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Abstract

There is a lot of information in literature on animals succumbing to disease(s) after exposure to stressors. But little information is available on what happens to corticosterone hormones when animals are exposed to a pathogen as a stressor. In this study, the effect of Malignant catarrhal fever viral challenge on the kinetics of corticosterone hormone in vaccinated and un-vaccinated cattle was investigated. Animals were randomly allocated into five (5) treatment groups each containing eight (8) animals. The animals were vaccinated against Malignant catarrhal fever using the attenuated Alcelaphine herpes virus AHV1.0 vaccine in combination with either flagellin or emulsigen adjuvants as follows; Group 1 (vaccine +emulsigen), Group 2 (vaccine+flagellin), Group 3 (vaccine+emulsigen+flagellin), Group 4 (emulsigen only) and Group 5 (flagellin only). All animals were challenged with Malignant catarrhal fever virus (virulent C-500 strain AHV-1 virus) $tcid_{50}$ on day 77 after the primary vaccination. Stress response was determined by measuring fecal corticosterone levels using ELISA. Survival from viral challenge was 75% (Group 1), 50% (Group 2), 37.5% (Group 3), 50% (Group 4) and 12.5% (Group 5). The differences between survival curves was significant (p=0.0182).

The 2-way ANOVA was used to determine whether the different treatment regime (vaccine, adjuvant, viral challenge combinations) resulted in different corticosterone responses. Results showed that while there was significant time effect ($p < 0.001$), the group and group×time interaction did not have significant effect. Initially in all treatment groups, the mean

corticosterone concentrations decreased progressively from baseline to lowest levels on day 56 and then increased sharply to peak levels on day 77 just before viral challenge. However the mean peak levels were significantly higher than baseline levels only in Group 2 (vaccine+flagellin) and Group 5 (flagellin alone). After challenge with live virus, the mean corticosterone levels decreased progressively in all groups from peak levels on day 77 to lowest levels on day 133 and then stabilized. The decrease in corticosterone levels after challenge was however significant in Group 5 only. Compared to peak levels, corticosterone concentrations in group 5 were significantly lower on Day 133 ($p<0.05$), Day 147 ($p<0.001$) and Day 161 ($p<0.001$).

To determine whether there was difference in corticisterone levels between animals that died from the disease and those that survived, the unpaired t-test was used to determine difference. Mean terminal corticosterone hormone metabolites in dead animals (43.36 ± 3.796) was significantly higher ($p< 0.05$) than in survivors (29.32 ± 4.548) indicating higher stress response in animals with severe clinical signs.

Results from this study demonstrate that Malignant catarrhal fever virus challenge in cattle decreases corticosterone levels but in general corticosterone levels are higher in animals with severe clinical signs of the disease.

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Introduction

Animals, both free ranging and domesticated endure many stressors in their day to day lives. For example, those in the wild experience food shortages, dwell in areas where predator or parasite densities are high, engage in conflicts with neighbors or group members, and face fluctuations in food and water availability and temperature fluctuation (Millspaugh *et al.*, 2003; Preisser *et al.*,

2005; Bayazit, 2009). Domesticated animals also encounter stressors ranging from restricted movements, forced proximity to humans, absence of retreat space, routine husbandry, restricted feeding and foraging opportunities and many others (Campbell, 2001; Koch *et al.*, 2009; Pearce and Paterson, 1993; Morgan and Tromborg, 2007).

Vertebrate animals have evolved mechanisms for coping with stress which involves the adrenal gland-produced glucocorticoids hormones (Sapolsky *et al.*, 2000; Harvey *et al.*, 1984). These hormones prepare the animal to a state of emergency by increasing synthesis of glucose from various storage sources (gluconeogenesis) but also suppress the immune functions, which may predispose to succumbing to diseases (Wingfield *et al.*, 1998; Cyr, 2008).

