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# New Variant of *Vibrio cholerae* O1 from Clinical Isolates in Amazonia

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**A survey of pathogenic *Vibrio cholerae* O1 strains from the north of Brazil by using arbitrarily primed PCR fingerprints revealed a group of strains with similar fingerprint patterns that are distinct from those of the current El Tor epidemic strain. These strains have been analyzed by in vivo and in vitro techniques and the group has been denominated the Amazonia variant of *V. cholerae* O1.**

*Vibrio cholerae* is causing a severe epidemic in Latin America after being absent from the continent for about 100 years (30). The taxonomy of this species has been the object of our interest, and we recently developed a method for distinguishing pathogenic groups by using arbitrarily primed PCR (AP-PCR) fingerprints (5, 6) on the basis of the general methodology of AP-PCR (31, 32). By this technique a single oligonucleotide with an arbitrary sequence is used in a PCR with the DNA of the strain under analysis. Low-stringency conditions for hybridization are used, and the oligonucleotide can find regions of pairing, leading to the amplification of various genome fragments. Our original study (6) involved four groups of pathogenic *V. cholerae*: classical, El Tor, Gulf of Mexico endemic El Tor, and Bengal, all of which are distinguishable with the fingerprints.

When applied to strains causing the Latin American epidemics the results were similar to those for other Old World El Tor strains. However, when this method was used to study a group of strains from patients with diarrheal disease in the westernmost part of the Brazilian Amazon, a quite distinct fingerprint pattern emerged for some of these strains. In the work described here we further extended this observation by using other in vitro and in vivo techniques to evaluate the degree of relatedness between this group and the other pathogenic strains.

## MATERIALS AND METHODS

**Strains of *V. cholerae*.** The strains used in the present study are listed in Table 1.

**AP-PCR.** The AP-PCR mixtures consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 100 μM (each) deoxynucleoside triphosphate (dNTP), 30 pmol of one of the oligonucleotides, and 100 ng of DNA in a total volume of 25 μl. The mixture was overlaid with oil, and 1.5 U of *Taq* DNA polymerase was added. The program consisted of 45 cycles, and an annealing temperature of 32°C was used (6). Two sets of fingerprints were done, one of them with oligonucleotide 1 (5′-GGTGCGGGAA) and the other with oligonucleotide 3 (5′-CCAGATGCAC) (6).

Analysis of the amplified fragments was done on 1.4% agarose gels (GIBCO-Bethesda Research Laboratories) in Tris-borate buffer (TBE) (27) running at 130 V for 2.5 h.

**Multilocus enzyme electrophoresis.** The multilocus enzyme electrophoresis method used in the present study was the same as that described previously (24).

**Ribotyping.** Chromosomal DNA was prepared from 5 ml of an overnight culture in alkaline peptone water (6, 28). A total of 15 μg of chromosomal DNA from each of the strains was cut with 30 U of *Bgl*I for a period of 8 h. DNA fragments were separated in 0.7%, 22-cm agarose gels in TBE at 50 V for 15 h. The DNA was transferred to a nitrocellulose filter (GIBCO-Bethesda Research Laboratories) with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and by a traditional Southern blot protocol (27). The probe for the ribosomal DNA was the 7.5-kb *Bam*HI fragment of plasmid pKK3535 (4) containing the complete *Escherichia coli rrnB* RNA operon. [$\alpha^{32}$P]dATP was incorporated through a random primer protocol (GIBCO-Bethesda Research Laboratories). The hybridization mixture, which was added directly to the dry filter, contained 6× SSC, 50% formamide, and 0.5% sodium dodecyl sulfate (17a). The denatured radioactive probe was added after wetting the filter, and the mixture was left overnight at 42°C with gentle shaking.

**Analysis of presence of virulence genes by PCR.** The basic program for the PCRs for the specific genes included 35 three-step cycles at 94°C (1 min), 55°C (1.5 min), and 72°C (1.5 min). A total of 100 ng of DNA, 20 pmol of each primer, 0.25 mM dNTPs, and 1.5 U of *Taq* DNA polymerase were used, with a 1.5 mM $MgCl_2$ buffer, in a total volume of 50 μl. The oligonucleotides used for *toxT* amplification were 5′-TTCAGAGTAGAACGCAATGA (sense) and 5′-ACTTT ACTCCTCGAGACTCTA (antisense), which were designed for classical strains (11). The classical strain O395 *toxT* fragment, which was used as an internal probe, was amplified with the same antisense oligonucleotide plus 5′-CGTTCA CTAAAATCTTACATTC (sense).

