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Microscopic examination of pig masseter muscle by pooled sample digestion method to identify the presence of *Trichinella* spp. larvae

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Abstract: Infection with the nematode worm *Trichinella* spp. causes the disease Trichinellosis which is zoonotic and occurs worldwide. Trichinellosis cases in Indonesia are rarely reported. Pigs are susceptible to *Trichinella* spp. infection. Examination of the presence of *Trichinella* spp. worms in pigs is not sufficient by simply looking at the clinical symptoms that appear, or through examination of feces, or blood. A definitive diagnosis for morphological and anatomical identification of *Trichinella worms* or their larvae must be made through microscopic examination. This study aimed to determine the presence of *Trichinella* worms in the Masseter muscle organs of pigs through microscopic examination. A cross-sectional study was conducted on 330 muscle (meat) samples of pigs in slaughterhouses in Kupang City. Muscle samples were examined for the presence of Trichinella larvae by pooled sample digestion (33 pools from 330 muscle samples) where positive results were examined individually by compression method. The results showed that 5 pooled muscle samples were positive for Trichinella. Positive results from 5 muscle pooled samples were followed by microscopic examination through the compression method. The results of the examination using the compression method found that 3 samples out of 330 muscle samples (0.9%) contained *Trichinella spiralis* larvae. These results indicate the presence of *T. spiralis* larvae in pigs slaughtered in Kupang City and could pose a threat to people who consume uncooked pork meat.
Keywords: *Trichinella spp*., cross-sectional study, pooled sample digestion, compression method.

1. Introduction

Trichinellosis is a parasitic disease caused by various species of nematode worms of the genus Trichinella that are enzootic worldwide in some carnivores and omnivores, especially scavenging animals (CDC, 2019; Dupouy-Camet, 2006). Humans can becomeinfected after ingesting or eating raw or undercooked meat containing live *encysted* larvae of *Trichinella* spp.

Examination of meat for the detection of Trichinella larvae is designed to prevent clinical trichinellosis in humans, but not to prevent infection (ISO, 2015). Identification of Trichinella larvae in muscle samples of pigs and other animal species (e.g. horses, pigs, wildlife, and bears) is limited to postmortem examination (Gondek et al., 2021; Mayer-Scholl et al., 2017; Gamble et al., 2000). Direct examination is also applied in sanctuary surveillance, where indicator animals (e.g. foxes or raccoons) are examined for the prevalence of Trichinella infections in wildlife reservoirs and the risk of transmission to domestic animals (Ryser-Degiorgis, 2013). Testing for Trichinella larvae in muscle samples is highly sensitive and results are influenced by sample size, the type of muscle selected for sampling, and the specific method used (Mayer-Scholl et al., 2017; Mangmee et al., 2020).

Digestive tracts in host animals that contain large numbers of Trichinella larvae cannot be used as clinical symbionts when compared to large numbers of Trichinella larvae in humans (Gajadhar et al., 2019). Identification of predilection sites that are useful for diagnostic purposes, especially for predilection sites in animal species, has been carried out by several experimental studies using the same or equivalent dose as the natural infection. In local pigs, the three preferred sites of *Trichinella spiralis* are the tongue, the Masseter muscle, and the *Diaphragm cruss* (Pozio, 2022; Nöckler et al., 2000). In addition to the best choice of muscle diagnostics, sufficient sample size is also required to generate a sensitive confidence level in detecting *Trichinella* larvae (Hajian-Tilaki, 2014).

Systematic examination of meat in animals for human consumption has an infection rate of at least 1 to 3 larvae per gram (LPG) of tissue for the sensitivity of the method, which is considered a standard level of food safety (Geiker et al., 2021). In the routine inspection of pig carcasses, if the pooled sample digest method *is* used at least 1 g of tissue from predilection organs should be examined (Biasino et al., 2018). The use of conventional trichoscopy for a single examination of pig carcasses (examination of 28 core-size diaphragm muscle cuts corresponding to a 0.5 g sample) is no longer recommended. This is due to the insensitivity ofthe method as well as the fact that non-encapsulated larval species such as *T. pseudospiralis* is very difficult to detect (Gottstein et al., 2009; Nöckler et al., 2000). Trichinoscopy can contribute to safeguarding public health under certain conditions and constraints of field conditions, where tools and materials are difficult to perform more sensitive testing via artificial digestion. At significantly greater prevalence rates, trichoscopy insensitivity poses a greater public health risk. Better diagnostic sensitivity is needed, especially for unacceptable false-negative test risk results (Shreffler & Huecker, 2020; Radua *et al.* 2011).

