First Report of Pentastomiasis and the Lung Histopathological Changes in Ptyas mucosa in Sidoarjo, East Java, Indonesia

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Abstract. This study aimed to determine the prevalence of pentastomiasis, identify the parasite, and observe the pulmonary histopathology in *Ptyas mucosa* collected from snake slaughter in Sidoarjo (Indonesia). The identification of pentastomids was carried out by Semichen-Acetic Carmine, and histopathological preparations of the lungs were performed to observe the microscopic lesions. The results showed that 65% of the snakes had pentastomiasis, and the parasites were characterized as *Kirichephalus pattoni*. Microscopical examination revealed mild fibrosis in the pulmonary area as a result of pentastomid infestation. This is the first report of some pentastomiasis in *P. mucosa* in Sidoarjo, East Java, Indonesia.

Key words: *Kirichephalus pattoni*, Parasitic Pneumonia, Rat Snake, Zoonosis.

Abbreviations:

Running title: Pentastomiasis in Rat Snake

INTRODUCTION

Exotic animals such as reptiles, especially various types of snakes, are now popular as pets throughout the world (Okulewicz et al., 2014). However, they are also concerned with the increasing animal welfare and health issues through the animal trade in the market (Yudhana et al., 2021). Some species of snake are bred in captivity, while others are caught in the wild, and sold as pets or as bushmeat for consumption (Hardi et al., 2017; Yudhana et al., 2021).

Ptyas mucosa is one of the most common snake species found in Indonesia. This type of snake, known as the Oriental rat snake and Indian rat snake, belongs to the Colubridae family which is widely distributed throughout East, South, and Southeast Asia (Auliya, 2010; Muliya and Bhat, 2016; Yuan et al., 2022). *P. mucosa* is a diurnal and semi-arboreal snake species that lives on forest floors, agricultural fields, wet ground, and in close proximity to human habitats. It feeds on frogs, rats, and other small animals (Chittora et al., 2021).

The meat of *P. mucosa* is traded legally in China. The meat and internal organs are used as fresh or frozen food, the bile and blood are used in traditional medicine, and the skin is exported to several European countries as raw materials for the leather industry. Although it is illegal, most rural communities in Indonesia trade and consume the meat and bile of *P. mucosa* for some health reasons (Auliya, 2010; Pranashinta et al., 2017).

Wildlife meat, however, poses a threat of transmitting infection with novel and exotic pathogens to human hosts (Hardi et al., 2017). Then new zoonotic diseases are likely to emerge consequently. For instance, Bats (Chiroptera) are important reservoirs of several emerging diseases, including Ebola virus disease (EVD), severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS), as well as the current pandemic COVID-19 caused by SARS Coronavirus 2 (Irving et al., 2021). Many types of parasites are also the cause of zoonotic emerging diseases, resulting in a significant public health burden, particularly in tropical countries (Alasil and Abdullah, 2019).

Several parasites found in confined reptiles spend a portion of their life cycle in the respiratory system. The majority of respiratory involvement is asymptomatic and modest, generating mostly localized inflammation, with the exception of instances with excessive parasite loads. However, severe disease can result from high burdens or unusual host-parasite interactions, which is frequently made worse by secondary infections (Comolli, 2021). Reptile-borne pentastomiasis is an unusual tropical disease that is neglected and under-researched in Asia, Africa, and South America (Sulyok et al., 2014).

Pentastomiasis is a parasitic zoonosis disease caused by infection with pentastomids, belonging to the subclass Pentastomida, under the phylum Arthropoda (Chittora et al., 2021; Tongtako et al., 2013; WHA, 2019). Pentastomida are also known as tongue worms since the adult phase of the genus Linguatula has similarities with the mammalian tongues (Paré, 2008). Adult parasites are usually found in the respiratory tract of reptiles, birds, and mammals, including humans, while their larval forms are in the internal organs of insects, mammals, and amphibians (Paré, 2008; Chen et al., 2010; Kelehear et al., 2014). Some zoonotic pentastomida in snakes are Armillifer and *Porocephalus crotali* (Rataj et al., 2011). Humans are infected with pentastomids since they consume raw snake meat or drink water contaminated with parasitic eggs (Ayinmode et al., 2010; Kelehear et al., 2014; Tappe et al., 2016).

Pentastomiasis in snakes have been reported in many countries, such as poisonous *Bothrops asper* in Costa Rica (Alvarado et al., 2015), Royal Python snakes in Nigeria (Ayinmode et al., 2010), Rat snakes in Sri Lanka and India (Fernando and Fernando, 2014; Sundar et al., 2015), wild snakes in Australia (Kelehear et al., 2014), various species of snakes in Slovania and Pakistan (Rataj et al., 2011), *Agkistrodon acutus* and *Python molurus* in China (Chen et al., 2010), and *Crotalus totonacus* in Mexico (Tepos-Ramírez et al., 2022).

To the best of our knowledge, there are no reports of pentastomids infections in snakes in Indonesia. Therefore, this study reports the prevalence of pentastomiasis, the identification of pentastomids, and the pulmonary histopathological profiles in Rat snakes (*P. mucosa*) in Sidoarjo Sub-district, East Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

This study was reviewed and approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga (number 678-KE).

Parasites collection

The collection was conducted at a snake slaughterhouse in Tulangan District, Sidoarjo Regency, East Java Province, Indonesia. The presence of pentastomid parasites was observed in the respiratory tract of sixty slaughtered Ptyas mucosa snakes. Pentastomides were taken and cleaned with distilled water, then stored in sterile plastic pots. The lungs of the P. mucosa infected with the parasite were also isolated for histopathological preparation.

Prevalence calculation

The prevalence of pentastomiasis in snake samples was calculated by the following formula:

Prevalence (%) = (The number of snakes infected with pentastomids)/(Total sample of snakes) × 100%.

Semichen-acetic carmine staining

The collected pentastomids were stained with the Semichen-Acetic Carmine technique (Kuhlmann, 2006). Adult pentastomids were fixed on object glasses and soaked in 5% glycerin alcohol for 24 hours. Then they were soaked in 70% alcohol for five minutes. Subsequently, the object glasses with pentastomids were placed in Carmine alcohol solution (1:2) for eight hours. The pentastomids were soaked in acid alcohol for 10 minutes and moved to base alcohol for 20 minutes. Furthermore, graded dehydration was carried out with 70%, 85% and 95% alcohol for five minutes, respectively. The mounting step was done using Hung's I for 20 minutes. Pentastomids were taken and placed on new clean object glasses. The pentastomids were dripped with Hung's II solution and then covered with a cover glass.

Parasite identification

Characteristics of the head and body shape and body size of the parasite were identified based on Riley and Self (1980), Almedia et al. (2006), and Christoffersen and De Assis (2013; 2015). Identification of parasite egg size was carried out according to Keegan (1943).

The length of the fresh parasites was measured from the anterior edge to the posterior end. The head was observed under a stereo microscope and documented with a Canon Digital Single-Lens Reflex (DSLR) camera. Stained parasites were observed using a light microscope with 40-100× magnification and documented using Miconos Optilab. Some female parasites were incubated in PBS overnight at 37 °C to collect the eggs. The supernatants were centrifuged at 1,500 rpm for 10 minutes and the pellet was dripped onto a glass object and observed using a light microscope with 100× magnification. Eggs were measured using Miconos Optilab.

Histopathological preparations

The lungs of snakes with pentastomid infestation were fixed in 10% formalin. Then graded dehydration was carried out with serial alcohol concentrations (70%, 80%, 90%, 96%, and absolute) and clearing using xylol. Furthermore, paraffin embedding was performed to obtain a solid block of tissue. The tissue blocks were sliced (3 micrometers thick) using a microtome, mounted on an object glass, and stained using Hematoxylin and Eosin. Microscopic examination was performed using Nikon Eclipse Ci.

RESULTS AND DISCUSSIONS

The prevalence of pentastomiasis in *Ptyas mucosa* snakes

Thirty-nine or 65% of the respiratory tract of 60 were positively infected with adult pentastomids. Totally, 204 pentastomids were collected (Table 1). Pentastomida were found to be strongly attached to the trachea and lungs of snakes (Figure 1). Some pentastomids even buried their heads in the lungs of snakes. The parasites of all snakes positively infected with pentastomids were found in the lungs (39 snakes), while only 5 snakes which parasites spread to their tracheas.

Table 1. Prevalence of pentastomiasis in *Ptyas mucosa* based on site and number of parasites.

Sites	Positive (%)	Number of parasites
Lungs	57% (34 of 60)	2-9 with the average of 5 pentastomida
Trachea	0% (0 of 60)	-
Lungs and Trachea	8% (5 of 60)	7-9 with the average of 8 pentastomida
Total	**65% (39 of 60)**	**204 pentastomida**

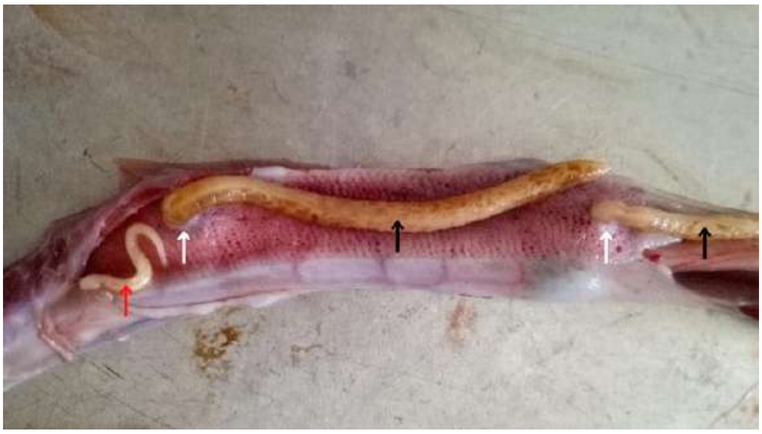

Figure 1. Pentastomid infestation in the lungs of *Ptyas mucosa*. The white arrows show areas with raised lesions around the attachment of pentastomids to the tissue. The black arrows show female pentastomids and the red arrow shows male pentastomids.

This is the first study to determine the prevalence of pentastomiasis in *Ptyas mucosa* in Sidoarjo, East Java Indonesia. As shown in our results, pentastomids were present in 39 of 60 (65%) snakes. Based on this finding, the prevalence of pentastomiasis in *P. mucosa* in this study was quite high. In this case, it was slightly the same as the prevalence of pentastomiasis in Brazil, 8 of 15 or 53.33% of snakes were positively infected with pentastomid. The high prevalence in this research was probably caused by the food consumed by the snakes. The type of food is very influential on the prevalence of infection and the type of pentastomida that infected the snakes (Almeida et al., 2007). *P. mucosa* snakes usually feed on rats, frogs, toads, lizards, and even smaller non-venomous and smaller snakes (Sidik, 2006). According to Esslinger (1962), rats, frogs, toads, lizards, and even non-venomous snakes can act as the intermediate hosts of pentastomida.

Identification of pentastomida

The characteristics of pentastomid found in this study can be seen in Table 2 and Figure 2. Pentastomids have a long cylindrical body with a larger head, a flat shape on the ventral, and a tapered tail or posterior. Their body was soft and whitish with a colorless cuticle, which tends to be transparent so that the internal organs are visible. There were annuli throughout their body up to the posterior. There are two pairs of hooks (a total of four hooks) around the head that are used to attach to the host's lung tissue, and it had an ovoid-shaped mouth between the two pairs of hooks.

Table 2. The characteristics of pentastomida compared with *Kiricephalus pattoni* by Riley and Self (1980) and Keegan (1943).

Parameters	The current study		British Museum collection (Riley and Self, 1980)		Keegan, 1943
	Females	Males	Females	Males	
Total number of parasites	183	21	17	2	-
Body length	72-125 mm ($\bar{X}$ 94.5 mm)	17-28.5 mm ($\bar{X}$ 23.75 mm)	83-137 mm ($\bar{X}$ 100 mm)	28-29 mm ($\bar{X}$ 28.5 mm)	-
Number of	29-41	28-35	34-38	31-32	-

annuli	($\bar{X}$ 31.6)	($\bar{X}$ 31)	($\bar{X}$ 36.5)	($\bar{X}$ 31.5)	
Diameter of cephalothorax	4-5.5 mm ($\bar{X}$ 4.75 mm)	2-2.5 mm ($\bar{X}$ 2 mm)	4 mm	-	-
Neck	2-3 mm ($\bar{X}$ 2.5 mm)	-	2 mm	-	-
Diameter of abdomen	3.5-5 mm ($\bar{X}$ 4.2 mm)	1.8-2.2 mm ($\bar{X}$ 2 mm)	4 mm	-	-
Hook parameter:			-	-	-
a. AC					
b. CB	0.23-0.55 mm	0.22-0.35 mm			
c. AD	0.37-0.69 mm	0.29-0.35 mm			
	0.47-0.88 mm	0.29-0.45 mm			
Mouth:			-	-	-
a. Length	0.16-29 mm	0.14-0.38 mm			
b. Width	0.12-0.24 mm	0.12-0.18 mm			
Egg:		-	-	-	
a. Length	114-130 µm ($\bar{X}$ 118 µm)				125-154 µm ($\bar{X}$ 133 µm)
b. Width	108-124 µm ($\bar{X}$ 116 µm)				106-143 µm ($\bar{X}$ 123 µm)

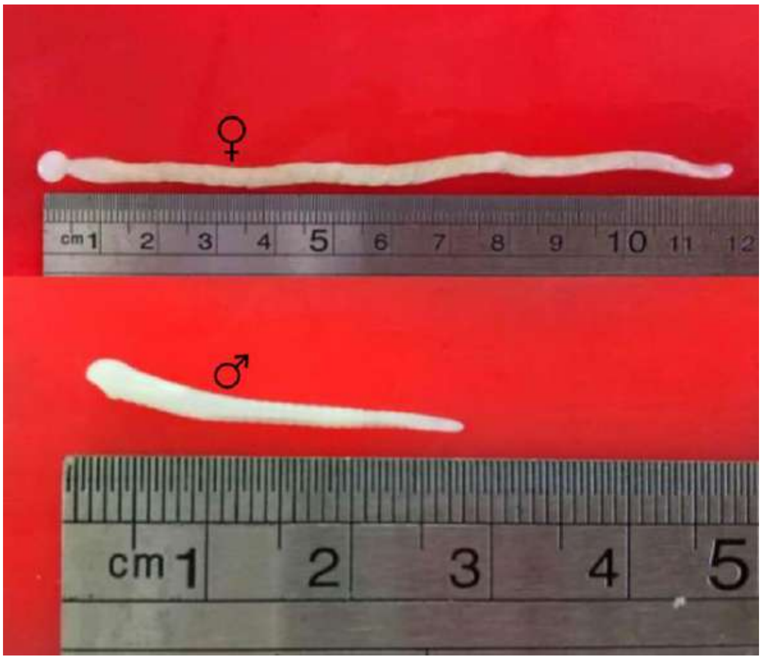

Figure 2. Differences in size between female (top) and male (bellow).

Both sexes, adult males and female pentastomids, were found in this research (Figures 1 and 2). The number of male pentastomid was less (21 heads) than that of females (183 heads) Table 2. Table 2 also describes the size of each pentastomid. The female pentastomids had longer and larger bodies than the males (Figures 1 and 2). The length of the female bodies was 72-125 mm with an average of 94.5 mm. Their abdominal diameter was 3.5-5 mm with an average of 4.2 mm. Furthermore, the anterior section or cephalothorax of the females was globular with a neck separating their head and their abdomen (Figure 3A). The average length and width of the female mouths were 0.24 mm and 0.16 mm. Furthermore, the results of the microscopy examination using Carmine staining showed that the female pentastomid had two spermathecae on the anterior part that connect directly to the uterus that contains the egg (Figures 3C, 4B, and 4C). Vagina was in the posterior body and around the anus (Figure 4B).

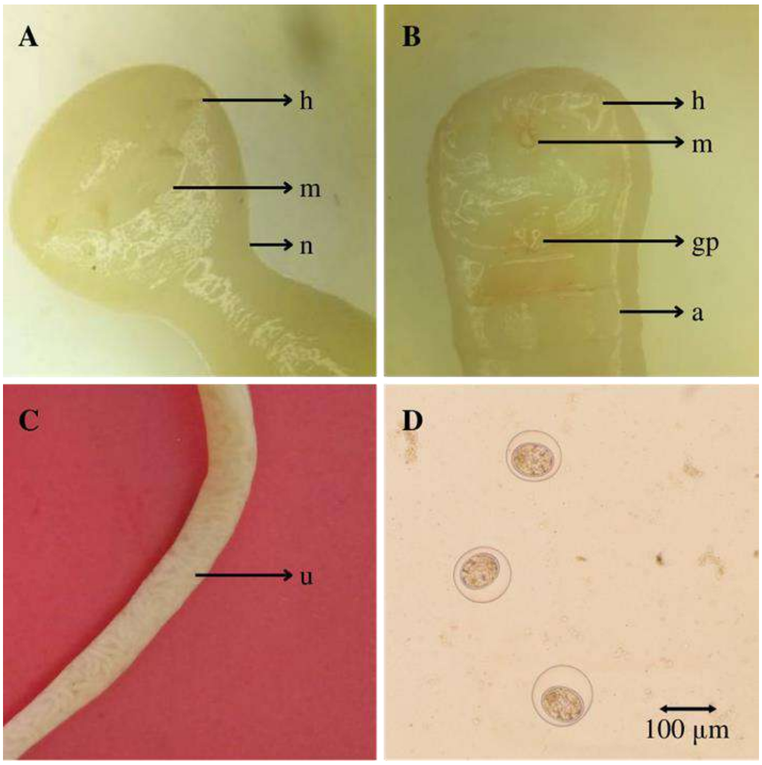

Figure 3. Pentastomida without staining. (A) The anterior section of the female. (B) The anterior section of the male. (C) Female body. (D) Pentastomid's eggs. h: hook, m: mouth, n: neck, gp: genital pore, a: first annuli, u: uterus.

On the other hand, the length of the males' bodies was 17-28.5 mm with an average of 23.75 mm. Their abdominal diameter was 1.8-2.2 mm with an average of 2 mm. The average length and width of the mouth were 0.23 and 0.14 mm, respectively. The male body was simple and straight without any curves in the neck (Figure 3B). The genital pore was identified in the first annulus and there was a pair of seminal vesicles, a pair of vasa deferens and cirrus (4D).

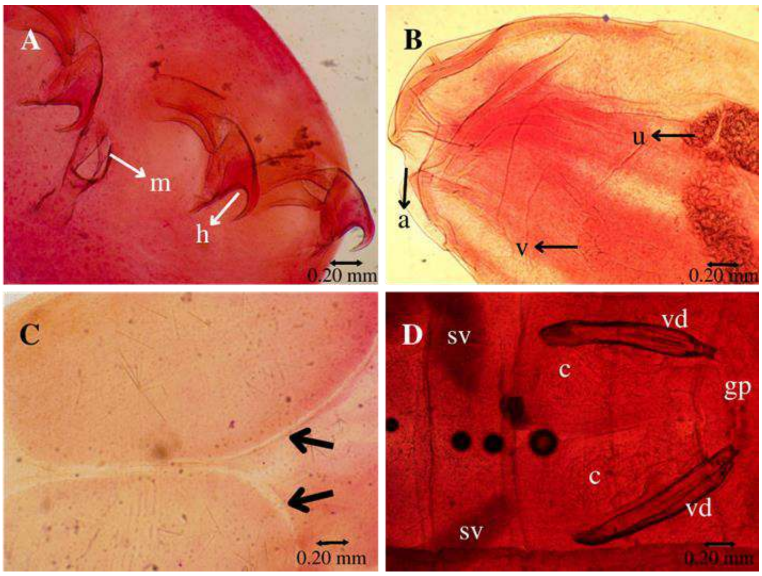

Figure 4. Carmine-stained pentastomida. (A) The anterior section. (B) The posterior section of the female. (C) A pair of female spermatheca. (D) Male reproductive organs. m: mouth, h: hook, a: anus, v: vagina, u: uterus, sv: seminal vesicle, c: cirrus, vd: vas deferens.

The eggs were oval and consisted of two layers of membrane that clearly could be distinguished. The first layer (outer membrane) was thin, while the second layer (inner membrane) was thick and yellowish. There was fluid-filled space between the first and second layers. The length of the eggs was 114-130 µm with an average of 118 µm and the width was 108-124 µm with an average of 116 µm (Figure 3D).

Pentastomida were mostly found in the lungs and the parasites also spread to the trachea in five snakes. This finding was similar to research by Riley and Self (1980) that revealed that adult *Kiricephalus pattoni* (one of the pentastomida

species) were only found in the lungs of *P. mucosa* snakes. In our observation, pentastomids that spread to the trachea were females. According to Sundar et al. (2015), the adult pentastomids with mature eggs are expelled from the trachea and eliminated from the definitive host by oral expulsion or may also be swallowed, resulting in shed eggs in the feces in which another intermediate mammal host will ingest to continue their life cycles.

The number of male pentastomids was fewer than the number of female ones. This case was also experienced by John and Nadakal (1988) and Riley and Self (1980) that only found a few males compared the female. We agree with Almeida et al. (2006) that pentastomids are not well known, in biology and the number of their species.

The taxonomy of pentastomida is still controversial. It has been known through molecular data that pentastomida are closely related to crustaceans and belong to class Maxillopoda, sub-phylum Crustacea, and the phylum Arthropoda (Paré, 2008). Based on the size of the egg (Almeida et al., 2007), the form of the female head that is globular with a neck separating the head from its body (Paré, 2008) and the size of the body length, the number of the annuli, the diameter of cephalothorax, neck, abdominal diameter, head, and body shape (Riley and Self, 1979; Riley and Self, 1980), the pentastomida found was classified as *K. pattoni*. *K. pattonii* can be classified into the order of porochepalida (Paré, 2008). According to Christoffersen and De Assis (2013; 2015), pentastomid in *P. mucosa* snakes are classified into *K. pattoni*.

K. pattoni has a long annulated body, a tapered tail, an anal canal, two pairs of hooks or a total of four hooks on its head, as well as a small ovoid-shaped mouth (Riley and Self, 1979). The female *K. pattoni* has a larger body than male one (Paré, 2008). Female *K. pattoni* also has a spiral body with a larger head than its body, and a curve of the neck, whereas male one has a straight body without a curve on the neck (Riley and Self, 1979; Riley and Self, 1980; John and Nadakal, 1988; Almeida et al., 2007). The spiral body in females is likely to facilitate their movement into the lung tissue of the definitive hosts (John and Nadakal, 1988).

The morphology of the eggs like the eggs of *Porocephalus crotali* described by Sundar et al. (2015), the eggs were composed of two-layer membranes of which the outer one is thin, and the inner membrane thick and there was a fluid-filled space between two membranes. But the average diameter of the eggs measured in this investigation is smaller (118 x 116 µm) than the eggs of *P. crotali* (144 µm) as described by Sundar et al. (2015).

Histopathological changes in the lungs of *Ptyas mucosa*

Squamates typically have two lungs, however, the left lung in most snakes is greatly reduced, never exceeding 85% of the right lung's size, or absent. The right lung usually runs from near the heart to the cranial to the right kidney. The cranial portion of a lung is vascularized and functions primarily as a gas exchanger (vascular lung), whereas the caudal primarily functions as an air sac (saccular lung). The vascular lung's wall is made up of honeycombed units called faveoli, which allow for gas exchange (Funk and Bogan, 2019). Microscopically, the faveoli in the snake lung parenchyma are lined mainly by squamous epithelial cells (type I cells) and, to a lesser degree, by cuboidal epithelial cells (type II) (Jacobson et al., 2021).

In this study, a nymphal stage of the parasite was found in the pulmonary tissue infected with pentastomida. The parasite is surrounded by some connective tissue (Figure 5). Finding the stages of the nymph in the histological lung of the snake and the stages of the parasite on the macroscopic lung organ means that the *P. mucosa* snake may act as an intermediate and final host of *K. pattoni*. Within the lung cavities of the definitive host, the nymphs develop into adults (Sundar et al., 2015). In this study, pentastomida infection in the lungs only caused a mild histological reaction in the form of fibrosis in the area around the parasite.

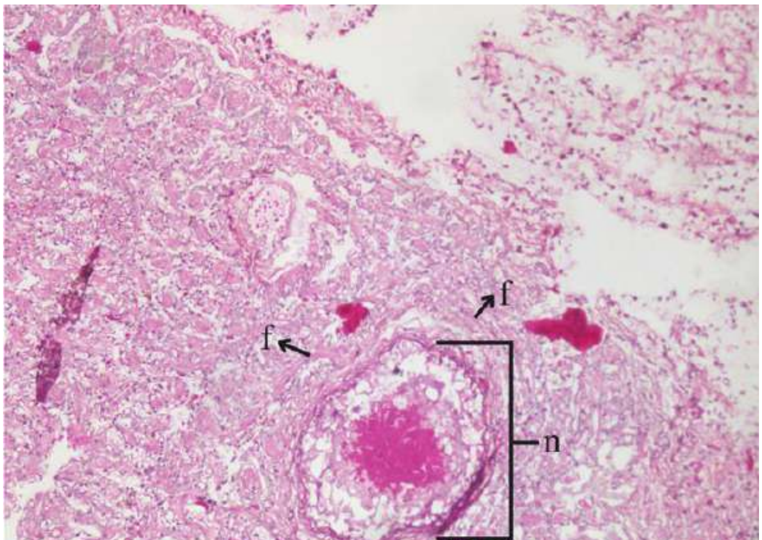

Figure 5. Histological microscopic picture of nymph-infected pulmo from pentastomida showed the presence of fibroblast around the area of infection. f: fibroblast, n: the body of the nymph stage pentastomida.

Pentastomida have an indirect life cycle that typically utilizes vertebrate hosts. Many of their species rely on reptiles as their definitive host, and the adults are usually found in the lungs (Wellehan and Walden, 2019). Pentastomida parasites migrate extensively through the visceral before settling in the lungs and, less typically, the trachea and nasal passageways. Pentastomida can produce localized lesions, whilst molting can elicit antigenic reactions. Adults feed on lung tissue fluids and pass embryonated eggs, which are coughed up through the oral cavity, swallowed, and passed in the feces (Comolli and Divers, 2021). In squamates, the pulmonary lumen is large, and the tracheal diameter may be smaller than that of the pentastomids. This poses a risk of respiratory obstruction by the adult pentastomes. However, the greatest pathological changes are seen in the intermediate host, where they may become embedded in tissues (Wellehan and Walden, 2019).

Pentastomiasis in reptiles, especially snakes, occurs subclinically, although the infection is large, and death is usually associated with severe pneumonia. Because pentastomids attach to the lung tissue, they can cause perforation, hemorrhaging, and serious inflammation in the host (Fernando and Fernando, 2014). In a Common Garter Snake (*Thamnophis sirtalis*), lung lesions were associated with one of the pentastomida species, *Kiricephalus coarctatus*, where the embedded parasite had gotten into the hypaxial muscle. Infections with *Armillifer armillatus* have also been linked to parasitic pneumonia in *Gaboon vipers*. Pentastomids occluding the trachea have been implicated in snake deaths. Bacterial infections could develop due to lung damage. Four of nine *Boa constrictors* with pulmonary damage from *Porocephalus dominicana* infection perished from bacteremia and pericarditis (Walden et al., 2021).

CONCLUSION

The prevalence of pentastomiasis in slaughtered *Ptyas mucosa* in Sidoarjo was 65% (39 of 60 snakes). The pentastomids that infect these snakes are classified as *Kiricephalus pattoni*. The pulmonary histological findings only show a light reaction in the form of mild fibrosis.

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First report of pentastomiasis and the lung histopathological changes in *Ptyas*[JB1][A2] *mucosa* in Sidoarjo, East Java, Indonesia

Abstract. This[JB3][A4] study aimed to determine the prevalence of pentastomiasis, identify the parasite, and observe the pulmonary histopathology in *Ptyas mucosa* collected from snake slaughter in Sidoarjo (Indonesia). The[JB5][A6] identification of pentastomids was carried out by Semichen-Acetic Carmine, and histopathological preparations of the lungs were performed to observe the microscopic lesions. The results showed that 65% of the snakes had pentastomiasis, and the parasites were characterized as *Kirichephalus pattoni*. Microscopical examination revealed mild fibrosis in the pulmonary area as a result of pentastomid infestation. This is the first report of some pentastomiasis in *P. mucosa* in Sidoarjo, East Java, Indonesia[JB7][A8].

Key words: *Kirichephalus pattoni*, Parasitic Pneumonia, Rat Snake, Zoonosis[JB9][A10].

Abbreviations:

Running title: Pentastomiasis in Rat Snake

INTRODUCTION

Exotic animals such as reptiles, especially various types of snakes, are now popular as pets throughout the world (Okulewicz et al., 2014). However, they are also concerned with the increasing animal welfare and health issues through the animal trade in the market (Yudhana et al., 2021). Some species of snake are bred in captivity, while others are caught in the wild, and sold as pets or as bushmeat for consumption (Hardi et al., 2017; Yudhana et al., 2021). [JB11][A12]

Ptyas mucosa is one of the most common snake species found in Indonesia[JB13][A14]. This type of snake, known as the Oriental rat snake and Indian rat snake, belongs to the Colubridae family which is widely distributed throughout East, South, and Southeast Asia (Auliya, 2010; Muliya and Bhat, 2016; Yuan et al., 2022). *P. mucosa* is a diurnal and semi-arboreal snake species that lives on forest floors, agricultural fields, wet ground, and in close proximity to human habitats. It feeds on frogs, rats, and other small animals (Chittora et al., 2021).

The meat of *P. mucosa* is traded legally in China[JB15][A16]. The meat and internal organs are used as fresh or frozen food, the bile and blood are used in traditional medicine, and the skin is exported to several European countries as raw materials for the leather industry[JB17][A18]. Although it is illegal, most rural communities in Indonesia trade and consume the meat and bile of *P. mucosa* for some health reasons (Auliya, 2010[JB19][A20]; Pranashinta et al., 2017).

Wildlife meat, however, poses a threat of transmitting infection with novel and exotic pathogens to human hosts (Hardi et al., 2017). Then new zoonotic diseases are likely to emerge consequently. For instance, Bats (Chiroptera) are important reservoirs of several emerging diseases, including Ebola virus disease (EVD), severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS), as well as the current pandemic COVID-19 caused by SARS Coronavirus 2 (Irving et al., 2021). Many types of parasites are also the cause of zoonotic emerging diseases, resulting in a significant public health burden, particularly in tropical countries (Alasil and Abdullah, 2019).

Several parasites found in confined reptiles spend a portion of their life cycle in the respiratory system. The majority of respiratory involvement is asymptomatic and modest[JB21][A22], generating mostly localized inflammation, with the exception of instances with excessive parasite loads. However, severe disease can result from high burdens or unusual host-parasite interactions, which is frequently made worse by secondary infections (Comolli, 2021). Reptile-borne pentastomiasis is an unusual tropical disease that is neglected and under-researched in Asia, Africa, and South America (Sulyok et al., 2014).

Pentastomiasis is a parasitic zoonosis disease [JB23][A24]caused by infection with pentastomids, belonging to the subclass Pentastomida, under the phylum Arthropoda (Chittora et al., 2021; Tongtako et al., 2013; WHA, 2019). Pentastomida are also known as tongue worms since the adult phase of the genus Linguatula[JB25][A26] has similarities with the mammalian tongues (Paré, 2008). Adult parasites are usually found in the respiratory tract of reptiles, birds, and mammals, including humans, while their larval forms are in the internal organs of insects, mammals, and amphibians (Paré, 2008; Chen et al., 2010; Kelehear et al., 2014). Some zoonotic pentastomida in snakes are Armillifer and *Porocephalus crotali* (Rataj et al., 2011). Humans are infected with pentastomids since they consume raw snake meat or drink water contaminated with parasitic eggs (Ayinmode et al., 2010; Kelehear et al., 2014; Tappe et al., 2016).

Pentastomiasis in snakes have been reported in many countries, such as poisonous *Bothrops asper* in Costa Rica (Alvarado et al., 2015), Royal Python[JB27][A28] snakes in Nigeria (Ayinmode et al., 2010), Rat snakes in Sri Lanka and India (Fernando and Fernando, 2014; Sundar et al., 2015), wild snakes in Australia (Kelehear et al., 2014), various species of snakes in Slovania and Pakistan (Rataj et al., 2011), *Agkistrodon acutus* and *Python molurus* in China (Chen et al., 2010), and *Crotalus totonacus* in Mexico [JB29][A30](Tepos-Ramírez et al., 2022).

To the best of our knowledge, there are no reports of pentastomids infections in snakes in Indonesia. Therefore, this study reports the prevalence of pentastomiasis, the identification of pentastomids, and the pulmonary histopathological profiles in Rat snakes (*P. mucosa*) in Sidoarjo Sub-district, East Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

This study was reviewed and approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga (number 678-KE).

Parasites collection[JB31][A32]

The collection was conducted at a snake slaughterhouse in Tulangan District, Sidoarjo Regency, East Java Province, Indonesia. The presence of pentastomid parasites was observed in the respiratory tract of sixty slaughtered Ptyas mucosa [JB33][A34]snakes. Pentastomides were taken and cleaned with distilled water, then stored in sterile plastic pots. The lungs of the P[JB35][A36]. mucosa infected with the parasite were also isolated for histopathological preparation[JB37][A38][A39].

Prevalence calculation

The prevalence of pentastomiasis in snake samples was calculated by the following formula:

Prevalence (%) = (The number of snakes infected with pentastomids)/(Total sample of snakes) × 100%.

Semichen-acetic carmine staining

The collected pentastomids were stained with the Semichen-Acetic Carmine technique[JB40] (Kuhlmann, 2006). Adult pentastomids were fixed on object glasses and soaked in 5% glycerin alcohol for 24 hours. Then they were soaked in 70% alcohol for five minutes. Subsequently, the object glasses with pentastomids were placed in Carmine alcohol solution (1:2) for eight hours. The pentastomids were soaked in acid alcohol for 10 minutes and moved to base alcohol for 20 minutes. Furthermore, graded dehydration was carried out with 70%, 85% and 95% alcohol for five minutes, respectively. The mounting step was done using Hung's I for 20 minutes. Pentastomids were taken and placed on new clean object glasses. The pentastomids were dripped with Hung's II solution and then covered with a cover glass.

Parasite identification

Characteristics of the head and body shape and body size of the parasite were identified based on Riley and Self (1980), Almedia et al. (2006), and Christoffersen and De Assis (2013; 2015). Identification of parasite egg size was carried out according to Keegan[JB41][A42] (1943).

The length of the fresh parasites was measured from the anterior edge to the posterior end. The head was observed under a stereo microscope and documented with a Canon Digital Single-Lens Reflex (DSLR) camera. Stained parasites were observed using a light microscope with 40-100× magnification and documented using Miconos Optilab. Some female parasites were incubated in PBS overnight at 37 °C to collect the eggs. The supernatants were centrifuged at 1,500 rpm for 10 minutes and the pellet was dripped onto a glass object and observed using a light microscope with 100× magnification. Eggs were measured using Miconos Optilab.

Histopathological preparations

The lungs of snakes with pentastomid infestation were fixed in 10% formalin. Then graded dehydration was carried out with serial alcohol concentrations (70%, 80%, 90%, 96%, and absolute) and clearing using xylol. Furthermore, paraffin embedding was performed to obtain a solid block of tissue. The tissue blocks were sliced (3 micrometers thick) using a microtome, mounted on an object glass, and stained using Hematoxylin and Eosin. Microscopic examination was performed using Nikon Eclipse Ci.

RESULTS AND DISCUSSIONS

The prevalence of pentastomiasis in *Ptyas mucosa* snakes

Thirty-nine or 65% of the respiratory tract of 60 were positively infected with adult pentastomids. Totally[JB43][A44], 204 pentastomids were collected[JB45][A46] (Table 1). Pentastomida were found to be strongly attached to the trachea and lungs of snakes (Figure 1). Some pentastomids even buried their heads in the lungs of snakes. The parasites of all snakes positively infected with pentastomids were found in the lungs (39 snakes), while only 5 snakes which parasites spread to their tracheas.

Table 1. Prevalence of pentastomiasis in *Ptyas mucosa* based on site and number of parasites.

