

Antea Tramontana



**Detection of the degree of contamination of Gutta-Percha Points by strains  
of *Staphylococcus aureus* (MRSA/MSSA)**

Universidade Fernando Pessoa  
Faculdade de Ciências da Saúde  
Porto, 2023



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Dissertação apresentada à Faculdade de Ciências da Saúde  
da Universidade Fernando Pessoa como parte dos requisitos  
para obtenção do grau de Mestre em Medicina Dentaria

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### Abstract

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a bacteria considered one of the most harmful to human health. Dentistry is, as in all Health fields, extremely careful in avoiding situations that can lead to infection. Several tested materials suitable for filling the root canal system are available, still being gutta-percha the gold standard.

**Objective:** Analysis *in vitro* of the contamination of gutta-percha points, regarding the bacteria Methicillin-Resistant (MRSA) and Methicillin-Sensitive (MSSA) *Staphylococcus aureus*, by classical microbiology methods and molecular biology techniques.

**Method:** 315 gutta-percha points of two different brands from opened packed, already in use for 1 month were analysed, in Brain Heart Infusion medium to check the presence of microbial growth. Positive results were plated on a chromogenic selective medium for MRSA/MSSA strains, and the strains were confirmed by Polymerase Chain Reaction (PCR). In case of microbial growth, the gutta-percha point was submitted to a disinfection protocol.

**Results:** From the 315 collected gutta-percha points, only 6 (1.9%) resulted microbial growth positive. After confirmation by PCR, only 1 sample of the 6 cones was a MRSA contamination, and the remaining 5 cones were MSSA. The disinfection protocol was effective in all of the contaminated GP points.

**Conclusions:** Gutta-percha points from opened presterilized packed did show very little amount of contamination by MRSA/MSSA. However, the accurate handling of this filling material must be kept in mind soon since the pre-graduation period, as this methodology should be the gold standard for all professional activity.

**Keywords:** *Staphylococcus aureus*; MRSA/MSSA; Gutta-Percha; Contamination; Endodontics

## Resumo

*Staphylococcus aureus* Resistente à Meticilina (MRSA) é considerada uma das bactérias mais nocivas à saúde humana. A Odontologia é, como em todas as áreas da Saúde, extremamente cuidadosa para evitar situações que possam levar ao contágio. Vários materiais testados e adequados para a obturação do sistema de canais radiculares estão disponíveis, sendo ainda os cones guta-percha (GP) o padrão-ouro.

**Objetivo:** Análise *in vitro* da contaminação dos cones de guta-percha, quanto à bactéria *Staphylococcus aureus* Resistente à Meticilina (MRSA) e Sensível à Meticilina (MSSA), por métodos clássicos de microbiologia e técnicas de biologia molecular.

**Método:** Foram analisados 315 cones de guta-percha de duas marcas diferentes de embalagens abertas, já em uso há 1 mês, em meio Brain Heart Infusion para verificar a presença de crescimento microbiano. Os resultados positivos foram semeados num meio cromogénico seletivo para estirpes MRSA/MSSA, e as colónias suspeitas confirmadas por Reação em Cadeia da Polimerase (PCR). Em caso de crescimento microbiano, os cones de guta-percha foram submetidos a um protocolo de desinfecção.

**Resultados:** Dos 315 cones analisados, apenas 6 (1,9%) mostraram um crescimento microbiano positivo. Após a confirmação por PCR, apenas 1 amostra foi de MRSA, e os 5 cones restantes foram de MSSA. O protocolo de desinfecção foi eficaz em todos os cones de GP contaminados.

**Conclusões:** Os cones de guta-percha de embalagens pré-esterilizadas abertas mostraram muito pouca contaminação por MRSA/MSSA. No entanto, o manuseio correto desse material deve ser lembrado desde a pré-graduação, pois essa metodologia deve ser o padrão ouro para toda atividade profissional.

**Palavras-chave:** *Staphylococcus aureus*; MRSA/MSSA; C Guta-Percha; Contaminação; Endodontia

## **Dedication**

Alla mia Vera Famiglia, che mi ha insegnato le varie forme dell'Amore.

### **Acknowledgements**

Sinceramente pensando a chi sarebbe da ringraziare, mi sento di poter dire con orgoglio, che le persone sono molteplici e di molti il nome mi è estraneo; questo perché tutti coloro che hanno fatto parte della mia vita, anche solo per un'ora o un minuto, mi hanno portato ad essere come sono, imperfetta e consapevole di voler sempre migliorare, imparando finalmente a farlo essendo più buona e tollerante con me stessa.

Grazie a Nonno, Nonna, Zia, Papà, Mamma e a mio fratello Davide, per avermi dato fiducia, per avermi sopportato nei momenti in cui continuavo a dire che non ce l'avrei mai fatta... Grazie per avermi dato la parte migliore di voi.

Grazie a tutti i maestri e professori che ho avuto nel mio percorso fino ad ora, sia quelli che mi dicevano che non valevo nulla perché mi avete dato la grinta di dimostrarvi che eravate nel torto e un Grazie di cuore a tutti i professori che invece mi hanno fatto capire che la verità era ben altra.

Grazie alla UFP e ai miei compagni di corso e non, che avete rappresentato una nuova famiglia in questo paese così sconosciuto all'inizio, ma oramai casa.

Grazie ad António, Maria, Beatriz, Sandra, Telmo, Cristina, Cidália, Joana e Afonso che mi avete trattato come una figlia, nonostante il legame sia 'solo' di cuore.

Grazie alla mia binomia Maria, che mi ha dato l'onore di condividere questo percorso con lei.

Grazie alle Professoressa Cristina, Inês, Ana e Conceição per aver creduto in me.

Grazie a Rossana, Serena e Valentina per avermi dato amore e sostegno anche quando io stessa non ero in grado di darmelo da sola; mi avete aiutato anche senza saperlo, siete speciali.

