Selective Medium for Isolation of *Bacteroides nodosus*¹

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Received for publication 8 March 1977

The sensitivity of *Bacteroides nodosus*, the causative agent of sheep foot rot, to 24 selected antimicrobial agents was tested. Many contaminants ordinarily associated with foot rot lesions were sensitive to lincomycin, whereas *B. nodosus* demonstrated resistance to this antibiotic. A concentration of 1 μ g of lincomycin per ml of basal medium optimally inhibited contaminants while allowing growth of *B. nodosus*. The basal medium was Eugon agar with 0.2% yeast extract and 10.0% defibrinated horse blood. Parallel inoculations of 31 foot rot lesion specimens onto basal medium and basal medium containing lincomycin (selective medium) were performed. *B. nodosus* was isolated from 16 of the specimens cultured on the selective medium and from only 3 of the specimens cultured on the basal medium. The concentration of agar in the standard Eugon medium was found to influence the growth of *B. nodosus* in the presence of lincomycin. Trimethoprim also exhibited potential selectivity for *B. nodosus*.

Isolation of *Bacteroides nodosus*, the causative agent of foot rot in sheep (2, 4, 8), is extremely difficult, partially because of the fastidious nature of this strict anaerobe but also because of the vast numbers of other bacteria comprising the foot rot lesion microflora. This report describes a selective medium that permits the growth of *B. nodosus* while inhibiting the growth of many extraneous or contaminant anaerobes associated with the foot rot lesion.

MATERIALS AND METHODS

The basal medium used was Eugon agar (Baltimore Biological Laboratory [BBL], Cockeysville, Md.) containing 0.2% yeast extract (Pfizer Inc., New York, N.Y.) and 10% defibrinated horse blood. This medium adequately supported *B. nodosus* growth when inoculated plates were incubated at 37°C in anaerobic jars gassed with 90% hydrogen and 10% CO₂. An incubation period of 5 to 6 days was used for all cultures on basal medium as well as media containing the various antimicrobial agents.

Antimicrobial agents were tested for their ability to select *B. nodosus* from a pool of unidentified contaminant anaerobes that were isolated from foot rot lesions. Sensitivity was determined primarily by using antimicrobial disks placed on the surface of inoculated basal medium plates; however, some antimicrobials were incorporated into the basal medium. Commercial sensitivity disks utilized were: bacitracin, 2 U; cephaloridine, 30 μ g; colistin, 10 μ g; doxycycline, 5 μ g; furadantin-macrodantin, 100 μ g; garamycin, 10 μ g; kanamycin, 5 μ g; neomycin, 5 μ g; polymyxin B, 50 U; and sulfamethoxypyridazine,

0.25 mg (BBL); clindamycin, 2 μ g; and lincomycin, 2 μ g (The Upjohn Co., Kalamazoo, Mich.); and sulfadiazine, 150 μ g (Difco Laboratories, Detroit, Mich.). Sensitivity disks were also prepared by impregnating sterile blank disks with various concentrations of other antimicrobial agents. These agents and their respective concentrations were: 5, 50, 100, and 500 μ g of brilliant green; 0.5 and 5 μ g of crystal violet; 50, 500, 1,000, and 5,000 μ g of sodium azide; 50 and 500 μ g of sodium deoxycholate; 50 μ g of sodium deoxycholate with 0.2 μ g of sodium citrate; 500 μ g of sodium deoxycholate with 2 μ g of sodium citrate; 50, 500, 1,000, and 5,000 μ g of sodium lauryl sulfate; 50, 500, 1,000, and 5,000 μ g of sodium taurocholate; and 1.2, 2.4, 4.8, 12, 24, and 48 μ g of trimethoprim. Agents tested by incorporation into the basal medium and their respective concentrations were: 1, 1.5, 1.8, and 3.6 μ g of lincomycin (Upjohn) per ml; 1, 2, 4, 6, and 8 μ g of virginiamycin (Smith Kline Animal Health Products, West Chester, Pa.) per ml; 10% bile (Oxgall, Difco); and 0.25% phenethylalcohol. Antimicrobial agents reported to have therapeutic value for foot rot were not included in this study.

