Effectiveness of Crystallization Test in Screening of Potentially Malignant Oral Disorders and Oral Cancer

Abstract
Aim: This study aims to determine the effectiveness of crystallization test in screening of oral potentially malignant disorders (PMDs) and oral cancer. Materials and Methods: Thirty patients of oral PMD, 30 patients of oral cancer and 40 normal healthy people were selected. One drop of blood was collected and added to 1 cc of double-distilled water at room temperature to get a final dilution of 6% hemolyzed blood. 0.1–0.2 cc of this blood sample is added to 10 cc of 20% cupric chloride solution and further is subjected to crystallization test. Results: In the normal healthy group, the pattern was typical with an eccentrically placed center of gravity with needles arranged in radiating fashion. Whereas in oral PMD and cancer groups, there was “transverse form” formation. This test had sensitivity and specificity of about 83.33% and 86.84% for PMDs group and 96.30% and 86.84% for oral cancer group respectively. Conclusion: Crystallization test was found to be sensitive, reliable, economical and less-invasive procedure for screening of oral PMDs and oral cancer.

Keywords: Crystallization test, histopathological grading, oral cancer, oral potentially malignant disorder, oral squamous cell carcinoma

Introduction
Oral cancer remains a significant cause of morbidity and mortality with an unaltered 5-year survival rate. Oral squamous cell carcinoma (OSCC) accounts to about 90% of all oral cancers and is often not diagnosed until advanced. The survival rates can be improved if detected, diagnosed, and treated at an early stage. There has been growing demand in the recent years towards developing new noninvasive tools in the early detection of oral cancer.

Most of the OSCCs develop from potentially malignant disorders (PMDs). The term PMD is preferred over precancer as not all of them terminate into malignancy. Instead, there is a series of morphological alterations with some exhibiting greater potential for malignant transformation. They are not only site-specific predictors as they also indicate the risk of future malignancy in clinically normal mucosa reflecting their wide anatomic distribution. Lack of awareness of the signs and symptoms of the PMDs among the general population attributes to the delay in diagnosis. Proper diagnosis and treatment of PMDs would prevent their further malignant transformation.

Although there are many methods available for early detection, the need of the hour is a method that is simple, economical, reliable, less time consuming and less invasive. Pfeiffer in 1938 introduced a new technique for detecting cancer by observing the crystallization pattern of cupric chloride on admixture with blood of cancer patient. According to Kopaczewski (1933), the different patterns of crystallization produced by organic and inorganic salts on the addition of colloidal solutions were attributed to different rates and amplitudes of molecular movements involved in the process of evaporation. Gruner in 1940 emphasized the role of colloidal proteins in dilute form of blood contributing to different crystallization patterns. Gulati et al. and Kuczkowski et al. performed this test in various head and neck malignancies and concluded its usage in mass screening programs.

The present study is an attempt to validate the efficacy of crystallization test in screening of oral PMDs and oral cancer.
Materials and Methods
The study was carried out in the Department of Oral Pathology and Microbiology. Thirty cases of clinically diagnosed oral PMDs and thirty cases of clinically suspected oral cancer which were further subjected to confirmatory histopathological analysis were present. The cases did not receive any therapy before the study and were free of any systemic disease. Control group included forty healthy subjects matched for age and sex having no obvious oral lesions or systemic disease. Informed consent has been obtained after explaining the procedure. Ethical clearance has been obtained from the institutional review board.

All the individuals of the study were in the age range of 30–75 years. The most common anatomical sites for OSCC were buccal mucosa, vestibule, and tongue.

In the potentially malignant group, leukoplakia (12 cases), followed by oral submucous fibrosis (7 cases), was more common.

Crystallization test
The procedure involved collecting a drop of blood by pricking the ring finger under aseptic conditions. The blood drop was added to 1 cc of double-distilled water, a final dilution of 6% hemolyzed blood was achieved. 0.1–0.2 cc of blood sample was then added to 10 cc of 20% Cupric chloride solution. Immediately, the mixture was poured in the prewarmed Petri dish of 10 cm size.

The Petri dish was placed in BOD incubator (temperature: 28°C–32°C and humidity of 35% to 55%) in an isolated room without any vibratory disturbances for about 18–19 h. Later, the crystallization patterns were carefully observed using magnifying lens against daylight. The presence of transverse form (TF) was considered as positive crystallization test. Further, the number of TFs was counted to correlate with the histological grades of OSCC.

