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Case Report

Detection of the highly leucotoxic JP2 clone of *Aggregatibacter actinomycetemcomitans* in members of a Caucasian family living in Sweden

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Abstract

Background: Carriers of the JP2 clone of *Aggregatibacter actinomycetemcomitans* exhibit an enhanced risk for developing aggressive periodontitis compared with individuals carrying non-JP2 clones. While the JP2 clone is almost exclusively detected among adolescents of African descent, reports on Caucasians colonized with the JP2 clone are remarkably few.

Objective: The aim of this paper is to report on the history of periodontal disease and microbiological findings in a Caucasian family.

Material and Methods: *A. actinomycetemcomitans* and other periodontitis-associated bacterial species in subgingival plaque samples were quantified by conventional culture technique. Leucotoxin promoter typing, serotyping and further characterizations of *A. actinomycetemcomitans* isolates were performed by PCR. DNA sequencing of the pseudogene, *hbpA* was performed to determine the origin of the detected JP2 clones. Further, genetically ancestry testing of family members was carried out.

Results: The JP2 clone was detected in samples from two of the family members, a 33-year-old daughter and her 62-year-old mother. Relationship of their JP2 clones with JP2 clone strains from the Mediterranean area of Africa was indicated. Genotyping confirmed the Caucasian origin of all family members.

Conclusions: Caucasian JP2 carriers exist and older subjects can carry the JP2 clone of *A. actinomycetemcomitans*.

Key words: aggressive periodontitis; ancestry; Caucasian; genetics; genetic testing; haemoglobin-binding protein; leucotoxin

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The onset and progression of periodontitis, a disease characterized by destruction of alveolar bone and other supportive periodontal tissues, involve complex bacterial–host interactions (Pihlstrom et al. 2005). Within the oral microbiota comprising about 1000 species, half of which are cultivable (Ten Cate 2006), *Porphyromonas gingivalis, Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* are considered to be the most important periodontal pathogens (Consensus-Report 1996). Among these bacterial species, *A. actinomycetemcomitans* is associated with aggressive periodontitis, a rapidly progressing variant of the disease (Zambon 1985, Van der Velden et al. 2006, Fine et al. 2007, Haubek et al. 2008).

Among a range of virulence factors of *A. actinomycetemcomitans*, its leucotoxin is the most studied (Fine et al. 2006, Kachlany 2010). This toxin affects human leucocytes by cell lysis and by

Conflict of interest and source of funding statement

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induction of degranulation and apoptosis (Korostoff et al. 1998, Johansson et al. 2000). Thus, this gram-negative bacterial species may affect important parts of the immune system. In addition, the leucotoxin induces an activation and massive release of IL-1β, a cytokine closely correlated to bone resorption from macrophages (Kelk et al. 2005, 2008). A specific clone (JP2) of *A. actinomycetemcomitans*, characterized by a leucotoxin promoter type lacking a 530-bp DNA-fragment, has an enhanced production of leucotoxin compared with other strains with a full-length promoter region of the leucotoxin gene (Brogan et al. 1994). In the Moroccan population, carriers of the JP2 clone exhibit higher risk for developing aggressive periodontitis compared with individuals colonized with non-JP2 clonal types of *A. actinomycetemcomitans* (Haubek et al. 2008). While the JP2 clone is almost exclusively detected among adolescents of African descent, reports on Caucasians colonized with the JP2 clone are remarkably few (Haubek et al. 1995, Bueno et al. 1998, Haraszthy et al. 2000, Kaplan et al. 2002, Fine et al. 2007).

