New Microbiologica, 42, 3, 171-175, 2019, ISN 1121-7138

FULL PAPER

Predictors of time to sputum smear conversion in patients with pulmonary tuberculosis under treatment

Francesco Bisognin[1#], Francesco Amodio[2#], Giulia Lombardi[1*], Maria Letizia Bacchi Reggiani[3], Elisa Vanino[2], Luciano Attard[2], Marina Tadolini[2], Maria Carla Re[1], Paola Dal Monte[1]

[1]*Department of Experimental, Diagnostic and Specialty Medicine - Unit of Microbiology, S. Orsola-Malpighi University Hospital, Bologna, Italy;*
[2]*Department of Medical and Surgical Sciences - Unit of Infectious Diseases, S. Orsola-Malpighi University Hospital, Bologna, Italy;*
[3]*Department of Experimental, Diagnostic and Specialty Medicine, S. Orsola-Malpighi University Hospital, Bologna, Italy*
[#]*These authors equally contributed to this work*

SUMMARY

Sputum acid-fast bacilli smear conversion is a fundamental index of treatment response and reduced infectivity in patients with pulmonary tuberculosis (P-TB). To date, there are no models to predict the time to sputum conversion based on patient characteristics. This study aims to ascertain the time to sputum conversion in patients with smear-positive P-TB under treatment, and the variables associated with time to smear conversion.

We retrospectively evaluated the time to sputum smear conversion of 89 patients with smear-positive P-TB undergoing treatment at the S. Orsola-Malpighi University Hospital, Bologna (Italy), a referral centre for the diagnosis of TB. Multivariate Cox regression analysis was performed to document variables independently associated with time to conversion.

Median time to sputum smear conversion was 24 days (IQR 12-54); the sputum smear converted within the first 2 months of treatment in 78.7% patients. Multivariate Cox regression analysis showed that older age, high baseline mycobacterial load detected by Xpert MTB/RIF, and severity of lung involvement are predictors of persistent smear positivity.

The identification of risk factors delaying smear conversion allowed us to develop predictive models that may greatly facilitate the management of smear-positive patients in terms of the duration of respiratory isolation and treatment.

Received December 12, 2018 *Accepted* April 16, 2019

INTRODUCTION

Tuberculosis (TB) currently represents a major global health problem, both in developing countries and in Europe, in correlation with migratory flows from high incidence countries. According to the latest WHO estimates, the global incidence of TB in 2017 was 10 million new cases and TB accounted for about 1.6 million deaths worldwide per year, including 300,000 deaths among people living with HIV (PLHIV). Therefore, although TB mortality rate fell by 42% between 2000 and 2017, the disease is still one of the top ten causes of death worldwide (World Health Organization, 2018).

Sputum smear microscopy is a fast, simple and cheap test to assess the presence of acid-fast bacilli (AFB) in the respiratory tract of patients with pulmonary TB (P-TB). Sputum AFB smear conversion is a fundamental index of treatment response and reduced infectivity (Pefura-Yone *et al.*, 2014). Patients with prolonged time to sputum smear conversion may require close monitoring, longer hospitalization and protracted intensive treatment. However, there are no models to date that predict time to sputum conversion based on patient characteristics.

In this study, we evaluated the time to sputum AFB smear conversion in patients with smear-positive P-TB under treatment, and the variables associated with time to smear conversion. In addition, we developed a predictive model of sputum smear conversion time based on demographic, clinical and microbiological data.

Key words:
Pulmonary tuberculosis (P-TB), sputum smear conversion, mycobacterial load, Xpert MTB/RIF, cavitary disease, predictor factors.

Corresponding author:
Giulia Lombardi
E-mail address: g.lombardi@unibo.it

MATERIALS AND METHODS

Study design

In this retrospective observational study, we analyzed data from all sputum smear positive P-TB patients being treated at the Infectious Disease Unit of the S. Orsola-Malpighi University Hospital in Bologna (Italy), a referral centre for the diagnosis of TB, from January 2013 to April 2017. Inclusion criteria consisted of:

1) age ≥18 years old;
2) at least one quality sputum sample found to be smear-positive before treatment;
3) culture and Xpert MTB/RIF confirmed TB.

