EXPERT REVIEWS

Challenges and perspectives in the diagnosis of extrapulmonary tuberculosis

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Extrapulmonary tuberculosis (EPTB) accounts for a significant proportion of tuberculosis cases worldwide. Nevertheless, the diagnosis is often delayed or even missed due to insidious clinical presentation and poor performance of diagnostic tests. Culture, the classical gold standard for tuberculosis, suffers from increased technical and logistical constraints in EPTB cases. In this review the authors outline current diagnostic options for the main forms of EPTB. The authors also discuss the opportunities and challenges linked in particular to microbiological diagnostics and to the attempts to find a new gold standard test for EPTB. Finally, new biomarkers and tests currently under evaluation are hopefully on the way to introduce significant improvements in EPTB diagnosis, for which clinical suspicion will nevertheless be essential.

Keywords: circulating biomarkers • clinical diagnosis • extrapulmonary tuberculosis • gold standard • microbiological diagnosis • Xpert MTB/RIF

Tuberculosis (TB) represents one of the major challenges to global health. It is estimated that in 2012, 8.6 million new cases occurred worldwide and 1.3 million people died from TB (940.000 deaths among HIV-negative and 320,000 among HIV-positive people) [1]. The strategies implemented to address the global TB epidemic have to face a complex and multifaceted disease; the most challenging biological-clinical aspects of TB are the complex interplay between the status of latent TB infection (LTBI) and active disease, the association between TB and HIV-AIDS, the emergence of multidrug-resistant and extensively drug-resistant strains of *Mycobacterium tuberculosis* (MTB) and the lack of solid diagnostic tests and biomarkers for therapy monitoring and for good-quality drugs and vaccine trials [2–4]. In particular, the association between the HIV-AIDS and TB epidemics, starting in the last two decades of the last century, not only boosted the resurgence of TB at the global level, but also widened the spectrum of clinical presentations of the disease [5]. In AIDS patients, indeed, pulmonary TB (PTB) is aggressive, often with atypical features and extrapulmonary manifestations [1]. As defined in the WHO Global TB report 2013, extrapulmonary TB (EPTB) is a form of TB affecting any organ other than the lung parenchyma. In many cases, pulmonary and extrapulmonary lesions are both present in the same patient, who is nevertheless classified as PTB [1]. Overall, extrapulmonary TB accounts for a variable proportion of notified TB cases. In a European survey, 16,116 (22%) of 72,334 TB cases notified in the EU in 2011 had EPTB, with a wide variability range (4–48%) among the reporting countries [6]. Those recent data are consistent with other studies performed in Germany and USA, in which the proportion of TB cases was respectively 21.6% in the period 1996–2000 and 18.7% in the period 1993–2006 [7,8]. WHO reported that at a global level, among the 8.6 million people affected with TB for the first time in 2013, 0.8 million (9.3%) had EPTB [1]. The data gap mentioned above has to be attributed to substantial under-diagnosis and under-notification of EPTB cases worldwide. Hence, although historically neglected because of its scarce epidemiological impact on the disease transmission (with the notable exceptions of laryngeal TB and intrathoracic lymph node TB with bronchial fistulization), EPTB accounts for a significant proportion of TB cases. Moreover, EPTB is frequently diagnosed in PTB patients, carrying additional

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challenges as for disease burden, diagnostic workup and clinical management [9].

The focus of this review is on the diagnosis of EPTB: on current diagnostic practices, but also on the challenges and opportunities outlined by the forthcoming and future technologies.

EPTB: current diagnostic tools & practices

EPTB can potentially affect any organ in the body. In particular, there are districts where EPTB localizes more frequently: superficial and deep lymph nodes, pleura, bone and joints, CNS and abdomen (gastrointestinal [GI] and/or genitourinary [GU] TB). A small number of cases (0–3% in a European study) present with disseminated TB [6]. The relative proportion of cases in whom EPTB affects each organ or system varies widely according to age, gender, ethnicity and geographic basin of the study population and the presence of predisposing factors (HIV infection, immunosuppressive therapies, high-prevalence countries, previous history of TB, etc.). In general, lymph node and pleural TB are the commonest forms of EPTB [6,10,11].

As per WHO definition of EPTB, diagnosis of EPTB should be made on the basis of:

- One culture-positive specimen, OR;
- Positive histology OR;
- Strong clinical evidence consistent with active EPTB [1].

EPTB may present itself with an extremely wide spectrum of signs and symptoms depending on the organ affected, the aggressiveness of the disease and the immune response of the host. In many cases, the diagnosis is made with a significant delay from the time of presentation simply because EPTB is lately put into the differential diagnosis panel. This often implies that diagnostic tests are performed too late, and in some cases even after the empirical course of antibiotic treatment has failed. Furthermore, even when the clinical suspect of EPTB is correctly formulated, diagnosis is not straightforward: clinical findings suffer from lack of specificity, and the results of tests assessing TB infection (tuberculin skin test [TST] or IFN-γ release assays [IGRAs]) may be misleading [12]. Moreover, EPTB is often pauci-bacillary, and the sites of infection may not be easily accessible for the collection of specimens suitable for microscopy, histology, culture or molecular tests [11,13].

To date, no form of EPTB can count on reliable single rule-out tests (i.e., test with minimal or absent false-negative results). The diagnosis of EPTB is thus often made by the integration of several non-specific clues from various investigations. In the next paragraphs we overview, for each of the main types of EPTB, hints of pathogenesis, epidemiology and clinical presentation as clues to a correct diagnostic hypothesis. Table 1 depicts current EPTB diagnostic tools and practices. It is useful to remind that EPTB diagnosis needs the exclusion of concomitant PTB.