By characterization of point mutations within housekeeping genes and pseudogenes of JP2 clone strains, isolated from individuals living in various geographical areas, a model of the global spreading of the JP2 clone has been suggested (Haubek et al. 2007). The patterns of mutations suggest that the JP2 clone emerged as a distinct genotype in the Mediterranean Africa approximately 2400 years ago and subsequently spread to West Africa. Later, it was transferred to other parts of the world. Apparently, JP2 clone strains are almost exclusively detected among individuals of African descent (Haubek et al. 1997, 2007). Limited information is available regarding the periodontal and microbiological status of Caucasians from Europe colonized with the clone (Haraszthy et al. 2000, Guthmiller et al. 2001, Orrù et al. 2006). In this report, we present findings of the JP2 clone in two related Swedish female adult Caucasians with genetically confirmed European origin and with a history of periodontal disease.

Case Report

In 2008, subgingival plaque samples were collected from periodontal pockets in a 33-year-old Caucasian female at the Department of Periodontology at Skanstull, Stockholm County Council, Sweden. The reason for subgingival plaque sampling was refractory periodontitis and disease progression despite 20 years of given periodontal treatment (Fig. 1). Measurements of the probing pockets depths showed that deep pockets were observed at teeth 16, 13, 11, 21, 22, 38 and 37. Subgingival plaque samples were collected with sterile paper points from teeth 13, 11 and 22 and transported in vials containing VMGA III medium (Möller 1966), which were sent to a microbiological laboratory at the Dental School in Umeå, Sweden, for analysis with regard to the presence of the periodontitis-associated bacterial species, *P. gingivalis, T. forsythia and A. actinomycetemcomitans*. After a procedure involving cultivation on blood agar, containing Columbia base, haemin (0.05 mg/ml) and K-vitamin (0.01 mg/ml), and on *A. actinomycetemcomitans*-specific plates (Tryptic soy–bacitracin–vancomycin, serum was omitted) (Slots 1982), it was found that the subgingival plaque sample contained high proportions of *A. actinomycetemcomitans* (Table 1). Samples were incubated on blood agar plates for 1 week in an anaerobic box and on *A. actinomycetemcomitans*-specific plates in 5% CO_2 in air at 37° for 3–5 days. Based on further characterization of the isolates, including determination of the leucotoxin promoter type according to methods described by Poulsen et al. (2003), it was concluded that the patient was colonized with the JP2 clone of *A. actinomycetemcomitans*. This clone was also detected in a second sample collected from the patient 3 weeks later (Table 1).

For evaluation of a putative transmission of the JP2 clone of *A. actinomycetem-comitans* within the family, the mother and the father of the 33-year-old patient were invited for clinical examination and subgingival plaque sampling. The diagnosis was chronic periodontitis, the mother generalized (Fig. 2) and the father local type. The sample analysis showed that in addition to the daughter the mother was colonized with the JP2 clone, while the

