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ENDOSYMBIONT BACTERIA OF *PHERETIMA* SP. EARTHWORMS (ANNELIDA: OLIGOCHAETA) POSSESSES ANTIBACTERIAL ACTIVITY

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Abstract– Earthworms are used as drugs, not only the compound earthworms but also endosymbiont microorganisms in earthworms suspected of having antibiotic activities. This research is aimed to identify the ability of several endosymbiont bacteria in *Pheretima* sp. earthworms to inhibit the growth of the bacteria *Salmonella typhi* and *Staphylococcus aureus.* Isolation of bacteria from *Pheretima* sp. earthworms was carried out on NA (nutrient agar medium. The observed characteristics of bacterial isolates from *Pheretima* sp.earthworms consisted of colony morphology, gram staining, and the presence of endospores. This research demonstrates the variety of colors, shapes, edges, and elevations of the isolates. The result showed that there are 2 isolate that was isolation form *Pheretemia* sp. namely *Bacillus choshinensis* and *Bacillus brevis*. Based on the test results of isolation and inhibition of *Pheretima* sp. earthworms, all isolate effectively inhibited the growth of the bacteria *S. thypi* and *S. aureus,* and they have the potential to act as antibiotics. Isolates *Bacillus choshinensis* had the largest inhibitory zone diameter of 21.32 mm against *S. thypi* after 15 days of incubation. Inhibitory properties of all isolates that were isolated from *Pheretima* sp. indicate that bacteria could be described as bactericidal.

INTRODUCTION

Earthworms (Phylum: Annelida, Order: Oligochaeta, Class: Clitellata) are known to be the most important soil fauna biomass in humid soils of temperate and tropical regions (Leveque *et al.*, 2013; Singh *et al.*, 2016). These animals are most often found in moist soil and contain numerous beneficial organic compounds and minerals from natural sources or waste disposal bins, their natural habitats (Chachina *et al.*, 2016). Earthworm species consist of three major groups, namely Polychaeta, Oligochaeta, and Harudinea, as well as two small groups, namely Aeolosamata and Branchiobdella (Klarica *et al.*, 2012; Carnovale *et al.*, 2015).

Polychaeta is the largest group of invertebrates, comprising approximately 8000 species; the largest group is found in the sea (Fiege *et al.*, 2010; Bohlen, 2001; Metcalfe and Glasby, 2008). The typical form of Polychaeta is characterized by a jointed body shape, and on every segment, there is a pair of parapodia (Bohlen. 2001; Omena *et al.*, 2012; Choi *et al.*, 2011). The potential of polychaetaearthworms as medicinal raw materials and ingredients for healing various diseases has been expressed by many researchers (Li *et al.*, 2011; Cho *et al.*, 2014).

Polychaeteearthworms are used as drugs, not only the compound polychaeta earthworms but also endosymbiont microorganisms in polychaete earthworms suspected of having antibiotic activities (Fiolka *et al.*, 2010). As reported in earlier studies, earthworms are used as drugs, not only the compound earthworms but also endosymbiont microorganisms in earthworms suspected of having antibiotic activities. Therefore, further screening of the potential bacterial endosymbionts contained in the worm *Pheritma* sp. needs to be done. This study is aimed to investigate antibiotic compounds from endosymbionts of *Pheretima* sp. earthworms (polychaetes) as inhibitors ofthe growth of *Salmonella thypi* and *Staphylococcus aureus*.

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MATERIAL AND METHODS

Isolation of Bacteria

Samples of *Pheretima* sp. earthworms were obtained from garbage and washed with sterile distilled water until the soil and dirt attached to the skin surface of the earthworms was washed away. The earthworms were then slowly killed by washing with 70% alcohol. Earthworms were measured, crushed using a mortar, and serially diluted by adding 0.1 mL to 0.9 mL of diluent to a maximum dilution of 10-6. A total of 1 mL of each dilution was then inoculated on NA (nutrient agar) medium by using the casting method, then incubated at 37°C for 48 h. Each colony that grew was selected for the purification step.

Purification of Bacterial Culture

This stage of purification began with selecting individual colonies. Round, sterile needles were touched to the surface of bacterial colonies and then inoculated on the surface of NA medium by the scratch method to obtain isolated colonies.It took several attempts to obtain pure colonies, which were then incubated at 37°C for 48 h. The purification step can be performed 2–3 times to ensure the complete purity of the selected colonies.

Bacterial Identification

Observation of Morphology

In the morphological observation,each colony formed after purification was observed. Observations made include colony form, color, edge, center, diameter, and surface morphology, as well as cell morphology and gram-positive or negative status of bacteria and their spores to classify isolates.

