

Effect of *Anoplocephala perfoliata* derived toxins on the epithelial integrity, and the intestinal pathology of infected horses.

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## *Anoplocephala perfoliata.*

- The most prevalent of the three equine cestodes worldwide, affecting an estimated 50% of UK horses<sup>1</sup>.
- It has an indirect life cycle, spread by pasture dwelling Oribatid mites. The mite feeds on eggs excreted with the faeces and becomes infectious as the cysticercoid develops within it; horses at pasture inadvertently ingest the mite and become infected. The adult worm uses four suckers on the scolex to attach to the mucosa of the ileocaecal junction (Fig.1,2,3).
- At post mortem, immature worms are found attached diffusely in late summer to early autumn. Gravid adults are found clustered together in late spring to early summer, and are generally accompanied by more severe pathology (Fig.1).
- Once thought to be benign, we now recognise that infections can be severe and potentially fatal.

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1: Pittaway, C. E., A. L. Lawson, G. C. Coles, and A. D. Wilson. 2014. Systemic and mucosal IgE antibody responses of horses to infection with *Anoplocephala perfoliata*. *Veterinary Parasitology* 199: 32-41.

Figure 2: Blanchard, T.W. (1998). *A.perfoliata* attachment to intestinal mucosa [Pathology slide]. Retrieved from: <https://www.askjpc.org/wsco/wsc/wsc97/97wsc16.htm>

Figure 3: *Anoplocephala perfoliata*, H&E staining [Pathology slide].

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The pathology caused extends well past the point of attachment and increases in severity with burden level; from erythema & mucosal erosion to widespread oedema, immune cell infiltration, loss of mucosal structure and damage to the myenteric plexus (Fig.4). This is achieved through the production of toxins. **Burden level has a positive correlation with an increased risk for spasmodic and ileal impaction colic, as well as intussusception<sup>2</sup>.**

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40x magnification | H&E staining

2: Proudman, C. J., N. P. French, and A. J. Trees. 1998. Tapeworm infection is a significant risk factor for spasmodic colic and ileal impaction colic in the horse. *Equine Veterinary Journal* 30: 194-199.

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## *Anoplocephala perfoliata*: E/S antigens

- Like many parasites, immature and mature worms produce a range of excretory/secretory antigens (E/S antigens) that are thought to promote survival and mediate pathogenesis (Fig.5).
- The 12/13 kD antigen (Fig.5) is unique to *A. perfoliata* and used as a diagnostic marker in IgG<sub>(T)</sub> serum and saliva ELISAs.
- E/S antigens have been found to demonstrate cholinesterase, aminopeptidase and alkaline phosphatase activity.
- *A. perfoliata* modulates the host immune system to its benefit through E/S antigens.
  - The antiparasitic Th<sub>2</sub> response was found to be suppressed at the lamina propria of infected horses, and IL-2 transcription is reduced in concanavalin A stimulated equine peripheral blood lymphocytes exposed to E/S antigen<sup>3</sup>.
  - E/S antigens are cytotoxic to Jurkat E6 cells (laboratory models for T-lymphocytes).

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### Attempts to fractionate the antigens and isolate its activities.

Heat inactivation and dialysing against a 3.5 kD cut-off membrane did not remove the activity. Neither did ultrafiltration with a 3 kD cut-off membrane, though it did remove all proteins visible on SDS page. Reverse phase chromatography does not inhibit activity either, with maximal function restored at 75% MeOH. When testing filtrate and retentate from a 30 kD cut-off membrane, both fractions inhibited Jurkat viability.

Overall, data collected suggested that the active component is **small**, may be associated with a larger **carrier molecule** and is not necessarily a protein<sup>3</sup>.

**To date, the exact structure(s) and function(s) of the E/S antigens are unknown.**

Whilst there is an association with infection and colic, no causal relationship has been found. Even so, identifying the mechanism by which *A. perfoliata* modulates the immune system or any links it has with known causes of colic is in the interest of parasitologists. If the immunomodulatory mechanism is synthetically replicable then it may lead to the development of immunosuppressive drugs.

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## Project aims.