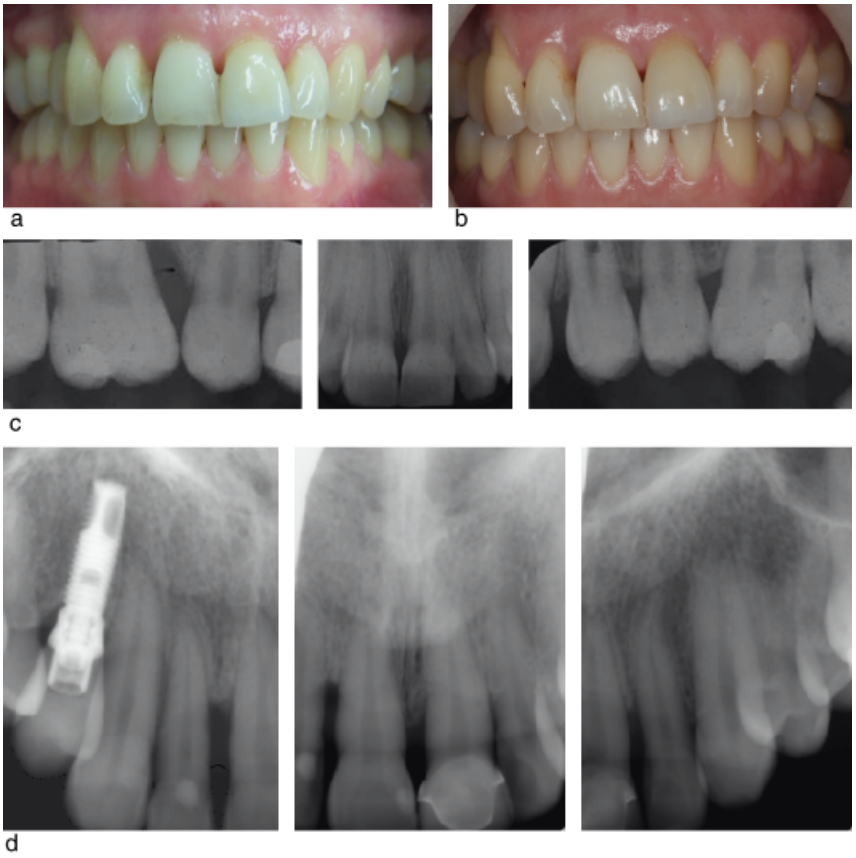

Fig. 1. JP2 clone-positive daughter. (a) Clinical photos before systemic antibiotic treatment and (b) clinical photos 1 year after systemic antibiotic treatment. (c) Radiographs illustrating marginal bone conditions in the upper jaw in 1988 and (d) in 2008.

Table 1. Aggregatibacter actinomycetemcomitans in samples detected by cultivation

	TVC	*A. a.*		Pocket depths
	million/sample	million/sample	(%)	
Daughter				
First sampling (year 2008)				
Sites 11, 13, 22 (pooled)	1.1	0.9	78	6 mm/6 mm/5 mm
Second sampling (year 2008)				
Sites, 11, 13, 21 (pooled)	3.2	2.8	88	6 mm/6 mm/5 mm
Site 37	1.2	0.7	58	6 mm
Third sampling (year 2008)				
Site 13	0.04	ND		3 mm
Site 11	0.05	ND		4 mm
Site 21, 22 (pooled)	0.9	ND		3 mm/3 mm
Site 16, 37 (pooled)	3.5	ND		4 mm/4 mm
Fourth sampling (year 2009)				
Site 16	29	ND		4 mm
Mother				
First sampling (year 2008)				
Site 32	*	0.6	*	8 mm
Sites 24, 25, 26 (pooled)	*	ND		5 mm/5 mm/5 mm
Second sampling (year 2008)				
Site 32	2.7	ND		6 mm
Sites 25, 26 (pooled)	7.0	ND		5 mm/5 mm
Third sampling (year 2009)				
Site 32	0.6	ND		5 mm
Father				
Sampling (year 2008)				
Sites 12, 21 (pooled)	5.6	0.02	0.4	5 mm/5 mm
Site 25	3.6	0.01	0.3	6 mm

*The samples were contaminated by *Stenotrophomonas maltophila*, an aquatic environment bacterial species. Thus, neither the total number of bacteria, nor the proportion of *A.a. (A. actinomycetemcomitans)* could be calculated.
TVC, total viable count; ND, not detected.

