

DEVELOPMENT OF A NEW CLASS OF VIRAL
DISINFECTANTS: ENZYMATIC INACTIVATION OF SA-11
ROTAVIRUS

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ABSTRACT

DEVELOPMENT OF A NEW CLASS OF VIRAL DISINFECTANTS: ENZYMATIC INACTIVATION OF SA-11 ROTAVIRUS

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The non-enveloped, pH- and heat resistant rotavirus (RV), which is cross species-infective among cattle, swine and humans may cause dehydration and high mortality in the young. Rotaviruses are inactivated only by corrosive and toxic disinfectants. In this study, the effects of bacterial proteases as a new type of disinfectants on simian rotavirus (SA-11) were analyzed. SA-11 rotavirus replicates in cells causing cytopathic effect (CPE) and is similar in protein composition to cattle and swine RV. Preliminary experiments tested the temperature and pH sensitivity of SA-11 rotavirus. At pH 8.5, 45°C was the highest temperature at which no loss in viral titer was seen, and the virus was still infective following treatment at 65°C for 2 hrs. pH sensitivity tests were then conducted for two hours at 45°C, with pH 5 being the lowest and pH 8.5 being the highest at which no loss in titer was observed. Four proteases were then tested for effectiveness at inactivating SA-11 rotavirus at their pH optimal at 45°C. Alcalase was selected as the most efficient protease. Alcalase was found to inactivate SA-11 at 25°C, and pH 8.5 in 3 days, indicating that enzymes were relatively effective at lower temperatures. SA-11 rotavirus virus was then tested for sensitivity to pH at 25°C and 15°C in absence of enzyme. At pH 2, 25°C a ~4 log reduction was seen following 15 min

of treatment, with viable virus still remaining after 8 hrs, at 15°C a ~1.75 log reduction was seen following 2 hrs, and a ~4 log reduction following 8 hrs of treatment. At pH 4 and 6, at 25°C and 15°C no effect on SA-11 titer was seen after 120 hrs treatment. The enzyme was then tested at 1.0% and 0.1% enzyme concentration, at 15°C and 25°C, and pH 6 to determine efficacy of enzyme at sub-optimal conditions. Following treatment with 1.0% Alcalase at 25°C a ~3.25 log reduction, and at 15°C, 1.0% Alcalase, a ~1.75 log reduction was seen at 120 hrs. At 15°C, 1.0% Alcalase a ~1.75 log reduction was seen at 120 hrs. Treatment with 0.1% Alcalase at 25°C, pH 6 resulted in ~2.25 log reduction after 120 hrs. At 15°C, 0.1% Alcalase a ~1.25 log reduction was seen following 120 hrs. The results showed that proteases, used as viral disinfectants, were not as effective at inactivating rotaviruses under simulated field conditions as originally hoped, nevertheless the ease of application and moderate but definite efficacy against rotaviruses may help reduce rotaviral infections and severity of clinical signs in young animals.

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LIST OF ABBREVIATIONS

SA-11	= Simian-11 rotavirus
CV-1	= African green monkey kidney cell
PBS	= Phosphate buffered saline solution
HBSS	= Hanks Balanced salt solution
FCS	= Fetal calf serum
EMEM	= Eagles minimal essential media
FPS	= Fungazone penicillin and streptomycin mixture
TCID _{50/ml}	= Tissue culture infective dose 50/ml
ul	= Microliters
ml	= Milliliters
CPE	= Cytopathic Eeffect

INTRODUCTION

Rotaviral (RV) infections are manifested as an acute infection of the small intestine, characterized by a short incubation period followed by anorexia, occasional vomiting and the development of diarrhea sometimes followed by death due to dehydration (14). Following rotaviral infection in cows, gross histopathological lesions with mild infections, and thin and distended intestines are observed. RV replication in all species leads to death of mature enterocytes, which atrophy and are replaced by immature cells that lack absorptive capabilities, resulting in diarrhea (2). Infection occurs as a sudden and rapidly spreading epizootic in young animals and humans, transmitted by the fecal oral route (15). Infection spreads in housed animals very rapidly. The majority of animals in contact with the active cases will develop diarrhea in 2-3 days. The proportion of rotaviral antibody positive animals often reaches 100%. Asymptomatic carriers of rotavirus are the main cause of disease spread (13).

In cattle, rotavirus is a major contributor to neonatal calf diarrhea syndrome worldwide (2), causing up to 50% mortality on dairy farms, cow-calf ranches, and veal-growing operations. In dairy cows approximately 10-20% of live-born calves die before reaching 2 months of age due to rotavirus, however, mortality among range-born beef cattle is usually considerably less. In veal-raising operations when either beef or dairy cattle are assembled, mortality rates occasionally reach 90%. The majority of these deaths are associated with neonatal calf diarrhea (13). Viral titers up to 10^8 infectious virions per ml of feces were observed in an outbreak with 100% morbidity and 50 %

mortality (1). Calf colostral and serum antibody titers did not reach high levels during infection. This is why calves are susceptible to reinfection each year (1).

Rotavirus is one of the most important etiologic agents of diarrheal illness in piglets. The disease is endemic on swine farms and usually appears in piglets weaned at 2 - 4 weeks of age, when passive immunity wanes. However, exposure to large numbers of RV may overwhelm high levels of maternal immunity prior to weaning. Rotaviruses isolated from humans, horses, and sheep have been shown to infect piglets. Rotaviruses result in acute loss of body weight (10-15%) in piglets, followed by a variable time of 7 - 14 days or longer for recovery to normal growth rate. Mortality rates of piglets due to porcine rotavirus range from 0-50%. In fecal preparations the virus will survive for at least 7 - 9 months at 18 - 20°C (33).

Human rotavirus causes staggering amounts of diarrheal diseases in developing countries. According to the World Health Organization diarrheal diseases account for as many as 15-34% of all deaths annually in certain developing countries, with most deaths occurring during infancy and childhood. It was estimated that 450,000,000 diarrheal episodes occurred in 1975, in children less than 5 years of age in Asia, Africa, and Latin America, of which 1-4% (5-18 million diarrheal episodes) were estimated to be fatal (21). More recently (1977-1978), it was estimated that between 3-5 billion cases of diarrhea and 5-10 million deaths were associated with diarrhea in a 1-year period in these regions, ranking diarrhea first in frequency and mortality (31). In the United States, non-bacterial infectious gastroenteritis was highlighted by the Cleveland Family Study, which found it to be the second most common disease, accounting for 16% of approximately 25,000 illnesses over a period of about 10 years (12). In the U.S. between 1973-1983, 5,539

children 1 month to 4 years of age died from diarrheal diseases, an average of 504 deaths per year, which is 10% of the preventable portion of post-neonatal deaths (12). In the US in the 1-4 age group rotaviruses cause an estimated one million cases of severe diarrhea and up to 150 deaths. The burden of rotaviruses in infants and young children below the age of 5 was analyzed in developing countries, and it was estimated that rotavirus is responsible for 125 million cases of diarrheal disease; over 18 million of these considered moderately severe or severe. Testing in Washington D.C., showed that 90% of children by their third year of life have acquired antibodies to rotavirus. Also in D.C., it was found that in a defined population of 29,000 infants and children, that 1 in 272 infants less than 1 year of age and 1 in 451 children between 12-24 months of age were hospitalized for rotaviral infections. In a study in Virginia during a 29-month surveillance period rotaviral infections were identified in 51% of the families (14). Rotaviruses are also important nosocomial (hospital acquired) diseases. Another study in D.C. indicated over an 8 year period that 34.5% of 1,537 individuals admitted for diarrhea shed rotavirus(14). In Japan a similar study was conducted and it was found that over a 6 year period that 45% of 1,910 patients admitted for diarrheal diseases were rotavirus positive. One study indicated that 17% of 60 children hospitalized for non-diarrheal diseases developed a diarrheal illness associated with rotavirus exposure. About one out of every five-rotavirus infections appears to be hospital-acquired (14). In adults, rotavirus gastroenteritis has been described in army recruits in Finland as well as in patients and staff in hospitals in England, Sweden, Canada, Finland, South America, Polynesia, and China. Several outbreaks have been seen in geriatric groups, which have

had high attack rates and some fatalities. Although rotaviral outbreaks occur in adults, because of the high level of rotaviral immunity most adults develop, it is seen rarely (14).

SA-11 rotavirus was first isolated in 1963 from a rectal swab of a healthy vervet monkey (17). Rotaviruses derive their name from the Latin word “rota” meaning wheel, a structural feature observed in rotavirions by negative staining. Rotaviruses belong to the Reoviridae family along with Reovirus, Orbivirus, Phytoreovirus, Fijivirus, and a presently unnamed group of cytoplasmic polyhedrosis viruses. Antigenically, rotaviruses are distinct from reoviruses and orbiviruses, but have not been tested serologically against the other members of Reoviridae. By negative-stain electron microscopy the distinct morphological appearance of rotaviruses can clearly be seen, but morphology alone cannot separate it from reoviruses and orbiviruses which have a similar appearance (14). A complete rotavirus particle has a double-layered icosahedral protein capsid, which consist of an inner and outer capsid (Figure 1, 2, and 3). The complete particles are designated “smooth” because of their well defined, smooth circular appearance. The complete rotavirus particle measures ~70 nm in diameter. The inner core contains 11 segments of double stranded RNA. The single shelled particles measure ~55 nm in diameter and are designated “rough” because of their “bristly” appearance. The core measure ~37 nm in diameter (4).

The recommended measures for the control of rotaviral infections in porcine and bovine includes vaccination, early suckling of colostrum, and proper sanitation (33). Vaccination efficacy in calves is questionable and it may interfere with passive maternal immunity obtained from the colostrum by neonates (33). Passive maternal immunity obtained from early suckling greatly reduces the risk of a rotaviral infection, but in order

for it to be effective it must occur immediately after birth, which is often hard to achieve (33). Proper sanitation is the most important of all the control measures (18). Disinfectants for equipment and buildings include formaldehyde, chloramine T, 70% ethanol, and possibly hexachlorophene, H₂O₂, Lysol and triclosan (33). Use of these disinfectants can be very costly and labor intensive. These disinfectants, if inappropriately used, can be hazardous to piglets, calves, and humans. All animals must be removed from the holding area prior to application of these disinfectants and residual disinfectant has to be washed away before the animals can be returned.

Rotavirus was selected for proteolytic inactivation studies because of the pandemic distribution and significant pathogenicity of the virus. Rotavirus is a gastroenteritis virus, which is in constant contact with a low pH environment and various pancreatic proteolytic enzymes. Rotaviruses as a family are fairly temperature and pH stable. They are also highly resistant to many of the currently used disinfectants. One interesting phenomena of the rotavirus family is that when treated with trypsin, a pancreatic protease, the rotavirus viral protein 3 (VP 3) is cleaved into VP 5 and VP 8 which increases viral uptake, thus increasing viral infectivity and titer (7). Since the mechanism expected to inactivate the virus, i.e. disruption of proteins by proteases, actually increases the titer of these particular viruses, it would appear to confound the formulation of a hypothesis as to what effect Alcalase will have on RV titer. Will Alcalase, itself a proteolytic enzyme, increase, decrease, or have no effect on viral titer?

For the rotavirus inactivation experiments performed in this study, the Simian-11 Rotavirus (SA-11) strain was selected for two reasons. First, it readily replicates and causes cytopathic effects (CPE) in cultured cells; making it suitable for *in vitro* studies.

Production of cytopathic effect allows titrating of the virus visually using an ordinary inverted light microscope (6). Reading of CPE was found to be one log more sensitive than either FA (fluorescence antibody) and 2 logs more sensitive than ELISA (10). Second, cross neutralization studies demonstrated that SA-11, bovine, and porcine rotavirus are serologically indistinguishable (1), indicating similar antigen composition of these viruses. This is important, because particular proteases, each of them attacking specific peptide bonds, will likely act similarly on SA-11 as on other strains of rotaviruses, making the first a suitable model for enzymatic inactivation of the latter.

SA-11 virus retains infectivity between pH 3.5-10, although some titer loss is observed. It is stable following treatment with ether, genetron, chloroform, repeated freeze/thawing, sonication, or incubation for 1 hour at 37°C or ambient temperatures. Following incubation at 50°C the SA-11 virus loses 80% of its infectivity after five minutes, and 99% of its infectivity after 30 minutes., and is inactivated following a 1 hour treatment with 1% formaldehyde, 50 % ethanol, 2% Lysol and 1% H₂O₂ (8). SA-11 loses most of its infectivity following treatment with 2M MgCl₂, CaCl₂, or NaCl for 15 min. at 50°C, and after freezing in 2M MgCl₂, CaCl₂, or NaCl. SA-11 infectivity is stable in a MgSO₄ solution (23). Human rotavirus outer capsid collapses below pH 3 and above pH 10, and is removed upon exposure to chelating agents such as EDTA, which convert the double capsid particle to a single capsid (8). Ethanol at 95% concentration, and Biogram 5% v/v, which are hospital disinfectants, are more effective than 4% or 10% w/v formalin (24). Lamb rotaviruses showed no loss in titer following treatment with a 3% sodium hypochlorite solution containing 11% available chlorine (14). A loss of hemagglutination activity is seen following treatment with chloroform, repeated freezing and thawing, or

incubation at a pH below 2, and a complete loss of hemagglutination was observed following treatment with ethanol or methanol. No loss in viral titer was found following treatment with alkaline solutions (pH 10.6) or diethylether (14).

Different virus families have extremely varied physico-chemical characteristics. Less resistant, often enveloped viruses, such as retrovirus (HIV), filovirus (Ebola), remain infectious outside their host only for a few minutes and are easily inactivated by common disinfectants. Other highly resistant viruses, often non-enveloped, such as parvovirus (canine parvovirus), picornavirus (human poliovirus), rotavirus (human, porcine, bovine, simian rotavirus), can remain viable in the environment for a long time. These viruses are often very difficult to inactivate and only by disinfectants which are typically corrosive and/or toxic. Such disinfectants can and are being used to clean various animal holding facilities. However, many are hazardous when applied on or near live animals or human beings. The development of a new class of viral disinfectants, combining low toxicity, high tolerability by animals and humans and high inactivating efficacy against various viruses, including the most resistant ones, would be beneficial in combating viral diseases.

During the development of an innovative method for the treatment of infectious hospital waste it was discovered that bacterial proteases were effective in inactivating viruses (25, 26). The concept is based on the very simple principle that proteases are capable of breaking peptide bonds which are the links between the amino acids that form the proteins. The protease should disrupt the viral protein structure of the virion rendering them non infectious. This principle has been tested on human polio virus-2 (complete inactivation following 60 minutes exposure to 0.05% enzyme, pH 9.0 at 50°C)

(25), infectious bovine rhinotracheitis virus (herpes virus) (complete inactivation following 15 min. exposure to 0.1% enzyme, pH 8.5 at 50°C), vaccinia virus (complete inactivation following 30 min. exposure to 1.0% enzyme, pH 8.5, at 50°C), bovine parainfluenza-3 virus (complete inactivation following 5 min. exposure to 1.0% enzyme, pH 8.5 at 50°C), and canine parvo virus (complete inactivation following 60 min. exposure to 0.1% enzyme, pH 8.5 at 50°C) (26).

Bacterial proteases, which are used in numerous household-cleaning products and are commercially available in large quantities and at a low cost, used in low concentrations, would be a safe and cost-effective alternative to currently used disinfectants against rotavirus that could be used on or around young animals. One advantage of proteases over the currently used disinfectants is the ability of these enzymes to digest the sloughed-off pieces of intestinal tissue which harbor rotaviruses, thus eliminating an important reservoir which protects infectious virions against standard disinfectants. In addition, proteases may predispose rotaviruses to the effects of other, natural factors (exposure to various enzymes or chemicals that may be contained in fecal matter, drying) that would otherwise inactivate rotaviruses only with low efficiency. The enzymes would also provide an environmentally friendly alternative to currently harmful disinfectants, since the enzymes are biodegradable (27).

Even in absence of complete inactivation, decreasing the levels of rotaviruses in the environment would decrease the exposure levels of young animals, which in turn would decrease the severity of rotavirus diarrhea and the magnitude of economic losses. It is uncertain what level of rotavirus is necessary for clinical disease, since the severity of infection depends on numerous factors such as general status of health of the animal,

stress, passive maternal immunity, etc. The same low level rotaviral infection that may cause relatively mild or no clinical signs in a healthy animal, may cause severe diarrhea, and dehydration leading to death in an immuno-compromised animal under more stressful conditions. Complete inactivation may not have to be achieved for the enzymes to be deemed effective, if titer decreases are significant enough to lessen the clinical signs of rotavirus. In such instances, although rotaviral infections may still be present, the magnitude of financial losses effecting farmers may greatly be reduced. The goal of this project was to determine if Alcalase would be effective as a disinfectant against rotavirus at temperatures and pHs consistent with field conditions, but suboptimal for enzyme activity (30).

The group of enzymes to be analyzed was obtained from Novo Nordisk (2880 Bagsvaerd, Denmark) one of the largest enzyme producing companies in the world. The enzymes selected for viral inactivation studies were three alkaline bacterial proteases - Durazyme, Alcalase, and Savinase, and one neutral bacterial protease - Neutrase. These enzymes are used in household detergent formulations; they are used to remove protein-based stains (i.e.. blood, mucus, grass, feces and food stains such as gravy and egg). The enzymes selected posses a wide range of specificity for various peptide bonds; this explains why one enzyme may be more effective against one virus and less against another (19).

