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Efficacy of TK Swordsman disinfectant when inactivating *Cryptosporidium parvum* oocysts.

Validation Study Report

Moredun Scientific Limited Study Number: MS1320-C

Study Location:

Moredun Scientific Limited (MSL)
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1. Objective

Moredun Scientific was contracted by Shepherd Animal Health to conduct a study to test the efficacy of the TK Swordsman disinfectant (herein named "TK Swordsman") when rendering *Cryptosporidium parvum* oocysts non-infectious, following exposure.

2. Justification

The study client wishes to confirm that the TK Swordsman is effective in the reduction of *C.parvum* oocysts infectivity, within a set exposure time, as to be adhered to within this study.

2. Test Material

TK Swordsman disinfectant sample (Shepherd Animal Health, Sample)

Cryptosporidium parvum oocyst suspensions (181118 IOWA Isolate, University of Arizona)

4. Experimental Design

4.1 Moredun Scientific were contracted by Shepherd Animal Health to conduct a study to test the efficacy of their "TK Swordsman" disinfectant (supplied by client) with regards to rendering *Cryptosporidium parvum* oocysts non-infectious following exposure.

In order to determine the effectiveness of the product a known number of oocysts were exposed to the disinfectant, specifically in a foaming state (as requested by sponsor), for a set period of time. In total five replicates of testing were carried out, along with a control, to allow accurate estimation of the disinfection efficacy. Following this, oocysts were observed using a microscope and an existing excystation protocol, commonly used at Moredun Scientific, was utilized on the samples to determine outcome of the disinfectant's effects on *C.parvum* oocyst viability/damage.

4.2 Before commencing work, approximately $1.5x10^6$ (150µl of stock) *C. parvum* oocysts were aliquoted into each of the six, 50ml centrifuge tubes. 25ml of disinfectant at a 1/30 dilution, was added to 5 of the 6 tubes, in a foam form.

The final tube contained *C.parvum* oocysts along with ultrapure water only (as used to dilute the disinfectant), which was tested in parallel with disinfectant treated samples. Each tube was incubated at room temperature for 12 hours.

4.3 Following the incubation period, disinfectant was removed from samples prior to excystation as this could have interfered with the process. To remove disinfectant foam, 25ml of ultrapure water was added to each 25ml sample. The tubes were then centrifuged at 500g for 15 minutes, and the supernatant was removed so that only 2-3ml remained. This washing process was repeated 3 times to ensure that the disinfectant had been reduced to a low dilution level. Following this a 10µl aliquot from each sample was placed onto a clean microscope slide and viewed at x40 magnification using a DIC Microscope, to assess damage to oocyst structure/integrity caused by the exposure. The supernatant from each sample was carefully removed down to a level which could fit into a 0.4ml

Eppendorf tube for the excystation assay. When removing this final supernatant care was taken to ensure that the pellet was not disturbed. If it was disturbed then the sample was centrifuged again.

4.4 The 0.4ml Eppendorf tubes containing the samples were centrifuged at 500g for 7 minutes. The supernatant from the pellet was carefully removed and discarded into an appropriate waste container. Care was taken to avoid disruption of the pellet in the bottom of the 0.4ml tube. If the pellet was disturbed then the tube was centrifuged again. The pellet was then re-suspended in 40µl of Hanks Buffered Salt Solution (HBSS).

50µl of 1% Trypsin (pH 3.0) was added to the re-suspended pellet and this was vortexed thoroughly. The pH of the trypsin, was tested prior to use with pH indicator paper, and was adjusted as required with 2% Hydrochloric Acid or 2.2% Sodium Bicarbonate.

The samples were incubated at +37 °C for 60 minutes in a water bath. This water bath contained a calibrated thermometer and was allowed to stabilize at the required temperature before use. Prior to use the thermometer was reset and the 0.4ml Eppendorf placed into the water bath housed in a float.

Following incubation, the mixture was centrifuged at 500g for 7 minutes and the supernatant was discarded, with care taken as to not disturb the pellet. The pellet was re-suspended in 90µl HBSS. 10µl of 2.2% Sodium Bicarbonate and 10µl of 1% Sodium Deoxycholate (in HBSS) was added to the suspension and was vortexed thoroughly.

The samples were incubated at +37°C in a water bath containing a calibrated thermometer for 40 minutes. The same process as detailed above with regards to resetting the thermometer before placing the 0.4ml Eppendorf tube into the float inside the bath was then followed.

After incubation the samples were immediately removed from the water bath and then centrifuged at 500g for 7 minutes with the supernatant discarded on completion. The pellets were resuspended in 50µl of Phosphate Buffered Saline (PBS) for examination.