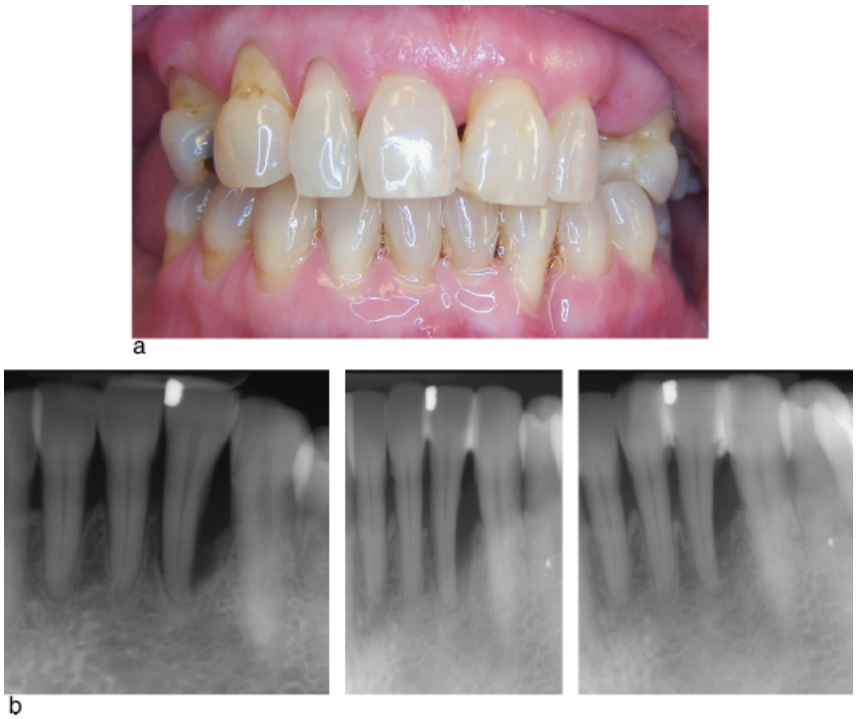

Fig. 2. JP2 clone-positive mother. (a) Clinical photos of the mother at the age of 62 years. (b) Radiographs illustrating marginal bone-level changes in teeth 32; years 2005, 2008; 2009, note bonefill 32 distally in year 2009.

father harboured the non-JP2 clonal type of *A. actinomycetemcomitans* only (Table 1, Fig. 3). None of the patients were found to harbour more than one clonal type when both subgingival plaque samples and isolated strains were characterized (Fig. 3).

After detection of the JP2 clone of *A. actinomycetemcomitans* in the periodontal pockets of the daughter and the mother, both were re-scaled and prescribed a mixture of amoxicillin/clavulanic acid (500 mg, three times a day) and metronidazole (400 mg, three times a day) for 10 days, combined with chlorhexidine rinse twice a day for 6 weeks. In addition to systemic antibiotics and continued supportive therapy, the mother has got one tooth extracted and the father had five teeth extracted for periodontal and prosthetic reasons.

All family members were followed 1 year after treatment. Periodontal destruction of the father and the daughter was now arrested (Fig. 1b), and the mother had only one pocket remaining at tooth 32, which remarkably showed bone-fill in newly taken radiographs (Fig. 2b). When the daughter and the mother were re-sampled 3 months and 1 year, respectively, after the treatment, *A. actinomycetemcomitans* was not detected (Table 1).

When *A. actinomycetemcomitans* strains from the three patients were tested for the leucotoxin promoter type (6–10 strains per patient), followed by determination of serotype according to methods described by Suzuki et al. (2001), it was shown that the two JP2 clone strains isolated from the daughter and the mother were, as expected serotype b, while the non-JP2 clonal type of *A. actinomycetemcomitans* from the father was serotype c.

Genotyping of the strains by arbitrarily primed PCR (AP-PCR), performed by means of a Ready to Go-Kit® (GE Healthcare, Little Chalfont, UK) and primers and temperature profile according to Paju et al. (1998), revealed a similar banding pattern for the two JP2 clone strains, distinguished from the banding pattern of the non-JP2 clonal type of *A. actinomycetemcomitans* obtained from the father (Fig. 4).

Sequencing of the *hbpA* pseudogene of JP2 clone strains from the daughter and the mother according to Haubek et al. (2007) revealed a specific point mutation at position 525,077, which most likely indicates that these strains have a North African origin.

