**Parasite-derived circulating microRNAs as biomarkers for the detection of human *Schistosoma* *japonicum* infection: lessons learned from a low-intensity infection setting in the Philippines**

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**Abstract**

Schistosomiasis remains a major public health problem afflicting more than 200 million people worldwide. Novel tools for early diagnosis and monitoring of schistosomiasis are urgently needed. This study aimed to validate parasite-derived miRNAs as potential novel biomarkers for the detection of *Schistosoma japonicum* infection. A total of 21 miRNAs were initially validated by RT-PCR using serum samples of *S. japonicum*-infected BALB/c mice as a result of which 12 miRNAs were further tested with a small number of clinical samples. Of these, 6 miRNAs were further validated with a human cohort of individuals from a schistosomiasis-endemic area of the Philippines; these included fifty-three Kato-Katz (KK) positives and twenty-five KK and SjSAP4 + Sj23-LHD-ELISA negatives. RT-PCR analysis showed that two parasite-derived miRNAs (sja-miR-2b-5p and sja-miR-2c-5p) could detect infected individuals with low infection intensity with moderate sensitivity/specificity values of 66%/68% and 55%/80%, respectively. Analysis of the combined data for the two parasite miRNAs revealed a specificity of 77.4% and a sensitivity of 60.0%; however, a duplex RT-PCR targeting both sja-miR-2b-5p and sja-miR-2c-5p did not result in an increased diagnostic performance compared with the singleplex assays. Furthermore, the serum level of sja-miR-2c-5p correlated significantly with faecal egg counts, whereas the other five miRNAs did not. In summary, targeting *S. japonicum*-derived miRNAs in serum resulted in a moderate diagnostic performance when applied to [a low schistosome infection intensity](https://www.researchgate.net/publication/331503574_Comparison_of_Kato_Katz_antibody-based_ELISA_and_droplet_digital_PCR_diagnosis_of_schistosomiasis_japonica_Lessons_learnt_from_a_setting_of_low_infection_intensity) setting. Consequently, assay optimization, such as increasing the starting serum volume for RNA extraction and using locked nucleic acid (LNA) primers, will be required to improve overall diagnostic accuracy using this approach.

**Keywords:**

Schistosomiasis; *Schistosoma japonicum*; biomarker; circulating miRNAs; diagnosis; Philippines

**1. Introduction**

Schistosomiasis, a debilitating, often fatal, disease, caused by trematode blood fluke parasites of the genus *Schistosoma,* afflicts over 230 million people in 78 countries [1]. Three species of schistosomes, *Schistosoma mansoni*, *S.* *haematobium* and *S. japonicum*,arethe most clinically relevant. The zoonotic *S. japonicum* is currently endemic in P.R. China, the Philippines with small foci occurring in Indonesia [2]. In the Philippines, *S. japonicum* is prevalent in 28 provinces in 12 regions of the country, with an estimated 28 million individuals at risk of infection [3]. Currently, schistosomiasis control relies predominantly on mass praziquantel (PZQ) drug administration (MDA) programs [3]. However, MDA on its own is insufficient to provide long term sustainable control of the disease if no additional integrated interventions are implemented [4, 5]. Indeed, a rebound in schistosome infection prevalence and morbidity several years after cessation of MDA has already been reported in several endemic areas [6, 7]. Accurate diagnostic tools are required in the context of integrated schistosomiasis control programs in the Philippines and other endemic areas.

Currently, three major types of diagnostic methods are available for schistosomiasis: parasitological detection (e.g. the Kato-Katz (KK) test and urine filtration); serology, including antibody-detection (AbD) and antigen-detection (AgD); and molecular methods (e.g., circulating nucleic acids detection) [8, 9]). The different methods have different advantages and disadvantages. For example, the traditional KK parasitological technique shows high specificity but an insufficient level of sensitivity, particularly in areas with reduced disease burden [9-12]. AbD-based methods are usually cost-effective and have considerable accuracy yet they have limited ability to discriminate past from active infections. Compared with Ab-based detection assays, AgD-based methods, in the format of lateral flow assays targeting urine samples, provide a rapid and non-invasive diagnostic, but suffer from limited sensitivity in low endemic settings, a relatively high false positive rate and high cost. Molecular techniques, notablypolymerase chain reaction (PCR)\_based-methods [10, 13, 14]), exhibit high accuracy for the detection of schistosome infections; however; their current high cost presents an obstacle for their application in widespread surveillance screening campaigns and in resource-limited endemic areas.

