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

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Cite this article: Mu Y, Cai P, Olveda RM, Ross AG, Olveda DU, McManus DP (2020). Parasite-derived circulating microRNAs as biomarkers for the detection of human Schistosoma japonicum infection. Parasitology 147, 889-896. https://doi.org/10.1017/S0031182019001690

Introduction

Schistosomiasis, a debilitating, often fatal, disease, caused by trematode blood fluke parasites of the genus Schistosoma, affects 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 and Gray, 2019). Currently, schistosomiasis control relies predominantly on mass praziquantel (PZQ) drug administration (MDA) programs (Olveda and 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 (Ross et al., 2015; Mutapi et al., 2017). 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 (Weerakoon et al., 2015; 2017b; Oliveira et al., 2018; Cai et al., 2019). 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 PCR-based methods (Weerakoon et al., 2016, 2017a, b), 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 (Peng and Croce, 2016; Anvarnia et al., 2019). As potential targets for novel diagnosis (Li and 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; Jia et al., 2017; Meningher et al., 2017; Jamal et al., 2018; Schonauer...
et al., 2018; Tengda et al., 2018; Filipów and Laczmański, 2019; Xue et al., 2019). Circulating miRNAs also have been proposed as having potential to detect parasitic helminth infections (Hoy et al., 2014; Tritten et al., 2014; Cai et al., 2015; Dong et al., 2017; Guo and Zheng, 2017). 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; Zhu et al., 2016a; Samoil et al., 2018) 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 the 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

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 microscopy. 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 (Epiconen 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 X RT buffer, 1 μL ATP (10 μM), 1 μl universal RT primer (1 μM), 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. 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 to essential protocols as described previously (Cai et al., 2015, 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 a 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 1.

Statistical analysis

Unpaired student’s t-test (two tails) was used for comparing the serum levels of miRNAs in naive and S. japonicum-infected BALB/c mice. The Mann–Whitney U-test was used for the 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 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 \( \geq 4 \) 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). ROC curve analysis was performed and the AUC levels were calculated to evaluate the diagnostic potential of each miRNA (Supplementary Fig 1). 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 \( \geq 0.80 \) 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 53 KK (+) individuals and 25 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-36-3p and sja-miR-2a-3p respectively (\( P = 0.0574, 0.0696, 0.0256, 0.1989, 0.0121 \) and 0.2542, 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 0.80, specificity 0.66).
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 control individuals 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 (Utzinger et al., 2015; Weerakoon et al., 2015; Cai et al., 2017; Oliveira et al., 2018). 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
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 (Cheng et al., 2013; Hoy et al., 2014; Cai et al., 2015; Meningher et al., 2017). However, there had been no reports hitherto of their use in the clinical diagnosis of schistosomiasis japonica.

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

<table>
<thead>
<tr>
<th>miRNA combination</th>
<th>AUC (95% CI)</th>
<th>P</th>
<th>Sens. (%)</th>
<th>Spec. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sja-miR-277 + sja-miR-125a</td>
<td>0.6257 (0.4961–0.7552)</td>
<td>0.0747</td>
<td>62.3</td>
<td>64.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-2b-5p</td>
<td>0.6460 (0.5167–0.7754)</td>
<td>0.0383</td>
<td>69.8</td>
<td>64.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-2c-5p</td>
<td>0.6657 (0.5415–0.7898)</td>
<td>0.0188</td>
<td>49.1</td>
<td>84.0</td>
</tr>
<tr>
<td>sja-miR-125a + sja-miR-2b-5p</td>
<td>0.6400 (0.5111–0.7689)</td>
<td>0.0471</td>
<td>43.4</td>
<td>88.0</td>
</tr>
<tr>
<td>sja-miR-125a + sja-miR-2c-5p</td>
<td>0.6642 (0.5375–0.7908)</td>
<td>0.0199</td>
<td>52.8</td>
<td>80.0</td>
</tr>
<tr>
<td>sja-miR-2b-5p + sja-miR-2c-5p</td>
<td>0.6906 (0.5645–0.8166)</td>
<td>0.0069</td>
<td>77.4</td>
<td>60.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-125a + sja-miR-2b-5p</td>
<td>0.6430 (0.5147–0.7714)</td>
<td>0.0425</td>
<td>41.5</td>
<td>88.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-125a + sja-miR-2c-5p</td>
<td>0.6619 (0.5359–0.7879)</td>
<td>0.0217</td>
<td>54.7</td>
<td>76.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-2b-5p + sja-miR-2c-5p</td>
<td>0.6867 (0.5422–0.7952)</td>
<td>0.0168</td>
<td>73.6</td>
<td>60.0</td>
</tr>
<tr>
<td>sja-miR-125a + sja-miR-2b-5p + sja-miR-2c-5p</td>
<td>0.6792 (0.5541–0.8044)</td>
<td>0.0110</td>
<td>55.8</td>
<td>80.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-125a + sja-miR-2b-5p + sja-miR-2c-5p</td>
<td>0.6672 (0.5409–0.7935)</td>
<td>0.0178</td>
<td>49.1</td>
<td>84.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-125a + sja-miR-36-3p + sja-miR-2b-5p + sja-miR-2c-5p</td>
<td>0.6483 (0.5195–0.7711)</td>
<td>0.0354</td>
<td>69.8</td>
<td>60.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-125a + sja-miR-36-3p + sja-miR-2b-5p + sja-miR-2c-5p</td>
<td>0.6121 (0.4817–0.7425)</td>
<td>0.0119</td>
<td>64.2</td>
<td>60.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-125a + sja-miR-36-3p + sja-miR-2c-5p + sja-miR-2a-3p</td>
<td>0.6468 (0.5205–0.7730)</td>
<td>0.0374</td>
<td>62.3</td>
<td>72.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-125a + sja-miR-2b-5p + sja-miR-2c-5p + sja-miR-2a-3p</td>
<td>0.6211 (0.4930–0.7402)</td>
<td>0.0858</td>
<td>37.7</td>
<td>88.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-125a + sja-miR-36-3p + sja-miR-2c-5p + sja-miR-2a-3p</td>
<td>0.6294 (0.5017–0.7572)</td>
<td>0.0664</td>
<td>37.7</td>
<td>88.0</td>
</tr>
<tr>
<td>sja-miR-277 + sja-miR-125a + sja-miR-2b-5p + sja-miR-2c-5p + sja-miR-36-3p + sja-miR-2a-3p</td>
<td>0.6294 (0.5013–0.7576)</td>
<td>0.0664</td>
<td>37.7</td>
<td>88.0</td>
</tr>
</tbody>
</table>

*Note: 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.

indicators (Manzano-Roman and Siles-Lucas, 2012; Cai et al., 2016a). 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 (Cheng et al., 2013; Hoy et al., 2014; Cai et al., 2015; 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 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 be 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 introduced 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. (2017) when detecting S. mansoni, S. haematobium and S. mekongi infections in 26 returning travellers 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 (Lagaté 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 harboured 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; Weerakoona 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 and Granados-Lopez, 2017), an active sorting mechanism of exosomal miRNA


Lagatie O, Batse Debrah L, Debrah A and Stuyver LJ (2017) Plasma-derived parasitic microRNAs have insufficient concentrations to be used as diagnostic biomarker for detection of *Oxudercia volvulus* infection or treatment monitoring using LNA-based RT-qPCR. *Parasitology Research* 116, 1013–1022.