Sites	Positive (%)	Number of parasites
Lungs[JB47][A48]	57% (34 of 60)	2-9 with the average of 5 pentastomida
Trachea	0% (0 of 60)	-
Lungs and Trachea	8% (5 of 60)	7-9 with the average of 8 pentastomida
Total	**65% (39 of 60)**	**204 pentastomida**

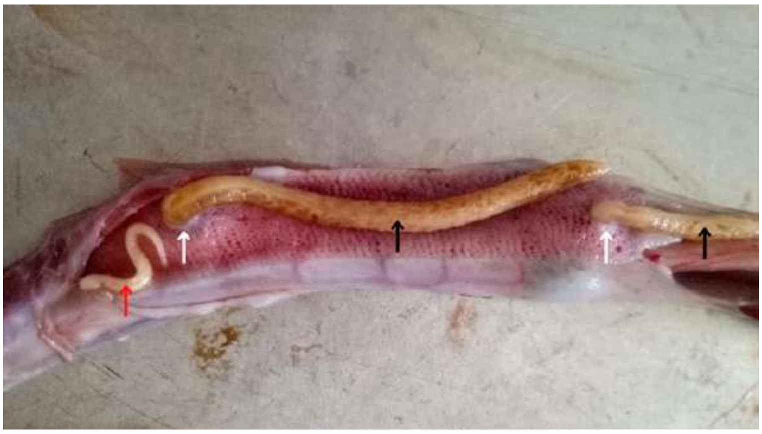

Figure 1. Pentastomid infestation in the lungs of *Ptyas mucosa*. The white arrows show areas with raised lesions around the attachment of pentastomids to the tissue. The black arrows show female pentastomids and the red arrow shows male pentastomids.

This is the first study to determine the prevalence of pentastomiasis in *Ptyas mucosa* in Sidoarjo, East Java Indonesia. As shown in our results, pentastomids were present in 39 of 60 (65%) snakes. Based on this finding, the prevalence of pentastomiasis in *P. mucosa* in this study was quite high. In this case, it was slightly the sa[JB49][A50]me as the prevalence of pentastomiasis in Brazil, 8 of 15 or 53.33% of snakes were positively infected with pentastomid. The high prevalence in this research was probably caused by the food consumed by the snakes. The type of food is very influential on the prevalence of infection and the type of pentastomida that infected the snakes (Almeida et al., 2007). *P. mucosa* snakes usually feed on rats, frogs, toads, lizards, and even smaller non-venomous and smaller snakes (Sidik, 2006). According to Esslinger (1962), rats, frogs, toads, lizards, and even non-venomous snakes can act as the intermediate hosts of pentastomida.

Identification of pentastomida

The characteristics of pentastomid found in this study can be seen in Table 2 and Figure 2. Pentastomids have a long cylindrical body with a larger head, a flat shape on the ventral, and a tapered tail or posterior. Their body was soft and whitish with a colorless cuticle, which tends to be transparent so that the internal organs are visible. There were annuli throughout their body up to the posterior. There are two pairs of hooks (a total of four hooks) around the head that are used to attach to the host's lung tissue, and it had an ovoid-shaped mouth between the two pairs of hooks[JB51][A52].

Table 2. The characteristics of pentastomida compared with *Kiricephalus pattoni* by Riley and Self (1980) and Keegan (1943).

Parameters	The current study		British Museum collection (Riley and Self, 1980)		Keegan, 1943
	Females	Males	Females	Males	
Total number of parasites	183	21	17	2	-
Body length	72-125 mm (X̄ 94.5 mm)	17-28.5 mm (X̄ 23.75 mm)	83-137 mm (X̄ 100 mm)	28-29 mm (X̄ 28.5 mm)	-
Number of	29-41	28-35	34-38	31-32	-

annuli	($\bar{X}$ 31.6)	($\bar{X}$ 31)	($\bar{X}$ 36.5)	($\bar{X}$ 31.5)	
Diameter of cephalothorax	4-5.5 mm ($\bar{X}$ 4.75 mm)	2-2.5 mm ($\bar{X}$ 2 mm)	4 mm	-	-
Neck	2-3 mm ($\bar{X}$ 2.5 mm)	-	2 mm	-	-
Diameter of abdomen	3.5-5 mm ($\bar{X}$ 4.2 mm)	1.8-2.2 mm ($\bar{X}$ 2 mm)	4 mm	-	-
Hook parameter:			-	-	-
a. AC					
b. CB	0.23-0.55 mm	0.22-0.35 mm			
c. AD	0.37-0.69 mm	0.29-0.35 mm			
	0.47-0.88 mm	0.29-0.45 mm			
Mouth:			-	-	-
a. Length	0.16-29 mm	0.14-0.38 mm			
b. Width	0.12-0.24 mm	0.12-0.18 mm			
Egg:		-	-	-	
a. Length	114-130 µm ($\bar{X}$ 118 µm)				125-154 µm ($\bar{X}$ 133 µm)
b. Width	108-124 µm ($\bar{X}$ 116 µm)				106-143 µm ($\bar{X}$ 123 µm)

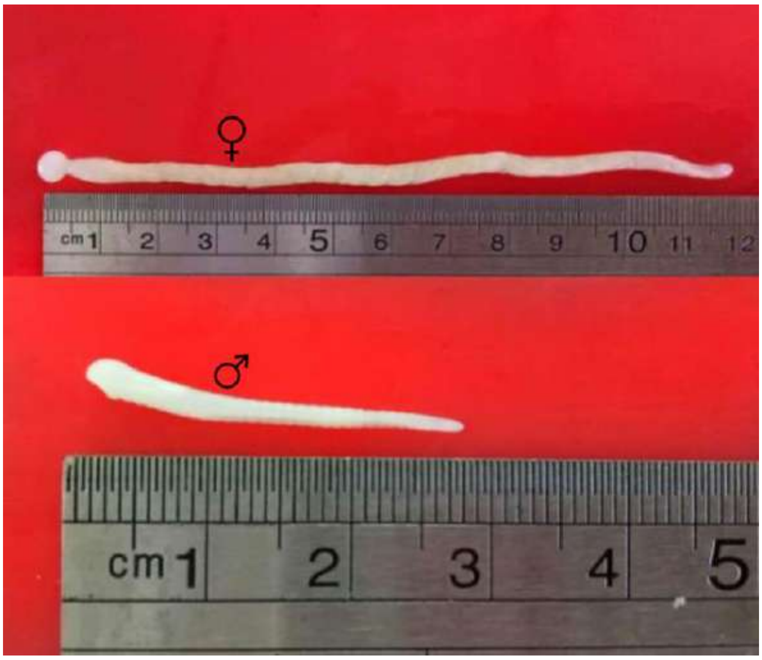

Figure 2. Differences in size between female (top) and male (bellow).

Both sexes, adult males and female pentastomids, were found in this research (Figures 1 and 2). The number of male pentastomid was less (21 heads) than that of females (183 heads) Table 2. Table 2 also describes the size of each pentastomid. The female pentastomids had longer and larger bodies than the males (Figures 1 and 2). The length of the female bodies was 72-125 mm with an average of 94.5 mm. Their abdominal diameter was 3.5-5 mm with an average of 4.2 mm. Furthermore, the anterior section or cephalothorax of the females was globular with a neck separating their head and their abdomen (Figure 3A). The average length and width of the female mouths were 0.24 mm and 0.16 mm. Furthermore, the results of the microscopy examination using Carmine staining showed that the female pentastomid had two spermathecae on the anterior part that connect directly to the uterus that contains the egg (Figures 3C, 4B, and 4C). Vagina was in the posterior body and around the anus (Figure 4B).

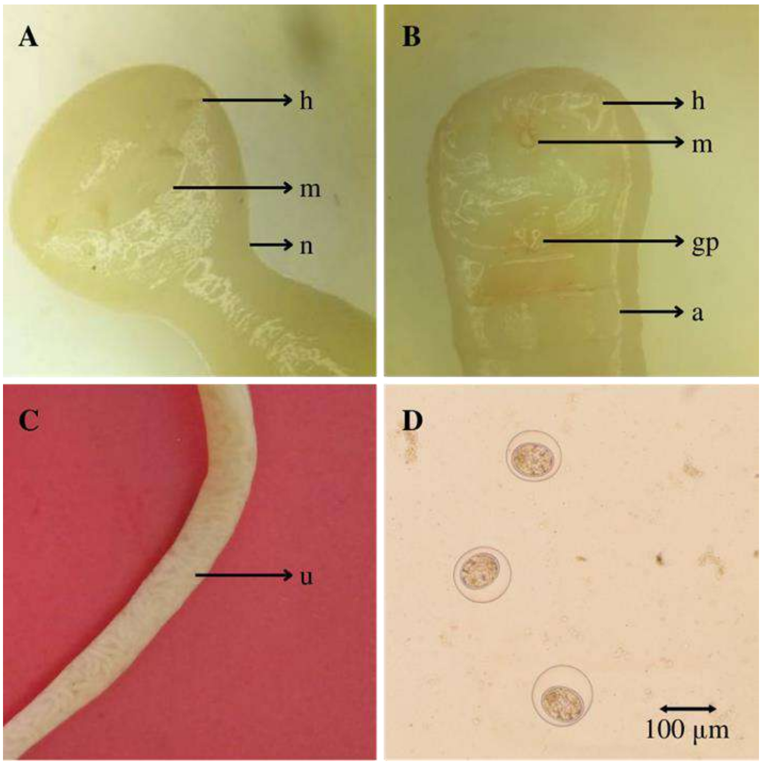

Figure 3. Pentastomida without staining. (A) The anterior section of the female. (B) The anterior section of the male. (C) Female body. (D) Pentastomid's eggs. h: hook, m: mouth, n: neck, gp: genital pore, a: first annuli, u: uterus.

On the other hand, the length of the males' bodies was 17-28.5 mm with an average of 23.75 mm. Their abdominal diameter was 1.8-2.2 mm with an average of 2 mm. The average length and width of the mouth were 0.23 and 0.14 mm, respectively. The male body was simple and straight without any curves in the neck (Figure 3B). The genital pore was identified in the first annulus and there was a pair of seminal vesicles, a pair of vasa deferens and cirrus (4D).

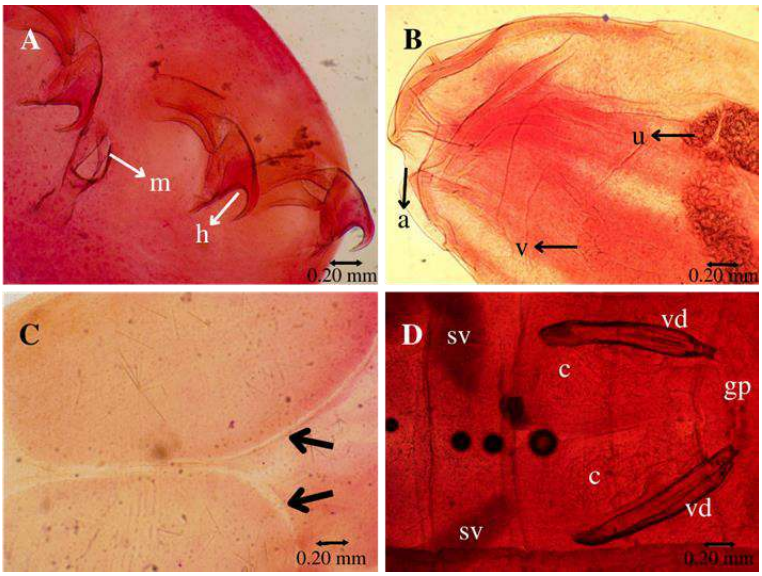

Figure 4. Carmine-stained pentastomida. (A) The anterior section. (B) The posterior section of the female. (C) A pair of female spermatheca. (D) Male reproductive organs. m: mouth, h: hook, a: anus, v: vagina, u: uterus, sv: seminal vesicle, c: cirrus, vd: vas deferens.

The eggs were oval and consisted of two layers of membrane that clearly could be distinguished. The first layer (outer membrane) was thin, while the second layer (inner membrane) was thick and yellowish. There was fluid-filled space between the first and second layers. The length of the eggs was 114-130 µm with an average of 118 µm and the width was 108-124 µm with an average of 116 µm (Figure 3D).

Pentastomida were mostly found in the lungs and the parasites also spread to the trachea in five snakes. This finding was similar to research by Riley and Self (1980) that revealed that adult *Kiricephalus pattoni* (one of the pentastomida

species) were only found in the lungs of *P. mucosa* snakes. In our observation, pentastomids that spread to the trachea were females. According to Sundar et al. (2015), the adult pentastomids with mature eggs are expelled from the trachea and eliminated from the definitive host by oral expulsion or may also be swallowed, resulting in shed eggs in the feces in which another intermediate mammal host will ingest to continue their life cycles.

The number of male pentastomids was fewer than the number of female ones. This case was also experienced by John and Nadakal (1988) and Riley and Self (1980) that only found a few males compared the female. We agree with Almeida et al. (2006) that pentastomids are not well known, in biology and the number of their species.

The taxonomy of pentastomida is still controversial. It has been known through molecular data that pentastomida are closely related to crustaceans and belong to class Maxillopoda, sub-phylum Crustacea, and the phylum Arthropoda (Paré, 2008). Based on the size of the egg (Almeida et al., 2007), the form of the female head that is globular with a neck separating the head from its body (Paré, 2008) and the size of the body length, the number of the annuli, the diameter of cephalothorax, neck, abdominal diameter, head, and body shape (Riley and Self, 1979; Riley and Self, 1980), the pentastomida found was classified as *K. pattoni*. *K. pattonii* can be classified into the order of porochepalida (Paré, 2008). According to Christoffersen and De Assis (2013; 2015), pentastomid in *P. mucosa* snakes are classified into *K. pattoni*.

K. pattoni has a long annulated body, a tapered tail, an anal canal, two pairs of hooks or a total of four hooks on its head, as well as a small ovoid-shaped mouth (Riley and Self, 1979). The female *K. pattoni* has a larger body than male one (Paré, 2008). Female *K. pattoni* also has a spiral body with a larger head than its body, and a curve of the neck, whereas male one has a straight body without a curve on the neck (Riley and Self, 1979; Riley and Self, 1980; John and Nadakal, 1988; Almeida et al., 2007). The spiral body in females is likely to facilitate their movement into the lung tissue of the definitive hosts (John and Nadakal, 1988).

The morphology of the eggs like the eggs of *Porocephalus crotali* described by Sundar et al. (2015), the eggs were composed of two-layer membranes of which the outer one is thin, and the inner membrane thick and there was a fluid-filled space between two membranes. But the average diameter of the eggs measured in this investigation is smaller (118 x 116 µm) than the eggs of *P. crotali* (144 µm) as described by Sundar et al. (2015).

Histopathological changes in the lungs of *Ptyas mucosa*

Squamates typically have two lungs, however, the left lung in most snakes is greatly reduced, never exceeding 85% of the right lung’s size, or absent[JB53][A54]. The right lung usually runs from near the heart to the cranial to the right kidn[JB55][A56]ey. The cranial portion of a lung is vascularized and functions primarily as a gas exchanger (vascular lung), whereas the caudal primarily functions as an air sac (saccular lung). The vascular lung's wall is made up of honeycombed units called faveoli, which allow for gas exchange (Funk and Bogan, 2019). Microscopically, the faveoli in the snake lung parenchyma are lined mainly by squamous epithelial cells (type I cells) and, to a lesser degree, by cuboidal epithelial cells (type II) (Jacobson et al., 2021).

In this study, a nymphal stage of the parasite was found in the pulmonary tissue infected with pentastomida. The parasite is surrounded by some connective tissue (Figure 5). Finding the stages of the nymph in the histological lung of the snake and the stages of the parasite on the macroscopic lung organ means that the *P. mucosa* snake may act as an intermediate and final host of *K. pattoni*. Within the lung cavities of the definitive host, the nymphs develop into adults (Sundar et al., 2015). In this study, pentastomida infection in the lungs only caused a mild histological reaction in the form of fibrosis in the area around the parasite.

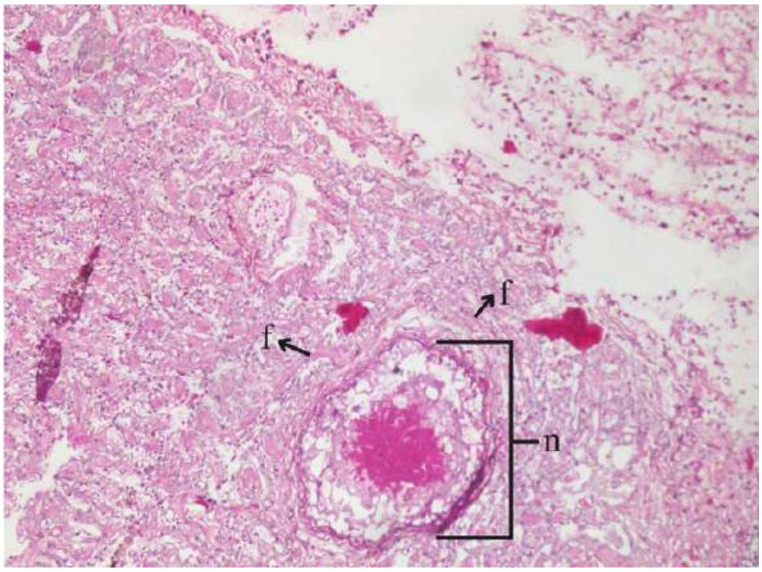

Figure 5. Histological microscopic picture of nymph-infected pulmo from pentastomida showed the presence of fibroblast around the area of infection. f: fibroblast, n: the body of the nymph stage pentastomida.

Pentastomida have an indirect life cycle that typically utilizes vertebrate hosts. Many of their species rely on reptiles as their definitive host, and the adults are usually found in the lungs (Wellehan and Walden, 2019). Pentastomida parasites migrate extensively through the visceral before settling in the lungs and, less typically, the trachea and nasal passageways. Pentastomida can produce localized lesions, whilst molting can elicit antigenic reactions. Adults feed on lung tissue fluids and pass embryonated eggs, which are coughed up through the oral cavity, swallowed, and passed in the feces (Comolli and Divers, 2021). In squamates, the pulmonary lumen is large, and the tracheal diameter may be smaller than that of the pentastomids. This poses a risk of respiratory obstruction by the adult pentastomes. However, the greatest pathological changes are seen in the intermediate host, where they may become embedded in tissues (Wellehan and Walden, 2019[JB57][A58]).

Pentastomiasis in reptiles, especially snakes, occurs subclinically, although the infection is large, and death is usually associated with severe pneumonia[JB59][A60]. Because pentastomids attach to the lung tissue, they can cause perforation, hemorrhaging, and serious inflammation in the host (Fernando and Fernando, 2014). In a Common Garter Snake (*Thamnophis sirtalis*), lung lesions were associated with one of the pentastomida species, *Kiricephalus coarctatus*, where the embedded parasite had gotten into the hypaxial muscle. Infections with *Armillifer armillatus* have also been linked to parasitic pneumonia in *Gaboon vipers*. Pentastomids occluding the trachea have been implicated in snake deaths. Bacterial infections could develop due to lung damage. Four of nine *Boa constrictors* with pulmonary damage from *Porocephalus dominicana* infection perished from bacteremia and pericarditis (Walden et al., 2021).

CONCLUSION

The prevalence of pentastomiasis in slaughtered *Ptyas mucosa* in Sidoarjo was 65% (39 of 60 snakes). The pentastomids that infect these snakes are classified as *Kiricephalus pattoni*. The pulmonary histological findings only show a light reaction in the form of mild fibrosis.

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First report of pentastomiasis and the lung histopathological changes in *Ptyas*[JB1] *mucosa* in Sidoarjo, East Java, Indonesia

Abstract. This[JB2] study aimed to determine the prevalence of pentastomiasis, identify the parasite, and observe the pulmonary histopathology in *Ptyas mucosa* collected from snake slaughter in Sidoarjo (Indonesia). The[JB3] identification of pentastomids was carried out by Semichen-Acetic Carmine, and histopathological preparations of the lungs were performed to observe the microscopic lesions. The results showed that 65% of the snakes had pentastomiasis, and the parasites were characterized as *Kirichephalus pattoni*. Microscopical examination revealed mild fibrosis in the pulmonary area as a result of pentastomid infestation. This is the first report of some pentastomiasis in *P. mucosa* in Sidoarjo, East Java, Indonesia[JB4].

Key words: *Kirichephalus pattoni*, Parasitic Pneumonia, Rat Snake, Zoonosis[JB5].

Abbreviations:

Running title: Pentastomiasis in Rat Snake

INTRODUCTION

Exotic animals such as reptiles, especially various types of snakes, are now popular as pets throughout the world (Okulewicz et al., 2014). However, they are also concerned with the increasing animal welfare and health issues through the animal trade in the market (Yudhana et al., 2021). Some species of snake are bred in captivity, while others are caught in the wild, and sold as pets or as bushmeat for consumption (Hardi et al., 2017; Yudhana et al., 2021). [JB6]

Ptyas mucosa is one of the most common snake species found in Indonesia[JB7]. This type of snake, known as the Oriental rat snake and Indian rat snake, belongs to the Colubridae family which is widely distributed throughout East, South, and Southeast Asia (Auliya, 2010; Muliya and Bhat, 2016; Yuan et al., 2022). *P. mucosa* is a diurnal and semi-arboreal snake species that lives on forest floors, agricultural fields, wet ground, and in close proximity to human habitats. It feeds on frogs, rats, and other small animals (Chittora et al., 2021).

The meat of *P. mucosa* is traded legally in China[JB8]. The meat and internal organs are used as fresh or frozen food, the bile and blood are used in traditional medicine, and the skin is exported to several European countries as raw materials for the leather industry[JB9]. Although it is illegal, most rural communities in Indonesia trade and consume the meat and bile of *P. mucosa* for some health reasons (Auliya, 2010[JB10]; Pranashinta et al., 2017).

Wildlife meat, however, poses a threat of transmitting infection with novel and exotic pathogens to human hosts (Hardi et al., 2017). Then new zoonotic diseases are likely to emerge consequently. For instance, Bats (Chiroptera) are important reservoirs of several emerging diseases, including Ebola virus disease (EVD), severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS), as well as the current pandemic COVID-19 caused by SARS Coronavirus 2 (Irving et al., 2021). Many types of parasites are also the cause of zoonotic emerging diseases, resulting in a significant public health burden, particularly in tropical countries (Alasil and Abdullah, 2019).

Several parasites found in confined reptiles spend a portion of their life cycle in the respiratory system. The majority of respiratory involvement is asymptomatic and modest[JB11], generating mostly localized inflammation, with the exception of instances with excessive parasite loads. However, severe disease can result from high burdens or unusual host-parasite interactions, which is frequently made worse by secondary infections (Comolli, 2021). Reptile-borne pentastomiasis is an unusual tropical disease that is neglected and under-researched in Asia, Africa, and South America (Sulyok et al., 2014).

Pentastomiasis is a parasitic zoonosis disease [JB12]caused by infection with pentastomids, belonging to the subclass Pentastomida, under the phylum Arthropoda (Chittora et al., 2021; Tongtako et al., 2013; WHA, 2019). Pentastomida are also known as tongue worms since the adult phase of the genus Linguatula[JB13] has similarities with the mammalian tongues (Paré, 2008). Adult parasites are usually found in the respiratory tract of reptiles, birds, and mammals, including humans, while their larval forms are in the internal organs of insects, mammals, and amphibians (Paré, 2008; Chen et al., 2010; Kelehear et al., 2014). Some zoonotic pentastomida in snakes are Armillifer and *Porocephalus crotali* (Rataj et al., 2011). Humans are infected with pentastomids since they consume raw snake meat or drink water contaminated with parasitic eggs (Ayinmode et al., 2010; Kelehear et al., 2014; Tappe et al., 2016).

Pentastomiasis in snakes have been reported in many countries, such as poisonous *Bothrops asper* in Costa Rica (Alvarado et al., 2015), Royal Python[JB14] snakes in Nigeria (Ayinmode et al., 2010), Rat snakes in Sri Lanka and India (Fernando and Fernando, 2014; Sundar et al., 2015), wild snakes in Australia (Kelehear et al., 2014), various species of snakes in Slovania and Pakistan (Rataj et al., 2011), *Agkistrodon acutus* and *Python molurus* in China (Chen et al., 2010), and *Crotalus totonacus* in Mexico [JB15](Tepos-Ramírez et al., 2022).

To the best of our knowledge, there are no reports of pentastomids infections in snakes in Indonesia. Therefore, this study reports the prevalence of pentastomiasis, the identification of pentastomids, and the pulmonary histopathological profiles in Rat snakes (*P. mucosa*) in Sidoarjo Sub-district, East Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

This study was reviewed and approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga (number 678-KE).

Parasites collection[JB16]

The collection was conducted at a snake slaughterhouse in Tulangan District, Sidoarjo Regency, East Java Province, Indonesia. The presence of pentastomid parasites was observed in the respiratory tract of sixty slaughtered Ptyas mucosa [JB17]snakes. Pentastomides were taken and cleaned with distilled water, then stored in sterile plastic pots. The lungs of the P[JB18]. mucosa infected with the parasite were also isolated for histopathological preparation[JB19].

Prevalence calculation

The prevalence of pentastomiasis in snake samples was calculated by the following formula:

Prevalence (%) = (The number of snakes infected with pentastomids)/(Total sample of snakes) × 100%.

Semichen-acetic carmine staining

The collected pentastomids were stained with the Semichen-Acetic Carmine technique[JB20] (Kuhlmann, 2006). Adult pentastomids were fixed on object glasses and soaked in 5% glycerin alcohol for 24 hours. Then they were soaked in 70% alcohol for five minutes. Subsequently, the object glasses with pentastomids were placed in Carmine alcohol solution (1:2) for eight hours. The pentastomids were soaked in acid alcohol for 10 minutes and moved to base alcohol for 20 minutes. Furthermore, graded dehydration was carried out with 70%, 85% and 95% alcohol for five minutes, respectively. The mounting step was done using Hung’s I for 20 minutes. Pentastomids were taken and placed on new clean object glasses. The pentastomids were dripped with Hung’s II solution and then covered with a cover glass.

Parasite identification

Characteristics of the head and body shape and body size of the parasite were identified based on Riley and Self (1980), Almedia et al. (2006), and Christoffersen and De Assis (2013; 2015). Identification of parasite egg size was carried out according to Keegan[JB21] (1943).

The length of the fresh parasites was measured from the anterior edge to the posterior end. The head was observed under a stereo microscope and documented with a Canon Digital Single-Lens Reflex (DSLR) camera. Stained parasites were observed using a light microscope with 40-100× magnification and documented using Miconos Optilab. Some female parasites were incubated in PBS overnight at 37 °C to collect the eggs. The supernatants were centrifuged at 1,500 rpm for 10 minutes and the pellet was dripped onto a glass object and observed using a light microscope with 100× magnification. Eggs were measured using Miconos Optilab.

Histopathological preparations

The lungs of snakes with pentastomid infestation were fixed in 10% formalin. Then graded dehydration was carried out with serial alcohol concentrations (70%, 80%, 90%, 96%, and absolute) and clearing using xylol. Furthermore, paraffin embedding was performed to obtain a solid block of tissue. The tissue blocks were sliced (3 micrometers thick) using a microtome, mounted on an object glass, and stained using Hematoxylin and Eosin. Microscopic examination was performed using Nikon Eclipse Ci.

RESULTS AND DISCUSSIONS

The prevalence of pentastomiasis in *Ptyas mucosa* snakes

Thirty-nine or 65% of the respiratory tract of 60 were positively infected with adult pentastomids. Totally[JB22], 204 pentastomids were collected[JB23] (Table 1). Pentastomida were found to be strongly attached to the trachea and lungs of snakes (Figure 1). Some pentastomids even buried their heads in the lungs of snakes. The parasites of all snakes positively infected with pentastomids were found in the lungs (39 snakes), while only 5 snakes which parasites spread to their tracheas.

Table 1. Prevalence of pentastomiasis in *Ptyas mucosa* based on site and number of parasites.

Sites	Positive (%)	Number of parasites
Lungs[JB24]	57% (34 of 60)	2-9 with the average of 5 pentastomida
Trachea	0% (0 of 60)	-
Lungs and Trachea	8% (5 of 60)	7-9 with the average of 8 pentastomida
Total	**65% (39 of 60)**	**204 pentastomida**

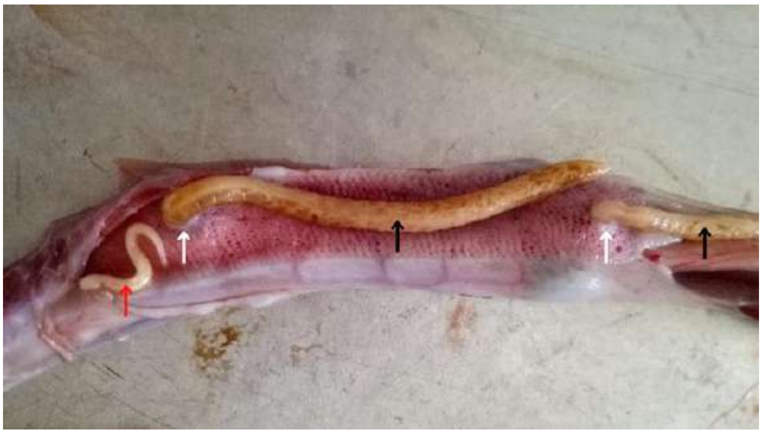

Figure 1. Pentastomid infestation in the lungs of *Ptyas mucosa*. The white arrows show areas with raised lesions around the attachment of pentastomids to the tissue. The black arrows show female pentastomids and the red arrow shows male pentastomids.

This is the first study to determine the prevalence of pentastomiasis in *Ptyas mucosa* in Sidoarjo, East Java Indonesia. As shown in our results, pentastomids were present in 39 of 60 (65%) snakes. Based on this finding, the prevalence of pentastomiasis in *P. mucosa* in this study was quite high. In this case, it was slightly the sa[JB25]me as the prevalence of pentastomiasis in Brazil, 8 of 15 or 53.33% of snakes were positively infected with pentastomid. The high prevalence in this research was probably caused by the food consumed by the snakes. The type of food is very influential on the prevalence of infection and the type of pentastomida that infected the snakes (Almeida et al., 2007). *P. mucosa* snakes usually feed on rats, frogs, toads, lizards, and even smaller non-venomous and smaller snakes (Sidik, 2006). According to Esslinger (1962), rats, frogs, toads, lizards, and even non-venomous snakes can act as the intermediate hosts of pentastomida.

Identification of pentastomida

The characteristics of pentastomid found in this study can be seen in Table 2 and Figure 2. Pentastomids have a long cylindrical body with a larger head, a flat shape on the ventral, and a tapered tail or posterior. Their body was soft and whitish with a colorless cuticle, which tends to be transparent so that the internal organs are visible. There were annuli throughout their body up to the posterior. There are two pairs of hooks (a total of four hooks) around the head that are used to attach to the host’s lung tissue, and it had an ovoid-shaped mouth between the two pairs of hooks[JB26].

Table 2. The characteristics of pentastomida compared with *Kiricephalus pattoni* by Riley and Self (1980) and Keegan (1943).

Parameters	The current study		British Museum collection (Riley and Self, 1980)		Keegan, 1943
	Females	Males	Females	Males	
Total number of parasites	183	21	17	2	-
Body length	72-125 mm (X̄ 94.5 mm)	17-28.5 mm (X̄ 23.75 mm)	83-137 mm (X̄ 100 mm)	28-29 mm (X̄ 28.5 mm)	-
Number of	29-41	28-35	34-38	31-32	-

annuli	(X̄ 31.6)	(X̄ 31)	(X̄ 36.5)	(X̄ 31.5)	
Diameter of cephalothorax	4-5.5 mm (X̄ 4.75 mm)	2-2.5 mm (X̄ 2 mm)	4 mm	-	-
Neck	2-3 mm (X̄ 2.5 mm)	-	2 mm	-	-
Diameter of abdomen	3.5-5 mm (X̄ 4.2 mm)	1.8-2.2 mm (X̄ 2 mm)	4 mm	-	-
Hook parameter:			-	-	-
a. AC					
b. CB	0.23-0.55 mm	0.22-0.35 mm			
c. AD	0.37-0.69 mm	0.29-0.35 mm			
	0.47-0.88 mm	0.29-0.45 mm			
Mouth:			-	-	-
a. Length	0.16-29 mm	0.14-0.38 mm			
b. Width	0.12-0.24 mm	0.12-0.18 mm			
Egg:		-	-	-	
a. Length	114-130 µm (X̄ 118 µm)				125-154 µm (X̄ 133 µm)
b. Width	108-124 µm (X̄ 116 µm)				106-143 µm (X̄ 123 µm)

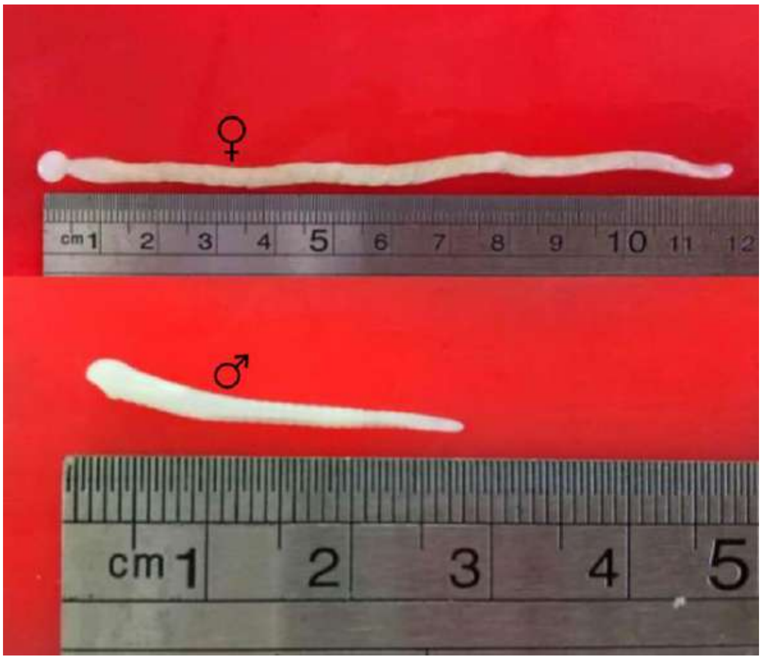

Figure 2. Differences in size between female (top) and male (bellow).

Both sexes, adult males and female pentastomids, were found in this research (Figures 1 and 2). The number of male pentastomid was less (21 heads) than that of females (183 heads) Table 2. Table 2 also describes the size of each pentastomid. The female pentastomids had longer and larger bodies than the males (Figures 1 and 2). The length of the female bodies was 72-125 mm with an average of 94.5 mm. Their abdominal diameter was 3.5-5 mm with an average of 4.2 mm. Furthermore, the anterior section or cephalothorax of the females was globular with a neck separating their head and their abdomen (Figure 3A). The average length and width of the female mouths were 0.24 mm and 0.16 mm. Furthermore, the results of the microscopy examination using Carmine staining showed that the female pentastomid had two spermathecae on the anterior part that connect directly to the uterus that contains the egg (Figures 3C, 4B, and 4C). Vagina was in the posterior body and around the anus (Figure 4B).

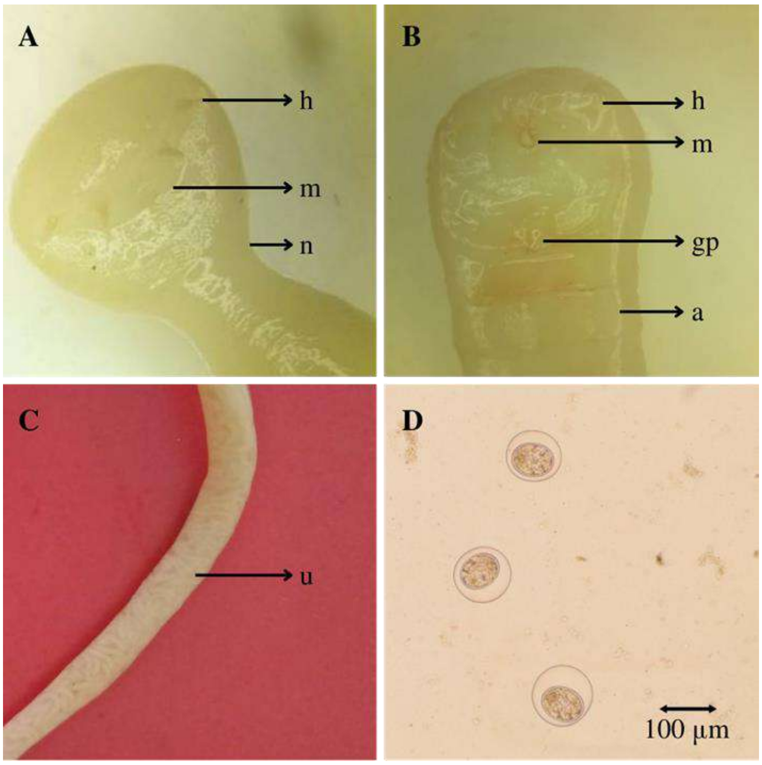

Figure 3. Pentastomida without staining. (A) The anterior section of the female. (B) The anterior section of the male. (C) Female body. (D) Pentastomid's eggs. h: hook, m: mouth, n: neck, gp: genital pore, a: first annuli, u: uterus.

