

Haematophagic *Caenorhabditis elegans*

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ABSTRACT

Caenorhabditis elegans is a free-living nematode that resides in soil and typically feeds on bacteria. We postulate that haematophagic *C. elegans* could provide a model to evaluate vaccine responses to intestinal proteins from hematophagous nematode parasites, such as *Necator americanus*. Human erythrocytes, fluorescently labelled with tetramethylrhodamine succinimidyl ester, demonstrated a stable bright emission and facilitated visualization of feeding events with fluorescent microscopy. *C. elegans* were observed feeding on erythrocytes and were shown to rupture red blood cells upon capture to release and ingest their contents. In addition, *C. elegans* survived equally on a diet of erythrocytes. There was no statistically significant difference in survival when compared with a diet of *Escherichia coli* OP50. The enzymes responsible for the digestion and detoxification of haem and haemoglobin, which are key components of the hookworm vaccine, were found in the *C. elegans* intestine. These findings support our postulate that free-living nematodes could provide a model for the assessment of neutralizing antibodies to current and future hematophagous parasite vaccine candidates.

KEYWORDS: *Caenorhabditis elegans*, *Necator americanus*, aspartic proteinase, epitope, erythrocyte, glutathione-S-transferase, hematophagy, vaccine.

INTRODUCTION

'The blood is the life!' Dracula (Stoker, 1897)

Caenorhabditis elegans is a nonparasitic free-living nematode (Sulston, 1976). It has become a powerful tool to model complex biological processes in genetics (Brenner, 1974), neurology (Chalfie *et al.*, 1994) and cell survival (Adams and Cory, 1998), due to its relative ease of growth and maintenance (Stiernagle, 2006). More recently, *C. elegans* has been identified as a suitable model to study parasitic behaviour (Crisford *et al.*, 2013) and facilitate the discovery of anthelmintic drugs (Burns *et al.*, 2015).

Parasites infect a quarter of the global population (Bethony *et al.*, 2006). Infections have a negative impact on human health and productivity (Keiser and Utzinger, 2010) as well as economic output, due to the infection of crops (Fuller *et al.*, 2008) and livestock (Besier, 2007). Bearing this in mind international initiatives, such as the Human Hookworm Vaccine Initiative targeted against *Necator americanus* (Sabin Vaccine Institute, 2014), have been established to develop and test vaccines to prevent infection of humans. The complex life-cycles of parasitic nematodes, which rely on a host for propagation (Chauhan *et al.*, 2017), may serve as a barrier to the development of therapeutics to prevent and treat infections (Holden-Dye and Walker, 2007). Therefore, *C. elegans*, which has an easily maintained lifecycle (Lightfoot *et al.*, 2016) that is independent of host interaction, may provide an alternative model to study parasitic infections.

In the present study, we investigated if *C. elegans* could ingest and then survive on a diet of human erythrocytes. These experiments were performed as a prelude to nominating a hematophagous *C. elegans* as a model to further understand haem metabolism in nematodes, coupled with the interrogation of immune responses to vaccines currently under development, and to identify new vaccine candidate molecules involved in the intestinal biochemical pathways of hematophagous nematodes.

Aspartic proteinases (APRs) and glutathione-S-transferase (GST) have assumed prominence in vaccine development due to their ability to digest haemoglobin and neutralize the toxic by-products of haemoglobin digestion, respectively. In this context, the current vaccine under development to combat necatoriasis is bivalent (Hotez *et al.*, 2010), comprising of an aspartic haemoglobinase (*Na*-APR-1) and a GST (*Na*-GST-1) (Brophy and Pritchard, 1992; Brown *et al.*, 1995; Williamson *et al.*, 2002). These enzymes function in tandem in the hookworm gut to process human haemoglobin then detoxify haem. Furthermore, neutralizing antibodies raised to the hookworm enzymes have been linked to the protective capacity of the *Na*-APR-1 component of the vaccine, indicating that sequence identity around the active sites and other epitopic regions of the *C. elegans* and *N. americanus* enzymes could be of immunological relevance (Pearson *et al.*, 2010). Therefore, the demonstration of hematophagy in *C. elegans* would pave the way for unambiguous experiments to test the modes of action of these neutralizing antibodies and to search for new gastrointestinal tract associated vaccine candidates.

In order to observe the haematophagic *C. elegans*, erythrocytes were harvested from a human blood donor. Erythrocytes were labelled with the fluorophores [carboxyfluorescein succinimidyl ester (FAM-SE), fluorescein isothiocyanate (FITC) and tetramethylrhodamine succinimidyl ester (TAMRA-SE)] to facilitate the visualization of erythrocyte ingestion and digestion. The viability of *C. elegans* fed on erythrocytes alone, when compared with nematodes fed on *E. coli* alone and a mixture of erythrocytes and *E. coli*, was monitored as a function of their motility. Furthermore, databases were screened to identify if *C. elegans*, like the parasitic *N. americanus*, translated proteins capable of the enzymatic processing of haemoglobin.

