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Occurrence of *Aggregatibacter actinomycetemcomitans* serotypes in subgingival plaque from United States subjects

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SUMMARY

This study examined the distribution pattern of *Aggregatibacter actinomycetemcomitans* serotypes in the subgingival plaque of subjects residing in the United States. *A. actinomycetemcomitans* was identified in 256 subgingival plaque samples from 161 subjects. For 190 of the 256 samples, the total cultivable bacteria and selected periodontal pathogenic species were determined. *A. actinomycetemcomitans* isolates were confirmed by a16S rDNA-based PCR analysis, genotyped by arbitrarily-primed PCR, and serotyped by PCR analysis of serotype-specific gene clusters. A total of 82 distinct *A. actinomycetemcomitans* strains were identified. The serotype distribution pattern of the strains was 21 (25.6%) serotype a, 12 (14.6%) b, 41 (50%) c, 6 (7.3%) e, 1 (1.2%) f, and 1 (1.2%) non-typeable. For 14 subjects where multiple colonies of *A. actinomycetemcomitans* were identified, 11 subjects (78.6%) were each infected by a single serotype, while the remaining three subjects (21.3%) were each infected by two serotypes of *A. actinomycetemcomitans*. There was an inverse relationship between the level of cultivable *A. actinomycetemcomitans* and *Porphyromonas gingivalis*. Within subgingival plaque of study cohort *A. actinomycetemcomitans* serotype c was the dominant serotype and comprised 50% of all strains, followed by (in order of detection frequency) serotypes a and b. Serotypes d, e, and f strains were either not detected or less frequently found. Serotype distribution patterns of subgingival *A. actinomycetemcomitans* may vary among subjects of different race orethnicity.

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a major pathogen in aggressive periodontitis and chronic periodontitis. The natural population of *A. actinomycetemcomitans* comprises distinct clonal lineages that exhibit little recombination between clones (Kaplan *et al.*, 2002; Kilian *et al.*, 2006). Six serotypes are distinguished among *A. actinomycetemcomitans* based on the structural genetics characteristics of the O-antigen of LPS (Kaplan *et al.*, 2002; Kilian *et al.*, 2006). Strains of the same serotypes appear to represent distinct clones, which may be further distinguished by other genotyping methods (Asikainen *et al.*, 1995).

Several lines of evidence have suggested that *A. actinomycetemcomitans* isolates exhibit variable virulence potential. A serotype b JP-2 clone, characterized by a 530-bp deletion in the promoter region of the leukotoxin operon, has been shown to be highly

virulent (Haubek *et al.*, 1996, 1997, 2008). It primarily affects subjects of African descent, presumably due to restricted transmission of *A. actinomycetemcomitans* between long-term cohabitants. Infection with JP-2 clone is a risk factor for aggressive periodontitis and active periodontal tissue breakdown. Other supporting evidence of clonal variability of virulence was loosely based on the observations that individuals may be colonized by *A. actinomycetemcomitans* without apparent tissue destruction, and that some genotypes of *A. actinomycetemcomitans* are frequently identified in subgingival sites with periodontal disease. However, the interpretation of the association of *A. actinomycetemcomitans* serotypes with disease requires knowledge of the serotypes distribution patterns of the study populations. For example, it has been proposed that serotype c strains of *A. actinomycetemcomitans* have questionable pathogenic potential because of their frequent detection in subgingival plaque of Asian populations irrespective of periodontal disease status (Rylev & Kilian, 2008). This conclusion may not be justified in a different population where a strong association between serotype c strains and aggressive periodontitis is found. It is worth noting that few studies have examined the occurrence of *A. actinomycetemcomitans* serotypes in United States (US) subjects (Yang *et al.*, 2005; Fine *et al.*, 2007). To further clarify the significance of serotype association with disease, we examined the serotype distribution of *A. actinomycetemcomitans* in subjects residing in the United States.

