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Comparison of Six Typing Methods for *Actinobacillus actinomycetemcomitans*

T. J. M. VAN STEENBERGEN,[1]* C. J. BOSCH-TIJHOF,[1] A. J. VAN WINKELHOFF,[1] R. GMÜR,[2] AND J. DE GRAAFF[1]

Department of Oral Microbiology, Academic Centre for Dentistry Amsterdam, Amsterdam, The Netherlands,[1] and Department of Oral Microbiology and General Immunology, Institute of Dentistry, University of Zurich, Zurich, Switzerland[2]

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***Actinobacillus actinomycetemcomitans* is an important pathogen in the etiology of severe periodontitis. For epidemiological studies on the prevalence of certain pathogenic clones and transmission of this bacterium, adequate typing methods are necessary. The purpose of this study was to compare six different typing methods for *A. actinomycetemcomitans*. Five reference strains and 27 fresh clinical isolates from periodontitis patients were used. Serotyping showed 12 serotype a strains, 13 type b strains, 6 type c strains, and 1 nontypeable strain. Biotyping on the basis of the fermentation of mannose, mannitol, and xylose resulted in six biotypes. Antibiogram typing was evaluated by measuring the inhibition zones of seven antibiotics in agar diffusion tests. With this method eight main types which could be further differentiated into 15 subtypes were found. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of outer membrane proteins were similar among all isolates tested. Restriction endonuclease analysis (REA) of whole chromosomal DNA resulted in five main types. These five main types were further differentiated into 24 subtypes on the basis of DNA fragment differences in the high-molecular-weight region. Hybridization of DNA fragments with ribosomal DNA (ribotyping) resulted in 22 to 24 different types, depending on the restriction endonuclease used. Ribotype patterns were easy to interpret and provided an univocal distinction between different strains compared with REA results. When applied to epidemiologically related isolates, all methods were able to discriminate two clonal types among five isolates from five children from one family. We conclude that serotyping, biotyping, and outer membrane patterns were reproducible but had a low discriminatory potential. REA and ribotyping were reproducible and gave the highest number of distinct types. When the DNA typing methods were compared, all strains tested could be distinguished. These findings confirm the heterogeneity found within the species *A. actinomycetemcomitans*.**

Actinobacillus actinomycetemcomitans has been implicated in the etiology of severe periodontitis in juveniles and adults (12, 15). Current epidemiological questions relate to, among other topics, the number of genetically distinct clones that exist in nature and in individuals, the possible existence of virulent and avirulent strains, and the mode of transmission of this bacterium. To be able to answer such epidemiological questions, adequate typing methods are necessary. Classical typing methods, such as serotyping and biotyping, have identified a limited number of different types within the species, thereby providing a low sensitivity. Antibiogram typing and typing by means of analysis of differences in outer membrane composition have not been described for *A. actinomycetemcomitans*. DNA fingerprinting by restriction endonuclease analysis (REA) of whole genomic DNA has shown varying results. Zambon et al. (18) reported three main types for *A. actinomycetemcomitans*, whereas van Steenbergen et al. (17) and Han et al. (8) found considerable heterogeneity with this method. Other typing methods using molecular techniques, including hybridization of DNA fragments with ribosomal DNA, ribotyping (1, 11), have been reported for *A. actinomycetemcomitans*.

The aim of the present investigation was to compare six different typing methods, including both classical typing methods and methods based on molecular techniques, for 32 independent and five epidemiologically related strains of *A. actinomycetemcomitans*.

* Corresponding author. Mailing address: Department of Oral Microbiology, Academic Centre for Dentistry Amsterdam, v.d. Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. Phone: 31-20-4448678. Fax: 31-20-4448318. Electronic mail address: TJM.van_Steenbergen.OMB.ACTA@med.vu.nl.

MATERIALS AND METHODS

Bacterial strains. The following reference strains of *A. actinomycetemcomitans* were used: ATCC 29522 and ATCC 29523 (obtained from the American Type Culture Collection, Rockville, Md.), NCTC 9710 (from the National Collection of Type Cultures, London, United Kingdom), Y4 (from S. S. Socransky, Forsyth Dental Center, Boston, Mass.), and 366 (from J. J. Zambon, State University of New York at Buffalo, Buffalo, N.Y.). Also, 27 fresh clinical isolates of *A. actinomycetemcomitans* from The Netherlands were used; these strains were isolated from 27 randomly selected, unrelated patients with severe *A. actinomycetemcomitans*-associated periodontitis. Furthermore, five isolates from *A. actinomycetemcomitans* which were obtained from five children from a single family with various degrees of periodontal disease were tested.

