Molecular diagnosis of suspected tuberculosis from archived smear slides from the Balimo region, Papua New Guinea

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A B S T R A C T

Background: Tuberculosis (TB) is a serious health problem in Papua New Guinea (PNG) with an estimated 30 000 new cases and 3800 deaths each year. In the Balimo region of the Western Province, diagnosis relies on clinical manifestations and on the microscopic detection of acid-fast bacilli (AFB) in sputum smears, a technique with limited sensitivity.

Methods: A molecular diagnosis assay targeting DNA extracted from archived sputum smear slides collected from the Balimo region (2012–2014) was conducted, without the need for a viable culture. The presence of Mycobacterium sp on 1162 slides prepared from 345 sputum samples was assessed using a real-time PCR (qPCR) approach.

Results: The qPCR technique identified the presence of mycobacteria in 35.4% of the smear slides and 59.7% of the tested sputum samples. Poor agreement was observed between the two diagnosis methods (smear AFB microscopy versus qPCR), with 100 AFB-positive sputum samples compared to 206 qPCR-positive sputum samples overall. Treatment was initiated in 90.2% of the smear-positive cases. Unnecessary treatment of ‘false-positive’ TB cases (AFB-negative/qPCR-negative) was very low (8.6%) and was even lower when the nine patients diagnosed with extrapulmonary TB were excluded from the analysis. However, the prevalence of false-negatives (AFB-negative/qPCR-positive) was high (28.5%).

Conclusions: Undetected smear-negative TB is occurring in the Balimo region of PNG, as well as some unnecessary empirical treatment. Molecular methods of diagnosis could greatly reduce the frequency of inappropriate clinical assessment, as well as providing point-of-care diagnosis. This may provide substantial patient and programmatic benefits, including lowering the economic burden on patients from rural areas seeking medical diagnosis in Balimo.

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Introduction

Tuberculosis (TB) is a serious health problem in Papua New Guinea (PNG) where it is estimated to cause more than 30 000 new cases and 3800 deaths each year, the second highest cause of mortality country-wide after lower respiratory infections (World Health Organization, 2015). In 2015, the estimated incidence rate for TB in PNG was 432 cases per 100 000 people (95% confidence interval (CI) 352–521 per 100 000) (World Health Organization, 2015). In comparison, the mean TB incidence rates in the World Health Organization (WHO) South-East Asia and Western Pacific regions were respectively 211 (95% CI 192–232) and 85 (95% CI 80–89) cases per 100 000 people per year. Overall, this makes PNG one of the top-10 countries for TB incidence worldwide, and the third highest TB incidence country in the South-East Asia and Western Pacific region, after Kiribati and Timor-Leste (Viney et al., 2015; World Health Organization, 2016). However, PNG is the only country of the three with more than 10 000 cases per year. Furthermore, evidence from rural areas of PNG has shown that TB rates may be much higher than officially reported, with incidence...
estimates of 550 cases per 100 000 people in Western Province, and 1290 cases per 100 000 people in Gulf Province (Cross et al., 2014; McBryde, 2012). This rate of TB is remarkably high, particularly as HIV (the modern driver of TB in Sub-Saharan Africa) appears to account for only about 5–10% of cases in PNG (McBryde, 2012).

Early diagnosis, effective treatment, and successful cessation of transmission are major strategies in the control of TB. But most of all, it is crucial to estimate accurately the prevalence of bacteriologically positive pulmonary TB (PTB), because these cases are responsible for spreading infection in the community (World Health Organization, 2016). Unfortunately, PNG has the lowest case-detection rate in the WHO Western Pacific Region (Ongugo et al., 2011), and the directly observed treatment, short course (DOTS) coverage was only 51% in 2010 (Anon, 2011; Ley et al., 2014), more than 10 years after the introduction in 1997 of the internationally recommended strategy in PNG (Levy et al., 1998).