**Biochemical identification and biotyping.** The biochemical characterization of Amazonia strains was done by a battery of standard tests (9) including oxidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, requirement for $Na^+$ (growth in nutrient broth with 0, 1, and 8% NaCl), motility, indole, gas from glucose, susceptibility to O/129 (150-μg discs), and acid production from D-glucose, L-arabinose, cellobiose, lactose, maltose, D-mannitol, salicin, and sucrose. Identification of biotypes was performed by detection of acetylmethylcarbinol (Voges-Proskauer test) and determination of susceptibility to polymyxin B (Oxoid) by spot inoculation onto Mueller-Hinton agar (Difco) containing 15 μg of polymyxin B per ml (21), hemolysis of sheep erythrocytes, and hemagglutination activity for human (O group) and chicken erythrocytes.

**HA and HAI tests.** Tests of hemagglutination (HA) and hemagglutination inhibition (HAI) by D-mannose and L-fucose were performed as described previously (14). Briefly, the HA test was done in U-bottom microtitration plates with doubling dilutions of bacterial suspensions (starting from $10^{10}$ CFU/ml) and chicken or human (O group) erythrocytes. The titer was recorded as the reciprocal of the highest dilution of bacteria giving a strong HA reaction. HAI tests were carried out on glass slides by mixing bacteria with a 0.05 M solution of D-mannose or L-fucose for 30 s before the erythrocyte suspension was added. *V. cholerae* O1 strains of the classical and the El Tor biotypes were used as controls.

**O1 somatic antigen characterization.** Expression of O1 antigen by Amazonia strains was further evaluated by tube agglutination tests against polyvalent O1 antisera prepared by immunizing rabbits with heat-killed cells of Inaba 569B or Ogawa O395 and then absorption of the antisera with the heat-killed cells of the heterologous serotype. Polyvalent O1 antiserum was obtained by mixing the two monospecific antisera. Tests were performed with live cultures grown for 3 h (1).

**Antimicrobial susceptibility tests.** Antimicrobial susceptibility testing was carried out on Mueller-Hinton agar (Difco) by the disc diffusion method (3) for cephalothin, cefoxitin, nalidixic acid, ampicillin, sulfamethoxazole-trimethoprim, chloramphenicol, furazolidone, gentamicin, tetracycline, amikacin, ceftriaxone, and norfloxacin.

**Cytotoxicity assays.** Cytotoxicity assays were done with culture supernatants of Amazonia strains grown in the following media: Syncase (10), AKI (12) and Casamino Acid yeast extract medium supplemented with 90 μg of lincomycin per ml (CAYE-L) (33). Vero and Y-1 mouse adrenal cells were grown in 96-well flat-bottom multiplates (Corning), and assays were conducted (23). Cholera toxin (CT)-producing classical 569B and El Tor 026 strains were used as controls. Bacterial cultures were centrifuged, and the supernatants were filtered through 0.22-μm-pore-size filters (Millex-GV; Millipore). Sterile filtrates were tested in final dilutions ranging from 1:20 to 1:5,120. Vero cells grown on coverslips were

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TABLE 1. Strains of *V. cholerae* analyzed in the study