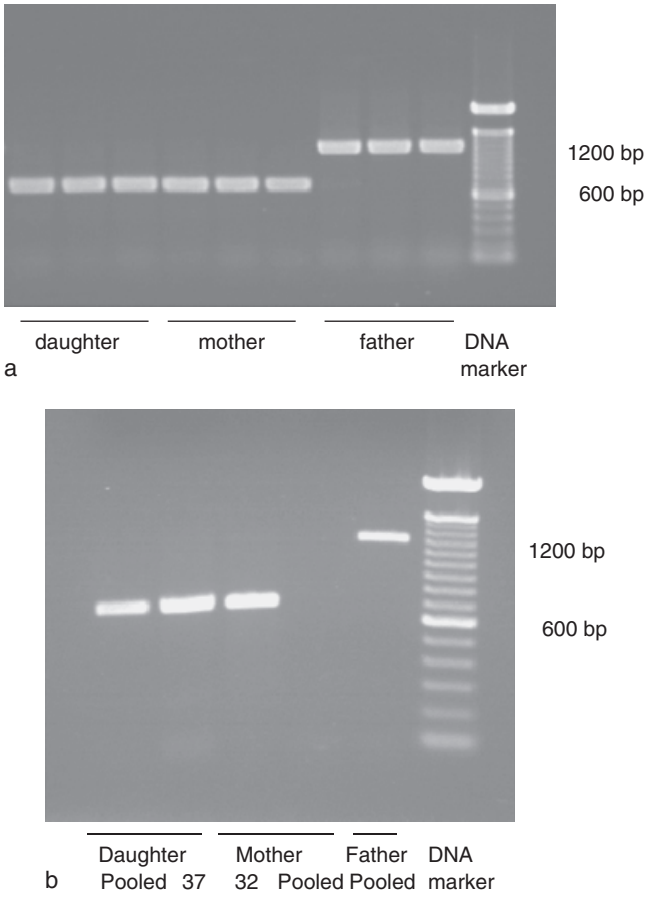

Fig. 3. Detection of DNA bands specific for JP2- and non-JP2 clonal types of *Aggregatibacter actinomycetemcomitans* (686 and 1216 bp, respectively), revealed by PCR amplification of the leucotoxin promoter gene in *A. actinomycetemcomitans* – strains isolated from a 33-year-old Caucasian female, her 62-year-old mother, and her 69-year old-father (a), or in plaque samples from the three subjects (b). Results from three isolates per subject are shown (a).

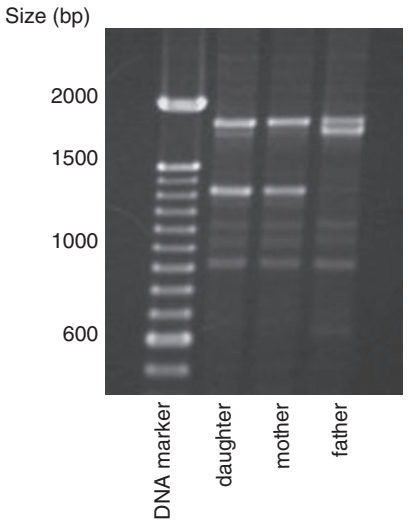

Fig. 4. Arbitrarily primed PCR banding patterns among *Aggregatibacter actinomycetemcomitans* strains isolated from the daughter (JP2), the mother (JP2), and the father (non-JP2). Patterns from the mother and the daughter are identical, while the pattern for the *A. actinomycetemcomitans*-isolate from the father differs.

While *A. actinomycetemcomitans* was detected in samples from the three patients, *P. gingivalis* and *T. forsythia* were not found in any of the samples when they were analysed according to established methods (Lakio et al. 2002).

The population genetic analysis (population stratification) of the family members by iGENEA DNA-genealogie (Zhürich, Switzerland) confirmed that they were Caucasians with a European origin (Table 2).

In the view of possibly additional routes of transmission of the JP2 clone, the carriers informed that they have not been in Africa.

It was not planned to follow this family over time when they were initially referred to the periodontal specialist clinic for treatment of their periodontal diseases. However, based on the microbiological findings of the family members, their history of disease is presented in a retrospective view.

