Litomosoidessigmodontis: A Jird Urine Metabolome Study

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ABSTRACT

The Neglected Tropical Disease Onchocerciasis affects more than 35 million people worldwide with over 95% in Africa. Disease infection initiates from the filarial parasitic nematode *Onchocerca volvulus*, which is transmitted by the blackfly vector *Simulium* sp. carrying infectious L3 larvae. New treatments and diagnostics are required to eradicate this parasitic disease. Herein, we describe that a previously discovered biomarker for onchocerciasis *N*-acetyltyramine-*O*-glucuronide (NATOG) is also present in urine samples of jirds infected with the onchocerciasis model nematode *Litomosoides sigmodontis*. Increased NATOG values paralleled a progressing infection and demonstrated that quantification of NATOG in this *L. sigmodontis* rodent model can be utilized to track its infectivity. Moreover, our findings suggest how NATOG monitoring may be used for evaluating potential drug candidates.

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Onchocerciasis, commonly referred to as river blindness, is a neglected tropical disease (NTD) caused by the parasitic nematode *Onchocerca volvulus*. Onchocerciasis is transmitted as L3 larvae to the human host during a blood meal by the blackfly vector (*Simulium* spp.).^[1] This parasitic disease affects more than 35 million people worldwide, predominantly in Africa.^[1] An additional 120 million people are at risk of becoming infected with *O. volvulus*. Onchocerciasis can cause severe eye lesions in about 10% of infections through migrating *O. volvulus* larvae into the host's eyes thus limiting the ability to work in many African countries with high poverty rates. Research to control and eradicate this parasitic disease has been hampered due to: (1) Adult *O. volvulus* nematodes are not viable outside the human host; (2) all rodent models are non-permissive to *O. volvulus*.^[1] Thus, a reliable substitute nematode model to investigate new drug candidates against *O. volvulus* is required in a simplified laboratory setting to mimic *O. volvulus* infection.^[2-4] This animal model is also crucial to overcome another major limitation, which is the diagnosis of the disease. Field evaluations are unable to distinguish between an ongoing and a past infection. Moreover, lacking is a robust, reliable and reproducible diagnostic to distinguish each parasitic developmental stage as multiple nematode stages may be present in an infected host.

Several accepted surrogate animal models for onchocerciasis have been developed and established within the last 25 years including two major models.^[5-7] The first filarial model nematode *Litomosoidessigmodontis* can produce patent infections in rodent hosts in 55-60 days upon initial infection.^[2, 6, 8-11] Wherein the

natural host is the cotton rat, *Sigmodon hispidus* and the tropical rat mite *Ornithonyssus bacoti* is the vector. The ability of *L. sigmodontis* to achieve patency in rodents has already been demonstrated and thus allows for comparisons with human *O. volvulus* studies, which is exclusive for this rodent-nematode model.^[4, 12] The natural filarial parasite of cattle, *O. ochengi* is the second commonly used parasite model and is the genetically closest related nematode to *O. volvulus*.^[5] However, the *L. sigmodontis* model is more advantageous with a shorter patency time, 7-8 weeks, which compared to *O. ochengi* takes several months.^[6] The reduced patency time permits more proficient analyses of infection. Moreover, tested drugs can be applied at reduced quantities compared to the bovine model.^[4]

A rodent model is of high value as it could grant both precise information on infection time and parasite stage development. Furthermore, it should be suited to evaluate new treatment options, while enabling host-parasite immune pathology to progress throughout all stages of the parasite's development.^[4, 13] In short, we hoped that *L. sigmodontis* would mitigate the challenges associated with an *O. volvulus* model, yet, provide a reliable means to monitor parasite development from a metabolomic stance.^[14-16] With this basis, a recently identified biomarker, tyramine, discovered in urine from *O. volvulus* infections, and excreted as *N*-acetyltyramine-*O*-glucuronide (NATOG) would be examined as a potential cross-over biomarker in *L. sigmodontis* infections in jirds.^[17]

