hemolysis in an area between the bacterial colonies at the end of the cut and the central streak (Fig. 3); however, where the strong beta-hemolysis was evident, there often were a few bacteria along the edges of the cut where the strong beta-hemolysis was evident, but visible bacterial colonies were rare.

If motile bacteria produced colonies along the edge of the cut where *S. hyodysenteriae* were present, the bacterial colonies were small and the *S. hyodysenteriae* moved lateral to the cut and into an area between the cuts where strong beta-hemolysis was seen and few motile bacteria migrated; occasionally these areas were free of motile bacteria. When scrapings from these areas containing both *S. hyodysenteriae* and motile bacteria were restreaked on sliced agar, one or more cuts in the restreaked plate usually contained *S. hyodysenteriae* free of the motile bacteria. It appeared that if strong beta-hemolysis was present, the growth of the other motile bacteria was partially inhibited. If a pure population of *S. hyodysenteriae* was streaked on sliced agar, the *S. hyodysenteriae* often did not migrate to the end of the cut as they usually did when streaked with contaminated rectal and colonic swabs. On rare occasions, a swarming *Proteus* sp. would spread over the area of strong hemolysis, but frequently the *S. hyodysenteriae* could be freed of this organisms with restreaking on sliced agar.

Although strong beta-hemolysis was seen both along the edges of the cuts in the sliced agar and where a plug of agar was removed in the conventionally streaked plates of all rectal and colonic swabs of swine with acute SD diarrhea (sources. 1 to 4, Table 1), it was easier to see the strong beta-hemolysis on the sliced agar then the hemolysis on the surface of the conventionally streaked plates before removing a plug of agar. Frequently a confirmative diagnosis was made 24 h after incubation with the sliced agar streaked with swabs from swine with acute SD diarrhea and areas of strong beta-hemolysis were often found which were free of motile bacteria.

In the sliced blood agar streaked with rectal and colonic swabs from swine with chronic SD diarrhea, strong beta-hemolysis developed along the edges of the cuts in one to six cuts, but occasionally more, after 48 to 96 h of incubation. The hemolysis involved fewer cuts and took longer to become evident than with inoculum from swine with acute SD diarrhea. There also were fewer *S. hyodysenteriae* isolates and more motile bacteria in the swabs from swine with chronic SD diarrhea.

When comparing streaking on sliced agar with conventional streaking for isolating *S. hyodysenteriae* from rectal and colonic swabs of swine with chronic SD diarrhea (sources. 5 to 8, Table 1), strong beta-hemolysis was seen along the edges of the cuts and *S. hyodysenteriae* was isolated in all streakings on the sliced agar; whereas, with conventional streaking, strong beta-hemolysis was seen and *S. hyodysenteriae* isolated in 9 of 16 rectal swabs of live swine, 5 of 6 colonic swabs of dead swine, 0 of 1 rectal swab of a farm outbreak, and 12 of 20 colonic swabs of frozen infected colon. Even though there was a greater problem with motile bacteria migrating in the cuts from the fecal material of swine with chronic SD diarrhea than with acute SD diarrhea; still, there remained areas of strong beta-hemolysis in the sliced agar which were free of bacterial colonies.

With the specimens from the nondiarrhetic shedder swine, strong beta-hemolysis developed in one to three cuts, but occasionally more, after 4 to 8 days of incubation. The hemolysis involved fewer cuts and took longer to develop. Frequently the band of strong beta-hemolysis that developed along the edges of the cuts was narrower since these specimens had fewer *S. hyodysenteriae* isolates than did the specimens from swine with acute or chronic SD diarrhea (Fig. 4). When the number of cuts was doubled by cutting between the first cuts as an afterthought, the incidence of developing hemolysis and isolating *S. hyodysenteriae* was increased with no failures after six attempts. However, with the narrower strips of agar, bacterial cross-contamination occasionally occurred. Infrequently, after 8 days of incubation, strong beta-hemolysis was not easily detected because the blood had degraded; yet *S. hyodysenteriae* had migrated from the central streak and were located on the edges of the cuts. On many of the agar plates, incubated for over 48 h, one or more cuts had widened because of agar shrinkage (Fig. 3), and occasionally there were small half-

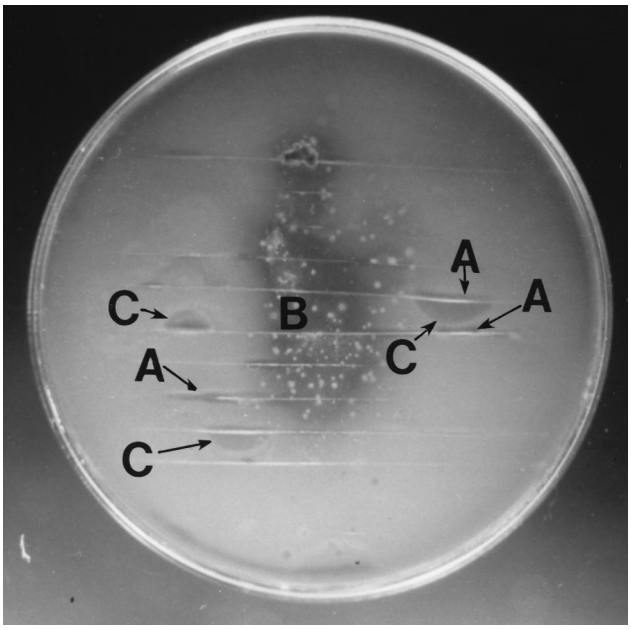

FIG. 4. Sliced blood agar plate streaked with a rectal swab from a pig that had developed an SD diarrhea, but the diarrhea had ceased 2 weeks previously. Plate had been incubated for 8 days. Notice narrower band of strong beta-hemolysis (A) along edges of cuts. Central streak (B). Half-moon-shaped areas where agar had become detached from the plastic plate (C).

moon-shaped areas along the edges of the cuts where the agar had become detached from the plastic plate (Fig. 4).

