Analysis of Quantification Chlamydospore in Normal Controls, Potentially Malignant and Malignant Patients in 8 and 16 Hours by Using Serum Milk and Corn Meal Broth +5% Milk As Culture Medias

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Abstract

The present study evaluates the association of Candida albicans with normal control group, potentially malignant and malignant lesions of oral cavity by using two different liquid culture media. Saliva was collected and biopsy was taken only from those clinically suspected potentially malignant and malignant lesions for histopathological diagnosis. Saliva samples were inoculated for fungal growth in Sabouraud's dextrose agar and culture-positive samples had undergone for Germ tube test. Germ tube-positive samples were further taken for quantification of chlamydospore production in liquid media at 8 and 16 hours. In normal control groups no fungus growth was found

Introduction

The present study comprised of total 75 patients, which included, 30 potentially malignant (15-leukoplakia and 15- oral submucous fibrosis), 30 oral squamous cell carcinoma and normal controls group comprised of 15 healthy volunteers who were not having any relevant medical, dental & habit history.

Source and method of collection of data

The subjects included in this study were randomly selected from Oral Medicine, Oral Surgery and Gujarat cancer research institute, Ahmadabad who were coming for various investigations in above respective department. Patient detail history was recorded in the proforma (Annexure I). The study was conducted in the department of Oral Pathology, Oral Medicine and Oral Surgery (Pacific Dental College and Hospital) in collaboration with department of Microbiology (Geetanjali Medical College and Hospital).

Methodology

All the subjects including normal control, premalignant and malignant groups were informed regarding the purpose of the study and asked patient to fill consent form (Annexure II). The oral examination was carried out and relevant findings were entered in proforma. The subjects were asked to rinse the mouth with distilled water thoroughly to remove any food debris. After 10 minutes phosphate buffer solution was used as oral rinse method for saliva collection. Samples were obtained by requesting subjects to keep and swirl the solution for one minute, and then expectorate all saliva into pre sterilized container without swallowing. Once sample collected, the container was placed in an ice carrier box and transferred to the laboratory for microbiological study. After collection of saliva, biopsy was taken only from those who were clinically suspected as potentially malignant and malignant lesions for histopathological diagnosis.

Preparation of Culture Medias
For Fungus Growth

Sabouraud's dextrose agar- 65 grams of the media was suspended in one liter of distilled water. It was mixed well until a uniform suspension was obtained. It was heated with frequent agitation and boiled for one minute and then sterilized at 118-121°C for 15 minutes. To prepare a selective culture medium aseptically 40mg Streptomycin was added for every milliliter of medium before use.

For Germ Tube Test

Serum- human venous blood was collected and allowed to fully clot. The specimen was placed vertically in a test tube rack to speed up the clotting action. After clotting the specimen was placed in a centrifuge machine at 3000 rpm for 10 minutes.

For Chlamydospore

Corn meal broth + 5% Milk- corn meal agar media was modified to prepare the broth. It was mixed with cold distilled water, stirred at 6°C. Next day insoluble components were removed by filtration, then 5% pasteurized milk was added and autoclaved at 115°C for 30 minutes. It was allowed to cool and then poured into the test tubes.

Milk Serum - was prepared from non-pasteurized cow's milk. Sulphuric acid was added drop by drop in milk until it curdifyies. The supernatant was removed and left over was filtered by filter paper until it became clear. Autoclaved at 115°C for 30 minutes. It was allowed to cool and then poured into the flask.

Saliva Culture Technique for Candida

All samples were centrifuged at 1700rpm for 10 min. Now the supernatant was discarded and sediment material was carried with pipette and inoculated in Sabouraud's Dextrose Agar (S.D.A) media. The sample was streaked using inoculating loop and incubated in 37°C for 48 hours. The growth appeared in 48 hours as cream/ white colored, smooth and pasty colonies.

Identification of Yeast Cells

Very small inoculum from an isolated candida colony was picked up with a sterile inoculating loop and was suspended on a glass slide, then a drop of water was added on it and with circular movement of the loop a thin smear was formed and air dried. The smear was fixed by quickly passing on the bunsen burner in to and fro motion holding slide at one then it was stained by Gram's staining method.

