Introduction

Ciguatera fish poisoning (CFP) is an illness resulting from eating fish that contain toxins collectively named ciguatoxins (CTXs) (1). CFP is prevalent in tropical or subtropical areas affecting several ten thousands of people annually. Thus, the illness is suggested to be the largest food-borne disease of a natural toxin origin. In the Pacific, the benthic dinoflagellate *Gambierdiscus toxicus* was identified as the primary source of the toxins that accumulated in fish via the food chain (2). In view of the multiple fish species involved, the diverse symptoms of patients and wide genetic variations among the *Gambierdiscus* spp., the divergence of the toxins was assumed. Adding to the complexity, toxins undergo structural modifications, mainly in an oxidative manner, in fish. Thus, as many as 23 toxin analogs were identified from fish and *G. toxicus* collected in French Polynesia (1). They are separable based on skeletal structures into two types, ciguatoxin-1B (CTX1B) type and ciguatoxin-3C (CTX3C) type (Figure 1). Previously, using 14 reference toxins, we carried out LC-MS/MS analysis on fish from waters around Japan and reported that the toxin profiles in the fish were essentially species-specific (3). We also inferred by comparing the toxin profiles between Okinawa Archipelago and Miyazaki, Kyushu Island, that the toxin profiles of *G. toxicus* must be different in the two areas. The information is not only important for implementation of monitoring but also interesting from an eco-toxicological point of view.

The present study was undertaken firstly to further prove the species-specific and the region-specific toxin profiles with increased number of fish, and secondly to prove compatibility of mouse bioassays (MBA) with LC-MS/MS so that possible contributions of other toxins to MBA results could be ruled out. Since the paucity of reference toxins severely hampered the validation of our method, preparation of toxin standards was initiated. Although the most laborious part of this study, preparation of CTX1B and CTX3C was successful, providing sufficient amounts for use in our future validation studies.

Materials and Method

Reference Ciguatoxins

As in the previous study, the following fourteen reference toxins were used: ciguatoxin-4A (CTX4A), ciguatoxin-4B (CTX4B), M-*seco*-ciugatoxin-4A/B (M-*seco*-CTX4A/B), 52-*epi*-54-deoxyciguatoxin-1B (52-*epi*-54-deoxyCTX1B), 54-deoxyciguatoxin-1B (54-deoxyCTX1B), ciguatoxin-1B (CTX1B), ciguatoxin-3C (CTX3C), M-*seco*-ciguatoxin-3C (M-*seco*-CTX3C), 49-*epi*ciguatoxin-3C (49-*epi*CTX3C), 2-hydroxyciguatoxin-3C (2-hydroxyCTX3C), 2,3-dihydroxyciguatoxin-3C (2,3-dihydroxyCTX3C), 51-hydroxyciguatoxin-3C (51-hydroxyCTX3C), M-*seco*-ciguatoxin-3C methyl acetal (Figure 1), and gambierol. The chromatographic and spectral data to support the structures were presented in the previous articles and the references therein (1, 2).

LC-MS/MS

The LC-MS/MS analysis was carried out using an Agilent 1200 Series LC coupled to an Agilent 6460 Triple Quadrupole LC/MS instrument according to the method reported in detail previously (3). The chromatogram for 14 reference toxins obtained with a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm) is shown in Figure 2. The flow rate was 0.4 mL/min with a gradient starting at 73% B, increased to 90% B in 11 min and held for 4 min (A = 5 mM ammonium formate and 0.1% formic acid in water, B = MeOH). Solvents and other chemicals were purchased from Wako Pure Chemical Industries, Ltd., Tokyo. For LC-MS/MS analysis, 5 μL each of sample solutions in MeOH was injected.

Fish Specimens

The snappers, *Lutjanus bohar* (36 specimens), *Lutjanus monostigma* (5 specimens), and the groupers, *Epinephelus fuscoguttatus* (11 specimens), *Variola louti* (17 specimens), *Plectropomus laevis* (6 specimens), *Anyperodon leucogrammicus* (2 specimens), and the spotted knifejaws, *Oplegnathus punctatus* (3 specimens) were collected in Okinawa Prefecture, the Ryukyu Islands. Of these, three specimens of *L. bohar* and one specimen of *V. louti* were collected in 1990, and others during recent 10 years. These fish are the most frequently implicated species in CFP in Okinawa (4). Additionally, an acetone extract prepared from the viscera of the amberjacks, *Seriola dumerili*, implicated in CFP in Hawaii was kindly supplied by Professor Y. Hokama, University of Hawaii. The effect of autoclaving

was tested using *L. monostigma* and *L. bohar*. The geographical locations of the fish analyzed in this study as well as in the previously study are shown in the map (Figure 3). To test the method performance at low toxin levels, the flesh of *L. monostigma* and *V. louti* judged as negative by MBA were used.