These enzymes were tested for effectiveness at 25°C and 15°C, far below the 45°C temperature used in initial tests. At these temperatures the enzymes lose between 80-90% of their activity (19). These temperatures were selected as an average ambient temperature range for normal calving facilities (20). The experiments were conducted at

pH 6 a reasonable estimate of diarrheic fecal matter (20). The virus was exposed to the enzyme for up to 5 days. An excess of 5 days was deemed unexceptable for field use. It was our hope that the proteases would provide an economically feasible and safe alternative to commonly used disinfectants. Although complete inactivation is desirable effectiveness should not be solely based on this criteria but on the reduction of viral titer to the point where an economic reward is achieved.

METHODS AND MATERIALS

Standard Protocol for Enzymatic Inactivation of SA-11 Rotavirus

1. MATERIALS

- 1.1 Simian rotavirus (SA-11) obtained from American Type Culture Collection (ATCC) with a titer of $10^{5.5}$ - 10^6 tissue culture infective dose 50/ml (TCID₅₀/ml).
- 1.2 Enzymes: Alcalase, Durazym, Neutrase, and Savinase, in detergent formulation obtained from Novo Nordisk, Denmark.
- 1.3 CV-1 cells (monkey kidney cells) obtained from ATCC for growth and propagation of SA-11. CV-1 cells grown and cultured in 1 X Eagles Minimal Essential Media (EMEM), 10% fetal calf serum (FCS), 1 X L-Glutamine, titration conducted in presence of antibiotic at 1 X concentration from prepared mixture containing streptomycin, amphotericin and penicilin.
- 1.4 Buffers: To control pHs of reactions use the buffers for enzymatic studies published in Handbook of Biochemistry and Molecular Biology. pp. 370-379. All solutions are at a 0.05-0.2M concentration. Buffer selected based on pH needed for individual experiment
- 1.5 Falcon Microtest III plates (Fisher Scientific) used for growth of cells in presence of virus.
- 1.6 15 ml centrifuge tube.
- 1.7 Heating/cooling waterbath.

- 1.8 1.5 ml snap cap microfuge tubes.
- 1.9 Trypsin (Sigma).
- 1.10 .22 um sterile syringe tip filters.
- 1.11 10 cc syringes.

2. PROCEDURES, ENZYME/PH/HEAT/TIME TREATMENT

One ml of frozen SA-11 virus was removed from the -80°C freezer and allowed to thaw at room temperature. For temperature and pH sensitivity test 1 ml of SA-11 virus was mixed with 9 mls of the sterile pH buffer selected for the particular experiment, in a 15 ml centrifuge tube, the virus was at a 10^{-1} dilution. For treated samples (enzyme exposed) the enzyme was first filtered with a .22 um syringe filter and placed on ice. One ml of virus and 9 mls of the selected pH buffer with the appropriate amount of enzyme for either 0.1% or 1.0% concentration was placed in a 15 ml centrifuge tube. The tubes were then placed in a heating or cooling waterbath at the selected temperature for the given experiment (15°C , 25°C or 45°C). At selected time points a tube was removed from the waterbath, and a 1 ml sample was removed from the tube. The remaining portion was placed back into the water bath for additional timepoints if any remained. For temperature and pH sensitivity test the samples were immediately frozen for later titration. Treated samples were immediately detoxified (separate protocol) and placed on ice for titration later. All samples were diluted serially ten-fold from 10^{-1} through 10^{-7} by the addition of 900 uls of cell culture media containing 10% antibiotics to a series of 1.5 ml microfuge tubes, 0.1 ml of virus sample was then added to the first

tube. The micropipeter tip was changed and the suspension was mixed, 0.1 ml was added to the next tube. The process was repeated until the 10^{-7} dilution was created. The original detoxified virus suspension represented 1:10, or 10^{-1} dilution. Then 0.1 ml of each dilution was added to quadruplicate wells of a 96-well cell culture microtiter plate. Three samples were placed on one plate, identical dilutions were added to letter defined wells of the same row (i.e. 10^{-1} to A1, B1, C1, D1; 10^{-2} to A2, B2, C2, D2; etc.). A 150 cm^2 flask, which contained replicating CV-1 cells was taken from the incubator, all the media was removed from the flask and discarded. The flask was then treated with 500 μl of trypsin, and rocked rapidly until all the cells were detached from the flask. The cells were then removed from the flask, counted and diluted in normal cell culture media, without antibiotics, to a concentration of 10,000 cells per 100 μl s. A multichannel pipettor was used for aliquots of 100 μl s of cells, which were placed in wells containing virus and in the bottom row of the plate for a cell control. The plates were then incubated at 37°C , 5% CO_2 for at least 10 days. They were periodically observed for correct morphology and lack of cell deterioration in cell control wells, and for the development of cytopathic effect in the virus inoculated wells. Observation of cells continued for 10 days to allow viral CPE to develop in all wells at all dilutions.

Protocol for Detoxification of Rotavirus Samples

1. BASIC CONCEPTS

Viruses replicate only in living cells. Demonstration of viral inactivation requires titration of treated and control virus samples in living cells. However, enzymes used may be toxic to cells, damaging or killing the cells, thus eliminating the ability to demonstrate replication of viruses. To detoxify samples gel filtration, with beads of appropriate fractionation characteristics are used. On the one hand, these beads permit entry and retention of small molecules (enzymes, toxic inorganic compounds) into the beads, thus detoxify the samples. On the other, the beads exclude the much larger virions which are present in the void volume, available for sampling and titration.

2. MATERIALS

- 2.1 Gel Filtration Resin = Sephacryl S-200 HR (Sigma) in 20% ethanol, preswollen, with fractionation range for globular proteins between 5,000-250,000MW. As most enzymes are below the upper fractionation limit, and the MW of SA-11 is above $\sim 1.5 \times 10^6$, these beads have the appropriate separation characteristics.
- 2.2 Empty 15 ml PD-10 disposable columns with porous glass disc to enclose bottom of column.
- 2.3 45 ml conical centrifuge tubes.
- 2.4 CRU-5000 centrifuge.

- 2.5 0.1 M phosphate buffered saline (PBS).
- 2.6 Sterile Hank's balanced salt solution (HBSS)
- 2.7 Samples to be titrated.

3. PROCEDURES FOR DETOXIFICATION

The S-200 HR beads were equilibrated by resuspending 1 to 1 in 0.1 M PBS, allowing the beads to settle, and repeating twice, each time the buffer was poured off once the resin had settled. Approximately 10 ml of the equilibrated S-200 HR beads were then added to the column. The column was then centrifuged at 1500 RPM (500xG) for 2 minutes to settle the resin. Filling and centrifugation of the column was repeated until the S-200 HR fills the syringe to the 9 ml mark. The columns were then autoclaved for 20 minutes on liquid cycle to sterilize. In a sterile hood, 1 ml HBSS was added to the top of the column, and the column was centrifuged at 1,500 RPM, for 3 minutes, at 4° C. This was repeated once, and the eluate was discarded. This was done to stabilize the beads to a salt concentration closely resembling the media in the viral samples. The 1.0 ml virus sample obtained from the enzyme treated test was then placed in the top of column and the column was centrifuged at 1,500 RPM, for 3 minutes. The 1 ml eluate: detoxified sample was collected and titrated.

4. RECYCLING USED COLUMNS

The used columns were autoclaved for 20 minutes on liquid cycle to kill any residual virus. Next 3 ml of 0.2 M NaOH was added to the top of the column, and centrifuged at 1,500 RPM, for 3 minutes, which caused the release of the enzymes from

the beads (repeated once). Then, 3 ml of 0.1 M PBS was added to the column and centrifuge at 1,500 RPM, for 3 minutes. This was repeated until the eluate pH became neutral. Approximately four to seven PBS rinses and centrifugations were necessary. The columns were then autoclaved, and equilibrated twice with HBSS, and the column was ready for the next sample.

Data Analysis

Procedures for determining which of the enzymes was most effective in inactivating SA-11 at 45°C, and for determining the efficacy of Alcalase at lower temperatures were conducted only once. The remaining experiments were all performed in triplicates. Microsoft Excel was used to calculate the mean and the standard deviation of the results. Jandel Scientifics, Sigma Plot was used to calculate a curve fit. On the graphs the data points are plotted as the mean of the replica experiments with their standard deviation bars.

RESULTS

Analysis of SA-11 Rotavirus Temperature Sensitivity

The initial temperature sensitivity studies were conducted to determine the effects of temperature extremes alone on SA-11 infectivity. The studies were conducted at pH 8.5 (the optimal pH for alkaline enzymes) using 5°C temperature intervals from 45°C-65°C for a two-hour period, samples being taken at 0, 30, 60, 90, and 120 minutes. 45°C was the highest temperature at which no viral inactivation was found following two-hours incubation (Figure 6). Temperatures of 50°C and 55°C reduced viral titer ~2 logs following 30 minutes of exposure, which remained the same at 120 minutes. This was the first time an apparent stabilization of SA-11 virus appeared. At 30 minutes of treatment a 99% reduction in viral load was noticed, followed by no reduction for the next hour and thirty minutes (Figures 7, 8). The virus appeared to be effected by these temperatures at the first time point, but with increased exposure very little to no additional loss in titer was found. This tailing effect was also seen in two papers, one of which used chlorine and the other ozone for rotavirus inactivation (28, 29). A large initial drop in titer from 1-3 logs was noticed followed by a long tailing period with gradual or no inactivation in extended exposure times, 2-3 times as long as the initial exposure. Loss of chlorine and ozone over the treatment time could have explained this observation, but no conclusions were made or studies conducted to determine what caused the tailing effect. In our study at 60°C and 65°C viral titers were reduced ~2.75 logs following 30 minutes exposure and ~3.25 logs at 120 minutes. These results demonstrated that at a pH of 8.5, 45°C was the

highest temperature at which the virus could be tested in the presence of the enzyme, without having a decrease in viral titer caused by variables other than the enzyme.

Analysis of SA-11 Rotavirus pH Sensitivity

Based on preliminary heat-resistance studies, SA-11 was tested for pH resistance at 45°C for a total of two hours. Samples were obtained at 0, 30, 60, 90 and 120 minutes. The highest pH at which no titer loss was observed after 2 hrs exposure was at pH 8.5 (Figure 11). This pH was selected for further enzyme experiments because it was the closest pH to the enzyme optimum with no effect on viral titer. At pH 9 a slight reduction in viral titer appeared, but by 120 minutes a ~1 log loss in titer was discovered, which was beyond the standard error values (Figure 12). The lowest pH at which no viral loss was seen was pH 5, viral titer appeared to be very stable, the line almost being straight (Figure 13). At pH 4, a ~1.5 log reduction was seen following 30 minutes and a ~2.5 log reduction following 120 minutes of exposure (Figure 14).

Selection of the Most Effective and Cost Efficient Protease Against

SA-11 Rotavirus

The next step was to determine which bacterial protease would be most effective at inactivating SA-11 virus. For this experiment the alkaline proteases Durazym, Alcalase, and Savinase were utilized at pH 8.5, and the neutral protease, Neutrase was utilized at pH 6.0 (all proteases were used at their pH optimum). This experiment was conducted for 120 minutes, the only sample being taken at this time point. Neutrase was found to be the least effective; it caused a ~1 log reduction in titer following 120 minutes

of exposure. This was quite disconcerting since Neutrase had a pH optimal close to 6, which would have been ideal for field conditions. Savinase and Durazym both caused a ~2.25 log reduction in SA-11 titer, close to the efficacy of Alcalase, but since they had slower activity rates than Alcalase at the conditions under which the enzyme was tested changed (based on enzyme data sheets), they were not selected. Thus, Alcalase was found to be the most effective of the proteases, reducing viral titer ~2.75 logs following 2 hrs exposure (Table 1). Thus, based on its effectiveness, lower cost, and higher enzyme activity at a wide range of temperatures and pH's, Alcalase was selected for further studies.

Analysis of Inactivation of SA-11 Rotavirus by 1.0% Alcalase at Lower Temperatures

Alcalase was tested at longer exposure times, to determine its effectiveness against SA-11 rotavirus at 45°C and 25°C, but still using the enzyme's optimal pH of 8.5. At 45°C, without enzyme, the virus showed a ~0.75 log reduction following 24 hours of exposure and a ~2.75 log reduction following 72 hours. This loss of titer was solely due to the pH and temperature interaction. With 1.0% Alcalase, at 45°C the virus showed a ~3.0 log reduction following 24 hours and a ~4 log reduction, complete inactivation following 48 hours of exposure. At 25°C, without enzyme, no decline in titer was seen following 72 hours of exposure. With the addition of 1.0% enzyme a ~1.25 log reduction was seen at 24 hours, a ~2.75 log reduction at 48 hours, and complete inactivation, a ~4.25 log reduction following 72 hours exposure. Thus, Alcalase was determined to be

somewhat effective at inactivating SA-11 rotavirus at lower temperatures closer to field conditions (Figure 15).

Analysis of SA-11 Viral Sensitivity to Low pH at 15°C and 25°C

Testing the efficacy of Alcalase against the SA-11 virus on lower temperatures necessitated the testing of SA-11 also for resistance to pH alone at these temperatures, pH 2, 4, and 6 were selected for review. No individual temperature sensitivity test was conducted (the pH 6 sensitivity test also served as the 25°C and 15°C temperature sensitivity test). At pH 2 and 25°C, SA-11 titer was reduced by ~4.5 logs (99.99% reduction in viral titer) following two hours exposure, with no further decrease in titer at 8 hrs (Figure 16). A large initial drop in titer of ~3 logs (99.9% reduction in virus) is seen within 15 min followed by only a ~1.5 log reduction (an additional .01% reduction in virus) over the next 1.25 hrs with no additional decline for the next 6 hrs. This rate of inactivation was similar to those experienced in prior experiments. At pH 2 and 15°C, SA-11 titer was reduced by ~2 logs (99% titer reduction) following 2 hrs exposure, and ~4 logs (99.99% reduction in viral load) following 8 hrs (Figure 17). Viral tailing did not appear as prominent in the 15°C as in the 25°C pH exposure test. At pH 4 and 6, at 15°C and 25°C no loss in SA-11 viral titer was found following 120 hrs exposure (Figures 18, 19, 20, and 21).

Analysis of SA-11 Rotavirus Inactivation by 1.0% Alcalase at 15°C and 25°C at pH 6

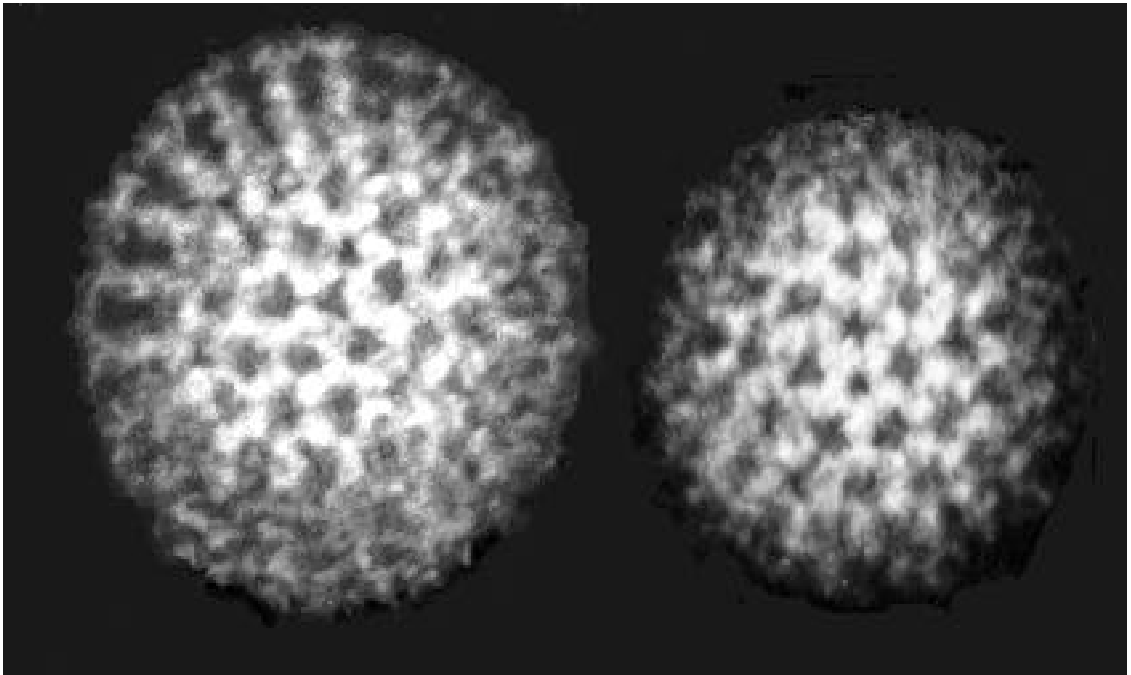
Alcalase was tested for effectiveness at 25°C and 15°C, at 1.0% enzyme concentration. Although pH 6 being well below the pH optimal for the enzyme, it was selected because it represents a reasonable average for diarrheic fecal matter (20). Decreasing the pH to 6 decreases enzyme efficacy 55-60% (19). SA-11 viral titer was reduced by ~2.75 logs following 24 hrs treatment with 1.0% enzyme at 25°C and by ~3.25 logs at 120 hrs. The enzyme was capable of reducing viral load by 99.9% over the 120 hr period (Figure 22). At 15°C, using 1.0% enzyme, a ~1.5 log reduction (95% reduction in infectious virions) was seen following 24 hrs exposure, and only an additional ~1.75 log at 120 hrs (Figure 23).

