Determination of copy number and circularization ratio of Tn916-Tn1545 family of conjugative transposons in oral streptococci by droplet digital PCR

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ABSTRACT

Background: Tn916 and Tn1545 are paradigms of a large family of related, broad host range, conjugative transposons that are widely distributed in bacteria and contribute to the spread of antibiotic resistance genes (ARGs). Variation in the copy number (CN) of Tn916-Tn1545 elements and the circularization ratio (CR) may play an important role in propagation of ARGs carried by these elements.

Objectives and Design: In this study, the CN and CR of Tn916-Tn1545 elements in oral streptococci were determined using droplet digital PCR (ddPCR). In addition, we investigated the influence of tetracycline on the CR of Tn916-Tn1545 elements.

Results: The ddPCR assay designed in this study is a reliable way to rapidly determine CN and CR of Tn916-Tn1545 elements.

Conclusions: Our data also suggest that Tn916-Tn1545 elements are generally stable without selective pressure in the clinical oral Streptococcus strains investigated in this study.

Introduction

The oral cavity is among the most microbiologically diverse environments in the human body and has been shown to contain over 1100 different bacterial species [1] of which Streptococcus species are the most abundant [2]. Although the majority of the Streptococcus species are not considered pathogenic, some species such as Streptococcus mutans are responsible for oral diseases and others, such as viridans group streptococci, can cause infections (such as pneumonia, endocarditis, and intra-abdominal infection) at other body sites [3].

There has been an increase in the number of antibiotic-resistant streptococcal strains over the last few decades [4], and recent studies suggest that the oral cavity functions as a reservoir for transferable antibiotic resistance genes [5–8] including genes encoding resistance to macrolides [9], beta-lactams, and tetracyclines [10]. One of the most common tetracycline resistance genes within oral isolates and metagenomes is tet(M) [8,11]. The broad distribution of tet(M) has frequently been linked to its association with mobile genetic elements (MGE) from the Tn916-Tn1545 family of conjugative transposons/Integrative Conjugative Elements [ICEs] [12–16].

The Tn916 conjugative transposons/ICE

Tn916 (accession number: U09422.1) is an 18-kb broad host range ICE [17] first isolated from Enterococcus faecalis DS16 [18]. Tn916 contains 24 ORFs (open reading frames) which are arranged in functional modules. These modules are responsible for conjugal transfer, transcriptional regulation, excision and insertion reactions (transposition), and accessory functions such as antibiotic and antiseptic resistance [17]. The transfer of Tn916 from a donor cell to a recipient cell involves the excision of the element from its original replicon to form a circular intermediate (CI) molecule [19], which has also recently been shown to autonomously replicate [20]. Tn916 and many related elements of the Tn916-Tn1545 family are frequently able to insert into multiple sites within a host genome [21].

Variations in ICE CN (copy number) may have an impact on their stability (of the ICE and the host genome) and conjugation potential thereby influencing the level of antibiotic resistance within bacterial populations [22–24].

Evaluation of the CN of the Tn916-Tn1545-like elements in oral streptococci can be achieved by Restriction Fragment Length Polymorphism (RFLP) followed by southern blot hybridization [25], full genome sequencing and assembly [26], and by real-time quantitative PCR (qPCR) [20]. The first two methods are not only time consuming and labor intensive but they also require high quantities of pure, high molecular weight DNA. The qPCR has become a common method in determining CN of target genes [27], however it does have some
limitations such as performance variation in and between assays [28] and artificial qPCR data resulting from samples with low target concentration but high levels of impurity [29]. These limitations can be overcome by droplet digital PCR (ddPCR) as it has been shown to produce more precise and reproducible results when compared to qPCR [30]. In the QX200 ddPCR (Bio-Rad, USA), a single PCR reaction is divided into approximately 20,000 droplets which are treated as individual reactions. Each reaction contains the relevant forward and reverse primer, the probes to detect the target gene and template DNA molecule.

In this study, we present an assay that rapidly reports CN of integrated and CI of Tn916-Tn1545 alike elements in various clinical oral Streptococcus species. In addition, the CR of CI in the study strains is reported (percentage of CI molecules detected within the bacterial population as a function of the total number of host genomes).

