Cureus
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Open Access Original Article

Diagnostic Yield of Modified Bleach-Assisted Ziehl-Neelsen Microscopy in Cervical Tuberculous Lymphadenitis: A Prospective Paired Diagnostic Accuracy Study

Review began 10/04/2026
Review ended 10/31/2026
Published 11/02/2026

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DOI: 10.7759/cureus.119025

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Abstract

Background: Direct Ziehl-Neelsen (ZN) microscopy is accessible but has limited sensitivity in paucibacillary tuberculous lymphadenitis. We evaluated whether a modified bleach concentration method increases the detection of microbiologically confirmed acid-fast bacilli (AFB) when used as an adjunct to direct ZN microscopy.

Materials and methods: This prospective, single-center, paired diagnostic accuracy study included 124 consecutive patients with clinically suspected cervical tuberculous lymphadenitis. For each participant, two separate microscopy slides were prepared from aliquots of the same lymph node aspirate: a direct ZN smear without bleach pretreatment and a second smear after modified bleach pretreatment and concentration, followed by the same ZN staining protocol. A composite microbiological reference standard based on cartridge-based nucleic acid amplification testing and/or mycobacterial culture was available for 116 participants. The primary outcome was paired sensitivity among reference-positive participants. Secondary outcomes included incremental confirmed yield; rescue, defined as modified-bleach positivity among specimens negative by direct ZN microscopy; specificity and other accuracy measures among participants with an available composite microbiological reference result; the cytomorphological distribution of discordant results; and exploratory scenario-based laboratory cost. Accuracy estimates used exact 95% confidence intervals, and paired differences were tested with the two-sided exact McNemar test.

Results: Direct ZN microscopy detected AFB in 21/124 specimens (16.9%), whereas modified bleach-assisted ZN microscopy detected AFB in 26/124 specimens (21.0%). Among 38 reference-positive participants, sensitivity was 55.3% (95% CI, 38.3-71.4) for direct ZN and 68.4% (95% CI, 51.3-82.5) for modified bleach-assisted ZN microscopy, a numerical difference of 13.2 percentage points. The prespecified two-sided exact McNemar comparison did not reach the conventional threshold for statistical significance ($p = 0.0625$). No false-positive result was observed among 78 reference-negative participants, corresponding to an observed specificity of 100% (95% CI, 95.4-100.0%) for each method. Modified bleach-assisted ZN microscopy detected 5/103 ZN-negative specimens (4.9%; 95% CI, 1.6-11.0), and all five had a positive composite microbiological reference result. It rescued 5/17 reference-positive cases missed by ZN (29.4%; 95% CI, 10.3-56.0). Four discordant cases occurred in necrotizing suppurative lymphadenitis, and one occurred in necrotizing granulomatous lymphadenitis. An exploratory scenario using locally reconstructed process-cost inputs estimated a cost of approximately Rs 256 per additional confirmed detection.

Conclusions: Modified bleach-assisted ZN microscopy increased microbiologically confirmed AFB yield and the point estimate of sensitivity, without an observed loss of specificity. Because inference was driven by only five discordant pairs and reference testing was incomplete in eight participants, the method should be considered a low-cost adjunct to direct ZN microscopy rather than a replacement for WHO-recommended nucleic acid amplification testing.

Categories: Pathology, Epidemiology/Public Health, Infectious Disease
Keywords: acid-fast bacilli, fine-needle aspiration cytology, lymph node tuberculosis, sodium hypochlorite, ziehl-neelsen stain

Introduction

Tuberculous lymphadenitis is a common extrapulmonary manifestation of tuberculosis and frequently presents as chronic cervical lymphadenopathy. Fine-needle aspiration cytology (FNAC) is rapid and minimally invasive, but granulomatous or necrotizing cytomorphology is not specific for *Mycobacterium tuberculosis*. Microbiological confirmation is therefore desirable, particularly before prolonged therapy or when alternative infections and malignancy remain possible [1,2].

Direct Ziehl-Neelsen (ZN) microscopy remains available in laboratories where molecular testing is delayed or inaccessible. Its sensitivity is constrained by low bacillary density, uneven organism distribution, and

obscuring necrotic material. Sodium hypochlorite digestion can clear background debris, while centrifugation concentrates bacilli into the sediment. Earlier lymph node studies reported substantially higher smear positivity after bleach processing, but most compared positivity within cytologically selected cases and did not use an independent microbiological reference standard [3-6].

Landmark systematic reviews of bleach processing reached a more cautious conclusion: sensitivity may improve, but effect size varies with specimen processing, centrifugation or sedimentation, and study quality; specificity and operational burden must be evaluated rather than assumed [7,8]. Previous lymph node diagnostic studies have emphasized prospective recruitment, transparent reporting of patient flow, clearly defined reference standards, paired comparisons, confidence intervals, and detailed analyses of discordant specimens [9,10].

We therefore evaluated modified bleach-assisted ZN microscopy as an adjunct to direct ZN microscopy in a prospective paired study. We hypothesized that bleach digestion and concentration would increase AFB detection without increasing false-positive microscopy. The primary objective was to compare the paired sensitivities of modified bleach-assisted ZN microscopy and direct ZN microscopy among participants with a positive composite microbiological reference result based on CBNAAT and/or mycobacterial culture. The secondary objectives were to evaluate: (1) incremental confirmed yield; (2) rescue, defined as modified-bleach positivity among specimens negative by direct ZN microscopy; (3) specificity and other accuracy measures among participants with an available composite reference result; (4) the cytomorphological distribution of discordant results; and (5) exploratory local laboratory-process costs. Participants without an available reference result contributed to the paired-yield analysis but not to the diagnostic-accuracy analyses.

Materials And Methods

Study design and participants

This prospective, single-center, paired diagnostic accuracy study was conducted in the Department of Pathology at a tertiary-care teaching hospital in central India from May 2024 through October 2025. Consecutive patients of all ages, including adults and children, with clinically suspected cervical tuberculous lymphadenitis who underwent FNAC were eligible. Patients receiving antitubercular therapy, those who had received such therapy within the preceding six months, those with inadequate aspirates, and those for whom aspiration was contraindicated were excluded. Written informed consent was obtained from adult participants; for minors, written consent was obtained from a parent or legal guardian and written assent from the child where applicable. The study was approved by the Institutional Ethics Committee of Gandhi Medical College and Associated Hospitals, Bhopal (IEC Protocol No. 192/1EC/2024; Letter No. 16304/MC/IEC/2024; dated 6 May 2024).

Specimen collection and cytomorphology

FNAC was performed using a 23-25-gauge needle and 10-mL syringe. For each participant, material from the same lymph node aspirate was divided for paired microscopy: one aliquot was used to prepare a direct ZN slide without bleach pretreatment, and a separate aliquot underwent modified bleach pretreatment and concentration before preparation of a second slide. Additional material collected during the same clinical episode was allocated to routine cytology and reference testing, as requested, as part of clinical care. Cytomorphology was classified as reactive, granulomatous, suppurative, necrotizing granulomatous, or necrotizing suppurative lymphadenitis.