Using a pipette, 10µl of the suspensions were added to a clean microscope slide which was gently covered with a cover slip. The edges of the cover slip were coated with nail varnish to prevent movement of the specimen caused by evaporation and using an appropriate microscope, the specimen was examined using a x40 objective, with the number of shells and oocysts recorded.

4.5 Calculation of the percentage viability and sporozoite to shell ratio was carried out using the equations below:

$$\text{Percentage Excystation (Viability)} = \frac{\text{No. of shells + partially excysted oocysts}}{\text{Total No. of oocysts (shells + oocysts)}} \text{ X 100}$$

$$\text{Sporozoite Ratio} = \frac{\text{No. of free sporozoite}}{\text{No. of shells + partially excysted oocysts}}$$

5. Experimental Acceptance Criteria

5.1 Acceptance criteria within this study was that, following exposure of *C.parvum* oocysts to TK Swordsman disinfectant foam, the oocysts would not excyst when an in-vitro excystation assay was performed on them. Furthermore it was expected that on observation, the oocysts would be damaged.

The excystation assay that was used evaluates oocyst quality, in the form of viability percentage (the number of oocysts which are alive, thus able to excyst) and sporozoite ratio (the number of sporozoites which each oocyst releases on excystation). Viability percentage is generally expected to be 80%+ for healthy oocysts. The value for sporozoite ratio can only exist between 0-4, as each oocyst only contains 4 sporozoites, and can therefore only release a maximum of 4 sporozoites. Generally a value of >2-3.5 is expected for healthy oocysts within this calculation.

7. Results

7.1 Results of the study can be seen below.

Observations using Olympus BX50F Microscope with DIC optics at x40 magnification

Observations from viewing the oocysts under magnification revealed that there did appear to be damage to the oocysts following exposure to the TK Swordsman disinfectant. Oocysts were mostly still intact, however the majority of intact oocysts contained contents which were visibly retracting from the outer shell. Some of these oocysts were also observed to have ruptured following exposure, with internal structures spilling completely or partially out of the shell.

In-vitro excystation assay to determine oocysts viability/sporozoite ratio

Results in Table 1 confirm that the ability of the oocysts to excyst was not affected by the exposure to the disinfectant. The contents of the oocysts however, did appear damaged by the TK Swordsman exposure, as observed above. The numbers of sporozoites identifiable following excystation were dramatically reduced, or entirely absent following exposure to the disinfectant.. The viability of the oocysts initially appears to be of high levels (71.3%-87.7%), however this

viability most likely only applies to the ability to actually excyst due to environmental cues (replicated by assay) and is not in this case indicative of the actual infectivity level of the oocysts themselves. The control sample results were of the expected quality level, confirming that untreated oocysts were all of a high quality/viability. Intact oocysts in the disinfected samples were also observed in higher numbers, suggesting that these oocysts were killed by the disinfectant or at the least their ability to excyst was compromised by the disinfectant, meaning they were non-infectious regardless.

Table 1. In-Vitro Excystation Results

Replicate Number	No. of Intact Oocysts	No. of Partially intact oocysts + shells	No. of Sporozoites	Percentage Viability	Sporozoite Ratio
1	39	234	1	85.7%	0.004
2	33	237	6	87.7%	0.025
3	84	227	0	72.9%	0
4	94	234	0	71.3%	0
5	37	217	3	85.4%	0.013
Control	7	76	209	91.6%	2.75

Results Summary

Although the experimental acceptance criteria for this study was not met, and oocysts did excyst following exposure to the disinfectant; from the above results it would appear that the disinfectant "TK Swordsman", created by Shepherd Animal Health, does have an ability to damage the internal contents of oocysts (when exposed for at least 12 hours as determined by this study). Intact sporozoites, which are responsible for infection once the parasite excysts within the host gut, were found to be badly damaged/destroyed. This means that when sporozoites were released by the oocysts in response to environmental cues (replicated by the study assay), radically reduced numbers of healthy sporozoites were able to be released intact, reducing oocyst infectivity.

Further in-vivo work would be required to determine whether the excysted sporozoites are still infective, as there is no in-vitro method to determine infectivity of these currently.

However purely based on this in-vitro assay, which shows a radically reduced sporozoite ratio in comparison to the control, the results would suggest that "TK Swordsman" could be an effective method to reduce *Cryptosporidium parvum* infectivity, following a set exposure time (12 hours minimum).

10. Archiving of Raw Data

All raw data generated during the study will be retained in the Moredun Scientific archive for a period of three years

12. Approvals

Signed on Behalf of Moredun Scientific Limited

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Ben Horton, BSc (Hons), MSc — Date

Study Director