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LEUKOCYTE RESPONSE IN ZEBU CATTLE EXPERIMENTALLY INFECTED WITH *CLOSTRIDIUM CHAUVOEI*

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Summary

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The molecular mechanisms and specific roles of toxins and neuraminidase produced by *C. chauvoei* in the pathogenesis of leukopenia in blackleg are unknown. In this study with Zebu cattle experimentally infected with *C. chauvoei*, its toxins or neuraminidase, all they produced leukopenia ($P<0.05$) due to neutropenia, lymphopenia, monocytopenia and eosinopenia. Neuraminidase and toxins produced by *C. chauvoei in vivo* acted synergistically to produce leukopenia of higher magnitude in the *C. chauvoei*-infected cattle, compared to the toxin and neuraminidase-administered animals. It was concluded that future therapeutic protocols against blackleg should target toxins and neuraminidase produced by *C. chauvoei.*

Key words: *Clostridium chauvoei,* leukocyte response, neuraminidase, toxins, Zebu cattle

INTRODUCTION

Blackleg is a fatal disease of cattle, sheep and other ruminants, caused by *Clostridium chauvoei* (Kijima-Tanaka *et al.,* 1998). In Nigeria, the disease was first reported in 1929, and has remained a major problem of cattle in the country (Osiyemi, 1975). Although vaccination has been carried out against it since 1930, sporadic outbreaks are recorded annually. It is an endemic and economically important disease of ruminants in both developed and developing countries (Adams, 1998). The economic losses resulting from blackleg have not been quantified in most parts of the world, but in Nigeria, losses of Zebu cattle alone to the disease have been estimated at US dollar 4.3 million annually (Useh *et al.,* 2006a).

There is no consensus on the pathogenesis of blackleg in ruminants, although toxins and neuraminidase produced by *C. chauvoei in vivo* have been reported to play important complimentary roles in the mechanisms of the disease (Useh *et al.,* 2003). Recent studies show that natural infection of Zebu cattle with *C. chauvoei* caused statistically significant leukopenia, but not significant changes in differential WBC counts, except for lymphocytes (Useh *et al*., 2008).

In this study, we have documented for the first time, the possible effect of *C. chauvoei* neuraminidase on leukocyte response in Zebu cattle experimentally infected with *C. chauvoei.*

MATERIALS AND METHODS

Animals and experimental design

Fourteen (14) Zebu bull calves were purchased, acclimatized, grazed, aged and grouped into 4 groups. Groups A (n=4), B (n=3) and C (n=4) were infected with *C. chauvoei* (Jakari strain), toxins and neuraminidase from the bacteria respectively, while group D (n=3) served as control. During the period of acclimatization, the animals were grazed on free range, because of the abundant pasture that characterizes the rainy season in Zaria, Nigeria, but when the experiment commenced they were confined in experimental pens and fed a combination of groundnut hay and hay prepared from *Andropogon gayanus, Hyprrhenia rufens, Pennisetum pedicellatum* and *Elionurus probeguinii* until the experiment was terminated. They were supplied feed commensurate with 4% of their individual body weights daily and water *ad libitum*. The weights of the animals were estimated using waist band and ranged between 80–140 kg. The animals were aged using dental eruption (Wosu, 2002) and their ages ranged between 19–23 months. There was no significant difference ($P>0.05$) between the mean age and the mean weights of all animal groups on day zero of the experiment. Although there is no ethical committee regulating the use of animals for research at the Faculty of Veterinary Medicine, Ahmadu Bello University, all the experimental animals were treated most humanely.

Packed cell volume (PCV) was determined once weekly (Schalm *et al.,* 1975) and used as a basis for grouping the animals, so that there was no statistically significant difference ($P>0.05$) between the mean PCVs of the control, neuraminidase, toxin and *C. chauvoei*-infected groups on day zero of infection.

Cultivation of C. chauvoei for infection

Lyophilized *C. chauvoei* (Jakari strain) donated by the National Veterinary Research Institute (NVRI), Vom, Plateau state, Nigeria was used for the experiment. The organism was first isolated from Zebu cattle with blackleg and its pathogenicity indices have been fully determined (Princewill, 1965). The preparation of the bacteria and infection of Zebu bull calves was carried out using the method described by Singh *et al*. (1993) and the experiment lasted for 21 days. The animals (experimental group A) were administered 40 mL of 10% $CaCl_2$ intramuscularly to create muscle damage and simultaneously administered 40 mL (11.0×10^9 cfu/mL) of 36-hour old culture of *C. chauvoei* (Jakari strain) in reinforced clostridial medium intramuscularly.

