RESEARCH ARTICLE

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# Optimizing Antimicrobial Treatment to Cryopreserve and Establish Oral Squamous Cell Carcinoma *In-Vitro* Models Derived from Habitual Tobacco Chewers

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## Abstract

Objectives: Cryopreservation and establishment of reliable in vitro models of oral squamous cell carcinoma (OSCC) are frequently compromised by microbial contamination arising from the oral microbiota of habitual tobacco users. This study aimed to optimize an antimicrobial strategy prior to biobanking to eliminate resistant microbial contaminants and improve the reliability of OSCC primary cultures. Methods: OSCC tissues obtained from habitual tobacco chewers were subjected to either conventional penicillin–streptomycin (Pen-Strep) treatment or a stepwise antimicrobial treatment before cryopreservation. The optimized protocol employed a combination of cell culture-grade antibiotics to selectively eliminate Pen-Strep-resistant microbial taxa. Post-thaw tissue viability and epithelial outgrowth were assessed. Microbial profiling of conventionally treated tissues was performed using 16S and 18S rRNA gene sequencing, and antibiotic susceptibility testing was carried out to determine resistance profiles. Results: Conventional Pen-Strep treatment alone was insufficient to eliminate microbial contamination, whereas the stepwise antimicrobial treatment effectively eradicated resistant microorganisms, enabling sterile tissue preservation. Post-thaw assessments demonstrated epithelial-like outgrowth from day 3 onward, confirming preserved tissue viability and integrity. Microbial profiling identified contaminants including *Neisseria elongata*, *Streptococcus gallolyticus*, and *Candida albicans*. Antibiotic susceptibility testing highlighted the necessity of tailored combinatorial antimicrobial strategies to overcome resistance. Conclusion: The refined biobanking protocol integrating a stepwise antimicrobial strategy prior to cryopreservation effectively mitigates microbial contamination while preserving OSCC tissue integrity. This approach enhances the reliability of OSCC primary cultures derived from tobacco chewers and provides a robust platform for translational oncology research and personalized medicine.

**Keywords:** Oral squamous cell carcinoma (OSCC)- biobanking- antimicrobial resistance- primary culture

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## Introduction

Oral squamous cell carcinoma (OSCC) is a malignant tumor that originates from epithelial cells of the oral cavity. In the United States, OSCC accounts for nearly 3% of all cancer cases as reported by the National Cancer Institute. While OSCC is the most frequent cancer in males and the third most common in women, with about 77,000 new diagnoses and 52,000 deaths per year, nearly a quarter of the global incidence, India bears a disproportionately greater burden [1-3]. This high incidence is largely attributed to the prevalent use of tobacco and betel quid, both well-established etiological factors [4].

Advancements in oral oncology research have paralleled the growing integration of biobanking into oral health studies. Biobanking, which entails the organized collection and long-term storage of human biospecimens accompanied by relevant clinical data, plays a vital role in facilitating translational cancer research [5]. Specimens such as saliva, deciduous and permanent teeth, dental pulp cells, oral biopsies, buccal swabs, and oral rinses are among the most commonly preserved in oral health biobanks [6]. However, tissue biobanking in OSCC poses unique challenges and cross contamination due to the complex and dense microbial environment of the oral cavity.

The oral microbiome harbors an extensive array of microorganisms, including *Streptococcus mutans*, *S. oralis*, Actinomyces gerencseriae, and Fusobacterium nucleatum, which are typically susceptible to antibiotics such as penicillin-streptomycin (Pen-Strep) used in cell culture [7, 8]. Yet, in OSCC patients based on the habitual

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users such as tobacco chewers,significant alterations in microbial composition occur, often involving Pen-strep-resistant strains such as Veillonella spp. and S. sanguinis [9]. Shifts in microbial prevalence have also been noted, with organisms such as Streptococcus anginosus, Prevotella melaninogenica, and Pseudomonas aeruginosa demonstrating varied patterns of antibiotic sensitivity [10, 11]. Such microbial imbalances, influenced by risk factors including tobacco use, alcohol consumption, human papillomavirus (HPV) infection, and host genetics can interfere with the successful establishment of primary cultures by fostering the persistence of antibiotic-resistant microbes in the oral cavity [12, 13]

Additionally, long-term storage in liquid nitrogen biobanks poses a risk of cross-contamination, threatening sample sterility [14]. These challenges can lead to overgrowth by resistant bacteria, or fungi, ultimately compromising the success and integrity of primary epithelial cell cultures [15].

To address these challenges, we developed a methodology incorporating microbial profiling, tailored antimicrobial treatments, and contamination control measures keeping the tissue viability to ensure reliable biobanking of OSCC tissues from chronic tobacco chewers for downstream clinical and translational research.

## Materials and Methods

The methodology adopted in this study is outlined in Figure 1. For this study, subjects were selected based on thorough clinical examination conducted by a maxillofacial prosthodontist and a surgical oncologist. Among the 15 individuals screened, (n = 10) were categorized as tobacco consumers. The samples were obtained from patients at the MS Ramaiah Institute, Bangalore, India. None of the selected subjects exhibited discernible signs of inflammation, mucosal diseases, or underlying systemic conditions such as diabetes or genetic disorders. Upon obtaining consent from the subjects for clinical examination and sampling, biopsies were performed to obtain tissue samples. Subsequently, these tissue samples were analyzed and oral carcinoma was confirmed by a pathologist. Exclusion criteria for this study excluded individuals under 18 years of age, medically unfit individuals, completely edentulous individuals, and those who have previously undergone treatment for cancer.

*Ethical Consideration*

The study adhered to the World Medical Association's Code of Ethics (Declaration of Helsinki). Data regarding medical history, age, gender, employment, smoking and alcohol habits, and general oral hygiene were documented. All participants provided written, informed consent before sample collection. Ethics approval for the study was obtained from the Ethics Committee of M S Ramaiah University of Applied Sciences (EC-24/95-F-FDS).

*Oral squamous carcinoma Tissue Samples: Procurement*

Prior to sample collection, individuals were instructed to refrain from eating, drinking, or performing oral hygiene routines for a minimum of one hour. During the sample collection, patients were asked to rinse their mouth with sterile normal saline for 30 seconds. Oral tissue samples (n = 10) were collected from individuals with a history of tobacco chewing, and the presence of cancer was confirmed via biopsy. The samples were transported in a medium comprising basal DMEM supplemented with 2 % glucose, 1 % penicillin-streptomycin, adjusted to a pH of 7.2, and appropriately labeled for identification. Samples were then transported in an insulated cold storage unit at 4°C from the operating room to the culture laboratory within a two-hour window for further processing.