| Strain[a] | Place[b] | Date | Serotype | Type |
|---|---|---|---|---|
| 3218 | São Paulo de Olivença | December 1991 | Ogawa | Amazonia |
| L-34 | ? | 1991 | Ogawa | Amazonia |
| 3439 | Tonantins | January 1992 | Ogawa | Amazonia |
| 3501 | São Paulo de Olivença | January 1992 | Ogawa | Amazonia |
| 3506 | São Paulo de Olivença | January 1992 | Ogawa | Amazonia |
| 4010 | São Paulo de Olivença | April 1992 | Ogawa | Amazonia |
| 4132 | Sto. Antonio do Içá | May 1992 | Ogawa | Amazonia |
| 026 | ? | 1991 | Ogawa | El Tor |
| 027 | ? | 1991 | Ogawa | El Tor |
| 028 | ? | 1991 | Ogawa | El Tor |
| 029 | ? | 1991 | Ogawa | El Tor |
| 218 | Sta. Rosa (Peru) | May 1991 | Ogawa | El Tor |
| 002 | Sta. Rosa (Peru) | April 1991 | Inaba | El Tor |
| 048 | Sta. Rosa (Peru) | April 1991 | Inaba | El Tor |
| 172 | ? | 1991 | Inaba | El Tor |
| 177 | ? | 1991 | Inaba | El Tor |
| 178 | ? | 1991 | Inaba | El Tor |
| 220 | Tabatinga | May 1991 | Inaba | El Tor |
| 240 | Sta. Rosa (Peru) | May 1991 | Inaba | El Tor |
| 243 | Sta. Rosa (Peru) | May 1991 | Inaba | El Tor |
| 254 | Tabatinga | May 1991 | Inaba | El Tor |
| 1562 | Barra do Solimões | September 1991 | Inaba | El Tor |
| 1975 | Benjamim Constant | September 1991 | Inaba | El Tor |
| 1977 | Benjamim Constant | September 1991 | Inaba | El Tor |
| 1979 | Benjamim Constant | September 1991 | Inaba | El Tor |
| 3189 | São Paulo de Olivença | December 1991 | Inaba | El Tor |
| 3729 | São Paulo de Olivença | 1992 | Ogawa | Amazonia |
| 3730 | São Paulo de Olivença | 1992 | Ogawa | Amazonia |
| 3731 | São Paulo de Olivença | February 1992 | Ogawa | Amazonia |
| 3775 | Sto. Antonio do Içá | February 1992 | Ogawa | Amazonia |
| 4007 | São Paulo de Olivença | April 1992 | Ogawa | Amazonia |
| 4008 | São Paulo de Olivença | April 1992 | Ogawa | Amazonia |
| 4009 | São Paulo de Olivença | April 1992 | Ogawa | Amazonia |

[a] The 26 first strains correspond to the original group of strains analyzed. The seven Amazonia strains included in this group were used in the tests described in the text. The last seven Amazonia strains were identified later. All isolates except isolates 172, 177 and 178, came from patients; isolates 172, 177, and 178 were obtained from water.

[b] All locations are in Brazil unless indicated otherwise.

exposed to cytotoxic filtrates, fixed with methanol, stained with Giemsa, and examined under a light microscope (magnification, ×1,000).

**$GM_1$-ELISA procedure.** The reagents and procedures used for the ganglioside $GM_1$-enzyme-linked immunosorbent assay (ELISA) were as described previously (22), except that a peroxidase-conjugated second antibody was used instead of an alkaline phosphatase conjugate. The wells of polyvinyl microtiter plates were coated with a solution of $GM_1$ (2 μg/ml) in phosphate-buffered saline (PBS). This was followed by the consecutive addition of test filtrates, rabbit anti-CT antiserum, and peroxidase-conjugated goat anti-rabbit immunoglobulin G antiserum (Sigma). Rabbit anti-CT antiserum was kindly donated by A. F. Pestana de Castro (UNICAMP) and was produced by immunizing New Zealand White rabbits with purified CT (Sigma). Titration curves for purified cholera toxin (lot no. 129F0543; C-3012; Sigma) showed that the sensitivity of the $GM_1$-ELISA was 280 pg/ml.

**Rabbit ileal loop assay.** Two Amazonia strains (3506 and 4010) and a classical vibrio (569B) were used in ileal loop assays. Rabbits were challenged with bacteria grown to the mid-log phase and resuspended to $10^8$ CFU/ml in PBS or with sterile filtrates from 18- to 20-h Syncase cultures. Bacterial suspensions or filtrates were injected into 10-cm ileal loops in 1-ml volumes. After 18 to 20 h the animals were sacrificed and the ratio between fluid accumulation and the length of the loop was calculated.

## RESULTS AND DISCUSSION

We studied 26 *V. cholerae* O1 strains (Table 1) isolated in 1991 and 1992 in northern Brazil and, in particular, from the villages bordering the Solimões River next to the Brazilian border with Colombia and Peru (Fig. 1). Most of them came from patients with diarrhea, and their coprocultures did not show other enteropathogenic bacteria.