Index tests

For direct ZN microscopy, a smear was prepared from an aliquot of the aspirate without bleach pretreatment. The smear was air-dried and heat-fixed. According to the departmental ZN staining protocol, the slide was covered with 1% carbol fuchsin, gently heated until vapor appeared without boiling, and left in contact with the stain for five minutes. It was then rinsed with water, decolorized with acid-alcohol consisting of 3% v/v hydrochloric acid in 95% ethanol for two to three minutes or until the smear ran clear, rinsed again, and counterstained with 0.1% methylene blue for one to two minutes. After a final rinse and air-drying, the slide was examined using a 100× oil-immersion objective. At least 100 oil-immersion fields were examined before a smear was classified as negative.

For the modified bleach method, a separate aliquot of the same aspirate was rinsed into a standard screw-capped 15-mL conical tube containing 1 mL of 5% sodium hypochlorite and 1 mL of normal saline. The suspension was incubated for 15 minutes at room temperature with intermittent agitation and then diluted with 2 mL of distilled water. It was centrifuged at approximately 1790 × g (3000 rpm; Eppendorf Centrifuge 5810 R, A-4-81 swing-bucket rotor with a 15-mL conical-tube adapter; centrifugation radius, 17.8 cm) for 15 minutes at room temperature. Standard screw-capped tubes were used without specialized aerosol-tight bucket enclosures. The supernatant was discarded, and a second smear was prepared from the sediment and stained using the same departmental ZN protocol as the direct smear. Thus, the paired index tests differed in specimen pretreatment and concentration rather than in the staining method.

Microscopy interpretation and AFB grading

Multiple trained microscopists participated in slide interpretation. Readers were blinded to the result of the other index test and to the available CBNAAT/culture result at the time of microscopy interpretation. Uncertain or discordant microscopy findings were reviewed by a senior pathologist.

AFB grading followed the criteria of the National Tuberculosis Elimination Programme (NTEP). A smear was classified as negative when no AFB were identified in 100 oil-immersion fields; scanty when 1-9 AFB were identified per 100 fields, with the exact number recorded; 1+ when 10-99 AFB were identified per 100 fields; 2+ when an average of 1-10 AFB per oil-immersion field was identified over at least 50 fields; and 3+ when more than 10 AFB per oil-immersion field were identified over at least 20 fields. For the primary binary analysis, scanty and all graded positive results were classified as AFB-positive.

The NTEP grading scale ends at 3+. Archived entries recorded as 4+ represented recording errors by observers documenting extremely heavy bacillary loads. For the exploratory grade-category analysis, scanty and 1+ results were categorized as low-grade, whereas 2+, 3+, and the non-standard 4+ entries were categorized as high-grade. The 4+ entries were therefore harmonized with the 3+ category. This harmonization did not affect the primary binary positive-versus-negative analysis.

Laboratory biosafety

All pre-fixation specimen processing was performed by trained laboratory personnel in a Class II Type A2 biological safety cabinet while wearing an N95 respirator, disposable gloves, and a laboratory gown. Specimens were processed in standard screw-capped 15-mL conical tubes. Centrifugation was performed using the A-4-81 swing-bucket rotor without specialized aerosol-tight bucket enclosures. Work surfaces and reusable equipment were decontaminated using 1% sodium hypochlorite with a contact time of 20-30 minutes. Infectious waste was autoclaved or treated with 1% sodium hypochlorite before disposal in designated biomedical-waste bags according to the institutional biomedical-waste procedure.

Reference standard and discordant-case adjudication

Reference testing was performed during routine clinical care at the request of the treating clinician, using additional material obtained during the same clinical episode. Reference testing comprised cartridge-based nucleic acid amplification testing using the Cepheid Xpert MTB/RIF assay and/or mycobacterial culture on solid Löwenstein-Jensen medium.

The archived dataset retained the CBNAAT and culture information in a single combined field. Composite-reference positivity was defined by a positive result on either recorded modality. For the 78 participants classified as composite-reference negative, both Xpert MTB/RIF and Löwenstein-Jensen culture were documented as negative. Among the 38 composite-reference-positive participants, the combined field did not identify whether positivity was established by Xpert MTB/RIF, culture, or both. Modality-specific diagnostic accuracy could therefore not be reconstructed.

Eight participants had no reference result because reference testing had not been requested during routine clinical care. All eight were negative by both microscopy methods. These participants were retained in the paired diagnostic-yield analysis but excluded from calculations of sensitivity, specificity, predictive values, and accuracy. Blinding of reference-standard assessors to the microscopy results was not separately documented. Bleach-positive/direct-ZN-negative specimens are therefore described as microbiologically or composite-reference confirmed rather than specifically CBNAAT-confirmed or culture-confirmed.

Outcomes and statistical analysis

The primary outcome was the sensitivity of each index test among participants with a positive composite microbiological reference result. Paired sensitivities were compared using the two-sided exact McNemar test. Secondary outcomes were specificity, positive and negative predictive values, accuracy, overall diagnostic yield, absolute incremental yield, and rescue among direct ZN-negative specimens and among reference-positive direct ZN-negative specimens. Rescue was defined as modified-bleach positivity following a negative direct ZN result. Diagnostic-accuracy analyses were restricted to participants with an available composite microbiological reference result; all 124 participants were retained for the paired-yield analysis. Accuracy estimates and rescue proportions were reported with two-sided Clopper-Pearson 95% confidence intervals. Because each index test was binary and no decision threshold was varied, receiver-operating-characteristic analysis was not performed. The absolute difference in paired sensitivity was expressed in percentage points, consistent with paired-proportion reporting principles [11]. Mid-P and asymptotic McNemar results, ordinal grade transitions, lesion-stratified results, and extreme-case missing-reference analyses were treated as sensitivity or exploratory analyses [12]. No multivariable model was fitted because only five incremental detections occurred.

Scenario-based cost analysis

An exploratory laboratory-perspective micro-costing considered resources additional to direct ZN

microscopy: a processing tube, sodium hypochlorite, normal saline, distilled water, five minutes of active technician time, and a nominal allowance for centrifuge electricity and depreciation. Cost per additional confirmed detection was calculated by dividing the estimated incremental process cost per specimen by the observed incremental confirmed yield. The individual resource inputs and calculations are presented transparently in the Appendices.

The cost inputs were reconstructed from the study workbook and represent local process assumptions rather than prospectively measured resource use. Downstream patient outcomes and comparative economic consequences were not evaluated. Accordingly, this exploratory calculation should not be generalized to other settings or interpreted as a formal cost-effectiveness evaluation [13].

Reporting and reproducibility

The report follows the Standards for Reporting Diagnostic Accuracy Studies (STARD) 2015 guidelines and STARD for Abstracts [14,15]. Analyses were reconstructed from the de-identified study workbook. Main and supplementary tables and figures are embedded after the first citation in this manuscript, and a corrected STARD 2015 checklist is provided in the Appendices.