Cultivation of C. chauvoei (Jakari strain) for toxin production

The method of Jayaraman *et al.* (1962) was used to cultivate the bacteria and produce the toxins which were administered to one experimental group. The protocol, including the amount of toxins administered is described elsewhere (Useh *et al*., 2007). Exactly 40 IU of *C. chauvoei* (Jakari strain) toxin was administered intramuscularly to the experimental animals (experimental group B).

Cultivation of C. chauvoei (Jakari strain) for neuraminidase production

Lyophilized *C. chauvoei* (Jakari strain) was cultivated and neuraminidase was isolated as described previously (Useh *et al*., 2004a). The neuraminidase was partially purified as described earlier (Useh *et al*., 2006b) and administered to experimental group C using conventional protocols (Useh *et al*., 2007). About 20

IU of partially purified *C. chauvoei* (Jakari strain) neuraminidase was administered to each calf intramuscularly. The experiment lasted for 21 days.

Determination of total and differential leukocyte counts

Total (WBC) and differential leukocyte counts (lymphocytes, neutrophils, monocytes and eosinophils) were determined using conventional procedures (Schalm *et al*., 1975).

Statistical analysis

Data obtained from the study were computed as mean ± standard deviation (SD), and analyzed using analysis of variance (ANOVA, Duncan multiple range test). Values of $P<0.05$ were considered statistically significant (Chatfield, 1983).

RESULTS

On day zero of infection, total leukocyte counts (WBC) of all experimental animals varied between 15.6 and 19.0 $\times 10^9$/L, with mean values of 17.88±1.24, 17.63± 1.04, 17.7±1.18, and 17.67±1.20 $\times 10^9$/L for neuraminidase, *C. chauvoei,* toxin-administered and control groups respectively. There was no statistically significant difference between the mean total leukocyte counts of all experimental groups on day zero of infection. However, 18 h post-infection, leukopenia appeared in all groups except the control, and the lowest levels were attained on day 3 (72 h) post-infection (Fig. 1) with mean leukocyte counts 5.75±1.65, 4.53±1.08 and 8.37±1.47 $\times 10^9$/L for neuraminidase, *C. chauvoei*-infected and toxin administered groups respectively, representing 68%, 74% and 54% decrease. The *C. chauvoei*-infected group recorded the highest degree of leukopenia, followed by the neuraminidase and toxin-administered groups in that order. Thereafter, mean WBC counts began to increase in all infected groups up to when the experiment was terminated (day 21 or 413 h post-infection). Mean pre-infection WBC counts were attained on day 21 (413 h) post-neuraminidase administration, while the *C. chauvoei*-infected and toxin-administered groups still recorded leukopenia at 15% and 17% respectively (Fig. 1). Mean total and differential leukocyte response in the bacteria-infected, neuraminidase and toxin-administered groups were statistically significantly different ($P<0.05$) from the control group as from day 1 (18 h) post administration with peak values attained on day 3 (72 h) of the experiment. The statistically significant variation ($P<0.05$) persisted until the experiment was terminated on day 21 (413 h) post commencement.

The response of leukocyte classes (lymphocytes, neutrophils, eosinophils and monocytes) followed a similar pattern as total WBC (Tables 1–4). Although the pre-infection mean values of the differential leukocytes were not attained on day 21 (413 h) post-administration of neuraminidase, toxins and *C. chauvoei*, there was no statistically significant difference between mean values on days 0 and 21.