When comparing streaking on sliced agar with conventional streaking for isolating *S. hyodysenteriae* from rectal and colonic swabs of nondiarrhetic shedder swine (source nos. 9 and 10; Table 1), strong beta-hemolysis was seen on sliced agar with 10 of the 12 samples of rectal swabs from live nondiarrhetic swine and 16 of 20 colonic swabs of frozen infected colons; whereas strong beta-hemolysis was seen and *S. hyodysenteriae* was isolated from only one of the 12 live swine and from 4 of the 20 colonic swabs of frozen infected colons with conventional streaking. With conventional streaking, areas of strong beta-hemolysis on the surface of the blood agar were difficult to detect because numerous colonies of other bacteria had overgrown the blood agar.

The spirochetes isolated on sliced blood agar from the two samples of swine not exposed to SD but having a soft stool (source 11; Table 1) migrated similarly to that of *S. hyodysenteriae* but produced a weak beta-hemolysis similar to that of *S. innocens* (2, 5). However, only 1 of the 2 samples produced weak beta-hemolysis with conventional streaking. It was easier to see the weak beta-hemolysis on the sliced agar than the hemolysis on conventionally streaked plates before removing a plug of agar. No strong or weak beta-hemolysis was observed along the edges of the cuts in the sliced agar streaked with swabs from the frozen colon of the nondiarrhetic pig not exposed to SD (source 12; Table 1) or from the rectal swabs of the 10 live nondiarrhetic swine not exposed to SD (source 13; Table 1), and only motile bacteria migrated in the cuts. Swabs containing *S. hyodysenteriae* and streaked on SBJ blood agar migrated away from the center streak and grew and produced strong beta-hemolysis; however, the additional antibiotics in this media markedly inhibited the growth of the *S. hyodysenteriae.*

DISCUSSION

The presence of strong beta-hemolysis which developed along the edges of the cuts in the sliced agar and the finding of large spirochetes in stained smears of scrapings in these areas was indicative that *S. hyodysenteriae* had migrated from the streaked inoculum in the center of the petri dish. The advantages of using sliced blood agar for isolating *S. hyodysenteriae* in comparison with conventional streaking were as follows: (i) the cut assisted the *S. hyodysenteriae* in migrating away from the other fecal bacteria in the center streak and the motile bacteria in the cut; (ii) strong beta-hemolysis was easier to see along the edges of the cut when free or relatively free of other bacteria; (iii) a confirmative diagnosis frequently could be made by using only one petri dish with sliced agar where no motile bacteria were present, conserving the time taken for restreaking and saving media; (iv) *S. hyodysenteriae* was occasionally isolated free of other bacteria; (v) restreaking *S. hyodysenteriae* on sliced agar often resulted in a culture free of other bacteria; and (vi) it was more effective in isolating *S. hyodysenteriae* from swine with chronic SD diarrhea and nondiarrhetic shedders of *S. hyodysenteriae*, in which the numbers were low. Using sliced blood agar reduced the need for media containing additional antibiotics. If one is accustomed to distinguishing between strong and weak beta-hemolysis by removing a small round plug of agar in an area suspected of being hemolytic, it may take a little practice to distinguish between the two types of hemolysis when they develop along the edge of a cut.

The cut in the agar appeared to give traction for the serpentine movement of the *S. hyodysenteriae* and the flagellar action of the motile bacteria in their migration. The rubbing of the swab over the edge of the cut in streaking was thought to assist in detaching the *S. hyodysenteriae* bacteria from the swab and depositing them in the cut. From the development of strong beta-hemolysis it appeared that the *S. hyodysenteriae* was always attempting to migrate away from the other bacteria in the inoculum. However, if the *S. hyodysenteriae* was free of contaminants, there was less inclination to migrate in the cuts. Increasing the number of cuts by increasing the size or changing the shape of the agar plates may be a means of increasing the chances of isolating *S. hyodysenteriae* from a single swab of suspected nondiarrhetic SD carriers and shedders. If *S. hyodysenteriae* established along a cut with the development of strong beta-hemolysis and there were other bacteria present, the growth of other bacteria appeared to be partially inhibited in the area of the hemolysis. Even though *S. hyodysenteriae* may be dependent upon other bacteria for growth factors, this propensity to separate from other bacteria in vitro may be because the normal habitat of *S. hyodysenteriae* in the pig, which is in the crypts of the colon, is relatively free of bacteria. Although freezing the infected colons (source nos. 4, 8, and 10) probably resulted in the killing of some *S. hyodysenteriae* bacteria, no marked decreases in the live population of *S. hyodysenteriae* were observed. Frozen colons infected with *S. hyodysenteriae* and stored at −75°C at the University of Missouri have been found to be infective for over 25 years.

With conventional streaking, both the *S. hyodysenteriae* and accompanying bacteria were diluted with each restreaking. If there was a higher proportion of *S. hyodysenteriae* to the accompanying bacteria as occurred with acute SD diarrhea, areas of strong beta-hemolysis hemolysis were seen in the most diluted area of the conventionally streaked plate, making conventional streaking equally effective in this study for the isolation of *S. hyodysenteriae*. However, in swine with chronic SD diarrhea or nondiarrhetic shedders, there were fewer *S. hyodysenteriae* and more other bacteria, and these bacteria could

not be diluted sufficiently with restreaking to prevent them from obliterating the growth of the *S. hyodysenteriae.*

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