Understanding the true burden of TB cases (and drug resistance) in PNG is made challenging by local constraints, both socio-cultural and structural (Ongugo et al., 2011). First, PNG has the lowest number of health care workers per capita in the region (Anon, 2011), and there is a genuine lack of laboratory diagnostics, especially in rural PNG where 80% of the population live. Second, the national TB control programme, which previously encouraged active surveillance (including mobile patrols) rather than passive diagnosis from within health facilities, was strongly affected by the establishment in 1995 of the “New Organic Law on Provincial Governments and Local Level Governments” in which the management and service delivery of rural health services were handed over from the National Department of Health to the provincial and local governments (Day, 2009); since then, TB control has mostly relied on passive case finding among individuals self-presenting to health care facilities. Unfortunately, accessing health facilities can be very challenging in rural areas, as a result of the geographic isolation, extremely limited road networks, and poverty, with predominantly subsistence-based livelihoods (Ongugo et al., 2011). Furthermore, because serial sputum specimens are required for TB diagnosis, as per WHO recommendations, people are asked to make repeated visits to the health care facility for sampling, collection of results, and initiation of treatment, which can be an additional constraint. Long delays in receiving a TB diagnosis have been experienced in PNG with, for example in the rural Balimo region of the Western Province, evidence of prolonged symptomatic periods prior to diagnosis and treatment initiation (Diefenbach-Elstob et al., 2017).

In the field of TB, the diagnosis itself is challenging. Conventional TB diagnosis relies on medical history, tuberculin skin test, chest X-rays, and bacteriological examination, methods that have inherent limitations. The poor sensitivity of sputum smear microscopy is a major concern (Steingart et al., 2007), and culture-based TB diagnosis remains complex, expensive, slow, and technically demanding, and requires expensive biocontainment facilities (Doughty et al., 2014). A Brazilian study showed that as many as 26.7% of PTB cases remained undiagnosed when traditional methods were used (Mello, 2001).

In the Balimo region of PNG, TB diagnosis is based either on the microscopic detection of acid-fast bacilli (AFB) in Ziehl–Neelsen-stained sputum smears, or on assessment of presenting signs and symptoms by the local clinician. In this case, it is very challenging to ascertain whether a clinical TB diagnosis is accurate (clinician assessment is a true-positive and smear is a false-negative) or inaccurate (clinician assessment is a false-positive and smear is a true-negative). Should the patient not have TB, this could result in unnecessary treatment, hence exposure to toxic drugs and significant costs to the health care system. At worst, it may exert a selective pressure leading to antibiotic resistance. Because they are rapid and highly sensitive, molecular techniques have significantly improved the diagnosis of TB, especially in patients with paucibacillary infection (Cheng et al., 2004; Helb et al., 2010).

In this study, suspected TB cases were identified using molecular testing by real-time PCR on DNA extracted from archival sputum smears on slides from PNG. The aims of this study were (1) to confirm the usefulness of already available smear slides for the extraction of mycobacterial DNA and molecular analysis, (2) to compare the sensitivity of the microscopic versus molecular methods for TB diagnosis, and (3) to assess the clinical diagnosis and response based on currently available techniques in the Balimo region of PNG.

Methods

Study setting

The Middle Fly District of Western Province, PNG, has a population of approximately 80 000 people. Of these, nearly half live in the greater Balimo region, including about 4400 people in the Balimo town area (National Statistical Office; https://www.nso.gov.pg/). Mostly subsistence gardeners, fisherman, and hunters, the people of this region are scattered along the Aramia River and its associated floodplain, with the majority of the people being part of the Gogodala language group. Air travel is expensive and road transport is extremely limited, so waterways are used extensively for transport of both goods and people between Balimo and outlying villages. Balimo has health facilities, although there is currently no physician at the hospital, and clinical services are provided by health extension officers and nursing staff.

Ethics statement

The study was conducted with the permission and support of the Middle Fly District Health Services and the Church Health Services, and at all times permission was obtained prior to sampling activities. Ethics approval was obtained from the PNG Medical Research Advisory Council and registered under the reference MRAC No. 17.02.

Sample collection

Passive case detection for TB is conducted at Balimo District Hospital, Western Province, and sputum samples are collected routinely for diagnostic purposes from patients with clinical signs of TB. Sputum samples are examined for AFB by light microscopy after Ziehl–Neelsen staining by a trained microscopist, and the slides are then stored in a dry area. AFB reporting is semi-quantitative, with positive AFB sputum quantified as 1+ to 3+ (1+: 0–1 AFB/100 fields; 2+: 1–9 AFB/10 fields; 3+: 1–9 AFB/field) (Anon, 1991).

