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Performance of nested RT-PCR on CSF for tuberculous meningitis diagnosis in HIV-infected patients

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SUMMARY

SETTING: Timely diagnosis of tuberculous meningitis (TBM) in patients with human immunodeficiency virus (HIV) infection remains a challenge. Despite the current scale-up of the Xpert® MTB/RIF assay, other molecular diagnostic tools are necessary, particularly in referral centres in low- and middle-income countries without Xpert testing.

OBJECTIVE: To determine the diagnostic performance of nested real-time polymerase chain reaction (nRT-PCR) in HIV-infected TBM patients categorised according to standardised clinical case definitions.

DESIGN: Based on clinical, laboratory and imaging data, HIV-infected patients with suspected TBM were prospectively categorised as 'definite TBM', 'probable TBM', 'possible TBM' or 'not TBM'. We evaluated nRT-PCR sensitivity and specificity in diagnosing TBM among definite TBM cases, and among definite + probable TBM cases.

RESULTS: Ninety-two participants were enrolled in the study. nRT-PCR sensitivity for definite TBM ($n = 8$) was 100% (95%CI 67–100) and 86% (95%CI 60–96) for both definite and probable TBM ($n = 6$). Assuming that 'not TBM' patients ($n = 74$) were true-negatives, nRT-PCR specificity was 100% (95%CI 95–100). The possible TBM group ($n = 4$) had no nRT-PCR positives.

CONCLUSIONS: The nRT-PCR is a useful rule-in test for HIV-infected patients with TBM according to international consensus case definitions. As nRT-PCR cannot exclude TBM, studies comparing and combining nRT-PCR with other assays are necessary for a rule-out test.

KEY WORDS: tuberculosis; central nervous system; TBM; meningeal tuberculosis; diagnosis; polymerase chain reaction; acquired immunodeficiency syndrome

TUBERCULOUS MENINGITIS (TBM), the most severe clinical presentation of tuberculosis (TB), can cause high mortality and morbidity, particularly in patients with human immunodeficiency virus (HIV) infection in resource-limited settings.[1–4] Conventional microbiological detection (direct smear examination and/or culture identification) is suboptimal for a timely diagnosis,[3–6] indicating the need for rapid and accurate diagnostic tests.

Commercial and in-house methods for the detection of *Mycobacterium tuberculosis* DNA in cerebrospinal fluid (CSF) have shown a sensitivity of 64–83% and a specificity of >98%.[5,6] More recently, the Xpert® MTB/RIF test (Cepheid, Sunnyvale, CA, USA) for detecting *M. tuberculosis* in CSF yielded a sensitivity of 29–85% and a specificity of >98%.[6–10] However, no nucleic-acid amplification assay has been approved by the US Food and Drug Administration for the testing of CSF specimens for TBM,[4,5] and some authors have raised concerns about World Health Organization recommendations of the use of Xpert.[9,10] In addition, the implementation of Xpert testing presents limitations and challenges, particularly in resource-limited settings.[8] Referral centres in some settings have laboratories with molecular diagnostic support, but in many laboratories the Xpert test is not available. Studies evaluating optimised polymerase chain reaction (PCR) methods thus remain relevant.

Two interesting PCR-based methods for the diag-

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nosis of TBM are nested PCR and real-time PCR, which target the *M. tuberculosis*-specific *mpt*64 gene. Information about their clinical value in HIV-related TBM is scarce.[5] Nested PCR has the advantage of increased sensitivity and specificity due to second-step amplification of the chosen target within the first-step PCR product. Real-time PCR is simpler, less time-consuming and more easily applied in the routine diagnostic laboratory testing.[5]

In 2010, an international consensus established a uniform case definition for TBM. Although not primarily intended to aid the diagnosis in clinical practice, this provided a valuable tool for standardising diagnostic research.[11,12] We conducted a study to determine the sensitivity and specificity of nested real-time PCR (nRT-PCR) in HIV-associated TBM patients categorised according to this standardised definition.

METHODS

We conducted a prospective observational study over 3 years at the Emílio Ribas Institute of Infectious Diseases (ERIID), São Paulo, SP, Brazil, a 250-bed public referral hospital for the management of patients with meningitis and a catchment population of 11 million. Hospitalised HIV-infected patients aged >12 months were eligible.

The inclusion criteria were 1) clinical suspicion of TBM, defined by the presence of one or more of the following symptoms: headache, fever, irritability, vomiting, neck stiffness, convulsions, focal neurological deficits, altered mental status or lethargy; and 2) availability of computed tomography (CT) of the brain and lumbar puncture for comprehensive analyses of CSF.