On the other hand, the length of the males' bodies was 17-28.5 mm with an average of 23.75 mm. Their abdominal diameter was 1.8-2.2 mm with an average of 2 mm. The average length and width of the mouth were 0.23 and 0.14 mm, respectively. The male body was simple and straight without any curves in the neck (Figure 3B). The genital pore was identified in the first annulus and there was a pair of seminal vesicles, a pair of vasa deferens and cirrus (4D).

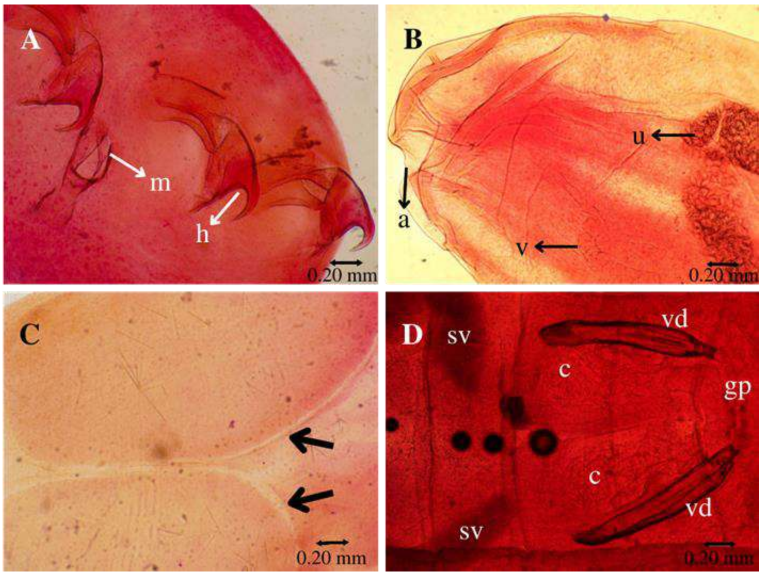

Figure 4. Carmine-stained pentastomida. (A) The anterior section. (B) The posterior section of the female. (C) A pair of female spermatheca. (D) Male reproductive organs. m: mouth, h: hook, a: anus, v: vagina, u: uterus, sv: seminal vesicle, c: cirrus, vd: vas deferens.

The eggs were oval and consisted of two layers of membrane that clearly could be distinguished. The first layer (outer membrane) was thin, while the second layer (inner membrane) was thick and yellowish. There was fluid-filled space between the first and second layers. The length of the eggs was 114-130 µm with an average of 118 µm and the width was 108-124 µm with an average of 116 µm (Figure 3D).

Pentastomida were mostly found in the lungs and the parasites also spread to the trachea in five snakes. This finding was similar to research by Riley and Self (1980) that revealed that adult *Kiricephalus pattoni* (one of the pentastomida

species) were only found in the lungs of *P. mucosa* snakes. In our observation, pentastomids that spread to the trachea were females. According to Sundar et al. (2015), the adult pentastomids with mature eggs are expelled from the trachea and eliminated from the definitive host by oral expulsion or may also be swallowed, resulting in shed eggs in the feces in which another intermediate mammal host will ingest to continue their life cycles.

The number of male pentastomids was fewer than the number of female ones. This case was also experienced by John and Nadakal (1988) and Riley and Self (1980) that only found a few males compared the female. We agree with Almeida et al. (2006) that pentastomids are not well known, in biology and the number of their species.

The taxonomy of pentastomida is still controversial. It has been known through molecular data that pentastomida are closely related to crustaceans and belong to class Maxillopoda, sub-phylum Crustacea, and the phylum Arthropoda (Paré, 2008). Based on the size of the egg (Almeida et al., 2007), the form of the female head that is globular with a neck separating the head from its body (Paré, 2008) and the size of the body length, the number of the annuli, the diameter of cephalothorax, neck, abdominal diameter, head, and body shape (Riley and Self, 1979; Riley and Self, 1980), the pentastomida found was classified as *K. pattoni*. *K. pattonii* can be classified into the order of porochepalida (Paré, 2008). According to Christoffersen and De Assis (2013; 2015), pentastomid in *P. mucosa* snakes are classified into *K. pattoni*.

K. pattoni has a long annulated body, a tapered tail, an anal canal, two pairs of hooks or a total of four hooks on its head, as well as a small ovoid-shaped mouth (Riley and Self, 1979). The female *K. pattoni* has a larger body than male one (Paré, 2008). Female *K. pattoni* also has a spiral body with a larger head than its body, and a curve of the neck, whereas male one has a straight body without a curve on the neck (Riley and Self, 1979; Riley and Self, 1980; John and Nadakal, 1988; Almeida et al., 2007). The spiral body in females is likely to facilitate their movement into the lung tissue of the definitive hosts (John and Nadakal, 1988).

The morphology of the eggs like the eggs of *Porocephalus crotali* described by Sundar et al. (2015), the eggs were composed of two-layer membranes of which the outer one is thin, and the inner membrane thick and there was a fluid-filled space between two membranes. But the average diameter of the eggs measured in this investigation is smaller (118 x 116 µm) than the eggs of *P. crotali* (144 µm) as described by Sundar et al. (2015).

Histopathological changes in the lungs of *Ptyas mucosa*

Squamates typically have two lungs, however, the left lung in most snakes is greatly reduced, never exceeding 85% of the right lung's size, or absent[JB27]. The right lung usually runs from near the heart to the cranial to the right kidn[JB28]ey. The cranial portion of a lung is vascularized and functions primarily as a gas exchanger (vascular lung), whereas the caudal primarily functions as an air sac (saccular lung). The vascular lung's wall is made up of honeycombed units called faveoli, which allow for gas exchange (Funk and Bogan, 2019). Microscopically, the faveoli in the snake lung parenchyma are lined mainly by squamous epithelial cells (type I cells) and, to a lesser degree, by cuboidal epithelial cells (type II) (Jacobson et al., 2021).

In this study, a nymphal stage of the parasite was found in the pulmonary tissue infected with pentastomida. The parasite is surrounded by some connective tissue (Figure 5). Finding the stages of the nymph in the histological lung of the snake and the stages of the parasite on the macroscopic lung organ means that the *P. mucosa* snake may act as an intermediate and final host of *K. pattoni*. Within the lung cavities of the definitive host, the nymphs develop into adults (Sundar et al., 2015). In this study, pentastomida infection in the lungs only caused a mild histological reaction in the form of fibrosis in the area around the parasite.

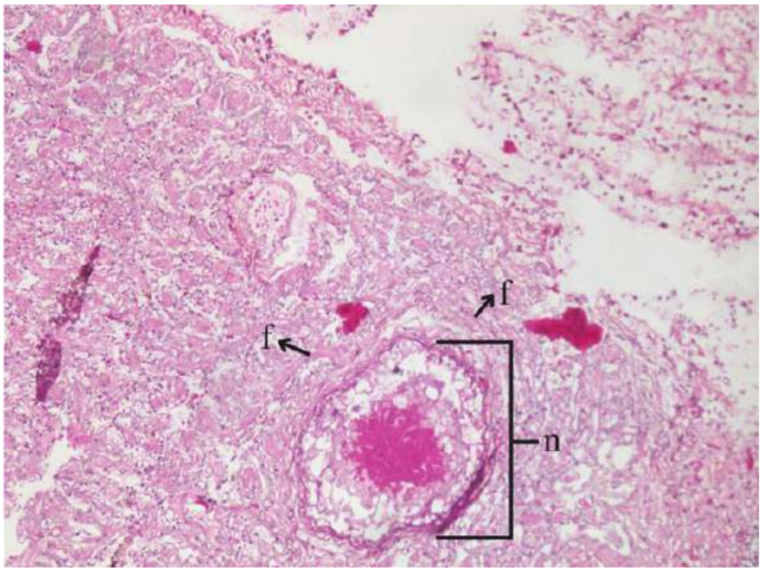

Figure 5. Histological microscopic picture of nymph-infected pulmo from pentastomida showed the presence of fibroblast around the area of infection. f: fibroblast, n: the body of the nymph stage pentastomida.

Pentastomida have an indirect life cycle that typically utilizes vertebrate hosts. Many of their species rely on reptiles as their definitive host, and the adults are usually found in the lungs (Wellehan and Walden, 2019). Pentastomida parasites migrate extensively through the visceral before settling in the lungs and, less typically, the trachea and nasal passageways. Pentastomida can produce localized lesions, whilst molting can elicit antigenic reactions. Adults feed on lung tissue fluids and pass embryonated eggs, which are coughed up through the oral cavity, swallowed, and passed in the feces (Comolli and Divers, 2021). In squamates, the pulmonary lumen is large, and the tracheal diameter may be smaller than that of the pentastomids. This poses a risk of respiratory obstruction by the adult pentastomes. However, the greatest pathological changes are seen in the intermediate host, where they may become embedded in tissues (Wellehan and Walden, 2019[JB29]).

Pentastomiasis in reptiles, especially snakes, occurs subclinically, although the infection is large, and death is usually associated with severe pneumonia[JB30]. Because pentastomids attach to the lung tissue, they can cause perforation, hemorrhaging, and serious inflammation in the host (Fernando and Fernando, 2014). In a Common Garter Snake (*Thamnophis sirtalis*), lung lesions were associated with one of the pentastomida species, *Kiricephalus coarctatus*, where the embedded parasite had gotten into the hypaxial muscle. Infections with *Armillifer armillatus* have also been linked to parasitic pneumonia in *Gaboon vipers*. Pentastomids occluding the trachea have been implicated in snake deaths. Bacterial infections could develop due to lung damage. Four of nine *Boa constrictors* with pulmonary damage from *Porocephalus dominicana* infection perished from bacteremia and pericarditis (Walden et al., 2021).

CONCLUSION

The prevalence of pentastomiasis in slaughtered *Ptyas mucosa* in Sidoarjo was 65% (39 of 60 snakes). The pentastomids that infect these snakes are classified as *Kiricephalus pattoni*. The pulmonary histological findings only show a light reaction in the form of mild fibrosis.

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First Report of Pentastomiasis and the Lung Histopathological Changes in Oriental Rat Snake (*Ptyas mucosa*) in Sidoarjo, East Java, Indonesia

Abstract. Currently, reptiles, especially snakes, are increasingly popular as pets around the world and are often consumed by several communities in Indonesia. This trend needs attention from a public health perspective because it has the potential to cause zoonotic diseases, one of which is parasitic disease, pentastomiasis. This study aimed to determine the prevalence of pentastomiasis, identify the parasite, and observe the pulmonary histopathology in Oriental rat snakes (*Ptyas mucosa*) collected in Tulangan, Sidoarjo, Indonesia. The methods used were the calculation of the prevalence of pentastomiasis, semichen-acetic carmine staining, and identification of parasites and eggs based on references as a comparison. Furthermore, histopathological preparation and hematoxylin-eosin staining were performed on the lungs infected with the parasite to observe the microscopic lesions. The results showed that 65% of the snakes had pentastomiasis, and the parasites were characterized as *Kirichephalus pattoni*. Microscopical examination revealed mild fibrosis in the pulmonary area as a result of pentastomid infestation. This is the first reported study of pentastomiasis in P. mucosa in Sidoarjo, Indonesia, with the implication that the snakes that were caught and commonly consumed were infected with zoonotic pentastomides.
Key words: Colubrid Snake, Parasitic Pneumonia, Tongue Worms, Zoonotic Parasitoses.

Abbreviations:

Running title: Pentastomiasis in Rat Snake

INTRODUCTION

Exotic animals such as reptiles, especially various types of snakes, are now popular as pets throughout the world (Okulewicz et al. 2014). However, they are also concerned with the increasing animal welfare and health issues through the animal trade in the market (Yudhana et al. 2021). Some species of snake are bred in captivity, while others are caught in the wild, and sold as pets or as bushmeat for consumption (Hardi et al. 2017; Yudhana et al. 2021).

Ptyas mucosa is a well-known snake species found in Indonesia (Pranashinta et al. 2017). This type of snake, known as the Oriental rat snake and Indian rat snake, belongs to the Colubridae family which is widely distributed throughout East, South, and Southeast Asia (Auliya 2010; Muliya and Bhat 2016; Yuan et al. 2022). *P. mucosa* is a diurnal and semi-arboreal snake species that lives on forest floors, agricultural fields, wet ground, and in close proximity to human habitats. It feeds on frogs, rats, and other small animals (Chittora et al. 2021).

The meat of *P. mucosa* is used in the illegal trade to China (Auliya 2010). The meat and internal organs are used as fresh or frozen food, the bile and blood are used in traditional medicine, and the skin is exported to several European countries as raw materials for the leather industry (Pranashinta et al. 2017). Although it is illegal, most rural communities in Indonesia trade and consume the meat and bile of *P. mucosa* for some health reasons (Auliya 2010; Pranashinta et al. 2017).

Wildlife meat, however, poses a threat of transmitting infection with novel and exotic pathogens to human hosts (Hardi et al. 2017). Then new zoonotic diseases are likely to emerge consequently. For instance, Bats (Chiroptera) are important reservoirs of several emerging diseases, including Ebola virus disease (EVD), severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS), as well as the current pandemic COVID-19 caused by SARS Coronavirus 2 (Irving et al. 2021). Many types of parasites are also the cause of zoonotic emerging diseases, resulting in a significant public health burden, particularly in tropical countries (Alasil and Abdullah 2019).

Several parasites found in confined reptiles spend a portion of their life cycle in the respiratory system. The majority of respiratory involvement is asymptomatic and mild, generating mostly localized inflammation, with the exception of instances with excessive parasite loads. However, severe disease can result from high burdens or unusual host-parasite interactions, which is frequently made worse by secondary infections (Comolli 2021). Reptile-borne pentastomiasis is an unusual tropical disease that is neglected and under-researched in Asia, Africa, and South America (Sulyok et al. 2014).

Pentastomiasis is a parasitic zoonosis caused by infection with pentastomids, belonging to the subclass Pentastomida, under the phylum Arthropoda (Chittora et al. 2021; Tongtako et al. 2013; WHA 2019). Pentastomida are also known as tongue worms since the adult phase of the genus *Linguatula* has similarities with the mammalian tongues (Paré 2008).

Adult parasites are usually found in the respiratory tract of reptiles, birds, and mammals, including humans, while their larval forms are in the internal organs of insects, mammals, and amphibians (Paré 2008; Chen et al. 2010; Kelehear et al. 2014). Some zoonotic pentastomida in snakes are Armillifer and *Porocephalus crotali* (Rataj et al. 2011). Humans can be infected with pentastomids since they consume raw snake meat, have direct contact with the snake, drink water contaminated with parasitic eggs, or are contaminated through the feces (Kelehear et al. 2014; Tappe et al. 2016; Mendoza-Roldan et al. 2020).

Pentastomiasis in snakes have been reported in many countries, such as poisonous Terciopelo (*Bothrops asper*) in Costa Rica (Alvarado et al. 2015), Royal python (*Python regius*) snakes in Nigeria (Ayinmode et al. 2010), Rat snakes (*Ptyas mucosa*) in Sri Lanka and India (Fernando and Fernando 2014; Sundar et al. 2015), wild snakes in Australia (Kelehear et al. 2014), various species of snakes in Slovania and Pakistan (Rataj et al. 2011), Chinese moccasin (*Agkistrodon acutus*) and Indian python (*Python molurus*) in China (Chen et al. 2010), and Totonacan rattlesnake (*Crotalus totonacus*) in Mexico (Tepos-Ramírez et al. 2022).

To the best of our knowledge, there are no reports of pentastomids infections in snakes in Indonesia. Therefore, this study reports the prevalence of pentastomiasis, the identification of pentastomids, and the pulmonary histopathological profiles in Oriental rat snakes (*P. mucosa*) in Sidoarjo Sub-district, East Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

This study was reviewed and approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga (number 678-KE).

Parasite collection

The sample used comes from the Oriental rat snake (*Ptyas mucosa*), which is often caught and sold for its meat and skin through collectors. The collection was conducted at a snake slaughterhouse in Tulangan District, Sidoarjo Regency, East Java Province, Indonesia. The presence of pentastomid parasites was observed in the respiratory tract of sixty slaughtered snakes. Pentastomides were taken and cleaned with distilled water, then stored in sterile plastic pots. The lungs of the snakes infected with the parasite were also isolated for histopathological preparation.

Prevalence calculation

The prevalence of pentastomiasis in snake samples was calculated by the following formula:

Prevalence (%) = (The number of snakes infected with pentastomids)/(Total sample of snakes) × 100%.

Semichen-acetic carmine staining

The collected pentastomids were stained with the Semichen-Acetic Carmine technique (Kuhlmann 2006). Adult pentastomids were fixed on object glasses and soaked in 5% glycerin alcohol for 24 hours. Then they were soaked in 70% alcohol for five minutes. Subsequently, the object glasses with pentastomids were placed in Carmine alcohol solution (1:2) for eight hours. The pentastomids were soaked in acid alcohol for 10 minutes and moved to base alcohol for 20 minutes. Furthermore, graded dehydration was carried out with 70%, 85% and 95% alcohol for five minutes, respectively. The mounting step was done using Hung's I for 20 minutes. Pentastomids were taken and placed on new clean object glasses. The pentastomids were dripped with Hung's II solution and then covered with a cover glass.

Parasite identification

Characteristics of the head and body shape and body size of the parasite were identified based on Riley and Self (1980), Almedia et al. (2006), and Christoffersen and De Assis (2013; 2015). Identification of parasite egg size was carried out according to Keegan (1943).

The length of the fresh parasites was measured from the anterior edge to the posterior end. The head was observed under a stereo microscope and documented with a Canon Digital Single-Lens Reflex (DSLR) camera. Stained parasites were observed using a light microscope with 40-100× magnification and documented using Miconos Optilab. Some female parasites were incubated in PBS overnight at 37 °C to collect the eggs. The supernatants were centrifuged at 1,500 rpm for 10 minutes and the pellet was dripped onto a glass object and observed using a light microscope with 100× magnification. Eggs were measured using Miconos Optilab.

Histopathological preparations

The lungs of snakes with pentastomid infestation were fixed in 10% formalin. Then graded dehydration was carried out with serial alcohol concentrations (70%, 80%, 90%, 96%, and absolute) and clearing using xylol. Furthermore, paraffin embedding was performed to obtain a solid block of tissue. The tissue blocks were sliced (3 micrometers thick) using a microtome, mounted on an object glass, and stained using Hematoxylin and Eosin. Microscopic examination was performed using Nikon Eclipse Ci.

RESULTS AND DISCUSSIONS

The prevalence of pentastomiasis in *Ptyas mucosa* snakes

Thirty-nine or 65% of the respiratory tract of 60 were positively infected with adult pentastomids. In total, 204 pentastomids were collected (Table 1). Pentastomida were found to be strongly attached to the trachea and lungs of snakes (Figure 1). Some pentastomids even buried their heads in the lungs of snakes. The parasites of all snakes positively infected with pentastomids were found in the lungs (39 snakes), while only 5 snakes which parasites spread to their tracheas.

Table 1. Prevalence of pentastomiasis in *Ptyas mucosa* based on site and number of parasites.

Sites	Positive (%)	Number of parasites
Lungs only	57% (34 of 60)	2-9 with the average of 5 pentastomida
Trachea only	0% (0 of 60)	-
Lungs and Trachea	8% (5 of 60)	7-9 with the average of 8 pentastomida
Total	**65% (39 of 60)**	**204 pentastomida**

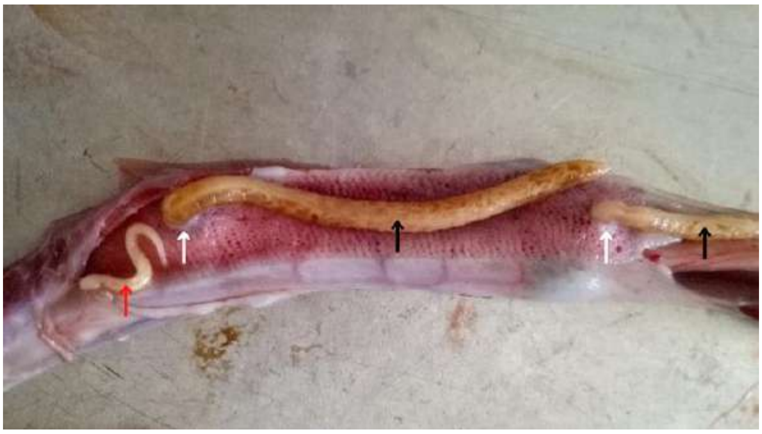

Figure 1. Pentastomid infestation in the lungs of *Ptyas mucosa*. The white arrows show areas with raised lesions around the attachment of pentastomids to the tissue. The black arrows show female pentastomids and the red arrow shows male pentastomids.

This is the first study to determine the prevalence of pentastomiasis in *Ptyas mucosa* in Sidoarjo, East Java Indonesia. As shown in our results, pentastomids were present in 39 of 60 (65%) snakes. Based on this finding, the prevalence of pentastomiasis in *P. mucosa* in this study was quite high. In this case, it was similar as the prevalence of pentastomiasis in Brazil, 8 of 15 or 53.33% of snakes were positively infected with pentastomid. The high prevalence in this research was probably caused by the food consumed by the snakes. The type of food is very influential on the prevalence of infection and the type of pentastomida that infected the snakes (Almeida et al. 2007). *P. mucosa* snakes usually feed on rats, frogs, toads, lizards, and even smaller non-venomous and smaller snakes (Sidik 2006). According to Esslinger (1962), rats, frogs, toads, lizards, and even non-venomous snakes can act as the intermediate hosts of pentastomida.

Identification of pentastomida

The characteristics of pentastomid found in this study can be seen in Table 2 and Figure 2. Pentastomids have a long cylindrical body with a larger head, a flat shape on the ventral, and a tapered tail or posterior. Their body was soft and whitish with a colorless cuticle, which tends to be transparent so that the internal organs are visible. There were annuli throughout their body up to the posterior. There are two pairs of hooks (a total of four hooks) around the head that are used to attach to the host's lung tissue, and it had an ovoid-shaped mouth between the two pairs of hooks.

Table 2. The characteristics of pentastomida compared with *Kiricephalus pattoni* by Riley and Self (1980) and Keegan (1943).

Parameters	**The current study**		**British Museum collection (Riley and Self 1980)**		**Keegan 1943**
	Females	**Males**	**Females**	**Males**	
Total number of parasites	183	21	17	2	-
Body length	72-125 mm (X̄ 94.5 mm)	17-28.5 mm (X̄ 23.75 mm)	83-137 mm (X̄ 100 mm)	28-29 mm (X̄ 28.5 mm)	-
Number of *annuli*	29-41 (X̄ 31.6)	28-35 (X̄ 31)	34-38 (X̄ 36.5)	31-32 (X̄ 31.5)	-

Diameter of cephalothorax	4-5.5 mm ($\bar{X}$ 4.75 mm)	2-2.5 mm ($\bar{X}$ 2 mm)	4 mm	-	-
Neck	2-3 mm ($\bar{X}$ 2.5 mm)	-	2 mm	-	-
Diameter of abdomen	3.5-5 mm ($\bar{X}$ 4.2 mm)	1.8-2.2 mm ($\bar{X}$ 2 mm)	4 mm	-	-
Hook parameter:			-	-	-
a. AC					
b. CB	0.23-0.55 mm	0.22-0.35 mm			
c. AD	0.37-0.69 mm	0.29-0.35 mm			
	0.47-0.88 mm	0.29-0.45 mm			
Mouth:			-	-	-
a. Length	0.16-29 mm	0.14-0.38 mm			
b. Width	0.12-0.24 mm	0.12-0.18 mm			
Egg:		-	-	-	
a. Length	114-130 µm ($\bar{X}$ 118 µm)				125-154 µm ($\bar{X}$ 133 µm)
b. Width	108-124 µm ($\bar{X}$ 116 µm)				106-143 µm ($\bar{X}$ 123 µm)

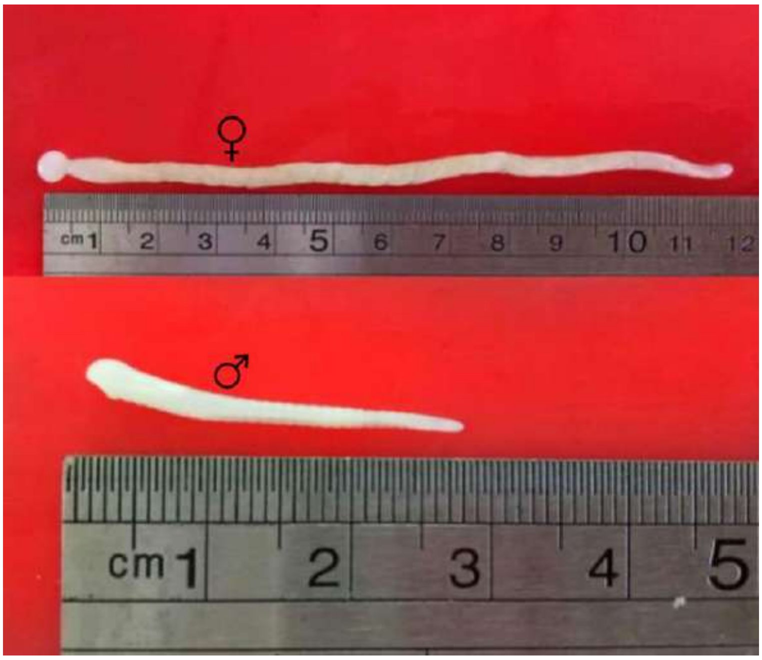

Figure 2. Differences in size between female (top) and male (bellow).

Both sexes, adult males and female pentastomids, were found in this research (Figures 1 and 2). The number of male pentastomid was less (21 heads) than that of females (183 heads) Table 2. Table 2 also describes the size of each pentastomid. The female pentastomids had longer and larger bodies than the males (Figures 1 and 2). The length of the female bodies was 72-125 mm with an average of 94.5 mm. Their abdominal diameter was 3.5-5 mm with an average of 4.2 mm. Furthermore, the anterior section or cephalothorax of the females was globular with a neck separating their head and their abdomen (Figure 3A). The average length and width of the female mouths were 0.24 mm and 0.16 mm. Furthermore, the results of the microscopy examination using Carmine staining showed that the female pentastomid had two spermathecae on the anterior part that connect directly to the uterus that contains the egg (Figures 3C, 4B, and 4C). Vagina was in the posterior body and around the anus (Figure 4B).

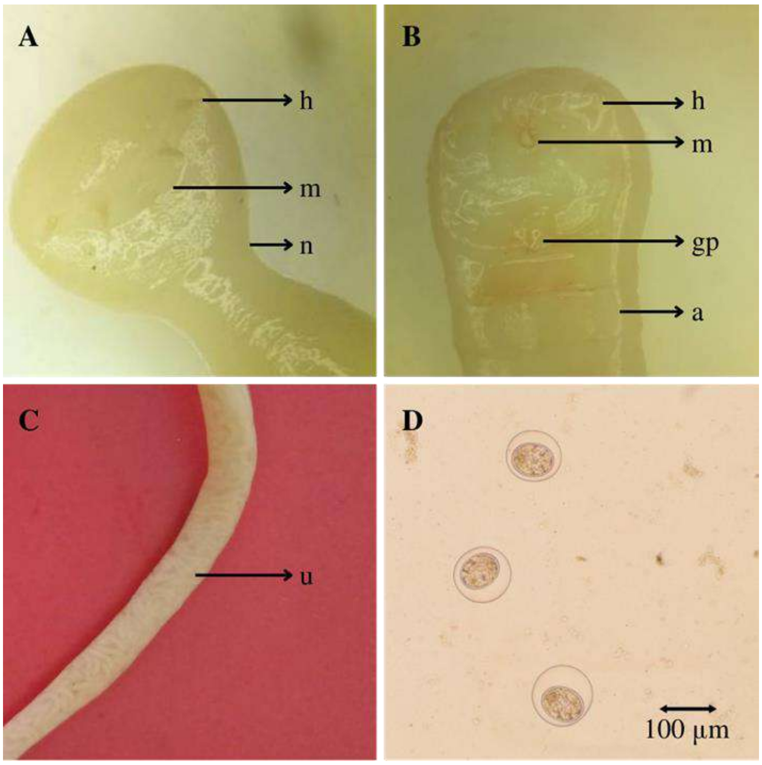

Figure 3. Pentastomida without staining. (A) The anterior section of the female. (B) The anterior section of the male. (C) Female body. (D) Pentastomid's eggs. h: hook, m: mouth, n: neck, gp: genital pore, a: first annuli, u: uterus.

On the other hand, the length of the males' bodies was 17-28.5 mm with an average of 23.75 mm. Their abdominal diameter was 1.8-2.2 mm with an average of 2 mm. The average length and width of the mouth were 0.23 and 0.14 mm, respectively. The male body was simple and straight without any curves in the neck (Figure 3B). The genital pore was identified in the first annulus and there was a pair of seminal vesicles, a pair of vasa deferens and cirrus (4D).

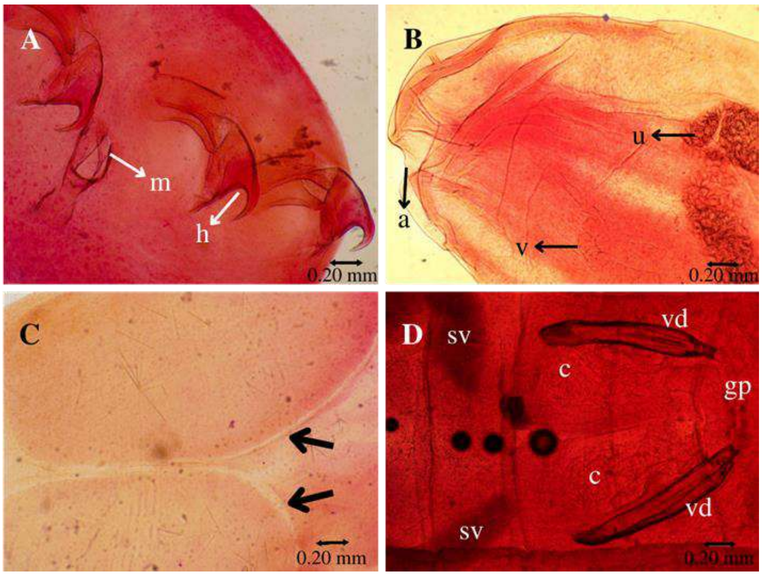

Figure 4. Carmine-stained pentastomida. (A) The anterior section. (B) The posterior section of the female. (C) A pair of female spermatheca. (D) Male reproductive organs. m: mouth, h: hook, a: anus, v: vagina, u: uterus, sv: seminal vesicle, c: cirrus, vd: vas deferens.

The eggs were oval and consisted of two layers of membrane that clearly could be distinguished. The first layer (outer membrane) was thin, while the second layer (inner membrane) was thick and yellowish. There was fluid-filled space between the first and second layers. The length of the eggs was 114-130 µm with an average of 118 µm and the width was 108-124 µm with an average of 116 µm (Figure 3D).

Pentastomida were mostly found in the lungs and the parasites also spread to the trachea in five snakes. This finding was similar to research by Riley and Self (1980) that revealed that adult *Kiricephalus pattoni* (one of the pentastomida

species) were only found in the lungs of *P. mucosa* snakes. In our observation, pentastomids that spread to the trachea were females. According to Sundar et al. (2015), the adult pentastomids with mature eggs are expelled from the trachea and eliminated from the definitive host by oral expulsion or may also be swallowed, resulting in shed eggs in the feces in which another intermediate mammal host will ingest to continue their life cycles.

The number of male pentastomids was fewer than the number of female ones. This case was also experienced by John and Nadakal (1988) and Riley and Self (1980) that only found a few males compared the female. We agree with Almeida et al. (2006) that pentastomids are not well known, in biology and the number of their species.

The taxonomy of pentastomida is still controversial. It has been known through molecular data that pentastomida are closely related to crustaceans and belong to class Maxillopoda, sub-phylum Crustacea, and the phylum Arthropoda (Paré 2008). Based on the size of the egg (Almeida et al. 2007), the form of the female head that is globular with a neck separating the head from its body (Paré 2008) and the size of the body length, the number of the annuli, the diameter of cephalothorax, neck, abdominal diameter, head, and body shape (Riley and Self 1979; Riley and Self 1980), the pentastomida found was classified as *K. pattoni*. *K. pattonii* can be classified into the order of porochepalida (Paré 2008). According to Christoffersen and De Assis (2013; 2015), pentastomid in *P. mucosa* snakes are classified into *K. pattoni*.

K. pattoni has a long annulated body, a tapered tail, an anal canal, two pairs of hooks or a total of four hooks on its head, as well as a small ovoid-shaped mouth (Riley and Self, 1979). The female *K. pattoni* has a larger body than male one (Paré 2008). Female *K. pattoni* also has a spiral body with a larger head than its body, and a curve of the neck, whereas male one has a straight body without a curve on the neck (Riley and Self 1979; Riley and Self 1980; John and Nadakal 1988; Almeida et al. 2007). The spiral body in females is likely to facilitate their movement into the lung tissue of the definitive hosts (John and Nadakal 1988).

The morphology of the eggs like the eggs of *Porocephalus crotali* described by Sundar et al. (2015), the eggs were composed of two-layer membranes of which the outer one is thin, and the inner membrane thick and there was a fluid-filled space between two membranes. But the average diameter of the eggs measured in this investigation is smaller (118 x 116 µm) than the eggs of *P. crotali* (144 µm) as described by Sundar et al. (2015).

Histopathological changes in the lungs of *Ptyas mucosa*

Squamates typically have two lungs, however, the left lung in most snakes is greatly reduced, never exceeding 85% of the right lung's size, or absent (Funk and Bogan 2019). The right lung usually runs from near the heart to the cranial to the right kidney (Funk and Bogan 2019). The cranial portion of a lung is vascularized and functions primarily as a gas exchanger (vascular lung), whereas the caudal primarily functions as an air sac (saccular lung). The vascular lung's wall is made up of honeycombed units called faveoli, which allow for gas exchange (Funk and Bogan 2019). Microscopically, the faveoli in the snake lung parenchyma are lined mainly by squamous epithelial cells (type I cells) and, to a lesser degree, by cuboidal epithelial cells (type II) (Jacobson et al. 2021).

In this study, a nymphal stage of the parasite was found in the pulmonary tissue infected with pentastomida. The parasite is surrounded by some connective tissue (Figure 5). Finding the stages of the nymph in the histological lung of the snake and the stages of the parasite on the macroscopic lung organ means that the *P. mucosa* snake may act as an intermediate and final host of *K. pattoni*. Within the lung cavities of the definitive host, the nymphs develop into adults (Sundar et al. 2015). In this study, pentastomida infection in the lungs only caused a mild histological reaction in the form of fibrosis in the area around the parasite.

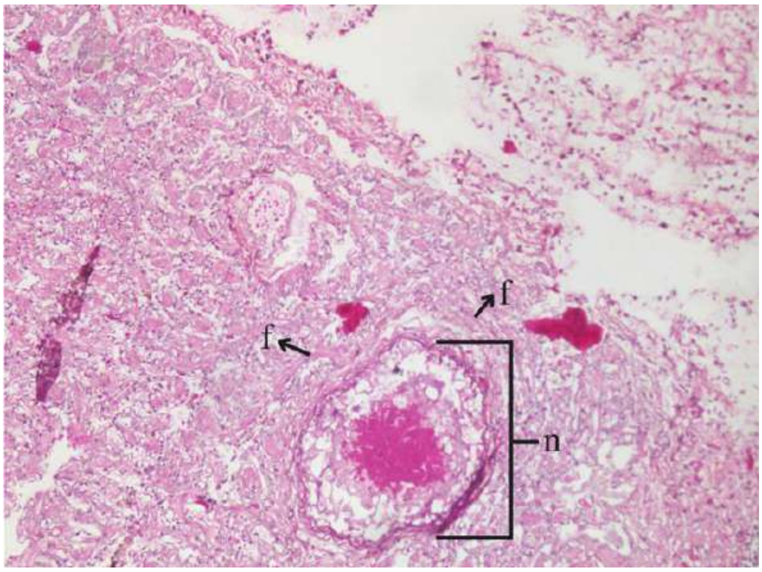

Figure 5. Histological microscopic picture of nymph-infected pulmo from pentastomida showed the presence of fibroblast around the area of infection. f: fibroblast, n: the body of the nymph stage pentastomida.