Exclusion criteria were:

1) fewer than 3 sputum samples collected in the first month of treatment;

2) treatment interruption longer than 2 weeks before sputum smear conversion.

All patients initially received a first or second line standard daily anti-TB treatment based on rifampicin sensibility determined by Xpert MTB/RIF assay, then tailored according to a drug susceptibility test (DST) as soon as it was available (World Health Organization, 2017). Consecutive sputum samples were analyzed at least every other day for hospitalized patients and at least once a week for outpatients. Demographic, clinical, microbiological and biochemical findings were recorded and analyzed. All patients were tested for HIV.

The study was approved by the Ethics Committee of S. Orsola-Malpighi University Hospital (protocol code N. 170/2017/O/OssN).

Microbiological tests

Smear microscopy

All specimens were directly stained for acid-fast microscopic examination using Ziehl-Neelsen stain. The degree of AFB positivity was assigned to one of four categories (1+, 2+, 3+, 4+) as per Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2008). Sputum smear conversion was defined as three consecutive negative smear samples (Jensen *et al*., 2005); the sample collection date of the first negative smear was used as the date of conversion.

Culture

After decontamination with N-acetyl-L-cysteine/sodium hydroxide (NALC-NaOH) (MycoPrep, Becton Dickinson, USA), specimens were re-suspended in phosphate-buffered saline (PBS), 0.5 ml were used to inoculate MGIT tubes (Mycobacteria growth indicator tube; Becton Dickinson, USA) according to the manufacturer's instructions, and 0.25 ml were inoculated in Lowenstein Jensen solid medium (Heipha Diagnostika Biotest, Germany) for culture isolation. Positive cultures were identified as *Mycobacterium tuberculosis* (MTB) by MGIT TBC Identification Test (Becton Dickinson, USA). MTB isolates were tested for susceptibility to first-line drugs (isoniazid, rifampicin, ethambutol, pyrazinamide) with the gold-standard automatic BACTEC MGIT 960 system (Becton Dickinson, USA).

Xpert MTB/RIF

The Xpert MTB/RIF (Cepheid, USA) test was performed on all samples before starting therapy following the manufacturer's recommended protocol: 500 µl of decontaminated concentrated sample were pre-treated with Sample Solution at a 1:3 ratio for 15 minutes at room temperature. Then 2 ml of sample were poured into a single-use disposable cartridge (Cepheid, 2016). The Xpert MTB/RIF system automatically measured the fluorescent signal and interpreted the results as:

1) invalid, if PCR inhibitors were detected with amplification failure;
2) negative
3) positive.

Positive results were divided into 4 categories (very low, low, medium, high) depending on cycle-threshold (Ct) as declared by the manufacturer (Cepheid) and susceptibility or resistance to rifampicin depending on the detection of mutations in the *rpoB* gene.

Case classification

New, relapse and re-treatment TB cases were classified according to WHO definitions (World Health Organization, 2014).

Radiological classification

Baseline chest high-resolution computed tomography (HRCT) was performed on all patients and findings were divided into 3 categories:

1) no cavities,
2) mono-lateral cavities,
3) bilateral cavities.

Statistical analysis

Statistical analysis was performed using Stata/SE 14.2 for Windows (College Station, USA). Continuous variables were expressed as mean ± standard deviation or median and interquartile range; categorical data were expressed as numbers (percentages). To assess the independent predictors of sputum smear conversion, a univariate analysis, a Kaplan Meier method and a multivariate Cox proportional hazard model were fitted to the data. Variables not significantly associated with outcome were removed from the model using a step-down procedure based on the likelihood-ratio test. The proportional-hazards assumption of the variables in the models were assessed on the basis of Schoenfeld residuals. Harrell's C statistic was calculated to evaluate the models' concordance probability.

All p values refer to two-tailed tests of significance; $p<0.05$ were considered significant.

