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Microbiology Section

Frequency of *bspA* and *mutS* Genes in *Tannerella forsythia* Isolated from Patients with Aggressive Periodontitis: A Cross-sectional Study

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ABSTRACT

Introduction: *Tannerella forsythia (T. forsythia)* is a predominant periodontal pathogen contributing to the progression of periodontitis. Among its virulence factors, the *bspA* gene, which encodes cysteine protease and the *mutS* gene, associated with DNA mismatch repair, is considered significant.

Aim: To determine the frequency of the *bspA* and *mutS* genes in clinical strains of *T. forsythia* isolated from periodontitis patients and to evaluate their antibiotic resistance profiles.

Materials and Methods: This cross-sectional study was conducted at Saveetha Dental College and Hospital, Chennai, Tamil Nadu, India, from January 2023 to May 2023, including 45 patients categorised into three groups: Gingivitis (Group I), Periodontitis Stage II (Group II), and Aggressive Periodontitis (Group III). The inclusion criteria were patients aged 18-60 years with periodontal disease, while smokers, pregnant women and those undergoing orthodontic treatment were excluded. Demographic parameters, including age, gender and periodontal health status, were recorded. Subgingival plaque samples were cultured anaerobically to identify *T. forsythia*, which was confirmed by Polymerase Chain Reaction (PCR). Genomic DNA was extracted for the detection of the *bspA* and *mutS* genes. Antibiotic susceptibility was tested using the E-strip method. Data analysis included descriptive statistics, Kruskal-Wallis test and Analysis of Variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) software, with statistical significance set at p-value <0.05.

Results: *T. forsythia* was detected in four out of 15 samples (26.7%) from Group III and in one out of 15 samples (6.7%) from Group II, while no samples from Group I showed its presence. The *bspA* and *mutS* genes were detected in three out of four strains (75%) and two out of four strains (50%), respectively. All isolates (100%) demonstrated resistance to metronidazole and clindamycin.

Conclusion: This study highlights the prevalence and virulent characteristics of *T. forsythia* in periodontal diseases, particularly in aggressive periodontitis. The detection of specific virulence genes and the antibiotic resistance patterns observed underscore the pathogenic potential of this bacterium. Regular monitoring of *T. forsythia* and its antimicrobial susceptibility is crucial for effective periodontal disease management. The findings suggest that targeted treatment strategies, considering the resistance profiles of *T. forsythia*, are necessary for better clinical outcomes in periodontal care.

Keywords: Antimicrobial resistance, Dental plaque, Periodontal health

INTRODUCTION

Periodontitis is a chronic inflammation initiated by the polymicrobial biofilm that adheres to the surface of teeth as dental plaque and is common in most of the adult and elderly population [1]. Periodontitis is caused by a conglomeration of different bacterial aetiologies, with an initial colonisation and further progression of the disease resulting in significant damage to the surrounding tissues of the tooth structures [2]. Periodontitis is graded into different stages, with chronic periodontitis and aggressive periodontitis being the two common stages [3].

The microbial aetiology of periodontitis encompasses bacterial flora categorised into various complexes. While bacteria of the orange and red complexes have been significantly identified as periodontal pathogens, bacteria of the purple, yellow and green complexes have also been highly associated with periodontal health. Red complex bacteria, comprising *Porphyromonas gingivalis*, *T. forsythia* and *Treponema denticola*, are considered the highly prevalent bacterial aetiologies associated with periodontitis [4]. These bacteria are typically detected in the periodontal pockets, in association with other bacteria, indicating a synergistic role in the destruction of the periodontal tissues [5].

Among the red complex pathogens, *T. forsythia* is reported as one of the pathogens strongly associated with aggressive periodontal diseases [6]. *T. forsythia*, in association with various other primary and secondary colonisers such as *Streptococcus* spp., *Actinomyces odontolyticus*, *Veillonella parvula*, *Campylobacter spp*., *Fusobacterium spp*., and *Prevotella spp*., progresses through different stages of periodontal disease [7]. Studies have shown a high prevalence (88.4%) of *T. forsythia*, followed by *P. gingivalis* (76.9%) and *T. denticola* (73.6%) from the subgingival plaque samples taken from periodontitis patients [8]. The progression of the disease is associated with various virulence factors with vital functions produced by *T. forsythia*, such as α-sialidase, trypsin-like proteases, surface layer protein A, methylglycosylsynthase and the proteins encoded by *mutS*, *glyA* and *bspA* [9].

