RESEARCH ARTICLE

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Use of Oral Rub and Rinse Technique for Oral Cancer Screening: Results from a Community-based Program in an LMIC

Audrey Madonna DCruz[1], Pushparaja Shetty[2]*, Urvashi Ashwin Shetty[2], Pandyanda Nanjappa Dechamma[3], Vinayak Kamath[4], Shaila M Bhat[5], Anirban Chakraborty[3]*

Abstract

Background: Oral cancer screening programs can aid in the early identification of potentially malignant oral lesions. The objective of the present study was to evaluate the effectiveness of the Oral Rub and Rinse (ORR) technique as an oral cancer screening tool and to test its potential in detecting genetic alterations in exfoliated cells obtained through ORR. **Methods:** The screening programs were conducted in rural Dakshina Kannada and Udupi districts in Karnataka. All adults with red and/or white lesions were included in the study. Samples were collected using the ORR technique, and smears were prepared. In parallel, Conventional Exfoliative cytology (CEC) smears were also prepared. ORR samples and tissue biopsy were obtained from Classes III, IV, and V cases. Seven Oral Squamous Cell Carcinoma (OSCC) tissue samples, 7 OSCC ORR samples, and five control samples were randomly selected. The presence of single nucleotide polymorphisms (SNP) in *USP9X* and *DDX3X* were checked through Sanger sequencing. Diagnostic agreement between the ORR and CEC technique was assessed using the McNemar test. **Results:** A total of 2514 individuals were screened, of which 217 patients with red/ white lesions were included in the study. There was good agreement between the exfoliative cytology and ORR. The sensitivity of the ORR was 84.54%, specificity 85.83%, positive predictive value 82.83%, and negative predictive value 87.29%. Overall, the diagnostic accuracy of the ORR technique was 85.25%. Although none of the samples showed the presence of target SNPs in *USP9X* and *DDX3X* genes, the utility of the ORR technique as a tool for conventional and molecular studies was confirmed. **Conclusion:** The present study highlights the usefulness of the ORR technique as an effective tool in population-based oral cancer screening programs. Further, the cells obtained from ORR are an excellent source of DNA and can potentially identify genetic alterations with high accuracy.

Keywords: Oral cancer- Oral rinse- Exfoliative cytology- oral cancer screening

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Introduction

Oral cancers are a significant concern across the world and are particularly prevalent in Asian countries. Globally, oral and oropharyngeal cancers rank sixth among all cancers [1]. As per the reports by GLOBOCAN 2020, the worldwide incidence of lip and oral cavity cancer was around 377,713, and that of oropharynx was around 98,412 [2]. Asia had the highest incidence of lip and oral cavity cancers (65.8%), followed by Europe (17.3%), North America (7.3%), Latin America and the Caribbean (4.7%), Africa (3.8%) and Oceania (1.2%). Oral Squamous Cell Carcinoma (OSCC) is a multifactorial and multistage disease, which can develop from 'precancerous lesions,' now termed Oral Potentially Malignant Disorders (OPMD), or can occur de novo [3]. The worldwide prevalence of OPMD is reported to be about 4.4% [4], of which leukoplakia is the most common, with a global incidence of 2 to 4% [5]. The severity of dysplasia is usually associated with the clinical type of leukoplakia. About 15% of leukoplakia show moderate dysplasia in contrast to 41% of speckled leukoplakia, which manifests

*[1]Nitte (Deemed to be University), AB Shetty Memorial Institute of Dental Sciences, Department of Public Health Dentistry, Mangaluru, Karnataka, India. [2]Nitte (Deemed to be University), AB Shetty Memorial Institute of Dental Sciences, Department of Oral Pathology and Microbiology, Mangaluru, Karnataka, India. [3]Nitte (Deemed to be University), Nitte University Centre for Science Education and Research (NUCSER), Division of Molecular Genetics and Cancer, Mangaluru, Karnataka, India. [4]Goa Dental College and Hospital, Department of Public Health Dentistry, Goa, India. [5]KVG Dental College and Hospital, Department of Oral Pathology and Microbiology, Sullia, India. *For Correspondence: anirban@nitte.edu.in, drpushparaj@nitte.edu.in*

as severe dysplasia [6, 7]. Tobacco, mainly the smokeless forms, areca nut use, and increased consumption of alcohol are considered risk factors for the occurrence of oral cancer and potentially oral malignant disorders [8]. It has been reported that the incidence of oral cancer increases with age and is highest over 60 years, even though cases in people younger than 40 years are growing [9]. Buccal mucosa and gingiva are the most frequently diagnosed sites of OSCC.

