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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 ×100 magnification. All suspicious oval-shaped elements was examined at ×400 magnification and measured with a micrometer. Those between 80 and 120 μm 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).

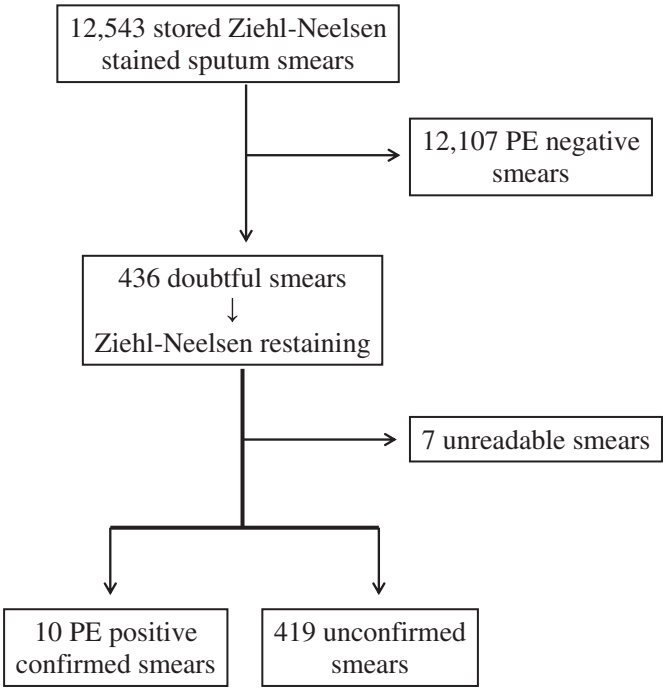

Fig. 1. Study flow chart: Microscopic screening of *Paragonimus* eggs on Zeehl-Nielsen stained sputum smears collected during the national Lao TB prevalence survey.

2.2. Data analysis

Data were entered using Microsoft Access 2007 software and statistical analyses were made using Stata version 11 (StataCorp LP).

2.3. Ethics statement

The national prevalence survey for tuberculosis in 2010–2011 was conducted by the National Tuberculosis Centre (NTC). Each subject participating in the prevalence survey was identified only by a reference number reported on the chest radiograph, smears and cultures. As sputum smears were anonymous, the identification numbers of patients detected with paragonimiasis were communicated to the NTC so that they were treated with praziquantel. The project has been approved by the National Ethics Committee for Health Research, Ministry of Health, Vientiane (No. 023/NECHR).

3. Results

3.1. Detection of Paragonimus eggs

The 12,543 sputum smears stored at the national TB reference laboratory for 6 to 19 months were stored at room temperature in a wooden slide box without a cover slip. Most of them had lost their stain reflecting the counterstaining with methylene blue. Restaining of 436 doubtful slides by ZN gave disappointing results throughout with non-contrasting colours and in 7 cases, a deterioration of the preparation with disappearance of the smear. *Paragonimus* eggs did not appear bright red on a blue background, but colored yellow to brown, sometimes distorted and without visible operculum, often with a longitudinal folding. The morphological alterations were even more important when the smears had been stored for a long time (Fig. 2). The number of eggs per slide ranged from 2 to 45, the spot sputum smear was more often positive than the smear of the next morning (Table 1).