Statistical analysis
Analysis was done using SPSS version 18. Entire values were expressed as percentages and mean ± standard deviation. Correlation between number of TFs and histopathological grades of oral cancer was observed using independent sample t-test. Chi-square test was applied and a \( P < 0.05 \) was considered statistically significant.

Results and Observations
The pattern of crystallization with cupric chloride solution alone included thick crystals with needles arranged randomly in a haphazard manner [Figure 1]. Such pattern was called as muddle formation by Sabarth and Williams.\(^9\) The needles exhibited side branching with secondary and tertiary branches.

The pattern of crystallization in the control group was that of an eccentrically placed center of gravity with orderly arranged needles radiating from centre towards periphery [Figure 2].

Among the PMDs and cancer group, there was presence of “transverse bar” or “TF” which consisted of transverse needles with wing-like formation on either or both sides [Figures 3–8]. The needles emerging from the center failed to pierce the TFs. These TFs did not exhibit any secondary or tertiary branching.

Among 30 PMDs, 25 were positive and 5 were negative for crystallization test. Sensitivity and specificity of crystallization test in screening PMDs were calculated. The positive and negative predictive values for oral potentially malignant group were found to be 83.33 and 86.84 respectively [Table 1].

TFs in various clinically diagnosed PMDs are shown in Table 2.

Crystallization test for three of the oral cancer patients and two of the normal group exhibited irregular or ill-defined patterns and were excluded from statistical analysis.

The OSCC group consisted of 22 (73.3%) females and 8 (26.67%) males. Buccal mucosa (9 cases) was a more common site in oral cancer group [Table 3]. Out of 27 cases of oral cancer, 26 were positive, and 1 was negative. The positive and negative predictive values for oral cancer group were found to be 83.87 and 97.06, respectively [Table 1].

Among the 38 normal cases, 33 were negative, and 5 were positive. Among the clinically suspected oral cancer patients, three were not operated as patients were unwilling. The Broder’s grading was done for histologically proven cases of OSCC, among which 14 cases were well differentiated, and 8 were moderately differentiated. The number of TFs was counted and was expressed as TF frequency in both the potentially malignant and oral cancer groups. Expression of TFs
was analyzed in PMDs in comparison to normal and OSCC in comparison to normal respectively. The Chi-square test was applied and “P” value was found to be <0.05. This indicates that crystallization test was statistically significant for the detection of oral PMDs and oral cancer [Table 1].

Table 1: Positive/negative crystallization test with statistical analysis in all the three groups (potentially malignant disorders, normal and oral cancer)

| Crystallization test | PMDs group, n (%) | Normal group, n (%) | Oral cancer group, n (%) |
|----------------------|------------------|--------------------|-------------------------|
| Positive             | 25 (83.3)        | 5 (13.2)           | 26 (96.3)               |
| Negative             | 5 (16.7)         | 33 (86.8)          | 1 (3.7)                 |
| Total                | 30               | 38                 | 27                      |

Parameter                  | Formula       | Normal versus PMDs | Normal versus oral cancer | PMDs versus oral cancer |
|---------------------------|---------------|---------------------|---------------------------|-------------------------|
| Sensitivity               | a/(a + b)     | 83.33               | 96.3                      | 83.33                   |
| Specificity               | d/(c + d)     | 86.84               | 86.8                      | 3.7                     |
| Positive predictive value | a/(a + c)     | 83.33               | 83.87                     | 49.02                   |
| Negative predictive value | d/(b + d)     | 86.84               | 97.06                     | 16.67                   |
| Disease prevalence        | (a + b)/(a + b + c + d) | 0.44 | 0.42 | 0.53 |
| $\chi^2$                  | 33.4817       | 43.7341             | 2.5325                   |
| $P$                       | 0.0001        | 0.0001              | 0.111                    |

PMDs=Potentially malignant disorders

Figure 2: Crystallization test in a normal individual exhibiting a pattern of eccentric centre of gravity with radiating needles

Figure 3: Crystallization test in an oral submucous fibrosis patient exhibiting transverse form

Figure 4: Crystallization test in an oral leukoplakia patient exhibiting transverse forms

Figure 5: Crystallization test in another leukoplakia patient exhibiting transverse forms
Further within OSCC group, TF frequency was correlated to various histopathological grades of oral cancer [Table 4].