MicroRNAs (miRNAs) are small non-coding RNAs (~22 nt), which are dysregulated in a wide array of biological processes including carcinogenesis [15, 16]. MiRNAs can be stably detected in a wide range of body fluids, including plasma, serum and urine [17] Three mechanisms contribute to the high stability of circulating miRNAs in bio-fluids. Firstly, they are complexed with high-density lipo-proteins or Argonaute proteins [18, 19]. Secondly, they can be incorporated into microvesical or exosomes [20]. Thirdly, they are small in size and RNase-resistant. As potential targets for novel diagnosis [21], circulating and/or extracellular vesicle (EV)-derived miRNA signatures have been tested as biomarkers for different types of diseases and disorders including cancers, infectious and inflammatory diseases [22-29].

Circulating miRNAs have been proposed as having potential to detect parasitic helminth infections [30-34]. To date, a number of parasite-derived miRNAs in plasma/serum have been validated for the purpose of schistosomiasis diagnosis [27, 31]. However, these investigations focused on the diagnosis of *S. mansoni* and *S. haematobium* infection by testing a limited number of patient samples; currently there are no data on the potential of detecting circulating miRNAs in individuals infected with *S. japonicum.* Recent advances in characterizing miRNA profiles in extracellular vesicles secreted by *Schistosoma* species [35-38] have raised the possibility for validating more parasite-derived miRNAs as potentially novel biomarkers for schistosomiasis detection.

In this study, we evaluated the potential of detecting circulating parasite-derived miRNAs in *S. japonicum* infected human subjects. Initially we employed the BALB/cmouse as a schistosomiasis model to validate 21 parasite-derived miRNA candidates in serum during *S. japonicum* infection. Then, following another step of screening, six candidate miRNAs were selected for further validation, individually or in combination, using human sera from a cohort of residents in an area in rural Philippines endemic for schistosomiasis japonica. We presented the diagnostic performance of parasite-derived miRNA signatures in a *S. japonicum*-endemic setting with a low-intensity infection.

**2. Results**

*2.1. Detection of parasite-derived miRNAs in the serum of BALB/c mice at 9 weeks post-S. japonicum infection*

Twenty-one miRNAs were selected to assess their potential for detection of *S. japonicum* infection based on prior published studies of schistosome circulating and extracellular vesicles/exosomes associated miRNAs (Supplementary Table S1). The expression of these 21 miRNAs was tested in naïve and *S. japonicum*-infected (9 weeks post-infection) BALB/cmice by RT-PCR (Figure 1). A total of 12 miRNAs (sja-miR-277, sja-miR-3479-3p, sja-miR-125a sja-miR-61, sja-miR-2b-5p, sja-miR-2162-3p, sja-miR-36-3p, sja-miR-3489, sja-miR-3487, sja-miR-2c-5p, sja-miR-2a-3p and sja-miR-10) were selected for further investigation based on a fold change cut-off value ≥ 4 and a *p* value cut-off < 0.05.

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**Figure 1.** The expression levels of 21 miRNAs in the sera of naïve and infected (9 wks p.i.) BALB/cmice determined using qRT-PCR (Control, n = 3; 9 wks p.i., n = 3). *P* values were calculated using the unpaired student’s *t*-test (\*, *p*<0.05; \*\*; *p*<0.01; \*\*\*, *p*<0.001).

*2.2. Initial screening of 12 miRNAs for the diagnosis of human S.* *japonicum* *infection*

In the next stage of screening qRT-PCR was used to determine the expression levels of the 12 *miRNAs* selected in serum samples from KK-positive (KK (+)) patients (n = 5) and control individuals (KK and SjSAP4 + Sj23-LHD-ELISA negative [39]) (n = 5) (Supplementary Figure S1). Receiver operating characteristic (ROC) curve analysis was performed and the area under the curve (AUC) levels were calculated to evaluate the diagnostic potential of each miRNA (Supplementary figure S1). As a result, six miRNAs (sja-miRNA-277, sja-miR-125a, sja-miR-2b-5p, sja-miR-36-3p, sja-miR-2c-5p and sja-miR-2a-3p) with an AUC value > 0.80 were selected for further validation.

