Section: Anatomy

Original Research Article

DETECTION OF HUMAN PAPILLOMA VIRUS TYPE 16 DNA IN CLINICALLY SUSPECTED CASES OF CERVICAL DYSPLASIA BY PCR AND TO CORELATE THE FINDINGS WITH TUBERCULOSIS OF CERVIX

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ABSTRACT

Background: Cervical cancer is a leading cause of cancer-related morbidity and mortality among women in India, with human papillomavirus type 16 (HPV16) being a major etiological factor. Tuberculosis of the cervix, although rare, can coexist with cervical lesions in high TB prevalence regions. Early detection of HPV16 and Mycobacterium tuberculosis DNA through molecular methods can aid in diagnosis and prevention strategies. The aim is to detect HPV16 DNA in clinically suspected cases of cervical dysplasia by polymerase chain reaction (PCR) and to correlate the findings with tuberculosis of the cervix.

Materials and Methods: Fifty cervical samples were collected from symptomatic patients of cervical dysplasia and carcinoma attending the Gynecology and Obstetrics OPD, S.N. Medical College, Agra. Samples were prepared on Whatman filter paper and stored at –70°C until analysis. PCR was performed using type-specific primers from the upstream regulatory region of HPV16 and IS6110-based primers for Mycobacterium tuberculosis. Amplified products were analyzed by agarose gel electrophoresis under strict contamination control.

Results: HPV16 DNA was detected in 78% of cervical carcinoma cases, 40% of high-grade intraepithelial lesions, 22% of inflammatory smears, and 12.5% of normal smears. Mycobacterium tuberculosis DNA was identified in 10% of inflammatory smears and 20% of CIN cases, with some cases showing coinfection with HPV16.

Conclusion: HPV16 infection shows a strong association with cervical carcinoma and CIN, but its detection in inflammatory and normal smears suggests additional cofactors in carcinogenesis. PCR is a sensitive tool for early diagnosis, and the coexistence of tuberculosis highlights the need to consider dual pathology in endemic areas.

Keywords: HPV16, cervical dysplasia, carcinoma cervix, PCR, tuberculosis of cervix.

INTRODUCTION

Cancer of cervix is the most common form of cancer in females of developing countries and the second most common cancer, ranking after breast cancer in females of developed world.[1] Cervical cancer is the best example of common human malignancy with a proven infectious etiology.[2] The data linking human papilloma virus (HPV) infection with the epidemiology and pathogenesis of cervical neoplasia is convincing while there are no absolute in medicine, the fact that HPVs are biologically involved in causing cervical cancer of virtually all histologic types comes very close. In addition to HPV DNA, other environmental risk factors have been shown to be associated with cervical cancer. Because of the universal presence of viral DNA, these factors should be considered as cofactors in the sense that their independent effect on cervical carcinogenesis in the absence of HPV cannot be observed.[3]

Human papilloma virus belongs to the group Papova virus[4]. It has a size of 45–55 nm, is a non-enveloped virus, with an icosahedral capsid having 72 capsomers. The genetic material is double-stranded DNA, 8000 base pairs in length.[4] In humans, 85 types of papilloma viruses have been characterized and fully sequenced and more than 120 putative novel types have been partially characterized.[5] Approximately 40 different types infect the epithelium of the anogenital tract. Papilloma viruses are epitheliotropic viruses that predominantly infect skin and mucous membrane and produce epithelial proliferation at the site of infection.[6]

Oncogenic risk grouping of anogenital human papilloma virus is as follows: low oncogenic risk types include 6, 11, 42, 43, 44, 53; high oncogenic risk types include 16, 18, 45, 56, 58; and other high-risk types include 31, 33, 35, 39, 51, 59, 68.[7]

Polymerase chain reaction (PCR) is the most common procedure used for the in vitro amplification of DNA. The idea of PCR was conceived by Dr. Kerry Mullis in 1983; however, some pioneering work was also done by Govind Khurana in 1971, who described the basic principle of replicating a piece of DNA using two primers. PCR has been extensively used for the detection of HPV virus DNA.[8]

As the causes of cervical dysplasia may vary from population to population, such information should be generated from different areas. Also, it is important to correlate the findings with other chronic inflammatory diseases of the cervix like cervical tuberculosis. The aim of the present study is to detect HPV-16 DNA and IS6110 gene of Mycobacterium tuberculosis in abnormal cervical smears of patients of Agra division using polymerase chain reaction.