Clinical, CSF and imaging data were summarised in an electronic chart and categorised under 21 criteria, each of which were scored based on an international consensus establishing a uniform case definition for the evaluation of diagnostic tests for TBM.[11] Patients were first categorised as 'probable' (score $\geqslant$12), 'possible' (score 6–11) or 'uncategorised' (score <6). Based on more conclusive test results, patients were finally categorised as follows: 1) definite TBM—the gold standard category, with Ziehl-Neelsen (ZN) staining showing acid-fast bacilli (AFB) or CSF culture positive for *M. tuberculosis*; 2) probable TBM—those with a score of $\geqslant$12, indicating a higher risk of a TBM diagnosis; 3) possible TBM—those with a score of 6–11, indicating a lower risk of a TBM diagnosis; and 4) not TBM—those with a confirmed alternative diagnosis. TBM severity was graded at presentation according to the British Medical Research Council (BMRC) system.[13] All patients were followed until hospital discharge or in-hospital death.

CSF samples of 10–12 ml were sent for biochemical analyses, and bacterial, fungal and mycobacterial culture. ZN staining and mycobacterial culture were used as the reference/gold standard. Culture was performed using BACTEC MGIT™ 960™ (BD, Cockeysville, MD, USA) and solid medium (Löwenstein-Jensen). A second portion of the CSF sample (1–2 ml) was submitted to the Adolfo Lutz Institute, São Paulo, SP, Brazil, the main public reference laboratory for meningitis diagnosis in São Paulo State, for the performance of the index test, nRT-PCR. Standard test and index text results were read by two and three researchers, respectively. Readers of one test did not have access to the results of the other test, clinical data, or to other laboratory results.

DNA from CSF samples was extracted using silica membrane commercial kits: 1) CSF (1–2 ml) was centrifuged at 12 000 $\times g$ for 15 min and 500 µl of the supernatant then discarded; and 2) incubation was performed using mutanolysin (350 U/ml) and lysozyme (0.2 mg/ml) at 37°C for 2 h.

nRT-PCR was performed using two pairs of primers capable of specifically amplifying the *mpt*64 gene sequence, as described by Takahashi et al.[5] All reactions were performed in duplicate; a positive control with *M. tuberculosis* DNA (H37Rv strain) and four negative control reactions lacking DNA were included in each assay. A positive result was defined as a cycle threshold (Ct) value of $\leqslant$20, and a negative result as a Ct value of 0 or $\geqslant$21. As a control for PCR inhibitors, and to monitor the efficiency of nucleic-acid extraction, each CSF sample was tested using RT-PCR for the presence of the human ribonuclease *P* gene. The nRT-PCR result was not used for case classification to avoid incorporation bias.

Alternative aetiologies were also evaluated in all participants. Patients underwent diagnostic testing for cryptococcal meningitis (India ink, cryptococcal antigen and culture in CSF and blood), acute bacterial meningitis (Gram stain, culture and PCR for the detection of *Streptococcus pneumoniae* and *Neisseria meningitides* in CSF) and neurosyphilis (venereal disease research laboratory test and *Treponema pallidum* haemagglutination assay in CSF). For the differential diagnosis, the CSF samples were tested using HIV-RNA for cytomegalovirus, *Toxoplasma gondii* and John Cunningham virus, and a search for neoplastic cells was undertaken.

nRT-PCR results for the definite TBM (gold standard) and not TBM categories were compared to determine the sensitivity and specificity of the test, with 95% confidence intervals (95%CIs). We also evaluated the proportion of positive nRT-PCR results in patients in the probable and possible categories. As microbiological confirmation could miss some TBM cases (definite TBM category), we also combined the definite and probable categories to assess nRT-PCR

Table 1 Main characteristics of HIV-infected patients with suspected TBM by final diagnostic categories