METHODS

Subjects and microbial sampling

Two sets of subgingival samples were examined. The first set (A) was obtained from 44 subjects without periodontal disease and from 51 subjects with aggressive periodontitis, as described in a previous study (Fujise *et al.*, 2004). The demographic and clinical characteristics of the subject groups are presented in Table 1. The subjects without periodontitis had a minimum of 26 teeth, showed no radiographic evidence of alveolar bone loss and had no periodontal sites with probing pocket depths (PD) ≥4 mm. Localized aggressive periodontitis subjects were 30 years old or younger, with at least two teeth, either central incisors and/or first molars, displaying ≥2 mm of clinical attachment loss (AL), and with fewer than four teeth (other than central incisors or first molars) exhibiting clinical attachment loss due to periodontitis. Generalized aggressive periodontitis subjects were 35 years old or younger, with at least two teeth with ≥2 mm of AL in each of the four quadrants. All subjects were systemically healthy and had not received antibiotic therapy for 3 months or any periodontal therapy, including scaling and root

Table 1 Demographic and clinical characteristics of study subjects

	No periodontitis (*N* = 44)	Localized aggressive periodontitis (*N* = 20)	Generalized aggressive periodontitis (*N* = 31)
Age (year)	25.2 ± 3.9 range: 11–30	21.5 ± 4.7 range: 14–30	26.7 ± 3.3 range: 16–32
Ethnicity (*N*)[1]			
African-American	0	5	7
Asian-American	23	8	9
Caucasian	12	0	3
Hispanic	4	7	12
Unidentified	5	0	0
Gender (*N*)			
Male	28	8	11
Female	16	12	20
Pocket depth (mm)			
Single	3.0 ± 0.4 range: 1-4	7.2 ± 1.8 range: 4–12	7.0 ± 2.0 range: 4–13
Pooled	2.7 ± 0.3 range: 2–3.7	5.7 ± 1.4 range: 3.7–8	5.2 ± 1.2 range: 3.3–7.3
Attachment loss (mm)			
Single	0 ± 0 range: 0	5.2 ± 2.5 range: 2–11	5.3 ± 2.5 range: 2–13
Pooled	0.0 ± 0.1 range: 0–0.67	3.7 ± 1.8 range: 0.67–7	3.5 ± 1.8 range: 1.0–9.3

[1]Self-identified.

planing, for 6 months prior to microbial sampling. Two subgingival plaque samples were obtained from each subject. One sample was obtained from a periodontal site with the deepest periodontal pockets in the mouth. The other sample was a pooled sample from three subgingival sites, each selected from the deepest pocket in the remaining three quadrants.

The second set of samples (B) from subjects with periodontitis was submitted by extramural dentists to the Oral Microbiology Testing Laboratory at the University of Southern California School of Dentistry.

Microbial culture

All samples were collected in vials containing VMGAIII and processed in the laboratory, according to established methods (Ashimoto *et al*., 1996) to identify cultivable *A. actinomycetemcomitans*. In addition, the total cultivable bacteria and key periodontal pathogens (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, *Campylobacter* spp., *Eubacterium* spp., *Fusobacterium* spp., *Peptostreptococcus micros*, Enteric Gram negative rods, *Eikenella corrodens*, *Staphylococcus* spp., and β-hemolytic *Streptococcus* spp.) were also determined for sample set A.

Multiple colonies (up to 20) of presumptively identified *A. actinomycetemcomitans* were recovered from each sample in set A. In contrast, one *A. actinomycetemcomitans* colony was included for analysis in each sample for set B. *A. actinomycetemcomitans* isolates were routinely cultured on trypticase soy broth supplemented with 0.1% yeast extract, 5% heat-inactivated horse serum, and 1.5% agar at 37°C in 5% CO_2. All confirmed *A. actinomycetemcomitans* isolates were subcultured and stored at −80°C until use.

16S rDNA PCR analysis and AP-PCR clonal analysis

The presumptively identified *A. actinomycetemcomitans* isolates were confirmed with a 16S rDNA-base PCR analysis, as previously described (Ashimoto *et al*., 1996). Multiple *A. actinomycetemcomitans* isolates from the same samples were distinguished with an AP-PCR genotyping protocol (Asikainen *et al*., 1995; Chen & Ashimoto, 1996).

PCR analysis of *A. actinomycetemcomitans* serotypes

Serotype analysis was performed with PCR protocols, as described previously (Kaplan *et al*., 2001; Suzuki *et al*., 2001; Fujise *et al*., 2004). Multiplex PCR was performed to test the serotype identity of isolates a–e. Isolates that showed no reaction were then tested for serotype f by PCR analysis. Genomic DNA from serotypes a–f strains of *A. actinomycetemcomitans* (ATCC29523, ATCC29524, ATCC33384 SA508, SA2149 and SC29) were used as references.