Materials and method

Control strains

The fully sequenced Bacillus subtilis BS4A (NZ_LN680001.1), B. subtilis BS49 (NZ_LN649259.1), Enterococcus faecium OrEc1, and E. faecium OrEc2 derivatives containing different CN of Tn916 were used as control strains in ddPCR (Table 1).

Clinical oral streptococcus strains

A selection of 10 antibiotic resistant oral Streptococcus strains collected by the National Advisory Unit for Detection of Antimicrobial Resistance (K-RES), University Hospital of North Norway were used in this study. These 10 strains tested PCR positive for tet(M) and the Tn916 integrase (intTn916) and excisionase (xisTn916) genes. The 10 strains were further identified at the species level using MALDI-TOF. MALDI-TOF identification was carried out at the National Advisory Unit for Detection of Antimicrobial Resistance (K-RES) laboratories, University Hospital of North Norway. These strains were used in the determination of CN and CR of Tn916-Tn1545 like elements in oral streptococci.

Bacterial cultivation

B. subtilis and E. faecium strains were cultivated on Luria-Bertani (LB) agar at 37°C under aerobic conditions whereas the oral Streptococcus strains (Table 1) were cultivated in anaerobic conditions on Todd Hewitt (TH) agar at 37°C overnight using the Anaerocult® System (Merck, Germany).

Determination of tetracycline MIC

The MIC of tetracycline for the oral streptococci was determined by E-test (BioMerieux, France) on Mueller-Hinton agar supplemented with 5% sheep blood and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org). The S. pneumoniae ATCC 49619 was included in all the runs as a positive control.

DNA extraction and DNA concentration measurement

The QIAcube (Qiagen, Hilden, Germany) automated system was used to extract DNA with a prepogrammed protocol using the QIAamp DNA Mini Kit (Qiagen, Germany) to obtain DNA from all bacterial strains in this study according to the manufacturer’s instructions. The quality and yield of extracted genomic DNA were

| Bacteria | Relevant properties (MIC Tet) | Reference or Source |
|----------|-----------------------------|---------------------|
| B. subtilis BS34 | Tetracycline resistant bacterium (32μg/ml) | [26] |
| (Control strain containing one copy of Tn916) | | |
| B. subtilis BS49 | Tetracycline resistant bacterium (48μg/ml) | [26] |
| (Control strain containing two copies of Tn916) | | |
| E. faecium OrEc1 | Tetracycline resistant (96μg/ml) (Transconjugant) | This study |
| (Control strain containing five copies of Tn916) | | |
| E. faecium OrEc2 | Tetracycline resistant (48μg/ml) (Transconjugant) | This study |
| (Control strain containing one copy of Tn916) | | |
| S. pneumonia (control strain for Tetracycline MIC) | Tetracycline susceptible (≤1μg/ml) | ATCC 49619 |
| S. mitis SM28 | Tetracycline resistant clinical isolate (64μg/ml) | This study |
| S. mitis SM29 | Tetracycline resistant clinical isolate (32μg/ml) | This study |
| S. sanguinus SS33 | Tetracycline resistant clinical isolate (24μg/ml) | This study |
| S. sanguinus SS41 | Tetracycline resistant clinical isolate (32μg/ml) | This study |
| S. oralis SO44 | Tetracycline resistant clinical isolate (2μg/ml) | This study |
| S. oralis SO47 | Tetracycline resistant clinical isolate (4μg/ml) | This study |
| S. oralis SO62 | Tetracycline resistant clinical isolate (24μg/ml) | This study |
| S. oralis SO67 | Tetracycline resistant clinical isolate (32μg/ml) | This study |
| S. gordonii SG71 | Tetracycline resistant clinical isolate (2μg/ml) | This study |
| S. oralis SO74 | Tetracycline susceptible clinical isolate (2μg/ml) | This study |
analyzed by agarose gel electrophoresis before determining the DNA concentration with the Qubit 3.0 fluorometer (Life Technologies, USA) according to the manufacturer’s instructions.