To test the importance of tyramine as a biomarker in *L. sigmodontis* we engaged a metabolomics-based approach using liquid chromatography mass spectrometry (LC-MS). For the rodent, we have chosen jirds as mammalian host because they are permissive to infections and display similar parasite development to that in the natural host, the cotton rat.^[6, 10, 18] Furthermore, jirds represent an advanced infection model as they develop equivalent parasite burden and microfilariae load, and encapsulate worms faster than cotton rats.^[6] Developmental stages of *L. sigmodontis* in jirds have been documented.^[2, 6, 18] Upon infection of jirds with *L. sigmodontis* L3 larvae, a migration is seen to the plural cavity within 4-5 days post infection (p.i.). Transition of L3 to L4 larvae can be observed within 4-10 days p.i., in which they mature to adult worms starting after 3-4 weeks p.i.. Full patency develops after ~50 days p.i. with microfilariae circulating in the blood. Maximum microfilariae load is reached after 20-24 weeks p.i..^[6]

Figure 1: PCA score plots of urine sample LC-MS experiments A) $m/z = 200-1400$ Da; B) $m/z = 200-660$ Da. Positive mode analysis demonstrates the metabolite composition difference between early infected jirds (4-11 days p.i.) and jirds at 8 weeks p.i. with *L. sigmodontis*.

For our biomarker exercise urine was collected and extracts analyzed at well-defined time points upon *L. sigmodontis* L3 larvae infection to cover all developmental stages of the parasite's lifecycle. Thus, jirds were infected with *L. sigmodontis* by exposure to *Ornithonyssus bacoti* mites containing infectious *L. sigmodontis* L3 larvae.^[19] Urine samples were collected every two weeks in 10-12 h periods overnight and during daytime using metabolic cages (Techniplast, Italy). Initial sample collection was performed on a daily basis from 4 to 11 days p.i., followed by sample collection every two weeks thereafter. Urine samples were collected at room temperature and subsequently stored at -80°C . Metabolites were extracted from 50-100 μL urine aliquots using a standard cold methanol-based extraction protocol for protein precipitation and the soluble metabolites were then analyzed by LC-MS analysis in positive analysis mode.^[17]

In order to detect metabolite composition differences between infected and uninfected jird urine samples a multivariate analysis

was utilized for urinary LC-MS spectra using *XC-MS online*.^[20, 21] The mass spectrometric analysis range was chosen between $m/z = 200-1400$ Da, note NATOG $m/z = 356.1340$. We accompanied unsupervised principal component analysis (PCA), which provides initial experimental information on the dataset relationship of all LC-MS spectra.^[20, 22] The corresponding loading plot (PC1/PC2) for mean-centered data analysis of 4-11 days post-infection (N=19) and 8 weeks post-infected jirds (N=19) is shown in Figure 1. Consequently, we selected 8 weeks as our sample set as the jirds would be fully patent.^[6] Positive mass spectrometric analysis revealed a 24% variance in the first dimension PC1 and 11% variance in PC2 demonstrating a significantly different metabolite composition between early and late stage infections (Figure 1A). These results warranted an additional analysis in which the mass range was narrowed so as to only focus on small molecules within the molecular mass range of metabolized neurotransmitters ($m/z = 200-660$ Da). The analysis of these low molecular mass experiments led to an increased separation of both sample sets with a 33% variance in the first dimension PC1 and 10% variance in PC2 (Figure 1B). Strikingly, this metabolomics analysis reveals a high probability to identify a metabolic marker and to distinguish infection stages. Moreover, the two separated sample sets indicate a significant influence of adult worms on the rodent metabolite composition.

The list generated revealed multiple differences in the metabolite composition between both sample sets. Within the TOP 30 up-regulated hits found to be statistically significant, we identified our onchocerciasis biomarker NATOG ($p = 2.36198 \times 10^{-6}$; 3.1-fold) as shown in Figure 2. This biomarker is based on the *O. volvulus* neurotransmitter tyramine, which is further metabolized by the nematode through *N*-acetylation and glucuronidation by the human host.^[17] As a point of reference, acetylated and glucuronidated serotonin, a common metabolite in mammals and a potential neurotransmitter used by nematodes was found to be essentially equal in both sample sets with peak intensities of 1.1 fold similarity and no significant differences.^[23]

Figure 2: Extracted mass spectrometric chromatogram for NATOG in early (4-11d) and late stage (8 weeks) infections.

