

Article

Phenotypic Biofilm-Forming Capacity and Antimicrobial Resistance Profiles of Culturable Subgingival Bacteria in Various Stages and Grades of Periodontitis in Iraqi Adults

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Citation: About E. A. Phenotypic Biofilm-Forming Capacity and Antimicrobial Resistance Profiles of Culturable Subgingival Bacteria in Various Stages and Grades of Periodontitis in Iraqi Adults. American Journal of Biology and Natural Sciences 2026, 3(10), 21-35.

Received: 15th Aug 2026

Revised: 17th Aug 2026

Accepted: 21th Sep 2026

Published: 03th Oct 2026


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Abstract: Background: There is a lack of phenotype-based data associated with the 2017 stage-grade framework for periodontal disease in Iraq, and subgingival biofilms maintain the periodontal dysbiosis and may include clinically relevant antimicrobial non-susceptibility bacteria. Objective: To evaluate if the ability to form biofilm and antimicrobial resistance profiles of culturable subgingival bacterial isolates vary with periodontitis stage and grade and if there is an independent association between strong biofilm-forming ability and antimicrobial non-susceptibility. Design: This is a multicenter analytical cross-sectional study comprised of 200 adults with Stage I-IV periodontitis. Plaque from four subgingival deepest sites, which were not adjacent, was collected. Isolates were grown aerobically, capnophilically, and anaerobically and phenotype with automated and/or mass-spectrometric confirmation, when available. Biomass levels of biofilms were measured in 96 well plates by crystal violet and the OD-c cutoff method. Broth microdilution methods were used for aerobic/facultative isolates and agar dilution methods for anaerobes were used to determine minimum inhibitory concentrations. Population-averaged logistic models with patient clustering and clinical and demographic covariates. Results: 645 isolates were recovered. Biofilm formation was found to be strong in 45.9% of the isolates (296) and it increased with an increase in periodontitis stage and grade. The highest amount of biofilm biomass was found with *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Staphylococcus aureus*, enterococci, and opportunistic Gram-negative isolates. Testing of the individual isolates of the biofilms showed a higher number of antimicrobial non-susceptible phenotypes on the tested panel and a higher number of multidrug-resistant phenotype/multiple-antimicrobial non-susceptibility phenotype in strong biofilms. Strong biofilm formation (aOR 2.10, 95% CI 1.04–4.25) was associated with stage III–IV disease after adjustment, and strong biofilm formation was an independent predictor of the composite resistance outcome (aOR 5.06, 95% CI 3.06–8.39). Conclusions: Stronger recovery of subgingival bacteria with higher ability to form biofilms, and less susceptible to the culture conditions, was correlated with advanced and rapidly progressed periodontitis. Species-resolved MIC surveillance could be useful in antimicrobial stewardship when there is a clinical need for adjunctive antibiotic use.

Keywords: antimicrobial resistance; biofilm; minimum inhibitory concentration; periodontitis; stage and grade; subgingival bacteria.

Introduction

Periodontitis is a multifactorial chronic inflammatory condition associated with dysbiotic dental plaque biofilm and continues to be a progressive loss of the tooth supporting apparatus. It is still an important cause of tooth loss and makes up a significant proportion of the burden of oral diseases globally[1][2]. The World Workshop classification 2017 added staging to indicate the severity and management complexity and grading to indicate the rate of progression and risk modifiers such as smoking and diabetes[3][4]. It is important to apply this framework to microbiological investigations for reasons that organisms recovered from a deep pocket in advanced disease may differ in composition and phenotype from those recovered in earlier disease. Subgingival plaque is a multi-species biofilm and not simply a bunch of planktonic organisms. The dysbiotic communities are affected by ecological succession, interspecies coaggregation, nutrient exchange, and host-derived inflammation, leading to the enrichment of anaerobic, capnophilic, and other bacteria such as *P. gingivalis*, *Prevotella* spp., *F. nucleatum*, and *P. micra*[5][6][7]. The persistence under environmental and antimicrobial stress is boosted by the extracellular matrix, spatial gradients, and altered physiology of the cells associated with a biofilm[8][9][10]. The crystal-violet microtiter assay is useful as a phenotypic measurement of the ability to form a biofilm when cutoffs are standardized and repeated measurements are taken, but it is not a direct measure of the viability of the cells[11]. Differences in oral biofilm formation have been reported among species, such as *Prevotella* species[12]. Oral cavity also serves as a reservoir of PheARD. Culture-based, genomic, and metagenomic studies have revealed significant interspecies and interclass differences as well as geographic and previous antimicrobial use differences [13][14][15][16]. Studies on species level have identified heterogeneous susceptibility among the *Prevotella*, and subgingival staphylococci[17][18]. Notably, amoxicillin–clavulanate and metronidazole continue to have activity against many periodontal anaerobes, while surveillance studies have shown the susceptibility to clindamycin, macrolides, and tetracyclines to be highly variable in some populations, and in some populations, amoxicillin is similarly variable[19][20][21]. Also, a recent synthesis revealed the presence of tetracycline, macrolide–lincosamide, and β -lactam resistance determinants in the subgingival microbiome and stressed significant methodological heterogeneity[22]. Patterns should not be indiscriminately combined because the resistance, drug panels and interpretive criteria vary for each taxon. MIC distributions, MIC50 and MIC90 are thus important in cases of lack of validated clinical breakpoints. In the absence of current guidelines, the use of systemic antibiotics routinely for all patients is not recommended; however, their use may be considered in selected cases of severe or rapidly progressing presentations, in combination with mechanical disruption of the biofilm[23]. Observational MIC data can thus be used to guide stewardship in a region-specific, species-specific manner, while avoiding the translation of MICs into treatment recommendations. There are still limited evidence linking periodontitis stage and grade with phenotypic biomass of the biofilm and antimicrobial non-susceptibility particularly in the Iraqi population. In this study, culturable subgingival bacteria were recovered from Iraqi periodontitis adult population and quantitative subgingival biofilm formation capacity at the isolate level, species-appropriate MIC profile and the association between quantitative subgingival biofilm formation capacity and antimicrobial non-susceptibility and disease stage and grade were evaluated.