Pentastomida have an indirect life cycle that typically utilizes vertebrate hosts. Many of their species rely on reptiles as their definitive host, and the adults are usually found in the lungs (Wellehan and Walden 2019). Pentastomida parasites migrate extensively through the visceral before settling in the lungs and, less typically, the trachea and nasal passageways. Pentastomida can produce localized lesions, whilst molting can elicit antigenic reactions. Adults feed on lung tissue fluids and pass embryonated eggs, which are coughed up through the oral cavity, swallowed, and passed in the feces (Comolli and Divers 2021). In squamates, the pulmonary lumen is large, and the tracheal diameter may be smaller than that of the pentastomids. This poses a risk of respiratory obstruction by the adult pentastomes. However, the greatest pathological changes are seen in the intermediate host, where they may become embedded in tissues (Wellehan and Walden 2019).

Pentastomiasis in reptiles, especially snakes, occurs subclinically, although the infection is large, and death is usually associated with secondary septicemia and pneumonia (Tappe and Büttner 2009). Because pentastomids attach to the lung tissue, they can cause perforation, hemorrhaging, and serious inflammation in the host (Fernando and Fernando 2014). In a Common Garter Snake (*Thamnophis sirtalis*), lung lesions were associated with one of the pentastomida species, *Kiricephalus coarctatus*, where the embedded parasite had gotten into the hypaxial muscle. Infections with *Armillifer armillatus* have also been linked to parasitic pneumonia in *Gaboon vipers*. Pentastomids occluding the trachea have been implicated in snake deaths. Bacterial infections could develop due to lung damage. Four of nine *Boa constrictors* with pulmonary damage from *Porocephalus dominicana* infection perished from bacteremia and pericarditis (Walden et al. 2021).

Bigger implications, in terms of possible impacts on human health, may be anticipated based on the histopathological changes found in this study. Humans are the final hosts of pentastomes, which can be contracted from improperly cooked meat from infected snakes or from drinking water contaminated with pentastomida eggs. In addition, humans have the possibility of getting the infection when handling and harvesting snake skin or having direct contact with the mouth of a pet snake (Latif et al. 2016). The pathological consequences to the clinical symptoms experienced by infected humans may be the same or even more severe, depending on the number of parasite eggs ingested, the duration from the larvae migrating to various organs to maturity and reproduction, and how severe the parasite infestation is in the human viscera.

Personal hygiene measures, such as avoiding drinking river water directly or boiling the water before drinking, are required to prevent human infections. Authorities should provide health education to warn people about eating undercooked snake meat, the possibility of transmission after handling snakes, and avoiding contact with snake excretions.

CONCLUSION

The prevalence of pentastomiasis in slaughtered *Ptyas mucosa* in Sidoarjo was 65% (39 of 60 snakes). The pentastomids that infect these snakes are classified as *Kiricephalus pattoni*. The pulmonary histological findings only show a light reaction in the form of mild fibrosis.

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First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (*Ptyas mucosa*) in Sidoarjo, East Java, Indonesia

Abstract. Currently, reptiles, especially snakes, are increasingly popular as pets ~~around the world~~worldwide and are often consumed by several communities in Indonesia. This trend needs attention from a public health perspective because it ~~has the potential to~~can potentially cause zoonotic diseases, ~~one of which is~~including parasitic disease~~,~~ and pentastomiasis. This study aimed to determine: the prevalence of pentastomiasis, identify the parasite~~,~~ and observe the pulmonary histopathology in Oriental rat snakes (*Ptyas mucosa*) collected in Tulangan, Sidoarjo, Indonesia. The methods used were ~~the calculation of the prevalence of pentastomiasis, semichen-acetic carmine staining, and identification of~~calculating the prevalence of pentastomiasis, semichen-acetic carmine staining, and identifying parasites and eggs based on references as a comparison. Furthermore, histopathological preparation and hematoxylin-eosin staining were performed on the lungs infected with the parasite to observe the microscopic lesions. The results showed that 65% of the snakes had pentastomiasis, and the parasites were c~~haracterized as~~alled *Kirichephalus pattoni*. ~~Microscopical~~ Furthermore, a microscopical examination revealed mild fibrosis in the pulmonary area due to ~~as a result of~~ pentastomid infestation. T~~h~~his study is the first reported ~~study of~~ pentastomida~~sis~~ in *P. mucosa* in Sidoarjo, Indonesia, showing~~, with the implication~~ that~~that the~~ snakes ~~that were~~ caught and commonly consumed were infected with zoonotic penta~~stomid~~tomic~~es~~.

Key~~-~~words: Colubrid Snake, Parasitic Pneumonia, Tongue Worms, Zoonotic Parasitoses.

Abbreviations:

Running title: Pentastomiasis in Rat Snake

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INTRODUCTION

Exotic animals such as reptiles, especially various ~~types of~~ snakes, are now popular as pets ~~throughout the world~~worldwide (Okulewicz et al. 2014). However, they are also concerned with the increasing animal welfare and health issues through the animal trade in the market (Yudhana et al. 2021). Some species of snake are bred in captivity, while others are caught in the wild~~,~~ and sold as pets or ~~as~~ bushmeat for consumption (Hardi et al. 2017; Yudhana et al. 2021).

Ptyas mucosa is a well-known snake species ~~found~~ in Indonesia (Pranashinta et al. 2017). This type of snake, known as the Oriental rat snake and Indian rat snake, belongs to the Colubridae family which is widely distributed throughout East, South, and Southeast Asia (Auliya 2010; Muliya and Bhat 2016; Yuan et al. 2022). *P. mucosa* is a diurnal and semi-arboreal snake species that live~~s~~ on forest floors, agricultural fields, wet ground, and ~~in close proximity to~~near human habitats. It feeds on frogs, rats, and other small animals (Chittora et al. 2021).

The meat of *P. mucosa* is used in the illegal trade to China (Auliya 2010). The meat and internal organs are used as fresh or frozen food, the bile and blood are used in traditional medicine, and the skin is exported to several European countries as raw materials for the leather industry (Pranashinta et al. 2017). Although ~~it is~~ illegal, most rural communities in Indonesia trade and consume the meat and bile of *P. mucosa* for some ~~health~~ reasons (Auliya 2010; Pranashinta et al. 2017).

Wildlife meat, however, poses a threat of transmitting infection with novel and exotic pathogens to human hosts (Hardi et al. 2017). Then new zoonotic diseases are likely to emerge consequently. For instance, Bats (Chiroptera) are important reservoirs of several emerging diseases, including Ebola virus disease (EVD), S~~s~~evere acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS), as well as the current pandemic COVID-19 caused by SARS Coronavirus 2 (Irving et al. 2021). Moreover, many ~~types of~~ parasites a~~re also the cause of~~lso cause ~~zoonotic emerging~~emerging zoonotic diseases, resulting in a significant public health burden, particularly in tropical countries (Alasil and Abdullah, 2019).

Several parasites ~~found~~ in confined reptiles spend a portion of their life cycle in the respiratory system. ~~The majority of respiratory involvement is asymptomatic and mild, generating mostly localized inflammation, with the exception of~~Most respiratory involvement is asymptomatic and mild, generating mostly localized inflammation except for ~~instances with~~ excessive parasite loads. However, severe disease can result from high burdens or unusual host-parasite interactions, ~~which~~

~~is~~ frequently ~~made worse~~worsened by secondary infections (Comolli 2021). Reptile-borne pentastomiasis is an unusual tropical disease ~~that is~~ neglected and under-researched in Asia, Africa, and South America (Sulyok et al. 2014).

Pentastomiasis is a parasitic zoonosis caused by infection with ~~pentastomids~~pentatomic, belonging to the subclass Pentastomida, under the phylum Arthropoda (Chittora et al. 2021; Tongtako et al. 2013; WHA 2019). Pentastomiasis~~da~~ ~~are~~ is also known as tongue worms since the adult phase of the genus *Linguatula* has similarities with ~~the~~ mammalian tongues (Paré 2008). Adult parasites are usually found in the respiratory tract of reptiles, birds, and mammals, including humans, while their larval forms are in the internal organs of insects, mammals, and amphibians (Paré 2008; Chen et al. 2010; Kelehear et al. 2014). Some zoonotic ~~pentastomida~~ Pentastomida in snakes ~~are~~ is Armillifer and *Porocephalus crotali* (Rataj et al. 2011). Humans can be infected with ~~pentastomids~~ pentatomic after~~since~~ they consume raw snake meat, have direct contact with the snake, drink water contaminated with parasitic eggs, or are contaminated through the feces (Kelehear et al. 2014; Tappe et al. 2016; Mendoza-Roldan et al. 2020).

Pentastomiasis in snakes have been reported in many countries, such as poisonous Terciopelo (*Bothrops asper*) in Costa Rica (Alvarado et al. 2015), Royal python (*Python regius*) snakes in Nigeria (Ayinmode et al. 2010), Rat snakes (*Ptyas mucosa*) in Sri Lanka and India (Fernando and Fernando 2014; Sundar et al. 2015), wild snakes in Australia (Kelehear et al. 2014), various species of snakes in ~~Slovania~~ Slovenia and Pakistan (Rataj et al. 2011), Chinese moccasin (*Agkistrodon acutus*) and Indian python (*Python molurus*) in China (Chen et al. 2010), and Totonacan rattlesnake (*Crotalus totonacus*) in Mexico (Tepos-Ramírez et al. 2022).

Moreover, t~~T~~o ~~the best of~~ our best knowledge, ~~there are no reports of pentastomids infections in snakes in Indonesia~~snakes in Indonesia have no reports of pentatomic infections. Therefore, ~~therefore, t~~his study reported~~s~~ the prevalence and ~~of pentastomiasis, the~~ identified~~ication~~ pentastomidae~~s of pentastomids,~~ and the pulmonary histopathological profiles in Oriental rat snakes (*P. mucosa*) in Sidoarjo Sub-district, East Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

Th~~is study was reviewed and approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga (number 678-KE)~~e Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga (number 678-KE) reviewed and approved this study.

Parasite collection

The sample used comes from the Oriental rat snake (*Ptyas mucosa*), which is often caught and sold for its meat and skin through collectors. The collection was conducted at a snake slaughterhouse in Tulangan District, Sidoarjo Regency, East Java Province, Indonesia. The presence of pentastomid parasites was observed in the respiratory tract of 60~~sixty~~ slaughtered snakes. Pentastomides were taken and cleaned with distilled water, then stored in sterile plastic pots. The lungs of the snakes infected with the parasite were also isolated for histopathological preparation.

Prevalence calculation

The ~~prevalence of pentastomiasis in snake samples was calculated by the following formula~~following formula calculated the prevalence of pentastomiasis in snake samples:

Prevalence (%) = (The number of snakes infected with pentastomids)/(Total sample of snakes) × 100%.

Semichen-acetic carmine staining

The collected pentastomids were stained with the Semichen-Acetic Carmine technique (Kuhlmann 2006). Adult pentastomids were fixed on object glasses and soaked in 5% glycerin alcohol for 24 hours. Then they were soaked in 70% alcohol for five minutes. Subsequently, the object glasses with pentastomids were placed in Carmine alcohol solution (1:2) for eight hours. ~~The~~ Next, the pentastomids were soaked in acid alcohol for 10 minutes and moved to base alcohol for 20 minutes. Furthermore, graded dehydration was carried out with 70%, 85%, and 95% alcohol for five minutes~~, respectively~~. The mounting step was done using ~~Hung's~~ Hung's I for 20 minutes. ~~Pentastomids~~ Finally, pentastomids were taken and placed on new clean object glasses. The pentastomids were dripped with ~~Hung's~~ Hung's II solutions and then covered with a cover glass.

Parasite identification

Characteristics of the head and body shape and body size of the parasite were identified based on Riley and Self (1980), Almedia et al. (2006), and Christoffersen and De Assis (2013; 2015). Identification of parasite egg size was carried out according to Keegan (1943).

The ~~length of the~~ fresh parasite's length was measured from the anterior edge to the posterior end. The head was observed under a stereo microscope and documented with a Canon Digital Single-Lens Reflex (DSLR) camera. Furthermore, s~~S~~tained parasites were observed using a light microscope with 40-100× magnification and documented using Miconos Optilab. Some female parasites were incubated in PBS overnight at 37 °C to collect the eggs. The supernatants were centrifuged at 1,500 rpm for 10 minutes, and the pellet was dripped onto a glass object and observed using a light microscope with 100× magnification. Those e~~E~~ggs were then measured using Miconos Optilab.

Histopathological preparations

The lungs of snakes with pentastomid infestation were fixed in 10% formalin. Then graded dehydration was carried out with serial alcohol concentrations (70%, 80%, 90%, 96%, and absolute) and clearing using xylol. Furthermore, paraffin embedding was performed to obtain a solid block of tissue. The tissue blocks were sliced (3 micrometers thick) using a microtome, mounted on an object glass, and stained using Hematoxylin and Eosin. Microscopic examination was performed using Nikon Eclipse Ci.

RESULTS AND DISCUSSIONS

The prevalence of pentastomiasis in *Ptyas mucosa* snakes

Thirty-nine (or 65%) of the respiratory tract of 60 samples were positively infected with adult pentastomids; a~~. In~~ total of~~,~~ 204 pentastomids samples were collected (Table 1). Pentastomida ~~were~~ was found to be strongly attached to the trachea and lungs of snakes (Figure 1). Some pentastomids even buried their heads in the lungs of snakes. The parasites of all snakes positively infected with pentastomids were found in the lungs (39 snakes), while only ~~5~~ five (5) snakes which parasites spread to their tracheas.

Table 1. Prevalence of pentastomiasis in *Ptyas mucosa* based on site and the number of parasites.

Sites	Positive (%)	Number of parasites
Lungs only	57% (34 of 60)	2-9 with ~~the~~ an average of 5 ~~pentastomida~~Pentastomida
Trachea only	0% (0 of 60)	-
Lungs and Trachea	8% (5 of 60)	7-9 with ~~the~~ an average of 8 ~~pentastomida~~Pentastomida
Total	**65% (39 of 60)**	**204 pentastomida**

Figure 1. Pentastomid infestation in the lungs of *Ptyas mucosa*. The white arrows show areas with raised lesions around the attachment of pentastomids to the tissue. The black arrows show female pentastomids, and the red arrow shows male pentastomids.

This is the first study to determine the prevalence of pentastomiasis in *Ptyas mucosa* in Sidoarjo, East Java, Indonesia. ~~As shown in our results,~~Our results showed that pentastomids were present in 39 of 60 (65%) snakes (Table 1). ~~Based on t~~This finding shows~~,~~ the prevalence of pentastomiasis in *P. mucosa* ~~in this study~~ was quite high. ~~In~~ T~~t~~his study's~~case, it was similar as the~~ prevalence is similar ~~of~~ to pentastomiasis in Brazil~~,~~ that 8 of 15 or 53.33% of snakes were positively infected with pentastomida. The ~~high prevalence in this research was probably caused by the food consumed by the snakes~~food consumed by the snakes probably caused the high prevalence in this research. The type of food is very influential on the prevalence of infection and the type of pentastomida that infected the snakes (Almeida et al. 2007). *P. mucosa* snakes usually feed on rats, frogs, toads, lizards, and even smaller non-venomous and smaller snakes (Sidik 2006). In addition, a~~A~~ccording to Esslinger (1962), rats, frogs, toads, lizards, and even non-venomous snakes can act as the intermediate hosts of pentastomida.

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Identification of pentastomida

The characteristics of pentastomid found in this study can be seen in Table 2 and Figure 2. Pentastomids have a long cylindrical body with a larger head, a flat shape on the ventral, and a tapered tail or posterior. Their body was soft and whitish with a colorless cuticle, which ~~tends~~ tended to be transparent ~~so that the internal organs are~~to make the internal organs visible. There were annuli throughout their body up to the posterior. There are two pairs of hooks (a total of four

~~hooks~~) around the head that ~~are used to~~ attach to the ~~host's~~ host's lung tissue, and it ~~had~~ has an ovoid-shaped mouth between the two pairs of hooks.

Table 2. The characteristics of pentastomida compared with *Kiricephalus pattoni* by Riley and Self (1980) and Keegan (1943).

Parameters	**The current study**		**British Museum collection (Riley and Self 1980)**		**Keegan 1943**
	Females	**Males**	**Females**	**Males**	
Total number of parasites	183	21	17	2	-
Body length	72-125 mm (X̄ 94.5 mm)	17-28.5 mm (X̄ 23.75 mm)	83-137 mm (X̄ 100 mm)	28-29 mm (X̄ 28.5 mm)	-
Number of *annuli*	29-41 (X̄ 31.6)	28-35 (X̄ 31)	34-38 (X̄ 36.5)	31-32 (X̄ 31.5)	-
Diameter of cephalothorax	4-5.5 mm (X̄ 4.75 mm)	2-2.5 mm (X̄ 2 mm)	4 mm	-	-
Neck	2-3 mm (X̄ 2.5 mm)	-	2 mm	-	-
Diameter of abdomen	3.5-5 mm (X̄ 4.2 mm)	1.8-2.2 mm (X̄ 2 mm)	4 mm	-	-
Hook parameter:			-	-	-
a. AC					
b. CB	0.23-0.55 mm	0.22-0.35 mm			
c. AD	0.37-0.69 mm	0.29-0.35 mm			
	0.47-0.88 mm	0.29-0.45 mm			
Mouth:			-	-	-
a. Length	0.16-29 mm	0.14-0.38 mm			
b. Width	0.12-0.24 mm	0.12-0.18 mm			
Egg:		-	-	-	
a. Length	114-130 µm (X̄ 118 µm)				125-154 µm (X̄ 133 µm)
b. Width	108-124 µm (X̄ 116 µm)				106-143 µm (X̄ 123 µm)

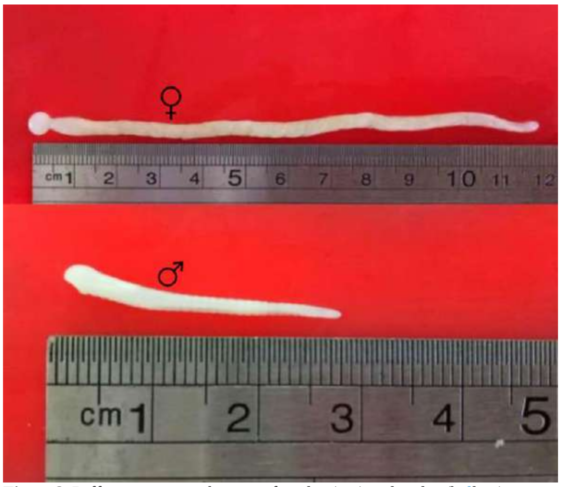

Figure 2. Differences in size between females (top) and males (below).

Both sexes, adult males and female pentastomids~~,~~ were found in this research (Figures 1 and 2). The number of male pentastomid was less (21 heads) than that of females (183 heads) Table 2. Table 2 also describes the size of each pentastomid. The female pentastomids had longer and larger bodies than the males (Figures 1 and 2). The length of the female bodies was 72-125 mm, with an average of 94.5 mm. Their abdominal diameter was 3.5-5 mm with an average of, averaging 4.2 mm. Furthermore, the anterior section or cephalothorax of the females was globular, with a neck separating their head and their abdomen (Figure 3A). The average length and width of the female mouths were 0.24 mm and 0.16 mm. Furthermore, the results of the microscopy examination using Carmine staining showed that the female pentastomid had two spermathecae on the anterior part. Tthhat connects directly to the uterus that contains the egg (Figures 3C, 4B, and 4C). In addition, the vVagina was in the posterior body and around the anus (Figure 4B).

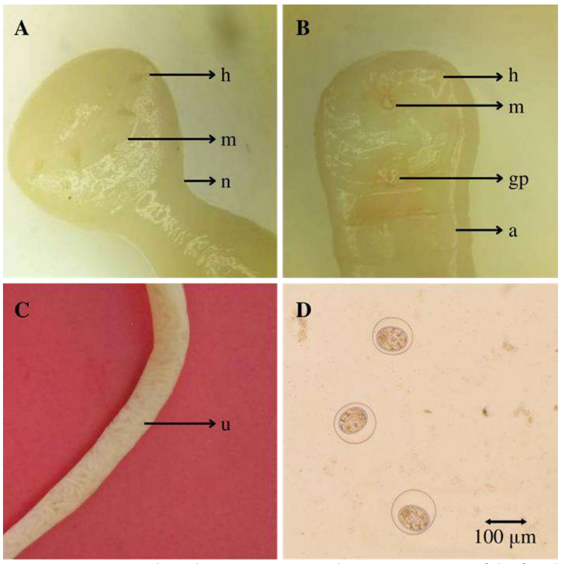

Figure 3. Pentastomida without staining. (A) The anterior section of the female. (B) The anterior section of the male. (C) Female body. (D) Pentastomid's Pentastomid's eggs. h: hook, m: mouth, n: neck, gp: genital pore, a: first annuli, u: uterus.

On the other hand, the length of the males' males' bodies was 17-28.5 mm, with an average of 23.75 mm. Their abdominal diameter was 1.8-2.2 mm with an average of, averaging 2 mm. The average length and width of the mouth were 0.23 and 0.14 mm, respectively. The male body was simple and straight without any curves in the neck (Figure 3B). The genital pore was identified in the first annulus, and there was a pair of seminal vesicles, a pair of vasa deferens, and cirrus (4D).

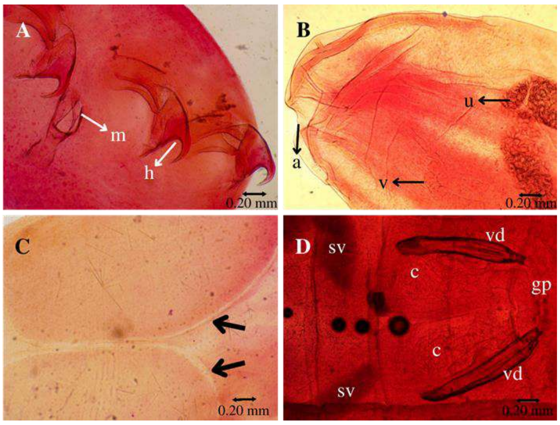

Figure 4. Carmine-stained pentastomida. (A) The anterior section. (B) The posterior section of the female. (C) A pair of female spermatheca. (D) Male reproductive organs. m: mouth, h: hook, a: anus, v: vagina, u: uterus, sv: seminal vesicle, c: cirrus, vd: vas deferens.

The eggs were oval and consisted of two layers of membrane that clearly could be distinguished. The first layer (outer membrane) was thin, while the second layer (inner membrane) was thick and yellowish. There was fluid-filled space between the first and second layers. The length of the eggs was 114-130 µm with an average of 118 µm, and the width was 108-124 µm with an average of 116 µm (Figure 3D).

Pentastomida ~~were~~ was mostly found in the lungs, and the parasites also spread to the trachea in five snakes. This finding was similar to research by Riley and Self (1980) that revealed that adult *Kiricephalus pattoni* (one of the pentastomida species) were only found in the lungs of *P. mucosa* snakes. In our observation, pentastomids that spread to the trachea were females. According to Sundar et al. (2015), the adult pentastomids with mature eggs are expelled from the trachea and eliminated from the definitive host by oral expulsion or may also be swallowed. That results~~, resulting~~ in shed eggs in the feces, ~~in~~ which another intermediate mammal host will ingest to continue their life cycles.

The number of male pentastomids was fewer than the number of female ones. This case was also experienced by John and Nadakal (1988) and Riley and Self (1980), ~~that~~ who only found a few males compared the female. This study result follows~~We agree with~~ Almeida et al. (2006), whose number of pentastomids and~~that pentastomids are not well known, in biology and the number of their species~~ their species is unknown in biology.

The taxonomy of pentastomida is still controversial. Moreover, i~~I~~t has been known through molecular data that pentastomida are closely related to crustaceans and belong to the class Maxillopoda, sub-phylum Crustacea, and the phylum Arthropoda (Paré 2008). Based on the ~~size of the~~ egg size (Almeida et al. 2007);~~,~~ the ~~form of the~~ female head form that ~~is~~ globular with a neck separating the head from its body (Paré 2008); and the size of the body length, the number of the annuli, the diameter of the cephalothorax, neck, abdominal diameter, head, and body shape (Riley and Self 1979; Riley and Self 1980);~~,~~ ~~the~~ pentastomid~~a~~a ~~found~~ was classified as *K. pattoni*. *K. pattoni*~~i~~ can be classified ~~into the order of~~as P~~p~~orochepalida (Paré 2008). In addition, a~~A~~ccording to Christoffersen and De Assis (2013; 2015), pentastomid in *P. mucosa* snakes are classified into *K. pattoni*.

K. pattoni has a long annulated body, a tapered tail, an anal canal, two pairs of hooks or ~~a total of four hooks on its head, as well as~~four hooks on its head, and a small ovoid-shaped mouth (Riley and Self, 1979). The female *K. pattoni* has a larger body than the male one (Paré 2008). Female *K. pattoni* also has a spiral body with a larger head than its body, and a curve of the neck, whereas male one has a straight body without a curve on the neck (Riley and Self 1979; Riley and Self 1980; John and Nadakal 1988; Almeida et al. 2007). The spiral body in females is likely to facilitate their movement into the lung tissue of the definitive hosts (John and Nadakal 1988).

The egg morphology is similar ~~of the eggs like~~to the eggs of *Porocephalus crotali* described by Sundar et al. (2015). T~~,~~ ~~t~~hesee eggs were composed of two-layer membranes, of which the outer one ~~is~~ was thin~~,~~ and the inner membrane thick, and there was a fluid-filled space between those two membranes. But the average diameter of the eggs measured in this investigation is smaller (118 x 116 µm) than the eggs of *P. crotali* (144 µm), as described by Sundar et al. (2015).

Histopathological changes in the lungs of *Ptyas mucosa*

Squamates typically have two lungs~~,~~; however, the left lung in most snakes is greatly reduced, never exceeding 85% of the right ~~lung's~~ lung's size~~,~~ or ~~absent~~ absence (Funk and Bogan 2019). The right lung usually runs from near the heart to the cranial to the right kidney (Funk and Bogan 2019). The cranial portion of a lung is vascularized and functions primarily as a gas exchanger (vascular lung), whereas the caudal primarily functions as an air sac (saccular lung). The vascular lung's wall ~~is made up of~~comprises honeycombed units called faveoli, ~~which allow for~~allowing gas exchange (Funk and Bogan 2019). ~~Microscopically,~~ T~~t~~he faveoli in the snake lung parenchyma are lined ~~mainly~~ microscopically by squamous epithelial cells (type I cells). In addition ~~and,~~ to a lesser degree, by cuboidal epithelial cells (type II) (Jacobson et al. 2021).

In this study, a nymphal stage of the parasite was found in the pulmonary tissue infected with pentastomida. The parasite is surrounded by some connective tissue (Figure 5). Finding the stages of the nymph in the ~~histological lung of the snake and the stages of the parasite~~snake's histological lung and the parasite's stages ~~on~~ in the macroscopic lung organ means that the *P. mucosa* snake may act as an intermediate and final host of *K. pattoni*. Within the lung cavities of the definitive host, the nymphs develop into adults (Sundar et al. 2015). In this study, pentastomida infection in the lungs only caused a mild histological reaction in the form of fibrosis in the area around the parasite.

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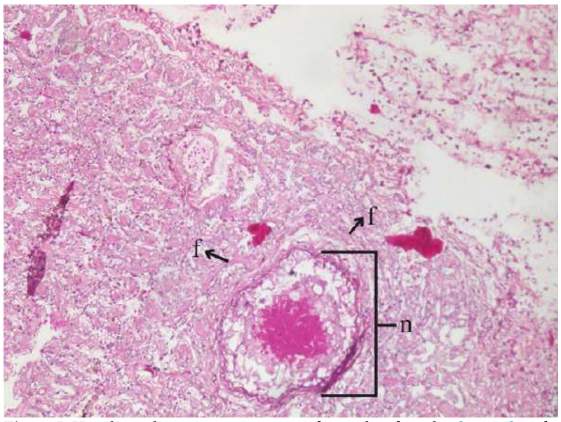

Figure 5. Histological microscopic picture of nymph-infected ~~pulmo~~ Pulmo from pentastomida showed the presence of fibroblast around the area of infection. f: fibroblast, n: the body of the nymph stage pentastomida.

Pentastomida ~~have~~ has an indirect life cycle that typically utilizes vertebrate hosts. ~~Many of~~Consequently, t~~-~~hese~~their~~ species rely on reptiles as their definitive host. ~~, and t~~The adults are usually found in their lungs (Wellehan and Walden 2019). Pentastomida parasites migrate extensively through the visceral before settling in the lungs and, less typically, the trachea and nasal passageways. Pentastomida can produce localized lesions, ~~whilst~~ while molting can elicit antigenic reactions. Adults feed on lung tissue fluids and pass embryonated eggs, which are coughed up through the oral cavity, swallowed, and passed in the feces (Comolli and Divers 2021). In squamates, the pulmonary lumen is large, and the tracheal diameter may be smaller than that of the pentastomids. That~~is~~ poses a risk of respiratory obstruction by the adult pentastomides. However, the greatest pathological changes are seen in the intermediate host, where they may become embedded in tissues (Wellehan and Walden 2019).

Pentastomiasis in reptiles, especially snakes, occurs subclinically. A~~, a~~lthough the infection is large, ~~and~~ death is usually associated with secondary septicemia and pneumonia (Tappe and Büttner 2009). Because pentastomids attach to the lung tissue, they can cause perforation, hemorrhaging, and serious inflammation in the host (Fernando and Fernando 2014). In a Common Garter Snake (*Thamnophis sirtalis*), lung lesions were associated with one of the pentastomida species, *Kiricephalus coarctatus*, where the embedded parasite had gotten into the hypaxial muscle. Infections with *Armillifer armillatus* have also been linked to parasitic pneumonia in *Gaboon vipers*. Pentastomids occluding the trachea have been implicated in snake deaths. Bacterial infections could develop due to lung damage. For example, there are f~~F~~our out of nine *Boa constrictors* with pulmonary damage from *Porocephalus dominicana* infection ~~-~~perished~~ed~~ due to~~from~~ bacteremia and pericarditis (Walden et al. 2021).

Bigger implications~~,~~ ~~in terms of~~regarding possible impacts on human health~~,~~ may be anticipated based on the histopathological changes found in this study. Humans are the final hosts of ~~pentastomes~~pentatomic, which can be contracted from improperly cooked meat from infected snakes or ~~from~~ drinking water contaminated with pentastomida eggs. In addition, humans ~~have the possibility of getting~~can get the infection when handling and harvesting snake~~-~~skin or having direct contact with the mouth of a pet snake (Latif et al. 2016). The pathological consequences ~~to~~ of the clinical symptoms experienced by infected humans may be the same or even more severe. That~~,~~ ~~depending~~ depends on the number of parasite eggs ingested, the duration from the larvae migrating to various organs to maturity and reproduction, and how severe the parasite infestation is in the human viscera.

Personal hygiene measures~~, such as avoiding drinking river water directly or boiling the water before drinking,~~ are required to prevent human infections. For example, avoid drinking river water directly or boiling the water before drinking. ~~Authorities~~ In addition, authorities should provide health education to warn people about eating undercooked snake meat, the possibility of transmission after handling snakes, and avoiding contact with snake excretions.

CONCLUSION

The prevalence of pentastomiasis in slaughtered *Ptyas mucosa* in Sidoarjo was 65% (39 of 60 snakes). The pentastomids that infect these snakes are classified as *Kiricephalus pattoni*. The pulmonary histological findings only show a light reaction in the form of mild fibrosis.

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First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (*Ptyas mucosa*) in Sidoarjo, East Java, Indonesia

Abstract. Currently, reptiles, especially snakes, are increasingly popular as pets worldwide and are often consumed by several communities in Indonesia. This trend needs attention from a public health perspective because it can potentially cause zoonotic diseases, including parasitic disease and pentastomiasis. This study aimed to determine: the prevalence of pentastomiasis, identify the parasite, and observe the pulmonary histopathology in Oriental rat snakes (*Ptyas mucosa*) collected in Tulangan, Sidoarjo, Indonesia. The methods used were calculating the prevalence of pentastomiasis, semichen-acetic carmine staining, and identifying parasites and eggs based on references as a comparison. Furthermore, histopathological preparation and hematoxylin-eosin staining were performed on the lungs infected with the parasite to observe the microscopic lesions. The results showed that 65% of the snakes had pentastomiasis, and the parasites were called *Kirichephalus pattoni*. Furthermore, a microscopical examination revealed mild fibrosis in the pulmonary area due to pentastomid infestation. This study is the first reported pentastomida in *P. mucosa* in Sidoarjo, Indonesia, showing that snakes caught and commonly consumed were infected with zoonotic pentatomic.
Key-words: Colubrid Snake, Parasitic Pneumonia, Tongue Worms, Zoonotic Parasitoses.

Abbreviations:

Running title: Pentastomiasis in Rat Snake

INTRODUCTION

Exotic animals such as reptiles, especially various snakes, are now popular as pets worldwide (Okulewicz et al. 2014). However, they are also concerned with the increasing animal welfare and health issues through the animal trade in the market (Yudhana et al. 2021). Some species of snake are bred in captivity, while others are caught in the wild and sold as pets or bushmeat for consumption (Hardi et al. 2017; Yudhana et al. 2021).

Ptyas mucosa is a well-known snake species in Indonesia (Pranashinta et al. 2017). This type of snake, known as the Oriental rat snake and Indian rat snake, belongs to the Colubridae family which is widely distributed throughout East, South, and Southeast Asia (Auliya 2010; Muliya and Bhat 2016; Yuan et al. 2022). *P. mucosa* is a diurnal and semi-arboreal snake species that live on forest floors, agricultural fields, wet ground, and near human habitats. It feeds on frogs, rats, and other small animals (Chittora et al. 2021).

The meat of *P. mucosa* is used in the illegal trade to China (Auliya 2010). The meat and internal organs are used as fresh or frozen food, the bile and blood are used in traditional medicine, and the skin is exported to several European countries as raw materials for the leather industry (Pranashinta et al. 2017). Although illegal, most rural communities in Indonesia trade and consume the meat and bile of *P. mucosa* for some reasons (Auliya 2010; Pranashinta et al. 2017).

Wildlife meat, however, poses a threat of transmitting infection with novel and exotic pathogens to human hosts (Hardi et al. 2017). Then new zoonotic diseases are likely to emerge consequently. For instance, Bats (Chiroptera) are important reservoirs of several emerging diseases, including Ebola virus disease (EVD), Severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS), as well as the current pandemic COVID-19 caused by SARS Coronavirus 2 (Irving et al. 2021). Moreover, many parasites also cause emerging zoonotic diseases, resulting in a significant public health burden, particularly in tropical countries (Alasil and Abdullah 2019).

Several parasites in confined reptiles spend a portion of their life cycle in the respiratory system. Most respiratory involvement is asymptomatic and mild, generating mostly localized inflammation except for excessive parasite loads. However, severe disease can result from high burdens or unusual host-parasite interactions, frequently worsened by secondary infections (Comolli 2021). Reptile-borne pentastomiasis is an unusual tropical disease neglected and under-researched in Asia, Africa, and South America (Sulyok et al. 2014).

Pentastomiasis is a parasitic zoonosis caused by infection with pentatomic, belonging to the subclass Pentastomida, under the phylum Arthropoda (Chittora et al. 2021; Tongtako et al. 2013; WHA 2019). Pentastomiasis is also known as tongue worms since the adult phase of the genus *Linguatula* has similarities with mammalian tongues (Paré 2008). Adult parasites are usually found in the respiratory tract of reptiles, birds, and mammals, including humans, while their larval forms are in the internal organs of insects, mammals, and amphibians (Paré 2008; Chen et al. 2010; Kelehear et al. 2014). Some zoonotic Pentastomida in snakes is Armillifer and *Porocephalus crotali* (Rataj et al. 2011). Humans can be infected with pentatomic after they consume raw snake meat, have direct contact with the snake, drink water contaminated with parasitic eggs, or are contaminated through the feces (Kelehear et al. 2014; Tappe et al. 2016; Mendoza-Roldan et al. 2020).