*Cryopreservation and Establishment of Primary Culture of OSCC tissue samples Conventional Method*

Initially, five OSCC tissue specimens (~6 mm) were obtained from chronic tobacco users and processed immediately under sterile conditions. Samples were washed with phosphate-buffered saline (PBS) and debrided, then incubated in 1% penicillin-streptomycin for 30 minutes. Each tissue was aseptically divided into two equal portions. First portion (~3 mm) was cryopreserved as described [16]. Briefly, it was placed in cryovials with 500 µl of cryoprotectant (10% DMSO, 10% propylene glycol, 10% FBS), subjected to slow freezing at 1°C/min using Mr. Frosty, initially held at −20°C, then −80°C, and finally stored in liquid nitrogen vapor (−196°C) for at least three months.

The second portion (~3 mm) of five tissues was minced into ~1 mm fragments and seeded into 35-mm culture dishes (HiMedia) containing complete growth medium: DMEM/Ham's F-12 (1:1) supplemented with 10% FBS, 2% glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 6 mM L-glutamine, 10 µg/ml insulin, 20 ng/ml EGF, and 0.5 µg/ml bFGF. Cultures were maintained at 37°C in 5% $CO_2$, with medium changes every three days. Cell outgrowth was monitored microscopically. After 24, 48, and 72 hours, microbial contamination was observed in three samples (S1–S3), which were used for microbial culture, isolation, and identification, whereas two samples remained free of contamination. Complete media without tissue served as a negative control.

*Establishment of microbial cultures from oral cancer tissue*

Microbial cultures were established according to the protocol as described with few modifications [17]. The cultured medium collected from S1, S2 and S3 at 24, 48, and 72 hours of cell culture were inoculated in YEPD and nutrient broth and incubated for 24 hours at 37°C. The population of pen-strep resistant microbial culture obtained in the respective broths were then streaked on YEPD agar plates with 1 µl/ml Ampicillin for fungal isolation and nutrient agar plates for bacterial isolation and incubated at 370C for 24 hours. Colonies obtained were morphologically identified and streaked onto the fresh plates to obtain pure culture. Pure colonies from the samples S1, S2, and S3 were picked from the streaked plates of YEPD and nutrient agar and further streaked onto the fresh plates respectively by quadrant streaking method. The pure colonies obtained from S1 to S3 were

morphologically assessed and subjected to staining for further confirmation. Periodic acid-Schiff (PAS) staining was performed on the colonies isolated from S1 and S2 YEPD plates wherein, isolates from S2 and S3 of nutrient plates were subjected to Gram staining to differentiate Gram-positive and Gram-negative bacteria. Morphometric analysis was conducted on pure colonies isolated from S1, S2 and S3.

*Morphometric Analysis of Microbial Structures Using Jenoptik Gryphax Image Analysis*

Morphological parameters including dimensions of spores, cocci, and bacilli were assessed using the Jenoptik Gryphax image analysis program v2.1 Jena, Germany. The line-measuring, free-form, and 3-point-circle measurement tools were utilized for this analysis. For each organism, the widths of the hyphae were measured, as well as the spore sizes. The diameter of fungal spores and cocci was determined using the three-point-circle measuring method. Bacilli width was measured by drawing a line along each cell using the line-measurement tool. Morphometric analysis was conducted on 50 high-power fields, and the average values were tabulated. Based on these values, a size range for each parameter was established for the three organisms under investigation [18].

*Identification of microbial isolates using genomic sequencing*

Genomic sequencing was employed to identify the pure microbial isolates cultured on YEPD and nutrient agar plates. Since both colonies in S1 and S2 on YEPD plates showed candida-like colonies, only S1 was sequenced. Isolates from nutrient agar plates of both S2 and S3 were sequenced. The DNA isolation and sequencing methods were conducted as described [19]. Initially, DNA isolation was performed using the EXpure Microbial DNA Kit developed by Bogar Bio Bee Pvt Ltd as per the manufacturer's guidelines. The DNA concentrations were measured by Qubit Fluorometer 3.0. Bacterial 16S rRNA were amplified for the S2 & S3 via PCR with specific primer sets - 27F (5' AGAGTTTGATCTGGCTCAG 3') and 1492R (5' TACGGTACCTTGTTACGACTT 3'). Amplification of the fungal 18S rRNA was achieved through PCR using specific primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') ITS4 (5' TCCTCCGCTTATTGATATGC 3'). For the sequencing phase, PCR products were purified using Montage PCR Clean to eliminate unincorporated primers and dNTPs, and sequenced using ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (AppliedBiosystems). Single-pass sequencing was performed on each template using above 16S rRNA and 18S rRNA universal primers respectively. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer.

*Data preprocessing*

The 16S rRNA and 18S rRNA data was subjected to BLAST using the NCBI similarity tool. Phylogenetic analysis was performed on the query sequence along with closely related sequences from the BLAST results, following multiple sequence alignment using MUSCLE 3.7 [20]. The aligned sequences were subsequently refined using Gblocks 0.91b to remove poorly aligned positions and divergent regions, thereby enhancing the quality of the alignment for downstream phylogenetic analysis [21]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.1. PhyML has been demonstrated to be as accurate as, if not more accurate than, other phylogenetic programs while being significantly faster. The program TreeDyn 198.3 was used for tree rendering [22].

*Determination of the optimal concentrations of antibiotic combinations*

The antibiotic susceptibility testing was performed to determine antibiotic combinations based on the established Kirby-Bauer protocol with a few modifications [23]. Pure colonies of bacteria and fungi which were identified by Genome sequencing were streaked on the Mueller-Hinton agar plates for antibiotic susceptibility testing. Firstly, recommended concentrations of antibiotics and antimycotics, namely Chlorhexidine gluconate (1 µg/ml) (Himedia), Gentamicin (30 µg/ml) (Himedia), and Amphotericin B (10 µg/ml) (Himedia), were infused into blank antibiotic disks respectively, air dried in aseptic conditions and stored at 4°C until use. A 0.5 McFarland standard inoculum was prepared from overnight cultures, and each isolate was streaked evenly onto Mueller-Hinton agar plates according to established protocols. The antibiotic disks were then placed onto the inoculated plates, gently pressed, and incubated at 28°C for 72 hours. The subsequent measurement of zones of inhibition around each antibiotic and antimycotic disk was measured in mm after 72 hours, and the results were analyzed adhering to established guidelines for antibiotic susceptibility testing.