Materials and Methods

The setting and design of the study

The present multicenter analytical cross-sectional study was carried out at three specialist periodontal services: College of Dentistry/University of Babylon, governmental specialist dental center and a private specialist periodontal clinic in Babylon Governorate, Iraq. The recruitment and laboratory

work were carried out consecutively from 1st October 2025 to 30th of June 2026. The manuscript was written in line with STROBE and STROBE-AMS reporting guidelines[24][25].

Sample size

The main comparison was the percentage of patients with at least one strong biofilm producer in early (Stage 1-2) and advanced (Stage 3-4) periodontitis. With the assumed proportions of 25% and 45%, respectively, and two-sided $\alpha = .05$, power = .80, and approximately equal group sizes, the minimum needed were 176 participants. Acknowledging the presence of non-evaluable cultures, and incomplete data, raised the recruitment target to 220. 200 participants with complete clinical, culture and biofilm data were analyzed. Data were analyzed in G*Power 3.1.

Participants

Adults (aged 18–70 years) were eligible if they had enough teeth for full mouth examination and subgingival sampling, had been diagnosed with periodontitis according to the 2017 classification, had an interpretable radiograph, had not sought periodontal treatment in the previous three months, and were able to provide written informed consent. Systemic antibacterial therapy within 3 months prior to the visit, antimicrobial mouthwash within 4 weeks of the visit, pregnancy or lactation, chemotherapy or clinically important immunosuppression, severe hematological or immune disease, acute periodontal abscess at the time of sampling, long-term systemic corticosteroid therapy, need for antibiotic prophylaxis prior to dental examination, or inability to complete the examination or obtain a valid sample were all excluded. Smoking and diabetes were kept as clinically important grade modifiers and possible confounders and cigarettes per day, diabetes duration, diabetes therapy, and HbA1c if available, were recorded.

Examination, staging and grading of periodontal disease

All measurements were made by a calibrated periodontist using a standardized periodontal probe at six sites on each tooth throughout the entire mouth. Measures taken were probing pocket depth (PPD), clinical attachment loss (CAL), bleeding on probing (BOP), plaque positive sites, gingival recession, mobility, furcation involvement, teeth lost due to periodontitis, radiographic bone-loss pattern and complexity factors. Stage I – IV and grade A – C were determined by CAL, radiographic bone loss, tooth loss, complexity, direct or indirect progression evidence, smoking, glycemic control and not only by PPD. The examiner re-measured 20 non-study patients prior to recruitment and intra-examiner intraclass correlations were 0.87 for PPD and 0.89 for CAL. Re-examination was performed on 10% of the participants during the study, and both coefficients were >0.80.

Subgingival sampling

Samples were taken prior to periodontal therapy, and prior to probing the selected sites. The four sites that were deepest were isolated with cotton rolls, supragingival plaque gently removed without going into the pocket and air dried. A sterile paper point was placed in the pocket base for 30 seconds each time at each site. The four points were placed in reduced transport fluid, reducing conditions during transport and processed within 02 hours. Each pocket sampled was recorded for PPD, CAL, BOP, tooth number and location.

Culture and bacterial ID

The specimens were vortexed, serially diluted and spread on enriched non-selective and selective media for oral anaerobic, capnophilic and aerobic/facultative bacteria. Anaerobic plates were placed in an anaerobic atmosphere and cultured for 5 to 7 days, capnophilic plates were cultured in 5 to 10 percent CO₂, and the aerobic/facultative plates were examined after 24 to 48 hours. Different morphotypes of colonies were subcultured to purity. Identification relied upon MALDI-TOF MS if available, automatic identification for taxa included in the database, and conventional Gram-specific stain, aerotolerance, catalase, oxidase, indole, nitrate, carbohydrate-utilization, and enzyme tests were used as needed. Calls that were not made by the automated callers within the manufacturer's confidence level or outside of the manufacturer's supported database were not considered to be

definitive species identification. The protocol used was culture based, so the results reported are culturable subgingival bacteria and not the entire microbiome.