DISCUSSION

For many decades, blackleg was mistaken for an exclusively animal disease (Radostits *et al*., 2000) because of the non availability of precise diagnostic tools. The report by Nagano *et al*. (2008) that *C. chauvoei* also caused human disease and that it was fatal to humans has placed blackleg in the list of hot topics for research today. These authors reported the death of a 58-year old Japanese national caused by *C. chauvoei* infection. The

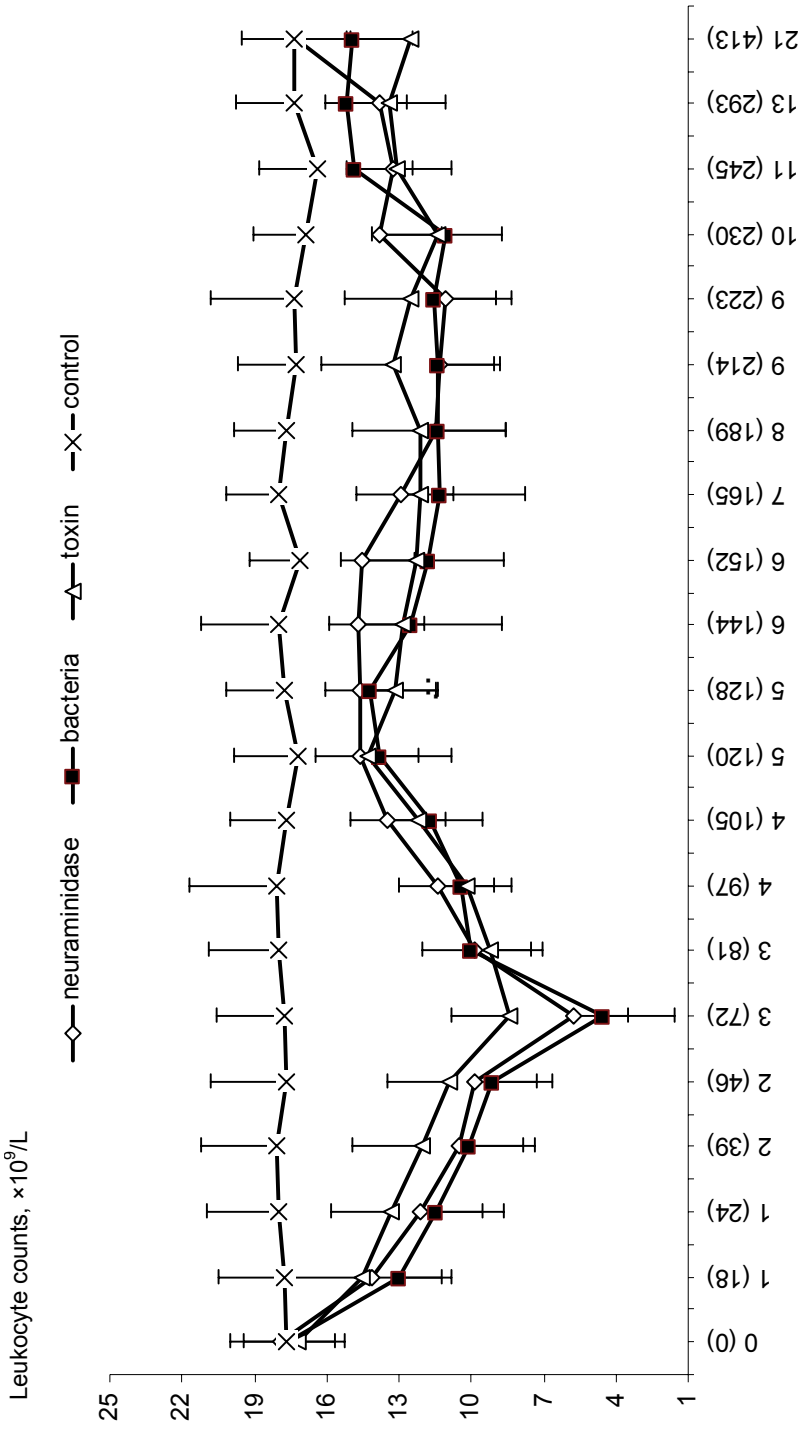

Fig. 1. Variation in mean total leukocyte counts of Zebu cattle experimentally administered *C. chauvoei*, its toxins and neuraminidase (mean±SD).

Table 1. Variation in mean lymphocyte counts in different groups of Zebu cattle experimentally administered *C. chauvoei*, its toxins and neuraminidase (mean±SD).