MATERIALS AND METHODS

Fifty samples were obtained from symptomatic patients of cervical dysplasia and cervical cancer, attending the outpatient department of Gynecology and Obstetrics, S.N. Medical College, Agra.

Sample collection, transport and storage

Cervical scrapes were obtained by Ayer's spatula and smeared onto a 3MM Whatman paper cut to the size of a small glass slide (5 cm × 2 cm). Smearing was done within a 0.5–1.0 cm diameter in the middle of the filter-paper slide. All the paper smears were performed in duplicate and put into individual auto-seal (ziplock) polythene bags. The spatula and the rest of the scraped cell materials were transferred to a 15-ml collection vial containing 5 ml of phosphate buffer solution (PBS) (pH 7.2) on ice. In case of cancerous lesions, tumor cell suspensions were blotted onto the paper slide. The remaining biopsy was also collected in PBS. All the samples were then transferred to Central JALMA Institute where they were stored at –70°C until DNA extraction.

DNA extraction and PCR analysis

All the samples were subjected to PCR analysis for both HPV16 DNA and Mycobacterium tuberculosis.

DNA extraction for HPV16: A 0.5–1.0 cm piece of filter paper was cut with a new sterile scalpel blade and transferred to a 0.5 ml centrifuge tube containing 50 µl distilled water. The tubes were boiled for 5 minutes in a microwave oven (360 W), cooled at room temperature, and then subjected to PCR.

Polymerase chain reaction for HPV16: Oligonucleotide primers were used for amplification of HPV16. For a 50 µl reaction volume, 9.00 µl of PCR mix was added to 41 µl of sample DNA in PCR tubes. Each PCR reaction included a positive and a negative control. The positive control consisted of formalin-fixed, paraffin-embedded tissue of squamous cell carcinoma of cervix, previously confirmed positive for HPV16 by immunoperoxidase technique. DNA extraction from the positive control involved cutting a 10 µm paraffin wax section, dewaxing in xylene, washing twice in 95% ethanol, drying, adding 250 µl water, and boiling for 30 minutes. A 40 µl aliquot of this solution was used for PCR. The negative control consisted of PCR mix without DNA.

Amplification was performed for 35 to 40 cycles in a DNA thermal cycler. The first cycle included denaturation at 94°C for 5 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. This was followed by 38 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes, with a final extension at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 7 minutes. Strict laboratory precautions were followed to prevent cross-contamination.

Gel Electrophoresis: A 2% agarose gel was prepared in 1X TBE buffer by heating in a microwave until dissolved. When cooled enough to pour, 5 µl of ethidium bromide was added and the gel was poured into a platform with combs to create wells. After solidification, the combs were removed, and the gel was placed in buffer containing distilled water, TBE buffer, and ethidium bromide. Processed PCR products mixed with loading dye were loaded into the wells, and electrophoresis was carried out at 50 V for 1 hour. Bands were visualized in a Gel Doc system and photographed using a Polaroid camera.

DNA extraction for Mycobacterial tuberculosis

Samples were transferred into microcentrifuge tubes containing 400 µl of TE buffer. DNA was isolated using a standard physio-chemical procedure. Samples were heat-killed at 100°C for 7 minutes, snap-chilled in ice at –20°C for 30 minutes, treated with lysozyme for 2 hours at 37°C, then with proteinase K and SDS at 65°C for 30 minutes, followed by NaCl and CTAB-NaCl at 65°C for 30 minutes. Chloroform–isoamyl alcohol was added, vortexed, and centrifuged to separate layers. The aqueous layer was transferred to a fresh tube and DNA was precipitated with isopropanol at –20°C for 1 hour, centrifuged, washed with 70% ethanol, air-dried, dissolved in TE buffer, and stored at –20°C.

PCR amplification and amplicon detection

IS6110-based primers were used for amplification of Mycobacterium tuberculosis. The PCR reaction

included a negative control with no DNA and a positive control with MTB standard strain H37Rv. Amplification was carried out for 35–40 cycles. The first cycle involved denaturation at 94°C for 5 minutes, annealing at 36°C for 1 minute, and extension at 72°C for 2 minutes, followed by 34 cycles at 94°C for 1 minute, 68°C for 1 minute 30 seconds, and 72°C for 1 minute, with a final extension at 94°C for 1 minute, 36°C for 1 minute, and 72°C for 10 minutes.