*2.3. The potential value of serum levels of six miRNAs by singleplex qRT-PCR for the diagnosis of human schistosomiasis*

The expression levels of sja-miRNA-277, sja-miR-125a, sja-miR-2b-5p, sja-miR-36-3p, sja-miR-2c-5p and sja-miR-2a-3p were further probed using sera from a human cohort of low-intensity infected individuals from a schistosomiasis-endemic area, Northern Samar, the Philippines (Table 1) by qRT-PCR. The cohort included fifty-three KK (+) individuals and twenty-five KK and SjSAP4 + Sj23-LHD-ELISA negatives as controls. The levels of two miRNAs, sja-miR-2b-5p, and sja-miR-2c-5p, were significantly higher in patients than in control individuals (*p* = 0.0251 and *p* = 0.0114, respectively), while the serum abundance of the other four miRNAs, sja-miRNA-277, sja-miR-125a, sja-miR-36-3p and sja-miR-2a-3p failed to differentiate the two groups (*p* < 0.05) (Figure 2a). Using optimal cut-off points, sja-miR-2b-5p and sja-miR-2c-5p could detect *S. japonicum* infected individuals with a specificity/sensitivity of 66.0%/68.0% and 54.7%/80.0%, respectively (Figure 2a). The ROC curve analysis for the six individual miRNAs in discriminating the KK (+) from the controls showed AUC values of 0.6340, 0.6279, 0.6574, 0.5906, 0.6770 and 0.5804 for sja-miRNA-277, sja-miR-125a, sja-miR-2b-5p, sja-miR-36-3p, sja-miR-2c-5p and sja-miR-2a-3p, respectively (*p* = 0.0574, 0.0696, 0.0256, 0.1989, 0.0121 and 0.02542, respectively) (Figure 2b).

Table 1. Basic information of study participants in the control and KK (+) groups

|  |  |  |
| --- | --- | --- |
| Group | Control (25) | KK (+) (53) |
| M / F | 9 / 16 | 45 / 8 |
| Age (years) | 39.4 ± 14.1 | 40.7 ± 16.0 |
| Kato-Katz |  |  |
| EPG | 0 | (3.3-746.7) |
| Light (1-99) | 0 | 41 |
| Moderate (100-399) | 0 | 8 |
| Heavy (>400) | 0 | 4 |
| Serological test (+ / -)\* | 0 / 25 | 50 / 3 |

\*ELISA assay detecting IgG antibodies against a combination of recombinant *S. japonicum* proteins (rSjSAP4 and rSj23-LHD) [39].

EPG: Eggs per gram of faeces.

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**Figure 2.** Discrimination of *S. japonicum*-infected and non-infected individuals by detection of parasite-derived miRNAs in serum. (a) The serum levels of the six candidate miRNAs in the KK (+) and control subjects. Boxes represent the interquartile range of the data with lines across the boxes indicate the median values. The hash marks below and above the boxes indicate the 10th and 90th percentiles for each group, respectively. (b) ROC curve analysis for the six candidate miRNAs was performed to evaluate the capabilities in differentiating the KK (+) and control participants.

ROC curve analysis was performed to evaluate the ability of combinations of the miRNAs to distinguish the KK (+) from the control participants (Table 2). Using the combined data for six miRNAs, the combination of sja-miR-2b-5p and sja-miR-2c-5p was best able to differentiate between the two groups with an AUC value of 0.6906 (95% CI 0.5645-0.8166; *p* = 0.0069; sensitivity 77.4%, specificity 60.0%;), followed by the combination of sja-miR-125a, sja-miR-2b-5p and sja-miR-2c-5p (AUC 0.6792; 95% CI 0.5541-0.8044; *p* = 0.0110; sensitivity 55.8%, specificity 80.0%).