Of late, fecal glucocorticoids metabolites are becoming the preferred target analytes for stress in different animals. It is a minimally invasive method compared to the blood sampling methods (Cook, 2012). The procedure has been applied in many animal species such as sheep (Coburn, 2010), white and black rhinoceros (Turner Jr *et al.*, 2002), elephants (Foley *et al.*, 2001), farm animals (Palme, 2012) and ground squirrels (Bosson, 2009).

There are many publications on how stress predisposes to diseases but little information is available on how infectious agents or disease affect the stress response. It is well established that infectious agents or parasites can be stressors (Muehlenbein, 2006; Frandsen, 1987; Sapolsky *et al.*, 2000; Cook, 2012; Agarwal and Marshall, 2001; Peterson *et al.*, 1991; Martin, 2009).

This study attempted to investigate the level of stress induced by inoculation of cattle with a viral infectious agent and also test whether animals that are protected by vaccination will have diminished stress response.

Materials and Methods

Experimental Animals

The study recruited 40 Tanzania shorthorn zebu purchased from cattle owners in local markets around Simanjiro district, Arusha, Tanzania**.** The experimental animal ages ranged from 24-30 months. The Animals were taken to a Holding Area located in Emboret village close to the

Simanjiro plain. Each animal was ear-tagged with unique identification number at the start of the trial. The animals received regular health checks and were administered with Ivermectin, a broad-spectrum anti-parasitic agent to treat gastrointestinal round worms, lungworm, grub and suckling lice. Also cattle were vaccinated against East Coast Fever one week before the trial.

Experimental animal groups

The animals were randomly assigned into five treatment groups, each containing eight animals. Depending on the group, the animals were either vaccinated with Malignant catarrh fever vaccine in combination with adjuvant or adjuvant only as described in Table 1.

Table 1: Experimental animals groups

Group	Treatment (inoculum)	
	Vaccine	Adjuvant
1	+	Emulsigen
2	+	Flagellin
3	+	Emulsigen+Flagellin
4	-	Emulsigen
5	-	Flagellin

Vaccination and challenge

Vaccine and adjuvant were prepared and administered according to the treatment groups. The propagated Malignant catarrhal fever virus C500 strain passaged more than 1000 times in bovine turbinatecells (Haig, 2008) was used as vaccine. The Emulsigen adjuvant used in this study was an licensed adjuvant from MVP laboratories (Omaha, USA) while the Flagellin used

in this study was extracted from bacterial flagellum as previously described (Lee *et al.*, 2006). For primary vaccination on day 0, cattle were arranged according to their respective treatment groups, confined in a crush then injected with 1.2 mL of inoculum intramuscular around the neck region. Booster vaccination was administered in the same way as prime vaccination on day 28. Animals in all groups were challenged with live Malignant catarrhal fever virus virus on day 77 by intranasal inoculation with 10 mLs virus of suspension containing approximately 10^4 $TCID_{50}$/mL.

Fecal sample collection

Fecal samples were collected just before vaccination (day 0), and after vaccination on days 28, 42, 56, 77, 105, 119, 133, 147 and 161 for determination of fecal corticosterone metabolite levels. Fresh fecal sample were collected from each cattle at approximately 9:00 am to avoid diurnal corticosterone sample variation (Touma and Palme, 2005). Cattle were confined in cattle chute then samples were directly collected from rectum after recto-stimulation. Each sample was stored in small plastic bag which was correctly identified with respect to animal ID and date. The fecal samples were stored in cool box with ice blocks then shipped to Veterinary Investigation Center Laboratory in Arusha town and frozen at-20 ^oC until use.

Monitoring of animals

The health status of the cattle was monitored through daily health checks and rectal temperatures were recorded every other day. Any cattle showing signs of disease (fever, depression, lack of appetite, ocular lesions, nasal discharge etc.) were monitored and the severity of clinical signs scored based on the Malignant catarrh fever clinical sign score sheet as previously described (Russell *et al.*, 2012). Animals with very severe clinical signs (score above 6), were judged not fit to continue with the trial and were therefore euthanized. Terminal fecal sample was collected before euthanization.