Early diagnosis can help reduce the burden of oral cancer and improve the patient's quality of life. Indeed, oral cancer screening programs that use specific diagnostic tools in asymptomatic patients with suspicious oral lesions can aid in the identification of potentially malignant/ malignant oral lesions [10]. Population-based screening programs for oral malignant and potentially malignant lesions have proven helpful in the early identification of asymptomatic patients with suspicious oral lesions, with a 32% reduction in mortality among high-risk individuals [11]. Numerous diagnostic aids are available that serve as adjuncts to the visual examination. They either aid in detecting early mucosal changes that could be missed during the visual examination or assess the biological potential of clinically abnormal mucosal lesions [12-14].

Exfoliative cytology has been used as an adjunct tool in oral cancer screening programs. Conventional exfoliative cytology using a spatula to scrape the oral mucosa and smear the cells onto a slide for cytologic evaluation is an accepted method. Although this method is widely used in low-resource settings to detect malignant and potentially malignant lesions [15], it requires some expertise. It can lead to procedural errors if not performed adequately. A variant of conventional exfoliative cytology is using oral rinses to collect the exfoliated cells and prepare the smears in a laboratory-based setting. The 'Oral Rub and Rinse (ORR) technique is one such technique. A study on a comparison of oral rinse–based smears and conventional smears for diagnosis of oral cancer and oral leukoplakia revealed that although the oral rinse–based smears were diagnostically reliable, the oral rinse-based method smears had reduced cellular clumping, better cellular clarity, and improved sample adequacy [16]. However, the usefulness and efficacy of this technique in screening the population for oral malignant and potentially malignant lesions in community settings need to be determined. Hence, the objective of the present study was to compare the 'Oral Rub and Rinse' technique with 'Conventional Exfoliative Cytology' as an oral cancer screening tool for malignant and potentially malignant oral lesions for use in community settings. In addition, the study aimed to determine the technique's efficacy in performing molecular investigations, including determining genetic alterations in rare genes that could serve as accurate biomarkers for early detection of malignant or potentially malignant lesions. It has been reported that the ORR technique successfully identified genetic alterations in the TP53 gene, one of the most commonly altered tumor suppressor genes in oral cancer, tissue, and exfoliated cells of oral cancer patients [17].

This study targeted two rare genes implicated in oral cancer: ubiquitin-specific peptidase 9, X linked (*USP9X*) and DEAD box helicase 3, X linked (*DDX3X*). *USP9X* is a deubiquitinase for SMAD4, a protein required for TGF-b signaling. *USP9X* regulates signaling pathways implicated in the initiation and progression of Oral squamous cell carcinoma [18], and an increased *USP9X* expression was found to be significantly correlated with poor prognosis in oesophageal squamous cell carcinoma [19] and non-small cell lung cancer [20]. *DDX3X* is a gene essential for apoptosis [21]. In a recently conducted genome-wide SNP genotyping and exome sequencing in 50 OSCC-GB patients, novel somatic mutations in *USP9X* and *DDX3X* were reported. However, the study did not carry out Sanger confirmation of the NGS data [22].

Materials and Methods

Screening for Oral malignant and potentially malignant lesions

Oral cancer screening programs were conducted at the rural health centers of Dakshina Kannada and Udupi districts of Karnataka, India. All adults above the age of 18 years who attended the screening programs were enquired about their socio-demographic details and tobacco use habits. A clinical examination was conducted, and the soft and hard tissues of the oral cavity were examined. In the case of red and/or white lesions, the patients were informed about the same, and those who were willing to sign the informed consent form were included as the study population. For ethical reasons, patients who did not have any lesions but had tobacco habits were educated on the harmful effects of tobacco use and were advised to quit tobacco. Ethical Clearance for the study was obtained from the Central Ethics Committee, Nitte (Deemed to be University) [Ref No: NU/CEC/2016-2017/052].

