Tuberculosis: lights and shadows in the current diagnostic landscape

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SUMMARY

Despite the improvements in the global fight against tuberculosis (TB), critical points still remain and fuel the epidemic. Even today, only 30% of the estimate number of people suffering from TB worldwide are correctly diagnosed, and lower proportions of cases are diagnosed in high-TB-burden, low-resource settings.

Current TB diagnostics are still suboptimal in their performance for childhood TB, smear-negative TB, extrapulmonary TB, HIV-TB and drug-resistant TB. Furthermore, there is no gold standard test for the identification of latent TB infection status. Improving diagnosis is therefore a strategic goal in TB research, and the pipeline of diagnostic tools is rapidly growing: new ways of performing "old" tests (e.g. sputum smear microscopy) and completely innovative tools (e.g. new technologies for molecular diagnosis) are under investigation or have already been endorsed by WHO. Some of the structural limits of current TB diagnostics are likely to be overcome by such new tools, but research is still needed.

Finally, the roll-out of new technologies and the development of newer ones will necessarily have to take into account the diagnostic needs of each context they are directed to (point-of-need testing approach), together with the logistic, economic and technical constraints present in the majority of high-TB-burden settings.

KEY WORDS: Tuberculosis, Diagnosis, Point-of-need testing, Diagnostic algorithms.

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Tuberculosis (TB) is among the major causes of mortality and morbidity for infectious disease worldwide. When *Mycobacterium tuberculosis* (MTB) meets its host, three conditions can occur:

a) the host immune response operates a complete clearance of MTB bacilli;
b) MTB replication is not controlled by the host immune response, producing a clinical disease called *primary TB*;
c) the host immune response is effective in walling off MTB live organisms into granulomas, but is not sufficient to sterilize the lesion. In this latter condition, no clinical disease is evident (*Latent TB infection status, LTBI*), but there is a lifetime risk of 5-10% of MTB escaping immune surveillance, producing a disease called *post-primary TB* (Horsburgh *et al.*, 2010; Walzl *et al.*, 2011).

One third of the global population is latently infected with MTB (WHO, 2008). As for active clinical TB, the most common form of tubercular disease is pulmonary TB (PTB); nevertheless, in a widely variable proportion of cases, TB can also affect extra-pulmonary sites within the body (Extra-Pulmonary TB, EPTB), exclusively or in combination with PTB. For the year 2011 the World Health Organization (WHO) reported 8.7 million estimated active TB incident cases and 12 million estimated prevalent cases; 990,000 HIV-negative and 430,000 HIV-positive people are estimated to have died of TB in 2011. Most cases occurred in Asia (59%) and Africa (26%) (WHO, 2012).

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Although the reliability of epidemiological assessments has progressively improved in recent years, no more than 30% of the estimate number of people suffering from TB are actually diagnosed with a diagnostic method of proven efficacy (WHO, 2012). This review focuses on the current TB/LTBI diagnostic landscape: the state-of-the-art, and which critical aspects are still unresolved.

Diagnosis of TB

Diagnosis has only recently been identified as a priority in TB research, mostly focused on new drugs and prognostic markers (McNerney *et al.*, 2012; Weyer *et al.*, 2011).

Although strict biological borders between LTBI and TB may be waning, the distinction between these two conditions is still crucial from a clinical and epidemiological point of view, and different diagnostic/therapeutic/epidemiological approaches are required when facing LTBI or TB. On the one hand, the subject with LTBI does not have a disease but a risk factor, and is not even infectious; on the other, TB patients suffer from a serious and progressive disease whose infectiousness depends on the localization within the body and the bacillary load (Mack *et al.*, 2009; Walzl *et al*., 2011).