Hormone Extraction

The corticosterone metabolites were extracted from fecal samples as previously described (Santymire *et al.*, 2012; Loeding *et al.*, 2011). Samples were defrosted and weighted using weight balance. To each 0.5g of sample was added 5 mLs of 90% ethanol. The mixture was homogenized for one (1) minute and centrifuged at 210(×g) for 20 minutes. The liquid supernatant was transferred into a clean plastic tube from which one (1) mL sample was removed and transferred to a test tube and left to evaporate and dry for one week. Then the dry sample was capped and stored at room temperature ready for corticosterone quantification.

Corticosterone Enzyme Immunoassay

The corticosterone concentration in samples was quantified using the DetectX® Corticosterone Immunoassay kit (Arborassay Company, USA) according to manufacturer's instructions. The kit comes with plate wells that are already pre-coated with donkey anti sheep IgG. Briefly, dried fecal samples were reconstituted by adding one (1) mL of assay buffer solution. Then 50µL of the sample and standard were added into respective wells in the plate. The DetectX® Corticosterone Conjugate (25uL) was added to wells and plates tapped to ensure adequate mixing. The plates were then covered with plate sealer and shaken for one hour at room temperature to ensure maximum binding.

The plates were then washed four times, by adding 300 µL of wash buffer and then taped on dry clean absorbent towels. Then 100 µL of TMB substrate were added to each well using micro channel pipette and incubated at room temperature for 30 minutes without shaking. Finally 50 µL of stop solution were added to each well. The optical density generated from each wells in the plate were measured by micro plate reader at the wavelength of 450 nm.

Based on the manufacturer's instructions, before samples were tested, validation of assay was carried out. This involved serial dilutions of fecal sample pool along with known concentration of standard dilutions of corticosterone to obtain respective optical density readings.

Data analysis

Datasets were analyzed using Graphpad prism version 6 (La Jolla, Ca, USA). Comparison of temperature responses between groups was done using One-way ANOVA with repeated measures. The Two-way ANOVA with repeated measures was used to determine differences between treatment groups over the experimental period. The student t-test was used to compare differences in corticosterone concentrations between animals that survived the challenge and those who died from challenge. Differences of $p < 0.05$ were deemed significant.

Results

Clinical outcome following vaccination and viral challenge

After viral challenge, clinical signs of Malignant catarrhal fever infection were first detectable around day 97 (20 days after challenge). The average incubation period was 30 days (Group 1), 51 days (Group 2), 51 days (Group 3), 47 days (Group 4) and 30 days (Group 5).

Animals in all groups developed clinical signs of Malignant catarrh fever but the signs were more severe in Group 5 and least severe in Group 1. The clinical signs included serous nasal discharge, ocular discharges, swelling of pre-scapular lymph nodes, bilateral corneal opacity, shivering, photophobia, aggression in some animals, incoordination, tremor, head pressing, body shivering, and convulsion.

Rectal temperature differences were apparent between groups after challenge with Malignant catarrhal fever. Temperatures were highest in Group 5 and lowest in Group 1 and the differences between groups was significant (Fig. 1).

