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The Use of the Xylose Tolerance Test as an Indicator of Malabsorption in Cryptosporidiosis

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THE USE OF THE XYLOSE TOLERANCE TEST AS AN INDICATOR OF MALABSORPTION IN CRYPTOSPORIDIOSIS

BY

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HONORS 480 RESEARCH PROJECT PRESENTATION

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ABSTRACT: *Cryptosporidium parvum*, a protozoan parasite infecting epithelial cells lining the intestinal tract of animals and humans, causes fulminate diarrhea and malabsorption of essential nutrients following damage to the cellular brush border. The present study was undertaken to evaluate the use of a pentose sugar (D-xylose) in a standardized tolerance test as an indicator of malabsorption due to cryptosporidiosis in the dexamethasone (DEX)-immunosuppressed adult C57BL/6N mouse model. One group containing 14 experimental mice (in addition to appropriate control groups) was immunosuppressed using daily intraperitoneal injections of DEX (125 µg/mouse). On day 7 postimmunosuppression, mice were inoculated orally with 10^4 *C. parvum* oocysts/mouse. Approximately 1 month postinoculation, feces from individual mice were checked for oocyst

shedding using an indirect immunofluorescent assay to confirm patent infections. After 24 hours of fasting, infected mice were administered D-xylose (2.5 mg/g body weight) by gavage. Mice were killed at either 45 or 90 minutes post-D-xylose administration, blood was collected from the chest cavity, and the serum was harvested. Sections of the proximal duodenum and distal ileum of each mouse were histologically prepared for quantitation of *C. parvum* using brightfield microscopy. Serum was analyzed with a spectrophotometer (520 nm) by employing a calorimetric reaction as a measure of the absorptive capacity of D-xylose in the small intestine of *C. parvum*-infected mice. Statistical analysis revealed no significant correlation between the number of parasites present and intestinal absorption of D-xylose. However, administration of DEX was positively correlated to D-xylose absorption. These findings indicate that the xylose tolerance test is inappropriate in the adult C57BL/6N mouse model for evaluating malabsorption due to cryptosporidiosis in the presence of DEX.

INTRODUCTION

Cryptosporidium parvum is a widespread protozoan parasite shown to cause diarrhea in humans and animals. A common route of infection is through fecal-oral means (17). Diarrhea ranges from moderately severe in immunocompetent individuals to a life threatening, cholera-like illness in immunocompromised individuals. This parasite has recently received greater attention due to its impact on patients with acquired immunodeficiency syndrome. *C. parvum* commonly manifests itself with clinical symptoms of fever associated with abdominal pain and chronic diarrhea causing weight loss (3). The differences between immunocompromised host and immunocompetent host reactions to *C. parvum* infection suggest that significant differences exist between intestinal tract responsiveness of the two groups.

Currently there are no effective treatments for *C. parvum* infection and consequently an accelerated effort is underway to develop methods of studying the organism. Improved understanding of *C. parvum* will certainly help develop effective treatments in the near future. *In vitro* and *in vivo* methods of investigating biological properties of this organism in our laboratory at Utah State University will undoubtedly contribute towards an effective treatment. An effective chemotherapy directed towards gastrointestinal tract treatment could offer hope of preventing magnification of the disease.

Infection has been observed in several regions of the intestinal tract in different species but it is most commonly found in the distal intestine and in the colon. The detrimental effects of this infection on the villi of the intestine have been well documented (5, 12). Such effects as intestinal dysfunction, villous atrophy and crypt hypertrophy have been noted. These effects have also been related to decreased absorption of nutrients by intestinal cells (11). An effective agent to study malabsorption will aid further studies of the effects of this parasite by revealing nutrient deficiencies that may be interfering.

LITERATURE REVIEW

History

In 1912, E. E. Tyzzer identified *Cryptosporidium parvum* as a protozoan parasite in the phylum Apicomplexa, class Sporozoasida, order Eucoccidiorida, and family Cryptosporidiidae(18). For many years after the initial identification, little research was done on *C. parvum* until it was identified as a possible cattle pathogen (14). This led to increased interest in studies of this parasite. Recently, more interest has developed due to increased number of immunodeficient humans such as those with AIDS. An infection with *C. parvum* in these individuals is very detrimental and often life-threatening.