Identification of Candida Species

Method

Very small inoculum from an isolated candidal colony was picked up with a sterile inoculating loop and was suspended in a test tube containing normal human serum (0.3- 0.5 ml) by rubbing the inoculated loop against the wall of the test tube. This helps in diluting the pasty colonies by giving the serum turbid appearance. The mixture was incubated at 42°C and examined after 2-3 hrs. A drop of mixture was placed in a clean glass slide and covered with a clean cover slip. This was first examined under a low power objective to locate the group of cells and later, the presence of germ tube was confirmed under high power objective of the microscope.

Comparison of Culture Medias for Rapid and Quantitatively More Production of Chlamydospore

Only the germ tube positive samples were preceded for further study and to compare the rapid production of chlamydospore with two different Culture Medias (corn milk broth + 5% milk and serum milk). Very small inoculum was picked up with a sterile inoculating loop and was suspended in same manner as it was done for Germ tube test. The two different test tubes containing corn meal broth + 5% milk and serum milk were placed in water bath at 45°C and results were read at 8 and 16 hours respectively.

Chlamydospores were observed under wet mounts. A drop of inoculated broth media was placed onto the slide, over it a drop of lactophenol cotton blue stain was added then a cover slip was put over it. This was first examined under a low power objective to locate. the group of cells and later, the presence of chlamydospores were confirmed and quantified (Annexure XI- XV) in by two observers in 10 fields (high power view) in both media (8 and 16 hours).

Histopathological diagnosis

In order to study the association of Candida albicans and the types of mucosal lesions all the consecutive biopsies of oral mucosa were processed according to the standardized laboratory method in the Department of Oral Pathology, Pacific Dental College. The histological sections were stained with Hematoxylin and Eosin for histopathological diagnosis of the Periodic Acid Schiff's stain, for the visualization of fungal hyphae.

Statistical Analysis
Formula used for analysis:

Mean:

$$\overline{X} = \frac{\sum_{i=1}^{N} X_i}{N}$$

Standard deviation:

$$\sigma = \sqrt{\frac{\sum (X_i - \overline{X})}{(N-1)}}$$

X_i= mid point of class interval
N= total of all frequency

Analysis of Variance

The analysis of variance frequently referred to as the ANOVA is a statistical technique specially designed to test whether the means of more than two quantitative populations are equal. This technique was developed by, R. A. Fisher in 1920s and is capable of fruitful application to a diversity of practical problems. Basically, it consists of classifying and cross classifying statistical results and testing whether the means of a specified classification differ significantly. In this way it is determined whether the given classification is important in affecting the results.

Technique of Analysis of Variance

The ANOVA can be one-way, two-way, three-way or N-way. In one-way classification the data are classified according to only one criterion. It is customary to summarize calculations for sums of squares, together with their number of degrees of freedom and mean square in a table called the

analysis of variance table, generally abbreviated ANOVA. The specimen of ANOVA table is given below:

Analysis of variance (ANOVA) table: one-way classification model

Source of Variation	SS (Sum of Squares)	V (degree of Freedom	MS (Mean Square)	Variance Ratio of F
Between samples	SSC	V1	MSC	F
Within samples	SSE	V2	MSE	

Where,

SST = Total sum of squares of variations
SSC = Sum of squares between samples
SSE = Sum of squares within samples
MSC = Mean sum of squares between samples
MSE = Mean sum of squares within sample

Student's t-test

In case of small sample Student's t-test is applied instead of z-test. It was designed by W. S. Gossett whose pen name was Student. Hence this test is known as Student's t-test. In case of means for two independent samples the hypothesis takes the following form

$$H_0: \mu_1 = \mu_2$$

$$H_1: \mu_1 \neq \mu_2$$

The two populations are sampled and means and the variances are computed based on samples of sizes n_1 and n_2. If the both samples are found to have same variance, a pooled variance estimate is computed from the two sample variances as follows:

$$s^2 = \frac{\sum(y_{1i} - \bar{y}_1)^2 + \sum(y_{2j} - \bar{y}_2)^2}{n_1 + n_2 - 2} = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}$$