**Table 2: Expression of transverse forms in potentially malignant disorders with mean and standard deviations**

| PMD                  | Transverse forms | n  |
|----------------------|------------------|----|
| Leukoplakia          | 1.00±0.74        | 12 |
| Chronic candidiasis  | 0.67±0.58        | 3  |
| Oral lichen planus   | 1.80±0.45        | 5  |
| OSMF                 | 1.29±0.76        | 7  |
| Smokers palate       | 1.50±0.71        | 2  |
| Tobacco pouch keratosis | 1.00±0.0  | 1  |

SD=Standard deviation, n=Number of cases, PMDs=Potentially malignant disorders, OSMF=Oral submucous fibrosis

**Table 3: Most common site in oral cancer group**

| Site                  | n (%) |
|-----------------------|-------|
| Buccal mucosa         | 9 (30.0) |
| Alveolar ridge        | 3 (10.0) |
| Floor of mouth        | 2 (6.7) |
| Lower lip             | 1 (3.3) |
| Oropharynx            | 1 (3.3) |
| Retromolar            | 1 (3.3) |
| Tongue                | 6 (20.0) |
| Vestibule             | 7 (23.3) |

n=Number of cases

**Table 4: Correlation between number of transverse forms and histopathological grades of oral cancer using independent sample t-test**

| Condition (mean±SD) | P       |
|---------------------|---------|
| Well                | 2.00±0.68 | >0.99; NS |
| Moderate            | 2.00±0.76 |         |

NS=Nonsignificant, SD=Standard deviation

There was no any correlation found between TF frequencies to various grades of oral cancer.

**Discussion**

Biochemical alterations occurring in malignancy can be detected in the blood as it serves as an excellent medium reflecting alterations in the body during malignancy.[10]

A specific method for cancer detection was employed by Pfeiffer (1938). According to this method, cupric chloride on admixture with the blood of a cancer patient exhibited specific pattern of crystallization. Pfeiffer’s crystallization test is based on the principle that colloidal proteins existing in the dilute form of human blood behave as an impurity when mixed with cupric chloride solution and get transformed into an orderly pattern with radiating needle-like crystals.[6] These patterns were found to be distinctive in health and disease by Pfeiffer. In experimental animals, these patterns were found to be specific and characteristic for each disease.[6]
These biochemical changes occurring in blood have a molecular basis at grass root level. There are certain forces at the molecular level maintaining the integrity of molecular structure. In case of malignancy, biochemical alterations bring about change in these physical forces involved in maintaining cohesion of molecules in crystalline pattern. This in turn is responsible for the peculiar crystalline pattern forming tendency with chemical substances. It can, therefore, be expected that these physical forces get altered in any kind of malignancy causing disturbance in molecular integrity.[9,11]

In malignancies, it has been noticed that there is increase in the levels of diamines and polyamines in blood which are intermediate products of protein metabolism.[12] These degraded products of protein metabolism might be responsible for the particular precancer and cancer-specific patterns in the crystallization test.

In the present study, crystallization patterns observed were quite similar to the previous studies. Certain variations in patterns mentioned by Sarode et al. like the presence of two or more centers of gravity with wing-like formations were present. In the present study, side branching arising from central radiating needles were differentiated from TFs by closer examination with magnifying lens similar to study by Sarode et al.[13]

In the present study, sensitivity and specificity of crystallization test for the oral potentially malignant group were 83.33 and 86.84%, respectively. In fact, this is the first study to evaluate the effectiveness of crystallization test in the group of oral PMDs. Crystallization test carried out on precancerous conditions of female genital tract had a sensitivity of 84.61% in a study by Shaikh et al.[9]

Sensitivity and specificity of the test for oral cancer group in the present study were 96.30 and 86.84%, respectively, whereas those obtained by Sarode et al. were, 96 and 96.66% respectively.[13] Sensitivity of the test carried out on oral cancer patients by Gulati et al. and Kuczkowski et al. were 88 and 71.5, respectively whereas in genital cancer carried out by Shaikh et al. was 94.7%.[5,7,8]

The mean TFs were further correlated with the histopathological grades of oral cancer. No statistically significant correlation was found. This went against the study by Sarode et al. where the correlation was found. Evaluation of this correlation further requires a larger sample performed under aseptic conditions.

