Establishment Of Chronic *Strongyloides ratti* Infection Model And Its Diagnostic Significance

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Abstract

Infection with *Strongyloides stercoralis* is associated with asymptomatic chronic disease involving the gut and respiratory systems and can persist for long period undetected leading to development of fatal hyperinfection syndrome. The severity is even more in immunocompromised individuals or patients undergoing immune suppressive therapy. Unfortunately the disease is difficult to diagnose due to low parasite load and the irregular larval output. Development of efficient diagnostic techniques requires large supply of infective larvae and this can be achieved through *in vivo* maintenance of the parasite. In this regard, *S. ratti* is being used as a model instead of *S. stercoralis* due to difficulty and risk of infection. In this study, a significant amount of previously characterized filariform (iL3) larvae of *S. ratti* was collected. Chronic infection was successfully established and maintained by experimentally induced immunosuppression of Wistar rats following oral administration of prednisolone. This model can serve as a good source of infective larvae for production of monoclonal antibodies and development of diagnostic assays for prevention of the disease. It can also provide room for further research in diagnostics of human strongyloidiasis.

Introduction

Strongyloides stercoralis is a soil borne helminth infection reported to affect over one million people globally, with majority residing in the tropics and subtropical regions of the world. However, the disease is beginning to emerge in Europe and the U.S due to increased frequency of international travel and migration due to war and civil unrest in mostly the Middle Eastern countries (Luis *et al*., 2008; Siddiqui and Berk 2001). Strongyloidiasis is a potentially fatal parasitic infection, particularly among the immunocompromised patients whose infection is associated with the development of severe gastrointestinal and pulmonary symptoms as well as secretion of large number of infective larvae in stool and or sputum (Al Maslamani *et al*., 2009). Chronic infection which manifest by relatively mild symptoms following *S. stercoralis* infection mostly follow the characteristics ability of the parasite to cause a cycle of autoinfection within its host and this can persist in an individual for decades (Grove, 1989). However, in immunocompromised patients, an uncontrolled autoinfection may occur leading to hyperinfection syndrome. Hyperinfection syndrome is characterized by migration of larvae to other organs beyond the confines of the pulmonary autoinfection route (Keiser & Nutman, 2004). Treatment with corticosteroids tend to exacerbate the situation and produces an immunosuppressive effect that increases the risk of acquiring chronic strongyloidiasis and development of hyperinfection syndrome leading to disseminated strongyloidiasis, which has a high mortality ratio (Fardet *et al*., 2007; Al Maslamani *et al*., 2009; Marcos *et al*., 2011).

Reports have highlighted the diagnostic challenges of *S. stercoralis* hyperinfection which has warranted the urgent need to develop improved techniques for efficient diagnosis of the disease. While conventional fecal egg counts is not reliable and require repeated sampling, serological assays have the problem of cross reactivity with other helminthes parasites, which tend to affect the specificity of the tests. Hence, one of the most reliable approaches is the use of monoclonal antibodies developed from larval extract in the development of ELISA kits.

This study was undertaken in order to establish and maintain experimental animal model of chronic *Strongyloides ratti* infection based on corticosteroid immune suppression. This is to allow for further research in diagnostics of human strongyloidiasis.

Materials and Methods

Animals and source of infective larvae

Approximately 70 wild rats (*Rattus norvegicus*) were captured using steel mouse traps from eateries and restaurants and were

used as natural source of the parasites. Fresh faecal samples were collected per rectum of the rats and analyzed by direct fecal smear and microscopy to detect parasite eggs and rhabditiform larvae. To study the structural features of the parasite, drops of formalin were used to kill the larvae and slides were stained with Lugol's iodine in order to observe the internal structures of the detected stage. The different stages were analyzed using an Image Analysis Software (Nikon Corporation, Japan) for morphological confirmation. All animal experiment was conducted according to the approved protocol of the Institutional Animal Care and Use Committee IACUC of the Universiti Putra Malaysia, (UPM/IACUC/AUP-R026/2015).

