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Short communication

Profiling circulating miRNAs in serum from pigs infected with the porcine whipworm, *Trichuris suis*

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ABSTRACT

microRNAs (miRNAs) are recently discovered as key regulators of gene translation and are becoming increasingly recognized for their involvement in various diseases. This study investigates the miRNA profile in pig serum during the course of an infection with the gastrointestinal parasite, *Trichuris suis*. Of this panel, the expression of selected miRNAs in serum from *T. suis* infected and uninfected pigs were determined by quantitative real time PCR using Exiqon Human Panel assays at 0, 2, 4, 6, 8 and 10 weeks post first infection (wpi). One miRNA, ssc-let-7d-3p, was significantly up-regulated in infected pigs 8 wpi. Interestingly, ssc-let-7d-3p shows high complementary to tsu-let-7a, which is the most highly transcribed miRNA in *T. suis*. The let-7 family miRNAs have been shown to post-transcriptionally regulate the translation of the helminth-controlling cytokine, IL-13, in a murine model for asthma and we hypothesize possible interactions between these host- and parasite-derived miRNAs and their immunomodulating roles.

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The porcine whipworm, *Trichuris suis*, is a parasitic nematode living in the large intestine of pigs worldwide. Prevalence is highly dependent on pig management system e.g., in-door versus out-door production. In most cases infections are sub-clinical but worm loads are associated with reduced growth rates, reduced feed efficiency, hemorrhagic diarrhea and death (Roepstorff et al., 2011). *Trichuris* spp. infections can be diagnosed by detection of eggs in faeces, but this method is laborious and only allows diagnosis from approximately 6–7 weeks after first infection with *T. suis* (Kringel and Roepstorff, 2006).

microRNA (miRNA) is a class of evolutionarily conserved small non-coding RNAs, encoded as a hairpin structure producing a functional (mature) miRNA of ~22 nucleotides in length that have been found in at least twelve different kinds of human body fluids, including serum and plasma (Weber et al., 2010). They play an important function in post-transcriptional regulation of genes involved in a range of biological processes, which primarily results from cleavage and destruction of mRNA by miRNA bound to a RNA-induced Silencing Complex (RISC) (Gregory et al., 2005). For example, miR-146a, miR-9 and miR-125b play a role in innate immunity, whereas miR-155 is crucial in adaptive immune responses and in regulation of differentiation, activation and function of T cells and B cells (Gracias and Katsikis, 2011).

miRNAs are transported inside extracellular vesicles which provides them a high degree of stability (Gallo et al., 2012). Therefore, the potential of using miRNAs as biomarkers of disease has in recent years been studied extensively and miRNA profiles, providing information on expression patterns of disease-associated miRNAs, have been obtained for various human conditions. Among these are different types of cancers (Lu et al., 2005) but also infectious diseases such as leishmaniasis caused by the protozoan parasite *Leishmania major* (Lemaire et al., 2013). In domestic animals most miRNA profiling research has focused on the expression patterns of miRNAs associated with important traits related to milk, meat, and egg production (Fatima and Morris, 2013). In pigs, few studies have been carried out focusing on miRNA profiles in different tissues associated with infectious diseases. Of these, one study has profiled miRNAs in lung tissue from pigs infected with the bacteria *Actinobacillus pleuropneumoniae* (Podolska et al., 2012) and other studies have focused on miRNAs associated with viral infections, such as with Porcine circovirus type 2 (Hong et al., 2015) and Porcine reproductive and respiratory syndrome virus (PRRSV)

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Table 1
Target miRNAs included in the customized Pick & Mix panel (Exiqon A/S, Denmark) and information about whether differential expression in infected versus uninfected pigs complies with the set criteria for significance. FC = Fold change, wpi = weeks post first infection.

