# Serotypic and Biochemical Characterization of Bacteroides nodosus Isolates from Oregon

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## ABSTRACT

Ninety-seven Bacteroides nodosus isolates were characterized by the tube agglutination test. Fourteen serotypes were identified including isolates that were serologically similar to Australian serotypes A, B and C. One additional isolate remains untyped and possibly represents another serotype. The isolates were cultured from 20 different flocks. Multiple isolates were obtained from 15 of the flocks and 13 of these had two to seven different B. nodosus serotypes.

Eleven B. nodosus isolates representing one Australian and ten Oregon serotypes were nonfermentative in various carbohydrates and did not produce indole. These isolates all exhibited proteolytic activity. The prototype strains of 12 of the 14 serotypes demonstrated virulence as assessed by an elastase production assay.

## RÉSUMÉ

Cette étude consistait à caractériser, par l'épreuve d'agglutination lente, 97 souches de Bacteroides nodosus. On en identifia ainsi 14 sérotypes dont certains se révélèrent semblables aux sérotypes australiens A, B et C. Une souche additionnelle qui correspondait peut-être à un autre sérotype demeura non identifiée. On isola ces souches chez des moutons qui appartenaient à 20 troupeaux; on en obtint plusieurs à partir de 15 des 20 troupeaux et 13 d'entre eux fournirent de deux à sept sérotypes de B. nodosus.

Onze souches de B. nodosus, qui représentaient un sérotype australien et dix de l'Oregon, n'attaquèrent pas certains sucres et ne produisirent pas d'indol; toutes s'avérèrent cependant protéolytiques. Une épreuve de production d'élastase permit de démontrer la virulence des souches prototypes de 12 des 14 sérotypes.


## INTRODUCTION

Ovine foot rot is a debilitating disease characterized by inflammation of the interdigital skin and hoof matrix. The minimum requirements for this disease are the fastidious anaerobic bacteria *Bacteroides nodosus* and *Fusobacterium necrophorum*. The transmitting agent is regarded as *B. nodosus*, whereas *F. necrophorum* is a normal inhabitant of the ovine environment (6, 12).

Australian investigators have classified *B. nodosus* isolates according to their K antigens and designated the three prevalent serotypes as types A, B and C (2). They also reported both field and experimental studies that indicate a strong relationship between the *B. nodosus* K antigen and immunogenesis (3, 18, 19). Serological typing of *B. nodosus* isolates in England has also been reported (21), but no such work has been reported from North America.

Vaccination trials with bacterins of *B. nodosus* have shown immunogenic and therapeutic value in sheep in Australia (3-5, 7, 18), New Zealand (16) and England (10, 13); however, an Australian-produced vaccine used in two small trials in the United States gave poor results (11, 17). The reason for the poor vaccine efficacy in the U.S. trials may have been due to variation in serotype of

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*B. nodosus* between the vaccine strain and the infecting field strain. The primary purpose of the present report is to give results of serotyping of 97 isolates of *B. nodosus* from sheep in Oregon, U.S.A. These data are of significance in the development of vaccine effective against the *B. nodosus* serotypes prevalent in North America.

*Bacteroides nodosus* has been reported to exhibit little biochemical activity (1, 9). Since *B. nodosus* is extremely fastidious, the media used for the biochemical tests may have influenced the results. For this reason, more recently described techniques (14, 15), which do not require active proliferation of the organism in the test system, were used to determine certain biochemical characteristics of the organism. Elastase production capability, as a measure of virulence (20), was determined for each prototype strain.

## MATERIALS AND METHODS

### CULTURES

*Bacteroides nodosus* serotypes A, B and C were obtained from Australia.[1] All other isolates of *B. nodosus* (OSU 1-OSU 97) were obtained in Oregon from foot rot-affected sheep utilizing culture techniques described previously (8). The *B. nodosus* isolates were identified by their characteristic colony and cell morphology and gram stain reaction. Isolates selected as prototype strains of each serotype were further identified using fluorescein-labeled anti-*B. nodosus* antiserum.[2]

### MEDIA

The agar medium used was Eugonagar[3] containing 10% defibrinated horse blood and 0.2% yeast extract. After inoculation with *B. nodosus*, the plates were placed in anaerobic jars fitted with gas exchange valves and evacuated four times — each time the atmosphere in the jar being replaced with 10% $CO_2$ in $H_2$ gas.