The standard deviation of test statistics can be estimated as:

$$S_{(\bar{x}_1 - \bar{x}_2)} = \sqrt{s^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

The appropriate value of t can be calculated as:

$$t = \frac{(\bar{X}_1 - \bar{X}_2)}{S_{(\bar{x}_1 - \bar{x}_2)}}$$

The degrees of freedom in this case are $(n_1 + n_2 - 2)$

If the calculated't' value exceeds the value given under desired level of significance it is said to be significant at that level of significance and hence null hypothesis ($H_{0)}$ is rejected and alternative hypothesis (H_1) is accepted.

Results & Observations

The study group comprised a total of 75 patients, which includes 30 potentially malignant (15 leukoplakia and 15 oral sub mucous fibrosis), 30 oral squamous cell carcinoma and 15 normal controls. From all patients unstimulated salvia samples were collected to study the association of Candida albicans with these mucosal lesions using Sabouraud's dextrose agar media, germ tube test was carried out in serum and further quantification of chlamydospore was done by using two different medias i.e. corn meal broth + 5% milk and serum milk. After collection of salvia samples biopsy were taken for histopathological diagnosis by using H & E staining and for identification of Candidal hyphae P.A.S staining was done.

The collected salvia samples were utilized for culture for fungal growth in SDA media In normal control groups no fungus growth was found, in potentially malignant 8 cases showed fungus growth out of 15 leukoplakia cases and 3 cases showed fungus growth out of 15 cases in oral sub mucous fibrosis cases where as in malignant (OSCC) 20 cases showed fungus growth out of 30 cases

Now fungus positive cases further carried for germ tube test in serum media .The result reveled that all leukoplakia and oral sub mucous fibrosis gave positive germ tube test but in malignant, 14 cases showed positive germ tube test for Candida albicans and 6 cases showed non albicans species out of total cases .Further the confirmatory test for candida albicans were carried by using two different medias i.e. corn meal broth + 5% milk and serum milk for production of chlamydospore. The quantification of chlamydospore was observed in 8 and 16 hours in both the lesions. In 8 hours no growth was observed in potentially malignant lesions whereas chlamydospore formation occurred only 4 malignant cases in both medias .Total chlamydospore formation in potentially cases was highly significant ($p < 0.001$) in corn meal broth + 5%milk (mean 41.36) in comparison to serum milk (mean 16.73,). In malignant cases total chlamydospore formation was also highly significant ($p < 0.001$) in corn meal broth + 5% milk media (mean 39.00) in comparison to serum milk media (mean 13.43,). These results reveled that corn meal broth + 5% milk media produced more number of chlamydospore in potentially malignant (71.21%) and malignant (74.39%) cases in comparisonto comparison to serum milk media (28.79% and 25.61% respectively) butquantitatively more in malignant cases .

This confirmatory test of chlamydospore showed that the association of candida albicans with potentially malignant (36-67% and malignant (46.67%) were statically significant but highly significant ($p < 0.001$) in malignant patients in comparison to normal controls.In potentially malignant and malignant cases chlamydospore formation in both media at 8 and 16 hours showed no statically significant results with age .In clinical staging of leukoplakia out of 15 cases, 8 were homogenous and 7 were non homogenous leukoplakia .In both variant only 4 cases showed positive fungal growth, germ tube formation and chlamydospore formation .Result showed no stastical significant result with clinical staging of leukoplakia was observed.In potentially malignant lesions histopathologically diagnosed leukoplakia cases divided into mild, moderate and severe group. In these groups there was no statically significant value found in relation to total production of

chlamydospore in serum milk media with mean 13.00, 17.75, 11.00 and corn meal broth + 5% milk with mean 29.00, 42.00, 56.00 respectively (table 20, graph 16). Similar result were obtain in histopathological diagnosed oral sub mucous fibrosis cases which were divided into mild, moderate and severe dysplasia (Table 21, 22 and graded as well differentiated and moderate differentiated Most of the potentially malignant (76.67%) and malignant cases (60%) were found in buccal mucosa. After than in tongue, angle of mouth, lower lip, gingiva and very few in alveolus and soft palate,

TABLE 5: Fungal growth with Sabouraud's agar media in normal control, potentially malignant and malignant cases.