EXPERIMENTAL

Materials

C. elegans Bristol N2 and *E. coli* OP50 were purchased from *Caenorhabditis* Genetics Center (CGC). Agar, protease peptone, cholesterol, gentamicin sulphate, EDTA, Alsever's, Dulbecco's and sodium hypochlorite solutions were obtained from Sigma-Aldrich (Gillingham, UK). Fluorescein isothiocyanate, carboxyfluorescein-SE, TAMRA-SE and BD Vacutainer® blood collection tubes were obtained from Thermo-Fisher Scientific (Loughborough, UK). Blood was donated by DIP (blood group B Rh negative). Deionized water (18.2 MΩ) was generated by Elga Purelab Ultra (ULXXXGEM2).

Methods

Blood collection

BD Vacutainer® tubes containing ethylenediaminetetraacetic acid (EDTA) (1.8 mg mL⁻¹ of blood) were used to collect blood from a healthy volunteer (4.5 mL). Initially, centrifugation was used to separate erythrocytes from plasma and leucocytes (800 rpm, 8 min). Erythrocytes were resuspended and washed a further 3 times in Alsever's solution using centrifugation (800 rpm, 8 min); discarding the supernatant, containing plasma and EDTA, and any remaining leucocytes and after each wash. After the final wash, the pellet was made up to 10 mL with Alsever's solution (~ 6 × 10⁸ cells mL⁻¹) and stored at 4 °C.

Establishing erythrocyte concentrations

Aliquots of the stock solution (10 µL) were serially diluted with Alsever's solution (90 µL), to create solutions at concentrations 6 × 10⁸, 6 × 10⁷, 6 × 10⁶, 6 × 10⁵, 6 × 10⁴, 6 × 10³, 6 × 10², 6 × 10¹ and 6 × 10⁰ cells mL⁻¹. Microscopy was used to determine a cell density that would permit free imaging of both erythrocytes and *C. elegans*.

Fluorescent labelling of erythrocytes

Erythrocytes (500 µL, 3 × 10⁸ cells mL⁻¹) were added to fluorescein isothiocyanate, carboxyfluorescein-SE or TAMRA-SE in Dulbecco's phosphate buffered saline solution (1 mg mL⁻¹, 500 µL, pH 8.0) and delicately inverted [2h, 1 rpm, HulaMixer™ (Invitrogen), 4 °C]. Erythrocytes were washed with Dulbecco's solution (15 × times, 10 mL⁻¹) and collected with centrifugation (800 rpm, 8 min). Labelling was confirmed with fluorescence microscopy using a Nikon Eclipse TE300 equipped with a Plan Fluor 40 × 0.75 NA objective and CoolLED pE-4000 and pE-100 light source. Labelled erythrocytes were suspended in Alsever's solution and stored in a light protected container at 4 °C.

Signal-to-noise ratio

The signal-to-noise ratio for erythrocytes was determined by drawing a line profile at the centre of erythrocytes labelled with either FAM-SE, FITC and TAMRA-SE. A Nikon Eclipse T1 and QIMAGING optiMOS camera equipped with CoolLED pE-4000 fluorescence illumination and pE-100 bright field illumination and 10 × (0.30 NA) objectives were used to image samples. Fluorescence was captured through excitation at 490 nm and 540 nm collecting at emission between 519 ± 26 nm 595 ± 33 nm (Exposure times 100 µs, LED power 50%). Images were analysed with FIJI open source software.

Growth and maintenance

Caenorhabditis elegans were maintained on NGM agar and *Escherichia coli* (OP50) at 20 °C. Synchronized growth cycles of *C. elegans* were prepared by harvesting eggs from gravid nematodes (Stiernagle, 2006). Briefly, high numbers of gravid nematodes were collected by rinsing NGM agar plates with ultra-pure sterile deionized water (4 mL). Sodium hydroxide (5 M, 0.5 mL) and sodium hypochlorite (5%, 1 mL) was added to the nematode suspension and vortexed (10 min) to release eggs and eliminate bacterial traces. The eggs were pelleted using centrifugation (1500 rpm, 1 min) and the supernatant discarded. The eggs were washed with ultra-pure sterile deionized water and collected using centrifugation (1500 rpm, 1 min). The egg suspensions were aspirated to 0.1 mL and *C. elegans*

and were plated on freshly prepared NGM agar seeded with an *E. coli* lawn and incubated at 20 °C. The generation time of *C. elegans* under these conditions was 4–5 days.