A screening was done with these strains by using AP-PCR fingerprints. Two oligonucleotides, oligonucleotides 1 and 3, were used in separate reactions. Each of the oligonucleotides showed that there were two markedly different groups of strains in the sample. One of these groups yielded the fingerprints found with other El Tor strains (6), and the other group, comprising seven strains and denominated the Amazonia variant, produced fingerprints different from those of the four pathogenic groups studied previously. The fingerprints of the strains within the group were identical. The results with oligonucleotide 1 (Fig. 2) showed a completely different pattern for the Amazonia strains, in which a 1.3-kb band seemed to be the only band common to the bands for the El Tor strains. In the case of oligonucleotide 3, a prominent 0.55-kb El Tor band was absent from the fingerprints of the Amazonia strains; the other bands were the same (data not shown). These same oligonucleotides have been used against representative El Tor, classical, Gulf Coast, and Bengal strains (6). A serotype difference is not detected with these oligonucleotides. All of the Amazonia strains tested belonged to the Ogawa serotype, but other Ogawa strains in the sample were normal El Tor isolates, producing their characteristic AP-PCR fingerprints. The Amazonia variant strains came from the small town of São Paulo de Olivença and a few other villages scattered 200 km downstream on the Solimões River. A further search of our collection revealed seven other Amazonia strains among the Ogawa strains from the same region.


FIG. 1. Map of Latin America with an insert showing the area around São Paulo de Olivença from which the Amazonia strains were isolated (latitudes, 0 to 5°S; longitudes, 75 to 65°W).

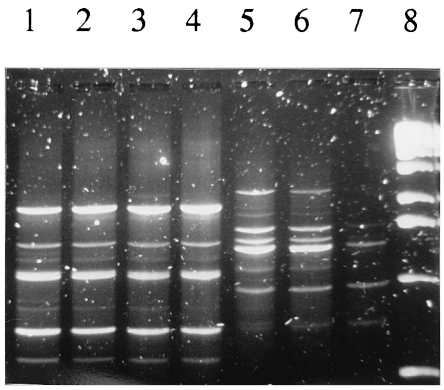


FIG. 2. Pathogenic *V. cholerae* O1 fingerprints obtained by AP-PCR with oligonucleotide 1. Lanes 1 to 4, Amazonia variant strains 3501, 3506, L-34, and 4010, respectively; lanes 5 to 7, biotype El Tor strains 1975, 3189, and 027, respectively; lane 8, 1-kb ladder size marker (GIBCO Bethesda Research Laboratories).

The method of multilocus enzyme electrophoresis (zymovar analysis) developed by Salles and Momen (24) places these strains in a zymovar different from those known for the pathogenic clones (Table 2). There is a difference in four enzymes from the pattern for classical strains, five enzymes from the pattern for El Tor strains, and four enzymes from the pattern for Gulf Coast strains.

Ribotyping was done on the basis of published techniques (16, 20), but with an *E. coli* RNA operon (4) used as a probe (Fig. 3). The seven original Amazonia strains and three El Tor isolates from the same sample of 26 strains were tested. These El Tor strains gave a ribotype 5 according to the scheme of Popovic et al. (20) or a ribotype B5 following the scheme of Koblavi et al. (16). The Amazonia isolates produced a single ribotype which did not correspond exactly to any of those obtained in the schemes of Popovic et al. (20). Ribotype 19, with a 3.5-kb band missing, was the closest match. They proposed this ribotype on the basis of results for a single nontoxigenic strain of human origin and isolated in Brazil in 1992. It is interesting that a strain with a different ribotype would not draw attention in a study of many strains, because various ribotypes are produced from a worldwide collection of El Tor strains as well (20). The AP-PCR fingerprints used in the present study are tuned at a less discriminative level that groups together the El Tor strains (except the Gulf Coast strains that form a separate group). This grouping occurs because of the choice of oligonucleotides, which were selected to distinguish between broad pathogenic groups and not strains within a group (6). A more conspicuous distinction between the Amazonia variant and the El Tor strains was produced with AP-PCRs than with ribotypes.

The Amazonia strains behaved in the biochemical tests as typical representatives of *V. cholerae*. Biological markers such as O-antigen specificity and antimicrobial susceptibility were also evaluated. It was shown that all Amazonia strains but one exhibited O1 agglutination titers of 1,024 (four strains) or 2,048 (two strains). The homologous titer for the classical Ogawa O395 strain was 4,096. Amazonia strain L-34 was autoagglutinable, preventing its testing. Further testing with monospecific antiserum showed that Amazonia strains reacted only with Ogawa antiserum. Antimicrobial susceptibility tests showed that the Amazonia and El Tor strains isolated from the same geographical area were equally susceptible to all antimicrobial agents tested.