Table 2. Discrimination of *S.* ***japonicum*** infected individuals from controls using serum levels of combined miRNAs

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| miRNA combination# | AUC  (95% CI) | *p* | Sens.  (%) | Spec.  (%) |
| sja-miR-277 + sja-miR-125a | 0.6257 (0.4961-0.7552) | 0.0747 | 62.3 | 64.0 |
| sja-miR-277 + sja-miR-2b-5p | 0.6460 (0.5167-0.7754) | 0.0383 | 69.8 | 64.0 |
| sja-miR-277 + sja-miR-2c-5p | 0.6657 (0.5415-0.7898) | 0.0188 | 49.1 | 84.0 |
| sja-miR-125a + sja-miR-2b-5p | 0.6400 (0.5111-0.7689) | 0.0471 | 43.4 | 88.0 |
| sja-miR-125a + sja-miR-2c-5p | 0.6642 (0.5375-0.7908) | 0.0199 | 52.8 | 80.0 |
| sja-miR-2b-5p + sja-miR-2c-5p | 0.6906 (0.5645-0.8166) | 0.0069 | 77.4 | 60.0 |
| sja-miR-277 + sja-miR-125a + sja-miR-2b-5p | 0.6430 (0.5147-0.7714) | 0.0425 | 41.5 | 88.0 |
| sja-miR-277 + sja-miR-125a + sja-miR-2c-5p | 0.6619 (0.5359-0.7879) | 0.0217 | 54.7 | 76.0 |
| sja-miR-277 + sja-miR-2b-5p + sja-miR-2c-5p | 0.6687 (0.5422-0.7952) | 0.0168 | 73.6 | 60.0 |
| sja-miR-125a + sja-miR-2b-5p + sja-miR-2c-5p | 0.6792 (0.5541-0.8044) | 0.0110 | 55.8 | 80.0 |
| sja-miR-277 + sja-miR-125a + sja-miR-2b-5p + sja-miR-2c-5p | 0.6672 (0.5409-0.7935) | 0.0178 | 49.1 | 84.0 |
| sja-miR-277 + sja-miR-125a + sja-miR-36-3p + sja-miR-2b-5p + sja-miR-2c-5p | 0.6483 (0.5195-0.7771) | 0.0354 | 69.8 | 60.0 |
| sja-miR-277 + sja-miR-125a + sja-miR-36-3p + sja-miR-2b-5p + sja-miR-2a-3p | 0.6121 (0.4817-0.7425) | 0.1119 | 64.2 | 60.0 |
| sja-miR-277 + sja-miR-125a + sja-miR-36-3p + sja-miR-2c-5p + sja-miR-2a-3p | 0.6468 (0.5205-0.7730) | 0.0374 | 62.3 | 72.0 |
| sja-miR-277 + sja-miR-125a + sja-miR-2b-5p + sja-miR-2c-5p + sja-miR-2a-3p | 0.6211 (0.4930-0.7492) | 0.0858 | 37.7 | 88.0 |
| sja-miR-277 + sja-miR-36-3p + sja-miR-2b-5p + sja-miR-2c-5p + sja-miR-2a-3p | 0.6294 (0.5017-0.7572) | 0.0664 | 37.7 | 88.0 |
| sja-miR-125a + sja-miR-36-3p + sja-miR-2b-5p + sja-miR-2c-5p + sja-miR-2a-3p | 0.6294 (0.5013-0.7576) | 0.0664 | 37.7 | 88.0 |
| sja-miR-277 + sja-miR-125a + sja-miR-36-3p +  sja-miR-2b-5p + sja-miR-2c-5p + sja-miR-2a-3p | 0.6264 (0.4981-0.7547) | 0.0730 | 39.6 | 88.0 |

#Sja-miR-36-3p and sja-miR-2a-3p were excluded from analysis for the combinations of 2, 3, and 4 miRNAs.

CI, confidence interval

*2.4. The diagnostic performance of serum miRNA levels determined by duplex* *and multiplex* *qRT-PCR assays for human schistosomiasis*

The serum miRNA levels were also probed using a duplex (designated as 2P, targeting sja-miR-2b-5p and sja-miR-2c-5p) and three multiplex qRT-PCR assays with the same cohort. The multiplex qRT-PCR assays were designated as 3P (targeting sja-miR-277, sja-miR-2b-5p, and sja-miR-2c-5p), 5P (targeting sja-miR-125a, sja-miR-2b-5p, sja-miR-36-3p, sja-miR-2c-5p and sja-miR-2a-3p) and 6P (targeting sja-miRNA-277, sja-miR-125a, sja-miR-2b-5p, sja-miR-36-3p, sja-miR-2c-5p and sja-miR-2a-3p). In the 2P and 5P assays, the serum levels of targeted miRNAs were significantly higher in the KK (+) than control individuals (*p* = 0.0491 and *p* = 0.0202, respectively), while no significant difference was observed between the two groups in the 3P and 6P assays (Figure 3a). The ROC curve analysis for discriminating the KK (+) from the controls yielded AUC values of 0.6385, 0.6302, 0.6630, and 0.6185, for the 2P, 3P, 5P and 6P assays, respectively (*p* = 0.0495, 0.0648, 0.0208 and 0.0928, respectively) (Figure 3b).