Preparation of Test Solutions for LC-MS Analysis

Because of the necessity to compare MBA and LC-MS results, crude extracts were prepared following the manual guide for MBA. Further clean-up of the extract was performed using Florisil and PSA cartridges (Figure 4). Muscle samples were taken respectively from the dorsal, caudal, and ventral parts to compare the toxin contents. The efficacy of clean-up on Florisil and PSA cartridges was examined using crude extracts of MBA-negative *L. bohar* spiked with two different doses of CTX1B (0.16 and 0.32 μg/kg).

Large Scale Preparation of Toxins

Fish were autoclaved before separating muscles from other organs and tissues. In preliminary tests, loss of toxins during autoclaving was indicated to be insignificant. Only muscles were used for extraction. The viscera were kept separately for future use. In addition to the fish collected in Okinawa, crude extracts accumulated over the past years from fish caught in other parts of the Pacific were also used. Purification of the toxins was carried out basically following the methods in the literature (1). The ^{1}H NMR spectra were recorded at RIKEN with a Bruker AV800 spectrometer equipped with a cryo-probe. The

samples were dissolved respectively in 300 μL of CD_3OD (99.95%, Aldrich), placed in a tube of 5 mmΦ, and measured at 10 °C without spinning.

Assessment of Bioactivities to Convert LC-MS Data to Mouse Lethality

The mouse bioassays (MBA) were carried out following the method officially recommended in Japan (5). The ion-influx activity through voltage-sensitive sodium channels (VSSC) was measured using radioactive [^{14}C] guanidine (6). The potency to bind VSSC was measured using radioactive [^{3}H] PbTx-3 following the published method (7). The neuroblastoma-2A assay also followed the published method (8). Further details of measurements were reported in 1994 in the MS thesis by K. Sugiyama, the Faculty of Agriculture of the Graduate School of Tohoku University.

Results and Discussion

The chromatogram for 14 standard toxins is shown in Figure 2. The solutions for injection were 1 ng/mL. Test solutions prepared from different parts of the body suggested an even distribution of CTX1B: dorsal part, 0.84; caudal part, 1.06; ventral part, 0.98 (μg/kg flesh). Same trend was observed for 52-*epi*-54-deoxyCTX1B and 54-deoxyCTX1B. Recoveries of CTX1B spiked at two different doses (0.8 ng and 1.6 ng) to a crude extract of a MBA-negative fish were 90.3% at the lower dose and 78.6% at the higher dose (n=2). The use of Florisil and PSA cartridges for clean-up seemed justifiable. The limits for detection (S/N >

3) and quantification (S/N > 10) of CTXs were determined to be 0.25 pg and 1.0 pg on column in the previous study (3). For further validation of the method toward low level samples, three specimens of fish judged as marginal or negative by MBA were tested. As shown in Figure 5, toxins were clearly detected. The calculated CTX1B contents were 0.181 μg/kg flesh in *L. monostigma* and 0.079 μg/kg flesh in *V. louti*. Judging from the intensities of the ion peaks, detection of CTX1B at one order lower levels seems possible, by changing the dilution of the sample solution or adding one step of cartridge treatment. With adequate amounts of CTX1B and CTX3C at hand, as will be mentioned later, we plan to implement a validation study using spiked fish flesh taken from different parts of fish of different species.

The toxin profiles of seven representative fish species in Okinawa are shown in Figure 6. CTX1B, 54-deoxyCTX1B, and 52-*epi*-54-deoxyCTX1B were dominant in snappers and groupers, and the relative abundance of toxins was in support of the species-specific profiles observed previously. Interestingly, the specimens of *L. bohar* and *V. louti* collected in 1990 produced toxin profiles similar to that of the latest catch. Hence, unchanged toxin profile during the past 20 years was suggested. The species-specific toxin profiles revealed in the previous study were further supported in this study with increased the number of fish. Perceivably, the inherent enzymes to metabolize the toxins are species-specific in fish. Hence, the species-specific toxin profiles are produced. If that is the case, most likely to be so from the present results, toxin profiles could be predicted by testing fewer specimens than used