Molecular characterization

Fecal samples positive for eggs of *S. ratti* were prepared and the DNA was extracted using the Qiagen DNA extraction kit (QIAamp® DNA stool MiniKit; Germany). The procedure for the DNA extraction was as per the instruction of the manufacturer. Briefly, about 200 mg of positive fecal sample was suspended in 1.4 mL of ASL buffer. The suspension was thoroughly mixed by vortexing (1 min) until completely homogenized. The procedure involves lysis and washing with suitable buffers as well as incubation and binding to the spin column provided by the manufacturer. The final step entails elution of the bound DNA from the columns in to a collection tube. The concentration (ng/µL) and purity of the extracted DNA was analyzed using NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific, USA). DNA samples were aliquoted (10 µL) in eppendorf tubes and stored at -30°C until required.

PCR Amplification of 28S rDNA gene

Two designed primer sets were used to amplify partial ribosomal DNA (28S) of *S. ratti* genome. Forward StroS (5´-TTAGAGTCGTGTTGCTTGGAA-3´) and reverse StroAS (5´-GTGCAACTGGCTCTGTATGC-3´) based on rRNA sequences related to *S. ratti*, found in GenBank (Accession SRU39490) to amplify a 180 bps target (Kramme *et al*., 2011). PCR reactions were achieved using the following reaction mix: 12 µL of 1x GoTaq Green Master Mix (Promega, USA), 1µL of each primer pair (0.1 - 1.0 M), 1µL of template DNA and nuclease-free water up to final volume of 25 µL. One tube without a DNA template was used as a negative control.Thermal cycling (Biometra, Germany) conditioned for an initial denaturation at 95ºC for 5 minutes (time-delay), 35 cycles at 95ºC for 5 seconds (denaturation), 50ºC for 20 seconds (annealing) and 72°C for 30 seconds (extension), followed by a final extension of 72ºC for 5 minutes.

Gel Electrophoresis of PCR Products

The PCR products were loaded in 1.5 % agarose gel (Vivantis Incorp, USA), 1x TBE (Vivantis Incorp, USA) buffer) pre-stained with GelRed (Biotium, Hayward, USA). Electrophoresis was carried out at 70 Volts for 65 minutes (Thermo, USA). Molecular markers of 100 bp (Axil Scientific, Singapore) were run in parallel well with the DNA samples and gels were visualized under UV light transilluminator (Major Science, USA), and photographed.

Experimental design

Twenty Sprague dawley rats (six weeks-old) were assigned into two groups: control and prednisolone group and inoculated with approximately 2,000 infective filariform (iL3) larvae of *S. ratti* in pure water (2000 iL3 / mL) by subcutaneous injection on the dorsum of the neck. The infection was allowed to set in for a period of ten (10) weeks. After observation of signs of disease and evaluation of fecal egg counts using Modified McMaster technique and culture, fecal samples were collected (per rectum) daily from one week post-infection in order to detect and harvest the larvae.

Upon confirmation of active infection in the experimental rats, it was maintained by oral prednisolone administration at a dose of 0.35 mg/kg body weight per day for a maximum of four weeks. This was to induce an experimental immunosuppression in the rats in order to maintain the infection. Drug administration was performed anytime the infection status is examined to be low due to low egg output. The drug was administered orally using 18 gauge rodent feeding needle following manual restrain.

Propagation and maintenance of infective larvae of *Strongyloides ratti*

At the end of the 10 weeks experiment, the 20 rats with chronic infection were divided into two groups of 10 rats each. The first group was given oral prednisolone solution at a dose of 0.25 mg/kg body weight daily for four weeks. While the second group was given 0.35mg/kg prednisolone orally daily for four weeks. The two experiments were performed concurrently and during this period, daily worm-egg counts were recorded. Subsequently, a third group containing five rats among the second group (0.35mg/kg) were re-infected with 2,000 infective larvae and maintained on same dose of prednisolone for four weeks. The induction of experimental immunosuppression was done to mimic natural infection leading to establishment of hyperinfection and disseminated strongyloidiasis. The rats were monitored daily for signs of discomfort and illness, daily worm-egg count was recorded for the period.

