Parasite-derived circulating microRNAs as biomarkers for the detection of human *Schistosoma japonicum* infection

Running title: Using circulating miRNAs for schistosomiasis diagnosis

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SUMMARY:

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 human *Schistosoma japonicum* infection. A total of 21 miRNAs were initially validated by RT-PCR using serum samples of *S. japonicum*-infected BALB/c mice. Of these, 6 miRNAs were further validated with a human cohort of individuals from a schistosomiasis-endemic area of the Philippines. 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% with an AUC value of 0.6906 (p = 0.0069); 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. Targeting *S. japonicum*-derived miRNAs in serum resulted in a moderate diagnostic performance when applied to a low schistosome infection intensity setting.

Keywords:

Schistosomiasis; Schistosoma japonicum; Biomarker; Circulating miRNAs; Diagnosis; Philippines

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 (Colley *et al.*, 2014). Three species of schistosomes, *Schistosoma mansoni, S. haematobium* and *S. japonicum,* are the most clinically relevant. The zoonotic *S. japonicum* is currently endemic in P.R. China, the Philippines with small foci occurring in Indonesia (Gordon *et al.*, 2019). 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 (Olveda & Gray, 2019). Currently, schistosomiasis control relies predominantly on mass praziquantel (PZQ) drug administration (MDA) programs (Olveda & Gray, 2019). However, MDA on its own is insufficient to provide long term sustainable control of the disease if no additional integrated interventions are implemented (Mutapi *et al.*, 2017; Ross *et al.*, 2015). 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) (Cavalcanti *et al.*, 2013; Weerakoon *et al.*, 2015)). 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 (Cai *et al.*, 2019; Oliveira *et al.*, 2018; Weerakoon *et al.*, 2015; Weerakoon *et al.*, 2017b). 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, notably polymerase chain reaction (PCR)_based-methods (Weerakoon *et al.*, 2017a; Weerakoon *et al.*, 2016; Weerakoon *et al.*, 2017b)), exhibit high accuracy for the detection of schistosome infections, but the current costs remain high.

MicroRNAs (miRNAs) are small non-coding RNAs (~22 nt), which are dysregulated in a wide array of biological processes including carcinogenesis (Anvarnia *et al.*, 2019; Peng & Croce, 2016). As potential targets for novel diagnosis (Li & Kowdley, 2012), 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 (Correia *et al.*, 2017; Filipów & Łaczmański, 2019; Jamali *et al.*, 2018; Jia *et al.*, 2017; Meningher *et al.*, 2017; Schonauen *et al.*, 2018; Tengda *et al.*, 2018; Xue *et al.*, 2019). Circulating miRNAs also have been proposed as having potential to detect parasitic helminth infections (Cai *et al.*, 2015; Dong *et al.*, 2017; Guo & Zheng, 2017; Hoy *et al.*, 2014; Tritten *et*

al., 2014). To date, a number of parasite-derived miRNAs in plasma/serum have been validated for the purpose of schistosomiasis diagnosis (Hoy *et al.*, 2014; Meningher *et al.*, 2017). 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 (Nowacki *et al.*, 2015; Samoil *et al.*, 2018; Zhu *et al.*, 2016a; Zhu *et al.*, 2016b) 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/c mouse 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.

MATERIALS AND METHODS

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.

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.

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.

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 (Olveda *et al.*, 2017; Ross *et al.*, 2015). 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.

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).

RNA extraction, polyadenylation and reverse transcription (RT)

For each mouse, total RNA was extracted from 100 μL serum samples, and for each human subject, total RNA was extracted from 200 μL 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 30 μL 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 10 μL RT reaction comprised: 1 μL 10 × RT buffer, 1 μL ATP (10 mM), 1 μl universal RT primer (1 mM), 0.1 μL dNTPs (25 mM each), 0.13 μL RNase inhibitor, 0.2 μL poly(A) polymerase, 0.5 μL MultiScribe MuLV and 5 μL RNA and 1.07 μL 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 (Cai *et al.*, 2015). The reaction system was the same as that for the Poly(A) method except that it incorporated 1 μL 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 1.

