Short communication

The effect of normal human serum on the mouse trypanosome *Trypanosoma musculi in vitro* and *in vivo*.

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Abstract:

Trypanosoma musculi, a common blood flagellate found in mice, is similar in morphology and life cycle to the rat trypanosome *T. lewisi*. Both species belong to the subgenus *Herpetosoma* and, as *T. lewisi* has recently been shown to be a zoonotic pathogen, there is concern that *T. musculi* could also be potentially infective to humans. To test this hypothesis, a well-established method, the normal human serum (NHS) incubation test, was carried out which distinguishes human and non-human infective trypanosomes. We found that *T. musculi* could grow in 0.31% NHS *in vitro*, and even kept their infectivity to mice after incubation with 10% NHS for 24 hours. In *in vivo* experiments, *T. musculi* were only slightly affected by NHS injection, confirming that it was less sensitive to the NHS than *T. b. brucei*, but more sensitive than *T. lewisi*. This resistance probably does not rely on a restricted uptake of ApoL-1. Due to this partial resistance, we cannot definitively confirm that *T. musculi* has the potential for infection to humans. As resistance is less than that of *T. lewisi*, our data suggest that it is unlikely to be a zoonotic pathogen although we would advise caution in the case of immunocompromised people such as AIDS and cancer patients.

1. Introduction

Trypanosoma lewisi is a rat-specific haemoflagellate that is distributed globally. When a number of human infection cases were reported, *T. lewisi* was suggested as a potential human pathogen (Lun, et al., 2009, Truc, et al., 2013). Currently, experimental evidence demonstrates that this rat trypanosome was resistant to lysis by normal human serum (NHS) and apolipoprotein L-1 (ApoL-1) both *in vitro* and *in vivo* and was therefore considered an opportunistic zoonotic trypanosome (Lun, et al., 2015). However, an important question is whether any other animal trypanosomes are also human infective.

The answer is yes, although it is considered to have occurred by accident. For instance, *Trypanosoma evansi*, which is a member of subgenus *Trypanozoon* alongside the two well-known human pathogens *T. b. gambiense* and *T. b. rhodesiense*, can infect humans possessing both the normal ApoL-1 gene and a mutated version

(Vanhollebeke, et al., 2006; Van Vinh Chau, et al., 2016; WHO, 2005). Patients have also been found with mixed infections of *T. congolense* and *T. b. brucei* (Truc et al., 1998). However, we were interested whether *T. musculi*, a mouse trypanosome with morphological characteristics similar to those of *T. lewisi* can naturally infect humans. Both *T. musculi* and *T. lewisi* belong to the subgenus *Herpetosoma* and they share many biological characteristics. In fact, some human cases infected with trypanosomes have only been identified as *T. lewisi*-like trypanosomes with the correct species being unknown (Sarataphan, et al., 2007, Verma, et al., 2011). Identification of these trypanosomes were mainly based on morphology and molecular diagnostics (Howie, et al., 2006). However, previous studies from our lab have shown that it is very difficult to distinguish *T. lewisi* from *T. musculi* either by morphological characteristics or by 18S DNA barcoding (Hong, et al., 2017). Therefore, whether these cases were caused by *T. lewisi* or *T. musculi* infection remains questionable. To address the question of human infection, the use of the NHS trypanolysis assay on *T. musculi* is the best option.

In fact, resistance to trypanolysis by NHS has long been considered the distinctive feature that can define the human infective trypanosomes from non-human infection species. It has been demonstrated that *T. b. gambiense* and *T. b. rhodesiense*, are naturally resistant to lysis by NHS (Capewell, et al., 2013, De Greef, et al., 1992, Laveran and Mensnil, 1912). The active component, ApoL-1, can permeabilize the lysosomal and mitochondrial membranes of trypanosomes resulting in death for the NHS sensitive species (Perez-Morga, et al., 2005, Thomson and Finkelstein, 2015, Uzureau, et al., 2013； Vanhamme, et al., 2003, Vanwalleghem, et al., 2015; Xong, et al., 1998). In order to determine the potential risk that *T. musculi* poses for human infection, we investigated the effect of NHS and ApoL-1 on this rodent trypanosome.

2. Materials and methods

2.1. Animals and Trypanosomes

All mice (Swiss Webster, 20-25 g) and rats (Sprague Dawley, 150-200 g) were purchased from the Center of Experimental Animals for the Guangdong province.

They were handled strictly according to protocols approved by the Laboratory Animal Use and Care Committee of Sun Yat-Sen University under the license for grant Number 31672276. The Partinico II strain of *T. musculi* used in this investigation was originally obtained from the London School of Hygiene and Tropical Medicine (Krampitz, 1969). It was gifted by Prof. Philippe Vincendeau of Université de Bordeaux, France. *T. musculi* (Partinico II) and *T. lewisi* (CPO02) were harvested from the infected blood of mice and rats respectively by centrifugation. In brief, red blood cells were pelleted at 170 g for 10-15 min, the parasite-containing supernatant was carefully transferred to a clean tube and spun at 800 g for 5 min. The trypanosomes were washed twice with serum-free RPMI-1640.