We aim to further current knowledge of the E/S antigens and their functions. Ongoing work aims to develop a reliable and practical diagnostic test to reduced the risk of anthelmintic resistance development within *A. perfoliata*; current options lack specificity, sensitivity and convenience so blanket treatment is often used. It also looks at identifying antigen structure and mechanism of action for assessment of pharmacological potential.

- To explore neuropathy as a mechanism by which *A. perfoliata* causes colic, through histological staining of healthy and infected gut tissue for acetylcholinesterase (AChE) activity.
- To test the presence of a larger carrier molecule by pre-treating the E/S antigen sample with methanol before fractionating by reverse phase chromatography. If there is an equilibrium of the active component bound to a carrier in an inactive state, the methanol should shift the equilibrium towards the unbound state and we should see increased activity.
- To expand and improve the data we have on the ability of E/S antigens to kill Jurkat cells.
- To investigate whether the yellow peak in the E/S antigen absorbance is associated with cytotoxicity to Jurkat cells.
- To use cell cycle analysis through quantitative flow cytometry to evaluate cellular arrest as a pathological mechanism of T-lymphocyte death.

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## Methods

### **Preparing the summer 2018 E/S antigen, and mucosal sample.**

*A. Perfoliata* was harvested in early August from the intestines of 5 freshly slaughtered horses at F. Drury & Sons abattoir, Swindon. The worms were washed in three changes of sterile PBS, placed in a sterile flask and covered with serum-free Gibco® RPMI 1640 without phenol red ([www.invitrogen.co.uk](http://www.invitrogen.co.uk)), containing 50 µg/ml gentamicin ([www.sigmaaldrich.com](http://www.sigmaaldrich.com)), and placed in a shaking incubator for 6 hours at 37 °C. Afterwards, the RPMI was removed and spun at 3000 g for 10 minutes. The supernatant was then filtered through a 0.2 µm mesh to sterilise. As a negative control, supernatant from a sample of healthy caecal mucosa near the ileocaecal valve of an infected horse was also prepared in the same way.

### **Maintenance of Jurkat cell line.**

Growth medium: Gibco® Advanced RPMI 1640 with laboratory-added 5 % FCS, 2 mM glutamine ([www.invitrogen.co.uk](http://www.invitrogen.co.uk)).

Jurkat E6 Cells ([www.atcc.org](http://www.atcc.org)) were seeded at 10<sup>5</sup> cells/ml into a 25 cm<sup>2</sup> cell culture flask ([www.sigmaaldrich.com](http://www.sigmaaldrich.com)) with 10 ml RPMI, and split 1:10 with fresh RPMI every 3-4 days when the suspension began to look cloudy.

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## Methods

### **Exposing Jurkat E6 cells to E/S antigen and mucosal supernatant.**

Using flat-bottomed 96 well plates ([www.sigmaaldrich.com](http://www.sigmaaldrich.com)), triplicate columns of wells were filled with 100 µl RPMI. Then, serial dilutions (using 100 µl of E/S antigen or mucosal supernatant) were done to one triplicate column (Fig.8) before adding 100 µl of  $10^5$  cells/ml. Plates were incubated at 37 °C for 3-4 days. The empty outer wells were filled with 200 µl of sterile water to lessen the effects of evaporation. At final dilution, the concentration of E/S antigen in the wells ranged from 1:10 to 1:320.

### **Exposing Jurkat E6 cells to LPS/IL-2, +/- E/S antigen.**

**LPS** (*Escherichia coli* 0111:B4, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)): final dilutions/ concentrations of 1:40 (25 µg/ml) to 1:640 (1.5625 µg/ml) or 1:1280 (0.78125 µg/ml), or added to the cell suspension to get a constant final concentration of 5 µg/ml.

**IL-2** (recombinant human, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)) added to the cell suspension to get a constant, final concentration of 20 units/ml.

**Measuring LPS content** - Pierce™ LAL Chromogenic Endotoxin Quantitation Kit protocol ([www.thermofisher.com](http://www.thermofisher.com)).