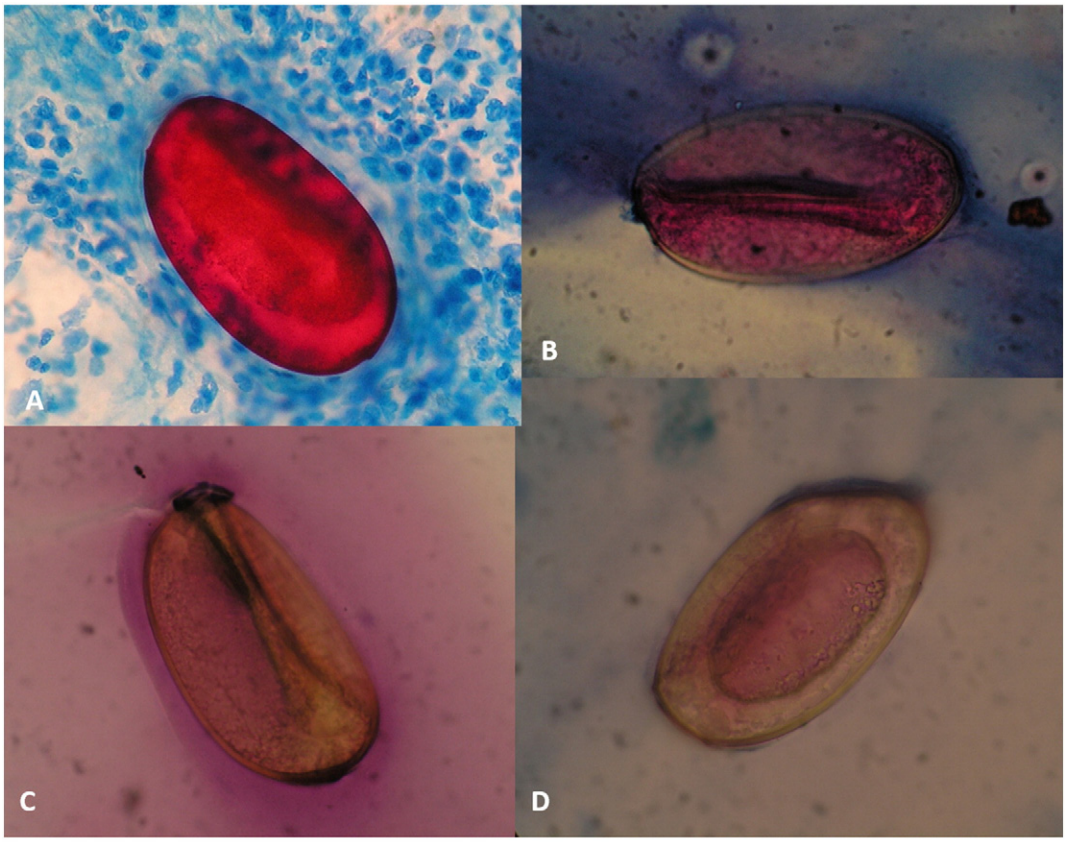

Fig. 2. *Paragonimus* eggs in Zeehl-Nielsen stained sputum smears according to the length of storage, Vientiane, Lao PDR 2012. [A: Fresh smear (reference); B: 6 months of storage; C: 17 months of storage; D: 19 months of storage].

Table 1
Characteristics of eight paragonimiasis cases.

Cases	Sex	Age (years)	Cough	Chest radiographic finding	AFB & Mtb cultures	Storage time* (months)	*Paragonimus* eggs/slide		Village District Province
							Day†	Morning‡	
1	M	42	10 days	Microcystic opacities in both lung fields, basal bilateral reticulo-nodular opacities, microcalcifications	negative	19	14	0	Dongkham Sanakham Vientiane
2	M	32	No	Large right pleural effusion, heterogeneous opacities of the left lower lobe with cystic pictures	negative	6	10	6	Napoung Boualapha Khammouane
3	M	64	No	Small cysts in the right base, small stellate opacities in the left base, microcalcifications	negative	11	2	0	Phoxai Champassak Champassak
4	F	50	3 days	Microcystic opacities in both lung fields, basal bilateral reticulo-nodular opacities, minimal right pleural effusion	negative	15	3	0	Poung Namtha Luang Namtha
5	M	32	45 days + haemoptysis	Microcystic opacities in the two bases, small stellate opacities in the left base, microcalcifications	negative	17	0	7	Parkxeng Parkxeng Luang Prabang
6	F	30	60 days + haemoptysis	Bilateral heterogeneous opacities, cystic or linear hilifuges	negative	17	45	12	Parkxeng Parkxeng Luang Prabang
7	M	40	No	Bilateral diffuse reticulonodular opacities, cystic center opacities in the two bases, microcalcifications	negative	17	6	0	Parkxeng Parkxeng Luang Prabang
8	F	46	>3 months + haemoptysis	Microcystic diffuse opacities in the right field, minimal right pleural effusion, microcalcifications	negative	17	17	0	Buamphasaeng Parkxeng Luang Prabang