Serotyping. Serotyping was performed by indirect immunofluorescence with monoclonal antibodies (MAbs) 150AA1.1 (specific for serotype a), 151AA1.2 (serotype a), 141AA1 (serotype b), 145AA1.1 (serotype b), 138AA1.1 (serotype c), and 146AA1.1 (serotype c) as described previously (4, 5). In addition, all strains were tested for binding to MAb 144AA2.1, which detects an *A. actinomycetemcomitans*-specific antigen expressed by all strains of the species. Strains that could not be

TABLE 1. Antibiogram types of 32 isolates of *A. actinomycetemcomitans* with seven antibiotics

Type	*n*	Mean (range) diam (mm) of inhibition zone with[a]:						
		Mecillinam	Methicillin	Metronidazole	Penicillin	Trimethoprim	Lincomycin	Bacitracin
1a	1	15 (12–18)	10 (10)	11 (11)	10 (10)	27 (26–28)	15 (12–18)	13 (12–13)
1b	1	12 (10–14)	10 (10)	10 (10)	10 (10)	43 (42–44)	10 (10–11)	10 (10)
2a	3	17 (12–25)	10 (10)	10 (10)	11 (10–15)	49 (42–54)	25 (18–40)	10 (10–11)
3a	1	13 (11–16)	10 (10–11)	15 (13–17)	13 (12–14)	27 (25–29)	11 (10–16)	16 (16–17)
3b	2	11 (10–15)	10 (10–13)	17 (15–25)	11 (10–16)	29 (21–38)	15 (10–25)	10 (10–13)
3c	10	19 (13–31)	16 (10–35)	22 (13–42)	17 (12–20)	35 (25–54)	23 (10–44)	19 (12–42)
3d	1	17 (15–18)	21 (20–23)	48 (42–52)	20 (13–56)	54 (48–60)	23 (20–32)	18 (13–21)
3e	1	19 (18–20)	21 (18–26)	20 (18–24)	27 (23–32)	37 (30–40)	25 (21–30)	12 (10–15)
4a	3	24 (17–38)	11 (10–15)	27 (15–62)	11 (10–18)	48 (30–60)	27 (19–40)	20 (10–40)
4b	1	18 (17–20)	13 (11–15)	33 (27–40)	11 (11)	46 (45–48)	42 (40–45)	16 (16)
5a	1	10 (10)	16 (15–17)	19 (17–22)	18 (17–20)	20 (18–22)	10 (10–11)	11 (11)
6a	2	11 (10–13)	27 (25–32)	22 (18–28)	27 (17–32)	37 (24–46)	29 (24–40)	13 (11–17)
7a	1	19 (15–22)	31 (26–34)	12 (11–15)	30 (30–31)	31 (26–35)	29 (28–30)	18 (17–18)
7b	1	17 (15–20)	32 (28–35)	10 (10–11)	30 (28–32)	49 (46–51)	32 (29–35)	17 (16–17)
8a	3	18 (10–25)	37 (32–42)	28 (23–33)	36 (25–43)	37 (30–46)	30 (25–33)	22 (12–30)

[a] Growth inhibition zones around the disks with antibiotics (disk diameter, 10 mm). Tests were performed three times in duplicate on each strain.

assigned to serotype a, b, or c were further assessed with serotype-specific polyclonal sera and with MAbs 248AA4.4 and 260AA3, reactive with serotype e and a serovar of serotype a, respectively (3a, 5).

Biotyping. Sugar fermentation was evaluated in peptone-yeast broth (9) supplemented with 1% maltose, mannitol, or xylose. A pH decrease of ≥0.5 pH unit was considered positive. A reproducible pH decrease ranging from 0.45 to 0.55 pH unit was recorded as ±.