Results

Participant flow and characteristics

All 124 enrolled participants completed both index tests. An available composite microbiological reference result was recorded for 116 participants (93.5%): 38 were reference-positive, and 78 were reference-negative. Both Xpert MTB/RIF and Löwenstein-Jensen culture were documented as negative in the 78 reference-negative participants. For the 38 reference-positive participants, the archived combined field did not indicate whether Xpert MTB/RIF, culture, or both established positivity. Eight participants had no reference result; all eight were negative by both index tests. These eight participants were excluded from the diagnostic-accuracy calculations but retained in the paired-yield analysis (Figure *1*). Mean age was 27.2 ± 13.4 years, and 82 participants (66.1%) were female. Necrotizing lesions comprised 52/124 specimens (41.9%) (Table *1*). No test-related adverse events occurred.

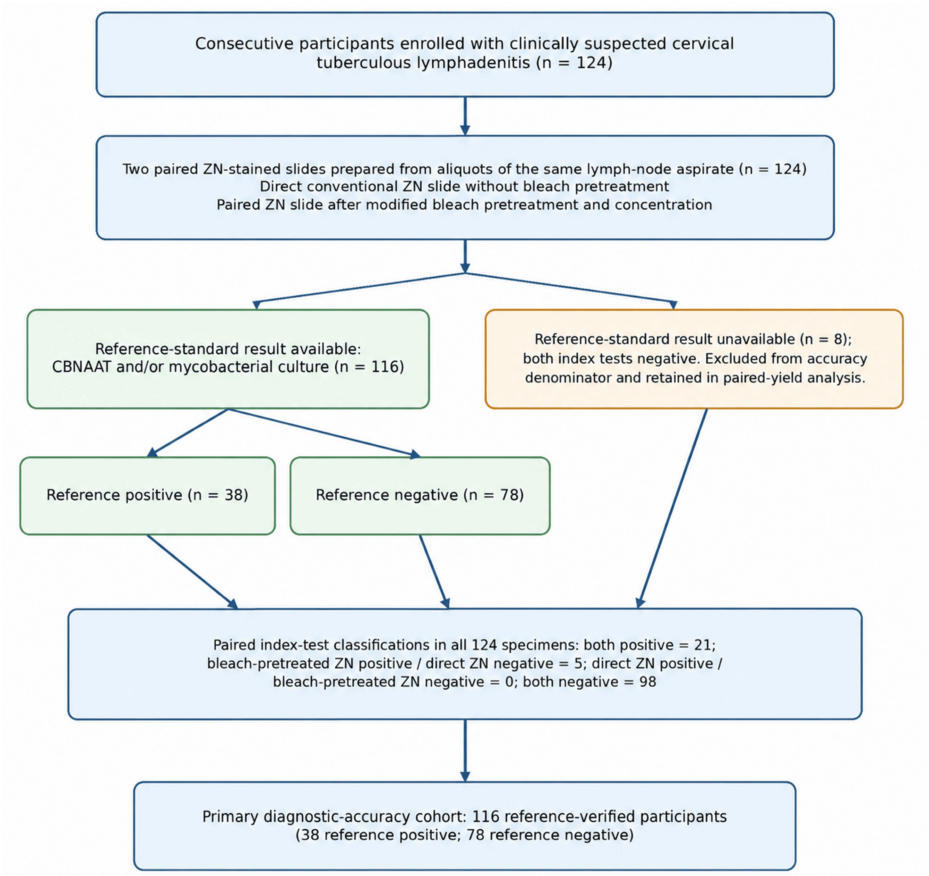


FIGURE 1: STARD participant-flow diagram

All 124 participants completed both index tests and were included in the paired diagnostic-yield analysis. An available composite microbiological reference result was recorded for 116 participants, including 38 reference-positive and 78 reference-negative participants; these 116 participants were included in the diagnostic-accuracy analysis. Eight participants had no reference result and were negative by both microscopy methods.

ZN: Ziehl-Neelsen, STARD: Standards for Reporting Diagnostic Accuracy Studies

Characteristic	Value
Age, years	27.2 ± 13.4
Female sex	82 (66.1)
Single lymph node	88 (71.0)
Multiple lymph nodes	36 (29.0)
Right cervical site	58 (46.8)
Left cervical site	54 (43.5)
Bilateral cervical site	12 (9.7)
Reactive lymphadenitis	25 (20.2)
Granulomatous lymphadenitis	42 (33.9)
Suppurative lymphadenitis	5 (4.0)
Necrotizing granulomatous lymphadenitis	23 (18.5)
Necrotizing suppurative lymphadenitis	29 (23.4)
Reference result available	116 (93.5)
Reference positive among verified	38/116 (32.8)

TABLE 1: Participant and specimen characteristics (n = 124)

Values are mean ± SD or n (%), unless otherwise specified.

SD: standard deviation

Paired diagnostic yield and accuracy

Direct ZN detected AFB in 21/124 specimens (16.9%), whereas modified bleach-assisted ZN microscopy detected 26/124 (21.0%). All ZN-positive specimens were bleach-positive. Five specimens were bleach-positive/ZN-negative, and none were ZN-positive/bleach-negative (Figure *2*). The incremental confirmed yield was 5/124 (4.0%; 95% CI, 1.3-9.2%). The two-sided exact McNemar test yielded a p-value of 0.0625.

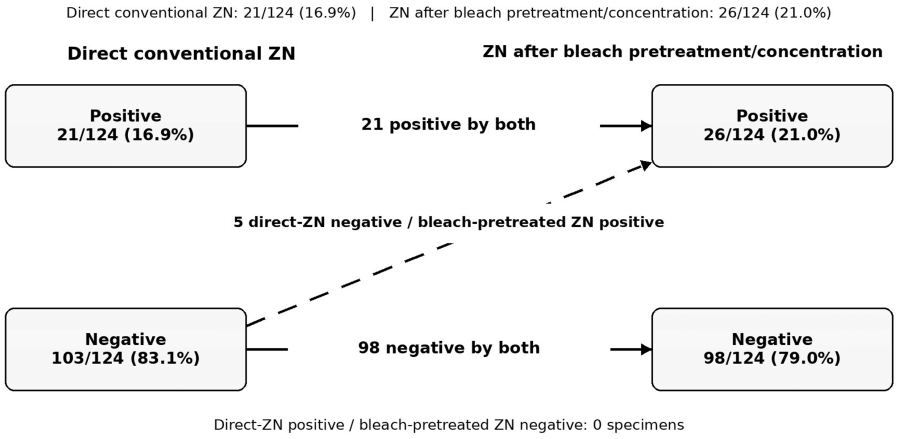


FIGURE 2: Paired classification of AFB detection among 124 lymph node aspirates

Solid arrows show concordant classifications; the dashed arrow represents the five specimens classified as negative on the direct ZN slide and positive on the paired ZN-stained slide after modified bleach pretreatment and concentration. All five had a positive composite microbiological reference result.