Grazie al mio Mr Panettone, Gianmarco, che nonostante la distanza è per me un pilastro.

Grazie a Michelle, che è e rimarrà sempre un pezzo di cuore.

Specialmente sento di dover dire un Grazie importante all'Antea di anni fa, per non aver mollato e non aver ceduto a quei pensieri, sono Orgogliosa.

In più voglio solo specificare che, anche se il vostro nome non compare nero su bianco su questa pagina, sappiate che se vi considero parte della mia Vera Famiglia, questo traguardo è dedicato anche a voi; mi avete dato tanto e io ne farò tesoro, ve lo prometto. Grazie a chi c'è stato, Grazie a chi c'è e Grazie a chi ci sarà.

Detection of the degree of contamination of Gutta-Percha Points by strains of *Staphylococcus aureus*  
(MRSA/MSSA)

*“ La teniamo sempre accesa  
Quest'ansia leggerissima che abbiamo poi  
Di vivere la vita piu' che puoi ”*

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## **LIST OF ABBREVIATIONS AND ACRONYMS**

BHI: Brain Heart Infusion

CPD: Chairside Disinfection Protocol

CPMD-UFP: Pedagogical Clinic of Dentistry – Fernando Pessoa University

ECDC: European Centre for Disease Prevention and Control

FDA: Food and Drug Administration

GP: Gutta-Percha

MRSA: Methicillin-Resistant *Staphylococcus aureus*

MSSA: Methicillin-Sensitive *Staphylococcus aureus*

NSRCT: Non-Surgical Root Canal Treatment

PBP: Penicillin-Binding Protein

PCR: Polymerase Chain Reaction

RCS: Root Canal System

SCCmec: Staphylococcal Cassette Chromosome mec

## I. INTRODUCTION

*Staphylococcus aureus* (*S. aureus*) is a commensal Gram-positive bacterium, specific of the skin and nasal region (Grice *et al.*, 2009; Grice & Segre, 2011; Byrd *et al.*, 2018). It is not a microorganism that simply colonizes these two regions, but also affects other parts of the body, such as the oral cavity. Even being *S. aureus* a well-known bacterium, its role in the pathology of the oral cavity is not yet fully understood (McCormack *et al.*, 2015). Over the years, it has been possible to find correlations between *S. aureus* and certain non-physiological oral conditions such as acute suppurative parotitis (Brook, 1992; Spiegel *et al.*, 2004), angular cheilitis (Oza & Doshi, 2017) and Staphylococcal mucositis (Bagg *et al.*, 1995; Subramaniam & Muthukrishnan, 2019). It has also been detected in oral infections such as jaw cysts (Iatrou *et al.*, 1988) and orofacial abscess (Labriola *et al.*, 1983; Lewis *et al.*, 1990; Jagadish Chandra *et al.*, 2017).

Therefore, despite its presence in the oral cavity and also its highly pathogenic potential (Koukos *et al.*, 2015; Blanco *et al.*, 2017; Cheung *et al.*, 2021), *S. aureus* manages to coexist in a totally harmless way in the human environment thanks to the mechanism known as homeostasis, which allows to maintain an innocuous equilibrium (Marsh, 2006; Coyte *et al.*, 2015; Lamont *et al.*, 2018; Deo & Deshmukh, 2019). However, when this balance is corrupted, creates the situation known as dysbiosis, which is a sharp shift that can be directly related to systemic and metabolic diseases such as diabetes, cardiovascular disease, periodontitis and obesity, among many others (Sudhakara *et al.*, 2018; Thomas *et al.*, 2021). The causes of oral dysbiosis are varied and multifactorial. Among them, a recognized cause is the use of antibiotics with systemic functionality (Abeles *et al.*, 2016; Swanson *et al.*, 2021). As an example, amoxicillin (belonging to the class of antibiotics called penicillins), is one of the most widely used and, within its applications approved by the Food and Drug Administration (FDA), is precisely against *Staphylococcus* spp. (Akhavan *et al.*, 2022).

Penicillin was discovered by Fleming in 1928, paving the way for the 'antibiotic era', yet its first use was more than a decade after its revelation (Gaynes, 2017). However, only a few years after its introduction into clinical use, resistance to this antibiotic was found, thus leading to the discussion of a possible abuse of penicillin as early as 1947 (Hoffman, 1947). Towards the end of the 60s decade, in XX century, 80% of the *S. aureus* isolated both in a community and in a hospital environment, were penicillin resistant (Lowy, 2003). Despite an attempt to halt this rapid increase of penicillin-resistant bacteria through the introduction of

semi-synthetic methicillin (second generation) in 1959 (Harkins *et al.*, 2017), the resistance mechanism arose shortly after, but this process was only discovered towards the 1980s, slightly more than 20 years after the introduction of methicillin in the market (Hartman & Tomasz, 1981). Finally, this discovery gave definition to this methicillin resistant bacterium, which is still called today *Methicillin resistant Staphylococcus aureus* or MRSA. The carriage of the staphylococcal cassette chromosome mec (SCCmec), that is a mobile cassette of genes, is the basis of the mechanism of methicillin resistance (Katayama *et al.*, 2000). A gene that is crucial for this mechanism is called *mecA* and resides on the *S. aureus* genome. *MecA* gene encodes an altered form of Penicillin-Binding Proteins (PBPs), called PBP-2a. This protein shows a reduced affinity to penicillin, making the bacteria presenting this gene resistant to  $\beta$ -lactam antibiotics (Matsushashi *et al.*, 1986; Fishovitz *et al.*, 2014; McCarthy *et al.*, 2015; Milheiriço *et al.*, 2017; Cikman *et al.*, 2019). MRSA has also the property of being a multi-resistant strain because it exhibits resistance to tetracyclines, macrolides, in addition to the well-known stubbornness to  $\beta$ -lactams antibiotics (penicillin, methicillin, carbapenems, and cephalosporins) (Gonçalves *et al.*, 2020). The strains of *S. aureus* that have retained sensitivity to this type of antibiotics, are called Methicillin Sensitive *Staphylococcus aureus* or MSSA.