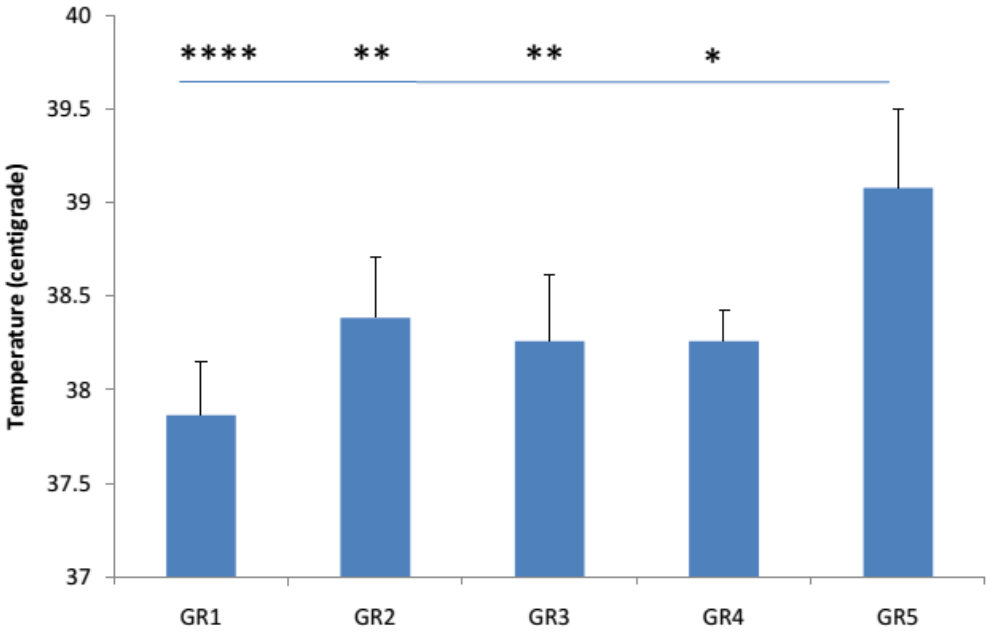

Figure 1: Mean temperatures between day 109 and day119. (*p<0.05, **p<0.01, ***p<0.001).

Effect of vaccination regimes on protection from MCF challenge

The proportion of surviving animals was 75% (Group 1), 50% (Group 2), 37.5% (Group 3), 50% (Group 4) and 12.5% (Group 5). The difference between groups was significant (p=0.0182) in survival from challenge were observed

Parallelism of the fecal sample and standard

The biochemical validation to analyze cattle corticosterone metabolites concentration using enzyme immunoassay was carried out. Serial dilutions of fecal sample pools yielded the displacement curve which was parallel to that of corticosterone standard curves with the linear regression R^2=0.999 (Fig. 2).