Biofilm biomass (phenotypic) assay

Biofilm-forming capacity was assessed using an adapted crystal-violet microtiter assay that took into account the growth requirements of each isolate. Standardized suspensions were triplicated and incubated under species appropriate atmospheric conditions, washed to remove non-adherent cells, fixed, stained with 0.1% crystal violet, and destained with 33% acetic acid. Absorbance was measured at 595 nm. Two independent trials were performed with each isolate. The cut off optical density (OD_c) was calculated as the mean of the negative controls plus 3 standard deviations (0.064 in this study). Categories were non-producer (OD ≤ OD_c), weak (OD_c < OD ≤ 2×OD_c), moderate (2×OD_c < OD ≤ 4×OD_c), and strong (OD > 4×OD_c). Crystal violet was used to measure total adherent biomass, but was not considered to be a viable-cell count.

Antimicrobial susceptibility testing

MIC testing was performed by broth microdilution for aerobic and facultative and the reference agar dilution for obligate anaerobes. Aerobic organisms were tested according to Clinical and Laboratory Standards Institute (CLSI) guidelines (M07/M100) and anaerobes were tested according to CLSI guidelines (M11) (Clinical and Laboratory Standards Institute [CLSI][26][27]. The panel of periodontal anaerobes included amoxicillin, amoxicillin-clavulanate, metronidazole, clindamycin, doxycycline, azithromycin, and moxifloxacin. Facultative and opportunistic organisms were grouped into organism appropriate panels; no organism was grouped against an intrinsically inactive agent. Susceptible, intermediate/susceptible increased exposure, and resistant interpretations were only made when there was a current, taxon-appropriate criterion. In other cases, MIC range, MIC50 and MIC90 were not provided with a clinical resistance label.

Resistance definitions

Multidrug resistance (MDR) was defined for taxa for which sufficient drug category coverage and interpretive criteria were available, as acquired non-susceptibility to ≥3 antimicrobial categories, which include at least one agent in each category[28]. The neutral term "multiple antimicrobial non-susceptibility" (MANS) was employed to describe the analogous observed phenotype for periodontal anaerobes for which there are no well-established category-specific breakpoints. Only the combined MANS/MDR was used for association modelling and did not represent that the same breakpoints were used across species.

Laboratory quality control

The CLSI recommended reference strains for each organism and method were included in each MIC testing run. Biofilm plates consisted of a positive biofilm control, a non-biofilm control, negative control media wells and blank wells. A 10% random sample of the isolates was identified, tested for biofilm production, and tested for the MICs once again by the second investigator. Lab personnel was masked for periodontitis stage and grade. Media and reagent lot number, anaerobic environment, incubator temperature, plate-reader calibration and quality-control limits were recorded, and runs that failed quality-control limits were repeated.

Statistical analysis

The analyses were performed at both patient and isolate level. Continuous variables were reported as mean ± standard deviation or median (interquartile range) whereas categorical variables were reported as number (%). χ^2 or Fisher's exact tests were used for comparisons between stage and grade for categorical variables and Kruskal-Wallis tests were used for continuous variables. Where appropriate, linear trends were evaluated in ordered stage and grade categories. Spearman coefficients assessed the relationship between biofilm OD and PPD, CAL and the number of non-susceptible agents. Species-antibiotic comparisons were limited to the applicable tests and across antibiotic-specific comparisons, the false-discovery rate was controlled for using Benjamini-Hochberg correction. Population-averaged logistic models were used, accounting for multiple isolates from the same

participant by using patient-clustered robust standard errors (generalized estimating equations [GEE] assuming working independence). Model 1 compared strong biofilm formation, taking into account stage, grade, species group, age, sex, smoking, diabetes, plaque level and previous year antibiotic exposure. In model 2, MANS/MDR was evaluated with the categorical variables of biofilm and the same clinically relevant variables. Adjusted odds ratios (aORs), 95% confidence intervals (CIs), and two-sided p values are presented. Variance inflation factors (VIFs) were used to screen for collinearity, and area under the receiver-operating-characteristic curve (AUC) and calibration error, summarized by the Brier score. A p value of less than .05 was considered to be statistically significant.

Ethical considerations

The protocol was carried out according to the Declaration of Helsinki and approved by the Scientific Research and Ethics Committee of the College of Dentistry/ University of Babylon and the approval of the concerned authority of the Babylon Health Directorate. Written informed consent was obtained from all the participants prior to examination and sampling.

Results

The analytical sample consisted of 200 adults (44, 50, 58 and 48 subjects with Stage I, II, III and IV periodontitis, respectively). There was a difference between the percentage of grade distribution (25 Grade A, 105 Grade B, and 70 Grade C) with less favorable clinical parameters with increase in the stage of the disease (Table 1).

Table 1. Demographic and clinical characteristics of the participants according to the periodontal stage.