Two broth media were used. Broth A, prepared in 90 mL volumes, contained 2.0 g gelatin, 2.0 g trypsin 1:250[4], 1.0 g galactose, 0.2 g yeast extract, 1.0 g proteose peptone, 0.05 g sodium thioglycollate, 3.0 g Eugonbroth[5] and 0.4 mL resazurin solution.[6] After incubation at 5°C for 18 hours, the medium was adjusted to pH 7.5, boiled for 15 minutes, cooled in an ice bath, filtered through a double thickness of medium qualitative filter paper and readjusted to pH 7.5. The medium was dispensed into 250 mL break-resistant Erlenmeyer flasks and prereduced according to the VPI method (9), substituting a mixture of 90% $H_2$ and 10% $CO_2$ for all gassing procedures and omitting all further pH adjustments. When cool, 0.05 g L-cysteine HCL • $H_2O$ was added and the flasks autoclaved in a safety shielded press at 121°C (15 pounds per square inch) for 25 minutes (slow exhaust).

Broth B was an improved form of Broth A and also a modification of the enriched Eugonbroth[7] medium used by Stewart (19). Gelatin hydrolysate[8] (5%) was substituted for the pancreatic extract used by Stewart, 0.4% resazurin solution was added as an anaerobic indicator and the medium was adjusted to pH 8.5 before dispensing and prereducing as in broth A above. The final pH after autoclaving was approximately 7.5.

### ANTIGEN

Antigen for the agglutination test was obtained by seeding agar plates with *B. nodosus*. After four to six days of anaerobic incubation at 37°C, the resultant growth was washed from two or three plates with 0.5 mL of broth medium and transferred into 90 mL of broth medium while maintaining an anaerobic environment within the flask (9). When marked turbidity of the medium was observed,

[1]Egerton, J.R., CISRO, McMaster Laboratory, Glebe, NSW, Australia.

[2]Burroughs-Wellcome, England.

[3]Baltimore Biological Laboratory, Cockeysville, Maryland.

[4]Difco Laboratories, Detroit, Michigan.

[5]Baltimore Biological Laboratory, Cockeysville, Maryland.

[6]One resazurin tablet (ca 11 mg) in 44 mL water.

[7]Baltimore Biological Laboratory, Cockeysville, Maryland.

[8]Grand Island Biological Company, Madison, Wisconsin.

usually after two to five days incubation at 37°C, the broth was centrifuged at 12,100 x g for 20 min and the cells resuspended in 0.25% formalized 0.03 M phosphate buffered saline (FPBS) pH 7.2.

### ANTISERUM PRODUCTION

The antigen for antiserum production was obtained in the manner described for the tube agglutination test or harvested directly from agar plates. Adult New Zealand white rabbits were injected intravenously with 0.25 mL of approximately 2.5 x $10^9$ *B. nodosus* cells/mL FPBS twice weekly for three weeks. Antiserum was harvested two weeks after the last injection.

Antisera against Australian serotypes A, B and C as well as OSU 9 and 11 were made initially. Additional antisera were made from any *B. nodosus* isolates that failed to agglutinate with any of the existing antisera or whenever two-way agglutination tests were indicated.

### AGGLUTINATION TESTS

Agglutination tests were performed in glass tubes (13 by 100 mm) containing 0.5 mL of antiserum serially diluted in FPBS starting at a dilution of 1:2. An equal volume of antigen suspension (approximately 5 x $10^8$ *B. nodosus* cells/mL) was added to 0.5 mL of the diluted antiserum. The final, reported titer was the dilution of antiserum before addition of antigen. After incubation for four to five hours in a 37°C water bath, the K-type agglutination (2) was graded macroscopically on a scale of 4+ to negative: 4+, heavy clumping; 3+, moderate clumping; 2+, light clumping; 1+, questionable clumping; and negative, no reaction. The highest antiserum dilution yielding a 2+ reaction was considered positive. If the agglutination titer of an isolate was identical to or within one dilution of the titer of a prototype and its homologous sera, the isolate was assigned to that serotype.

### BIOCHEMICAL CHARACTERIZATION

The prototype strains of OSU serotypes I through X and Australian serotype B were characterized using the following tests: indole production, gelatin hydrolysis and fermentation tests for glucose, maltose, sucrose, lactose and starch. Conventional methods were used for the detection of indole production (9); however, modifications of rapid methods (14, 15) were used for the remaining tests.