Among these virulence factors, the surface and secreted *bspA* (Bacteroides surface protein A) encodes a cell surface-associated protein with an apparent molecular mass of 98 kDa, which is considered highly antigenic. Similarly, the *mutS* gene and its encoded protein in *T. forsythia* are involved in DNA mismatch repair in the bacterium's response to oxidative stress. This is evident because a mutant strain of *T. forsythia* is less vulnerable to oxidative damage than the wild-type strain, indicating that

mutS is essential for *T. forsythia's* capacity to survive in the host environment [10]. The *mutS* gene in *T. forsythia* is also responsible for its pathogenic ability to maintain genomic stability and further adapt to environmental stress.

The rationale for this research was based on the need for a deeper understanding of the molecular mechanisms by which *T. forsythia* contributes to periodontal disease, as current literature does not fully elucidate the specific roles of these genes in the bacterium's virulence and resistance. The novelty of the study lies in its exploration of how *bspA*, which encodes a surface-associated protein and *mutS*, involved in DNA mismatch repair and oxidative stress response, contribute to *T. forsythia's* pathogenicity and survival in the host environment. This study enhances the existing body of knowledge by offering new insights into the genetic factors that govern *T. forsythia's* resistance to environmental stressors, genomic stability and antibiotic resistance. The hypothesis of this study posits that the expression of *bspA* and *mutS* plays a pivotal role in the bacterium's pathogenic potential and its resistance to antimicrobial agents, which could lead to novel therapeutic strategies for managing periodontal diseases.

The present study aimed to assess the prevalence of *T. forsythia* in patients with aggressive periodontitis and to evaluate its antibiogram profile. The primary objective was to determine the frequency of *T. forsythia* in different periodontal conditions, specifically aggressive periodontitis and its resistance to common antibiotics. The secondary objective was to genetically characterise the *T. forsythia* isolates for the presence of the *bspA* and *mutS* genes using PCR, to investigate their potential association with periodontal disease pathogenesis. This study will provide insights into the molecular characteristics and antimicrobial resistance patterns of *T. forsythia*, offering valuable information for the management of periodontal diseases.

MATERIALS AND METHODS

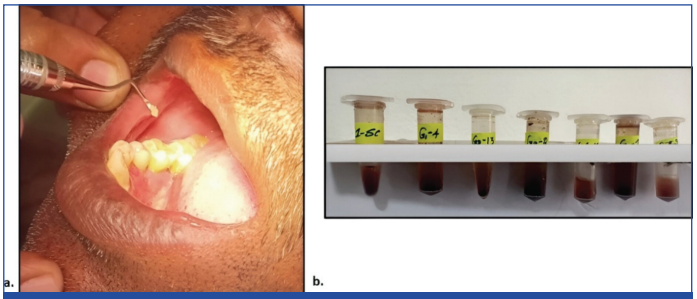
This cross-sectional study was conducted at the Department of Periodontology, Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India, from January 2023 to May 2023. Ethical approval for the study was obtained from the Institutional Ethics Committee (IEC) (IHEC/SDC/Faculty/23/Micro/002 dated 3.01.2023), and informed consent was obtained from all participants before their inclusion. As a time-bound study, 45 patients were enrolled to provide preliminary data.

Inclusion criteria: Patients aged 18 to 60 years, diagnosed with any stage of periodontitis, were included in the study.

Exclusion criteria: Individuals who had received antibiotic treatment in the last three months, those with systemic illnesses, pregnant women, smokers and individuals undergoing orthodontic treatment were excluded from the study. A total of 45 patients were enrolled and classified into three groups based on clinical examination: Group I (Gingivitis), Group II (Periodontitis Stage II), and Group III (Aggressive Periodontitis). Periodontal assessments were performed by a clinical periodontist and subgingival plaque samples were collected aseptically for microbiological analysis.

