1988

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JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1988, p. 2357–2360
0095-1137/88/112357-04$02.00/0

Vol. 26, No. 11

Improved Selective Medium for the Isolation of *Treponema hyodysenteriae*

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Received 14 March 1988/Accepted 3 August 1988

An agar medium with improved selection for *Treponema hyodysenteriae* was developed. Cultures of *T. hyodysenteriae* and *T. innocens*, feces from 11 clinically normal pigs, and colonic contents from 6 pigs with gross lesions consistent with swine dysentery were diluted in phosphate-buffered saline and plated on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) with 5% citrated bovine blood (TSA), TSA with 400 μg of spectinomycin per ml (TSA-S400), TSA-S400 with 25 μg each of colistin and vancomycin per ml, and TSA with 5% pig feces extract and five antimicrobial agents (spiramycin, rifampin, vancomycin, colistin, and spectinomycin) (BJ). Viable numbers of *T. hyodysenteriae* grown on BJ were virtually identical to those for TSA, TSA-S400, and TSA-S400 with colistin and vancomycin. Pure cultures of four isolates of *T. hyodysenteriae* and three isolates of *T. innocens* were sustained through six subcultures on BJ. Fecal floras were completely inhibited on BJ for 14 of 17 fecal samples from both groups of pigs. A total of 461 colonic specimens from naturally occurring cases of porcine intestinal disease were plated on TSA-S400 and BJ. *T. hyodysenteriae* was isolated on both TSA-S400 and BJ for 69 specimens and on BJ alone for an additional 19 specimens.

Treponema hyodysenteriae is the etiologic agent of swine dysentery (SD), a severe mucohemorrhagic diarrheal disease of weanling to finishing pigs (4). Definitive diagnosis of the disease is dependent on the isolation and identification of *T. hyodysenteriae*, which are especially important because a morphologically similar nonpathogenic organism, *T. innocens*, is present in the ceca and colons of pigs (11). Determining the species of these organisms is possible on primary isolation media if the other fecal flora are sufficiently inhibited to allow observation of the hemolytic pattern produced (17; J. M. Kinyon, M.S. thesis, Iowa State University, Ames 1974). Further determination of species is possible by using biochemical and serological tests (2, 6, 10, 11, 15, 18; Kinyon, M.S. thesis) and live-animal inoculation (4, 9; Kinyon, M.S. thesis). Selective isolation of *T. hyodysenteriae* is also of epidemiological significance in the detection of asymptomatic carrier pigs and other vectors.

Isolation methods for *T. hyodysenteriae* have included the use of membrane filter plates (20), serial filtration through filters of decreasing pore size (5), serial dilution and plating (11), and selective media (8, 22, 24). The most widely used selective medium, Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.) with 400 μg of spectinomycin per ml (TSA-S400), was developed by Songer et al. (22) in 1976. This medium inhibited fecal flora 10^3 to 10^4 CFU/g, did not inhibit *T. hyodysenteriae*, and made isolation of the organism possible in veterinary research and diagnostic facilities throughout the world (1, 3, 7, 16–19, 21, 26).

Other selective media have been reported, including a medium made by Jenkinson and Wingar and containing colistin, vancomycin, and spectinomycin (CVSBA) (8); Szynkiewicz and Binek have reported a medium containing spectinomycin and vancomycin (24).

We have developed a selective medium (BJ) which contains pig feces extract (14) to promote the growth of *T. hyodysenteriae* and five antimicrobial agents to inhibit the fecal flora. The antimicrobial agents used in this study all have MICs greater than 100 μg/ml against *T. hyodysenteriae* (13). Various combinations of antimicrobial agents used in MIC trials resulted in the formulation of BJ.

MATERIALS AND METHODS

Antimicrobial solutions. Antimicrobial agents were purchased from Sigma Chemical Co., St. Louis, Mo. Stock solutions of spectinomycin, colistin methanesulfonate, and vancomycin were dissolved in sterile distilled water. Stock solutions of rifampin (23) and spiramycin were dissolved in 100% ethanol and diluted with sterile distilled water to a final concentration of 20% ethanol. Stock solutions were stored at 4°C for 6 months.