RESULTS

Study population

Ninety-nine positive smear P-TB cases were identified during the study period. Four of them were excluded due to treatment interruption more than 2 weeks before sputum AFB smear conversion and six were excluded because fewer than 3 sputum samples were collected in the first month of treatment. Therefore, the data from 89 patients were analyzed (study population characteristics are summarized in *Table 1*). None were co-infected with HIV; 76 subjects had drug susceptible TB, 9 had mono-resistant TB (1 rifampicin-resistant TB, 6 isoniazid-resistant TB and 2 ethambutol-resistant TB) and 4 were MDR-TB cases.

Time to sputum smear conversion

The mean number of sputum samples collected was 10.3±5.9 (range 4-36) per patient.

Median time to sputum AFB smear conversion in our study population was 24 days (IQR 12-54). *Figure 1* shows the proportion of patients with positive sputum smear in relation to days of treatment: at the end of intensive phase (60 days), 21.3% patients were still sputum smear-positive.

Factors associated with time to sputum smear conversion and predictive models of time to sputum smear conversion

Table 1 shows the results of univariate analysis. Variables with p-value <0.1 (age, baseline sputum AFB smear grading, Xpert MTB/RIF result category, radiological classification and treatment interruption ≤14 days in the in-

tensive phase) were selected for Cox regression analysis. We found that age, baseline sputum smear grading, Xpert MTB/RIF result category and radiological classification are associated with time to sputum smear conversion, as defined by Harrell's C value (0.71) (*Table 2*).

Based on results of Cox regression analysis, we elaborated models to predict the time to sputum smear conversion related to radiological classification of disease and to Xpert MTB/RIF result category (*Figure 2*). Kaplan-Meier curves show that severity of radiological findings and increase of semi-quantitative Xpert MTB/RIF result category correspond to a delay of smear conversion. For example, after

Table 1 - *Study population characteristics and univariate analysis of variables associated with sputum smear conversion time.*

Study population N=89	*Prevalence (%)*	*p*	*HR (95% CI)**
Gender			
Male	60 (67.4)	Reference	Reference
Female	29 (32.6)	0.43	0.83 (0.53 - 1.31)
Nationality			
Italian	13 (14.6)	Reference	Reference
European (except ITA)	21 (23.6)	0.59	1.21 (0.60 - 2.45)
African	29 (32.6)	0.10	1.74 (0.90 - 3.38)
Asiatic	21 (23.6)	0.42	1.33 (0.66 - 2.69)
South American	5 (5.6)	0.56	1.41 (0.45 - 4.36)
Age (years)			
18-34	49 (55.1)	Reference	Reference
35-64	33 (37.1)	0.05	0.63 (0.40 - 1.00)
≥65	7 (7.8)	0.13	0.53 (0.23 - 1.20)
Alcohol abuse			
No	85 (95.5)	Reference	Reference
Yes	4 (4.5)	0.85	0.91 (0.33 - 2.48)
Classification based on history of previous TB treatment			
New cases			
Relapse cases	83 (93.3)	Reference	Reference
Treatment after loss to follow-up cases	5 (5.6)	0.66	0.82 (0.33 - 2.03)
	1 (1.1)	0.43	2.23 (0.30 - 16.33)
Baseline sputum smear grading1		<0.001	0.54 (0.42 - 0.67)
1+	13 (14.6)		
2+	25 (28.1)		
3+	26 (29.2)		
4+	25 (28.1)		
Xpert MTB/RIF result category1		<0.001	0.38 (0.26 - 0.56)
Very low/Low	10 (11.2)		
Medium	52 (58.4)		
High	27 (30.3)		
Radiological classification[1]		<0.001	0.42 (0.28 - 0.62)
Absence of cavities	11 (12.4)		
Monolateral cavities	60 (67.4)		
Bilateral cavities	18 (20.2)		
Any first-line anti-TB drugs resistance			
No	76 (85.4)	Reference	Reference
Yes	13 (14.6)	0.73	1.12 (0.59 - 2.12)
Treatment interruption due to side effects (≤14 days within intensive phase)			
No	79 (88.8)	Reference	Reference
Yes	10 (11.2)	0.01	0.55 (0.27 - 1.12)
Haemoglobinemia (Hb) <120 g/l (F), <133 g/l (M)			
No	26 (29.2)	Reference	Reference
Yes	63 (70.8)	0.80	0.94 (0.58 - 1.51)
Erytrocyte sedimentation rate (ESR) ≥15 mm/h (F), ≥20 mm/h (M)			
No	2 (2.2)	Reference	Reference
Yes	87 (97.8)	0.35	0.51 (0.12 - 2.11)
Diabetes mellitus			
No	76 (85.4)	Reference	Reference
Yes	13 (14.6)	0.22	0.68 (0.37 - 1.26)
Other chronic therapies			
No	48 (53.9)	Reference	Reference
Yes	41 (46.1)	0.18	0.74 (0.48 - 1.14)