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 (Cai *et al.*, 2015; Cai *et al.*, 2018). Briefly, the 5 μL PCR reaction contained 2.5 μL TaqMan Universal Master Mix II (Life Technologies, Carlsbad, CA), 0.5 μL of RT products, 1 μL primer mixture (forward and universal reverse primers) (final concentration: 0.2 μM), 0.5 μL universal double quenched probe (final concentration: 0.25 μM) (IDT, Coralville, IA), and 0.5 μL nuclease-free water. The assays were performed on an ABI Quantstudio 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) 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 $2^{-\Delta\Delta Ct}$ 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 1.

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 capability 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.

RESULTS

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 2). The expression of these 21 miRNAs was tested in naïve and *S. japonicum*-infected (9 weeks post-infection) BALB/c mice by RT-PCR (Fig 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.

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 (Cai *et al.*, 2017)) (n = 5) (Supplementary Fig 1). 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 (S1 Fig). 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.

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 ($p < 0.05$) (Fig 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 (Fig 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) (Fig 2B).

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%).

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 (Fig 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) (Fig 3B).

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 (Fig 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 Fig 2).

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 (Cai *et al.*, 2017; Oliveira *et al.*, 2018; Utzinger *et al.*, 2015; Weerakoon *et al.*, 2015). 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 (Cai *et al.*, 2016a; Manzano-Roman & Siles-Lucas, 2012). 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 (Cai *et al.*, 2015; Cheng *et al.*, 2013; Hoy *et al.*, 2014; Meningher *et al.*, 2017). However, there had been no reports hitherto of their use in the clinical diagnosis of schistosomiasis japonica.

Of the 12 initially selected miRNAs, based on results obtained in the animal model of schistosomiasis japonica, the majority were unable to discriminate infected from uninfected individuals in a clinical cohort (Fig 2 and Supplementary Fig 1), although an increased volume of serum was used for RNA extraction from clinical samples and miRNAs extracted from human samples are subjected to RT with the more sensitive S-Poly(T) method (Kang *et al.*, 2012). This

may have been due to the facts 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 positive, since 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 (Cai *et al.*, 2016b), 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, $p = 0.0121$) (Fig 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, duplex/multiplex qRT-PCR 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 singleplex assays targeting all six miRNAs failed to show any discrimination ability in the diagnosis of clinical *S. japonicum* infections (Fig 3 and Table 2). However, the multiplex assay 5P exhibited a superior diagnostic power than that obtained by the combination targeting the same miRNAs (AUC 0.6630, $p = 0.0208$ vs 0.6294, $p = 0.0664$) (Fig 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.* (Meningher *et al.*, 2017) 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. 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 (Lagatie *et al.*, 2017). 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 (Gordon *et al.*, 2015; Ross *et al.*, 2015; Weerakoon *et al.*, 2018). 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 ability to measure elevated diagnostic scores.

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 (Zhu *et al.*, 2016a), indicating that serum and serum-exosomal miRNAomes are significantly different in terms of miRNA numbers, types and expression profiles (Zhao *et al.*, 2016). Although accumulating evidence indicates that extracellular miRNAs are mainly found bound to AGO proteins (Lopez & Granados-Lopez, 2017), an active sorting mechanism of exosomal miRNA may enrich specific miRNA members in extracellular vesicles/exosomes (Gon *et al.*, 2017; Janas *et al.*, 2015; Villarroya-Beltri *et al.*, 2013; Zhang *et al.*, 2015). Further detection of parasite-derived miRNAs from serum EV composition 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 (Cai *et al.*, 2015). 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. It is worth noting that the accuracy of the KK test may be affected by the uneven distribution of eggs in the fecal samples and the daily fluctuation in the number of eggs discharged; particular when most of the tested KK (+) individuals in the cohort harbored light infections. Also, 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.