AFB: acid fast bacilli; Mtb: *Mycobacterium tuberculosis*.
* : Storage time after Ziehl-Neelsen staining.
† :Smear of the day, at the moment of the survey.
‡ : Smear collected in the morning of the following day of the survey.

3.2. Paragonimiasis cases detected

In total, *Paragonimus* eggs were found on 10 sputum smears (0.08%) corresponding to 8 participants (0.13%). There were five men and three women, aged 42 ± 11 years (range 30–64). All were rice farmers, with the exception of a retiree. Among these 8 confirmed cases, 3 complained of chronic cough for over two weeks and reported bloody sputum. At the chest X-ray, all the 8 patients had microcystic opacities in both lung fields associated with various other abnormalities, mainly calcifications (5 cases), reticulo-nodular opacities (3 cases) or pleural effusion (3 cases) (Table 1; Fig. 3). Seven of the eight had been initially classified as suggestive of TB according to their chest X-ray. Beside a prevalence of smear-positive and culture-positive TB estimated

Fig. 3. Chest X-ray of a 40 years man, suggestive of TB infection, tested negative for acid-fast bacilli but positive for *Paragonimus* eggs in sputum microscopy.

to 278 per 100,000 and 595 per 100,000 respectively in the national survey [Law I, 2015], these 8 patients tested negative for AFB detection by smear microscopy and culture.

3.3. Geographical distribution

Four patients lived in two villages of the Pakxeng district (province of Luang Prabang), one in the Sanakham district (province of Vientiane), one in the Namtha district (province of Luang Namtha), one in the Boualapha district (province of Khammouane), and one in the Champassak district (province of Champassak) (Table 1; Fig. 4).

4. Discussion

The first national survey of TB prevalence conducted in Laos in 2010–2011 provided an unexpected opportunity to detect unrecognized paragonimiasis cases by a simple rereading of the ZN stained sputum smears. According to the results of the 2004 survey conducted in 3 neighboring villages in the province of Vientiane (Odermatt et al., 2007), expected rates of PP prevalence were around 12.7% in suspect patients. Our results are 100 times lower with rates of 127 per 100,000. How to explain such a low prevalence rate while paragonimiasis has been reported in almost all provinces of Lao PDR?

A first explanation lies in the sampling method of the national TB prevalence survey which aimed a good territorial representation of the entire population with 15 of the 50 clusters selected in urban areas (Law et al., 2015). In both cases, the sampling methodology was targeted, but not for the same purposes. While TB is mainly correlated with population density, the distribution

