

METHYLENE BLUE-TRIPHENYL TETRAZOLIUM CHLORIDE TEST
TUBE METHOD IN THE EARLY DIAGNOSIS OF CANCER OF THE
CERVIX UTERI AND ITS CO-RELATION WITH EXFOLIATIVE
CYTOLOGY AND BIOPSY RESULTS

by

V. P. MITAL*, M.D.

USHA K. LUTHRA** M.D., Ph.D., M.C. (Path.) (Lond.), M.A.M.S.,
F.I.A.C.

and

V. P. BHARADWAJ*** Ph.D.

Introduction

Carcinoma cervix is a major problem in our country. Several methods have been employed in the easy diagnosis of cervical carcinoma from time to time. Papanicolaou's staining technique of exfoliated vaginal cells, fluorescent microscopy, colposcopy, phase contrast microscopy and histological examination of cervical tissue are the commonly used methods.

Papanicolaou's staining technique requires experience in the cell morphology, a team of trained workers and large equipment for the laboratory. The time required is more. It works at the cellular level where the morphological changes have started.

Fluorescence microscopy, colposcopy and phase contrast microscopy can be used as a first screening test but require

confirmation by Papanicolaou's staining technique and histo-pathological examination of the tissue biopsy.

In recent years great interest and emphasis has been laid on cytochemistry to enable an early detection of cervical carcinoma. This facet of science is yet in its infancy. However, it has been increasingly apparent that morphologic and biologic difference between normal, dysplastic and neoplastic cells have their basis in fundamental biophysical and biochemical differences of the cellular level when apparent morphological changes have not had enough time to set in. Therefore, it is presumed that a cell undergoing a neoplastic change can be detected at an early stage using cytochemical techniques.

A method simple enough which does not entail any operative procedure, is thus to be evaluated which could be used for screening of large number of cases as well as for detection of the early carcinoma of the uterine cervix.

The present study was carried out with an aim that Triphenyl-Tetrazolium chloride (T.T.C.) test tube method may fulfil the above criteria as it is a simple screening test and thus could be more

(Postgraduate Department of Pathology and Bacteriology, S. N. Medical College, Agra).

*Reader in Pathology, S. N. Medical College Agra.

**Professor of Pathology, at present Dy. Director-General, Indian Council of Medical Research, Ansari Nagar, New Delhi-16.

***Research Officer, Department of Pathology, S. N. Medical College, Agra.

Received for Publication on 16-1-1971.

easily adopted to the mass screening survey.

Material and Methods

The material for the present study was collected from 580 patients, who came to the outpatient department of gynaecology ward and radium institute of S. N. Hospital, Agra. T.T.C. method was done in all cases, Papanicolaou's staining in 540 cases, and cervical punch biopsies in 67 cases. The patient was put in lithotomy position, Sim's speculum was inserted and anterior vaginal wall retracted so that the cervix could be viewed. Then 1-2 cc of T.T.C. solution was taken in a glass pipette and poured on the mouth of the cervix. The cervix was then rinsed 2 to 3 times with the solution collected in the posterior fornix of the vagina and the solution was aspirated and kept in a labelled test tube. The test tubes containing vaginal aspirates were immediately kept in a water bath at 37°C for an hour. The sediment at the bottom of the test tube was examined. If it was red coloured, a drop on the slide was taken, covered with a coverslip and examined under light microscope for type A or type B cells. If there was no colour change, the case was not examined microscopically and treated as negative.

Criteria for Type A or Malignant Cells

- (1) Type A cells are usually round in shape, a few may be deformed.
- (2) The size of these cells is about 3 to 5 times that of a polymorphonuclear leucocyte.
- (3) The cytoplasm is filled with strawberry-like coarse red, granules with high refractive properties.
- (4) The nucleus is generally hidden by these granules but occasionally it may appear as a completely empty space or sparsely scattered with a few granules.

- (5) The colour of the granules is generally bright red or orange red. In the cases having bleeding, the granules are coloured brownish or yellowish.

Criteria for Type B Cells

- (1) The cells are of small, size, approximately equal to the neutrophilic leucocytes regardless of the appearance of the granules.
- (2) The cells contain fine granules distributed around the nucleus in a basal cell.
- (3) The squamous cells with too few granules.

Observations

First Screening Test: Microscopic Examination of the Test Tube Sediment

Out of 580 cases examined by TTC method, 290 cases showed change in the colour of deposit to red, while there was no change in the colour in the remaining 290 cases.