Concluding comments

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 infection burden schistosomiasis-endemic area. The best diagnostic performance was achieved using a combination of sja-miR-2b-5p and sja-miR-2c-5p (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.

ACKNOWLEDGEMENTS

We thank Mary Duke for maintenance of the *S. japonicum* lifecycle at QIMRB. We also appreciate the efforts of the local field and clinical staff in Palapag, Northern Samar for the collection of the clinical samples.

FINANCIAL SUPPORT

This work was funded by the National Health and Medical Research Council (NHMRC) of Australia (ID APP1160046, APP1037304, APP1102926 and APP1098244). D.P.M. is an NHMRC Senior Principal Research Fellow and Senior Scientist at QIMRB. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Figure Legends

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

Fig 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.

Fig 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.

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

Table S1. Primers and probe used in this study.

	Target miRNA	Sequence (5'-3')
Universal RT primer used in the Poly(A) method		CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTTVN
RT primer pool used in the S-Poly(T) method	ath-miR-159a	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTTAGAGCT
	sja-miR-277	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTTACGGG
	sja-miR-3479-3p	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTCAAGGC
	sja-miR-125a	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTGGCAAT
	sja-miR-61	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTGAAGTG
	sja-miR-2b-5p	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTGGCTCA
	sja-miR-2162-3p	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTAGAGTG
	sja-miR-36-3p	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTGCGAAT
	sja-miR-3489	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTCGTCCT
	sja-miR-3487	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTTGGCCA
	sja-miR-2c-5p	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTACATCA
	sja-miR-2a-3p	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTGTTCAT
	sja-miR-10	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTCCAAAC
Forward primer used in singleplex assays	ath-miR-159a	GGTTTGGATTGAAGGGAGCT
	sja-bantam	GGTGAGATCGCGATTAAAGC
	sja-miR-277	CGGTAAATGCATTTTCTGGCC
	sja-miR-3479-3p	GGTATTGCACTTACCTTCGC
	sja-miR-125b	CGGTCCCTGAGACTGATAATT
	sja-miR-125a	TGTCCCTGAGACCCTTTGAT
	sja-miR-61	GGTGACTAGAAAGTGCACTC
	sja-miR-2b-5p	TGCGTCTCAAAGGACTGTGA
	sja-miR-2162-3p	CGGTATTATGCAACGTTTCAC
	sja-let-7	TGGGAGGTAGTTCGTTGTGT
	sja-miR-36-3p	TTCCACCGGGTAGACATTCA
	sja-miR-3489	TTGCCACAACAGTTCGAGGA
	sja-miR-2d-3p	GGTATCACAGTCCTGCTTAG
	sja-miR-3487	TTTCCTCGAACTGTTGTGGC
	sja-miR-2c-5p	TGACCCTTGTTCGACTGTGA
	sja-miR-2a-3p	GGTCACAGCCAGTATTGATG
	sja-miR-71a	GGTGAAAGACGATGGTAGTG
	sja-miR-3488	TTGCTCCGGTAGCTTAGTTG
	sja-miR-3492	TGATCCGTGCTGAGATTTCG
	sja-miR-71b-5p	CGGTGAAAGACTTGAGTAGTG
	sja-miR-307	CGGTCACAACCTACTTGATTG
	sja-miR-10	TTAACCCTGTAGACCCGAGT
Forward primers used in the duplex assay 2P	sja-miR-2b-5p	TGCGTCTCAAAGGACTGTGA
	sja-miR-2c-5p	TGACCCTTGTTCGACTGTGA
Forward primers used in the multiplex assay 3P	sja-miR-2b-5p	TGCGTCTCAAAGGACTGTGA
	sja-miR-36-3p	TTCCACCGGGTAGACATTCA
	sja-miR-2c-5p	TGACCCTTGTTCGACTGTGA
Forward primers used in the multiplex assay 5P	sja-miR-125a	TGTCCCTGAGACCCTTTGAT
	sja-miR-2b-5p	TGCGTCTCAAAGGACTGTGA
	sja-miR-36-3p	TTCCACCGGGTAGACATTCA
	sja-miR-2c-5p	TGACCCTTGTTCGACTGTGA
	sja-miR-2a-3p	GGTCACAGCCAGTATTGATG
Forward primers used in the multiplex assay 6P	sja-miR-277	CGGTAAATGCATTTTCTGGCC
	sja-miR-125a	TGTCCCTGAGACCCTTTGAT
	sja-miR-2b-5p	TGCGTCTCAAAGGACTGTGA
	sja-miR-36-3p	TTCCACCGGGTAGACATTCA
	sja-miR-2c-5p	TGACCCTTGTTCGACTGTGA
	sja-miR-2a-3p	GGTCACAGCCAGTATTGATG
Universal reverse primer		CAGTGCAGGGTCCGAGGT
Universal double-quenched probe		56-FAM/CAGAGCCAC/ZEN/CTGGGCAATTT/3IABkFQ