Dosing *C. elegans* with erythrocytes

Synchronized nematodes were collected by washing NGM plates with sterile deionized water. *C. elegans* were washed in deionized water (10 mL) and collected using centrifugation (3 times, 1500 rpm, 1 min). Nematodes were re-suspended in gentamicin (500 µg mL⁻¹, 10 mL, 30 min) to remove traces of *E. coli*. *C. elegans* were washed again in sterile deionized water (10 mL) and collected using centrifugation to remove traces of gentamicin (3 times, 1500 rpm, 1 min). Pelleted *C. elegans* were added to fluorescently labelled erythrocytes (1 × 10⁶ cells mL⁻¹, TAMRA). Observations of erythrocyte ingestion were made using fluorescence microscopy using AMG F1 Microscope equipped with an AMG Plan Fluor 10 × 1.2 NA objective and Epiphan DVI2USB 3.0 (30 fps, 1920 × 1200 pixels) to capture video. Aliquots of nematode and blood suspending media (10 µL) were added to sterile tryptone soya broth media and incubated overnight to check for microbial contamination.

Motility fraction half-life (*Mft*₅₀) derivation

To help describe the viability of nematode populations, using motility of nematode population as an indicator, the *Mft*₅₀, the time required to reduce the motility of population of nematodes by 50%, was derived for *C. elegans* treated with (1) erythrocytes, (2) *E. coli*, and (3) erythrocytes and *E. coli*. For each data point, 100 nematodes were evaluated in triplicate and the standard deviation was calculated. Statistical analyses were conducted using Student's t-test, where P < 0.05 indicated statistical significance.

Protein sequence analysis

The known sequences for *N. americanus* aspartic haemoglobinase (Necepsin II/*Na*-APR-1, Uniprot Q9N9H3) and GST (*Na*-GST-1, Uniprot D3U1A5) were searched on UniProt for sequence identity in *C. elegans*. Sequences were aligned using the National Institute for Health's National Center for Biotechnology Information blastp tool. The sequences were annotated, where available, with respect to the signal peptide sequence, active site aspartic acids using Clustal Omega.

RESULTS

Fluorescent labelling of erythrocytes

In order to observe the haematophagic *C. elegans*, erythrocytes were harvested from a human blood donor. Erythrocytes were separated from whole blood by centrifugation, using EDTA as an anticoagulant and stored in Alsever's solution, to enable preservation and long-term storage of erythrocytes (Li *et al.*, 2007). *C. elegans* and erythrocytes, when visualized using a brightfield microscope, are optically transparent, such that only refracted light, due the curvature of the nematode anatomy and torus geometry of the red blood cell, permits their visualization. Therefore, to enhance the contrast between *C. elegans* and erythrocytes and to augment visualization of hematophagy events using fluorescence microscopy, red blood cells were fluorescently labelled with either FAM-SE, FITC or TAMRA-SE. Succinimidyl esters and isothiocyanates readily conjugate to biological protein rich structures that contain amine functional groups, typically found in lysine residues, *via* stable carboxamide and thiourea bonds, respectively (Haugland, 2005).

FAM-SE, FITC and TAMRA-SE were all able to label erythrocytes (Fig. S1). TAMRA-SE demonstrated highly effective labelling of erythrocytes, Fig. 1A. This is because TAMRA-SE labelled erythrocytes, when subjected to the same excitation power and exposure time for imaging, demonstrated, 1.6 × and 6.7 × greater signal to noise ratio, when compared with FAM-SE and FITC labelled erythrocytes, respectively (Fig. 1B). These observations could be attributed to a combination of factors, which include greater labelling efficiency and stability of succinimidyl esters, when compared with

isothiocyanates (Banks and Paquette, 1995), and the superior quantum yield of TAMRA-SE in comparison with FAM-SE and FITC (Haugland, 2005). Furthermore, the utility of TAMRA-SE labelled erythrocytes permits imaging of *C. elegans* in the absence of unwanted substantial age-related green lipofuscin auto-fluorescence (Forge and Macguidwin, 1986; Pincus *et al.*, 2016) (Fig. 1C) and unwanted blue excitation light dependent phototaxis (Ward *et al.*, 2008), thus augmenting imaging capabilities of haematophagic events. This is because lipofuscin auto-fluorescence could generate unwanted imaging artefacts that could be interpreted as internalized erythrocytes. In addition, blue light, which is used to excite FAM-SE and FITC fluorescence emission, initiates heightened light dependent *C. elegans* motility that renders continual high frame imaging of haematophagic events challenging.