Life cycle

Cryptosporidium parvum is a small (2-5)*u*m depending on stage in life cycle) coccidian parasite that, for the most part, infects the respiratory tree, the gastrointestinal tract, and associated organs (8). Infection can be identified by watery diarrhea, emaciation, and intestinal villous atrophy (8, 10, 12). The infective stage of its life is the thick-walled oocyst produced from fertilization of the macrogamete (an equivalent of an ovum) by the microgamete (the equivalent of sperm) (8). The life cycle is illustrated in Figure 1. The mode of transmission is typically through food and/or water contaminated by feces of an infected host. Once the oocysts are ingested, they develop into the sporozoite which develops into the trophozoite that infects a new host's

epithelial cells. This develops into a Type I meront which either continues through the cycle and develops into a Type II meront or returns to infect a different epithelial cell in the host. The infection of a host cell requires that the sporozoites excyst from the oocyst and attach to the intestinal villi. The host cell membrane then encapsulates the sporozoite and thin membranes envelop the parasite. The encapsulation process of the oocysts can lead to decreased microvillar disaccharidases and forms vacuoles containing clear fluid. This is thought to cause damage to the absorptive capacities of the villi (4, 13).

Though many agents have been tested as possible treatments, as of yet, there is no known effective chemotherapeutic treatment for cryptosporidiosis.

Analysis of Malabsorption

A nutrient absorption analysis can be used to analyze damage in the intestinal tract of an individual. Several agents, such as fat and vitamin A, are commonly used to test malabsorption (6, 7, 19). To ensure proper analysis, it is necessary to use agents which can be easily identified (16). This can be done by either radiologically labeling the test agent or by using one that is not commonly found in the system. Both methods have been used but their reliability can be influenced by physiological variations of the patient. Specifically, the absorption site can vary greatly depending on the species (9). This differentiation can be due to such things as the individual environment and the particular diet of the test organism.

A common analysis of malabsorption can be done by employing a calorimetric reaction. The method was first detailed by Roe and Rice (15). Simply, this method utilizes deproteinizing reagents added to the serum sample to reduce interfering proteins and a colorizing reagent (*p* - bromoanaline) to produces a color when heated to 70 ° C for ten minutes. Following the color conversion, the sample is then placed in a spectrophotometer at 520 nm and the absorption is read.

Cryptosporidium parvum commonly infects the small intestine and can disrupt the nutrient absorption of its host (5). As was mentioned previously, disruption can occur from the damage caused to the villi through the infection process. Presently, malabsorption of carbohydrates from mouse intestine infected with *C. parvum* is not well understood.

OBJECTIVES AND HYPOTHESES

The uncertainties of malabsorption previously mentioned have led to the following objectives and hypotheses for this study.

Objective 1: Determine any correlation between infection of the villi and decreased absorption of nutrients.

Hypothesis 1: Infection of the brush border of the small intestine leads to damage of absorptive capacities of cells, which causes nutrient contents to be greater in feces than in the blood.

Objective 2: Evaluate the potential of D-xylose, an easily monitored carbohydrate not commonly found in blood, to measure intestinal absorption alterations caused by *C. parvum* infection.

Hypothesis 2: A readily detected monosaccharide, such as D-xylose, will reveal that its serum blood levels are measurably lowered in infected mice.

Objective 3: Monitor the effects of dexamethasone, an immunosuppressant, on absorptive alterations caused by *C. parvum.*

Hypothesis 3: The effects of dexamethasone, a glucocorticosteriod, will not significantly alter absorption of D-xylose in *C. parvum* infected mice.

Objective 4: Develop an effective diagnostic, if possible, to detect *C. parvum* affected malabsorption and therefore reduce production losses.

Hypothesis 4: Early *C.parvum* infection of the small intestine alters absorption and can be monitored by monosaccharide administration.