Selection of study population for sample collection

Patients above 18 years of age visiting rural centers/ dental treatment camps with a red/ white lesion in the oral mucosa and who were willing to participate by signing the informed consent form were included in the study. Patients with previous history of malignancy and treated for cancers (surgery, chemotherapy, radiotherapy) were excluded from the study.

Collection of samples and preparation of smears

First, the Oral Rub and Rinse (ORR) technique was used to collect oral cells, followed by the conventional exfoliative cytology (CEC). For the ORR, subjects were asked to swish their mouths with water and expectorate. Then, the clinician rubbed the suspected oral lesion firmly with the gloved forefinger in the accessible areas for 30 seconds. Pressure was exerted such that it caused blanching of the oral mucosa without any pain or discomfort to the patient. They were then asked to swish with 10mL of phosphate buffered saline (pH 7.2) and expectorate the solution into a sterile falcon tube. The sample thus obtained was labeled and stored in cold boxes containing gel packs. After sample collection using the ORR technique, the same lesion was scraped with a sterile stainless steel spatula, and scrapings were smeared on labeled glass slides for CEC. All the slides thus prepared

were immediately fixed in absolute alcohol.

The ORR samples and the CEC samples were transported to the Oral Pathology laboratory from the collection site in cooler boxes with ice gel packs. The ORR samples were either processed immediately or were stored at –20°C till further processing. The ORR samples were centrifuged at 1000 rpm for five minutes. The supernatant fluid was discarded, a micropipette was used to collect the cells from the cell plug, and smears were prepared and fixed. The remainder of the cell plug was stored at -20°C and used to extract genomic DNA. Both smears prepared by CEC and ORR were stained with a Papanicolaou (PAP) stain using the RAPID-PAPTM stain kit per the manufacturer's instructions.

Smear Assessment

Two trained pre-calibrated cytopathologists assessed all slides through a total-blind approach. The cytologic specimens were classified as follows [23]:

Class I (Normal): Only normal cells are observed.

Class II (Atypical): Presence of minor atypia but no evidence of malignant changes.

Class III (Indeterminate): Wider atypia that maybe suggestive of cancer, but they are not clear-cut and may represent precancerous lesions or carcinoma-in-situ. A biopsy is recommended.

Class IV (Suggestive of cancer): A few cells with malignant changes or many cells with borderline characteristics. Biopsy is mandatory.

Class V (Positive for cancer): Cells that are obviously malignant. Biopsy is mandatory.

For analysis, Classes 1 and 2 were considered negative, and Classes 3-5 were positive.

Extraction of genomic DNA and sequencing of target regions in USP9X and DDX3X

ORR and tissue biopsy samples were obtained from Classes III, IV, and V cases. Genomic DNA was extracted from oral tissues and exfoliated cells of ORR using the DNeasy Blood & Tissue Kit (QIAGEN, Germany), according to the manufacturer's instructions [24]. Further, the genomic DNA extracted was checked for purity and concentration (ng/µl) by measuring the absorbance at 260nm using a UV spectrophotometer (Eppendorf, Germany). The DNA was stored at -20°C till further use. (Figure 1)

Based on a previous report [22], a total of 6 SNPs in *USP9X* and 1 in *DDX3X* were targeted, and the location of these target SNPs was identified from the SNP database (https://www.ncbi.nlm.nih.gov/snp/). The location of target SNPs in *USP9X* and *DDX3X* genes is shown in Table 1. Forward and reverse primers were designed to encompass the mutation sites using an online primer designing tool (Primer 3 input, https://primer3.ut.ee/), and the sequence details of the primers are shown in Table 2. PCR amplified the gene fragments encompassing the target SNPs for *USP9X* and *DDX3X*. The amplicon size and optimized annealing temperature for each set of PCR are shown in Supplementary Table 1.

Seven OSCC tissue samples, 7 OSCC ORR samples, and five control samples were randomly selected for sequencing of target areas of interest of *USP9X* (exon 6, intron between exon13 and 14, exon 29, exon 33, exon 37 and exon 43) and *DDX3X* exon 17. The selection process of samples for sequencing was random and based on good concentration and quality of the DNA and not on any other factors. The gene amplification was done in an automated thermal cycler (Eppendorf, Germany). The PCR products were stored at -20°C until further use. The amplified products were run on agarose gel, purified, and sequenced by capillary sequencing. The sequencing was outsourced to commercial vendors (Eurofins, Bangalore). The chromatograms obtained were compared with the reference gene sequences available at NCBI to identify the variations (www.ncbi.nih/nlm/gov).