2.2. DNA extraction, PCR amplification and sequencing of 18S rDNA

DNA samples of trypanosomes were isolated as described previously (Hong et al., 2017). Primers (F: 5'-GGATAACAAAGGAGCAGC-3', R: 5'-GGATAACAAAGGAGCAGC-3') were designed using Primer Premier 5 to amplify an 834-bp specific region of the 18S rDNA gene of *T. lewisi*. PCR was carried out in a 25 μl reaction mixture containing 12.5 μl PrimeSTAR Max Premix (TAKARA, Japan), 6.25 pmol of each primer, 100 ng template DNA. Amplification was performed for 35 cycles (98 ºC for 10 s, 55 ºC for 15 s, and 72 ºC for 10 s). PCR products were analyzed by gel electrophoresis using a 1% (w/v) agarose gel for 20 min at 110 V and sent for sequencing. Sequenced amplicons were aligned with reference sequences of *T. rangeli* (EU867803), *T. musculi* (AJ223568) and *T. lewisi* (KP098535, GU252209-GU252215, HQ437158, AJ009156, AB242273 and KP098536), using MEGA 5.2 software. Haplotype network analyses were performed using POPART.

2.3. Normal human sera and trypanocidal assays

A pool of freshly collected serum from healthy adult volunteers was prepared using standard procedures. The trypanolytic activity of the NHS was confirmed by using an *in vitro* assay with *T. b. brucei* 427 strain where over 90% were lysed after an incubation at 37 ºC for 4 h (Lun, et al., 2015). NHS was aliquoted and stored at -80 ºC before use.

In vitro cultured trypanosomes (2×10^5/ml) were incubated with RPMI-1640 medium complemented with a serial dilution of NHS (10%, 5%, 2.5%, 1.25%, 0.63%, 0.31%, and 0%) at 37°C with 5% CO_2. The numbers of trypanosomes were counted using a haemocytometer by microscopy.

After being supplemented with 10% NHS or 10% FBS (control) at 37°C for 24 hours, trypanosomes were then injected into mice intraperitoneally. Parasitemia was monitored daily and parasites were found in all mice on the eighth (FBS) and tenth (NHS) day post-infection.

In vivo assays were also carried out. Each mouse was intraperitoneally inoculated with 1×10^5 *T. musculi*. The parasitemia usually reached 1×10^6 trypanosomes/ml in 3 days, and then each mouse was i.p. injected with 0.5 ml fresh NHS. Parasitemia was then monitored daily, until sacrificed 30 days later. Each control mouse was treated with 0.5 ml FBS instead of NHS.

2.4. ApoL-1 endocytosis

Parasites (5×10^6) were incubated in RPMI-1640 medium supplemented with 5% NHS or FBS at 37°C for 1 hour. All trypanosomes were harvested by centrifugation and washed twice with PBS and analyzed by SDS-PAGE. After electrophoresis, samples were blotted onto PVDF membranes and proteins were detected with an ApoL-1 polyclonal antibody (1:1000 dilution, Proteintech, China), HRP-conjugated 2^{nd} antibody (Proteintech, China) and the reaction was detected using ECL (Thermofisher, USA).

2.5. Statistical analysis

Statistical analysis was performed using SPSS statistical software 10.0 (SPSS Inc, Chicago). The time of disappearance and relapse of parasitaemia in each group was analyzed by one-way-ANOVA, independent sample t test, and paired-sample t test. For all analyses, $p < 0.05$ was considered significant.

3. Results

Firstly, we aligned the 18S rDNA of *T. lewisi* and *T. musculi* with the published sequences from human infective *T. lewisi*-like trypanosomes (Howie, et al., 2006). We

amplified the 18S fragment from *T. lewisi* CPO02, CPO04, CPO06, CPO07, *T. musculi* Partinico II, ATCC 30148, ATCC 30181, and ATCC 30182. Other available 18S sequences of *T. lewisi, T. lewisi*-like and *T. musculi* were downloaded from the NCBI, while *T. rangeli* was used as an outgroup. There are 3 polymorphic sites among the *T. musculi*, *T. lewisi* and *T. lewisi*-like trypanosomes (Fig.1A), and these represent 4 nodes in a haplotype network (Fig.1B). Interestingly, one *T. lewisi* (WC365 (accession number KP098535)) showed 100% identity to four other *T. musculi* sequences. These findings confirmed the difficulty in distinguishing these two parasites from each other using the 18S rDNA sequence. More importantly, the human infective *T. lewisi*-like trypanosomes showed 100% identity to four known *T. lewisi* sequences, but not to the one isolated from rodents in the same house where the patient lived.