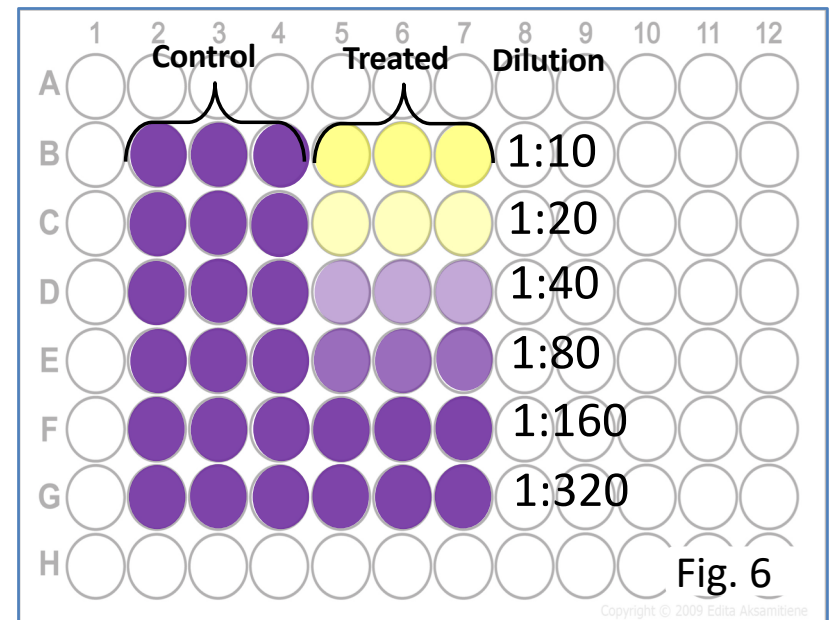


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## Methods

### Measuring Jurkat viability.

The abcam® MTT assay protocol for non-adherent cells was used to measure Jurkat cell viability ([www.abcam.com](http://www.abcam.com)). This assay quantifiably measures the amount of purple formazan dye produced by metabolically active viable cells reducing the MTT reagent, through reading the absorbance at 570 nm (Fig.6). OD values are standardised to % average control on that plate.



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## Methods

### **Cell cycle analysis using flow cytometry.**

Using a 12 well flat-bottomed plate ([www.sigmaaldrich.com](http://www.sigmaaldrich.com)), 1 ml of  $10^6$  cells/ml was added to eight wells. Two control wells, three with a final concentration of 1:20 summer 2011 E/S antigen and three with a final concentration of 1:10 autumn 2018 E/S antigen. Cells treated with antigen were harvested at 24, 48 and 72 hours of incubation at 37 °C, the controls were harvested at 24 and 48 hours; the samples were fixed in 70 % ethanol and stained with propidium iodide (PI) using the protocol outlined by University College London ([www.ucl.ac.uk](http://www.ucl.ac.uk)). The samples were stored at -4 °C for five weeks until the flow cytometer was available for use.

A NovoCyte® flow cytometer ([www.aceabio.com](http://www.aceabio.com)) was used to quantitatively measure cell cycle distribution, by measuring PI in a linear scale. Forward and side scatter identified the single cells and pulse shape analysis identified doublets and debris; the gates were combined and applied to the PI histogram.

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## Methods

### **Reverse phase chromatography.**

- Two columns were run, one with 10 ml of E/S antigen containing MeOH at 20 % and one containing deionized water at 20 %. Both samples were spun at 15000 g for 10 minutes prior to use.
- Supelclean™ Ultra C18 columns ([www.sigmaalrich.com](http://www.sigmaalrich.com)) were washed with 2 ml 100 % MeOH, 2 ml sterile PBS and 2 ml deionized water. Then 1 ml of E/S antigen sample was added at a time, the first 5 drops were discarded. The columns were then washed with 2 ml PBS then 2 ml of 25 %, 50 %, 75% and 100 % MeOH, the first 5 drops being discarded each time.
- 1 ml of each of the 7 samples (E/S antigen sample, flow through, PBS wash, 25 %, 50 %, 75 % and, 100 % MeOH) were freeze-dried and reconstituted in 1 ml of serum-free Gibco® RPMI 1640 without phenol red, to remove the MeOH.
- Each one was then spun at 10000 g for 5 minutes in a sterile Eppendorf. Jurkats were exposed in the same way as before and the MTS assay measured any retention of activity in the samples.
- Each methanol wash was also spectrophotometrically analysed using a FLUOstar® Omega.