Characteristic	Stage I (n = 44)	Stage II (n = 50)	Stage III (n = 58)	Stage IV (n = 48)	P
Age, years	35.2 ± 8.0	41.4 ± 8.9	51.5 ± 7.2	55.3 ± 7.4	< .001
Male sex	19 (43.2)	27 (54.0)	32 (55.2)	28 (58.3)	.497
Current smoking	5 (11.4)	9 (18.0)	15 (25.9)	19 (39.6)	.010
Diabetes mellitus	3 (6.8)	0 (0.0)	13 (22.4)	10 (20.8)	.001
BMI, kg/m ²	24.9 ± 3.0	25.0 ± 2.9	26.2 ± 2.9	26.6 ± 3.4	.012
Antibiotic exposure in previous year	9 (20.5)	10 (20.0)	18 (31.0)	30 (62.5)	< .001
Plaque-positive sites, %	32.9 ± 7.3	44.2 ± 8.2	59.2 ± 8.9	68.4 ± 8.9	< .001
Bleeding on probing, %	28.2 ± 10.1	40.5 ± 7.5	53.1 ± 8.2	64.7 ± 8.0	< .001
Mean probing pocket depth, mm	3.8 ± 0.5	4.8 ± 0.5	6.3 ± 0.4	7.3 ± 0.5	< .001
Mean clinical attachment loss, mm	1.9 ± 0.6	3.5 ± 0.6	5.8 ± 0.5	7.3 ± 0.6	< .001
Natural teeth, n	27.2 ± 1.7	25.9 ± 1.9	23.9 ± 2.2	20.5 ± 2.0	< .001

Note. Data shown are mean ± SD or n. Kruskal–Wallis or Pearson χ^2 test was used to obtain the total p values. BMI is a person's weight to height ratio.

Sixty-four (645) were recovered (median 3.0, 2.0–4.0 species per participant). The most common groups of organisms recovered were oral streptococci, *Prevotella intermedia/nigrescens*, *F. nucleatum*, *P. gingivalis* and *P. micra*. The prevalence of *P. gingivalis*, *Prevotella* and *F. nucleatum* was found to be increasing according to the stages while *Actinomyces* showed a reverse distribution. Opportunistic staphylococci, enterococci and Gram-negative rods were rarely detected and were primarily seen in the advanced disease (Table 2).

Table 2. Periodontitis is classified into stages and grades, and the distribution of culturable subgingival bacterial species in each stage and grade is shown.

Species/group	Stage I	Stage II	Stage III	Stage IV	P	Grade A	Grade B	Grade C	P
Oral <i>Streptococcus</i> spp.	26 (59.1)	33 (66.0)	36 (62.1)	30 (62.5)	.922	16 (64.0)	63 (60.0)	46 (65.7)	.736
<i>Prevotella intermedia/nigrescens</i>	15 (34.1)	27 (54.0)	29 (50.0)	24 (50.0)	.232	10 (40.0)	46 (43.8)	39 (55.7)	.220
<i>Fusobacterium nucleatum</i>	18 (40.9)	16 (32.0)	23 (39.7)	23 (47.9)	.457	12 (48.0)	31 (29.5)	37 (52.9)	.006
<i>Porphyromonas gingivalis</i>	6 (13.6)	13 (26.0)	30 (51.7)	30 (62.5)	< .001	5 (20.0)	35 (33.3)	39 (55.7)	.001
<i>Parvimonas micra</i>	7 (15.9)	15 (30.0)	21 (36.2)	20 (41.7)	.048	4 (16.0)	36 (34.3)	23 (32.9)	.200
<i>Capnocytophaga</i> spp.	8 (18.2)	15 (30.0)	16 (27.6)	12 (25.0)	.591	3 (12.0)	30 (28.6)	18 (25.7)	.232
<i>Actinomyces</i> spp.	14 (31.8)	12 (24.0)	11 (19.0)	10 (20.8)	.466	5 (20.0)	27 (25.7)	15 (21.4)	.732
<i>Aggregatibacter actinomycetemcomitans</i>	8 (18.2)	10 (20.0)	13 (22.4)	8 (16.7)	.893	5 (20.0)	23 (21.9)	11 (15.7)	.598
<i>Campylobacter rectus</i>	5 (11.4)	8 (16.0)	7 (12.1)	8 (16.7)	.829	5 (20.0)	13 (12.4)	10 (14.3)	.612
<i>Staphylococcus aureus</i>	4 (9.1)	3 (6.0)	7 (12.1)	6 (12.5)	.673	0 (0.0)	12 (11.4)	8 (11.4)	.204
<i>Enterococcus faecalis</i>	2 (4.5)	2 (4.0)	3 (5.2)	3 (6.2)	.963	0 (0.0)	4 (3.8)	6 (8.6)	.173
<i>Enterobacterales/non-fermenters</i>	0 (0.0)	3 (6.0)	4 (6.9)	1 (2.1)	.253	1 (4.0)	5 (4.8)	2 (2.9)	.820

Note. Values are the number of patients with the species/group n (number of patients in the stage or grade). Patients were also able to donate more than one species, p values represent all stages/grades of the species and were calculated by Pearson χ^2 or Fisher's exact test as appropriate.

A high proportion (45.9%) of the 645 isolates (296) produced strong biofilm. The OD595 and categorical biofilm capacity values were significantly different for each species (global Kruskal–Wallis and χ^2 $p < .001$). The highest proportions of strong biofilm producers were found for the opportunistic Gram-negative group, followed by the species *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans*, *S. aureus* and *E. faecalis* (Table 3).

Table 3. According to the species, the phenotypic Biofilm-Forming Capacity of subgingival bacterial isolates is reported.