**Conventional PCR**

Conventional PCR was conducted with primers listed in Table 2 and depicted in Figure 1S (see supplementary data). All reactions were performed in a final volume of 25 μl containing 12.5 μl of 2 x Dream Taq Green PCR master mix (Thermo Scientific, USA), 1 μl each of the forward and reverse primer (20 μM), 2.5 μl DNA sample, and 8 μl water. The PCR conditions were: initial denaturation at 94°C for 10 min, 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 2 min, and a final extension step at 72°C for 10 min. A volume of 5 μl was analyzed with a 1-KB DNA ladder on a 1% agarose gel containing GelRed™ (Biotium, USA) for visualization of the amplicon.

**Genetic linkage between tet(M) and intTn and xisTn**

In order to analyze the genomic proximity between tet (M) and intTn and xisTn, indicating that the genes are located on the same genetic element, linkage analysis by a duplex ddPCR was carried out. Intact bacterial cells were boiled for 5 min in molecular grade water to obtain a DNA template. The concentration of the DNA was determined using the Qubit 3.0 fluorometer (Life Technologies) and accordingly the optimal DNA amount was used for further analysis. The digestion reactions were performed in a 20 μl reaction mixture, which contained 2.5 μl of the bacterial DNA, 10 U of restriction enzyme, 2 μl of 10 x buffer, and water. The digestion was conducted at 37°C for 4 hrs prior to inactivation at 80°C for 20 min. The restriction enzymes used were BsuRI (which does not cleave Tn916 between tet(M) and intTn and xisTn genes) and HincII which cuts Tn916 between the tet(M) and xisTn at position 14,934 bp in U09422.1. The HincII enzyme was used as a control for the genetic linkage analysis. The initial linkage between tet(M), intTn, and xisTn was automatically calculated by the Quantalife™ software as a ‘linkage’ score. This is the estimate of the total number of molecules (copies/μl) in the assay that contain fragments on which the two targets are physically linked. The linkage percentage is calculated by normalizing the linkage score for differences in DNA input between the two assays as described by Roberts et al. [31]. The linkage percentage (L%) between tet(M), intTn and xisTn was calculated as follows: 

\[
L\% = \left( \frac{2 \times \text{tet(M) copies per one droplet} \times \text{intTn/xisTn regions}}{\lambda} \right) \times 100
\]

where 

\[
\lambda = \frac{\text{tet(M)} \times \text{intTn/xisTn regions}}{\text{tet(M) copies per one droplet}}
\]

intTn/xisTn regions is the average number of intTn and xisTn genes droplets linkage, \( \lambda \) tet(M) is the average number of tet(M) copies per one droplet and \( \lambda \) intTn/xisTn regions is the average number of intTn and xisTn genes copies per one droplet.

**Evaluation of amyE, intTn, and xisTn genes as a representative gene and representative region for genome CN and Tn916 CN, respectively**

The known genome size of *B. subtilis* BS49 and different amounts of DNA inputs based on Qubit 3.0 fluorometer (Life Technologies) readings were used to evaluate the suitability of amyE as a representative gene for detection of the genome CN in ddPCR.

The formula used to calculate the expected genome CN of *B. subtilis* BS49 with a DNA input ranging from 0.6 pg to 40 pg in the ddPCR reaction mixture is as follows: Genome equivalents/copy = A/HGW where A is the input DNA concentration and HGW is the genome weight of the bacterial genome that was