DIAGNOSIS OF ACTIVE TB DISEASE: CURRENT PRACTICE AND CHALLENGES

The first essential step to diagnose TB is clinical suspicion (Schaaf e*t al*., 2010; Sharma & Mohan, 2004). TB can present with almost any symptom, and must be considered in differential diagnosis especially (but not only) in patients with epidemiologic risk (exposure to infectious patients, travel to or residence in a high prevalence area, previous TB). The clinical suspicion of TB is then further investigated through (Table 1):

- imaging: infiltrates, fibrosis, cavitation at chest X-ray; calcified lymph nodes on CT scan, other imaging findings;
- microbiology: sputum smear positive for acid-fast bacilli (AFB), MTB-positive culture of sputum or tissue/body fluid specimens, positive molecular tests for MTB;
- histopathology: caseous granulomas, AFB in the specimen.

The state-of-the-art diagnostic path often uses the tests mentioned above in combination, and additional diagnostic tools/procedures are sometimes performed such as induced sputum, advanced imaging techniques, bronchoscopy with lavage and tissue biopsies (Sia & Wieland, 2011). Nevertheless, confirmation of the diagnosis of TB requires laboratory identification of AFBs by smear microscopy, and/or MTB positive culture (liquid culture is currently the *gold standard* method both in clinics and in research). MTB-specific Nucleic Acid Amplification tests (NAATs) can be a valid surrogate to direct observation or to isolation of tubercular bacilli (Lawn & Zumla, 2011). In addition to identifying the presence and extent of TB, it is nowadays essential to be able to determine in an effective and reliable way the drug susceptibility of MTB especially in those Patients presenting with a treatment failure or a recurrence: *phenotypical* (*gold standard*) or *molecular based Drug Susceptibility Tests (DSTs)* are cur-

TABLE 1 - *Current diagnostic tools for TB/LTBI.*

	TB disease	*Latent TB infection*
"Pathogen-side"	- Sputum smear microscopy (light/fluorescence/LED) - Culture (*Liquid culture: gold standard*) - NAATs - Histopathology	
"Host-side"	- History collection and physical exam - CXR (Digital CXR preferred) → advanced imaging - (TST/IGRAs) - (Serology)	- History of TB contacts, physical exam - CXR (exclusion of PTB) - TST - IGRA

CXR: Chest X-ray; IGRA: Interferon-Gamma Release Assay; NAATs: Nucleic Acid Amplification Test; TST: Tuberculin Skin Test. For comments, see text.

rently available for this scope (Bwanga *et al.*, 2009; Koh *et al.*, 2012; Miotto *et al.*, 2009).
Although often performed in the initial management of suspect TB cases, neither Tuberculin Skin test (TST) nor Interferon-Gamma Release Assay (IGRA) are currently recommended for the diagnosis of tubercular disease since they simply investigate the immune response against MTB antigens and do not provide information on the presence of clinical TB (Lange & Sester, 2012). TB serological assays are widely used and merchandised in the field, especially in the private healthcare sector of low-resource Asian countries; nevertheless, they showed unsatisfying diagnostic performance (low specificity and sensitivity) in randomized controlled trials and their use has been advised against by WHO (Steingart *et al.*, 2011; WHO, 2011a).
Global challenges to effective and universal diagnosis of TB rely mainly on forms of TB which are intrinsically difficult to diagnose, or on the poor performance of current diagnostic tests. They are represented by:

- Childhood TB: the diagnosis of TB in children requires high level of clinical suspicion given the atypical clinical manifestations and reduced information on symptoms; children are *per se* more likely to develop active disease following MTB infection, and such risk skyrockets in the presence of TB-HIV co-infection (Marais *et al.*, 2004; Schaaf *et al.*, 2010). The mycobacterial load, on the other hand, is often low in paediatric cases, and it is extremely difficult to obtain sputum samples suitable for analysis from younger children (Perez-Velez & Marais, 2012; Schaaf *et al.*, 2010). Those two factors together contribute to reduce the performance of all diagnostic tests, which are meant to identify the pathogen (e.g. sputum smear microscopy, culture, NAATs). As a result, a *gold standard* diagnostic test for paediatric TB is still missing (Pearce *et al.*, 2012).
- Smear-negative pulmonary TB (S-PTB) and EPTB: the first hurdle in the diagnosis of these forms of tubercular disease is in the great variety of clinical manifestations those conditions can present with, thereby requiring a high index of suspicion to be included in the differential diagnosis. Moreover, the backbone of TB diagnosis worldwide still consists in methods intended to find/isolate the pathogen, and this is a major limit when the mycobacterial load is low or the district of infection is not easily accessible. For these reasons, the diagnosis of S-PTB and EPTB is often a late one, and - even in the hi-tech, high-resources western countries - such diagnosis is often made *ex juvantibus*, based on the clinical response to empiric antitubercular treatment without microbiological confirmation (Sharma & Mohan, 2004; Tortoli *et al.*, 2012).
- TB in HIV/AIDS patients: TB-HIV co-infection is one of the main factors that fuel the TB epidemic worldwide (Harries *et al.*, 2010). When TB occurs in HIV/AIDS patients, it is not only more severe at a clinical level, it is also more difficult to diagnose because of the decreased bacillary load, the higher proportion of atypical cases and the possibility to co-exist with Immuno-Reconstitution Syndrome (IRIS). On the other hand, due to the increased biological aggressiveness of the disease, such a difficult diagnosis needs to be prompt and precise.
- Drug-resistant TB: the management of patients with multidrug-resistant (MDR) TB, extensively drug-resistant (XDR) TB or total-resistant TB requires an appropriate and rapid diagnosis. The process should start with the identification of high-risk groups, followed by microbiological confirmation and the appropriate treatment (Kalokhe *et al.*, 2012; Saleri *et al.*, 2010). DSTs are often performed in centralized reference centres, and many of the available technologies require skilled staff and high-level biosafety laboratories, conditions that are costly and not universally achievable. The turnaround time required for a culture-based DST on solid media is often too long to be useful in the patient's management, and it is not a remote possibility that a patient dies from MDR TB before the DST on its MTB isolate has been completed. In December 2010 the closed, automated molecular assay Xpert MTB/RIF (Cepheid. Sunnyvale, CA 94089 USA), capable of detecting mutations associated with Rifampicin resistance, was endorsed by WHO for the diagnosis of Rifampicin-resistant TB, with the potential to represent a breakthrough in the diagnosis of TB and MDR TB. The situation may be slowly changing, but in 2011 only 5% of the reported TB cases were tested for drug resistance (WHO, 2012). Moreover, DST is often too expensive for

the patient or the health system, especially in high-burden countries, and in many settings it is simply overlooked because of the lack of second-line drugs (McNerney *et al.*, 2012; Sia & Wieland, 2011).

In addition to the structural limitations analysed above, it has to be considered that the already existing and well-experienced diagnostics are not always used to their full potential (Raviglione & Pio, 2002; Raviglione & Uplekar, 2006; WHO, 2012). Indeed, the current economic downturn, together with financial policies aimed at reducing public expenditure, can potentially impact on the implementation of state-of-the-art diagnostic algorithms, and on the long-term sustainability of new diagnostic tools.

Adverse geographical, social, epidemiological situations, in specific contexts, constitute additional hurdles towards the implementation and correct utilization of old and new TB diagnostic devices:

- Logistical challenges: in many areas of the world an inconstant electricity supply prevents the execution of both simple culture systems and sophisticated modern diagnostic assays which require constant electric power to run. Resistance of modern hi-tech devices to heat, humidity, shocks and dust is another issue to consider when trying to implement such diagnostic instruments in rural areas. Moreover, the supply of reagents and the availability of the know-how required for maintenance are not granted nor easy worldwide (Batz *et al.*, 2012; Mauch *et al.*, 2011; Ongugo *et al.,* 2011).
- Health policy challenges: in Africa and Southeast Asia, the WHO regions most heavily affected by TB, per capita government expenditure on health in 2007 was only $34 and $15, respectively. Although TB *treatment* is generally free, patients are sometimes required to pay for some of the diagnostic tests even in the public sector, and some patients opt to consult private practitioners at their own expense (Abramovitz & Zelnick, 2010; McNerney *et al.*, 2012).
- Social/cultural challenges: TB still is, as it has always been, a disease of poverty (Sia & Wieland, 2011). On the one hand many of the social risk factors for TB are favoured by poverty or represent themselves conditions of destitution (Lonnroth *et al.*, 2009); on the other, in addition to the physical suffering, the diagnosis of TB (like the diagnosis of HIV) usually implies social marginalization, stigma, and even blame (Somma *et al.*, 2008). Other cultural aspects to consider are the level of training and education of health workers operating at a community level, the accuracy of their knowledge and the thoroughness of their supervision (Nansera *et al.*, 2010). Moreover, in many rural areas of the world, the reference points for people's healthcare at a peripheral level still consist in deep-rooted cultural beliefs, traditional healers or private practitioners non-integrated in an organized and supervised healthcare system: "official" healthcare workers, thus, often have to fight cultural barriers or prejudice from the population they serve while carrying out their role of health assistance and education (Farmer, 2005; Ongugo *et al.*, 2011).
- Political challenges: nation-level, widespread governmental institutions aimed at supporting TB patients and their families, as well as educating the population on how to prevent the transmission of the disease, proved to be effective already in the pre-antibiotic era. Even today, TB control requires commitment from the political class: strongly advised are measures for strengthening the peripheral healthcare system, fighting the social injustice situations in which TB nests, for implementing quality-assurance mechanisms among healthcare providers and for promoting TB research (Ongugo *et al*., 2011). Initiatives for integrating the management of TB, HIV and other associated epidemics are also needed (Matseke *et al.*, 2012; Ongugo *et al.*, 2011).

DIAGNOSIS OF LTBI: CURRENT PRACTICE AND CHALLENGES

While in high-prevalence countries most TB cases result from recent transmission from other PTB patients, in non-endemic countries a significant proportion of the cases is represented by post-primary re-activation of TB in people carrying LTBI (Amanatidou *et al.*, 2012). It is thus clear that the control of the disease in the "western world" - and worldwide, definitely - needs to target LTBI. Preventive treatment with 6 months of Isoniazid or with other regimens is able to reduce by up to 90% the odds of re-activation of TB

(Leung *et al.*, 2011): the crucial point is therefore to provide this treatment to the portion of LTBI population at risk of developing post-primary TB. Several screening programs, targeting social groups most at risk of carrying LTBI and thus of developing TB, were implemented decades ago into the national healthcare system of many high income countries: their backbone consists of a physical examination (comprehensive of careful anamnesis on previous TB and possible/proven contacts with PTB cases), TST - or, more recently, IGRAs - and chest X-ray to rule out PTB (Sia & Wieland, 2011) (Table 1). As in every screening, the testing for LTBI has to be driven by *intention-to-treat*, and is useless if there is neither the will nor the opportunity to administer the efficacious preventive therapy to positive cases (Amanatidou *et al.,* 2012; Linas *et al.*, 2011; Pollock *et al.*, 2012; Schluger & Burzynski, 2010). The targeted screening programs have been effective in reducing the burden of TB in the "western world"; nevertheless, their positive effect is now being put in the shadows by at least two factors which are driving TB/LTBI epidemiology in low-TB-prevalence settings. On the one hand, a growing number of people from low-income, high-TB-burden countries move to rich western societies, carrying a likely status of LTBI (Ricks *et al.*, 2011). On the other, longer life, better survival with chronic diseases and more powerful immunosuppressive drugs increase the odds of reactivation of TB in the population the "western world" latently infected by MTB (i.e. elderly subjects, patients suffering from diabetes or malignancies, immune-depressed or immuno-suppressed subjects, transplant recipients, patients in haemodyalisis) (Dobler *et al.*, 2011; Holty *et al.*, 2009; Kim *et al.*, 2012; Minguez *et al.*, 2012; Schaaf *et al.*, 2010).