*Determination of microbial sensitivity to combined antimicrobial concentrations*

Antibiotic susceptibility testing was performed based on the established Kirby-Bauer protocol on Mueller-Hinton agar [23]. Antibiotic and antimycotic combinations, Amphotericin B (10 µg/ml), Pen-strep (100 U/ml penicillin, 100 µg/ml streptomycin), and Gentamicin (30 µg/ml) were incorporated into single blank disks. The disks were air-dried under aseptic conditions and stored until use. A 0.5 McFarland standard inoculum was prepared from overnight bacterial and fungal cultures. Each isolate was uniformly streaked onto Mueller-Hinton agar plates following established protocols. The prepared antibiotic disks were carefully placed onto the inoculated plates, which were subsequently incubated at 28°C for 72 hours. Post-incubation, zones of inhibition surrounding each disk were measured in millimeters. The results were interpreted according to standard criteria and classified as susceptible (S), intermediate (I), or resistant (R).

*Cryopreservation and Establishment of Primary Culture of OSCC tissue samples: Optimisation of a Stepwise Protocol*

Following antimicrobial susceptibility profiling, five newly collected OSCC tissue samples were aseptically divided into three equal portions (~3mm). Firstly, each tissue was centrifuged at 1500 rpm for 5 minutes in DMEM supplemented with 10% fetal bovine serum (FBS) to remove blood and debris. The first portion was subjected to conventional antibiotic treatment. The second and the third portion underwent a stepwise antimicrobial pretreatment: the tissue pellet was treated with 1 µg/ml chlorhexidine gluconate for 30 minutes, followed by three washes with sterile PBS. It was then incubated for 2 hours in basal medium supplemented with 30 µg/ml gentamicin and 10 µg/ml amphotericin B. After incubation, the tissue was again washed with PBS. The second portion was immediately cultured in 35-mm tissue culture dishes containing DMEM/Ham's F-12 medium (1:1), supplemented with 10% FBS, 1× L-glutamine, 5 µg/ml insulin, 10 ng/ml basic fibroblast growth factor (bFGF), and 20 ng/ml epidermal growth factor (EGF). An optimized antimicrobial cocktail comprising 100 U/ml penicillin, 100 µg/ml streptomycin, 30 µg/ml gentamicin, and 10 µg/ml amphotericin B was added to the growth medium. Cultures were incubated at 37 °C with 5% $CO_2$ for 72 hours without disturbance. Daily observations were performed, and the medium was refreshed every three days. Once cell outgrowth from the tissue was observed, the culture medium was switched to basal medium containing only penicillin and streptomycin, excluding gentamicin and amphotericin B. The third portion was cryopreserved using the modified protocol and stored at –196 °C for three months [16].

*Thawing of cryopreserved OSCC tissue*

Conventional and stepwise antimicrobial-treated OSCC cryopreserved tissue samples were thawed rapidly by placing the cryovials in a 37°C water bath for 3 minutes with gentle agitation. Immediately after thawing, 1 ml of pre-warmed DMEM/Ham's F-12 (1:1) medium was added directly into each cryovial. The contents were then transferred to a sterile 15 ml conical tube containing 5 mL of complete growth medium. The tubes were centrifuged at 1,500 rpm for 5 minutes at room temperature to pellet the tissue fragments. Following centrifugation, the supernatant was discarded, and the pellet was washed twice with sterile PBS to remove residual DMSO and propylene glycol. The washed tissue was then processed and cultured as described above.

## Results

*Microbial Contamination in Conventionally Treated and Cryopreserved OSCC Tissues*

Among the five samples analyzed using the conventional method, 3 samples (S1, S2, and S3) exhibited microbial contamination resistant to penicillin–streptomycin, while two samples (S4 and S5) were free from contamination. At 24, 48, and 72 hours, microbial structures from the contaminated samples (S1, S2, and S3) were collected and examined under a phase-contrast microscope at 10× magnification.

Fungal contamination was identified in S1 and S2, as evidenced by the presence of hyphae, characterized by branching, thread-like tubular structures, and spores (Figure 2 A,B). Bacterial contamination was confirmed in S2 and S3, indicated by the observation of cocci and rod-shaped bacterial structures (Figure 2 C, D). Similarly, upon thawing and subsequent culture of conventionally cryopreserved OSCC tissues, both fungal and bacterial contamination became evident within 72 hours. This suggests that the conventional treatment with penstrep prior to cryopreservation does not inherently eliminate microbial contaminants (Figure 2 E, F,G,H). These findings reinforce the necessity for implementing preemptive antimicrobial treatment strategies before cryopreservation to ensure the sterility and reliability of cryopreserved OSCC tissue models for downstream applications.

*Morphological Insights into Conventionally Treated OSCC Tissues*

Microbial contaminants were successfully isolated from conventionally treated OSCC tissue samples, exhibiting morphologically distinct fungal and bacterial colonies on YEPD and nutrient agar plates. These colonies were preliminarily characterized based on their macroscopic appearance, color, and microscopic staining properties.

Fungal colonies obtained from samples S1 and S2 appeared as dome-shaped, white, velvety growths on YEPD agar (Supplemental Figure SIA) and demonstrated periodic acid–Schiff (PAS)-positive staining, indicative of Candida albicans spores (Supplemental Figure SIB). In contrast, S2 produced smooth, shiny colonies on nutrient agar, which were composed of Gram-positive cocci (Supplemental Figures SIC and SID). S3 displayed irregular, yellow-pigmented colonies with Gram-negative bacilli morphology (Supplemental Figures SIE and SIF). Morphometric analysis revealed spore sizes in S1 and S2 ranging from 2.581 to 10.070 µm, cocci in S2 ranging from 0.4042 to 0.8172 µm, and bacilli in S3 measuring between 1.438 and 1.653 µm (Table 1). These morphological differences highlight the diversity and complexity of microbial contaminants encountered in primary OSCC cultures.

*Microbial Profiling and confirmation through 16S and 18S rRNA gene Sequencing and BLAST Analysis*

In this study, 16S rRNA and 18S rRNA gene sequencing was employed from isolates of S1,S2 and S3 to characterize the microbial composition, to further elucidate and confirm the identities of these microbial entities, a subsequent BLAST analysis (Basic Local Alignment Search Tool Nucleotide) was conducted. The outcomes of the BLAST analysis provide a molecular fingerprint, verifying the presence of *Neisseria elongata*, *Streptococcus gallolyticus*, and *Candida albicans* and shedding light on their genetic characteristics and

Table 1. Morphometric Analysis of Mean Size of the Spores/Rod Shape/Cocci of 50 Microscopic Fields (P Value)

| | Sample 1 | | Sample 2 | | Sample 2 | | Sample 3 | |
|---|---|---|---|---|---|---|---|---|
| | Spores (µm) | | | | Cocci (µm) | | Bacilli (µm) | |
| | Smallest | Largest | Smallest | Largest | Smallest | Largest | Smallest | Largest |
| | 2.581 | 10.07 | 2.022 | 3.347 | 1.438 | 1.653 | 0.4042 | 0.8172 |
| Average | 6.3255 | | 2.6845 | | 1.5455 | | 0.6107 | |

potential implications within the context of the specific environment or host from which the samples were derived.