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## Methods

### **Enzyme histochemistry.**

Samples of caecal mucosa near the ileocaecal valve were taken from infected and uninfected horses. The samples were fixed in paraffin wax and slides prepared using standard histological techniques. After dewaxing, endogenous peroxidase was quenched by submerging in methanol with 0.3 % H<sub>2</sub>O<sub>2</sub> for 10 minutes. Slides were then stained with MBL® Acetylcholinesterase Rapid Staining Kit ([www.mblintl.com](http://www.mblintl.com)) and counterstained with haematoxylin and eosin before dehydration and mounting.

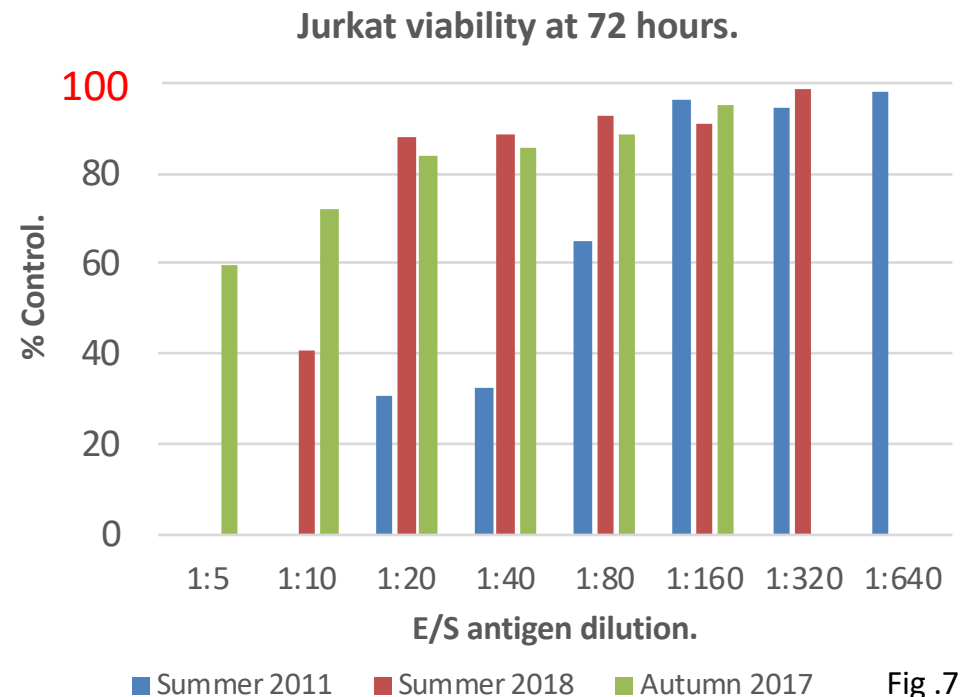
Some slides of healthy tissue were also submerged in either E/S antigen or PBS after dewaxing, then incubated at 37 °C for 30 minutes before staining.

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## Results – effects on Jurkat viability.

Every sample collected so far demonstrates a consistent dose-response, of decreasing Jurkat inhibition with increasing sample dilution, and a delayed pattern of death peaking at around 72 hours (Fig.7). The degree of inhibition varies with the condition of the original culture, Jurkat cells are more resistant to killing when cultured at higher densities, demonstrating some kind of community effect.

Growth of Jurkat cells is also affected by well conditions. Despite filling the outer wells with water, some wells had a visibly reduced volume. And, when using media with phenol red, were visibly more acidic. Future work aims to reduce these effects and build a large and reproducible dataset.



E/S antigen sample preparation has not been standardised between projects. The 2011 sample has always been far more cytotoxic but this has not been reproducible. Future work aims to correct this.