Lymph node TB

Lymph node TB is the most common form of EPTB at a worldwide level, accounting for approximately 35% of cases [14]. Although observed in people from all age groups, the greater proportion of lymph node TB is seen in patients under 14 years; additional predisposing factors are HIV infection, female gender and Asian ethnicity [10,14]. The commonest clinical presentation is cervical lymphadenitis (60–90% of lymph node TB cases), which is historically referred to as *scrofula* (from the Latin, 'glandular swelling'); other common sites of TB involvement are mediastinal, axillary, mesenteric, perihepatic and inguinal lymph nodes. The localization of TB to the lymphatic system reflects a systemic involvement of TB infection, since MTB bacilli follow the lymphatic drainage routes from a primary complex (most commonly located in the lung) to the systemic lymphatic circulation [15]. The common clinical picture is made of unilateral single or multiple painless masses developing over weeks to months, whose typical location is the posterior cervical/supraclavicular region, with a classic triad of multiplicity, matting and caseation. Systemic symptoms like low-grade fever, weight loss, fatigue and night sweats may or may not be present, and medical history is positive for TB contacts in 22% of cases and for TB infection in 16% [14]. Clinical criteria for the classification of TB lymphadenitis were described by Jones and Campbell [16,17]. The main complications are fistulization and rupture, compression of adjacent structures, secondary bacterial infection and local extension of TB infection to the skin (*scrofuloderma*) or to other organs. Lymph node enlargement with compressive symptoms can be dangerous and even life-threatening especially when paratracheal, mediastinal or hilar hepatic lymph nodes are involved. The differential diagnosis is wide and includes infections, neoplasms, non-specific reactive lymph node hyperplasia, sarcoidosis and connective tissue diseases. Among infectious differential diagnoses, it is important, in particular in childhood, to differentiate MTB from non-tubercular mycobacterial (NTM) lymphadenitis [14]. The diagnostic panel for lymph node TB is shown in Table 1.

The mainstay of lymph node TB treatment is the prompt initiation of the appropriate anti-TB treatment. Surgery is seldom indicated in drug-susceptible TB lymphadenitis due to the prompt response to appropriate chemotherapy (whereas NTM infection response is usually mild); the usefulness of corticosteroids is controversial [11,14].

Pleural TB

Pleural TB accounts for a significant proportion of EPTB cases. In an Indian study, pleural TB has been reported as the commonest form of EPTB, especially in the over 65 years' age group; in a European epidemiological survey, pleural TB is the second form of EPTB [6,10]. It has also been reported that in high-incidence countries, 20–25% of PTB patients have TB pleural effusion, and that TB is the leading cause of pleural effusion in some areas of the world [18]. HIV-infected individuals, unlike patients with immune suppression from other causes, have an increased risk of developing the disease. TB pleurisy can occur either as a sequel of primary infection or as a manifestation of TB reactivation, even in the absence of patent PTB. The disease is thought to follow the rupture of a

pulmonary caseous focus into the sub-pleural space: the presence of mycobacterial antigens into the pleural space induces a delayed hypersensitivity reaction with lymphocytic pleuritis and lymphocyte-rich exudative fluid production [19]. The clinical picture is often an acute febrile illness whose hallmarks are cough, pleural chest pain and dyspnea. Diagnostic tools are listed in Table 1; it is noteworthy that, due to its specific pathogenesis, culture and microscopy of pleural fluid are of poor diagnostic value, because they are intended to find MTB in a fluid in which those organisms are present only in scarce quantity [20]. Hence, in a patient with a lymphocytic pleural effusion, the easiest way to diagnose TB is by dosing adenosine deaminase (ADA) level, whose levels above 40 U/l are highly suggestive for TB pleurisy [19]. Pleural TB generally resolves spontaneously without treatment; nevertheless, the patient at this point is at high risk of developing a recurrence of TB, which has historically been reported in 45–65% of cases [19]. Other rare complications include bronchopleural fistulas, empyema and fibrothorax. TB pleurisy responds well to standard anti-TB therapy, with resorption of pleural fluid in 1–3 months.

CNS tuberculosis

CNS involvement is observed in a minority of TB cases; among the different forms of CNS TB, TB meningitis (TBM) is the commonest. Nevertheless, TBM is the most severe form of TB in terms of morbidity and mortality and represents a medical emergency [11,21,22]. TBM pathogenesis resides in the inflammation of the meninges following the presence of MTB into the subarachnoid space after the rupture of a sub-ependymal tubercle. If not recognized and treated, TBM is almost always fatal. The clinical findings of cranial nerve palsies, focal neurologic deficits, impairment of consciousness and seizures are all direct or indirect consequences of the CNS inflammatory state, leading to hydrocephalus, cerebral vasculitis and infarctions, inflammatory encasement of nerves and direct damage to the cerebral parenchyma [21]. The delay in appropriate anti-TB therapy is a poor prognostic factor in TBM. Table 1 depicts the landscape of current first-level and confirmatory tests useful in TBM diagnosis; it is crucial to suspect the disease on a clinical basis (history, symptoms and signs, first-line blood tests, CSF findings, imaging of brain and thorax) in order to promptly introduce appropriate empiric therapy. Indeed, anti-TB four-drug regimen must not be delayed while waiting for microbiological or molecular diagnostic information [22]. The complete course of treatment of drug-susceptible TBM lasts at least 12 months and includes HRZE (isoniazid, rifampicin, pyrazinamide, ethambutol) for 2 months followed by HR (isoniazid, rifampicin) for 10 months. Adjunctive corticosteroids (dexamethasone or prednisolone) are recommended in all patients regardless of the disease severity at presentation. Hydrocephalus and TB cerebral abscesses are indications for neurosurgical referral [22].