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**Figure 3.** The diagnostic performance for detecting human schistosomiasis japonica using serum parasite-derived miRNAs quantified by duplex and multiplex qRT-PCR assays. (a) The serum levels of parasite-derived miRNA combinations in the control and KK (+) individuals. Boxes represent the interquartile range of the data with lines across the boxes indicating the median values. The hash marks below and above the boxes indicate the 10th and 90th percentiles for each group, respectively. (b) ROC curve analysis was performed for the levels of different miRNA combinations determined by the duplex and multiplex qRT-PCR assays to evaluate the capabilities in discriminating the KK (+) from the control individuals.

*2.5. Correlations of the serum miRNA levels with egg burden in the KK (+) individuals*

The associations between the levels of the six miRNA signatures (individually or in combination) in serum and egg burden were then investigated in the KK (+) group. The serum level of miRNA-2c-5p correlated with EPG (*r* = 0.3222, *p* = 0.0186), whereas the serum levels of the other 5 miRNAs did not show a significant correlation with infection intensity determined by the KK method (Figure 4). Also, no significant correlation was observed between the serum miRNA levels determined by the duplex (2P) or multiplex assays (3P, 5P and 6P) and faecal egg burden(Supplementary Figure S2).

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**Figure 4.** Correlations between the serum abundance of six miRNAs and faecal egg burden (EPG) in the KK (+) individuals using Pearson’s correlation coefficient.

**3. Discussion**

Accurate diagnosis of schistosomiasis, especially in low intensity areas following MDA and other control programs, remains a great challenge. Nevertheless, the development and deployment of novel diagnostic tools, with the requisite accuracy, for the purpose of monitoring control efforts in endemic areas to ensure schistosomiasis elimination will be critical [9, 12, 39, 40]. The realization that detection of parasite-derived miRNAs in the host circulatory system during an infection is possible has generated much interest in their application as diagnostic indicators [41, 42]. The utility of using circulating miRNAs as biomarkers for the detection of schistosome infections has been shown in several recent pioneering investigations using animal models of schistosomiasis and/or with clinical samples [27, 31, 32, 43]. However, there had been no reports hitherto of their use in the clinical diagnosis of schistosomiasisjaponica.

Of the 12 initially selected miRNAs, based on results obtained in the the animal model of schistosomiasis japonica, the majority were unable to discriminate infected from uninfected individuals in a clinical cohort (Supplementary Figure S1 and Figure 2), although an increased volume of human serum samples was used for RNA extraction and a more sensitive assay, the S-Poly(T) method was used the reverse transcription of human samples [44]. This may have been due to the fact that: 1) the severity of a schistosome infection is far more pronounced in the experimental murine model of schistosomiasis than is found in subjects who are KK (+) in that even a single worm pair in a mouse represents a high infection burden when body weight is taken into consideration; and 2) *S. japonicum* adult worm pairs digest a considerable number of erythrocytes daily in order to obtain essential amino acids [45], and in so doing this results in the release of a high concentration of small RNA signatures of host origin, which may readily cause non-specific amplification in the samples obtained from individuals with a high burden of infection, as was the case with the BALB/c mouse model utilised here.