Characteristics	TBM definite ($n = 8$) *n* (%)	TBM probable ($n = 6$) *n* (%)	TBM possible ($n = 4$) *n* (%)	Not TBM ($n = 74$) *n* (%)	Total ($n = 92$) *n* (%)
Male	6 (75)	5 (83)	1 (25)	54 (73)	65 (71)
Age, years, median [IQR]	32.5 [28–44]	38 [32–46]	36 [33–43]	37 [30–43]	37 [31–43]
Previous TB	5 (63)	3 (50)	2 (50)	25 (34)	35 (38)
Previous HIV treatment use	4 (50)	3 (50)	3 (75)	40 (54)	50 (54)
CD4 cells/mm^3, median [IQR]	26 [22–52]	152 [85–270]	111 [65–213]	79 [19–190]	73 [23–189]
Nadir CD4 cells/mm^3, median [IQR]	17 [17–52]	106 [41–268]	86.5 [40–155]	46 [14–112]	46 [17–130]
HIV viral load, $\log_{10}$ copies/ml, median [IQR]	4.30 [4.1–4.9]	4.89 [3.8–5.3]	4.45 [4.3–4.5]	4.53 [2.7–5.3]	4.45 [3.2–5.2]
Haemoglobin, g/dl, median [IQR]	9.5 [8.7–10.3]	9.8 [9.2–11]	13.9 [12.2–14.1]	11.4 [9.4–12]	11.2 [9–12]
Symptoms					
Headache	6 (75)	2 (33)	3 (75)	40 (54)	51 (55)
Altered consciousness	7 (88)	3 (50)	3 (75)	23 (31)	36 (39)
Seizures	0	1 (17)	0	9 (12)	10 (11)
Cranial nerve palsies	0	0	1 (25)	16 (22)	17 (19)
Meningeal signs	1 (13)	0	0	9 (12)	10 (11)
Duration of symptoms, days, median [IQR]	30 [15–30]	40 [33–55]	25 [18–38]	15 [4–30]	20 [5–30]
Fever	3 (38)	5 (83)	2 (50)	18 (24)	27 (29)
Glasgow Coma Scale, mean (min–max)	12.4 (6–15)	14.3 (14–15)	14.3 (14–15)	14.2 (7–15)	14.1 (6–15)
BMRC Grade I, *n*	1	2	1	37	41
BMRC Grade II, *n*	4	4	3	35	46
BMRC Grade III, *n*	3	0	0	2	5
Death, *n*	3 (38)	2 (33)	0	16 (22)	21 (23)
CSF parameters, median [IQR]					
White cells, /mm^3	78 [43–211]	101 [52–120]	38 [30–248]	6 [1–45]	16 [2–78]
Neutrophil, %	49 [25–70]	19 [3–34]	15 [1–16]	0 [0–3]	1 [0–10]
Lymphocyte, %	30 [19–54]	66 [45–77]	85 [70–89]	31 [0–88]	42 [0–88]
Protein, mg/dl	187 [157–306]	98 [82–110]	187 [176–196]	71 [42–148]	85 [50–178]
Glucose ratio, CSF/serum	0.23 [0.18–0.39]	0.32 [0.31–0.38]	0.41 [0.34–0.43]	0.51 [0.37–0.63]	0.48 [0.35–0.60]
Adenosine deaminase, IU/l	3.1 [2.7–3.8]	6.2 [4.8–7.1]	6.5 [5.45–8.8]	3.3 [0.6–8.8]	3.9 [1.1–8.1]
Cerebral imaging					
Cerebral atrophy	5 (63)	4 (67)	3 (75)	41 (55)	53 (58)
Focal lesion(s)	1 (13)	3(50)	1 (25)	30 (41)	35 (38)
Hydrocephalus	2 (25)	2 (33)	1 (25)	6 (8)	11 (12)
Basal exudates	2 (25)	0	0	3 (4)	5 (5)
Infarcts	1 (13)	3 (50)	0	1 (1)	5 (5)
TBM diagnostic score, points, median [IQR]	14.5 [14.8–15.3]	14.5 [14–15]	9.5 [8.8–10.3]	8.0 [6.0–9.0]	8 [6.8–10.3]

HIV = human immunodeficiency virus; TBM = tuberculous meningitis; IQR = interquartile range; TB = tuberculosis; BMRC = British Medical Research Council; CSF = cerebrospinal fluid; IU = international unit.

sensitivity (a composite gold standard). Continuous variables were expressed as mean and standard deviation (SD) if they had a normal distribution, and median with interquartile range (IQR) if otherwise.

The ERIID Research Ethics Committee, São Paulo, SP, approved the study protocol. Written informed consent was provided by all participants or their legal representative.

RESULTS

A total of 92 HIV-infected patients with suspected TBM upon hospital admission were included in the study. The median age was 37 (IQR 31–43) years and 71% were male. The median CD4 count upon hospital admission was 73 (IQR 23–189) cells/mm^3. Comprehensive patient data are presented in Table 1.