MRSA strains are one of the major causes of nosocomial infections, leading to high mortality (Shorr, 2007; Pantosti & Venditti, 2009; Ahmad Bhat & Tenguria, 2014). According with the European Centre for Disease Prevention and Control (ECDC) and its annual report of the Priority Program of Infections and Resistances to Antimicrobials, Portugal is considered one of the countries with the highest MRSA percentage in Europe (ECDC, 2023). Although the risk of infection in dental clinics is lower than in hospitals, *S. aureus* strains are still responsible for a lot of pathologies at oral and perioral level, making this bacterial strain a public health problem (Gonçalves *et al.*, 2020).

Dentistry is, as in all Health fields, extremely careful in avoiding situations that can lead to infection. The aim of Non-Surgical Root Canal Treatment (NSRCT) is the prevention or handling (if already present) of apical periodontitis thorough debridement and cleaning of the root canal system (RCS). In this way, the RCS must be cleaned, shaped and, disinfected at the higher possible level (Wong *et al.*, 2021), before three-dimensional filling, to prevent or minimize any chances of infection or reinfection by avoiding re-penetration of microorganisms or their products, all of this, under aseptic means (Siqueira, 2001; Teles *et al.*, 2013; Tabassum & Khan, 2016; Wong *et al.*, 2021). In fact, despite the absence of pulpal

tissue, the RCS continues to communicate with the outside environment, by apical foramen, accessory root canals and dentinal tubules. This surely implies that sealing must be assured apically, laterally, and coronally (Siqueira, 2001).

Several tested materials suitable for filling the RCS are available, being gutta-percha (GP) the gold standard. Among others, the GP points' merits are their dimensional stability, radiopacity, being inert and, for this reason biocompatible. Moreover and of notice, GP points have, in their composition, zinc oxide which is not to be considered an inert component, but acts as a potential and most-proven antibacterial agent (Moorer & Genet, 1982). Whilst for the main demerits, it can be pointed out, the lack of rigidity, and of adhesive capacity, the reason why an endodontic sealer is recommended in order to achieve the desirable three-dimensional sealing of the irregular anatomic surface of all RCS (Vishwanath & Rao, 2019).

Although GP points are manufactured under aseptic conditions and present potential antimicrobial properties especially owing to the zinc oxide component (Moorer & Genet, 1982), the risk of GP contamination either by handling or aerosols and from physical sources during the storage process, among others, do not guarantee sterility (da Motta et al., 2001). In fact, there are several studies that testify the presence of bacterial activity even in freshly opened packages (Kayaoglu et al., 2009; Demiryürek et al., 2012; Saeed et al., 2017).

Hence, the aim of this *in vitro* study was to analyse the contamination of GP points from commercial packages, already in use for a (one) month (in a clinical setting), regarding the bacteria Methicillin-Resistant (MRSA) and Methicillin-Sensitive (MSSA) *Staphylococcus aureus*, by classical microbiology methods and by molecular biology techniques.

The following null hypothesis was formulated:

- 1) For the presence of contamination detected in the GP points: H0 - the degree of contamination is very low.

## II. MATERIALS AND METHODS

This is an observational study, conducted, after approval of Ethics Committee of Fernando Pessoa University (see Annex 1).

All the collection and laboratory techniques were performed by the dissertation author (student), under supervision of the respective advisors.

### 1.1. Collection and evaluation of contamination of GP points

This work is a follow-up of a previous *in vitro* study, conducted by Bracciale *et al.* (2020), but with a specific *in vitro* essay, that is the analysis of the degree of contamination of GP points by strains of *S. aureus* (MRSA/MSSA).

#### 1.1.1. Sampling of GP points

315 GP points collected from packages already in use for 1 month, in the Pedagogical Clinic of Dentistry of Fernando Pessoa University (CPMD-UFP), used by pre-graduation students, of six distinct International Organization for Standardization (ISO) gauges (k20, k25, k30, k35 belonging to R&S<sup>®</sup>, and A and B belonging to Dentsply<sup>®</sup>) (Table 1).

Brand and GP point sizes	Number of GP points
Dentsply <sup>®</sup> 142 (45.1%)	
A	70 (22.2%)
B	72 (22.9%)
R&S <sup>®</sup> 173 (54.9%)	
k20	48 (15.2%)
k25	46 (14.6%)
k30	41 (13.0%)
k35	38 (12.1%)

Table 1: Sampling of GP points (n = 315) divided by brands and sizes.

The GP points were collected, each one with a sterile tweezers, from 4 commercial packages (Figure 1). Used conditions include that all tested GP boxes were in use for, at least 4 weeks, each box supported an average of 8 appointments per week, and the storage of the box respected the ideal requirements of temperature and humidity, indicated by the manufacturers. These conditions were the same used in the *in vitro* study by Bracciale *et al.* (2020).

Detection of the degree of contamination of Gutta-Percha Points by strains of *Staphylococcus aureus* (MRSA/MSSA)



Figure 1: Examples of commercial boxes chosen to collect GP points for analysis and a tweezers used for each GP point picking.

Each GP point was inserted in one different sterile test tube and adequately labelled. Each sample was coded as “A” or “B”, according to the commercial box in test, for cases of GP points with the ISO gauge classification between K15 to K40, and, similarly, coded as “1” or “2”, corresponding to the 2 commercial boxes containing GP points defined as “accessory”, according to their gauge by “A”, “B”, “C” and “D”.