Bone & joint TB

It has been calculated that 1–3% of patients suffering from TB have involvement of the skeletal system. Vertebral TB is the most common form of bone TB, accounting for approximately 50% of the cases [23]. Spinal TB, also named Pott's disease, affects mainly the thoracic spine and the thoracolumbar junction [24]. It occurs via hematogenous dissemination of MTB to the vertebral bodies as a result of bacillemia; the infection then spreads to the adjacent discs and vertebral bodies under the longitudinal ligaments [23]. Clinical presentation of spine TB can vary, the commonest symptom being back pain, not always accompanied by general symptoms (fever, fatigue, weight loss, night sweats). The pain typical of spine TB is not as intense as occurs in pyogenic spinal infections and is often localized to the affected area. Local pain, swelling and functional limitation of joint movements may precede radiological changes by 4–8 weeks [24]. The main complications of spine TB are structural damages to the axial skeleton producing deformities and compression on nerves and spinal cord. Affected bone can fracture and produce spinal cord compressions, and untreated bone TB can spread to the adjacent soft tissues and into the epidural space, producing neurological symptoms as well, by compression or even dissemination to the CNS [25]. Moreover, a vertebral abscess can spread along the psoas muscle causing retroperitoneal masses sometimes progressing into cutaneous inguinal fistulas. Diagnosis of Pott's disease is based on clinical and radiological clues, and on histological/microbiological confirmation (Table 1). As in any form of TB, the isolation of MTB from clinical samples is useful both for confirmation of clinical-radiological diagnosis and for determination of drug susceptibility. Due to the insidious clinical features of spine TB, diagnostic delay is often prolonged (mean 6.5 months from presentation, with a time range from 3 to 12 months) [24]. Late diagnosis impacts on the overall prognosis: morphological and functional changes in the skeletal system, once established, are often irreversible even if appropriate anti-TB therapy is introduced, resulting in various degrees of disability. Surgery is not indicated as first-line treatment unless severe spinal instability or progressive neurological symptoms occur, with evidence of cord compression or deformation. In skeletal TB with structural damage, reparative-reconstructive surgery may be needed after chemotherapy [25]. A third indication for surgery is to obtain a bioptic sample when microbiological/histological confirmation of diagnosis is needed, and less invasive methods (needle aspiration/biopsy) are not feasible or proved unsatisfactory [24].

Genitourinary TB

GU TB is another common form of EPTB. It includes a variety of clinical conditions, from single-organ TB localization to complex GU conditions producing renal failure, infertility or chronic pelvic pain. GU TB is almost always secondary to the spread of TB localized elsewhere in the body: MTB bacilli reach, mainly via hematogenous spread, the kidney, the epididymis or the female genital organs. From the kidneys, the spread towards the GU system is via continuous structures (calyx, renal basin, ureter, bladder, urethra, reproductive organs, sometimes contralateral kidney), while from the epididymis or

Table 1. Current diagnostic tools and practices for extrapulmonary tuberculosis.

	History	Clinical findings	Imaging
Lymphatic TB	History of TB or active TB, or history of TB contacts Cervical swelling: unilateral single/multiple painless slow growing mass ± SCS ± symptoms due to compression to adjacent structures	Enlarged lymph nodes, fixed to surrounding tissue, ± central softening due to colliquation, ± sinus tract formation (classification by Jones and Campbell)	CXR: present/past PTB US: single/multiple hypoechoic cystic lesions with thick capsule CT: conglomerated nodal masses with central lucency, thick irregular rim of contrast enhancement, peri-lesional signs of inflammation MR: discrete, matted and confluent masses ± necrotic foci + soft tissue edema 18F-FDG PET: unable to discriminate TB from malignancy, possible use in response monitoring to rx
Pleural TB	History of TB or active TB Young (primary) or elderly Pts (reactivation) HIV+ve Acute febrile illness with cough, pleuritic chest pain, dyspnea Less commonly, subacute presentation	Unilateral pleural effusion Pleural thickening	CXR: small-moderate unilateral pleural effusion CT: pleural thickening
CNS TB (TBM)	History of TB or active TB, or PTB contacts Headache, fever, anorexia, vomiting, photophobia, blurred consciousness Hyperacute presentation can occur, or subacute ones mimicking dementia	Stiff neck Confusion/coma Focal neurological signs (any, mostly cranial n. palsy, hemiplegia) Seizures (up to 50% of children)	CXR: present/past PTB CT head with contrast: hydrocephalus, basal contrast enhancing, infarction MRI: high definition on infra-tentorial lesions and early TBM changes. False + findings for other CNS diseases ± spectroscopy
Bone and joint TB	History of TB or active TB Elderly Pts (in low-prevalence countries) (Back) pain (localized) + functional limitation	± local swelling	CXR: present/past PTB Spinal XR: disc narrowing, less definite paradiscal vertebral margins (after 3–6 months) CT: patterns of bone destruction (fragmentary, osteolytic, subperiosteal and lytic with sclerotic margins) ± soft tissue/epidural TB involvement MR: ↓ T1, ↑ T2. Patterns: Para-discal, anterior, central ± soft tissue/epidural TB involvement, detection of spinal cord damage 67Ga-citrate SPECT: as sensitive as bone scintigraphy for bone infection, complementary to CT

>: Presentation in the majority of cases; ADA: Adenosine deaminase; C: Culture; CSF: Cerebrospinal fluid; CT: Computed tomography; CXR: Chest x-ray; DST: Drug symptoms (fever – usually low-grade, night sweats, weight loss, feeling unwell); ss: Sensitivity; sp: Specificity; TB: Tuberculosis; TBM: Tuberculous meningitis; TST: Tuberculin Data taken from [11,14,19,22,24,25,30,103–110].

Microbiology: microscopy and culture	Microbiology: molecular methods	Clinical chemistry/ cytology/histology	Other methods	Gold standard
M on material from draining sinus or FNA specimen. ss 88%, sp 96% C of material from draining sinus, FNA specimen or biopsy	PCR on material from draining sinus, FNA specimen or biopsy	Lymph node biopsy: Langerhans giant cells, caseating necrosis, granulomatous inflammation ± calcification Immunocytochemistry	TST: identifies mycobacterial infection, not useful for disease	C, histology
M/C of pleural fluid: poor performance unless tuberculous empyema. M/C of pleural biopsy specimens or induced sputum: better results	PCR for MTB on biopsy specimens or induced sputum or pleural fluid	Pleural fluid: exudative (proteins >5 g/dl, high LDH levels), lymphocyte predominance >50%, scarce mesothelial cells Pleural biopsy specimens histology + M + C à ss >90%	ADA in pleural fluid: if >40 U/l in lymphocytic pleural effusion à suggestive for TB pleurisy if <40 U/l à not likely TB Lysozyme in pleural fluid: under investigation IFN-γ in pleural fluid: under investigation	C, histology on pleural specimans (NOT on pleural fluid)
M: useful for rapid diagnosis on concentrated CSF specimens C of concentrated CSF specimens for confirmation and DST	PCR on CSF: confirmatory test (cannot rule out TBM)	CSF: leukocytosis (>lymphocytes), ↑ proteins, CSF/plasma glucose ratio <50%	ADA on CSF: not recommended for routine use IGRA: does not distinguish TB infection from TB disease	C
M/C on lesion biopsies/ needle aspirate specimens	PCR on biopsy/needle aspirate specimens	Biopsies (needle or open): necrotizing epithelioid cell granulomas		C, histology

susceptibility testing; FNA: Fine-needle aspiration; GI: Gastrointestinal; GU: Genito-urinary; LN: Lymph node; M: Microscopy; Pts: Patients; SCS: Systemic constitutional skin test; US: Ultrasonographic imaging study.