Medium preparation. TSA, TSA-S400, and CVSBA were prepared as described previously (8, 22). BJ was prepared by combining TSA (BBL) prepared according to the directions of the manufacturer, deionized distilled water, and 5% pig feces extract. After being autoclaved (121°C, 15 lb/in^2, 15 min), the medium was cooled to 45 to 50°C, the antimicrobial solutions and 5% sterile citrated bovine blood were added, and the medium was poured into sterile disposable petri plates. The final concentrations of antimicrobial agents in BJ (in micrograms per milliliter) were as follows: spiramycin, 25; rifampin, 12.5; vancomycin, 6.25; colistin, 6.25; and spectinomycin, 200.

TSA, TSA-S400, BJ, and CVSBA were stored at 4°C and were inoculated within 1 week of preparation. All plates were air-dried for 30 min prior to storage and use.

Isolates. *T. hyodysenteriae* isolates B204, B78, and B169 and *T. innocens* isolates B1555A, B256, and B359 were obtained from the culture collection of the authors (11, 12). *T. hyodysenteriae* isolate A1 was supplied by D. J. Taylor, University of Glasgow, Glasgow, Scotland (25). *T. hyodysenteriae* isolate 26153 was isolated from a naturally occurring case of SD and was supplied by the Iowa Veterinary Diagnostic Laboratory (IVDL), Iowa State University, Ames. The criteria for identification of this isolate and the isolates recovered during the study included colony morphology, growth characteristics, hemolytic pattern, and cell

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morphology. Cultures of *T. hyodysenteriae* and *T. innocens* were grown in an autoclaved liquid medium, F/S or F/C (14), supplemented with 5% fetal bovine serum, and incubated for 24 to 48 h at 37°C prior to inoculation onto agar medium. All cultures were determined to be pure by dark-field microscopy and inoculation onto TSA incubated aerobically and anaerobically.

Pure cultures of *Treponema* spp. (i) Dilution trials. Broth cultures of *T. hyodysenteriae* and *T. innocens* were serially diluted in phosphate-buffered saline (0.01 M; pH 7.4), and 0.01-ml samples of each dilution were plated by lawn streaking onto duplicate sets of TSA, TSA-S400, and BJ. CFU were counted after 2-, 4-, and 6-day incubation periods. The trials were performed twice.

(ii) Serial subculture trials. Cultures of *T. hyodysenteriae* isolates B204 (initially in vitro passage 25), B169 (initially in vitro passage 9), B78 (initially in vitro passage 24), and A1 (initially in vitro passage 9) and cultures of *T. innocens* isolates B256 (initially in vitro passage 16), B1555A (initially in vitro passage 27), and B359 (initially in vitro passage 8) were transferred from broth culture onto BJ and maintained on BJ through a series of subcultures.

Swine intestinal content trials. (i) Colonic contents of normal and dysenteric pigs. Samples of feces (1 g) from seven clinically normal pigs with no previous exposure to SD, four clinically normal pigs with unknown SD exposure status, and six pigs with gross lesions consistent with SD were diluted in phosphate-buffered saline, and 0.01-ml samples were lawn streaked onto TSA, TSA-S400, BJ, and CVSBA. Samples were also plated in undiluted form by placing a heavy inoculum in the primary area of the plates and using sterile loops to streak for routine isolation. The number of CFU per milliliter was recorded after 2-, 4-, and 6-day incubation periods. The identities of the fecal flora isolates (other than *Treponema* spp.) were not determined. The trials were performed once.