Annotation	$P < 0.05$	P *adjusted* <0.0031	FC ($2^{-\Delta\Delta Ct}$) >±1.5
ssc-miR-16	No	No	No
ssc-miR-191	No	No	No
ssc-miR-214	No	No	No
ssc-miR-328	No	No	No
ssc-miR-429	No	No	No
ssc-miR-451	No	No	No
ssc-miR-125b	Yes (8 wpi)	No	Yes (10 wpi)
ssc-miR-199a-5p	No	No	No
ssc-miR-214-3p	No	No	No
ssc-miR-27a	No	No	No
ssc-miR-28-5p	No	No	No
ssc-miR-374b-5p	No	No	No
ssc-miR-885-5p	No	No	No
ssc-miR-99a	No	No	No
ssc-let-7d-5p	No	No	No
ssc-let-7d-3p	Yes (8 wpi)	Yes (8wpi)	Yes (8 wpi)

(Li et al., 2015). However, to our knowledge miRNA levels in pig serum associated with parasitic infections have not been profiled.

The aim of this study was to find miRNAs of importance in *T. suis* infections in pigs by analyzing the levels of a number of pre-selected miRNAs in serum samples from infected and uninfected pigs at several time points post first infection. Identification and characterization of miRNAs associated with *T. suis* infections, could provide basic knowledge of host-parasite interactions, i.e. how the host responds to the infection, which cellular processes are induced and how these are regulated. Furthermore, it may allow us to identify potential miRNA biomarkers for prepatent infections. Even though infection with *T. suis* can have severe consequences for pigs and farmers, intentional infections with *T. suis* in humans may have ameliorating effects on autoimmune diseases, i.e. in helminth therapy (e.g. Summers et al., 2005). Thus, miRNA biomarkers for whipworm infections in pigs may also be useful in humans. In addition, as *T. suis* is very similar to the human whipworm, *T. trichiura*, these biomarkers may also be relevant for detecting infections with this parasite species.

A mix of female and castrated male Landrace/Yorkshire/Duroc piglets obtained from a commercial specific pathogen-free (SPF) swine herd were 5–6 weeks of age and weighed 17.3± 1.9 kg (mean ± S.D.) as previously described (Petersen et al., 2014). Briefly, the pigs were randomly allocated into two groups after stratification by sex and body weight. One group (n = 6) was trickle infected with 10 *T. suis* eggs/day/kg and the other group (n = 6) were kept as uninfected controls. Serum samples from all 12 pigs were collected at 0, 2, 4, 6, 8 and 10 weeks post first infection (wpi) and the faecal egg counts estimated every third-fourth day pi. The Danish Animal Experiments Inspectorate (Permission 2005/561-1060) approved the experiment and used procedures.

Total RNA was purified from serum with a commercial miRNeasy Serum/Plasma kit (Qiagen, Hilden, Germany) and RNA was stored at −80 °C prior to reverse transcription. cDNA synthesis and subsequent RT-qPCR was performed at Exiqon A/S (Vedbaek, Denmark) using the miRCURY LNA™ Universal RT microRNA PCR system. A pre-screen of the pooled serum samples from each group at 0, 6, 8 and 10 wpi was performed to determine the miRNA-composition of a larger, refined panel of pig miRNAs. This refined Customized Pick & Mix panel (Exiqon A/S), containing 22 primer sets, was used for the detailed miRNA profiling of all the serum. Of these, six stably expressed miRNAs identified in the pre-screen were used as internal references for data normalization (ssc-miR-423-5p, ssc-miR-19b, ssc-let-7c, ssc-miR-21, ssc-let-7a and ssc-miR-23a) leaving 16 target miRNAs (Table 1). Assay signals below 40Ct's were included in the analysis. In both runs (pre-screen and refined miRNA profiling) primers of the spike-in templates, added during the miRNA isolation, were included in the PCR panel in order to test the technical quality of RNA isolation, cDNA synthesis and the presence of PCR inhibitors in the samples. Two replicas were made for each serum sample. Normalization of the quantitative RT-qPCR data was carried out for each target miRNA by subtracting the Ct of a target miRNA from the mean Ct of stable miRNAs (ΔCt). Ct values were linearized ($2^{-\Delta Ct}$) and an Analysis of Variance (ANOVA) was performed using the PROC GLM procedure with repeated measures in SAS version 9.4 for Windows (SAS institute, Cary NC, USA). Normalized miRNA expression level values were used as response variable with infection status (infected vs. uninfected), time, sex and weight at week zero as explanatory variables. A significance level of 0.05 was used, as well as a Bonferroni corrected significance level of 0.0031 taking multiple testing (n = 16) into account. A Kolmogorov–Smirnov test was used to test for normality. Fold changes (FC) of expression levels between infected and uninfected pigs were calculated as $2^{-\Delta\Delta Ct}$ according to Schmittgen and Livak (2008). A 1.5-fold-change threshold was used as in previous studies (e.g. Kuchenbauer et al., 2008).

