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Molecular Diagnosis of Schistosomiasis in Feces Samples From Napu Valley Community of Poso Regency, Central Sulawesi, Indonesia

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Abstract

Schistosomiasis is infection caused by Schistosoma worms. Schistosomiasis in Indonesia caused by a worm of *Schistosoma japonicum*, which is an endemic disease and it is only found in Central selawesi, in high land of Napu Valley, Lindu, and Bada villages. Prevalence of schistosomiasis during 2001-2010 experienced fluctuation, which the lowest prevalence was in 2003, it was 0.70%, and the highest prevalence was in 2010, it was 5.68%. In 2012, the proportion of schistosomiasis case in Napu was 1.44%. From 15 Sub-districts examined in Napu Highland area, there were 12 sub-districts which had the prevalence above the WHO standard of 1%. Routine inspection was conducted by the Department of Health-Central Sulawesi microscopically based on Kato-Katz method. Target of this study is to find out comparation accuration data resulted from molecular examination by PCR method, compare to the results of microscopic examination based on Kato-Katz method. This research was an observational descriptive research. Molecular examination by PCR method was done using primers sequencing of forward 5'-TCT AAT GCT ATT GGT TTG AGT-3' and reverse 5'-TTC CTT ATT TTC ACA AGG TGA-3'. The target umplification was DNA of SjR2 gene, at 230 bp band. Preserved feces samples was done using ethanol 96% at Dodolo village, that have been previously examined microscopically using Kato-Katz method. Based on microscopic examination on 70 samples, 19 sample was positive infected by the worm of *S japonicum*, and 51 sample of them was shown negative result. Upon further investigation molecularly by PCR, there were 40 people positively detected for infection by the worm *S. japonicum*, which was shown by appearing on the target band of 230 bp, while 30 others samples were declared negatively. Molecular examination data showed two times more likely in detecting schistosomiasis, compared to microscopic examination by the Kato-Katz method. Our data also showed that Molecular examination using PCR method can be used for 70-96% ethanol-preserved facel samples.

INTRODUCTION

Schistosomiasis in Indonesia was first discovered by Muller and Tesch in 1937, it caused by *Schistosoma japonicum* with an intermediate host of Oncomelania. The snail was first discovered in the rice field, lake Lindu in 1971 by Carney *et al.*, And was identified as *Oncomelania hupensis lindoensis* (Gunawan *et al.*, 2014).

In Indonesia, schistosomiasis in humans is only found in Central Sulawesi, especially in Napu, Lindu, and Bada. Central Sulawesi is the only province in Indonesia that is endemic to schistosomiasis. This disease was found in 2 of 11 regencies (Sigi and Poso) in Central Sulawesi, (Dinas Kesehatan Provinsi Sulteng, 2013).

According to the data based on the measurements by the Kato-Katz method, there were 19 cases of schistosomiasis, 12 of them were current cases in 2015 and 7 others were newc cases. This indicating that the medical treatments to patiens were not going well. Praziquantel is an effective drug for the treatment of schistosomiasis. However, the treatment of schistosomiasis in these area were not done properly, especially in terms of taking medication. (Dinas Kesehatan Provinsi Sulteng, 2015)

Microscopic measurements (Kato-Katz method), were done routinelyt by the local Public Health Officer every year, in order to detect the presence of the eggs of *Schistosoma japonicum*, but the worm eggs were not be detected under microscope when they are in few number (Satrija *et al*, 2008). More sensitive and specific diagnostic tools are needed in controlling and prevention program of schistosomiasis, in order to reduce the number of infected person by *S. japonicum* in endemic areas of Central Sulawesi.

Molecular measurement by PCR (Polymerase Chain Reaction) method is an option, as a diagnostic tool for schistosomiasis that recommended by the World Health Organization (WHO, 2010). PCR method has a high level of sensitivity, because the DNA fragment can be amplified and will produce 106-109 times, so it will be easier in detection, eventhough the amount of eggs from *S. japonicum* is low (<5 eggs per gram of feces).