(ii) Samples from naturally occurring cases of intestinal disease. Personnel in the bacteriology section of IVDL were provided with BJ so that it could be compared with TSA-S400 in the recovery of *Treponema* spp. from submitted specimens. A total of 461 undiluted colonic content samples were plated onto TSA-S400 and BJ. The relative amounts of *T. hyodysenteriae*, *T. innocens*, and other colonic flora on the media were observed and recorded. Specimens that did not yield growth of *Treponema* spp. after 6 days of incubation were recorded as negative for *T. hyodysenteriae* and *T. innocens*.

(iii) Incubation. After inoculation, plates were incubated for 6 days at 42°C in GasPak jars (BBL) with a palladium catalyst and H_2-CO_2 atmosphere provided by a GasPak generator envelope (BBL).

RESULTS

Cultures of *T. hyodysenteriae* (isolates B204, A1, B78, B169, and 26153) and *T. innocens* (isolates B256, B359, and B1555A) were serially diluted in phosphate-buffered saline and plated on TSA, TSA-S400, and BJ. The number of CFU per milliliter was unaltered on BJ compared with those for TSA and TSA-S400 for six of the eight isolates tested. A depression of viable cell numbers on BJ compared with those for TSA and TSA-S400 was noted for *T. hyodysenteriae* isolate B78 and for *T. innocens* isolate B256 (Table 1).

T. hyodysenteriae isolates B204, B169, B78, and A1 and *T. innocens* isolates B256, B359, and B1555A were transferred without diminished growth through a series of six subcultures on BJ. All *T. hyodysenteriae* isolates were observed, with each subculture, to produce a typical beta-hemolytic pattern. With continued incubation, an enhanced hemolytic pattern was noted immediately around areas where agar plugs had been removed 2 days previously (ring phenomenon (11, 17). This phenomenon was not observed with continued incubation of *T. innocens* isolates treated in the same manner. *T. innocens* isolates were observed to produce a typical weak beta-hemolytic pattern. The presence of these hemolytic patterns serves as a criterion for distinguishing *T. hyodysenteriae* from *T. innocens* (11).

TABLE 1. Cultures of *T. hyodysenteriae* and *T. innocens* plated on TSA, TSA-S400, and BJ media

Isolate	CFU[a] of *Treponema* spp./ml on:		
	TSA	TSA-S400	BJ
T. hyodysenteriae			
26153	5×10^8	2×10^8	2×10^8
B204	3×10^8	4×10^8	6×10^8
A1	7×10^6	1×10^7	2×10^7
B169	3×10^8	ND[b]	2×10^8
B78	1×10^8	1×10^8	2×10^5
T. innocens			
B359	2×10^8	2×10^8	2×10^8
B1555A	7×10^7	ND	5×10^9
B256	3×10^8	ND	$<10^3$

[a] Mean result of duplicate trials.
[b] ND, Not done.

Fecal samples from seven clinically normal pigs with no previous exposure to SD were plated in diluted and undiluted forms on TSA, TSA-S400, and BJ (Table 2). Fecal samples from four clinically normal pigs with an unknown SD exposure status were plated in diluted and undiluted forms on TSA, TSA-S400, BJ, and CVSBA (Table 3). The number of CFU of fecal flora other than *T. hyodysenteriae* per gram of sample was depressed 10^1 to 10^2 on TSA-S400, 10^4 to 10^7 on CVSBA, and 10^4 to 10^{11} on BJ, compared with that on TSA. Growth of fecal flora other than *T. hyodysenteriae* was completely inhibited on BJ for 9 of the 11 plated samples. *T. hyodysenteriae* was isolated on BJ and CVSBA media, but not on TSA or TSA-S400, from one of the four fecal samples collected from pigs with unknown SD exposure status.

Colonic content samples collected postmortem from 6 pigs with gross lesions consistent with SD were plated in diluted and undiluted forms on TSA, TSA-S400, BJ, and CVSBA (Table 4). The number of CFU of fecal flora other than *T.*

TABLE 2. Cultures on TSA, TSA-S400, and BJ media of fecal samples from clinically normal pigs with no previous exposure to SD

Sample no.	CFU of fecal flora/g of sample on:		
	TSA	TSA-S400	BJ
1	1×10^9	3×10^7	3×10^5
2	3×10^9	6×10^7	NG[a]
3	3×10^9	3×10^7	NG
4	2×10^9	6×10^7	NG
5	4×10^9	3×10^8	NG
6	5×10^9	3×10^7	NG
7	9×10^9	6×10^7	NG

[a] NG, No growth.