Days (h)	Neuraminidase	Bacteria	Toxins	Control
0	13.71±1.19	13.53±1.15	12.73±1.18	13.73±1.18
1 (18)	10.81±1.17	9.98±1.16	11.22±1.14	13.52±1.21
1 (24)	8.78±1.20	8.47±1.18	8.96±1.17	13.36±1.19
1 (39)	7.31±1.18	6.91±1.14	8.40±1.19	12.87±1.16
2 (46)	6.55±1.16	6.02±1.17	7.63±1.19	12.37±1.18
3 (72)	4.03±1.20	3.17±1.18	5.86±1.17	12.41±1.19
3 (81)	6.90±1.18	7.04±1.17	6.44±1.16	12.58±1.14
3 (97)	7.96±1.17	7.28±1.18	7.14±1.19	12.65±1.16
4 (105)	9.43±1.14	7.96±1.15	8.54±1.16	12.39±1.19
5 (120)	10.24±1.18	9.66±1.17	10.96±1.19	12.01±1.17
5 (128)	10.24±1.12	9.66±1.19	10.96±1.15	12.01±1.18
6 (144)	10.31±1.19	8.75±1.17	8.96±1.17	12.58±1.15
6 (152)	9.01±1.13	8.26±1.18	8.61±1.17	11.99±1.19
7 (165)	9.03±1.17	7.91±1.19	8.51±1.16	12.58±1.14
8 (189)	8.03±1.19	7.96±1.17	8.51±1.18	12.39±1.16
9 (214)	7.89±1.21	7.98±1.19	9.28±1.20	12.09±1.19
9 (223)	7.77±1.16	8.10±1.20	8.58±1.18	12.16±1.20
10 (230)	9.68±1.19	7.93±1.21	7.98±1.20	11.83±1.19
11 (245)	9.29±1.17	10.42±1.20	9.14±1.15	11.48±1.19
13 (293)	9.67±1.18	10.64±1.17	9.38±1.19	12.13±1.20
21 (413)	12.11±1.85	10.48±1.89	8.75±1.91	12.48±1.78

16S–23S rRNA gene intergenic spacer region of the pathogen isolated from the patient was amplified by polymerase chain reaction (PCR), generating a 522-bp *C. chauvoei* specific product, coinciding with that of *C. chauvoei* ATCC 10092, confirming that the 58-year old man actually died of *C. chauvoei* infection.

Decreased mean total leukocyte counts (leukopenia) and mean differential leukocyte counts (lymphopenia, neutropenia, eosinopenia and monocytopenia) were observed in the Zebu cattle experimentally administered *C. chauvoei*, its toxins and neuraminidase. However, the molecular mechanisms of the leukopenia are poorly understood. Leukocytes have sialic acid as a terminal sugar, which is involved in anti-recognition (Woodruff & Woodruff, 1976a,b). Neuraminidases (sialidases, EC 3.2.1.18) are glycosyl hydrolases that release terminal N-acetyl neuraminic (sialic) acid residues from glycoproteins, glycolipids and polysaccharides (Roggentin *et al.,* 1993). Although the sialic acid concentration of the total and differential leukocytes were not investigated in this study, it is believed that the leukopenia, lymphopenia, neutropenia, eosinopenia and monocytopenia observed in the neuraminidase-administered and *C. chauvoei*-infected groups may partly be attributed to the role of neuraminidase, which may have cleaved sialic acids from the leukocytes, leading to their removal from peripheral circula-

tion (leukophagocytosis), especially with the detection of neuraminidase activity in the plasma of these animals *in vivo*. This is strongly supported by Mackenzie & Cruickshank (1973) who reported leukophagocytosis in the liver of sheep infected with *Trypanosoma congolense*. The findings of Esievo & Saror (1983) in experimental bovine trypanosomiasis, where a similar peripheral blood leukocyte response was observed in Zebu cattle and attributed to the action of neuraminidase produced by *Trypanosoma vivax in vivo,* also provides support to the findings reported in the present study.