Application of the artificial digestion method is carried out by examination of groups of muscle samples up to 100 carcasses (Jiang et al., 2012). Muscle or meat samples susceptible to *Trichinella* spp. should undergo examination by the artificial digestion method (Mayer-Scholl et al., 2017; Nöckler et al., 2000). Application of the artificial digestion method requires a lot of technical equipment, but the method is efficient, cost-effective, highly sensitive, and non-encapsulated Trichinella larvae can be detected

through microscopic examination of digestive juices. Larvae can be excreted after digestion in muscle tissue with the use of artificial digestion fluids of 1% pepsin (1:10000; US National Formulary) and 1% hydrochloric acid, and subject to selective screening, filtration or sedimentation and microscopic examination (OIE 2004).

Several procedures for digestion methods are used in pooled samples digestions such as the stomacher method and Trichomatic 35 for the identification of Trichinella in meat, but the magnetic stirrer method is considered the gold standard because it is a method specifically designed for meat samples and has been validated (Nokler et al., 2009; OIE, 2008; Webster et al., 2006).

2. Materials e Methods

2.1. Research Location

Muscle samples were collected from Oeba slaughterhouse, Kupang City, East Nusa Tenggara Province, Indonesia. Examination and identification of muscle samples were carried out at the Microbiology Laboratory in the Animal Health Study Program of the State Agricultural Polytechnic of Kupang, Indonesia.

2.2. Research Sample Design and Population

This study was conducted with a cross-sectional study. The muscle selected for direct examination was the pig Masseter muscle. Muscle sampling was done by simple random sampling. The sample size was determined using Win Episcope 2.0 software, with a confidence level of 95%, an expected prevalence of 30%, as well as an error rate of 5%, with a totalof 330 Masseter muscle samples. Testing of muscle samples for *Trichinella* spp. larvae were carried out by pooled sample digestion with the magnetic stirrer method and if positive results were found, the identification of individual samples by the compression method was continued.

2.2.1. First Stage: Pooled Sample Digestion Through Magnetic Stirrer Method Examination Procedure

A total of 50 g of Masseter muscle from 10 pigs (5 g/head each) was minced in a blender using a special knife for meat to be digested with artificial digestive juices. The International Commission on Trichinellosis (ITC) recommends a muscle sample of 5 g per pig for endemic areas (Gamble et al., 2000). The artificial digestion fluid consisted of water (44 - 46 °C), 37% HCl, and pepsin (1:10000 NF/1:12500 BP/2000 FIP). The digestion was stirred for 30 min at 44 - 46 °C in a glass beaker (2-liter volume) using a hot plate magnetic stirrer (during this process the larvae were expected to detach from the muscles). The digestive fluid was then poured through a metal sieve (hole diameter ± 0.18 mm) into a glass funnel covered with a rubber hose clamp. The larvae were allowed to stay for 30 minutes and then 40 ml of sample liquid was quickly poured into a 50 ml tube, then left for more than 10 minutes to allow sedimentation (suspension). A total of 30 ml of supernatant was taken and the remaining 10 ml of sediment was poured into a petri dish for further examination for at least 8 minutes with a stereomicroscope to visualize or see the presence of *Trichinella* spp. larvae.

2.2.2. Second Stage: Compression Method

The examination was conducted using the digestion method with positive results for the presence of Trichinella larvae followed by individual examination of each sample using the compression method. The compression technique is mainly used to distinguish encapsulated and non-encapsulated *Trichinella* spp. larvae. The muscle samples were cut into pieces with each piece being as long as the muscle fiber with the thinnest possible cut. The muscle pieces were compressed between two glass plates (compression) until they were translucent, then examined individually for the presence of Trichinella larvae with a stereomicroscope through various magnifications (low range, mid-range, and high range). Morphological features of encapsulated and non-encapsulated *Trichinella* spp. larvae, are different and vary in shape (Sharma et al., 2020; Sacchi et al., 2001).