Microbial Processing of the Plaque Samples

Isolation of *T. forsythia*: Plaque samples [Table/Fig-1] were plated onto a sterile anaerobic blood agar base supplemented with 5% sheep blood, 5% hemin and vitamin K. The plates were placed in an anaerobic jar with a gas pack system and incubated at 37°C for 48-72 hours. After incubation, colonies with grey coloured colonies, characteristic of *T. forsythia*, were identified and further analysed. Gram staining was performed to confirm the gram-negative morphology of the isolates. The grey coloured colonies obtained from the anaerobic blood agar plates were subjected to a series of phenotypic tests to ensure comprehensive characterisation and accurate identification of *T. forsythia*. These tests included motility assessments using semisolid agar to observe bacterial movement, sugar fermentation assays to evaluate carbohydrate metabolism and urease activity tests to detect the hydrolysis of urea into ammonia and carbon dioxide. Haemolysis patterns were assessed on blood agar plates to determine the ability of the isolates to lyse blood cells, while the Voges-Proskauer (VP) test was performed to evaluate acetoin production. Catalase testing was conducted to detect the presence of catalase enzyme activity by observing bubble formation upon exposure to hydrogen peroxide. Collectively, these phenotypic evaluations provided a detailed and confirmatory profile of the isolates, ensuring their accurate identification as *T. forsythia*.



[Table/Fig-1]: a) Collection of plaque specimen from periodontal patients with different stages of periodontitis; and b) Plaque samples in eppendorf tubes with anaerobic blood broth.

Molecular Characterisation of *T. forsythia*

Genomic DNA isolation: Fresh broth suspensions of *T. forsythia* were prepared and genomic DNA was extracted following the manufacturer's instructions provided in the Helini Pure Fast Bacterial DNA Mini Spin Prep Kit (Helini Biomolecules, Chennai, India) [11]. The DNA was stored at -20°C for future analysis after a final centrifugation at 13,000 rpm for one minute. In-vitro amplification for the confirmation of *T. forsythia* was performed using specifically designed primers (F: GGGTGAGTAACGCGTATGTAACCT; R: GCC CATCCGCAACCAATAAA) and PCR. The reaction setup for a 20 µL master mix included the following conditions: initial denaturation at 94°C for five minutes, followed by 36 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 20 seconds, with a final extension at 72°C for 20 seconds [12].

Antibiotic susceptibility test using E-strip method: Fresh broth suspensions of confirmed *T. forsythia* strains were prepared and inoculated as lawn cultures on an anaerobic blood agar base supplemented with 5% sheep blood, 5% hemin and 5% vitamin K. Antibiotic susceptibility profiling was performed using the disk diffusion method, following the Clinical and Laboratory Standards Institute (CLSI, 2022) guidelines. The antibiotics tested included tetracycline (Ezy 0.016-256 mcg/mL, Himedia EM056-10ST), imipenem (Ezy 0.002-32 mcg/mL, Himedia EM104-10ST), cefoperazone (Ezy 0.016-256 mcg/mL, Himedia EM112-10ST), chloramphenicol (Ezy 0.016-256 mcg/mL, Himedia EM016-10ST), clindamycin (Ezy 0.016-256 mcg/mL, Himedia EM019-10ST), moxifloxacin (Ezy 0.002-32 mcg/mL, Himedia EM033-10ST), and metronidazole (Ezy 0.016-256 mcg/mL, Himedia EM128-10ST). The antibiotic strips were placed on the agar surface using sterile forceps, ensuring the 'E-end' faced the edge of the plate with the scale visible. The plates were incubated anaerobically in a gas pack system at 37°C for 5-7 days. After incubation, the Minimum Inhibitory Concentration (MIC) zones of clearance were measured, and the resistance patterns were recorded in accordance with CLSI standards [13].

Molecular detection of *bspA* and *mutS* gene in *T. forsythia* As described earlier, PCR was performed to detect the frequency of the *bspA* and *mutS* genetic determinants from the clinical strains of *T. forsythia* using specific primers. The primers include F: ATGGCTTTACCGTCAACTGG and R: GGTTTGTCCGTAAGCGTAG (*bspA*), as well as F: TACAGCTGAAAACGGCATTG and R: GTGGGTCGTTGCAAATTTCT (*mutS*). The specificity of the primers against *T. forsythia* was verified using the Basic Local Alignment Search Tool (BLAST). An in-silico PCR approach was also employed

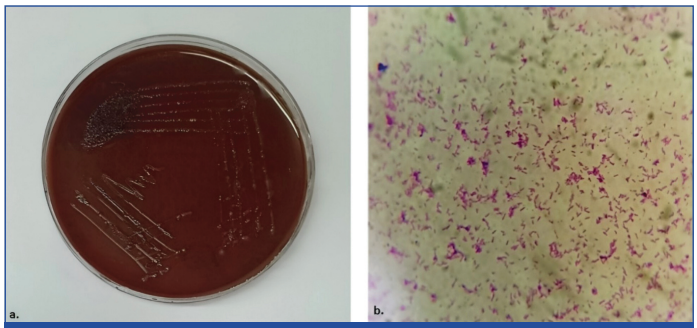
to further confirm the primer selection for *T. forsythia*. The forward and reverse primer sequences were inputted and queried against *Tannerella* species available in the database. The output indicated amplification for strains of *T. forsythia* with an amplicon size of 127 bp. Once the primer selection was completed, 20 µL of the PCR master mix was prepared and run for nearly 36 cycles, with annealing at 56° for 30 seconds for *bspA* and at 64° for 30 seconds for *mutS*, as described earlier [14].

