JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1990, p. 2456–2461
0095-1137/90/112456-06$02.00/0

Vol. 28, No. 11

Polymerase Chain Reaction Amplification of the Constant and Variable Regions of the *Bacteroides nodosus* Fimbrial Gene

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Received 20 March 1990/Accepted 14 August 1990

Serogrouping of *Bacteroides nodosus* is based on antigenic differences in fimbriae of the different New Zealand prototype strains. Because of the time needed to isolate and grow pure cultures of *B. nodosus* and the difficulty in distinguishing between different serogroups because of cross-agglutination, a new DNA-based diagnostic approach based on the fimbrial gene sequence of *B. nodosus* was developed. Published nucleotide sequences of the fimbrial genes for serogroups A, G, D, and H showed conservation at the 5′ end, coding for the N terminus, and variability at the 3′ end, coding for the C terminus. The polymerase chain reaction was used to amplify both the constant and variable regions of the fimbrial genes. Constant-region oligonucleotide primers were used to amplify a 100-base-pair fragment from the constant regions of the fimbrial genes of 10 New Zealand serogroups. Serogroup-specific oligonucleotide primers for serogroups A and H allowed amplification of a 282-base-pair fragment from serogroup A and a 363-base-pair fragment from serogroup H. Thus, amplification of the constant and variable regions of the fimbrial gene allows rapid detection and grouping of *B. nodosus*.

Bacteroides nodosus is the causative agent of foot rot in sheep (1, 7). Foot rot remains a constant economic burden for many Western United States sheep ranchers. Serious cases of foot rot result in lameness, poor wool production, and susceptibility to other infectious diseases. The economic impact in the past few years has been substantial. Annual losses of $50,000 or more for individual ranchers due to added labor, feed, and antibiotics and to lower lamb prices have been reported (2, 13).

B. nodosus is an anaerobic, gram-negative, rod-shaped bacterium with characteristic knobs at both ends (6, 14). Surface fimbriae and high levels of stable extracellular proteases are thought to allow the bacteria to colonize the interdigital epithelial tissue of sheep feet (10). Although foot rot is not a fatal disease, it is very infectious. Infected sheep can easily spread the bacteria throughout a herd, especially during the wet season. Vaccines based on whole cells or purified fimbriae induce antibodies to the surface fimbriae, resulting in some immunoprotection against *B. nodosus* (15, 26, 27). Ten major *B. nodosus* serogroups found in infected sheep herds in Australia and New Zealand are included in the vaccine currently available in the United States.

The current method of grouping *B. nodosus* is based on antigenic epitopes located on the surface fimbriae (24, 31). Agglutination (slide or tube) or enzyme-linked immunosorbent assay (ELISA) is currently used to identify unknown strains (3, 5, 16, 28). Serogrouping is very time consuming since it normally takes about 3 to 4 weeks to grow and harvest enough pure cultures of *B. nodosus* from agar plates to perform an agglutination or an ELISA. In addition, these immunoassays show some cross-reactivity between serogroups, making it difficult to identify a specific serogroup. Our current grouping system is based on 10 New Zealand prototype strains: A, B1, B2, B4, C, D, E, F, G, and H.

Published amino acid sequences of the fimbriae of Australian strains (A, B, C, D, E, F, G, and H), which are similar to if not the same as those of the New Zealand strains, have shown conservation at the amino-terminal regions and variability at the carboxy termini (8, 20, 21). Isolation from several strains of *B. nodosus* of the genes encoding fimbrial protein allowed the determination of the nucleotide sequences of the fimbrial gene from serogroups A (strain 198), G (strain 238), D (strain 340), and H (strain 265) (8–12). Comparison of the gene sequences also showed conservation at the N termini and variability at the C termini.

