

***Aggregatibacter actinomycetemcomitans* serotype prevalence and antibiotic resistance in a UK population with periodontitis**

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ABSTRACT

Objectives: *Aggregatibacter actinomycetemcomitans* is a recognised pathogen involved in aggressive periodontitis. Seven serotypes of *A. actinomycetemcomitans* exist with a range of virulence and distribution dependent on ethnicity and geography. The ability of *A. actinomycetemcomitans* to invade soft tissue can necessitate the use of systemic antibiotics for treatment, however variations in its antibiotic susceptibility exist dependent on geographical location.

Methods: Serotypes of *A. actinomycetemcomitans* isolates from a UK cohort of 50 patients with aggressive periodontitis were determined by PCR. Resistance of the isolates to eight antibiotics [penicillin (1 U), amoxicillin (2 µg), amoxicillin/clavulanic acid (30 µg), metronidazole (5 µg), clindamycin (2 µg), tetracycline (10 µg), ciprofloxacin (5 µg) and ceftazidime (30 µg)] were determined by disk diffusion according to BSAC guidelines.

Results: Prevalences of serotypes a, c, b, e and mixed serotypes were 48%, 22%, 2%, 2% and 12%, respectively. The serotype of isolates from seven patients (14%) could not be deduced by PCR. Of the 56 isolates tested, 100% were resistant to penicillin and metronidazole, 87.5% to clindamycin, 83.9% to amoxicillin and 76.8% to ceftazidime. Low rates of resistance to tetracycline (8.9% resistant) and amoxicillin/clavulanic acid (14.3% resistant) were observed, whereas no isolates were resistant to ciprofloxacin.

Conclusions: As in a number of publications the suggested treatment of aggressive periodontitis includes the combined use of amoxicillin with metronidazole, these results highlight the need for culture and antimicrobial susceptibility investigations in patients with aggressive periodontitis prior to systemic use of antibiotics concomitantly to periodontal therapy.

1. Introduction

Periodontitis, the most prevalent chronic inflammatory disease in humans [1], compromises the integrity of the tooth-supporting tissues, including the gingivae, periodontal ligament and alveolar bone [2]. *Aggregatibacter actinomycetemcomitans* is a recognised periodontal pathogen involved in the onset of aggressive periodontitis [3,4]. There are seven serotypes of *A. actinomycetemcomitans* (named a–g) classified by differences in surface antigens, with certain serotypes and clones being more pathogenic, e.g. serotype b JP2 clone [5]. The distribution of these serotypes varies depending on geography and/or the patient's ethnic background [6].

Patients suffering from aggressive periodontal disease are initially treated by provision of oral hygiene instructions as well as scaling and root planing together with the concomitant use of systemic antibiotics, more specifically with the combined use of amoxicillin and metronidazole [7–9]. Tetracycline, shown to be effective against *A. actinomycetemcomitans* [10,11], is also frequently used in treating localised aggressive periodontitis. However, large variations in the antibiotic susceptibility profiles of *A. actinomycetemcomitans* exist depending on geographical location [12,13], perhaps reflecting different antibiotic usage patterns. Little is known about the *A. actinomycetemcomitans* serotype prevalence and antibiotic resistance profiles in the UK. This study aimed to determine the prevalence of serotypes and the antibiotic resistance profiles of *A. actinomycetemcomitans* associated with aggressive periodontitis in 50 UK patients.

2. Materials and methods

2.1. Isolation of bacteria

Aggregatibacter actinomycetemcomitans clinical isolates from 50 patients who had attended the Periodontology Clinic at the Eastman Dental Hospital (London, UK) with suspected aggressive periodontitis and prior to their routine hospital treatment were analysed in this study. These isolates were obtained from routinely collected diagnostic subgingival plaque samples taken from the patients' four deepest periodontal pockets that had been serially diluted in sterile tryptone–soya broth followed by culture onto tryptone–soya–bacitracin–vancomycin agar [14] and incubation anaerobically for 5–7 days as part of the routine diagnostic service.

2.2. PCR confirmation and serotyping

All presumptive *A. actinomycetemcomitans* colonies were counted and subcultured and their identity was confirmed by PCR targeting a specific region found in *A. actinomycetemcomitans* on the 16S RNA gene (see Table 1) [15] using the following conditions: 35 cycles of 95 °C for 1 min (10 min for the first cycle), 61 °C for 1 min and 72 °C for 5 min (10 min for the final cycle).