Table S2. Twenty-one miRNAs selected based on published studies of schistosome circulating and extracellular vesicles/exosomes associated miRNAs

miRNA	Sequence[#]	Ref
bantam	UGAGAUCGCGAUUAAAGCUGGU	[1-7]
miR-277	UAAAUGCAUUUUCUGGCCCGU	[1, 2, 4, 6, 8]
miR-3479-3p	UAUUGCACUUACCUUCGCCUUG	[4, 6-8]
miR-125b	UCCCUGAGACUGAUAAUUGCUC	[1-3]
miR-125a	UCCCUGAGACCCUUUGAUUGCC	[3]
miR-61	UGACUAGAAAGUGCACUCACUUC	[1-3, 6]
miR-2b-5p	CGUCUCAAAGGACUGUGAGCC	[1]
miR-2162-3p	UAUUAUGCAACGUUUCACUCU	[3, 4, 6]
let-7	GGAGGUAGUUCGUUGUGUGGU	[1]
miR-36-3p	CCACCGGGUAGACAUUCAUUCGC	[1, 2, 6]
miR-3489	GCCACAACAGUUCGAGGACG	[1]
miR-2d-3p	UAUCACAGUCCUGCUUAGGUGACG	[1, 6]
miR-3487	UCCUCGAACUGUUGUGGCC	[1]
miR-2c-5p	ACCCUUGUUCGACUGUGAUGU	[1, 2]
miR-2a-3p	UCACAGCCAGUAUUGAUGAAC	[2, 4, 6]
miR-71a	UGAAAGACGAUGGUAGUGAGAUG	[1-3, 6]
miR-3488	GCUCCGGUAGCUUAGUUGGU	[2, 5]
miR-3492	AUCCGUGCUGAGAUUUCGUCU	[2, 3]
miR-71b-5p	UGAAAGACUUGAGUAGUGAGACG	[2, 3]
miR-307	UCACAACCUACUUGAUUGAGGGG	[6]
miR-10-5p	AACCCUGUAGACCCGAGUUUGG	[1-4, 7]

[#] S. japonicum miRNA sequences are shown

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2. Nowacki FC, Swain MT, Klychnikov OI, Niazi U, Ivens A, Quintana JF, et al. Protein and small non-coding RNA-enriched extracellular vesicles are released by the pathogenic blood fluke *Schistosoma mansoni* . J Extracell Vesicles. 2015;4:28665.

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6. Zhu S, Wang S, Lin Y, Jiang P, Cui X, Wang X, et al. Release of extracellular vesicles containing small RNAs from the eggs of Schistosoma japonicum. Parasit Vectors. 2016;9(1):574.