Table 1. Current diagnostic tools and practices for extrapulmonary tuberculosis (cont.).

	History	Clinical findings	Imaging
GU (kidney) TB	History of TB or active TB Male, HIV+, hemodialysis, end-stage renal failure Asymptomatic/irritative urinary symptoms	Urine analysis: hematuria, proteinuria, 'sterile' pyuria (= no bacterial growth at std cultures) Renal failure (late stages)	CXR: present/past PTB x-ray: calcification Intravenous urography: (early): changes in single calyx (late):calyces distortion, multiple ureter strictures, bladder fibrosis CT: calcifications; strictures, thickening, fibrosis and ulceration of the urinary tract and bladder
GU (male genital) TB	History of TB or active TB Scrotal/epididymal mass ± scrotal fistula discharging watery odorless pus		CXR: present/past PTB US: enlarged, hypoechoic epididymis Vasography if obstructive infertility
GU (female genital) TB	History of TB or active TB Asymptomatic or Infertility, menses irregularity, abdominal pain, pelvic inflammatory disease ± specific S/S depending on the site of involvement (see text for details) Postmenopausal women with bleeding, persistent leucorrhoea, pyometra		CXR: present/past PTB Hysterosalpingogram: tubal occlusion, constrictions, scarring (not to be performed if TB diagnosed by other means)
GI TB	History of TB or active TB Variable presence of chronic abdominal pain/discomfort, weight loss, fever, night sweats, loss of appetite, change in bowel habits, nausea/vomiting	± abdominal mass(es) ± ascites	CXR: present/past PTB US: asymmetric thickening of the bowel wall (especially ileocecal region) + abdomen lymphadenopathy ± ascites CT: asymmetric thickening of the bowel wall (esp. ileocecal region) + abdomen lymphadenopathy ± ascites. Better than US for LN calcifications

>: Presentation in the majority of cases; ADA: Adenosine deaminase; C: Culture; CSF: Cerebrospinal fluid; CT: Computed tomography; CXR: Chest x-ray; DST: Drug symptoms (fever – usually low-grade, night sweats, weight loss, feeling unwell); ss: Sensitivity; sp: Specificity; TB: Tuberculosis; TBM: Tuberculous meningitis; TST: Tuberculin
Data taken from [11,14,19,22,24,25,30,103–110].

female reproductive organs the spread occurs both per *contiguity* (testis vs peritoneum and pelvic structures) and *continuity* routes (vas deferens, ejaculatory ducts, seminal vesicles, prostate vs fallopian tubes, uterus, vagina). In both males and females, rare cases have been described, in which ascending spread from external genital organs took place [26,27].

At a worldwide level, GU TB affects patients of any age, with a peak incidence in women in child-bearing age (20–40 years old) being responsible for extensive morbidity especially when not promptly diagnosed and treated. Risk factors for GU TB are male gender (prevalence twice as women), HIV infection, hemodialysis and end-stage renal failure. Subjects recipients of renal transplant are at increased risk of any form of TB, of which 7–15% is represented by GU TB [26].

Kidneys are the main route of entry of MTB into the GU tract and the most common site of GU TB. Renal involvement can be completely asymptomatic; if symptoms develop, those are mainly irritative voiding symptoms (urinary frequency, urgency, dysuria and nocturia) and hematuria. Other complaints can be abdominal, flank or urethral pain. At urine analysis, the classic triad is hematuria, proteinuria and 'sterile pyuria', which means no bacterial growth in standard urine cultures. Almost 5.7% of untreated patients eventually develop end-stage renal failure [28].

Male genital tract TB can present either as insidious condition with minimal pain or as acute epididymitis or epididymo-orchitis. If undiagnosed and untreated, the condition may evolve towards caseation and possibly creation of a fistula. The characteristic thin, watery and odorless pus coming from the sinus tract is useful for diagnostic exams. In addition, genital tract TB can evolve to chronic tubercular prostatitis and/or extensive scarring (epididymis, ejaculatory ducts, seminal vesicles), resulting in male infertility [29].

75% of women with GU TB are aged between 20 and 45. The disease can be asymptomatic or present itself with general and local symptoms and signs. General symptoms are

Microbiology: microscopy and culture	Microbiology: molecular methods	Clinical chemistry/ cytology/histology	Other methods	Gold standard
C of three early morning urine samples M/C of samples taken from the site of suspected TB	PCR for MTB on urinary and bioptic specimens	Kidneys: necrotizing epithelioid cell granulomas. Early: small bilateral cortical Late: granulomatous destruction of renal parenchyma Rarely interstitial nephritis Ureter, bladder: strictures, fibrosis		C (urine, tissue sample)
M/C of samples taken from the site of suspected TB/on purulent discharge	PCR of specimens from the site of suspected TB	Necrotizing epithelioid cell granulomas		C (tissue sample)
M/C of samples taken from the site of suspected TB/on purulent discharge	PCR of specimens from the site of suspected TB	Necrotizing epithelioid cell granulomas		C (tissue sample)
M/C on peritoneal fluid: poor performance, better if performed on concentrated specimens C on mucosal biopsies positive in a minority of cases (33%)	PCR on peritoneal fluid (for peritoneal disease) PCR on bioptic samples useful for discriminating between Crohn's disease and TB PCR on fecal samples: under evaluation	Peritoneal fluid: exudative Biopsies: caseous necrosis and AFBs pathognomonic. Suggestive of TB: confluent large granulomas and/or ulcers lined by epithelioid hystiocytes, submucosal inflammation	ADA in peritoneal fluid ss 93% sp 96% (for TB peritonitis, see text for details) Endoscopy (all patients with suspected GI TB) > segmental lesions, rare pancolitis. Perform biopsies	Clinical-microbiological/ hystopathology

susceptibility testing; FNA: Fine-needle aspiration; GI: Gastrointestinal; GU: Genito-urinary; LN: Lymph node; M: Microscopy; Pts: Patients; SCS: Systemic constitutional skin test; US: Ultrasonographic imaging study.