2.2.3. Third Stage: Larval Identification

The results of the examination were carried out using the pooled sample digestion method, if Trichinella larvae were found, followed by the compression method to determine the speciesof Trichinella larvae and calculate the prevalence.

The most distinguishing appearance of Trichinella larvae is the stichosome which consists of a series of discoid cells lining the esophagus and occupying the front half of the worm body (Gajadhar et al., 2019). Trichinella larvae may appear coiled (when cold), motile (when warm), or C-shaped (when dead) (Gajadhar et al., 2019a). If in doubt the larvae should be viewed at higher magnification and further tissue should be examined. Larvae recovered from muscle digestion can be stored in 70-75% ethanol (or 95% for long-term storage). This storage is useful for subsequent genotyping by polymerase chain reaction (PCR). Laboratories using artificial digestion methods should maintain appropriate quality assurance systems to ensure test sensitivity(Gajadhar et al., 2019; OIE 2012).

3. Results

3.1. Pooled Sample Digestion with Magnetic Stirrer Method

Trichinella spp. larvae were found in 5 *pooled samples of* pig muscle (sample codes E, I,K, L, and T). Microscopic images of *Trichinella* spp. larvae found can be seen in Figure 1.

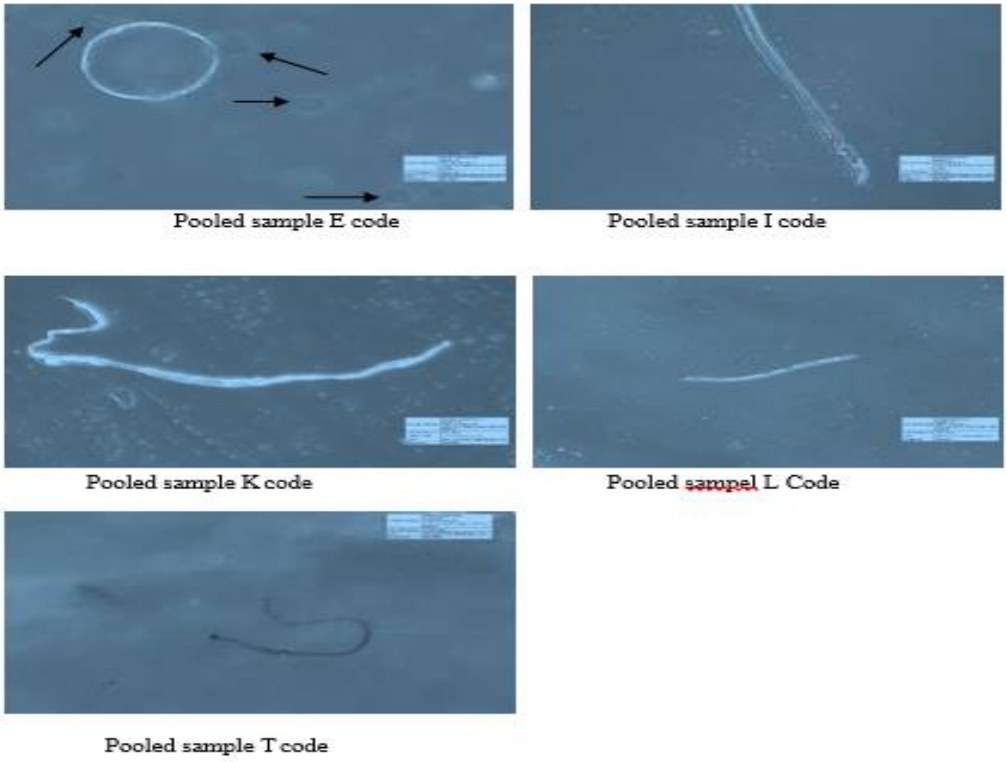

Figure 1 – *Trichinella* spp. larvae from *pooled sample digestion* examination.

The results of the examination conducted: pooled sample Code E is likely the larvae in a state of low temperature (circular shape). Pooled samples Code I, Code K, and Code L larvae aremotile, while for Code T it is likely that the larvae found are dead.