The Figure shows the initial and final categorisation of the patients as definite TBM ($n = 8$), probable TBM ($n = 6$), possible TBM ($n = 4$) and not TBM ($n = 74$). More severe disease (i.e., BMRC Grade III disease) was more common among those classified as definite TBM (38%) than in the probable and possible TBM groups (0%).

All eight definite TBM participants had a positive nRT-PCR result, giving a sensitivity of 100% (95%CI 67–100). Conversely, all 74 participants in the not TBM group had negative nRT-PCR (specificity 100%, 95%CI 95–100). Among those with probable TBM, nRT-PCR was positive in 4/6 (67%, 95%CI 30–90); none of the four patients in the possible TBM group had a positive result. When the definite and probable TBM categories were combined, nRT-PCR sensitivity was 86% (95%CI 60–96).

The differential diagnoses of patients in the not TBM category are shown in Table 2. Among persons with aseptic meningitis, five were discharged home with unequivocal improvement of symptoms with non-specific treatment only (analgesics and adjustment of epilepsy treatment). One individual with idiopathic meningitis died while hospitalised due to renal failure and sepsis.

Although treatment and outcome were not used for

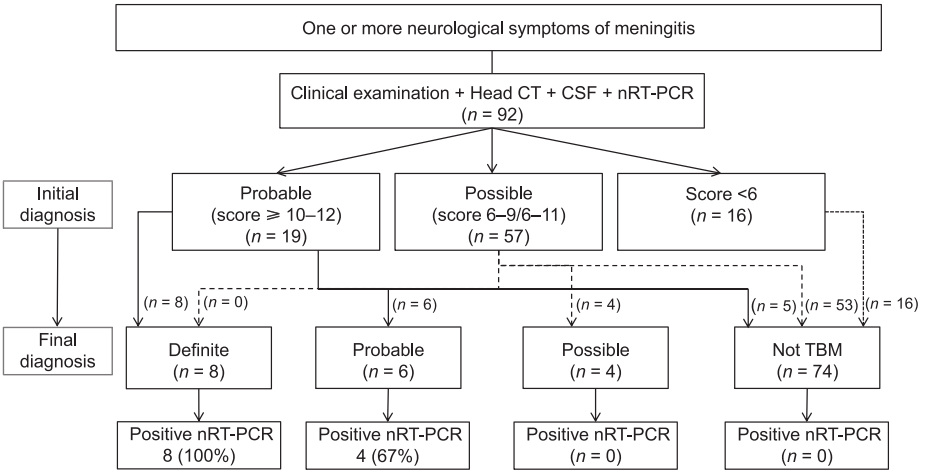

Figure Initial and final diagnostic categories of patients with suspected TBM upon hospital admission. Ninety-two patients received a score and primary diagnostic category (probable TBM, $n = 19$; possible TBM, $n = 57$). Sixteen patients received a diagnostic score of <6. The final diagnostic categories were adjudicated according to subsequent results of more conclusive examinations (e.g., culture, histopathology findings). nRT-PCR results for each final category are indicated. CT = computed tomography; CSF = cerebrospinal fluid; nRT-PCR = nested real-time polymerase chain reaction; TBM = tuberculous meningitis.

classification purposes, we observed that all patients with a final categorisation of definite TBM, probable TBM or possible TBM received treatment for TBM ($n = 18$); five patients died (definite TBM, $n = 3$; probable TBM, $n = 2$). Those who died were nRT-PCR-positive. The two individuals in the probable TBM group who died were AFB-positive on pulmonary smear and were also receiving ceftriaxone for suspected bacterial sepsis following clinical deterioration.

The two persons in the probable TBM group who were PCR-negative were discharged from hospital after clinical improvement and underwent anti-tuberculosis treatment only (one had disseminated TB, with no other alteration of CSF parameters except for protein). We could not ascertain whether the four patients categorised in the Possible TBM group received any other treatment during hospitalisation and after discharge.

Table 2 Diagnosis in the 'not TBM' category

	n
Cerebral toxoplasmosis	16
Cryptococcal meningitis	15
Cytomegalovirus encephalitis	9
Bacterial sepsis	9
HIV encephalopathy	6
Neurosyphilis	4
CNS lymphoma	3
Progressive multifocal leukoencephalopathy	2
Disseminated *Mycobacterium avium* infection	2
CNS schistosomiasis	1
Ischaemic stroke	1
Aseptic meningitis	6
Total	74

TBM = tuberculous meningitis; HIV = human immunodeficiency virus; CNS = central nervous system.