MATERIALS AND METHODS

Oocysts used in this investigation were generated by infecting a one day old Holstein calf using procedures outlined by Yang and Healey (21). The feces were mixed with 5% potassium dichromate solution and stored at 4 °C. Fecal collections were strained and oocysts purified using discontinuous sucrose gradient centrifugation (2). Briefly, the process of discontinuous sucrose gradients involves two contrasting sugar solutions: a 4:1 water/sucrose and a 2:1 water/sucrose. Sugar solutions were layered in a centrifuge tube and 20 ml of strained feces were overlaid and centrifuged at 1500 x *g* for 25 minutes. Oocysts were recovered from the interface layer of the two sucrose solutions. Collections were saline-washed and resuspended in 2.5% potassium dichromate. This process was repeated to purify the oocysts, and the final collection was suspended in 2.5% potassium dichromate and refrigerated at 4° C.

Female C57BL/6N mice approximately six to eight weeks of age were purchased from Taconic Inc. and housed in the Laboratory Animal Research Center at Utah State University. Mice were housed in plastic cages (5 mice/cage) with wire tops and received food and water *ad libitum.*

Mice were randomly assigned to one of the following treatment groups (Figure 1.): 1) Non-immunosuppressed, non-infected, given D-xylose

2) Immunosuppressed, non-infected, given D-xylose

3) Immunosuppressed, infected, given D-xylose

4) Immunosuppressed, infected, not given D-xylose.

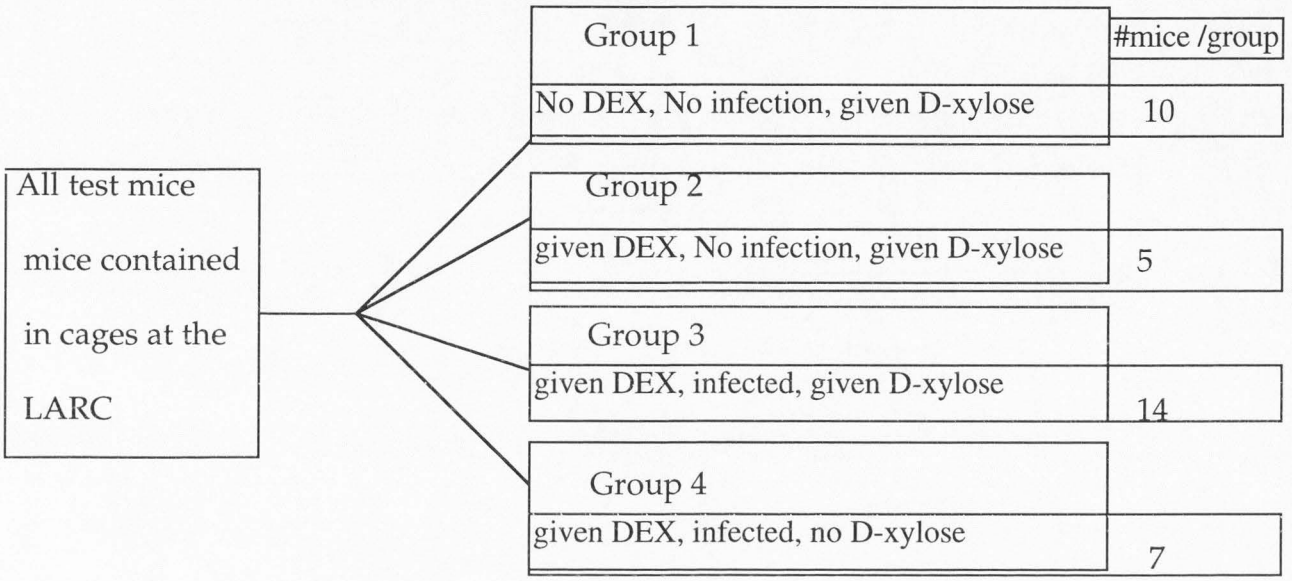


Figure 1. Identification of four test groups of mice randomly assigned dexamethasone, *C. parvum* infection and D-xylose administration including the number of mice per group. Mice were immunosuppressed and infected but were not given D-xylose until the day of the digestion experiment.

