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THE USE OF ZIEHL-NEELSEN STAINING AND NAOH CONCENTRATION TECHNIQUES IN THE DETECTION OF *PARAGONIMUS* OVA IN SPUTUM: IMPLICATIONS FOR ENHANCED POLICY AND SERVICE DELIVERY

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ABSTRACT

Pulmonary paragonimiasis is a parasitic disease with signs and symptoms that mimic pulmonary tuberculosis (PTB). Current laboratory methods for diagnosis of paragonimiasis rely on ova detection in sputum specimen by microscopic examination through sodium hydroxide (NaOH) concentration technique, but studies have shown that the *Paragonimus* ova can also be seen using the Ziehl-Neelsen (ZN) staining technique for acid fast bacilli. This study aimed to revisit the use of both ZN staining and NaOH concentration techniques in the diagnosis of paragonimiasis and compare their detection rates. Sputum samples were collected from patients exhibiting signs and symptoms of PTB and were processed and examined using both acid-fast staining and NaOH techniques by trained microscopists. A total of 479 patients were included in this study. *Paragonimus* ova were seen in 14.2% of the patients by ZN technique, 7.5% by NaOH concentration technique, 19.2% by either technique, and 2.5% were positive by both techniques. Coinfection with PTB was seen in 0.4% of the patients. Using positivity for any one technique as reference standard, sensitivity for ZN technique and NaOH concentration technique were 73.9% and 39.1%, respectively. ZN technique shows a higher sensitivity (73.9%) than NaOH concentration technique (39.1%). In known co-endemic areas, using the acid fast staining techniques offer the benefit of screening for both PTB and paragonimiasis at the same time.

Keywords: Paragonimiasis, acid-fast staining, sodium hydroxide concentration

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INTRODUCTION

Pulmonary paragonimiasis, also known as lung fluke disease, is a parasitic diseases that presents clinically with signs and symptoms that mimic pulmonary tuberculosis (PTB) or even lung cancer (Mendenhall, 1937Under the broader umbrella of foodborne trematodiases (FBTs), paragonimiasis is considered to be a neglected tropical disease (NTD) targeted for control (WHO, 2020b). It is caused by *Paragonimus* spp. and is transmitted by consumption of raw, pickled or undercooked freshwater crabs and crayfish or boar (paratenic host) meat that contain the metacercariae which is the infective stage (WHO, 2020a). Because the signs and symptoms of paragonomiasis mimic PTB, it is often misdiagnosed (Mendenhall, 1937). As such, paragonimiasis should be considered in patients who come from areas co-endemic for PTB and paragonimiasis (Mendenhall, 1937; Toscano et al., 1994; Belizario et al., 1997).

About one million people are estimated to be infected with paragonimiasis every year (Yoshida et al., 2019), and 293 million people are at risk for infection (Kaiser & Utzinger, 2009). Paragonimiasis is known to be endemic across East Asia, Southeast Asia, and South Asia including India with prevalence rates ranging from 0.2-11.3% in Vietnam to 26.4% in Lao PDR (Yoshida et al., 2019). The species of *Paragonimus* known to cause human infection in these regions is *Paragonimus westermani* (Blair, 2022). In the Philippines, paragonimiasis is known to be endemic in 12 out of the 81 provinces with infection rates of 12.5-55.6% based on historical research data (Cabrera & Fevidal, 1974; Belizario et al., 1997). More recently, prevalence levels of 5.24% in the provinces of Zamboanga del Norte and 22.06% in Zamboanga del Sur have been reported (delos Trinos et al., 2020).