Second Screening Test: Microscopic Examination of the Coloured Deposits

For convenience the exfoliated vaginal cells in the red coloured sediment of test tube were divided into the following:

1. Type A or malignant cells.
2. Type B cells.
3. Unclassified cells.

Type A Cells or Malignant Cells

These exfoliated cells were found in the patients suffering from uterine carcinoma. Cells were round with uniform outline 3 to 5 times the size of the polymorphonuclear leucocyte. They were filled with coarse red granules, varying from 30 to 140 in number per cell. The shape of the granules was more or less round or oval having bright red colour. In cases accompanied with vaginal bleeding, the granules were yellowish orange.

The granules had a characteristic distribution inside the cell. Some cells were completely full of granules, while in most of the cells a clear space representing the nuclear mass was left. In a few cells, the granules were arranged in a horse-shoe fashion.

The number of type A or malignant cells varied according to the nature of malignancy. In one of the cases type A cells were from 8 to 15 in number per 1 to 2 high power field. This case was later proved anaplastic carcinoma by cervical biopsy. In this case the number of granules varied from 60 to 120 per cell (Fig. 1).

In 8 cases, later proved to be early invasive epidermoid carcinoma, type A cells were 2 to 5 cells per high power fields. The number of granules varied from 40 to 100 per cell.

In 3 cases of preinvasive carcinoma, type A or malignant cells were found with difficulty, one cell was seen per 30 to 50 high power field. Number of granules was comparatively less varying from 30 to 60 per cell, scattered diffusely or at the peripheral part of the cell (Fig. 2).

Type B Cells

These cells were seen in 177 cases suffering from benign lesions of the cervix and the vagina viz., cervicitis, endocervicitis and vaginitis.

The exfoliated cells were superficial squamous cells and parabasal cells showing diffuse distribution of fine granules. These fine granules were also seen extracellularly (Fig. 3).

Out of 177 cases in which type B cells were encountered, 70 cases clinically diagnosed as vaginitis, showed intense red deposit in the test tube fluid within 15 minutes. These cases were examined within half an hour of incubation, as the deposition of the granules increases in

number and most of the granules change colour from red to blue or purple on further incubation.

Unclassified Cells

These cells were seen in 7 cases showing change in the colour of the test tube sediment. These cells were round, polygonal or oval in shape, usually appearing in groups or sometimes singly. The cell outline was uniform with coarse and fine red granules (Fig. 4).

Using Papanicolaou's staining technique, 2 cases out of the 7 were confirmed as dysplasia grade II while the remaining 5 showed dysplasia grade III.

In the present study, a total of 580 cases were examined by TTC method and a correlative study was done in 540 cases by Papanicolaou's staining technique while cervical punch biopsy was done in 67 cases. Out of these 580 cases, 540 cases had gynaecological symptoms, while 40 were asymptomatic which served as controls giving negative results on first screening.

The 540 cases with gynaecological symptoms showed type A cells in 106 cases, Type B cells in 177 cases and unclassified cells in 7 cases, while the remaining 250 cases did not show any colour change (Table 1).

Out of the 106 cases positive for type A cells by T.T.C., 104 cases were confirmed to be of cervical carcinomas by Papanicolaou's staining technique or cervical biopsies. In one of the remaining two cases, the Papanicolaou's staining technique revealed dysplasia grade II while the smear was unsatisfactory in the other.

Using T.T.C. method type B cells were observed in all the cases of dysplasia grade I, and also in 22 cases of dysplasia grade II and in 2 cases of dysplasia grade III. Unclassified cells were found in two of the dysplasia grade II cases and 5 cases

TABLE I

Showing Analysis of 540 Cases with Gynaecological Symptoms with Respect to T.T.C. Test

No. of cases	Type A cells or malignant cells	Type B cells	Unclassified cells	No. colour change
540	106	177	7	250
Percentage	19.63	32.78	1.30	46.30

of dysplasia grade III. In 3 cases of dysplasia grade III, T.T.C. method revealed type A cells. These cases were confirmed by cervical biopsy as carcinomas. These features are detailed in Table II.

Further analysis was done for comparative accuracy of T.T.C. method, Papanicolaou's staining technique and cervical biopsies. This is detailed in Table III.

Sixty-five cases, out of 105 cases positive for malignant or type A cells by T.T.C. revealed carcinoma on histological

examination of the cervical biopsy material. The analysis of these 65 positive cases of carcinoma is given in Table IV.

The above table shows that the incidence of advanced invasive epidermoid carcinoma was highest amongst all the other types of carcinoma, constituting 76.92 per cent.