Analysis of SA-11 Rotavirus Inactivation by 0.1% Alcalase at 15°C and 25°C at pH 6

Alcalase was tested for effectiveness at 25°C and 15°C, at pH 6, and 0.1% enzyme concentration. SA-11 viral titer was reduced by ~2.0 logs (99% reduction in viral load) following 24 hrs treatment with 0.1% enzyme at 25°C, and by a total of ~2.5 logs reduction (an additional 0.5% reduction) at 120 hrs (Figure 24). At 15°C, using 0.1% enzyme, a ~0.75 log reduction (<90% reduction in viral load) was seen following 24 hrs exposure and a ~1.0 log reduction (90% reduction in viral load) at 120 hrs (Figure 25).

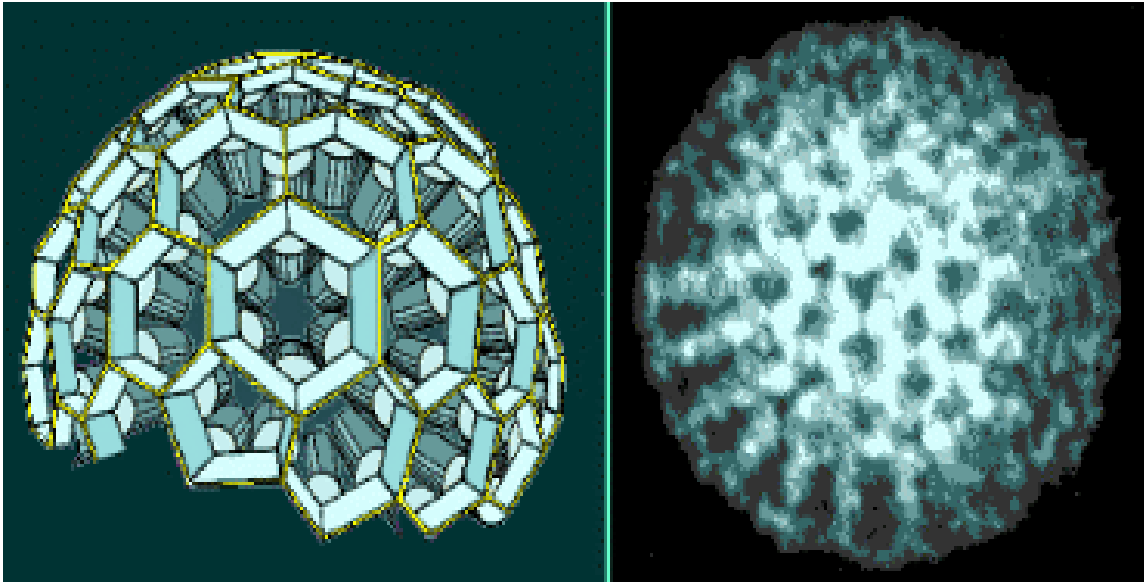
Table 1. SA-11 vs Enzyme At pH Optimal For Enzyme, At 45°C, After 120 Minutes

1.0% Enzyme	pH Tested	Logs Reduction
Neutrase	6.0	~1.0
Savinase	8.5	~2.25
Durazym	8.5	~2.25
Alcalase	8.5	~2.75



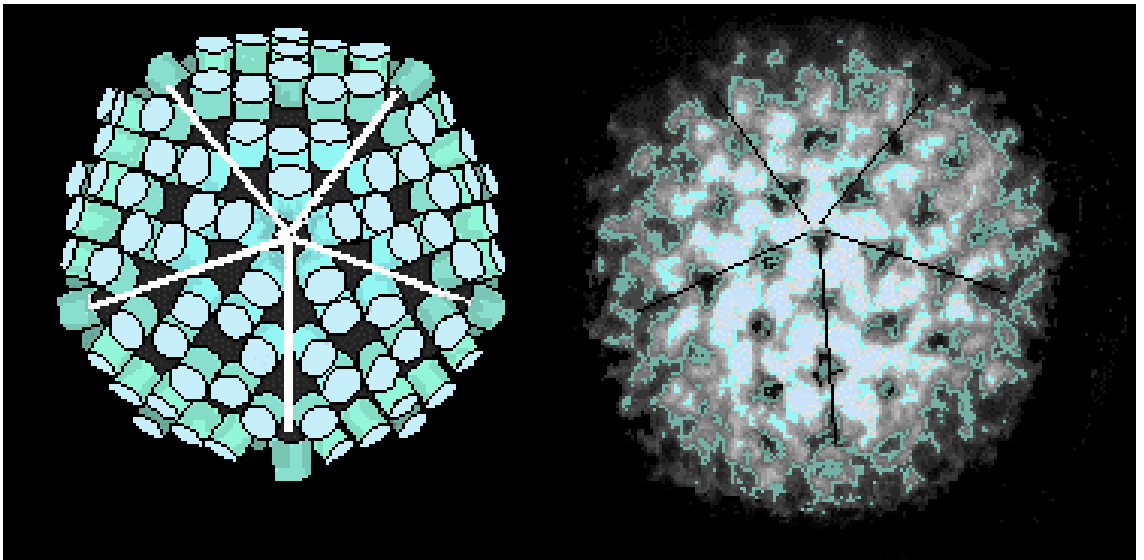
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Figure 1 EM photograph of double shelled rotavirus virion on the right and the single shelled virion on the left.



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Figure 2 Computer generated depiction of the outer capsid of rotavirus.



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Figure 3 Computer generated depiction of the inner capsid of rotavirus.



Figure 4 Uninfected CV-1 cells (Monkey Kidney).

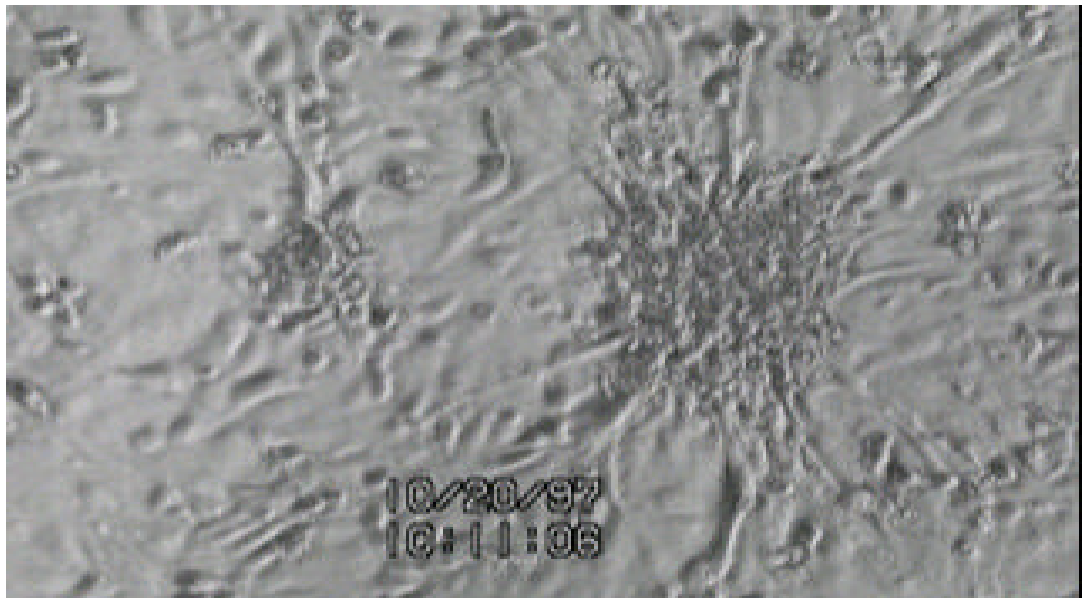


Figure 5 Late cytopathic effect in CV-1 cells (Monkey Kidney) infected with SA-11 rotavirus.

● SA-11 vs 45°C

SA-11 vs 45°C, pH 8.5

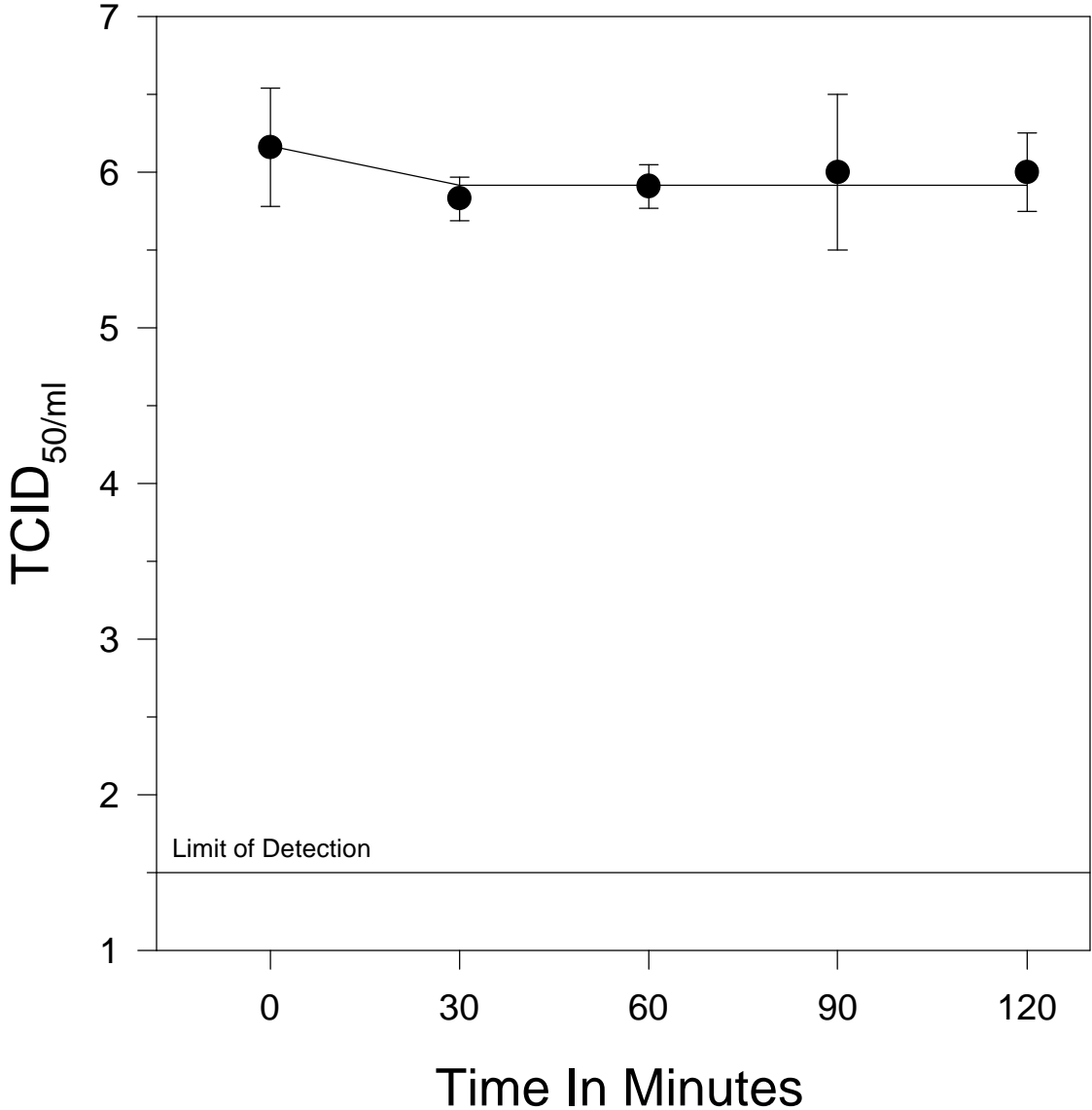


Figure 6. Simian-11 rotavirus tested against 45°C, at pH 8.5 for 2 hrs.

● SA-11 vs Alcalase

SA-11 vs 50°C, pH 8.5

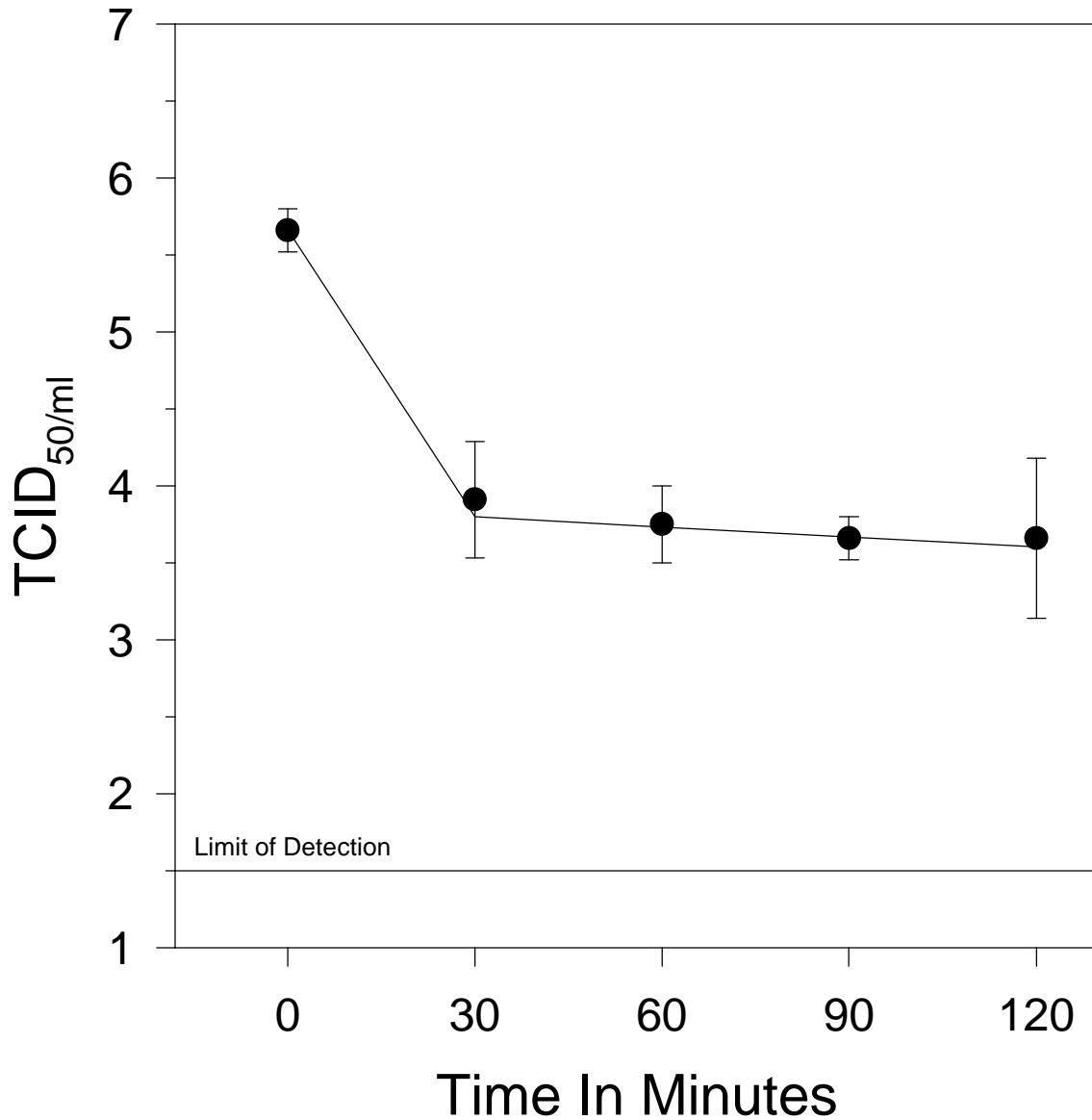


Figure 7. Simian-11 rotavirus tested against 50°C, at pH 8.5 for 2 hrs.