AFB: acid-fast bacilli, ZN: Ziehl-Neelsen

Among the 38 reference-positive participants, ZN sensitivity was 55.3% (21/38; 95% CI, 38.3-71.4), and modified bleach-assisted ZN microscopy sensitivity was 68.4% (26/38; 95% CI, 51.3-82.5), an absolute increase of 13.2 percentage points. Both tests were negative in all 78 reference-negative participants, yielding an observed specificity of 100% (95% CI, 95.4-100.0) for each method. Negative predictive value estimates were 82.1% for direct ZN and 86.7% for modified bleach; corresponding complete-case accuracy estimates were 85.3% and 89.7%, respectively (Table *2*; Figure *3*).

Measure	Method/category	Result
Paired outcome, all 124	Both positive	21
	Bleach positive/ZN negative	5
	ZN positive/bleach negative	0
	Both negative	98
Diagnostic yield	Direct ZN	21/124 (16.9%)
	Modified bleach	26/124 (21.0%)
Incremental confirmed yield	Bleach minus ZN	5/124 (4.0%; 95% CI, 1.3-9.2)
Sensitivity	Direct ZN	21/38 (55.3%; 95% CI, 38.3-71.4)
	Modified bleach	26/38 (68.4%; 95% CI, 51.3-82.5)
Paired sensitivity comparison	Absolute difference	13.2 percentage points; exact McNemar p = 0.0625
Specificity	Direct ZN	78/78 (100%; 95% CI, 95.4-100.0)
	Modified bleach	78/78 (100%; 95% CI, 95.4-100.0)
Positive predictive value	Direct ZN	21/21 (100%; 95% CI, 83.9-100.0)
	Modified bleach	26/26 (100%; 95% CI, 86.8-100.0)
Negative predictive value	Direct ZN	78/95 (82.1%; 95% CI, 72.9-89.2)
	Modified bleach	78/90 (86.7%; 95% CI, 77.9-92.9)
Accuracy	Direct ZN	99/116 (85.3%; 95% CI, 77.6-91.2)
	Modified bleach	104/116 (89.7%; 95% CI, 82.6-94.5)
Rescue among ZN-negative specimens	All paired specimens	5/103 (4.9%; 95% CI, 1.6-11.0)
Rescue among reference-positive ZN-negative cases	Reference-positive subgroup	5/17 (29.4%; 95% CI, 10.3-56.0)

TABLE 2: Paired diagnostic yield and diagnostic accuracy

Accuracy calculations used the 116 participants with an available reference-standard result. Exact Clopper-Pearson confidence intervals are shown.

ZN: Ziehl-Neelsen, CI: confidence interval

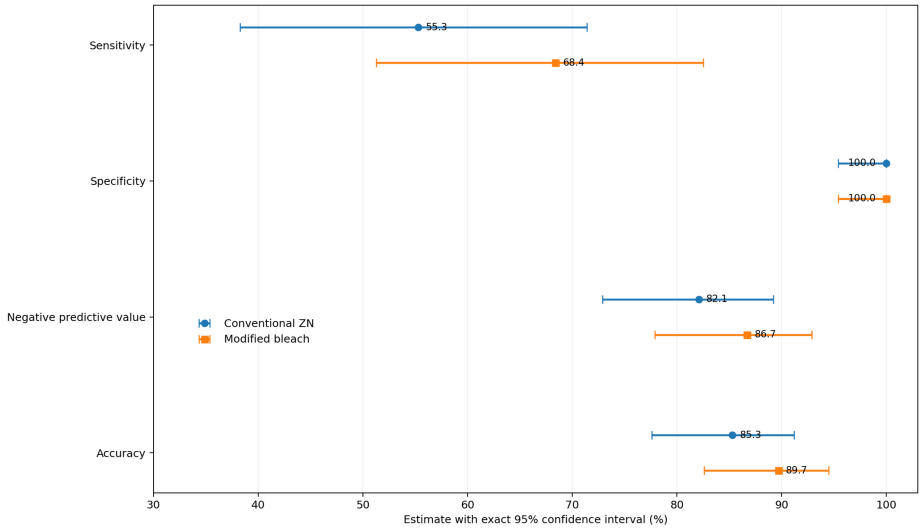


FIGURE 3: Diagnostic performance against the available CBNAAT/culture reference standard

Points show estimates, and bars show exact two-sided 95% confidence intervals.

ZN: Ziehl-Neelsen

Among 103 direct ZN-negative specimens, modified bleach-assisted ZN microscopy detected five additional specimens (4.9%; 95% CI, 1.6-11.0). Among 17 reference-positive cases missed by direct ZN, modified bleach-assisted ZN microscopy detected five (29.4%; 95% CI, 10.3-56.0).

Discordant cases and cytomorphology

All five bleach-positive/ZN-negative specimens had a positive combined CBNAAT/culture record. Four occurred in necrotizing suppurative lymphadenitis and one in necrotizing granulomatous lymphadenitis; each was graded 1+ after bleach processing (Table *3*). Cytomorphology-stratified detection and harmonized AFB-grade transitions are presented in the Appendices. Because the archived dataset stored CBNAAT and culture in a single combined field, the discordant specimens are described as microbiologically confirmed rather than modality-specific CBNAAT-confirmed or culture-confirmed.

Code	Age/sex	Aspirate	Cytomorphology	Bleach grade	Combined CBNAAT/culture record
D1	28/M	Blood mixed	Necrotizing suppurative	1+	Positive
D2	62/F	Scanty	Necrotizing suppurative	1+	Positive
D3	27/F	Blood mixed	Necrotizing suppurative	1+	Positive
D4	50/M	Pus	Necrotizing suppurative	1+	Positive
D5	25/F	Pus	Necrotizing granulomatous	1+	Positive

TABLE 3: Microbiological adjudication of bleach-positive/ZN-negative cases

ZN: Ziehl-Neelsen, M: male, F: female

Representative cytomorphological and microscopic fields are shown in Figures *4*-*5*. Figure *4* provides morphological context for the lesion categories used in the stratified analysis. Figure *5* shows paired slides prepared from the same patient aspirates and illustrates the effects of modified bleach pretreatment and concentration before ZN staining, including increased AFB concentration and reduced background debris that could obscure bacilli. These photomicrographs are descriptive only and were not used to reclassify or quantitatively grade the primary study outcomes.

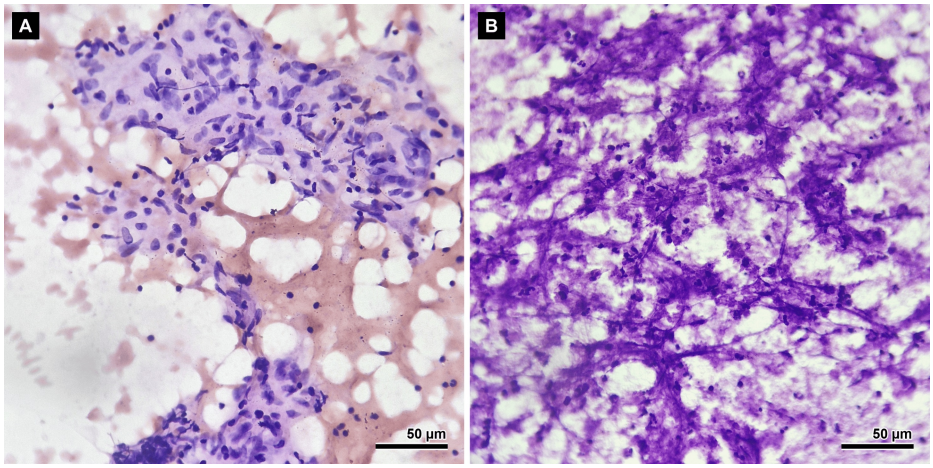


FIGURE 4: High-power cytomorphology of lymph node aspirates

Representative cytomorphological findings at ×400 total magnification (40× objective and 10× ocular). (A) Hematoxylin and eosin-stained smear showing a granulomatous lesion. (B) May-Grünwald-Giemsa-stained smear showing a necrotic and suppurative/abscess-like background. Scale bars = 50 µm.