Therefore, we consider that *T. musculi* may share other similar biological characteristics with *T. lewisi*, e.g. the resistance/tolerance to fresh NHS. In this study, we tested four available *T. musculi* strains, Partinico II, ATCC 30148, ATCC 30181 and ATCC 30182. During a 6-hour incubation in 2.5% NHS, over half (51.1% - 82.9%) of the trypanosomes (10^5) were lysed, but 16.2% - 24.1% trypanosomes survived even 24 hours after incubation (Table 1 & Fig. S1). Therefore, the resistance of *T. musculi* strains to the lysis by NHS *in vitro* was considered to be at an intermediate level when compared with *T. b. brucei* and *T. lewisi* (Table 1). In order to further understand the resistance in *T. musculi*, we performed a detailed comparison with *T. b. brucei* and *T. lewisi*. As shown in Fig. 1, *T. b. brucei* was highly sensitive to NHS and was lyzed even in 0.31% NHS, while *T. lewisi* was completely tolerant to 10% NHS and exhibited continuous growth in 5% NHS. Interestingly, the *T. musculi* (Partinico II strain) was tolerant to 0.63% NHS and continuously grew in 0.31% NHS (Fig. 2) further supporting the notion that *T. musculi* has an intermediate level of resistance.

To test the effect of NHS on infectivity of *T. musculi* (Partinico II strain) in mice, parasites were pre-incubated with both 10% NHS and 10% FBS (control) *in vitro* for 24 hours and then inoculated into mice, respectively. Parasitemia in the mice

inoculated with NHS-treated *T. musculi* was observed from day 10 to day 25 after inoculation but was delayed by 2-3 days compared with the group of mice inoculated with 10% FBS treated *T. musculi*. Moreover, in comparison with the *T. musculi* treated with FBS, a significantly lower parasitemia was observed in the mice inoculated with the *T. musculi* treated with NHS serum (Fig. 3). An *in vivo* assay was also performed by inoculation of 500 µl NHS into each mouse infected with *T. musculi* (Partinico II strain) when the parasitemia of the infected animal reached 2×10^6 trypanosomes/ml. A significantly lower parasitemia was found in the mice inoculated with NHS than those inoculated with FBS, while the duration of the parasitemia was similar (Fig. 4). These results fall into a similar category which were defined as sub-resistance in previous studies on *T. brucei* (Hawking, 1976). Unfortunately, we do not have *in vivo* NHS resistance data for the three *T. musculi* ATCC strains as they were unable to infect mice. However, our results from the *T. musculi* Partinico II strain suggest that *T. musculi* has a much lower sensitivity to lysis by NHS than *T. b. brucei* but a higher sensitivity than *T. lewisi*.

In order to understand the potential mechanism of resistance of *T. musculi* to NHS, we compared the endocytotic activity of ApoL-1 in these trypanosomes. After incubation with 5% NHS for 1 hour, *T. b. brucei*, *T. lewisi* and *T. musculi* (Partinico II strain) lysates were resolved by SDS-PAGE. Western blot analysis revealed a similar amount of ApoL-1 protein at 44 kDa, during endocytosis in all tested trypanosomes (Fig. 5). Using known quantities of NHS, these experiments indicated that 5×10^6 trypanosomes would take up ApoL-1 at an equivalent of 0.31 µl of NHS. Interestingly, an unexpected band was observed in the trypanosome samples, which was about 55 kDa, much larger than ApoL-1. The origins or identity of this protein is unclear.

4. Discussion

Emerging infectious diseases have been a major problem in global public health in recent times. Many of them are caused by pathogens from wild animals, for example, HIV and Zika from wild primates (Bush and Tebit, 2015, Dick, et al., 1952), SARS from bats (Li, et al., 2005), and avian flu from birds (Tan et al 2015). For these

reasons, human infections caused by animal trypanosomes such *T. evansi* and *T. lewisi* have also attracted great attention by the World Health Organization (WHO, 2005). These infections not only cause severe clinical signs but these pathogens are also distributed globally. The normal human serum incubation test, using target trypanosomes is a classic method for identifying the potential for human infectivity (Laveran and Mensnil, 1912). According to the literature, ApoL-1 occurs at a concentration of about 1070 - 2140 μg/ml in human serum (Da Silva, et al., 2011, 2012). One μg/ml (0.1% NHS) ApoL-1could easily kill *T. brucei*, while *T. lewisi* could resist even 100 μg/ml (10% NHS) (Lun et al., 2015). Although, to our knowledge, *T. musculi* has not previously been reported as human infective, the risk of potential human infection is worrying due to similarities to the zoonotic trypanosome *T. lewisi*. Our results demonstrate that *T. musculi* is partially resistant to NHS both *in vitro* (0.31% NHS, corresponding to 3.3 - 6.6 μg/ml ApoL-1) and *in vivo*, which falls in to the category defined as sub-resistant (Hawking, 1976), unlike resistant *T. lewisi* or sensitive *T. b. brucei*. Therefore, those *T. lewisi*-like human infection cases that have been reported should not be attributed to *T. musculi*.

A degree of resistance has also been reported in other animal trypanosomes, such as *T. evansi* (Da Silva, et al., 2011, 2012; Otto, et al., 2010) and *T. congolense*, whose resistance could be even further enhanced when continuously stimulated with non-lethal NHS (Lai, et al., 2010, Xong, et al., 2002). In addition, some other animal trypanosomes such as *T. b. brucei* and *T. congolense* can also infect humans for, as yet, unknown reasons (Truc, et al., 1998). Although the mechanisms of human infection by some animal trypanosomes, such as *T. evansi*, have been elucidated and shown to be due to a mutation of the ApoL-1 gene in patients (Joshi, et al., 2005, Vanhollebeke, et al., 2006), this is not always the case (Van Vinh Chau, et al., 2016).