STATISTICAL ANALYSIS

The Kruskal-Wallis test (at 95% CI; 0.05) was performed to assess the plaque index, gingival index, probing depth and calculus among the study population in the three groups and the p-value was found to be significant. Similarly, ANOVA was performed to compare the prevalence of the clinical strains and the frequency of the genes among the study groups.

RESULTS

Among the 15 samples analysed from Group I, *T. forsythia* was not detected in any sample. In Group II, *T. forsythia* was detected in one of the 15 samples analysed (6.7%), identified as grey coloured colonies on CDC anaerobic blood agar under anaerobic conditions. In Group III, *T. forsythia* was detected in four out of the 15 samples (26.7%), with colonies identified in grey colour on CDC anaerobic blood agar [Table/Fig-2a]. Gram staining of positive samples from Group II and III revealed gram-negative, rod-shaped bacteria consistent with the morphology of *T. forsythia* [Table/Fig-2b].



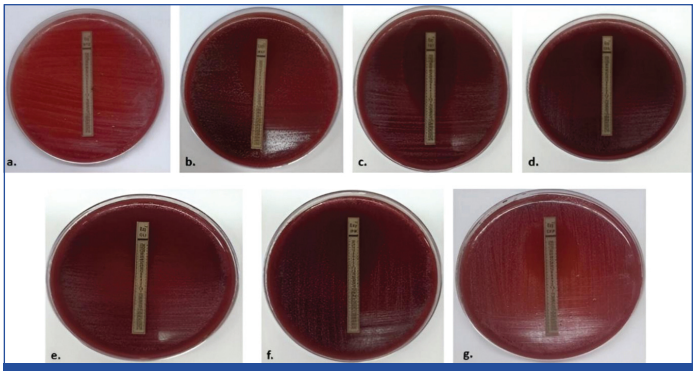
[Table/Fig-2]: a) Grey coloured colonies of *T. forsythia* on anaerobic blood agar; and; b) Gram staining showing the typical gram-negative pleomorphic bacilli under 100× magnification.

Molecular confirmation was performed for positive colonies using PCR with species-specific primers that target the 16S rRNA gene. DNA extraction and amplification produced the expected amplicon size, thereby confirming the identification. The prevalence of *T. forsythia* was predominantly observed in individuals aged 30 to 60 years [Table/Fig-3].

Groups (N=45)	*T. forsythia*	Resistance	*bspA*	*mutS*	Statistics
I: Gingivitis (n=15)	-	-	-	-	Kruskal wallis test (p-value <0.045; 95% CI (0.02, 0.08)
II: Periodontitis (Stage 2) (n=15)	1 (6.7%)	1 (100%)*	-	-	
III: Periodontitis Stage 3/(Aggressive periodontitis) (n=15)	4 (26.7%)	4 (100%)*	3 (75%)	2 (50%)	

[Table/Fig-3]: Distribution of the resistant and virulent strains of *T. forsythia* among periodontal patients with three different stages of the disease.
*Resistance to metronidazole and clindamycin

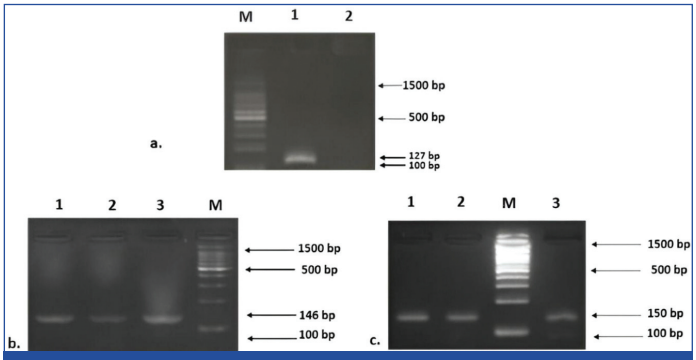
Determination of antibiotic-resistant pattern in *T. forsythia*: All five isolates of *T. forsythia* (one from Group II and four from Group III) were subjected to Antimicrobial Susceptibility Testing (AST) using the E-strip method. The antibiotics tested included tetracycline, imipenem, cefoperazone, chloramphenicol, clindamycin, moxifloxacin, and metronidazole. The E-strips, placed on anaerobic blood agar inoculated with *T. forsythia* suspensions, displayed elliptical zones of inhibition, indicating MICs. The results revealed that all five isolates were resistant to metronidazole and clindamycin, while susceptibility was observed to cefoperazone, tetracycline, imipenem, chloramphenicol and moxifloxacin. These findings emphasise the antibiotic resistance profile of *T. forsythia*, particularly in individuals with aggressive periodontitis [Table/Fig-4].