Purified oocysts were prepared for mouse infection by washing once in bleach, twice in sterilized Hank's balanced saline solution, and once with sterile RPMI 1640 (Sigma Chem. Co.) to remove the potassium dichromate. Seven days postimmunosuppression, mice were infected with *C. parvum* by intragastric administration of 10^4 oocysts/mouse, using a 22-gauge straight feeding needle.

D-xylose administration. Twelve hours prior to D-xylose administration, mice were fasted to prevent foodstuffs from interfering in the

trial. To determine D-xylose quantities to be administered, mice were weighed 24 hr prior to the digestion experiment. The calculated dosage was 25 mg D-xylose per gram body weight. D-xylose samples were prepared in advance and appropriate dosage for each individual mouse placed in a prelabeled 2 ml microcentrifuge tube and administered to each animal using a 22 gauge feeding needle when the trial was initiated.

Sacrificing mice and sample collection. Immediately prior to sacrificing, mice were removed from their cages and held briefly in a laminar flow hood to allow collection of feces. Collections were placed in labeled 2 ml microcentrifuge tubes and suspended in potassium dichromate. Collected feces were stored at 4° C until Immunoflourescent assay (IFA) could be completed.

Mice were sacrificed in two divisions to allow a more accurate analysis since the D-xylose absorption curve had not been completely determined at this time. Randomly selected mice from each group were sacrificed at either 45 or 90 minutes postadministration. Blood was immediately extracted from the chest cavity and placed into 2 ml microcentrifuge tubes, labeled, and allowed to clot. The entire gastrointestinal tract was harvested and placed in 15 ml of 10% formalin in a 50 ml conical centrifuge tube.

Processing blood samples. Microcentrifuge tubes containing clotted blood were centrifuged at 1000 x g for 5 min. The serum was collected and placed in a new microcentrifuge tube and stored at 0°C. Analysis of serum samples was carried out according to the procedure outlined by Dahlqvist (7)

with some modifications to adjust for reduced sample quantity. The analysis procedure began with 10μl serum samples being pipetted into 70 μl of water plus 10μl of barium hydroxide and 10μl of zinc sulfate. This was centrifuged at 1000 x g for 5 min. Sixty microliters of the supernatant were placed into duplicate tubes and 300 μl of para-bromoanaline (Sigma Chem. Co.) added. One of the duplicate tubes was placed in a 70° C water bath for 10 min and the other was set aside and utilized as a spectroscopy blank. The heated tube was removed from the water bath and all tubes were placed in darkness at room temperature for 70 minutes. D-xylose concentration was spectrophotometrically measured at 520 nm. The absorption was measured using the unheated tube as a blank and calibrating the spectrophotometer for each sample. Tubes were read within 30 min of removal from darkness. A calculation of absorbance of the sample to absorbance of a standard D-xylose preparation was performed on the results to determine the blood D-xylose concentration.

Fecal analysis. Fecal collections were analyzed for oocyst concentration using the IFA staining technique (1). This was achieved by first using an inoculating loop to apply smears on glass slides approximately one inch in diameter. Smears were allowed to dry for 30 min and were then flame fixed. Smears were treated with 80 μl of anti-oocyst IgM and incubated at 37° C for 30 min. Slides were washed three times using phosphate buffered saline (PBS). The second antibody, a fluorescent labeled anti-mouse IgM, was applied at 80

μl per slide. Slides were again incubated at 37° C for 30 minutes and washed three times with PBS. Two drops of glycerol/PBS were applied and cover slips were placed on the slides and preparations were examined using an ultraviolet light microscope (Carl Zeiss, Germany) with a 40x objective. The oocysts were quantified with a single pass through the center of the smear.