Egg and Larval Counts

The experimental infection was monitored by daily examination of rat feces and data was presented as a daily mean of egg per gram of feces (EPG) using modified McMaster technique. Larvae were collected from cultured feces and counted. On completion of the experiments, the rats were euthanized using chloroform and the relevant organs (intestines, lung, liver, kidneys, heart, and spleen) were collected.

Results

Morphological Characteristics of the Different Stages of *Strongyloides ratti*

Parasitological examinations of fecal samples from infected rats revealed the different stages in the life cycle of this nematode parasite to be *Strongyloides ratti* based on the their morphological characteristics. Eggs and rhabditiform larvae were encountered through the fecal smearing and wet mount methods respectively while infective larvae (filariform larvae) and free-living adults were observed only after fecal culture technique (Figure 1).

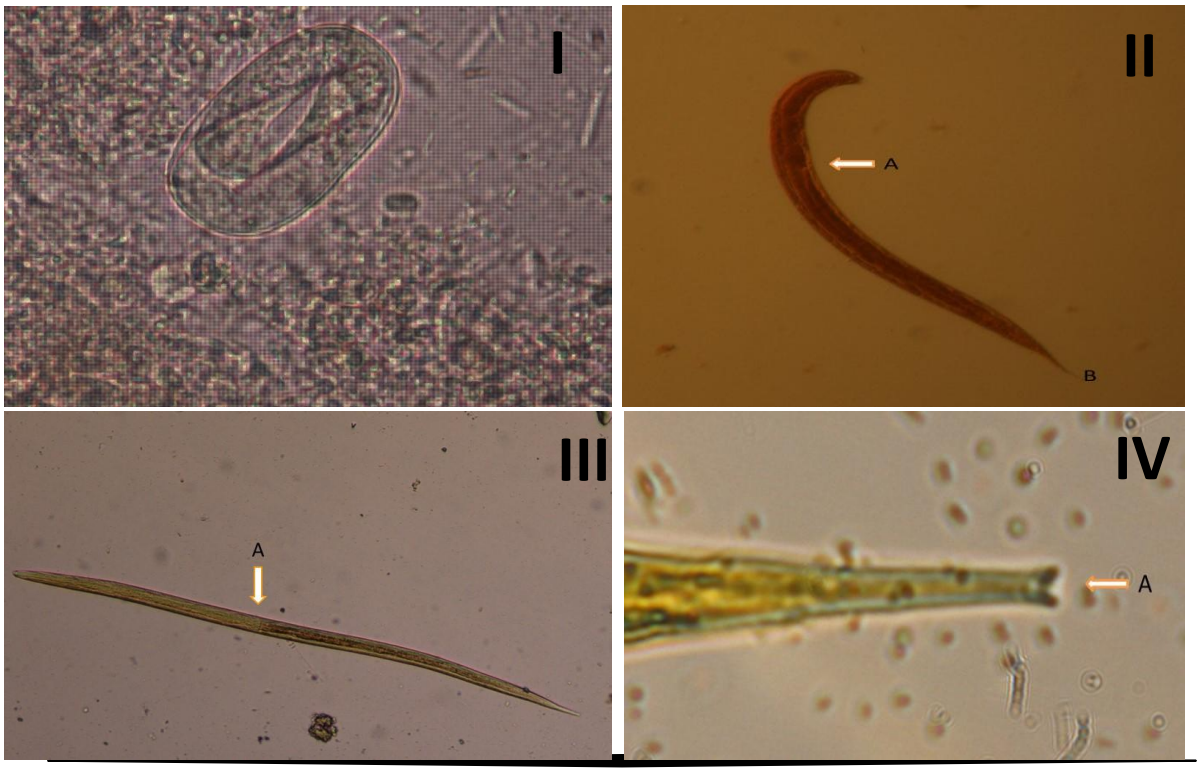

Figure 1:
I. Egg-containing larva of *S. ratti*.
II. Rhabditiform larva. A: Rhabditiform oesophagus; B: Pointed tail tip.
III. Filariform larva showing eosophagus (A).
IV. Filariform larva showing characteristic notched-tail.