	NORMAL CONTROL	POTENTIALLY MALIGNANT				MALIGNANT	
		Leukoplakia	%	OSMF	%	OSCC	%
Positive growth	0	8	53.3	3	20	20	66
Negative growth	0	7	46.6	12	80	10	33
Total	15	15	100	15	100	30	100

TABLE 6: Germ tube test with serum in potentially malignant and malignant cases.

	POTENTIALLY MALIGNANT				MALIGNANT	
	Leukoplakia	%	ÐSMF	%	OSCC	%
Candida albicans	8	100	3	100	14	46
Non albicans species	0	0	0	0	6	20
Total	8	100	3	100	20	100

TABLE 7: Chlamydospore formation with corn meal broth + 5% milk in potentially malignant and malignant cases.

	POTENTIALLY MALIGNANT				MALIGNANT	
	Leukoplakia	%	OSMF	%	OSCC	%
8 hours	0	0	0	0	4	28
16 hours	8	100	3	100	14	100

TABLE 8: Chlamydospore formation with serum milk in potentially malignant and malignant cases.

	POTENTIALLY MALIGNANT				MALIGNANT	
	Leukoplakia	%	OSMF	%	OSCC	%
8 hours	0	0	0	0	0	0
16 hours	8	100	3	100	14	100

TABLE 9: Total chlamydospore formation in potentially malignant (Leukoplakia + OSMF) cases after 16 hours.

	No. of patients	Mean	SD	t	Df	Result
Serum milk	11	16.73	5.92	5.58	10	***
Corn meal broth + 5% milk	11	41.36	16.32			

TABLE 10: Chlamydospore formation in malignant (OSCC) cases after 16 hours.

	No. of patients	Mean	SD	t	Df	Result
Serum milk	14	13.43	6.43	4.828	13	***
Corn meal broth + 5% milk	14	39.00	18.37			

TABLE 11: Total chlamydospore formation with serum milk and corn meal broth + 5 % milk in potentially malignant and malignant cases.

	Potentially Malignant		Malignant		Total	
	N	%	N	%	N	%
Serum milk	184	28.79	188	25.61	372	27.09
Corn meal broth + 5% milk	455	71.21	546	74.39	1001	72.91
Total	639	100.00	734	100.00	1373	100.00

N- Total number of Chlamydospore formation

TABLE 12- Association of Candida albicans with normal controls, potentially malignant and malignant cases.

	Normal Controls		Pot. Malignant		Malignant	
	N	%	N	%	N	%
Positive cases	0	0.00	11	36.67	14	46.67
Negative cases	15	100.00	19	63.33	16	53.33
Total	15	100.00	30	100.00	30	100.00

Chi Sqr = 10.05; df = 2; Result: p < 0.01 (Highly Significant)

TABLE 13: Chlamydospore formation in serum milk after 16 hours in potentially malignant cases with respect to different age group.

Age Group	No. of patients	Mean	SD	F	df	Result
<= 30 yrs	3	22.00	5.000	2.281	2,8	NS
31-40 yrs	4	13.50	5.972			
41-50 yrs	4	16.00	4.690			

TABLE 14: Chlamydospore formation in corn meal broth + 5% milk after 16 hours in potentially malignant cases with respect to different age group.

Age Group	No. of patients	Mean	SD	F	df	Result
<= 30 yrs	3	44.33	10.693	0.767	2,8	NS
31-40 yrs	4	33.25	15.628			
41-50 yrs	4	47.25	20.597			

TABLE 15: Chlamydospore formation in serum milk after 16 hours in malignant cases with respect to different age group.