non-specific: fever, night sweats, weight loss and feeling unwell; the symptoms related to the genital tract can be non-specific (acute pelvic inflammatory disease determining Fitz-Hugh-Curtis syndrome) or specific depending on the site of involvement, and may imply various differential diagnosis scenarios. Lesions involving the cervix often present with post-coital bleeding and resemble neoplastic masses at the examination; vulvar and vaginal TB ulcers may be painful especially for secondary bacterial infections, and may mimic sexually transmitted infections because of the bloodstained purulent discharge. TB of the Bartholin's glands presents as a local infection typically resistant to common empiric antibiotic treatments. Involvement of the ovaries may result in tubo-ovaric abscesses and adnexal masses mimicking ovarian cancer, mostly in presence of peritoneal TB involvement and when levels of CA-125 marker are raised (see below the paragraph on peritoneal TB). Besides the clinical presentation, the main complication of asymptomatic and symptomatic disease is infertility. Indeed, it has been calculated that most women are diagnosed GU TB during investigations for primary or secondary infertility. Even after complete anti-TB treatment for genital TB, the conception rate and the live-birth rate are unsatisfactory (19.2 and 7.2%, respectively) [27].

As all forms of EPTB, also GUTB requires a high index of suspicion to be diagnosed, and current diagnostic tools are outlined in Table 1. The gold standard is the microbiological confirmation of MTBs in samples from the affected sites. Urine or pus culture can prove useful in some cases, but in some others more invasive procedures (up to diagnostic laparoscopy) are needed [27].

GI & peritoneal TB

Even if less common than other forms of EPTB, GI TB nevertheless represents an important portion of EPTB cases worldwide [30]. GI involvement by TB disease can occur either in

primary or in post-primary disease: (i) by swallowing of infected sputum in PTB patients, (ii) by ingestion of contaminated food (especially unpasteurized milk or dairy products), (iii) by lymphatic or (iv) hematogenous spread and, rarely, (v) by spread from adjacent organs (e.g., fallopian tubes). Thus, the disease can affect any part of the digestive tract from the esophagus to the rectum, but the characteristic site of involvement is the ileocecal area, which is rich in lymphoid tissue and which is a region of physiologic stasis of the bowel content [30]. Women have been reported to be more frequently affected than men. Clinical findings are often non-specific with variable presence of chronic abdominal pain, weight loss, fever, night sweats, loss of appetite, change in bowel habits, nausea/vomiting, abdominal mass(es) and ascites [31,32]. Complications include obstruction, malabsorption, intestinal hemorrhage, perforation and fistulization [30]. According to the protean clinical manifestations, the differential diagnosis is wide. Distinguishing GI TB from Crohn's disease is often challenging due to common clinical presentation and radiological, pathological and endoscopic features [33]. Other conditions often in differential diagnosis are solid and lymphoid malignancies, *Yersinia enterocolitica* infections, amebiasis, histoplasmosis and fungal infections. Diagnostic tools are analyzed in Table 1. As any other form of TB, the recommended therapy for GI TB is combined anti-TB therapy, usually with the schema 2HRZE+4HR. Given the inadequacy of current diagnostic test in the confirmation/exclusion of GI TB, a full course of empiric anti-TB therapy is recommended when suggestive findings are present and the diagnosis cannot be ruled out [30]. Surgery is reserved for acute complications or for complications refractory to medical therapy.

Peritoneal TB is an uncommon form of EPTB resulting often from the re-activation of latent peritoneal TB foci. Three forms have been described: 'wet' type with ascites, 'dry' type with adhesions and fibrotic type with omental thickening and loculated ascites [34]. Classical risk factors include HIV infection, cirrhosis and continuous ambulatory peritoneal dialysis; additional risk factors are diabetes mellitus, underlying malignancy and therapy with anti-TNF agents [35]. Clinical presentation is insidious with variable presence of abdominal pain, fever and ascites. Classically, the ascitic fluid is exudative, with a serum-ascites albumin gradient lower than 1.1 g/dl and leukocyte count variable from 150 to 4000/mm^3 with a lymphocytic predominance, although neutrophylic pleocytosis can be seen in cases undergoing peritoneal dialysis [11]. On diagnostic laboratory tests, findings are highly non-specific with elevated erythrocyte sedimentation rate, c-reactive protein, anemia, lymphopenia or lympohcytosis. A potential diagnostic trap comes from the finding of raised levels of CA-125 marker in some patients with peritoneal TB: in female subjects with peritoneal TB nodules, this finding can mislead the diagnosis supporting the hypothesis of abdominal spread of an ovarian neoplasm [36,37]. As for laboratory investigations potentially useful in discriminating abdominal TB from other conditions, it has been reported that ADA levels in TB ascitic fluid are characteristically elevated, with a pooled sensitivity and specificity of respectively 93 and 96% in a recent meta-analysis [38]. Yields of microscopy and culture are enhanced by centrifugation of 1 L of peritoneal fluid; in doubtful cases, laparoscopy is the diagnostic procedure of choice [11,31,35].

Microbiological diagnostics for EPTB

A deep analysis of clinical, radiological and histopathological diagnostic tools used in EPTB diagnosis goes beyond the scope of this review. In this section, we focus on the microbiological aspects of the diagnostic process, with particular attention to recent tools like IGRAs and molecular methods, including nuclear acid amplification technologies (NAATs). Finally, we provide an overview of the diagnostic tools in development or under evaluation, which are hopefully going to introduce substantial improvements in the diagnosis of EPTB in the short to medium term.

Microscopy

Direct visualization of acid-fast bacteria (AFB) is still the first microbiological test to be performed when samples from a site of suspected TB (either PTB or EPTB) are collected. Light-emitting diode (LED) technology has been demonstrated to be a cheaper and viable alternative to traditional Ziehl–Neelsen microscopy and to fluorescence microscopy based on mercury-vapor or halogen lamps. LEDs, with low power consumption, are able to excite auramine-rhodamine stain without the need of UV lamps, producing high-quality images even outside dark rooms [39]. For these reasons, in 2007 WHO issued policies for the widespread roll-out of this technology [40,41]. Fluorescence microscopy proved to be more sensitive than conventional Zihel–Neelsen microscopy [41]. Moreover, the examination of fluorochrome-stained smears proved to take less time than ZN-stained ones, after appropriate training [41].