Current laboratory diagnosis for paragonimiasis relies on the detection of *Paragonimus* eggs in sputum and/or stool by microscopic examination (Singh, 2012). The World Health Organization (WHO) recommends that sputum be examined for *Paragonimus* ova using 3% sodium hydroxide (NaOH) concentration technique (1980), while the Ziehl-Neelsen (ZN) staining technique used for acid-fast bacilli (AFB) has been recommended as an alternative (Slesak et al., 2011; delos Trinos et al., 2020). These techniques have limited sensitivity with the NaOH concentration technique having a sensitivity of only 30-40% (Procop, 2009), and the ZN technique having variable reported sensitivity, from 27.59% to 76.90% (Slesak et al., 2011; delos Trinos et al., 2020). In the Philippines, based on previous studies (Belizario et al., 2014a), the Department of Health (DOH) recommends the use of the ZN technique as a screening method for diagnosis of paragonimiasis in known endemic provinces with NaOH concentration technique to be used for confirmation of ZN slides negative for paragonimiasis (2018).

Challenges with using the ZN technique include the potential destruction to the *Paragonimus* ova via the heating step (Sadun & Buck, 1960). This study used a cold modification of the ZN staining technique removes the prolonged heat mordant step (Kinyoun, 1915).

As more sensitive immunodiagnostic techniques and molecular assays are currently not available, microscopic techniques may still have considerable usefulness for laboratory confirmation of paragonimiasis in endemic areas. This study aimed to revisit the use of both ZN staining and NaOH concentration techniques in the diagnosis of paragonimiasis and compare their detection rates. Results of the study could provide basis

for enhancement of disease control policy and service delivery.

METHODS

Ethical considerations

The study protocol was reviewed and approved by the UP Manila Research Ethics Board (UPMREB 2021-0630-01). Informed consent was obtained prior to collection of sputum samples. All submitted sputum samples were processed and examined by trained medical technologists from the research team, DOH ZP-CHD, and partner LGUs. Patients who tested positive for paragonimiasis or PTB were referred to the respective rural health units (RHUs) for appropriate treatment following Philippine DOH guidelines.

Study site and participants

The study was conducted in selected municipalities of Zamboanga del Norte (Figure 1), a province known to be endemic for paragonimiasis (Belizario et al., 2007). The selection of municipalities was based on historical research data and willingness of local government units (LGUs) to cooperate. The selected municipality of President Manuel Roxas had previously been shown to have a paragonimiasis prevalence of 6.8-14.8% while Katipunan had been shown to have a paragonimiasis prevalence of 6.5% (Belizario et al., 2007, 2014). Study participants were selected based on the criteria for presumptive PTB which "refers to any person having: 1) two weeks or longer of any of the following – cough, unexplained fever, unexplained weight loss, night sweats; or 2) chest X-ray finding suggestive of TB" as stated in the National Tuberculosis Program Manual of Procedures (DOH, 2020).