**Table 2.** Sequence of primers and probes used in the study.

| Target       | Forward primer       | Reverse primer       | Probe sequence     | Amplicon size | Label (chlorophore) | Annealing temperature |
|--------------|----------------------|----------------------|--------------------|---------------|---------------------|----------------------|
| **Tet(M)**   | GGT TTC TCT TGG ATA  | CCA ACC ATA CAA TCC  | ATG CAG TTA TGG AAG AAG GTA TCG GAT TCG GAA CGA TGG | 88bp          | HEX/FAM             | 56°C                 |
| **anyE B. subtilis** | TGC AGA CGG AAT TTA CAC | CCG AGT CAT TAT ATA AAC CA | AAT ACT CGA AAG AAG CAT A | 146bp         | HEX                 | 56°C                 |
| **Circular Intermediate (CI) PCR** | | | | | | |
| **intTn and xisTn regions** | GCG GTA GTA CTA GGA CAA | TAC A | AGT TCC ACC CCT GCA TGG | 88bp         | HEX                 | 56°C                 |
| **amyE S. sanguinusa** | GGC GGA TGT CTA GGA CAA | CAA TAG ACC TAC GCT TTA | TGG ATT GCC TTC CGT CTT | 67bp           | HEX                 | 56°C                 |
| **amyE S. oralis** | GCG ATC ATA GTC GTG ACC TAT G | ACG GCC TGG ACT TAC ATC TTT ACC A | ACG GCC TGG ACT TAC ATC TTT ACC A | 98bp          | HEX                 | 56°C                 |
| **amyE S. mitis** | GCA TCT ACG CGG AAA CC | GAC CTA GAC TTG AAA CAT CCT GAA CTA CTG CTA TTT CTG | CAG TTG CAG GCA CATT CAA TTA GAA TTA GGC | 98bp          | HEX                 | 56°C                 |
| **amyE S. gordonii** | ATA AAT ACC AGG GCG TCG ACT T | GGG ATG TGA GGA CGA TGA T | CTT AAA CCA TTG ATG CTT AGC AAT CAA GCA | 148bp         | HEX                 | 56°C                 |
| **amyE E. faecium** | GCG GTA GTA TCT CCA TAC | ACG CTT GAT AAA GTG TGA TAA | ACG CTT GAT AAA GTG TGA TAA | 56°C         | N/A                 | 56°C                 |
calculated according to the genome size in Mb multiplied by 0.001096.

In addition, the intTn and xisTn in *E. faecium* OrEc1 and *B. subtilis* BS49, which harbor five and two copies of Tn916 respectively, were evaluated for being a representative region in Tn916 for determination of the CN of Tn916-Tn1545 like elements by ddPCR. The primers and probes used for the *amyE* and the *intTn* and *xisTn* genes were designed and labeled with either FAM or HEX as listed in Table 2.

**Calculation of the CN of Tn916-Tn1545-like elements and their CR by ddPCR**

The QX200™ Droplet Digital™ PCR system (Bio-Rad, Pleasanton, CA) was used in the current study to determine the CN of Tn916-Tn1545-like elements in the genome. In addition, the CR of Tn916-Tn1545-like elements that formed the CI in the *B. subtilis*, *E. faecium* and *Streptococcus* species populations were evaluated. The primers and probes used in the ddPCR assays in this study are listed in Table 2. The reaction mixture for CN experiments consisted of 10 µl ddPCR™ Supermix for Probes (No dUTP), 1 µl of 20 x *intTn/xisTn* regions (target gene) primers and probes, 1 µl 20 x of *amyE* primers and probe, 0.5 µl restriction enzyme (5 Units per reaction), 8 µl water, and 60 pg DNA template.

A total volume of 21 µl of the reaction mixture was transferred into the sample well of the cartridge, and 70 µl of droplet generation oil was applied to the correspondent oil well prior to placing the gasket over the cartridge and transferring it into the droplet generator. After droplet generation, 40 µl of the sample emulsion was transferred into a 96-well PCR plate (Eppendorf, Germany) and then sealed with pierceable foil (Bio-Rad). PCR amplification was done in a C1000™ Thermal cycler (Bio-Rad). In all experiments, a non-template control (NTC) and positive controls were used to rule out any primer dimer or contamination issues. The amplification parameters consisted of an initial activation step at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, and varying the annealing temperature (depending on the primers annealing temperatures as listed in Table 2) for 30 s. An additional inactivation step at 98°C for 10 min was used at the end of the cycles. The temperature ramp was set to 2°C per second and the lid was heated to 105°C. Upon completion of the PCR, the 96-well plate was transferred to the QX200 Droplet Reader (Bio-Rad) and the generated data were analyzed using the QuantaSoft software version 1.7.4.0971 (Bio-Rad). The threshold to distinguish positive droplets from the negative ones was set for each reaction automatically by the software if not stated otherwise. If needed, further analysis of the data was done using the QuantaSoft™ PRO software (version 1.0).