Resolution of amplified dna by agarose gel electrophoresis

A 2% agarose gel containing ethidium bromide was prepared, and PCR products mixed with loading dye were loaded into the wells. Electrophoresis was carried out at 50 V for 1 hour. The amplified DNA bands were visualized in a Gel Doc system and photographed using a Polaroid camera.

RESULTS

[Table 1] depicts the sample distribution. About 9 samples were of carcinoma, 5 were CIN, 28 were inflammatory smears and 8 were normal smears by cytology.

Table 1: Sample distribution

Type	Number
1. Normal pap smear	8
2. Inflammatory pap smear	28
3. Carcinoma cervix	9
4. CIN	5
Total	50

Table 2: Distribution Of Cases According To Age Groups

Age interval in years	Normal cervical smears	Infflamatory smears	Squamous cell carcinoma	HSIL/CIN
21-30	3	6	0	0
31-40	3	9	1	1
41-50	2	8	2	2
51-60	0	5	4	1
61-70	0	0	2	1
Total	8	28	9	5

[Table 2] depicts age wise distribution of all cases. Majority of the cases of squamous cell carcinoma and CIN were in 41–60 years age group interval. Most of the normal cervical smears cases were in 21–40 age group interval and most of the inflammatory cases were in 31–50 age group interval.

Table 3: Percentage Positivity For HPV16

Type of sample	No of cases	No of positive cases	% positivity
Normal Cervical smear	8	1	12.5%
Inflammatory smear	28	6	22%
CIN/Cervical dysplasia	5	2	40%
Carcinoma cervix	9	7	78%

[Table 3] depicts percentage of samples positive for HPV16 by PCR. In case of cervical carcinoma 78% of samples were positive by PCR. In CIN positivity rate was 40%, in inflammatory smears rate was 22% and in case of normal smears it was 12.5%.

Table 4: Percentage Positivity For Mycobacterium Tuberculosis

Type of samples	No of cases	No of positive cases	% positivity
Normal cervical smears	8	0	0%
Inflammatory smears	28	3	10.7%
Cervical carcinoma	9	0	0%
CIN	5	1	20.0%

Table 5: PCR Results for Mycobacterium Tuberculosis

S.NO	SAMPLES	PCR REPORT
1	C1	+ve
2	C2	-ve
3	C3	+ve
4	C4	-ve
5	C5	-ve
6	C6	-ve
7	C7	-ve
8	C8	+ve
9	C9	-ve
10	C10	-ve

11	C11	-ve
12	C12	-ve
13	C13	-ve
14	C14	-ve
15	C15	-ve
16	C16	-ve
17	C17	-ve
18	C18	-ve
19	C19	-ve
20	C20	-ve
21	C21	-ve
22	C22	-ve
23	C23	-ve
24	C24	-ve
25	C25	-ve
26	C26	-ve
27	C27	-ve
28	C28	-ve
29	C29	-ve
30	C30	-ve
31	C31	-ve
32	C32	-ve
33	C33	-ve
34	C34	-ve
35	C35	-ve
36	C36	-ve
37	C37	-ve
38	C38	-ve
39	C39	-ve
40	C40	-ve
41	C41	-ve
42	C42	-ve
43	C43	-ve
44	C44	-ve
45	C45	-ve
46	C46	-ve
47	C47	-ve
48	C48	-ve
49	C49	-ve
50	C50	-ve

[Table 5] depicts the cases which were positive for Mycobacterium tuberculosis by PCR.

Electrophoresis results of PCR products of DNA isolated from clinical samples:

1. Positive samples Lane No.: 1, 3, 6 and 8.
2. Negative samples Lane No.: 2, 4, 5, 7, 9 and 10–50.
3. Positive control (H37 Rv): 11.
4. Negative control (D/W): 10.