We used the polymerase chain reaction (PCR) technique (22, 23) for rapid and accurate detection of *B. nodosus* by amplifying the constant region of the fimbrial gene. In addition, selected serogroups of *B. nodosus* isolates can be quickly identified by amplifying the unique variable regions of the fimbrial genes. PCR requires two primers: an upstream primer, which anneals to one strand of the DNA, and a downstream primer, which anneals to the opposite strand. One cycle involves denaturation of double-stranded DNA, annealing of the two primers, and extension of the two primers in opposite directions, thus doubling the amount of target DNA. Repetition of this cycle results in an exponential amplification of the fragment. The length of the newly synthesized fragment of DNA can be determined easily by gel electrophoresis. The ability to amplify a target sequence to a specific length allows for a very specific, sensitive, and rapid diagnostic test.

MATERIALS AND METHODS

Preparation and isolation of DNA. Samples of exudate from infected sheep feet were plated for *B. nodosus* isolation onto hoof agar (sheep hoof powder, 1.5%; beef extract, 0.5% [Difco, Detroit, Mich.]; proteose peptone, 1% [Difco]; sodium chloride, 0.5% [Sigma Chemical Co., St. Louis, Mo.]; yeast extract, 0.1% [Difco] [adjusted to pH 7.8 to 8.0 before autoclaving]) and grown anaerobically (90% H_2–10% CO_2) for 5 to 7 days at 37°C. Following isolation, *B. nodosus* was inoculated onto eugon agar (eugon agar, 4.5% [Difco]; yeast extract, 0.2% [Difco]; 10% defibrinated horse blood [Colorado Serum Co., Denver, Colo.]) and collected after anaer-

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obic growth in a 90% H_2–10% CO_2 gas mixture for 5 to 7 days at 37°C. *Clostridium perfringens* (grown anaerobically) and *Pseudomonas aeruginosa* (grown aerobically) were collected from Trypticase soy agar containing 5% sheep blood agar (TSA II; BBL Microbiology Systems, Cockeysville, Md.) after 24 to 36 h of growth at 37°C. Bacteria (two plates, 100 by 15 mm) were collected into 250 μl of TE buffer (10.0 mM Tris hydrochloride, pH 8.0, 1.0 mM EDTA). Lysozyme (Sigma) (4 mg/ml) was added, and the mixture was put on ice for 20 min. Sodium dodecyl sulfate (SDS; 1%) was added, the mixture was stirred gently, and 200 μg of protease K per ml (Sigma) was added with gentle mixing; the mixture was incubated at 45°C for 4 h. The mixture was extracted twice with an equal volume of phenol-chloroform (1:1). Nucleic acids were precipitated from the aqueous phase with 2 volumes of ice-cold 90% ethanol at −20°C for 1 h. The precipitate was dissolved in 400 μl of TE buffer. RNase A (Sigma) (100 μg/ml) was added, and the mixture was incubated for 30 min at 37°C. Proteinase K (100 μg/ml) and 0.5% SDS were added, and the mixture was incubated for 1 h at 50°C. Two phenol-chloroform extractions were performed, as described above, and the DNA was precipitated with ice-cold 90% ethanol–1/10 volume of 3 M sodium acetate at −20°C for 1 h. The precipitate was dissolved in 250 μl of TE buffer.

PCR. Oligonucleotides C1 (5'GGTTTCACCTTAATCGA ACTC3'), C2 (5'CTTGTGAACGAGCGATGTAGT3'), and C3 (5'GATTGTAGTTGCAATTATCGGTATCTTAGC3') were synthesized by Vance Vorndam at the Centers for Disease Control Branch Laboratory at Fort Collins, Colo. The oligonucleotides A1 (5'AAGGGAGATGCGAACCCAG CT3'), A2 (5'TTTAACAGCATTCGGAATAAA3'), and H2 (5'TTTCTTACCAACGAT3') were synthesized by Research Genetics Corp., Huntsville, Ala. Fragments were amplified from chromosomal DNA in 50-μl reactions containing 16.6 mM $(NH_4)_2SO_4$, 6.7 mM Tris hydrochloride (pH 8.8 at 25°C), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 200 μM each dATP, dCTP, dGTP, and dTTP; 8.5 μg of bovine serum albumin per ml, 0.3 μg of each primer, and 2.5 U of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin Elmer Corp., Norwalk, Conn., or New England BioLabs, Beverly, Mass.) were added to the PCR buffer. The chromosomal DNA was denatured at 95°C for 1 min, incubated at 40°C for 1 min to allow the primers to anneal, and then incubated at 65°C for 1 min to activate the polymerase, thus completing one cycle. An average of 25 to 50 cycles were performed in the temperature cycler (Ericomp, Inc., San Diego, Calif.) on nanogram levels of DNA. When amplifying femtogram levels of DNA, a 1:100 dilution of the primer concentration used in PCRs, when the starting DNA concentration was in the nanogram-or-higher range, was used for the first 10 cycles. Additional primers were then added to yield the normal concentration for the next 30 to 50 cycles. For amplification of whole-cell bacterial DNA, 10% dimethyl sulfoxide was added to the PCR buffer and the initial denaturing cycle was 5 min at 95°C.