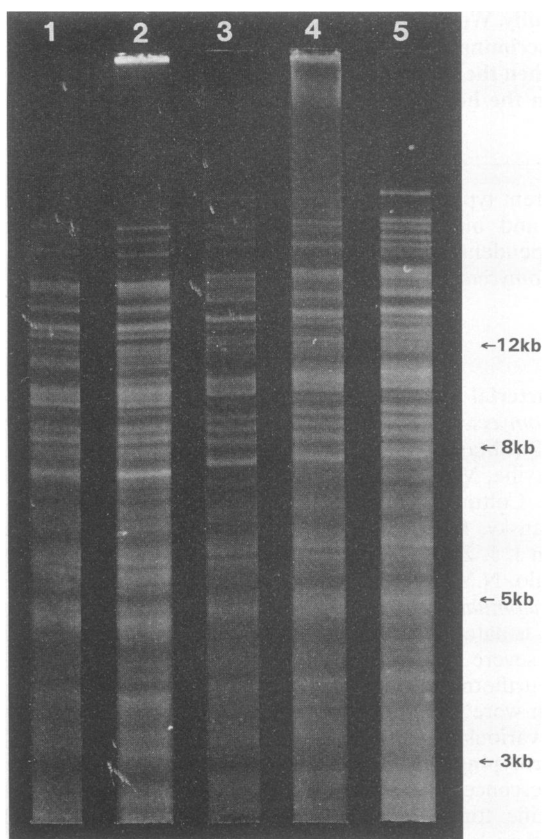

FIG. 1. Restriction endonuclease patterns (*Bam*HI-*Pst*I digests of whole chromosomal DNA) of five strains of *A. actinomycetemcomitans* serotype a. Lane 1, ATCC 29523; lane 2, HG 1228; lane 3, HG 1075; lane 4, HG 1215; lane 5, HG 1233.

Antibiogram typing. Antibiogram typing was evaluated by measuring the inhibition zones of 35 different antibiotics in agar disk diffusion tests. The antibiotics mecillinam, methicillin, metronidazole, penicillin, trimethoprim, lincomycin, and bacitracin showed useful differences between the strains and were therefore further evaluated. Strains were inoculated onto Columbia agar plates (BBL), and inhibition zones were measured after 2 days of incubation in air plus 5% CO_2. These tests were performed three times in duplicate. Strains differing in inhibition zones of at least two antibiotics were assigned different numbers, and strains that differed for one antibiotic were given a subtype designation.

Outer membrane proteins. Bacteria were grown in brain heart infusion broth, washed in HEPES buffer (100 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 15 mM $MgCl_2$, 4 mM EDTA, pH 7.2), and lysed with 1% sarcosyl in HEPES buffer. Membrane fragments were isolated after filtration through a 0.45-μm-pore-size filter by centrifugation for 30 min at 27,000 × *g*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the outer membrane proteins was carried out on the Phastgel System (LKB-Pharmacia).

REA. Total genomic DNA was isolated essentially as described previously (17). Cells were grown in brain heart infusion broth (Difco), washed in Tris-EDTA-sodium chloride buffer, and lysed with lysozyme, proteinase K, and SDS in the presence of 0.5 μg of RNase per ml. DNA was purified by phenol-chloroform extractions and ethanol precipitation.

Two micrograms of DNA was digested to completion in a volume of 20 μl with a combination of the restriction endonucleases *Pst*I and *Bam*HI for 2 h. DNA fragments were separated for 20 h at 25 V in a horizontal gel containing 0.6% agarose in TAE (40 mmol of Tris-acetate per liter, 1 mmol of EDTA per liter, pH 8.0). The DNA was stained for 1 h in 1 mg of ethidium bromide per liter and photographed with a Polaroid 35 camera.

Ribotyping. Ribotyping was performed essentially as described by van Steenbergen et al. (16). Chromosomal DNA was isolated and digested with restriction endonucleases. The enzymes *Bam*HI and *Bgl*I (11) were found to provide useful