DISCUSSION

nRT-PCR sensitivity for definite TBM ($n = 8$) was 100% (95%CI 67–100) and was 86% (95%CI 60–96) for definite and probable TBM ($n = 6$). The specificity for not TBM ($n = 74$ cases) was 100% (95%CI 95–100).

After lumbar puncture, nRT-PCR was used to confirm the TBM diagnosis in 63% of the patients initially categorised as probable TBM (Figure) based on international consensus clinical criteria.[11] nRT-PCR enabled timely confirmation of the diagnosis of TBM in this group with high suspicion of TBM. The use of nRT-PCR helped reduce the total assay time and risk of contamination using two-step amplification and control reactions, and facilitated a routine diagnosis compared with conventional and nested PCR.[5]

Our study showed higher sensitivity and specificity for definite TBM than other studies in HIV-infected populations.[14–16] This finding was probably due to the improved performance of the nRT-PCR assay and specificity of the PCR target (*mpt*64). Scarpellini et al. reported similar results among HIV-infected patients with TBM; however, their control group was smaller ($n = 24$).[17] Some recent studies using Xpert in predominantly HIV-infected patients showed comparable results with larger study populations in Viet Nam, South Africa and Uganda.[18–20]

The proportions of patients in the probable TBM (67%) and possible TBM (0%) groups with positive nRT-PCR were similar to findings from studies that used Xpert testing.[19,20] In contrast to the high sensitivity for definite TBM cases, those results could be explained, at least in part, by the fact that none of

the patients in those groups had severe disease according to the BMRC system. Culture-positive patients generally have a higher burden of *M. tuberculosis*.[21] Cases with both positive culture and nRT-PCR may have more severe clinical disease due to a greater *M. tuberculosis* burden. It is also possible that the case definition for possible TBM is less specific than that for probable TBM, which may have led to the inclusion of patients without TB.

Several factors can influence PCR sensitivity, such as the presence of PCR inhibitors, inappropriate mycobacterial lysis and uneven distribution of mycobacteria in clinical specimens.[22] However, we have no evidence to suggest that such factors were responsible for the differences in test positivity across the different categories in our study.

The diagnosis of TBM is challenging, particularly in HIV-infected patients.[1,23] International consensus clinical criteria are useful for the identification of presumptive cases. However, as neurological manifestations in HIV-infected individuals are generally non-specific,[23] most of the patients initially classified as probable and possible TBM had a confirmed diagnosis other than TBM (Figure). Nevertheless, further studies are needed for a better understanding of the probable and possible TBM categories in HIV-infected patients with suspected TBM.

The definition of a TBM clinical case, although intended for standardisation of research studies, could also be used as a screening method to identify patients who should be tested using nRT-PCR. This strategy could be particularly important in settings with limited resources, as no patient in the possible and not TBM categories were diagnosed with TBM in the present study.

The study had several limitations. First, it was conducted at a single urban referral centre. Nevertheless, we believe that our results can be generalised to other settings, as we included patients with a high diversity of HIV-associated neurological conditions, including diseases that mimic TBM. Second, the number of patients in the definite TBM category was smaller than that in other studies.[16,18–20] This led to relatively wide CIs for our point estimate of test sensitivity. Third, the amount of CSF submitted for centrifugation (1–2 ml) was not optimal. Other studies have used larger volumes that yielded better results in the probable and possible TBM categories.[18–20] Fourth, nRT-PCR is more complex than Xpert testing, and requires adequate infrastructure and trained staff.[7,10] Nevertheless, our study had a relatively large, well-defined control group without TBM, which allowed us to calculate the specificity of the assay with good precision. The study design, the rigorous investigation of each patient and the differential diagnoses were the strengths of our findings.

Study results should be translated to clinical practice in the context of current challenges in the management of HIV-associated TBM. Although a positive nRT-PCR result confirms the diagnosis, TBM cannot be ruled out by a negative test result, despite the high test sensitivity observed in the present study.[3,4,9,10] TBM should be considered a medical emergency, and treatment should be initiated in a timely manner (and often empirically) to reduce the risk of mortality.[4,9,10] Even with the advent of Xpert, and given the controversy about the lipoarabinomannan lateral flow assay, ruling out TBM remains a challenge.[9,10,24] A positive nRT-PCR associated with high suspicion of TBM allows physicians to reduce the number of broad-spectrum antibiotics[10] in the already polypharmacy-exposed HIV-infected patient. As any single test is likely to miss TBM cases, a combination of more than one method could be an alternative approach for the diagnosis of HIV-related TBM.