Gel electrophoresis. Five microliters of the 50-μl PCR sample was added to a 1.8% agarose gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) (pH 8.0)–0.5 μg of ethidium bromide per ml. Two microliters of tracking dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in H_2O) was added to the 5-μl PCR sample. Electrophoresis was at 70 V for 90 min. A 123-base-pair DNA ladder (3 μg) (Bethesda Research Laboratories, Gaithersburg, Md.) was used as standard size markers. The gel was observed over a UV transilluminator and photographed with a Polaroid MP4 camera.

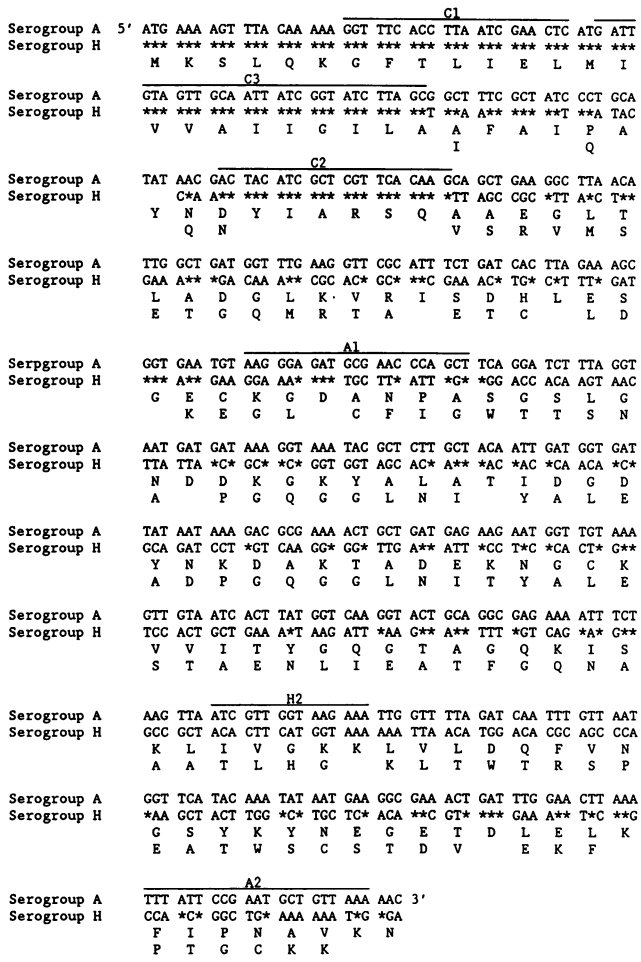

FIG. 1. Comparison of the nucleotide and amino acid sequences of pilin genes from *B. nodosus* serogroup A (strain 198) and serogroup H (strain 265). Identical nucleotides are indicated by an asterisk. The full amino acid sequence of serogroup A is shown; positions which differ in serogroup H are indicated under the A sequence. C1 (21-mer) and C2 (21-mer) represent the constant upstream and downstream primers, A1 (21-mer) and A2 (21-mer) represent the variable upstream and downstream primers for serogroup A, and C1 and H2 (15-mer) represent the variable upstream and downstream primers for serogroup H. C3 (30-mer) represents the ^{32}P-labeled probe.