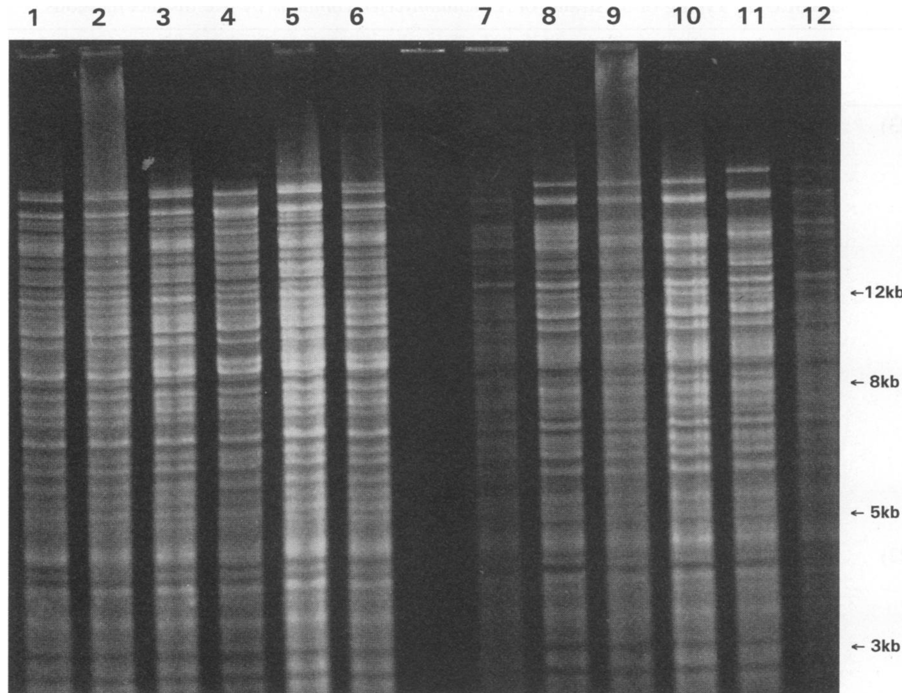

FIG. 2. Restriction endonuclease patterns (*Bam*HI-*Pst*I digests of whole chromosomal DNA) of 12 strains of *A. actinomycetemcomitans* of serotype b. Lane 1, Y4; lane 2, HG 1229; lane 3, HG 1232; lane 4, HG 1220; lane 5, HG 1184; lane 6, ATCC 29522; lane 7, HG 1236; lane 8, 366; lane 9, HG 1182; lane 10, HG 1183; lane 11, HG 1217; lane 12, HG 1235.

patterns. DNA fragments were separated by electrophoresis, transferred to nylon filters, and fixed by UV illumination. Hybridization was performed with plasmid pKK 3535, which contains the rRNA operon of *Escherichia coli*. The digoxigenin-labelled plasmid was detected with antidigoxigenin antibodies labelled with alkaline phosphatase and the luminescent substrate Lumigen-PPD (Boehringer Mannheim).

RESULTS

Serotyping. Serotyping with the MAbs showed 11 serotype a strains, 13 serotype b strains, 6 serotype c strains, and 2 strains which did not react with any of the serotype-specific MAbs. One of the MAb-nontypeable strains, HG 1224, reacted by Western blot (immunoblot) analysis with 8 of 12 polyclonal human serum specimens specific for serotype a and not with human sera specific for serotypes b, c, and e (5). Therefore, this strain was recognized as a new serovar of serotype a. Strain HG 1186 was nontypeable with MAbs or with polyclonal antisera. This strain could not be assigned to any of the five serotypes of *A. actinomycetemcomitans*. One strain (HG 1236) was composed of an apparently stable mixed cell population of approximately 99% nontypeable, but 144AA2.1-positive, bacteria and approximately 1% serotype b cells, as detected by immunofluorescence. Attempts to separate these two cell types by recloning, i.e., repeated subculturing on agar plates, were not successful.

Biotyping. Biotyping on the basis of the fermentation of maltose, mannitol, and xylose resulted in six distinct biotypes. All serotype b strains fermented xylose, whereas none of the other strains was positive for this sugar. Five of six serotype c strains were unable to ferment maltose, whereas only one strain of serotypes a and b each was unable to ferment maltose. The four mannitol-negative strains all belonged to serotype b.

Antibiogram typing. By the antibiogram typing method eight main types could be discriminated, with inhibition zones that differed for at least two antibiotics. These eight main types could be further differentiated into 15 subtypes, which were defined as strains with a different inhibition zone for one antibiotic (Table 1). The variation found in the individual measurements of the inhibition zones amounted to approximately 2 to 5 mm, depending on the particular strain and antibiotic.

Some correlation of antibiogram type and serotype was found. Type 3c was found most often in serotypes a and c, whereas for serotype b strains a larger variation in the antibiogram types was found.