The main limitation of smear microscopy is sensitivity [42]. As for EPTB, Tortoli *et al.* reported a 48% sensitivity of microscopy compared with culture as reference standard [43]. The sensitivity hurdle is relevant both at a biological and at a technical level. At a biological level, sensitivity of microscopy is further lowered by the pauci-bacillary nature of EPTB; at a technical level, in certain forms of EPTB, the collection of samples suitable for microscopy is not straightforward because of the limited anatomic accessibility of the site of infection or the risks associated with the sampling procedure (e.g., lumbar puncture, biopsy of deep lymph nodes, etc.). In some cases, tricks like concentration of large volumes of sampled fluid (CSF, ascites, etc.) and repeated analyses can increase the diagnostic yield [22]. The empiric antibiotic therapies often administered for non-specific symptoms constitute another technical encumbrance that undermines the diagnosis of EPTB in many cases. Microscopy, as also culture, may be indeed affected by the rapid mycobacterial killing operated by some antibiotic agents like fluoroquinolones, resulting in false-negative results [44].

Culture

Liquid culture is the mainstay for the diagnosis of EP mycobacterial disease [45]. In 2007, WHO endorsed the phased-manner implementation of automated liquid culture systems in low- and middle-income countries because of the advantages in terms of increased sensitivity and reduced delays over solid media culture [46]. Solid cultures are most commonly performed on the egg-based Löwenstein–Jensen medium, whereas the most commonly used commercially available automated liquid culture system is the BACTEC MGIT 960 based on modified Middlebrook 7H9 Broth with an oxygen-sensitive fluorescent detection technology [47]. The advantages of liquid culture over other diagnostic tools are its sensitivity, the possibility to identify the *Mycobacterium* species and to perform phenotypic drug-susceptibility tests (DSTs) as well as genotyping for molecular epidemiology studies. The main drawback of both solid and liquid culture is the time needed for mycobacteria to grow. Indeed, liquid cultures require at least 9–10 days for positive results and 6 weeks for being considered negative; in Löwenstein–Jensen cultures, the minimum time-to-positivity is even longer, up to 4–8 weeks. When DSTs are required, mean turnaround times are 1–3 weeks for liquid culture and 3–4 weeks for solid culture [48]. Such long time-to-results may not be acceptable in the management of aggressive TB cases like TBM or HIV-associated TB [22,46,49]. A peculiar challenge in EPTB is the low mycobacterial load of some EPTB lesions. In addition, pre-analytical aspects have to be considered: samples from biopsy or fine-needle aspiration may be submitted for analyses in incorrect media (e.g., formalin) or insufficient volume, undermining the possibility to perform culture tests [42]. In other cases, the sampling procedure is not directed to the proper target, as is in case of TB pleurisy, when culture of pleural fluid is often requested in place of culture/histology of pleural biopsies which are much more sensitive to reveal MTBs [19]. The detrimental effect of empiric broad-range antibiotic therapies for aspecific symptoms has already been discussed in microscopy paragraph.

IFN-γ release assays

IGRAs are *in vitro* immunodiagnostic based on the assessment of the IFN-γ released after stimulation of sensitized T-cells by highly MTB-specific antigens, including early secreted antigenic target 6 and culture filtrate protein 10, both encoded within the region of difference 1, a portion of the MTB genome that is absent in BCG strain and most of NTM species [50,51]. There are two commercially available IGRAs: QuantiFERON-TB Gold In Tube assay (QIAGEN corp., Hilden, Germany), which utilizes an ELISA technique to measure the amount of IFN-γ secreted, and the T.SPOT-TB (Oxford Immunotec, Abingdon, UK), which uses an ELISpot assay to quantify the number of IFN-γ-producing cells.

IGRAs cannot distinguish between latent infection and active TB; therefore, they are not suited to diagnose active TB. According to the European CDC, they may however have an ancillary role in supporting the diagnosis of particular forms of TB difficult to recognize, among which EPTB [52]. In this ancillary role IGRAs maintain their advantages over the TST: they are not influenced by BCG vaccination and present higher sensitivity for detecting LTBI status [53].

Recently, several studies have focused on testing IGRAs on specific body fluids and on site-specific leukocytes. A small multicentre study conducted using T-SPOT.TB revealed 95% sensitivity and 76% specificity for active pleural TB when mononuclear cells from pleural effusions were directly tested [54]. In a study aiming to diagnose TBM, the ELISpot performed both on cerebrospinal mononuclear cells and peripheral blood lymphocytes revealed a sensitivity of 94% [55].

Such studies lead to hypothesize that mononuclear cells are compartmentalized at the site of infection, where they show higher early secreted antigenic target 6-specific IFN-γ response in comparison with peripheral mononuclear cells. Once confirmed, such studies could have important implications for the diagnosis of EPTB [56].

Nucleic-acid amplification tests

In the last 20 years, the amplification methods have been increasingly used to diagnose TB directly on clinical specimens. Most of them are intended by producers for testing respiratory specimens only and repeatedly the CDC asserted that their use on extrapulmonary samples is not supported by sufficient evidence [57]. A number of papers have however been published and among them three on large number of specimens [43,58,59], reporting satisfactory results in particular with lymph nodes, tissues and CSFs. One of them (Tortoli *et al.* [2012]) was carried out using the Xpert MTB/RIF system, a fully automated platform which had produced excellent sensitivity and specificity in diagnosing PTB [60–62].

Very recently, the WHO reviewed about 20 relevant studies concerning the use of the Xpert system for the diagnosis of EPTB. Excellent sensitivity and specificity emerged from pooled data on CSFs, gastric fluids and biopsies: for lymph nodes sensitivity was 84.9% and specificity 92.5%, for gastric fluid 83.8 and 98.1% respectively, for tissue 81.2 and 98.1%, respectively, and for CSF 79.5 and 98.6%, respectively. Not satisfactory was in contrast the use of the Xpert system on pleural fluids to diagnose TB pleurisy [63].