Statistical analysis

The data obtained was entered in Microsoft Excel for Windows. SPSS statistical software 24.0 (SPSS Inc, Chicago) was used to analyze the data. Descriptive analysis (frequency and percentages) was used. Diagnostic agreement between the ORR and CEC technique was assessed using the McNemar test. A p-value < 0.05 was considered significant. The test's diagnostic accuracy was compared in terms of sensitivity and specificity, positive predictive value, and negative predictive value. The frequency and percentages of the genetic variations were assessed in each study group.

Results

A total of 2514 individuals were screened, of which the majority (n=1438, 57.2%) were 21 – 40 years of age (Table 3). About 60% of the participants (n= 1508) were males. About 26.6% of the screened population (n=669) used tobacco, of which smokers comprised 42.5% (n=284), smokeless tobacco users 40.2% (n=269), and 17.3% (n=116) used both types (Table 3). Among

Table 1. Location of Target Single Nucleotide Polymorphisms (SNPs) for *USP9X* and *DDX3X*

Gene	Allele	Genomic position
USP9X Exon 6	A/G	NC_000023.11: 41136904
USP9X Intron between Exon 13 & 14	G/T	NC_000023.11: 41151058
USP9X Exon 29	C/T	NC_000023.11: 41197451
USP9X Exon 33	G/A	NC_000023.11: 41210555
USP9X Exon 37	A/G	NC_000023.11: 41218446
USP9X Exon 43	G/T	NC_000023.11: 41229606
DDX3X Exon 17	G/T	NC_000023.11: 41346863

Table 2. Primer details for *USP9X* and *DDX3X* Genes

USP9X Exon 6	F	5'-TCGTCTGGTGGAGCTATGTG - 3'
	R	5'- CAACACACCTTTGGTGATCG - 3'
USP9X Intron between Ex13 & 14	F	5'-AAATGGGTTATTCCCGCACT - 3'
	R	5'- AACTGTGGCTTACTTCCTCATT - 3'
USP9X Exon 29	F	5'- CCTCCAGCTCTGTCTCGTTC - 3'
	R	5'- GCACCTCCTTTTTCACAACC - 3'
USP9X Exon 33	F	5'- TGTTGTTTGTGGTCTTGTTGC - 3'
	R	5'- TACCTATGTGGGCAGCCTTG - 3'
USP9X Exon 37	F	5'- ACCTTTGCTGGTTCTCTCCA - 3'
	R	5'- GGCACTCCAGAAGGTATTCG - 3'
USP9X Exon 43	F	5'- GCAGGGCAATGGAGATCTTA - 3'
	R	5'- CTGCACACTGGTGGCTTTTT - 3'
DDX3X Exon 17	F	5'- TGACAAAGATTTTGCTCAAAGC - 3'
	R	5'- CGGCACTTCTTGTTTAGCTTC - 3'

smokers, about 189 (66.5%) smoked cigarette and 95 (33.5%) smoked bidi. Among the users of smokeless forms of tobacco, the majority of them (n= 75, 27.9%) chewed tobacco, followed by ghutka (n=68, 25.3%) and hans (n=50, 18.6%).

Of the 2514 screened population, about 217 participants who, on clinical examination, had red and/or white lesions were included in the study and hence comprised the study population. Most of the study population (n= 114, 52.6%) were 41-60 years old, and about 71% were males (Table 4). Most of the study population (n=188, 86.7%) were current smokeless tobacco users, among which 40.6% were betel quid chewers with tobacco. Only 51 participants (23.5%) were current smokers.

Conventional exfoliative cytology diagnosis was compared with the oral rub and rinse technique (Table 5). About 26 smears were classified as Grade I, 53 as Grade II, 31 as Grade III, 31 as Grade IV, and eight as Grade V by exfoliative cytology and the ORR technique.