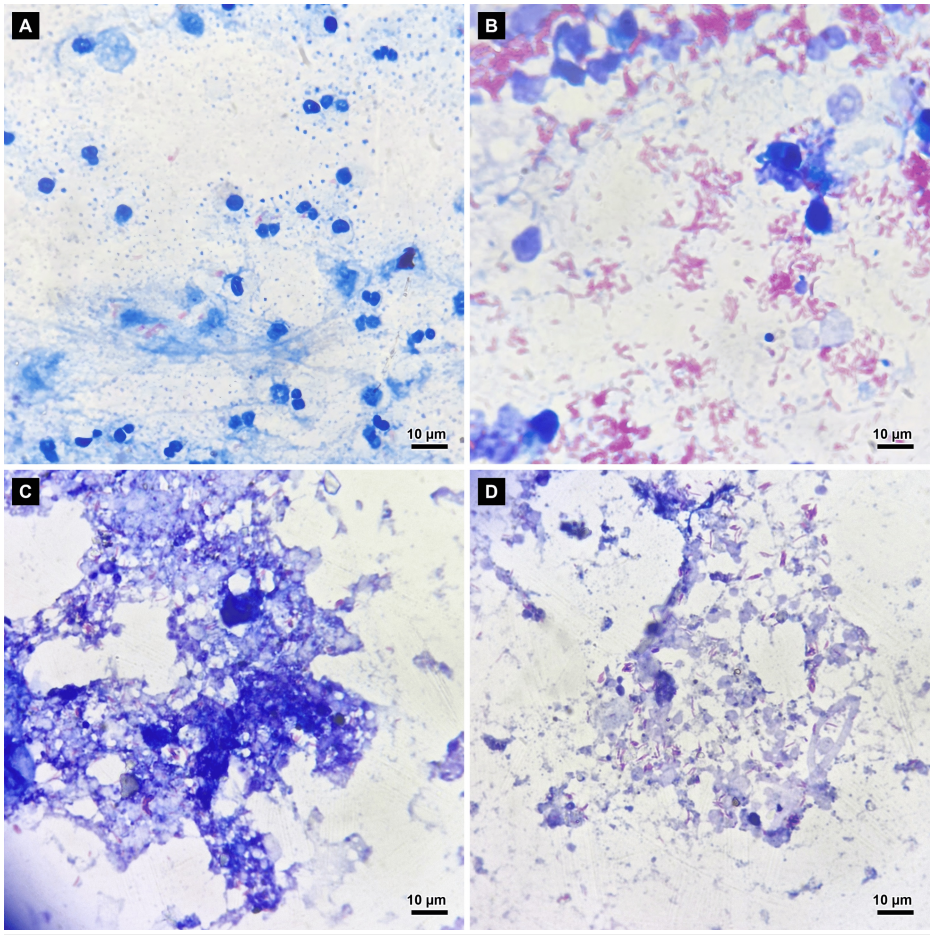


FIGURE 5: Paired direct and modified bleach-assisted ZN microscopy

Representative paired ZN photomicrographs at ×1000 total magnification (100× oil-immersion objective and 10× ocular). Panels A and B were prepared from aliquots of the same lymph node aspirate: (A) direct ZN smear without bleach pretreatment, showing sparse AFB and a low apparent bacillary burden; (B) smear prepared after modified bleach pretreatment and concentration, followed by the same ZN staining protocol, showing greater apparent AFB density. Panels C and D constitute a second paired example prepared from aliquots of the same aspirate: (C) direct ZN smear without bleach pretreatment, with bacilli partly obscured by background debris; (D) smear prepared after modified bleach pretreatment and concentration, followed by the same ZN staining protocol, showing a cleaner background and improved AFB visualization. Scale bars = 10 µm.

AFB: acid-fast bacilli, ZN: Ziehl-Neelsen

Because the archived dataset combined CBNAAT and culture in a single field, Table *3* supports microbiological confirmation of the five bleach-positive/ZN-negative cases but does not permit modality-specific labeling as CBNAAT-confirmed or culture-confirmed.

Sensitivity and cost analyses

The primary exact McNemar result was sensitive to the statistical method used to analyze the small number of discordant pairs: mid-P p = 0.0312, uncorrected asymptotic p = 0.0253, and continuity-corrected p = 0.0736 (Appendices). This method dependence supports interpretation of effect size and case-level confirmation rather than a binary claim of statistical superiority. If all eight participants without reference testing were assumed to have tuberculosis, sensitivity would be 45.7% for ZN and 56.5% for modified bleach-assisted ZN microscopy; the direction of the difference would remain unchanged (Appendices).

Using the locally reconstructed process-cost inputs presented in the Appendices and the observed incremental confirmed yield of 5/124 (4.0%), the exploratory estimate was approximately Rs 256 per additional confirmed detection. Varying the assumed per-specimen processing cost from Rs 8 to Rs 15 and the incremental yield across its exact confidence limits produced a scenario range of approximately Rs 87-Rs 1,135 per additional detection. These estimates reflect the local assumptions used, exclude downstream clinical outcomes, and should not be generalized or interpreted as cost-effectiveness results.

Discussion

Principal findings

Modified bleach-assisted ZN microscopy identified five additional AFB-positive lymph node aspirates beyond direct ZN microscopy, and all five had a positive composite microbiological reference result. Among the 38 reference-positive participants, the sensitivity point estimate was 13.2 percentage points higher with modified bleach-assisted ZN microscopy than with direct ZN microscopy. Modified bleach-assisted ZN microscopy also detected five of the 17 reference-positive cases missed by direct ZN. No direct-ZN-positive/modified-bleach-negative specimens or false-positive modified-bleach results were observed among the 78 reference-negative participants. The primary exact paired test remained just above the conventional 0.05 threshold because the inference was driven by only five discordant pairs.

Comparison with previous evidence

Earlier lymph node cytology studies reported much larger increases in positivity, for example, from 27% to 72%, 33% to 63%, and 13% to 61% after bleach concentration [3-5]. A more recent evaluation from Ethiopia also reported improved AFB detection after modified bleach processing [16]. Those studies established biological and operational plausibility but often included patients with cytologically diagnosed tuberculosis and did not consistently use an independent microbiological reference standard. Our smaller incremental yield should therefore be interpreted in the context of a consecutively recruited cohort of patients with clinically suspected tuberculosis, for whom a microbiological reference result was available in most cases.