Each day, the collection of GP points consisted in taking: 4 GP points of two different gauge, from the same commercial package (for example: 4 K20 points – A1K20, A2K20, A3K20 and A4K20, respectively, and, similarly, 4 more of K25 gauge) and the same number from the other commercial box in test (B1K20, B2K20, B3K20 and B4K20 and 4 more for gauge K25) -, performing a total of 16 GP points. Likewise, for the accessory points, 4 points of gauge A and 4 points of gauge B were taken from 2 different boxes, denominated “1” and “2”, always completing a total of 16 GP accessory points, per day, of sampling. This sample collection was repeated, in different days, until a similar number of GP points gauge was attempted, based on Bracciale *et al.* (2020) regarding the number of GP points/gauge (Table 2).

Table 2: Total of sample (all GP points collected) distribution according to each commercial box and gauge.

GP point gauge	Number of GP Points	GP point gauge	Number of GP Points
- GP points from box A	88 (13.95%)	- GP points from box B	86 (13.64%)
k20	24 (3.8%)	k20	24 (3.8%)
k25	23 (4.44%)	k25	23 (4.44%)
k30	23 (3.17%)	k30	18 (2.85%)
k35	15 (2.54%)	k35	21 (2.54%)
- GP points from box 1	77 (13.64%)	- GP points from box 2	65 (9.83%)
A	43 (6.98%)	A	27 (4.44%)
B	34 (6.66%)	B	38 (5.39%)

### 1.1.2. Evaluation of contamination of GP points

Each GP point collected was, immediately, placed in one different sterile test tube containing 5 mL of Brain Heart Infusion (BHI) medium and incubated, in the microbiology laboratory of Fernando Pessoa University, at 37°C for 72 hours. After this period, tubes were checked for the possible presence of turbidity (Figure 2), corresponding to bacterial growth.

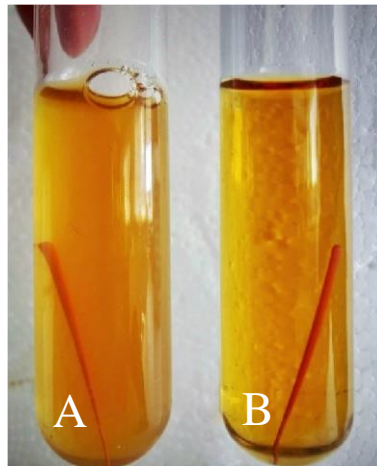


Figure 2: Positive (A) and negative (B) results for bacterial growth in BHI medium containing one GP point.

These results are perfectly comparable with the control samples created at the beginning of the experiment in order to have a visual representation of what to expect from a positive and a negative result. The positive control (C+ SA) corresponds to a tube of BHI medium with culture of *S. aureus* ATCC, while the negative control (C-) contains the pure BHI medium. Both samples were incubated at 37°C and observed after 72h (Figure 3).

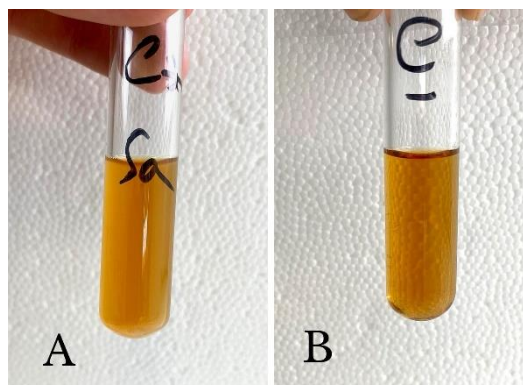


Figure 3: Control samples in BHI medium: positive (A) and negative (B).

## 1.2. Identification of *S. aureus* (MRSA/MSSA)

### 1.2.1. Classical Microbiology

In each tube that presented turbidity, the contaminated BHI medium (Merck) (Figure 4) was used to evaluate the bacterial nature responsible for the observed growth. All these samples were cultured in biplates of selective chromogenic medium – chromID®MRSA/chromID *S. aureus* (following manufacturer instructions (Biomérieux, Figure 5) and incubated at 37°C for 48 hours. The blue colonies are equivalent to MRSA, while beige corresponds to MSSA.

<b>Composition (BHI Broth, Brain Heart Broth, Merck)</b>
beef heart (infusion from 250g), 5 g/L
calf brains (infusion from 200g), 12.5 g/L
disodium hydrogen phosphate, 2.5 g/L
D(+)-glucose, 2 g/L
peptone, 10 g/L
sodium chloride, 5 g/L
<i>Technique(s)- microbiological culture: suitable</i>

Figure 4: BHI medium composition (Merck).

#### **Media for the screening of methicillin-resistant *Staphylococcus aureus* (MRSA) (biomerieux)**

Multi-drug resistant organisms (MDRO) and related risk of healthcare associated infection (HAI) outbreaks are among today's most challenging public health issues. bioMérieux has an overall commitment to helping address these challenges in innovative ways, such as with our AMS Program initiative and our SMART range of media, which is developed specifically to target pathogens involved.

Methicillin-resistant *Staphylococcus aureus* (MRSA) are a prime concern – listed as a “serious threat” by the Centers for Disease Control<sup>1</sup>. MRSA (and other *Staphylococci*) cause a range of infections including pneumonia and bloodstream infections that can lead to sepsis. Rapid identification of MRSA is therefore essential to improved patient care, and infection control and outbreak management.

Figure 5: Technical specifications: CHROMID® MRSA (Biomérieux).

The Gram staining procedure (Figure 6), catalase test (Figure 7) and coagulase test (Figure 8), were performed on typical colonies of *S. aureus*.