Although animal trypanosome infection of humans is not a major problem for public health at the present, there is a potential for risk in immunosuppressed members of the population. In fact, immunosuppressed patients infected with some insect trypanosomes, e.g. *Leptomonas* spp., have been reported (Ghosh, et al., 2012, Pacheco, et al., 1998, Srivastava, et al., 2010). This indicates that wild animal and

even insect trypanosomes can potentially naturally infect humans who are immunosuppressed or have other health problems. *T. musculi* may be unlikely to be able to infect humans in normal situations, but, due to the partial resistance to NHS, it could be a potentially high risk for people with immunodeficiency, such as patients with cancer, AIDS and organ transplantation. Since it is a globally distributed species in its mammalian hosts, there would be no spatial or environmental restrictions on its potential emergence.

The mechanism of partial resistance of *T. musculi* against lysis by NHS is an interesting topic to consider. Based on our results from the endocytosis analysis, *T. musculi* and *T. b. brucei* as well as *T. lewisi* take up a similar amount of ApoL-1 suggesting that the resistance of *T. lewisi* and *T. musculi* may not rely on a restriction in uptake of ApoL-1. Thus, it is probable that their resistance may depend on some mechanism which blocks the trypanolytic activity of ApoL-1. However, we were not able to subcellularly localize the ApoL-1 within *T. musculi*. Trypanosome membranes could bind a lot of host molecules by different mechanisms, without further endocytosis (Vincendeau and Daeron, 1989, Vincendeau, et al., 1986). This is an alternative explanation for the tolerance of *T. musculi* against ApoL-1.

One well-characterized factor that is known to directly block the trypanolytic effect of ApoL-1 is the SRA protein. This is a truncated VSG-like protein and has been found to be expressed in *T. b. rhodesiense* (De Greef, et al., 1992, Xong, et al., 1998). Attempts to identify SRA homologs in *T. musculi* and *T. lewisi* by PCR amplification were unsuccessful, indicating that a divergent protein(s) or mechanism is (are) operating in these rodent trypanosomes. Another molecular mechanism, which is active against NHS and found in *T. b. gambiense*, is linked to a VSG-like glycoprotein (TgsGP) and protects this trypanosome from ApoL-1 by stiffening endosomal membranes (Capewell, et al., 2013, Uzureau, et al., 2013). However, this mechanism is also unlikely to be operating in *T. musculi* and *T. lewisi*, as it was excluded by our endocytosis experiments. Although our findings suggest that *T. musculi* is unlikely to be a zoonotic pathogen, the identity of other *T. lewisi*-like human infective trypanosomes urgently needs to be investigated. In addition to the

morphological characteristics, molecular data (e.g. nuclear DNA or kinetoplast DNA barcodes) are necessary for species identification within the subgenus *Herpetosoma* (Hong, et al., 2017). Finally, genomic sequences of these two parasites may help to reveal their strategies that enable them to survive, or partially survive, in the presence of ApoL-1.

Based on our results, we conclude that *T. musculi* was less sensitive to NHS than *T. b. brucei*, while it is more sensitive to NHS than *T. lewisi*. Therefore, those reported *T. lewisi*-like human infection cases should not be attributed to *T. musculi*. Although it is unlikely to be a zoonotic pathogen, we would advise caution in the case of immunocompromised people.

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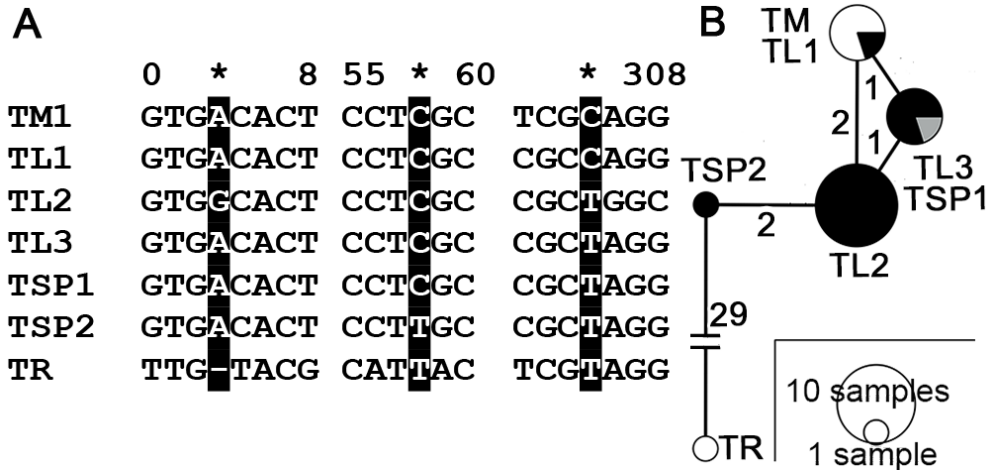
Figure legends.