All PCR-confirmed *A. actinomycetemcomitans* isolates were further characterised by deducing their serotype by PCR using specific primers (see Table 1). Multiplex PCR was used to deduce serotypes b, c and f, and individual PCR reactions were used for serotypes a, d and e [16]. Serotype g was not tested for. Both reactions used the following PCR conditions: 30 cycles at 95 °C for 30 s (10 min for the first cycle), 55 °C for 30 s and 72 °C for 30 s (10 min for the last cycle).

Aggregatibacter actinomycetemcomitans serotype b isolates were analysed by PCR to determine whether they were of the JP2 clone using in-house primers LeukF and LeukR (see Table 1) under the following conditions: 30 cycles at 95 °C for 1 min (10 min for the first cycle), 56 °C for 1 min and 72 °C for 1 min (10 min for the last cycle).

Genomic DNA extracted from pure cultures of reference *A. actinomycetemcomitans* serotypes a–e (HK 929, SUNY 465, HK 914, HK 928 and HK 972, respectively), JP2 [SUNY 465 (JP2) and SUNY 462 (non-JP2)] and serotype f (clinical isolate courtesy of Mogens Kilian, University of Aarhus, Aarhus, Denmark) strains were used as controls.

2.3. Antibiotic sensitivity and interpretation

Antibiotic sensitivity was determined by disk diffusion on Iso-Sensitest agar plates (Oxoid Ltd., Basingstoke, UK) supplemented with 5% defibrinated horse blood (E&O Laboratories Ltd., Bonnybridge, UK) according to British Society of Antimicrobial Chemotherapy (BSAC) guidelines [17] using the direct inoculation method. Eight antibiotic disks [penicillin (1 U), amoxicillin (2 µg), amoxicillin/clavulanic acid (AMC) (30 µg), metronidazole (5 µg), clindamycin (2 µg), tetracycline (10 µg), ciprofloxacin (5 µg) and ceftazidime (30 µg); all from Oxoid Ltd.] were placed onto the surface of the agar plates within 15 min of inoculation and were incubated in air supplemented with 5% carbon dioxide for 2 days. Metronidazole sensitivity was also tested on fastidious anaerobic agar (FAA) plates (Lab M Ltd., Heywood, UK) supplemented with 5% defibrinated horse blood (E&O Laboratories Ltd., Bonnybridge, UK) incubated for 2 days at 37 °C under anaerobic conditions. Quality control strains

(*Staphylococcus aureus* NCTC 6571, *Escherichia coli* NCTC 12241, *Haemophilus influenzae* NCTC 11931 and *Bacteroides fragilis* NCTC 9343; Public Health England, London, UK) were tested simultaneously and their zone diameters were determined to ensure they were within acceptable ranges before interpreting tests. Measured zone diameters were interpreted as being susceptible (S), intermediate-susceptible (I) and resistant (R) according to BSAC [17]. For *A. actinomycetemcomitans*, the interpretive criteria for the HACEK group were applied for amoxicillin, AMC, ceftazidime, ciprofloxacin and tetracycline. *Pasteurella multocida* criteria were used for penicillin. No interpretive criteria exist for clindamycin and metronidazole, therefore the interpretive criteria for anaerobes (*B. fragilis*) were applied as used by Kulik et al. [18].

2.4. Statistical analysis

Any differences in the number of antibiotic-resistant *A. actinomycetemcomitans* isolates between the different serotypes was assessed using the χ^2 test. Data were analysed using SPSS software version 14.0, and the 5% level of statistical significance was used throughout the analyses.

3. Results

3.1. Serotype prevalence

The median total viable count of the subgingival plaque microbiota was 9.18×10^6 CFU/mL (range 6.4×10^3 to 2.5×10^8 CFU/mL) and the median viable count of *A. actinomycetemcomitans* was 5.0×10^4 CFU/mL (range 5.5×10^1 CFU/mL to $6.6 \times$

10⁶ CFU/mL). The proportion of the cultivable plaque microbiota that comprised *A. actinomycetemcomitans* ranged from 0.001% in one subject to a maximum of 81.25% (median 0.63%). Of the 50 patients, 24 (48%) harboured *A. actinomycetemcomitans* serotype a, 11 (22%) serotype c, 1 (2%) serotype b and 1 (2%) serotype e. Mixed serotype profiles were observed in six of the patients (12%), five of which were a combination of serotypes a and c and one subject harboured a combination of serotypes b and c. Seven patients (14%) carried untypeable isolates as no product was amplified with the PCR primer sets. The serotype b isolate was not a JP2 clone as no deletion in the promoter region was detected by PCR analysis of the leukotoxin gene.