The archived slides available for this study were prepared with sputum collected from June 2012 to March 2014, but exhaustive only from September 2012. The demographic (age, sex, location) and clinical data for the corresponding patients (including AFB results) were collected from the laboratory registers at Balimo District Hospital. One sputum sample was smeared on several slides, generally at least one prepared using direct/fresh sputum, and one prepared from decontaminated and concentrated sputum using the modified Petroff method (Petroff, 1915), with up to five slides per sputum sample. In addition to these duplicate slides from a single sputum sample, duplicates from different sputum samples collected at different time points (e.g., on the spot, on the following morning, or during treatment follow-up) were sometimes available for an individual patient (see Results section).
A second register, i.e., the hospital patient TB register, includes information only on patients placed on treatment. The suspected TB patients were classified as ‘treated’ versus ‘not treated’ by comparing the two registers, making the assumption that the patients who appeared in the laboratory register but not on the TB register were those who were not placed on treatment. TB register data were available only for the period from June 2013 onwards.

DNA extraction

All experiments were performed in biosafety level 2 laboratories. DNA was extracted from archived stained slides using a modified version of a previously described Chelex DNA extraction method (Van Der Zanden et al., 2003). Briefly, for the permanent slides (with a coverslip mounted), a preliminary step of 24 h in a xylene bath was included to remove the mounting medium. For slides without a coverslip, 80 μl of filtered Tris–ethylenediaminetetraacetic acid (EDTA) was added to the microscopic preparations and material was scraped off the slides. In the microcentrifuge tube containing the material, 150 μl of 10% Chelex suspension was added, and after thorough mixing, the samples were incubated at 95 °C in a water bath for 30 min. The samples were then sonicated for 5 min and centrifuged at 15 000 g for 15 min at 4 °C. The supernatant was transferred to a fresh microcentrifuge tube and used directly for PCR.

Detection of Mycobacterium species

The presence of mycobacteria in the extracted DNA was confirmed using a TaqMan real-time PCR following a published protocol (Broccolo et al., 2003) using the primers TAQM3 (5'-AGGCGAACCCTGCC-AG-3') and TAQM4 (5'-GATCCGGCA-3') and a probe of 30 bp (5'-FAM-TGTGGTGAGCA-GACCTACATGTGCAG-BHQ-1-3'). The expected amplicon was a 122-bp fragment selected within a central region of the IS6110 multicopy element sequence, highly conserved among Mycobacterium species. Because non-tuberculous mycobacteria (NTM) are rare in the Balimo region of PNG (see Discussion section), this assay is considered a diagnosis method for suspected TB.

All reactions were performed in a total volume of 20 μl containing 10 μl of GoTaq Probe qPCR Master Mix 1000 rxn (Promega), 1.6 μl of each primer (0.8 μM), 0.2 μl of the probe (0.1 μM), 4.6 μl of DNase-free water, and 2 μl of DNA template from the previous extraction from smear slides. Real-time PCRs were performed on a Rotor-Gene Q6000 (Qiagen, Hilden, Germany) using the following conditions: initial denaturation at 95 °C for 2 min, followed by 45 cycles of denaturation for 5 s at 95 °C and annealing/elongation for 15 s at 60 °C (Broccolo et al., 2003). All qPCRs were run with negative and positive controls (H37rv reference strain). Samples were considered to be specifically reacting with mycobacteria when the quantification cycle (Cq) value was ≤40. A duplicate qPCR was performed for samples with Cq >35. At the individual level, a patient was considered TB-positive when showing at least two positive results, either from the same slide (duplicate qPCR) or from different slides (but smeared from a single sputum sample).

Statistical analyses

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the AFB smear technique were calculated with 95% CI, assuming that qPCR is the gold standard. The kappa index was used to assess the degree of agreement between the two diagnosis techniques. The two smear methods (fresh sputum versus NaOH decontaminated and concentrated sputum) were compared to assess a possible correlation between the smear method and the qPCR results. For each smear method, a sample was considered to be specifically reacting when one qPCR result was clearly positive (Cq < 35) or at least two qPCR results were positive (Cq ≤ 40). Results per individual per smear method were compared with each other using the McNemar test. p-Values of < 0.05 were considered statistically significant. The statistical analyses were performed using Stata software version 14.2 (StataCorp, College Station, TX, USA).