The diagnostic agreement between the conventional exfoliative cytology and the ORR technique was observed (Table 6). There was good agreement (kappa statistic value = 0.70) between the exfoliative cytology and ORR technique. The sensitivity of the ORR was 84.54%, specificity 85.83%, positive predictive value 82.83%, and negative predictive value 87.29%. Overall, the diagnostic

Table 3. Demographic Characteristics and Tobacco Use among the Screened Population

Variable		Frequency	Percent
Age	20 years and below	133	5.3
	21 – 40 years	1438	57.2
	41 – 60 years	679	27
	61 years and above	264	10.5
	Total	2514	100
Gender	Males	1508	60
	Females	1006	40
	Total	2514	100
Tobacco use	Yes	669	26.6
	No	1845	73.4
	Total	2514	100
Smoke tobacco	Cigarette	189	7.5
	Bidi	95	3.8
	Total	284	42.5
Smokeless tobacco	Betel quid with tobacco	38	1.5
	Ghutka	68	2.7
	Hans	50	2
	Tobacco chewing	75	3
	Pan masala	38	1.5
	Total	269	40.2
Both smoke and smokeless tobacco		116	17.3

Table 4. Demographic Characteristics and Tobacco Use among the Study Population

Variable		Frequency	Percent
Age	20 years and below	0	0
	21 – 40 years	45	20.7
	41 – 60 years	114	52.6
	61 years and above	58	26.7
	Total	217	100
Gender	Males	154	71
	Females	63	29
	Total	217	100
Smoking	No	165	76.1
	Yes	51	23.5
	Yes, past, quit 20 years back	1	0.4
	Total	217	100
Type	None	165	76.1
	Beedi	25	11.5
	Cigarette	27	12.4
	Total	217	100
Smokeless	Non-User	27	12.4
	Yes, Past	2	0.9
	Yes, Present	188	86.7
	Total	217	100
Type	None	27	12.4
	Pan masala	4	1.8
	Betel quid with tobacco	91	42
	Ghutka	54	24.9
	Hans	10	4.6
	Tobacco chewing	31	14.3
	Total	217	100

Table 5. Comparison of Conventional Exfoliative and the Oral Rub and Rinse Techniques

ORR Diagnosis	Exfoliative Cytology Diagnosis					Total
	1	2	3	4	5	n (%)
1	26	15	9	0	0	50 (23.0%)
2	9	53	6	0	0	68 (31.3%)
3	12	3	31	4	0	50 (23.0%)
4	0	2	4	s	0	37 (17.1%)
5	0	0	3	1	8	12 (5.5%)
Total	47 (21.70%)	73 (33.6%)	53 (24.4%)	36 (16.6%)	8 (3.7%)	217 (100%)

accuracy of the ORR technique was 85.25% (Table 7).

The Sanger sequencing of the representative samples did not show the presence of SNPs in OSCC tissue or ORR samples in our regions of interest (*USP9X* Exon 6, *USP9X* Intron between Exon13 and 14, *USP9X* Exon 29, *USP9X* Exon 33, *USP9X* Exon37 and *USP9X* Exon 43 and *DDX3X* Exon 17). The representative electropherograms of Sanger sequencing are shown in supplementary Figures 1 – 7.

Table 6. Diagnostic Agreement of Conventional Exfoliative Technique v/s the Oral Rub and Rinse Technique

ORR Diagnosis	Exfoliative Cytology Diagnosis		Total	McNemar Test	Kappa Statistic
	Class 1+2	Class 3+4+5		p-value	
Class 1+2	103 (85.8%)	15 (15.50%)	118 (54.40%)	0.86 (NS)	0.7 (Good)
Class 3+4+5	17 (14.20%)	82 (84.50%)	99 (45.60%)		
Total	120 (100%)	97 (100%)	217 (100%)		