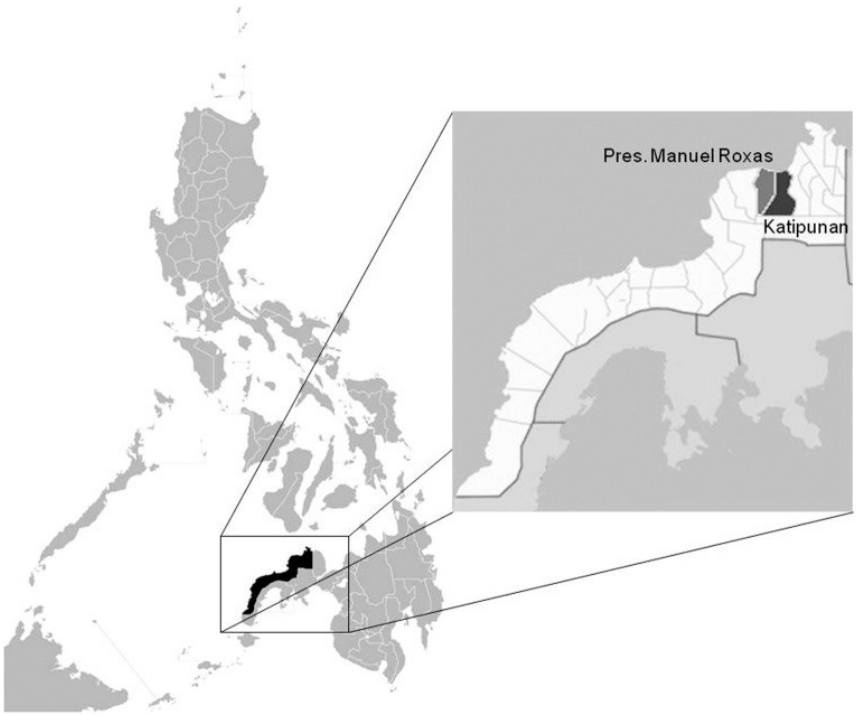


Figure 1 Map of study sites. Shaded areas represent the municipalities of Katipunan and President Manuel Roxas, Zamboanga del Norte, Philippines which are known to be endemic for paragonimiasis (Belizario et al., 2014).

Data collection and inclusion criteria

Data was obtained through active surveillance conducted by the study team together with local medical technologists who were trained on the microscopic diagnosis of *Paragonimus* ova in sputum using both NaOH concentration and ZN techniques in collaboration with the Department of Health Zamboanga Peninsula Center for Health Development (DOH ZP-CHD).

Patients were instructed to provide two sputum samples, one early morning (taken between 6 A.M. and 10 A.M.) and one spot sample (taken during specimen submission) with each sample labeled accordingly. Sputum samples were collected in transparent, screw-capped, sterile containers. Sputum quality was determined by trained personnel to ensure samples submitted were sputum and not saliva or nasal secretions. Insufficient samples and samples containing only saliva were not processed and examined (WHO, 2003).

Laboratory procedures

Laboratory procedures were done following standard laboratory safety guidelines. All research staff and medical technologists who handled specimens had personal protective equipment including gloves and a KN95 facemask. All laboratory procedures were done in a field laboratory with an open-air set-up.

For the ZN technique, sputum samples were processed using a modification of the cold method (Kinyoun, 1915). Samples were smeared and heat fixed. Smears were then stained with carbol fuchsin for 10-15 minutes at room temperature before washing and decolorizing with acid alcohol. Methylene blue was used as a counterstain for 2-5 minutes (Vasanthakumari et al., 1986).

For the NaOH technique, a 3% NaOH solution was prepared following standard laboratory procedures (WHO, 1980). Sputum samples were dissolved in the solution and centrifuged at high speed for five minutes and the sediment was examined under the microscope.

Smears processed from both ZN technique and NaOH concentration technique were read using 100x magnification for scanning and the 400x magnification for confirmation of identified *Paragonimus* ova. Demonstration of more than one identified ova in a single ZN slide constituted a positive case. Quality control was performed by agreement and by trained medical technologists on identified ova and digital capture of images for verification by a reference microscopist from the Department of Parasitology, College of Public Health, University of the Philippines Manila. The slides processed using ZN technique were also read for acid-fast bacilli by trained TB microscopists. Quality control for TB slides was performed by the blinded rechecking method (WHO, 2003). Results for TB reading will not be discussed in detail as it is not within the scope of this paper. Each sample was processed and examined in duplicate for both techniques. A positive result for at least one of the duplicate smears after quality control was considered as a positive result for the sample.

Data processing and analysis

Results from laboratory diagnosis for both ZN technique and NaOH concentration technique were double encoded for data validation, and positivity rates for tuberculosis for the former and paragonimiasis for each and either one of the two techniques were computed. Two by two tables comparing the rate of detection between ZN technique and NaOH technique was prepared to derive sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) using positivity for either one technique as the reference standard. Data were encoded and analyzed using Microsoft Excel.

RESULTS

In this study, the ZN technique commonly used for direct sputum smear microscopy to detect acid fast bacilli, was used to detect both PTB and paragonimiasis while the NaOH concentration technique is the current WHO recommended method to detect paragonimiasis through sputum concentration.