Results

Characteristics of the presumptive TB patients

The collected smear slides originated from a total of 310 presumptive TB patients. For most patients, a single test result was available, either from the initial diagnosis or during treatment; however, 29 patients appeared repeatedly in the database, at different time points, leading to a total of 345 sputum samples. The age at collection date was available for 196 of the 310 patients; the others were reported as adult (n = 98), child (n = 3), or unknown (n = 13). The mean age of the patients for whom age data were available was 34.2 ± 2.1 years, ranging from 10 months to 70 years of age. More than half of the patients were aged 16–40 years (n = 115, 58.7%), and 31.6% (n = 62) were aged 41–60 years. Only a small proportion of the patients occurred at the extremes, i.e., below 16 years of age (n = 15, 7.7%) or above 60 years of age (n = 4, 2%). When taking into account the patients classified as child vs. adult, the proportion of young patients was even lower (n = 18, 6%). Females represented 47% of the study population (145/310). Among the 345 sputum samples collected, 268 were tested at initial diagnosis (77.7%), while 65 (18.8%) were tested during treatment follow-up. The remaining 3.5% were samples for which the status was unknown.

Each sputum smear was smeared on several slides, but only one microscopy result was available per sputum sample (i.e., per slide set) by laboratory registers. Microscopy results were available for 309 of the 345 sputum samples (89.6%). Among these, AFB were observed in 100 samples (32.4%), and positive AFB stains were quantified as follows: 45 AFB ·‘3’, 17 AFB ·‘2’, 22 AFB ·‘1’, and 13 ·‘scanty’; there were also three smear-positive samples that had two different microscopy results (2/3 or 3/1), probably from readings of duplicate slides.

Detection rate of Mycobacterium species by qPCR

In total, 1161 slides were available for testing, originating from the 345 sputum samples; 574 were prepared using direct/fresh sputum, while 587 were prepared using decontaminated and concentrated sputum. The diagnostic value of a TaqMan assay in the diagnosis of TB from archived sputum smears on slides was evaluated. Detailed results of the qPCR assay, including the Cq values, are available in the Supplementary Material Table S1. Overall, 35.4% (95% CI 32.7–38.2%) of the tested slides and 59.7% (95% CI 54.3–64.9%) of the tested sputum samples specifically reacted with mycobacteria based on the qPCR assay (Table 1) and were considered ‘suspected TB’. Poor agreement was found between the two TB diagnosis methods (AFB microscopy vs. qPCR, kappa = 0.443) (see Table 1 for details). Using the molecular method as the gold standard, the overall sensitivity and specificity of the AFB microscopy technique was 52% (CI 44.7–59.5%) and 98% (CI 94.2–99.8%), respectively, with a PPV of 98% (CI 93–99.8) and an NPV of 57% (CI 49.9–63.8%). Hence the poor agreement was predominantly due to smear negativity and qPCR positivity.

The AFB results (quantified as 1+, 2+ or 3+) were plotted against the quantity of DNA after extraction (indirectly provided by the Cq

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AFB, acid-fast bacillus; qPCR, real-time PCR.

* The results are provided at the patient level. Each patient was tested using up to five slides, generally including both fresh sputum and decontaminated and concentrated sputum. A sample was molecularly confirmed for the presence of mycobacteria (AFB-positive) when a quantification cycle value (Cq) of ≤40 was obtained for at least two qPCR replicates.

<table>
<thead>
<tr>
<th>Method</th>
<th>AFB-positive</th>
<th>AFB-negative</th>
<th>Unknown</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR-positive</td>
<td>98</td>
<td>90</td>
<td>18</td>
<td>206</td>
</tr>
<tr>
<td>qPCR-negative</td>
<td>2</td>
<td>119</td>
<td>18</td>
<td>139</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>209</td>
<td>36</td>
<td>345</td>
</tr>
</tbody>
</table>

AFB results and clinical decision

The laboratory register included 220 samples with a collection date starting from June 2013, allowing a comparison with the hospital TB register. Of these, 156 were initial diagnostic samples available to compare the laboratory diagnosis (AFB microscopy results) with the management decision (to treat or not to treat the patient). These 156 samples included 51 AFB-positive samples and 105 AFB-negative samples.