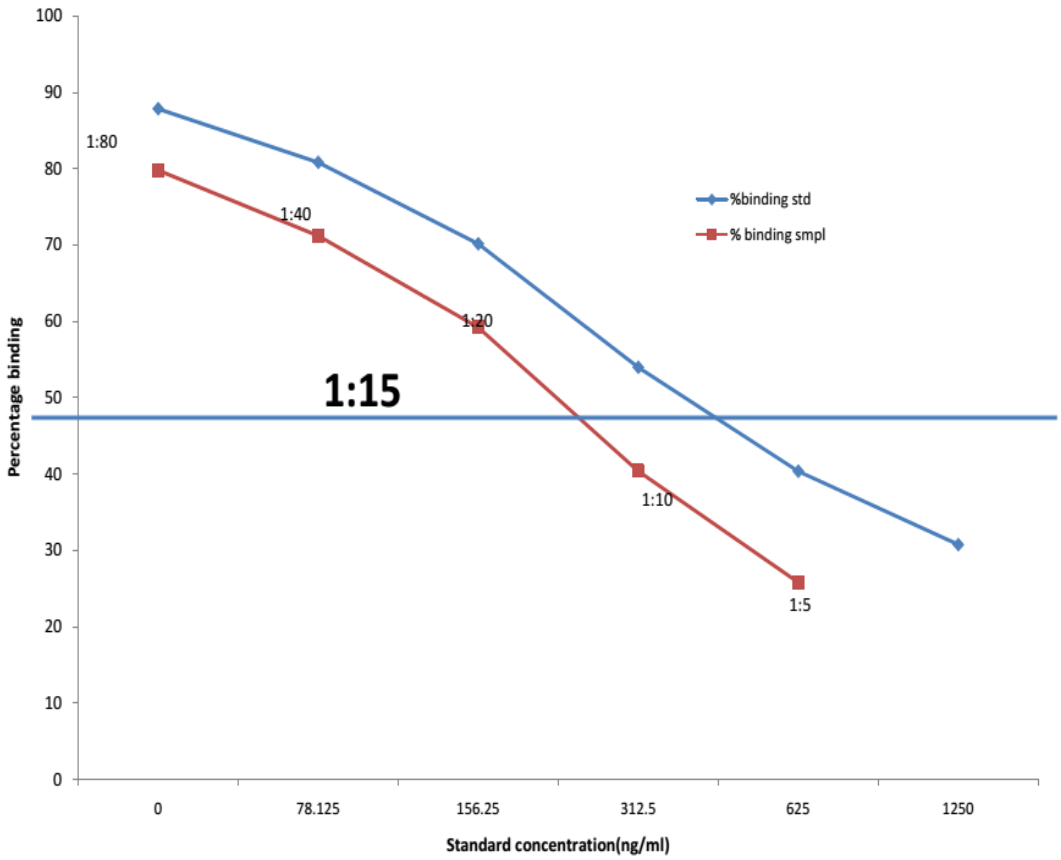

Figure 2: Parallelism of serial dilutions of fecal corticosterone in samples and corticosterone standard concentrations.

Effect of vaccination regimes and challenge on concentration of fecal corticosterone

The two-way ANOVA with repeated measures was used to determine whether the different treatment regimens (vaccine, adjuvant, viral challenge combinations) induced different corticisterone responses. Results showed that while there was significant time effect ($p < 0.001$), the group and group×time interaction did not have significant effect (Figure 3). Initially in all treatment groups, the mean corticosterone concentrations decreased progressively from baseline to lowest levels on 56 day and then increased sharply to peak on 77 day, just before challenge. However the mean peak levels were significantly higher than baseline levels only in two groups; Group 2 (vaccine+flagellin) and Group 5 (flagellin alone).

After challenge with live virus, the mean corticosterone levels decreased progressively in all groups from peak levels on day 77 to lowest levels on day 133 and then stabilized. The decrease in corticosterone levels after challenge was however significant in group 5 only. Compared to peak levels (Day 77), corticosterone concentrations were significantly lower in group 5 on Day 133 ($p<0.05$), Day 147 ($p<0.01$) and Day 161 ($p<0.01$).