Table 6: PCR Results for HPV16

S.NO	SAMPLES	PCR REPORT FOR HPV16
1	C1	+ve
2	C2	-ve
3	C3	-ve
4	C4	-ve
5	C5	+ve
6	C6	-ve
7	C7	-ve
8	C8	+ve
9	C9	-ve
10	C10	-ve
11	C11	+ve
12	C12	-ve
13	C13	-ve
14	C14	+ve
15	C15	-ve
16	C16	-ve
17	C17	-ve
18	C18	-ve
19	C19	+ve
20	C20	+ve
21	C21	-ve
22	C22	-ve

23	C23	-ve
24	C24	-ve
25	C25	-ve
26	C26	-ve
27	C27	-ve
28	C28	+ve
29	C29	-ve
30	C30	-ve
31	C31	-ve
32	C32	-ve
33	C33	+ve
34	C34	+ve
35	C35	+ve
36	C36	-ve
37	C37	-ve
38	C38	-ve
39	C39	-ve
40	C40	+ve
41	C41	+ve
42	C42	-ve
43	C43	-ve
44	C44	+ve
45	C45	+ve
46	C46	-ve
47	C47	-ve
48	C48	+ve
49	C49	-ve
50	C50	+ve

Table 6: depicts the cases which were positive for HPV16 by PCR.

Electrophoresis results of PCR products of DNA isolated from clinical samples:

1. Positive samples Lane No.: 1, 5, 8, 11, 14, 19, 20, 28, 33, 34, 35, 40, 41, 44, 45 and 48.
2. Negative samples Lane No.: 3, 4, 6, 7, 9, 10, 12–13, 15–18, 21–27, 29–32, 36–39, 42, 43, 46–47 and 49–50.

TYPE OF CASES — CASE DISTRIBUTION

- **Normal Smears:** C4, C12, C19, C22, C26, C37, C39, C43
- **Inflammatory Smears:** C2, C3, C5, C7, C11, C13, C15, C18, C24, C25, C26, C27, C28, C29, C30, C32, C36, C37, C38, C39, C40, C42, C43, C44, C46, C47, C48 and C50
- **Cervical Dysplasia:** C1, C9, C16, C23 and C35

Carcinoma Cervix: C6, C8, C10, C14, C20, C33, C34, C41 and C45

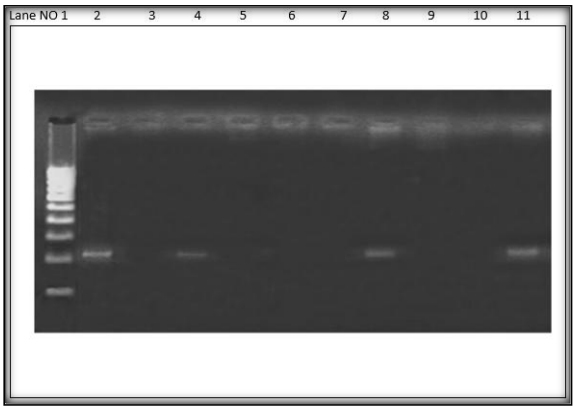

Figure 1: PCR amplification of HPV type 16. Amplimers of 217 base pair are seen in Lane-4,8and 11.Lane2 is Positive control. Lane3 is Negativ e control.

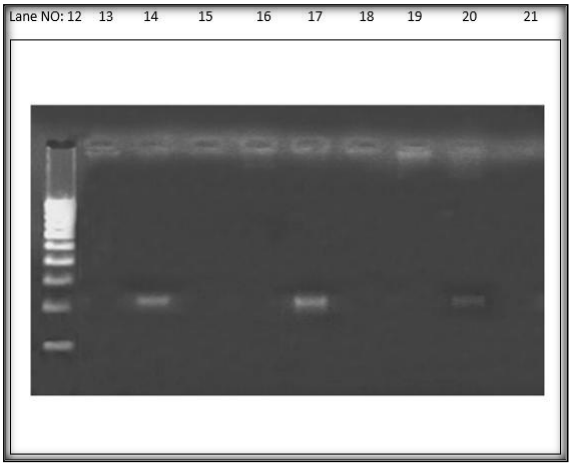

Figure 2: PCR amplification of HPV type 16.Positive samples in lane 17 and 20. Lane-14 Positive control. Lane-13 Negative control.