Initially, the antimicrobial agents were tested against pure cultures of *B. nodosus* and/or pools of contaminant anaerobes. Agents that gave preliminary promise of inhibiting contaminants were further tested by using mixed cultures containing both *B. nodosus* and contaminant organisms. Finally, lincomycin, the antimicrobial agent that exhibited the best selectivity for *B. nodosus*, was incorporated into the basal medium. Basal media containing various concentrations of lincomycin were inoculated with composite suspensions of *B. nodosus* and contaminant anaerobes and with foot rot lesion specimens to determine the lincomycin concentration demonstrating optimal selectivity for *B. nodosus*.

The foot rot lesions cultured were graded from 1 to 4 based on the severity of the lesion. A "1" denoted

¹ Oregon Agricultural Experiment Station Technical Paper no. 4461.

an early, mild interdigital dermatitis, a "2" was a more advanced lesion with erosion of the bulbs of the hoof, a "3" denoted underrunning of the sole of the hoof, and a "4" specified an extensive lesion with separation of the sole and the hoof wall.

Specimens of sheep foot rot lesions were obtained by scraping necrotic material from the lesion with a no. 20 Baird-Parker blade (Becton, Dickinson & Co., Rutherford, N.J.) and placed in a transport medium consisting of a suspension of Stuart medium base (Difco) with 0.5% glycerol. The culture specimens were completely submerged in the transport medium to minimize oxygen contact and kept in an ice-cooled insulated container until processed. In the laboratory, foot rot lesion specimens were triturated in a Ten Broeck tissue grinder with 0.5 to 2.0 ml of 0.25 M sucrose (depending on the size of the specimen), and Gram-stained smears of the suspensions were examined for *B. nodosus* cells. A total of 31 parallel cultures of foot rot lesion specimens were made by inoculation of 10 plates each of basal and selective media. A filter paper was inserted into the lid of these primary plates to absorb condensation.

With a stereoscopic microscope at $\times 14$ magnification and a fluorescent illuminator, the plates were examined for colonies of typical *B. nodosus* morphology (11). Suspect colonies were Gram stained and transferred to fresh basal medium plates. Preliminary identification of *B. nodosus* isolates was made on the basis of colony morphology, cellular morphology, and Gram reaction. Confirmation of *B. nodosus* was based on the tube agglutination test (3) by using antisera made against Australian *B. nodosus* serotypes (courtesy of J. R. Egerton, CSIRO, McMaster Laboratory, Glebe, N.S.W., Australia) or serotypes isolated in our laboratory and identified by fluorescent antibody conjugate (Wellcome Research Laboratories, Beckenham, England) (9).

Parallel inoculations of seven *B. nodosus* strains were made onto basal and selective media containing normal (1.5%) and increased (2.2%) agar concentrations to determine the influence of these modifications, singly and in combination, on the growth of *B. nodosus*. The cultures were diluted in 0.25 M sucrose and 0.025-ml aliquots streaked onto plates of each of the four types of media. After 5 to 6 days of incubation, the colonies on each plate were enumerated. Further study of the growth of *B. nodosus* on the selective medium was done by subculturing each of 44 *B. nodosus* strains four times on the selective medium.

RESULTS

B. nodosus was sensitive to all of the commercial antimicrobial disks tested except those containing lincomycin and sulfamethoxypyridazine to which it was resistant and partially sensitive, respectively. The sensitivity tests using the laboratory-prepared disks indicated that brilliant green, sodium deoxycholate, sodium deoxycholate with sodium citrate, and trimethoprim could inhibit foot rot contaminants to a greater degree than *B. nodosus*.

When incorporated into basal medium, bile, phenethylalcohol, and virginiamycin each inhibited *B. nodosus* growth.

Lincomycin was the most promising selective antimicrobial agent for *B. nodosus* isolation, and further trials revealed that a concentration of 1 μg of lincomycin per disk inhibited contaminants in a zone around the disk while allowing growth of *B. nodosus* colonies within this zone (Fig. 1). When incorporated into the basal medium, lincomycin partially inhibited *B. nodosus* growth at 1.5 μg but only slightly at 1 $\mu\text{g}/\text{ml}$; consequently the latter concentration was used in the selective medium.

Parallel inoculations of 31 foot rot lesion specimens on basal and selective media resulted in isolation of *B. nodosus* from 3 of the specimens cultured on basal medium and from 16 of the specimens cultured on selective medium. The three successful cultures on basal medium were also positive on the selective medium (Table 1).