Histological examination of tissues. Two regions of the small intestine were examined, the terminal ileum and proximal duodenum. The regions were approximately 1 cm in length and embedded in paraffin. Thin (4μm) sections of the tissues were stained with hematoxylin and eosin. The prepared slide was read using a light microscope with a 100x oil immersion objective. Infection was noted by the colonization of the villi with small (4μm in diameter) spherical oocysts attached to the lumenal side of the villi or located within the crypts. The level of infection was calculated using ten random countings of oocysts in the villi. Countings were made from the apex of one villous to the apex of an adjacent villous.

RESULTS AND DISCUSSION

Fecal analysis. Mouse fecal collections were examined using the IFA technique to determine parasites intensity in each of the four treatment groups. Significant differences existed between fecal oocyst concentrations of treated and control mice (Figure 2). Groups 3 and 4 had well established infections, while groups 1 and 2 remained uninfected or showed little infection.

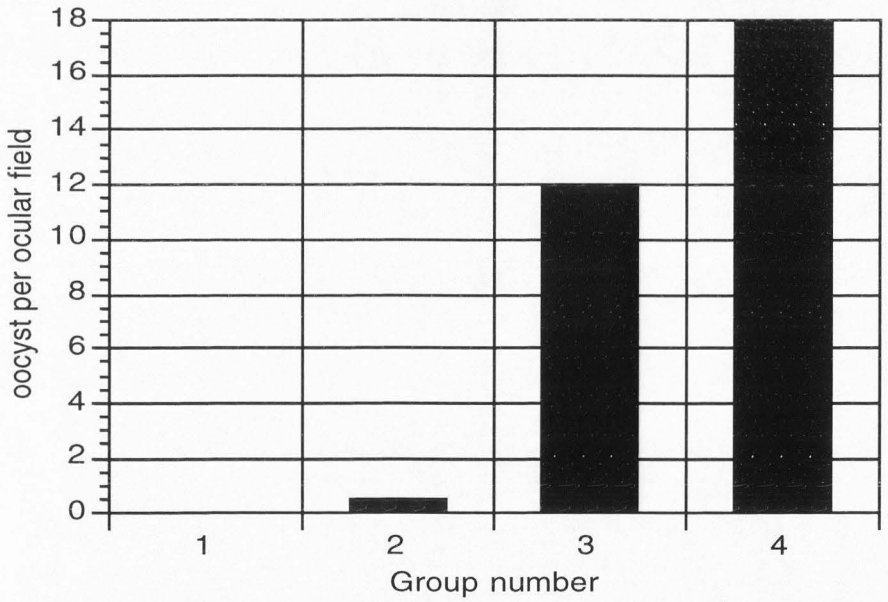


Figure 2. Number of oocysts per ocular field using the IFA technique to analyze a sample fecal smear.

Histological examination of tissues. The site of greatest colonization by the parasite is typically the terminal regions of the small intestine. The histological examination confirmed that there were highly significant differences between the colonization of the terminal ileum and the proximal duodenum (Figure 3). There was also significant swelling of the villi which resulted in a loss of the finger-like morphology. The terminal ileum of

infected groups had an average oocysts per villi ratio of 40 (Figure 3). Differences among the infected groups were also seen in the significant variation in infection intensities noted in the graph.