Type A cells or malignant cells were seen in 79 cases, out of 80 cases which were clinically diagnosed as cervical carcinoma. These were confirmed by cervical biopsy or Papanicolaou's staining

TABLE II

Analysis of Cases Showing Dysplastic Change Using Papanicolaou's Staining Technique and Cervical Biopsies

Report of Papanicolaou's staining method	No.	T.T.C. Test		
		Type A cells	Type B cells	Unclassified cells.
Dysplasia grade I	36	-	36	-
Dysplasia grade II	24	-	22	2
Dysplasia grade III	10	3	2	5

TABLE III

Showing Comparative Accuracy of T.T.C. Method, Papanicolaou's Staining Technique and Cervical Biopsies

Method of examination	No. of cases	Correct diagnosis		False negative		False positive	
		Number	Percentage	Number	Percentage	Number	Percentage
T.T.C.	105	104	99.05	-	-	1	0.95
Papanicolaou's staining	95	92	96.84	3	3.16	-	-
Cervical biopsy	67	65	97.01	2	2.99	-	-

TABLE IV

Showing Analysis of 65 Positive Cases of Carcinoma Confirmed by Biopsy for Nature of the Malignancy

Histopathological diagnosis	Total Number of cases	Percent-age
Intra-epithelial carcinoma	3	4.62
Early invasive epidermoid carcinoma	8	12.30
Advanced invasive epidermoid carcinoma	50	76.92
Cervical adenocarcinoma	3	4.62
Endometrial adenocarcinoma	1	1.54

technique. The remaining one case was proved free from the malignant lesion, both by cervical biopsy and T.T.C. method. The above mentioned findings are shown in Table V.

TABLE V

Showing Analysis of 80 Cases, Diagnosed Clinically as Cervical Carcinoma

Detailed analysis	Total no. of cases	Percent-age
Cervical biopsy done	46	-
Cervical biopsy positive for carcinoma	45	97.86
Papanicolaou's staining technique done	70	-
Malignant cells seen	70	100.00
T.T.C. method done	80	-
Type A cells seen	79	98.75

The above table shows that the clinical diagnosis was correct in 79 out of the 80 cases.

Fourteen cases were suspected clinically as carcinoma, out of these, 9 cases were confirmed to be carcinoma by cervical biopsy, while in 2 cases the biopsy material was inadequate for the diagnosis. The detailed analysis of these cases is shown in Table VI.

TABLE VI

Showing Analysis of 14 Cases Suspected Clinically of Carcinoma Cervix

Detailed analysis	Number of cases	Percent-age
Cervical biopsy done	14	-
Cervical biopsy positive for carcinoma	9*	64.28
Papanicolaou's staining technique done	14	-
Smears positive for malignant cells	11	78.75
T.T.C. method done	14	-
Type A cells seen	11	78.75

Note:—*2 cases showed inadequate tissue in the biopsy material.

The above table shows that the clinical diagnosis was correct in 78.75 per cent cases. It also shows that the 3 cases who were suspected to be suffering from cervical carcinoma were confirmed free of the malignant lesion of the cervix. Lastly, it shows that T.T.C. and Papanicolaou's staining technique revealed an equal and maximum percentage of accuracy.

Out of 5 follow up cases of cervical carcinoma, who came for check-up, 2 cases treated by radium and in 1 case, Wertheims hysterectomy was done showed malignant cells by T.T.C. method and were confirmed by Papanicolaou's staining technique for recurrence of malignancy.

Four hundred and thirty-six cases were confirmed clinically to be of benign cervical lesions, out of these 11 were confirmed to be carcinoma by cervical biopsy, Papanicolaou's staining technique and T.T.C. method.

Discussion

In the present study a method simple enough which does not entail any operative procedure, is thus to be evaluated which could be used for screening, of

large number of cases as well as for detection of the early carcinoma of the uterine cervix. From the present study Triphenyl-tetrazolium chloride (T.T.C.) test tube smear method seems to fulfil the above criteria. It is a simple screening test; diagnosis can be made upon the appearance of test tube sediment with change of colour and further recognition of the Type A or malignant cells on smear examination. It can be more easily adopted to the mass screening survey.

In order to test the accuracy of this method, a total of 580 cases were examined from the gynaecological Out-patient Department, Radium out-patient department of S. N. Hospital, Agra, and Out-patient Departments of Lady Lyall Hospital, Agra. A correlative study of 540 cases with Papanicolaou's staining technique and 67 cervical biopsies were undertaken. The accuracy figures of T.T.C. method were 99.05 per cent for Papanicolaou's staining technique 96.84 per cent and 97.01 per cent for the cervical biopsy.