Species/group	Isolates	OD595, median (IQR)	Non- producer	Weak	Moderate	Strong
Oral <i>Streptococcus</i> spp.	125	0.120 (0.069–0.243)	30 (24.0)	38 (30.4)	31 (24.8)	26 (20.8)
<i>Prevotella intermedia/nigrescens</i>	95	0.242 (0.113–0.409)	8 (8.4)	18 (18.9)	28 (29.5)	41 (43.2)
<i>Fusobacterium nucleatum</i>	80	0.258 (0.179–0.478)	3 (3.8)	8 (10.0)	29 (36.2)	40 (50.0)
<i>Porphyromonas gingivalis</i>	79	0.417 (0.244–0.528)	2 (2.5)	3 (3.8)	16 (20.3)	58 (73.4)
<i>Parvimonas micra</i>	63	0.312 (0.146–0.511)	3 (4.8)	11 (17.5)	13 (20.6)	36 (57.1)
<i>Capnocytophaga</i> spp.	51	0.256 (0.142–0.482)	3 (5.9)	7 (13.7)	16 (31.4)	25 (49.0)
<i>Actinomyces</i> spp.	47	0.149 (0.093–0.252)	7 (14.9)	13 (27.7)	15 (31.9)	12 (25.5)
<i>Aggregatibacter actinomycetemcomitans</i>	39	0.383 (0.165–0.556)	2 (5.1)	3 (7.7)	12 (30.8)	22 (56.4)
<i>Campylobacter rectus</i>	28	0.191 (0.099–0.439)	6 (21.4)	3 (10.7)	9 (32.1)	10 (35.7)
<i>Staphylococcus aureus</i>	20	0.355 (0.225–0.501)	0 (0.0)	0 (0.0)	7 (35.0)	13 (65.0)
<i>Enterococcus faecalis</i>	10	0.267 (0.144–0.482)	0 (0.0)	2 (20.0)	2 (20.0)	6 (60.0)
<i>Enterobacteriales</i> /non-fermenters	8	0.574 (0.395–0.687)	0 (0.0)	0 (0.0)	1 (12.5)	7 (87.5)

Note. Unless noted, results are dried biomass (biofilm). OD_c = 0.064. Both the general between species differences in OD595 and category were $p < .001$.

Biofilm was observed to have a progressive increase in strength from stage to grade. Resultant 48.9% of the Stage I-II patients and 84.9% of the Stage III-IV patients had at least one strong isolate. Isolate OD correlated with PPD (Spearman $\rho = 0.44$, $p < .001$) (Table 4).

Table 4. shows the distribution of categories of biofilm formation by periodontitis stages and grades.

Stage/grade	Isolates	Non-producer	Weak	Moderate	Strong
Stage I	113	28 (24.8)	37 (32.7)	31 (27.4)	17 (15.0)
Stage II	157	24 (15.3)	38 (24.2)	54 (34.4)	41 (26.1)
Stage III	200	10 (5.0)	23 (11.5)	64 (32.0)	103 (51.5)
Stage IV	175	2 (1.1)	8 (4.6)	30 (17.1)	135 (77.1)
Grade A	66	23 (34.8)	14 (21.2)	15 (22.7)	14 (21.2)
Grade B	325	34 (10.5)	71 (21.8)	110 (33.8)	110 (33.8)
Grade C	254	7 (2.8)	21 (8.3)	54 (21.3)	172 (67.7)

Note. Values are at a particular level in each stage / grade (n within each stage / grade %). The total χ^2 p is $< .001$ for stage, and $< .001$ for grade; the ordered trend p is $< .001$ for each.

MICs were different for each species and drug. The *Prevotella*, *P. gingivalis*, oral streptococci, and *S. aureus* strains demonstrated higher numbers of elevated MICs for clindamycin and azithromycin, while amoxicillin–clavulanate had the lowest and most consistent MIC50/MIC90 values. The MICs for metronidazole were low for most obligate anaerobes. Table 5 includes the results for organism–drug combinations for which there are no applicable interpretive criteria, as well as the results for these organism–drug combinations that were not determined as resistant.

Table 5. The antimicrobial susceptibility and the MIC distribution of major subgingival bacterial species were summarized.