As statistical metabolic analysis can only provide semi-quantitative data, we sought to quantify NATOG in each urine sample using deuterium labeled D_3 -NATOG as an internal standard (Figure 3, Figure S1). The internal standard was added to each urine sample at a known concentration, followed by metabolite extraction.^[17] We identified NATOG in infected jirds (16-18 wks p.i.) at increased levels of $c = 23.1 \mu\text{M}$ compared to the uninfected control group with a concentration of $c = 9.9 \mu\text{M}$ (Figure 3A). The more than two-fold increased NATOG levels follows a similar trend to that of our previously reported NATOG values of *O. volvulus* infected patients in human urine samples

with a 6-fold increase in infected patients. Quantification of

assessment of NATOG values in *L. sigmodontis* at distinct time

Figure 3:A) Mean quantitative values of NATOG in jird urine samples collected at different time points post-infection using a D₃-NATOG internal standard. Error bars represent SEM values; N = Number of samples. Data were analyzed by ANOVA, followed by Tukey's multiple comparison method to determine significance; * p < 0.05, ** p < 0.01, *** < 0.001. B) Box-and-whisker plot of quantitative data. N = Same numbers as in 3A.

NATOG in urine samples collected at different p.i. time points revealed a steady increase in NATOG levels with detectable elevated values after 2 wks with $c=13.2 \mu\text{M}$ (Figure 3). A statistical significant increase in NATOG values compared to early infection stage (4-11d) was already observed at 6 wks p.i. ($c=16.7 \mu\text{M}$, $p < 0.05$). At 8 weeks p.i., in which the *L. sigmodontis*-jird model reaches full patency, average values attained levels of $c=21.3 \mu\text{M}$, and statistical analysis revealed high significance when compared to the control group ($p < 0.001$). The slightly reduced values after 10 weeks p.i. will require further investigation as urine samples from this set presented a widespread range of values from below $10 \mu\text{M}$ to above $40 \mu\text{M}$ depending on the jird analyzed (Figure 3B). The sequence of events culminating in enriched NATOG values in patent jirds demonstrate that quantification of NATOG in urine samples can be utilized to identify *L. sigmodontis* infections and may furthermore be useful as a measure of the infection stage.

To investigate if NATOG values are dependent on the sample collection time, we quantified urine samples, which were collected at day- or night separately. Sample collection at specific daytimes are required for an accurate microfilariae count for nematodes such as *Loa loa*, for which blood samples must be collected between 10 am and 2 pm; and *Brugia spp./Wuchereria bancrofti* that requires sample collection after 8 pm.^[24, 25] Our analysis revealed no statistical significance in four out of five different infection time points (Figure S2). Only at 8 wksp.i. was statistical significance observed with elevated levels at $c=28 \mu\text{M}$ for night collection compared to daytime collection at $c=19 \mu\text{M}$ ($p < 0.05$). Whether this difference is meaningful to disease state monitoring will require a larger sample set. Finally, analysis of NATOG in urine samples collected at 4 °C and room temperature did not reveal any statistically significant differences (data not shown).

To the best of our knowledge, research detailing monoaminergic neurotransmitters in *L. sigmodontis* has not been reported.^[26] Increased NATOG values found to be statistically important as the infection progresses allows us to posit that tyramine in *L. sigmodontis* is required for larvae and microfilariae development similar to *O. volvulus*.^[27] Quantitative

points allow previously inaccessible detailed insights at which developmental phase NATOG is produced (Table 1). On the basis of this we can compare data obtained in the *L. sigmodontis*-jird model with the *O. volvulus* lifecycle in humans.^[28] NATOG values are significantly elevated upon 6 weeks p.i. in jirds, which corresponds to a complete maturation process of *L. sigmodontis*. Furthermore, during this time the rodent host begins to encapsulate adult worms. This time point corresponds to about 2-4 months p.i. in *O. volvulus* infections.