[1]Variable treated as incremental value.
*Hazard ratio (HR) and relative 95% CI refer to the probability to convert the sputum smear microscopy.

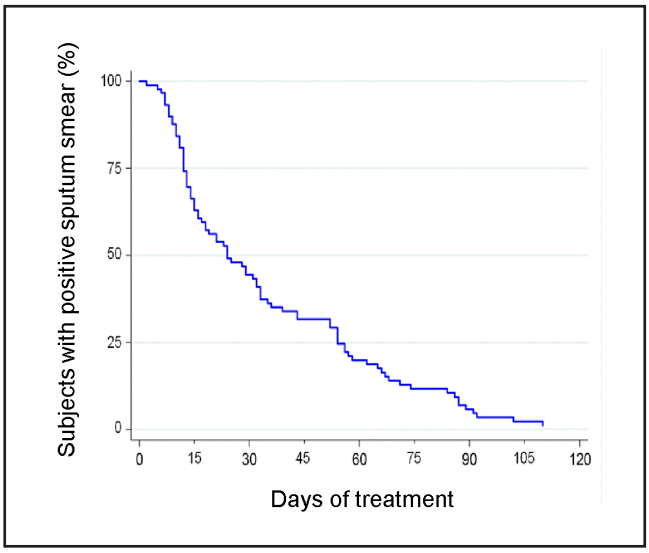

Figure 1 - *Proportion of patients with positive sputum smear during anti-TB treatment (days).*

1 month of anti-TB treatment about 20% of patients with an initial "low" Xpert result category still had not experienced sputum smear conversion, compared to about 60% of patients with initial "high" Xpert result.

DISCUSSION

Factors related to time to sputum smear conversion were previously described, finding correlation between male gender, age >40 years old, smoker, new TB case, high baseline sputum smear grading (3+/4+), multiple lung cavities documented by chest X-ray, altered baseline erythrocyte sedimentation rate (ESR) value or anemia at TB diagnosis, diabetes mellitus, HIV co-infection and treatment interruption (Telzak *et al.*, 1997; Domínguez-Castellano *et al.*, 2003; Singla *et al.*, 2003; Güler *et al.*, 2006; Wang *et al.*, 2009; Caetano *et al.*, 2012; Nagu *et al.*, 2014; Mlotshwa *et al.*, 2016). Our study describes for the first time the direct comparison of Xpert result category with time to sputum smear conversion.

Table 2 - *Cox regression analysis results.*

Variable	*p*	*HR (95% CI)**
Age	0.001	0.52 (0.36 - 0.76)
Baseline sputum smear grading[1]	0.07	0.76 (0.56 - 1.02)
Xpert MTB/RIF mycobacterial load[1]	0.03	0.58 (0.35 - 0.95)
Radiological classification[1]	<0.001	0.42 (0.26 - 0.68)

[1]Variable treated as incremental value.
*Hazard ratio (HR) and relative 95% CI refer to the probability to convert the sputum smear microscopy.