A total of 479 patients submitted sputum samples and were screened for paragonimiasis and PTB. Table 1 shows that, of the 479 patients examined, 68 (14.2%) were positive for *Paragonimus* spp. ova by ZN technique, 36 (7.5%) were positive by NaOH technique, 92 (19.2%) were positive by either technique, and 12 (2.5%) were positive by both techniques. Of the participants tested, only 11 (2.3%) were positive for TB, 2 (0.4%) of whom had coinfections with paragonimiasis.

Table 1 Paragonimiasis positivity rate by Ziehl-Neelsen and NaOH concentration techniques, tuberculosis positivity rate by Ziehl-Neelsen technique and coinfection rates

Selected municipalities, Zamboanga del Norte, December 2022 to May 2023

Parameter	No. (%)
Patients examined	479 (100%)
Patients positive for paragonimiasis by ZN technique	68 (14.2%)
Patients positive for paragonimiasis by NaOH concentration	36 (7.5%)
Patients positive for paragonimiasis by either NaOH concentration or ZN technique	92 (19.2%)
Patients positive for paragonimiasis by both NaOH concentration and ZN technique	12 (2.5%)
Patients positive for PTB by ZN technique	14 (2.9%)
Patients positive for both PTB and paragonimiasis	2 (0.4%)

Only 13.0% (12/92) of the patients with paragonimiasis were positive for both techniques. Of the 92 participants positive for paragonimiasis using either technique, 60.9% (56/92) were positive by ZN technique only and 26.1% (24/92) were positive by NaOH concentration technique only (Table 2-3).

Table 2 Comparison of ZN technique with ZN/NaOH techniques in detecting *Paragonimus* ova in sputum
Selected municipalities, Zamboanga del Norte, December 2022 to May 2023

	Ziehl-Neelsen (ZN)/NaOH concentration technique		
ZN technique	No. of patients positive for *Paragonimus* sp.	No. of patients negative for *Paragonimus* ova	**TOTAL**
No. of patients positive for *Paragonimus*	68	0	**68**
No. of patients negative for *Paragonimus*	24	387	**411**
TOTAL	**92**	**387**	**479**

Table 3 Comparison of NaOH technique with ZN/NaOH techniques in detecting *Paragonimus* ova in sputum
Selected municipalities, Zamboanga del Norte, December 2022 to May 2023

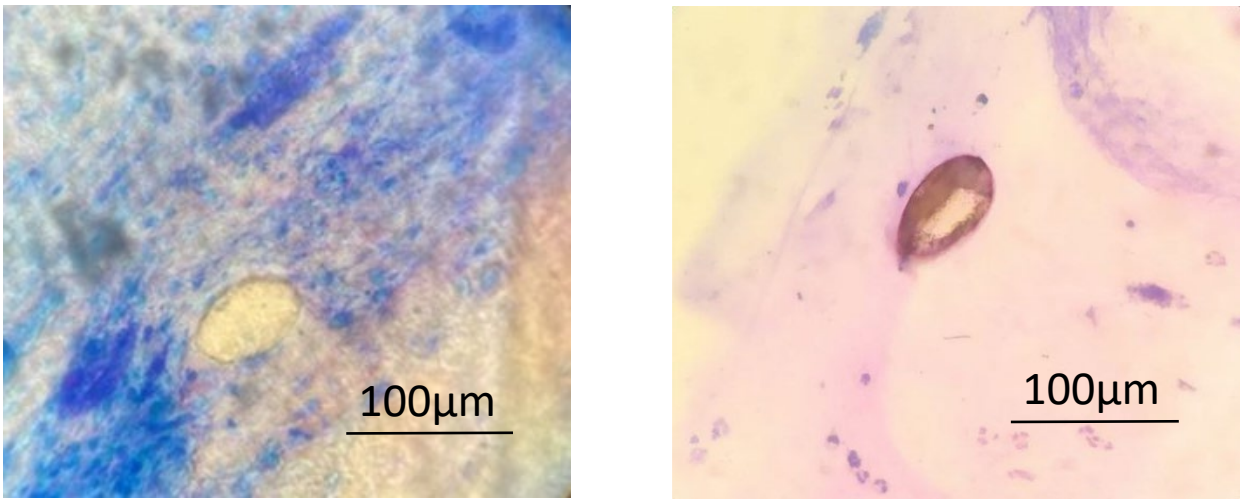
	Ziehl-Neelsen (ZN)/NaOH concentration technique		
NaOH technique	No. of patients positive for *Paragonimus*	No. of patients negative for *Paragonimus*	**TOTAL**
No. of patients positive for *Paragonimus*	36	0	**36**
No. of patients negative for *Paragonimus*	56	387	**443**
TOTAL	**92**	**387**	**479**