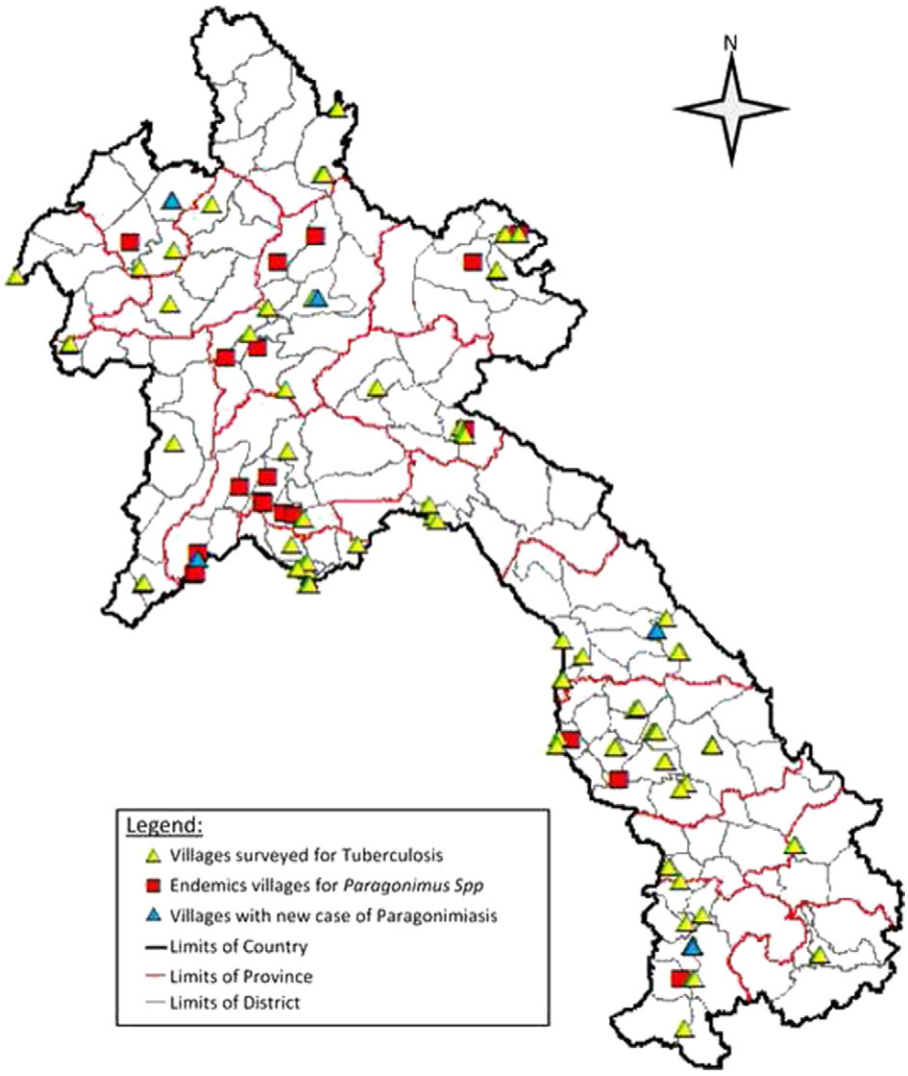

Fig. 4. Map of Lao PDR (1: location of the national TB survey's sites; 2: location of the sites previously known as endemic for paragonimiasis; 3: location of new paragonimiasis cases detected by retrospective eggs' screening in sputum smears).

of paragonimiasis is extremely focused in areas where intermediate hosts of the trematode (i.e. mollusks and crabs) coexist with traditional eating habits. Only by chance could the clusters of the national TB survey coincide with the foci of paragonimiasis.

In addition, the national TB survey only included people aged over 15 years, while the age group under 15 years is the most affected by PP. Indeed, in some villages, raw freshwater crabs and crustaceans are mostly eaten by children and their consumption decreases with age (WHO, 2009). Of 178 patients paragonimiasis cases diagnosed in North Vietnam, 68.5% were under 15 years (De et al., 2000). By excluding this population, the prevalence of the disease may have been underestimated.