Observation of haematophagic events

To permit effective visualization of red blood cells and nematodes in a single field of view, erythrocyte counts were performed by serially diluting the stock solution. A concentration of 1×10^6 cells mL^{-1} was identified as a concentration that would enable observation of hematophagy events using fluorescence microscopy.

The occurrence of hematophagy was confirmed by feeding erythrocytes to axenic *C. elegans* for up to 24 h. Internalized erythrocytes were visualized in the pharynx and intestinal tract of nematodes after 5 h, Fig. 2. After 24 h, virtually all red blood cells had been consumed. From our observations, all stages of *C. elegans* were able to consume erythrocytes, but the labelling of the intestinal tract was limited to a select number of nematodes.

The diameter of an erythrocyte ($\sim 10 \mu\text{M}$, Fig. 1B) is approximately twice the diameter of an adult *C. elegans* mouth opening [$\sim 3\text{--}4 \mu\text{M}$ (Altun and Hall, 2018)] and more than 3 times the size of *E. coli* [$\sim 2\text{--}3 \mu\text{M}$ (Reshes *et al.*, 2008)], such that during feeding *C. elegans* could be unable to ingest whole red blood cells. Therefore, to decipher the mechanism of erythrocyte ingestion nematodes were continually imaged using fluorescence microscopy (see Supporting Movie 1). Time-lapse images (Fig. 3) show the ingestion of erythrocytes by *C. elegans* that occurs *via* a 5-step process: (1) *C. elegans* survey their immediate vicinity for sustenance (1.35 s). These observations highlight that *C. elegans* are capable of adapting, by modifying their mechanism of food ingestion, rather than limiting their diet to smaller bacterial organisms (Fang-Yen *et al.*, 2009).

The loss of erythrocyte integrity visualized during *C. elegans* digestion (Fig. 3, Supporting Movie 1) would release haemoglobin, which is toxic to organisms upon the release of haem. Therefore, to cope with potential haem toxicity, nematodes use enzymatic pathways that include APRs and GSTs to neutralize the toxic by-products of haemoglobin digestion (Perally *et al.*, 2008).

Viability of *C. elegans* during erythrocyte feeding

The motility of *C. elegans* can be used to predict viability as nematode body movement gradually declines and stops completely with age (Collins *et al.*, 2008). Using *C. elegans* motility as an absolute parameter, where motile and non-motile nematodes were classified as *viable* and *non-viable* the effects of restricting the nematode diet to erythrocytes alone, *E. coli* alone or a mixture of erythrocytes and *E. coli* was investigated. The motility fraction (*Mf*), as an indicator for *C. elegans* viability (Chauhan *et al.*, 2013), showed the three diets did not affect the overall *viability* (Fig. 4, $P > 0.05$) and were comparable with previously reported survivorship data (Wood *et al.*, 2004).

To determine the effect of different diets on the viability of nematodes, the Mft_{50} , the time required to reduce the motility of population of nematodes by 50%, was derived. The Mft_{50} for *C. elegans* fed on erythrocyte alone, *E. coli* alone and erythrocyte and *E. coli* were 7.07 (± 0.96 S.D.) days, 8.71 (± 0.13 S.D.) days and 7.68 (± 0.57 S.D.) days, respectively, and were not statistically different ($P > 0.05$).

Therefore, under an erythrocyte diet was not affected when compared with the control groups of *E. coli* and erythrocytes and *E. coli*.

Enzyme sequence identities between *C. elegans* and *N. americanus*

A high degree of sequence identity was confirmed. In particular, peptide A291Y, an epitope in *Na*-APR-1 (Necepsin II) recognized by enzyme neutralizing and host-protective antibodies, shares 71% identity and 10/13 active site amino acids with *C. elegans* Asp-4 (Fig. 5). The sequence identities between the respective GSTs are shown in Fig. S2.

DISCUSSION

The scientific community is keen to develop vaccines against parasitic nematodes of humans (Noon and Aroian, 2017) and livestock (Nisbet *et al.*, 2016). During vaccine development, APRs have assumed prominence given their ability to digest haemoglobin. It is apparent that neutralizing antibodies that interfere with the activity of these enzymes contribute to host protection. High levels of sequence identity, between enzymes involved in hematophagy, have also been identified with *Schistosoma mansoni*, *Onchocerca volvulus*, *Strongyloides stercoralis*, *Ancylostoma spp* and *Haemonchus contortus*. Therefore, the development of a high-throughput model, using a nematode species that is simple to manipulate, to investigate parasite hematophagy could become a high priority for the parasitological research community (Buckingham and Sattelle, 2009).