● SA-11 vs 55°C

SA-11 vs 55°C, pH 8.5

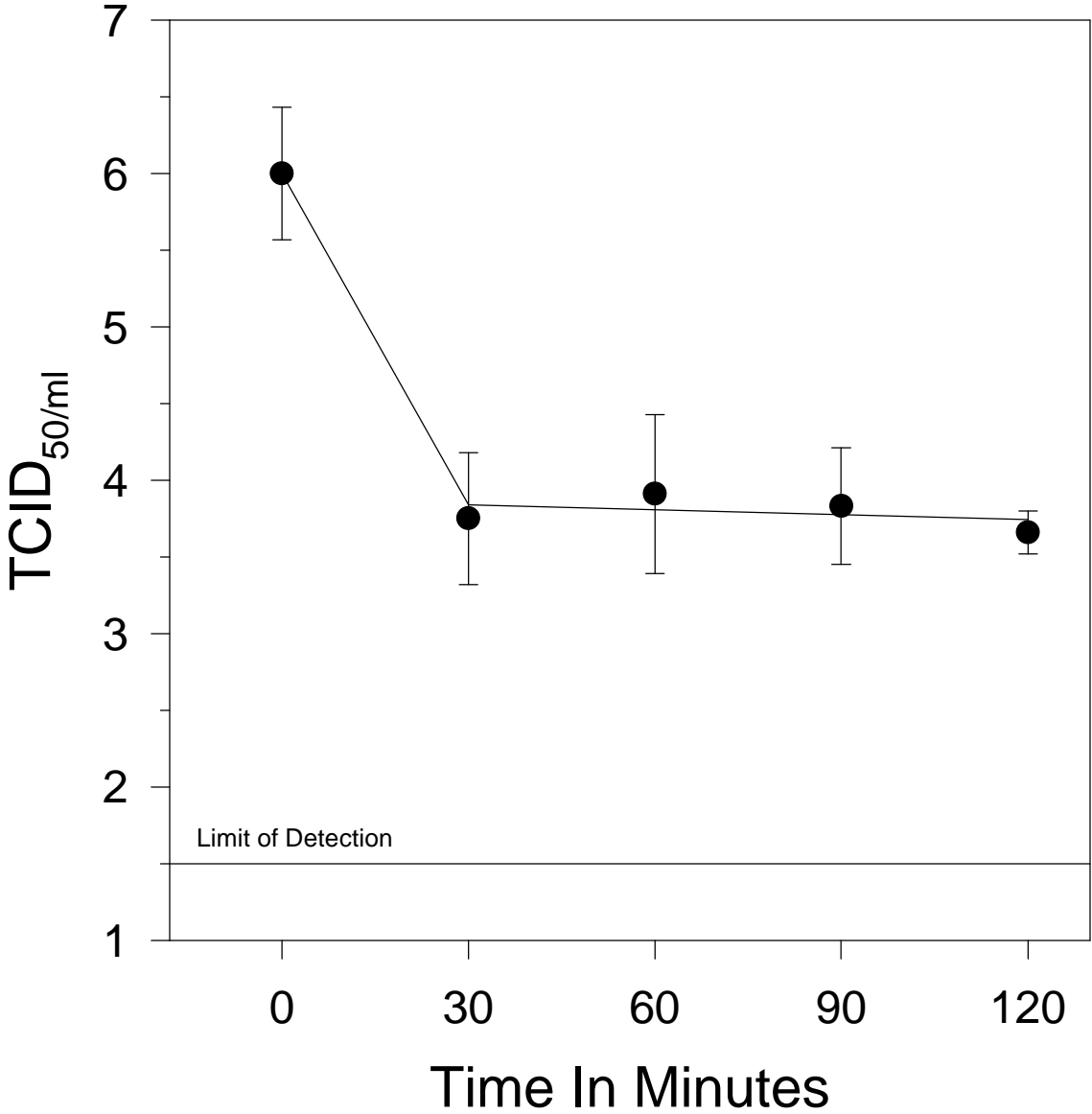


Figure 8. Simian-11 rotavirus tested against 55°C, at pH 8.5 for 2 hrs.

● SA-11 vs 60°C

SA-11 vs 60°C, pH 8.5

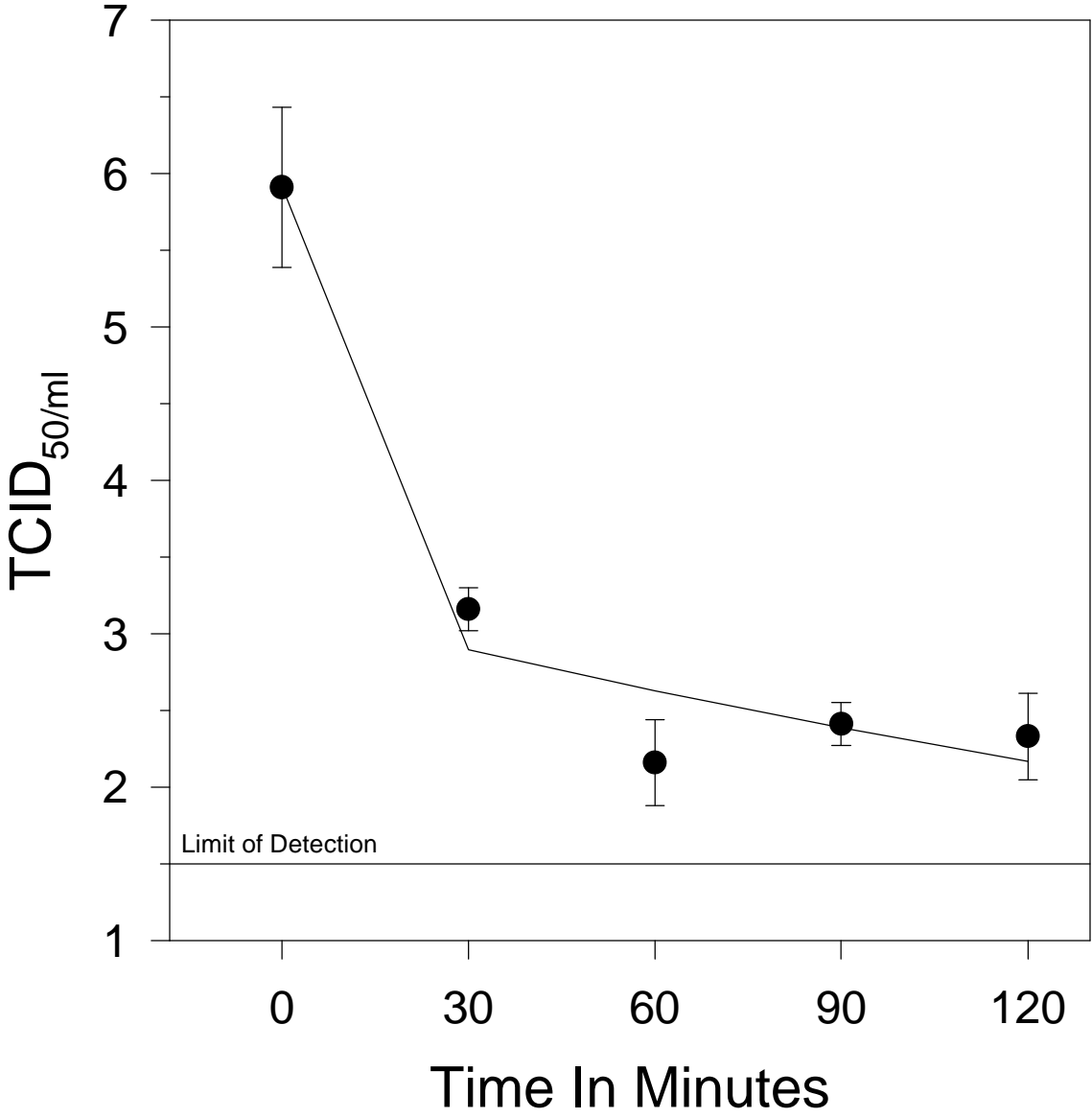


Figure 9. Simian-11 rotavirus tested against 60°C, at pH 8.5 for 2 hrs.

● SA-11 vs 65°C

SA-11 vs 65°C, pH 8.5

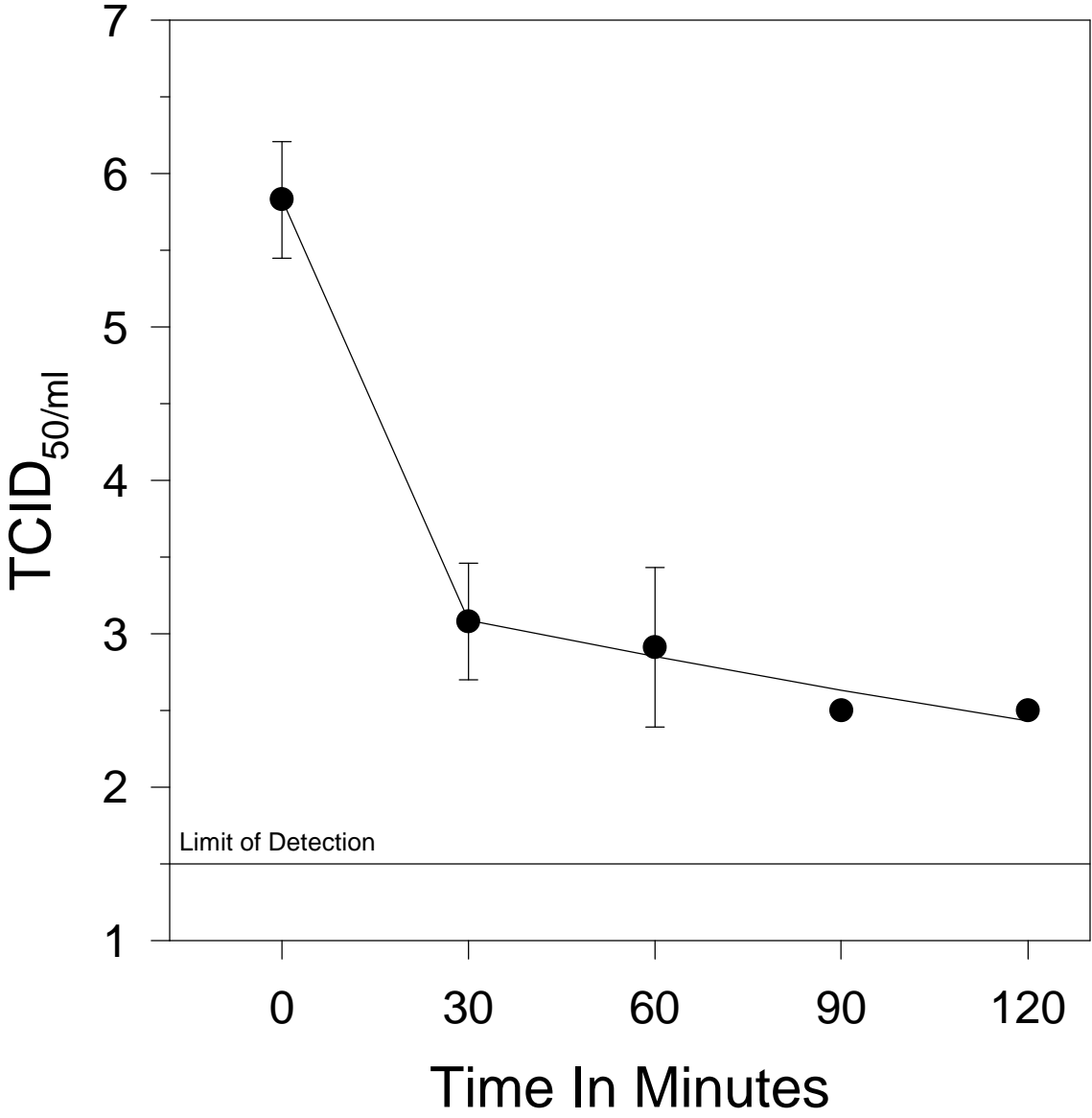


Figure 10. Simian-11 rotavirus tested against 65°C, at pH 8.5 for 2 hrs.

● SA-11 vs pH 8.5

SA-11 vs pH 8.5, 45°C

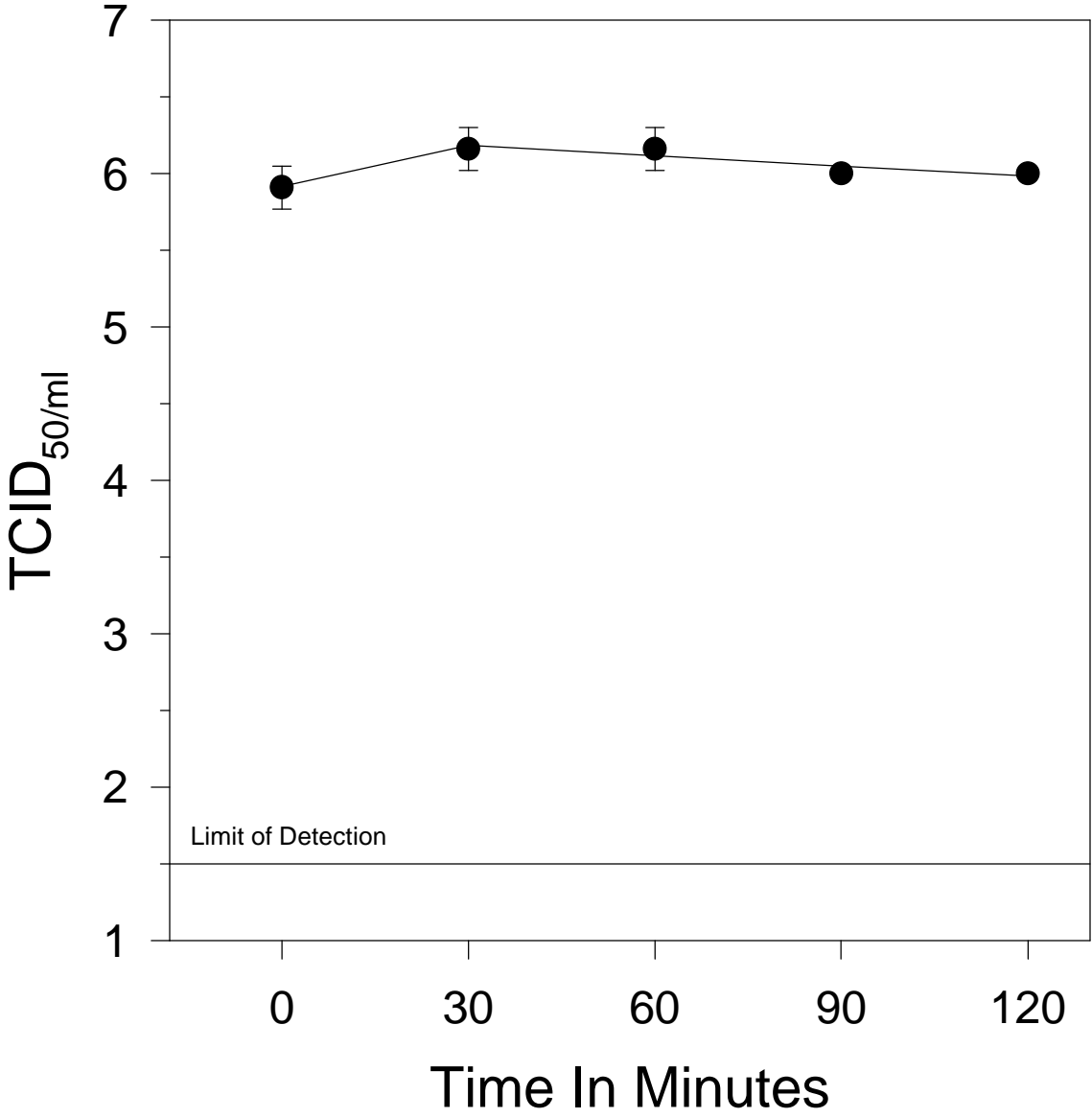


Figure 11. Simian-11 rotavirus tested against pH 8.5, at 45°C for 2 hrs.

● SA-11 vs pH 9

SA-11 vs pH 9, 45°C

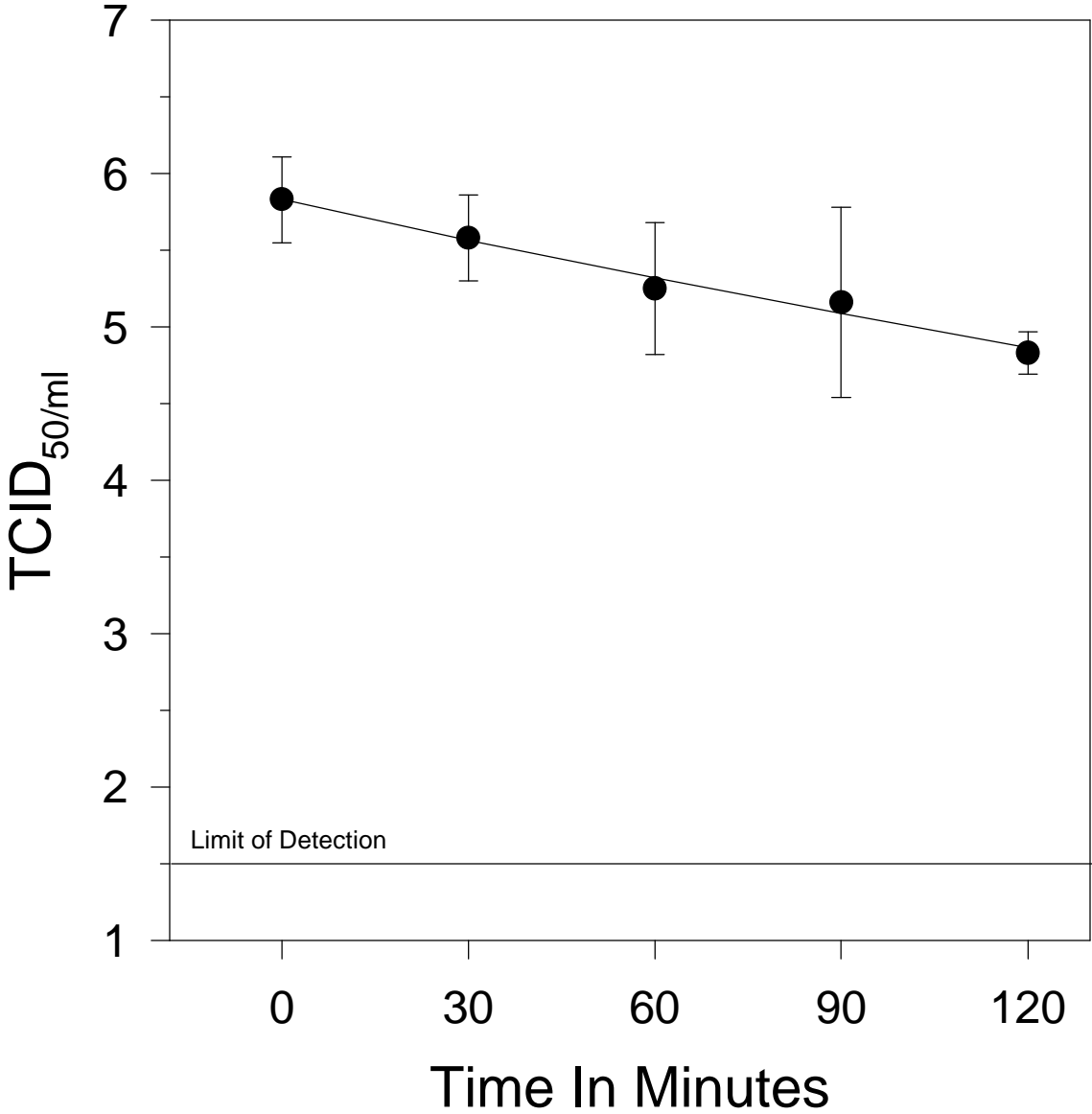


Figure 12. Simian-11 rotavirus tested against pH 9, at 45°C for 2 hrs.