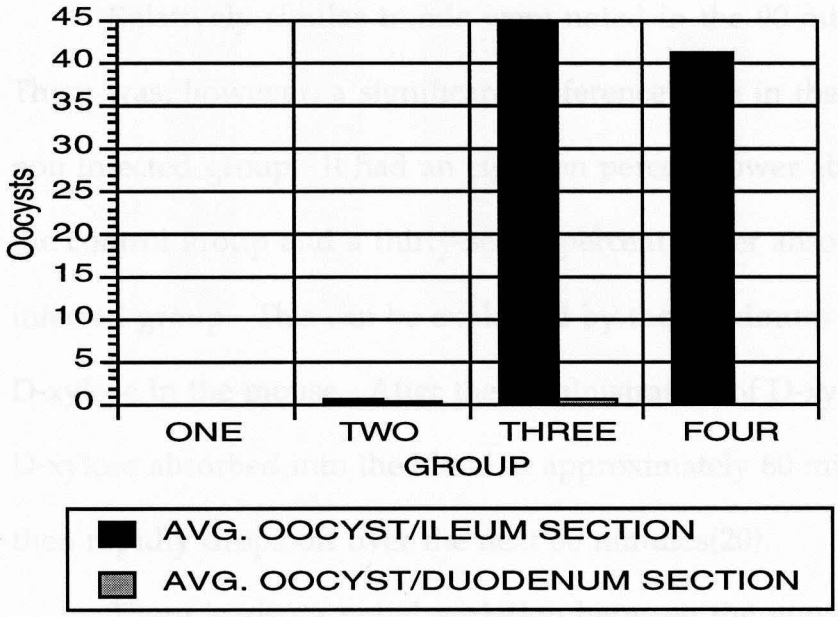


Figure 3. *Cryptosporidium parvum* colonization in the proximal duodenum and the terminal ileum. Note the overwhelming differences in infection rates.

D-xylose analysis . Positive absorption of D-xylose was correlated with the specific treatment groups given D-xylose (Figure 4). There was no significant difference of D-xylose absorbed between the 45 and 90 minute collection groups. The control groups of this experiment returned expected low values for D-xylose absorption.

Within the 45-minute collection group, there was a significantly higher amount of D-xylose absorbed from the immunosuppressed non-infected

group two than the non-immunosuppressed, non-infected control group one (Figure. 4). The greatest observed amount absorbed occurred in the immunosuppressed, infected group 3.

Relatively similar trends were noted in the 90-minute collection group. There was, however, a significant difference seen in the immunosuppresed, non infected group. It had an eighteen percent lower absorption value than the control group and a thirty-seven percent lower absorption value than the infected group. This can be explained by the maximum absorption times for D-xylose in the mouse. After the administration of D-xylose there is a peak of D-xylose absorbed into the blood at approximately 60 minutes, the absorption then rapidly drops off over the next 60 minutes(20).

There is also a noted variation between the nonimmunosuppressed, noninfected groups at both 45 and 90 minutes and the immunosuppressed infected groups. The immunosuppressed groups were an average 11 percent higher then nonimmunosuppressed groups. The individual groups along with the number in each group, the time of sampling and the type of treatment administered are shown in Table 1.