In one of the cases, we performed crystallization test pre- and post-treatment and found a reduction in the number of TFs posttreatment. Role of crystallization test in assessing the remission of the lesion can further be analyzed in future studies.

Crystallization test in this study was also carried out on a long-term areca nut chewer, and a smoker without any clinical lesions and TFs were observed [Figures 9 and 10]. This however needs further validation by carrying over larger populations.

In the potentially malignant group, highest number of TFs was observed in leukoplakia and oral submucous fibrosis as they formed the majority of cases. Histopathological features such as epithelial dysplasia are most commonly used indicators for malignant transformation. Other parameters are needed for accurate assessment of malignant transformation apart from histopathological assessment.[14] In this regard, number of TFs in oral PMDs can be considered for risk assessment.

In the present study, 5 patients of oral PDMs group and 1 patient of oral cancer group exhibited false negative. On the other hand, 5 of the normal group people exhibited false-positive result. Further, the irregular or ill-defined patterns observed for few of the study could be due to the technical sensitivity. As this test is purely based on physical phenomenon and is technically highly sensitive, it should be carried out under strict physical conditions to avoid false positives and negatives as shortcomings.

**Conclusion**

Thus our study concludes crystallization test to be an effective, simple, less invasive, and less time-consuming screening method in detection of oral PMDs and oral cancer. This test can be used at community level in mass screening of high-risk individuals who are more prone to develop potentially malignant and malignant oral lesions. These high-risk individuals should be subjected to more confirmatory tests to evaluate their malignancy potential. However further studies are required on larger samples to validate the effectiveness of crystallization test in screening of oral PMDs and oral cancer.

**Acknowledgments**

We want to acknowledge the Efforts of laboratory technician - Sheri and attenders – Vijayamma and Neelappa...
of Oral Pathology department, Bapuji Dental College and Hospital for their support while carrying out study.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

References
1. Mercadante V, Paderni C, Campisi G. Novel non-invasive adjunctive techniques for early oral cancer diagnosis and oral lesions examination. Curr Pharm Des 2012;18:5442-51.
2. Feller L, Lemmer J. Oral squamous cell carcinoma: Epidemiology, clinical presentation and treatment. J Cancer Ther 2012;3:263-8.
3. Mortazavi H, Baharvand M, Mehdipour M. Oral potentially malignant disorders: An overview of more than 20 entities. J Dent Res Dent Clin Dent Prospects 2014;8:6-14.
4. Warnakulasuriya S, Johnson NW, Van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med 2007;36:575-80.
5. Shaikh SI, Kawale DN, Diwan CV, Quadeer A, Kharkar AR. Crystallization test for detection of malignancy of female genital tract. Int J Basic Med Sci 2012;3:118-24.
6. Gruner OC. Experience with the Pfeffer crystallization method for diagnosis of cancer. Can Med Assoc J 1940;43:99-106.
7. Gulati SP, Sachdeva OP, Sachdeva A, Adlakha RP, Kakkar V. Crystallization test for the detection of head and neck cancer. ORL J Otorhinolaryngol Relat Spec 1994;56:283-6.
8. Kuczkowski J, Zaorski P, Betlejewski A. Crystallization test in patients with head and neck neoplasms. Otolaryngol Pol 1995;49 Suppl 20:121-4.
9. Sabarth E, Williams HN. Sensitive Crystallization Process as Demonstration of Formative Forces in the Blood. 2nd ed. Spring Valley, New York: Anthroposophic Press; 1975.
10. Burkhardt A. Advanced methods in the evaluation of premalignant lesions and carcinomas of the oral mucosa. J Oral Pathol 1985;14:751-78.
11. Quadeer A. New Approach for Detection of Malignancy by Crystallization Test [Ph.D. Thesis] for (Medicine) Degree in Anatomy. Nagpur University; 1988.
12. Savory J, Shipe JR. Serum and urine polyamines in cancer. Ann Clin Lab Sci 1975;5:110-4.
13. Sarode SC, Sarode GS, Barpande S, Tupkari JV. Efficacy of crystallization test for screening of oral squamous cell carcinoma with clinico-pathological correlation. Indian J Dent Res 2013;24:464-7.
14. Reibel J. Prognosis of oral pre-malignant lesions: Significance of clinical, histopathological, and molecular biological characteristics. Crit Rev Oral Biol Med 2003;14:47-62.