Southern blotting and hybridization. Gels were blotted onto a GeneScreen Plus membrane (Dupont, Boston, Mass.). Briefly, DNA was denatured in the gel for 30 min with 0.4 N NaOH and 0.6 M NaCl and neutralized for 30 min with 1.5 M NaCl and 0.5 M Tris hydrochloride, pH 7.5. Prior to blotting, the GeneScreen membrane was soaked in 10× SSC (1.5 M NaCl, 0.15 M citric acid) for 15 min. Blotting for transfer of DNA was allowed to continue for 16 to 24 h. After transfer, the membrane was immersed in 0.4 N NaOH for 30 to 60 s to ensure denaturation of DNA. The membrane was then immersed in excess 0.2 M Tris hydrochloride (pH 7.5)–2× SSC and allowed to dry at room temperature. The dried membrane was prehybridized in a sealable bag with a 6× SSC–10% SDS solution for 4 h at room temperature with gentle agitation. The ^{32}P-labeled oligonucleotide probe, C3 (5×10^6 cpm), was added to the solution in the sealable bag

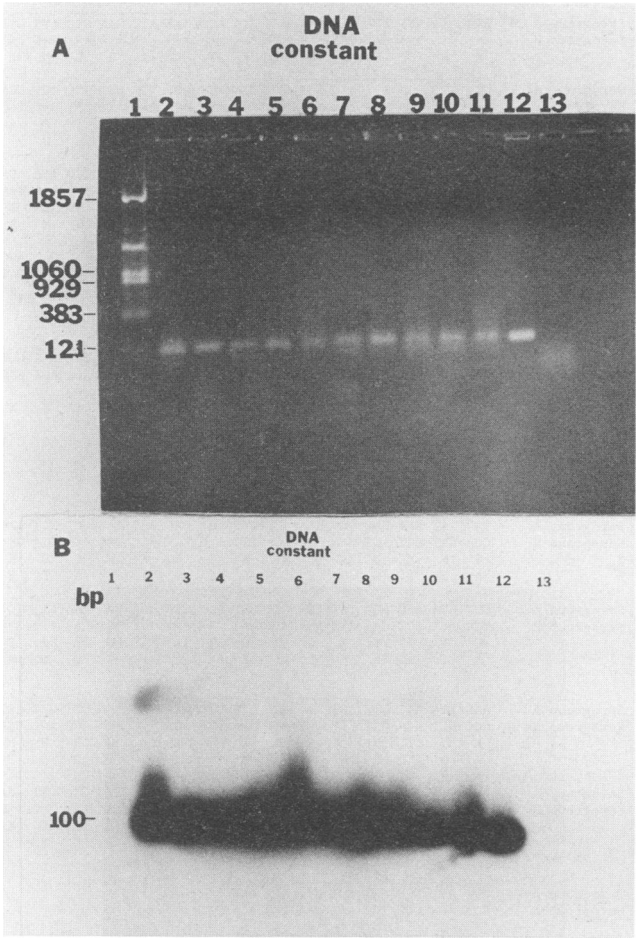

FIG. 2. (A) Ethidium bromide-stained gel of the constant-region amplification (30 cycles) of pure DNA from all 10 standard serogroups. Lanes: 1, pBR322 DNA *Bst*N1 digest; 2, serogroup A; 3, B1; 4, B2; 5, B4; 6, C; 7, D; 8, E; 9, F; 10, G; 11, H; 12, plasmid (7AIE) containing the fimbrial gene for serogroup A (strain 198); 13, blank (no DNA). (B) Autoradiogram of a Southern blot of the gel described above probed with ^{32}P-labeled C3 probe.

and hybridized for 18 to 24 h at room temperature. The membrane was removed from the hybridization solution and washed twice with 3× SSC–1% SDS for 20 min with gentle agitation at room temperature. The membrane was then washed twice with 3× SSC for 20 min each time with gentle agitation at room temperature. The membrane was wrapped in plastic wrap and exposed to X-ray film for 6 to 12 h.