Biotyping showed that these strains were Voges-Proskauer test positive and susceptible to polymyxin, and all but one of the strains were negative for hemolysis of sheep erythrocytes. The expression of cell-bound hemagglutinins (by the HA test) and HAI by D-mannose (MSHA) and L-fucose (FSHA) has been associated with the El Tor and classical biotypes of *V. cholerae*, respectively (13, 14). Amazonia strains, like the classical vibrios, preferentially agglutinated human rather than chicken erythrocytes. Most (four of seven) showed fourfold higher HA titers against human O group erythrocytes. However, unlike the classical and El Tor vibrios, Amazonia strains express hemagglutinins, which are inhibited by 0.05 M concentrations of D-mannose and L-fucose (MSHA/FSHA pattern). Taken together these results support the previous findings of AP-PCR, zymovar, and ribotyping analyses that Amazonia strains are a separate group distinct from the El Tor (polymyxin resistant, Voges Proskauer test positive, mostly hemolytic, and producing MSHA of chicken erythrocytes) and the classical (polymyxin susceptible, Voges-Proskauer test negative, nonhemolytic, and producing FSHA of human O group erythrocytes) biotypes of *V. cholerae* O1.

The presence of CT was investigated in various ways in the Amazonia strains. PCR amplifications were done (26), with negative results. The restriction fragment length polymorphisms of the *ctx* genes were tested. A cholera toxin DNA fragment of 982 bp was used as the radioactive probe. This DNA fragment was produced by PCR amplification from an El Tor strain, and it includes most of the *ctxA* and *ctxB* genes (26). A very weak hybridization was found with a *Hin*dIII band of 8.5 kb, while El Tor counterparts in the same filter hybridized strongly. This indicates that the toxin genes, if present, have a very divergent sequence.

The supernatants of all Amazonia strains elicited morphological alterations and cell death on Y-1 and Vero cells when

TABLE 2. Zymovar analysis showing the electromorphs (alleles) present in the Amazonia variant compared with those present in the other pathogenic *V. cholerae* O1

| Type | Zymovar | Enzyme loci[a] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ACO | ADH | IDH | ME | NSE | PGD | MDH | PGM | GPI | G6P | PD | P1 | P2 |
| Amazonia variant | 99 | 1[b] | 1 | 1.5 | 2 | 5 | 4 | 3 | 1 | 3 | 5 | ND | ND | ND |
| Classical | 13 | 1 | 1 | 1 | 2 | 4 | 2 | 3 | 2 | 3 | 5 | 4 | 2 | 1 |
| El Tor | 14 | 1 | 1 | 1 | 2 | 4 | 3 | 3 | 2 | 2 | 5 | 4 | 2 | 1 |
| Gulf Coast | 71 | 1 | 1 | 1 | 2 | 5 | 3 | 3 | 2 | 2 | 5 | 4 | 2 | 1 |

[a] Abbreviations: ACO, aconitase; ADH, alanine dehydrogenase; IDH, isocitrate dehydrogenase; ME, malic enzyme; NSE, carboxylesterase; PGD, 6-phosphogluconate dehydrogenase; MDH, malate dehydrogenase; GPI, glucose phosphate isomerase; G6P, glucose 6-phosphate; PD, proline dipeptidase; P1, peptidase, substrate leucyl leucyl leucine; P2, peptidase, substrate leucyl glycil glycine; ND, not done.
[b] The numbering of the specific alleles is the same as reference 24.

grown in Syncase or CAYE-L medium. Only three strains (L-34, 3439, and 4010) were cytotoxic for Y-1 cells when grown in AKI medium. The cytotoxic activities of bacterial filtrates heated to 100°C for 30 min were completely abolished. Y-1 cells were rounded with a somewhat granular aspect and poorly defined edges. Vero cells observed after 6 to 8 h were rounded or irregularly shaped showing bulging projections. Giemsa-stained cell monolayers showed intense pyknosis and cell vacuolation. After 20 h most of the cells detached from the plastic and were floating in the medium. Attempts to neutralize the cytotoxic effect of Amazonia filtrates with cholera antitoxin or 12.5 μg of $GM_1$ (Sigma) per ml did not succeed. A cytotoxic effect has been observed with filtrates from many O1 and non-O1 *V. cholerae* strains and has been associated with the production of soluble hemolysins or even Shiga-like toxin (18). Cytotoxic filtrates of Amazonia strains were tested against sheep and human erythrocytes in dilutions as low as 1:4 without evidence of hemolysis. On the other hand the occurrence of a cytocidal effect on Y-1 and Vero cells argues against the identity between cytotoxic factor and Shiga-like toxin, because the latter toxin is recognized as inactive against Y-1 cells (17, 29). The nature of this cytotoxin will be analyzed further.