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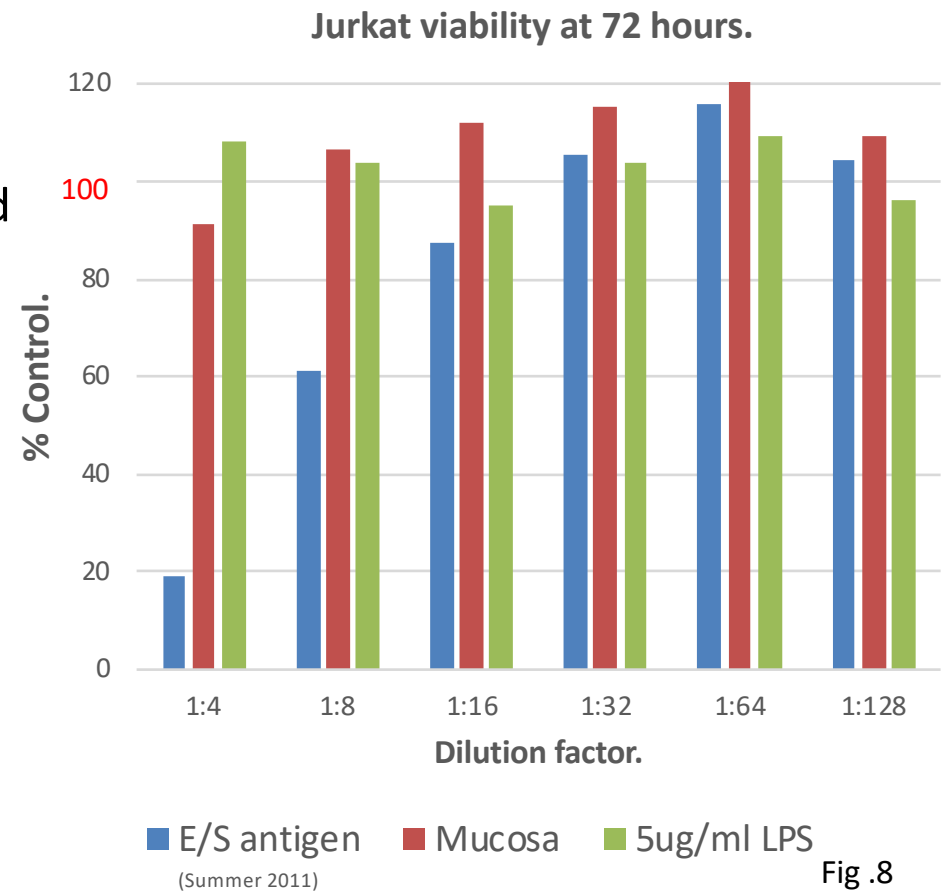
## Results – is cytotoxicity due to the antigens?.

A referee questioned the roles of mucosal factors and symbiotic or contaminating bacteria as confounding factors in the effects we demonstrate. The mucosal preparation was used as a negative control and showed little evidence of cytotoxicity (Fig.8).

The Lipopolysaccharide (LPS) content of undiluted summer 2011 was found to be 0.794 µg/ml (data not shown). Furthermore, 5 µg/ml LPS did not significantly affect Jurkat viability (Fig.8).

These results support the claim that the toxicity to Jurkat cells and the immunomodulation seen in the mucosa of infected horses is due to the presence of *A. perfoliata*, whether that be as a direct or indirect result.

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## Results – cytotoxic mechanism.

Cells treated with E/S antigen had an increased % population in the sub-G1 phase, showing apoptosis. %SubG1 increased with time, all summer 2011 treated cells were dead by 72 hours. Structural and functional similarities to small immunosuppressive drugs such as cyclosporine<sup>5</sup> (arrests T lymphocytes at G0/G1 and inhibits IL-2 expression) or colchicine<sup>6</sup> (yellow, and arrests T lymphocytes at G2/M) prompted this work, however we found no evidence of cellular arrest significant enough to account for the cell death seen at any point.

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5: Naujokat, C., V. Daniel, T. M. Bauer, M. Sadeghi, and G. Opelz. 2003. Cell cycle- and activation-dependent regulation of cyclosporin A-induced T cell apoptosis. *Biochemical and Biophysical Research Communications* 310: 347-354.

6: Kim, S. K., S. M. Cho, H. Kim, H. Seok, S. O. Kim, T. K. Kwon, and J. S. Chang. 2013. The colchicine derivative CT20126 shows a novel microtubule-modulating activity with apoptosis. *Experimental and Molecular Medicine* 45.

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## Results – cytotoxic mechanism.