In the present paper, we have demonstrated that *C. elegans* were able to ingest and digest fluorescently labelled erythrocytes. Ingestion would appear to begin with erythrocyte rupture at the mouth and could be followed by mechanical degradation by the pharyngeal grinder (Avery and Thomas, 1997), with cell membrane rupture complemented by potential haemolysins, such as the *C. elegans* saponins and amoebapores (Banyai and Patthy, 1998), which are activated by a low pH microenvironment (McGhee, 2007). Haemoglobin digestion and haem detoxification could be conducted by *C. elegans* Aspartyl Protease 4 and GST.

At this stage, we feel that sufficient initial evidence has been attained to support experiments to investigate antibodies raised against protein homologues from parasites, to assess their effects on the blood feeding and associated viability and survival of *C. elegans*. Proof of principle data on the value of the model would pave the way for the exploration of new targets associated with haem metabolism in nematodes (Chen *et al.*, 2012; Sinclair and Hamza, 2015) and identify alternate gastrointestinal associated vaccine candidates

With respect to candidate selection, gastrointestinal associated molecules with corresponding homologues in parasites would be selected, then cloned and expressed, enabling the production of mono-specific antibodies against the candidate molecule. The ability of this antibody to inhibit hematophagy by *C. elegans*, and have a negative impact on its survival, would be indicative of the value of the target molecule as a potential vaccine candidate. For example, the intestinally expressed lipases (Behm, 2002) the NUC-1 nuclease (Lyon *et al.*, 2000), for processing cell free DNA from nucleated tissue cells and leucocytes, and calreticulin (CRT-1) could be considered as new vaccine candidates (Park *et al.*, 2001; Winter *et al.*, 2005).

In conclusion, Walker stated in 2005 that it was ‘difficult to disagree with the lament that unfortunately the biology of digestion (in *C. elegans*) represents something of a blind spot in this remarkably well-characterised organism’ (Walker *et al.*, 2005). The conversion of *C. elegans* to hematophagy will hopefully promote new interest in the functioning of its intestine, where parallels may be drawn with the biochemistry of hematophagic parasites.

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Author contributions

VMC and DIP conceptualized experiments and wrote the manuscript. VMC conducted experiments, prepared figures and supporting information.

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Conflict of interest

The authors declare no conflict of interest.

Ethical standards

Not applicable.

Supplementary material

The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182018001518>

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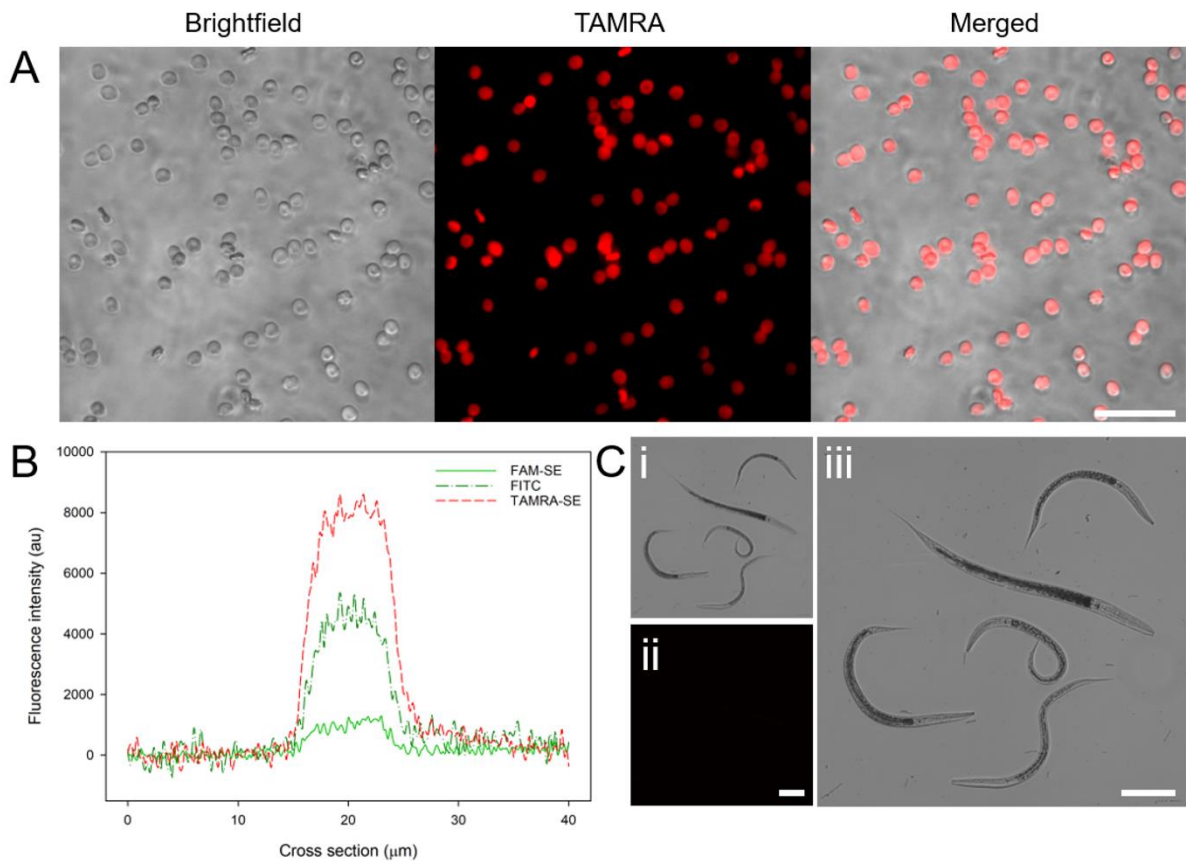