Upon extraction of the genomic DNA from S1, PCR was used to amplify the 18S rRNA gene. The internal transcribed spacer (ITS) region was sequenced and was then subjected to BLAST to identify the fungal species. BLASTn analysis results revealed 100 hits with a similarity greater than or equal to 200 of alignment scores (Figure 3A). The query sequence, comprising 488 bases, exhibited alignment with a subject sequence of 522 bases.The overall alignment showed 99 % identities with no gaps or missing residues, showcasing a high degree of sequence conservation and structural integrity with Candida sp. Additionally, the E-value for this alignment was zero, signifying a very low probability of encountering false positives and indicating a high confidence in the observed alignment scores. (Supplemental Figure SII). Following the identification of Candida sp., a phylogenetic relationship analysis was conducted. This analysis identified various strains of Candida sp., shedding light on the genetic relatedness and diversity within the Candida genus (Figure 3B).

Following the extraction of the bacteria's genomic DNA from S2, the 16S rRNA gene was amplified by PCR. Upon purification, the resultant 16S amplicons were sequenced. Sequences acquired from single-pass sequencing were subjected to BLAST. The BLAST analysis of the 16S ribosomal RNA gene from S2 was conducted against 100 subject sequences. A total of 169 hits were identified. The alignment showcased Streptococcus sp. and a remarkable 98 % identity between the sequences, with only a minimal 1 % occurrence of gaps, implying a *S. gallolyticus* strain and the sequence similarity between the query (1073 bases) and the subject sequence (819,994 bases) yielded an alignment score of 1929 bits (Figure 3C). The E-value was determined to be zero. High degree of sequence conservation with limited structural variations (Supplemental Figure SIII). The phylogenetic tree identified distinct branches, with S. pasterurianus clustered in one branch and *S. gallolyticus*, *S. gallolyticus*, and S. macedonicus in another. This branching pattern elucidates the genetic relatedness among these Streptococcus sp., highlighting both shared ancestry and potential divergences (Figure 3D).

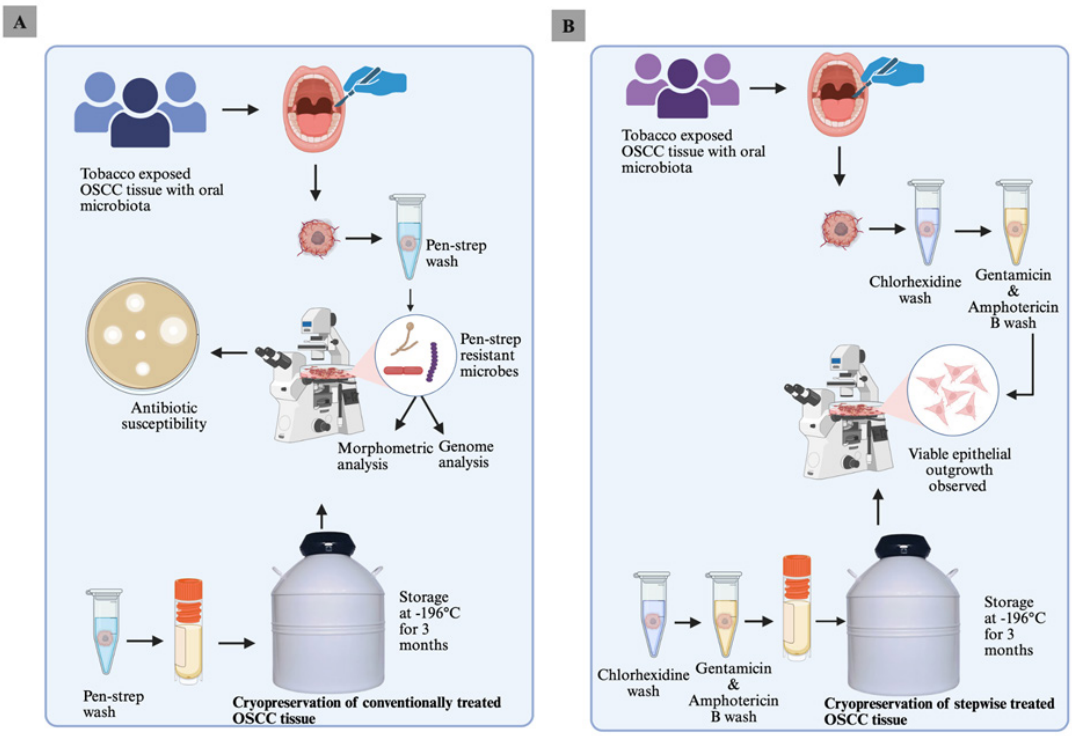


Figure 1. Schematic Representation of Conventional versus Stepwise Treatment Approaches for Culturing and Cryopreservation of Tobacco Exposed OSCC Tissues. (A) Conventional pen-strep treatment during culture, prior to cryopreservation, and after thawing resulted in the survival of resistant microbes (B) Stepwise treatment with chlorhexidine, gentamicin, and amphotericin B during culture, prior to cryopreservation, and after thawing preserved viable epithelial tissue without microbial contamination. Created in BioRender. Ganapathy, K (2025) https://BioRender.com/0s1cwtk