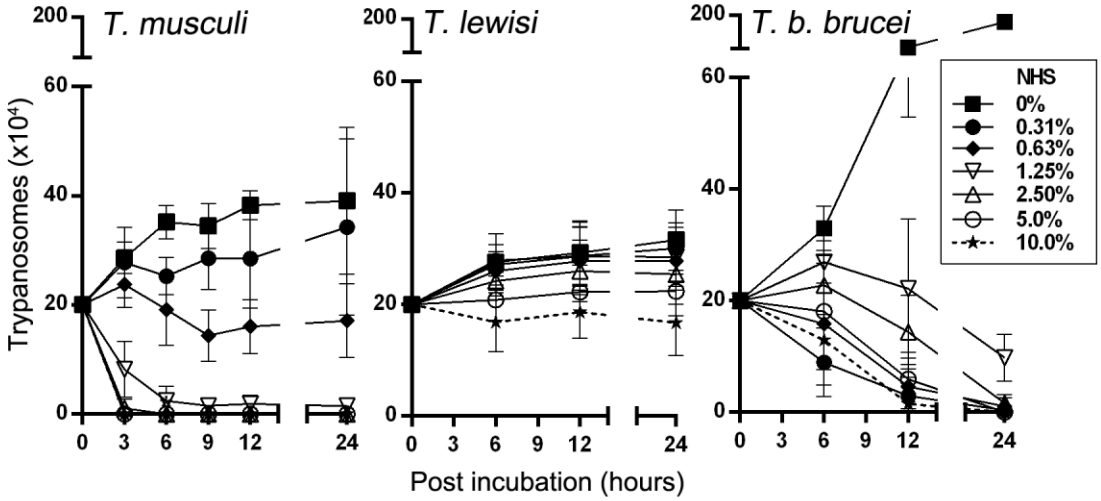
Fig. 1. The alignment of sequences of the 18S fragment (A) and haplotype networks (B) from *T. lewisi*, *T. musculi* and *T. rangeli*.



A: PCR amplification of the 834-bp region of the 18S rDNA gene from *T. lewisi* and *T. musculi* for 35 cycles (98 ^{o}C for 10 s, 55 ^{o}C for 15 s, and 72 ^{o}C for 10 s). PCR products were grouped into 4 haplotypes, which were aligned using MEGA 5.2 software with *T. rangeli* TryCC643 (EU867803) as an outgroup (TR). Polymorphic sites are indicated with an asterisk (*). TM includes three PCR sequences of *T. musculi* strains Partinico II, ATCC 30181 and ATCC 30182, and Genbank sequence of AJ223568. TL1 includes only *T. lewisi* WC365 (KP098535). TL2 includes four PCR sequences of *T. lewisi* CPO02, CPO04, CPO06, CPO07, and 7 Genbank sequences of *T. lewisi* TryCC34 (GU252210), TryCC35 (GU252211), TryCC43 (GU252212), TryCC44 (GU252213), TryCC1148 (GU252215), TryCC124 (GU252214) and Af (GU252209). TL3 includes 4 Genbank sequences of *T. lewisi* (HQ437158), Molteno B3 (AJ009156), TryRaIDN202 (AB242273) and LC244 (KP098536). TSP1 includes 1 Genbank sequence of *T. lewisi*-like (DQ011519). TSP2 includes 1 Genbank sequence of *T. lewisi*-like in the patient's house (DQ011520).

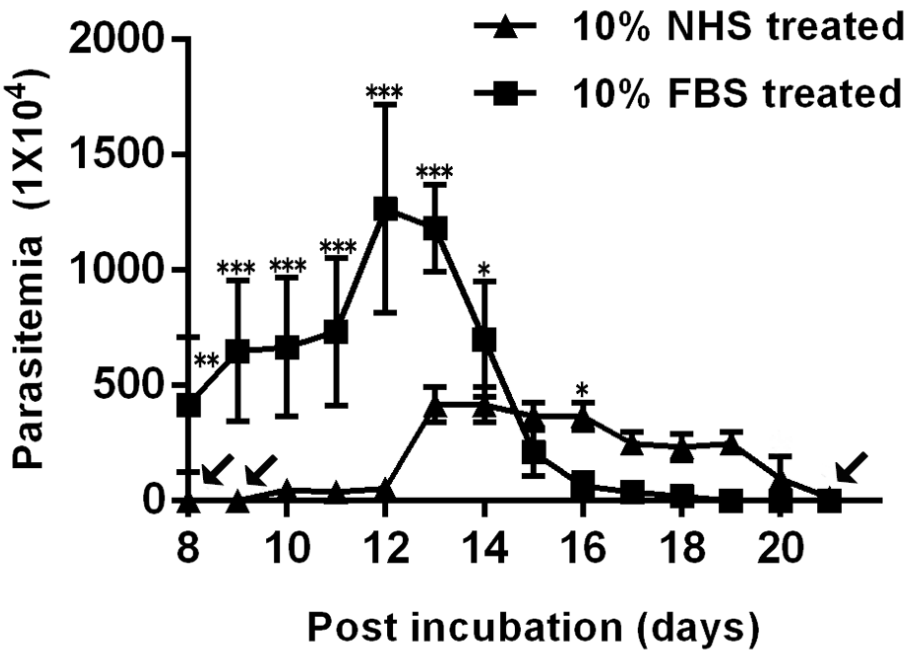
B: Median-joining network inferred from *T. lewisi*, *T. musculi* and *T. rangeli* sequences. The haplotypes are indicated by the nodes. *T. lewisi* was split into 3 clades, *T. musculi* T. sp1, T. sp2 and *T. rangeli* assembled into 1 clade each, respectively.