The present findings align more closely with systematic reviews of bleach-treated sputum, which found small average sensitivity gains and emphasized between-study heterogeneity, protocol standardization, and possible specificity trade-offs [7,8]. They also follow the presentation principles used in influential lymph node molecular diagnostic studies: report the exact analysis cohort, use paired head-to-head comparisons, show numerator/denominator with confidence intervals, depict participant flow, and investigate each discordant result rather than relying only on aggregate p-values [9,10].

Clinical and laboratory implications

The World Health Organization (WHO) recommends low-complexity automated nucleic acid amplification tests on lymph node aspirate or tissue as the initial diagnostic test for suspected extrapulmonary tuberculosis [17]. Systematic evidence also supports nucleic acid amplification as the principal route to microbiological confirmation in extrapulmonary specimens [18]. Modified bleach-assisted ZN microscopy was not directly compared with molecular testing in this study. Unlike molecular assays, it cannot provide species confirmation or drug-resistance information and should not replace WHO-recommended nucleic acid amplification testing. Its potential role is adjunctive: improving AFB visualization when residual aspirate is available, molecular testing is delayed or inaccessible, or rapid morphological-microbiological correlation is required. The modified bleach procedure also adds a 15-minute incubation, a 15-minute centrifugation step, about five minutes of active technician time, and additional specimen handling under appropriate biosafety conditions. Its operational feasibility will therefore depend on laboratory workload, availability of trained personnel, centrifuge access, and biosafety infrastructure. In an exploratory scenario using locally reconstructed process-cost inputs, an assumed incremental cost of Rs 10.34 per specimen and the observed 4.0% incremental confirmed yield produced an estimated cost of Rs 256 per additional confirmed detection. The reported cost calculation represents an exploratory estimate of incremental local laboratory-process cost and should not be interpreted as evidence of cost-effectiveness.

All five rescued cases occurred in necrotizing lesions, consistent with the proposed mechanism by which bleach digestion clears proteinaceous and inflammatory debris and releases bacilli from necrotic material. This pattern is hypothesis-generating rather than a basis for selective testing because only five incremental events occurred. A future multicenter study should prospectively preserve separate CBNAAT and culture results, HIV status, screening time, and patient-management consequences while using a standardized concentration protocol.

Strengths and limitations

Study strengths included consecutive prospective recruitment, within-patient paired testing, complete ascertainment of both index tests, separate analyses of diagnostic yield and reference-standard accuracy, exact confidence intervals, participant-flow reporting consistent with STARD guidelines, and case-level evaluation of every discordant result. All five additional bleach-positive detections were supported by positive composite microbiological reference results. The rescue fraction provides an intuitive clinical measure that is not reported in most earlier bleach studies.

Limitations include the single-center design, the modest number of reference-positive participants, and only five discordant pairs. Eight participants negative on both index tests lacked reference testing, creating possible partial-verification bias. Although both Xpert MTB/RIF and Löwenstein-Jensen culture were documented as negative in the 78 composite-reference-negative participants, the archived combined field did not identify which modality established positivity among the 38 reference-positive participants. This

prevented modality-specific accuracy analysis and introduced possible reference-classification uncertainty. Blinding of reference-standard assessors to the microscopy results was not separately documented.

Although the NTEP grading scale ends at 3+, a small number of archived entries were recorded as 4+ and were harmonized with the 3+ category for the exploratory high-grade analysis. The primary binary analysis was unaffected. Specimen processing was performed in a Class II Type A2 biological safety cabinet using screw-capped tubes and appropriate personal protective equipment; however, specialized aerosol-tight centrifuge bucket enclosures were not used. Laboratories seeking to implement this procedure should undertake a local biosafety risk assessment and use validated centrifuge containment consistent with applicable institutional and national requirements.

Finally, the cost inputs were reconstructed from local assumptions rather than prospectively measured resource use. These limitations preclude an unqualified claim of statistical superiority, equivalent specificity, broad generalizability, or cost-effectiveness.

Conclusions

Modified bleach-assisted ZN microscopy identified five additional microbiologically confirmed AFB-positive lymph node aspirates and produced a numerically higher sensitivity estimate than direct ZN microscopy. The prespecified exact paired comparison did not establish statistical superiority, and no false-positive modified-bleach results were observed among the 78 reference-negative participants. The concentration of the five additional detections in necrotizing lesions should be regarded as hypothesis-generating.

Because the findings were based on only five discordant pairs, eight participants lacked reference verification, and the archived composite reference field did not permit modality-specific interpretation, the results should be considered preliminary and setting-specific. Modified bleach-assisted ZN microscopy may serve as an adjunct to direct ZN microscopy where appropriate laboratory and biosafety infrastructure is available, but it should not replace WHO-recommended molecular testing. Larger, multicenter, fully reference-verified studies using standardized processing parameters are required.

Appendices

Cytomorphology	n	Reference positive	ZN positive (sensitivity)	Bleach positive (sensitivity)	Additional
Reactive lymphadenitis	25	0	Not estimable	Not estimable	0
Suppurative lymphadenitis	5	0	Not estimable	Not estimable	0
Granulomatous lymphadenitis	42	8	4 (50.0%)	4 (50.0%)	0
Necrotizing granulomatous	23	8	4 (50.0%)	5 (62.5%)	1
Necrotizing suppurative	29	22	13 (59.1%)	17 (77.3%)	4

TABLE 4: Cytomorphology-stratified detection

ZN: Ziehl-Neelsen

Direct ZN	Bleach negative	Bleach low-grade	Bleach high-grade
ZN negative	98	5	0
ZN low-grade	0	10	6
ZN high-grade	0	0	5

TABLE 5: Harmonized AFB grade-category transitions

Low-grade comprises scanty and 1+ results; high-grade comprises 2+, 3+, and non-standard 4+ entries. Because 4+ is not an NTEP category, these entries were considered observer-recording errors indicating extremely heavy bacillary loads and were harmonized with 3+ for this exploratory summary. The primary binary diagnostic-accuracy analysis was unaffected.

AFB: acid-fast bacilli, ZN: Ziehl-Neelsen, NTEP: National Tuberculosis Elimination Programme

Method	p-value	Interpretation
Two-sided exact conditional McNemar	0.0625	Primary; conservative with sparse discordance
Mid-P McNemar	0.0312	Sensitivity analysis
Uncorrected asymptotic McNemar	0.0253	Approximation; unstable with five discordances
Continuity-corrected asymptotic McNemar	0.0736	Sensitivity analysis

TABLE 6: Statistical-method sensitivity for five discordant pairs

Variation in p-values is expected when all five discordant pairs favor one method. Interpretation therefore emphasizes paired counts, effect size, confidence intervals, and microbiological adjudication rather than dichotomous significance.