The test for gelatin hydrolysis was performed on exposed X-ray film strips (3.0 x 20.0 mm) which were aseptically added to actively growing cultures in 5.0 mL of the enriched eugon broth A previously described. The tests were incubated at 37°C and observed at 24 and 48 hours. The removal of the gelatin emulsion from the strips indicated a positive test.

The media for the fermentation tests were made as indicated (15); however, since *B. nodosus* growth is sparse, a modification was made in the inoculation procedure. All bacteria were harvested from eugon agar plates using phosphate buffered saline (pH 7.2) and standardized to a MacFarland No. 6 nephelometer. Eight Pasteur pipet drops (about 0.2 mL) of the bacterial suspension were added to each substrate tube and the tubes incubated aerobically at 37°C. At four and 24 hours, each tube was colorimetrically compared to a set of fermentation base medium standards ranging from pH 5.8 and 7.2 in 0.2 increments. Fresh bromthymol blue solution (0.05 mL) was added as needed to aid in the interpretation of questionable reactions (15). *Fusobacterium necrophorum* and *Clostridium perfringens* were inoculated in parallel to the *B. nodosus* isolates and provided a positive and negative control for each test. At the conclusion of each test, the medium was Gram-stained to verify purity.

All prototype strains, including Australian serotypes A, B and C, were further examined for elastase production by clearing of elastin particles in TAS agar medium as described by Stewart (20).

## RESULTS

The tube agglutination tests on 97 *B. nodosus* isolates revealed at least 14 different serotypes of *B. nodosus* in Oregon. These were designated OSU serotypes I through XIV. One isolate did not agglutinate with any of the 14 serotypes and may represent an additional serotype. Australian serotypes A, B and C appear to be serologically identical to Oregon serotypes XIV, IX and VI, respectively (Table I). In addition, OSU serotype X appears to share antigens with Australian

TABLE I. Tube Agglutination Reactions (K Type) between *Bacteroides nodosus*[a] Isolates and Antisera Prepared in Rabbits

| Antigen | Prototype[b] Strain | Antisera | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | V | VI | C | VII | VIII | IX | B | XII | XIII | XIV | A | X | XI |
| I | 2 | 512[c] | o[d] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 32 | 0 | 0 | 0 | 0 | 0 | 0 |
| II | 4 | 0 | 512 | 0 | 0 | 16 | 16 | 0 | 0 | 0 | 128 | 16 | 0 | 16 | 0 | 0 | 0 | 0 |
| III | 6 | 0 | 0 | 1024 | 0 | 0 | 0 | 0 | 0 | 0 | 16 | 64 | 0 | 0 | 0 | 0 | 0 | 0 |
| IV | 9 | 4 | 0 | 2 | 512 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 16 | 0 | 2 | 0 |
| V | 11 | 0 | 8 | 0 | 0 | 1024 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| VI | 18 | 0 | 2 | 0 | 0 | 0 | 2048 | 1024 | 0 | 4 | 4 | 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| C | 141 | 0 | 0 | 0 | 0 | 0 | 2048 | 1024 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 16 |
| VII | 22 | 8 | 0 | 4 | 0 | 0 | 0 | 0 | 2048 | 4 | 16 | 16 | 2 | 0 | 128 | 32 | 8 | 0 |
| VIII | 26 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 512 | 4 | 16 | 0 | 4 | 0 | 0 | 0 | 0 |
| IX | 27 | 0 | 64 | 4 | 0 | 0 | 0 | 0 | 8 | 4 | >2048 | 1024 | >2048 | 128 | 0 | 0 | 0 | 0 |
| B | 206 | 0 | 32 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1024 | 2048 | 2048 | 128 | 0 | 0 | 0 | 0 |
| XII | 74 | 0 | 2 | 4 | 0 | 0 | 0 | 0 | 8 | 4 | 16 | 0 | >2048 | 128 | 0 | 0 | 0 | 0 |
| XIII | 75 | 0 | 32 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 256 | 256 | 512 | 2048 | 4 | 0 | 0 | 0 |
| XIV | 76 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 64 | 0 | 0 | 2 | 0 | 0 | 2048 | 512 | 128 | 0 |
| A | 198 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 32 | 0 | 0 | 2 | 0 | 4 | 1024 | 512 | 32 | 0 |
| X | 40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 | 0 | 0 | 0 | 0 | 0 | 128 | 512 | 512 | 2 |
| XI | 72 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 2 | 4 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1024 |
| OSU 95 | | 2 | 4 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 128 |