Nucleotide sequence accession numbers. The GenBank/EMBL accession numbers for serogroups A and H are K02662 and M13765, respectively.

RESULTS

Fimbrial proteins from all of the serogroups (A to H) have identical amino acid sequences for the first 30 amino acids. Primers were chosen on the basis of homology between these sequences. A comparison of the fimbrial gene sequences for serogroups A and H is shown in Fig. 1, in which the identical amino acid sequence and nucleotide sequence are displayed at the amino terminus.

A 21-mer constant upstream primer (C1) was selected and was 100% homologous with the published sequences from the fimbrial gene for serogroups A, G, D, and H (8, 9, 11, 12). The homology of the 21-mer constant downstream primer (C2) with serogroups A, G, D, and H was 100% (A), 86% (G), 100% (D), and 100% (H).

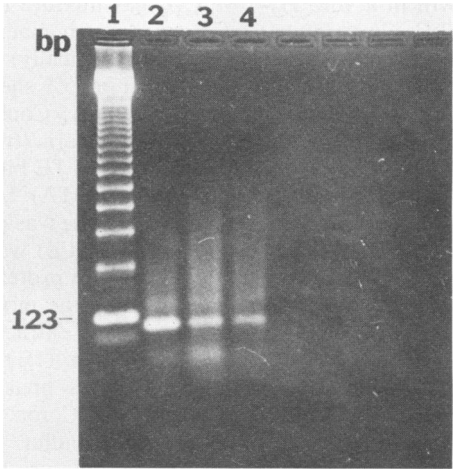

FIG. 3. Amplification of dilutions of pure *B. nodosus* DNA. Lanes: 1, 123-base-pair ladder; 2, 10 ng; 3, 100 pg; 4, 10 fg. Amplification involved 40 cycles.

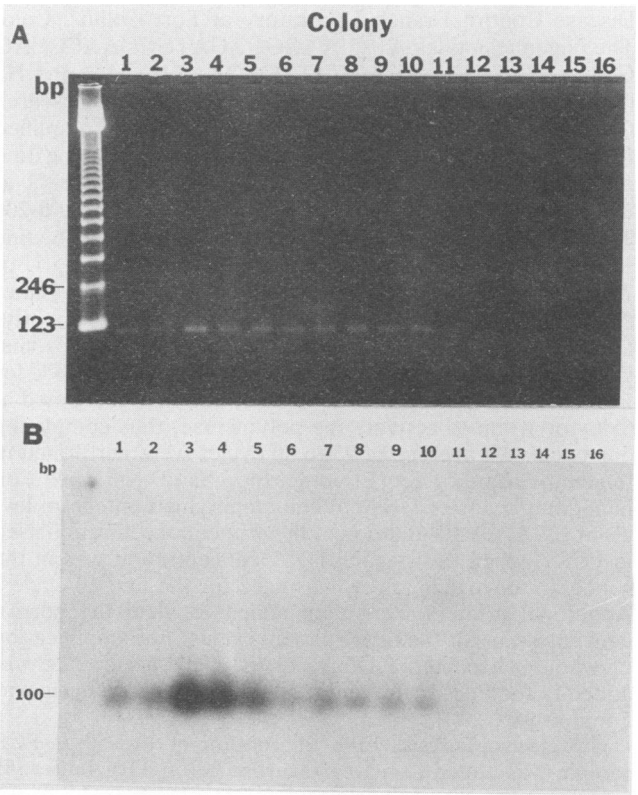

FIG. 4. Ethidium bromide-stained gel following direct amplification (30 cycles) of the constant region from whole-cell bacteria of all 10 serogroups. (A) Lanes: 1, serogroup A; 2, B1; 3, B2; 4, B4; 5, C; 6, D; 7, E; 8, F; 9, G; 10, H; 11, plasmid (7AIE); 12, *P. aeruginosa*; 13, *C. perfringens*; 14, contaminant (c) 1; 15, c 2; 16, c 3. (B) Autoradiogram of a Southern blot of the gel described above probed with ^{32}P-labeled C3 probe.