TB treatment was initiated in 90% (46/51) of the patients with AFB-positive samples. Twenty-three percent (24/105) of the patients whose samples were read as AFB-negative were also started on treatment as a result of empirical clinical evidence. Out of these patients, nine were reported as having PTB, 14 as having extrapulmonary TB (EPTB), and one as having both. Three patients were retreated after default from treatment (one PTB and two EPTB cases). The remaining 86 patients tested were not put on treatment, in accordance with an AFB-negative result (n = 81), or despite an AFB-positive result (n = 5). For these five patients, possible reasons were that they were mistakenly not registered in the TB register, or perhaps did not return to the hospital for the laboratory result after collection of the sputum specimen and were thus lost to follow-up.

Using molecular diagnosis as the standard, i.e. considering qPCR-positive samples as real positives and qPCR-negative samples as real negatives, the ‘clinical case assessment’ among the samples for which clinical information was available (n = 156) was analysed (Figure 2). Despite a negative microscopy result, 24 patients received treatment, of whom 11 were qPCR-positive (46% PPV). On the other hand, 81 AFB-negative patients received no treatment, of whom 38 were qPCR-negative (47% NPV). This left 43 patients who were not treated despite being qPCR-positive and 13 patients who were treated despite being qPCR-negative, thus potentially treated unnecessarily. Not taking into account the five cases that were probably lost to follow-up, overall 62.9% of the patients received an appropriate clinical assessment in relation to their ‘real’ TB status (i.e., qPCR result).

Discussion

Archived sputum smears on slides were analysed for the molecular detection of Mycobacterium species. The first aim was to confirm that DNA isolated from sputum smear microscopy slides can be used at the time of collection or retrospectively for TB diagnosis. Despite the small quantity of material harvested for a smear, the method proved to be effective for TB diagnosis in PNG, even a long time after collection. Based on the literature, stained smear microscopy slides could be a safe system for the transportation of sputum specimens from remote health centres to reference TB laboratories for further molecular TB or multidrug-resistant (MDR)-TB detection, and at no extra cost since smear slides are performed routinely (Rakotosamimanana et al., 2017). Archived slides could also be used to reassess the true burden of TB in areas or countries where the available information is not always dependable.

The sensitivity of the AFB microscopy technique for TB diagnosis in the Balimo region was also assessed. The genomic target IS6110 is one of the most utilized PCR targets of Mycobacterium tuberculosis, and is of high sensitivity, being a multicopy insertion element. The sensitivity of the assay had to be
high, due to the amount of material available from a smear. However, this target does not allow a specific identification of M. tuberculosis (Brocolo et al., 2003). The specificity of qPCR for the diagnosis of TB is a common issue, and even with the GeneXpert MTB/RIF assay, some misdiagnosis of TB has been observed in five NTM species at a high bacterial load (Pang et al., 2017). Because drug regimens for TB may differ from those for other mycobacterial infections, it is important that the species be identified correctly. Although tests have been developed for Mycobacterium species identification, they are very laboratory-intensive. In PNG, one study that tested 1182 smear-positive sputum samples from four different PNG provinces reported a single NTM (Aia et al., 2016), while a second study focusing on 225 sputum samples grown in cultures from three sites in PNG reported nine NTM (Ley et al., 2015). Despite the limitation of the present study method regarding differentiating M. tuberculosis from NTM, because NTM are rare in PNG, this method is reliable in identifying ‘suspected TB’ in the local context and in the absence of cultures.

The original total of 100 TB patients detected by AFB microscopy was doubled with the introduction of a molecular technique, confirming the low sensitivity of AFB microscopy used alone. However, because in Balimo the clinical decision to treat or not to treat a TB suspect also relies on clinical signs and symptoms, a further evaluation of the concordance between treatment and TB status was performed. The analysis showed that some of the AFB-negative/qPCR-positive TB cases were identified through the clinical evaluation and appropriately put on treatment. Overall, the clinical assessment, i.e., the decision to treat or not a patient based on available evidence, was appropriate for 63% of the TB suspects evaluated. The remainder were split into two categories: TB cases that were not treated and non-TB cases that were treated. As empirical treatment of TB is common in high-burden settings (Dheda et al., 2013), a high proportion of unnecessary treatment was expected, and as a result, a low number of untreated TB cases. In fact, the number of unnecessary treatment of false-negative TB cases was very low (8.6%), and could be even lower as some of these cases were clinically diagnosed as EPTB, which could explain their negative qPCR results. On the other hand, a greater number of AFB-negative patients who were retrospectively confirmed as TB cases by qPCR were not treated (28.5%), demonstrating the challenges in the management of TB in this setting. Although smear-positive patients are considered to be the most infectious, some smear-negative PTB may contribute to transmission of the disease at the population level (Behr et al., 1999; Hernandez-Garduno et al., 2004; Tostmann et al., 2008). The relative transmission rate from patients with smear-negative compared with smear-positive PTB is around 22% (Behr et al., 1999). When half of all cases of PTB are smear-negative (51.4% in the present study), the overall burden of infection caused by this group is substantial.