The daughter, a Caucasian female, medically healthy and non-smoking, was referred to Department of Periodontology at Skanstull, Stockholm Country Council, Sweden in 1988. A periodontal examination was carried out and the diagnosis localized juvenile periodontitis (now called aggressive periodontitis) was given. Initial therapy consisted of oral hygiene instructions, scaling, root planing, and surgical intervention of the deepest periodontal pocket, supplemented by a prescription of systemic antibiotics in terms of penicillin (2×88 mg, twice a day) for 10 days. Despite maintenance care during the following years, progression of the disease was found at several occasions, and flap surgeries were necessary. Several types of antibiotics, like tetracycline and amoxicillin (systemic administration) and metronidazole gel (local administration) were used as a supplement to conventional periodontal therapy. Choice of antibiotics was, however, not based on microbiological diagnostics before referral to our lab in 2008.

The mothers' history is summarized as follows: The mother was 17 years ago referred to the same specialist clinic in periodontology for treatment as the daughter. At that point of time, she was 45 years old, medically healthy, non-smoking and without medication. Before referral, treatment of deep periodontal pockets by both a dentist and a dental hygienist during the last 5 years had been performed without improvements. Initial treatment at the specialist clinic consisted of oral hygiene instruction, flap surgery in the maxilla, and scaling in the lower jaw. Despite continued maintenance therapy two to three times per year, flap surgery was necessary to perform. Also the prognosis of several teeth got worse over the years. A splint was made and worn every night. Supportive care was intensified by visits three to four times per year. At one of the check-up visits, local treatment with doxycycline of all deep pockets were performed and resulted in improvements observed as reduced bleeding on probing. Never the less, 2 years later re-evaluation indicated refractory periodontitis. At this point of time, three teeth were extracted and six teeth had dubious prognosis.

Table 2. The population stratification test (iGENEA*) of the three subjects

Subject	Haplogroup	Primitive tribe	Country of origin
Maternal lineage			
Daughter (JP2 carrier)	T	Teuton	Germany
Mother (JP2 carrier)	T	Teuton	Germany
Father (non-JP2 carrier)	W	Balts	Sweden
Paternal lineage			
Father (non-JP2 carrier)	I1	Vikings	Iceland

*For information of the tests see http://www.igenea.com/

The haplogroup shows the origins in pre-history, the indigenous people group (primitive tribe) shows the ancestry in antiquity (900 BC–AD 900) and the country of origin shows where the ancestors lived between the 11th and 13th centuries.

The father was referred to the same specialist clinic in periodontology as his wife and daughter when he was at the age of 54 years. At this point of time, the father was medically healthy with no prescribed medications, former pipe-smoker. The latter habit, he had quit a year earlier. Given treatment had been oral hygiene instruction, scaling and root planing, and a flap surgery. Oral hygiene level had seldom been perfect and despite regular supportive care until today, the goal, resolution of deep periodontal pockets, has not been reached.

All participants gave their informed consent, and the study was approved by the Ethics Committee of Umeå University, Sweden.

Discussion

This case report demonstrates that the JP2 clone of *A. actinomycetemcomitans* can be found in subgingival plaque collected from Caucasians. These findings are remarkable as the JP2 clone is almost exclusively detected in individuals of African descent (Haubek et al. 1996, 1997, 2007). Further, adults and not only adolescents can be colonized with the JP2 clone. Previous reports have suggested that this clone preferentially infects younger individuals, and that the clone with its increased leucotoxin production may disappear at increasing age of the host (Zambon 1996, Haraszthy et al. 2000, Guthmiller et al. 2001).