Fig. 1. (A) Brightfield, fluorescent red (TAMRA-SE) and merged images for TAMRA labelled red blood cells. (B) Cross-sectional intensity line plot across the centre of red blood cells labelled with carboxyfluorescein succinimidyl ester (FAM-SE), fluorescein isothiocyanate (FITC) and tetramethylrhodamine succinimidyl ester (TAMRA-SE). (Ci) Brightfield, (Cii) fluorescent red and (Ciii) merged image of *C. elegans*. Scale bars = 100 μm .

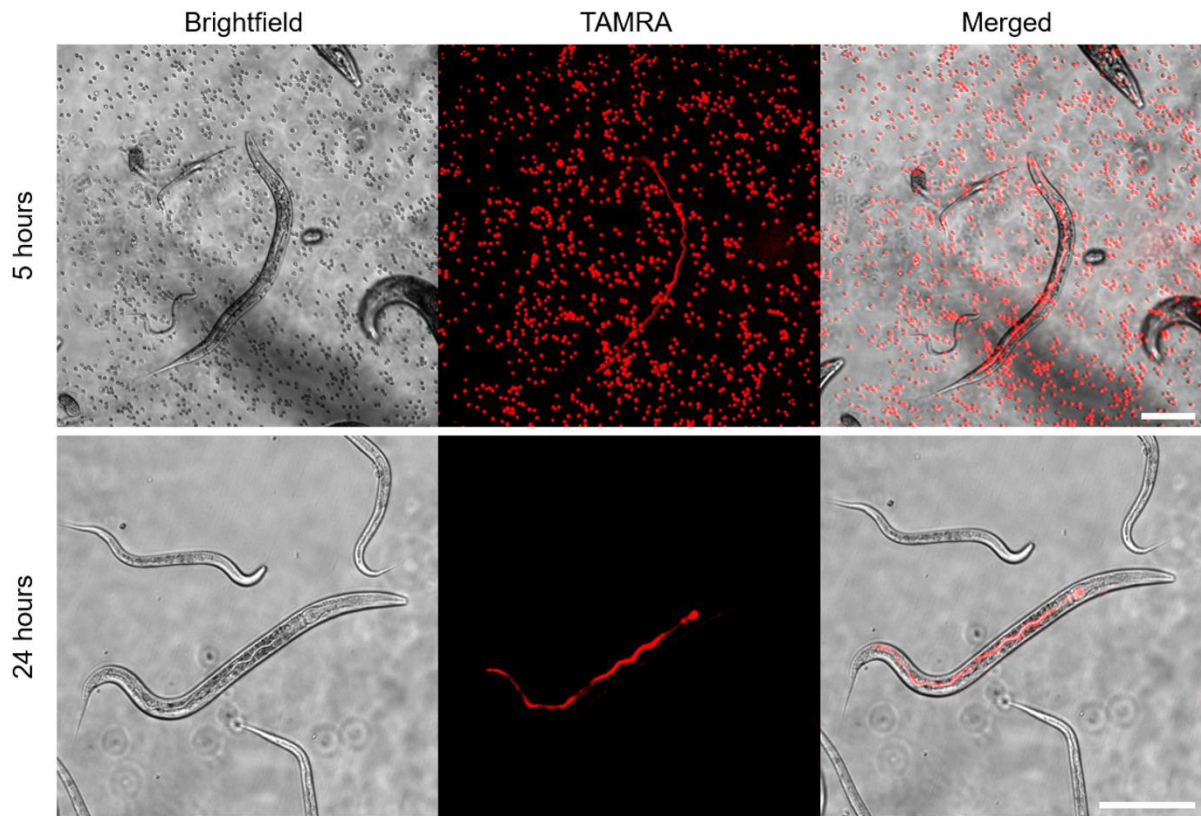


Fig. 2. Brightfield, fluorescent (TAMRA-SE) and merged images for *C. elegans* feeding on fluorescently labelled (TAMRA-SE) red blood cells at 5 h and 24 h after red blood cell introduction. Scale bars = 100 μm .