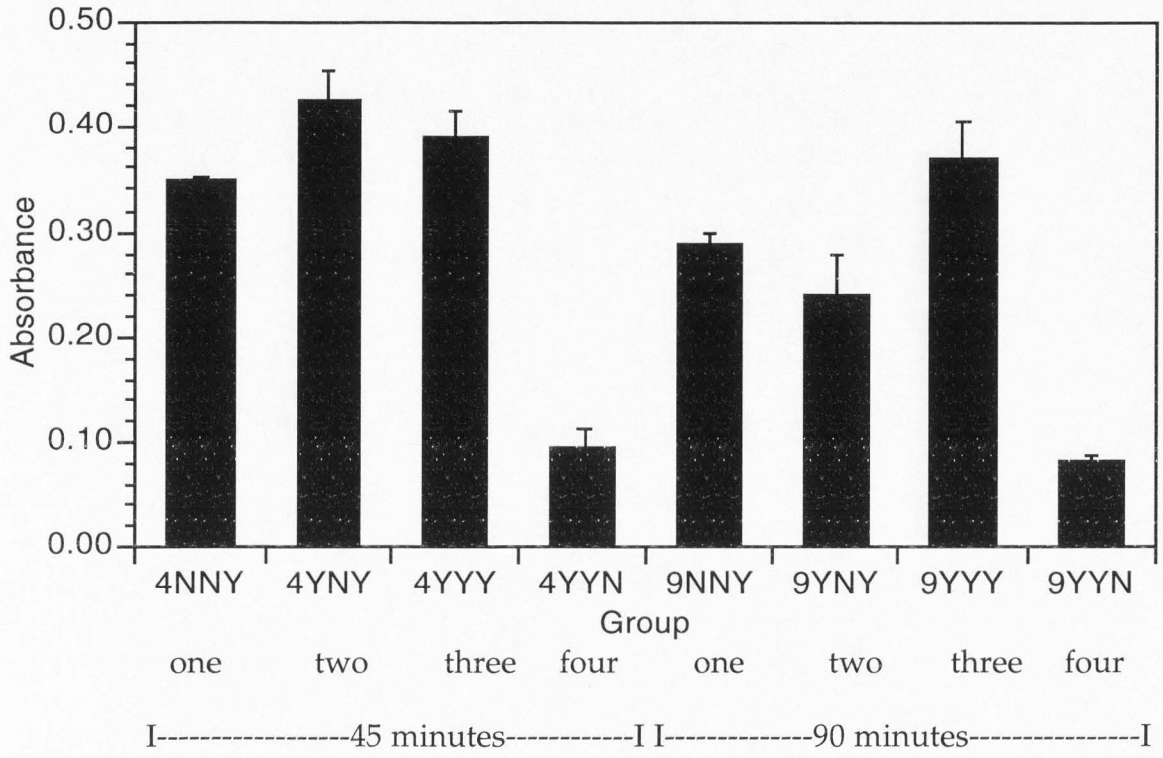
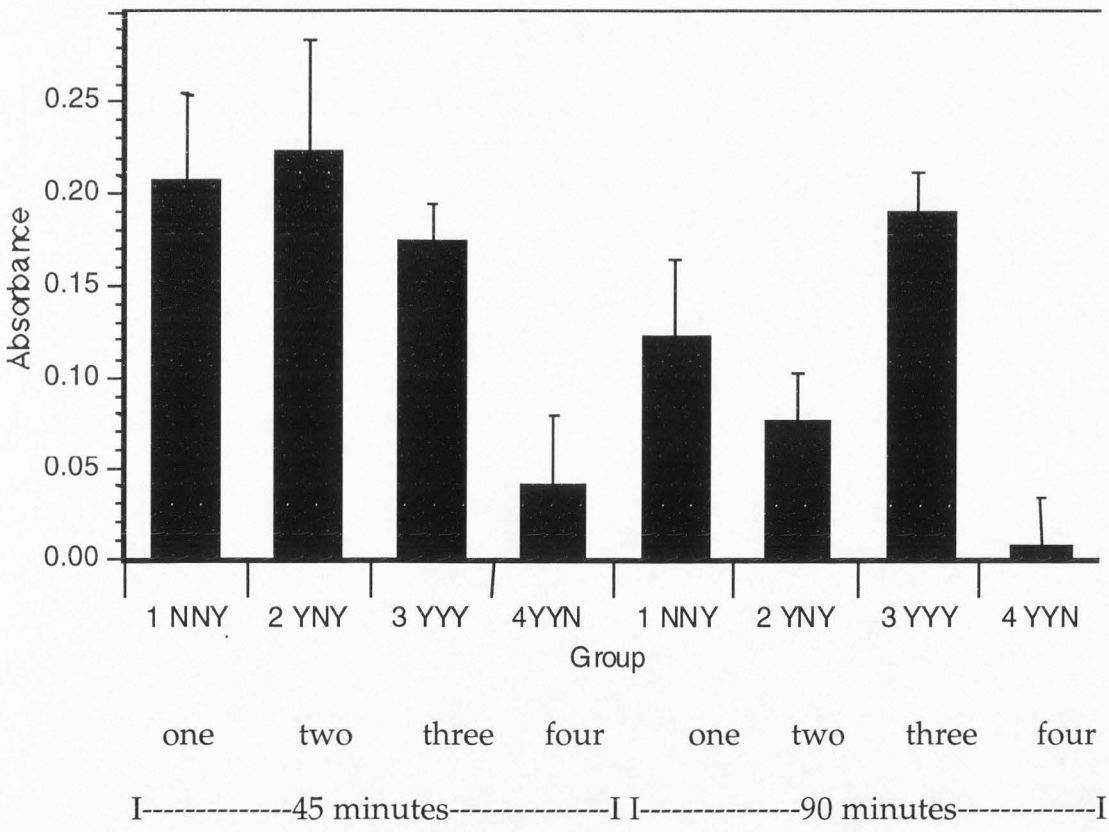


Figure 4. Comparison of the D-xylose absorbance among the different treatment groups.