Molecular Characteristics of *Strongyloides ratti*

The concentration and purity of the extracted DNA was found to be 65.1 ng/uL and 2.15 (A260/A280) respectively. These were analyzed using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA) and the result of the A260/A280 ratio was approximately above 2, indicating pure DNA that is free from contaminants. The PCR analysis revealed a 180 bp band which was obtained via electrophoresis of the PCR product. Absence of bands in the negative control sample is an indication that there was no contamination in the reaction (Figure 4).

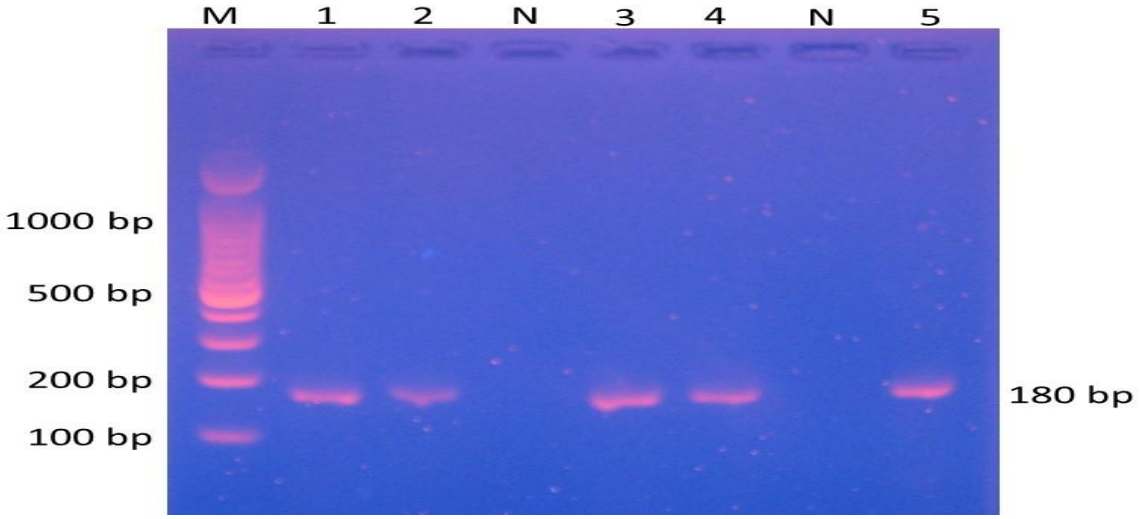

Figure 4: Agarose gel electrophoresis: **PCR amplification of 28S rDNA gene using universal primer; M: 100 bp DNA Ladder; 1 - 5: PCR products from selected samples (180 bp); N: Negative control.**

Establishment of chronic *Strongyloides ratti* infection

This experiment was able to establish chronic infection model of *S. ratti* in rats. Infective larvae were successfully generated following sub-cutaneous inoculation with an initial 2,000 infective larvae (iL3). Analysis by the Modified Faecal Filtration Technique (MFFT) revealed that a significant increase in larval output resulted after prednisolone administration. The harvested infective larvae were also found to be very active and contained minimum contamination from fecal debris, bacteria and fungus. The daily mean egg output peaked at 15,510 eggs per gram (EPG) of feces two weeks post-infection, and then declined gradually to zero by week 10 as shown in (Figure 3).