Table 1. Overview of the *L. sigmodontis* life cycle with corresponding time post infection in comparison with the *O. volvulus* life cycle.

<i>L. sigmodontis</i> lifecycle	Time post infection	Comparison with <i>O. volvulus</i> life cycle
Transition of L3 to L4 larvae	4-10 d	3-12 weeks
Larvae in pleural cavity		
Adult worms	24-30 d	1-3 months
in pleural cavity	(3-4 weeks)	(4-12 weeks)
Encapsulation	4-12 weeks	2-4 months
Microfilariae	50 d	6-12 months
in blood	(7-8 weeks)	(24-54 weeks)
Microfilariae	20-24 weeks	-
Maximum Microfilaremia		

Further validation of NATOG as a cross-over biomarker for *O. volvulus* and *L. sigmodontis* infections was investigated by searching the available genome databases for both nematodes. Since the genome of *O. volvulus* is better characterized than *L. sigmodontis* and in many cases the translation and function of the proteins encoded by the genes have been predicted, we initiated our analysis in *O. volvulus*. Previously, two *O. volvulus* enzymes were proposed to be involved in the biosynthesis of NATOG: 1) a decarboxylase that converts L-tyrosine to tyramine and 2) an *N*-acetyltransferase that converts tyramine to *N*-acetyltyramine.^[17] First, the WormBase (WS248) was used as a source for finding a decarboxylase that could catalyze the

production of tyramine. Through this search a protein, labeled as a putative aromatic L-amino acid decarboxylase was found in the *O. volvulus* database, which we propose could potentially catalyze the decarboxylation of L-tyrosine.^[29] Using this amino acid sequence as a guide, an almost identical protein was identified in the *L. sigmodontis* database,^[30] as indicated by an alignment of the two protein's amino acid sequences (Figure S3). Based on these positive findings, an analogous approach was taken in search of an acetylCoA-dependent *N*-acetyltransferase. Two candidate proteins were identified for *O. volvulus* that contain the highly conserved acetyl-CoA binding motif Arg/Gln-X-X-Gly-X-Gly/Ala, which should have the capability to catalyze the acylation of tyramine. Upon analysis of *L. sigmodontis*, two proteins were also discovered that contain similar sequences based on amino acid alignments (Figure S4). Ultimately, a decarboxylase and two *N*-acetyltransferases that are highly similar between *O. volvulus* and *L. sigmodontis* were identified that could potentially catalyze the conversion of L-tyrosine to tyramine followed by *N*-acetyltyramine formation.

The gold standard diagnostic for onchocerciasis is the highly invasive skin snip test, which can detect microfilariae with excellent sensitivity at 6-12 months p.i. Yet, for this diagnostic to confirm an ongoing *O. volvulus* infection, a threshold of

microfilaria numbers must be present, which can take up to 1.5 years. As outlined, the production of NATOG at increasing levels begins during the development of adult worms and presents NATOG as a disease state biomarker at an earlier developmental stage than skin snips, i.e. before microfilariae are detectable in blood (jirds) or skin snips (humans). Besides pre-patent infection recognition, patent infections during microfilarial production (8 weeks p.i.) can be distinctly distinguished from uninfected samples through analysis of NATOG.

In conclusion, we have identified that the onchocerciasis urine biomarker NATOG is also a marker for *L. sigmodontis* infections in a jird model, which is a prevailing animal model for onchocerciasis. Detailed analysis of NATOG within the *L. sigmodontis* lifecycle discloses that the biosynthesis of NATOG begins at an early infection stage, values increase during the maturation process and are significantly increased at the stage of adult worms. At the clinical level our efforts reveal that NATOG holds great potential to predict disease progression. As a next logical step, urine samples of infected jirds will be analyzed during drug treatment to evaluate if NATOG can be quantified to monitor successful treatments. Success here would be particularly valuable for future screening of new *O. volvulus* drug candidates in this *L. sigmodontis*-jird model.

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