[a]Serotypes I-XIV are Oregon serotypes; A, B and C are Australian serotypes
[b]Oregon State University or Australian isolation number
[c]Reciprocal of the antibody titer
[d]No reaction in antiserum diluted 1:2 or greater

serotype A; and OSU serotypes XII and XIII share antigens with Australian serotype B. Low level cross agglutination reactions were also detected between serotypes II and IX and between serotypes VII and XIV. One *B. nodosus* isolate, OSU 95, did not agglutinate with any of the 14 OSU serotypes at a level high enough to be classified; however, it did agglutinate at 1:128 with OSU serotype XI, indicating some shared antigens with this serotype. Rabbit antiserum against OSU 95 is being produced for further characterization of this isolate.

**TABLE II. Tube Agglutination Reaction (K Type) of *Bacteroides nodosus* Isolates Identified as OSU Serotype IV with Antisera of Selected Serotypes**

| Number of Isolates | *Bacteroides nodosus* serotype antisera | | | | |
|---|---|---|---|---|---|
| | OSU[a] | | Australian | | |
| | IV | V | A | B | C |
| 16[b] | ⩾512[c] | 0[d] | 0 | 0 | 0 |
| 3 | ⩾512 | 8 | 0 | 16 | 0 |
| 2 | ⩾512 | 0 | 0 | 16 | 4 |
| 1 | ⩾512 | 0 | 0 | 8 | 0 |
| 1 | ⩾512 | 0 | 0 | 32 | 0 |
| 1 | ⩾512 | 0 | 0 | 64 | 0 |
| 1 | ⩾512 | 8 | 0 | 8 | 0 |
| 1 | ⩾512 | 16 | 0 | 4 | 0 |
| 1 | ⩾512 | 16 | 0 | 8 | 0 |
| 1 | ⩾512 | 16 | 0 | 32 | 0 |
| 1 | ⩾512 | 8 | 4 | 32 | 4 |

[a]Oregon State University
[b]Includes OSU 9, the prototype stain of serotype IV
[c]Reciprocal of the antibody titer
[d]No reaction in antisera diluted 1:4 or greater

Not all *B. nodosus* isolates classified within one serotype reacted equally with the prototype antisera of other serotypes. This is illustrated in Table II which shows the range of agglutination titers of all isolates classified as OSU serotype IV when tested with four heterologous antisera. These cross agglutination titers ranged from 1:4 to 1:64.

The 97 *B. nodosus* isolates tested were obtained from 20 flocks. Five flocks each yielded a single isolate while multiple isolates were obtained from 15 flocks. Thirteen of the 15 flocks yielding multiple isolates had two to seven different *B. nodosus* serotypes while only one serotype was identified among isolates from each of the remaining two flocks. The distribution of the *B. nodosus* isolates among the 14 sero-

**TABLE III. Number and Distribution of *Bacteroides nodosus* Serotypes Isolated from 20 Oregon Sheep Flocks**

| Flock Designation | Total Isolates | *Bacteroides nodosus* OSU Serotype | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | V | VI | VII | VIII | IX | X | XI | XII | XIII | XIV | untyped |
| WM | 22 | | | 1[a] | 8 | | | | 3 | 6 | | 2 | 1 | 1 | | |
| VM | 13 | | | 1 | 4 | 2 | | | | 1 | 4 | | | | 1 | |
| AR | 8 | | | 1 | 6 | | 1 | | | | | | | | | |
| LK | 7 | | | | | | | | | 3 | 4 | | | | | |
| SL | 6 | | | | | | | | 1 | | 5 | | | | | |
| SC | 6 | | | | 4 | 1 | | | | | | | | | | 1 |
| CS | 5 | | | | 2 | 2 | | | | 1 | | | | | | |
| JK | 5 | | | | 5 | | | | | | | | | | | |
| WS | 4 | | | 1 | 1 | | | 2 | | | | | | | | |
| LA | 4 | 3 | | | | 1 | | | | | | | | | | |
| DG | 3 | 1 | | 1 | | 1 | | | | | | | | | | |
| IJ | 3 | | | | | | | | 1 | 2 | | | | | | |
| JB | 2 | 1 | | | | | | | 1 | | | | | | | |
| EL | 2 | | | 1 | | 1 | | | | | | | | | | |
| BH | 2 | | | | | 2 | | | | | | | | | | |
| HK | 1 | | 1 | | | | | | | | | | | | | |
| HH | 1 | | | | | | | | | 1 | | | | | | |
| AW | 1 | | | | | | | | 1 | | | | | | | |
| JG | 1 | | | | | | | | 1 | | | | | | | |
| DN | 1 | | | 1 | | | | | | | | | | | | |
| Totals | 97 | 5 | 1 | 7 | 30 | 10 | 1 | 2 | 8 | 14 | 13 | 2 | 1 | 1 | 1 | 1 |