3.2. Antibiotic susceptibility

All 56 *A. actinomycetemcomitans* isolates tested were resistant to metronidazole (both in CO₂ and anaerobic conditions) and penicillin. Of the 56 isolates, 49 (87.5%), were resistant to clindamycin, 47 (83.9%) to amoxicillin and 43 (76.8%) to ceftazidime. Most of the isolates were susceptible to tetracycline, with only 5 (8.9%) of 56 isolates being resistant. Moreover, 8 (14.3%) of the 56 *A. actinomycetemcomitans* isolates were resistant to AMC. All of the *A. actinomycetemcomitans* isolates (100%) were susceptible to ciprofloxacin (Table 2).

The χ^2 analysis of the antibiotic resistance profiles of the two *A. actinomycetemcomitans* serotypes found to be most prevalent in this study (serotypes a, $n = 29$; and serotype c, $n = 17$) only demonstrated a significant difference when comparing the number isolates exhibiting resistance to amoxicillin ($P = 0.036$), with 17 (100%) serotype c isolates resistant to this antibiotic compared

with 22 (75.9%) of the serotype a isolates demonstrating resistance to this antibiotic (Table 2; Supplementary Table S1).

4. Discussion

Serotype a was mostly frequently identified (48% of patients) in a UK cohort of patients with aggressive periodontitis, followed by serotype c (22%). Serotypes b and e were recovered from single patient samples. In contrast, in Brazilian, US and Korean samples, serotype c was the most prevalent [52.9% ($n = 85$), 42% ($n = 21$) and 61.9% ($n = 21$), respectively] [19–21], whereas serotype b [33.3% ($n = 24$)] was the most common serotype detected in German isolates [21]. In 6 (12%) of the 50 patients in the current study, two serotypes were detected, similar to the findings of van der Reijden et al. (12.2%) and Roman-Torres et al. (9.3%) [19,22]. In the study by van der Reijden, the prevalence of *A. actinomycetemcomitans* serotypes shifted over time [22].

The b serotype isolated in the current study was not a JP2 clone, which concurs with the finding that this highly toxic JP2 clone is predominately recovered in North African [5] rather than in European populations.

4.1. Antibiotic susceptibilities and resistance

This study also tested the susceptibility of *A. actinomycetemcomitans* to antibiotics either commonly or infrequently used to treat periodontal disease.

4.1.1. Tetracycline

Tetracycline was found to be one of the most effective antibiotic against the isolates in this study, with 51 (91.1%) of 56 isolates being susceptible, slightly lower than found with French (96%; $n = 50$) [23], Swiss (99.2%; $n = 125$) [18], Dutch and Spanish isolates (100%; $n = 18$ and $n = 10$, respectively) [24]. In contrast, high tetracycline concentrations were required to kill 90% ($MIC_{90} = 4$ mg/L) of Japanese isolates ($n = 11$) [25].

4.1.2. Clindamycin

Clindamycin resistance was noted in 87.5% of the 56 isolates, in keeping with high resistance rates in the USA (93.8%; $n = 81$) [26], Colombia (83.33%; $n = 18$) [13] and Switzerland (88%; $n = 125$) [18]. However, Van Winkelhoff et al. reported lower clindamycin resistance rates for Dutch (22%) and Spanish (30%) isolates [24].

4.1.3. Ceftazidime

Only 13 (23.2%) of the 56 *A. actinomycetemcomitans* isolates tested were susceptible to ceftazidime, a third-generation cephalosporin frequently used as a first-line antibiotic in treating *A. actinomycetemcomitans*-associated infective endocarditis [27]. These results contrast with a study of HACEK organisms [28] that found third-generation cephalosporins to be most effective, with ceftriaxone having an MIC range of 0.006–0.023 mg/L ($n = 5$).