In conclusion, nRT-PCR showed high sensitivity and specificity in HIV-related TBM. This method appears to be a useful test to rule in a TBM diagnosis, particularly in HIV-infected patients. More studies are necessary to confirm our results, including a comparison with the Xpert test.

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Conflicts of interest: none declared.

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RESUME

SETTING : Un diagnostic rapide de la méningite tuberculeuse (TBM) chez les patients infectés par le virus de l'immunodéficience humaine (VIH) reste un défi. En dépit de l'accélération actuelle de la mise en œuvre du test Xpert® MTB/RIF, d'autres outils de diagnostic moléculaire restent nécessaires, particulièrement dans les centres de référence des pays à revenu faible et moyen ne disposant pas du test Xpert.
OBJECTIF : Déterminer la performance diagnostique d'une réaction polymérase nichée en chaîne en temps réel (nRT-PCR) chez les patients atteints de tuberculose (TB) maladie et infectés par le VIH selon la définition clinique internationale uniforme du cas.
SCHÉMA : En se basant sur les données cliniques, biologiques et radiographiques, les patients infectés par le VIH avec suspicion de TBM ont été prospectivement classés en «TBM certaine», «TBM probable», «TBM possible» et «pas de TBM». Nous avons évalué la sensibilité et la spécificité de la nRT-PCR pour le diagnostic de la TBM par rapport au cas de TBM certaine et TBM certaine + probable.
RÉSULTATS : Nous avons enrôlé 92 participants. La sensibilité de la nRT-PCR pour la TBM certaine ($n = 8$) a été de 100% (IC95% 67–100) et pour la TBM certaine + probable ($n = 6$), elle a été de 86% (IC95% 60–96%]. Si l'on considère la catégorie non TBM ($n = 74$) comme de vrais négatifs, la spécificité a été de 100% (IC95% 95–100%). La catégorie TBM possible ($n = 4$) n'a eu aucun résultat positif à la nRT-PCR.
CONCLUSION : La nRT-PCR est un test utile pour confirmer le diagnostic des patients infectés par le VIH et atteints de TBM selon la définition de cas de recherche par consensus. Comme la nRT-PCR ne peut pas exclure la TBM, il est nécessaire de réaliser des études comparatives et combinées avec d'autres tests à la recherche d'un test d'élimination.

RESUMEN

MARCO DE REFERENCIA: El diagnóstico oportuno de la meningitis tuberculosa (TBM) en los pacientes aquejados de infección por el virus de la inmunodeficiencia humana (VIH) plantea aún dificultades. Pese a la actual ampliación de escala de utilización de la prueba Xpert® MTB/RIF, es necesario contar con otros medios diagnósticos moleculares, sobre todo en los centros de referencia de los países con ingresos bajos y medianos que no cuentan con esta prueba.
OBJETIVO: Determinar el rendimiento diagnóstico de una reacción en cadena de la polimerasa, anidada ultrarrápida (nRT-PCR), en los pacientes infectados por el VIH con diagnóstico internacional de TBM.
MÉTODO: En función de los datos clínicos, de laboratorio y radiográficos se asignó de manera prospectiva a los pacientes infectados por el VIH con presunción clínica de TBM la categoría de TBM definitiva, probable, posible o de ausencia de TBM. Se evaluó la sensibilidad y la especificidad de la nRT-PCR en el diagnóstico de la TBM y se comparó con la definición clínica de las diferentes categorías de casos de TBM.
RESULTADOS: Participaron en el estudio 92 pacientes. La sensibilidad de la nRT-PCR en el diagnóstico definitivo ($n = 8$) de TBM fue 100% (IC95% 67–100) y en los diagnósticos definitivo y probable ($n = 6$) fue 86% (IC95% 60–96). Al considerar la categoría sin TBM ($n = 74$) como casos negativos verdaderos, la especificidad de la prueba fue 100% (IC95% 95–100). En los casos con TBM posible ($n = 4$) no hubo resultados positivos de la nRT-PCR.
CONCLUSIÓN: La nRT-PCR constituye una prueba 'definitoria' útil en los pacientes infectados por el VIH que presentan TBM, según el consenso internacional de definiciones de caso con fines de investigación. Dado que la nRT-PCR no permite excluir la TBM, se precisan aún estudios comparativos y estudios asociados con otras pruebas con el propósito de buscar una prueba que permita descartar el diagnóstico.