The CN of Tn916-Tn1545-like elements per bacterial genome was calculated by using the ratio between the Tn916 target region, that is *intTn* and *xisTn* and the single copy reference gene (*amyE*). For strain specificity, the variable region of the reference gene was used to design species-specific primers and probes for the studied species (Figure 25 in the supplementary data). The accession numbers of the *amyE* used in the current study are given in Table 3. In the CR experiments, the reaction mixture was prepared as described above with the exception of the primers and probes used, which in this case, only produced a signal if and when the element was in the circular form. The sequence of the primers, probes, product size and annealing temperatures are shown in Table 2.

The CR was measured by calculating the percentage of the CI molecules detected within the bacterial population, that is the number of detected copies of CI molecules in the bacterial population to the number of bacterial genomes represented by the *amyE* CN in the same population. Based on screening for the presence of CI by conventional PCR, seven oral *Streptococcus* strains; *S. oralis* (*n* = 4), *S. mitis* (*n* = 2), and *S. gordonii* (*n* = 1) were selected for the CR analysis. In addition to investigate the CR, this study also assessed the effect of varying the tetacycline concentration below the MIC levels on the excision of Tn916-Tn1545-like elements in the control strains, that is *E. faecium* and *B. subtilis*, and the oral *Streptococcus* strains.

It has been recently reported in *B. subtilis* that Tn916 can replicate autonomously [20]. In order to determine whether the element in our strains was replicating autonomously we compared the ratio of detected CI to the detected copies of bacterial genome, represented by *amyE* CN.

**DNA sequencing of the promoter region upstream of tet(M)**

DNA Sanger sequencing was used to investigate the DNA sequence of the promoter region upstream of tet(M) in oral streptococci. In brief, two primers (Table 2) were designed to yield a PCR fragment of 595 bp that covers the promoter region upstream of tet(M). The PCR fragment was subjected to BigDye terminator v 3.1 (Thermo Scientific) cycle sequencing prior to DNA sequencing by capillary electrophoresis using the SeqStudio sequencing platform (Thermo Scientific). Sequencing data were aligned against the wild type Tn916 using the Lasergene Molecular Biology Suite software (DNASTAR, USA).

**Table 3. Accession numbers and genetic regions of the reference gene *amyE***

| Bacterial species | Accession number | *amyE* genetic region |
|------------------|------------------|-----------------------|
| *B. subtilis*     | NZ_LN680001      | 327604.329583         |
| *E. faecium*      | CP012522.1       | 178531.1787153        |
| *Streptococcus sanguinis* | CP000387      | 1041272.1042738       |
| *Streptococcus oralis*  | FR720602        | 723983.725431         |
| *Streptococcus mitis* | FN568063        | 702427.703881         |
| *Streptococcus gordonii* | CP000725      | 1119068.1120519       |
Results

Bacterial strains

The 10 oral Streptococcus species included in this study were identified by MALDI-TOF as follows: S. mitis (n = 2), S. sanguinis (n = 2), S. oralis (n = 5), and S. gordonii (n = 1).

Evaluation of amyE as a representative gene for genome CN by ddPCR

The accuracy and sensitivity of using the amyE as a reference gene for detecting genome CN by ddPCR was achieved by analyzing varying concentrations of B. subtilis BS49 DNA (obtained from cultures that were cultivated in the absence of selection pressure). B. subtilis BS49 is known to harbor two copies of Tn916 [26] and, as shown in Figure 1, the detected CN of Tn916 using amyE as a reference gene over a two-fold increase in DNA concentration was on average 2.00, SD 0.24. The detected CN of Tn916 measured up to the expected theoretical CN even at a low DNA input of 0.6 pg/µl.