[Table/Fig-4]: Antibiogram profiling using the E-strip method. Antibiotic susceptibility results for *T. forsythia* isolates are shown as follows: Metronidazole (>256 µg/mL; Resistant), Moxifloxacin (0.064 µg/mL; Sensitive), Tetracycline (0.125 µg/mL; Sensitive), Chloramphenicol 0.5 µg/mL; Sensitive), Clindamycin (>256 µg/mL; Resistant), Imipenem 0.032 µg/mL; Sensitive), and Cefoperazone (0.25 µg/mL; Sensitive).

Genomic DNA isolation: *T. forsythia* primers selected were specific by in-silico PCR and BLAST analysis. Confirmation of the strains was done by PCR with an amplicon size of 127 bp [Table/Fig-5a].

Molecular detection of *bspA* and *mutS* gene in *T. forsythia*: *T. forsythia* was checked for the presence of the *bspA* and *mutS* genes by PCR. The amplicon sizes were observed using specific primers, with an amplicon size of 146 bp for the *bspA* gene and 150 bp for the *mutS* gene, respectively. Among the four strains in Group III, three strains (75%) showed the presence of the *bspA* gene, and two strains (50%) showed the presence of the *mutS* gene. The single isolate from Group-II did not show the presence of either gene [Table/Fig-5b,c].



[Table/Fig-5]: Agarose gel electrophoretogram showing: (a) genotypic confirmation of *T. forsythia* with an amplicon size of 127 bp; (b) amplification of the *bspA* gene with an amplicon size of 146 bp; and (c) amplification of the *mutS* gene with an amplicon size of 150 bp. Lane M=100 bp DNA marker. Panel (c) shows distinct bands at 150 bp in Lanes 1 and 2. Lane 3 shows the positive amplicon from the control strain of *T. forsythia* (ATCC 43037) confirming the presence of the *mutS* gene in the test samples.

DISCUSSION

Periodontitis is a condition that initially affects the gingival tissue and may rapidly spread to deeper tissues, affecting the bone's normal homeostasis and ultimately leading to tooth loss [15]. Periodontitis is considered a polymicrobial conglomerate of aetiologies resulting in an inflammatory response that progress to various stages of the disease [16]. Among the many microbes, *T. forsythia* is one of the prominent periodontal pathogens, ranging from causing mild gingivitis to aggressive periodontitis. *T. forsythia* can be cultivated with significant difficulty in anaerobic culture medium and confirmation always relies on molecular methods.

In the present study, an attempt was made to evaluate the prevalence rate of *T. forsythia* in patients with different grades of periodontitis. The strains were specifically cultured in standard anaerobic culture medium and all strains were confirmed by PCR.

Among the three groups studied, *T. forsythia* exhibited a prevalence rate of 26.7% in aggressive periodontitis cases (Group III) and 6.7% in Stage II periodontitis cases (Group II). Previous studies have reported a wide range of *T. forsythia* prevalence, ranging from 49% to 87.5% in patients diagnosed with aggressive periodontitis. These findings are consistent with earlier research highlighting a higher prevalence of *T. forsythia* in aggressive periodontitis compared to other stages of periodontal disease [17,18].

The prevalence rate may vary based on the demographics, geography, associated risk factors, sampling method and sample size. Significant prevalence of *T. forsythia* in patients with periodontitis, in comparison with healthy controls, has also shown a higher prevalence in the periodontitis group [19]. This evidence substantiates that *T. forsythia* may be an important pathogen in the establishment and progression of periodontitis. The identification of the pathogen by molecular methods, such as PCR, reveals greater sensitivity and the present investigation confirmed 26.7% of the isolates. Earlier studies have also confirmed *T. forsythia* using PCR methods with specific primers, suggesting that molecular methods are the gold standard for confirming anaerobic pathogens [20].