In addition to those challenging points, the diagnosis of LTBI presents critical issues itself. The most important one is that we lack a g*old standard* test for LTBI: both TST and IGRAs investigate lasting immune response against MTB antigens, and cannot actually verify the presence/absence of dormant bacteria still able to reactivate. Moreover, it is not possible to stratify TST/IGRA-positive patients for the risk of progression to active disease (Lawn & Zumla, 2011; Pai & O'Brien, 2008; Pollock *et al.*, 2012). Finally, there are categories of patients (children under 5 years of age and immuno-compromised patients including HIV-infected subjects) in which the test performances are particularly poor, and that urgently need a better test for LTBI (Amanatidou *et al.*, 2012; Pollock *et al.*, 2012).

MOVING TOWARDS INCISIVE DIAGNOSTIC INNOVATIONS

The countries where the diagnostic challenges described above are greatest are often the same as those in which TB epidemics are heaviest, as illustrated by the overlapping of high incidence rates and poor diagnostic rates in Figures 1 and 2. It is therefore of paramount importance for any diagnostic technology intended to ameliorate TB control at a global level to be suited to operating in the settings where it is most needed, namely high-TB-burden, poor-resource countries (McNerney *et al.*, 2012). In addition to this, in the context of the renewed worldwide interest in TB diagnosis research, it is becoming increasingly clear that the formula "one-size-fits-all" is no longer suitable to effective TB management. Thus, at the state-of-the-art technology, no current or forthcoming diagnostic tool is likely to become *the only* ultimate test for TB; indeed, a proper diagnostic system can be built through the *integration* of *existing and new diagnostics* operating at different levels of the healthcare system, with optimization of the operational algorithms according to the various population settings (as recently outlined by the Stop TB Partnership and WHO among the highest-priority topics in TB research (WHO, 2011b)). In relation to this direction undertaken by TB diagnostics research, the WHO global tuberculosis report 2012 divides the forthcoming and future TB diagnostic tools according to the healthcare level they are best suited for operating in (Figure 3).

It is noticeable that the novelties under validation or already recommended by WHO include:

1. new operational protocols applied to already existing consolidated tools (2-specimen approach for sputum smear microscopy);
2. new devices performing "old" tests (culture in liquid media and automated systems, sputum smear examination through LED microscopy, Xpert MTB/RIF and LPA assays for nucleic-acid amplification testing);