The strong cytopathic effect detected in filtrates of Amazonia strains could hide the more subtle effects of CT on cell cultures. Filtrates were screened for CT by the microtiter $GM_1$-ELISA. Filtrates of all Amazonia strains tested were negative for CT in this assay.

Experimental infection by Amazonia strains was evaluated in ligated ileal loops in rabbits (7). The loops injected with sterile filtrates or live suspensions of the 569B classical strain were positive for fluid accumulation (≥2.0 ml/cm). Ileal loops infected with live suspensions or challenged with sterile filtrates of strains 3506 and 4010 did not produce significant fluid accumulation. The mucous membrane of the infected loops, however, was entirely covered by a dark brown or bright red gelatinous material. Aliquots of this material plated onto TCBS (thiosulfate-citrate-bile salts-sucrose agar) or blood agar medium resulted in pure cultures of the inoculated strains.

Histological sections of infected loops were stained with hematoxylin-eosin. Microscopic observation revealed that the intestinal lesions caused by strains 3506 and 4010 were similar, differing only in severity. There was an almost complete loss of mucosal architecture and diffuse necrosis of enterocytes, without evidence of neutrophil infiltration in the lamina propria or epithelium. Numerous bacteria were seen adhering to the epithelial surface and embedded together with erythrocytes and sloughed epithelial cells in a dense mucous coat covering the mucous layer.

The presence of other *V. cholerae* virulence genes was investigated by PCR. Oligonucleotides specific for *sto* (heat-stable enterotoxin) (19), *zot* (zona occludens toxin) (2), and *tcpA* (15) were used. The test for *tcpA* used a pair of oligonucleotides for the classical or the El Tor biotypes (25). All of these amplifications gave negative results. Positive controls with an El Tor strain were included in all of the experiments. On the other hand, amplification with a pair of oligonucleotides for the *toxT* regulatory gene (8, 11) did produce a band of the appropriate size (653 bp); this band hybridized to an internal DNA fragment of the classical strain O395 *toxT*.

Preliminary phenetic analysis places the Amazonia clone at a considerable distance from other pathogenic O1 clones (El Tor, classical, and Gulf Coast). The Amazonia variant seems to be restricted, for the time being, to a small area of the Amazon Basin and is probably unable to compete with the invading El Tor strains. A parallel may be traced with the early isolates of El Tor from the quarantine station in the Sinai Desert. Their epidemiological relevance is, at present, negligible, but as in

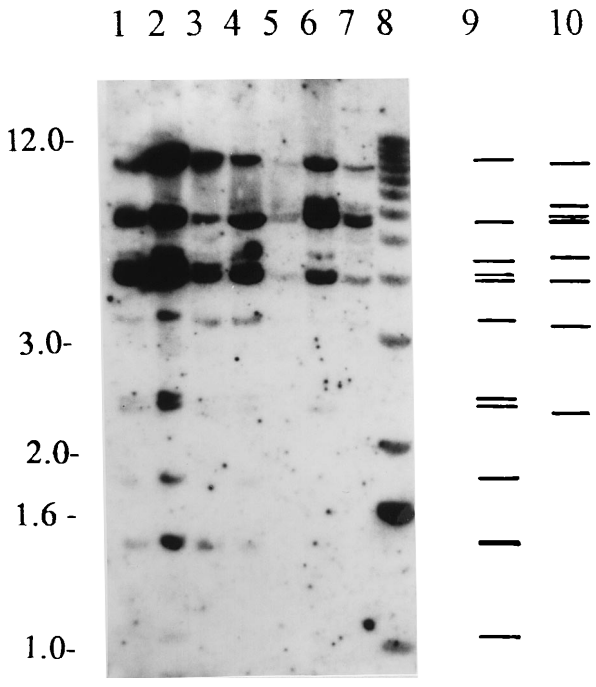


FIG. 3. *Bgl*I ribotypes of *V. cholerae* O1 strains from the north of Brazil. Lanes 1 to 4, Amazonia variant strains 3501, 3506, 4010, and L-34, respectively; lanes 5 to 7, biotype El Tor strains 3189, 1975, and 048, respectively; lane 8, 1-kb ladder size marker; lanes 9 and 10, schemes for the Amazonia and El Tor strains, respectively. Molecular sizes (in kilobases) are indicated on the left.

the latter case, future developments of cholera in Latin America may outline its importance.

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