Determination of the CN of Tn916-Tn1545-like elements

Prior to determining the CN of Tn916-Tn1545-like elements in Streptococcus species, we used previously published genome data of B. subtilis BS34A and B. subtilis BS49 (which contain one and two copies of Tn916, respectively) [26] and E. faecium OrEc1 and E. faecium OrEc2 (sequenced transconjugants produced in our laboratory which contain five copies and one copy of Tn916, respectively) to validate our ddPCR CN determination assay. In B. subtilis BS49 and E. faecium OrEc1 the observed number of Tn916 corresponded to the expected CN (2 and 5 respectively). In B. subtilis BS34A, the observed CN was 0.72 as more amyE were detected than intTn/xisTn. The validated CN determination assay was used to screen a panel of 10 oral streptococci for determination of the CN of Tn916-Tn1545-like elements. Figure 2 shows that all the tested oral streptococci strains harbored only one copy of a Tn916-Tn1545-like element.

Linkage between tet(M) and intTn and xisTn

The linkage percentage between tet(M) and intTn and xisTn regions, which represents the percentage of droplets containing both targets suggesting that they are physically linked on the same fragment of DNA. This linkage percentage ranged from 88% to 6% in the bacterial cells that were digested with BsuRI as shown in Figure 3(c). In the control samples that were digested with HincII which not only cuts between the tet(M) and the intTn and xisTn genes but produces six fragments in Tn916, we observed a 3–10-fold drop in linkage (ranging from 2–26%). Figure 3(a, b) illustrates that the reduction in droplets that contain the double targets; tet(M) and the intTn and xisTn genes when digested with the two targets are physically delinked.

The CR of Tn916-Tn1545-like elements

The CN of the circular form of Tn916-Tn1545-like elements and the CN of amyE in the bacterial population were used to calculate the CR, that is the percentage of CI molecules detected within the bacterial population. Based on our findings, the values of CI vary among
Figure 2. A graphical illustration of the copy number of Tn916-Tn1545 family detected in bacteria involved in this study. The bars represent the copies of the Tn916-Tn1545 family per bacterial genome. The error bars represent 95% confidence intervals.

Figure 3. An illustration of linkage between tet(M) and IntTn/XisTn regions. 3(a, b) show 2-D amplitude plot in which each axis represents the amplitude axis of either FAM or HEX. The blue droplets represent FAM targets (tet(M)), the green droplets represent HEX targets (IntTn/XisTn regions), the orange droplets are those that contain both FAM and HEX, and the gray represents the droplets with no target molecules. Image 3(a) shows the droplets distribution of undigested B. subtilis BS49 whereas as image 3(b) illustrates the target distribution when the template is digestion with HincII which cut between tet(M) and IntTn/XisTn regions in Tn916. 3C shows linkage percentage between tet(M) and IntTn/XisTn regions. The blue bars represent the linkage percentage between tet(M) and IntTn/XisTn regions. The orange bars show the drop of the linkage percentage when the two targets have been physical separated by restriction enzyme digestion.
the bacterial species. In the selected clinical oral Streptococcus species, that is S. oralis, S. sanguinis, and S. mitis, the CR of Tn916-Tn1545-like elements was influenced by the presence and concentration of tetracycline. In the absence of tetracycline, the CR ranged from 0% to 0.036% while in the presence of 5 and 10 μg/ml tetracycline, the observed CR ranged from 0.004% to 0.17% and from 0.008% to 3.19%, respectively (Figure 4(a)). Interestingly, in E. faecium OrEc1, B. subtilis BS34A and B. subtilis BS49, the levels of CI were higher than in oral streptococci and influenced by the presence and concentration of tetracycline. In the absence of tetracycline, the observed CRs were, 9.9%, 0.4%, and 9.7% for E. faecium OrEc1, B. subtilis BS34A and B. subtilis BS49, respectively. In the presence of 5 μg/ml tetracycline the detected levels of CI increased to 11.8%, 9.8%, and 244% for E. faecium OrEc1, B. subtilis BS34A, and B. subtilis BS49, respectively (Figure 4(b)). When E. faecium OrEc1 was cultivated in the presence of 10 μg/ml tetracycline, it was observed that the percentage of CI molecules detected within the bacterial population exceed 50%. In both B. subtilis BS34A and B. subtilis BS49, the percentage of CI molecules detected within the bacterial population were 113% and 239%, respectively, exceeding the number of bacterial genomes that were detected in the assay.