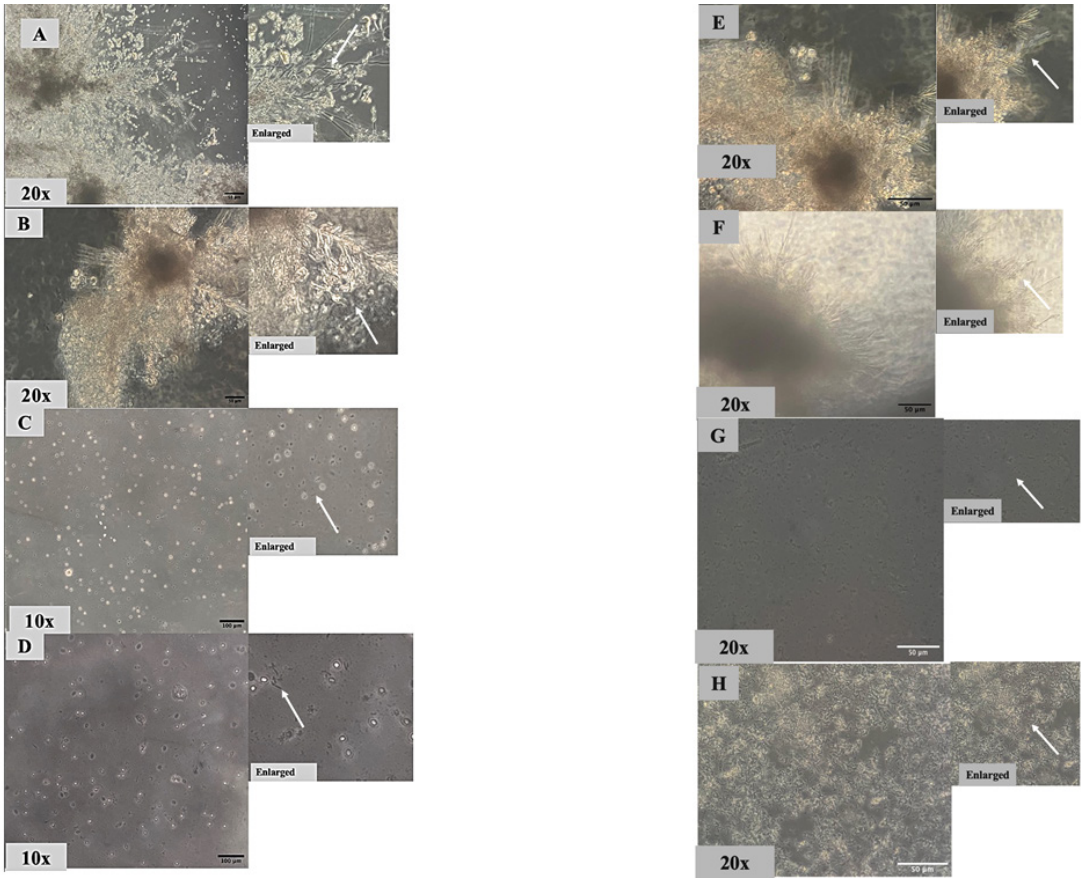


Figure 2. Phase-Contrast Microscopy Images of Microbial Contaminants Observed in Fresh and Cryopreserved OSCC Tissue Sample Cultures Isolated from Tobacco Chewers after 72 Hours of Incubation in Complete Medium Containing Penicillin-Streptomycin (Pen-Strep).(A–D) Fresh tissue cultures: (A) Tissue from S1 and (B) tissue from S2 exhibit hyphae and spores, as highlighted in the magnified insets. (C) Tissue from S2 displays coccoid microbial morphology, while (D) tissue from S3 shows rod-shaped bacterial forms. Images A and B were captured at 20× magnification, and images C and D at 10×.(E–H) Cryopreserved tissue cultures: (E) Tissue from S1 and (F) tissue from S2 display extensive hyphal growth with spore-like structures, shown in the enlarged insets. (G) Tissue from S2 reveals coccoid morphology, and (H) tissue from S3 exhibits rod-shaped bacterial forms. All images were captured at 20× magnification.

Similarly, genomic DNA from bacteria isolated from sample S3 was extracted and subjected to BLAST analysis of the 16S ribosomal RNA gene against 100 subject sequences revealed 145 hits, with a similarity greater than or equal to 200, represented by red lines (Figure 3E). The alignment exhibited 100 % identities to *N. elongata* strain 14 with no gaps or missing residues, suggesting a high degree of conservation. The query sequence, with 1190 bases, aligned with a subject sequence of 1260 bases, resulting in an alignment score of 2198 bits. The E-value (Expected value) for the alignment was zero. This implies a very low probability of obtaining the observed alignment scores by chance. In practical terms, an E-value of zero indicates high confidence in the alignment, suggesting a significant match between the query and subject sequences. (Supplemental Figure SIV). The phylogenetic tree results provide a comprehensive insight into the evolutionary relationships among Neisseria species, particularly highlighting the placement of *N. elongata* strain 14 within the broader phylogenetic context. The identified branches in the tree reflect genetic relatedness based on the analyzed 16S ribosomal RNA gene sequences (Figure 3F).

Table 2. Kirby-Bauer Disk Diffusion Antibiotic Susceptibility Test: Antibiotic susceptibility of *Streptococcus gallolyticus, Neisseria elongata*, and *Candida albicans* was assessed using the Kirby-Bauer disk diffusion method. The table presents the zone of inhibition (diameter in mm) for each antimicrobial agent tested at the specified disk concentrations. Susceptibility was categorized as S (Susceptible), R (Resistant), or I (Intermediate) based on standard interpretative criteria.

| Antimicrobial | Disk concentration | S. gallolyticus | | N. elongata Subsp | | C. albicans | |
|---|---|---|---|---|---|---|---|
| | | Zone Dia (mm) | S, R or I | Zone Dia (mm) | S, R or I | Zone Dia (mm) | S, R or I |
| Chlorhexidine gluconate | 1µg/mL | 8 | Intermediate | 8 | Intermediate | 0.4 | Resistant |
| Gentamicin | 30µg/mL | 20 | Susceptible | 12 | Susceptible | 0 | Resistant |
| Amphotericin B | 10µg/mL | 0 | Resistant | 0 | Resistant | 12 | Susceptible |