Protein patterns. SDS-PAGE patterns of both outer membrane proteins and total protein were evaluated for seven strains belonging to the three serotypes. The patterns obtained were similar for all strains investigated. Therefore, this method was not evaluated any further.

REA of whole chromosomal DNA. REA of whole chromosomal DNA resulted in high numbers of visible bands, with a size range of approximately 2 to 20 kb, for strains of serotypes a and b, respectively (Fig. 1 and 2). Five main types could be distinguished: one main type for serotype a, three main types within serotype b, and one for serotype c (Table 2). The nontypeable strain HG 1186 showed a DNA pattern similar to the main type of the serotype a strains. The five main types were further differentiated into 24 subtypes on the basis of DNA fragment differences in the high-molecular-weight region between approximately 12 and 20 kb. Figure 2 shows the REA patterns of several serotype b strains. Besides the presence of minor differences in the high-molecular-weight region for some strains, this figure shows also that some strains have indistinguishable patterns.

TABLE 2. Typing of 32 strains of *A. actinomycetemcomitans* by five distinct methods

Strain (international code)	Serotype[a]	Biotype[b]	Antibiogram type[c]	REA type (*Bam*HI + *Pst*I)[d]	Ribotype[e]	
					*Bam*HI	*Bgl*I
HG 1223 (ATCC 29523)	a	+, +, −	5a	A-1	I	1
HG 1214	a	±, +, −	3c	A-2	II	1
HG 1215	a	±, +, −	3c	A-2	II	2
HG 1230	a	±, +, −	3a/c	A-3	III	3
HG 1221	a	+, +, −	3a	A-4	II	4
HG 1224	a[f]	+, +, −	3d	A-5	IV	5
HG 1233	a	+, +, −	3c	A-6	IV	6
HG 1234	a	+, +, −	3c	A-7	V	7
HG 1181	a	+, +, −	3c	A-8	VI	8
HG 1218	a	−, +, −	3c	A-9	VII	1
HG 1075	a	±, +, −	8a	A-10	VIII	1
HG 1228	a	+, +, −	3b	A-11	IX	9
HG 90 (Y4)	b	+, +, +	1a	B-1	X	10
HG 1229	b	±, +, +	7b	B-1	XI	11
HG 1232	b	+, +, +	3c/4a	B-1	X	12
HG 1220	b	+, +, +	2a	B-2	XI	12
HG 1184	b	+, +, +	2a	B-1	XI	12
HG 1222 (ATCC 29522)	b	+, +, +	2a	B-1	XII	12
HG 1185	b	+, +, +	4a	C-1	XIII	ND
HG 1236	NT-b	+, +, +	4a	C-1	XIV	13
HG 1080 (366)	b	−, −, +	8a	D-1	XV	ND
HG 1182	b	±, −, +	6a	D-2	XVI	13
HG 1183	b	+, −, +	6a	D-2	XVII	14
HG 1217	b	+, −, +	8a	D-3	XVIII	15
HG 1235	b	±, +, +	1a	D-4	XIX	16
HG 683 (NCTC 9710)	c	±, +, −	3e	E-1	XX	17
HG 1216	c	−, +, −	3c	E-2	XXI	18
HG 1226	c	−, +, −	3b	E-3	XXI	19
HG 1227	c	−, ±, −	7a	E-4	XXII	20
HG 1231	c	−, ±, −	4b	E-5	XXIII	21
HG 1225	c	−, +, −	3c	E-6	XXIV	22
HG 1186	NT	+, +, −	3c	A-8	ND	ND

[a] NT, nontypeable with MAbs and human sera.
[b] Fermentation of maltose, mannitol, and xylose, respectively. +, pH decrease of ≥0.55 pH unit; ±, pH decrease of 0.45 to 0.55 pH unit; −, pH decrease of <0.45 pH unit.
[c] Designations as indicated in Table 1.
[d] The main types are presented by a letter code (A to E).
[e] Distinct types were given different numbers. ND, not determined.
[f] HG 1224 belongs to a serovar of serotype a.