Pentastomiasis in snakes have been reported in many countries, such as poisonous Terciopelo (*Bothrops asper*) in Costa Rica (Alvarado et al. 2015), Royal python (*Python regius*) snakes in Nigeria (Ayinmode et al. 2010), Rat snakes (*Ptyas mucosa*) in Sri Lanka and India (Fernando and Fernando 2014; Sundar et al. 2015), wild snakes in Australia (Kelehear et al. 2014), various species of snakes in Slovenia and Pakistan (Rataj et al. 2011), Chinese moccasin (*Agkistrodon acutus*) and Indian python (*Python molurus*) in China (Chen et al. 2010), and Totonacan rattlesnake (*Crotalus totonacus*) in Mexico (Tepos-Ramírez et al. 2022).

Moreover, to our best knowledge, snakes in Indonesia have no reports of pentatomic infections. Therefore, this study reported the prevalence and identified pentastomida and the pulmonary histopathological profiles in Oriental rat snakes (*P. mucosa*) in Sidoarjo Sub-district, East Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

The Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga (number 678-KE) reviewed and approved this study.

Parasite collection

The sample used comes from the Oriental rat snake (*Ptyas mucosa*), which is often caught and sold for its meat and skin through collectors. The collection was conducted at a snake slaughterhouse in Tulangan District, Sidoarjo Regency, East Java Province, Indonesia. The presence of pentastomid parasites was observed in the respiratory tract of 60 slaughtered snakes. Pentastomides were taken and cleaned with distilled water, then stored in sterile plastic pots. The lungs of the snakes infected with the parasite were also isolated for histopathological preparation.

Prevalence calculation

The following formula calculated the prevalence of pentastomiasis in snake samples:

Prevalence (%) = (The number of snakes infected with pentastomids)/(Total sample of snakes) × 100%.

Semichen-acetic carmine staining

The collected pentastomids were stained with the Semichen-Acetic Carmine technique (Kuhlmann 2006). Adult pentastomids were fixed on object glasses and soaked in 5% glycerin alcohol for 24 hours. Then they were soaked in 70% alcohol for five minutes. Subsequently, the object glasses with pentastomids were placed in Carmine alcohol solution (1:2) for eight hours. Next, the pentastomids were soaked in acid alcohol for 10 minutes and moved to base alcohol for 20 minutes. Furthermore, graded dehydration was carried out with 70%, 85%, and 95% alcohol for five minutes. The mounting step was done using Hung's I for 20 minutes. Finally, pentastomids were taken and placed on new clean object glasses. The pentastomids were dripped with Hung's II solutions and then covered with a cover glass.

Parasite identification

Characteristics of the head and body shape and body size of the parasite were identified based on Riley and Self (1980), Almedia et al. (2006), and Christoffersen and De Assis (2013; 2015). Identification of parasite egg size was carried out according to Keegan (1943).

The fresh parasite's length was measured from the anterior edge to the posterior end. The head was observed under a stereo microscope and documented with a Canon Digital Single-Lens Reflex (DSLR) camera. Furthermore, stained parasites were observed using a light microscope with 40-100× magnification and documented using Miconos Optilab. Some female parasites were incubated in PBS overnight at 37 °C to collect the eggs. The supernatants were centrifuged at 1,500 rpm for 10 minutes, and the pellet was dripped onto a glass object and observed using a light microscope with 100× magnification. Those eggs were then measured using Miconos Optilab.

Histopathological preparations

The lungs of snakes with pentastomid infestation were fixed in 10% formalin. Then graded dehydration was carried out with serial alcohol concentrations (70%, 80%, 90%, 96%, and absolute) and clearing using xylol. Furthermore, paraffin embedding was performed to obtain a solid block of tissue. The tissue blocks were sliced (3 micrometers thick) using a microtome, mounted on an object glass, and stained using Hematoxylin and Eosin. Microscopic examination was performed using Nikon Eclipse Ci.

RESULTS AND DISCUSSIONS

The prevalence of pentastomiasis in *Ptyas mucosa* snakes

Thirty-nine (or 65%) of the respiratory tract of 60 samples were positively infected with adult pentastomids; a total of 204 pentastomids samples were collected (Table 1). Pentastomida was found to be strongly attached to the trachea and lungs of snakes (Figure 1). Some pentastomids even buried their heads in the lungs of snakes. The parasites of all snakes positively infected with pentastomids were found in the lungs (39 snakes), while only five (5) snakes which parasites spread to their tracheas.

Table 1. Prevalence of pentastomiasis in *Ptyas mucosa* based on site and the number of parasites.

Sites	Positive (%)	Number of parasites
Lungs only	57% (34 of 60)	2-9 with an average of 5 Pentastomida
Trachea only	0% (0 of 60)	-
Lungs and Trachea	8% (5 of 60)	7-9 with an average of 8 Pentastomida
Total	**65% (39 of 60)**	**204 pentastomida**

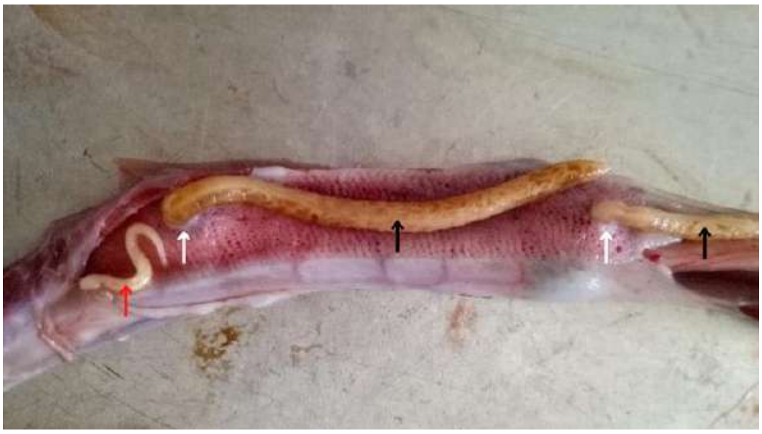

Figure 1. Pentastomid infestation in the lungs of *Ptyas mucosa*. The white arrows show areas with raised lesions around the attachment of pentastomids to the tissue. The black arrows show female pentastomids, and the red arrow shows male pentastomids.

This is the first study to determine the prevalence of pentastomiasis in *Ptyas mucosa* in Sidoarjo, East Java, Indonesia. Our results showed that pentastomids were present in 39 of 60 (65%) snakes (Table 1). This finding shows the prevalence of pentastomiasis in *P. mucosa* was quite high. This study's prevalence is similar to pentastomiasis in Brazil that 8 of 15 or 53.33% of snakes were positively infected with pentastomida. The food consumed by the snakes probably caused the high prevalence in this research. The type of food is very influential on the prevalence of infection and the type of pentastomida that infected the snakes (Almeida et al. 2007). *P. mucosa* snakes usually feed on rats, frogs, toads, lizards, and even smaller non-venomous and smaller snakes (Sidik 2006). In addition, according to Esslinger (1962), rats, frogs, toads, lizards, and even non-venomous snakes can act as the intermediate hosts of pentastomida.

Identification of pentastomida

The characteristics of pentastomid found in this study can be seen in Table 2 and Figure 2. Pentastomids have a long cylindrical body with a larger head, a flat shape on the ventral, and a tapered tail or posterior. Their body was soft and whitish with a colorless cuticle, which tended to be transparent to make the internal organs visible. There were annuli throughout their body up to the posterior. There are two pairs of hooks (a total of four) around the head that attach to the host's lung tissue, and it has an ovoid-shaped mouth between the two pairs of hooks.

Table 2. The characteristics of pentastomida compared with *Kiricephalus pattoni* by Riley and Self (1980) and Keegan (1943).

Parameters	The current study		British Museum collection (Riley and Self 1980)		Keegan 1943
	Females	Males	Females	Males	
Total number of parasites	183	21	17	2	-
Body length	72-125 mm ($\bar{X}$ 94.5 mm)	17-28.5 mm ($\bar{X}$ 23.75 mm)	83-137 mm ($\bar{X}$ 100 mm)	28-29 mm ($\bar{X}$ 28.5 mm)	-
Number of *annuli*	29-41 ($\bar{X}$ 31.6)	28-35 ($\bar{X}$ 31)	34-38 ($\bar{X}$ 36.5)	31-32 ($\bar{X}$ 31.5)	-

Diameter of cephalothorax	4-5.5 mm (X̄ 4.75 mm)	2-2.5 mm (X̄ 2 mm)	4 mm	-	-
Neck	2-3 mm (X̄ 2.5 mm)	-	2 mm	-	-
Diameter of abdomen	3.5-5 mm (X̄ 4.2 mm)	1.8-2.2 mm (X̄ 2 mm)	4 mm	-	-
Hook parameter:			-	-	-
a. AC					
b. CB	0.23-0.55 mm	0.22-0.35 mm			
c. AD	0.37-0.69 mm	0.29-0.35 mm			
	0.47-0.88 mm	0.29-0.45 mm			
Mouth:			-	-	-
a. Length	0.16-29 mm	0.14-0.38 mm			
b. Width	0.12-0.24 mm	0.12-0.18 mm			
Egg:		-	-	-	
a. Length	114-130 µm (X̄ 118 µm)				125-154 µm (X̄ 133 µm)
b. Width	108-124 µm (X̄ 116 µm)				106-143 µm (X̄ 123 µm)

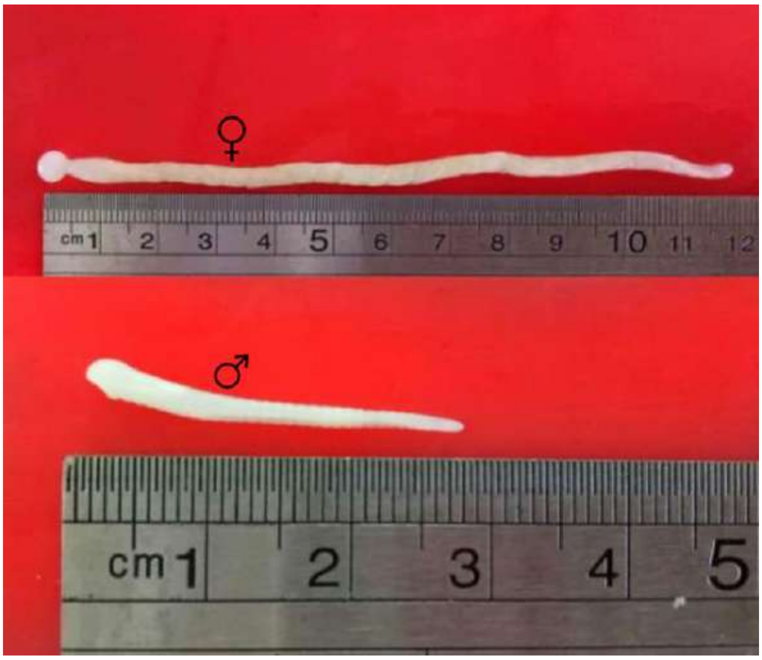

Figure 2. Differences in size between females (top) and males (below).

Both sexes, adult males and female pentastomids were found in this research (Figures 1 and 2). The number of male pentastomid was less (21 heads) than that of females (183 heads) Table 2. Table 2 also describes the size of each pentastomid. The female pentastomids had longer and larger bodies than the males (Figures 1 and 2). The length of the female bodies was 72-125 mm, with an average of 94.5 mm. Their abdominal diameter was 3.5-5 mm, averaging 4.2 mm. Furthermore, the anterior section or cephalothorax of the females was globular, with a neck separating their head and abdomen (Figure 3A). The average length and width of the female mouths were 0.24 mm and 0.16 mm. Furthermore, the results of the microscopy examination using Carmine staining showed that the female pentastomid had two spermathecae on the anterior part. That connects directly to the uterus that contains the egg (Figures 3C, 4B, and 4C). In addition, the vagina was in the posterior body and around the anus (Figure 4B).

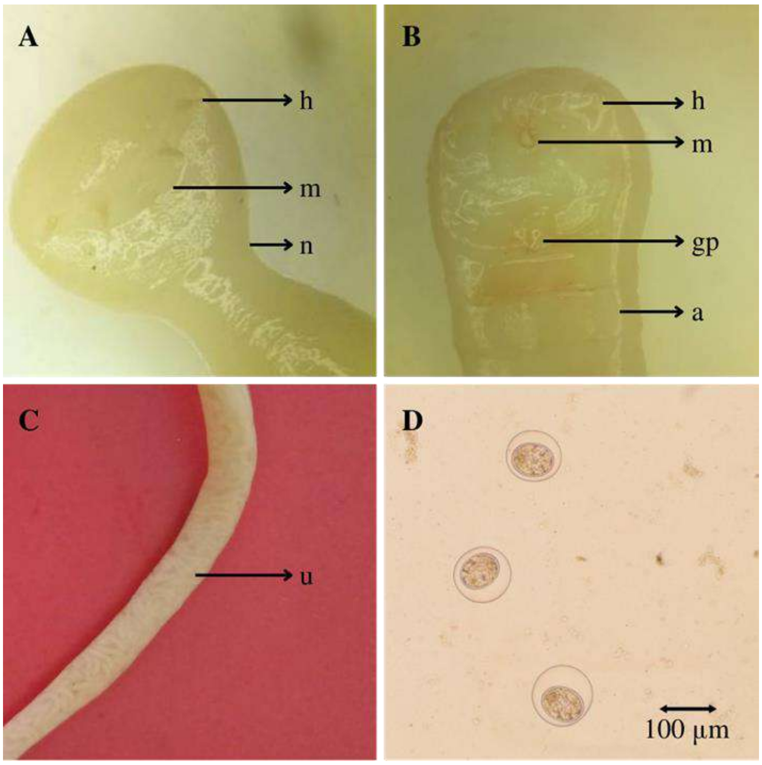

Figure 3. Pentastomida without staining. (A) The anterior section of the female. (B) The anterior section of the male. (C) Female body. (D) Pentastomid's eggs. h: hook, m: mouth, n: neck, gp: genital pore, a: first annuli, u: uterus.

On the other hand, the length of the males' bodies was 17-28.5 mm, with an average of 23.75 mm. Their abdominal diameter was 1.8-2.2 mm, averaging 2 mm. The average length and width of the mouth were 0.23 and 0.14 mm, respectively. The male body was simple and straight without any curves in the neck (Figure 3B). The genital pore was identified in the first annulus, and there was a pair of seminal vesicles, vas deferens, and cirrus (4D).

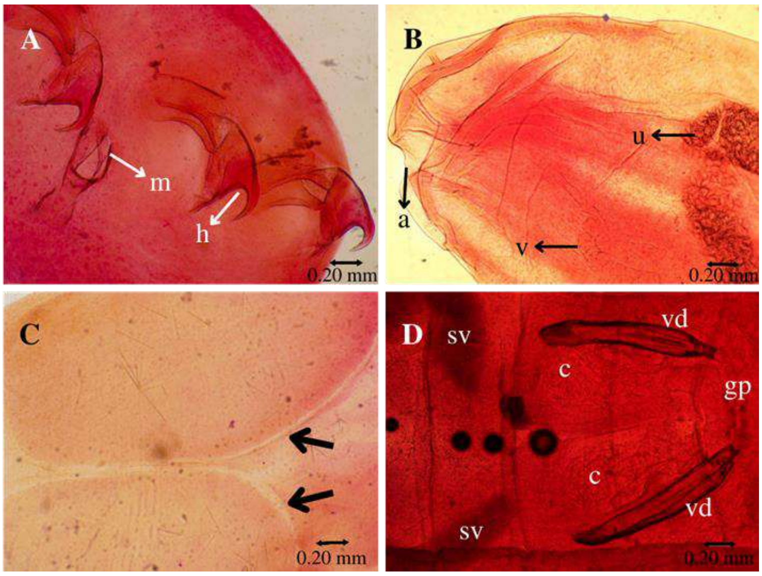

Figure 4. Carmine-stained pentastomida. (A) The anterior section. (B) The posterior section of the female. (C) A pair of female spermatheca. (D) Male reproductive organs. m: mouth, h: hook, a: anus, v: vagina, u: uterus, sv: seminal vesicle, c: cirrus, vd: vas deferens.

The eggs were oval and consisted of two layers of membrane that clearly could be distinguished. The first layer (outer membrane) was thin, while the second layer (inner membrane) was thick and yellowish. There was fluid-filled space between the first and second layers. The length of the eggs was 114-130 µm with an average of 118 µm, and the width was 108-124 µm with an average of 116 µm (Figure 3D).

Pentastomida was mostly found in the lungs, and the parasites also spread to the trachea in five snakes. This finding was similar to research by Riley and Self (1980) that revealed that adult *Kiricephalus pattoni* (one of the pentastomida species) were only found in the lungs of *P. mucosa* snakes. In our observation, pentastomids that spread to the trachea were females. According to Sundar et al. (2015), the adult pentastomids with mature eggs are expelled from the trachea and eliminated from the definitive host by oral expulsion or may also be swallowed. That results in shed eggs in the feces, which another intermediate mammal host will ingest to continue their life cycles.

The number of male pentastomids was fewer than the number of female ones. This case was also experienced by John and Nadakal (1988) and Riley and Self (1980), who only found a few males compared the female. This study result follows Almeida et al. (2006), whose number of pentastomids and their species is unknown in biology.

The taxonomy of pentastomida is still controversial. Moreover, it has been known through molecular data that pentastomida are closely related to crustaceans and belong to the class Maxillopoda, sub-phylum Crustacea, and the phylum Arthropoda (Paré 2008). Based on the egg size (Almeida et al. 2007); the female head form that globular with a neck separating the head from its body (Paré 2008); and the size of the body length, the number of the annuli, the diameter of the cephalothorax, neck, abdominal diameter, head, and body shape (Riley and Self 1979; Riley and Self 1980); pentastomida was classified as *K. pattoni*. *K. pattoni* can be classified as Porochepalida (Paré 2008). In addition, according to Christoffersen and De Assis (2013; 2015), pentastomid in *P. mucosa* snakes are classified into *K. pattoni*.

K. pattoni has a long annulated body, a tapered tail, an anal canal, two pairs of hooks or four hooks on its head, and a small ovoid-shaped mouth (Riley and Self, 1979). The female *K. pattoni* has a larger body than the male one (Paré 2008). Female *K. pattoni* also has a spiral body with a larger head than its body, and a curve of the neck, whereas male one has a straight body without a curve on the neck (Riley and Self 1979; Riley and Self 1980; John and Nadakal 1988; Almeida et al. 2007). The spiral body in females is likely to facilitate their movement into the lung tissue of the definitive hosts (John and Nadakal 1988).

The egg morphology is similar to the eggs of *Porocephalus crotali* described by Sundar et al. (2015). These eggs were composed of two-layer membranes, of which the outer one was thin and the inner membrane thick, and there was a fluid-filled space between those two membranes. But the average diameter of the eggs measured in this investigation is smaller (118 x 116 µm) than the eggs of *P. crotali* (144 µm), as described by Sundar et al. (2015).

Histopathological changes in the lungs of *Ptyas mucosa*

Squamates typically have two lungs; however, the left lung in most snakes is greatly reduced, never exceeding 85% of the right lung's size or absence (Funk and Bogan 2019). The right lung usually runs from near the heart to the cranial to the right kidney (Funk and Bogan 2019). The cranial portion of a lung is vascularized and functions primarily as a gas exchanger (vascular lung), whereas the caudal primarily functions as an air sac (saccular lung). The vascular lung's wall comprises honeycombed units called faveoli, allowing gas exchange (Funk and Bogan 2019). The faveoli in the snake lung parenchyma are lined microscopically by squamous epithelial cells (type I cells). In addition to a lesser degree, by cuboidal epithelial cells (type II) (Jacobson et al. 2021).

In this study, a nymphal stage of the parasite was found in the pulmonary tissue infected with pentastomida. The parasite is surrounded by some connective tissue (Figure 5). Finding the stages of the nymph in the snake's histological lung and the parasite's stages in the macroscopic lung organ means that the *P. mucosa* snake may act as an intermediate and final host of *K. pattoni*. Within the lung cavities of the definitive host, the nymphs develop into adults (Sundar et al. 2015). In this study, pentastomida infection in the lungs only caused a mild histological reaction in the form of fibrosis in the area around the parasite.

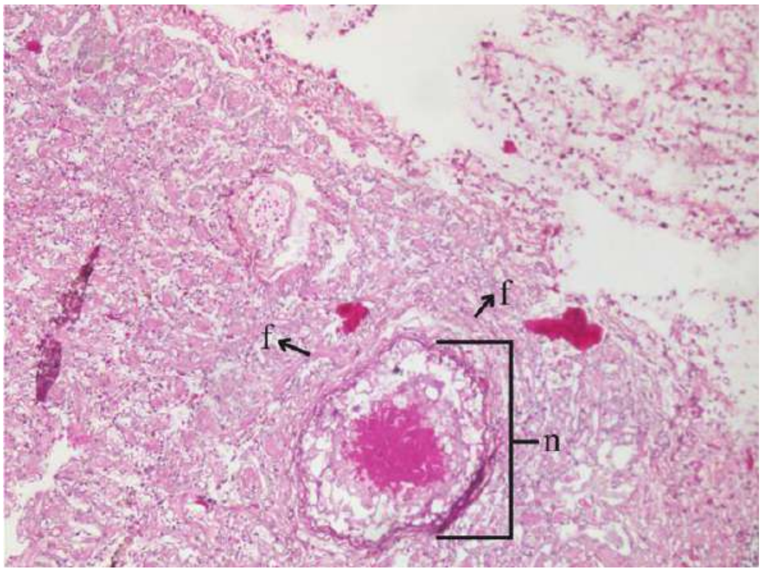

Figure 5. Histological microscopic picture of nymph-infected Pulmo from pentastomida showed the presence of fibroblast around the area of infection. f: fibroblast, n: the body of the nymph stage pentastomida.

Pentastomida has an indirect life cycle that typically utilizes vertebrate hosts. Consequently, these species rely on reptiles as their definitive host. The adults are usually found in their lungs (Wellehan and Walden 2019). Pentastomida parasites migrate extensively through the visceral before settling in the lungs and, less typically, the trachea and nasal passageways. Pentastomida can produce localized lesions, while molting can elicit antigenic reactions. Adults feed on lung tissue fluids and pass embryonated eggs, which are coughed up through the oral cavity, swallowed, and passed in the feces (Comolli and Divers 2021). In squamates, the pulmonary lumen is large, and the tracheal diameter may be smaller than that of the pentastomids. That poses a risk of respiratory obstruction by the adult pentastomids. However, the greatest pathological changes are seen in the intermediate host, where they may become embedded in tissues (Wellehan and Walden 2019).

Pentastomiasis in reptiles, especially snakes, occurs subclinically. Although the infection is large, death is usually associated with secondary septicemia and pneumonia (Tappe and Büttner 2009). Because pentastomids attach to the lung tissue, they can cause perforation, hemorrhaging, and serious inflammation in the host (Fernando and Fernando 2014). In a Common Garter Snake (*Thamnophis sirtalis*), lung lesions were associated with one of the pentastomida species, *Kiricephalus coarctatus*, where the embedded parasite had gotten into the hypaxial muscle. Infections with *Armillifer armillatus* have also been linked to parasitic pneumonia in *Gaboon vipers*. Pentastomids occluding the trachea have been implicated in snake deaths. Bacterial infections could develop due to lung damage. For example, there are four out of nine *Boa constrictors* with pulmonary damage from *Porocephalus dominicana* infection perished due to bacteremia and pericarditis (Walden et al. 2021).

Bigger implications regarding possible impacts on human health may be anticipated based on the histopathological changes found in this study. Humans are the final hosts of pentastomic, which can be contracted from improperly cooked meat from infected snakes or drinking water contaminated with pentastomida eggs. In addition, humans can get the infection when handling and harvesting snakeskin or having direct contact with the mouth of a pet snake (Latif et al. 2016). The pathological consequences of the clinical symptoms experienced by infected humans may be the same or even more severe. That depends on the number of parasite eggs ingested, the duration from the larvae migrating to various organs to maturity and reproduction, and how severe the parasite infestation is in the human viscera.

Personal hygiene measures are required to prevent human infections. For example, avoid drinking river water directly or boiling the water before drinking. In addition, authorities should provide health education to warn people about eating undercooked snake meat, the possibility of transmission after handling snakes, and avoiding contact with snake excretions.

CONCLUSION

The prevalence of pentastomiasis in slaughtered *Ptyas mucosa* in Sidoarjo was 65% (39 of 60 snakes). The pentastomids that infect these snakes are classified as *Kiricephalus pattoni*. The pulmonary histological findings only show a light reaction in the form of mild fibrosis.

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First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (*Ptyas mucosa*) in Sidoarjo, East Java, Indonesia

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Abstract. *Plumeriastuti H, Pranashinta GT, Suwanti LT, Kusnoto, Proboningrat A. 2023. First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (*Ptyas mucosa*) in Sidoarjo, East Java, Indonesia. Biodiversitas 24: xxxx.* Currently, reptiles, especially snakes, are increasingly popular as pets worldwide and are often consumed by several communities in Indonesia. This trend needs attention from a public health perspective because it can potentially cause zoonotic diseases, including parasitic disease and pentastomiasis. This study aimed to determine: the prevalence of pentastomiasis, identify the parasite and observe the pulmonary histopathology in Oriental rat snakes (*Ptyas mucosa* Linnaeus 1758) collected in Tulangan, Sidoarjo, Indonesia. The methods used were calculating the prevalence of pentastomiasis, semichen-acetic carmine staining, and identifying parasites and eggs based on references as a comparison. Furthermore, histopathological preparation and hematoxylin-eosin staining were performed on the lungs infected with the parasite to observe the microscopic lesions. The results showed that 65% of the snakes had pentastomiasis, and the parasites were called *Kirichephalus pattoni* Stephens 1908. Furthermore, a microscopical examination revealed mild fibrosis in the pulmonary area due to pentastomid infestation. This study is the first reported pentastomida in *P. mucosa* in Sidoarjo, Indonesia, showing that snakes caught and commonly consumed were infected with zoonotic pentatomic.

Keywords: Colubrid snake, parasitic pneumonia, tongue worms, zoonotic parasitoses

INTRODUCTION

Exotic animals such as reptiles, especially various snakes, are now popular as pets worldwide (Okulewicz et al. 2014). However, they are also concerned with the increasing animal welfare and health issues through the animal trade in the market (Yudhana et al. 2021). Some species of snake are bred in captivity, while others are caught in the wild and sold as pets or bushmeat for consumption (Hardi et al. 2017; Yudhana et al. 2021).

Ptyas mucosa Linnaeus 1758 is a well-known snake species in Indonesia (Pranashinta et al. 2017). This type of snake, known as the Oriental rat snake and Indian rat snake, belongs to the Colubridae family which is widely distributed throughout East, South, and Southeast Asia (Auliya 2010; Muliya and Bhat 2016; Yuan et al. 2022). *P. mucosa* is a diurnal and semi-arboreal snake species that live on forest floors, agricultural fields, wet ground, and near human habitats. It feeds on frogs, rats, and other small animals (Chittora et al. 2021).

The meat of *P. mucosa* is used in the illegal trade to China (Auliya 2010). The meat and internal organs are used as fresh or frozen food, the bile and blood are used in traditional medicine, and the skin is exported to several European countries as raw materials for the leather industry (Pranashinta et al. 2017). Although illegal, most rural communities in Indonesia trade and consume the meat and bile of *P. mucosa* for some reasons (Auliya 2010; Pranashinta et al. 2017).

Wildlife meat, however, poses a threat of transmitting infection with novel and exotic pathogens to human hosts (Hardi et al. 2017). Then new zoonotic diseases are likely to emerge consequently. For instance, Bats (Chiroptera) are important reservoirs of several emerging diseases, including Ebola virus disease (EVD), Severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS), as well as the current pandemic COVID-19 caused by SARS Coronavirus 2 (Irving et al. 2021). Moreover, many parasites also cause emerging zoonotic diseases, resulting in a significant public health burden, particularly in tropical countries (Alasil and Abdullah 2019).

Several parasites in confined reptiles spend a portion of their life cycle in the respiratory system. Most respiratory involvement is asymptomatic and mild, generating mostly localized inflammation except for excessive parasite loads. However, severe disease can result from high burdens or unusual host-parasite interactions, frequently worsened by secondary infections (Comolli 2021). Reptile-borne pentastomiasis is an unusual tropical disease neglected and under-researched in Asia, Africa, and South America (Sulyok et al. 2014).

Pentastomiasis is a parasitic zoonosis caused by infection with pentatomic, belonging to the subclass

Pentastomida, under the phylum Arthropoda (Chittora et al. 2021; Tongtako et al. 2013; WHA 2019). Pentastomiasis is also known as tongue worms since the adult phase of the genus *Linguatula* has similarities with mammalian tongues (Paré 2008). Adult parasites are usually found in the respiratory tract of reptiles, birds, and mammals, including humans, while their larval forms are in the internal organs of insects, mammals, and amphibians (Paré 2008; Chen et al. 2010; Kelehear et al. 2014). Some zoonotic Pentastomida in snakes is Armillifer and *Porocephalus crotali* Humboldt 1812 (Rataj et al. 2011). Humans can be infected with pentatomic after they consume raw snake meat, have direct contact with the snake, drink water contaminated with parasitic eggs, or are contaminated through the feces (Kelehear et al. 2014; Tappe et al. 2016; Mendoza-Roldan et al. 2020).

Pentastomiasis in snakes have been reported in many countries, such as poisonous Terciopelo (*Bothrops asper* Garman 1883) in Costa Rica (Alvarado et al. 2015), Royal python (*Python regius* Shaw 1802) snakes in Nigeria (Ayinmode et al. 2010), Rat snakes (*P. mucosa*) in Sri Lanka and India (Fernando and Fernando 2014; Sundar et al. 2015), wild snakes in Australia (Kelehear et al. 2014), various species of snakes in Slovenia and Pakistan (Rataj et al. 2011), Chinese moccasin (*Agkistrodon acutus*) and Indian python (*Python molurus* Linnaeus 1758) in China (Chen et al. 2010), and Totonacan rattlesnake (*Crotalus totonacus* Gloyd & Kauffeld 1940) in Mexico (Tepos-Ramírez et al. 2022).

Moreover, to our best knowledge, snakes in Indonesia have no reports of pentatomic infections. Therefore, this study reported the prevalence and identified pentastomida and the pulmonary histopathological profiles in Oriental rat snakes (*P. mucosa*) in Sidoarjo Sub-district, East Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

The Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga (number 678-KE) reviewed and approved this study.

Parasite collection

The sample used comes from the Oriental rat snake (*P. mucosa*), which is often caught and sold for its meat and skin through collectors. The collection was conducted at a snake slaughterhouse in Tulangan Sub-district, Sidoarjo District, East Java Province, Indonesia. The presence of pentastomid parasites was observed in the respiratory tract of 60 slaughtered snakes. Pentastomides were taken and cleaned with distilled water, then stored in sterile plastic pots. The lungs of the snakes infected with the parasite were also isolated for histopathological preparation.

Prevalence calculation

The following formula calculated the prevalence of pentastomiasis in snake samples:

Prevalence (%) = (The number of snakes infected with pentastomids)/(Total sample of snakes) × 100%.

Semichen-acetic carmine staining

The collected pentastomids were stained with the Semichen-Acetic Carmine technique (Kuhlmann 2006). Adult pentastomids were fixed on object glasses and soaked in 5% glycerin alcohol for 24 hours. Then they were soaked in 70% alcohol for five minutes. Subsequently, the object glasses with pentastomids were placed in Carmine alcohol solution (1:2) for eight hours. Next, the pentastomids were soaked in acid alcohol for 10 minutes and moved to base alcohol for 20 minutes. Furthermore, graded dehydration was carried out with 70%, 85%, and 95% alcohol for five minutes. The mounting step was done using Hung's I for 20 minutes. Finally, pentastomids were taken and placed on new clean object glasses. The pentastomids were dripped with Hung's II solutions and then covered with a cover glass.

Parasite identification

Characteristics of the head and body shape and body size of the parasite were identified based on Riley and Self (1980), Almedia et al. (2006), and Christoffersen and De Assis (2013; 2015). Identification of parasite egg size was carried out according to Keegan (1943).

The fresh parasite's length was measured from the anterior edge to the posterior end. The head was observed under a stereo microscope and documented with a Canon Digital Single-Lens Reflex (DSLR) camera. Furthermore, stained parasites were observed using a light microscope with 40-100× magnification and documented using Miconos Optilab. Some female parasites were incubated in PBS overnight at 37°C to collect the eggs. The supernatants were centrifuged at 1,500 rpm for 10 minutes, and the pellet was dripped onto a glass object and observed using a light microscope with 100× magnification. Those eggs were then measured using Miconos Optilab.

Histopathological preparations

The lungs of snakes with pentastomid infestation were fixed in 10% formalin. Then graded dehydration was carried out with serial alcohol concentrations (70%, 80%, 90%, 96%, and absolute) and clearing using xylol. Furthermore, paraffin embedding was performed to obtain a solid block of tissue. The tissue blocks were sliced (3 micrometers thick) using a microtome, mounted on an object glass, and stained using Hematoxylin and Eosin. Microscopic examination was performed using Nikon Eclipse Ci.

RESULTS AND DISCUSSIONS

The prevalence of pentastomiasis in *P. mucosa* snakes

Thirty-nine (or 65%) of the respiratory tract of 60 samples were positively infected with adult pentastomids; a total of 204 pentastomids samples were collected (Table 1). Pentastomida was found to be strongly attached to the trachea and lungs of snakes (Figure 1). Some pentastomids even buried their heads in the lungs of snakes. The parasites of all snakes positively infected with pentastomids were found in the lungs (39 snakes), while only five (5) snakes which parasites spread to their tracheas.

This is the first study to determine the prevalence of pentastomiasis in *P. mucosa* in Sidoarjo, East Java, Indonesia. Our results showed that pentastomids were present in 39 of 60 (65%) snakes (Table 1). This finding shows the prevalence of pentastomiasis in *P. mucosa* was quite high. This study's prevalence is similar to pentastomiasis in Brazil that 8 of 15 or 53.33% of snakes were positively infected with pentastomida. The food consumed by the snakes probably caused the high prevalence in this research. The type of food is very influential on the prevalence of infection and the type of pentastomida that infected the snakes (Almeida et al. 2007). *P. mucosa* snakes usually feed on rats, frogs, toads, lizards, and even smaller non-venomous and smaller snakes (Sidik 2006). In addition, according to Esslinger (1962), rats, frogs, toads, lizards, and even non-venomous snakes can act as the intermediate hosts of pentastomida.

Identification of pentastomida

The characteristics of pentastomid found in this study can be seen in Table 2 and Figure 2. Pentastomids have a long cylindrical body with a larger head, a flat shape on the ventral, and a tapered tail or posterior. Their body was soft and whitish with a colorless cuticle, which tended to be transparent to make the internal organs visible. There were annuli throughout their body up to the posterior. There are two pairs of hooks (a total of four) around the head that attach to the host's lung tissue, and it has an ovoid-shaped mouth between the two pairs of hooks.

Both sexes, adult males and female pentastomids were found in this research (Figures 1 and 2). The number of male pentastomid was less (21 heads) than that of females (183 heads) Table 2. Table 2 also describes the size of each pentastomid. The female pentastomids had longer and larger bodies than the males (Figures 1 and 2). The length of the female bodies was 72-125 mm, with an average of 94.5 mm. Their abdominal diameter was 3.5-5 mm, averaging 4.2 mm. Furthermore, the anterior section or cephalothorax of the females was globular, with a neck separating their head and abdomen (Figure 3A). The average length and width of the female mouths were 0.24 mm and 0.16 mm. Furthermore, the results of the microscopy examination using Carmine staining showed that the female pentastomid had two spermathecae on the anterior part. That connects directly to the uterus that contains the egg (Figures 3C, 4B, and 4C). In addition, the vagina was in the posterior body and around the anus (Figure 4B).