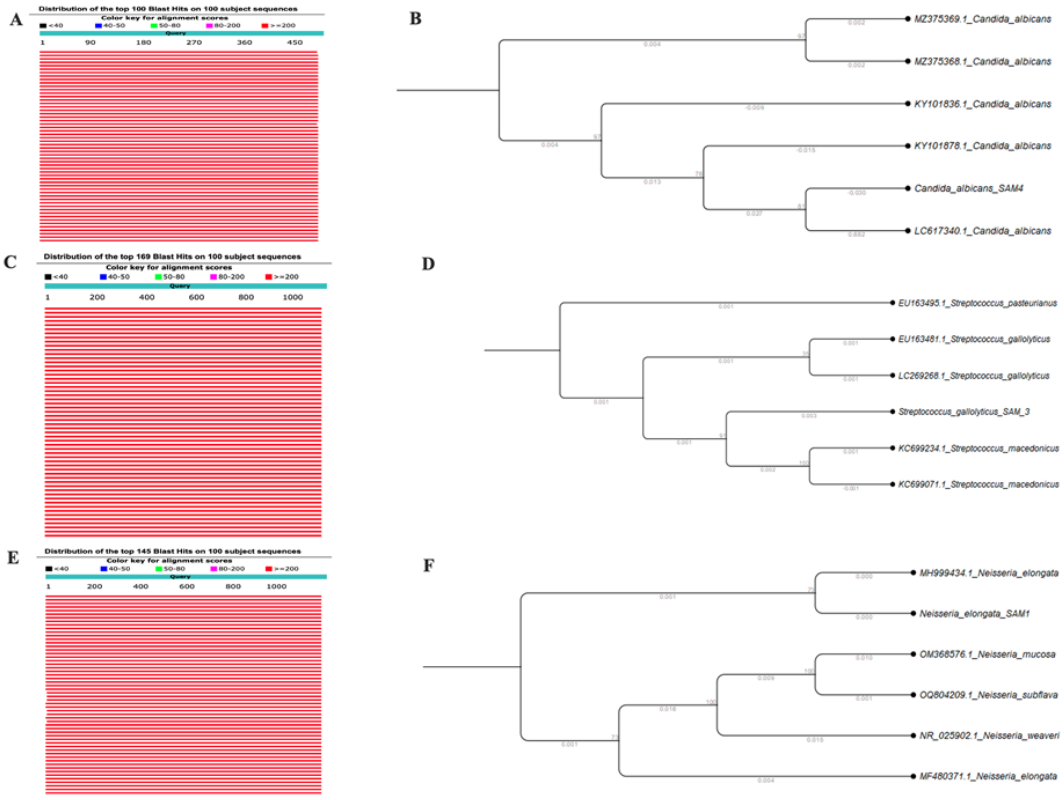


Figure 3. Represents the Sequencing Data of Microbial Contaminants from OSCC Tissue Samples (S1, S2 and S3) (A) Top 100 BLAST hits for sequencing data of S1; red lines indicate alignment scores ≥200. (B) Phylogenetic relationship of Candida sp. based on S1 sequences. (C) Top 169 BLAST hits for S2 sequences; red lines indicate alignment scores ≥200. (D) Phylogenetic relationship of Streptococcus sp. based on S2 sequences. (E) Distribution of the top 145 BLAST hits for S3 sequences; red lines indicate alignment scores ≥200. (F) Phylogenetic relationship of Neisseria sp. based on S3 sequences.

*Antibiotic Susceptibility Test*

The antimicrobial susceptibility assay was conducted to evaluate the inhibitory effects of various antimicrobial agents on *S. gallolyticus*, *N. elongata*, and *C. albicans* (Figure 4, Table 2). Amphotericin B, an antifungal agent, exhibited susceptibility against C. albicans, with a zone of inhibition (ZoI) of 15 mm, aligning with manufacturer-established susceptibility criteria (susceptible ≥15 mm, intermediate 10–14 mm, and resistant ≤10 mm). However, both *S. gallolyticus* and *N. elongata* subsp. demonstrated complete resistance to amphotericin B, highlighting its limited efficacy against these bacterial species. Gentamicin exhibited susceptibility against *N. elongata* subsp., with a ZoI of 12 mm, while *S. gallolyticus* demonstrated a ZoI of 20 mm, indicating susceptibility. In contrast, *C. albicans* was resistant to gentamicin. Chlorhexidine gluconate displayed intermediate resistance in *S. gallolyticus* and *N. elongata* subsp., with ZoI values of 8 mm for both species. However, *C. albicans* exhibited resistance, with a ZoI of 0.4 mm.These findings emphasize the diverse susceptibility patterns of different microorganisms, underscoring the importance of tailored antibiotic selection based on specific resistance mechanisms.

*Antimicrobial Susceptibility of S. gallolyticus, N. elongata, and C. albicans to a Combined Antibiotic Treatment*

Antimicrobial susceptibility testing was performed using a disk diffusion assay to evaluate the efficacy of a combination therapy comprising penicillin-streptomycin (100 µg), gentamicin (30 µg), and amphotericin B (10 µg) against *S. gallolyticus*, *N. elongata*, and C. albicans. The combination exhibited enhanced antimicrobial activity, as evidenced by the formation of clear inhibition zones around the disks. *S. gallolyticus* exhibited a ZoI of 39.5 mm, indicating susceptibility to the combination treatment. *N. elongata* demonstrated a ZoI of 25 mm, also

Table 3. Antimicrobial Susceptibility for *S. gallolyticus, N. elongata*, and *C. albicans* Using a Combination of Penicillin-Streptomycin (100 µg/mL), Gentamicin (30 µg/mL), and Amphotericin B (10 µg/mL). The table presents the zone of inhibition (in mm) and interpretation of susceptibility (S: Susceptible, R: Resistant, I: Intermediate) for each organism.

| Antimicrobial | Disk Concentration | S. gallolyticus | | N. elongata | | C. albicans | |
|---|---|---|---|---|---|---|---|
| | | Zone Dia (mm) | S, R or I | Zone Dia (mm) | S, R or I | Zone Dia (mm) | S, R or I |
| Penicillin-streptomycin, Gentamicin and Amphotericin B | 100µg/mL + 30µg/mL + 10µg/mL | 39.5 | Susceptible | 25 | Susceptible | 15 | Susceptible |

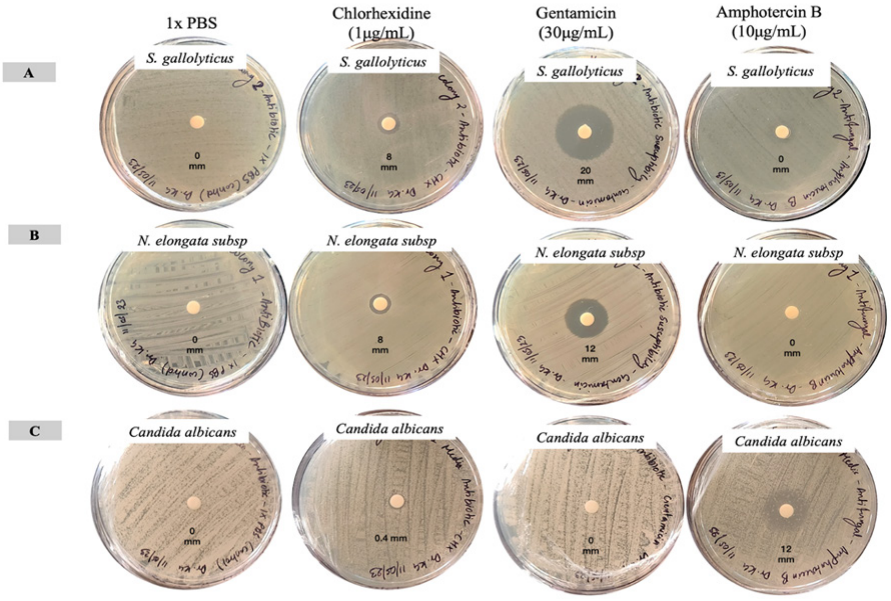


Figure 4. Kirby Bauer's Disk Test for Antibiotic Susceptibility A) *S.gallolyticus*, from S2 B) *N.elongata* from S3 and C) *C. albicans* from S1 grown on Muller-Hinton agar and tested against chlorhexidine (1 µg/mL), gentamicin (30 µg/mL) and Amphotericin B (10 µg/mL)