Statistical analysis

t-Test was performed to compare the mean % *A. actinomycetemcomitans* in samples with or without *P. gingivalis* or *T. forsythia.*

RESULTS

Microbial analysis of sample set A

Table 2 presents the microbial analysis of set A samples and the combined results for subjects with localized aggressive and generalized aggressive periodontitis. The prevalence and percentage of

Table 2 Microbial culture results of subject groups

	No periodontitis			Aggressive periodontitis		
	% Subject	% Sample	Mean % cultivable bacteria	% Subject	% Sample	Mean % cultivable bacteria (SD)
A. actinomycetemcomitans	4.5	2.3	1.65	23.5	18.6	9.1 (17.2)
P. gingivalis	2.3	1.1	0.45	43.1	43.1	13.4 (13.7)
T. forsythia	0	0	0	15.6	21.6	4.6 (4.0)

cultivable periodontal pathogens were much lower in subjects without periodontitis than in those with aggressive periodontitis. The mean percentages of cultivable microbiota were 9.1% for *A. actinomycetemcomitans*, 13.4% for *P. gingivalis*, and 4.6% for *T. forsythia*. As indicated by the large standard deviations of the mean, the percentages of cultivable pathogens varied widely among samples. Figure 1 displays the percentage of cultivable pathogens organized into three levels: <1, 1–10, and >10% of the total cultivable bacteria. In the majority of the samples *A. actinomycetemcomitans* comprised <1% of the total cultivable bacteria. In contrast, both the levels of *P. gingivalis* and *T. forsythia* commonly reached above 1 or 10% of the total cultivable bacteria.

There was a trend for an inverse relationship between the percent cultivable *A. actinomycetemcomitans* and *P. gingivalis* (Fig. 2A). For example, among 44 samples positive for *P. gingivalis*, none was co-colonized by *A. actinomycetemcomitans* at a level of 0.2% or greater. Conversely, among 10 samples with >0.3% cultivable *A. actinomycetemcomitans*, none contained cultivable *P. gingivalis*. The mean % *A. actinomycetemcomitans* in samples with or without *P. gingivalis* were 0.0073 and 14.05% respectively and were statistically significantly different ($P < 0.0001$). Similarly, an inverse association was also noted between *A. actinomycetemcomitans* and *T. forsythia*. The mean % *A. actinomycetemcomitans* in samples with or without cultivable *T. forsythia* were 0.009 and 10.75% ($P < 0.05$ by *t*-test). This

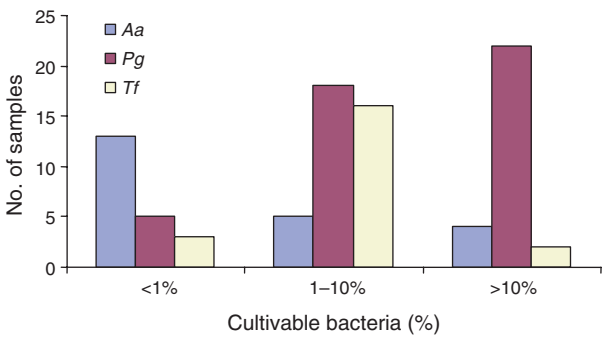

Figure 1 Levels of cultivable periodontal pathogens in subgingival plaque of sample set A. *Aa*: *A. actinomycetemcomitans*; *Pg*, *P. gingivalis*; *Tf*, *T. forsythia*. *A. actinomycetemcomitans* usually comprised <1% of the total cultivable bacteria. In contrast, *P. gingivalis* and *T. forsythia* commonly reached >1 or >10% of the total cultivable bacteria.