Species/group	n	AMX	AMC	MTZ	CLI	DOX	AZM	MOX
<i>Porphyromonas gingivalis</i>	79	0.125/16 [11/79; 13.9%]	0.032/0.064 [2/79; 2.5%]	0.125/0.25 [2/79; 2.5%]	8/32 [48/79; 60.8%]	0.125/16 [29/79; 36.7%]	0.125/16 [30/79; 38.0%]	0.064/8 [11/79; 13.9%]
<i>Prevotella intermedia/nigrescens</i>	95	1/32 [46/95; 48.4%]	0.064/0.125 [5/95; 5.3%]	0.25/32 [11/95; 11.6%]	16/32 [59/95; 62.1%]	0.25/16 [30/95; 31.6%]	0.5/8 [41/95; 43.2%]	0.125/8 [12/95; 12.6%]
<i>Fusobacterium nucleatum</i>	80	0.25/16 [13/80; 16.2%]	0.064/0.25 [5/80; 6.2%]	0.25/32 [8/80; 10.0%]	0.125/16 [21/80; 26.2%]	0.25/16 [20/80; 25.0%]	0.5/8 [23/80; 28.7%]	0.125/8 [8/80; 10.0%]
<i>Parvimonas micra</i>	63	0.25/0.5 [3/63; 4.8%]	0.064/0.125 [3/63; 4.8%]	0.25/64 [10/63; 15.9%]	0.25/16 [26/63; 41.3%]	0.25/16 [13/63; 20.6%]	0.25/8 [12/63; 19.0%]	0.125/4 [7/63; 11.1%]
<i>Aggregatibacter actinomycetemcomitans</i>	39	0.5/16 [10/39; 25.6%]	0.064/8 [5/39; 12.8%]	NA	0.25/32 [14/39; 35.9%]	0.5/32 [13/39; 33.3%]	0.25/8 [6/39; 15.4%]	0.125/16 [4/39; 10.3%]
Oral <i>Streptococcus spp.</i>	125	0.25/16 [32/125; 25.6%]	0.064/0.125 [7/125; 5.6%]	NA	0.25/32 [46/125; 36.8%]	0.25/16 [25/125; 20.0%]	0.5/16 [51/125; 40.8%]	0.125/8 [16/125; 12.8%]
<i>Staphylococcus aureus</i>	20	8/32 [12/20; 60.0%]	0.064/0.125 [0/20; 0.0%]	NA	16/32 [14/20; 70.0%]	0.5/32 [7/20; 35.0%]	4/8 [13/20; 65.0%]	0.125/8 [2/20; 10.0%]

Note. Each drug cell is: MIC50/MIC90, mg/L [n/valid denominator; % not susceptible]. NA means that the organism–drug pair was not interpreted due to the lack of testing interpretation for the organism and/or intrinsic inactivity of the drug. AMX = amoxicillin, AMC = amoxicillin – clavulanate, MTZ = metronidazole, CLI = clindamycin, DOX = doxycycline, AZM = azithromycin, MOX = moxifloxacin. Interpretations were made using species specific CLSI criteria, whenever no clinical breakpoints were available for a species, the summaries of the MICs should be prioritized.

The proportion of non-susceptible isolates was higher among strong biofilm producers for all the antimicrobials assessed, after taking into account the denominators. A total of 126 isolates (19.5%) were composite MANS/MDR phenotype, and this phenotype was found more often in strong biofilm former than non-strong biofilm former isolates. For the number of non-susceptible agents, OD595 correlated with the numbers (Spearman $\rho = 0.35$, $p < .001$) (Table 6).

Table 6. The correlation between the ability to form biofilm and antimicrobial non-susceptibility.

Outcome	Strong biofilm n/N (%)	Non-strong biofilm n/N (%)	Unadjusted (95% CI)	OR	Adjusted p
Amoxicillin	98/296 (33.1)	55/349 (15.8)	2.65 (1.82–3.85)		< .001
Amoxicillin–clavulanate	23/296 (7.8)	10/349 (2.9)	2.86 (1.34–6.10)		.009
Metronidazole	26/185 (14.1)	10/160 (6.2)	2.45 (1.14–5.26)		.021
Clindamycin	169/296 (57.1)	111/349 (31.8)	2.85 (2.07–3.94)		< .001
Doxycycline	113/296 (38.2)	61/349 (17.5)	2.92 (2.03–4.19)		< .001
Azithromycin	139/296 (47.0)	97/349 (27.8)	2.30 (1.66–3.19)		< .001
Moxifloxacin	44/296 (14.9)	31/349 (8.9)	1.79 (1.10–2.92)		.021
MANS/MDR phenotype	104/296 (35.1)	22/349 (6.3)	8.05 (4.92–13.19)		< .001

Note. Denominators only include isolates with an applicable organism–drug interpretation. Fisher's exact test with Benjamini–Hochberg correction are used for the generation of antibiotic-specific p values. The MANS/MDR row is a composite row that spans the entire range of drug categories, and is not a member of the multiplicity family. MANS = multiple antimicrobial non-susceptibility, MDR = multidrug resistance.

The prevalence of all strong biofilm producers, all non-susceptible and all MANS/MDR was greater with each step up in stage and grade. We found the MANS/MDR phenotype in 16.0% of Grade A patients and 62.9% of Grade C patients (Table 7).

Table 7. Patient Level Prevalence of strong Biofilm producers and non-susceptible bacterial isolates

Stage/grade	Patients	Species per patient, median (IQR)	Any strong isolate	Any non- susceptible isolate	Any MANS/MDR isolate
Stage I	44	3.0 (1.8–3.0)	15 (34.1)	39 (88.6)	5 (11.4)
Stage II	50	3.0 (2.0–4.0)	31 (62.0)	47 (94.0)	14 (28.0)
Stage III	58	3.0 (2.0–4.0)	46 (79.3)	56 (96.6)	27 (46.6)
Stage IV	48	4.0 (2.0–5.0)	44 (91.7)	48 (100.0)	35 (72.9)
Grade A	25	3.0 (2.0–4.0)	10 (40.0)	19 (76.0)	4 (16.0)
Grade B	105	3.0 (2.0–4.0)	62 (59.0)	102 (97.1)	33 (31.4)
Grade C	70	4.0 (2.2–5.0)	64 (91.4)	69 (98.6)	44 (62.9)

Note. Data are given as n (%). There were significant overall stage and grade comparisons ($p < .05$) for all three binary outcomes.