TABLE 3. Cultures on TSA, TSA-S400, BJ, and CVSBA media of fecal samples from clinically normal pigs with unknown SD exposure status

Sample no. and type	CFU of organisms/g of sample on:			
	TSA	TSA-S400	BJ	CVSBA
1				
FF[a]	4×10^{10}	2×10^{9}	1×10^{0}	5×10^{5}
T. hyodysenteriae	NG[b]	NG	NG	NG
2				
FF	2×10^{11}	5×10^{9}	NG	8×10^{4}
T. hyodysenteriae	NG	NG	NG	NG
3				
FF	5×10^{10}	3×10^{9}	NG	1×10^{6}
T. hyodysenteriae	NG	NG	NG	NG
4				
FF	1×10^{10}	4×10^{8}	NG	2×10^{5}
T. hyodysenteriae	NG	NG	1×10^{5}	4×10^{4}

[a] FF, Fecal flora other than *T. hyodysenteriae*.
[b] NG, No growth.

hyodysenteriae was depressed 10^1 to 10^3 on TSA-S400, 10^2 to 10^4 on CVSBA, and 10^4 to 10^{10} on BJ, compared with that on TSA. Fecal flora other than *T. hyodysenteriae* were recovered on BJ from only one of the six samples. *T. hyodysenteriae* was isolated from five of the six samples. The number of CFU of *T. hyodysenteriae* was not depressed on BJ compared with those on the other media, and in one case *T. hyodysenteriae* was recovered on BJ and not on TSA or TSA-S400.

Colonic content samples from naturally occurring disease specimens submitted to IVDL from June 1986 through January 1987 were plated in undiluted form onto TSA-S400

TABLE 4. Cultures on TSA, TSA-S400, BJ, and CVSBA media of colonic content samples from pigs affected by SD

Sample no. and type	CFU of organisms/g of sample[a] on:			
	TSA	TSA-S400	BJ	CVSBA
1				
FF[b]	2×10^{9}	8×10^{6}	NG	ND
T. hyodysenteriae	NG	NG	3×10^{6}	ND
2				
FF	1×10^{10}	4×10^{7}	2×10^{6}	ND
T. hyodysenteriae	NG	NG	NG	ND
3				
FF	1×10^{8}	3×10^{7}	NG	2×10^{7}
T. hyodysenteriae	1×10^{7}	1×10^{6}	2×10^{6}	2×10^{6}
4				
FF	3×10^{10}	6×10^{7}	NG	1×10^{7}
T. hyodysenteriae	2×10^{10}	1×10^{9}	2×10^{10}	3×10^{10}
5				
FF	2×10^{8}	2×10^{6}	NG	1×10^{6}
T. hyodysenteriae	2×10^{9}	2×10^{9}	3×10^{9}	1×10^{9}
6				
FF	2×10^{9}	3×10^{8}	NG	1×10^{7}
T. hyodysenteriae	2×10^{7}	2×10^{8}	1×10^{9}	2×10^{9}

[a] NG, No growth; ND, not done.
[b] FF, Fecal flora other than *T. hyodysenteriae*.