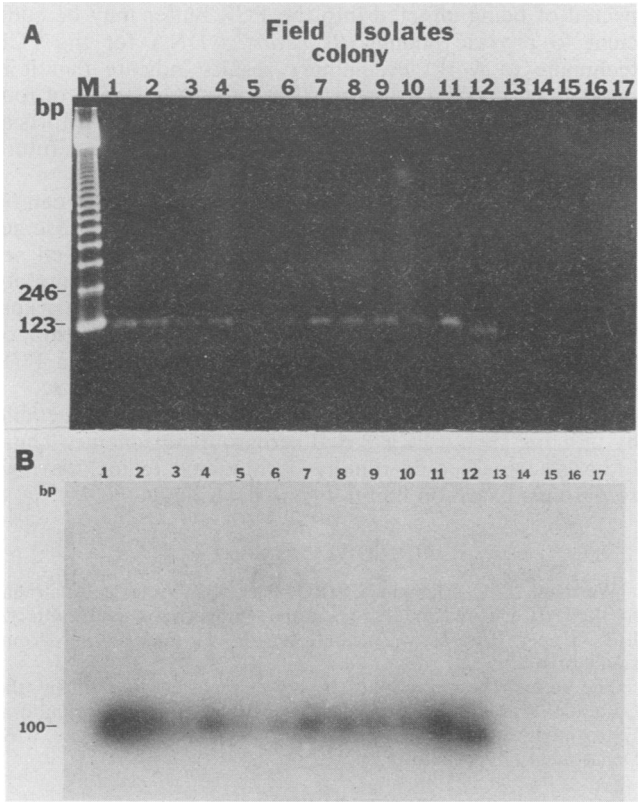

FIG. 5. (A) Ethidium bromide-stained agarose gel following direct amplification (30 cycles) of the constant region from whole-cell bacteria (field isolates). Lanes: 1, plasmid (7AIE); 2, 404; 3, G82; 4, G83; 5, G19; 6, G18; 7, G332; 8, G162; 9, 5A Moel.; 10, G84; 11, G338; 12, G72; 13, *P. aeruginosa*; 14, *C. perfringens*; 15, contaminant (c) 1; 16, c 2; 17, c 3. (B) Autoradiogram of a Southern blot of the gel described above probed with ^{32}P-labeled C3 probe.