In the patient-clustered model, the following were independently associated with the strong biofilm formation: stage III–IV disease, grade C, high-biofilm-associated species, plaque burden, and antibiotic exposure in previous year. Model discrimination was acceptable (AUC = 0.80), the maximum VIF was 3.41 (Table 8) and the Brier score was 0.180.

Table 8. Biofilm Formation was Patient-Clustered Multivariable Logistic Analysis of Factors Associated with Strong Biofilm Formation.

Predictor	Adjusted OR	95% CI	p
Stage III–IV (vs. I–II)	2.10	1.04–4.25	.038
Grade C (vs. A–B)	2.92	1.92–4.45	< .001
High-biofilm-associated species	2.95	2.01–4.34	< .001
Age (per 10 years)	1.23	0.97–1.57	.084
Male sex	1.09	0.74–1.59	.673
Current smoking	0.80	0.50–1.27	.338
Diabetes mellitus	1.16	0.73–1.85	.537
Plaque-positive sites (per 10%)	1.20	0.96–1.49	.106
Antibiotic exposure in previous year	1.54	1.01–2.35	.046

Note. Population-averaged logistic model, with patient-clustered robust standard errors (645 isolates within 200 patients). AUC = 0.80; Brier score = 0.180; maximum VIF = 3.41.

Biofilm formation alone was a significant predictor of MANS/MDR after accounting for stage, grade, species group, age, smoking, diabetes and antibiotic use in the year before the infection. Exposure to antibiotics before the test and identity of the opportunistic bacteria were also associated with the resistance result. Model AUC was 0.80, Brier score was 0.124, and maximum VIF was 2.28 (Table 9).

Table 9. Susceptibility in Children with Pneumonia.

Predictor	Adjusted OR	95% CI	p
Strong biofilm formation	5.06	3.06–8.39	< .001
Stage III–IV (vs. I–II)	1.51	0.79–2.87	.213
Grade C (vs. A–B)	1.28	0.83–1.98	.263
Opportunistic bacterial group	4.29	2.02–9.12	< .001
Age (per 10 years)	1.12	0.84–1.49	.440
Current smoking	1.56	1.00–2.44	.049
Diabetes mellitus	0.86	0.44–1.69	.662
Antibiotic exposure in previous year	1.94	1.24–3.01	.003

Note. Population-averaged logistic model with patient clustered robust standard errors (645 isolates within 200 patients). AUC = 0.80; Brier score = 0.124; maximum VIF = 2.28. The end product was MANS for periodontal anaerobes and MDR for the taxa for which there were a number of validated categories.

Discussion

In this multicenter study the 2017 periodontitis stage–grade system was related to two clinically relevant isolate phenotypes – adherent biofilm biomass and antimicrobial non-susceptibility. There were three main findings. First, stronger biofilm-producing isolates were found as disease was more severe and the disease stage and grade increased. Second, the species distribution changed towards *P. gingivalis*, *Prevotella*, *F. nucleatum* and some opportunistic species in advanced disease, organisms more usual of the early stages of colonization being relatively less common. Third, the ability to create a strong biofilm had an independent association with the composite MANS/MDR outcome, when considering the disease classification, species grouping, patient risk factors and antibiotic exposure.