However, it should be noted that some of these apparently untreated smear-negative TB cases might in fact have initiated treatment without being registered in the hospital patient TB register. This could occur because people coming from remote areas may receive their 6 months of treatment all at once, and would be seen just once, thus increasing the chance that registration may be missed. Also, some cases might have been diagnosed and/or registered elsewhere, but have attended Balimo for laboratory investigation and/or collection of treatment, and these cases would not have been registered in the Balimo TB register.

TB is a major public health issue in developing countries where increases in MDR- and extensively drug-resistant (XDR)-TB strains have been reported (World Health Organization, 2016). A delay in timely diagnosis of TB (and drug-resistant TB) due to non-availability of rapid, more sensitive and specific techniques, is a major concern in these countries. The problem can be intensified for countries such as PNG where the burden of EPTB and smear-negative PTB is also high (Viney et al., 2015). Before TB can be treated, a diagnosis needs to be made in an efficient and timely manner, preferably at the point of care (POC); this first step is of paramount importance in TB prevention, treatment, and control.

The definition of POC implies the ability to make a diagnosis at the point where patient consultation and presentation occurs, as well as the ability to translate the result into same-day treatment, if appropriate (Dheda et al., 2013). A significant proportion of patients in high burden settings fail to return to collect their smear microscopy results (Millen et al., 2008), and it is therefore assumed that providing a rapid result and initiating treatment while the patient is still within the confines of the health care facility will translate into continued adherence and completion of treatment (Dheda et al., 2013). In the present study, however, only five AFB-
positive TB cases were not treated, presumably because they did not return to the hospital after the initial sample collection. This seems to indicate that most of the patients arriving in Balimo for a first diagnosis are not lost before a clinical decision is made. However, it is still possible that some TB cases will never reach the hospital because of the diverse constraints already discussed. A social science study conducted in the Balimo region reported that there was a significant "economic burden created by the need to be away from home" for diagnosis and treatment (Diefenbach-Elstob et al., 2017). Other studies have reported that 24% of TB direct costs occur prior to diagnosis in Burkina Faso (Laokri et al., 2013) or up to 43.7% in Nigeria (Ukwaja et al., 2013). Providing a POC diagnosis would lower the economic burden on patients from rural areas seeking medical diagnosis in Balimo.

This study identified only two AFB-positive patients ("2+") for whom sputum samples did not specifically react with mycobacteria, despite eight qPCR tests being run in total for each sample (four slides per sample, each tested twice). Possible explanations could be (1) the loss of DNA during the extraction process, (2) the presence of inhibitors in the sputum sample, or (3) a low copy number (or no copy) of the IS6110 target in the genome of the bacteria (Lira et al., 2013). Broccolo et al. recommended a multiplex approach with a second qPCR able to detect M. tuberculosis strains that lack the IS6110 multicopy elements (Broccolo et al., 2003). However, these strains appear to be very rare in the Balimo region of PNG (unpublished data), and the low sensitivity of a qPCR targeting a single-copy gene was considered insufficient to test DNA extracted from smear slides, i.e., in very low concentrations.