The observed median time to sputum conversion (24 days) was in line with the literature (Telzak *et al.*, 1997; Domínguez-Castellano *et al.*, 2003) and sputum smear conversion occurred in most patients (78.7%) in the first 2 months of treatment. Similar results were obtained in other studies with proportions ranging from 60% to 93% (Singla *et al.*, 2003; Wang *et al.*, 2009; Caetano *et al.*, 2012; Nagu *et al.*, 2014; Mlotshwa *et al.*, 2016).

Our analysis shows that older age, high Xpert MTB/RIF result category, high smear grading and severe involvement of the lung on radiography are risk factors for persistent sputum smear positivity. In contrast, male gender, alcohol abuse, any first-line anti-TB drugs resistance, anemia, diabetes, chronic therapies and altered baseline ESR value are not associated with delayed smear conversion.

The causes of prolonged sputum smear positivity in elderly subjects could be:

1) progressive impairment of the immune system, which may affect the clearance of mycobacteria in the lungs;
2) an increase in comorbidities and, consequently, more concomitant therapies (Singla *et al.*, 2003; Caetano *et al.*, 2012; Nagu *et al.*, 2014; Mlotshwa *et al.*, 2016).

We may also suppose that poor tolerability of first-line an-

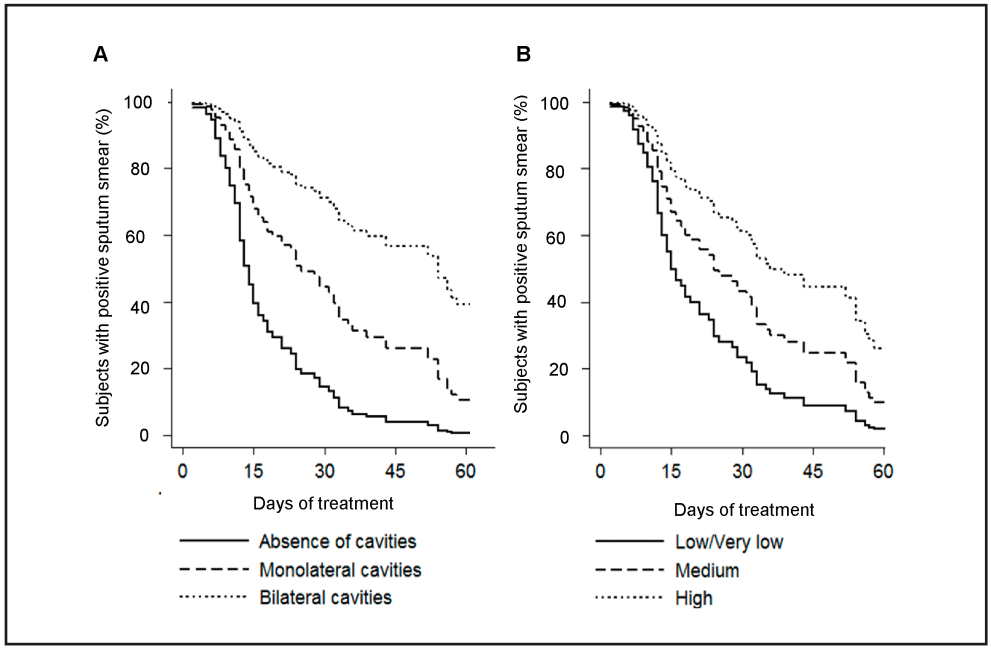

Figure 2 - *Sputum smear conversion time trend related to radiological classification of disease (A) and to Xpert MTB/RIF load (B).*

ti-TB drugs and drug interactions may play a significant role in delaying sputum smear conversion in this particular category of patients.