Table 1. Details the separate groups including the number of mice in each group, the time of sampling and the individual treatments given or not given.

	Number	Min. for Sampling	ImSup	Infection	Xylose
4NNY	4	45	No	No	Yes
4YNY	2	45	Yes	No	Yes
4YYY	7	45	Yes	Yes	Yes
4YYN	4	45	Yes	Yes	No
9NNY	6	90	No	No	Yes
9YNY	3	90	Yes	No	Yes
9YYY	7	90	Yes	Yes	Yes
9YYN	3	90	Yes	Yes	No

A repeat analysis was performed on the original serum samples using the same procedures and the results are shown in Figure 5. There were the same trends noted in the original examination though the absorption amounts were lower (average absorption value lower by 13%).



one two three four one two three four

I----------45 minutes----------I I----------90 minutes----------I

Figure 5. Additional comparison of the differences among the groups in the absorption of D-xylose. Note that the same trends are seen in the repeat analysis as were originally noted and the overall lower absorption percentage.

SUMMARY AND CONCLUSIONS

Both the fecal and the histological examinations gave positive results for infection. In all mice that had been infected, a significant colonization had occurred. Histological preparations exhibited definitive damage to the epithelial tissues including villous atrophy and hyperplasia. From these examinations it follows that previously documented malabsorption should have been measured in the tissues, provided the proper malabsorption test agent was used (5, 11, 12, 16).

D-xylose analysis. Data analysis revealed that no correlation existed between *C. parvum* infection and intestinal D-xylose absorption. There were subtle differences between 45 and 90-minute collection groups, although an absorption curve for humans exists in the literature which indicates that D-xylose absorption should be similar during these time periods.

Further analysis revealed that there was a positive correlation between DEX administration and intestinal D-xylose absorption. Results conflicted with our original hypothesis that DEX would not have interfering effects on the test. The effects of a glucocorticoid on the positive absorption of D-xylose from the intestine is great enough that its appearance masks the effects of cryptopsoridiosis on D-xylose absorption.

In agreement with published texts, there were differences in the site of absorption, depending on the species. In the mouse there is a greater D-xylose absorption in the proximal areas and this absorption decreased further down

the intestinal tract (9, 20). Since there was a greater infection in distal then proximal intestinal sections, there is a high probability that the absorption analysis of an agent absorbed from proximal region of the intestine would be altered. This explanation, in conjunction with the effects of DEX, provides some understanding for the absorption increase of D-xylose in infected compared to noninfected individuals. The exact reasons for absorption site differences are not fully understood at this time.

Conclusions . This experiment demonstrated that D-xylose absorption is not a good determinant to evaluate altered absorption in the adult C57BL/6N mouse model due to cryptosporidiosis. The interfering affects of DEX, in conjunction with physiological differences in the mouse model, confound the malabsorption results when D-xylose is used. The effects of the immunosuppressive agent DEX on the absorption of the pentose nutrient can be of assistance to future experiments. Increased absorption of D-xylose in subjects receiving DEX can explain the increased weight gain in mice immunosuppresed for some time and future tests examining the health of the patient need to take this into consideration.

For further malabsorption studies, results of this experiment suggest the following reagents; 1) Sucrose, because it has been shown greater amounts absorbed in the distal intestine sections than in proximal sections, therfore it would better detail any loss of absorptive capacity due to *C. parvum* infection and/or 2) Lipid, due to its absorption into fecal materials which would eliminate the variation caused by a glucocorticoid on absorption into

blood. It is also be suggested that further studies be completed on the physiological mechanisms of the mouse model before conclusions about *C. parvum* induced malabsorption can be reached.

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