Gram Staining

Observations of cell morphology were made using Gram staining techniques. First, bacteria were mounted and fixed on glass slides. A total of 2–3 drops of stain A (crystal violet) weredropped onto bacterial colonies and incubated for 60 s. Preparations were then washed under running water and dried. A total of 2–3 drops of stain B (lugol) were dripped over the preparation and left for 60 s. The mixture was washed under running water and dried. Preparations were then etched with 2–3 drops of stain C (alcohol-acetone), incubated for approximately 30 s, then washed again and dried. Further, 2-3 drops of spilled stain D (safranin) were added and allowed to stand for 60 s, then washed and dried. Observations were then made under light microscopy.

Endospore Staining

This test is used to determine the presence of bacterial spores in soil worms. A smear of bacteria from pure cultures was fixed on a sterile glass microscope slide, flooded with 5% malachite green, and placed on a hot plate that had been preheated to 200ºC for 10 min. After 10 min, the slide was cooled and then cleaned under running water. The smear of bacteria was flooded with safranin for 1 min and washed under running water. The slide was drained of excess water on absorbent paper and then observed under a microscope. Spores appeared green in color or refractive.

Biochemical Identificatiom

The isolated bacteria strains from *Pheretima* sp. gut were identified using biochemical tests API 20 E (Bio-Merieux).

Bacterial Isolates of Preculture Stage Symbionts

Isolates obtained were subsequently used to inoculate a preculture/starter culture in NB (nutrient broth) mediumand incubated with shaking for 24 h at 37°C. After it grew, the preculture was inoculated into NB (nutrient broth) medium at a 1:10 ratio.

Testing Antibiotics

Antibiotic activity was tested using MHA medium (Muller Histon order) by the agar diffusion method using a paper disk. Each paper disk was immersed in the supernatant of bacterial symbionts for 15 min and a positive control solution of chloramphenicol. A 1 mL suspension of test bacteria was added to a sterile petri dish and MHA medium was added at 45°C and allowed to solidify. Paper disks that had been soaked in MHA media solidified with a distance of 20 mm between each paper disk and then incubated at 37°C. Observations were made after 24 h, then the diameter of the inhibition zone formed was measured, and incubation was resumed for 48 h to determine the nature of the antibiotic compounds contained in the worm.

RESULTS

Microscopic Observation and Identification of Bacterial Isolates from *Pheretima* sp.

Based on the results of microscopic observation and

biochemical identification, there are 2 isolates:*Bacillus choshinensis*and *Bacillus brevis*. Based on the results of Gram staining, both of bacteria obtained were in the shape of gram-negative bacilli (rods). Endospore tests of the stained slidesrevealed that bothisolates capable of forming endospores. Isolate *Bacillus choshinensis*in the form of single bacilli, while isolate *Bacillus brevis* in the form of bacilli with two chain (Table 1).

Inhibitory Power Earthworm Test Against Bacterial Pathogens *Staphylococcus aureus* (gram-positive) and *Salmonella typhi* (gram-negative)

Earthworm play a critical role in the decomposition of organic matter. In organic materials, there are many types of bacterial, including pathogenic bacteria. This type of bacteria can affect the composition of the endosymbiont bacterial on earthworm. Inhibition test of bacteria is a method for determining the ability of bacteria to inhibit other bacteria. Table 2 shows the effect of incubation time on the inhibition zone on both test bacteria,*S. thypi*and *S. aureus*. After an incubation time of 15 days, 15.27 mm and 21.32 mm diameter zones of inhibition occurred around *Bacillus choshinensis* in the test bacteria *S. thypi* and *S. aureus*,respectively. The appearance of an inhibition zone around *Bacillus choshinensis*greater than 14 mm in diameter indicated effective inhibition ofbacterial growth, in accordance with Cappuccino & Shermthan[16]. Zones of inhibition with diameters of 10–11 mm and 9 mm are considered less effective and ineffective, respectively (Cappuccino and Sherman, 2011).

DISCUSSION

The inhibition zonesaround bacteria isolated from *Pheretima* sp. worms were greater than those around thecommercial antibiotic Amoxicillin (Table 2). This suggests that the bacteria isolated from *Pheretima* sp. worms have potentialas an antibiotic. The same phenomenon was reported by Fiolka *et al.* (2010), Brito-Vega and Espinosa-Victoria (2009), and Byzov *et al.* (2007) that some worm species have bacterial endosymbionts that can inhibitthe growth some types of pathogenic bacteria.