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1. Take a clean, grease free slide.
2. Prepare the smear of suspension on the clean slide with a loopful of sample.
3. Air dry and heat fix
3. Crystal Violet was poured and kept for about 30 seconds to 1 minutes and rinse with water.
4. Add lugol for 1 minute and wash with water.
5. Wash with 95% alcohol for about 10-20 seconds and rinse with water.
6. Add safranin for about 1 minute and wash with water.
7. Air dry, blot dry and observe under Microscope.

Figure 6: Procedure of Gram staining.

1. Use a loop or sterile wooden stick to transfer a small amount of colony growth in the surface of a clean, dry glass slide.
2. Place a drop of 3% H<sub>2</sub>O<sub>2</sub> in the glass slide.
3. Observe for the evolution of oxygen bubbles.

Figure 7: Procedure of slide test catalase method.

1. Place a drop of physiological saline on each end of a slide
2. With the loop emulsify a portion of the isolated colony in each drops to make two thick suspensions.
3. Add a drop of rabbit plasma to one of the suspensions, and mix gently.
4. Look for clumping of the organisms within 10 seconds.
5. No plasma is added to the second suspension to differentiate any granular appearance of the organism from true coagulase clumping.

Figure 8: Procedure of slide test coagulase method.

### 1.2.2. Molecular biology techniques

The presence of MRSA strains was confirmed by the detection of the *mecA* gene, that is considered the *gold standard* by literature, using the Polymerase Chain Reaction (PCR) (Datta *et al.*, 2011; Coban, 2012; Stegger *et al.*, 2012; Montazeri *et al.*, 2015; Chen *et al.*, 2017). Strains that tested negative for the presence of the *mecA* gene were subsequently examined for the presence of the *nuc* gene, which allows the identification of MSSA strains (Sahebnaasagh *et al.*, 2014; Chen *et al.*, 2017).

In this study, the PCR conditions employed were the same as presented in Gonçalves *et al.* (2020), which in turn refers to the work of Koukos *et al.* (2015). So, for the detection of the *mecA* gene, the DNA (5 µl) was amplified in a reaction mix that contained 10 µl of 5x PCR buffer solution, 3 µl of 25 mM MgCl<sub>2</sub>, 2 µl of 10 mM dNTP mixture, 5 µl of each 10 µM primer and 0.25 µl of Taq polymerase in a total volume of 50 µL (Koukos *et al.*, 2015). The primers used for the identification of the *mecA* gene were 5'-AACGATTGTGACACGATAGCC-3' and 5'-GGGATCATAGCGTCATTATTC-3'. PCR conditions were: 10 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 1 minute at 55°C and 1 minute at 72°C; final extension for 10 minutes at 72°C (Koukos *et al.*, 2015).

A similar technique, also based on the work of Koukos *et al.* (2015), was performed for the identification of the *nuc* gene. Primers used in the PCR were 5'-TCAGCAAATGCATCACAAACAG-3' and 5'-CGTAAATGCACTTGCTTCAGG-3' and the PCR conditions were: 10 minutes at 94°C; 35 cycles of 30 seconds at 94°C, 1 minute at 58°C and 1 minute at 72°C; final extension for 10 minutes at 72°C.

Finally, PCR products were visualized by electrophoresis in 1% agarose gel.

### **1.3. Chairside disinfection protocol (CPD)**

Based on Bracciale *et al.* (2020) a disinfection protocol was only performed for each GP point showing contamination. The CPD implemented started with the collection of the GP point from the tube with the aid of sterile tweezers, followings its incubation in an eppendorf tube filled with 10 mL of 3% sodium hypochlorite solution for 60 seconds. After that, the GP point was removed from the eppendorf, with another sterile tweezers, and then rinsed with 10 mL of sterile distilled water. Successively, the point was dried using a sterile gauze and then placed in a new sterile tube containing BHI medium and incubated as described above.

### **1.4. Statistical Analysis**

Statistical analysis of the result was conducted using the IBM® SPSS® Statistics vs. 25.0 software (IBM Corp, released 2017, Amonk, NY). Absolute and relative counts (n and %) were used to describe all qualitative variables. The results of dichotomic variables were conducted using the binomial test. All comparisons were performed using a level of significance of 0.05 (P= 0.05).

### III. RESULTS

From the 315 collected samples, the percentage of contaminated points (1.9%) is extremely trifling, when compared to the uncontaminated ones (98.1%).

Being so, the first null hypothesis was accepted. From the 6 samples that tested positive:

- 2 (0.6%) were from box A (1 – k25 and 1 – k35);
- 1 (0.3%) was from box B (k25);
- 1 (0.3%) was from box 1 (gauge B);
- 2 (0.6%) were from box 2 (gauge B).

All colonies suspected were Gram positive with cocci morphology, catalase positive, and coagulase positive, typical of *S. aureus* (Figure 9: A, B and C, respectively).

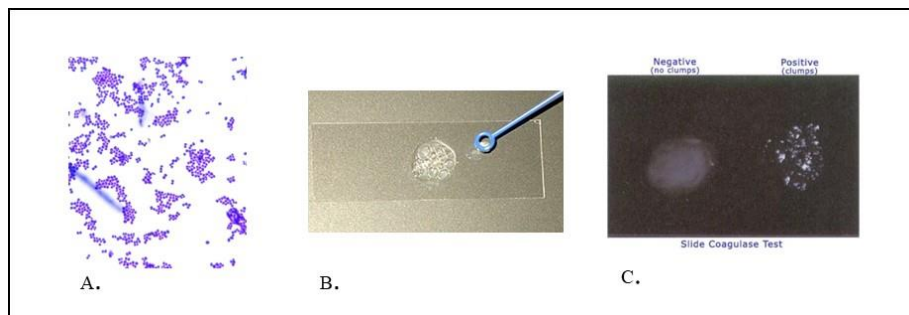


Figure 9: A- Gram-positive with cocci morphology bacteria; B- catalase positive test (<https://en.wikipedia.org/wiki/Catalase>); C- coagulase slide test (<https://laboratoryinfo.com/coagulase-test>).