The condition of the foot rot lesion appeared to markedly influence the success of *B. nodosus* isolation. On the selective medium all 5 "grade 1" lesions cultured were positive (100%), 3 of 5 "grade 2" lesions cultured were positive (60%), 1 of 3 "grade 3" lesions were positive (33.3%), and 7 of 18 "grade 4" lesions were positive (38.9%). All three *B. nodosus* strains isolated on the basal medium were from "grade 1" lesions (Table 1).

Gram-negative cells with the morphology of *B. nodosus* were seen in smears from 13 of the 16 specimens positive for *B. nodosus*. Conversely *B. nodosus*-like cells were present in nine specimens that were negative by culture; however, the identity of the cells in seven of these specimens was questionable (Table 1).

Parallel inoculations of *B. nodosus* cultures onto basal and selective media demonstrated the relative inhibition of *B. nodosus* growth on the selective medium. The decrease in the number of colonies on the selective medium relative to the basal medium ranged from 10 to 36% (Table 2).

Enhanced growth of six of the seven strains on basal medium containing increased agar was demonstrated with 4 to 25% more colonies present on plates of basal medium with 2.2% agar than on plates of basal medium containing the usual 1.5% agar. The medium containing lincomycin (1 $\mu\text{g}/\text{ml}$) and 2.2% agar was detrimental to *B. nodosus* in five of the seven strains, with the number of colonies on these plates being decreased 3 to 99% relative to the 2.2% agar medium without lincomycin and 19 to 52% relative to the 1.5% agar medium with lincomycin (Table 2).

In addition to reducing the number of colo-

FIG. 1. Lincomycin sensitivity disk (1 μ g/disk) on basal medium inoculated with a pool of *B. nodosus* and extraneous (contaminant) organisms. Notice the zone of inhibition of contaminant colonies and the presence of *B. nodosus* colonies (arrows) within this zone close to the disk (D). The bar represents 1 mm.

nies per plate, lincomycin altered the appearance and reduced the size of many of the colonies produced by certain strains. These colonies were very small and had a "snowflake" appearance. The agar concentration of the medium had no apparent influence on colony morphology.

Four consecutive passages of 44 *B. nodosus* strains, including Australian serotypes A, B, and C (courtesy of J. R. Egerton), on the selective medium, did not appear to deter growth of the organism. Some strains appeared to grow more readily than others on the selective medium; however, this characteristic was constant during the four passages in all cases. In no case did the relative growth of *B. nodosus* decrease from the first to the fourth subculture on the selective medium; indeed, in several cases there appeared to be some adaption to the selective medium as indicated by enhanced growth on the second, third, or fourth passage relative to earlier cultures.

DISCUSSION

Isolation of *B. nodosus* was significantly more successful ($P < 0.01$, chi-square test) on the selective medium than on the basal medium in this trial. This enhanced isolation success was related to suppression of contaminants, preventing their spread and overgrowth of *B. nodosus* colonies. Another apparent factor

was the suppression of a contaminant or contaminants that produced a gas that was unfavorable to *B. nodosus* growth. It was found that stock cultures of *B. nodosus* on basal medium failed to grow, or grew very poorly, when incubated in anaerobic jars with other plates that had been freshly inoculated with certain foot rot lesion specimens. In these cases an obnoxious odor was released when the anaerobic jar was opened, and persons working with these cultures invariably developed headaches soon after opening the jars. The agar in these jars developed a distinct darkened color similar to the appearance of chocolate blood agar medium. Colonies of *B. nodosus* were never observed on any of these discolored plates. Lincomycin apparently inhibited the organism(s) that produced this toxic gas since selective medium plates never developed this dark appearance when care was taken to exclude all basal medium plates inoculated with foot rot lesion specimens from the same anaerobic jar.

Earlier reports that *B. nodosus* isolation was more successful from early cases of foot rot (6) were supported by the results of this trial. The recommendation that isolation should be attempted only from specimens in which *B. nodosus* cells were present in abundance (6) was also supported to a degree, since *B. nodosus* cells were present, though not in large numbers, in 13 of the 16 positive specimens.

Several antimicrobial agents other than lincomycin exhibited some potential for selectively inhibiting contaminants while allowing growth of *B. nodosus*. Trimethoprim would merit special consideration since this agent, although not inhibitory to *B. nodosus*, inhibited some contaminants that demonstrated resistance to lincomycin. Perhaps a combination of both lincomycin and trimethoprim would further enhance selection as is the case in a selective medium for *Neisseria gonorrhoeae* (7).