On the other hand, the length of the males' bodies was 17-28.5 mm, with an average of 23.75 mm. Their abdominal diameter was 1.8-2.2 mm, averaging 2 mm. The average length and width of the mouth were 0.23 and 0.14 mm, respectively. The male body was simple and straight without any curves in the neck (Figure 3B). The genital pore was identified in the first annulus, and there was a pair of seminal vesicles, vas deferens, and cirrus (4D).

The eggs were oval and consisted of two layers of membrane that clearly could be distinguished. The first layer (outer membrane) was thin, while the second layer (inner membrane) was thick and yellowish. There was fluid-filled space between the first and second layers. The length of the eggs was 114-130 μm with an average of 118 μm, and the width was 108-124 μm with an average of 116 μm (Figure 3D).

Table 1. Prevalence of pentastomiasis in *P. mucosa* based on site and the number of parasites.

Sites	Positive (%)	Number of parasites
Lungs only	57% (34 of 60)	2-9 with an average of 5 Pentastomida
Trachea only	0% (0 of 60)	-
Lungs and Trachea	8% (5 of 60)	7-9 with an average of 8 Pentastomida
Total	**65% (39 of 60)**	**204 pentastomida**

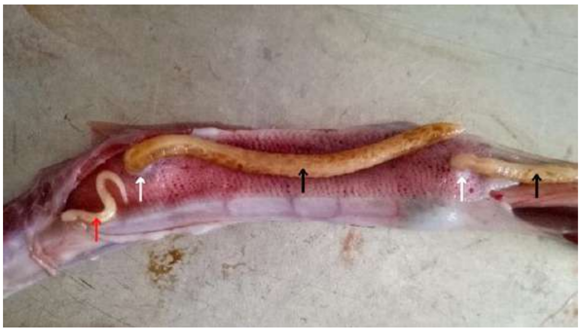

Figure 1. Pentastomid infestation in the lungs of *P. mucosa*. The white arrows show areas with raised lesions around the attachment of pentastomids to the tissue. The black arrows show female pentastomids, and the red arrow shows male pentastomids.

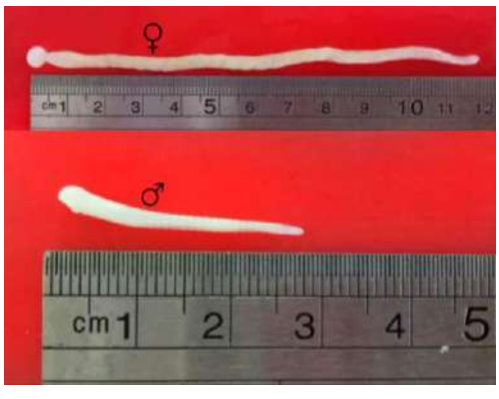

Figure 2. Differences in size between females (top) and males (below).

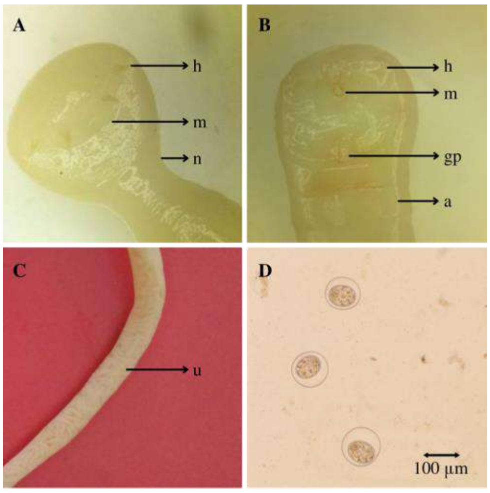

Figure 3. Pentastomida without staining. (A) The anterior section of the female; (B) The anterior section of the male; (C) Female body; (D) Pentastomid's eggs. h: hook, m: mouth, n: neck, gp: genital pore, a: first annuli, u: uterus.

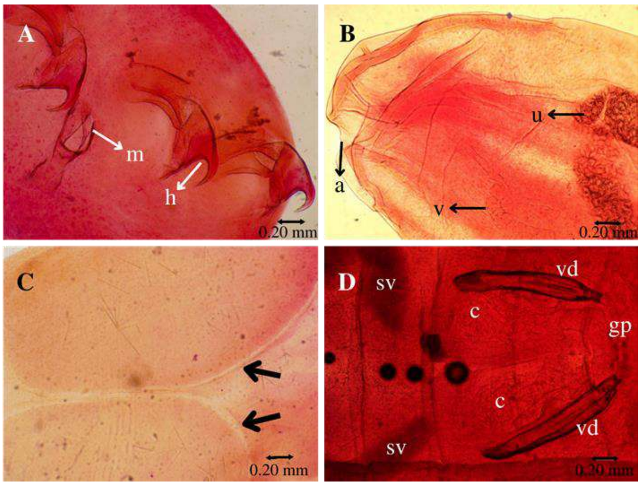

Figure 4. Carmine-stained pentastomida. (A) The anterior section; (B) The posterior section of the female; (C) A pair of female spermatheca; (D) Male reproductive organs. m: mouth, h: hook, a: anus, v: vagina, u: uterus, sv: seminal vesicle, c: cirrus, vd: vas deferens.

Table 2. The characteristics of pentastomida compared with *Kirichephalus pattoni* Stephens 1908 by Riley and Self (1980) and Keegan (1943).

Parameters	**The current study**		**British Museum collection (Riley and Self 1980)**		**Keegan 1943**
	Females	**Males**	**Females**	**Males**	
Total number of parasites	183	21	17	2	-
Body length	72-125 mm (X̄ 94.5 mm)	17-28.5 mm (X̄ 23.75 mm)	83-137 mm (X̄ 100 mm)	28-29 mm (X̄ 28.5 mm)	-
Number of *annuli*	29-41 (X̄ 31.6)	28-35 (X̄ 31)	34-38 (X̄ 36.5)	31-32 (X̄ 31.5)	-
Diameter of cephalothorax	4-5.5 mm (X̄ 4.75 mm)	2-2.5 mm (X̄ 2 mm)	4 mm	-	-
Neck	2-3 mm (X̄ 2.5 mm)	-	2 mm	-	-
Diameter of abdomen	3.5-5 mm (X̄ 4.2 mm)	1.8-2.2 mm (X̄ 2 mm)	4 mm	-	-
Hook parameter:			-	-	-
AC	0.23-0.55 mm	0.22-0.35 mm			
CB	0.37-0.69 mm	0.29-0.35 mm			
AD	0.47-0.88 mm	0.29-0.45 mm			
Mouth:			-	-	-
Length	0.16-29 mm	0.14-0.38 mm			
Width	0.12-0.24 mm	0.12-0.18 mm			
Egg:		-	-	-	
Length	114-130 µm (X̄ 118 µm)				125-154 µm (X̄ 133 µm)
Width	108-124 µm (X̄ 116 µm)				106-143 µm (X̄ 123 µm)

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Pentastomida was mostly found in the lungs, and the parasites also spread to the trachea in five snakes. This finding was similar to research by Riley and Self (1980) that revealed that adult *K. pattoni* (one of the pentastomida species) were only found in the lungs of *P. mucosa* snakes. In our observation, pentastomids that spread to the trachea were females. According to Sundar et al. (2015), the adult pentastomids with mature eggs are expelled from the trachea and eliminated from the definitive host by oral expulsion or may also be swallowed. That results in shed eggs in the feces, which another intermediate mammal host will ingest to continue their life cycles.

The number of male pentastomids was fewer than the number of female ones. This case was also experienced by

John and Nadakal (1988) and Riley and Self (1980), who only found a few males compared the female. This study result follows Almeida et al. (2006), whose number of pentastomids and their species is unknown in biology

The taxonomy of pentastomida is still controversial. Moreover, it has been known through molecular data that pentastomida are closely related to crustaceans and belong to the class Maxillopoda, sub-phylum Crustacea, and the phylum Arthropoda (Paré 2008). Based on the egg size (Almeida et al. 2007); the female head form that globular with a neck separating the head from its body (Paré 2008); and the size of the body length, the number of the annuli, the diameter of the cephalothorax, neck, abdominal diameter, head, and body shape (Riley and Self 1979; Riley and Self 1980); pentastomida was classified as *K. pattoni*. *K. pattoni* can be classified as Porochepalida (Paré 2008). In addition, according to Christoffersen and De Assis (2013; 2015), pentastomid in *P. mucosa* snakes are classified into *K. pattoni*.

K. pattoni has a long annulated body, a tapered tail, an anal canal, two pairs of hooks or four hooks on its head, and a small ovoid-shaped mouth (Riley and Self 1979). The female *K. pattoni* has a larger body than the male one (Paré 2008). Female *K. pattoni* also has a spiral body with a larger head than its body, and a curve of the neck, whereas male one has a straight body without a curve on the neck (Riley and Self 1979; Riley and Self 1980; John and Nadakal 1988; Almeida et al. 2007). The spiral body in females is likely to facilitate their movement into the lung tissue of the definitive hosts (John and Nadakal 1988).

The egg morphology is similar to the eggs of *P. crotali* described by Sundar et al. (2015). These eggs were composed of two-layer membranes, of which the outer one was thin and the inner membrane thick, and there was a fluid-filled space between those two membranes. But the average diameter of the eggs measured in this investigation is smaller (118x116 µm) than the eggs of *P. crotali* (144 µm), as described by Sundar et al. (2015).

Histopathological changes in the lungs of *P.mucosa*

Squamates typically have two lungs; however, the left lung in most snakes is greatly reduced, never exceeding 85% of the right lung's size or absence (Funk and Bogan 2019). The right lung usually runs from near the heart to the cranial to the right kidney (Funk and Bogan 2019). The cranial portion of a lung is vascularized and functions primarily as a gas exchanger (vascular lung), whereas the caudal primarily functions as an air sac (saccular lung). The vascular lung's wall comprises honeycombed units called faveoli, allowing gas exchange (Funk and Bogan 2019). The faveoli in the snake lung parenchyma are lined microscopically by squamous epithelial cells (type I cells). In addition to a lesser degree, by cuboidal epithelial cells (type II) (Jacobson et al. 2021).

In this study, a nymphal stage of the parasite was found in the pulmonary tissue infected with pentastomida. The parasite is surrounded by some connective tissue (Figure 5). Finding the stages of the nymph in the snake's histological lung and the parasite's stages in the macroscopic lung organ means that the *P. mucosa* snake may act as an intermediate and final host of *K. pattoni*. Within the lung cavities of the definitive host, the nymphs develop into adults (Sundar et al. 2015). In this study, pentastomida infection in the lungs only caused a mild histological reaction in the form of fibrosis in the area around the parasite.

Pentastomida has an indirect life cycle that typically utilizes vertebrate hosts. Consequently, these species rely on reptiles as their definitive host. The adults are usually found in their lungs (Wellehan and Walden 2019). Pentastomida parasites migrate extensively through the visceral before settling in the lungs and, less typically, the trachea and nasal passageways. Pentastomida can produce localized lesions, while molting can elicit antigenic reactions. Adults feed on lung tissue fluids and pass embryonated eggs, which are coughed up through the oral cavity, swallowed, and passed in the feces (Comolli and Divers 2021). In squamates, the pulmonary lumen is large, and the tracheal diameter may be smaller than that of the pentastomids. That poses a risk of respiratory obstruction by the adult pentastomids. However, the greatest pathological changes are seen in the intermediate host, where they may become embedded in tissues (Wellehan and Walden 2019).

Pentastomiasis in reptiles, especially snakes, occurs subclinically. Although the infection is large, death is usually associated with secondary septicemia and pneumonia (Tappe and Büttner 2009). Because pentastomids attach to the lung tissue, they can cause perforation, hemorrhaging, and serious inflammation in the host (Fernando and Fernando 2014). In a Common Garter Snake (*Thamnophis sirtalis* Linnaeus 1758), lung lesions were associated with one of the pentastomida species, *Kiricephalus coarctatus* Diesing 1850, where the embedded parasite had gotten into the hypaxial muscle. Infections with *Armillifer armillatus* Wyman 1845 have also been linked to parasitic pneumonia in gaboon vipers. Pentastomids occluding the trachea have been implicated in snake deaths. Bacterial infections could develop due to lung damage. For example, there are four out of nine *Boa constrictors* with pulmonary damage from *Porocephalus dominicana* Riley & Walters 1980 infection perished due to bacteremia and pericarditis (Walden et al. 2021).

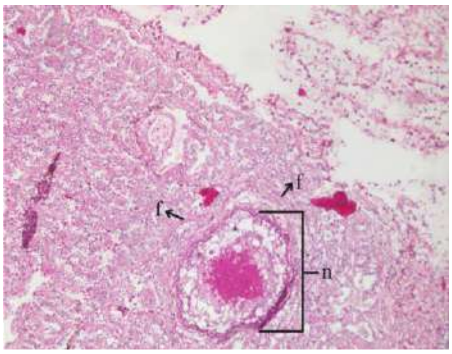

Figure 5. Histological microscopic picture of nymph-infected pulmo from pentastomida showed the presence of fibroblast around the area of infection. f: fibroblast, n: the body of the nymph stage pentastomida.

Bigger implications regarding possible impacts on human health may be anticipated based on the histopathological changes found in this study. Humans are the final hosts of pentastomic, which can be contracted from improperly cooked meat from infected snakes or drinking water contaminated with pentastomida eggs. In addition, humans can get the infection when handling and harvesting snakeskin or having direct contact with the mouth of a pet snake (Latif et al. 2016). The pathological consequences of the clinical symptoms experienced by infected humans may be the same or even more severe. That depends on the number of parasite eggs ingested, the duration from the larvae migrating to various organs to maturity and reproduction, and how severe the parasite infestation is in the human viscera.

Personal hygiene measures are required to prevent human infections. For example, avoid drinking river water directly or boiling the water before drinking. In addition, authorities should provide health education to warn people about eating undercooked snake meat, the possibility of transmission after handling snakes, and avoiding contact with snake excretions.

In conclusion the prevalence of pentastomiasis in slaughtered *P. mucosa* in Sidoarjo was 65% (39 of 60 snakes). The pentastomids that infect these snakes are classified as *K. pattoni*. The pulmonary histological findings only show a light reaction in the form of mild fibrosis.

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First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (*Ptyas mucosa*) in Sidoarjo, East Java, Indonesia

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Abstract. *Plumeriastuti H, Pranashinta GT, Suwanti LT, Proboningrat A, Kusnoto~~, Proboningrat A~~. 2023. First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (*Ptyas mucosa*) in Sidoarjo, East Java, Indonesia. Biodiversitas 24: xxxx.* Currently, reptiles, especially snakes, are increasingly popular as pets worldwide and are often consumed by several communities in Indonesia. This trend needs attention from a public health perspective because it can potentially cause zoonotic diseases, including parasitic disease ~~and~~ such as pentastomiasis. This study aimed to determine: the prevalence of pentastomiasis, identify the parasite and observe the pulmonary histopathology in Oriental ~~rat~~ Rat ~~snakes~~ Snakes (*Ptyas mucosa* Linnaeus 1758) collected in Tulangan, Sidoarjo, Indonesia. The methods used were calculating the prevalence of pentastomiasis, semichen-acetic carmine staining, and identifying parasites and eggs based on references as a comparison. Furthermore, histopathological preparation and hematoxylin-eosin staining were performed on the lungs infected with the parasite to observe the microscopic lesions. The results showed that 65% of the snakes had pentastomiasis, and the parasites were called *Kirichephalus pattoni* Stephens 1908. Furthermore, a microscopical examination revealed mild fibrosis in the pulmonary area due to pentastomid infestation. This study is the first reported pentastomida in *P. mucosa* in Sidoarjo, Indonesia, showing that snakes caught and commonly consumed were infected with zoonotic pentastomic.

Keywords: Colubrid snake, parasitic pneumonia, tongue worms, zoonotic parasitoses

INTRODUCTION

Exotic animals such as reptiles, especially various snakes, are now popular as pets worldwide (Okulewicz et al. 2014). However, they are also concerned with the increasing animal welfare and health issues through the animal trade in the market (Yudhana et al. 2021). Some species of snake are bred in captivity, while others are caught in the wild and sold as pets or bushmeat for consumption (Hardi et al. 2017; Yudhana et al. 2021).

Ptyas mucosa Linnaeus 1758 is a well-known snake species in Indonesia (Pranashinta et al. 2017). This type of snake, known as the Oriental ~~rat~~ Rat ~~snake~~ Snake and Indian ~~rat~~ Rat ~~snake~~Snake, belongs to the Colubridae family which is widely distributed throughout East, South, and Southeast Asia (Auliya 2010; Muliya and Bhat 2016; Yuan et al. 2022). *P. mucosa* is a diurnal and semi-arboreal snake species that live on forest floors, agricultural fields, wet ground, and near human habitats. It feeds on frogs, rats, and other small animals (Chittora et al. 2021).

The meat of *P. mucosa* is used in the illegal trade to China (Auliya 2010). The meat and internal organs are used as fresh or frozen food, the bile and blood are used in traditional medicine, and the skin is exported to several European countries as raw materials for the leather industry (Pranashinta et al. 2017). Although illegal, most rural communities in Indonesia trade and consume the meat and bile of *P. mucosa* for some reasons (Auliya 2010; Pranashinta et al. 2017).

Wildlife meat, however, poses a threat of transmitting infection with novel and exotic pathogens to human hosts (Hardi et al. 2017). Then new zoonotic diseases are likely to emerge consequently. For instance, Bats (Chiroptera) are important reservoirs of several emerging diseases, including Ebola ~~virus~~ Virus ~~disease~~ Disease (EVD), Severe ~~acute~~ Acute ~~respiratory~~ Respiratory ~~syndrome~~ Syndrome (SARS), and Middle East ~~respiratory~~ Respiratory ~~syndrome~~ Syndrome (MERS), as well as the current pandemic COVID-19 caused by SARS Coronavirus 2 (Irving et al. 2021). Moreover, many parasites also cause emerging zoonotic diseases, resulting in a significant public health burden, particularly in tropical countries (Alasil and Abdullah 2019).

Several parasites in confined reptiles spend a portion of their life cycle in the respiratory system. Most respiratory involvement is asymptomatic and mild, generating mostly localized inflammation except for excessive parasite loads. However, severe disease can result from high burdens or unusual host-parasite interactions, frequently worsened by secondary infections (Comolli 2021). Reptile-borne pentastomiasis is an unusual tropical disease neglected and under-researched in Asia, Africa, and South America (Sulyok et al. 2014).

Pentastomiasis is a parasitic zoonosis caused by infection with pentastomic, belonging to the subclass Pentastomida, under the phylum Arthropoda (Chittora et al. 2021; Tongtako et al. 2013; WHA 2019). Pentastomiasis is also known as tongue worms since the adult phase of the genus *Linguatula* has similarities with mammalian tongues (Paré 2008). Adult parasites are usually found in the respiratory tract of reptiles, birds, and mammals, including humans, while their larval forms are in the internal organs of insects, mammals, and amphibians (Paré 2008; Chen et al. 2010; Kelehear et al. 2014). Some zoonotic Pentastomida in snakes is Armillifer and *Porocephalus crotali* Humboldt 1812 (Rataj et al. 2011). Humans can be infected with pentastomic after they consume raw snake meat, have direct contact with the snake, drink water contaminated with parasitic eggs, or are contaminated through the feces (Kelehear et al. 2014; Tappe et al. 2016; Mendoza-Roldan et al. 2020).

Pentastomiasis in snakes have been reported in many countries, such as poisonous Terciopelo (*Bothrops asper* Garman 1883) in Costa Rica (Alvarado et al. 2015), Royal ~~python~~ Python Snakes (*Python regius* Shaw 1802) ~~snakes~~ in Nigeria (Ayinmode et al. 2010), Rat ~~snakes~~ Snakes (*P. mucosa*) in Sri Lanka and India (Fernando and Fernando 2014; Sundar et al. 2015), wild snakes in Australia (Kelehear et al. 2014), various species of snakes in Slovenia and Pakistan (Rataj et al. 2011), Chinese ~~moccasin~~ Moccasin (*Agkistrodon acutus*) and Indian ~~python~~ Python (*Python molurus* Linnaeus 1758) in China (Chen et al. 2010), and Totonacan ~~rattlesnake~~ Rattlesnake (*Crotalus totonacus* Gloyd & Kauffeld 1940) in Mexico (Tepos-Ramírez et al. 2022).

Moreover, to our best knowledge, snakes in Indonesia have no reports of pentastomic infections. Therefore, this study reported the prevalence and identified pentastomida and the pulmonary histopathological profiles in Oriental ~~rat~~ Rat ~~snakes~~ Snakes (*P. mucosa*) in Sidoarjo ~~Sub-district~~Regency, East Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

The Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga (number 678-KE) reviewed and approved this study.

Parasite collection

The sample used comes from the Oriental ~~rat~~ Rat ~~snake~~ Snake (*P. mucosa*), which is often caught and sold for its meat and skin through collectors. The collection was conducted at a snake slaughterhouse in Tulangan ~~Sub-district~~District, Sidoarjo ~~District~~Regency, East Java Province, Indonesia. The presence of pentastomid parasites was observed in the respiratory tract of 60 slaughtered snakes. Pentastomides were taken and cleaned with distilled water, then stored in sterile plastic pots. The lungs of the snakes infected with the parasite were also isolated for histopathological preparation.

Prevalence calculation

The following formula calculated the prevalence of pentastomiasis in snake samples:

Prevalence (%) = (The number of snakes infected with pentastomids) / (Total sample of snakes)~~-~~ × 100%.

Semichen-acetic carmine staining

The collected pentastomids were stained with the Semichen-Acetic Carmine technique (Kuhlmann 2006). Adult pentastomids were fixed on object glasses and soaked in 5% glycerin alcohol for 24 hours. Then they were soaked in 70% alcohol for five minutes. Subsequently, the object glasses with pentastomids were placed in Carmine alcohol solution (1:2) for eight hours. Next, the pentastomids were soaked in acid alcohol for 10 minutes and moved to base alcohol for 20 minutes. Furthermore, graded dehydration was carried out with 70%, 85%, and 95% alcohol for five minutes. The mounting step was done using Hung's I for 20 minutes. Finally, pentastomids were taken and placed on new clean object glasses. The pentastomids were dripped with Hung's II solutions and then covered with a cover glass.

Parasite identification

Characteristics of the head and body shape and body size of the parasite were identified based on Riley and Self (1980), Almedia et al. (2006), and Christoffersen and De Assis (2013; 2015). Identification of parasite egg size was carried out according to Keegan (1943).

The fresh parasite's length was measured from the anterior edge to the posterior end. The head was observed under a stereo microscope and documented with a Canon Digital Single-Lens Reflex (DSLR) camera. Furthermore, stained parasites were observed using a light microscope with 40-100× magnification and documented using Miconos Optilab. Some female parasites were incubated in PBS overnight at 37°C to collect the eggs. The supernatants were centrifuged at 1,500 rpm for 10 minutes, and the pellet was dripped onto a glass object and observed using a light microscope with 100× magnification. Those eggs were then measured using Miconos Optilab.

Histopathological preparations

The lungs of snakes with pentastomid infestation were fixed in 10% formalin. Then graded dehydration was carried out with serial alcohol concentrations (70%, 80%, 90%, 96%, and absolute) and clearing using xylol. Furthermore, paraffin embedding was performed to obtain a solid block of tissue. The tissue blocks were sliced (3 micrometers thick) using a microtome, mounted on an object glass, and stained using Hematoxylin and Eosin. Microscopic examination was performed using Nikon Eclipse Ci.

RESULTS AND DISCUSSIONS

The prevalence of pentastomiasis in *P. mucosa* snakes

Thirty-nine (or 65%) of the respiratory tract of 60 samples were positively infected with adult pentastomids; a

total of 204 pentastomids samples were collected (Table 1). Pentastomida was found to be strongly attached to the trachea and lungs of snakes (Figure 1). Some pentastomids even buried their heads in the lungs of snakes. The parasites of all snakes positively infected with pentastomids were found in the lungs (39 snakes), while only five (5) snakes which parasites spread to their tracheas.

This is the first study to determine the prevalence of pentastomiasis in *P. mucosa* in Sidoarjo, East Java, Indonesia. Our results showed that pentastomids were present in 39 of 60 (65%) snakes (Table 1). This finding shows the prevalence of pentastomiasis in *P. mucosa* was quite high. This study's prevalence is similar to pentastomiasis in Brazil that 8 of 15 or 53.33% of snakes were positively infected with pentastomida. The food consumed by the snakes probably caused the high prevalence in this research. The type of food is very influential on the prevalence of infection and the type of pentastomida that infected the snakes (Almeida et al. 2007). *P. mucosa* snakes usually feed on rats, frogs, toads, lizards, and even smaller non-venomous and smaller snakes (Sidik 2006). In addition, according to Esslinger (1962), rats, frogs, toads, lizards, and even non-venomous snakes can act as the intermediate hosts of pentastomida.

Identification of pentastomida

The characteristics of pentastomid found in this study can be seen in Table 2 and Figure 2. Pentastomids have a long cylindrical body with a larger head, a flat shape on the ventral, and a tapered tail or posterior. Their body was soft and whitish with a colorless cuticle, which tended to be transparent to make the internal organs visible. There were annuli throughout their body up to the posterior. There are two pairs of hooks (a total of four) around the head that attach to the host's lung tissue, and it has an ovoid-shaped mouth between the two pairs of hooks.

Both sexes, adult males and female pentastomids were found in this research (Figures 1 and 2). The number of male pentastomid was less (21 heads) than that of females (183 heads) Table 2. Table 2 also describes the size of each pentastomid. The female pentastomids had longer and larger bodies than the males (Figures 1 and 2). The length of the female bodies was 72-125 mm, with an average of 94.5 mm. Their abdominal diameter was 3.5-5 mm, averaging 4.2 mm. Furthermore, the anterior section or cephalothorax of the females was globular, with a neck separating their head and abdomen (Figure 3A). The average length and width of the female mouths were 0.24 mm and 0.16 mm. Furthermore, the results of the microscopy examination using Carmine staining showed that the female pentastomid had two spermathecae on the anterior part. That connects directly to the uterus that contains the egg (Figures 3C, 4B, and 4C). In addition, the vagina was in the posterior body and around the anus (Figure 4B).

On the other hand, the length of the males' bodies was 17-28.5 mm, with an average of 23.75 mm. Their abdominal diameter was 1.8-2.2 mm, averaging 2 mm. The average length and width of the mouth were 0.23 and 0.14 mm, respectively. The male body was simple and straight without any curves in the neck (Figure 3B). The genital pore was identified in the first annulus, and there was a pair of seminal vesicles, vas deferens, and cirrus (4D).

The eggs were oval and consisted of two layers of membrane that clearly could be distinguished. The first layer (outer membrane) was thin, while the second layer (inner membrane) was thick and yellowish. There was fluid-filled space between the first and second layers. The length of the eggs was 114-130 μm with an average of 118 μm, and the width was 108-124 μm with an average of 116 μm (Figure 3D).

Table 1. Prevalence of pentastomiasis in *P. mucosa* based on site and the number of parasites.

Sites	Positive (%)	Number of parasites
Lungs only	57% (34 of 60)	2-9 with an average of 5 Pentastomida
Trachea only	0% (0 of 60)	-
Lungs and Trachea	8% (5 of 60)	7-9 with an average of 8 Pentastomida
Total	**65% (39 of 60)**	**204 pentastomida**

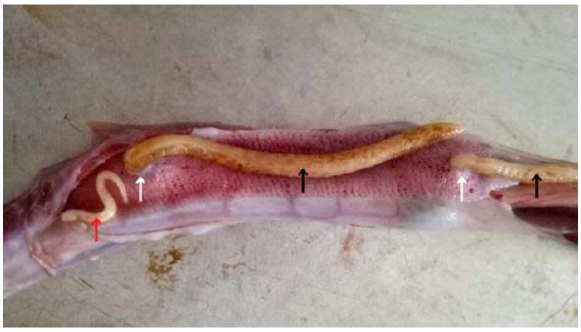

Figure 1. Pentastomid infestation in the lungs of *P. mucosa*. The white arrows show areas with raised lesions around the attachment of pentastomids to the tissue. The black arrows show female pentastomids, and the red arrow shows male pentastomids.

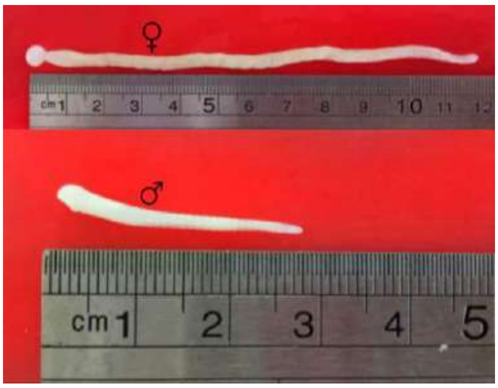

Figure 2. Differences in size between females (top) and males (below).

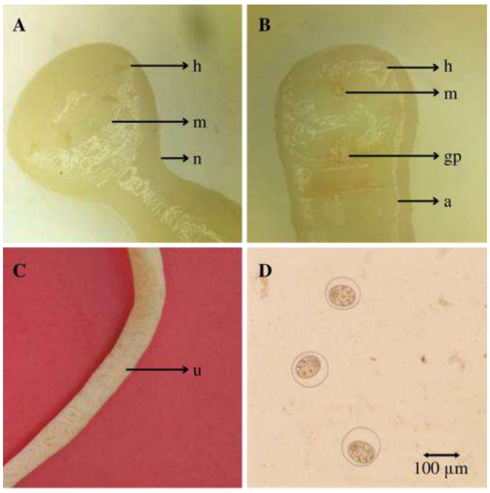

Figure 3. Pentastomida without staining. (A) The anterior section of the female; (B) The anterior section of the male; (C) Female body; (D) Pentastomid's eggs. h: hook, m: mouth, n: neck, gp: genital pore, a: first annuli, u: uterus.

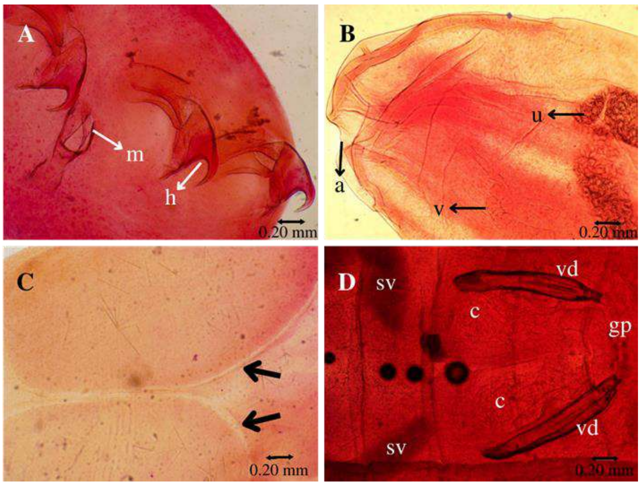

Figure 4. Carmine-stained pentastomida. (A) The anterior section; (B) The posterior section of the female; (C) A pair of female spermatheca; (D) Male reproductive organs. m: mouth, h: hook, a: anus, v: vagina, u: uterus, sv: seminal vesicle, c: cirrus, vd: vas deferens.

Table 2. The characteristics of pentastomida compared with *Kirichephalus pattoni* Stephens 1908 by Riley and Self (1980) and Keegan (1943).

Parameters	The current study		British Museum collection (Riley and Self 1980)		Keegan 1943
	Females	Males	Females	Males	
Total number of parasites	183	21	17	2	-
Body length	72-125 mm (X̄ 94.5 mm)	17-28.5 mm (X̄ 23.75 mm)	83-137 mm (X̄ 100 mm)	28-29 mm (X̄ 28.5 mm)	-
Number of *annuli*	29-41 (X̄ 31.6)	28-35 (X̄ 31)	34-38 (X̄ 36.5)	31-32 (X̄ 31.5)	-
Diameter of cephalothorax	4-5.5 mm (X̄ 4.75 mm)	2-2.5 mm (X̄ 2 mm)	4 mm	-	-
Neck	2-3 mm (X̄ 2.5 mm)	-	2 mm	-	-
Diameter of abdomen	3.5-5 mm (X̄ 4.2 mm)	1.8-2.2 mm (X̄ 2 mm)	4 mm	-	-
Hook parameter:			-	-	-
AC	0.23-0.55 mm	0.22-0.35 mm			
CB	0.37-0.69 mm	0.29-0.35 mm			
AD	0.47-0.88 mm	0.29-0.45 mm			
Mouth:			-	-	-
Length	0.16-29 mm	0.14-0.38 mm			
Width	0.12-0.24 mm	0.12-0.18 mm			
Egg:		-	-	-	
Length	114-130 µm (X̄ 118 µm)				125-154 µm (X̄ 133 µm)
Width	108-124 µm (X̄ 116 µm)				106-143 µm (X̄ 123 µm)

Pentastomida was mostly found in the lungs, and the parasites also spread to the trachea in five snakes. This finding was similar to research by Riley and Self (1980) that revealed that adult *K. pattoni* (one of the pentastomida species) were only found in the lungs of *P. mucosa* snakes. In our observation, pentastomids that spread to the trachea were females. According to Sundar et al. (2015), the adult pentastomids with mature eggs are expelled from the trachea and eliminated from the definitive host by oral expulsion or may also be swallowed. That results in shed eggs in the feces, which another intermediate mammal host will ingest to continue their life cycles.

The number of male pentastomids was fewer than the number of female ones. This case was also experienced by

John and Nadakal (1988) and Riley and Self (1980), who only found a few males compared the female. This study result follows Almeida et al. (2006), whose number of pentastomids and their species is unknown in biology

The taxonomy of pentastomida is still controversial. Moreover, it has been known through molecular data that pentastomida are closely related to crustaceans and belong to the class Maxillopoda, sub-phylum Crustacea, and the phylum Arthropoda (Paré 2008). Based on the egg size (Almeida et al. 2007); the female head form that globular with a neck separating the head from its body (Paré 2008); and the size of the body length, the number of the annuli, the diameter of the cephalothorax, neck, abdominal diameter, head, and body shape (Riley and Self 1979; Riley and Self 1980); pentastomida was classified as *K. pattoni*. *K. pattoni* can be classified as Porochepalida (Paré 2008). In addition, according to Christoffersen and De Assis (2013; 2015), pentastomid in *P. mucosa* snakes are classified into *K. pattoni*.

K. pattoni has a long annulated body, a tapered tail, an anal canal, two pairs of hooks or four hooks on its head, and a small ovoid-shaped mouth (Riley and Self 1979). The female *K. pattoni* has a larger body than the male one (Paré 2008). Female *K. pattoni* also has a spiral body with a larger head than its body, and a curve of the neck, whereas male one has a straight body without a curve on the neck (Riley and Self 1979; Riley and Self 1980; John and Nadakal 1988; Almeida et al. 2007). The spiral body in females is likely to facilitate their movement into the lung tissue of the definitive hosts (John and Nadakal 1988).

The egg morphology is similar to the eggs of *P. crotali* described by Sundar et al. (2015). These eggs were composed of two-layer membranes, of which the outer one was thin and the inner membrane thick, and there was a fluid-filled space between those two membranes. But the average diameter of the eggs measured in this investigation is smaller (118x116 µm) than the eggs of *P. crotali* (144 µm), as described by Sundar et al. (2015).

Histopathological changes in the lungs of *P.mucosa*

Squamates typically have two lungs; however, the left lung in most snakes is greatly reduced, never exceeding 85% of the right lung's size or absence (Funk and Bogan 2019). The right lung usually runs from near the heart to the cranial to the right kidney (Funk and Bogan 2019). The cranial portion of a lung is vascularized and functions primarily as a gas exchanger (vascular lung), whereas the caudal primarily functions as an air sac (saccular lung). The vascular lung's wall comprises honeycombed units called faveoli, allowing gas exchange (Funk and Bogan 2019). The faveoli in the snake lung parenchyma are lined microscopically by squamous epithelial cells (type I cells). In addition to a lesser degree, by cuboidal epithelial cells (type II) (Jacobson et al. 2021).

In this study, a nymphal stage of the parasite was found in the pulmonary tissue infected with pentastomida. The parasite is surrounded by some connective tissue (Figure 5). Finding the stages of the nymph in the snake's histological lung and the parasite's stages in the macroscopic lung organ means that the *P. mucosa* snake may act as an intermediate and final host of *K. pattoni*. Within the lung cavities of the definitive host, the nymphs develop into adults (Sundar et al. 2015). In this study, pentastomida infection in the lungs only caused a mild histological reaction in the form of fibrosis in the area around the parasite.