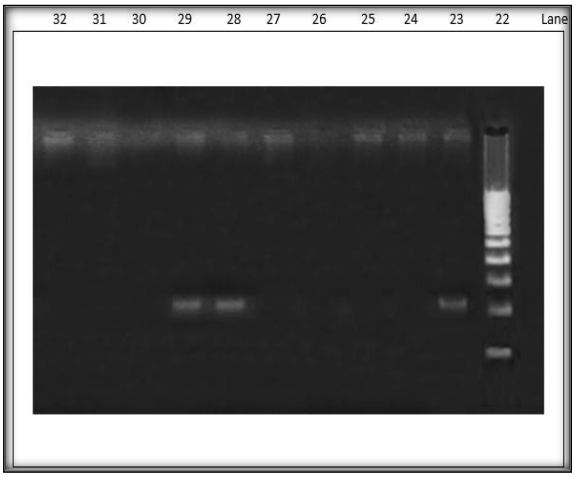

Figure 3: PCR amplification of HPV type 16. Positive samples in lane 28 and29. Lane 23 is positive control. Lane 24 is negative control.

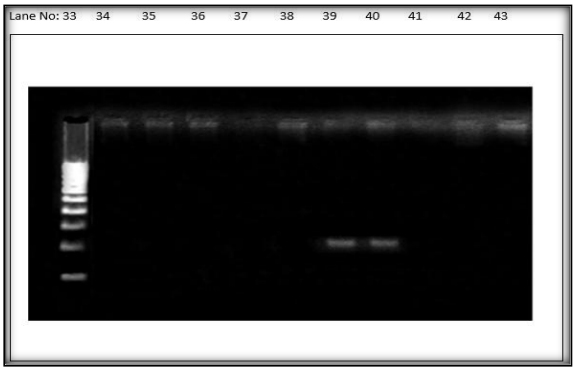

Figure 4: PCR amplification of HPV type 16.Positive samples in lane 40. Lane 39 is positive control. Lane 34 is negative control.

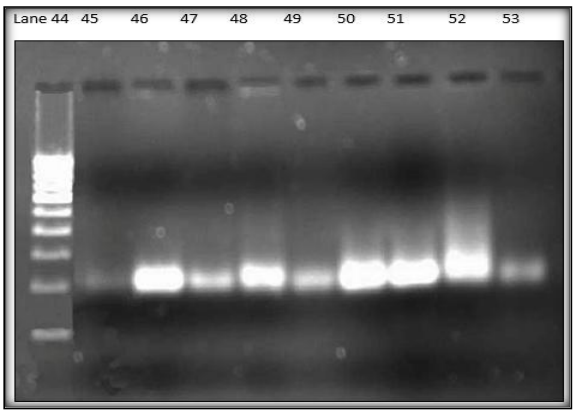

Figure 5: PCR amplification of HPV type 16.Positive samples in lane 46,48and 49. Lane 47is positive control.

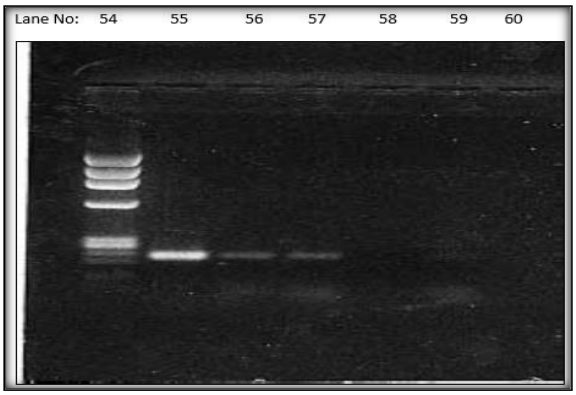

Figure 6: PCR amplification of HPV 16.Positive samples in lane 56 and 57. Lane 55 is positive control. Lane 60 is negative control.

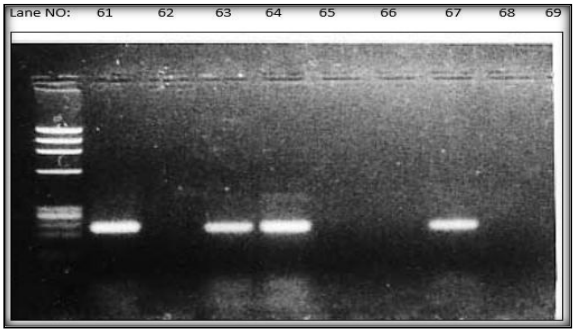

Figure 7: PCR amplification of HPV type 16. Positive samples in lane 64,65 and 68. Lane 62is positive control. Lane 63 is negative control.