Age Group	No. of patients	Mean	SD	F	df	Result
31-40 yrs	2	12.00	0.000	0.068	2, 11	NS
41-50 yrs	9	13.89	7.557			
> 50 yrs	3	13.00	6.083			

TABLE 16: Chlamydospore formation in corn meal broth + 5% milk after 16 hours in malignant cases with respect to different age group.

Age Group	No. of patients	Mean	SD	F	df	Result
31-40 yrs	2	42.50	21.920	0.209	2, 11	NS
41-50 yrs	9	40.33	20.439			
> 50 yrs	3	32.67	14.189			

TABLE 17: Chlamydospore formation in corn meal broth + 5% milk after 8 hours in malignant cases with respect to different age group.

Age Group	No. of patients	Mean	SD	F	df	Result
31-40 yrs	1	9.00	-	0.797	2, 11	NS
41-50 yrs	2	12.00	2.828			
> 50 yrs	1	8.00	-			

TABLE 18: Positive fungal growth, germ tube test and chlamydospore formation in Leukoplakia.

Clinical categories	No of Pt.	SDA +ve Fungal Growth	GTT (serum)	CHLAMYDOSPORE Serum Milk	CHLAMYDOSPORE Corn Meal+ 5% milk	Result
Homogeneous	8	4	4	71	149	NS
Non homogeneous	7	4	4	60	162	
Total	15	8	8	131	311	

TABLE 19: Chlamydospore formation in serum milk after 16 hours in different histopathological grades of Leukoplakia.

Leukoplakia	No. of patients	Mean	SD	F	df	Result
Mild	3	13.00	5.29	1.111	2,5	NS
Moderate	4	17.75	4.99			
Severe	1	11.00	0.00			

TABLE 20: Chlamydospore formation in corn meal broth + 5% milk after 16 hours in different histopathological grades of Leukoplakia.

Leukoplakia	No. of patients	Mean	SD	F	df	Result
Mild	3	29.00	10.39	0.978	2,5	NS
Moderate	4	42.00	21.46			
Severe	1	56.00	0.00			

TABLE 21: Chlamydospore formation in serum milk after 16 hours in different histopathological grades of Oral submucous fibrosis.

OSMF	No. of patients	Mean	SD	t	df	Result
OSMF with mild dysplasia	1	27.00	0.00	1.299	1	NS
OSMF with moderate dysplasia	2	18.00	5.66			

TABLE 22: Chlamydospore formation in corn meal broth + 5% milk after 16 hours in different histopathological grades of Oral submucous fibrosis.

OSMF	No. of patients	Mean	SD	t	df	Result
OSMF with mild dysplasia	1	56.00	0.00	0.77	1	NS
OSMF with moderate dysplasia	2	44.00	12.73			

TABLE 23: Chlamydospore formation in serum milk after 16 hours in malignant cases in different histopathological grades of OSCC.

OSCC	No. of patients	Mean	SD	t	df	Result
WDSCC	4	16.25	6.95	1.042	12	NS
MDSCC	10	12.30	6.22			

TABLE 24: Chlamydospore formation in corn meal broth + 5% milk after 16 hours in malignant cases in different histopathological grades of OSCC.

OSCC	No. of patients	Mean	SD	t	df	Result
WDSCC	4	35.00	9.27	0.5	12	NS
MDSCC	10	40.60	21.18			

Conclusion

The present study was carried out to evaluate the association of Candida albicans with normal controls, potentially malignant and malignant patients, using different culture medias. Saliva collection was done from all the patients and biopsy was taken from those who were clinically suspected as potentially malignant (leukoplakia and oral submucous fibrosis and malignant lesions (oral squamous cell carcinoma) for histopathological evaluation. The result showed that:

- The association of Candida albicans with normal controls, potentially malignant and malignant patients was highly significant.
- In potentially malignant the association of Candida albicans with leukoplakia was more significant in comparison with oral submucous fibrosis.
- The confirmatory test of candida albicans species was carried oput by the positive germ test and chlamydospore formation in normal controls. Potentially, malignant and malignant patients.
- The rapid production of chlamydospore formation was noted in corn meal broth + 5% milk in comparison of serum milk.

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