Table 1. Characterization of bacterial colony morphology earthworms *Pheretima* sp. and Gram's staining and endospore staining

Isolates	Forms colonies				Gram Staining		Endospore
	Color	Form	Edge	Elevation	Form	Gram	
Bacillus choshinensis	White	Circullar	Entire	Flat	Single basil	Positive	exist
Bacillus brevis	White	Circullar	Entire	Convex	Basil two chain	Positive	exist

Table 2. Inhibitory Zone of isolate bacteria that was isolated form *Pheretima* sp.

No.	Isolate	Incubation Time	Inhibitory Zone			
			Salmonella thypi		*Staphylococcus aureus*	
			24 hours	48 hours	24 hours	48 hours
1.	*B. choshinensis*	5 days	13,09 mm	13,60 mm	12,20 mm	12,42 mm
		10 days	14,24 mm	14,63 mm	13,20 mm	13,57 mm
		15 days	20,05 mm	21,32 mm	15,56 mm	16,88 mm
		20 days	13,40 mm	13,55 mm	13,01 mm	13,15 mm
2.	*B. brevis*	5 days	11,90 mm	12,60 mm	9,90 mm	10,10 mm
		10 days	9,97 mm	10,40 mm	10,20 mm	10,31 mm
		15 days	15,13 mm	15,25 mm	12,20 mm	12,40 mm
		20 days	10,70 mm	10,79 mm	10,20 mm	10,35 mm
3.	Amoxicillin	5 days	8,40 mm	9,05 mm	13,80 mm	13,98 mm
		10 days	8,40 mm	9,05 mm	13,90 mm	13,98 mm
		15 days	8,40 mm	9,05 mm	13,80 mm	13,98 mm
		20 days	8,40 mm	9,05 mm	13,80 mm	13,98 mm
4.	Chloramphenicol	5 days	25,00 mm	25,10 mm	13,09 mm	13,87 mm
		10 days	25,00 mm	25,10 mm	14,15 mm	14,50 mm
		15 days	25,20 mm	25,45 mm	14,15 mm	14,50 mm
		20 days	25,20 mm	25,45 mm	14,15 mm	14,50 mm

The results showed that the inhibitory zone around chloramphenicol was larger than those around bacteria isolated from *Pheretima* sp. worms (Table 2). This can occur because chloramphenicol is an antibiotic that is commonly used in the treatment of typhoid and contains active compounds that have been tested. However, the use of chloramphenicol is limited because it can damage the ribosomes of mitochondria in mammalian cells[19]. As with several compounds utilized in medicine that have a nitro group component, chloramphenicol may be toxic to animals and plants (Madigan *et al*., 2012).

Based on observations of inhibitory activity, the test showed that the bacterial inhibition isolated from *Pheretima* sp. earthworms (*Bacillus choshinensis* and *Bacillus brevis*) was capable of inhibiting bacterial growth after a 24-h incubation. After incubation for 48 h, the diameter of the inhibitory zone had increased from the from the first 24 h. According to Madigan *et al*., (2012), when the inhibition zone formed after an incubation period of 48 h remains the same or expansion of the inhibition zone diameter occurs, then the bacteria could be described as bactericidal. This is the case withbacteria isolated from *Pheretima* sp.

This result shows that incubation for 15 days effectively assessed inhibition of the growth of pathogenic bacteria, marked by the largest inhibition zone. These results agreewith Madigan *et al*., (2012), that secondary metabolites (antimicrobial) are produced by microorganisms in the late stationary phase of growth. This is because secondary metabolites are usually synthesized by the end of the growth cycle of the cell, namely the stationary phase, as the population remains stable because the number of cells that grow equal the

Table 3. Comparison of Isolated bacteria that was Isolated form Various Earthworm

Earthworm	Location	Identification Method	Isolated Bacteria	References
Dendrobaena veneta (Annelida)	Poland	Biochemical identification (API 20 E Bio-Merieux)	*Raoultella ornithinolytica*	Fiolka *et al.* (2010) [15]
Libyodrilus violaceus (Annelida)	Nigeria	Biochemical identification (Manual)	*Alcaligans faecalis, Bacillus brevis, Bacillus ceveus, Bacillus lalerosporus, Bacillus lichenoform, Bacillus maceraus*	Owa *et al.* (2013)[24]
Lampito mauritii (Annelida)	India	16S rRNA sequencing	*Bacillus cereus*	Biswas *et al.* (2014)[25]
Lumbricus terrestris (Annelida)	Ireland	Automated ribosomal intergenic spacer analysis of 16S and 23S genes	Proteobacteria, Firmicutes and an actinobacterium	Thakuria *et al.* (2010)[26]
Perionyx excavatus (Annelida)	India	Biochemical identification (Manual)	*Bacillus, Staphylococcus, Enterococci,* Micrococcus, Enterobacter, and Citrobacter	Samanta & Das (2016)[27]
Eisenia fetida (Annelida)	China	16S rRNA sequencing	Bacillus, Balneimonas, Cupriavidus, Hylemonella, Kaistobacter, Lysobacter, Thermomonas, Flavihumibacter, Flavisolibacter Pseudomonas, Stenotrophomonas, and Achromobacter	Ma *et al.* (2017)[28]
Aporrectodea caliginosa (Annelida)	Russia	16S rRNA sequencing	*Aeromonas encheleia*, *Bacillus thuringiensis*, *Acinetobacter* sp., *Aeromonas* sp. and *Streptomyces* sp.	Tikhonov *et al.* (2016)[29]