presently. The consistency of toxin profiles in fish collected from the wide area of Okinawa during past 20 years would be helpful, when applying ELISA kits or biosensors such as surface Plasmon resonance detectors for monitoring. The limited toxin profiles are also interesting from the ecological point of view, because the organism producing CTXs in Okinawa likely belongs to only one genetic type. That is in contrast to the phylogenetic diversity of *Gambierdiscus* spp. found in Japan (9). Prominently, *O. punctatus* differed from the snappers and groupers in dominantly containing CTX4A and CTX4B, less oxidized precursors of CTX1B congeners. Reportedly, *O. punctatus* feeds on sea urchins and marine snails, suggesting these grazers as the first link in the food chain. The occurrence of ciguatera poisoning in Minamitorishima after eating turban shells and subsequent detection of CTX-like toxins in the same species support the above hypothesis (10, 11). Previously, we showed that CTX3C, 49-*epi*CTX3C, 51-hydroxyCTX3C (main), and M-*seco*-CTX3C were the major toxins in *O. punctatus* caught at Miyazaki coast. It is suggested, therefore, that *G. toxicus* in the two areas belongs to two different genetic types.

The amberjack, *S. dumerili*, from Hawaii resembled *L. bohar* from Minamitorishima used in the previous study in containing both CTX1B-type and CTX3C-type toxins (Figure 7). Since the fish is of migratory behavior, the toxin profiles could vary depending on the place of catch.

Results obtained using the MBA are here treated as a reference method to evaluate the safety of fish, and for this reason it is critical to increase our knowledge of ciguatoxin profiles as well as the relative potencies of congeners to determine if LC-MS can predict total potency. One challenge in researching the ciguatoxins however is the requirement of both MBA and LC-MS for sufficient quantities of sample. Fortunately a variety of different micro scale assays can be used to study the relative potencies of the ciguatoxins, and it is remarkable that the results roughly parallel the MBA values among these methods, as shown in Table 1. Some of the discrepancies seen may have resulted from the inaccuracy of weighing small samples.

In the present study, good agreement was observed between the MBA and LC-MS/MS data using the individual congener mouse i.p. potencies given in Table 1, further demonstrating the potential of the LC-MS method as an alternative to MBA. To meet the very stringent hazard advisory levels of 0.01 μg/kg CTX1B equivalents recently published by the US Food and Drug Administration (12), however, additional optimization of the current LC-MS/MS method seems desirable.

The low toxin concentrations coupled with the absence of CTX3C-type toxins posed a great obstacle in using Okinawan fish as the toxin sources, however this difficulty was overcome by using crude material retrieved from past studies. Two important toxins, CTX1B

and CTX3C, were produced at around 120μg yields, respectively. Their purities were confirmed by LC-MS and ^{1}H NMR data (Figure 8). A parallel effort to purify other toxins, CTX4A, CTX4B, and 51-hydroxyCTX3C, is expected to produce these toxins, though in smaller amounts. They will be useful in future validation studies and promote the use of the LC-MS method.

Acknowledgments

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Figure captions,

Figure 1. Structures of reference ciguatoxins. Note that CTX1B is also referred to as P-CTX-1 (Pacific ciguatoxin 1) in some publications.

Figure 2. Chromatogram for 14 reference toxins. The structure of gambierol is omitted.

Figure 3. The places of catch of fish specimens.

Figure 4. Preparation of test solutions for LC-MS/MS analysis.

Figure 5. Toxin profiles of MBA-negative fish (a) *L. monostigma* and (b) *V. louti*

Figure 6. Toxin profiles of representative ciguatera fish in Okinawa.

Figure 7. Toxin profile of the amberjack, *Seriola dumerili*, from Hawaii. *2,3,51-trihydroxyCTX3C was deduced from $[M + Na]^+$ (*m/z* 1095) and Rt (2).

Figure 8. LC-MS chromatograms (insets) and ^1H NMR spectra of purified CTX1B (a) and CTX3C (b)

Table 1. Bioactivities of CTXs

Toxins	Mouse (i.p.)	Ion influx*1	Receptor binding*2	Neuro-2A*3
	p mole/kg	ED50 (pM)	Ki (pM)	ED50 (pM)
CTX1B	320	260	49	2.6
52-*epi*-54-deoxyCTX1B	640	150	21	25.0
51-hydroxyCTX3C	190	250	28	8.5
CTX3C	1200	390	87	20.0
CTX4A	1300	1010	73	150.0
CTX4B	3400	5400	340	220.0