Detection of the degree of contamination of Gutta-Percha Points by strains of *Staphylococcus aureus* (MRSA/MSSA)

After culturing on MSSA/MRSA selective medium, only 1 of the 6 positive samples (16.6%) showed blue colonies, typical of MRSA (Figure 10), leading to the suspicion of MRSA.



Figure 10: Typical colonies of MRSA and MSSA colonies in chromID®/MRSA/chromID *S. aureus* (Biomérieux) - blue is equivalent to MRSA, while beige corresponds to MSSA.

The suspected MRSA strains were then confirmed by PCR (presence of the *mecA* gene) (Figure 11). The remaining (83.3%) samples showed beige colonies typical of MSSA, confirmed by the presence of the *nuc* gene by PCR.

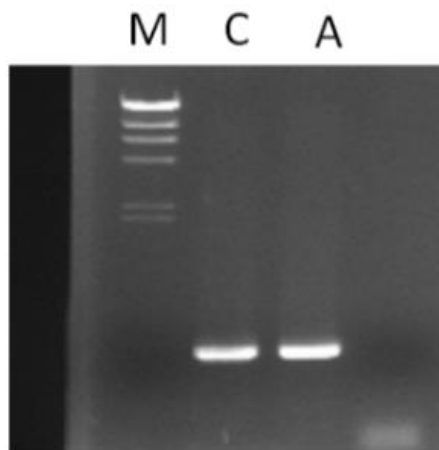


Figure 11: PCR products using primers for the *mecA* gene. M: molecular weight marker (lambda phage DNA cut with Hind III); C: control (MRSA).

The chairside disinfection protocol was effective in 100% (6/6) of the contaminated GP points.

#### IV. DISCUSSION

From the 315 collected samples, only 6 (1.9%) resulted microbial growth positive and 98.1% uncontaminated. After confirmation by PCR, only 1 sample of the 6 cones was a MRSA contamination, and the remaining 5 points were MSSA. These results were expected, since this study was a specific analysis for detection of the degree of contamination of GP points by strains of *S. aureus* (MRSA/MSSA). Also, the results obtained by classical methodology was in agreement with molecular biology techniques by PCR, and that is in accordance with other recent published studies (Gonçalves *et al.*, 2020).

*Staphylococcus aureus* Methicillin-Resistant (MRSA) was described for the first time in England in 1961 (Jevons, 1961), soon after the introduction of methicillin in clinical practice (Harkins *et al.*, 2017). In the beginning, this antibiotic was widely used, however, due to its toxicity, it is not commercialised nowadays for human use and was replaced by similar and more stable penicillins such as oxacillin. However, the term *S. aureus* Methicillin-Resistant is still used (Lee *et al.*, 2018). Due to the widely empirical prescription of antibiotics, bacteria acquire several antibiotic resistances through diverse genetic mechanisms. *Staphylococcus aureus*, especially its Methicillin-Resistant (MRSA) strain, acquires resistance to antibiotics, not only to  $\beta$ -lactams such as penicillin and its derivatives, methicillin/oxacillin, carbapenems and cephalosporins, but also to other classes like macrolides and tetracyclines, that makes it a multi-resistant strain (Gonçalves *et al.*, 2020). Nowadays, MRSA strains are endemic in several health units all over the world and, consequently, have become an important focus of global efforts of infection control. Due to the limited treatment options, these strains have become the biggest cause of nosocomial infections worldwide with high morbidity and mortality rates (Shorr, 2007; Pantosti & Venditti, 2009).

Some clones of *S. aureus* evolved to *S. aureus* Methicillin-Resistant (MRSA) by acquiring, through horizontal genetic transfer, the staphylococcal chromosomal cassette *mec* (SCC*mec*), a mobile genetic element that includes the *mecA* or *mecC* genes that confers resistance to most  $\beta$ -lactam antibiotics, including methicillin (García-Garrote *et al.*, 2013; Skov *et al.*, 2013; McCarthy *et al.*, 2015; Milheiriço *et al.*, 2017; Cikman *et al.*, 2019). In fact, MRSA has a remarkable capacity to acquire resistance to any antibiotic, not only to the  $\beta$ -lactams, that leads to important implications to the present and future options of treatment of infections by this pathogen (Chambers & Deleo, 2010; Lee *et al.*, 2018).

*S. aureus* is frequently associated with skin infections, pneumonia, surgery wounds, bacteraemia, osteomyelitis and endocarditis, being considered one of the most important pathogens of the human being, both at the community level and at nosocomial infections. This pathogenicity is related with a big number of virulence factors, that allow adhesion to surfaces, invasion or avoid the immune system and cause toxic effects in the host (Bien *et al.*, 2011; Shorr, 2007). Among the most important virulence factors are adhesins and exoproteins, such as exotoxins and enzymes (nucleases, proteases, lipases, hyaluronidases and collagenases) (Bien *et al.*, 2011; Merghni *et al.*, 2014; Blomqvist *et al.*, 2015).

The risk of a cross contamination with pathogens strains in clinics are a very serious and important problem in all health fields, like in Dentistry, and all the dental health care professionals must have attention and carefully in avoiding situations that can lead to infection. Cross-transmission of micro-organisms in dental offices via direct contact is an almost unavoidable problem, and one of well-studied micro-organisms is MRSA, which is responsible for a substantial amount of health care-associated infections which are difficult to treat. Both patients and dental health care professionals can serve as a host for micro-organisms; both patients and dental health care professionals can serve as a reservoir for pathogenic microorganisms and both can become infected because of their involvement in dental treatments. Transmission of micro organisms in the dental office may occur by direct contact (including blood-blood contact) or by inhalation/ingestion of the micro-organisms (in bio-aerosols) from dental unit water (Volgenant & de Soet, 2018).