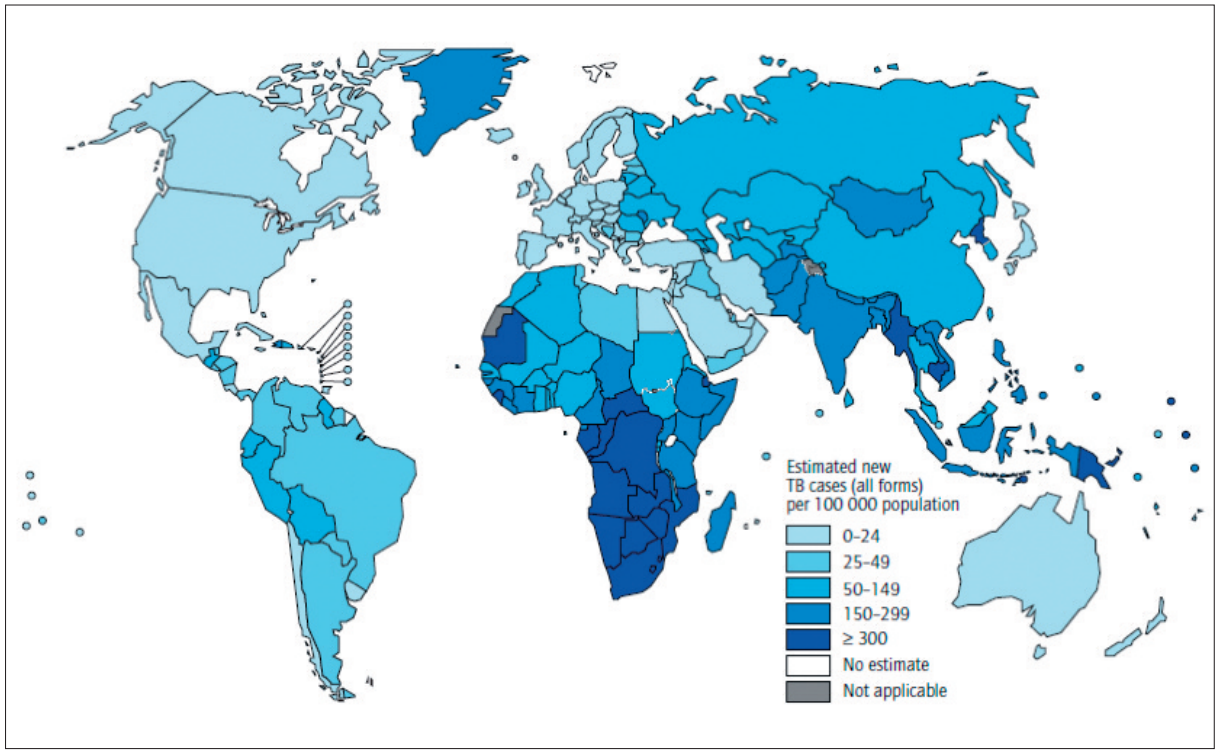

FIGURE 1 - *Estimated TB incidence rates, 2011. Reproduced with permission from (WHO, 2012).*

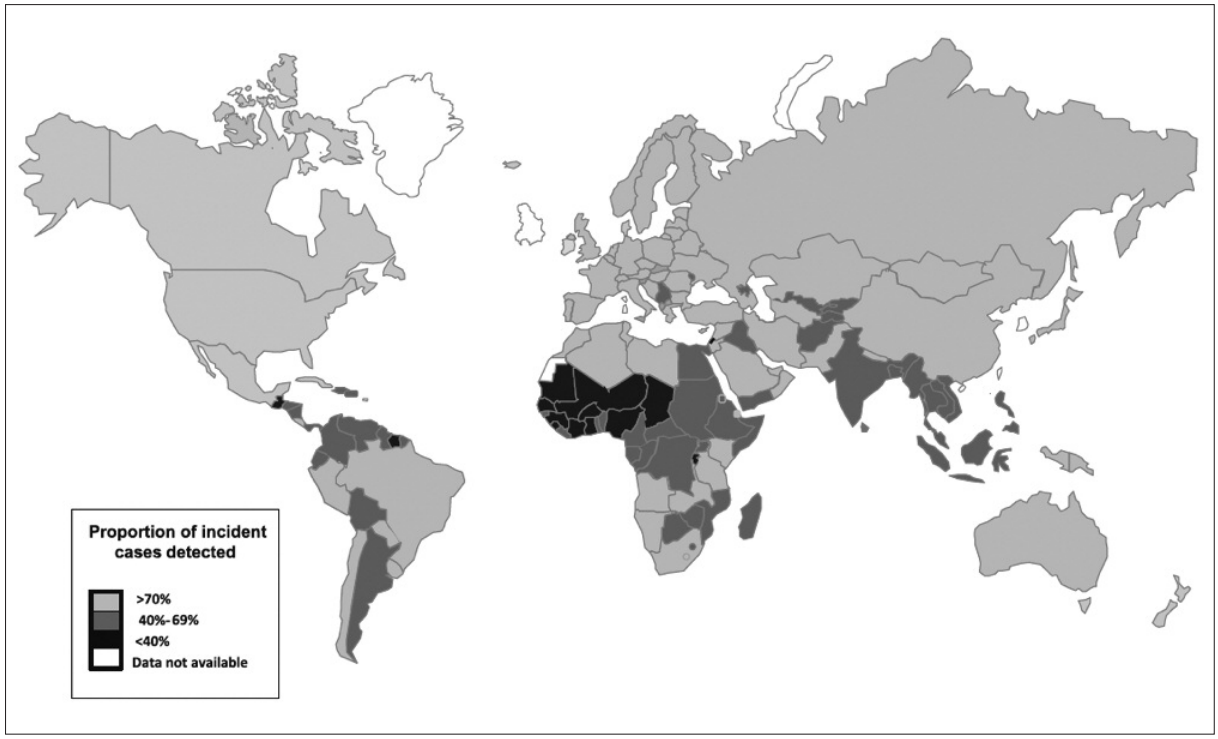

FIGURE 2 - *Estimated global TB case detection rates. Reproduced with permission from (McNerney et al., 2012).*