4.1.4. Penicillin

Although penicillin is commonly used against micro-organisms of the HACEK group, no *A. actinomycetemcomitans* isolates were susceptible to penicillin in this study, in line with the susceptibility rates in studies conducted in Germany (6.2%) [29] and Switzerland (12%) [18]. A French study found higher susceptible rates (60%; $n = 50$) [23], similar to those found in Dutch (55.6%; $n = 18$) and Spanish (60.6%; $n = 10$) isolates.

4.1.5. Amoxicillin

The majority (83.9%) of the *A. actinomycetemcomitans* isolates in the present study were resistant to amoxicillin, similar to rates in a Columbian study (77.7%; $n = 18$) [13]. A number of studies have reported high susceptibility rates (93.9–100%) of *A. actinomycetemcomitans* isolates to amoxicillin [23,24,26], however breakpoint concentrations in these studies were considerably higher than the BSAC definition of resistance in HACEK organisms [30], similar to an issue found in studies that showed 33.3% and 25% resistance rates [24,29] and therefore these results are not comparable.

4.1.6. Amoxicillin/clavulanic acid (AMC)

The combination AMC reduced the proportion of resistance from 83.9% for amoxicillin alone to 14.3% in isolates in the current study. In a Columbian study, similar reductions occurred (77.77% to 0%) [13]. Results from a Spanish group reported a resistance decrease from 33.3% to 10% with the addition of clavulanic acid [24].

4.1.7. Metronidazole

Metronidazole was one of the least effective antibiotics used in this study (100% resistance), results that are corroborated by other studies [13,23,25,31]. Lower resistance rates of 20.8% [18] and 37.5% [29] have been reported when performed under anaerobic conditions [32]. There was no increase in zone diameter when incubated anaerobically, implying that the variance in resistance rates in the abovementioned studies was probably due to geographical distribution or the breakpoint chosen as, e.g., the breakpoint chosen by Eick et al. was ≥ 32 mg/L [29].

4.1.8. Ciprofloxacin

All 56 *A. actinomycetemcomitans* isolates (100%) were shown to be susceptible to ciprofloxacin, in line with the high susceptibility rates in isolates found in Germany [29,31], France [23], Finland [33] and Japan [25].

4.2. Serotype-specific resistance

The only significant difference in the proportion of antibiotic-resistant serotype a and c isolates was found in susceptibility to amoxicillin, with 24.1% and 0% of serotype a and c isolates sensitive to this antibiotic, respectively ($P = 0.036$). Pajukanta et al. found only small differences in the MIC of ceftazidime between different serotypes ($n = 80$) [33], whereas a smaller study by Ihalin et al. ($n = 12$) reported substantial differences in sensitivities between different serotypes [34], although these results should be treated with caution owing to the sample size.

The large variation in antibiotic susceptibility rates between studies may be attributed to different levels of antibiotic consumption [24] as seen in data published in 2013 by the European Surveillance of Antimicrobial Consumption (ESAC) [35]. Total outpatient antibiotic use in 2013 varied by a factor of almost three between countries. Local observation data are decisive and should be utilised to direct clinical supervision, to modernise treatment procedures, to instruct prescribers and to conduct infection control policies [36].

To determine which antibiotic should be selected as an adjunct in *A. actinomycetemcomitans*-associated periodontitis therapy, most clinical laboratories, use the disk diffusion method. This method does not allow determination of the MIC or consider the influence of periodontal biofilms on susceptibility [37–39]. Other considerations include the ability of *A. actinomycetemcomitans* to invade and grow in human cells [40] and the ability to achieve a therapeutic level of the antibiotic in gingival crevicular fluid and saliva. Fluoroquinolones, which have higher bioavailability levels in saliva than in plasma [31] coupled with the high susceptibility rates of *A. actinomycetemcomitans* to this antibiotic class observed in this study, could make them an option for treating unresponsive *A. actinomycetemcomitans* periodontitis.

5. Conclusion

This is the first study examining the serotype prevalence and *A. actinomycetemcomitans* antibiotic resistance in a UK population of periodontitis patients. Caution is required prior to prescription of antibiotics for the treatment of

periodontal disease and, if necessary, microbial testing should be carried out before treatment commences.

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