Aerosols as one of the major route of direct or surface contamination, leading to the increase of these strains during patient attendance in dental medicine and consequently to a higher probability of cross infection (Bernardo *et al.*, 2005; Hallier *et al.*, 2010; Kobza *et al.*, 2018). Surfaces in clinics, as well as the attending uniform and the hands of the dental doctor can be MRSA reservoirs. Aiming to control and reduce infection standard measures are suggested including: disinfection of hands before and after patient attendance; use of gloves, mask, cap, glasses and work uniform (Williams *et al.*, 2003; Faden, 2018). Also, the implementation of several disinfection protocols is needed in several specific areas of dental medicine.

It has been recently reported that the transmission dynamics of MRSA within a hospital environment are complex and that the likelihood of contamination with MRSA is possibly associated with the period of time patients stay in the healthcare facility. In dentistry, where the contact time between patients and staff is relatively short, the transmission of MRSA is

expected to be less complex. Several studies in dentistry reported that MRSA is found more often in the nose or on hands of dental students compared to a control group without patient contact, although contradicting findings have been reported. The differences in prevalence may be due to differences between countries, e.g., in applying (hand) hygiene measures (Volgenant & de Soet, 2018).

Within the RCS, when NSRCT is performed, asepsis is impossible to be achieved because there are anatomical irregularities, smear layer formation during the shaping phase of this treatment which contains microorganisms and their products and, as it is attached to the root canal walls, acts as a physical barrier, preventing the entrance of irrigant solutions into dentinal tubules. To overcome this, although still nowadays slightly controversial, there are guidelines that recommend, after the GP point choice, confirmed by tugback and periapical radiography, the execution/application of a final irrigation protocol, to ensure a significant decrease of the microbial load inside the RCS, followed by three-dimensional filling of RCS, intending to create conditions compatible with the handling of periapical pathology of endodontic origin (Siqueira, 2001). According with this author claimed that it is impossible to guarantee the total absence of microorganisms inside the RCS after a NSRCT.

NSRCT has as primary target the creation of conditions to prevent tooth loss, safeguarding whenever possible, the restoration of the tooth and adequate periodontal support. Otherwise, it becomes an absolute contra-indication. An endodontic treated tooth must be functional and NSRCT must prevent secondary endodontic infections.

NSRCT execution has the oral cavity as a scenario, besides the handling of instruments and materials, and aerosols formation, as potential severe ways of cross contamination (Malmberg *et al.* 2016). This can be a hard task if the dentist does not adopt clinical protocols that prevent this undesirable infection. In this way, there is a high risk of GP points contamination. For this reason, this was the major goal of this study: to assess the contamination degree.

GP points contain approximately 20% GP (matrix), 66% zinc oxide (filler), 11% heavy metal sulphates (radiopacifier) and 3% waxes and/or resins (plasticizer) (Belsare *et al.*, 2015). For being biocompatible, this material has cost efficiency and long clinical usage and potential antimicrobial properties, especially owing to their zinc oxide component (Vishwanath & Rao, 2019), it is sale as ready to use, among others. These mentioned characteristics justify the fact that GP is still consider the gold-standard when filling of RCS is to be performed.

Nevertheless, as it has not adherence to root canal walls of dentine, and, moreover, as mentioned before the irregular anatomy of RCS is a handicap to face with, GP point soaked in an endodontic cement is recommended in order to achieve the most three-dimensional possible filling of the once pulpal space.

Particular attention must be paid on GP points handling. Considering that when a GP point is taken from the commercial box, all the others that will not be used in that specific NSRCT are naturally exposed, and that same commercial box of GP points is used for multiple NSRCT, the risk of cross-contamination is a real possibility.

A result that does not even reach 2% of the total number of tested GP points, is exactly what it was hoped, based on previous data of Bracciale et al. (2020).

The typology of GP points analysed is limited to those most frequently used in endodontic treatments in the clinic where the study was carried out, both for the 'main' GP points (size ranging from K20 to K35) and accessory ones (size A and B). Normally, larger cones are rarely used, also dictated by the fact that most NSRCTs involve teeth with closed root apex and/or in patients who are adults and the majority of endodontic treatments performed by pre-graduating students is manual, whose taper is lower than the one achieved with rotary systems.

Another detail that can justify such a minimal percentage of positive results is also the fact that the presence of a specific bacterium has been tested – this was the goal of this research, achieving to replicate the Bracciale earlier study, but focused on MRSA species; to be sure of limiting the possibility of growth errors of other bacteria, different from the one on which this study wanted to focus, the choice was made to use BHI and ChromAgar as valid methods; considering the fact that BHI is a medium that facilitates the growth of *S. aureus* (Wijesinghe et al., 2018), and ChromAgar is a reliable method for detections of Methicillin resistant *Staphylococcus aureus* (MRSA) and the results are available more quickly than other methods (Zurita et al., 2010). *S. aureus* has always been recognized as a possible pathogen of importance for human health, which in recent years has been obtaining even more recognition due to its duality both as asymptomatic bacteria which colonizes various epithelial surfaces, and as potentially deadly pathogen (Howden et al., 2023).

Another relevant aspect of the methodology adopted was that in this study, opposing to Bracciale et al. (2020), each Eppendorf tube contained only one GP point, in order to assess

and, subsequently, conclude which exactly GP points were contaminated. In the previous mentioned study, in each Eppendorf tube contained 4 GP points. Being so, it was impossible to identify what point was really contaminated.

Besides, the gauge of GP points was limited to the ones which are the most used in the clinical environment. These new focused approaches were considered as good and pertinent improvements.

Prevention of cross-infection during an endodontic session relies, among others, on the rubber dam use, disinfection of instruments, attention to minimize the GP packages to exposition, when they are in use (Zahran *et al.*, 2022).