Fig. 2. Trypanocidal effect of NHS on *T. musculi* Partinico II strain, *T. b. brucei* 427 and *T. lewisi in vitro.*



Trypanosomes ($2x10^5$/ml) were incubated with RPMI-1640 medium complemented with a serial dilution of NHS at 37°C with 5% CO_2. The numbers of trypanosomes were counted using a haemocytometer by microscopy. Each measurement was performed in triplicate and repeated three times (n = 3). Statistical analysis between these are shown in Table S1.

Fig. 3. Parasitemia of *T. musculi* Partinico II strain in mice after pre-incubation with 10% NHS or FBS.



Parasites were incubated in RPMI-1640 medium supplemented with 10% NHS or 10% FBS (control) at 37°C for 24 hours and trypanosomes were then injected into mice intraperitoneally. Parasitemia was monitored daily and parasites were found in all mice on the eighth (FBS) and tenth (NHS) day post-infection (n = 5). Arrows indicate when we were unable to detect a parasitaemia. Significant differences (paired-sample *t* test) were indicated by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Fig. 4. Trypanocidal effect of NHS on the *T. musculi* Partinico II strain *in vivo.* Infected mice, with a parasitemia of 10^6 trypanosomes/ml, were intraperitoneally injected with 0.5 ml fresh NHS. Parasitemia was then monitored daily (n = 5). Mice inoculated with FBS were used as a control. Parasites in the mice were found two weeks after injection with fresh NHS or FBS, but the parasitemia in the mice inoculated with NHS was found to be lower than those in the control group. Arrows indicate when we were unable to detect a parasitaemia. Significant differences (paired-sample *t* test) were indicated by asterisks (*, $p<0.05$; ***, $p<0.001$).

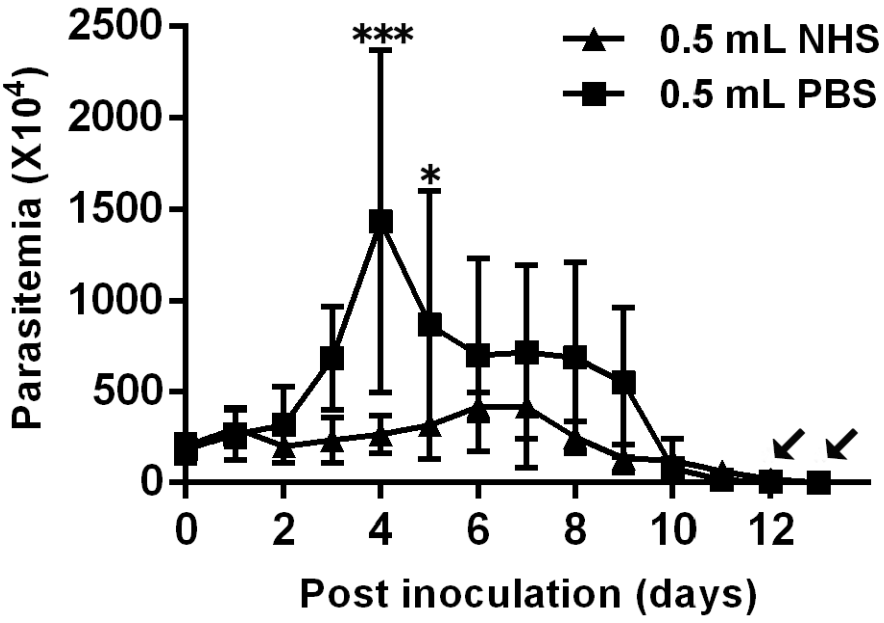
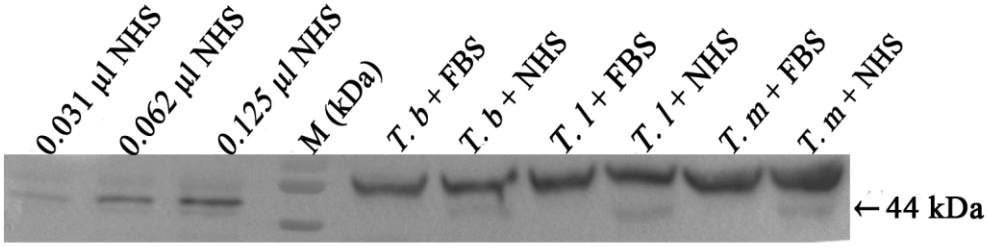
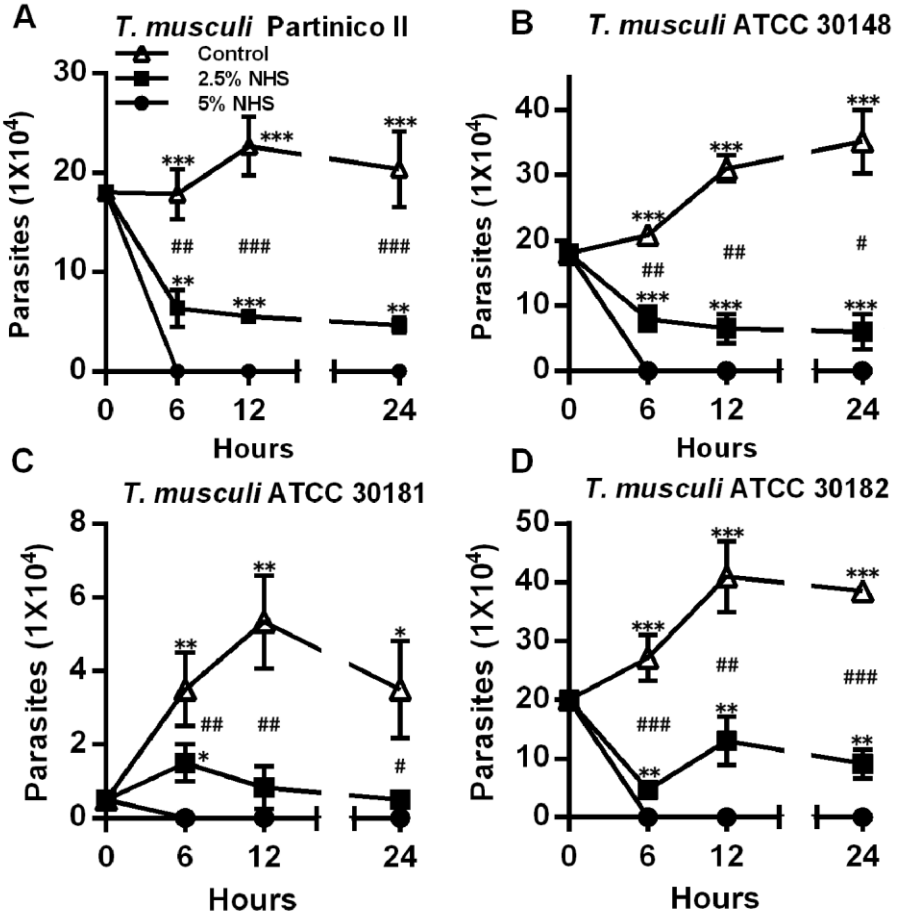