Pentastomida has an indirect life cycle that typically utilizes vertebrate hosts. Consequently, these species rely on reptiles as their definitive host. The adults are usually found in their lungs (Wellehan and Walden 2019). Pentastomida parasites migrate extensively through the visceral before settling in the lungs and, less typically, the trachea and nasal passageways. Pentastomida can produce localized lesions, while molting can elicit antigenic reactions. Adults feed on lung tissue fluids and pass embryonated eggs, which are coughed up through the oral cavity, swallowed, and passed in the feces (Comolli and Divers 2021). In squamates, the pulmonary lumen is large, and the tracheal diameter may be smaller than that of the pentastomids. That poses a risk of respiratory obstruction by the adult pentastomids. However, the greatest pathological changes are seen in the intermediate host, where they may become embedded in tissues (Wellehan and Walden 2019).

Pentastomiasis in reptiles, especially snakes, occurs subclinically. Although the infection is large, death is usually associated with secondary septicemia and pneumonia (Tappe and Büttner 2009). Because pentastomids attach to the lung tissue, they can cause perforation, hemorrhaging, and serious inflammation in the host (Fernando and Fernando 2014). In a Common Garter Snake (*Thamnophis sirtalis* Linnaeus 1758), lung lesions were associated with one of the pentastomida species, *Kiricephalus coarctatus* Diesing 1850, where the embedded parasite had gotten into the hypaxial muscle. Infections with *Armillifer armillatus* Wyman 1845 have also been linked to parasitic pneumonia in gaboon vipers. Pentastomids occluding the trachea have been implicated in snake deaths. Bacterial infections could develop due to lung damage. For example, there are four out of nine *Boa constrictors* with pulmonary damage from *Porocephalus dominicana* Riley & Walters 1980 infection perished due to bacteremia and pericarditis (Walden et al. 2021).

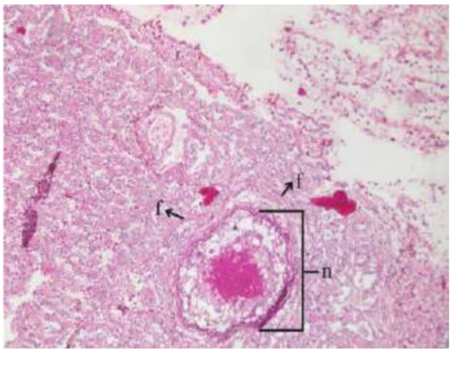

Figure 5. Histological microscopic picture of nymph-infected pulmo from pentastomida showed the presence of fibroblast around the area of infection. f: fibroblast, n: the body of the nymph stage pentastomida.

Bigger implications regarding possible impacts on human health may be anticipated based on the histopathological changes found in this study. Humans are the final hosts of pentastomic, which can be contracted from improperly cooked meat from infected snakes or drinking water contaminated with pentastomida eggs. In addition, humans can get the infection when handling and harvesting snakeskin or having direct contact with the mouth of a pet snake (Latif et al. 2016). The pathological consequences of the clinical symptoms experienced by infected humans may be the same or even more severe. That depends on the number of parasite eggs ingested, the duration from the larvae migrating to various organs to maturity and reproduction, and how severe the parasite infestation is in the human viscera.

Personal hygiene measures are required to prevent human infections. For example, avoid drinking river water directly or boiling the water before drinking. In addition, authorities should provide health education to warn people about eating undercooked snake meat, the possibility of transmission after handling snakes, and avoiding contact with snake excretions.

In conclusion the prevalence of pentastomiasis in slaughtered *P. mucosa* in Sidoarjo was 65% (39 of 60 snakes). The pentastomids that infect these snakes are classified as *K. pattoni*. The pulmonary histological findings only show a light reaction in the form of mild fibrosis.

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First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (*Ptyas mucosa*) in Sidoarjo, East Java, Indonesia

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Abstract. *Plumeriastuti H, Pranashinta GT, Suwanti LT, Proboningrat A, Kusnoto. 2023. First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (*Ptyas mucosa*) in Sidoarjo, East Java, Indonesia. Biodiversitas 24: 2045-2051.* Currently, reptiles, especially snakes, are increasingly popular as pets worldwide and are often consumed by several communities in Indonesia. This trend needs attention from a public health perspective because it can potentially cause zoonotic diseases, including parasitic disease such as pentastomiasis. This study aimed to determine: the prevalence of pentastomiasis, identify the parasite and observe the pulmonary histopathology in Oriental Rat Snakes (*Ptyas mucosa* Linnaeus 1758) collected in Tulangan, Sidoarjo, Indonesia. The methods used were calculating the prevalence of pentastomiasis, semichen-acetic carmine staining, and identifying parasites and eggs based on references as a comparison. Furthermore, histopathological preparation and hematoxylin-eosin staining were performed on the lungs infected with the parasite to observe the microscopic lesions. The results showed that 65% of the snakes had pentastomiasis, and the parasites were called *Kiricephalus pattoni* Stephens 1908. Furthermore, a microscopical examination revealed mild fibrosis in the pulmonary area due to pentastomid infestation. This study is the first reported pentastomida in *P. mucosa* in Sidoarjo, Indonesia, showing that snakes caught and commonly consumed were infected with zoonotic pentastomic.

Keywords: Colubrid snake, parasitic pneumonia, tongue worms, zoonotic parasitoses

INTRODUCTION

Exotic animals such as reptiles, especially various snakes, are now popular as pets worldwide (Okulewicz et al. 2014). However, they are also concerned with the increasing animal welfare and health issues through the animal trade in the market (Yudhana et al. 2021). Some species of snake are bred in captivity, while others are caught in the wild and sold as pets or bushmeat for consumption (Hardi et al. 2017; Yudhana et al. 2021).

Ptyas mucosa Linnaeus 1758 is a well-known snake species in Indonesia (Pranashinta et al. 2017). This type of snake, known as the Oriental Rat Snake and Indian Rat Snake, belongs to the Colubridae family which is widely distributed throughout East, South, and Southeast Asia (Auliya 2010; Muliya and Bhat 2016; Yuan et al. 2022). *P. mucosa* is a diurnal and semi-arboreal snake species that live on forest floors, agricultural fields, wet ground, and near human habitats. It feeds on frogs, rats, and other small animals (Chittora et al. 2021).

The meat of *P. mucosa* is used in the illegal trade to China (Auliya 2010). The meat and internal organs are used as fresh or frozen food, the bile and blood are used in traditional medicine, and the skin is exported to several European countries as raw materials for the leather industry (Pranashinta et al. 2017). Although illegal, most rural communities in Indonesia trade and consume the meat and bile of *P. mucosa* for some reasons (Auliya 2010; Pranashinta et al. 2017).

Wildlife meat, however, poses a threat of transmitting infection with novel and exotic pathogens to human hosts (Hardi et al. 2017). Then new zoonotic diseases are likely to emerge consequently. For instance, Bats (Chiroptera) are important reservoirs of several emerging diseases, including Ebola Virus Disease (EVD), Severe Acute Respiratory Syndrome (SARS), and Middle East Respiratory Syndrome (MERS), as well as the current pandemic COVID-19 caused by SARS Coronavirus 2 (Irving et al. 2021). Moreover, many parasites also cause emerging zoonotic diseases, resulting in a significant public health burden, particularly in tropical countries (Alasil and Abdullah 2019).

Several parasites in confined reptiles spend a portion of their life cycle in the respiratory system. Most respiratory involvement is asymptomatic and mild, generating mostly localized inflammation except for excessive parasite loads. However, severe disease can result from high burdens or unusual host-parasite interactions, frequently worsened by secondary infections (Comolli 2021). Reptile-borne pentastomiasis is an unusual tropical disease neglected and under-researched in Asia, Africa, and South America (Sulyok et al. 2014).

Pentastomiasis is a parasitic zoonosis caused by infection with pentastomic, belonging to the subclass Pentastomida, under the phylum Arthropoda (Tongtako et al. 2013; Chittora et al. 2021; WHA 2019). Pentastomiasis is also known as tongue worms since the adult phase of the genus *Linguatula* has similarities with mammalian tongues (Paré 2008). Adult parasites are usually found in the respiratory tract of reptiles, birds, and mammals, including humans, while their larval forms are in the internal organs of insects, mammals, and amphibians (Paré 2008; Chen et al. 2010; Kelehear et al. 2014). Some zoonotic Pentastomida in snakes is Armillifer and *Porocephalus crotali* Humboldt 1812 (Rataj et al. 2011). Humans can be infected with pentastomic after they consume raw snake meat, have direct contact with the snake, drink water contaminated with parasitic eggs, or are contaminated through the feces (Kelehear et al. 2014; Tappe et al. 2016; Mendoza-Roldan et al. 2020).

Pentastomiasis in snakes have been reported in many countries, such as poisonous Terciopelo (*Bothrops asper* Garman 1883) in Costa Rica (Alvarado et al. 2015), Royal Python Snakes (*Python regius* Shaw 1802) in Nigeria (Ayinmode et al. 2010), Rat Snakes (*P. mucosa*) in Sri Lanka and India (Fernando and Fernando 2014; Sundar et al. 2015), wild snakes in Australia (Kelehear et al. 2014), various species of snakes in Slovenia and Pakistan (Rataj et al. 2011), Chinese Moccasin (*Agkistrodon acutus*) and Indian Python (*Python molurus* Linnaeus 1758) in China (Chen et al. 2010), and Totonacan Rattlesnake (*Crotalus totonacus* Gloyd & Kauffeld 1940) in Mexico (Tepos-Ramírez et al. 2022).

Moreover, to our best knowledge, snakes in Indonesia have no reports of pentastomic infections. Therefore, this study reported the prevalence and identified pentastomida and the pulmonary histopathological profiles in Oriental Rat Snakes (*P. mucosa*) in Sidoarjo Regency, East Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

The Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga (number 678-KE) reviewed and approved this study.

Parasite collection

The sample used comes from the Oriental Rat Snake (*P. mucosa*), which is often caught and sold for its meat and skin through collectors. The collection was conducted at a snake slaughterhouse in Tulangan District, Sidoarjo Regency, East Java Province, Indonesia. The presence of pentastomid parasites was observed in the respiratory tract of 60 slaughtered snakes. Pentastomides were taken and cleaned with distilled water, then stored in sterile plastic pots. The lungs of the snakes infected with the parasite were also isolated for histopathological preparation.

Prevalence calculation

The following formula calculated the prevalence of pentastomiasis in snake samples:

Prevalence (%) = (The number of snakes infected with pentastomids) / (Total sample of snakes) × 100%.

Semichen-acetic carmine staining

The collected pentastomids were stained with the Semichen-Acetic Carmine technique (Kuhlmann 2006). Adult pentastomids were fixed on object glasses and soaked in 5% glycerin alcohol for 24 hours. Then they were soaked in 70% alcohol for five minutes. Subsequently, the object glasses with pentastomids were placed in Carmine alcohol solution (1:2) for eight hours. Next, the pentastomids were soaked in acid alcohol for 10 minutes and moved to base alcohol for 20 minutes. Furthermore, graded dehydration was carried out with 70%, 85%, and 95% alcohol for five minutes. The mounting step was done using Hung's I for 20 minutes. Finally, pentastomids were taken and placed on new clean object glasses. The pentastomids were dripped with Hung's II solutions and then covered with a cover glass.

Parasite identification

Characteristics of the head and body shape and body size of the parasite were identified based on Riley and Self (1980), Almedia et al. (2006), and Christoffersen and De Assis (2013; 2015). Identification of parasite egg size was carried out according to Keegan (1943).

The fresh parasite's length was measured from the anterior edge to the posterior end. The head was observed under a stereo microscope and documented with a Canon Digital Single-Lens Reflex (DSLR) camera. Furthermore, stained parasites were observed using a light microscope with 40-100× magnification and documented using Miconos Optilab. Some female parasites were incubated in PBS overnight at 37°C to collect the eggs. The supernatants were centrifuged at 1,500 rpm for 10 minutes, and the pellet was dripped onto a glass object and observed using a light microscope with 100× magnification. Those eggs were then measured using Miconos Optilab.

Histopathological preparations

The lungs of snakes with pentastomid infestation were fixed in 10% formalin. Then graded dehydration was carried out with serial alcohol concentrations (70%, 80%, 90%, 96%, and absolute) and clearing using xylol. Furthermore, paraffin embedding was performed to obtain a solid block of tissue. The tissue blocks were sliced (3 micrometers thick) using a microtome, mounted on an object glass, and stained using Hematoxylin and Eosin. Microscopic examination was performed using Nikon Eclipse Ci.

RESULTS AND DISCUSSIONS

The prevalence of pentastomiasis in *P. mucosa* snakes

Thirty-nine (or 65%) of the respiratory tract of 60 samples were positively infected with adult pentastomids; a

total of 204 pentastomids samples were collected (Table 1). Pentastomida was found to be strongly attached to the trachea and lungs of snakes (Figure 1). Some pentastomids even buried their heads in the lungs of snakes. The parasites of all snakes positively infected with pentastomids were found in the lungs (39 snakes), while only five (5) snakes which parasites spread to their tracheas.

This is the first study to determine the prevalence of pentastomiasis in *P. mucosa* in Sidoarjo, East Java, Indonesia. Our results showed that pentastomids were present in 39 of 60 (65%) snakes (Table 1). This finding shows the prevalence of pentastomiasis in *P. mucosa* was quite high. This study's prevalence is similar to pentastomiasis in Brazil that 8 of 15 or 53.33% of snakes were positively infected with pentastomida. The food consumed by the snakes probably caused the high prevalence in this research. The type of food is very influential on the prevalence of infection and the type of pentastomida that infected the snakes (Almeida et al. 2007). *P. mucosa* snakes usually feed on rats, frogs, toads, lizards, and even smaller non-venomous and smaller snakes (Sidik 2006). In addition, according to Esslinger (1962), rats, frogs, toads, lizards, and even non-venomous snakes can act as the intermediate hosts of pentastomida.

Identification of pentastomida

The characteristics of pentastomid found in this study can be seen in Table 2 and Figure 2. Pentastomids have a long cylindrical body with a larger head, a flat shape on the ventral, and a tapered tail or posterior. Their body was soft and whitish with a colorless cuticle, which tended to be transparent to make the internal organs visible. There were annuli throughout their body up to the posterior. There are two pairs of hooks (a total of four) around the head that attach to the host's lung tissue, and it has an ovoid-shaped mouth between the two pairs of hooks.

Both sexes, adult males and female pentastomids were found in this research (Figures 1 and 2). The number of male pentastomid was less (21 heads) than that of females (183 heads) Table 2. Table 2 also describes the size of each pentastomid. The female pentastomids had longer and larger bodies than the males (Figures 1 and 2). The length of the female bodies was 72-125 mm, with an average of 94.5 mm. Their abdominal diameter was 3.5-5 mm, averaging 4.2 mm. Furthermore, the anterior section or cephalothorax of the females was globular, with a neck separating their head and abdomen (Figure 3A). The average length and width of the female mouths were 0.24 mm and 0.16 mm. Furthermore, the results of the microscopy examination using Carmine staining showed that the female pentastomid had two spermathecae on the anterior part. That connects directly to the uterus that contains the egg (Figures 3C, 4B, and 4C). In addition, the vagina was in the posterior body and around the anus (Figure 4B).

On the other hand, the length of the males' bodies was 17-28.5 mm, with an average of 23.75 mm. Their abdominal diameter was 1.8-2.2 mm, averaging 2 mm. The average length and width of the mouth were 0.23 and 0.14 mm, respectively. The male body was simple and straight without any curves in the neck (Figure 3B). The genital pore was identified in the first annulus, and there was a pair of seminal vesicles, vas deferens, and cirrus (4D).

The eggs were oval and consisted of two layers of membrane that clearly could be distinguished. The first layer (outer membrane) was thin, while the second layer (inner membrane) was thick and yellowish. There was fluid-filled space between the first and second layers. The length of the eggs was 114-130 μm with an average of 118 μm, and the width was 108-124 μm with an average of 116 μm (Figure 3D).

Table 1. Prevalence of pentastomiasis in *P. mucosa* based on site and the number of parasites

Sites	Positive (%)	Number of parasites
Lungs only	57% (34 of 60)	2-9 with an average of 5 Pentastomida
Trachea only	0% (0 of 60)	-
Lungs and Trachea	8% (5 of 60)	7-9 with an average of 8 Pentastomida
Total	**65% (39 of 60)**	**204 pentastomida**

Figure 1. Pentastomid infestation in the lungs of *P. mucosa*. The white arrows show areas with raised lesions around the attachment of pentastomids to the tissue. The black arrows show female pentastomids, and the red arrow shows male pentastomids

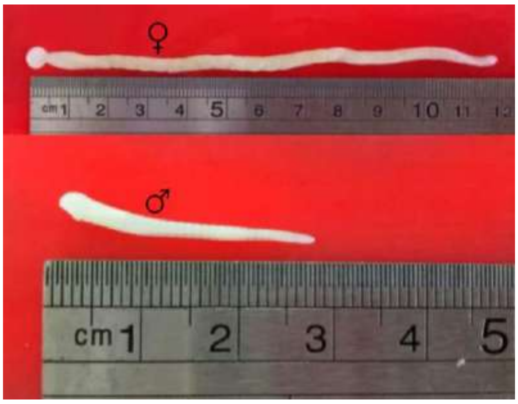

Figure 2. Differences in size between females (top) and males (below)

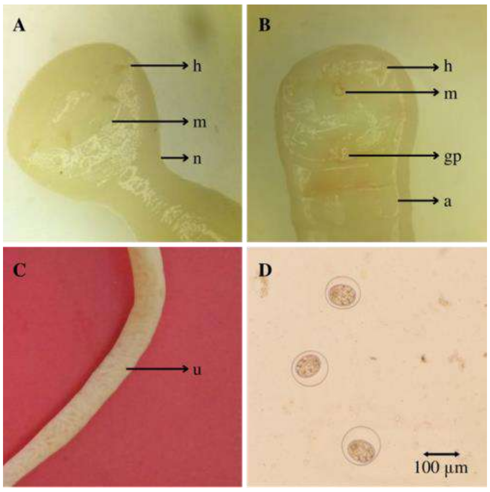

Figure 3. Pentastomida without staining. A. The anterior section of the female. B. The anterior section of the male. C. Female body. D. Pentastomid's eggs. h: hook, m: mouth, n: neck, gp: genital pore, a: first annuli, u: uterus

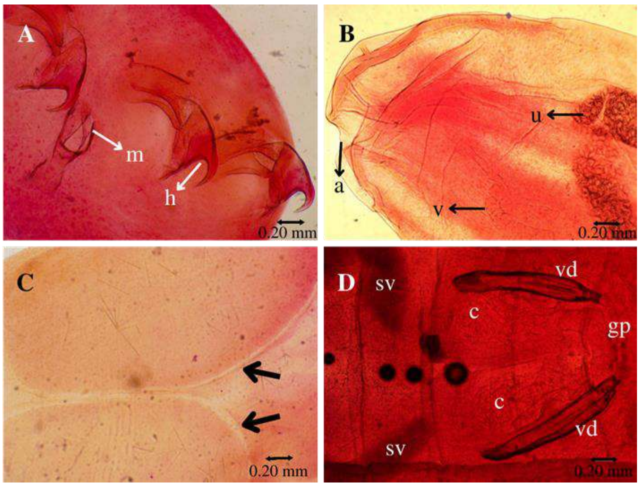

Figure 4. Carmine-stained pentastomida. A. The anterior section. B. The posterior section of the female. C. A pair of female spermatheca. D. Male reproductive organs. m: mouth, h: hook, a: anus, v: vagina, u: uterus, sv: seminal vesicle, c: cirrus, vd: vas deferens

Table 2. The characteristics of pentastomida compared with *Kirichephalus pattoni* Stephens 1908 by Riley and Self (1980) and Keegan (1943)

Parameters	**The current study**		**British Museum collection (Riley and Self 1980)**		**Keegan 1943**
	Females	**Males**	**Females**	**Males**	
Total number of parasites	183	21	17	2	-
Body length	72-125 mm (X̄ 94.5 mm)	17-28.5 mm (X̄ 23.75 mm)	83-137 mm (X̄ 100 mm)	28-29 mm (X̄ 28.5 mm)	-
Number of *annuli*	29-41 (X̄ 31.6)	28-35 (X̄ 31)	34-38 (X̄ 36.5)	31-32 (X̄ 31.5)	-
Diameter of cephalothorax	4-5.5 mm (X̄ 4.75 mm)	2-2.5 mm (X̄ 2 mm)	4 mm	-	-
Neck	2-3 mm (X̄ 2.5 mm)	-	2 mm	-	-
Diameter of abdomen	3.5-5 mm (X̄ 4.2 mm)	1.8-2.2 mm (X̄ 2 mm)	4 mm	-	-
Hook parameter:			-	-	-
AC	0.23-0.55 mm	0.22-0.35 mm			
CB	0.37-0.69 mm	0.29-0.35 mm			
AD	0.47-0.88 mm	0.29-0.45 mm			
Mouth:			-	-	-
Length	0.16-29 mm	0.14-0.38 mm			
Width	0.12-0.24 mm	0.12-0.18 mm			
Egg:		-	-	-	
Length	114-130 µm (X̄ 118 µm)				125-154 µm (X̄ 133 µm)
Width	108-124 µm (X̄ 116 µm)				106-143 µm (X̄ 123 µm)

Pentastomida was mostly found in the lungs, and the parasites also spread to the trachea in five snakes. This finding was similar to research by Riley and Self (1980) that revealed that adult *K. pattoni* (one of the pentastomida species) were only found in the lungs of *P. mucosa* snakes. In our observation, pentastomids that spread to the trachea were females. According to Sundar et al. (2015), the adult pentastomids with mature eggs are expelled from the trachea and eliminated from the definitive host by oral expulsion or may also be swallowed. That results in shed eggs in the feces, which another intermediate mammal host will ingest to continue their life cycles.

The number of male pentastomids was fewer than the number of female ones. This case was also experienced by John and Nadakal (1988) and Riley and Self (1980), who only found a few males compared the female. This study result follows Almeida et al. (2006), whose number of pentastomids and their species is unknown in biology

The taxonomy of pentastomida is still controversial. Moreover, it has been known through molecular data that pentastomida are closely related to crustaceans and belong to the class Maxillopoda, sub-phylum Crustacea, and the phylum Arthropoda (Paré 2008). Based on the egg size (Almeida et al. 2007); the female head form that globular with a neck separating the head from its body (Paré 2008); and the size of the body length, the number of the annuli, the diameter of the cephalothorax, neck, abdominal diameter, head, and body shape (Riley and Self 1979; Riley and Self 1980); pentastomida was classified as *K. pattoni*. *K. pattoni* can be classified as Porochepalida (Paré 2008). In addition, according to Christoffersen and De Assis (2013; 2015), pentastomid in *P. mucosa* snakes are classified into *K. pattoni*.

K. pattoni has a long annulated body, a tapered tail, an anal canal, two pairs of hooks or four hooks on its head, and a small ovoid-shaped mouth (Riley and Self 1979). The female *K. pattoni* has a larger body than the male one (Paré 2008). Female *K. pattoni* also has a spiral body with a larger head than its body, and a curve of the neck, whereas male one has a straight body without a curve on the neck (Riley and Self 1979; Riley and Self 1980; John and Nadakal 1988; Almeida et al. 2007). The spiral body in females is likely to facilitate their movement into the lung tissue of the definitive hosts (John and Nadakal 1988).

The egg morphology is similar to the eggs of *P. crotali* described by Sundar et al. (2015). These eggs were composed of two-layer membranes, of which the outer one was thin and the inner membrane thick, and there was a fluid-filled space between those two membranes. But the average diameter of the eggs measured in this investigation is smaller (118x116 µm) than the eggs of *P. crotali* (144 µm), as described by Sundar et al. (2015).

Histopathological changes in the lungs of *P.mucosa*

Squamates typically have two lungs; however, the left lung in most snakes is greatly reduced, never exceeding 85% of the right lung's size or absence (Funk and Bogan 2019). The right lung usually runs from near the heart to the cranial to the right kidney (Funk and Bogan 2019). The cranial portion of a lung is vascularized and functions primarily as a gas exchanger (vascular lung), whereas the caudal primarily functions as an air sac (saccular lung). The vascular lung's wall comprises honeycombed units called faveoli, allowing gas exchange (Funk and Bogan 2019). The faveoli in the snake lung parenchyma are lined microscopically by squamous epithelial cells (type I cells). In addition to a lesser degree, by cuboidal epithelial cells (type II) (Jacobson et al. 2021).

In this study, a nymphal stage of the parasite was found in the pulmonary tissue infected with pentastomida. The parasite is surrounded by some connective tissue (Figure 5). Finding the stages of the nymph in the snake's histological lung and the parasite's stages in the macroscopic lung organ means that the *P. mucosa* snake may act as an intermediate and final host of *K. pattoni*. Within the lung cavities of the definitive host, the nymphs develop into adults (Sundar et al. 2015). In this study, pentastomida infection in the lungs only caused a mild histological reaction in the form of fibrosis in the area around the parasite.

Pentastomida has an indirect life cycle that typically utilizes vertebrate hosts. Consequently, these species rely on reptiles as their definitive host. The adults are usually found in their lungs (Wellehan and Walden 2019). Pentastomida parasites migrate extensively through the visceral before settling in the lungs and, less typically, the trachea and nasal passageways. Pentastomida can produce localized lesions, while molting can elicit antigenic reactions. Adults feed on lung tissue fluids and pass embryonated eggs, which are coughed up through the oral cavity, swallowed, and passed in the feces (Comolli and Divers 2021). In squamates, the pulmonary lumen is large, and the tracheal diameter may be smaller than that of the pentastomids. That poses a risk of respiratory obstruction by the adult pentastomids. However, the greatest pathological changes are seen in the intermediate host, where they may become embedded in tissues (Wellehan and Walden 2019).

Pentastomiasis in reptiles, especially snakes, occurs subclinically. Although the infection is large, death is usually associated with secondary septicemia and pneumonia (Tappe and Büttner 2009). Because pentastomids attach to the lung tissue, they can cause perforation, hemorrhaging, and serious inflammation in the host (Fernando and Fernando 2014). In a Common Garter Snake (*Thamnophis sirtalis* Linnaeus 1758), lung lesions were associated with one of the pentastomida species, *Kiricephalus coarctatus* Diesing 1850, where the embedded parasite had gotten into the hypaxial muscle. Infections with *Armillifer armillatus* Wyman 1845 have also been linked to parasitic pneumonia in gaboon vipers. Pentastomids occluding the trachea have been implicated in snake deaths. Bacterial infections could develop due to lung damage. For example, there are four out of nine *Boa constrictors* with pulmonary damage from *Porocephalus dominicana* Riley & Walters 1980 infection perished due to bacteremia and pericarditis (Walden et al. 2021).

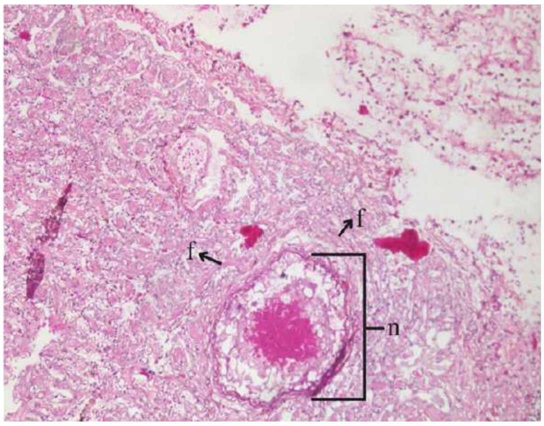

Figure 5. Histological microscopic picture of nymph-infected pulmo from pentastomida showed the presence of fibroblast around the area of infection. f: fibroblast, n: the body of the nymph stage pentastomida

Bigger implications regarding possible impacts on human health may be anticipated based on the histopathological changes found in this study. Humans are the final hosts of pentastomic, which can be contracted from improperly cooked meat from infected snakes or drinking water contaminated with pentastomida eggs. In addition, humans can get the infection when handling and harvesting snakeskin or having direct contact with the mouth of a pet snake (Latif et al. 2016). The pathological consequences of the clinical symptoms experienced by infected humans may be the same or even more severe. That depends on the number of parasite eggs ingested, the duration from the larvae migrating to various organs to maturity and reproduction, and how severe the parasite infestation is in the human viscera.

Personal hygiene measures are required to prevent human infections. For example, avoid drinking river water directly or boiling the water before drinking. In addition, authorities should provide health education to warn people about eating undercooked snake meat, the possibility of transmission after handling snakes, and avoiding contact with snake excretions.

In conclusion the prevalence of pentastomiasis in slaughtered *P. mucosa* in Sidoarjo was 65% (39 of 60 snakes). The pentastomids that infect these snakes are classified as *K. pattoni*. The pulmonary histological findings only show a light reaction in the form of mild fibrosis.

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First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (*Ptyas mucosa*) in Sidoarjo, East Java, Indonesia

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Abstract. *Plumeriastuti H, Pranashinta GT, Suwanti LT, Proboningrat A, Kusnoto. 2023. First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (*Ptyas mucosa*) in Sidoarjo, East Java, Indonesia. Biodiversitas 24: 2045-2051.* Currently, reptiles, especially snakes, are increasingly popular as pets worldwide and are often consumed by several communities in Indonesia. This trend needs attention from a public health perspective because it can potentially cause zoonotic diseases, including parasitic disease such as pentastomiasis. This study aimed to determine: the prevalence of pentastomiasis, identify the parasite and observe the pulmonary histopathology in Oriental Rat Snakes (*Ptyas mucosa* Linnaeus 1758) collected in Tulangan, Sidoarjo, Indonesia. The methods used were calculating the prevalence of pentastomiasis, semichen-acetic carmine staining, and identifying parasites and eggs based on references as a comparison. Furthermore, histopathological preparation and hematoxylin-eosin staining were performed on the lungs infected with the parasite to observe the microscopic lesions. The results showed that 65% of the snakes had pentastomiasis, and the parasites were called *Kiricephalus pattoni* Stephens 1908. Furthermore, a microscopical examination revealed mild fibrosis in the pulmonary area due to pentastomid infestation. This study is the first reported pentastomida in *P. mucosa* in Sidoarjo, Indonesia, showing that snakes caught and commonly consumed were infected with zoonotic pentastomic.

Keywords: Colubrid snake, parasitic pneumonia, tongue worms, zoonotic parasitoses

INTRODUCTION

Exotic animals such as reptiles, especially various snakes, are now popular as pets worldwide (Okulewicz et al. 2014). However, they are also concerned with the increasing animal welfare and health issues through the animal trade in the market (Yudhana et al. 2021). Some species of snake are bred in captivity, while others are caught in the wild and sold as pets or bushmeat for consumption (Hardi et al. 2017; Yudhana et al. 2021).

Ptyas mucosa Linnaeus 1758 is a well-known snake species in Indonesia (Pranashinta et al. 2017). This type of snake, known as the Oriental Rat Snake and Indian Rat Snake, belongs to the Colubridae family which is widely distributed throughout East, South, and Southeast Asia (Auliya 2010; Muliya and Bhat 2016; Yuan et al. 2022). *P. mucosa* is a diurnal and semi-arboreal snake species that live on forest floors, agricultural fields, wet ground, and near human habitats. It feeds on frogs, rats, and other small animals (Chittora et al. 2021).

The meat of *P. mucosa* is used in the illegal trade to China (Auliya 2010). The meat and internal organs are used as fresh or frozen food, the bile and blood are used in traditional medicine, and the skin is exported to several European countries as raw materials for the leather industry (Pranashinta et al. 2017). Although illegal, most rural communities in Indonesia trade and consume the meat and bile of *P. mucosa* for some reasons (Auliya 2010; Pranashinta et al. 2017).

Wildlife meat, however, poses a threat of transmitting infection with novel and exotic pathogens to human hosts (Hardi et al. 2017). Then new zoonotic diseases are likely to emerge consequently. For instance, Bats (Chiroptera) are important reservoirs of several emerging diseases, including Ebola Virus Disease (EVD), Severe Acute Respiratory Syndrome (SARS), and Middle East Respiratory Syndrome (MERS), as well as the current pandemic COVID-19 caused by SARS Coronavirus 2 (Irving et al. 2021). Moreover, many parasites also cause emerging zoonotic diseases, resulting in a significant public health burden, particularly in tropical countries (Alasil and Abdullah 2019).

Several parasites in confined reptiles spend a portion of their life cycle in the respiratory system. Most respiratory involvement is asymptomatic and mild, generating mostly localized inflammation except for excessive parasite loads. However, severe disease can result from high burdens or unusual host-parasite interactions, frequently worsened by secondary infections (Comolli 2021). Reptile-borne pentastomiasis is an unusual tropical disease neglected and under-researched in Asia, Africa, and South America (Sulyok et al. 2014).

Pentastomiasis is a parasitic zoonosis caused by infection with pentastomic, belonging to the subclass Pentastomida, under the phylum Arthropoda (Tongtako et al. 2013; Chittora et al. 2021; WHA 2019). Pentastomiasis is also known as tongue worms since the adult phase of the genus *Linguatula* has similarities with mammalian tongues (Paré 2008). Adult parasites are usually found in the respiratory tract of reptiles, birds, and mammals, including humans, while their larval forms are in the internal organs of insects, mammals, and amphibians (Paré 2008; Chen et al. 2010; Kelehear et al. 2014). Some zoonotic Pentastomida in snakes is Armillifer and *Porocephalus crotali* Humboldt 1812 (Rataj et al. 2011). Humans can be infected with pentastomic after they consume raw snake meat, have direct contact with the snake, drink water contaminated with parasitic eggs, or are contaminated through the feces (Kelehear et al. 2014; Tappe et al. 2016; Mendoza-Roldan et al. 2020).

Pentastomiasis in snakes have been reported in many countries, such as poisonous Terciopelo (*Bothrops asper* Garman 1883) in Costa Rica (Alvarado et al. 2015), Royal Python Snakes (*Python regius* Shaw 1802) in Nigeria (Ayinmode et al. 2010), Rat Snakes (*P. mucosa*) in Sri Lanka and India (Fernando and Fernando 2014; Sundar et al. 2015), wild snakes in Australia (Kelehear et al. 2014), various species of snakes in Slovenia and Pakistan (Rataj et al. 2011), Chinese Moccasin (*Agkistrodon acutus*) and Indian Python (*Python molurus* Linnaeus 1758) in China (Chen et al. 2010), and Totonacan Rattlesnake (*Crotalus totonacus* Gloyd & Kauffeld 1940) in Mexico (Tepos-Ramírez et al. 2022).

Moreover, to our best knowledge, snakes in Indonesia have no reports of pentastomic infections. Therefore, this study reported the prevalence and identified pentastomida and the pulmonary histopathological profiles in Oriental Rat Snakes (*P. mucosa*) in Sidoarjo Regency, East Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

The Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (number 678-KE) reviewed and approved this study.

Parasite collection

The sample used comes from the Oriental Rat Snake (*P. mucosa*), which is often caught and sold for its meat and skin through collectors. The collection was conducted at a snake slaughterhouse in Tulangan District, Sidoarjo Regency, East Java Province, Indonesia. The presence of pentastomid parasites was observed in the respiratory tract of 60 slaughtered snakes. Pentastomides were taken and cleaned with distilled water, then stored in sterile plastic pots. The lungs of the snakes infected with the parasite were also isolated for histopathological preparation.

Prevalence calculation

The following formula calculated the prevalence of pentastomiasis in snake samples:

Prevalence (%) = (The number of snakes infected with pentastomids) / (Total sample of snakes) × 100%.

Semichen-acetic carmine staining

The collected pentastomids were stained with the Semichen-Acetic Carmine technique (Kuhlmann 2006). Adult pentastomids were fixed on object glasses and soaked in 5% glycerin alcohol for 24 hours. Then they were soaked in 70% alcohol for five minutes. Subsequently, the object glasses with pentastomids were placed in Carmine alcohol solution (1:2) for eight hours. Next, the pentastomids were soaked in acid alcohol for 10 minutes and moved to base alcohol for 20 minutes. Furthermore, graded dehydration was carried out with 70%, 85%, and 95% alcohol for five minutes. The mounting step was done using Hung's I for 20 minutes. Finally, pentastomids were taken and placed on new clean object glasses. The pentastomids were dripped with Hung's II solutions and then covered with a cover glass.