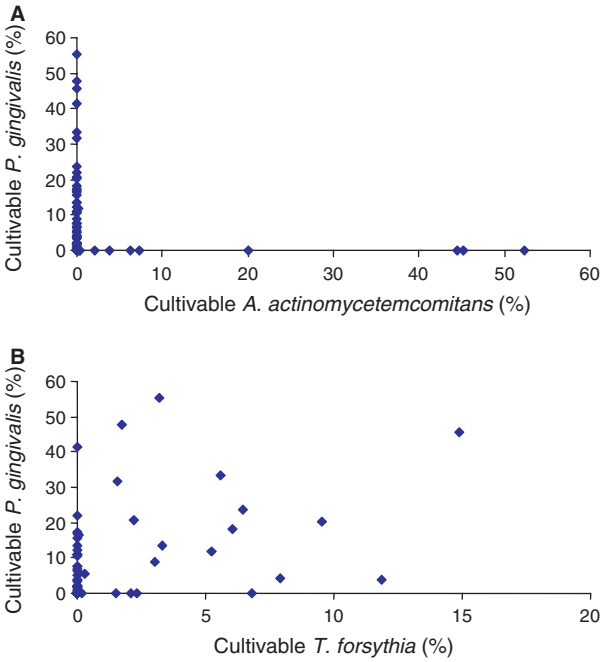

Figure 2 The levels of cultivable *A. actinomycetemcomitans* and *P. gingivalis* (A, upper panel) and *T. forsythia* and *P. gingivalis* (B, lower panel) in subgingival plaque samples. Each diamond represents the result of a sample. There was a trend for an inverse relationship in the cultivable levels between *A. actinomycetemcomitans* and *P. gingivalis*.

inverse relationship trend did not exist between *P. gingivalis* and *T. forsythia* (Fig. 2B).

Serotype distribution of *A. actinomycetemcomitans* isolates

A summary of results for serotype analysis of *A. actinomycetemcomitans* colonies from sample set A is presented in Table 3. An average of 16.2 colonies of *A. actinomycetemcomitans* was identified from each of 21 *A. actinomycetemcomitans*-positive samples. The colonies were genotyped by AP-PCR, and representative strains of each distinct genotype within individuals were determined for serotype. Of the 14 *A. actinomycetemcomitans*-positive subjects, three were colonized by two serotypes. In no cases were different serotypes of *A. actinomycetemcomitans* identified from the same sample sites (i.e., distinct serotypes of *A. actinomycetemcomitans* within individuals were found either in different samples [subject D11] or in samples pooled from different sites [subjects D17 and D18]). Isolates of the same serotypes within individuals exhibited indistinguishable

Table 3 Detection of distinct serotypes of *A. actinomycetemcomitans* (*Aa*) in sample set A

Subject	Race/ethnicity	Diagnosis[1]	Sample	*Aa* (%)	No. of colony	Serotype
H1	Hispanic	H	Single	0.45	19	c
			Pooled	0	–	–
H5	Asian-American	H	Single	0	–	–
			Pooled	2.85	20	Non-typeable
D3	Hispanic	GAP	Single	6.35	20	c
			Pooled	0.3	20	c
D7	African-American	GAP	Single	7.35	18	a
			Pooled	0	–	–
D9	Hispanic	GAP	Single	0.39	14	b
			Pooled	2.18	16	b
D10	African-American	GAP	Single	20	17	b
			Pooled	3.89	10	b
D11	African-American	GAP	Single	44.4	17	c
			Pooled	45.2	19	f
D15	Hispanic	GAP	Single	0.03	17	c
			Pooled	0.008	10	c
D17	Asian-American	LAP	Single	0.13	20	a
			Pooled	0.08	20	a (18), c (2)
D18	Asian-American	GAP	Single	0	–	–
			Pooled	0.16	17	f (16), c (1)
D28	African-American	LAP	Single	52.4	18	b
			Pooled	0	–	–
D37	Asian-American	GAP	Single	0.004	19	c
			Pooled	0.03	19	c
D40	Asian-American	GAP	Single	0	–	–
			Pooled	0.008	20	c
D45	Hispanic	GAP	Single	0.005	8	c
			Pooled	0	–	–

[1]H, healthy or no periodontal disease; GAP, generalized aggressive periodontitis; LAP, localized aggressive periodontitis.

Table 4 Serotype distribution patterns of all samples

Serotype	No. of strains in sample set A	No. of strains in sample set B	Total
a	2	19	21
b	3	9	12
c	9	32	41
d	0	0	0
e	0	6	6
f	1	0	1
Non-typeable	1	0	1

AP-PCR fingerprinting patterns, suggesting that they belonged to the same clonal types.