Leukopenia, lymphopenia, neutropenia, eosinopenia and monocytopenia were also observed in the toxin-administered group. The pathogenesis of these events is not well understood, although the finding is in agreement with previous reports (Singh *et al.,* 1993; Jubb *et al.,* 1993; Jones *et al.,* 1997) where toxins were reported to be responsible for leukopenia in clinical blackleg. However, toxic chemicals are known to impair cellular (mitochondrial) respiration in trypanosomes, leading to death of the trypanosomes (Nok, 2002). Whether the toxins produced by *C. chauvoei in vivo* in clinical blackleg impairs cellular (mitochondrial) respiration in leukocytes, leading to WBC necrosis and subsequently leukopenia has yet to be investigated. Also, bacterial endotoxins produced by Gram-negative bacteria *in vivo* are known to trigger the expression of leukocyte adhesion molecules on endothelial cells of blood vessels to

Table 2. Variation in mean neutrophil counts in different groups of Zebu cattle experimentally administered *C. chauvoei*, its toxins and neuraminidase (mean±SD)

Days (h)	Neuraminidase	Bacteria	Toxins	Control
0	4.83±0.98	4.91±0.97	4.64±1.02	4.77±0.99
1 (18)	3.67±1.21	3.41±1.00	3.55±1.06	4.61±0.89
1 (24)	3.16±1.65	2.98±1.46	3.62±1.68	4.67±1.05
1 (39)	2.73±1.87	2.63±1.47	3.12±1.22	4.70±1.39
2 (46)	2.44±0.98	2.38±1.22	2.84±1.43	4.59±1.68
3 (72)	1.49±0.11	1.18±0.18	2.17±0.17	4.61±0.19
3 (81)	2.56±0.66	2.61±0.45	2.39±0.33	4.67±0.28
3 (97)	2.96±0.17	2.70±0.43	2.66±0.22	4.70±0.62
4 (105)	3.50±0.22	2.96±0.39	3.18±0.54	4.60±0.49
5 (120)	3.80±0.41	3.59±0.44	3.72±0.35	4.46±0.39
5 (128)	3.72±0.55	3.70±0.35	3.44±0.44	4.61±0.39
6 (144)	3.83±0.37	3.25±0.33	3.33±0.42	4.67±0.44
6 (152)	3.79±0.46	3.07±0.51	3.20±0.33	4.46±0.38
7 (165)	3.36±0.22	2.94±0.34	3.16±0.45	4.67±0.48
8 (189)	2.99±0.42	2.96±0.35	3.16±0.43	4.60±0.45
9 (214)	2.94±0.32	2.96±0.41	3.45±0.29	4.49±0.48
9 (223)	2.89±0.29	3.01±0.38	3.19±0.44	4.51±0.36
10 (230)	3.58±0.44	2.98±0.49	2.97±0.36	4.39±0.28
11 (245)	3.45±0.33	3.87±0.41	3.39±0.34	4.26±0.45
13 (293)	3.59±0.26	3.98±0.43	3.49±0.28	4.51±0.39
21 (413)	4.50±0.55	3.89±0.48	3.25±0.65	4.64±0.51

Table 3. Variation in mean eosinophil counts in different groups of Zebu cattle experimentally administered *C. chauvoei*, its toxins and neuraminidase (mean±SD). ND=not determined

Days (h)	Neuraminidase	Bacteria	Toxins	Control
0	0.36±0.09	0.35±0.07	0.34±0.08	0.35±0.08
1 (18)	0.28±0.07	0.26±0.09	0.29±0.08	0.36±0.06
1 (24)	0.24±0.08	0.23±0.07	0.26±0.09	0.36±0.08
1 (39)	0.21±0.06	0.20±0.08	0.24±0.07	0.36±0.09
2 (46)	0.19±0.09	0.18±0.07	0.22±0.06	0.35±0.07
3 (72)	0.12±0.07	0.09±0.06	0.17±0.08	0.36±0.09
3 (81)	0.20±0.04	0.20±0.05	0.19±0.08	0.36±0.08
3 (97)	0.23±0.08	0.22±0.07	0.25±0.09	0.36±0.07
4 (105)	0.27±0.06	0.23±0.09	0.24±0.08	0.35±0.09
5 (120)	0.29±0.09	0.28±0.08	0.29±0.09	0.34±0.07
5 (128)	0.29±0.08	0.28±0.07	0.27±0.09	0.36±0.06
6 (144)	0.33±0.07	0.25±0.06	0.26±0.08	0.36±0.09
6 (152)	0.29±0.09	0.24±0.08	0.25±0.06	0.34±0.04
7 (165)	0.26±0.05	0.23±0.09	0.24±0.07	0.35±0.08
8 (189)	0.23±0.09	0.23±0.07	0.24±0.08	0.35±0.06
9 (214)	0.23±0.08	0.23±0.09	0.25±0.07	0.35±0.6
9 (223)	ND	ND	ND	ND
10 (230)	0.27±0.07	0.23±0.08	0.23±0.09	0.34±0.04
11 (245)	0.27±0.09	0.29±0.08	0.26±0.07	0.33±0.06
13 (293)	0.28±0.07	0.31±0.04	0.27±0.08	0.35±0.09
21 (413)	0.35±0.08	0.30±0.07	0.25±0.09	0.36±0.06