Of the six miRNAs tested, any individual miRNA provided only moderate diagnostic power for differentiating the KK (+) and control participants (AUC from 0.5804 to 0.6770); slightly higher to the most powerful singleplex test targeting sja-miR-2c-5p (AUC: 0.6770, *p* = 0.0121) (Figure 1), the best diagnostic performance was obtained with a combination of sja-miR-2b-5p and sja-miR-2c-5p (AUC: 0.6906, *p* = 0.0069), showing a sensitivity of 76% and specificity of 60% (Table 2). In order to improve the diagnostic potential by amplification two or multiple miRNAs simultaneously, two duplex/multiplex qRT-PCT assays were developed. The duplex assay 2P targeting the two most powerful miRNA signatures (sja-miR-2b-5p and sja-miR-2c-5p) only marginally discriminated the control and KK (+) individuals with an AUC of 0.6385 (*p* = 0.0495) and sensitivity/specificity values of 66.0%/60.0%. The multiplex assay 3P failed to differentiate the control and KK (+) subjects (AUC = 0.6302, *p* = 0.0648), while the accumulative data based on singleplex assay targeting the same miRNAs exhibited moderate diagnostic power with an AUC of 0.6687 (*p* = 0.0168). Furthermore, both the multiplex assay 6P and the combined data based on a singleplex assay targeting all six miRNAs failed to show any discrimination ability in the diagnosis of clinical *S. japonicum* infections (Figure 3 and Table 2). However, the multiplex assay 5P exhibited a superior diagnostic power than that by combined data targeting the same miRNAs (AUC 0.6630, *p* = 0.0208 vs 0.6294, *p* = 0.0664) (Figure 3 and Table 2). Nevertheless, the diagnostic power of the 5P assay was inferior to that of the singleplex assay detecting sja-miR-2c-5p. The failure of the duplex and multiplex assays to increase the diagnostic power may due to: 1) The data obtained with the duplex/multiplex assays still mainly depend on a highly expressed signature(s) within the target miRNAs; 2) a relatively higher noise background may be introduce by targeting two or multiple targets, especially when the samples from a low-intensity infection setting were tested.

Overall, the diagnostic performance of the assays (singleplex, duplex, and multiplex) developed in the current study for detecting *S. japonicum* miRNAs in serum, was moderate, but is consistent with the results obtained by Meningher *et al.* [27] when detecting *S. mansoni*, *S. haematobium* and *S. mekongi* infections in twenty-six returning travelers with schistosomiasis (based on the detection of eggs or the positive results of serologic tests) returning from either sub-Saharan Africa or Laos by amplification of miRNAs extracted from serum [27]. Furthermore, it has been reported that parasite miRNAs are not present in plasma at a sufficiently high level to be used as a biomarker for *Onchocerca volvulus* infection or for monitoring treatment using miRCURY Locked Nucleic Acid (LNA) primer-based RT-qPCR [46]. The modest AUC values we obtained in efforts to diagnose schistosomal infections in the human Philippines cohort may be attributable to the following factors: 1) Most of the KK (+) individuals tested harbored light schistosome infections (Table 1), a feature which itself poses a challenge for any of the currently available diagnostic tools for schistosomiasis; 2) We have previously shown that the targeted cohort is extensively co-parasitised with intestinal worms and intestinal protozoa [4, 47, 48]. These pathogens are likely to secrete RNA signatures with sequence similarity to the miRNAs detected here, thereby affecting the specificity of the assays we employed.; 3) The limited cohort sample number may also have impaired our e ability to measure elevated diagnostic scores. Nevertheless, the diagnostic power of these assay may be improved by including additional optimization steps such as increasing the initial volume of serum to 2 ml, as we recently used for successfully extracting cell-free DNA for the detection of *S. japonicum* infection by ddPCR assays [10], and by using LNA primer-based qRT-PCR for quantification [49].

Differential abundance of miRNAs has been observed in the EV contents from *S. mansoni* and *S. japonicum*. Sma-miR-125b, sma-bantam and sma-miR-71a were the most abundant miRNAs in the content of exosome-like vesicles derived from *S. mansoni* [36]. Sja-miR-36-3p, sja-miR-10-3p and sja-bantam were the top three miRNAs isolated from schistosomal egg EVs [38], while sja-miR-10-5p sja-miR-125b and sja-miR-61 were identified as the top three miRNAs associated with EVs from adult *S. japonicum* [35]. These observations indicate that the abundance of individual miRNAs in EV contents is not only species dependent, but is also developmental stage dependent. The two most powerful serum-based signatures identified here, sja-miR-2b-5p and sja-miR-2c-5p, were listed as the top fourth and fourteenth miRNAs associated with *S. japonicum* adult EVs [35]. These observations thus lend support that serum and serum-exosomal miRNAomes are significantly different in terms of miRNA numbers, types and expression profiles [50]. Although accumulating evidence indicates that extracellular miRNAs are mainly found bound to AGO proteins [51], an active sorting mechanism of exosomal miRNA may enrich specific miRNA members in extracellular vesicles/exosomes [52-55]. Further detection of EV-derived miRNAs represents another direction for the diagnosis of human *S. japonicum* infection.