● SA-11 vs 45°C

SA-11 vs pH 5, 45°C

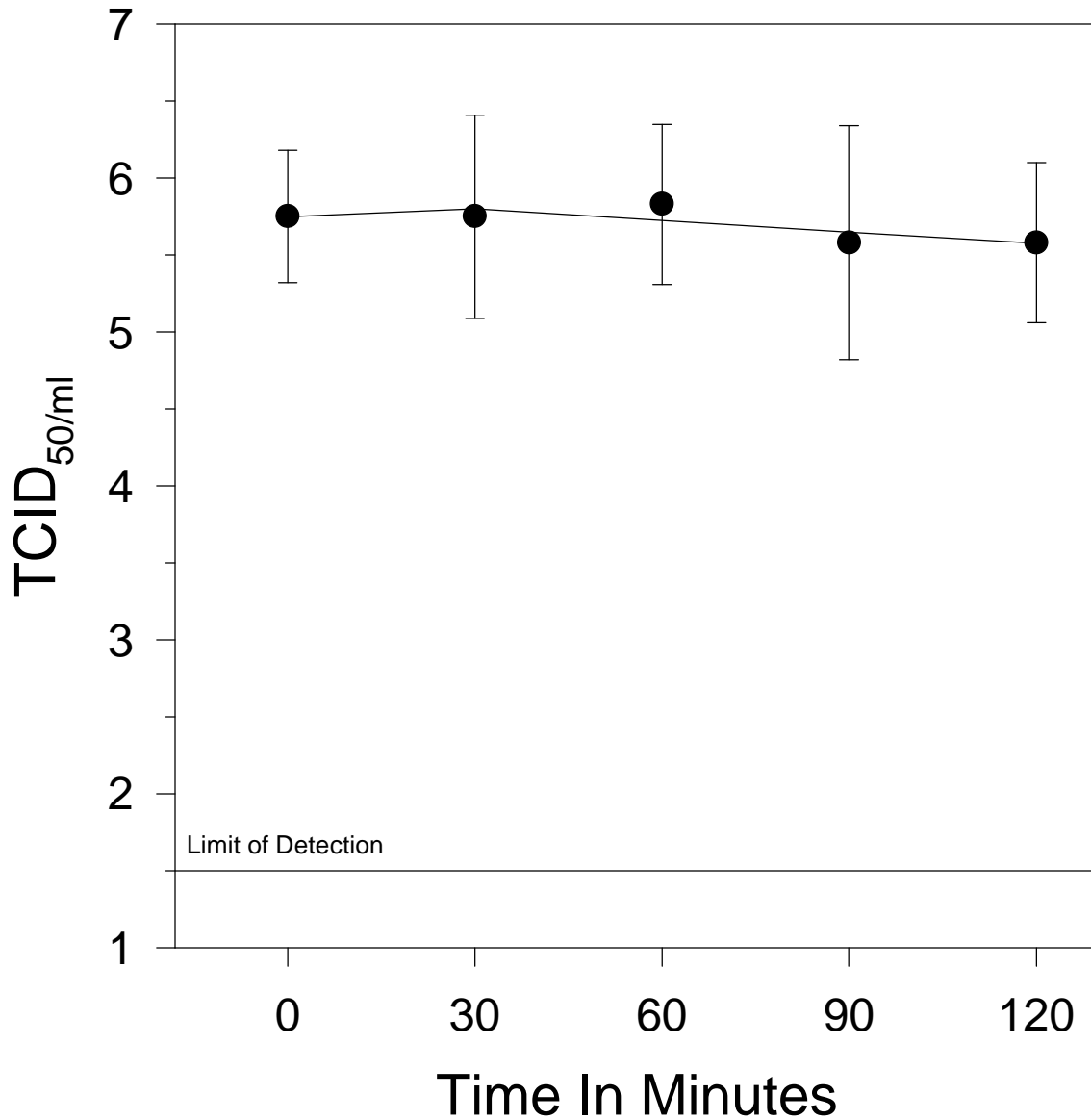


Figure 13. Simian-11 rotavirus tested against pH 5, at 45°C for 2 hrs.

● SA-11 vs pH 4

SA-11 vs pH 4, 45°C

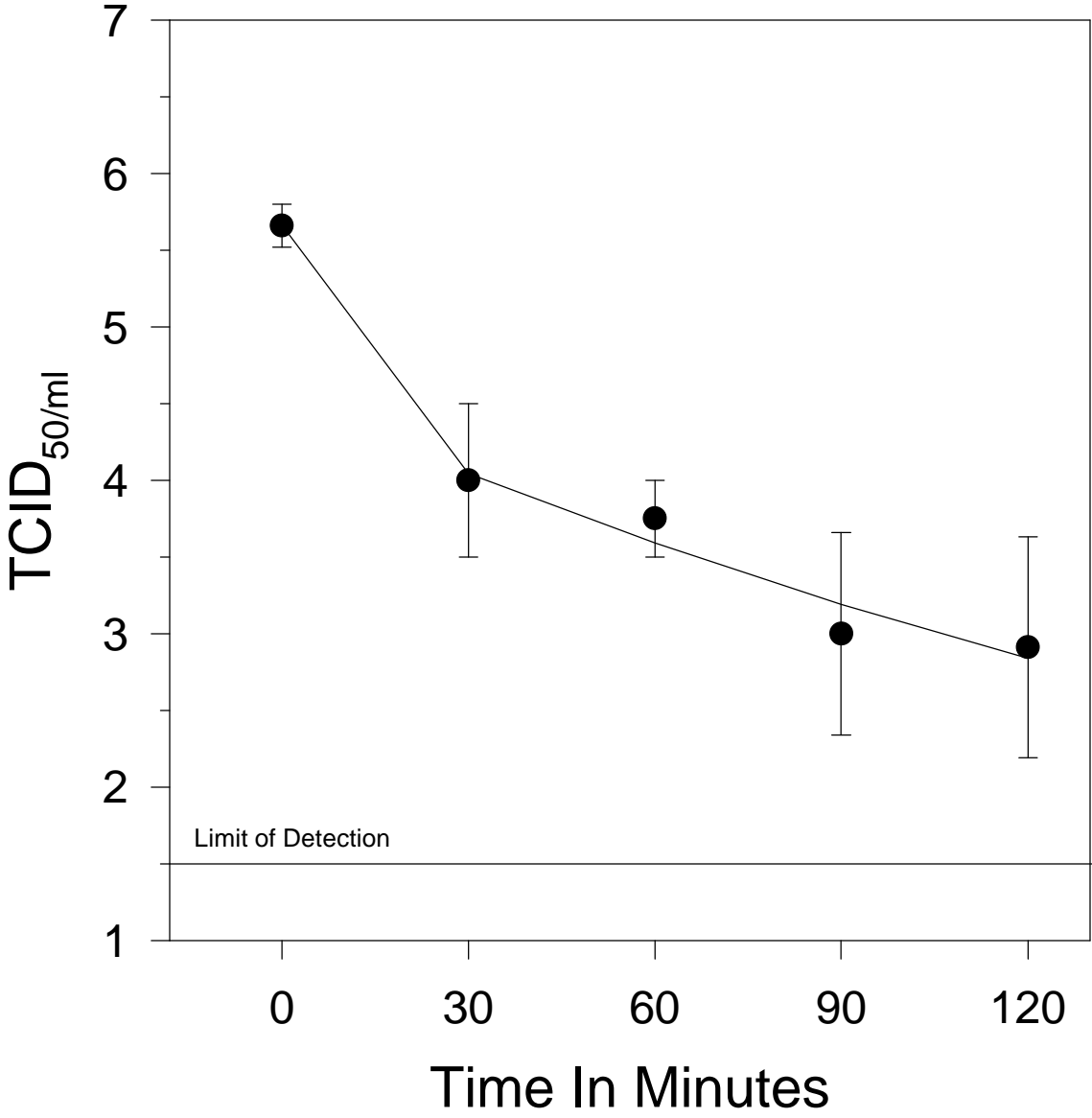


Figure 14. Simian-11 rotavirus tested against pH 4, at 45°C for 2 hrs.

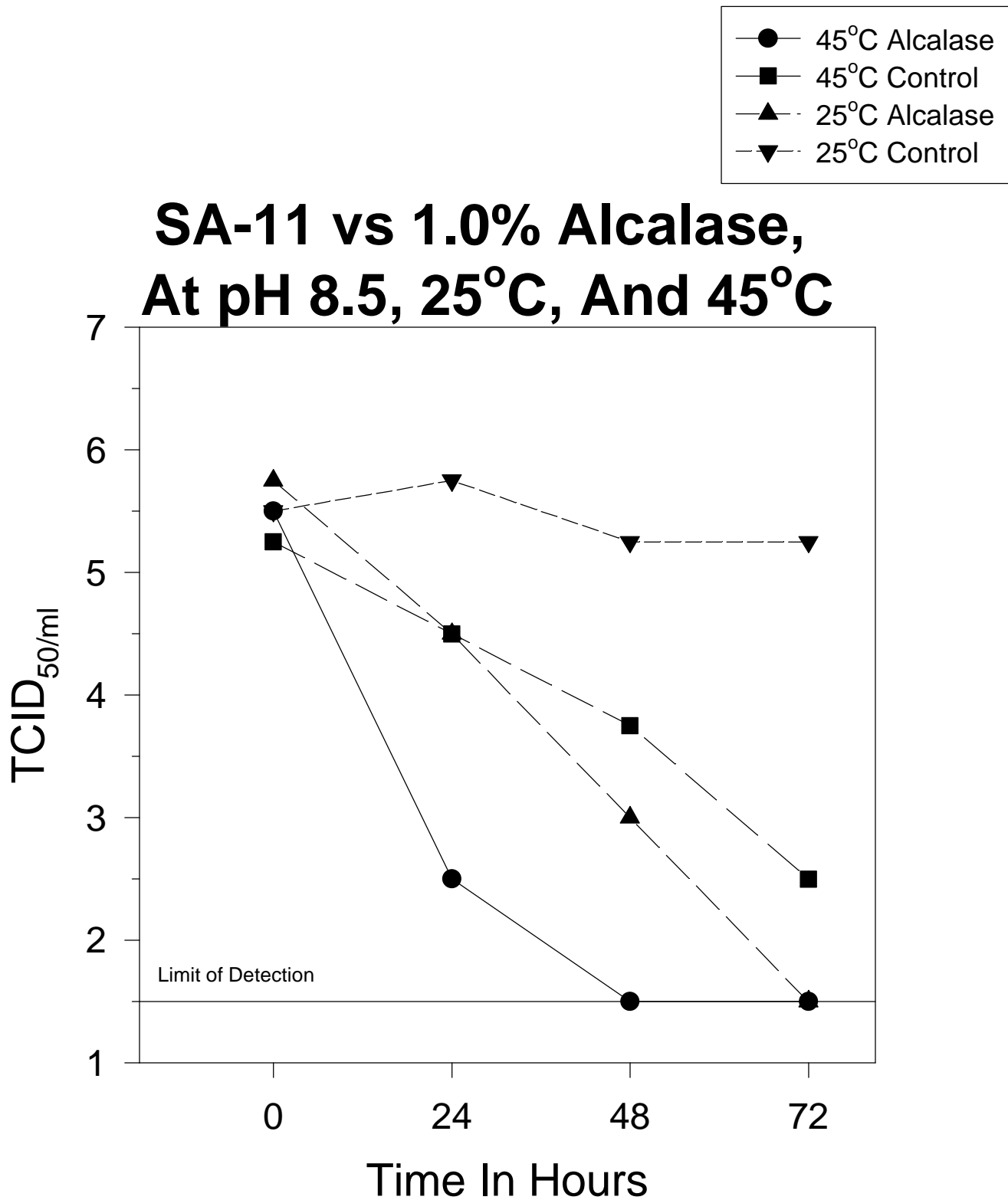


Figure 15. Simian-11 rotavirus tested against 1.0% Alcalase, at 25°C and 45°C, pH 8.5 for 3 days.

● SA-11 vs pH 2

SA-11 vs pH 2, 25°C

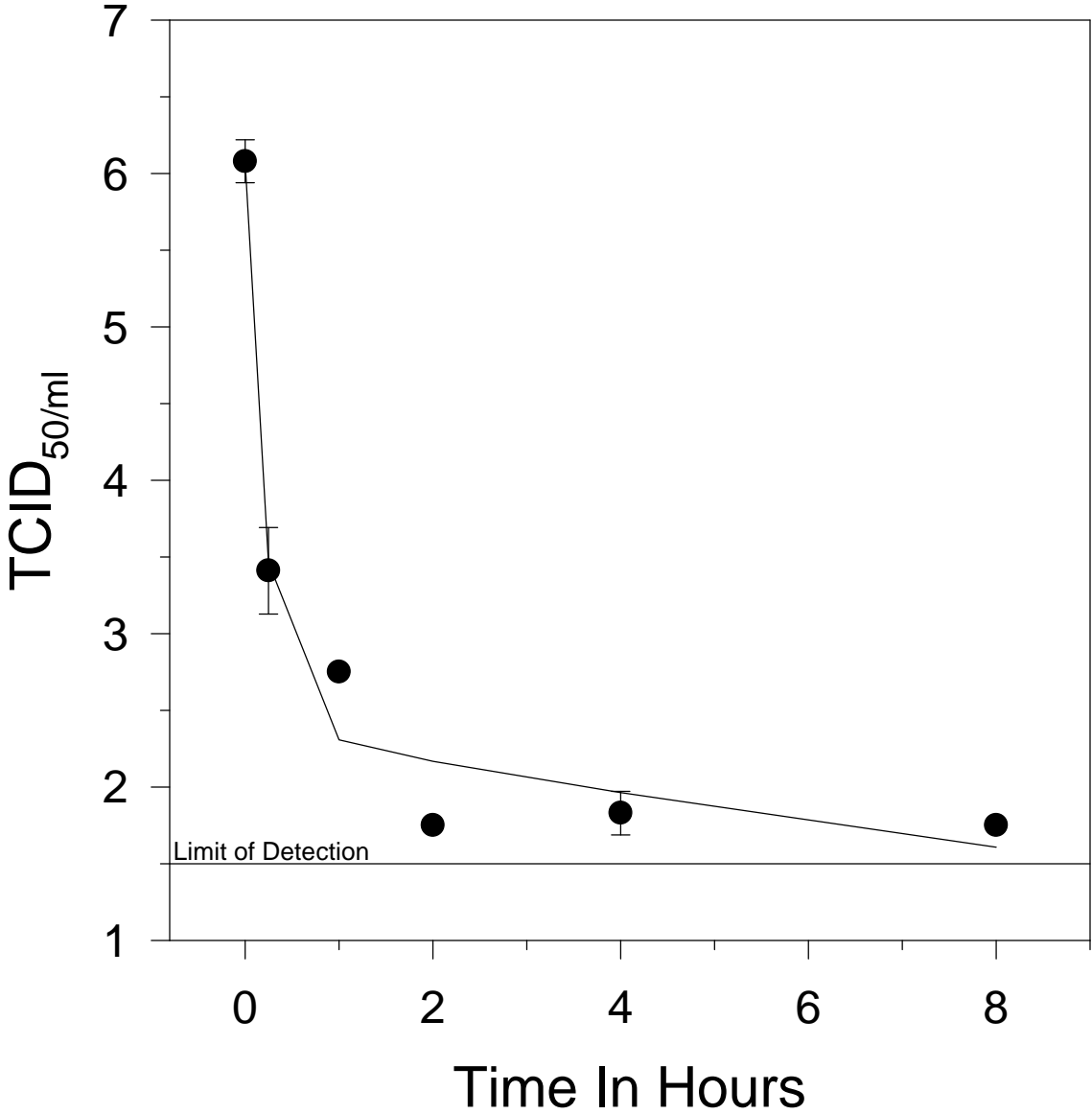


Figure 16. Simian-11 rotavirus tested against pH 2, at 25°C for 8 hrs.