Fig. 5. Western Blot detecting APOL-1 protein in *T. musculi* Partinico II strain after incubation with NHS.



Parasites (5×10^6) were incubated in RPMI-1640 medium supplemented with 5% NHS or FBS at 37°C for 1 hour. All trypanosomes were harvested by centrifugation and washed twice with PBS and analyzed by SDS-PAGE. After blotting onto PVDF membrane, signals were detected using ApoL-1 polyclonal antibody (1:1000 dilution, Proteintech, China), HRP-conjugated 2nd antibody (Proteintech, China) and the reaction was detected using ECL (Thermofisher, USA).

Figure S1. *In vitro* trypanolytic effect of NHS on *T. musculi* strains of Partinico II (A), ATCC 30148 (B), ATCC 30181 (C) and ATCC 30182 (D)



Trypanosomes were incubated with RPMI-1640 medium complemented with a serial dilution of NHS (5%, 2.5%, and 0%) at 37°C with 5% CO_2. The numbers of trypanosomes were counted using a haemocytometer by microscopy. Each measurement was performed in triplicate and repeated three times (n = 3). Significant differences (independent-sample *t* test) of 5% NHS vs 0% NHS and 5% NHS vs 2.5% NHS were indicated by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Significant differences (independent-sample *t* test) of 0% NHS vs 2.5% NHS were indicated by hash symbols (#, $p<0.05$; ##, $p<0.01$; ###, $p<0.001$).

Table 1. Origin of trypanosomes used in this work and their characteristics of lysis by normal human serum (NHS) *in vitro* over 24 hrs.

Stocks	Host	Origin	Isolate year	Survival ratio in 2.5% NHS	Phenotype
T. b. brucei lister 427	*Bos taurus*	Tanzania	1956	0.9 ± 0.2%	S
T. musculi Partinico II	Unknown	Partinico Italy	1969	23.2 ± 0.3%	R/R
T. musculi ATCC 30148	Unknown	Unknown	Unknown	16.6 ± 4.9%	R/R
T. musculi ATCC 30181	*Mus domesticus*	Partinico Italy	1962	16.2 ± 7.6%	R/R
T. musculi ATCC 30182	*Mus musculus*	USA	Unknown	24.1 ± 0.4%	R/R
T. lewisi CPO02	*Rattus rattus*	China	2008	80.5 ± 4.6%	R
T. lewisi CPO04	*Rattus rattus*	China	2008	79.9 ± 6.8%*	R
T. lewisi CPO06	*Rattus rattus*	China	2008	88.0 ± 11.6%*	R
T. lewisi CPO07	*Rattus norvegicus*	China	2008	153 ± 16.8%*	R

S, sensitive to NHS; R/R, relatively resistant to NHS; R, resistant to NHS. Values of *in vitro* lysis are expressed as percentage survival and represent means of experiments and ± SD. Each measurement was performed in triplicate and repeated three times (n = 3). *, data were obtained from 10% NHS incubations (Lun et al., 2015).