**DNA sequencing of the promoter region upstream of tet(M)**

The DNA sequencing results show distinct 58 bp deletions in two S. oralis strains, a 27 bp and 12 bp

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**Figure 4.** An image indicating percentage of CI per bacterial population. (a) CR in oral streptococci. The green circles represent the CR in the absence of tetracycline whereas the blue squares and the orange triangles represent the CR in the presence of 5 mg/ml and 10 mg/ml tetracycline, respectively.

![Graph](image-url)
deletion in other two *S. mitis* strains, and multiple SNPs in the upstream of *tet(M)* in the other oral *Streptococcus* strains included in the CR experiment compared to the wild type sequence of Tn916. The deletions effectively removed the predicted large terminator structure responsible for transcriptional attenuation (Supplementary data, Figure 3S).

**Discussion**

Antibiotic resistance in oral streptococci is an ever-growing problem [32]. Advances in molecular biological techniques and detection methods of resistance genes, have increased our knowledge of factors contributing to the propagation of MGEs carrying resistance genes in bacterial populations. The CN determination of MGEs carrying resistance genes in oral streptococci is of particular interest, as high CN might influence the propagation and spread of resistance due to availability of more than one element in any given genome. Furthermore, the presence of more than one copy of MGEs might influence bacterial biological fitness [33] and hence the reversibility of resistance. Our attempt to determine CN of MGEs, such as Tn916-Tn1545-like elements, is part of ongoing work to determine the biological cost of these elements in oral streptococci. This work has led to the development of an assay that can easily and accurately determine the CN of Tn916-Tn1545-like elements in *E. faecium*, *B. subtilis* and oral streptococci using ddPCR. We tested the sensitivity and reproducibility of our assay by analyzing varying amounts of input DNA that contained a predetermined number of target regions per genome. Our results illustrate that ddPCR is a sensitive and highly specific tool that can be used to determine CN of MGEs. The reproducibility and precision, even at very low input DNA concentration (0.6 pg/µl) is promising as it allows for analysis of samples with low DNA target concentrations and may be applicable therefore to analysis of bacteria directly from saliva samples and other body fluids.

Four sequenced bacterial strains; *B. subtilis* BS34A (NZ_LN680001.1), *B. subtilis* BS49 (NZ_LN69259.1) *E. faecium* OrEc1, and *E. faecium* OrEc2 (unpublished data) were used to determine the accuracy of ddPCR in detecting multiple copies of Tn916-Tn1545-like elements. In *B. subtilis* BS49, *E. faecium* OrEc1, and *E. faecium* OrEc2, we were able to accurately detect the expected number of elements using *amyE* as a chromosomally located, single copy, reference gene. In *B. subtilis* BS34A however, the ratio between Tn916-Tn1545-like elements (represented by the intTnxisTn genes) and the reference gene *amyE* was below one copy (approximately 0.75). The lower ratio may be explained by the chromosomal positioning of the two targets in relation to the origin of replication. In *B. subtilis* BS34A, the *amyE* gene (327,604–329,583 bp) is situated closer to the origin of replication in comparison to Tn916 which is in position 1,886,552–1,904,583 bp. The closer proximity of *amyE* to the origin of replication may result in more targets of the reference gene due to the occurrence of multiple replication forks within a cell prior to cell division, as has been previously reported in *B. subtilis* [34].

A few studies have reported the presence of more than one copy of Tn916-Tn1545-like elements in clinical strains [35–37]. Rice et al. (2005) reported that the presence of multiple copies Tn916-Tn1545 elements in clinical strains is rare [38]. The current study supports such findings as all our clinical oral streptococci contained one copy of Tn916-Tn1545-like elements.