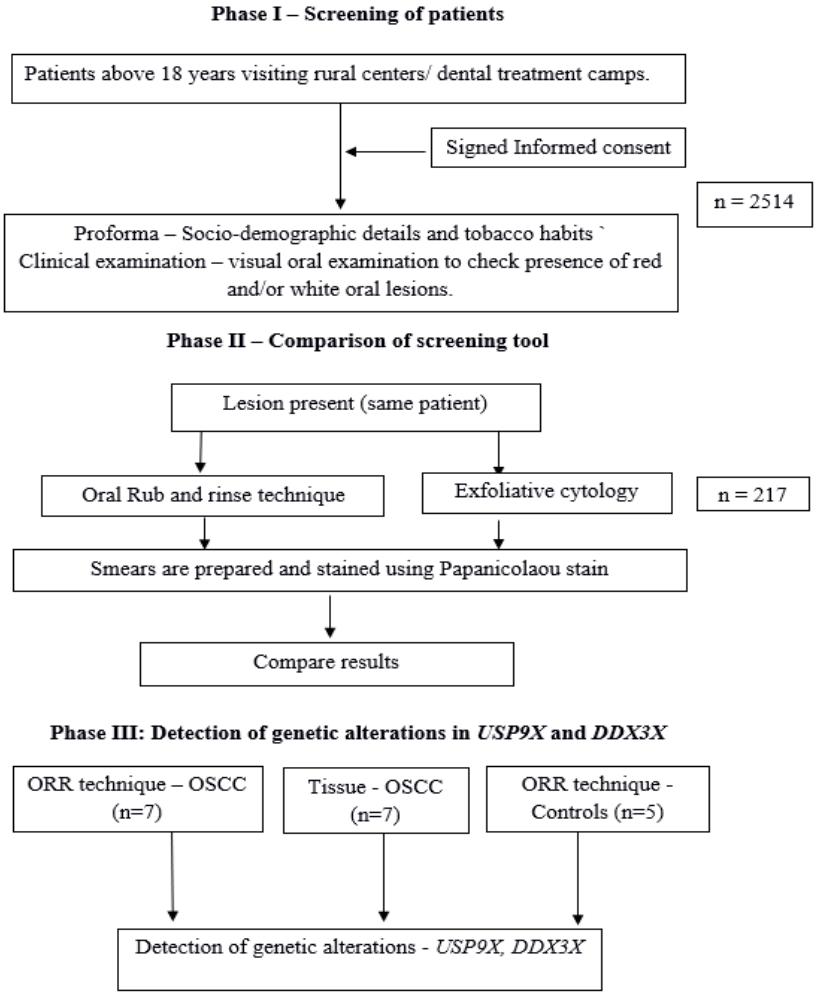

Figure 1. Flowchart of Mmethodology

Table 7. Diagnostic accuracy of Conventional Exfoliative Technique v/s the Oral Rub and Rinse Technique

		Exfoliative Cytology		Total	
		Positive	Negative		
ORR	Positive	82 (a)	17 (b)	99 (a+b)	Positive Predictive Value (PPV) a/(a+b) = 82.83% (74.21, 88.99)
	Negative	Sensitivity a/(a+c) = 84.54% (76.04, 90.4)	103 (d)	118 (c+d)	Negative Predictive Value (PPV) d/(c+d) = 87.29% (80.08, 92.14)
	Total	97 (a+c)	120 (b+d)	217 (a+b+c+d)	Overall Diagnostic Accuracy (a+d)/(a+b+c+d) = 85.25% (79.92, 89.36)
		Sensitivity a/(a+c) = 84.54% (76.04, 90.4)	Specificity b/(b+d) = 85.83% (78.48, 90.96)		

Discussion

Cancer is a multi-hit event where several genetic aberrations contribute to the malignant transformation. Oral cancer is one of the most prevalent cancers in the world, and among the various subtypes of oral cancer, squamous cell carcinoma is the most common one [25]. Despite advances in diagnostic and therapeutic modalities in managing OSCC, the patient prognosis remains poor. Early detection and diagnosis, followed by treatment, can help reduce morbidity and mortality.

Several diagnostic adjuncts, including exfoliative cytology, brush biopsy, vital staining methods, chemiluminescent techniques, saliva-based diagnostic tests, etc., are used alone or in combination with conventional oral examination to screen malignant and potentially malignant lesions [12-14]. Exfoliative cytology has been successfully used as an adjunct tool in oral cancer screening programs. Conventional exfoliative cytology uses a spatula to scrape the oral mucosa, and then the cells are smeared onto a slide, and cytologic evaluation is done. Although it is a widely used method in low-resource settings for detecting malignant and potentially malignant

lesions [15], it requires some level of expertise and, if not performed adequately, can lead to procedural errors. A variant of conventional exfoliative cytology is using oral rinses to collect the exfoliated cells and prepare the smears in a laboratory-based setting. One such technique is the 'Oral Rub and Rinse technique.' A study reported that the oral rinse-based method showed better cellular clarity and an overall improvement compared to conventional smears in diagnosing oral cancer and leukoplakia. However, the diagnostic reliability in both methods was similar [16]. The present study attempted to compare the Oral Rub and Rinse technique over conventional exfoliative cytology in population screening of potentially malignant and malignant oral disorders and evaluate the ORR technique's efficacy in identifying cancer-associated genetic changes in the extracted cells.