The gradual increase of recovery of *P. gingivalis*, *Prevotella* and *F. nucleatum* with the progression of disease is biologically plausible in the context of today's considerate of dysbiosis. *F. nucleatum* plays a role in spatial organization and bridging between species, whereas *P. gingivalis* is able to modify the interaction between host and microbes and the behaviour of the microbial community. Additionally, recent studies using pooled metagenomic analysis and geographic comparisons of culture-based subgingival bacterial profiles revealed differences in these profiles based on periodontal condition and population. However, the current culture-based paradigm shouldn't be viewed as a comprehensive microbiome profile as the fastidious and uncultivated taxa are underrepresented. It does however give viable isolates for phenotype testing. The stage-biofilm gradient is an expansion of the classification meaning that goes beyond destruction of tissue. Advanced pockets provide a protected niche that is often anaerobic, has a greater colonizable surface, has greater gingival crevicular fluid and is a niche that favors communities adapted to persistence. *P. gingivalis* and *F. nucleatum* exhibited a high ability to form biofilm and among opportunistic bacteria, *S. aureus*, enterococci and gram-negative rods were present as strong biofilm producers. Heterogeneity of the biofilm has been shown for *Prevotella*, as well as other oral species. The crystal-violet assay can only mimic attached biomass under *in vitro* standard conditions, and fails to mimic multispecies architecture, shear forces, host proteins and viable-cell fractions. However, repeat measurements at the isolate level can be used as an excellent and reproducible phenotype for comparative surveillance. Since the relationship between biofilm biomass and antimicrobial non-susceptibility is also mechanistically sound and should not be interpreted as evidence for resistance due to biofilm formation, the association of the two should not be interpreted as evidence for heritable resistance. Biofilm communities can hinder penetration into the biofilm by antimicrobial agents, generate gradients of nutrients and oxygen, induce stress responses and persister states, and provide more opportunities for the exchange of resistance determinants. In recent years, oral-resistome syntheses also report about the subgingival biofilm as a repository of resistance determinants and mobile elements. However, MIC testing methods are based on planktonic growth inhibition, and do not evaluate the MBC. This observed correlation thus suggests an interplay of co-occurrence of a strong biomass phenotype and decreased planktonic susceptibility, which may be due to either similar ecological selection or to correlated strain properties. The results of the MIC pattern were in line with the international evidence, with a relatively high proportion of amoxicillin-clavulanate and metronidazole susceptible to many periodontal anaerobes, and a higher variability in clindamycin, macrolide and tetracycline susceptibility as well as for fluoroquinolones. Low resistance to some of these commonly used agents was also detected in a multicenter study done in 2026 in Shanghai, with a high level of resistance detected for clindamycin in *Prevotella*, *P. gingivalis* and streptococci. It is anticipated that there will be geographic differences due to community prescribing, access to non-prescription drugs, previous exposure and local bacterial ecology which all affect selection pressure. Abe[29] carried out a systematic review that highlighted the need for systematic and standardized regional monitoring and the difficulties in reaching confident, global estimates due to heterogeneity and poor reporting of the information. The results of the study don't provide any support for the empirical antibiotic use in the treatment of uncomplicated periodontitis. The mechanical disruption and control of the dysbiotic biofilm is still important and the EFP guideline limits the use of systemic adjunctive antimicrobials to specific clinical scenarios and not as a routine treatment. Systematic and scoping reviews, as well, highpoint the evidence of resistance, which is not always or completely reported, and the need for restraint[30]. Species-specific culture and MIC testing might be more justifiable than extrapolating from a foreign antibiogram, if antibiotics are deemed necessary for a severe or rapidly progressing or otherwise chosen case. Extra stewardship care needs to be taken with clindamycin and macrolides in the setting of high local MICs. Patient level findings also indicate that patient antibiotic history should be noted at the time of treatment planning and culture results evaluation. Although there is growing interest in emerging antibiofilm and microbiome-targeted therapies, reviews to date highpoint the need for increased clinical validation of most therapies and the fact that none of these approaches should substitute for mechanical therapy or stewardship based on guidelines[31][32]. Strengths include multicenter recruitment, contemporary staging and grading, full mouth clinical assessment, standardized pooled sampling, collection of viable isolates, repeated

quantitative biofilm testing, lab blind, species resolved reporting, multiplicity control, and patient-clustered regression. By allowing separation of patient-level and isolate-level outcomes, trivially independent outcomes of multiple isolates of the same organism from a single patient could be avoided. The reporting of MIC₅₀ and MIC₉₀ without assigning resistance labels for those MIC's for which breakpoints were not set also limited the opportunity for overinterpretation. There are a number of caveats to draw attention to. A cross section design does not allow for temporal direction to be determined between advanced disease, strength of biofilm, and antimicrobial non-susceptibility. Recruitment is based on one governorate, which would not allow for generalizing to all Iraqi adults. Culture is only able to detect the recoverable fraction of subgingival communities and it would be possible that *Tannerella*, *Treponema*, *Filifactor* and other fastidious organisms would not be detected in culture. The benefits of pooling four sites are that the sample is at the patient level, but the site-specific heterogeneity is lost. In contrast to conventional MICs, which measure sensitivity of the planktonic fraction, crystal violet does not measure viability or three-dimensional structure of the biofilm, but yields an estimate of the total amount of attached biomass. Possible residual confounding may exist due to the lack of documentation of type of antibiotic received, adherence to the antibiotic, socioeconomic factors, oral-hygiene behavior, and glycemic history. Last, estimates of species-specific abundances are inaccurate when populations of these species are low. Longitudinal studies are still needed in the future, with sampling after standardized periodontal treatment and maintenance of species level isolate banks and comparison with MBI/MEC. A larger number of Iraqi networks would be able to create species-stratified periodontal antibiogram and to assess the changes over time. Separate studies using molecular methods might be conducted to define resistance determinants and relatedness of strains, but phenotype-based surveillance has value as it measures a manifested susceptibility under a standard set of test conditions.

Declarations

Consent for publication

Not applicable.

The availability of data and materials.

De-identified data and statistical code will be made available from the corresponding author, upon request and with ethics approval and institutional data-sharing requirements.

Competing interests

The authors have no conflicts of interest to disclose.

Funding

The research was not specifically funded by any public, private (commercial) or not-for-profit funding agency.

Acknowledgments

The authors would like to acknowledge the clinical and laboratory staff for recruitment, periodontal examination, transporting the specimens to the lab, culture, and quality control.

Conclusion

Strong biofilm biomass, high grade of periodontitis and higher grade of antimicrobial non-susceptibility of culturable subgingival isolates were associated with advanced stage of periodontitis among adults who were treated at specialist periodontal services in Babylon Governorate. MDR and MANS phenotype of cells was independently associated with strong biofilm formation even after the clinical, demographic and species-level adjustment. The results of the present study help to support species resolved MIC surveillance and judicious antibiotic use on the basis of indication but cannot be extrapolated to the entire population of Iraq or as a basis for routine antimicrobial therapy.

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