● SA-11 vs pH 2

SA-11 vs pH 2, 15°C

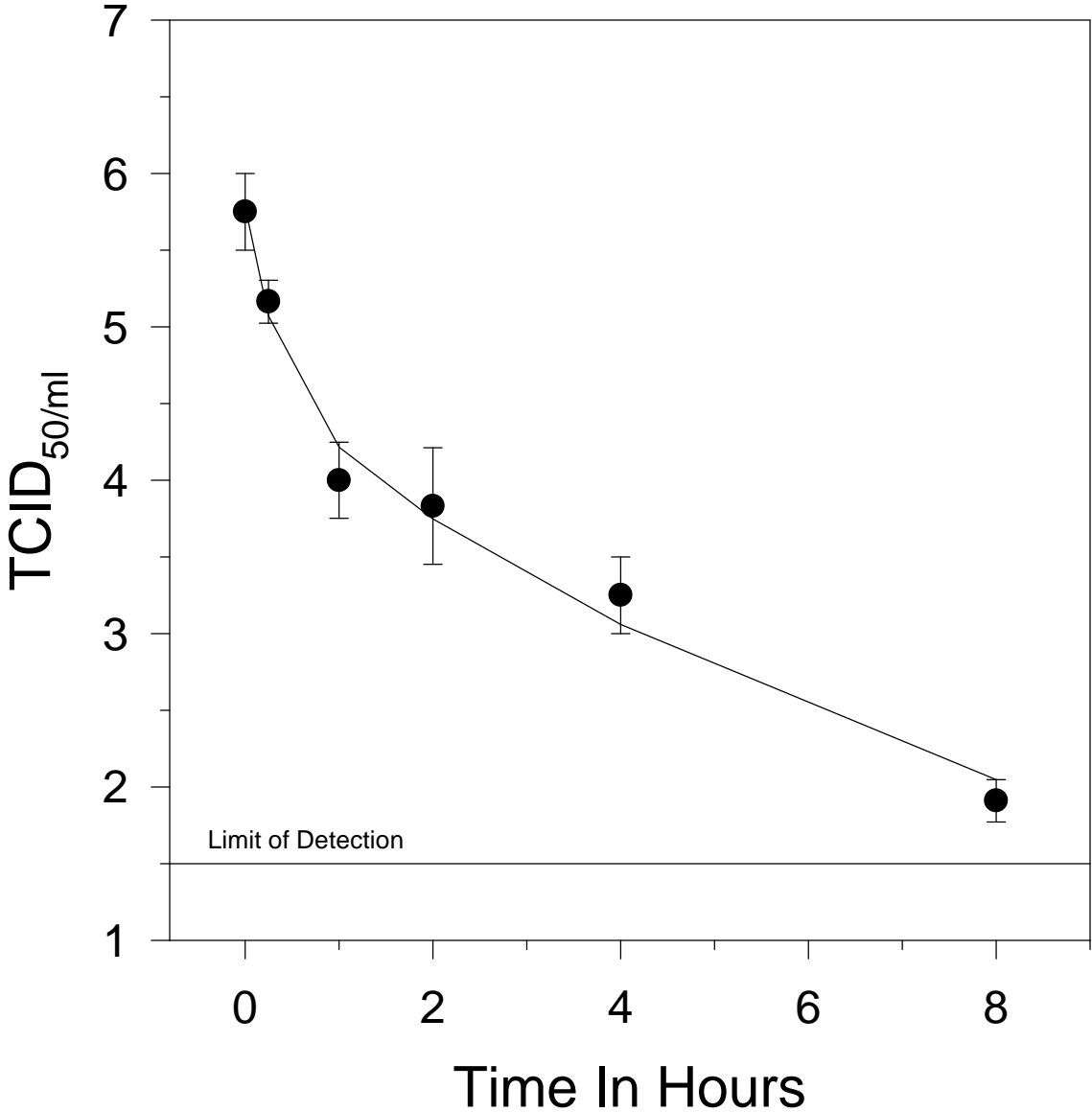


Figure 17. Simian-11 rotavirus tested against pH 2, at 15°C for 8 hrs.

● SA-11 vs pH 4

SA-11 vs pH 4, 25°C

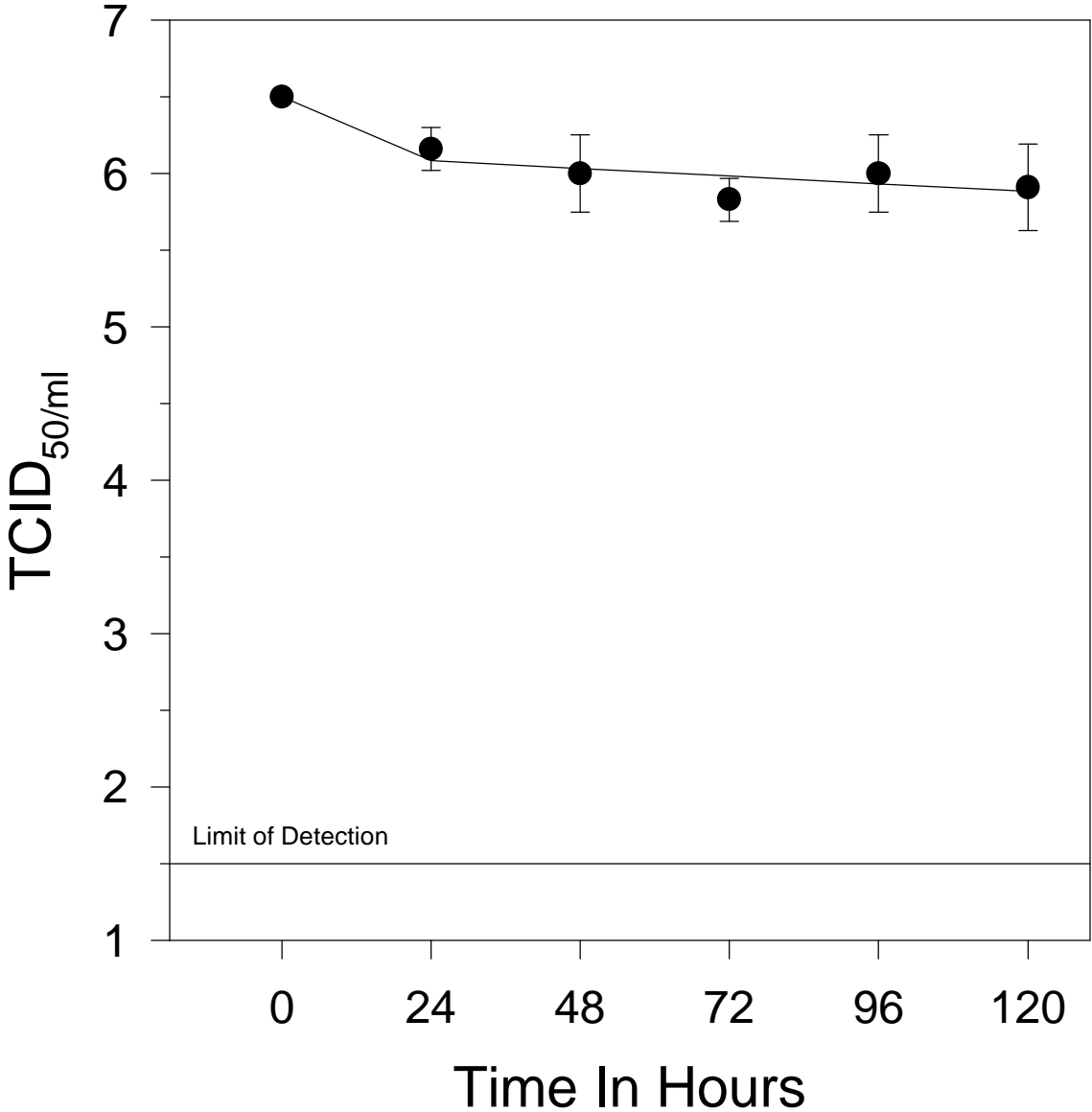


Figure 18. Simian-11 rotavirus tested against pH 4, at 25°C for 5 days.

● SA-11 vs pH 4

SA-11 vs pH 4, 15°C

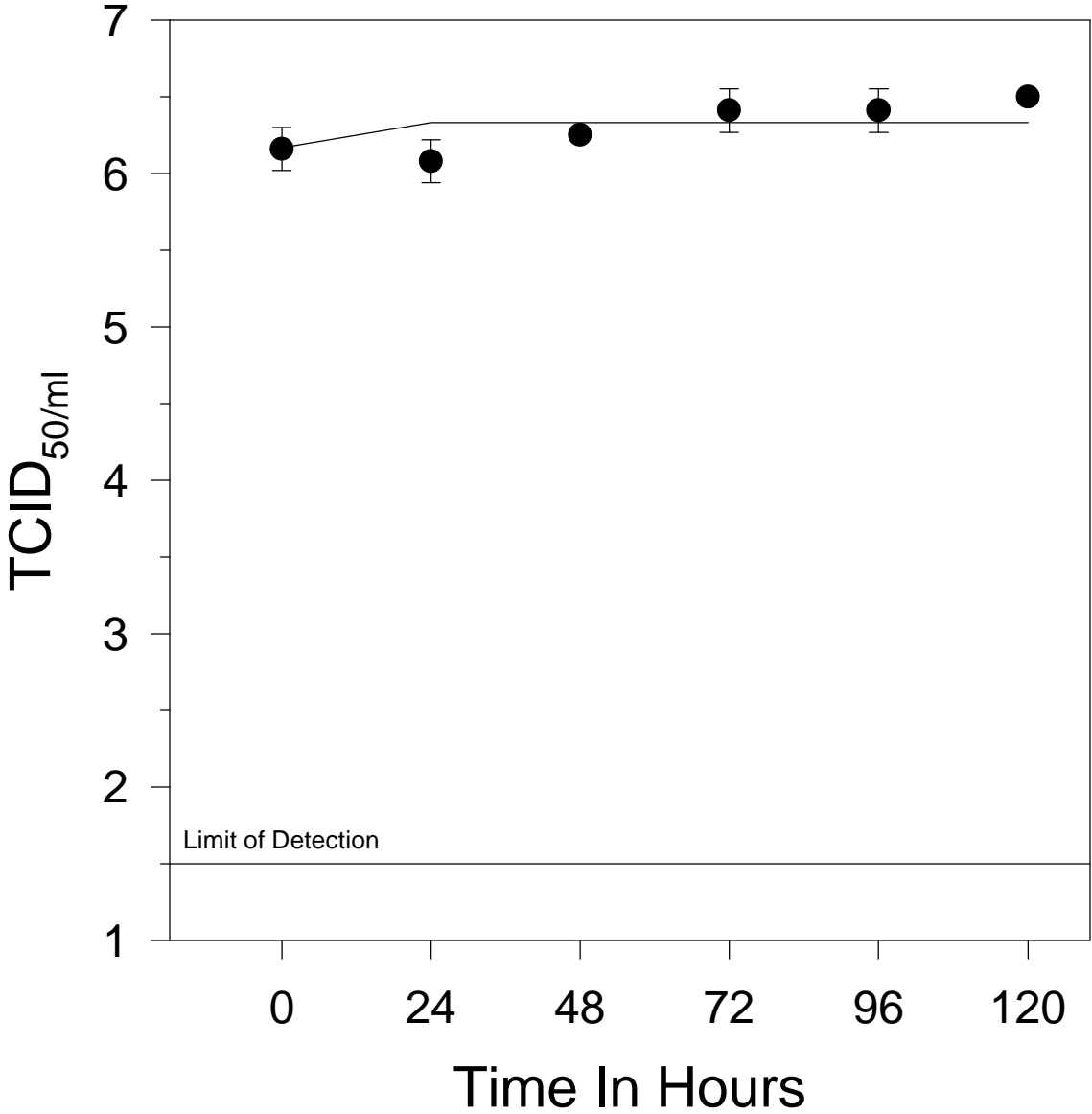


Figure 19. Simian-11 rotavirus tested against pH 4, at 15°C for 5 days.

● SA-11 vs pH 6

SA-11 vs pH 6, 25°C

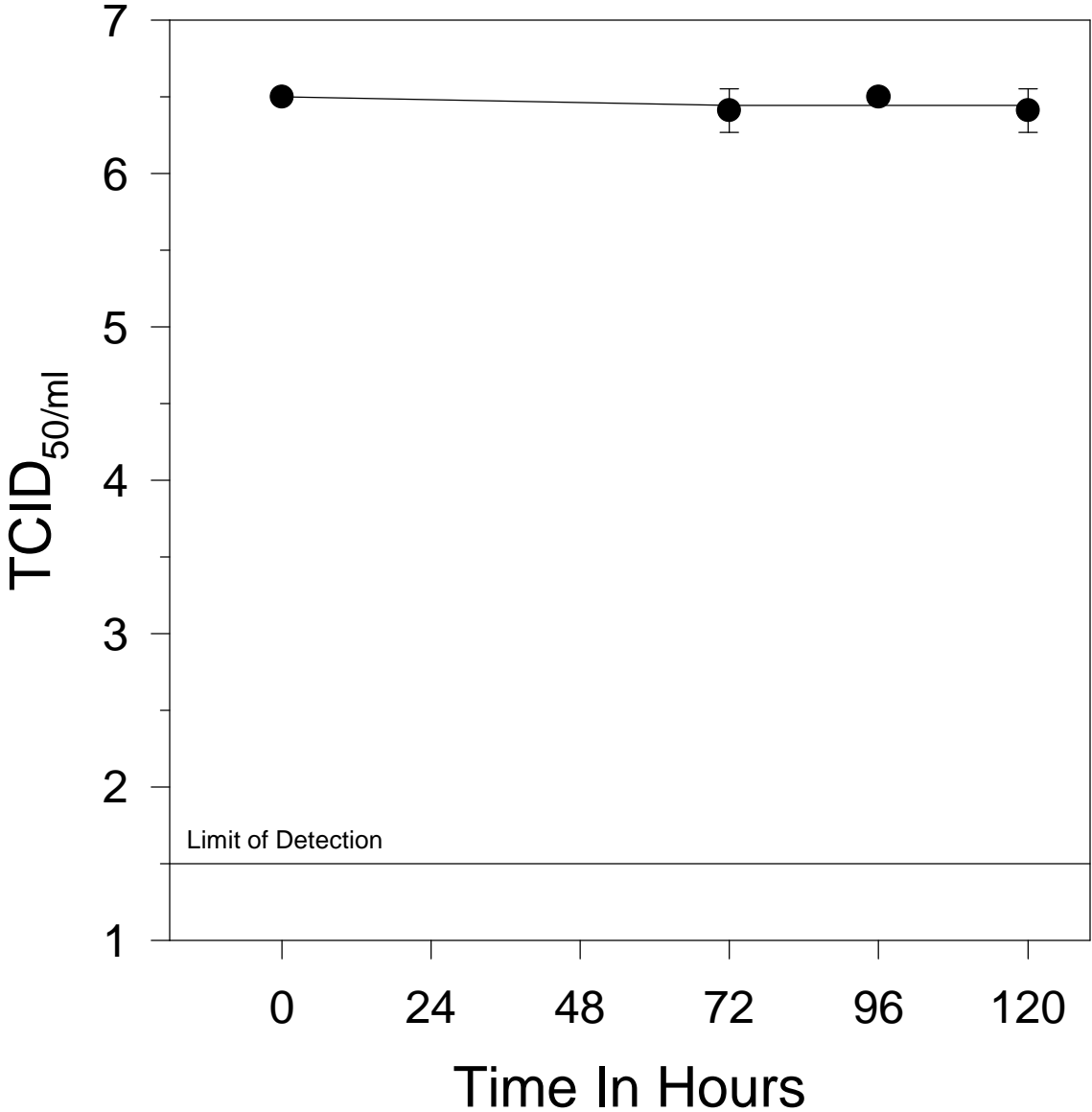


Figure 20. Simian-11 rotavirus tested against pH 6, at 25°C for 5 days.