cause massive leukocyte margination within blood vessels, leading to leukopenia (Kumar *et al.,* 2005). It is not clearly understood whether toxins produced by *C. chauvoei* also cause leukocyte margination, and this remains open to research. It is worth noting, however, that the mean leukopenia which occurred as a result of decrease in mean differential leukocyte counts and possibly attributed to the action of *C. chauvoei* neuraminidase and toxins may explain why the mean total and differential leukocyte values in the *C. chauvoei*-infected group were much lower than those of groups treated only with either neuraminidase or toxin. The findings reported in this study suggest that it is possible to target neuraminidase and toxins produced by *C. chauvoei* in the future chemotherapy of clinical blackleg. In a previous study (Useh *et al.,* 2004b), some herbal remedies used by the nomadic Fulanis of rural Nigeria, who are transhumance pastoralists, to treat blackleg were reported to inhibit the activity of *C. chauvoei* neuraminidase *in vitro*. Based on this finding, it was strongly speculated that the inhibition of neuraminidase activity *in vivo* may partly be the mechanism by which the aforementioned herbal remedies ameliorated clinical blackleg.

In conclusion, this study has shown that neuraminidase and toxins produced

Table 4. Variation in mean monocyte counts in different groups of Zebu cattle experimentally administered *C. chauvoei*, its toxins and neuraminidase (mean±SD). ND=not determined

Days (h)	Neuraminidase	Bacteria	Toxins	Control
0	0.36±0.06	0.35±0.04	0.34±0.09	0.35±0.08
1 (18)	0.28±0.07	0.26±0.09	0.29±0.08	0.36±0.07
1 (24)	0.24±0.05	0.23±0.08	0.26±0.07	0.36±0.08
1 (39)	0.21±0.04	0.20±0.09	0.24±0.08	0.36±0.06
2 (46)	0.19±0.08	0.18±0.09	0.22±0.05	0.35±0.07
3 (72)	0.12±0.04	0.09±0.06	0.17±0.03	0.36±0.08
3 (81)	0.20±0.08	0.20±0.04	0.19±0.05	0.36±0.08
3 (97)	0.23±0.03	0.22±0.06	0.25±0.07	0.36±0.09
4 (105)	0.35±0.06	0.27±0.09	0.23±0.05	0.24±0.07
5 (120)	0.35±0.08	0.29±0.08	0.28±0.07	0.29±0.06
5 (128)	ND	ND	ND	ND
6 (144)	0.36±0.07	0.33±0.09	0.25±0.07	0.26±0.06
6 (152)	0.34±0.04	0.29±0.05	0.24±0.08	0.25±0.09
7 (165)	0.35±0.03	0.26±0.08	0.23±0.06	0.24±0.08
8 (189)	0.36±0.08	0.25±0.06	0.23±0.08	0.24±0.07
9 (214)	0.23±0.09	0.23±0.09	0.25±0.06	0.35±0.02
9 (223)	0.34±0.04	0.27±0.06	0.23±0.07	0.23±0.01
10 (230)	ND	ND	ND	ND
11 (245)	0.27±0.02	0.29±0.04	0.26±0.07	0.33±0.05
13 (293)	0.28±0.07	0.31±0.09	0.27±0.05	0.34±0.01
21 (413)	0.35±0.07	0.30±0.07	0.25±0.06	0.36±0.09

by *C. chauvoei in vivo* in clinical blackleg acted synergistically to produce leukopenia, lymphopenia, neutropenia, eosinopenia and monocytopenia. It was hypothesized that desialylation of leukocytes, leading to leukophagocytosis, in combination with some other unknown mechanisms may possibly be responsible for the leukopenia observed. It is suggested that future therapeutic protocols against blackleg should target the neuraminidase and toxins produced by *C. chauvoei.*

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