The results showed that these methods were equally effective in grading the smears into different categories. Of the 217 participants with clinically suspected premalignant/ malignant lesions, categorization by conventional technique showed 47 as Class I smears, 73 as Class II smears, 53 as Class III smears, 36 as Class IV smears, and eight as Class V smears. On the other hand, the Oral Rub and Rinse technique classified smears as 50 Class I smears, 68 Class II smears, 50 Class III smears, 37 Class IV smears, and 12 Class V smears. Thus, although the ORR technique did not show any statistically significant difference when compared to the conventional exfoliative cytology in grading the samples, it is to be noted that the smear preparations were better in the ORR technique and hence had better cellular clarity. This finding was similar to that reported in the literature [16, 26]. So far, the studies published on the ORR technique have measured the cytonucleomorphometric changes in laboratory settings. To our knowledge, this is the first study that attempted to test this technique in community settings.

Being simple and non-invasive, the classic oral cytologic evaluation is most commonly used to detect malignant and potentially malignant lesions in population-based studies. However, it requires some level of expertise to prepare smears on-site. Also, negative results are not always reliable [15]. Hence, it needs to be followed by a scalpel biopsy for a confirmatory diagnosis. The scalpel biopsy followed by histopathology is considered the gold standard for diagnosing oral malignant and potentially malignant lesions and has been the first choice for diagnosing malignant lesions [27]. However, conventional biopsy is an invasive procedure that has its demerits and may not be feasible in all suspected cases. One of the reasons for poor prognosis in OSCC is late diagnosis of the cancer, and it is particularly evident in a country like India where most of the cancer cases are diagnosed at advanced stages of progression.

The sensitivity of the ORR in the present study was 84.54%, and the specificity was 85.83%. For studies reporting the sensitivity and specificity of a new technique, the novel technique must be compared to a gold standard. In this case, although histopathology is the gold standard for comparison, the class I and II smears do not warrant a biopsy, and class III smears it is recommended but not mandatory. Hence, for practical and ethical reasons, conventional exfoliative cytology, widely used for field settings, was considered the gold standard for comparison.

Detecting alterations at the molecular level is crucial as it aims to detect specific genetic anomalies [28]. Tumor biomarkers are substances produced by the tumor cells or the host immune mechanisms released in the body fluids. Molecular identification of these tumor markers and their monitoring in body fluids aid in the early detection, diagnosis, and management of cancers, consequently improving disease prognosis. Several such biomarkers have been identified for OSCC [29, 30]. Saliva, a storehouse of enzymes, nucleic acids, and proteins, is a promising, non-invasive, and easy source of tumor biomarkers for the diagnosis and prognosis of OSCC. Oral rinses allow the cytological investigation of normally shed oral cells, assisted by molecular analysis for oral cancer screening. The Oral Rub and Rinse technique is one such method of oral rinse. Identifying genetic biomarkers for OSCC using the exfoliated cells from the 'Oral Rub and Rinse' technique has further strengthened its usefulness.

In our earlier study, the ORR technique successfully identified common genetic variations such as TP53 in oral cancer patients [17]. Hence, we attempted to study variations in two novel genes - *USP9X* and *DDX3X*, which are not frequently reported in OSCC. These genes were chosen as little information was available in the literature regarding their variation pattern in OSCC, except for one study that reported a few SNPs in these two genes based on NGS data and other studies on their role in cancer [18-22]. Interestingly, the NGS data was not confirmed by Sanger sequencing. Thus, we wanted to check if these SNPs are associated with OSCC and whether the Sanger sequencing could identify the presence of those SNPs. Therefore, tissue and ORR samples from confirmed OSCC patients were studied to detect target variations in *USP9X* and *DDX3X*. However, we did not find any SNPs in the targeted regions of *USP9X* and *DDX3X*. The reasons for not detecting any SNPs in these two target genes could be many, including the possibility of the Sanger technique missing it due to the high amount of wild-type background DNA or missing some cases that might have had these SNPs, as we followed a random approach. Nevertheless, our results suggest that the SNPs reported earlier for these two genes may not be present in the cohort studied here. Moreover, the results indicate that molecular studies are possible with cells obtained through ORR, even when done in a community setting and transported to a lab later.