This publication certainly represents an important update to the previous policy guidance [64], but due to heterogeneity of published studies and the poor strength of available data, it only recommends the use of the assay as the first line on CSF specimens from patients presumed to have TBM (strong recommendation) and as a replacement test for usual practice on specific non-respiratory specimens (lymph nodes and other tissues) from patients presumed to have EPTB (conditional recommendation) [65].

Although Xpert MTB/RIF certainly represents an important advance in PTB and EPTB diagnosis, structural challenges and logistical hindrances prevent it from being the ultimate test for TB, and a true point-of-care (POC) test [13,66]. Among such challenges are its dependence on electric power to run, the

Table 2. Major drawbacks of current microbiological diagnostic methods for extrapulmonary tuberculosis, leading to false-positive or false-negative results.

Assay	False-positive result	False-negative result
Culture	Contamination[†] Wrong mycobacterium species identification[†,‡]	Low bacillary load MTB growth inhibitors (e.g., antibiotics) sample preparation[†] instrument failure
Smear microscopy	Reader's ability to distinguish AFBs from NTM[†] Sample cross-contamination	low bacillary load sample preparation[†] reader's ability to detect AFBs[†]
NAAT	Non-pathogenic MTB presence (e.g., old granuloma, LTBI) Carry-over contamination[†,§]	MTB not present in the specimen PCR inhibitors sample preparation[†] mutated MTB genoma

[†]Linked to the training level/experience of the staff.
[‡]This problem has been significantly reduced by the introduction of tests like p-nitrobenzoic acid, catalase and Capilia test, and new PCR-based MTB identification assays are under evaluation.
[§]A major advantage of closed, automated PCR systems like GeneXpert MTB/RIF is the potential elimination of this type of contamination.
AFBs: Acid-fast bacilli; LTBI: Latent TB infection; MTB: *Mycobacterium tuberculosis*; NTM: Non-tuberculous mycobacteria.
Data taken from [72,73,111].

need of appropriate (although basic) staff training, the cartridge supply and storage conditions and the difficulty to carry and implement the system in rural environments. The cost of the test, even if controlled by an agreement involving the FIND foundation and the manufacturer, can still be a problem for the widespread scale-up of the assay [66]. Other NAAT systems and technologies are currently under evaluation, among which particularly interesting are the ones based on loop-isothermal amplification [67]. This technology seems particularly suited for operating with little or no laboratory infrastructure and potentially represents an important step towards the creation of a real POC diagnostic test for TB, including EPTB [68–71].

Unresolved issues & future perspectives

The 'gold standard issue'

Culture has always been considered the 'gold standard' for EPTB diagnosis. However, culture – even liquid one – is not the ideal test for EPTB (Table 2). To better assess diagnostic tests, a 'combined' reference standard is increasingly used which integrates microbiology, clinical findings, other test results and follow-up evaluations to optimize the definition of TB cases. The combined reference standard finds its highest added value over microbiology only in particular in 'difficult' forms of TB like smear-negative TB and EPTB [43,72–74].

A major challenge of the combined reference standard is the lack of criteria for standardization: different combinations of clinical findings, histology, microbiology and response to treatment are used in different studies with non-uniform case definition among studies [75]. On a clinical point of view, the advantages of diagnosing some cases of TB also in presence of negative culture are counterbalanced by the impossibility to determine phenotypically the susceptibility pattern of such cases. As a consequence, some patients may be treated with ineffective therapy, exposed to worthless side effects, and broader drug-resistance patterns may be induced. This gap is partially filled by new NAATs which – likewise Xpert MTB/RIF – are suited not only to detect MTB, but to recognize the genotypic bases of drug resistance [76]. As previously mentioned, the diagnosis of EPTB is often based only on clinical evidence (e.g., full recovery after a course of anti-tubercular therapy). Few data are available on the direct comparison of microbiological tests, IGRAs, NAATs and other diagnostics against the clinical evidence-based diagnosis. Research is needed to better correlate diagnosis with clinical outcome and to elucidate the management of cases showing disagreement among different tests (e.g., PCR-positive and culture-negative) [73].

Perspectives

Diagnosis

Bacteriological hallmarks of EPTB diagnosis are smear microscopy, culture and NAATs. Table 2 reviews the major technical pitfalls of those assays. All those tests are dependent on the presence of viable or non-viable MTBs in the analyzed sample; this can be a problem in EPTB which is characteristically a pauci-bacillary disease and in which the sampling of the tubercular lesion is not always straightforward. On the other hand, diagnostic tools which are *per se* not linked to this constraint proved to have only an ancillary role (TST, IGRAs), or even no utility at all (serodiagnostic assays) in TB diagnosis [53,77].

To overcome this problem, many efforts in TB research are now directed to detect biomarkers, which are reviewed in a recent paper by Wallis *et al.* [4]. The majority of the candidate biomarkers for TB are directed at finding surrogate endpoints for treatment outcome and for assessing the efficacy of new anti-TB drugs and vaccines; nevertheless, there are interesting candidate biomarkers holding potential diagnostic advances. Among these, there are the detection of mycobacterial lipoarabinomannan (LAM) in urine, ADA in body fluids and the analysis of clinical samples through highly multiplexed assays (Transcriptomics, proteomics, metabolomics and miRNA

assays). ADA detection in pleural and peritoneal fluid has already been discussed in previous paragraphs and Table 1. Moreover, the analysis of MTB-specific volatile organic compounds in breath is another promising field of inquiry, but its utility seems limited only to PTB diagnosis and will not be discussed in this review.

LAM is one of the lipopolysaccharidic structures of the mycobacterial cell wall and it has been shown to play important roles in the survival and pathogenicity of MTB in the human host. This molecule is present and chemically stable in urinary samples from TB patients, and ELISA-based tests have been developed for urinary LAM identification for diagnostic purposes [78]. In particular, a lateral-flow assay named Determine TB-LAM Ag (Alere, Waltham, MA, USA) is a simple, rapid (readout in less than 30'), cheap, POC test which is currently under evaluation for TB diagnosis in specific population subgroups. Indeed, urinary LAM detection tests proved to perform satisfactorily only in those HIV-positive individuals with low CD4 counts. Therefore, Determine TB-LAM Ag may play important role in the prompt identification and treatment of TB in HIV-infected people who have low access to diagnostic facilities, have the poorest diagnostic performance with current tests, have the highest mortality risk and benefit most from same-day diagnosis and treatment initiation [56,78–80].