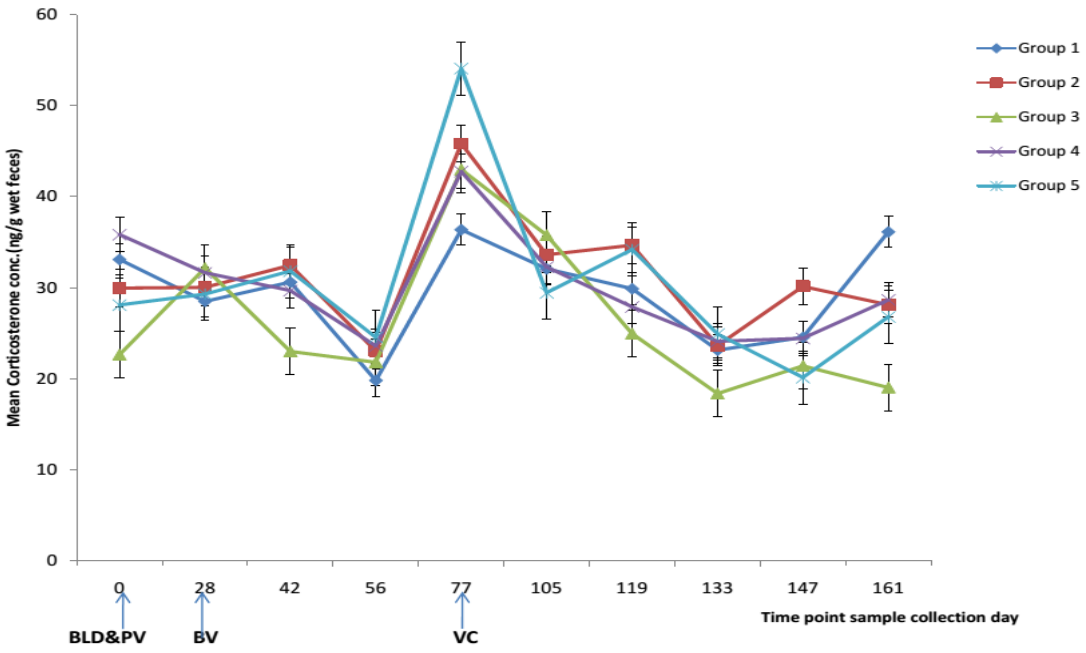

Figure 3: Changes in mean corticosterone concentrations (ng/g wet feces) in the five groups during the course of study. BLD-Base line data, PV-Primary vaccination, BV-Booster vaccination and VC-Viral challenge.

Terminal corticosterone levels in animals that died from viral challenge

The unpaired t- test was used to determine whether there were differences in corticosterone levels between animal which died from the disease and those that survived. Mean corticosterone hormone metabolites in dead 43.36 ± 3.796 (n=20) was significantly higher ($p< 0.05$) than for survivors 29.32 ± 4.548 (n=18).

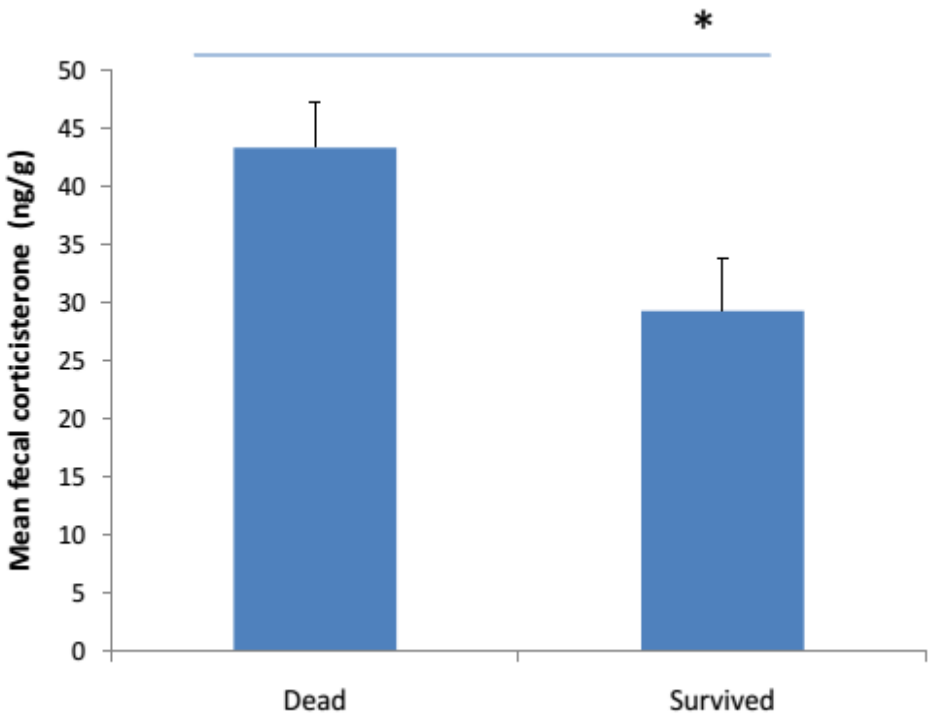

Figure 4: Corticosterone concentrations and animals that survived and those that died from malignant catarrhal fever challenge. (* p<0.05).