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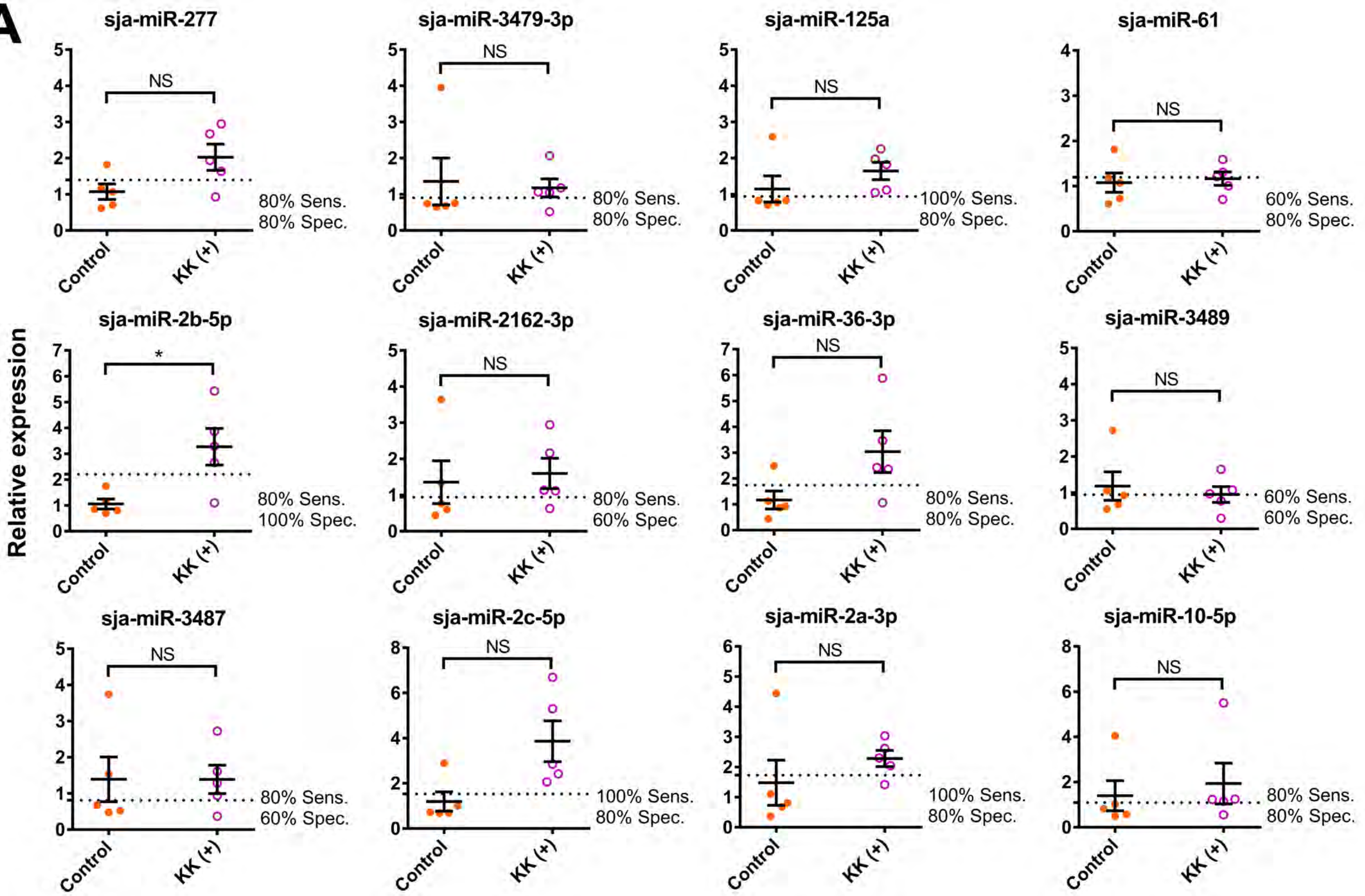
A