With positivity for any one technique as reference standard, the sensitivity of the ZN technique was almost twice as high (73.9%) than the sensitivity of NaOH concentration technique (39.1%). ZN technique also had a higher negative predictive value (94.2%) than NaOH concentration technique (87.4%) (Table 4). This suggests that ZN technique is able detect paragonimiasis better than the NaOH technique.

Table 4 Comparison of the measures of diagnostic accuracy of Ziehl-Neelsen and NaOH concentration techniques in detecting *Paragonimus* ova in sputum (n=479)

Measure of diagnostic accuracy	ZN (%, 95% CI)	NaOH (%, 95% CI)
Sensitivity	73.91 (63.71-82.52)	39.13 (29.12-49.86)
Specificity	100.00 (99.05-100.00)	100.00 (99.05-100.00)
Positive Predictive Value	100.00 (94.75-100.00)	100.00 (90.26-100.00)
Negative Predictive Value	94.16 (91.44-96.22)	87.36 (83.90-.31)

Figure 2 (a) Starw colored to clear, unstained and (b) yellowish-brown stained *Paragonimus* ova in ZN stain under HPO (400x magnification).

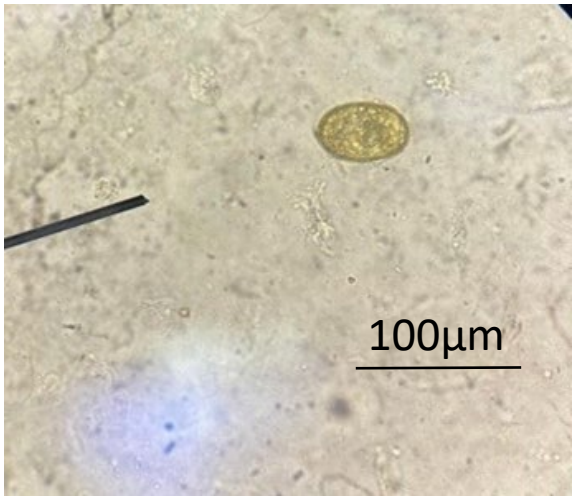


(a) (b)

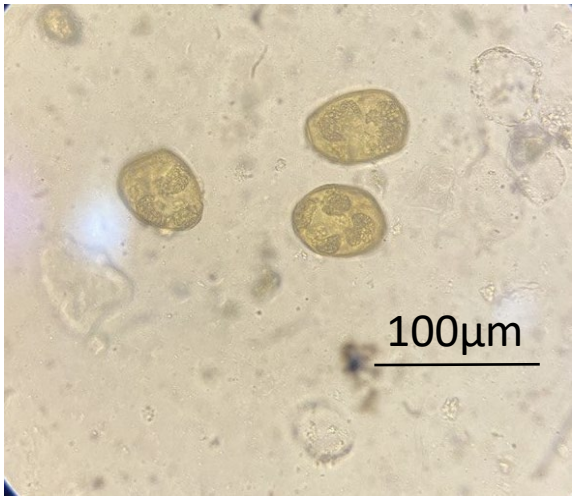
Egg morphology

In both techniques, the *Paragonimus* ova detected were 70-80 µm long and 40-45 µm wide. *Paragonimus* ova in ZN stain appears yellowish-brown, stained or straw-colored to clear unstained, ovoid or elongated, with a thick shell, and consistently have apparent operculum and abopercular thickening in a blue to purple background (Figure 2).In the slides examined using NaOH concentration technique, both typically bile-stained eggs and atypical pale yellow and broken eggs can be observed with a thick shell (Figure 3). Seemingly atypical eggs in NaOH concentration had comparable relative size and shape of ova with apparent operculum and abopercular thickening.