Two hundred and fifty cases out of 540 cases and the 40 control cases were found negative for cervical carcinoma on the first screening test of T.T.C. method (i.e. there was no colour change in them); therefore, half of the cases could be screened off on first screening test. These were confirmed by Papanicolaou's staining technique as having no malignant cells in the vaginal smear. One hundred and eighty-four cases out of 540 cases showing type B cells were also confirmed by Papanicolaou's staining technique as negative for malignant cells. Therefore, there was no false negative results by T.T.C. method.

Krishnamurthi, Shanta and Rao (1962) used slightly different technique of this method. They took a drop of vaginal fluid on a slide and added a drop of 1 per cent

tetrazolium solution and mixed. These slides were kept at 29° to 32°C for half an hour and then examined under the microscope. In their series of study, they took clinical diagnosis as the firmest criterion, and different methods were tested in relation to the former. Cervical biopsy results tallied with clinical carcinoma cervix cases in 92.7 per cent cases, Papanicolaou's staining technique tallied with 91.2 per cent cases while T.T.C. method tallied in 89.8 per cent cases of clinically diagnosed cases of cervical carcinoma.

Jian-ren, Zimin and Wenman (1963), reported 99.18 per cent accuracy figures by T.T.C. method and 0.82 per cent false negative figures. In our studies false negative figures were none.

Out of 105 cases positive for Type A cells by T.T.C. method, carcinoma of the cervix was confirmed either by Papanicolaou's staining technique or by cervical biopsies. Forty-five cases were confirmed by cervical biopsies, while Papanicolaou's staining technique revealed malignant cells in 92 cases. Of these 3 cases were of intra-epithelial carcinoma, 8 of early invasive epidermoid carcinoma and 50 cases of advanced invasive epidermoid carcinoma. Three cases were cervical adenocarcinoma and 1 case was of endometrial adenocarcinoma. These results paralleled to some extent with Jian-ren, Zimin and Wenman (*loc. cit.*) studies where 3 cases of intra-epithelial carcinoma, and 8 cases of early invasive carcinoma were found in 121 cases of clinically diagnosed cervical carcinoma cases.

Out of the 434 cases which were clinically considered as benign lesions of cervix such as cervicitis, vaginitis, endocervicitis, sterility or menstrual disorders, 11 cases were confirmed as cervical carcinoma by Papanicolaou's staining techni-

que or T.T.C. method. All these 11 cases revealed type A or malignant cells by T.T.C. method. Krishnamurthi, Shanta and Rao (loc. cit.) found best results by T.T.C. method when the temperature of the reaction was maintained at 29-32°C. On the other hand Jianren, Zimin and Wenman (loc. cit.) found 37°C as the suitable temperature for the reaction of T.T.C. test. According to them the reduction of T.T.C. test may be delayed or become false negative if the temperature was below 35°C. On the other hand, T.T.C. test may undergo a non-specific reduction with an increase in the temperature over 40°C.

We were also faced with the problem of regulating the temperature for incubation of the test tubes containing vaginal fluid in the beginning, but later on it was found that the optimum results were obtained at 37°C.

In our studies false negative results were none. In Jianren *et al's* (loc. cit.) studies, false negative results were as low as 0.82 per cent. In contrast to these, Krishnamurthi, Shanta and Rao (loc. cit.) found higher percentage (10.1 per cent) of the false negative results. They thought profuse cervical bleeding to be responsible for the false negative results and explained that large amount of haemoglobin produced as result of haemorrhage inhibited the activity of dehydrogenase enzyme system. Haemoglobin is a very active electron acceptor, therefore it captures the electrons and thus blocks the reduction of the Tetrazolium salt, with a result that a negative result was seen.

In one of our cases of cervical carcinoma, who had profuse vaginal bleeding at the time of first examination, T.T.C. method gave negative results. However, when the examination was repeated after 2 days on the same patient,

T.T.C. method gave positive results (as now the bleeding was controlled).

The percentage of false positive figures in our studies was comparatively lower (0.95 per cent) than those reported by Krishnamurthi *et al* (loc. cit.) (8.8 per cent) and Jianren, *et al* (loc. cit) (4.7 per cent). In 2 cases of dysplasia grade II and 5 cases of dysplasia grade III as studied by Papanicolaou's staining technique, T.T.C. method showed a different type of cells which neither belonged to the Type A cells, nor to type B cells. The size of the cells was equal to the size of the type A cells but filled with an admixture of red coarse and fine granules. These were termed as "Unclassified group of cells". Biopsy could not be done in these cases so the correct diagnosis could not be ascertained. It might be that these cases may regress to the type B cell or advance to type A cells with the increase of time or these cells might be a link between advance dysplasia and malignancy. These cases could not be followed up, as the study was confined to a short period of observations.