Scenario	Method	Sensitivity	NPV	Accuracy
Complete case: 8 excluded	ZN	55.30%	82.10%	85.30%
	Modified bleach	68.40%	86.70%	89.70%
All 8 assumed reference negative	ZN	55.30%	83.50%	86.30%
	Modified bleach	68.40%	87.80%	90.30%
All 8 assumed reference positive	ZN	45.70%	75.70%	79.80%
	Modified bleach	56.50%	79.60%	83.90%

TABLE 7: Extreme-case analysis for eight missing reference results

These are bounding scenarios, not a correction for verification bias. Both assumptions preserve the direction of the sensitivity difference.

ZN: Ziehl-Neelsen

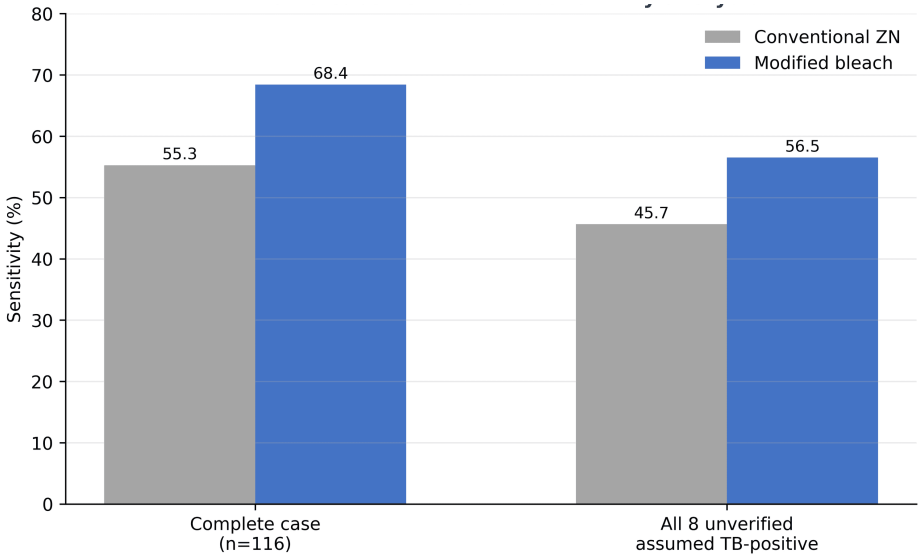


FIGURE 6: Extreme-case verification sensitivity analysis assuming that all eight participants without reference testing had TB

ZN: Ziehl-Neelsen, TB: tuberculosis

Resource	Quantity	Unit cost	Incremental cost (Rs)
15-mL tube	1	Rs 3.48	Rs 3.48
5% sodium hypochlorite	1 mL	Rs 0.182/mL	Rs 0.18
Normal saline	1 mL	Rs 0.0476/mL	Rs 0.05
Distilled water	2 mL	Rs 0.09/mL	Rs 0.18
Technician active time	5 min	Rs 1.189/min	Rs 5.95
Centrifuge electricity/depreciation	per test	assumption	Rs 0.50
Total incremental cost per specimen	-	-	Rs 10.34
Observed incremental confirmed yield	5/124	-	4.03%
Base-case cost per additional confirmed detection	-	-	Rs 256

TABLE 8: Scenario-based micro-costing

At per-test costs of Rs 8-Rs 15 and incremental yields spanning the exact 95% confidence limits for 5/124 (1.3-9.2%), the scenario range is approximately Rs 87-Rs 1,135 per additional detection. These values are reconstructed local process-cost assumptions rather than prospectively measured costs. The analysis excludes downstream clinical outcomes and comparative economic consequences and should not be interpreted as a cost-effectiveness evaluation.

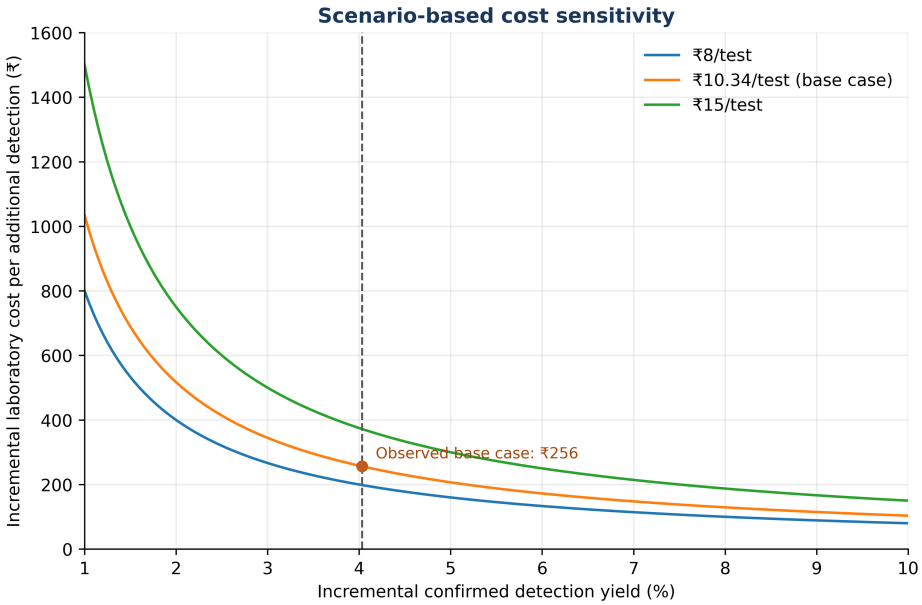


FIGURE 7: Scenario-based relationship between incremental laboratory cost, incremental confirmed yield, and cost per additional detection

Item	STARD 2015 requirement	Location/status
1	Identification as a study of diagnostic accuracy using at least one measure of accuracy	Title; Abstract
2	Structured summary of study design, methods, results, and conclusions	Abstract
3	Scientific and clinical background, including intended use and clinical role of the index test	Introduction

4	Study objectives and hypotheses	End of Introduction
5	Whether data collection was prospective or retrospective	Methods - Study design and participants
6	Eligibility criteria	Methods - Study design and participants
7	Basis for identifying potentially eligible participants	Clinically suspected cervical tuberculous lymphadenitis; Methods
8	Where and when potentially eligible participants were identified	Tertiary-care teaching hospital, central India; May 2024-October 2025
9	Whether participants formed a consecutive, random, or convenience series	Consecutive series; Methods
10a	Index test in sufficient detail to allow replication	Methods - Index tests, microscopy interpretation and AFB grading, and laboratory biosafety; centrifugation at approximately 1790 ×g for 15 minutes.
10b	Reference standard in sufficient detail to allow replication	Methods - Reference standard; Xpert MTB/RIF and/or Löwenstein-Jensen culture recorded in a combined field; modality establishing positivity unavailable.
11	Rationale for choosing the reference standard	Methods - Reference standard
12a	Definition and rationale for index-test positivity cut-offs or result categories	Any reported AFB grade counted positive for binary analysis
12b	Definition and rationale for reference-standard positivity cut-offs or result categories	Positive result on either available CBNAAT/culture modality
13a	Whether clinical information and reference-standard results were available to index-test performers/readers	Microscopy readers were blinded to the other index-test result and the available composite-reference result; uncertain or discordant microscopy findings underwent senior-pathologist review.
13b	Whether clinical information and index-test results were available to reference-standard assessors	Blinding of reference-standard assessors to the index-test results was not separately documented.
14	Methods for estimating or comparing diagnostic accuracy	Methods - Statistical analysis
15	Handling of indeterminate index-test or reference-standard results	Methods - Reference standard; no separate indeterminate category stored
16	Handling of missing index-test or reference-standard data	Complete-case accuracy analysis plus extreme-case sensitivity analysis
17	Analyses of variability in diagnostic accuracy	Supplementary lesion-stratified and missing-reference analyses
18	Intended sample size and how it was determined	124 participants
19	Participant flow, using a diagram	Figure 1; 124 participants in the paired-yield analysis, 116 in the diagnostic-accuracy analysis, and eight without reference testing.
20	Baseline demographic and clinical characteristics	Table 1
21a	Distribution of severity of disease in participants with the target condition	Table 1; Table 5
21b	Distribution of alternative diagnoses in participants without the target condition	Table 1; Table 5
22	Time interval and clinical interventions between index test and reference standard	Both index tests were performed on aliquots from the same lymph-node aspirate; reference testing used additional material from the same clinical episode when requested during routine care.
23	Cross-tabulation of index-test results by reference-standard results	Table 2
24	Estimates of diagnostic accuracy and precision	Table 2; Figure 3