Previously, we showed that the serum levels of two parasite-derived miRNAs, sja-miR-277 and sja-miR-3479-3p, exhibited a strong correlation with hepatic egg burden (*p* < 0.0001) during the course of *S. japonicum* infection in C57BL/6 and BALB/c mice [32]. In the current study, only the serum level of sja-miR-2c-5p weakly correlated with infection intensity based on the KK test, indicating that the infectious status or disease progression in schistosomiasis patients may be complicated. Also, as only a small quantity of feces was used in the KK test, the accuracy of the technique may be affected by the uneven distribution of eggs in the fecal samples and the daily fluctuation in the number of eggs discharged; additionally, most of the tested KK (+) individuals in the cohort harbored light infections. As stable markers in serum, the abundance of parasite-derived miRNAs may not only be associated with infection intensity but also with the infection phase. The miRNA concentration may accumulate over the infection course, although a metabolic balance may be achieved at the later stages of infection. Furthermore, as the target cohort was located in a medium-high prevalence schistosomiasis-endemic area, reinfection with *S. japonicum* was considered to be a frequent occurrence. Accordingly, the abundance of parasite-derived miRNAs in the sera of re-infected individuals has less chance to show a significant linear relationship with infection intensity.

**4. Materials and Methods**

*4.1. Ethics Statement*

Animal experiments were carried out according to the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition) and with the approval of the Animal Ethics Committee, QIMR Berghofer Medical Research Institute (QIMRB), Brisbane, Australia (Ethics Approval: Project P288). The human cohort study was approved by the Institutional Review Board of the Research Institute for Tropical Medicine (RITM), Department of Health, Manila, the Philippines (Approval No: 2012-13-0) and the Human Ethics Committee, QIMRB (Ethics Approval: Project P524), in accordance with the Declaration of Helsinki. When conducting the human study, written informed consent was obtained from all participants (for those aged less than 15 years, written informed consent was obtained from their legal guardians).

*4.2. Parasites*

*S. japonicum-*infected *Oncomelania hupensis hupensis* snails were purchased from Nanjing Municipal Center for Disease Control and Prevention, China, and transported to QIMRB, Brisbane, Australia. Cercariae were shed from the infected snails under light stimulation.

*4.3. Mouse infection and serum collection*

Three eight-week-old female BALB/c mice were percutaneously infected with a low-dose challenge of *S. japonicum* cercariae (16 ± 2). Mice were sacrificed at 9 weeks p.i. and ~1 ml blood was collected by cardiac puncture. Blood samples were then allowed to stand at room temperature for 2 h. After centrifugation at 3,000 *g* for 10 min, the serum samples were collected and stored at −80°C. Serum samples from three naive mice were used as controls.

*4.4. Study cohort and human sample collection*

The human subjects were recruited from schistosomiasis-endemic areas in Laoang and Palapag, Northern Samar, the Philippines. Additional information on the study population is available in previous reports [4, 56]. For each participant ~10 ml blood was drawn and serum was then collected after centrifugation, and stored at 2-8°C. The serum samples were transported to RITM, and stored at -80°C. Subsequently, a subset of samples was shipped to QIMRB, Australia, on dry ice.

*4.5. Parasitological detection (Kato-Katz)*

Each individual from the study cohort provided a stool specimen from which Kato-Katz thick smear slides were prepared. Slides were examined by experienced laboratory technicians under a light microscope. The burden of infection is presented as the number of eggs per gram of faeces (EPG).

*4.6. RNA extraction, polyadenylation and reverse transcription (RT)*

For each mouse, total RNA was extracted from 100μL serum samples, and for each human subject, total RNA was extracted from 200μL serum samples, using miRNeasy mini kits (Qiagen, Hilden, Germany) according to manufacturer's instructions. During the RNA extraction procedure, 3.2 fmoles *Arabidopsis thaliana* ath-miR-159a (IDT, Coralville, IA) was added to each sample as a spike-in control. The total RNA product was eluted with 30μL nuclease-free water.