number of cells that die. The synthesis of secondary metabolites is initiated when some of the nutrients in the microbial growth medium have been exhausted. Limitation of these nutrients causes the accumulation of secondary metabolites, enzyme inducers.

The observation that 15 days is the optimum incubation time for bacteria in this research to inhibit pathogenic bacteria is related to secondary metabolites. One of the factors that affects the production of secondary metabolites is the duration of growth of the bacteria. An indicator of the optimum time of production of anti-microbials is the time at which anti-microbial compounds produced are maximally characterized by the formation of the largest inhibition zone at a certain incubation time on the growth of gram-negative bacteria *S. thypi* and gram-positive bacteria *S. aureus*.

Based on the bacteria that have been isolated from *Pheretima* sp. earthworms, there are antibiotics that can inhibit the growth of pathogenic bacteria because they produce a large inhibition zone. This is in accordance with the study of Fiolka *et al.* (2010), who showed that a bacterium isolated from the midgut of earthworms has antimycobacterial activity and has shown activity against four strains of fast-growing mycobacteria. According to Li *et al.* (2011) and Fiolka *et al.*(2010), microbial soil can routinely produce antibiotics through a chemical process called secondary metabolism. It is believed that the production of antibiotics by microorganisms in the soil increases when they are likely to compete with other microbes in soil. This occurs in the form of competition for space or food (Omena *et al.*, 2012).

In this study, two bacterial isolates from *Pheretima* sp. worms namely *Bacillus choshinensis* and *Bacillus brevis*. Table 1 shows various types of bacteria that was isolated by several studies from various worms. Table 1 shows the different types of worms that have different endosymbiont bacteria. Endosymbiont bacteria that can be isolated by several studies dominated by the genus Bacillus (Owa *et al.*, 2013; Biswas *et al.*, 2014; Samanta and Das, 2016; Ma *et al.*, 2017; Tikhonov *et al.*, 2016). These results are in accordance with a recent study succeeded in isolating two species of bacteria of the genus Bacillus. This suggests that genus Bacillus is a genus generally be endosimbiont bacteria in the earthworm. According to Rahman *et al.*(2014), the genus Bacillus is commonly found in soil bacteria that is often found as endosymbion on earthworm.

Two bacterial isolates from *Pheretima* sp. worms namely *Bacillus choshinensis* and *Bacillus brevis*were shown to inhibit the growth of the bacteria *S. thypi* and *S. aureus*. Therefore, detailed studies of the role of bacterial *Bacillus choshinensis* and *Bacillus brevis* on the lives of worms *Pheretima* sp. worms should be further investigated to uncover benefits and reasons why it is endosymbiont with the worm *Pheretima* sp.

CONCLUSION

Based on the test results of isolation and inhibition of *Pheretima* sp. earthworms,it was concluded that *Bacillus choshinensis* and *Bacillus brevis*effectively inhibited the growth of the bacteria *S. thypi* and *S. aureus*, and they have the potential to act as antibiotics.Isolates *Bacillus choshinensis*had the largest inhibitory zone diameter of 21.32 mm against *S. thypi* after 15 days of incubation.Inhibitory properties of all isolates that were isolated from *Pheretima* sp. indicate that bacteria could be described as bactericidal.

Significance Statements

As reported in earlier studies, earthworms are used as drugs, not only the compound earthworms but also endosymbiont microorganisms in earthworms suspected of having antibiotic activities. Therefore, further screening of the potential bacterial endosymbionts contained in the worm *Pheritma* sp. Our findings revealed two bacterial isolates from *Pheretima* sp. worms namely *Bacillus choshinensis* and *Bacillus brevis*. It showed signicicant inhibitory activities as inhibitors of the growth of *Salmonella thypi* and *Staphylococcus aureus*. We postulate that *Bacillus choshinensis* and *Bacillus brevis* that was isolated from *Pheritma* sp. earthwormhave great potentials to become a source of therapeutic agent for phatogenic bacterial infection.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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