sja-miR-277
NS
80% Sens.
80% Spec.
sja-miR-3479-3p
NS
80% Sens.
80% Spec.
sja-miR-125a
NS
100% Sens.
80% Spec.
sja-miR-61
NS
60% Sens.
80% Spec.
sja-miR-2b-5p
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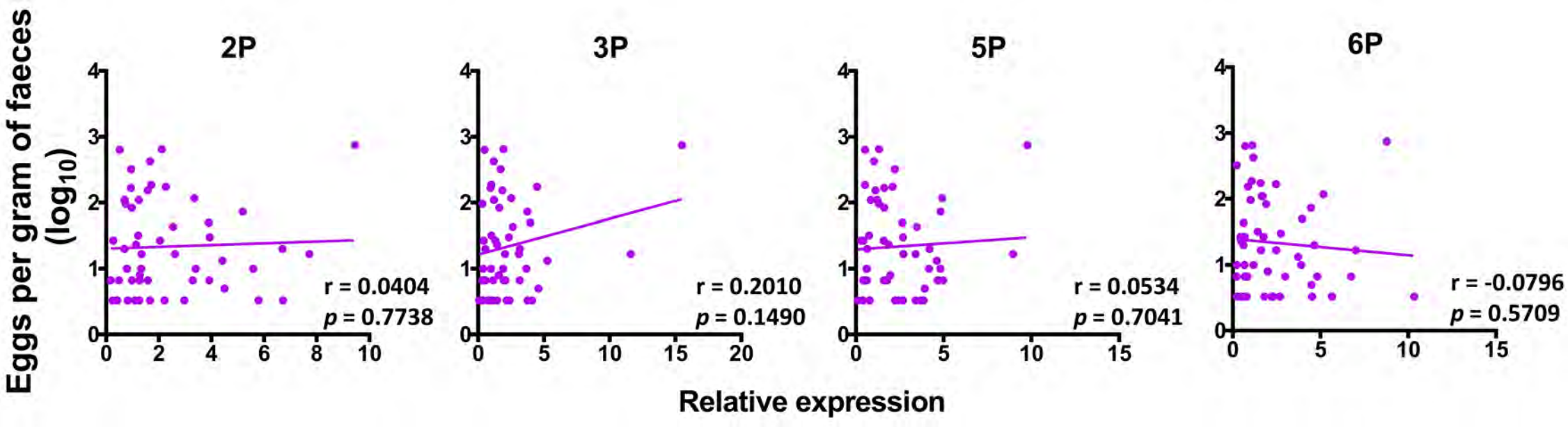
80% Sens.
100% Spec.
sja-miR-2162-3p
NS
80% Sens.
60% Spec
sja-miR-36-3p
NS
80% Sens.
80% Spec.
sja-miR-3489
NS
60% Sens.
60% Spec.
sja-miR-3487
NS
80% Sens.
60% Spec.
sja-miR-2c-5p
NS
100% Sens.
80% Spec.
sja-miR-2a-3p
NS
100% Sens.
80% Spec.
sja-miR-10-5p
NS
80% Sens.
80% Spec.
Control
KK (+)
Relative expression

B

sja-miR-277
AUC=0.84
p=0.0759
sja-miR-3479-3p
AUC=0.64
p=0.4647
sja-miR-125a
AUC=0.80
p=0.1172
sja-miR-61
AUC=0.60
p=0.6015
sja-miR-2b-5p
AUC=0.92
p=0.0283
sja-miR-2162-3p
AUC=0.64
p=0.4647
sja-miR-36-3p
AUC=0.84
p=0.0759
sja-miR-3489
AUC=0.52
p=0.9168
sja-miR-3487
AUC=0.56
p=0.7540
sja-miR-2c-5p
AUC=0.88
p=0.0473
sja-miR-2a-3p
AUC=0.80
p=0.1172
sja-miR-10-5p
AUC=0.72
p=0.2506
Sensitivity%
100% - Specificity%


Eggs per gram of faeces (log10)
2P
r = 0.0404
p = 0.7738
3P
r = 0.2010
p = 0.1490
5P
r = 0.0534
p = 0.7041
6P
r = -0.0796
p = 0.5709
Relative expression