Bile at a 10% concentration in the basal me-

TABLE 1. Isolation of *B. nodosus* from foot rot lesion specimens using basal and selective media

Specimen	<i>B. nodosus</i> cells in specimen ^a	Severity of foot rot lesion ^b	Isolation of <i>B. nodosus</i> ^c	
			Basal medium	Selective medium
IJ 918	—	4	—	+
IJ 060(1)	—	4	—	+
IJ 060(2)	—	3	—	+
IJ 222	—	4	—	—
AW 379	±	4	—	+
EL 467	—	4	—	—
EL 484	±	4	—	+
EL 528	±	3	—	—
EL 514	+	4	—	—
EL 474	—	4	—	—
JK 75(1)	++	2	—	+
JK 75(2)	++	1	+	+
CS 210	±	4	—	—
CS 256(1)	+++	1	+	+
CS 256(2)	++	1	+	+
CS 315	±	4	—	—
DG 635	±	4	—	—
DG 654	+	4	—	+
DG 718	±	4	—	+
DG 999	—	2	—	—
VM 129	±	4	—	—
VM 132	—	2	—	—
VM 195	+++	1	—	+
AW 359	±	3	—	—
AW 415	+	4	—	—
EL 467	±	4	—	+
EL 496	±	4	—	—
EL 510	—	4	—	—
CS 317	+	2	—	+
VM 36	++	2	—	+
VM 189	+	1	—	+

^a Gram-stained smears examined. —, No cells observed; ±, some questionable cells observed; +, few cells observed; ++, moderate number of cells observed; and +++, many cells observed.

^b 1, Mild; 2, mild to moderate; 3, severe; 4, extensive.

^c —, *B. nodosus* not isolated; +, *B. nodosus* isolated.

TABLE 2. Relative growth of *B. nodosus* on basal and selective media and on basal medium with increased agar concentration with and without lincomycin

Serotype ^a	No. of strains tested	No. of plates of each medium inoculated ^b	Medium		
			1.5% agar with lincomycin	2.2% agar without lincomycin	2.2% agar with lincomycin
4	1	2	90% ^c	74%	71%
9	3	4	64%	116%	17%
11	1	4	78%	114%	26%
A	1	2	90%	125%	117%
B	1	4	90%	104%	104%

^a Serotypes 4, 9, and 11 are Oregon serotypes; A and B are Oregon strains identified as Australian serotypes A and B.

^b Media inoculated: basal medium, basal medium with lincomycin (selective medium), basal medium with increased agar (2.2%), and basal medium with increased agar and lincomycin.

^c Mean number of colonies per plate expressed as percentage of the mean number of colonies on the basal medium.

dium inhibited all growth of *B. nodosus*, whereas this concentration has been shown to actually enhance the growth of certain other *Bacteroides* species (1). Phenethylalcohol, which has been used to select for *Fusobacterium necrophorum* (5), another member of the *Bacteroidaceae* family, also inhibited *B. nodosus* growth in this study.

A previous report that increased concentrations of agar (2.2%) enhanced the growth of *B. nodosus* (10) was substantiated in this study. The dramatic inhibition of certain *B. nodosus* strains on medium containing 2.2% agar and lincomycin at the standard 1-μg/ml concentration was unexpected and cannot be explained at this time. The "snowflake" colony appearance of certain *B. nodosus* strains grown on lincomycin media was not unique. Colonies of this type have also been observed occasionally on basal medium upon reconstitution of lyophilized *B. nodosus* cultures with 0.25 M sucrose or after incubation of inoculated plates under suboptimal anaerobic conditions.

When using selective media it is generally recommended that parallel inoculations of media with and without the antimicrobial agents be made. The results of this study indicate that, when culturing for *B. nodosus*, the optimal procedure would be parallel inoculations of the selective medium described here and the basal medium as described here, but with a 2.2% agar concentration.

ACKNOWLEDGMENTS

The assistance of the Oregon sheep producers who cooperated in the collection of specimens is gratefully acknowledged. We also thank Sheryl Nied and Dana Bearwood for excellent technical assistance.

This research was supported by the Veterinary Medicine Account of the Oregon Agricultural Experiment Station.

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