The PCR method uses several temperature levels and using Polymerase enzyme to amplify DNA fragment. The PCR process consists of three stages: denaturation, annealing, and extension. Denaturation is the process of separating double strand DNA into two single strands of DNA at 90°–95°C for 3-5 minutes. Annealing is a primary attachment process with a temperature of 50°-60°C for ± a minute. Extension is a polymerization reaction or elongation of DNA chains at 72°C for 5-7 minutes (Elrod and William, 2011).

This study "Molecular Diagnosis of Schistosomiasis in feces of Dodolo Village, Napu, Poso, Central Sulawesi, Indonesia was aimed to optimize the molecular detection on schistosomiasis case, using the PCR method and compare to the results of microscopic detection by the Kato-Katz method.

MATERIAL AND METHODS

This study was an observational descriptive using molecular analysis by PCR method of feces samples after preserved in 96% ethanol from Dodolo village, which had previously been examined microscopically using the Kato-Katz method.

a. Isolation DNA of *Schistosoma japonicum*

DNA isolation from *S. japonicum* using the Favor Prep Stool DNA Isolation Mini Sample Kit, with tools such as vortex, centrifuge, micropipette, blue tip, yellow tip, refrigerator, incubator, microwave, gloves, masks, bead tube, microtube. The materials used for DNA isloation were 100 mg stool samples, 200mg glass beads, Proteinase K 20 asel, SDE1 300 ul, SDE2 100 ul, SDE3 200 ul, SDE4 250 ul, SDE Column, Wash buffer 750 ul, Elution buffer 100 ul and SDE2 100 ul, SDE3 200 gl, SDE4 250 ul, SDE Column, Wash buffer 750 ul, Elution buffer 100 ul and ethanol 96-100% 250 u l.

b. PCR (Polymerase Chain Reaction)

PCR was conducted using the SjR2 (Retrotransposon) primary design with the target band at 230 bp. The primary sequences were (forward 5 '-TCTAATGCTATTGGTTTGAGT-3') and (reverse 5 '-TTCCTTATTT TCACAAGGTGA-3'). The components of the PCR were Mix 15 u l solution, 9H dH2O 9,, DNA sample 4 ul, SjR2 primers 2 ul. All materials were mixed using a vortex, then centrifuge for 1 minute at 10.00 rpm, then insert it into the PCR machine. PCR conditions were at 95°C for 5 minutes, 94°C for 1 minute, 54°C for 1 minute, 72°C for 1 minute, 72°C for 7 minutes and followed by 35 cycles. The PCR results will be amplified with 1.5% agarose gel.

c. Making Agarose Gel (1.5%)

1.5 grams agarose were dissolved to 100 ml TBE, heat it in the microwave (± 2 Minutes) until it was completely dissolved and the solution

looks clear. Ethidium Bromid was added for 5 ul. The agarose solution was poured on a plate that has been installed with a comb to form a well above the agarose gel (± 30 minutes) until it hardens and dries, then the comb was carefully removed from the mold.

d. Electrophoresis

The process of running DNA was using the mupid electrophorator. The preparation process was inserting a marker into the agarose wells in the first sequence for 2.5 ul, and each DNA sample in the next well for 7 ul. After all agarose wells were filled, close the electrophoresis chamber, the electode was connected to the power supply with a voltage of 100 V (Volt) for (± 30 minutes). Then observed the bands under the UV transiluminator light.

RESULTS AND DISCUSSION

The population of Dodolo village are 378 and 296 of them were checked. There were 70 samples that examined from 7 different places in Dodolo. Determination of sample based on the prevalence in 2015 from the first examination there were 10 people, and the second examination were 12 people. Based on the formula that used to determine the prevalence, a minimum limit of the number of samples was obtained as many as 30 samples. Samples were taken based on the main focus in the village of Dodolo. In Dodolo village there were 7 main focus and 10 samples will be taken randomly at each main focus. So the sample size that will be used were 70 samples, because it had exceeded the minimum sample limit.