TABLE 5. Isolation of treponemes from colonic content specimens plated on TSA-S400 and BJ media[a]

Specimen reaction and medium	No. of reacting samples/no. of samples tested (%)
Positive for *T. hyodysenteriae*	88/461 (19)
On BJ	88/88 (100)
On TSA-S400	69/88 (78)
Positive for *T. innocens*	11/461 (2)
On BJ	11/11 (100)
On TSA-S400	4/11 (36)
Negative for *T. hyodysenteriae* and *T. innocens* (on both media)	362/461 (79)

[a] We tested 461 specimens which had been submitted to IVDL from June 1986 through January 1987.

and BJ, and the culture results were recorded (Table 5). A total of 19% (88 of 461) of the submitted specimens were culture positive for *T. hyodysenteriae*, and 2% (11 of 461) were positive for *T. innocens*. There was no case in which isolation of treponemes was obtained on TSA-S400 and not on BJ. However, 22% of the positive *T. hyodysenteriae* cultures (19 of 88) and 64% of the positive *T. innocens* cultures (7 of 11) were isolated only on BJ.

DISCUSSION

T. hyodysenteriae was confirmed as the etiologic agent of SD (4, 25), and isolation of the organism was greatly improved by the development of TSA-S400 (22). This medium worked extremely well for isolation from acute nontreated naturally occurring cases of the disease. However, the inhibition of fecal flora on TSA-S400 was not complete, which often prevented thorough, accurate examination for the presence of *Treponema* spp. on the primary plate and made subsequent subculture difficult, especially if there was a low population of treponemes in the original sample. The medium described in this report (BJ) inhibited virtually all fecal flora so that pure cultures of *Treponema* spp. were often obtained on the primary isolation plate. This result facilitated continued observation of the plates through a 6-day incubation period and permitted testing for the ring phenomenon on the primary plate. The tremendous degree of inhibition of normal fecal flora observed on BJ was due to the inclusion of the selected antimicrobial agents spectinomycin, colistin, and vancomycin (which have previously been used for selective isolation of *T. hyodysenteriae*), rifampin, and spiramycin. The role of pig feces extract in BJ has not been determined; however, pig feces extract has been shown to contribute to improved growth of *T. hyodysenteriae* and *T. innocens* in a liquid medium (14).

The growth of *T. hyodysenteriae* isolate B78 was somewhat inhibited on BJ, compared with growth on TSA-S400 in the dilution trials; however, B78 was maintained without diminished growth through six subcultures on BJ. The other *T. hyodysenteriae* isolates used in the dilution trials were not inhibited on BJ, compared with growth on TSA-S400.

When used in dilution trials, four of six colonic content samples from pigs with gross lesions consistent with SD yielded essentially identical numbers of viable *T. hyodysenteriae* on BJ and TSA-S400. *T. hyodysenteriae* was isolated on BJ and not on TSA-S400 from one sample, and was not recovered on either medium from another of the six samples.

It is of interest that *T. hyodysenteriae* from a fecal sample from an apparently healthy pig was isolated on BJ (also on

CVSBA) and not on TSA-S400. The history of this animal was vague; the use of BJ allowed the identification of an asymptomatic carrier.

Isolation of *T. hyodysenteriae* and *T. innocens* was improved substantially when specimens form naturally occurring cases of intestinal disease were plated on BJ instead of TSA-S400. There was no case in which isolation was obtained on TSA-S400 and not on BJ. Furthermore, for the 8-month period studied, 19 cases of SD which would have been reported (falsely) negative by culture on TSA-S400 alone were successfully confirmed by culture on BJ.

The success of attempted eradication of SD is dependent on identification of all reservoirs of the disease. The data that have been collected suggest that 20% or more of naturally occurring cases of SD are not confirmed by current laboratory culturing procedures. This study demonstrates enhanced isolation of *T. hyodysenteriae* from naturally occurring infections. We believe that continued use of BJ may result in improved isolation of *T. hyodysenteriae* from all reservoirs of the disease, including swine with acute and chronic cases of SD, asymptomatic shedders, lagoons, pits, and mechanical and biological vectors.

ACKNOWLEDGMENTS

The assistance of B. J. Schmitt, L. J. McDeid, M. A. Person, M. A. Brodahl, M. T. Reiter, and J. L. Carr (Department of Veterinary Microbiology and Preventive Medicine) and L. M. Henderson, J. P. Morgan, and L. J. Hoffman (IVDL), all of Iowa State University, is gratefully acknowledged.

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