Discussion

This study attempted to investigate the level of stress induced by Malignant catarrhal fever in cattle via the quantification of fecal corticosterone metabolites. Animals were vaccinated with a Malignant catarrhal fever vaccine in combination with either emulsigen or Flegellin adjuvant and subsequently challenged with live Malignant catarrhal fever virus. Clinical signs characteristic of Malignant catarrhal fever (Cleaveland, 2001; Li *et al.*, 2011; Russell *et al.*, 2009; Schultheiss *et al.*, 2000) developed in all groups though with differing severity. Signs were more severe in unvaccinated control group (Group 5) from which 7/8 animals died and least severe in Group 1 (Vaccine +emulsigen) from which only 2/8 animals died. Considering that there is currently no

known vaccine for Malignant catarrhal fever, results from this study are very promising for a vaccine formulated with emulsigen adjuvant which protected 75% of animals from deaths.

Results from this study shows that the mean corticosterone levels in all groups increased sharply from day 56 and peaked on day 77 just before challenge. After challenge, the mean corticosterone levels progressively decreased to baseline levels between day 119 and day 133. It was interesting to note that although the increase in corticosterone levels occurred in all groups (treated and untreated), significant increase was only noted in Group 2 (vaccine +flagellin) and Group 5 (flagellin only) indicating that flagellin adjuvant played a role in the cortosterone increase. However, flagellin was last injected in animals during a booster vaccination which was done on day 28. Since peak levels were detected on day 77, which is 49 days after booster vaccination, it creates some doubts whether the increase was due to the adjuvant or the vaccine. Normally detectable increase in levels of fecal corticosterone appears in a few hours to few days after induction of stress (Santymire *et al.*, 2012; Möstl and Palme, 2002; Millspaugh and Washburn, 2004) which is contrary to what was seen in this study. An alternative explanation for this rise in cortisone is some environmental factors such water shortage, shortage of grass or even presence of predators as was observed in other studies (Laver *et al.*, 2012; Palme, 2005; Scheuerlein *et al.*, 2001; Boonstra, 1998; Millspaugh and Washburn, 2004). This latter explanation is more feasible because animals in all groups experienced increase in corticosterone levels regardless of the treatment administered.

It was observed that the cortisterone levels started decreasing progressively after animals were challenged with live virus and clinical signs associated with Malignant catarrh fever developed. This is in contrast to our hypothesis and what was observed in other studies involving viral challenge to animals where corticosterone levels increased soon after challenge (Sapolsky *et al.*, 2000; Webster and Sternberg, 2004). This may be due to different reasons; firstly, there is paucity of information on how cortisterone levels react to viral challenges in cattle in view of the fact that to our knowledge no previous Malignant catarrhal fever viral challenge experiment has been reported in this species. Secondly, it may be that the pathology associated with Malignant catarrhal fever interferes with the stress sensing or stress response mechanisms in cattle. Malignant catarrhal fever is associated with pathology in various tissue and organs including lymphocyte infiltration in various non-lymphoid organs and this may interfere with a number of

organs including the adrenal gland which secretes the corticosterone hormones. Alternatively since it is known that elevated corticosterone suppresses immune responses, the decrease in corticosterone levels may be due to an unknown inbuilt mechanism to boost the immune response.

We noted however that those animals that exhibited severe clinical signs and subsequently euthanized had significantly higher terminal corticosterone levels compared with those with mild clinical signs who survived the challenge. This is in agreement with our hypothesis and other studies involving viral challenge in different animal species (Webster and Sternberg, 2004). This also agrees with our argument that higher corticosterone levels may have compromised the immune responses in animals with severe clinical signs and left them susceptible to progression of disease.

Conclusion

This study demonstrated for the first time the stress responses in cattle following challenge with virulent Malignant catarrh fever virus. The results and methodology used will set the trend for further studies of stress responses in cattle.

Acknowledgements

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