In this study, the origin of the three family members has been determined not only by self-reporting of origin, but also the information has been confirmed by genetically ancestry testing. It is well known, that the presence of the JP2 clone of *A. actinomycetemcomitans* is strongly associated with individuals of African descent (Haubek et al. 1996, 1997, 2007). It could be suggested that Caucasians are absent or under-represented in studies on the JP2 clone. When summarizing the findings in different studies, it is, however, clear that a limited number of Caucasians have been included in several studies and that sporadically reporting on Caucasians positive for the JP2 clone can be found in the literature (Haraszthy et al. 2000, Guthmiller et al. 2001). Thus, detection of the JP2 clone appears to be rare among individuals of the Caucasian race, albeit not completely absent (Haubek et al. 1995, Bueno et al. 1998, Haraszthy et al. 2000). For example, three to four cases of the JP2 clone colonizing Caucasians are mentioned by Guthmiller et al. (2001), but without specific information on the origin of the carriers. Also, Orrù et al. (2006) reported that six JP2 clone carriers were detected among 81 patients from Sardinia, an Italian province not far from North Africa. In the present report, it was confirmed by population genetic analysis that the two family members, positive for the JP2 clone, belong to the Caucasian race. In other studies, this type of genetically based ancestry testing seems to be absent.

A number of JP2 clone strains, isolated from African carriers, have been characterized with regard to DNA sequences of various genes (Haubek et al. 2007). Specific point mutations within one of them, the one encoding haemoglobin-binding protein (*hpbA*-2), have been shown to separate JP2 strains originating from North Africa from variants of West African origin. After sequencing a part of the *hbpA*-2 gene in the JP2 clone strains from subjects of this study, it was suggested that the strains originate from North Africa. More attention on microbiological subtyping of *A. actinomycetemcomitans* may in the future reveal more Caucasians positive for the JP2 clone and provide a better understanding of the global spreading of the JP2 clone of *A. actinomycetemcomitans*.

For the authors of this report, the contact with cases presented in this report started at the point of time when the first samples from the daughter were analysed and the JP2 clone was detected, i.e., in 2008. However, in the light of this finding, it might be valuable to discuss the history of periodontal disease of the daughter. First to be commented is that the microbiological status concerning the presence of *A. actinomycetemcomitans* was unknown in 1988. At that point of time, no microbiological diagnosis was performed. Further, detailed information on the JP2 clone of *A. actinomycetemcomitans* was not yet available in the literature. Second, the daughter was over the years treated by different clinicians. This might have affected the treatment strategy, e.g. that different antibiotic regimes, however, not the combination of amoxicillin and metronidazole (Van Winkelhoff et al. 1989, 1992), were used as an adjunct to the periodontal therapy over the years. Third, the daughter was not sampled for analysis of the pocket microflora during a period of 20 years. This may, due to the refractory nature of the disease, be remarkable. In the absence of information on the pocket microflora, it might have been a difficult task to choose relevant antibiotics as an adjunct to the periodontal treatment carried out. Obviously, the different clinicians, based on clinical examinations, tested various treatment strategies. Some of the strategies might have lacked scientific basis (Fine 1994, Pavicic et al. 1994, Berglundh et al. 1998, Winkel et al. 2001). However, after we found the JP2 clone in samples from the daughter and the mother, treatment with metronidazole and amoxicillin was prescribed (Van Winkelhoff et al. 1989, 1992). At re-sampling one year later, the JP2 clone could not be detected.

Regarding acquisition of *A. actinomycetemcomitans*, it can occur throughout life (Asikainen et al. 1997). In the present case, it is not known at what age the subjects were infected by the JP2 clone. Patients were 33 and 62 years, respectively, when they were sampled and were found for the first time to be positive for the JP2 clone. Mother-to-child transmission of *A. actinomycetemcomitans* has been reported on before (Dogan et al. 2008). For the daughter, periodontitis was detected 20 years

earlier. The 33-year-old daughter may have adopted the JP2 clone from the mother early in life. A similar AP-PCR pattern, obtained when JP2 clone strains from the mother and the daughter were genotyped, supports the possibility that vertical transmission has occurred (Asikainen et al. 1997). However, this route of transmission cannot be proven.