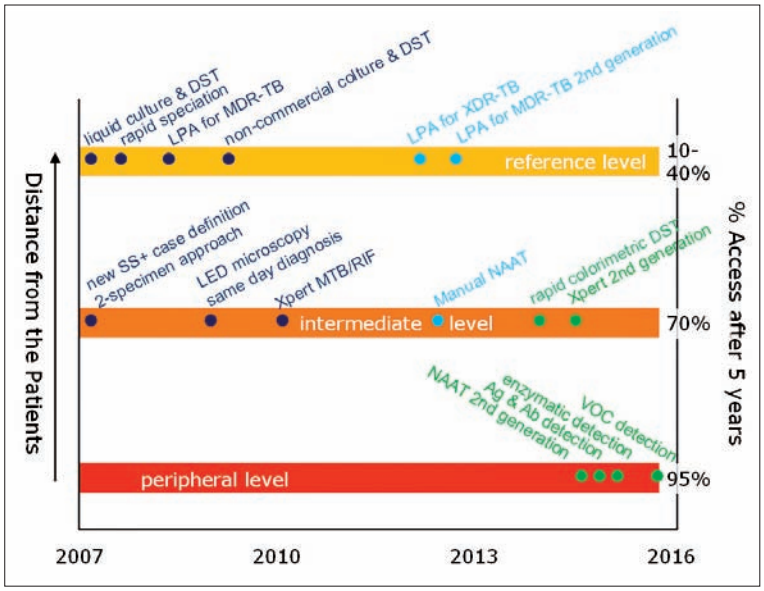

FIGURE 3 - *Diagnostic tests endorsed by WHO, or available in the near future. "Non-commercial culture & DST" refers to MODS, NRA and CRI. Ab: antibody; Ag: antigen; CRI: colorimetric redox indicator assay; DST: drug susceptibility testing; LED: light-emitting diode; LPA: line probe assay; MODS: microscopic observation drug-susceptibility; NAAT: nucleic acid amplification test; NRA: nitrate reductase assay; VOC: volatile organic compound. Colour legend: blue: technologies or methods endorsed by WHO; light blue: technologies commercialised, not yet endorsed by WHO; : technologies at feasibility stage; green: technologies at early stages of development. Reproduced with permission from (WHO, 2012).*

3. absolutely new approaches for testing TB (VOC detection, enzymatic detection, antigen-antibody detection, new nucleic-acid amplification technologies).

Xpert MTB/RIF is a fully automated, closed NAAT assay designed for operating on the GeneXpert® real-time PCR platform (Cepheid, Sunnyvale, CA 94089, USA). It is the first NAAT system designed for operating with minimal infrastructural requirements and minimally trained staff, and potentially represents a breakthrough in TB diagnosis since is suitable for use in district and sub-district laboratory facilities. Its capability to detect mutations conferring resistance to Rifampicin extends its usefulness beyond the diagnosis of TB (sensitivity 98.2% and 72.5% respectively for smear-positive and smear-negative samples; specificity 99.2%), also to first-line assessment of Rifampicin resistance (99.1% sensitivity and 100% specificity) and prediction of multi-drug resistance (99% sensitivity) (Boehme *et al.*, 2010). Although Xpert MTB/RIF was conceived for analyzing sputum samples, interesting data also come from studies on extra-pulmonary samples (especially biopsies, urines, pus and cerebrospinal fluids) for the diagnosis of EPTB (Tortoli *et al.*, 2012).