The overall serotype distribution patterns of strains from sample sets A and B are presented in Table 4. The distribution pattern in sample set B showed that serotype c is the dominant serotype among *A. actinomycetemcomitans*, while relatively few serotypes d, e and f were found. A similar distribution pattern was noted among *A. actinomycetemcomitans* of sample set A. Combining the isolates from these two sample sets clearly showed the following serotype distribution pattern: 21 (25.6%) serotype a strains, 12 (14.6%) b strains, 41 (50%) c strains, 0 d strain, 6 (7.3%) e strains, 1 (1.2%) f strain, and 1 (1.2%) non-typeable strain.

DISCUSSION

In this study of subgingival *A. actinomycetemcomitans*, the prevalence was 4.5% in the subjects without periodontal disease and 23.5% for subjects with aggressive periodontitis. Both appear to be in the lower range of the prevalence for subjects of similar disease diagnosis and age groups reported in the literature (Slots & Ting, 1999). For example, among seven studies of adolescent subjects with localized aggressive periodontitis, the median prevalence of subgingival *A. actinomycetemcomitans* was 80%

(range 39–100%) compared with a median prevalence of 15% (range 0–37%) among seven studies of non-diseased subjects of the same age group (Slots & Ting, 1999). Similarly to previous findings, our work has shown that, in most samples, *A. actinomycetemcomitans* comprised <1% of the total cultivable subgingival bacteria in contrast to *P. gingivalis*, which frequently comprised >1 to 10% of the total cultivable bacteria (Asikainen & Chen, 1999). We also noted a trend for an inverse relationship between the levels of *A. actinomycetemcomitans* and *P. gingivalis*. This finding is in agreement with the results of a previous study noting a negative correlation between the proportions of *A. actinomycetemcomitans* and bacteria within the so called red complex (*Tannerella forsythia*, *P. gingivalis*, *Treponema denticola*) in subjects with periodontitis (Faveri *et al.*, 2009). It is suggested that *A. actinomycetemcomitans* may be associated with the onset of periodontal disease and is found at high levels in shallow and intermediate pockets, while red complex pathogens, such as *P. gingivalis*, are associated with disease progression, the proportion of which increases with deepening pockets (Faveri *et al.*, 2009).

This study further examined the pattern of distribution of *A. actinomycetemcomitans* serotypes and included a larger but clinically less well-defined set of subgingival samples in the analysis. The results showed that serotype c is the dominant serotype, followed by serotypes a and b. Serotypes d, e, and f were either not detected or relatively rare.

The distribution patterns of *A. actinomycetemcomitans* serotypes have been examined in numerous studies (Rylev & Kilian, 2008), which have varied widely in study design (cross-sectional or longitudinal), subjects (race/ethnicity and geographic variations), periodontal disease diagnosis and status (non-diseased, chronic periodontitis, aggressive periodontitis), sampling protocols (single or multiple samples/subject, single or pooled samples, single colony or multiple colonies per sample or subject) and microbial detection methods and serotype analysis techniques (culture versus molecular techniques, PCR serotyping or immunodiffusion of pure culture or direct immunofluorescence detection of serotypes of bacteria in the samples). To permit comparison of the studies, some transformation of the published data is necessary based on the following principles. First, *A. actinomycetemcomitans* isolates are considered distinct among non-cohabitants. Second, multiple isolates of *A. actinomycetemcomitans* from the same individuals/samples are considered identical unless serotyping or clonal typing analysis proves otherwise.

The prevalence rates of single- and double-clone infection of *A. actinomycetemcomitans* in this study (78.6 and 21.4%, respectively) are similar to those reported in previous studies. The majority of the studies showed that among *A. actinomycetemcomitans*-positive individuals, 10–15% of the subjects were infected by more than one clonal type. An exception is the study by Yoshida *et al.* (2003) in which 33% of the subjects were infected by more than one clone of *A. actinomycetemcomitans*. The probability of multiple clone infection appears to be governed by the random chance of infection by different clonal types of *A. actinomycetemcomitans.* Nevertheless, it is possible that the colonization of additional *A. actinomycetemcomitans* strains may be difficult due to competition between the resident strain and the invading strain (Haubek *et al.*, 2009) or due to the suppression of the invading strain from the host immune response to the resident strain. This may explain our observation that no distinct serotypes of *A. actinomycetemcomitans* were recovered from the same sample sites.