[a]Number of isolates of this serotype recovered from this flock

types is shown in Table III. Each serotype group is comprised of one to 30 isolates obtained from one to seven flocks.

Multiple *B. nodosus* isolates were acquired from eight different sheep. In five of these sheep the isolates were all of the same serotype; however, in three cases the sheep were simultaneously infected with two different serotypes — serotypes III and VII, serotypes IV and IX and serotype VIII and XI. Furthermore, the sheep from which serotypes VIII and XI were simultaneously isolated had yielded an isolate of serotype XIII 130 days earlier.

Oregon serotypes I through X and Australian serotype B were examined for biochemical characteristics. All isolates digested gelatin, a manifestation of proteolytic activity, while none fermented carbohydrates or produced indole.

The prototype strains of serotype VI, XI and Australian serotype B were elastase negative. Serotypes II, IV, V, VIII, IX, X, XII, XIII and Australian serotype A were strongly elastase positive, while serotypes I, III, VII, XIV and Australian serotype C were only weakly positive. Thirteen serotype IV isolates were also tested and found to be strongly elastase positive.

## DISCUSSION

There are two previous reports of serological typing of *B. nodosus* isolates. Egerton (2) first described the K and O agglutination tests with *B. nodosus* and classified 33 of 46 Australian isolates into types A, B or C on the basis of K agglutination tests. The remaining 13 isolates studied could not be classified by this method. More recently, K agglutination reactions of nine English strains and two Australian strains (Types A and C) with homologous and heterologous antisera were determined. On the basis of these tests, nine serologically distinct groups were identified among the English isolates; these isolates were all serologically distinct from the two Australian serotypes. Four additional English isolates, for which no antisera were available, were also tested against the available *B. nodosus* antisera and none showed serological identity with the English or the Australian serogroups (21).

In contrast to the study conducted in England, *B. nodosus* isolates serologically similar to the Australian serotypes A, B and C were identified in Oregon, U.S.A. The previous reports, together with the present study indicate considerable serological heterogeneity among *B. nodosus* isolates. It is also possible that further serological differences could be demonstrated in the present study if antiserum to each isolate were produced. For example, if antisera for isolates OSU 40 (Serotype XIV) and 76 (Serotype X) had not been produced, they would both appear identical to Australian serotype A (Table I). Isolates could have also been further separated serologically in the present study on the basis of the minor cross reactions shown in Table II. However, current emphasis was placed on major serological reactions as this should be more useful in selection of strains for vaccine production.

The biochemical inactivity of *B. nodosus* reported previously was supported in this study. The virulence of 12 of the 14 Oregon *B. nodosus* prototype strains, as determined by elastase production, is not surprising as these isolates were obtained from flocks with serious foot rot problems. Further investigation will be necessary to establish any possible correlation between degree of virulence of an isolate and its serotypic classification.

The apparent antigenic heterogeneity of *B. nodosus* isolates is disappointing relative to the possible development of an effective sheep foot rot vaccine. The protective immunogen of *B. nodosus* bacterins has been shown to be an antigen either identical to, or closely associated with the K antigen (3, 18, 19). Serological differences between field strains and vaccine strains of *B. nodosus* appears to have caused the failure of a foot rot vaccine in several flocks in Australia (3). Experimentally it has been shown that cross protection occurs between Australian types A and B, but not between Australian types A and C (3). The finding of at least 14 different serotypes in Oregon, with up to seven different serotypes isolated from one flock indicates the necessity to determine cross immunogenesis between different *B. nodosus* serotypes and to clearly elucidate specific immunogenic antigens associated with *B. nodosus* organisms if effective vaccines are to be developed.

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