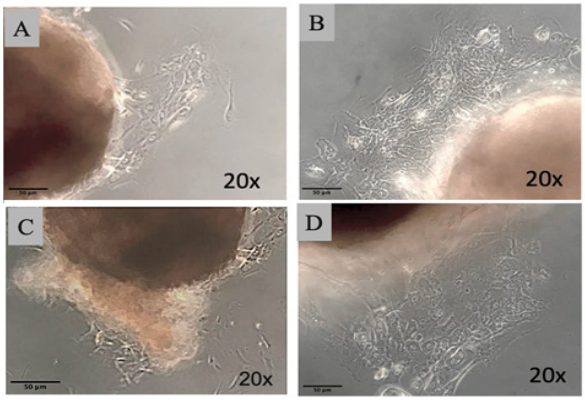


Figure 5. Represents Phase-Contrast Images of New Oral Tissues from Patient-Derived OSCC Samples S5 and S6 that are Cultured and Cryopreserved Using Stepwise Treatment. (A) Represents cell growth of fresh S5 tissue at Day 8 (B) Represents cell growth shown of fresh S6 tissue at Day 8 (C) Represents cell growth of cryopreserved S5 tissue at day 8 in culture (D) Represents cell growth of cryopreserved S6 tissue day 8 in culture. Control samples were contaminated hence was discarded on day 3. Images were captured at 20× magnification.

suggesting susceptibility, while *C. albicans* displayed a ZoI of 15 mm, confirming its sensitivity to the treatment. These inhibition zones were notably larger compared to those observed when the antibiotics were tested individually. These results emphasize the effectiveness of the combined approach in managing caused by these microbes, given the variability in susceptibility patterns often observed in clinical settings (Supplemental Figure SV, Table 3).

*Efficacy of Stepwise Antimicrobial Treatment in Establishing and Biobanking Contamination-Free OSCC Cultures*

Oral tissues from five different patient-derived OSCC samples were processed as three portions. The first portion, which was treated conventionally with penicillin-streptomycin, exhibited both bacterial and fungal contamination after 72 hours of culture, preventing the establishment of viable primary cultures.

Oral tissues from five different patient-derived OSCC samples were processed as three portions. The first portion, which was treated conventionally with penicillin-streptomycin, exhibited both bacterial and fungal contamination after 72 hours of culture, preventing the establishment of viable primary cultures (Supplemental Figure. SVI).

In contrast, the second portion underwent a stepwise antimicrobial pretreatment. These tissues were initially treated with 1 µg/ml chlorhexidine gluconate for 30 minutes and subsequently incubated for 2 hours in growth medium supplemented with a customized antimicrobial cocktail consisting of 100 µg/ml streptomycin, 100 U/ml penicillin, 30 µg/ml gentamicin, and 10 µg/ml amphotericin B. Initial epithelial-like outgrowth appeared as early as day 3, with rapid proliferation observed by day 8, in the absence of any microbial contamination. These cultures were successfully maintained long-term using only penicillin-streptomycin, without the need for additional antifungal or antibacterial agents (Figure 5A, B).

The third portion, cryopreserved after undergoing the same stepwise pretreatment, was thawed after three months and re-cultured under the same optimized conditions. Post-thaw cultures demonstrated healthy epithelial-like cell outgrowth from day 3 onward and remained free of microbial contamination (Figure 5C, D). These findings collectively demonstrate that the stepwise protocol is highly effective in eliminating penicillin-

streptomycin resistant contaminants and facilitates both contamination-free biobanking and reliable downstream culture of primary OSCC tissues.

## Discussion

In this study, we established a cryopreservation and primary culture protocol specifically tailored for OSCC tissues from chronic tobacco users. Cryopreservation is a commonly used approach in biobanking to store mammalian cells and tissues, maintaining their long-term viability and reducing the risk of genetic drift [24]. However, microbial contaminants may remain after clinical specimens are cryopreserved, which could complicate downstream cell processing and cause cross-contamination within biobanks. Due to their rich microbial diversity, human oral and cervical tissues are more vulnerable to microbial contamination [25]. Microorganisms exhibit remarkable resilience during cryogenic storage; as both bacteria and fungi remain viable under liquid nitrogen storage [26-28].

Therefore, tissues are generally processed and subjected to antimicrobial treatment prior to cryopreservation. Standard protocols typically employ penicillin–streptomycin, and in some cases gentamicin and amphotericin B, to suppress microbial contamination [29]. However, in samples with a high microbial load, greater resistance, or diverse microbial communities, these treatments may be insufficient [30]. In our study, microbial isolates from 3 out of 5 tobacco-associated tissue samples freshly cultured using the conventional method, exhibited resistance to penicillin and streptomycin, likely reflecting the resistant microbial populations. Tobacco chewers are known to host varied microbial communities enriched with resistant strains, with tobacco acting as a reservoir; among the predominant genera frequently identified are Bacillus, Pseudomonas, and Staphylococcus [31-34]. Similarly, it was reported that chewable tobacco samples showed a particularly high prevalence of Bacillus species, while other frequently detected genera included Staphylococcus, Virgibacillus, Halobacillus, Prevotella, Corynebacterium, and Streptococcus [35]. Several of these taxa have been associated with resistance to multiple antibiotic classes, such as methicillin, macrolides, tetracyclines, chloramphenicol, and streptomycin [36]. In our study, OSCC tissue samples from habitual tobacco chewers that were treated with penicillin-streptomycin prior to cryopreservation still exhibited microbial growth after thawing, mirroring the contamination observed in freshly cultured, conventionally treated samples. Microbial profiling using 16S and 18S rRNA sequencing identified *Neisseria elongata*, *Streptococcus gallolyticus*, and *Candida albicans* in the three samples as major microbial contaminants within OSCC cultures after treatment with pencillin-streptomycin. Based on these findings, we present data supporting a tailored antimicrobial combination that effectively eliminates penicillin-streptomycin resistant microbes while preserving tissue viability.