Unfortunately, Xpert MTB/RIF suffers a series of limitations: it requires an ambient temperature lower than 30°C and an uninterrupted stable electric power supply to operate; cartridge storage time, space and conditions, as well as waste disposal, often constitute a logistic problem; Xpert MTB/RIF is a relatively low-throughput technology and, finally, implementation studies showed that the minimal training requirements needed for operating the GeneXpert platform and for managing the software reporting system are often more difficult to achieve than expected. Therefore, Xpert MTB/RIF cannot be *the ultimate* test for TB, but its development nevertheless opened new pathways in the progress of TB diagnostics (Weyer *et al.*, 2011; Weyer *et al.*, 2012). The process that led to the endorsement of Xpert MTB/RIF by WHO in December 2010, and which is now driving the scale-up of this new diagnostic platform worldwide, is an interesting pathfinder in the development, production and evaluation of any new diagnostic test for TB: field-workers, academia, industry, FIND diagnostics, public and private funders productively engaged in collaborations and negotiations to develop and validate a system conceived for effectively responding to the users' needs at an affordable price.

Despite the potential advances stemming from implementation of the described forthcoming diagnostic tools, and in addition to their specific challenges, some of the weak points of current TB diagnostics still persist as structural problems potentially unresolved. Such unresolved issues are:

a) the low utilization of samples more easy to obtain than sputum;

b) the reliance of the test performance on the presence of MTB in the analysed specimen.

As for the first point, relying on samples other than sputum would be a major advance in TB testing in paediatric and HIV/AIDS populations, and in general would reduce the infectious risk for the healthcare operator. With reference to the second point, a test capable of diagnosing TB independently of the presence of live MTB or mycobacterial components in the analysed specimen would significantly improve the diagnosis of EPTB and smear-negative PTB cases.

In addition to the tools depicted in Figure 3, several new technologies are currently at an early stage of development (McNerney *et al.*, 2012). Some of these technologies seem particularly promising in addressing those "unresolved issues" mentioned above: in particular, we highlight the interesting diagnostic approach based on serum nucleic acids, with special reference to micro RNAs (miRNAs). Fu *et al.* reported having identified at least two serum miRNAs useful for discriminating PTB patients from healthy controls (Fu *et al.*, 2011); unpublished data from our laboratory confirm this finding and extend the discriminatory potential of serum miRNAs not only to the PTB condition, but also to EPTB, LTBI and other pulmonary infections (Miotto *et al.*, data presented at 33rd congress of the European Society of Mycobacteriology. Brasov, Romania, July 2012).

In conclusion, the diagnostic scenario that can be outlined will necessarily rely on a cheap, sensitive, easy-to-run test performed at a peripheral level, where the vast majority of TB cases are seen. Such test must have a short turnaround time to be able to link execution of the test to the start of possible anti-tubercular therapy in the same clinical encounter ("one-stop-shop" diagnostic-therapeutic path). Notwithstanding the importance of moving TB diagnostic tools as near to the patients as possible, the diagnostic-therapeutic management of peculiar cases (such as MDR TB cases) will require referral to secondary level healthcare facilities or even regional reference centres. For this reason, the expression *"point-of-care" testing*, widely used in recent years for indicating the ultimate need in TB diagnostics, is nowadays better reformulated as *"point-of-need" testing*.

Nevertheless, experience coming from the ongoing implementation of the Xpert MTB/RIF system teaches us that the creation of any new diagnostic tool is not the arrival point, but a point of departure. It is not sufficient that diagnostic devices are developed, produced, validated, endorsed and merchandized at cheap prices. To be effective against TB, they must *be wisely implemented* in healthcare systems and services (invariably with an economic effort and a cultural shift), they must *be used* in current clinical practice, and must be followed by appropriate, timely and correct *therapy*. Finally, in the current scenario of free market and competitive production of goods, a continuous surveillance has to be kept on manufacturers and on the commercial supply chain, in order to guarantee *quality standards* of diagnostics, as well as of drugs.

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