● SA-11 vs pH 6

SA-11 vs pH 6, 15°C

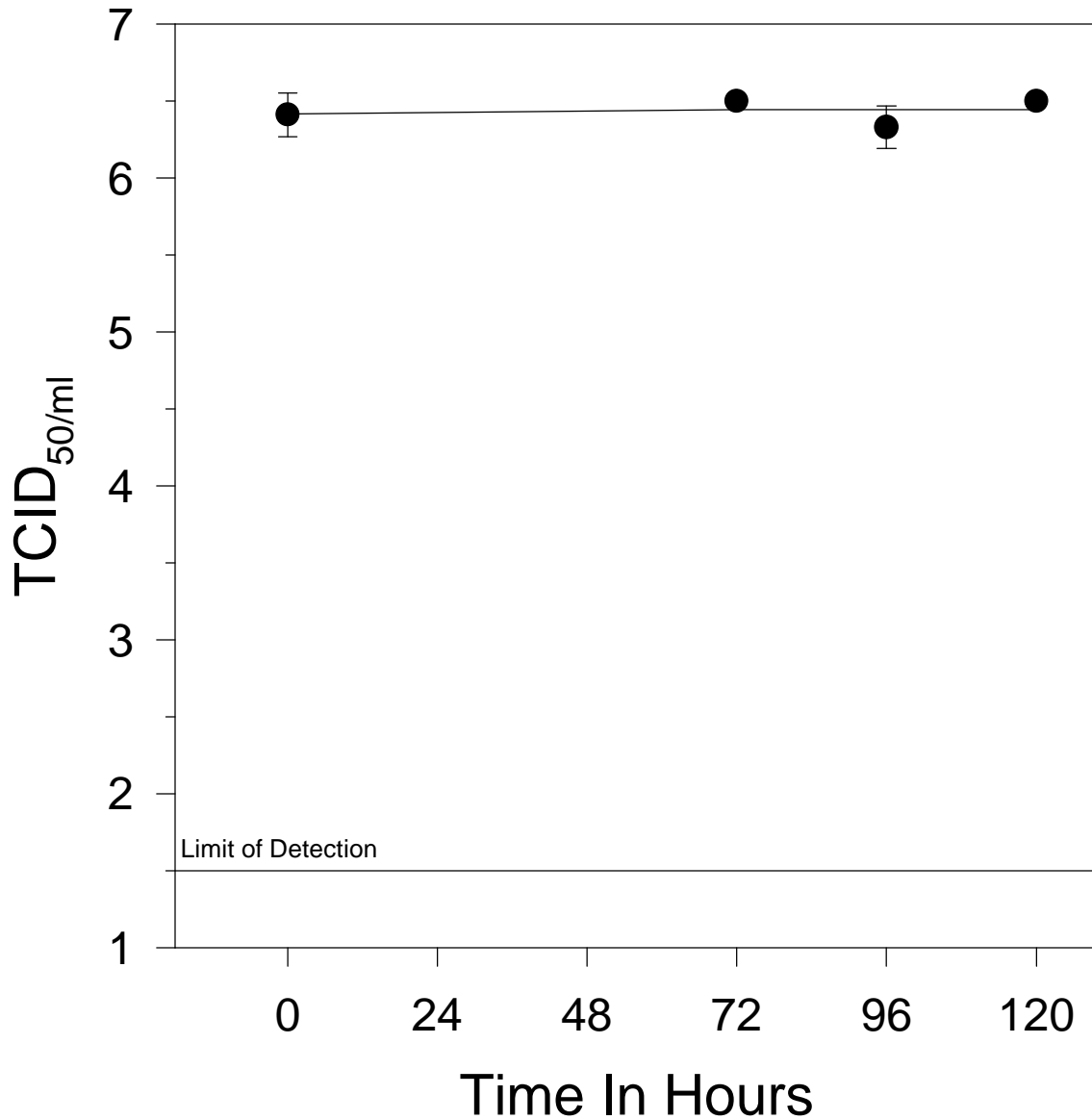


Figure 21. Simian-11 rotavirus tested against pH 6, at 15°C for 5 days.

● SA-11 vs Alcalase

SA-11 vs 1.0% Alcalase, 25°C, pH 6

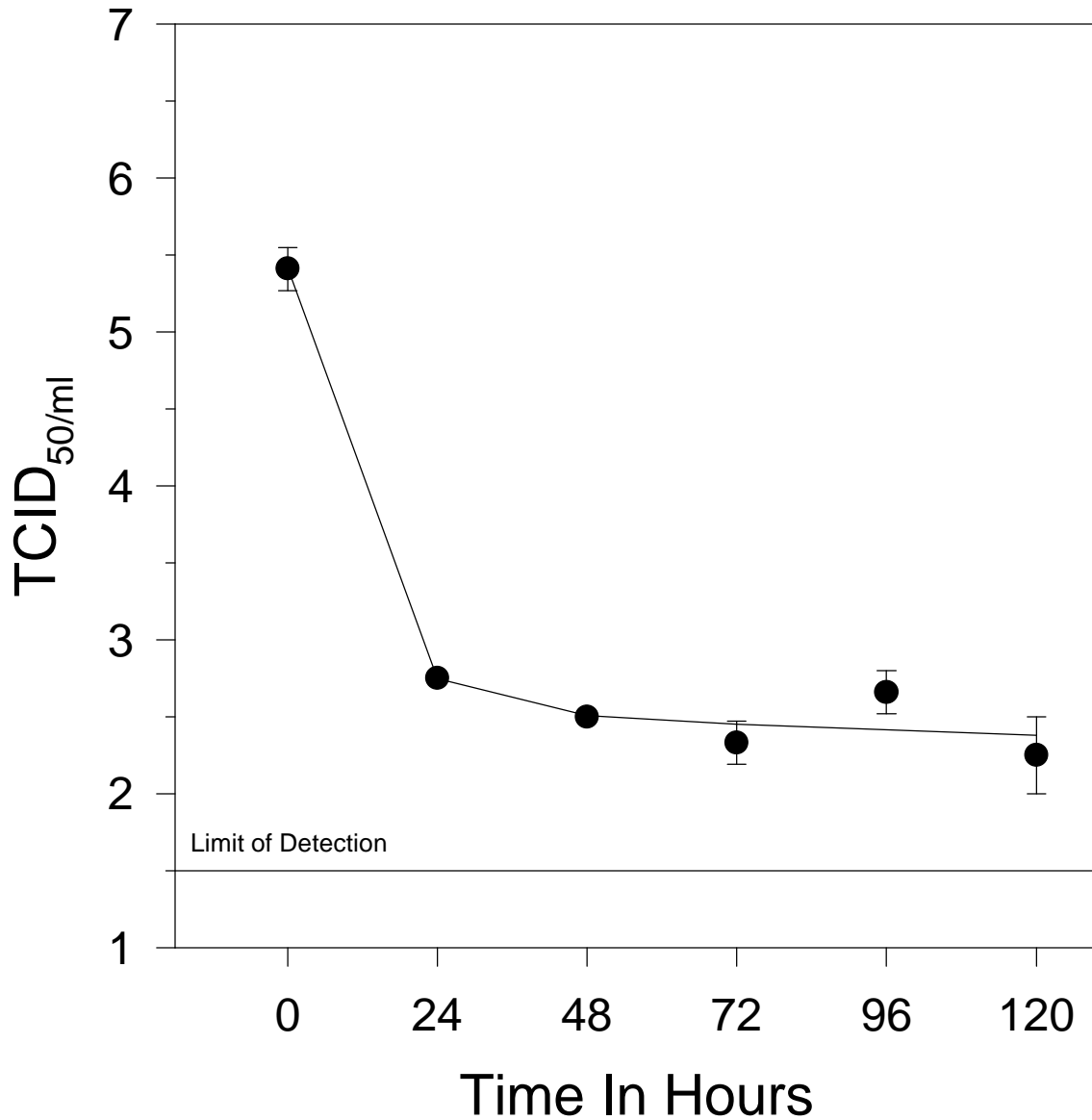


Figure 22. Simian rotavirus tested against 1.0% Alcalase, at 25°C, pH 6 for 5 days.

● SA-11 vs Alcalase

SA-11 vs 1.0% Alcalase, 15°C, pH 6

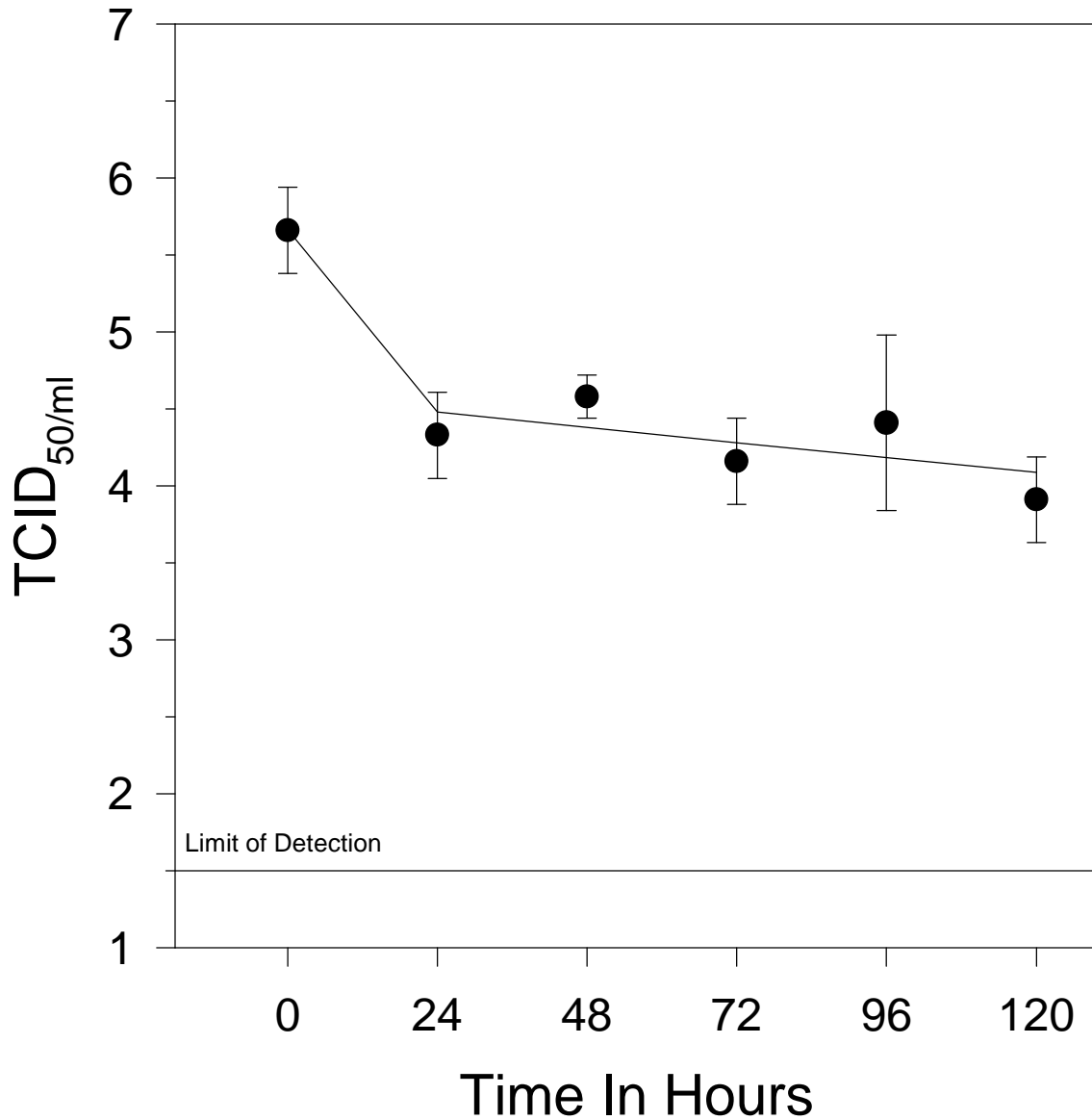


Figure 23. Simian-11 rotavirus tested against 1.0% Alcalase, at 15°C, pH 6 for 5 days.

● SA-11 vs Alcalase

SA-11 vs 0.1% Alcalase, 25°C, pH 6

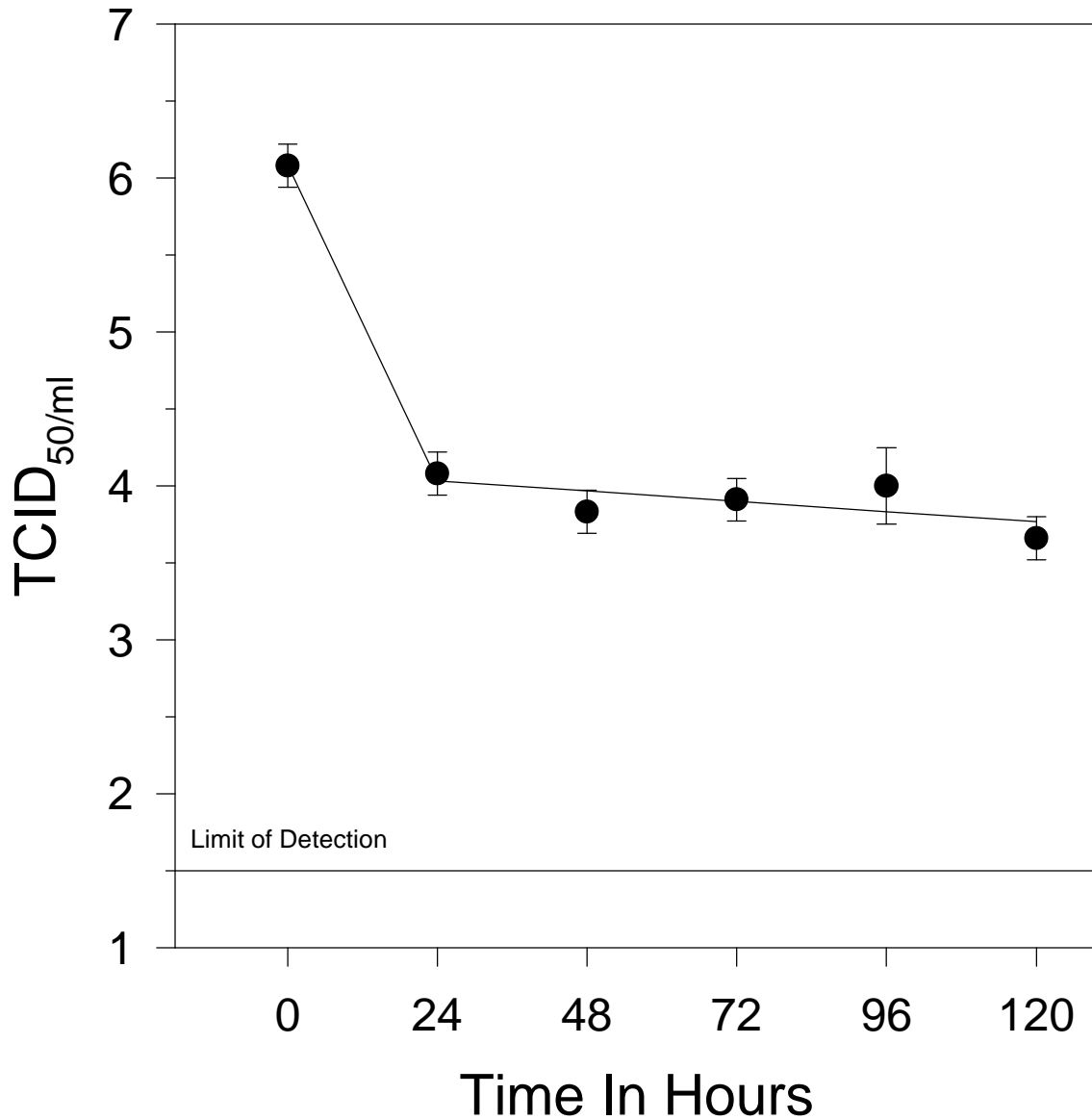


Figure 24. Simian-11 rotavirus tested against 0.1% Alcalase, at 25°C, pH 6 for 5 days.

● SA-11 vs Alcalase

SA-11 vs 0.1% Alcalase, 15°C, pH 6

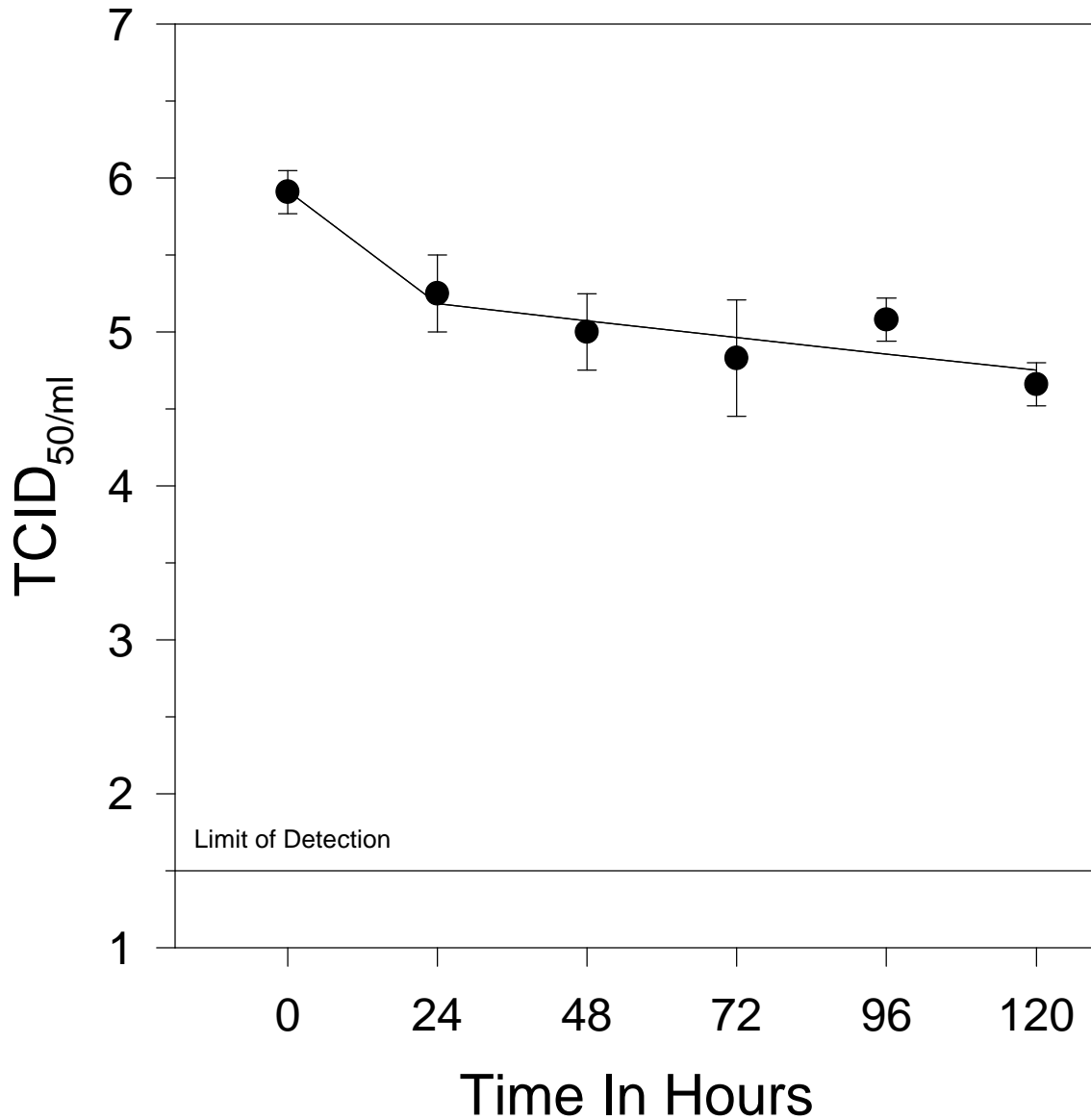


Figure 25. Simian-11 rotavirus tested against 0.1% Alcalase, at 15°C, pH 6 for 5 days.