Few studies have examined the distribution of *A. actinomycetemcomitans* serotypes among US subjects. Yang *et al.* (2004) identified 129 *A. actinomycetemcomitans* strains from 115 subjects with periodontitis in the Philadelphia area. The resultant serotype distribution pattern was 21.7% a, 42.6% b, 29.5% c, 2.3% d, 2.3% e, and 1.6% non-typeable. The relatively infrequent detection of serotypes d and e and non-typeable strains is a common finding (Yang *et al.*, 2004). However, our study showed a lower detection rate of serotype b, and a much higher detection rate of serotype c strains. Interestingly, in a study of *A. actinomycetemcomitans* in school students (ages 11–17 years), it was noted that serotypes a-c strains were more or less equally distributed among African-American subjects, while serotype c strains were found more frequently in Hispanic subjects (Fine *et al.*, 2007). We noted four of the five Hispanics subjects in sample set A harbored serotype c strains.

It is widely accepted that distribution patterns of *A. actinomycetemcomitans* vary among subjects of different race/ethnicity and geographic regions.

Saarela *et al.* (1992) examined 96 strains from Finnish subjects with or without periodontitis and found the serotype distribution pattern to be 26% a, 28.1% b, 31.3% c, 4.2% d, 5.2% e, and 5.2% non-typeable (Saarela *et al.*, 1992). The results suggest that serotypes a, b and c are equally dominant and collectively comprise 70% or more of all *A. actinomycetemcomitans* strains in Finland. Two studies have examined the serotype distribution patterns of *A. actinomycetemcomitans* in a Japanese population. In both studies serotypes c and e were two of the dominant serotypes, followed by serotype a, while serotype b was relatively rare (Yamamoto *et al.*,; Yoshida *et al.*, 2003). For 117 *A. actinomycetemcomitans* strains in Chinese adults with varying degrees of periodontal disease severity (Mombelli *et al.*, 1999). The strain distribution pattern shared some similarity to that in the Japanese population. Serotype c was the dominant serotype, and serotype b was relatively uncommon. Unlike within the Japanese population, serotype e was not one of the dominant serotypes. In addition, 176 *A. actinomycetemcomitans* strains from Chinese subjects without or with periodontitis in Taiwan were serotyped to 14.8% a, 46% b, 38% c, and 1.1% non-typeable (Yang *et al.*, 2005). The differences between the results from these two studies of Chinese populations shows that serotype distribution patterns may be affected by geographic variations, even between subjects of the same race/ethnicity. The serotype distribution pattern of *A. actinomycetemcomitans* within a local population may change over time, as documented van der Reijden *et al.* (2008) in Indonesian subjects with periodontitis between 1994 and 2002.

Some studies have suggested a strong association between specific clonal types of *A. actinomycetemcomitans* and periodontal disease. This was taken as evidence for the variability in virulence among *A. actinomycetemcomitans* clonal types. However, the interpretation of association between specific clonal types and periodontal disease should be taken with some caveat. The current genotyping/clonal methods distinguish clonal lineages of *A. actinomycetemcomitans* but provide little insight to the genome content of individual strains. Bacterial species are known to exhibit significant genomic variations among strains. We have sequenced the genomes of eight *A. actinomycetemcomitans* strains and found 10–30% of the genome content in each strain to be unique (unpublished data; C. Chen, W. Kittichotirat, R. Bumgarner). Genomic variations are often due to horizontal gene transfer and acquisition of genomic islands, which may carry factors that enhance the virulence/fitness and/or host adaptation of the bacteria (Hacker & Carniel, 2001). Here we postulate that most *A. actinomycetemcomitans* clonal types are capable of causing periodontal disease, but each may do so with different pathogenic mechanisms. This hypothesis is supported by the observation that the non-JP2 clone of *A. actinomycetemcomitans* strains are associated with aggressive periodontitis in subjects of non-African descent.

In conclusion, this study demonstrated that serotype c is the dominant serotype among *A. actinomycetemcomitans* from subjects with periodontitis in the United States. Serotype a strains were more frequently detected than serotype b strains. Serotypes d, e, f, and non-typeable strains were either not detected or were relatively infrequent in the examined population.

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