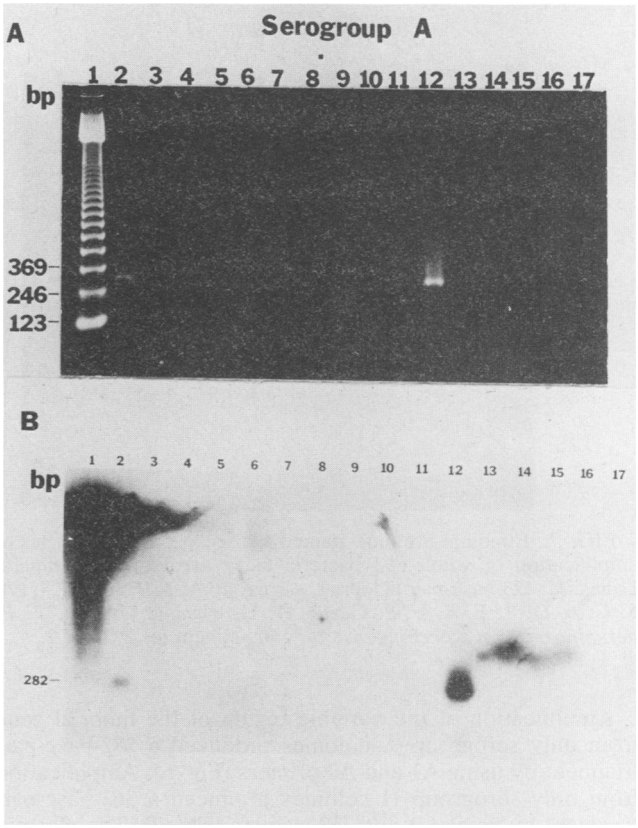

FIG. 6. (A) Ethidium bromide-stained agarose gel after 30 cycles of amplification of whole-cell bacteria using serogroup A-specific primers. Lanes: 1, 123-base-pair ladder; 2, serogroup A; 3, B1; 4, B2; 5, B4; 6, C; 7, D; 8, E; 9, F; 10, G; 11, H; 12, plasmid (7AIE); 13, *P. aeruginosa*; 14, *C. perfringens*; 15, contaminant (c) 1; 16, c 2; 17, c 3. (B) Autoradiograph of a Southern blot of the gel described above probed with ^{32}P-labeled plasmid 7AIE.

The C1 primer was 81%, 76%, and 81% homologous with the constant regions of the fimbrial genes of *P. aeruginosa* PAK, PA1, and PAO, with the last base at the 3′ end being noncomplementary (8, 18). The C2 primer was 71.4, 76, and 71.5% homologous with strains PAK, PA1, and PAO, respectively. It is expected that samples collected directly from sheep feet and subjected to PCR amplification would contain *P. aeruginosa*, since this bacterium is commonly found in soil and water. An annealing temperature of 40°C enabled the primers to specifically amplify only the standard *B. nodosus* serogroups, with no detectable amplification of *P. aeruginosa*.

The 21-mer variable-region upstream primer (A1) and the 21-mer downstream primer (A2) were 100% homologous with only serogroup A (Fig. 1). The A2 primer had less than 70% homology with the sequences available from other standard serogroups. The 15-mer variable-region primer (H2) for serogroup H was 100% homologous with serogroup H (Fig. 1) and less than 70% homologous with the remaining standard serogroups.

Approximately 30 pg of chromosomal DNA from each of the serogroups was amplified with these primers. The plasmid (7AIE) containing the fimbrial gene for serogroup A (strain 198) (9) served as the positive control, and *C. perfringens* served as the negative control. A 100-base-pair sequence from the constant region of the fimbrial gene from all serogroups and subgroups was amplified by using the C1 and C2 primers (Fig. 2). A Southern blot, using a ^{32}P-labeled detector probe (C3) for the constant region, confirmed that the correct fragments were amplified (Fig. 2B). Bacterial colonies morphologically similar to *B. nodosus* were frequently observed on initial isolation plates. These colonies yielded bacteria which were differentiable from *B. nodosus* on the basis of Gram stain characteristics. When selected colonies of these contaminants were subjected to PCR amplification, no amplified product was obtained.

A 10-fold serial dilution, starting with 1 μg of purified bacterial DNA, was created in order to test the sensitivity of PCR. After 40 amplification cycles, the last dilution (10 fg) was successfully amplified (Fig. 3). This dilution corresponds to the amount of DNA in two to three bacteria. Smaller amounts of DNA were more efficiently amplified by the biphasic amplification scheme described in Materials and Methods (data not shown). Additional *Taq* polymerase is usually not needed.

In addition to amplification of pure DNA, amplification directly from single colonies of *B. nodosus* was attempted (Fig. 4 and 5). Colonies from each sample were collected with an inoculating loop and put into the PCR buffer. The sample was subjected to 30 amplification cycles. Amplification of contaminants usually found in an infected sheep foot, plus amplification of *P. aeruginosa* and *C. perfringens*, was also attempted. The primers amplified only the *B. nodosus* DNA, thus indicating a high degree of specificity.