Parasite identification

Characteristics of the head and body shape and body size of the parasite were identified based on Riley and Self (1980), Almedia et al. (2006), and Christoffersen and De Assis (2013; 2015). Identification of parasite egg size was carried out according to Keegan (1943).

The fresh parasite's length was measured from the anterior edge to the posterior end. The head was observed under a stereo microscope and documented with a Canon Digital Single-Lens Reflex (DSLR) camera. Furthermore, stained parasites were observed using a light microscope with 40-100× magnification and documented using Miconos Optilab. Some female parasites were incubated in PBS overnight at 37°C to collect the eggs. The supernatants were centrifuged at 1,500 rpm for 10 minutes, and the pellet was dripped onto a glass object and observed using a light microscope with 100× magnification. Those eggs were then measured using Miconos Optilab.

Histopathological preparations

The lungs of snakes with pentastomid infestation were fixed in 10% formalin. Then graded dehydration was carried out with serial alcohol concentrations (70%, 80%, 90%, 96%, and absolute) and clearing using xylol. Furthermore, paraffin embedding was performed to obtain a solid block of tissue. The tissue blocks were sliced (3 micrometers thick) using a microtome, mounted on an object glass, and stained using Hematoxylin and Eosin. Microscopic examination was performed using Nikon Eclipse Ci.

RESULTS AND DISCUSSIONS

The prevalence of pentastomiasis in *P. mucosa* snakes

Thirty-nine (or 65%) of the respiratory tract of 60 samples were positively infected with adult pentastomids; a

total of 204 pentastomids samples were collected (Table 1). Pentastomida was found to be strongly attached to the trachea and lungs of snakes (Figure 1). Some pentastomids even buried their heads in the lungs of snakes. The parasites of all snakes positively infected with pentastomids were found in the lungs (39 snakes), while only five (5) snakes which parasites spread to their tracheas.

This is the first study to determine the prevalence of pentastomiasis in *P. mucosa* in Sidoarjo, East Java, Indonesia. Our results showed that pentastomids were present in 39 of 60 (65%) snakes (Table 1). This finding shows the prevalence of pentastomiasis in *P. mucosa* was quite high. This study's prevalence is similar to pentastomiasis in Brazil that 8 of 15 or 53.33% of snakes were positively infected with pentastomida. The food consumed by the snakes probably caused the high prevalence in this research. The type of food is very influential on the prevalence of infection and the type of pentastomida that infected the snakes (Almeida et al. 2007). *P. mucosa* snakes usually feed on rats, frogs, toads, lizards, and even smaller non-venomous and smaller snakes (Sidik 2006). In addition, according to Esslinger (1962), rats, frogs, toads, lizards, and even non-venomous snakes can act as the intermediate hosts of pentastomida.

Identification of pentastomida

The characteristics of pentastomid found in this study can be seen in Table 2 and Figure 2. Pentastomids have a long cylindrical body with a larger head, a flat shape on the ventral, and a tapered tail or posterior. Their body was soft and whitish with a colorless cuticle, which tended to be transparent to make the internal organs visible. There were annuli throughout their body up to the posterior. There are two pairs of hooks (a total of four) around the head that attach to the host's lung tissue, and it has an ovoid-shaped mouth between the two pairs of hooks.

Both sexes, adult males and female pentastomids were found in this research (Figures 1 and 2). The number of male pentastomid was less (21 heads) than that of females (183 heads) Table 2. Table 2 also describes the size of each pentastomid. The female pentastomids had longer and larger bodies than the males (Figures 1 and 2). The length of the female bodies was 72-125 mm, with an average of 94.5 mm. Their abdominal diameter was 3.5-5 mm, averaging 4.2 mm. Furthermore, the anterior section or cephalothorax of the females was globular, with a neck separating their head and abdomen (Figure 3.A). The average length and width of the female mouths were 0.24 mm and 0.16 mm. Furthermore, the results of the microscopy examination using Carmine staining showed that the female pentastomid had two spermathecae on the anterior part. That connects directly to the uterus that contains the egg (Figures 3.C, 4.B, and 4.C). In addition, the vagina was in the posterior body and around the anus (Figure 4.B).

On the other hand, the length of the males' bodies was 17-28.5 mm, with an average of 23.75 mm. Their abdominal diameter was 1.8-2.2 mm, averaging 2 mm. The average length and width of the mouth were 0.23 and 0.14 mm, respectively. The male body was simple and straight without any curves in the neck (Figure 3.B). The genital pore was identified in the first annulus, and there was a pair of seminal vesicles, vas deferens, and cirrus (Figure 4.D).

The eggs were oval and consisted of two layers of membrane that clearly could be distinguished. The first layer (outer membrane) was thin, while the second layer (inner membrane) was thick and yellowish. There was fluid-filled space between the first and second layers. The length of the eggs was 114-130 µm with an average of 118 µm, and the width was 108-124 µm with an average of 116 µm (Figure 3.D).

Table 1. Prevalence of pentastomiasis in *P. mucosa* based on site and the number of parasites

Sites	Positive (%)	Number of parasites
Lungs only	57% (34 of 60)	2-9 with an average of 5 Pentastomida
Trachea only	0% (0 of 60)	-
Lungs and Trachea	8% (5 of 60)	7-9 with an average of 8 Pentastomida
Total	65% (39 of 60)	204 pentastomida

Figure 1. Pentastomid infestation in the lungs of *P. mucosa*. The white arrows show areas with raised lesions around the attachment of pentastomids to the tissue. The black arrows show female pentastomids, and the red arrow shows male pentastomids

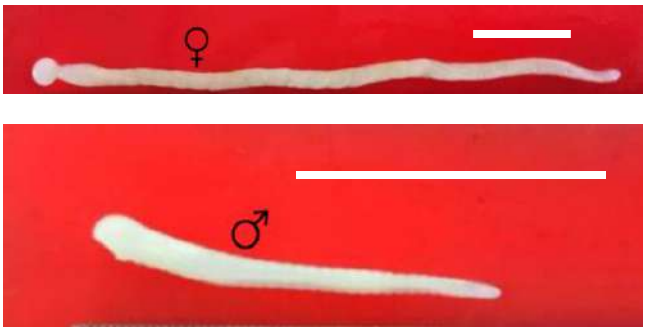

Figure 2. Differences in size between females (*top*) and males (*below*). Bar = 2 cm

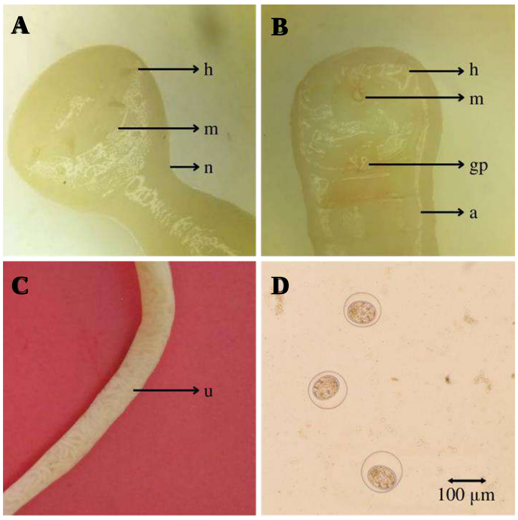

Figure 3. Pentastomida without staining. A. The anterior section of the female. B. The anterior section of the male. C. Female body. D. Pentastomid's eggs. h: hook, m: mouth, n: neck, gp: genital pore, a: first annuli, u: uterus

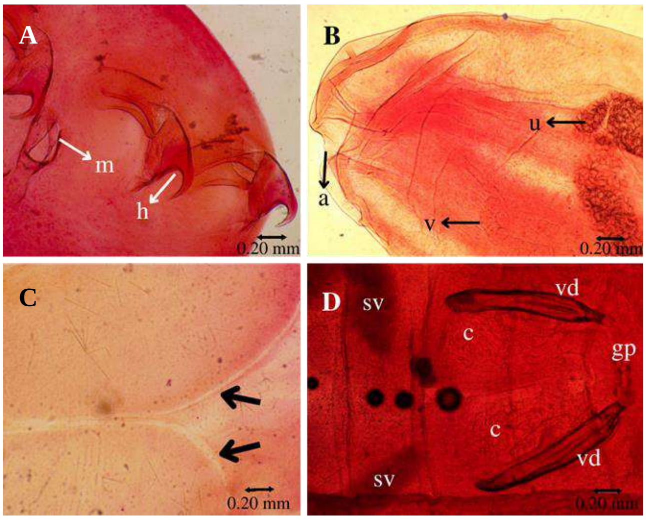

Figure 4. Carmine-stained pentastomida. A. The anterior section. B. The posterior section of the female. C. A pair of female spermatheca. D. Male reproductive organs. m: mouth, h: hook, a: anus, v: vagina, u: uterus, sv: seminal vesicle, c: cirrus, vd: vas deferens

Table 2. The characteristics of pentastomida compared with *Kirichephalus pattoni* Stephens 1908 by Riley and Self (1980) and Keegan (1943)

Parameters	The current study		British Museum collection (Riley and Self 1980)		Keegan 1943
	Females	Males	Females	Males	
Total number of parasites	183	21	17	2	-
Body length	72-125 mm (X̄ 94.5 mm)	17-28.5 mm (X̄ 23.75 mm)	83-137 mm (X̄ 100 mm)	28-29 mm (X̄ 28.5 mm)	-
Number of *annuli*	29-41 (X̄ 31.6)	28-35 (X̄ 31)	34-38 (X̄ 36.5)	31-32 (X̄ 31.5)	-
Diameter of cephalothorax	4-5.5 mm (X̄ 4.75 mm)	2-2.5 mm (X̄ 2 mm)	4 mm	-	-
Neck	2-3 mm (X̄ 2.5 mm)	-	2 mm	-	-
Diameter of abdomen	3.5-5 mm (X̄ 4.2 mm)	1.8-2.2 mm (X̄ 2 mm)	4 mm	-	-
Hook parameter:			-	-	-
AC	0.23-0.55 mm	0.22-0.35 mm			
CB	0.37-0.69 mm	0.29-0.35 mm			
AD	0.47-0.88 mm	0.29-0.45 mm			
Mouth:			-	-	-
Length	0.16-29 mm	0.14-0.38 mm			
Width	0.12-0.24 mm	0.12-0.18 mm			
Egg:		-	-	-	
Length	114-130 µm (X̄ 118 µm)				125-154 µm (X̄ 133 µm)
Width	108-124 µm (X̄ 116 µm)				106-143 µm (X̄ 123 µm)

Pentastomida was mostly found in the lungs, and the parasites also spread to the trachea in five snakes. This finding was similar to research by Riley and Self (1980) that revealed that adult *K. pattoni* (one of the pentastomida species) were only found in the lungs of *P. mucosa* snakes. In our observation, pentastomids that spread to the trachea were females. According to Sundar et al. (2015), the adult pentastomids with mature eggs are expelled from the trachea and eliminated from the definitive host by oral expulsion or may also be swallowed. That results in shed eggs in the feces, which another intermediate mammal host will ingest to continue their life cycles.

The number of male pentastomids was fewer than the number of female ones. This case was also experienced by John and Nadakal (1988) and Riley and Self (1980), who only found a few males compared the female. This study result follows Almeida et al. (2006), whose number of pentastomids and their species is unknown in biology.

The taxonomy of pentastomida is still controversial. Moreover, it has been known through molecular data that pentastomida are closely related to crustaceans and belong to the class Maxillopoda, sub-phylum Crustacea, and the phylum Arthropoda (Paré 2008). Based on the egg size (Almeida et al. 2007); the female head form that globular with a neck separating the head from its body (Paré 2008); and the size of the body length, the number of the annuli, the diameter of the cephalothorax, neck, abdominal diameter, head, and body shape (Riley and Self 1979; Riley and Self 1980); pentastomida was classified as *K. pattoni*. *K. pattoni* can be classified as Porochepalida (Paré 2008). In addition, according to Christoffersen and De Assis (2013; 2015), pentastomid in *P. mucosa* snakes are classified into *K. pattoni*.

K. pattoni has a long annulated body, a tapered tail, an anal canal, two pairs of hooks or four hooks on its head, and a small ovoid-shaped mouth (Riley and Self 1979). The female *K. pattoni* has a larger body than the male one (Paré 2008). Female *K. pattoni* also has a spiral body with a larger head than its body, and a curve of the neck, whereas male one has a straight body without a curve on the neck (Riley and Self 1979; Riley and Self 1980; John and Nadakal 1988; Almeida et al. 2007). The spiral body in females is likely to facilitate their movement into the lung tissue of the definitive hosts (John and Nadakal 1988).

The egg morphology is similar to the eggs of *P. crotali* described by Sundar et al. (2015). These eggs were composed of two-layer membranes, of which the outer one was thin and the inner membrane thick, and there was a fluid-filled space between those two membranes. But the average diameter of the eggs measured in this investigation is smaller (118x116 μm) than the eggs of *P. crotali* (144 μm), as described by Sundar et al. (2015).

Histopathological changes in the lungs of *P.mucosa*

Squamates typically have two lungs; however, the left lung in most snakes is greatly reduced, never exceeding 85% of the right lung's size or absence (Funk and Bogan 2019). The right lung usually runs from near the heart to the cranial to the right kidney (Funk and Bogan 2019). The cranial portion of a lung is vascularized and functions primarily as a gas exchanger (vascular lung), whereas the caudal primarily functions as an air sac (saccular lung). The vascular lung's wall comprises honeycombed units called faveoli, allowing gas exchange (Funk and Bogan 2019). The faveoli in the snake lung parenchyma are lined microscopically by squamous epithelial cells (type I cells). In addition to a lesser degree, by cuboidal epithelial cells (type II) (Jacobson et al. 2021).

In this study, a nymphal stage of the parasite was found in the pulmonary tissue infected with pentastomida. The parasite is surrounded by some connective tissue (Figure 5). Finding the stages of the nymph in the snake's histological lung and the parasite's stages in the macroscopic lung organ means that the *P. mucosa* snake may act as an intermediate and final host of *K. pattoni*. Within the lung cavities of the definitive host, the nymphs develop into adults (Sundar et al. 2015). In this study, pentastomida infection in the lungs only caused a mild histological reaction in the form of fibrosis in the area around the parasite.

Pentastomida has an indirect life cycle that typically utilizes vertebrate hosts. Consequently, these species rely on reptiles as their definitive host. The adults are usually found in their lungs (Wellehan and Walden 2019). Pentastomida parasites migrate extensively through the visceral before settling in the lungs and, less typically, the trachea and nasal passageways. Pentastomida can produce localized lesions, while molting can elicit antigenic reactions. Adults feed on lung tissue fluids and pass embryonated eggs, which are coughed up through the oral cavity, swallowed, and passed in the feces (Comolli and Divers 2021). In squamates, the pulmonary lumen is large, and the tracheal diameter may be smaller than that of the pentastomids. That poses a risk of respiratory obstruction by the adult pentastomids. However, the greatest pathological changes are seen in the intermediate host, where they may become embedded in tissues (Wellehan and Walden 2019).

Pentastomiasis in reptiles, especially snakes, occurs subclinically. Although the infection is large, death is usually associated with secondary septicemia and pneumonia (Tappe and Büttner 2009). Because pentastomids attach to the lung tissue, they can cause perforation, hemorrhaging, and serious inflammation in the host (Fernando and Fernando 2014). In a Common Garter Snake (*Thamnophis sirtalis* Linnaeus 1758), lung lesions were associated with one of the pentastomida species, *Kiricephalus coarctatus* Diesing 1850, where the embedded parasite had gotten into the hypaxial muscle. Infections with *Armillifer armillatus* Wyman 1845 have also been linked to parasitic pneumonia in gaboon vipers. Pentastomids occluding the trachea have been implicated in snake deaths. Bacterial infections could develop due to lung damage. For example, there are four out of nine *Boa constrictors* with pulmonary damage from *Porocephalus dominicana* Riley & Walters 1980 infection perished due to bacteremia and pericarditis (Walden et al. 2021).

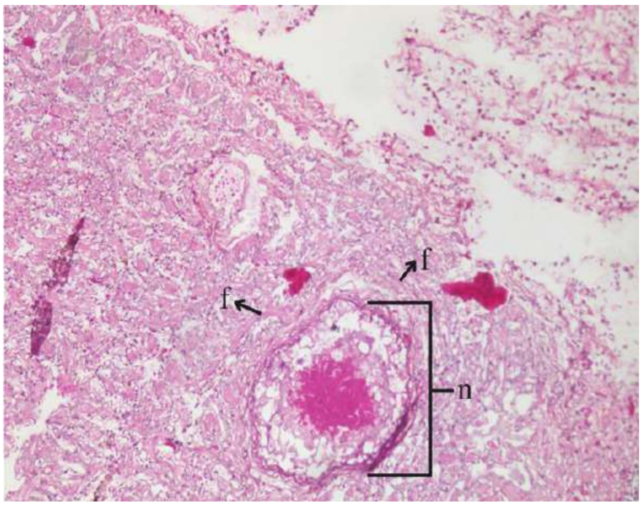

Figure 5. Histological microscopic picture of nymph-infected pulmo from pentastomida showed the presence of fibroblast around the area of infection. f: fibroblast, n: the body of the nymph stage pentastomida

Bigger implications regarding possible impacts on human health may be anticipated based on the histopathological changes found in this study. Humans are the final hosts of pentastomic, which can be contracted from improperly cooked meat from infected snakes or drinking water contaminated with pentastomida eggs. In addition, humans can get the infection when handling and harvesting snakeskin or having direct contact with the mouth of a pet snake (Latif et al. 2016). The pathological consequences of the clinical symptoms experienced by infected humans may be the same or even more severe. That depends on the number of parasite eggs ingested, the duration from the larvae migrating to various organs to maturity and reproduction, and how severe the parasite infestation is in the human viscera.

Personal hygiene measures are required to prevent human infections. For example, avoid drinking river water directly or boiling the water before drinking. In addition, authorities should provide health education to warn people about eating undercooked snake meat, the possibility of transmission after handling snakes, and avoiding contact with snake excretions.

In conclusion the prevalence of pentastomiasis in slaughtered *P. mucosa* in Sidoarjo was 65% (39 of 60 snakes). The pentastomids that infect these snakes are classified as *K. pattoni*. The pulmonary histological findings only show a light reaction in the form of mild fibrosis.

ACKNOWLEDGEMENTS

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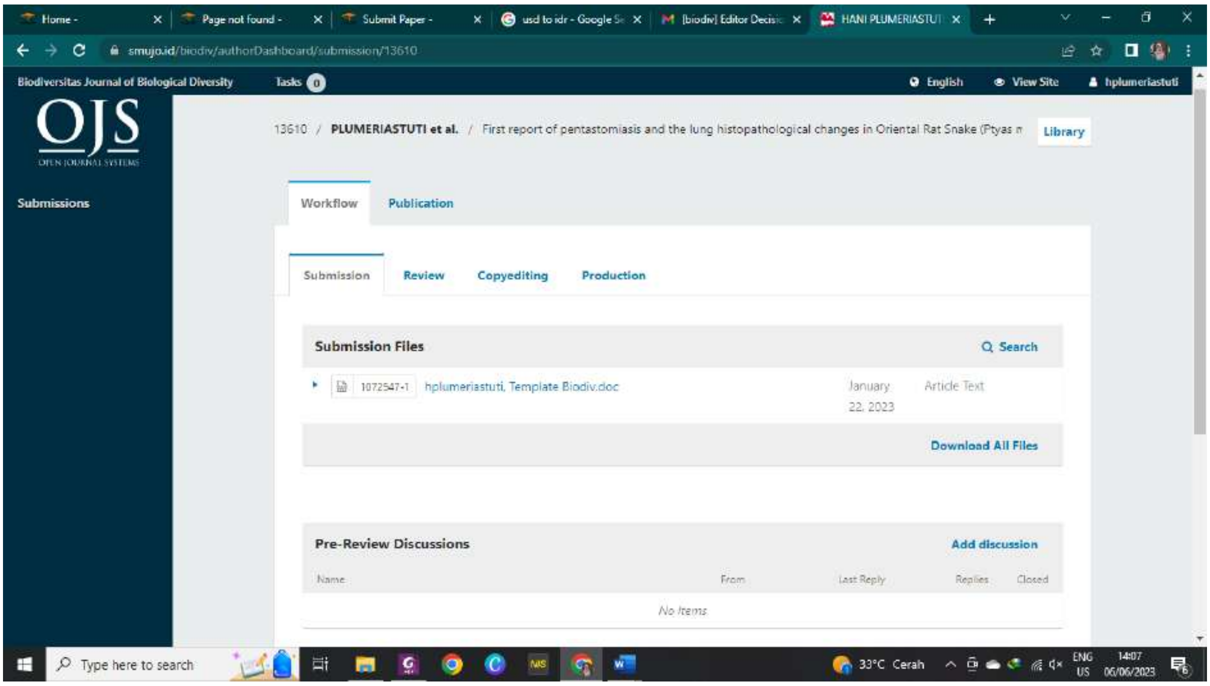
1. Article Submission



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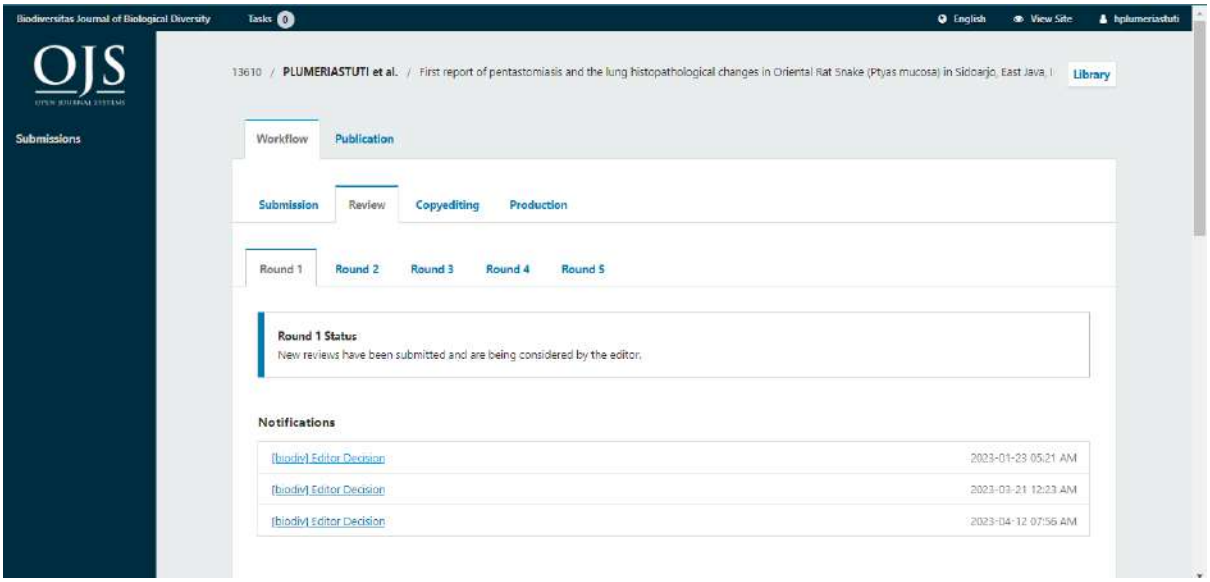
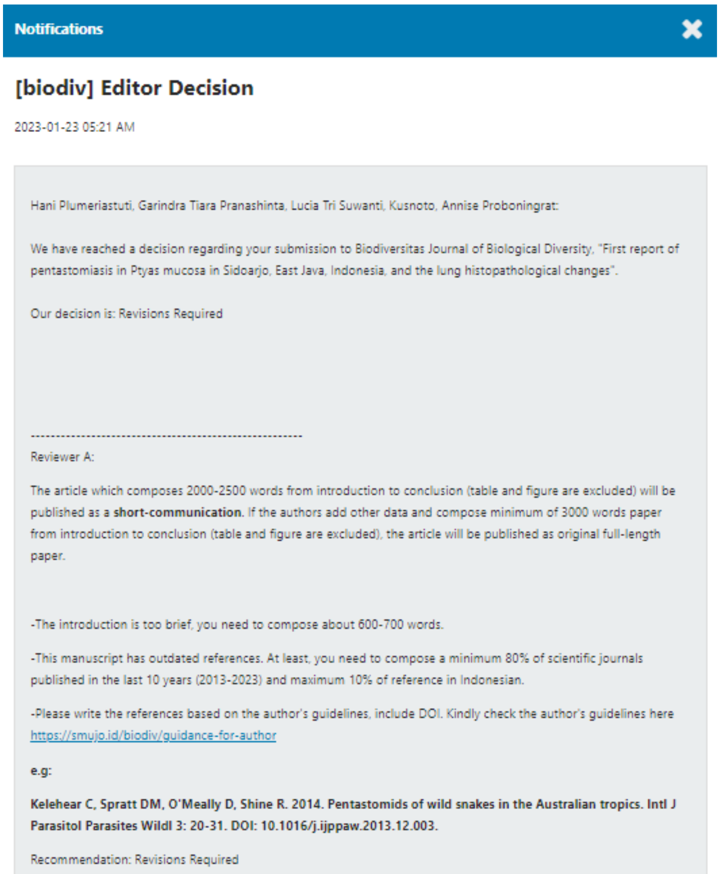
2. Article Review



First Revision


Notifications

[biodiv] Editor Decision

2023-01-23 05:21 AM

Hani Plumeriastuti, Garindra Tiara Pranashinta, Lucia Tri Suwanti, Kusnoto, Annise Proboningrat:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "First report of pentastomiasis in Ptyas mucosa in Sidoarjo, East Java, Indonesia, and the lung histopathological changes".

Our decision is: Revisions Required

Reviewer A:

The article which composes 2000-2500 words from introduction to conclusion (table and figure are excluded) will be published as a **short-communication**. If the authors add other data and compose minimum of 3000 words paper from introduction to conclusion (table and figure are excluded), the article will be published as original full-length paper.

-The introduction is too brief, you need to compose about 600-700 words.

-This manuscript has outdated references. At least, you need to compose a minimum 80% of scientific journals published in the last 10 years (2013-2023) and maximum 10% of reference in Indonesian.

-Please write the references based on the author's guidelines, include DOI. Kindly check the author's guidelines here https://smujo.id/biodiv/guidance-for-author

e.g:

Kelehear C, Spratt DM, O'Meally D, Shine R. 2014. Pentastomids of wild snakes in the Australian tropics. Intl J Parasitol Parasites Wildl 3: 20-31. DOI: 10.1016/j.ijppaw.2013.12.003.

Recommendation: Revisions Required

Second Revision

[biodiv] Editor Decision

2023-03-21 12:23 AM

Hani Plumeriastuti, Garindra Tiara Pranashinta, Lucia Tri Suwanti, Kusnoto, Annise Proboningrat:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "First report of pentastomiasis in Ptyas mucosa in Sidoarjo, East Java, Indonesia, and the lung histopathological changes".

Our decision is: Revisions Required

Reviewer A:

Dear Authors,

Thank you for submitting this manuscript that explores pentastomiases in snakes. This is an interesting and novel findings and has some potential application as a result.

There are some revisions required in order to consider this manuscript for publication. I have included specific feedback on the word document version of the manuscript, please find attached. Make sure that any changes to the manuscript are shown using highlighted text or tracked changes. Additionally, please address the following key areas when making revisions:

1. Methods. Be clear on exactly how and where the snakes were sourced as this could affect disease status. Provide some clearer informaiton on what proportion of the parasites were sampled and whether any other parasites were identified during the study.

2. Watch out for errors in citation and reference style. All citations are currently not formatted to the journal requirements so do revise these.

3. Implications. What are the implications and future direction for this research? Currently the discussion and abstract are very brief and as such they do not summarise the imprtance of the study's findings.

With these revisions, the work should be in a stronger position overall.

Recommendation: Revisions Required

[biodiv] Editor Decision External Inbox ×

Smujo Editors via SMUJO <mail@smujo.id>
to me, Garindra, Lucia, Kusnoto, Annise

Mar 21, 2023, 7:24 AM

Hani Plumeriastuti, Garindra Tiara Pranashinta, Lucia Tri Suwanti, Kusnoto, Annise Proboningrat:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "First report of pentastomiasis in Ptyas mucosa in Sidoarjo, East Java, Indonesia, and the lung histopathological changes".

Our decision is: Revisions Required

Reviewer A:

Dear Authors,

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There are some revisions required in order to consider this manuscript for publication. I have included specific feedback on the word document version of the manuscript, please find attached. Make sure that any changes to the manuscript are shown using highlighted text or tracked changes. Additionally, please address the following key areas when making revisions:

1. Methods. Be clear on exactly how and where the snakes were sourced as this could affect disease status. Provide some clearer informaiton on what proportion of the parasites were sampled and whether any other parasites were identified during the study.

2. Watch out for errors in citation and reference style. All citations are currently not formatted to the journal requirements so do revise these.

3. Implications. What are the implications and future direction for this research? Currently the discussion and abstract are very brief and as such they do not summarise the imprtance of the study's findings.

With these revisions, the work should be in a stronger position overall.

Recommendation: Revisions Required

Response for Reviewer Comment

Participants Edit

Smujo Editors (editors)

DEWI NUR PRATIWI (dewinurpratiwi)

Agustina Putri (aputri1)

Hani Plumeriastuti (hplumeriastuti)

Messages

Note	From
Dear editor, here is the initial manuscript with answers to the reviewer's comments. I have also uploaded the revised manuscript (with highlighted changes) in the revision box. hplumeriastuti, Answer to Reviewer's Comments.doc	hplumeriastuti 2023-04-02 06:35 PM

Add Message

Revision for Reviewer A

Participants Edit

Smujo Editors (editors)

DEWI NUR PRATIWI (dewinurpratiwi)

Agustina Putri (aputri1)

Hani Plumeriastuti (hplumeriastuti)

Messages

Note	From
Thanks for the corrections and valuable suggestions. We have made revisions according to the corrections from reviewers. We are waiting for further progress on our article. Thank you very much.	hplumeriastuti 2023-04-07 01:45 PM

Add Message

Third Revision

[biodiv] Editor Decision External Inbox ×

Smujo Editors via SMUJO <support@smujo.com> Apr 5, 2023, 9:29 AM
to me, Garindra, Lucia, Kusnoto, Annise

Hani Plumeriastuti, Garindra Tiara Pranashinta, Lucia Tri Suwanti, Kusnoto, Annise Proboningrat:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "First report of pentastomiasis in Ptyas mucosa in Sidoarjo, East Java, Indonesia, and the lung histopathological changes".

Our decision is: Revisions Required

Reviewer A:
Recommendation: Accept Submission

Uncorrected Proof

[biodiv] New notification from Biodiversitas Journal of Biological Diversity External Inbox ×

DEWI NUR PRATIWI via SMUJO <support@smujo.com> Sun, Apr 9, 1:37 PM
to me

You have a new notification from Biodiversitas Journal of Biological Diversity:

You have been added to a discussion titled "Uncorrected Proof" regarding the submission "First report of pentastomiasis in Ptyas mucosa in Sidoarjo, East Java, Indonesia, and the lung histopathological changes".

Link: https://smujo.id/biodiv/authorDashboard/submission/13610

Ahmad Dwi Setyawan

____
Biodiversitas Journal of Biological Diversity

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Uncorrected Proof

Participants

Smujo Editors (editors)

DEWI NUR PRATIWI (dewinurpratiwi)

Agustina Putri (aputri1)

Hani Plumeriastuti (hplumeriastuti)

Messages

Note	From
Dear Author(s), Pls, find attached file for an uncorrected proof (Copyedited file). The revised manuscript (Corrected proof) is awaited. Do not worry about layout changes due to revision: our staff will fix it again. Note: Kindly TURN ON track changes when you make improvements. dewinurpratiwi, Plumeriastuti-Colubrid snake.doc	dewinurpratiwi 2023-04-09 06:37 AM
Thanks for the information on the progress of my manuscript. Please find the corrected proof that I have attached in this discussion column. I will also send proof of payment for the manuscript soon. hplumeriastuti, 13610-Article Text-1079305-1-18-20230409 - Corrected Proof.doc	hplumeriastuti 2023-04-09 08:06 AM

Notifications

[biodiv] Editor Decision

2023-04-12 07:56 AM

HANI PLUMERIASTUTI, GARINDRA TIARA PRANASHINTA, LUCIA TRI SUWANTI, ANNISE PROBONINGRAT, KUSNOTO:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (Ptyas mucosa) in Sidoarjo, East Java, Indonesia".

Our decision is to: Accept Submission

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Biodiversitas Journal of Biological Diversity

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Agustina Putri via SMUJO <support@smujo.com> Wed, Apr 12, 2:56 PM
to me, GARINDRA, LUCIA, ANNISE, KUSNOTO

HANI PLUMERIASTUTI, GARINDRA TIARA PRANASHINTA, LUCIA TRI SUWANTI, ANNISE PROBONINGRAT, KUSNOTO:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "First report of pentastomiasis and the lung histopathological changes in Oriental Rat Snake (Ptyas mucosa) in Sidoarjo, East Java, Indonesia".

Our decision is to: Accept Submission

Biodiversitas Journal of Biological Diversity

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3. Payment

DEWI NUR PRATIWI via SMUJO <support@smujo.com> Apr 9, 2023, 1:45 PM
to me

You have a new notification from Biodiversitas Journal of Biological Diversity:

You have been added to a discussion titled "BILLING" regarding the submission "First report of pentastomiasis in Ptyas mucosa in Sidoarjo, East Java, Indonesia, and the lung histopathological changes".

Link: https://smujo.id/biodiv/authorDashboard/submission/13610

Ahmad Dwi Setyawan

...

Reply Forward

BILLING

Participants

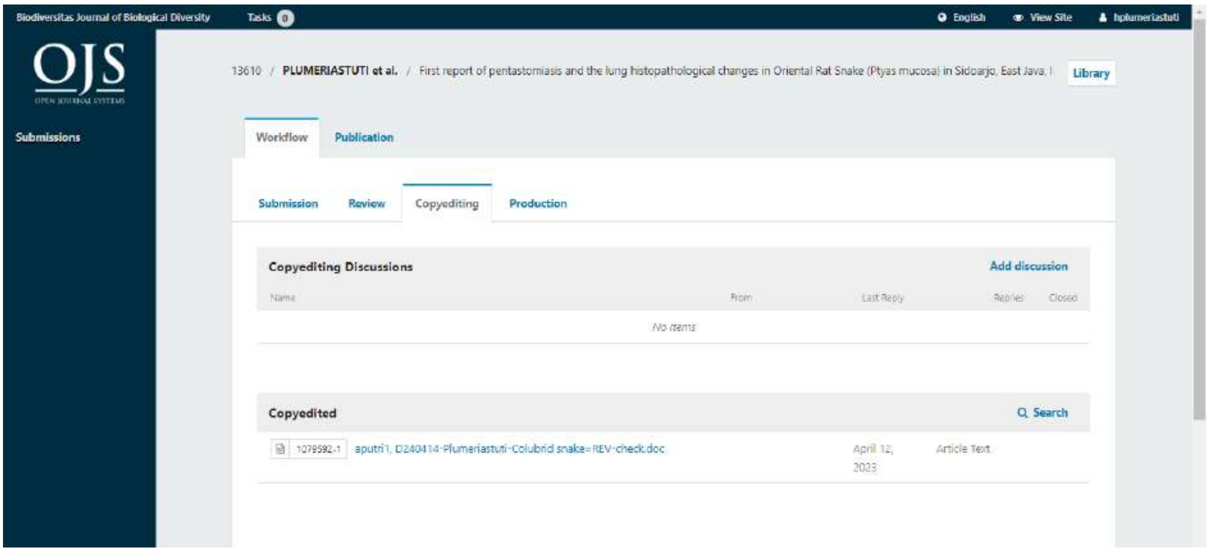
DEWI NUR PRATIWI (dewinurpratiwi)

Hani Plumeriastuti (hplumeriastuti)

Messages

Note	From
Dear Author(s). Kindly find attached an invoice for the publication of your manuscript. dewinurpratiwi, 143.HANI PLUMERIASTUTI.pdf	dewinurpratiwi 2023-04-09 06:44 AM
Dear Editor. Please find the attached proof of payment for my manuscript. Thank you very much. hplumeriastuti, Payment Proof.jpg	hplumeriastuti 2023-04-10 04:22 PM
payment received, thank you	dewinurpratiwi 2023-04-12 11:50 AM

4. Copyediting



5. Publication