Ribotyping. Ribotyping was performed by digestion with restriction endonuclease *Bgl*I or *Bam*HI and probing of the digests with ribosomal DNA of *Escherichia coli*. A typical example of the ribotypes obtained for representative strains of the three serotypes, demonstrating a clear pattern of approximately five bands, is shown in Fig. 3. The patterns obtained after digestion with *Bam*HI were more clearly distinct than those obtained after digestion with *Bgl*I. The sizes of the DNA fragments found with ribotyping ranged from five to 20 kb. Ribotyping was able to distinguish most strains: 24 and 22 distinct patterns were identified with *Bam*HI and *Bgl*I, respectively. Some strains that were clearly distinct with one enzyme were indistinguishable with the other and vice versa.

Epidemiologically related isolates. Within the group of five isolates from one family, two distinct clonal types could be discriminated with serotyping, biotyping, antibiogram typing, and the DNA typing methods (Table 3). The REA patterns were similar to the main types found for serotype b. Four isolates from the periodontal pockets of four children were indistinguishable with all these typing methods.

DISCUSSION

For epidemiological studies of bacteria, adequate typing methods are necessary. For the typing of the suspected periodontal pathogen *A. actinomycetemcomitans* several typing methods, including serotyping, biotyping, and methods based on molecular biological techniques, have been described. With the classical methods relatively few distinct types can be discriminated. New methods which have been described for *A. actinomycetemcomitans* include REA of whole chromosomal DNA (8, 17, 19), analysis of restriction fragment length polymorphisms with a genomic probe (2, 13) or a panel of probes (7), ribotyping (1, 11), arbitrary primed PCR (13), and analyzing restriction fragment length polymorphisms of the spacer region in the rRNA operon (6). Whereas these methods are able to discriminate 3 to 12 distinct types, their sensitivity and reproducibility have not always been evaluated with a large number of strains. The purpose of the present study was to compare several typing methods, both classical and based on molecular biological techniques, for their ability to discrimi-

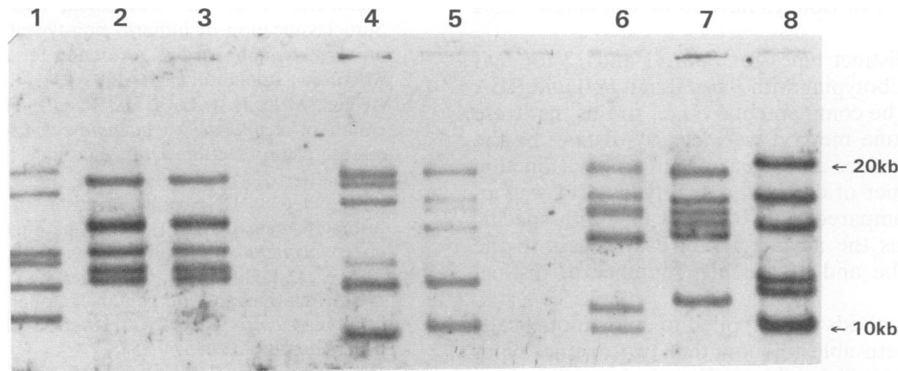

FIG. 3. Ribotype patterns (*Bam*HI digests) of DNA of eight strains of *A. actinomycetemcomitans* of three serotypes. Lanes 1 to 3, serotype a strains (ATCC 29523, HG 1224, and HG 1233, respectively); lane 4, serotype b strain HG 1232; lane 5, strain HG 1236 (mixture of nontypeable cells and serotype b cells); lanes 6 to 8, serotype c strains (NCTC 9710, HG 1216, and HG 1226, respectively).

nate between a total of 32 *A. actinomycetemcomitans* strains, including 5 reference strains and 27 randomly chosen fresh clinical isolates from The Netherlands. These clinical isolates were obtained from subgingival samples from both localized juvenile periodontitis and refractory adult periodontitis.

Until recently, three serotypes of *A. actinomycetemcomitans* were recognized. The majority of the clinical isolates in this study belonged to serotypes a and b. Recently, two additional serotypes, d and e, have been described (5, 10). In our group of 27 randomly chosen clinical isolates, serotypes d and e were not found. One of the strains was nontypeable with MAbs or with polyclonal human sera, which may indicate that additional not yet described serotypes exist within the species or that certain strains lack the expression of any serotype antigen. The finding that this strain closely resembled serotype a strains in REA typing suggests a genetic relationship of this strain to serotype a strains. Another strain, HG 1236, was composed of a mixed population of nontypeable cells and serotype b cells, which was detectable only by immunofluorescence due to single-cell resolution. It is unlikely that this was due to a contamination, since several attempts to separate the cell types by recloning were not successful. A possible explanation is a differential modulation of the expression of the serotype b antigen by individual bacteria. In REA typing this strain showed a close resemblance to another serotype b strain, suggesting a genetic relationship of this strain to serotype b strains.