Transcriptomics is another frontline in the research of candidate biomarkers for TB and EPTB. Little data is available for EPTB, but research in this booming field is likely to change the current diagnostic scenario in the next few years, thanks to new technologies like real-time multiplexed PCR and next-generation sequencing.

Several studies investigate the potential role of specific cytokines and chemokines as biomarkers for active TB and LTBI. Multi-cytokine expression profiling seems to be able to differentiate active disease from latent infection, and specific CD4+ T-cell cytokine expression profiles seem associated with the presence of live MTB [81]. High serum levels of IL-8, IP-10, MCP-1 and MIP-1 β have been associated to PTB, and modifications in specific cytokine profiles have been proposed as 'biomarker signature' for early treatment monitoring [82]. With regard to EPTB, Djoba *et al.* showed that pleural TB patients had an increased cytokine expression compared to PTB patients, while Yu *et al.* demonstrated increased levels of CCL1, CCL21 and IL-6 in pleural effusion of TB pleurisy patients [83,84]. Recently, research on heat shock proteins unveiled their potential biomarker role in the diagnosis of PTB and EPTB [85].

At a very early evaluation stage is the identification of TB-associated patterns of circulating miRNAs. miRNAs are hairpin-derived, 20- to 24-nucleotide long RNA sequences, which post-transcriptionally regulate the expression of target genes. Since miRNAs have been demonstrated to be present, stable and quantifiable in body fluids, studies attempting to find a correlation between single miRNAs (or miRNA patterns) and different physiological and pathological conditions (pregnancy, cancer, infection, etc.) have been conducted on different sample types using different techniques [86–88]. Few studies have been published on the correlation of circulating miRNAs with PTB, but interesting potential biomarkers have already been identified and are currently under investigation [89–94]. One single study has been conducted on pooled and single serum samples from PTB, EPTB, LTBI patients versus healthy subjects and patients suffering from non-tuberculous pulmonary infections [95]. The study identified a panel of 15 circulating miRNAs which showed 85.7% sensitivity and 78.7% specificity overall in the diagnosis of PTB, with better results if population-specific miRNA subsets were used. As for data on EPTB and LTBI patients, available results are only suggestive of a potential role of miRNA signatures in a rapid, pathogen-independent diagnosis of such diseases [95]. To date, the major challenges to miRNA profiling are: the lack of standardization of miRNA extraction and detection protocols, the role of population differences in miRNA profiling and normalization methods and expression cutpoints [95].

Clinical management

Recently, more and more focus was made on the need to improve diagnostic and treatment standards in agreement with the new patient-centered concept of TB management, and the International Standards for TB Care and the European Union Standards for TB Care, among others, have been developed [96–98]. Within this vision, the consultation of a team of experts is recommended in the management of difficult-to-treat cases of TB, to minimize errors and maximize the chances of positive outcome [99]. An electronic platform (available at [100]) allowing free consultation for any complicated case of TB has been successfully launched by WHO and the ERS (European Respiratory Society). This platform allows to obtain case management suggestions from global experts selected through stringent criteria in an average time of 24 h [101]. The first analysis of the queries reveals that a substantial proportion of request relates to EPTB [102]. This finding once again confirms the fact that for the diagnosis of EPTB microbiological data alone (as clinical data alone) are not sufficient; the best 'gold standard' we can recommend for everyday practice is therefore the wise integration of clinical medicine, microbiology and other diagnostic modalities, with the possibility to promptly refer doubtful cases to a team of experts.

Expert commentary & five year view

In conclusion, EPTB is a complex and multifaceted disease which constitutes a significant proportion of the global TB burden. Lack of POC test, able to diagnose EPTB and/or monitoring the response to treatment in an independent way from mycobacteriological testing, remains one of the major gaps in TB care and control. A prompt identification and treatment of cases is required to reduce morbidity, mortality and disability, in agreement with the new WHO post-2015 Strategy which focuses on TB elimination and emphasizes on research and innovation eventually translated into new vaccines, drugs and diagnostic tools. To date, the key to a proper diagnosis remains

clinical suspicion; confirmatory tests based on conventional TB diagnostics often perform poorly or are not feasible due to specimen collection hindrances and other challenges. The recent introduction and WHO endorsement of automated nucleic-acid identification assays may hold promises in the simplification and fastening of EPTB diagnosis, though a real breakthrough would be the introduction of a pathogen-independent test, following current studies on circulating biomarkers. A major global effort is needed to ensure that new effective diagnostic tools are available in the next few years.

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Key issues

- Extrapulmonary tuberculosis (EPTB) is defined as any form of tuberculosis (TB) in which the lung is not involved.
- EPTB accounts for a variable but significant proportion of TB cases (ranging from about 20 to 50%), the most frequent localization being the pleura, lymph nodes, bone and joints, CNS and abdomen (gastrointestinal and/or genitourinary system).
- EPTB diagnosis is difficult. Clinical presentation of EPTB is extremely variable due to the site of involvement and to the aggressiveness of the disease. Imaging findings are often non-specific, and serologic tests proved to lack utility in TB diagnosis. Sample collection is not always straightforward for histology and for microbiological tests.
- Microbiological methods are microscopy, culture and nucleic-acid amplification techniques. They are all useful in EPTB diagnosis, and developments of such techniques like automated liquid culture and closed automated nucleic-acid amplification technique devices showed satisfactory or even good results. Nevertheless, challenges still remain, and the performance of current microbiological methods for EPTB diagnosis is still suboptimal.
- The development of new diagnostics for EPTB is flawed by the absence of a true gold-standard test. New studies often rely, as a gold standard, on the combination of clinical and microbiological findings, but standardization of such compound classification systems is needed.
- To overcome current hurdles in EPTB diagnosis, research is focusing on biomarkers. Lipoarabinomannan detection in urine is under evaluation for TB diagnosis in immunocompromised patients. Cytokine and transcriptome profiling are other interesting fields of TB (and EPTB) biomarker research. In particular, serum miRNA profiling holds promises for TB detection.
- In conclusion, the diagnosis of EPTB highly depends on clinical suspicion. Confirmatory tests are of high value, but research is needed to produce significant improvements in EPTB biomarker discovery and to translate it into widespread diagnostic tools.

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