Among commonly used antiseptics, povidone-iodine and chlorhexidine gluconate are known for their distinct modes of action and areas of application. Povidone-iodine exerts its antimicrobial effect through the gradual release of free iodine, which acts as a potent oxidizing agent that interferes with microbial metabolism and damages cell membranes [37]. In contrast, chlorhexidine gluconate targets microbial cell membranes through electrostatic interactions, causing disruption and leakage of intracellular components [38]. While both agents are common in clinical decontamination protocols, their cytotoxicity at higher doses or with prolonged exposure must be carefully managed, particularly in sensitive tissue cultures [39, 40].

In our workflow, OSCC explants underwent a 30-minute treatment with 1 µg/mL chlorhexidine gluconate, followed by PBS rinses and a 2-hour incubation in a medium containing 30 µg/mL gentamicin and 10 µg/mL amphotericin B. This extended antimicrobial exposure successfully eliminated microbial growth post-thaw and during in-vitro culture. Compared to the one-minute Betadine-Povidone treatment used whose samples showed residual bacterial and fungal contamination, our longer, stepwise protocol using chlorhexidine gluconate proved more effective, emphasizing the importance of duration and agent selection in disinfection strategies [41].

Previously, It was demonstrated the successful transport and preservation of ovarian tissue using a formulation comprising DMEM supplemented with 10% (Fetal calf serum) FCS, 1% penicillin-streptomycin, 2.5 µg/mL Fungizone, and 50 µg/mL gentamicin [42]. In contrast, our protocol for OSCC tissue incorporated lower concentrations of chlorhexidine and gentamicin, along with 10 µg/mL amphotericin B, and emphasized extended exposure durations and combinatorial treatments tailored to the resistant and diverse oral microbiota. This divergence highlights the critical need to customize antimicrobial strategies based on tissue origin and the corresponding microbial landscape, particularly when addressing contamination risks in tissues derived from high-risk cohorts such as chronic tobacco users.

Notably, *N. elongata* exhibited intermediate resistance to chlorhexidine gluconate and resistance to penicillin-streptomycin and amphotericin B. However, a combination of penicillin-streptomycin, gentamicin, and amphotericin B was effective, highlighting the potential of combinatorial antimicrobial strategies. Interestingly, gentamicin demonstrated a smaller inhibition zone (12 mm with a 30 µg disk) against *N. elongata* in our study compared with previously reported values (17.8–21.8 mm with 10 µg disks) [43]. While this indicates reduced susceptibility, it also provides an important insight into the strain dependent variability of oral commensal Neisseria. This observation reinforces the clinical and experimental relevance of our findings: commensal species may harbor early signs of decreased susceptibility that could compromise standard antimicrobial regimens.

*C. albicans* is a pleomorphic opportunistic fungal pathogen, commonly found as a commensal organism within the human microbiota [44]. Since antibacterial agents such as penicillin and streptomycin target bacterial-specific structures namely peptidoglycan in the cell wall and distinct ribosomal subunits they lack molecular targets in *C. albicans* and thus have no antifungal effect [45, 46].

Consistent with this, in our study *C. albicans* demonstrated resistance to gentamicin and penicillin-streptomycin. Furthermore, resistance to chlorhexidine gluconate was observed, aligning with previous findings who reported the absence of inhibition zones in chlorhexidine susceptibility testing using the Kirby-Bauer disk diffusion method [47]. In contrast, amphotericin B was effective as a standalone treatment, and its use in combination therapies further enhanced antimicrobial activity, underscoring its potential in overcoming *C. albicans* resistance within the OSCC.

*S. gallolyticus* has been associated with colorectal cancer and is recognized as a potential biomarker for gastrointestinal malignancies [48]. However, its role in OSCC remains unclear. Its presence in our cultures suggests a possible association between oral cancer and systemic microbial dysbiosis, warranting further investigation into its pathogenic contribution to OSCC progression. In our study, *S. gallolyticus* subsp. gallolyticus exhibited intermediate resistance to chlorhexidine gluconate and penicillin-streptomycin but remained susceptible to gentamicin. These findings align with previous reports indicating that *S. gallolyticus* is generally susceptible to commonly used antibiotics, although resistance to macrolides (45–59%) and tetracyclines (56–78%) has been documented [49].

Considering the diverse microbial population of the oral cavity, we employed a stepwise protocol for culturing and biobanking. In newly obtained tissue samples, treatment with penicillin-streptomycin alone resulted in microbial contamination. In contrast, newly obtained tissues subjected to a stepwise, optimized antimicrobial treatment during fresh culture and prior to cryopreservation showed no detectable contamination, even after three months of storage and subsequent thawing. Notably, epithelial-like cell outgrowth was evident as early as day 3 post-thaw, confirming both the viability and proliferative capacity of the preserved OSCC tissues. These findings highlight the importance of incorporating pre-cryopreservation decontamination strategies, particularly for newly obtained OSCC tissues derived from chronic tobacco users who often harbor diverse and penicillin-streptomycin resistant oral microbiota. Optimizing cryopreservation workflows through tailored antimicrobial pretreatment not only ensures sterility but also enhances the reliability of downstream applications and supports robust long-term biobanking practices.

In conclusion, this study establishes an optimized biobanking protocol for oral squamous cell carcinoma (OSCC) tissues from chronic tobacco users, addressing microbial contamination challenges during cryopreservation. We identified Pen-Strep resistant microbes, including *N. elongata*, *S. gallolyticus*, and C. albicans, as key contaminants that compromise tissue integrity in primary OSCC cultures. Using a stepwise protocol with chlorhexidine gluconate, gentamicin, and amphotericin B, we successfully eliminated these resistant microbes prior to cryopreservation. The protocol ensured the long-term viability and sterility of the stored tissues, as evidenced by epithelial-like cell outgrowth post-thaw. Our approach mitigates the risks of microbial interference and cross-contamination in liquid nitrogen storage, offering a reliable method for preserving high-quality OSCC biospecimens. This protocol provides a valuable foundation for improving tumor tissue biobanks, supporting future advancements in oral cancer research and biomarker discovery.

## Author Contribution Statement

K.G. and S.SV., conceptualized and developed the methodology for the study. K.P., D.A., and A.L., collected the samples. V.VK., A.S., S.SV., and A.L., data curation as well as analysis. K.G., S.SV., V.VK., A.S., wrote the original draft of the main manuscript text, with K.G., and S.SV., editing the original draft. All authors reviewed the manuscript.

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*Competing Interests*

The authors have no competing interests to declare that are relevant to the content of this article.

*Ethics Approval*

The study has been approved by the Ethics Committee of M S Ramaiah University of Applied Sciences (EC-24/95-F-FDS).

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