The detection by kato-katz method showed that there were 19 samples out of 296 samples, that infected by *S. japonicum* and also showed a double infection of *S. japonicum* - *Ascaris lumbricoides*, and *S.japonicum* - Hookworm. The result of PCR showed that there were 40 out of 70 samples that infected by the *S. japonicum* which was characterized by appearance of230 bp band as shown in Fig.1.
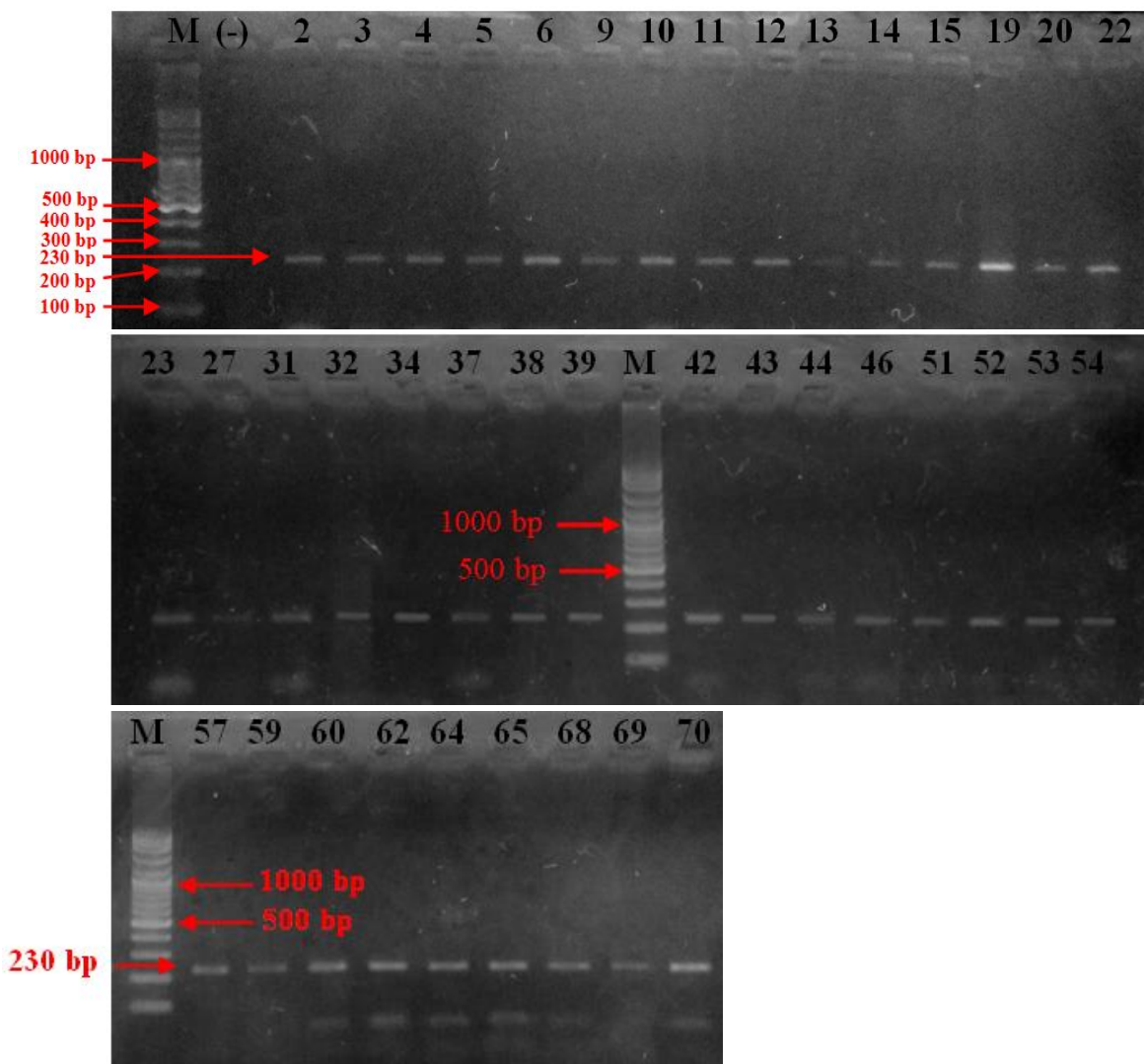

Figure 1. Electrophoresis of PCR Products with Primary Target of SjR2

There were 40 out of 70 samples that infected by the *Schistosoma japonicum* in agarose gel that had been electrophoresed by looking under UV Transiluminator.