Also for the mother, the scenario concerning acquisition of *A. actinomycetemcomitans* and infectious route is unclear. Based on her history of periodontitis, nothing can be suggested regarding time for the JP2 clone acquisition. If the mother infected her daughter by the JP2 clone, she may have carried it for at least 30 years before detection. Persistent colonization with *A. actinomycetemcomitans*, including the JP2 clone of *A. actinomycetemcomitans*, for years has been reported (Saarela et al. 1999, Haubek et al. 2009).

Of the three family members in this report, two were more than 60 years old and one of those was carrying the JP2 clone. Based on clinical investigation, their diagnosis was chronic periodontitis and for the mother chronic periodontitis of the refractory type. Concerning the father, he had lost more teeth than the other family members despite the fact that the microbiological analysis revealed that he was not carrying the JP2 clone. Despite extensive tooth loss, the destructive type of periodontitis of the father was not as severe and extensive as in the mother. He suffered from localized chronic periodontitis, and had in addition lost several teeth for prosthetic reasons. Concerning *A. actinomycetemcomitans*, low proportions of the non-JP2 clones were detected in samples from the father. Whether this fact has been involved in his disease progression is unknown. As mentioned in several reports and reviews, many different aetiological factors of periodontitis may be involved in disease development (Nunn 2003, Pihlstrom et al. 2005), and some of these may have been implicated in the development of the disease of the father. Albeit not detected in samples from the daughter or the mother, it cannot be excluded that non-JP2 clones or other factors, such as the total composition of the oral microflora as well as dentally related factors or specific host-related factors, may be involved in and partly explaining the disease status of these patients.

The finding of the JP2 clone in the mother is remarkable because association of the JP2 clone to aggressive periodontitis in particular has been shown for adolescents (Zambon 1996, Haraszthy et al. 2000, Guthmiller et al. 2001, Haubek et al. 2008). No reports are available regarding the association between older individuals with various types of periodontitis and the occurrence of the JP2 clone.

For treatment of patients with aggressive periodontitis testing of subgingival plaque for the presence *A. actinomycetemcomitans*, may be beneficial. Although only two cases of JP2 clone-positive individuals in Caucasians are presented in this report, it could be speculated if such a test should include a method for detection of the JP2 clone also when the patients belong to other populations than the African population. So far, evidence only indicates that individuals of African descent deserve particular attention in relation to microbial diagnosis for the presence of the JP2 clone. But as origin of individuals may in many cases be unknown or difficult to unravel and as unexpected JP2 clone-positive individuals of non-African origin have been found, the relevance of microbial diagnosis, with special attention given to the detection of the JP2 clone, need to be addressed further in future studies.

Conclusion

The present study provides further cases of JP2 clone-infected Caucasians to the almost negligible number of earlier reported cases of this kind. In addition, it is shown that the JP2 clone also may be detected in adults despite a suggested age predilection for infection with the clone in younger individuals.

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Clinical Relevance

Scientific rationale for the study: Carriers of the highly leucotoxic JP2 clone of *Aggregatibacter actinomycetemcomitans* exhibit high risk for developing aggressive periodontitis. For unknown reasons, the JP2 clone seems to almost exclusively colonize individuals of African descent. However, in this case report we circumvent the hypothesis that Caucasians may resist colonization by the JP2 clone.

Principal findings: A 33-year old Caucasian female with a 20-year history of refractory periodontitis was found to carry high proportions of the JP2 clone of *A. actinomycetem-comitans* in periodontal pockets. The 62-year-old mother was also found to be infected by the JP2 clone. After surgery and systemic treatment with amoxicillin/clavulanic acid and metronidazol, neither the JP2 clone nor other clonal types of *A. actinomycet-emcomitans* could be detected in any of the patients when they were sampled three months and one year after the treatment, respectively. By a ancestry testing of the JP2 carriers, it was confirmed that they were of Caucasian origin.

Practical implications: This case study lends support to the concept that microbial screening could provide a potentially useful tool in the clinical management of patients with aggressive periodontal disease.