

## Usefulness and limits of Ziehl-Neelsen staining to detect paragonimiasis in highly endemic tuberculosis areas



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### 1. Introduction

Tuberculosis (TB) and paragonimiasis are two endemic lung diseases in Lao People's Democratic Republic (Lao PDR). As pulmonary tuberculosis is the most common (70–80%) expression of *Mycobacterium tuberculosis* infection, pulmonary paragonimiasis (PP) is the most common (76–90%) clinical form of human infection with certain species of trematodes belonging to the genus *Paragonimus* (Singh et al., 2012). These two diseases share many common symptoms such as cough, hemoptysis, chest pain, sometimes accompanied by fever, asthenia and weight loss, while the lack of chest radio specificity does not allow to differentiate them from each other (Singh et al., 2012). In areas where the two endemics coexist, PP cases are often mistakenly considered as smear-negative TB and receive inappropriate treatment (Narain et al., 2004). In Lao PDR, sporadic cases of PP are reported from most provinces since 1947 (Kirkley, 1973), associated with traditional eating habits as the consumption of raw freshwater crabs or crayfishes containing metacercariae (Odermatt et al., 2009). Little is known about the distribution of this endemic parasitic disease, which appears scattered in multiple areas throughout the country. Thus, a cluster of 12 confirmed cases was identified in 2003 in a village of Hinheub District, Vientiane Province (Tran et al., 2004). The prevalence of PP is probably underestimated because it is often confused with TB or other lung diseases. It was estimated at 0.6% in a 2004 survey in three villages of Vientiane Province (Odermatt et al., 2007). Because misidentified and mistreated, paragonimiasis should be classified as neglected diseases in Laos (WHO, 2009).

So, it is recommended to rule out the diagnosis of PP by repeated egg search in sputum and pleural fluid before starting anti-TB antibiotic treatment (Singh et al., 2005). Although the shell of *Paragonimus* eggs is clearly revealed by the Ziehl-Neelsen (ZN) staining, this method has been rejected for the diagnosis of PP because it could damage the eggs in the sputum sample (Sadun &

**Abbreviations:** AFB, acid-fast bacilli; ELISA, enzyme-linked immunosorbent assay; ID, intradermal; IFMT, Institut de la Francophonie pour la Médecine Tropicale; Lao PDR, Lao People's Democratic Republic; NTC, National Tuberculosis Centre; PP, pulmonary paragonimiasis; TB, tuberculosis; WHO, World Health Organization; ZN, Ziehl-Neelsen.

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Buck, 1960). Various technical improvements to ZN staining have led to revise this opinion, concerning in particular the decolorisation solution and the heating time during the carbolfuchsin staining process (Slesak et al., 2011). Thus, a 2009 study in northern Laos demonstrated that the detection of *Paragonimus* eggs was more sensitive and less expensive with ZN stained sputum smears than with wet film (Slesak et al., 2011).

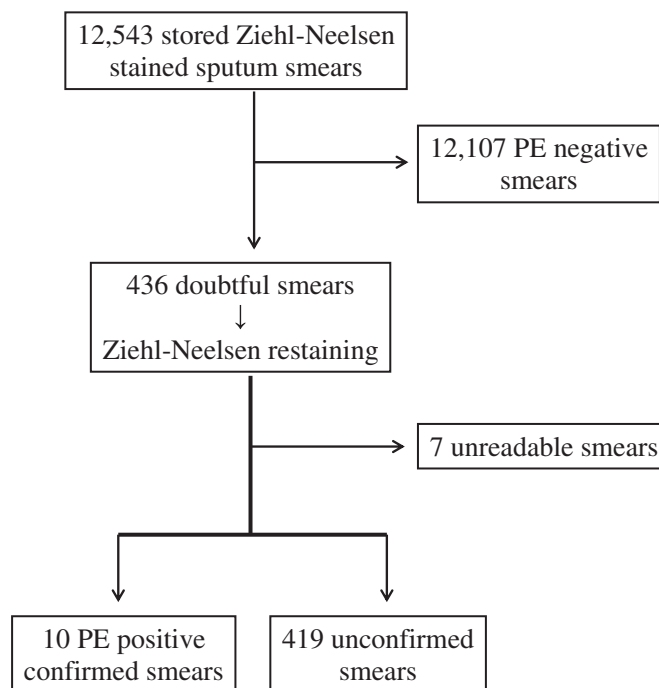
TB is a major public health problem in Laos. It ranks seventh leading cause of adult mortality. A first national TB prevalence survey was conducted in 2010 and 2011 by the National TB Programme supported by the World Health Organization (WHO) (Law et al., 2015). We took the opportunity of this national TB survey to retrospectively check for *Paragonimus* eggs by re-reading all the ZN stained smears.

## 2. Methods

### 2.1. Design of the study

The 2010–2011 national survey of TB prevalence had been performed on a representative sample (proportional to size of the 2005 population census) of 39,212 consenting participants aged 15 years and over, divided into fifty clusters screened by interview and chest X-ray. All participants with chronic cough for two weeks or more and/or haemoptysis in the previous month, and/or with radiological abnormalities suggestive of TB were asked to provide a spot and a morning sputum samples for microscopy and culture. At least one sputum was collected from each selected patient. All samples were sent to the TB reference laboratory. An aliquot of each sputum was set aside for the microscopic detection of acid-fast bacilli (AFB). Briefly, after heat fixation, hot ZN staining was performed: carbol fuchsin 1%, slow heating until steaming, rinsing after 10 min, destaining with 25% sulfuric acid for 3 min, rinsing, counterstaining with methylene blue 0.1% for 60 s, rinsing and drying (Lumb et al., 2013). The remaining sample was cultured without delay on Ogawa media.

We conducted a retrospective study on all sputum smears collected during this national survey, ie 12,543 ZN stained slides stored at the National Reference Laboratory for TB corresponding to 6290 participants (6253 provided two samples and 37 people provided only one sample). Light microscopy examinations were performed by nineteen trained students from the *Institut de la Francophonie pour la Médecine Tropicale* (IFMT) (Fig. 1). Each smear was directly read at  $\times 100$  magnification. All suspicious oval-shaped elements were examined at  $\times 400$  magnification and measured with a micrometer. Those between 80 and 120  $\mu\text{m}$  in length size were submitted to a senior biologist (YB) for validation and restained with ZN if necessary. For each confirmed case, the following data were collected: number of *Paragonimus* eggs identified in the slides, socio-demographics, geographic location of residence, symptoms of cough and haemoptysis, and chest X-ray findings that were secondarily reinterpreted by an expert (MS).



**Fig. 1.** Study flow chart: Microscopic screening of *Paragonimus* eggs on Ziehl-Neelsen stained sputum smears collected during the national Lao TB prevalence survey.

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