According to Figure 1, showed that the first agarose well (17 agarose wells) was the marker (M) of band sequences. The second well was a negative (-) control without the bands in 230 bp, the result of being infected by *Schistosoma japonicum* was marked by a band that appears in the target band of 230 bp.

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Table 1. Measurements of feces by Kato-Katz and PCR (Polymerase Chain Reaction)

No.	Kato-Katz (Average of eggs/gram) Negative	Kato-Katz (Average of eggs/gram) Positive	PCR Negative	PCR Positive	No.	Kato-Katz (Average of eggs/gram) Negative	Kato-Katz (Average of eggs/gram) Positive	PCR Negative	PCR Positive
1.	-		-		36.	-		-	
2.	-			✓	37.	-			✓
3.	-			✓	38.		20 EPG		✓
4.	-			✓	39.		67 EPG		✓
5.	-			✓	40.	-		-	
6.		13 EPG		✓	41.	-		-	
7.	-		-		42.		140 EPG		✓
8.	-		-		43.		73 EPG		✓
9.	-			✓	44.	-			✓
10.		140 EPG		✓	45.	-		-	
11.		27 EPG		✓	46.	-			✓
12.		33 EPG		✓	47.	-		-	
13.	-			✓	48.	-		-	
14.	-			✓	49.	-		-	
15.	-			✓	50.	-		-	
16.	-		-		51.	-			✓
17.	-		-		52.		47 EPG		✓
18.	-		-		53.	-			✓
19.		180 EPG		✓	54.	-			✓
20.		20 EPG		✓	55.	-		-	
21.	-		-		56.	-		-	
22.		127 EPG		✓	57.		93 EPG		✓
23.	-			✓	58.	-		-	
24.	-		-		59.	-		-	✓
25.	-		-		60.		153 EPG		✓
26.	-		-		61.	-		-	
27.	-			✓	62.		127 EPG		✓
28.	-				63.	-		-	
29.	-		-		64.		167 EPG		✓
30.	-		-		65.		127 EPG		✓
31.	-			✓	66.	-		-	
32.	-			✓	67.	-		-	
33.	-				68.	-			✓
34.		40 EPG		✓	69.	-			✓
35.	-		-		70.		280 EPG		✓

In Table 1 showed that the detection of Kato-katz and PCR. The data can be used as a standard that the less thick band has a small number of eggs. There were seven main focus (focus point) which are the habitat of snails. The place were different, some are watery, moist, grassy, and planted with cacao. The snails were not revealing its body in the moist soil, but it adhere to the grassy soil.

There were 19 out of 296 samples that infected by *S. japonicum* based on Kato-Katz detection method, ufter observed on eggs under microscope. The results of the kato-katz method also showed that there were double infection of worm, *S.japonicum* and STH (Soil transmitted helminth). STH was caused by several different species including *Ascaris lumbricoides*, whipworm (*Trichuris trichura*), and hookworm.

STH infection is one of the most widespread infections in the world, especially for people that living far away from the clean water sources, poor environmental sanitation, and lack of knowledge about healthy and clean living. Infection occurs through the eggs in human feces that contaminate the soil with poor sanitation (Nurwidayati *et al.*, 2015).

The results by the kato-katz method showed that a double infection between *Schistosoma japonicum-Ascaris lumbricoides*, and *Schistosoma japonicum*-Hookworm. These results were in line to data by Nurwidayati *et al.*, (2015), stated that *Ascaris lumbricoides* and Hookworm infections in those area were still high, above 2%. This was probably due to the fact that local residents in Dodolo village were majority as farmers, who work in the fields. The high STH infection also showed that the level of sanitation was not good enough for human living.

According to PCR method, showed that there were 40 out of 70 samples re-infected by the *S. japonicum*. The PCR method using SjR2 as the main target of DNA sequences, using the primary order (fordward 5'-TCT AAT GCT ATT GGT TTG AGT-3 ') and (reverse 5'-TTC CTT ATT TTC ACA AGG TGA-3') in the target band of 230 bp.