With biotyping on the basis of sugar fermentation, 10 different types have been distinguished (14). In the present study we were unable to differentiate for the fermentation of dextrin. Differences in the fermentation of maltose, mannitol, and xylose resulted in six distinct biotypes. Our finding that all serotype b strains were xylose positive and that all serotype c strains except one were maltose negative is largely in agreement with the findings of Slots et al. (14).

To our knowledge, antibiogram typing of *A. actinomycetemcomitans* has not been described earlier. The method showed more heterogeneity compared with serotyping and biotyping but less than the typing methods based on DNA. Typing by means of outer membrane protein pattern has been described for several bacteria. For *A. actinomycetemcomitans* outer membrane patterns showed low variation even between the different serotypes, which makes these methods not applicable for typing purposes. This finding is in agreement with the findings of DiRienzo and Spieler (3).

The results of REA typing of whole chromosomal DNA for *A. actinomycetemcomitans* have been confusing. Whereas van Steenbergen et al. (17) and Han et al. (8) found considerable heterogeneity within the species, Zambon et al. (19) found only three main DNA patterns. In the present investigation we found five main REA types, one for serotypes a and c and three within serotype b. This finding confirms the conclusion that a relatively low number of main REA types exist within the species. On the other hand, we found besides these main types a total of 24 subtypes for the 32 strains tested. A possible explanation for the discrimination between the subtypes in this study is the relatively high resolution of bands in the high-molecular-weight region, where the differences between the subtypes were found. For some strains indistinguishable DNA patterns were obtained, for instance, five strains with type B1, including laboratory strains Y4 and ATCC 29522, and three fresh isolates. Some of these strains could be further differentiated by antibiogram typing and ribotyping. A disadvantage of REA typing is the large number of bands which have to be compared, which can be difficult in nonadjacent lanes. When a

TABLE 3. Typing of epidemiologically related isolates of *A. actinomycetemcomitans* by five distinct methods[a]

Isolate	Age (yr) of isolate source	Serotype	Biotype	Antibiogram type	REA type (*Bam*HI + *Pst*I)	Ribotype	
						*Bam*HI	*Bgl*I
p 1.2	14	b	−, −, +	8a	D-5	XXV	13
p 4.1	17	b	−, −, +	8a	D-5	XXV	13
p 5.1	10	b	−, −, +	8a	D-5	XXV	13
p 6.1	13	b	−, −, +	8a	D-5	XXV	13
ts 3.3	20	NT	+, +, −	3c	C-2	XXVI	23

[a] See Table 2, footnotes *a* to *e*, for definitions of results.

relatively small number of isolates have to be evaluated, REA typing can be useful.

By ribotyping 24 distinct types for *Bam*HI and 22 for *Bgl*I could be identified. Ribotyping with *Bam*HI and *Bgl*I and REA typing were found to be complementary; i.e., strains that were indistinguishable by one method were clearly distinct by the other. Ribotyping provides an easier and univocal distinction between a large number of different clonal types of *A. actinomycetemcomitans*. Compared with RFLP typing with specific probes, ribotyping has the advantages of easy access to the ribosomal DNA probe and of the high number of distinct types.

When the typing methods were applied to epidemiologically related strains, we were able to show that two distinct types were present among the five children. Four isolates from four children were indistinguishable by all methods tested. This finding suggests a transmission of one clonal type between these family members. The possibility that this specific clonal type was also present in the other child cannot be excluded, as only one isolate per individual was included in the present investigation.

In conclusion, this study confirms that *A. actinomycetemcomitans* is a heterogeneous species and that many distinct clonal types can be found among patients with periodontitis. Serotyping, biotyping, and typing by outer membrane patterns were reproducible but had a low discriminatory potential. REA typing and ribotyping were reproducible and sensitive with a high number of distinct types. Antibiogram typing could be useful as an additional method. For epidemiological questions on, for instance, the mode of transmission of *A. actinomycetemcomitans*, a very sensitive typing method is required. This can be obtained by applying two or more typing methods.

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