3.2. Compression Method

The results of the examination by the compression method of 50 individual samples (from 5 pooled samples positive results in the digestion test) with a stereomicroscope for the presence of *encapsulated* and non-encapsulated *Trichinella* spp. larvae were found to be encapsulated larvae of the *T. spiralis* species. Three *T. spiralis* larvae were found in 330 muscle samples (Table 1).

Sample code number	Date of sampling	Pig breeds	Age / Gender	Inspection Result
42	16/11/2013	Mixed breed	Above 1.5 yrs/ Male	(+)
105	8/12/2013	Local	Above 3 yrs/female	(+)
193	18/01/2014	*Triple cross*	Above 3 yrs/female	(+)

Table 1 – Positive samples tested by the compression method.

The prevalence of *T. spiralis* from pig muscle was 0.9%. Morphological features of *T.spiralis* in pig muscle found in the Oeba abattoir can be seen in Figure 2.

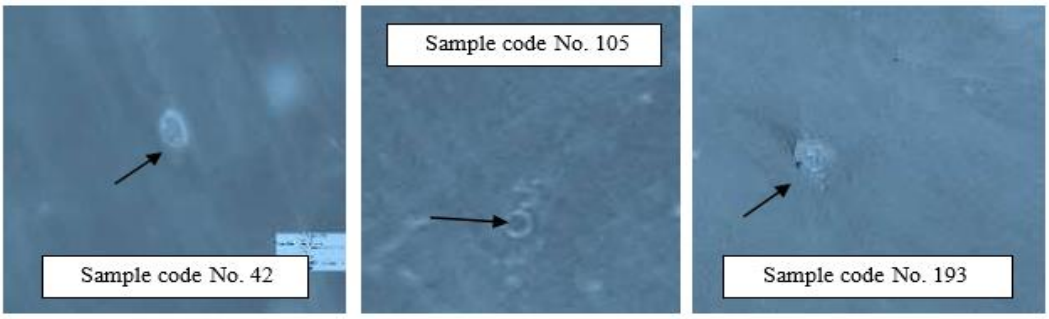

Figure 2 – *Trichinella spiralis* larvae examined by the compression method.

4. Discussion

The trichinellosis case found in the Oeba abattoir needs to be further investigated for its origin and source. The substandard condition of the abattoir with poor sanitation and hygiene means that small pieces of meat containing some larvae may be an important source of infection for other pigs. Lack of adequate hygiene practices in livestock production and weak implementation of Trichinella testing in abattoirs have contributed to the incidence of trichinellosis (Liu & Boireau, 2002; Wang et al., 2006). The artificial digestion method is the test of choice in diagnostics for Trichinella transmission control and eradication programs (Li et al., 2010; Jiang et al., 2012). Examination in pigs by the digestion method from infected farms is recommended ≥ 20 g of muscle tissue is used to improve the detection of animals at low levels of infection (Braasch et al., 2020; Murato et al., 2020).

T. spiralis, T. nativa, T. britovi, T. nelsoni and *T. murrelli* induce nurse cell formation in the striated muscle of the host, whereas the *non-encapsulated* species *T. pseudospiralis* and *T. papuae* are characterized by a lack of capsules around the larval muscle (Murrell *et al*. 2000). Non-encapsulated larval species are more difficult to detect by trichinoscopy or compression methods. All samples should first be examined by digestion, as the compression method cannot guarantee the presence of all *Trichinella* spp. (Pastusiak, 2006).

The incidence of *T. spiralis* in pig slaughterhouses in Kupang City is higher than that of pooled sample digestion in Thailand using the diaphragm muscle (0.05%) and in Chiang Mai (0.01%) (Takahashi et al., 2000). The prevalence in Kupang City could be related to farm management practices such asmost farmers still feeding leftover food from home or restaurants, as well as farmers' lack of knowledge about trichinellosis.

5. Conclusions

The results of the examination of the presence of *Trichinella* spp. larvae from pig muscle samples through pooled sample digestion with the magnetic stirrer method found *Trichinella*spp. Further examination of the presence of larvae found by the compression method leads to the larvae of the *T. spiralis* species, with a prevalence of 0.9%. The discovery of Trichinellaworms in Kupang's Oeba abattoir poses a zoonotic disease threat to the community in Kupang City, East Nusa Tenggara Province.

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