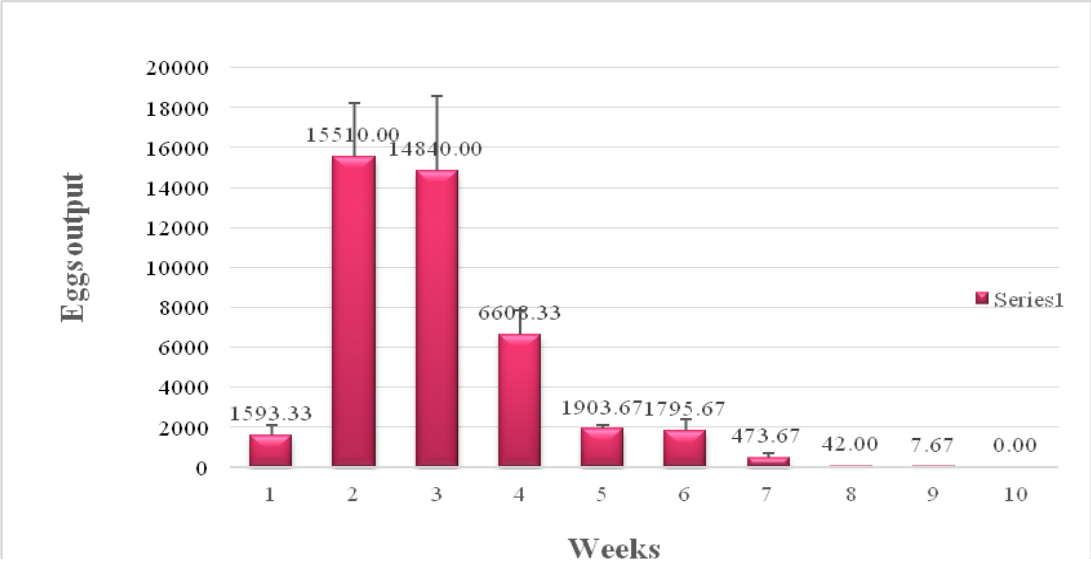

Figure 3: Eggs output of chronic *S. ratti* infection. Means with different numbers are significantly different (P< 0.05).

The daily mean egg output after treatment with 0.25 mg/kg of prednisolone increased from zero reaching a peak at 89,340 EPG in the fourth week. Similarly, a higher number of eggs were obtained 4 weeks after treatment with 0.35 mg/kg of prednisolone (17,976 EPG). However,

upon reinfection with 2,000 infective larvae a very statistically significant increase ($P < 0.05$) in daily egg output (232,566 EPG) was noticed during the third week. In each of the stages, none of the feces collected was contaminated by other parasites (Figure 4).

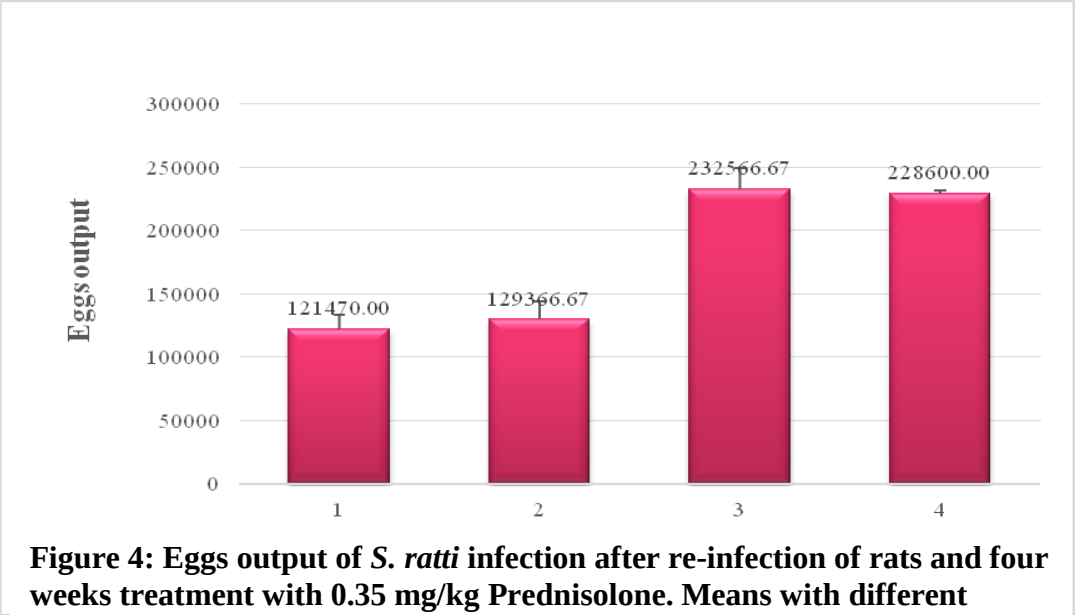

Figure 4: Eggs output of *S. ratti* infection after re-infection of rats and four weeks treatment with 0.35 mg/kg Prednisolone. Means with different numbers are significantly different (P< 0.05).