Virulence factors produced by *T. forsythia* play a major role in the progression and intermolecular interactions in periodontal tissues. In this note, the *bspA* protein is known for its proinflammatory mechanisms, as evidenced by an increase in the production of chemokines in preosteoblastic murine cells, which causes the release of bone-resorbing proinflammatory cytokines from monocytic cells through the TLR-2-dependent pathway. This mechanism influences the recruitment of neutrophils, inflammation and tissue deterioration in the tissues [21]. The leucine-rich repeat protein *bspA* of *T. forsythia* is described as both a cell-surface-associated and secreted protein and it has been proposed to play key roles in both bacterial adhesion and inflammation, resulting in bone resorption. This is experimentally proven by comparing the mutant strain to the wild-type strain, where the *bspA* mutant significantly reduced alveolar bone loss [22]. Based on these studies, present study aimed to detect the frequency of the *bspA* gene among the prevailing strains of *T. forsythia* in our dental settings. In earlier studies, the incidence of *bspA* among clinical strains ranged from 20-71% [23], and in present study, it was detected in up to 75%, correlating with the earlier studies.

In the same context, *mutS* is yet another virulence factor contributing to biofilm formation and further inflammatory conditions in the periodontal tissues. To maintain bacterial homeostasis in the periodontal tissues, the mismatch repair mechanism helps sustain bacterial survival. *MutS* is known to act on the DNA mismatch repair mechanism, aiding in the progression of periodontitis [24,25]. Thus, in the present study, authors also selected the *mutS* gene to detect its frequency among the *T. forsythia* clinical strains. Previous research has documented the prevalence of the *bspA* and *prtH* genotype genes as 88.40% and 53.50%, respectively, in subgingival plaque samples from aggressive periodontitis patients, highlighting their potential role in the pathogenesis of periodontitis [17]. Similarly, present study reports a *mutS* gene frequency of 50% among aggressive periodontitis patients, further supporting its potential association with the disease.

Although *T. forsythia* is highly associated with periodontitis, there have not been many studies related to the antimicrobial susceptibility patterns of the strains in clinics. Thus, in the present study, authors made an attempt to evaluate the antibiogram susceptibility profile using the E-strip method. *T. forsythia* strains that were positive for both the *bspA* and *mutS* genes were resistant to metronidazole and clindamycin. This observation suggests a major threat in all dental healthcare settings due to the emergence of clinical isolates of *T. forsythia* exhibiting both virulent and resistant traits. In an earlier study, a similar observation regarding metronidazole resistance was recorded among the *T. forsythia* strains, which were susceptible to moxifloxacin [26]. In another study, a similar observation was made regarding resistance to tetracycline [27]. In contrast, a study from Colombia recorded a lower resistance pattern among the *T. forsythia* strains prevalent among patients with periodontitis [28].

Limitation(s)

This study has several limitations that warrant consideration. The relatively small sample size, inherent to its pilot design, may affect the generalisability of the findings. Additionally, antibiotic susceptibility testing was performed using the E-strip method rather than the CLSI-recommended microbroth dilution technique, which may have introduced variability in the determination of MIC values. The cross-sectional design of the study limits the ability to infer causality between the presence of *T. forsythia* virulence genes and disease progression. Moreover, the study focused exclusively on detecting the *bspA* and *mutS* genes, leaving other potential virulence factors or resistance mechanisms unexamined. Future research with larger cohorts, longitudinal designs and a broader investigation of virulence determinants is essential to validate these findings and further elucidate the pathogenic and antimicrobial resistance profiles of *T. forsythia*.

CONCLUSION(S)

This study demonstrates a notable prevalence of *T. forsythia* in patients with aggressive periodontitis, with isolates effectively characterised through both culture-based methods and molecular confirmation via PCR. The identification of virulence-associated genes, *bspA* and *mutS*, in clinical strains highlights their relevance as genetic markers in the pathogenesis of periodontitis. The observed resistance to metronidazole underscores the need for alternative therapeutic options, with moxifloxacin emerging as a promising choice for effective treatment. These findings emphasise the importance of regular surveillance and antimicrobial resistance monitoring to mitigate the emergence and spread of resistant and virulent *T. forsythia* strains, ultimately enhancing strategies for periodontal healthcare.

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