Figure 3 Typical (a), atypical (b), broken (c), and pale (d) *Paragonimus* ova in unstained sputum wet film using 3% NaOH concentration under HPO (400x magnification)



(a)



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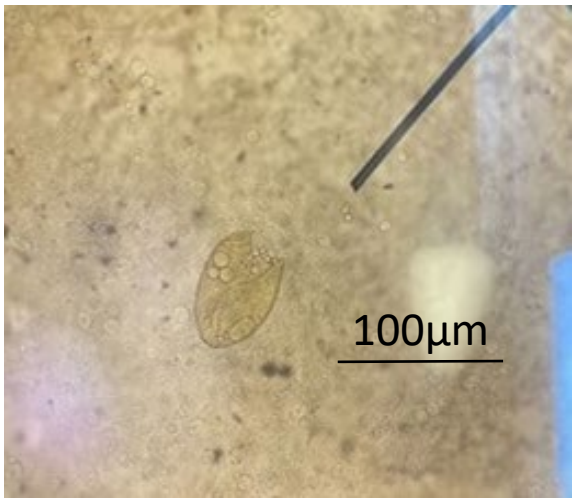
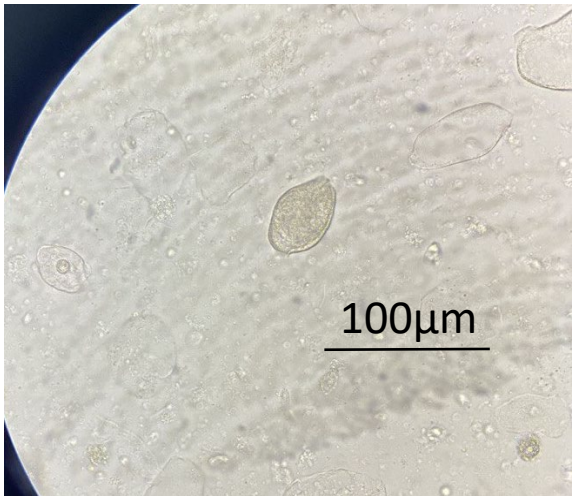


(c)



(d)

DISCUSSION

In this study, the ZN technique was shown to be able to detect *Paragonimus* ova with a sensitivity of 73.9%, which supports previous findings (Slesak et al., 2011). The appearance of *Paragonimus* ova using the standard hot method revealed eggs that were darkly stained (Slesak et al., 2011; delos Trinos et al., 2020), which is in contrast to the ova seen using ZN technique (cold method) in this study that showed eggs that were largely unstained and pale yellow, but still demonstrating the characteristic flattened operculum, thick shell, and abopercular thickening. Continuous heating of smears in the hot method could produce darkly stained eggs or fragments of eggs (Slesak et al., 2011). Using the cold method may increase the detection rate of paragonimiasis by the ZN technique. Coinfections of PTB with paragonimiasis were also seen in this study (0.4%). The difference in egg morphology between hot (possible fragmentation, darker staining) and cold (unstained pale yellow; lighter stained yellowish brown) ZN techniques should be noted during training for identification of *Paragonimus* ova which is highly recommended to be

incorporated with TB microscopic training for the simultaneous detection of PTB and paragonimiasis especially in co-endemic areas.