Discussion

Strongyloidiasis is one of the most neglected tropical diseases despite its high prevalence and health burden especially among individuals with compromised immune system. The disease is also frequently misdiagnosed due its asymptomatic nature and most routine diagnostic test is insufficient enough to detect infections due to poor sensitivity. However, significant progress has been recorded in terms of improving the sensitivity of serological assays including the indirect immunofluorescence microscopy (IFAT) which has demonstrated a sensitivity of up to 81–98% (Requena-Me´ndez *et al*., 2013; Boscolo *et al*., 2007). Nevertheless, the need for large number of whole living infective-larvae still represents a drawback. In this study, an experimental infection and immunosuppression was induced in order to develop a chronic infection rat model of strongyloidiasis. A large number of infective larvae was obtained five weeks after initial

inoculation with 2,000 iL3 larvae of *Stongyloides ratti* and maintained by administration of prednisolone. Following oral administration of prednisolone, a significant increase in egg per gram of feces was noted as well as yield in larvae. This observation is similar to earlier reports by Mahmuda *et al*. (2017) and Ohnishi *et al*. (2004) that used oral prednisolone to induce immunosuppression following *S. ratti* infection and the high number of *S. stercoralis* eggs recorded from AIDS patient respectively. Molecular analysis of the DNA obtained following experimental infection revealed similar morphological features of different stages of *S. ratti* and the phylogenetic analysis also show that *S. ratti* isolated was 97% similar to *S. ratti* and *S. stercoralis*.

The development of amenable animal models of infection for some parasitic nematodes, including *Strongyloides*, is borne out of the fact they represent important human and animal pathogens. In this regard, *S. ratti* and *S. venezuelensis* infections of mice have been established previously for the purpose of immunological studies (Viney and Kikuchi 2017). In all this, the important fact remains essentially it's for the sake of development of diagnostic techniques for *S. stercoralis* which is the human pathogen. And because of the characteristic unique feature of human *S. stercoralis* infection of occurrence of internal autoinfection, which does not occur in *S. ratti* neither can it be artificially induced. Hence, despite the obvious similarities of this specie to the human parasite, its application as a model in studying human strongyloidiasis should be tempered.

References

Al Maslamani Mona A, Hussam A. Al Soub, Abdel Latif M. Al Khal, Issam A. Al Bozom, Mohammed J. Abu Khattab and Kadavil C. Chacko (2009). *Strongyloides stercoralis* hyperinfection after corticosteroid therapy: a report of two cases. *Ann. Saudi Med.* 2009 Sep-Oct; 29(5): 397–401.

Boscolo M, Gobbo M, Mantovani W, Degani M, Anselmi M, et al. (2007). Evaluation of an indirect immunofluorescence assay for strongyloidiasis as a tool for diagnosis and follow-up. *Clin. Vaccine Immunol*. 14: 129–133.

Fardet, L., Généreau, T., Poirot, J.L., Guidet, B., Kettaneh, A. & Cabane, J. (2007). Severe Strongyloidiasis in corticosteroid-treated patients: Case series and literature review. *J. of Infection*, 54(1): 18-27.

Grove DI (1996). Human strongyloidiasis. Adv. Parasitol. 1996;38:251–309.

Keiser, P.B. & Nutman, T.B. (2004). *Strongyloides stercoralis* in the immunocompromised population. *Clinc. Microbiol. Rev.*, 17(1): 208-217.