Fifty-one 'non-TB' patients, confirmed by two negative diagnostic results (AFB-negative/qPCR-negative), were also identified. Thirteen of them were treated based on a suggestive clinical presentation for TB, of whom nine were classified as having EPTB. For these nine EPTB patients, the negative laboratory results based on a sputum sample would be expected. By removing the nine patients from the group and considering only those with a clinical suspicion of PTB, the rate of appropriate clinical assessment would rise from 63% to 69% of the TB suspects evaluated. Children generally also have reduced pulmonary bacillary loads, but in the present data (out of the 24 treated AFB-negative patients), the only two below 16 years of age were diagnosed as EPTB, thus already taken into account before. The remaining four patients (out of 13 treated 'non-TB') clinically diagnosed as PTB could either be false-positive clinically diagnosed TB cases, thus unnecessarily treated, or TB cases that were missed by the two diagnostic techniques combined; the sensitivity of the qPCR technique is not 100%, especially when testing DNA extracted from smear slides. Among the non-treated AFB-negative/qPCR-negative patients, seven were children, and some additional ones may have had EPTB. Although less likely, some might have been PTB cases that were missed by the two laboratory techniques, as well as by the clinical case assessment. There is anecdotal clinical evidence of increased rates of TB at non-pulmonary sites in Balimo, with symptoms such as lymphadenopathy (Tanya Diefenbach-Elstob, unpublished data). Extrapulmonary specimens (pleural fluid, ascitic fluid, pericardial fluid) and pus specimens (lymph nodes) provide better results than sputum samples for the diagnosis of EPTB (Ullah et al., 2017). Despite this, in the present dataset, which only contained sputum specimens and not site-specific specimens for EPTB, seven people clinically diagnosed with EPTB had sputum specimens that were qPCR-positive, of which six were AFB-negative. Thus, the implementation of molecular techniques on sputum specimens in the Balimo region could improve both PTB and EPTB diagnosis. Other pathogens may cause pulmonary disease, which in countries with a high TB prevalence can cause a diagnostic dilemma. In some specific settings, the saprophytic Gram-negative bacterium Burkholderia pseudomallei, which causes melioidosis in humans, can be incorrectly diagnosed and treated as 'clinical TB' because of a similar clinical presentation. In the Balimo region, melioidosis is an important cause of pneumonia and sepsis. In a previous study conducted in the Balimo region in 1998, three positive cultures for B. pseudomallei were confirmed out of 170 patients sampled, i.e. with a calculated prevalence of 1.8% (Warner et al., 2007). Furthermore, seroprevalence studies in different age groups showed that a feature of melioidosis in this region is the childhood predilection (Diefenbach-Elstob et al., 2015; Warner et al., 2007). For this reason, melioidosis should be considered in the differential diagnosis of patients presenting with a febrile illness from this region, particularly in smear-negative patients and/or patients refractory to standard TB treatment, as suggested previously (Warner et al., 2010; Warner et al., 2007).

Other pulmonary infections are reported by the WHO as the highest causes of mortality in PNG (World Health Organization, 2015), i.e. pneumonia, bronchiolitis, bronchitis, and bronchiectasis. Causative pathogens have been determined as Streptococcus pneumoniae and Haemophilus influenzae type B in acute infections, and non-typeable (non-capsular) H. influenzae in chronic infections (Hare et al., 2010). Having a rapid and effective (molecular) diagnosis of TB and possibly also melioidosis would allow the exclusion of these two pulmonary pathogens, and potentially better recognition of other sources of pulmonary infection in the Balimo region.

In conclusion, in many regions of the world, particularly in developing countries like PNG, cultures are not used to confirm a clinical TB diagnosis in a suspected infected individual. For those regions of the world, the secondary analysis of Ziehl–Neelsen-stained slides for the diagnosis of suspected TB cases by molecular methods such as a simple real-time PCR may contribute to a better and faster diagnosis, especially where clinical diagnosis is equivocal. Retrospective analysis of archived smear slides could also help to reassess TB diagnosis among suspected cases in some countries.

In the future, prospective analysis of direct sputum by molecular methods, either using the qPCR technique presented herein or a GeneXpert System technology, would greatly improve the diagnosis and proper treatment of TB in PNG. While rapid and accurate diagnosis represents only one facet of TB control and cannot be seen in isolation, there is no doubt that improving case finding through enhanced diagnostic methods is the first step in the fight against TB in rural Balimo. This advocates for the implementation of PCR-based techniques to improve TB diagnosis in the rural Balimo region of PNG.

Ethics approval and consent to participate

This retrospective research was conducted on data collected for clinical purposes. All data used in the study were previously anonymized and at all times permission was obtained prior to sampling activities. The study was conducted with the permission and support of the Middle Fly District Health Services and the Church Health Services. Ethics approval was obtained from the PNG Medical Research Advisory Council and registered under the reference MRAC No. 17.02.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**Conflict of interest**

The authors declare that they have no competing interests.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.ijid.2017.12.004.

**References**