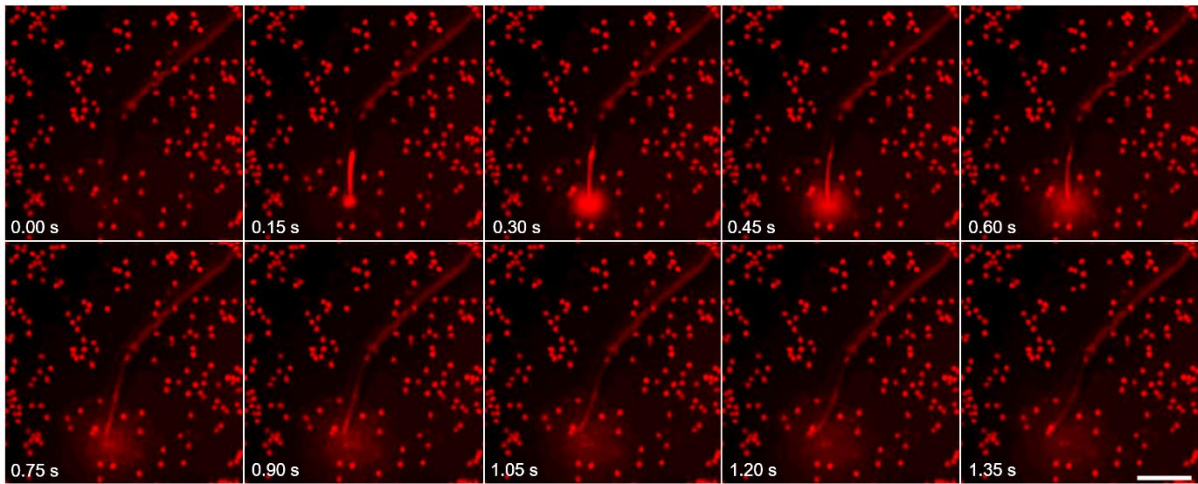


Fig. 3. Time-lapse images (0.00–1.35 s) for *C. elegans* ingesting fluorescently labelled red blood cells. For full-length video See Supporting Movie 1. Scale Bar = 100 μ m.

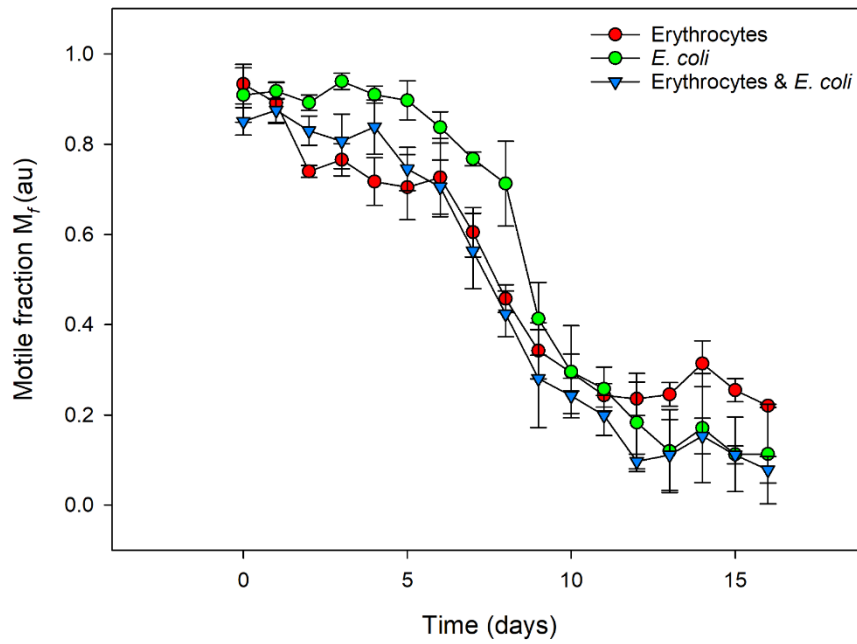


Fig. 4. Motility fraction as an indicator of nematode survival for *C. elegans* fed on erythrocytes (red), *E. coli* alone (green) and erythrocytes & *E. coli*, monitored for 15 days. Each data point evaluates the motility of ~100 nematodes each monitored in triplicate, where the error bars represent the standard deviation between experimental repeats.