DISCUSSION

The preliminary data showed that Simian-11 (SA-11) virus is a typical rotavirus as far as its resistance to pH and temperature are concerned. SA-11 rotavirus, while being sensitive to treatment at 50°C (Figure 7), was still infective following 2 hrs treatment at 65°C (Figure 10). During the initial test, rotaviral pH resistance was less than described in previously published literature, however all pH sensitivity tests in the literature were run at 25°C as opposed to 45°C tested here, which could account for the difference (Figures 11, 12, 13, and 14).

Through BLAST and FASTA tests (database comparison tools for determination of protein homology), and literature reviews, it was determined that SA-11 rotavirus possesses similar protein composition to human, porcine, and bovine rotaviruses while being the most resilient of them, thus representing a useful model for other rotaviruses in inactivation studies.

The initial tests of alkaline and neutral proteases, conducted at or near their pH optimum (pH 6.0 for the neutral protease and a pH 8.5 for the alkaline proteases), at 45°C, demonstrated considerable difference in their ability to inactivate SA-11. Even though SA-11 as a rotavirus is relatively resistant to temperature, pH extremes, and as an enteric virus resistant to the action of various gastrointestinal proteases, it proved to be susceptible to the action of various bacterial proteases. Savinase, Alcalase, and Durazym all were effective at decreasing rotaviral titer by more than 2 logs within two hours when used at high temperatures (45°C), and at optimal pH (Table 1). At 45°C the enzyme still retains about 70% of its activity (19), but this temperature far exceeds any that may be encountered in field conditions and obviously is highly dangerous to animals. This

cannot be considered for use on or near animals and would be very difficult to retain once the enzyme solution is applied in the field.

Testing of the enzyme at lower temperatures more closely related to field conditions (i.e. temperatures encountered in a calving facilities), was necessary. Temperatures of 25°C and 15°C were selected based on recommendations of clinicians as an average temperature range in which calving would take place. A temperature of 15°C being on the low end and 25°C on the higher end, using this range would allow for a more realistic comparison to field conditions (20). Testing at 25°C, pH 8.5 (enzyme pH optimal) showed that even with a decrease in temperature the enzyme was still capable of inactivating the virus. Representatives of the manufacturers of the enzymes, Novo Nordisk, informed us that for every 10°C drop in temperature experienced, the enzyme would lose ~50% of their activity. Alcalase has a temperature optimum of ~60°C and a pH optimum of pH ~8.5. By decreasing our temperatures to 25°C and 15°C, the enzymes would be expected to be working at 20% of their optimal at 25°C and at 15% at 15°C (19).

During testing for pH sensitivity at lower temperatures, we learned that as the temperature dropped, viral resistance to pH increased greatly. At 45°C and pH 4, SA-11 suffered titer loss in less than 2 hrs (Figure 14), but when the temperature was decreased to 25°C no titer loss was noticeable even after 120 hrs of treatment (Figure 18 and 19). It was postulated that this was due to the increased stability of the proteins as the temperature decreased (9). This means that the decrease temperature not only decreases efficacy of the enzyme but also increase stability of the virus.

Based on these experimental results, bacterial proteases do not appear to be an effective alternative to currently used disinfectants under field conditions and parameters that were tested, as was hypothesized. It appears that Alcalase while effective at inactivating SA-11 following 72 hrs exposure at 25°C, pH 8.5, and 1.0% enzyme concentration (Figure 15). It cannot be used to inactivate rotavirus at environmentally relevant temperatures and do not appear to be able to overcome all the variables introduced by the environment. Even under the most optimal conditions tested with the enzyme, 1.0% Alcalase, at 25°C, lowering pH to 6 stops the enzyme from being able to fully inactivate SA-11 following 5 days of treatment, only decreasing titer by ~3.75 logs (Figure 22). It is clear, that when the temperature declines to 15°C the enzyme is virtually inactive as a disinfectant. Accomplishing only a ~1.75 log reduction over 120 hrs at pH 6, and 15°C, with 1.0% Alcalase (Figure 23), and at pH 6, 15°C, 0.1% Alcalase a ~0.75 log reduction at 24 hrs and a ~1.0 log reduction at 120 hrs (Figure 25) is not sufficient to advocate use of Alcalase under field conditions.

Reviewing two journal articles, one of which reports about inactivation of rotavirus by chlorine (28), and the other about inactivation by ozone (29), a tailing effect was noticeable during viral inactivation. In these two studies, which only viewed inactivation over very short periods of time a large initial drop in titer by up to ~ 3 logs was seen after 20 seconds of exposure followed by little to no drop over the next 40 seconds. Currently it is unclear what causes the tailing effect of the virus; this effect was not discussed in the article. The same effect was found in our studies, after a short exposure a substantial titer reduction is seen followed by little to no reduction over the next couple of time points. If the enzyme was able to reduce the titer equally at a rate

equal to the ones observed between 0 and the first time point beyond the next couple of time points, complete inactivation would have occurred within 2-5 of days sometimes less, depending on conditions used.

Even though the enzyme did not achieve total viral inactivation, the use of enzymes as a disinfectant still may be applicable in certain situations. If the enzyme would have inactivated the virus enough to achieve a decrease in the severity of clinical signs among infected calves the project would have succeeded, but the rate of inactivation observed in this study, was found to be below acceptable standards for a disinfectant. In rotaviral infections where the titer often reaches 10^8 infectious virions/ml a 1-2 logs reduction will not likely reduce the number of infectious virions to such a degree that clinical signs will not occur or even be diminished. Our tests having been run in a clean system without the presence of organic matter that may interfere with the enzyme's activity, represent probably the best case scenarios for enzyme activity. In field conditions temperatures will probably often go below 15°C , the pH may also fall below what was given as an average, all these factors possibly contributing to a decrease in enzyme efficacy.

The idea of using bacterial proteases to inactivate rotaviruses even with these initial results is still one that should be analyzed further. It is quite evident that calves' active immunity cannot stop rotavirus and that passive maternal immunity alone, which often interferes with the development of active immunity of the calf, cannot be relied upon for protection. The only real solution is a combination of the three, active immunity, passive immunity, and disinfection. Because viral disinfection is the most important one, which the farmers have the most control over, this should be the initial

starting point. Use of the proteases, as a disinfection method may not have been as effective as first hoped but could still play an essential role in helping to reduce clinical signs or aid in stopping the transmission of rotaviral infections. What makes these enzymes so highly regarded is their low cost and ease of use. The enzyme may be used in the presence of animals and then left there indefinitely without ever needing to be washed away. More studies should be conducted to determine if repeated treatments, once a day or every other day might be effective at inactivating rotaviruses, since such a large initial drop is seen following the first day of treatment. While it is possible that the fecal matter may inhibit the enzyme, one may also hypothesize that the enzyme may actually predispose the virus to other environmental factors, such as substances in fecal matter that are not normally harmful to the virus.

The enzymes used under near optimal to optimal conditions are quite capable of inactivating rotaviruses. The enzymes tested were just a small subset of the overall enzyme population. A search for more enzymes should be conducted to determine if in fact other enzymes, with lower temperature optimums and pH optimums close to pH 6 could be discovered. Finding a protease with a pH optimal of 6 would stop any loss of enzyme activity by the pH of the fecal matter. Currently there is a 55-60% reduction in enzyme activity by using the protease at such a low pH (10), which greatly hinders the rate of inactivation. Further searches should also be conducted for other enzymes with lower temperature optimal. Every one of the enzymes tested here had an optimal temperature well above 45°C, which is far too high for field conditions. By using such an enzyme a large decrease in enzyme activity is seen from the start. Compounding the low temperature with a low pH is a great disadvantage to the protease. With addition of

increased stability of viral proteins with decreasing temperatures the enzyme is very much handicapped. With the discovery of an enzyme with lower pH and temperature optimal a much greater likelihood of inactivation would be seen.

Further tests should be conducted on the use of enzymes as an aid to currently used viral disinfectants. The enzymes are quite capable of freeing the virus from sloughed off intestinal tissue and should be tested in conjunction with other disinfectants. The enzymes may be capable of freeing up the virus so that other less noxious disinfectants, not normally capable of inactivating rotavirus, would do so. This may allow for the use of a disinfectant that could be used around the animals without first having to remove them.

Overall, the use of enzymes as a disinfectant or a disinfection aid should merit further evaluation. Very few parameters were actually analyzed in this study. Introduction of other proteases and more optimal conditions under which the protease would have to act would greatly increase the success of the project. The use of these enzymes would again be safe for the use on or around small animals, environmentally and cost efficient.

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APPENDIX A

Data from inactivation of SA-11 rotavirus

2 hr Temp. exposure, pH 8.5	Replica	Sampling timepoints (min.)				
45°C		0	30	60	90	120
	1	6.5	6	6	6.5	6.25
	2	6.25	5.75	5.75	5.5	5.75
	3	5.75	5.75	6	6	6
Mean		6.166667	5.833333	5.916667	6	6
Stdev		0.381881	0.144338	0.144338	0.5	0.25
50°C		0	30	60	90	120
	1	5.5	4	3.75	3.75	3.5
	2	5.75	3.5	3.5	3.5	3.25
	3	5.75	4.25	4	3.75	4.25
Mean		5.666667	3.916667	3.75	3.666667	3.666667
Stdev		0.144338	0.381881	0.25	0.144338	0.520416
55°C		0	30	60	90	120
	1	5.75	3.5	3.5	3.5	3.75
	2	5.75	3.5	3.75	3.75	3.5
	3	6.5	4.25	4.5	4.25	3.75
Mean		6	3.75	3.916667	3.833333	3.666667
Stdev		0.433013	0.433013	0.520416	0.381881	0.144338
60°C		0	30	60	90	120
	1	6.5	3.25	2	2.5	2.5
	2	5.75	3	2.5	2.5	2.5
	3	5.5	3.25	2	2.25	2
Mean		5.916667	3.166667	2.166667	2.416667	2.333333
Stdev		0.520416	0.144338	0.288675	0.144338	0.288675
65°C		0	30	60	90	120
	1	5.75	3	2.75	2.5	2.5
	2	5.5	2.75	2.5	2.5	2.5
	3	6.25	3.5	3.5	2.5	2.5
Mean		5.833333	3.083333	2.916667	2.5	2.5
Stdev		0.381881	0.381881	0.520416	0	0

2 Hour pH exposure, 45°C

pH 9		0	30	60	90	120
	1	6	5.75	5.5	5.75	4.75
	2	5.5	5.25	4.75	4.5	4.75
	3	6	5.75	5.5	5.25	5

Mean		5.833333	5.583333	5.25	5.166667	4.833333
Stdev		0.288675	0.288675	0.433013	0.629153	0.144338

pH 8.5		0	30	60	90	120
	1	5.75	6	6.25	6	6
	2	6	6.25	6	6	6
	3	6	6.25	6.25	6	6

Mean		5.916667	6.166667	6.166667	6	6
Stdev		0.144338	0.144338	0.144338	0	0

pH 5		0	30	60	90	120
	1	5.25	5	5.25	4.75	5
	2	6	6	6.25	5.75	5.75
	3	6	6.25	6	6.25	6

Mean		5.75	5.75	5.833333	5.583333	5.583333
Stdev		0.433013	0.661438	0.520416	0.763763	0.520416

pH 4		0	30	60	90	120
	1	5.75	3.5	3.5	2.5	2.5
	2	5.5	4.5	3.75	2.75	2.5
	3	5.75	4	4	3.75	3.75

Mean		5.666667	4	3.75	3	2.916667
Stdev		0.144338	0.5	0.25	0.661438	0.721688

1.0% Enz. 45°C, at pH optimal

	0	120	120C
Alcalase	5.5	2.75	5.5
Durazyme	5.5	3.25	5.5
Savinase	5.5	3.25	5.5
Neutrase	5.5	4.5	5.5

	Replica	Sampling timepoints (hrs.)			
1.0% Alcalase 25°C & 45°C, pH 8.5		0	24	48	72
45°C 1% enzyme		5.5	2.5	1.5	1.5
45°C Control		5.25	4.5	3.75	2.5
25°C 1% enzyme		5.75	4.5	3	1.5
25°C Control		5.5	5.75	5.25	5.25

pH 2		0	0.25	1	2	4	8
15°C	1	5.75	5.25	4.25	3.5	3	2
	2	5.5	5.25	3.75	3.75	3.25	1.75
	3	6	5	4	4.25	3.5	2
Mean		5.75	5.166667	4	3.833333	3.25	1.916667
Stdev		0.25	0.144338	0.25	0.381881	0.25	0.144338
pH 2		0	0.25	1	2	4	8
25°C	1	6	3.75	2.75	1.75	1.75	1.75
	2	6	3.25	2.75	1.75	1.75	1.75
	3	6.25	3.25	2.75	1.75	2	1.75
Mean		6.083333	3.416667	2.75	1.75	1.833333	1.75
Stdev		0.144338	0.288675	0	0	0.144338	0
pH 4		0	24	48	72	96	120
15°C	1	6.25	6	6.25	6.5	6.5	6.5
	2	6.25	6	6.25	6.25	6.5	6.5
	3	6	6.25	6.25	6.5	6.25	6.5
Mean		6.166667	6.083333	6.25	6.416667	6.416667	6.5
Stdev		0.144338	0.144338	0	0.144338	0.144338	0
pH 4		0	24	48	72	96	120
25°C	1	6.5	6.25	6	5.75	6.25	5.75
	2	6.5	6.25	6.25	6	5.75	6.25
	3	6.5	6	5.75	5.75	6	5.75
Mean		6.5	6.166667	6	5.833333	6	5.916667
Stdev		0	0.144338	0.25	0.144338	0.25	0.288675
pH 6		0	72	96	120		
15°C	1	6.5	6.5	6.25	6.5		
	2	6.25	6.5	6.25	6.5		
	3	6.5	6.5	6.5	6.5		
Mean		6.416667	6.5	6.333333	6.5		
Stdev		0.144338	0	0.144338	0		

pH 6 25°C		0	72	96	120	
	1	6.5	6.5	6.5	6.5	
	2	6.5	6.25	6.5	6.5	
	3	6.5	6.5	6.5	6.25	

Mean		6.5	6.416667	6.5	6.416667	
Stdev		0	0.144338	0	0.144338	

1.0% Alcalase pH 6 25°C		0	24	48	72	96	120
	1	5.5	2.75	2.5	2.25	2.75	2.5
	2	5.5	2.75	2.5	2.25	2.5	2
	3	5.25	2.75	2.5	2.5	2.75	2.25

Mean		5.416667	2.75	2.5	2.333333	2.666667	2.25
Stdev		0.144338	0	0	0.144338	0.144338	0.25

1.0% Alcalase pH 6 15°C		0	24	48	72	96	120
	1	6	4.5	4.5	4.5	4.75	3.75
	2	5.5	4	4.75	4	3.75	3.75
	3	5.5	4.5	4.5	4	4.75	4.25

Mean		5.666667	4.333333	4.583333	4.166667	4.416667	3.916667
Stdev		0.288675	0.288675	0.144338	0.288675	0.57735	0.288675

0.1% Alcalase pH 6 25°C		0	24	48	72	96	120
	1	6.25	4	3.75	4	3.75	3.5
	2	6	4	3.75	4	4.25	3.75
	3	6	4.25	4	3.75	4	3.75

Mean		6.083333	4.083333	3.833333	3.916667	4	3.666667
Stdev		0.144338	0.144338	0.144338	0.144338	0.25	0.144338

0.1% Alcalase pH 6 15°C		0	24	48	72	96	120
	1	6	5.5	5	5.25	5.25	4.75
	2	6	5.25	5.25	4.75	5	4.5
	3	5.75	5	4.75	4.5	5	4.75

Mean		5.916667	5.25	5	4.833333	5.083333	4.666667
Stdev		0.144338	0.25	0.25	0.381881	0.144338	0.144338

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