DISCUSSION

The epidemiology of cervical neoplasia suggests a role for a sexually transmitted infectious agent[2]. The data linking HPV infection with the epidemiology and pathogenesis of cervical cancer is continuing. Both in vitro and in vivo studies strongly suggest that high-risk HPV 16 genotypes influence the development of cervical cancer. The present study attempts to detect the presence of HPV16 in precancerous, cancerous, inflammatory, and normal smears of uterine cervix and to correlate the findings with other chronic inflammatory lesions of uterine cervix like cervical tuberculosis. In our study, 50 symptomatic cases of cervical dysplasia were included. Cytology was done and cases were described as 10 cases of squamous cell carcinoma, 5 of high grade intraepithelial lesions (HGIL/CIN-III), 28 inflammatory smears, and 8 normal smears [Table 1]. Regarding the age distribution of the cases, as depicted in Table 2, 6 out of 9 cases of cervical cancers i.e. 67% and 3 out of 5 i.e. 60% of cervical dysplasia I fall in the age group of 41–60 years. This is in accordance with the average age of cervical squamous carcinoma and CIN patients as studied by Reagen et al,[9] in a series of 66 cases which was 49.9 years. To substantiate it further, the average age for invasive squamous cell cancer as studied by Wright et al,[10] was found to be 51.4 years.

All the 50 cases were subjected to PCR analysis for HPV16. Type-specific oligonucleotide primers from the upstream regulatory region (URR) of HPV16 genome were used. An amplification of 217 base pair was obtained. This is in agreement with earlier reports of Das et al,[8] and Kailash et al,[11] who also reported an amplimer of 217 base pairs for HPV16 in 70 paraffin-embedded sections of cervical carcinoma and cervical dysplasia. In our study, 78% of cases of squamous cell carcinoma were positive for HPV16 DNA [Table 3]. This compares fairly well with the study of Iwasawa et al.[12] who detected a positivity rate of 78% in 352 cases of squamous cell carcinoma using nested-PCR. Our results are also comparable with the study conducted by Sarnath et al,[13] who reported 77% positivity of HPV16 in a study conducted on 337 Indian women with squamous cell carcinoma by PCR.

We observed the HPV16 positivity of 22% in inflammatory smears and 40% in high grade intraepithelial lesions [Table 3]. This is in agreement with the study conducted by Zhang et al,[14] who studied 28 biopsies of histologically confirmed cervicitis and 29 biopsies of cervical intraepithelial neoplasia, and reported positivity of 25% in inflammatory cervical smears (cervicitis) and positivity of 41% in high grade intraepithelial lesions by using PCR. We also reported that 12.5% of normal cervical smear cases were positive for HPV16. This can be compared with the results of the study conducted by Sarnath et al,[13] for PCR analysis of HPV16 in which a positivity of 15.2% was reported

in normal healthy cases. This is also in agreement with the study conducted by Griffin et al,[15] who reported positivity of 30% in normal cervical smears. Tornesello et al,[16] also reported positivity of 8.7% in normal subjects.

All the 50 samples were also analyzed by IS6110-based PCR system to detect Mycobacterium tuberculosis. An amplicon of 123 base pair was obtained. Ten percent of inflammatory smears and 20% of high grade CIN were found positive for both HPV16 and Mycobacterium tuberculosis (Table 4). Though we do not have any relevant data available, there are studies suggesting the correlation of cervical carcinoma with tuberculosis of cervix. Micha et al,[18] reported a case of cervical tuberculosis simulating cervical carcinoma clinically. Rajaram et al,[19] also reported a case of cervical carcinoma coexisting with cervical tuberculosis.

CONCLUSION

HPV16 infection is strongly associated with cervical carcinoma and CIN, but its presence in inflammatory and normal smears indicates that additional cofactors contribute to cervical carcinogenesis. PCR serves as a sensitive method for early detection, and the coexistence of Mycobacterium tuberculosis in some cases underscores the need to consider dual pathology in high TB prevalence areas.

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