We aren't seeing sudden cell death so there must be certain conditions, other than antigen exposure, which allows or promotes its induction. It is possible that between the 48 and 72 hour sample, the E/S antigen treated cells may have been arrested and died before they could be analysed; perhaps future work can measure samples at more frequent time points.

As IL-2 transcription is inhibited in stimulated equine lymphocytes, it was speculated that lymphocyte death was simply due to lack of growth factor. Exogenous IL-2 did not rescue the Jurkats (data not shown); however Jurkats are essentially leukaemic cells, so may behave unexpectedly. This experiment is best repeated using peripheral blood lymphocytes collected at slaughter.



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## Results – physical properties.

Cytotoxicity is present in both the flow-through (F.T.) and methanol washes, and maximum ability to induce Jurkat cell death returns in the 75 % fraction (Fig.10). This is consistent with previous work, and what would be expected if the active molecule was in an equilibrium between an unbound state and inactively bound to a more hydrophilic carrier.

We found no significant difference between the methylated and non-methylated samples. It is not yet clear whether this shifts the equilibrium or not, further repeats are needed.

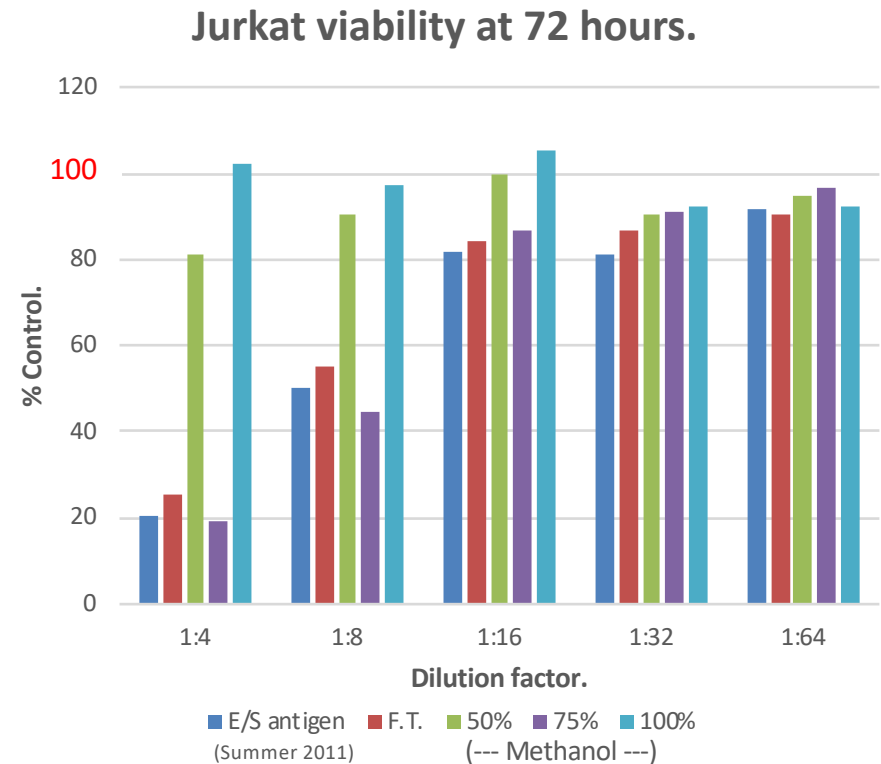


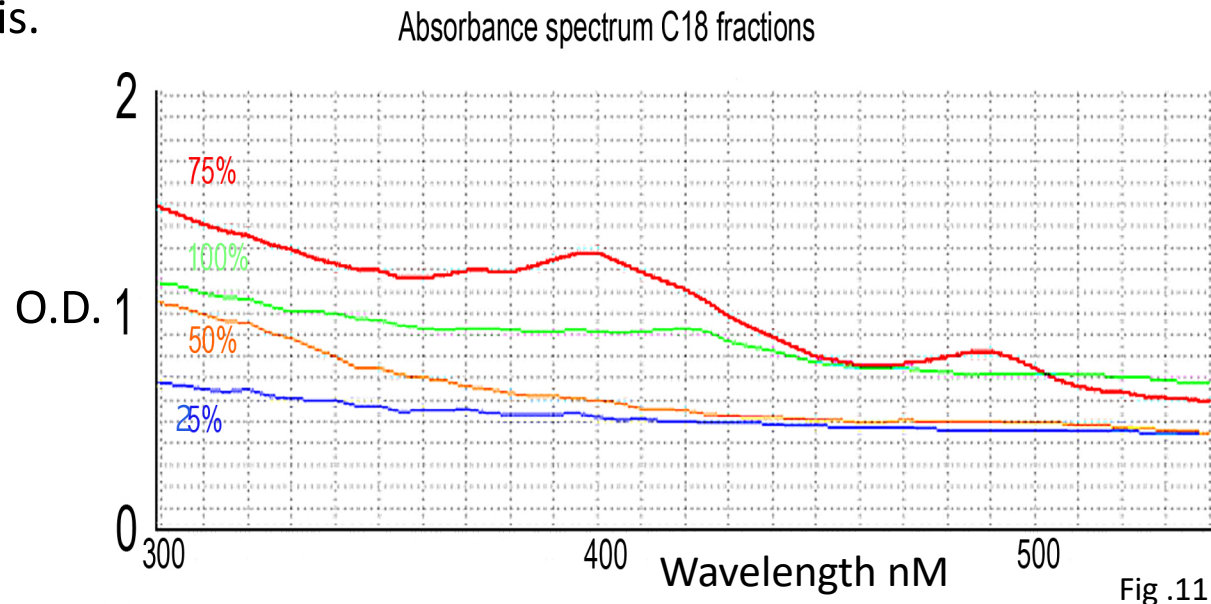
Fig.10: Non-methylated sample.

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## Results – physical properties.

Previous work found an unexpected peak in the violet absorbance range that was not present in the culture media, indicating a yellow colour in the whole E/S antigen sample. When repeated using the fractions obtained from reverse phase chromatography, we found that there are two large peaks (in the violet, and blue/green range) present only in the 75% fraction (Fig.11). Maximal cytotoxic function in the E/S antigen is restored in the 75 % fraction, we might associate the yellow or red/orange colouring with the function. Further work is needed to validate this.

These promising results indicate a more discriminating way to monitor the isolation of the active substance, enabling identification via mass spectroscopy.



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## Results - colic.