It is to be noted that the studies that have been reported in the literature so far have been conducted in the laboratory in a hospital setting and have been processed immediately. In contrast, the samples were obtained in community settings in this study. One of the major concerns in the present study was whether the cells would remain viable and if the cell cytomorphology would be maintained as several factors are to be considered, such as the time taken from obtaining the sample at the field to processing the sample in the laboratory, the temperature of the sample to be maintained, the integrity of the extracted DNA, etc. The results obtained from the present study highlight that the ORR technique works

well in community settings. Although the samples were transported in boxes with ice packs for 3 hours, it was still possible to extract DNA from the cells, amplify target regions of the genome by PCR, and sequence those target regions. Also, the cell cytonuclear morphology was maintained in the smears prepared.

The ORR technique, being a non-invasive method, has its advantages. Like the exfoliative cytology, it can be used in anxious patients who refuse a surgical biopsy and in patients in whom biopsy may be contraindicated. However, it has a few benefits and differences over conventional exfoliative cytology. As only finger pressure is used for rubbing the lesion, it gives psychological comfort to the patient, and patient cooperation is better during sample collection. The procedural time taken at the field is reduced as only the sample is collected in the tubes and processed at the laboratory later. Hence, more patients can be screened, and more samples can be collected. In conventional exfoliative cytology, the slides must be prepared and processed immediately at the sample collection site.

Elaborate equipment and materials needn't be taken to the field for screening since the slides are prepared in the laboratory. Only the tubes with 1% phosphate-buffered saline can be taken to the field for sample collection, which can be stored in an ice-lined sample collection box till further processing. The conventional exfoliative cytology technique requires some expertise to prepare the slides at the sample collection site. In comparison, any healthcare worker can collect the sample using the ORR technique, as the slides are later prepared in the laboratory.

In addition, multiple smear slides can be prepared from a single sample compared to conventional exfoliative cytology. If excess material is obtained, such as in erosive lesions, the specimen can be preserved by preparing tissue blocks. Also, the cell pellet obtained can be used as a source of salivary DNA, as proven in the present study.

The present study concludes that the ORR technique can screen malignant and potentially malignant lesions in population-based oral cancer screening programs in field settings. Although there was no significant difference between the ORR and CEC in grading and diagnosing the lesions, the smears prepared by the ORR technique were superior in quality concerning the sample adequacy and cellular clarity. Overall, the ORR technique has good diagnostic accuracy for screening oral cancer and pre-cancer in community settings. Also, the cells obtained from the method are a good source of DNA and can be used to detect alterations at the genetic level.

Author Contribution Statement

AD: Conceptualised the study, collected the clinical samples, carried out the clinical analysis, obtained funds, and prepared the original draft. PS: Assisted in study design, participated in discussion, assisted in clinical and cytological analysis, and reviewed the first draft. US: Carried out cytological analysis and helped in clinical and cytological data curation and compilation. DPN: Carried out molecular experiments and was involved in data analysis. VK: Assisted in planning the study design with sample size estimation and statistical analysis of the final data. SM: Assisted in study design and participated in discussion and cytological analysis. AC: Supervised the work, analyzed the data, edited the draft manuscript, and prepared the final manuscript.

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Availability of data

The datasets generated during the current study are available from the corresponding author upon reasonable request.

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Scientific Committee Approval

The research was a part of the doctoral thesis and was approved by the Scientific Research Committee, Nitte (Deemed to be University).

Ethical Approval and Consent to Participate

The study was approved by the Central Ethics Committee of Nitte (Deemed to be University); the approval ID is NU/CEC/2016-2017/052. Informed consent was obtained from all individual participants included in the study.

Statement Conflict of interest

The authors declare that they have no conflict of interest.

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