Other, easy and logical, accurate handling of GP points is to use a sterile clamp to pick up them out of the commercial box, instead of the tweezers which is part of the initial material of all Dentistry appointment and that is being used in the endodontic session. Careful must be taken to introduce the GP points directly into the shaped and cleaned root canal, avoiding their contact with any device in use within the operational field.

Because of their thermoplastic properties (Siqueira *et al.*, 2001) and owing to their physical and chemical nature, GP points are not amenable to physical methods of sterilization like hot air oven and autoclaving. To overcome this, it has been proposed by several studies a rapid chairside disinfection protocol resorting to chemical solutions before starting the filling stage of NSRCS (Stabholz *et al.*, 1987; Cardoso *et al.*, 2009; Athiban *et al.*, 2012; Pradeep *et al.*, 2013; Panuganti *et al.*, 2016; Alves *et al.*, 2018).

Already in 2012, the importance of GP decontamination to prevent any bacterial contamination of RCS during the filling procedure was widely recognized in endodontic practice (Athiban *et al.*, 2012). Clearly this disinfection protocol must be, in the best way possible, rapid, reliable, inexpensive and effective.

During the laboratory research, although not initially programmed, it looked worthen to assess a disinfection protocol in the very few positive cases (6) of contamination of GP points. The technique used in this study, based on the previous study already published (Bracciale *et al.*, 2020), is fast and easy to execute.

The “disinfection approach” applied in the laboratory, for the only 6 GP points (1.9%) that showed microbial growth, displayed the desired results. It should be emphasized that is it very

important to pay attention to the correct handling of the GP points during endodontic sessions and to the implementation of a disinfection protocol to avoid the potential risk of contamination and guarantee safer results in endodontic treatments, as well as to the correct storage of GP boxes.

The concentration used in this study (3%) was very effective, but the immersion time of the GP point in the liquid must be kept under control according that structural changes had been already detected with the formation of chloride crystals if after disinfection with NaOCl, the GP points are not washed with sterile water or alcohol. Moreover, loss of elasticity of the GP point can occur if it is immersed in NaOCl for a period longer than 1 minute (Valois *et al.*, 2005; Prado *et al.*, 2011; Tilakchand *et al.*, 2014; Nunes *et al.*, 2019). For this reason, in this study, the choice was to use 3% NaCl for a period of 60 seconds, according to the work of Bracciale *et al.* (2020), where its effectiveness had already been demonstrated. In accordance with the main goal of this research, assessment of potential contamination of GP points, the surface alterations already documented were not analysed.

Many chemicals such as hydrogen peroxide, chlorhexidine, ethyl alcohol, polyvinyl pyrodone iodine, quaternary ammonium compounds have been tried for decontamination.

In future research, it would be interesting to compare the efficacy of different chemical solutions, as well as, within each disinfectant, assess the percentage and time needed to obtain decontamination of GP points. Furthermore, with high significant clinical influence, the analysis of eventual surface alterations of GP points should be performed resorting to techniques of scanning electronic microscopy. For financial limitations, these suggestions were not possible to be executed and, so, do not constitute the main target of this *in vitro* study.

It is of the utmost importance that, among pre-graduating dentistry students, the worth of every effort to maintain and obtain the highest levels of disinfection is fully understood (Jalan & Sandeep, 2023).

## V. CONCLUSIONS

The purpose was to analyse the *S. aureus* (MRSA/MSSA) in the GP points most frequently used in the CMPD-UFP clinics.

The obtained results were what was hoped for, because although very specific when compared to the general area where this study fits, this was the initial intention. Gutta-percha points from opened presterilized packed did show very little amount of contamination by MRSA/MSSA (1,9%). The accurate handling of this filling material must be kept in mind soon since the pre-graduation period, as this methodology should be the gold standard for all professional activity.

The risk for transmission of pathogens, like MRSA strains, in a dental office is still unknown but cannot be considered negligible.

The conclusion drawn is that, even if the contamination is minimal, cases of bacterial growth were observed. Therefore, particular emphasis must be given to the correct handling of the GP points, during NSRCT, but especially to always adopt an effective disinfection protocol to avoid the potential risk of GP points' contamination and guarantee even higher success rates of this conservative treatment option.

A suggestion to continue this study could be the specific analysis of further microorganisms, such as *Enterococcus faecalis* for Gram positive, or *Fusobacterium nucleatum* for Gram negative, and for *Candida albicans* would also be interesting, as would also be to analyse the possible cross-contamination agents, trying to identify and test protocols that are doable in the clinical environment to minimize the risk of contamination.

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Detection of the degree of contamination of Gutta-Percha Points by strains of *Staphylococcus aureus*  
(MRSA/MSSA)

Annex 1



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Exma. Senhora  
Prof. Doutora Sandra Gavinha  
Directora da FCS

Porto, 10 de Dezembro de 2019

Exma. Senhora Prof. Doutora,

A Comissão de Ética, depois de receber, no dia 4 de Dezembro de 2019, o formulário de submissão de projectos à CE devidamente assinado (com data de 2 de Dezembro de 2019), como solicitado na comunicação emitida por esta mesma Comissão no dia 28 de Novembro, e relativa à primeira submissão, do dia 4 de Novembro, apreciou o projeto de investigação em Medicina Dentária de Ana Moura Teles, Cristina Pina e Maria Inês Cardoso, intitulado, "Deteção do grau de contaminação de cones de Gutcha-percha por estirpes de staphylococcus aureus". A Comissão de Ética não tem nada a opor à realização do mesmo.

Com os melhores cumprimentos.

A Presidente da  
Comissão de Ética da UFP

  
Teresa Toldy

*Das cc a' investigadora*

  
11-12-19



Fundação Ensino e Cultura "Fernando Pessoa"

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