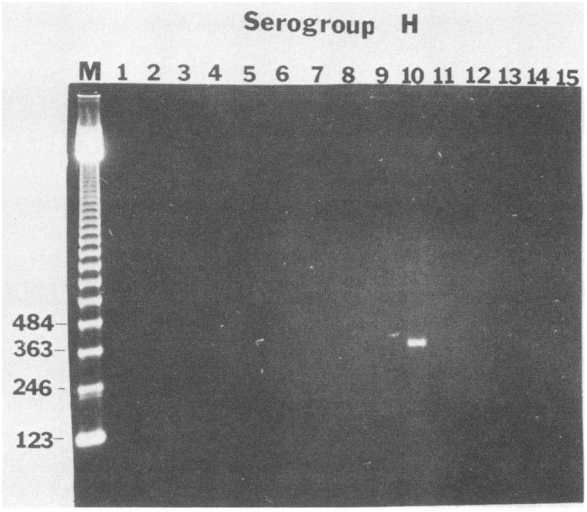

FIG. 7. Ethidium bromide-stained agarose gel after 30 cycles of amplification of whole-cell bacteria using serogroup H primers. Lanes: M, 123-base-pair ladder; 1, serogroup A; 2, B1; 3, B2; 4, B4; 5, C; 6, D; 7, E; 8, F; 9, G; 10, H; 11, plasmid (7AIE); 12, *P. aeruginosa*; 13, *C. perfringens*; 14, contaminant (c) 1; 15, c 2.

Amplification of the variable region of the fimbrial gene from only serogroup A colonies produced a 282-base-pair fragment by using A1 and A2 primers (Fig. 6). Amplification from only serogroup H colonies produced a 363-base-pair fragment by using C1 and H2 primers (Fig. 7). The plasmid (7AIE) which contains the gene for serogroup A (strain 198) was labeled by using the multiprime DNA labeling system (Amersham, Arlington Heights, Ill.) and was used as a detector probe for serogroup A in Southern blot hybridization. Both the A primer and the H primer were tested with several frequently observed bacterial contaminants and the remaining standard serogroups; no amplification was detected.

DISCUSSION

The current methods for identifying and serogrouping (A to H) *B. nodosus* isolates from an infected sheep are based on biochemical identification (19, 25, 29, 30) and either agglutination or ELISA (3–5, 17, 31). We took a new approach in identifying and grouping the bacteria because the current identification and serogrouping methods (agglutination, ELISA, etc.) are not completely specific and are very time consuming. The normal sequence of events involved in *B. nodosus* isolation and serogrouping involves collection of a sample from an infected foot, passage onto a hoof agar plate, and anaerobic incubation for 5 to 7 days. Additional passages are usually performed to obtain sufficient amounts of pure *B. nodosus* for agglutination or ELISA serogrouping. During this process, various biochemical reactions must also be performed to confirm *B. nodosus* identification. It normally takes 3 to 5 weeks to positively identify and serogroup *B. nodosus*.

The PCR technique can be used to shorten the time for identifying and serogrouping *B. nodosus* since the technique can be easily performed on single colonies from the initial plating. Since the assay is capable of amplifying 10 fg of DNA (the amount of DNA in two to three bacteria), an initial sample collection from an infected sheep foot or one suspected of being infected into the PCR buffer may be sufficient to provide enough *B. nodosus* DNA for the PCR technique to work. Preliminary results indicate that it is possible to amplify *B. nodosus* directly from swabs of foot rot lesions (data not shown). We are continuing our efforts at direct amplification and will report the results in a future publication.

Our findings have shown that amplified sequences can be detected easily on an ethidium bromide-stained agarose gel without the use of radioactive probes. The amplified sequence has a specific length and can be identified accurately by comparing it to a standard molecular weight marker. Thus, by selecting primers which yield amplification of fragments of specific lengths for each serogroup, a PCR serogrouping system can be developed for *B. nodosus*.

Emerging serogroup variation should be rapidly detectable by utilizing such a PCR-based serogrouping scheme. Thus, future vaccines could rapidly be modified to include new serogroups based on fimbrial gene heterology.

ACKNOWLEDGMENTS

We thank T. C. Elleman, CSIRO, Parkville, Victoria, Australia, for the 7AIE plasmid and D. M. Liardet, Coopers Animal Health NZ Ltd., Upper Hut, New Zealand, for the *B. nodosus* serogroup standard strains.

The research reported herein was supported in part by the Colorado Wool Growers Association, the Colorado Agricultural Experiment Station, and grant 86-CRSR-2-2907 from the U.S. Department of Agriculture.

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