Initial results were as expected, strong staining (brown in colour) seen in muscle layers (Fig. 12a, A), blood vessel walls (B) and weaker staining in nerve ganglia (C). However, no real difference was observed between the uninfected and infected tissues (not shown). Upon first look, slides treated with summer 2011 E/S antigen appeared to have significantly reduced AChE activity (Fig.12b), but on further investigation it was realised that the inhibition may be due to the low pH of the sample (5.6)<sup>4</sup>. When buffered to pH 7.4, to match PBS, the inhibition was not seen. These results do not support the argument that E/S antigen alters AChE expression or activity as a pathological mechanism.

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4: Dziri, L., Boussaad, S., Tao, N., Leblanc, R. m. 1998. Effect of pH on acetylcholinesterase Langmuir and Langmuir-Blodgett films studied by surface potential and atomic force microscopy. Thin Solid Films 327-329.

## Discussion.

Jurkat cell death is induced within 72 hours of exposure to E/S antigens, the death was not found to be due to the LPS content of the sample or through mucosal factors. This backs the claim that the E/S antigens themselves are immunomodulatory and cause pathology in the ileocaecal mucosa, whether that be through direct or indirect mechanisms.

We also conclude that induction of cellular arrest is not responsible for the delayed Jurkat cell death that is seen, but repeating this experiment at more frequent time points would be beneficial. Lack of growth factor IL-2 is not the cause of Jurkat cell death, though this experiment is not representative of equine T-lymphocytes.

We have found that the ability of E/S antigens to induce jurkat cell death may be associated with a large peak in the absorbance range indicating a yellow/orange/red colour and that the active cytotoxic antigen is likely to be in equilibrium with a state bound to a larger, more hydrophilic, carrier.

We have found no evidence supporting neuropathy, through inhibition of AChE expression or activity, as a pathological mechanism for colic.

This work will provide the basis for future projects with the aim to identify pathological mechanisms and isolation of the active component of *Anoplocephala perfoliata* excretory/secretory antigens.

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