Based on the relative expression levels of mir-23a and mir-451 it was concluded that no samples had been exposed to hemolysis (ΔCt < 5) (Blondal et al., 2013). One miRNA, ssc-let-7d-3p, was found to be significantly different expressed ($P = 0.0025$, FC = 1.7) between infected and uninfected pigs at 8 wpi (Fig. 1A) which coincides with a peak in egg excretion (Petersen et al., 2014). At 8 and 10 wpi ssc-miR-125b were increased 1.18 ($P = 0.04$) and 2.03 ($P = 0.1$) fold, respectively, as compared to controls. All infected pigs were *T. suis* egg positive 6–7 wpi (Petersen et al., 2014) which is in accordance with previous studies (Kringel and Roepstorff, 2006).

The let-7 family has previously been shown to down-regulate the expression of IL-13 in a murine model for asthma, resulting in alleviated asthma features (Kumar et al., 2011) presumably by repressing 'Suppressor of Cytokine Signaling' (SOCS) proteins (Hu et al., 2010). IL-13 is a cytokine secreted by T_H2-lymphocytes and other cells, which, in addition to modulating allergic reactions in asthma, promotes host defense in helminth infections, including infection with *T. suis* (Kringel et al., 2006).

miRNAs have recently been described in the characterization of the *T. suis* genome (Jex et al., 2014). In that study, tsu-let-7a was consistently the most highly transcribed miRNA encoded by the parasite and transcription was highest in the stichosome of the adult worm embedded within the pig gut epithelium (Fig. 1B). While this is to be expected, given the important role let-7 plays in worm biology (Reinhart et al., 2000), it is noteworthy that the seed sequence of tsu-let-7a shows perfect complementarity to the IL-13 3′UTR let-7 family target sequence (Polikepahad et al., 2010). Based on this we hypothesize that tsu-let-7a can target and block the synthesis of IL-13 which could serve as a sophisticated survival mechanism for the parasites. An in-depth analysis is needed to reveal the targets of ssc-let-7d-3p in pigs and which cellular processes this miRNA may interfere with, however, it is intriguing that ssc-let-7d-3p shows high complementarity to tsu-let-7a (Fig. 1C). We speculate, given the likely role of tsu-let-7a in regulating cytokine production during parasite infection, that ssc-let-7d-3p may have a counteracting role by binding to and blocking tsu-let-7a and thereby prevent IL-13 inhibition. Thus, the up-regulation of ssc-let-7d-3p in *T. suis* infected pigs observed in this study at 8 wpi, when egg excretion and presumably worm load peaks, could be an important mechanism for establishing an adequate IL-13 level in order to eliminate the parasites. That an elevated IL 13 level is reached in the pigs, despite this presumed attempt from the parasites to hinder it, is supported by Andreasen et al. (2015) who found a significant up-regulation of IL-13 (FC = 26,9) at day 35 post infection with *T. suis*. Although not

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