Table S1. Statistics analysis of trypanolytic assays on *T. brucei*, *T. lewisi* and *T. musculi* with serial dilutions of NHS for a 3-24 hours incubation. P values between groups were shown. While $0.01<p\leq0.05$ were shadowed in black, $0.001<p\leq0.01$ were shadowed in white, $p\leq0.001$ were black boxed in white. Groups with cells und countable that were unable to be subjected to the student *t* test are indicated with "-".

T. brucei	*Incubation*	*0.31%NHS*	*0.63%NHS*	*1.25%NHS*	*2.5%NHS*	*5.0%NHS*	*10%NHS*
0%NHS	6h	p<0.001	p<0.001	p=0.005	p<0.001	p<0.001	p<0.001
	12h	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
	24h	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
0.31%NHS	6h		p=0.1186	p<0.001	p<0.001	p=0.004	p=0.160
	12h		p=0.3432	p<0.001	p<0.001	p=0.140	p=0.251
	24h		p=0.2614	p<0.001	p=0.060	p=0.269	p=0.193
0.63%NHS	6h			p=0.011	p=0.105	p=0.588	p=0.486
	12h			p<0.001	p=0.002	p=0.527	p=0.043
	24h			p<0.001	p=0.456	p=0.062	p=0.049
1.25%NHS	6h				p=0.072	p<0.001	p<0.001
	12h				p=0.118	p=0.002	p<0.001
	24h				p<0.001	p<0.001	p<0.001
2.5%NHS	6h					p=0.072	p=0.001
	12h					p=0.007	p<0.001
	24h					p=0.010	p=0.008
5.0%NHS	6h						p=0.054
	12h						p=0.016
	24h						p=0.332

T. lewisi	*Incubation*	*0.31%NHS*	*0.63%NHS*	*1.25%NHS*	*2.5%NHS*	*5.0%NHS*	*10%NHS*
0%NHS	6h	p=0.922	p=0.152	p=0.509	p=0.012	p<0.001	p<0.001
	12h	p=0.817	p=0.595	p=0.814	p=0.233	p=0.012	p<0.001
	24h	p=0.535	p=0.101	p=0.242	p=0.028	p=0.001	p<0.001
0.31%NHS	6h		p=0.225	p=0.552	p=0.031	p<0.001	p=0.001
	12h		p=0.743	p=0.984	p=0.310	p=0.016	p=0.251
	24h		p=0.259	p=0.513	p=0.065	p=0.002	p=0.193
0.63%NHS	6h			p=0.785	p=0.366	p=0.005	p=0.001
	12h			p=0.774	p=0.521	p=0.046	p=0.002
	24h			p=0.739	p=0.286	p=0.012	p<0.001
1.25%NHS	6h				p=0.351	p=0.018	p=0.003
	12h				p=0.354	p=0.026	p=0.001
	24h				p=0.233	p=0.017	p<0.001
2.5%NHS	6h					p=0.025	p=0.004
	12h					p=0.142	p=0.007
	24h					p=0.216	p=0.005
5.0%NHS	6h						p=0.107
	12h						p=0.128
	24h						p=0.035

T. musculi	Incubation	0.31%NHS	0.63%NHS	1.25%NHS	2.5%NHS	5.0%NHS	10.0%NHS
0%NHS	3h	p=0.727	p=0.010	p<0.001	p<0.001	p<0.001	p<0.001
	6h	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
	9h	p=0.024	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
	12h	p=0.005	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
	24h	p=0.500	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
0.31%NHS	3h		p=0.131	p<0.001	p<0.001	p<0.001	p<0.001
	6h		p=0.024	p<0.001	p<0.001	p<0.001	p<0.001
	9h		p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
	12h		p=0.002	p<0.001	p<0.001	p<0.001	p<0.001
	24h		p=0.010	p<0.001	p<0.001	p<0.001	p<0.001
0.63%NHS	3h			p<0.001	p<0.001	p<0.001	p<0.001
	6h			p<0.001	p<0.001	p<0.001	p<0.001
	9h			p<0.001	p<0.001	p<0.001	p<0.001
	12h			p<0.001	p<0.001	p<0.001	p<0.001
	24h			p<0.001	p<0.001	p<0.001	p<0.001
1.25%NHS	3h				p=0.001	p<0.001	p<0.001
	6h				p=0.015	p=0.015	p=0.015
	9h				p=0.003	p=0.003	p=0.003
	12h				p=0.003	p=0.003	p=0.003
	24h				p=0.006	p=0.006	p=0.006
2.5%NHS and 5.0%NHS	3h					-	-
	6h					-	-
	9h					-	-
	12h					-	-
	24h					-	-