The histopathogenesis of the carcinoma in situ is not clearly understood. Two cases were followed up for 2 years who on first examination revealed dysplasia grade II. On the second examination the Papanicolaou's staining technique again revealed dysplasia grade II while the cervical biopsy showed dysplasia grade III. On the third examination the Papanicolaou's staining technique showed dysplasia grade III, while T.T.C. test was positive for type A cells. This time the biopsy examination of cervix revealed intra-epithelial carcinoma in both the cases. These observations seem to indicate a progression of the dysplastic lesion to the neoplastic state. This contention is also favoured by the observations of Wahi

et al that the dysplastic lesions of the advanced severity either progress to carcinoma or persist. The distinction between the advanced dysplastic lesions and the early malignant lesion is very narrow morphologically and this factor may explain the false positive results.

It has not been possible to define a cytomorphological pattern of carcinoma *in situ* as distinct from that of an invasive carcinoma by T.T.C. test. The same type of cells can be exfoliated from an invasive as well as preinvasive lesions. However, the population of "Type A" (malignant) cells has been found to be more (8-15 cells per high power field) in late invasive carcinoma and less in early invasive cases (2-5 cells per 8-15 high power field). In preinvasive carcinoma number of cells is very low (An occasional cell per 30-50 high power field). The number of granules also varied in the each type of carcinoma. These were maximum (60-120 per cell) in late invasive cases and minimum in preinvasive cases (30-60 per cell).

This quantitative difference in the number of type A cells and number of granules can be explained on the basis that a cancer cell derives more of its energy by anaerobic glycolysis and apart from these, the damage to the cellular wall and mitochondria also increases as the malignancy advances from early to a late stage. These factors lead to increased penetration of the tetrazolium salt in the cell and deposition of large amount of Formazan crystals. The immediate colour reaction was observed in 70 cases, with type B cells overcrowded with fine red granules.

The actual cause for this intense reaction could not be explained entirely on the basis of the T.T.C. test, but it has been reported in literature that trichomonas vaginitis is frequently seen to be associated with dysplastic lesions of the cervical

epithelium and treatment of the infection regresses the dysplastic change in the cervical epithelium. May be, in these cases T.T.C. method gave intense colouration due to the associated dysplastic change.

Summary

A total of 580 cases have been studied by T.T.C. method. Correlative studies were done in 540 cases by Papanicolaou's staining technique and in 67 cases by cervical biopsies, to evaluate the accuracy results of these methods in respect to T.T.C. method.

The Cytological accuracy by T.T.C. method was found to be 99.05 per cent; 96.84 per cent by Papanicolaou's staining technique and 97.01 per cent by cervical biopsy.

There were no false negative results for T.T.C. method, 3.16 per cent for Papanicolaou's staining technique and 2.99 per cent for cervical biopsy.

False positive figures for T.T.C. method were 0.95 per cent, while none for Papanicolaou's staining technique and cervical biopsies.

Eleven clinically unsuspected cases for cervical carcinoma revealed type A or malignant cells by T.T.C. method and were confirmed by cervical biopsy to be intra-epithelial carcinoma in 3 cases, early invasive epidermoid carcinoma in 6 cases, advanced invasive epidermoid carcinoma in one and cervical adenocarcinoma in one case.

T.T.C. method has given sensitive and more accurate results than Papanicolaou's staining technique. Three cases positive for type A or malignant cells by T.T.C. method showed dysplasia grade III by Papanicolaou's staining technique. Two of these were confirmed as intra-epithelial carcinoma while one as an early invasive epidermoid carcinoma.

Conclusions

From the present study it is concluded that T.T.C. method is an easy, sensitive and accurate test, requiring less time for screening. Therefore, it holds a promising future as a routine primary mass screening programme method in a mass survey of cervical carcinoma. It is also

useful for early detection of cervical carcinoma.

References

1. Jian-ren, K., Zimin, H. and Wen-man, T.: Chinese J. Med., 82: 1963.
2. Krishnamurthi, S., Shanta, V. and Rao, S. R. V.: J. Obst. Gyn. India, 12: 4, 1962.

See Figs. on Art Paper II