While the correlation between baseline sputum smear grading and smear conversion time is widely described in literature (Telzak *et al*., 1997; Ramarokoto *et al*., 2002; Wang *et al*., 2009; Caetano *et al*., 2012; Mlotshwa *et al*., 2016), there is currently no evidence of the association between the Xpert MTB/RIF result category and sputum smear conversion time. The objectivity of this molecular test and the possibility of rapidly identifying rifampicin resistance represent great advantages over smear microscopy (Opota *et al*., 2016; Tesfaye *et al*., 2017). From our analysis, the Xpert MTB/RIF result category also demonstrated a significant correlation with smear conversion time. To our knowledge, this is the first report describing a correlation between Xpert MTB/RIF result category and time to sputum smear conversion, which could be a useful parameter in the management of TB patients; previously Shenai *et al.* (2016) found a significant correlation between Xpert Ct and time to culture conversion (Shenai *et al*., 2016). Furthermore, Jayakumar *et al.* (2016) showed that quantitative outputs of the Xpert MTB/RIF assay may be useful as a dynamic measure of TB treatment response to Rifapentine (Jayakumar *et al*., 2016). Finally, we confirmed a significant association between the delay in sputum smear conversion and the severity of lung involvement. In fact, cavities are generally associated with a higher mycobacterial load in the pulmonary parenchyma (Matsuoka *et al*., 2004; Perrin *et al*., 2010), which requires more time to clear (Telzak *et al*., 1997; Domínguez-Castellano *et al*., 2003; Singla *et al*., 2003; Wang *et al*., 2009; Perrin *et al*., 2010).

We also developed predictive models to measure the time to sputum conversion based on patient characteristics. This model may greatly facilitate the management of smear-positive patients in terms of forecasting the duration of respiratory isolation and treatment. Nevertheless, prospective studies are needed in order to validate the predictive models in similar settings.

Our study has some limitations. First, it is a retrospective study, and so may be biased due to missing data (e.g., smoking) and the subjective evaluation of some variables. Second, there were no HIV co-infected patients in our study population, and this makes it impossible to apply our results to PLHIV. In addition, only the presence or absence of cavities and their distribution within the lungs (mono- or bilateral) were evaluated. Consequently, other pathological findings (i.e., nodules, tree-in-bud pattern, mediastinal lymphadenopathies) were not considered.

In conclusion, in this study we identified risk factors that delay sputum smear conversion: older age, high baseline sputum smear grading, high Xpert MTB/RIF result category and severe radiological findings.

Acknowledgements

The authors thank Dr. Paola Monari and Dr. Sonia Bonora for technical support and Jackie Leeder, BSc, for English language editing.

This work was partially supported by the contribution of the "Fondazione Del Monte of Bologna and Ravenna" (ID ROL FdM/6243, 2017).

References

Caetano Mota P., Carvalho A., Valente I., Braga R., Duarte R. (2012). Predictors of delayed sputum smear and culture conversion among a Portuguese population with pulmonary tuberculosis. *Rev Port Pneumol.* **18**, 72-79.

Cepheid. Xpert MTB/RIF - Package Insert. California, USA (2016).

Clinical and Laboratory Standards Institute. M48-A Laboratory detection and identification of Mycobacteria; approved guideline. Wayne, USA (2008). Available at: https://clsi.org/media/1447/m48a_sample.pdf

Domínguez-Castellano A., Muniain M.A., Rodriguez-Baño J., Garcia M., Rios M.J., et al. (2003). Factors associated with time to sputum smear conversion in active pulmonary tuberculosis. *Int J Tuberc Lung Dis.* **7**, 432-438.

Güler M., Ünsal E., Dursun B., AydIn Ö., Capan N. (2006). Factors influencing sputum smear and culture conversion time among patients with new case pulmonary tuberculosis. *Int J Clin Pract.* **61**, 231-235.

Jayakumar A., Savic R.M., Everett C.K., Benator D., Alland D., et al. (2016). Xpert MTB/RIF Assay Shows Faster Clearance of Mycobacterium tuberculosis DNA with Higher Levels of Rifapentine Exposure. *J Clin Microbiol.* **54**, 3028-3033.

Jensen P.A., Lambert L.A., Iademarco M.F., Ridzon R. Guidelines for preventing the transmission of Mycobacterium tuberculosis in healthcare settings, 2005. Centers for Disease Control. Atlanta, USA. 2005.