25	Adverse events from performing the index test or reference standard	None
26	Study limitations, including potential bias, statistical uncertainty, and generalisability	Discussion - Strengths and limitations
27	Implications for practice, including intended use and clinical role of the index test	Discussion; Conclusions
28	Registration number and name of registry	Not registered; prospective observational diagnostic accuracy study
29	Where the full study protocol can be accessed	Source thesis/protocol available from authors/institution subject to approval
30	Sources of funding and other support; role of funders	Disclosures and Data Availability

TABLE 9: STARD 2015 checklist

ZN: Ziehl-Neelsen, TB: tuberculosis

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: Kajol Pradhan, Archana Shrivastava

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Drafting of the manuscript: Kajol Pradhan, Maneesh Sulya

Critical review of the manuscript for important intellectual content: Kajol Pradhan, Archana Shrivastava, Maneesh Sulya, Pramila Jain

Supervision: Archana Shrivastava, Maneesh Sulya, Pramila Jain

Disclosures

Human subjects: Informed consent for treatment and open access publication was obtained or waived by all participants in this study. Institutional Ethics Committee of Gandhi Medical College issued approval 16304/MC/IEC/2024. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

Acknowledgements

The authors acknowledge the Department of Pathology, Gandhi Medical College and Associated Hospitals, Bhopal, Madhya Pradesh, India, for providing institutional support and laboratory facilities for conducting this study.

References

1. Fontanilla JM, Barnes A, von Reyn CF: Current diagnosis and management of peripheral tuberculous lymphadenitis. Clin Infect Dis. 2011, 53:555-62. 10.1093/cid/cir454
2. World Health Organization: WHO Consolidated Guidelines on Tuberculosis: Module 3: Diagnosis. World Health Organization, Geneva, Switzerland; 2025. https://www.who.int/publications/i/item/9789240107984.
3. Gangane N, Anshu, Singh R: Role of modified bleach method in staining of acid-fast bacilli in lymph node aspirates. Acta Cytol. 2008, 52:325-8. 10.1159/000325515
4. Annam V, Karigoudar MH, Yelikar BR: Improved microscopical detection of acid-fast bacilli by the modified bleach method in lymphnode aspirates. Indian J Pathol Microbiol. 2009, 52:349-52. 10.4103/0377-4929.54991

5. Chandrasekhar B, Prayaga AK: Utility of concentration method by modified bleach technique for the demonstration of acid-fast bacilli in the diagnosis of tuberculous lymphadenopathy. J Cytol. 2012, 29:165-8. 10.4103/0970-9371.101160
6. Krishna M, Gole SG: Comparison of conventional Ziehl-Neelsen method of acid fast bacilli with modified bleach method in tuberculous lymphadenitis. J Cytol. 2017, 34:188-92. 10.4103/JOC.JOC_84_16
7. Steingart KR, Ng V, Henry M, et al.: Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 2006, 6:664-74. 10.1016/S1473-3099(06)70602-8
8. Cattamanchi A, Davis JL, Pai M, Huang L, Hopewell PC, Steingart KR: Does bleach processing increase the accuracy of sputum smear microscopy for diagnosing pulmonary tuberculosis?. J Clin Microbiol. 2010, 48:2433-9. 10.1128/JCM.00208-10
9. Ligthelm LJ, Nicol MP, Hoek KG, et al.: Xpert MTB/RIF for rapid diagnosis of tuberculous lymphadenitis from fine-needle-aspiration biopsy specimens. J Clin Microbiol. 2011, 49:3967-70. 10.1128/JCM.01310-11
10. Minnies S, Reeve BW, Rockman L, et al.: Xpert MTB/RIF ultra is highly sensitive for the diagnosis of tuberculosis lymphadenitis in a high-HIV setting. J Clin Microbiol. 2021, 59:0131621. 10.1128/JCM.01316-21
11. Newcombe RG: Improved confidence intervals for the difference between binomial proportions based on paired data. Stat Med. 1998, 17:2635-50.
12. Fagerland MW, Lydersen S, Laake P: The McNemar test for binary matched-pairs data: mid-p and asymptotic are better than exact conditional. BMC Med Res Methodol. 2013, 13:91. 10.1186/1471-2288-13-91
13. Husereau D, Drummond M, Augustovski F, et al.: Consolidated health economic evaluation reporting standards 2022 (CHEERS 2022) statement: updated reporting guidance for health economic evaluations. BMJ. 2022, 376:067975. 10.1136/bmj-2021-067975
14. Bossuyt PM, Reitsma JB, Bruns DE, et al.: STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. BMJ. 2015, 351:5527. 10.1136/bmj.h5527
15. Cohen JF, Korevaar DA, Gatsonis CA, et al.: STARD for Abstracts: essential items for reporting diagnostic accuracy studies in journal or conference abstracts. BMJ. 2017, 358:3751. 10.1136/bmj.j3751
16. Mulugeta F, Tiruneh M, Abebe B, Yitayew G, Ayehubizu Z, Getahun M, Gelaw A: Evaluation of modified bleach technique for the detection of acid fast bacilli in lymph node aspirate at the University of Gondar Comprehensive Specialized Hospital, Northwest Ethiopia. J Clin Tuberc Other Mycobact Dis. 2022, 28:100328. 10.1016/j.jctube.2022.100328
17. World Health Organization: WHO Consolidated Guidelines on Tuberculosis. World Health Organization, Geneva, Switzerland; 2025. https://iris.who.int/bitstream/handle/10665/340242/9789240022706-eng.pdf..
18. Kohli M, MacLean E, Pai M, Schumacher SG, Denkinger CM: Diagnostic accuracy of centralised assays for TB detection and detection of resistance to rifampicin and isoniazid: a systematic review and meta-analysis. Eur Respir J. 2021, 57:2000747. 10.1183/13993003.00747-2020