Kramme, S., Nissen, N., Soblik, H., Erttmann, K., Tannich, E., Fleischer, B., Panning, M., & Brattig, N. (2011). Novel real-time PCR for the universal detection of *Strongyloides* species. *Journal of medical microbiology*, *60*(4), 454-458.

Luis A. Marcos, Angelica Terashim, Herbert L. DuPont, Eduardo Gotuzzo (2008). Strongyloides hyperinfection syndrome: an emerging global infectious disease. *Trans. Roy. Soc. Trop. Med. Hyg.* (2008) 102, 314—318.

Mahmuda, A., Al-Zihiry, K.J.K., Roslaini, A.M., Rukman, A.H., Abdulhaleem, N., Bande, F., Mohammed, A.A., Alayande, M.O., Abdullah, W.O. and Zasmy, U. (2017). Histopathological confirmation of disseminated larvae (iL3) of *Strongyloides ratti* in an immunosuppressed Wistar rat. *Tropical Biomedicine* 34(1): 212–223.

Marcos, L.A., Terashima, A., Canales, M. & Gotuzzo, E. (2011). Update on strongyloidiasis in the immunocompromised host. *Current Infectious Disease Reports*, 13(1): 35-46.

Requena-Me´ndez A, Chiodini P, Bisoffi Z, Buonfrate D, Gotuzzo E, Jose´ Munoz (2013). The Laboratory Diagnosis and Follow Up of Strongyloidiasis: A Systematic Review. *PLoS. Negl. Trop. Dis*. 7(1): e2002.

Siddiqui AA and Berk SL (2001). Diagnosis of *Strongyloides stercoralis* infection. *Clin Infect. Dis. 33(7):1040-7.*

Viney M and Kikuchi T (2017). *Strongyloides ratti* and *S. venezuelensis* - rodent models of Strongyloides infection. *Parasitology*. 144(3):285-294.

احداث اصابة مزمنة لطفيلي سترونجيلويديس راتي وتقييم مدى فعاليتها التشخيصية

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الخلاصة

ترتبط الاصابة بطفيلي سترونجلويديس ستيركورواليس بمرض مزمن بدون اعراض في الاصحاء مناعيا يشمل الجهازين الهظمي والتنفسي والذي من الممكن ان يبقى غير مكتشف لمدة طويلة من الزمن وقد يتحول الى اصابة شديدة مميتة. تزداد شدة المرض في الافراد الذين يعانون من نقص المناعة او المرضى الخاضعين لعلاجات مثبطة للمناعة. يتميز المرض بصعوبة تشخيصه وذلك لقة اعداد يرقات الطفيلي او عدم انتظام خروجها في براز المصاب. يعتمد تطوير وتطبيق اي طريقة تشخيصية لهذه الاصابة على توفر اعداد كافية من هذا الطفيلي والذي يكون بالدرجة الاساس معتمدا على ادامة الاصابة في الحيوانات المختبرية. في هذا الصدد يتم الاعتماد في التجارب المختبرية على طفيلي الجرذان سترونجلويديس راتي كنموذج وبديل مختبري ناجح وذلك لخطورة التعامل مع الطفيلي البشري فضلا عن فشل محاولات اصابته للحيوانات المختبرية. بناء على ذلك عزل هذا الطفيلي من الجرذان البرية وشخص بالطرق المظهرية والجزيئية واستخدم في الدراسة الحالية لاحداث اصابة مزمنة في الجرذان المختبرية وادامتها عن طريق كبح مناعة هذه الحيوانات باستخدام عقار البريدنيزيلون. الطفيلي المتوفر من الاصابة المختبرية في هذه الدراسة يمكن ان يعد نموذجا مثاليا ومصدرا ليرقات الطفيلي المعدية اللازم توفرها باعداد كبيرة لتطوير تقنيات تشخيصية حديثة ومنها تصنيع اجسام مضادة احادية النسيلة قادرة على تشخيص الطفيلي بدقة عالية باستخدام تقنية الاليزا وبالتالي السيطرة على الاصابة بالطفيلي المصيب للانسان.