Matsuoka S., Uchiyama K., Shima H., Suzuki K., Shimura A., et al. (2004). Relationship between CT findings of pulmonary tuberculosis and the number of acid-fast bacilli on sputum smears. *Clin Imaging.* **28**, 119-123.

Mlotshwa M., Abraham N., Beery M., Williams S., Smit S., et al. (2016). Risk factors for tuberculosis smear non-conversion in Eden district, Western Cape, South Africa, 2007-2013: a retrospective cohort study. *BMC Infect Dis.* **16**, 365.

Nagu T.J., Spiegelman D., Hertzmark E., Aboud S., Makani J., et al. (2014). Anemia at the initiation of tuberculosis therapy is associated with delayed sputum conversion among pulmonary tuberculosis patients in dar-es-salaam, Tanzania. *PLoS One.* **9**, e91229.

Opota O., Senn L., Prod'hom G., Mazza-Stalder J., Tissot F., et al. (2016). Added value of molecular assay Xpert MTB/RIF compared to sputum smear microscopy to assess the risk of tuberculosis transmission in a low-prevalence country. *Clin Microbiol Infect.* **22**, 613-619.

Pefura-Yone E.W., Kengne A.P., Kuaban C. (2014). Non-conversion of sputum culture among patients with smear positive pulmonary tuberculosis in Cameroon: a prospective cohort study. *BMC Infect Dis.* **14**, 138.

Perrin F.M., Woodward N., Phillips P.P., McHugh T.D., Nunn A.J., et al. (2010). Radiological cavitation, sputum mycobacterial load and treatment response in pulmonary tuberculosis. *Int J Tuberc Lung Dis.* **14**, 1596-1602.

Ramarokoto H., Randriamiharisoa H., Rakotoarisaonina A., Rasolovavalona T., Rasolofo V., et al. (2002). Bacteriological follow-up of tuberculosis treatment: A comparative study of smear microscopy and culture results at the second month of treatment. *Int J Tuberc Lung Dis.* **6**, 909-912.

Shenai S., Ronacher K., Malherbe S., Stanley K., Kriel M., et al. (2016). Bacterial loads measured by the Xpert MTB/RIF assay as markers of culture conversion and bacteriological cure in pulmonary TB. *PLoS One.* **11**, e0160062.

Singla R., Osman M.M., Khan N., Al-Sharif N., Al-Sayegh M.O., et al. (2003). Factors predicting persistent sputum smear positivity among pulmonary tuberculosis patients 2 months after treatment. *Int J Tuberc Lung Dis.* **7**, 58-64.

Telzak E.E., Fazal B.A., Pollard C.L., Turett G.S., Justman J.E., et al. (1997). Factors influencing time to sputum conversion among patients with smear-positive pulmonary tuberculosis. *Clin Infect Dis.* **25**, 666-670.

Tesfaye A., Fiseha D., Assefa D., Klinkenberg E., Balanco S., et al. (2017). Modeling the patient and health system impacts of alternative Xpert® MTB/RIF algorithms for the diagnosis of pulmonary tuberculosis in Addis Ababa, Ethiopia. *BMC Infect Dis.* **17**, 318.

Wang J.Y., Lee L.N., Yu C.J., Chien Y.J., Yang P.C. et al. (2009). Factors influencing time to smear conversion in patients with smear-positive pulmonary tuberculosis. *Respirology*. **14**, 1012-1019.

World Health Organization. Definitions and reporting framework for tuberculosis – 2013 revision. Geneva, Switzerland (2014). Available at: http://apps.who.int/iris/bitstream/10665/79199/1/9789241505345_eng.pdf

World Health Organization. Guidelines for the treatment of drug-susceptible tuberculosis and patient care. Geneva, Switzerland (update 2017). Available at: http://apps.who.int/iris/bitstream/10665/255052/1/9789241550000-eng.pdf?ua=1

World Health Organization. Global tuberculosis report 2018. Geneva, Switzerland (2018). Available at: http://apps.who.int/iris/bitstream/handle/10665/274453/9789241565646 -eng.pdf?ua=1