A	SCORE	EXPECT	METHOD	IDENTITIES	POSITIVES	GAPS
	649 bits(1674)	8e ⁻⁷⁷	Compositional matrix adjusted	307/434(71%)	358/434(82%)	6/434(1%)

B										
<i>C. ele</i>	1	MNRCILLLLGALLLVQGLH	---	VHKRQQLR	TVSLKKQPTL	RETL	LLQAGSFET	FAKRRHG	57	
		M R + LL+ L +H H+ ++ + +VSL +QPTLRE L+ +GS+E + K R+								
<i>N. ame</i>	1	MARLVFLLVLC	TLAAASV	HRRLFHQARR	HVTSVLSRQPTL	RLRERLIAS	GSWEDYQKQRYH		60	
<i>C. ele</i>	58	YKKYLKTNGNHHFDKYQALNVEGE	IDELLRN	YMDAQYFGT	ISIGTPAQNF	TVIF	ITGSSN		117	
		Y+K K + +K L EIDELLRN	YMDAQY+G I IGTPAQNF	TVIFD	TGSSN					
<i>N. ame</i>	61	YRK--KILAKYAANKASKLQ	SANEIDELLRN	YMDAQYYGVIQ	IGTPAQNF	TVIF	ITGSSN		118	
<i>C. ele</i>	118	LWI	PSKCPFYDIACMLH	HRYDSKSS	TYKEDGRKMAI	QYGTGSMKGF	ISKDSVCVAGVC		177	
		LW+PS+KCPFYDIACMLH	RYDS +SSTYKEDGRKMAI	QYGTGSMKGF	ISKD	VC+AG+C				
<i>N. ame</i>	119	LWV	PSRKCPFYDIACMLH	RYDSGAS	TYKEDGRKMAI	QYGTGSMKGF	ISKDIVCIAGIC		178	
<i>C. ele</i>	178	AEDQPFAEATSEPGITF	VAAKFDGILG	MAYPEIAVL	GVQPVFNTL	LFEQKKVPS	NLFSFWL		237	
		AE+QPFAEATSEPG+TF+AAKFDGILG	MA+PEIAVLGV	PVF+T	EQKKVPS	+F+FWL				
<i>N. ame</i>	179	AEQ	QPFAEATSEPG	LTFIAAKFDGILG	MAFPEIAVL	GVTPVFHTFIE	QKKVSPVFAFWL		238	
<i>C. ele</i>	238	NRNPDSEIGGEIT	FGGIDSRRYVE	PITYVPVTR	KGYWQFKMD	KVV-GSGVLG	CSNGCQAI		296	
		NRNP+SEIGGEIT	FGG+D+RRYVE	PIT+ PVTR+GYWQ	FKMD V GS + C	NGCQAI				
<i>N. ame</i>	239	NRN	PESEIGGEIT	FGGVDTRYVE	PITWTPVTR	RGYWQFKMD	MVQGGSSSIACPNGCQAI		298	
<i>C. ele</i>	297	A	ITGTSLI	AGPKAQIEAIQNE	IGAEPLIKGEY	MISCDKVP	TLPPVSFVIGGQEFSLKGED		356	
		ADTGTSLI	AGPKAQ+EAIQ	+IGAEPL+KGEYMI	CDKVP+LP	VSF+I G+ F+LKGED				
<i>N. ame</i>	299	A	ITGTSLI	AGPKAQVEAIQKY	IGAEPLMKGEYMI	PCDKVPSLPD	VSFIDGKTFTLKGED		358	
<i>C. ele</i>	357	Y	LKVSQGGKTICLSG	FMGIDLPERV	GELWILGDV	FIGRYSV	VDFDQNRVGF	AQAKTAD	416	
		YVL V GK+ICLSG	FMG+D PE++GELWILGDV	FIG+YY+VFD	Q RVGFAQAK+ D					
<i>N. ame</i>	359	Y	LTVKAAGK	ICLSGFMG	MDFPEKIGEL	WILGDV	FIGKYTVF	VDVQARVGF	AQAKSED	418
<i>C. ele</i>	417	GRP	VDPAPRPFRSV	430						
		G PV R FR +								
<i>N. ame</i>	419	GFP	VGTPVRTFRQL	432						

Fig. 5. (A) Search criteria and (B) amino acid overlay for *C. elegans* (*C. ele*, ASpartyl Protease 4, Query) and *N. americanus* (*N. ame*, Necepsin II, Subject). Highlighted regions indicate leader sequence (grey), Aspartic active sites (pink) and target epitopes [*C. elegans* (green), Overlay (77% match, yellow), *N. americanus* (red)].