A one-step procedure of polyadenylation and RT reaction was performed by the combined use of two kits: a Poly(A) polymerase tailing kit (Epicentre Biotechnologies, Madison, WI) and a TaqMan microRNA reverse transcription kit (Life Technologies, Carlsbad, CA). For mouse samples, the Poly(A) method was used. Briefly, a 10μL RT reaction comprised: 1μL 10 × RT buffer, 1μL ATP (10 mM), 1μl universal RT primer (1 mM), 0.1μL dNTPs (25 mM each), 0.13μL RNase inhibitor, 0.2μL poly(A) polymerase, 0.5μL MultiScribe MuLV and 5μL RNA and 1.07μL nuclease-free water. Reverse transcription (RT) reactions were carried out using a Veriti 96-well thermal cycler (ABI, Foster City, CA) under the following condition: 37°C for 30 min, 42°C for 30 min, and followed by enzyme inactivation at 85°C for 5 min. For human subjects, polyadenylation and RT reactions were performed using the S-Poly(T) method [32]. The reaction system was the same as that for the Poly(A) method except that it incorporated 1μL of miRNA-specific primer pool (25 nM of each primer). RT products were stored at −20°C prior to subsequent analysis. The RT primers are listed in Supplementary Table S2.

*4.7. qRT-PCR for miRNA quantification*

Quantification of the serum levels of miRNAs was performed by probe-based qRT-PCR according essentially to protocols described previously [32, 57]. Briefly, the 5μL PCR reaction contained 2.5μL TaqMan Universal Master Mix II (Life Technologies, Carlsbad, CA), 0.5μL of RT products, 1μL primer mixture (forward and universal reverse primers) (final concentration: 0.2μM), 0.5μL universal double quenched probe (final concentration: 0.25μM) (IDT, Coralville, IA), and 0.5μL nuclease-free water. The assays were performed on an ABI Quantstudio 5 Real-Time PCR System (Thermo Fisher Scientific, city?, country?) with the following cycling condition: pre-denaturation at 95°C for 10 min, followed by 50 cycles: 95°C for 15 sec, and 60°C for 1 min. For analyses, a cutoff Ct value of 40 was set as background for the purpose of calculating signal over noise. The expression levels were determined by the 2−ΔΔCt method with the spiked-in ath-miR-159a used as the normalization control. Three technical replicates were performed for each sample. The primer and probe sequences used are listed in Supplementary Table S2.

*4.8. Statistical analysis*

Unpaired student’s *t*-test (two tails) was used for comparing the serum levels of miRNAs in naïve and *S. japonicum*-infected BALB/c mice. The Mann-Whitney *U*-test was used for analysis of the cability of the serum levels of miRNAs in discriminating the KK (+) group from the control group. The receiver operating characteristic (ROC) curve analyses were performed and the area under the curve (AUC) was calculated to assess the potential of using the parasite-derived circulating miRNAs (individually or in combination) as novel biomarkers for schistosomiasis japonica. Cut-off values for determination of sensitivity and specificity were set by maximizing the Youden's index. Pearson’s correlation coefficient (r) was used for the assessment of the correlation between the serum levels of miRNAs and infection intensity (egg burden) in the KK (+) subjects. Statistical analysis was performed with GraphPad Prism Version 6.01 for windows.

**5. Conclusion**

In summary, we have developed singleplex, duplex and multiplex qRT-PCR assays for the diagnosis of *S. japonicum* infection by targeting parasite-derived serum miRNAs in a clinical cohort from a medium-high prevalence but low intensity schistosomiasis-endemic area. The duplex and multiplex assays did not show superior perfomance in terms of diagnostic power compared with the best performed singleplex assay targeting sja-miR-2c-5p. The best diagnostic performance was achieved using a combinations of sja-miR-2b-5p and sja-miR-2c-5p in accumulative?? form (AUC: 0.6906, *p* = 0.0069). The results here shed light on the diagnostic performance of parasite-derived serum miRNAs for the detection of schistosomiasis japonica by probing a human cohort with low infection burden. This study establishes a basis for developing a supplemental novel diagnosis of schistosomiasis japonica.

**Author Contributions:** Conceived and designed the experiments: Y.M., P.C. and D.P.M. Performed the experiments: Y.M. and P.C. Analyzed the data: Y.M., P.C. and D.P.M. Contributed reagents/materials/analysis tools: P.C., R.M.O., A.G.R., D.U.O. and D.P.M. Wrote the paper: Y.M., P.C. and D.P.M.

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