The second explanation of this unexpectedly low positivity rate is provided by the technical conditions surrounding the microscopic detection of *Paragonimus* eggs. After a 6 to 19 months storage of ZN stained sputum smears in a non-air conditioned atmosphere, the bright red color of eggs due to carbolfuchsin had virtually disappeared. A rapid fading of ZN stained AFB had previously been noted in the quality control of sputum smear microscopy for TB screening, especially in countries with hot and humid climate like Bangladesh, Comoros and Laos, the "false positives" results becoming "true positives" after ZN restaining (Van Deun et al., 1997). Experimentally, with close to 100% humidity, AFB became invisible after 2 to 3 weeks at 40 °C and after 3 to 4 weeks at 35 °C (Van Deun et al., 1997). In such case, the ZN restaining of sputum smears allows to rediscover AFB that were discolored, but in our experience, it was ineffective on *Paragonimus* eggs, likely due to the cleaning in xylene of slides covered with immersion oil before ZN restaining. These technical drawbacks may have produced false negative results since the diagnosis was confirmed only on the presence of ovular elements between 80 and 120 μm in length retaining a residual carbolfuchsin staining. The visualization of operculum, less clear in *P. heterotremus* than in *P. westermani* (Miyazaki & Fontan, 1970; Yahiro et al., 2008), was not necessary to confirm the identification of eggs.

If the sputum microscopy allows a definitive diagnosis of PP, this method has a poor sensitivity because the eggs' release may be discontinuous, which warrants repeat testing in suspect patients (Toscano et al., 1994). In our study, six of the eight patients screened had only one positive sample of the two tested.

On the other hand, several methods of immunodiagnosis have been developed (Singh et al., 2012) to detect pre-patent stages and extrapulmonary forms of paragonimiasis (Narain et al., 2005). ID tests appeared very sensitive, easy to perform, inexpensive, and well suited to mass screening in a survey conducted in the Luang Prabang province, Laos, from 2003 to 2005 (Song et al., 2008). However, the ID tests remain positive for several years after the cure and may cross-react with other trematodiasis such as schistosomiasis and clonorchiasis when performed with crude antigen (Singh et al., 2012). ELISA tests, highly specific but more expensive than ID tests, can be used for mass screening, in addition to the detection of parasitic eggs in sputum (Narain et al., 2005). In a multi-dot ELISA seroprevalence survey conducted in northern Vietnam, *Paragonimus* eggs were detected in only 6 (19%) of the 32 patients tested positive (Doanh, 2011), indicating that screening based only on sputum examination leads to a major underestimation of the actual PP prevalence.

This study has some weaknesses, apart from the technical limitations detailed above. One can argue the lack of training for junior doctors responsible for rereading sputum smear microscopy. Teaching endemic parasitic diseases in Southeast Asia is part of the Master "Tropical Medicine and International Health" prepared in IFMT. It includes practices in parasitology that were supplemented by specific training in *Paragonimus* eggs identification. Such teaching method including theoretical courses, laboratory practice and field experience has been successfully tested in the Third Military Medical University in China to train students in diagnosis, treatment and prevention of paragonimiasis (Zhang et al., 2013).

Despite the small number of cases detected, this study provides three useful informations. First, it clarifies the conditions for using ZN smears for the detection of *Paragonimus* eggs in the sputum: (i) reading of the freshly stained smears at ×100 magnification to detect the eggs, then at ×400 magnification to define their size and morphology; (ii) reading at ×1000 magnification to detect AFB. This process should be systematic in all endemic areas for both TB and PP (Narain et al., 2004). Second, ZN restaining recommended for the quality control of TB microscopy screening is not relevant for detecting *Paragonimus* eggs. Third, this study identified hitherto unknown endemic areas in the province of Luang Prabang, located in the Pakxeng district. Previous surveys with ID test among schoolchildren and villagers had located another endemic area in the neighboring district of Namback (Song et al., 2008) and led to identify metacercariae of *P. harinasutai* in rock crabs belonging to the species *Indocinamon ou* (Sohn et al., 2009). Further studies are needed to investigate this new cluster, to determine the prevalence of human paragonimiasis in the villages of the district, to identify the species of *Paragonimus* and intermediate hosts involved in the parasitic cycle. They will help to complete the geolocation of human paragonimiasis foci to concentrate efforts for screening, treatment and prevention through health education. Considering these data, a national program of human paragonimiasis screening using ID or ELISA tests completed with ZN sputum smear microscopy should be jointly carried out with the national fight against TB in Lao PDR.

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