The ability to detect linkage between two genetic targets; the likelihood that two genetic targets are in physical proximity of each other, is fundamental to our understanding of the likelihood of horizontal gene transfer of resistance genes, especially if the linked genetic target to the resistance determinants is responsible for mobility. Several Tn916-Tn1545-like elements that confer resistance to more than one antibiotic have been reported [2,36,39–40]. The ability to determine whether or not these resistance genes are on the same mobile element will shed light on the prevalence of these resistance determinants and the likelihood for co-transfer. In our study, we illustrate that ddPCR can be used to determine the linkage between *tet(M)* and *intTn* and *xisTn* genes of Tn916 in *B. subtilis* BS49, *E. faecium*, and in oral streptococci. Whilst most Tn916-Tn1545 elements share an extremely high degree of sequence homology we acknowledge that subtle variations in the DNA sequence of the genetic target for ddPCR will affect the efficiency of the reaction. This can be overcome by using multiple sets of primers to detect the element itself and previously published primers for various resistance genes based on conserved regions within these genes.

The ratio between CI and the number of bacterial genomes present in the bacterial population was used to investigate the CR of the elements. The basal rate of CI formation varied between and within species. In *B. subtilis* BS34A, *B. subtilis* BS49, and the *E. faecium* OrEc1, the CR ranged from 0.4% to 9.9% in the absence of tetracycline. In oral *Streptococcus* strains, the CR in the presence of tetracycline (5 µg/ml and 10 µg/ml) was observed to range from 0.004% to 3.19% (Figure 4(a)). In contrast, the CR in *B. subtilis* and *E. faecium* strains in the presence of the same tetracycline concentrations showed higher numbers of CI in the bacterial population. It can be hypothesized that the observed lower CR in oral streptococci may be due to the fact that these are clinical isolates containing Tn916-Tn1545-like elements that have evolved a mechanism to reduce the level of excision to reduce any fitness cost associated with acquisition of these elements, a theory that requires further investigation for these strains. Sequencing of DNA upstream of *tet*
(M) shows several SNPs and deletion when compared to the wild type sequence of Tn916. The presence of deletions results in the removal of the predicted large terminator structure responsible for transcriptional attenuation upstream of tet(M). The removal of the large terminator structure would suggest that these isolates would not respond to tetracycline in the same way as it is hypothesized for the wild type Tn916.

Another possible explanation for the low CR of Tn916-Tn1545 in oral streptococci is that tet(M) is not present on the Tn916-Tn1545 like elements in some strains (e.g. S. sanguinis SS41 in Figure 3(c)).

In both B. subtilis strains, cultivation in the presence of 10 μg/ml tetracycline resulted in levels of CI that were higher than the number of bacterial genomes detected. This suggests that at this concentration of tetracycline, Tn916 is undergoing autonomous replication; a phenomenon that has been recently demonstrated [20]. The observed increase in CI presumably occurred because tetracycline could result in increased mobility of Tn916 as shown by Scornec et al. [41]. Introduction of even higher concentrations of tetracycline did not result in more CR in our bacterial populations, but rather we observed a decrease of CI number in the bacterial population where the concentration of tetracycline reached higher values, although still under the MIC values for these resistant strains (Table 1). The latter observation could be attributed to an overall effect on protein synthesis thus leading to slower or nearly diminished replication [12].

It is reasonable to assume that the more copies of the Tn916-Tn1545 family present in the genome, the more CI molecules would be present in the bacterial cell. This assumption was true when CI levels in B. subtilis BS49 strain; containing two copies of Tn916, were compared to CI levels in B. subtilis BS34A, which harbors only one copy of Tn916. This is consistent with findings from previous studies [42].

In this study, we demonstrate that ddPCR can be used to study CN and CR of the Tn916-Tn1545 family in oral streptococci with and without the presence of antimicrobial challenge. In addition to detection of the CI, we have also demonstrated that ddPCR can be used to detect an increase in the CN of the target molecule compared to another, as would happen if the CI of Tn916 was autonomously replicating. The minimal skills requirements, and flexibility, requirements for small amounts of DNA sample, and good reproducibility illustrate the potential that ddPCR carries for the advancement of studying MGEs like Tn916-Tn1545 family and antibiotic resistance.

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