The electrophoresis data showed that there was band that look less thick in the well number 13 (Fig.1) compared with the other bands. This was because the result of microscop detection was negative, then a PCR detection was performed and the result was positive although the band on electrophoresis was less thick, due to the absence of the eggs in microscope. Furthermore, it might be caused by the amount of the eggs were few in feces (<5 eggs per gram of feces).

The bands that look thick indicated the high number of eggs in feces, and the other way around. In this study, the PCR method proved that the lowest number of eggs was 13 EPGs, showed a band that looked less thick, when compared to the high number of eggs, 280 EPGs.

In the electrophoresis, the volume of PCR products that inserted into the agarose well for each sample were 7 µl, and markers were 2,5 µl. The electrophoresis results showed that there were bands that looked less thick, so the electrophoresis was re-done by increasing the sample volume of 12 µl, to got more clear and thick bands, but not all showed the bands that look thick and clear. This was due to the the slightly DNA concentrations for the *S. japonicum*.

The PCR method showed that there were 40 people that infected with the *Schistosoma japonicum*, which was marked by bands that appeared at 230 bp. The results were higher it compared to Kato-Katz method. The high infection of *S. japonicum* were may be caused by the rice fields in Dodolo village, in which the focus point of the snail as an intermediary agent of the *S japonicum*. Therefore the farmers can be easily infected by schistosomiasis during cultivating activity without using protective equipments. That reason is in line to report of Hu *et al.*, (2004), stated that people infected with schistosomiasis in China were the people who lived near by the lake Poyang and more frequently exposed to the lake.

In Dodolo village, there were large areas that become transmigration locations, so that there more lands were used to be rice fields and plantation area by the migrants, which were the focus point of the intermediate snail.. This was supported by the report of Watts *et al.*, (2005), stated that the schistosomiasis in endemic areas were easily transmitted to migrants because they were less immunity to the disease.

The sensitivity of PCR method was 100%. This study was in line to Potens *et al.*, (2003), showed that 5 out of 12 samples were negative by Kato-Katz method. Then with the PCR method, 12 samples were positive. The sensitivity of PCR method was 96.7%. These results indicate that setection by PCR is a good alternative in diagnosing the infection of *S. Japonicum*. Another method that can be used to detect schistosomiasis infection was Nested-PCR. The Nested-PCR method is a DNA application technique that uses 2 primers. Guo *et al.*, (2012) showed that a high level of sensitivity and specificity by Nested-PCR but requires a long time, when compared with Quantitative-PCR.

Loop-Mediated Isothermal Amplification (LAMP) is the development of the PCR method, which is a DNA amplification process that uses DNA coloring or fluorescent tracers (Sudjadi, 2008). Schistosomiasis is better diagnosed molecularly using the PCR and LAMP methods, because of their high level of sensitivity. The LAMP process does not require a long time like Quantitative-PCR (Nurwidayati *et al.*, 2015). Wang *et al.*, (2011) reported that LAMP can detect plasmids at concentrations of 10-4ng, whereas PCR can detect plasmids in higher concentrations of 10-2 ng. LAMP was more sensitive in detecting pathogens, so it is more suitable for initial screening of schistosomiasis or in endemic areas with lower cases. However,

LAMP is less suitable when it used to detect schistosomiasis after treatment programs, because the high sensitivity, all samples will be detected positively infected even though the absence of the eggs (Wang *et al.*, 2011).

In this study the measurement of DNA concentration was done after the PCR product had been electrophoresed. The DNA concentrations of samples that positive were measured and showed that the DNA concentration of sample number 13 with a less thick band was 15 ng/μg. The highest DNA concentration was sample number 19 (357 ng/μg). Technique or methods with higher sensitivity and specificity are important to supporting the schistosomiasis control and preventive program. According to PCR method showed that several advantages, including to detect helminthiasis with a low number of parasetemia.

CONCLUSION

Based on the results of the molecular detection by PCR method, there were 40 out of 70 amples that infected by *Schistosoma japonicum*. The results of PCR method were higher compared to the Kato-Katz method. This proves that the PCR method was more sensitive for detecting *S. japonicum*

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