The use of 3% NaOH concentration was shown to have a sensitivity of 39.13%, which is similar to findings in previous studies (Procop, 2009). NaOH is a caustic agent which liquefies sputum but may also cause the eggs to appear pale, empty, or fragmented (Slesak et al., 2011). The correct concentration of the NaOH solution preparation may help reduce errors in specimen processing. Careful reading of slides by trained microscopists for possible pale, empty, fragmented, or altered ova will need to be considered. Direct wet smear without solution concentration may be used to detect *Paragonimus* ova in situations where NaOH is unavailable; however, sensitivity may be reduced (Toscano et al., 1994).

In the Philippines, the DOH has recommended the integration of the diagnosis of paragonimiasis with direct sputum-smear microscopy (DSSM) using ZN technique in seven regions with 16 provinces, taking advantage of the screening protocol for suspected PTB patients (2018); however, the recent guidelines recommend the use of GeneXpert as the first screening tool (DOH, 2020). The ZN technique can detect PTB in areas without access to GeneXpert or where there may be a lack of cartridges due to logistical challenges with the added benefit of being able to detect paragonimiasis in concerned endemic areas. The same guiding document recommends the use of NaOH concentration in the absence of *Paragonimus* ova in the ZN technique for confirmation (2018). As this study has demonstrated, 26.1% and 60.9% of the positive cases could be missed if ZN technique or NaOH concentration technique would be used in isolation, respectively; therefore, it is recommended that both techniques be used when available to increase likelihood of diagnosis of paragonimiasis. When only one of the techniques needs to be chosen, ZN technique may have a comparative advantage as it has a higher sensitivity (73.9%) and may also detect PTB.

In the clinical setting, medical technologists, especially TB microscopists and validators, need to be trained to identify typical and atypical *Paragonimus* ova morphology and size (70-80 µm long and 40-45 µm wide) in NaOH and ZN techniques. Training and capacity building of medical technologists to detect *Paragonimus* ova can enhance the ability to recognize ova (Belizario et al., 2014b). Having an ocular micrometer during training and capacity would be helpful in determining the relative size of the identified ova with respect to other objects in the field to help in verification. Increased capacity for detection of *Paragonimus* ova can enhance diagnosis for treatment and data collection to provide evidence for policy, planning and enhanced service delivery.

The use of ZN technique has the benefit of being fixed and can be read later by reference microscopists. This also reduces the risk of PTB transmission during processing which is present in NaOH concentration technique (Slesak et al., 2011). This study has demonstrated that ova seen in both NaOH and ZN slides can be confirmed by digital image capture of identified ova and referred to reference microscopists where an agreement between them has been used to constitute a confirmation of a positive result. While this study has shown that paragonimiasis can be detected through both ZN and NaOH concentration techniques, there still exists a need to develop more sensitive point of care immunoserologic and/or molecular test methods with more objective quality control measures to detect active cases of paragonimiasis especially among

asymptomatic patients and those having difficulty to produce sputum.

In the context of the COVID-19 pandemic where routine health services have been disrupted (Maravilla et al, 2023), it has been shown that COVID-19 presents similar symptoms as both paragonimiasis and PTB, and can be misdiagnosed (Blair, 2022). This presents a need for catch up with possibly more cases of paragonimiasis resulting from lack of case finding and treatment. Sputum microscopic examination by ZN technique and NaOH concentration may be useful for increased detection of paragonimiasis.

Both ZN and NaOH techniques are practical and feasible laboratory techniques for the detection of paragonimiasis. The ZN technique shows a higher sensitivity (73.9%) than NaOH technique (40.2%). The current guidelines used in the Philippines promote the use of GeneXpert as primary screening device which has lessened the role of DSSM and the ZN technique in PTB screening. In areas known to be endemic for both PTB and paragonimiasis, using the ZN technique offers the benefit of screening for both diseases at the same time. The findings of the study also support the reintegration of paragonimiasis detection using existing techniques in the National Tuberculosis Program in the light of the revision of previous guidelines and can be used to develop laboratory protocol guidelines for the inclusion of paragonimiasis in the national guidelines for PTB. Integrating paragonimiasis control with the existing national TB program is a more cost-effective way to implement control strategies for paragonimiasis (WHO, 2002).

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