*1 Radioactive guanidine was used to measure ion influx

*2 Radioactive PbTx-3 was used

*3 Neuroblastoma-2A cells were used for cytotoxicity assay

Fig. 1

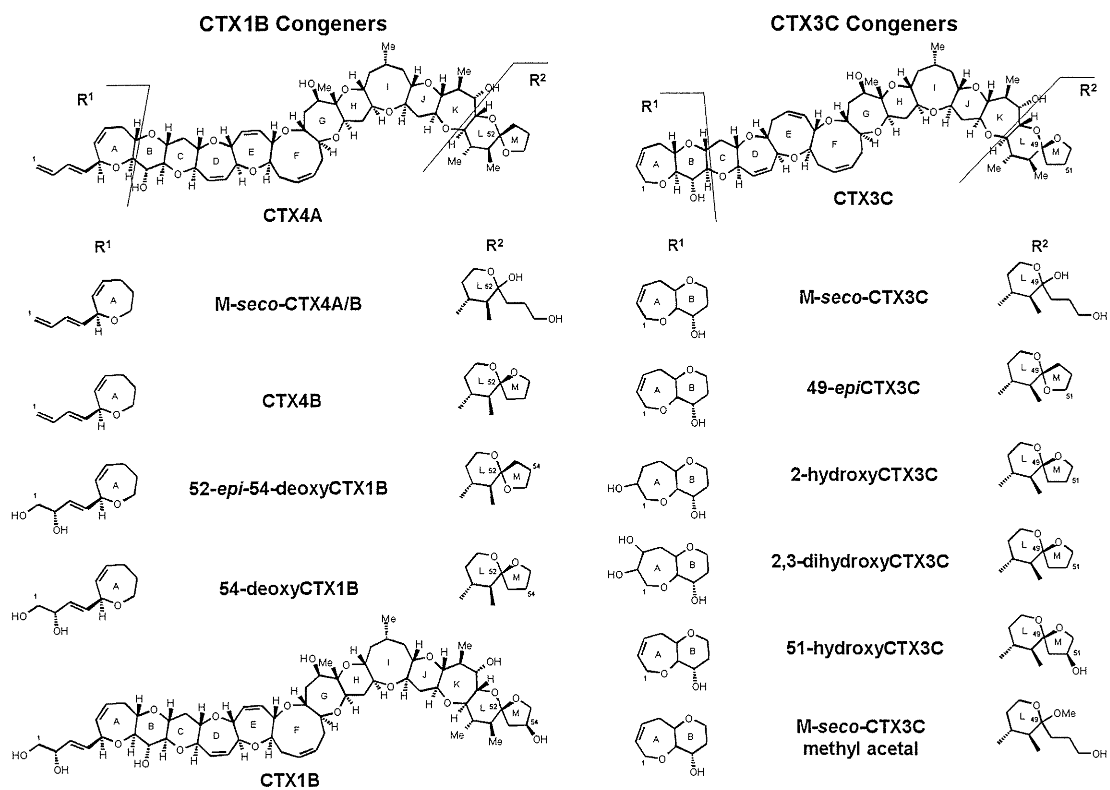


Fig. 2

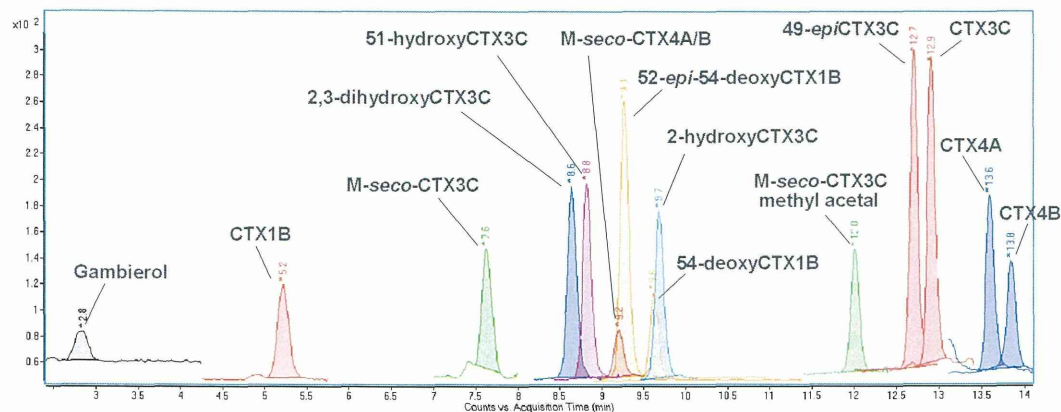


Fig. 3

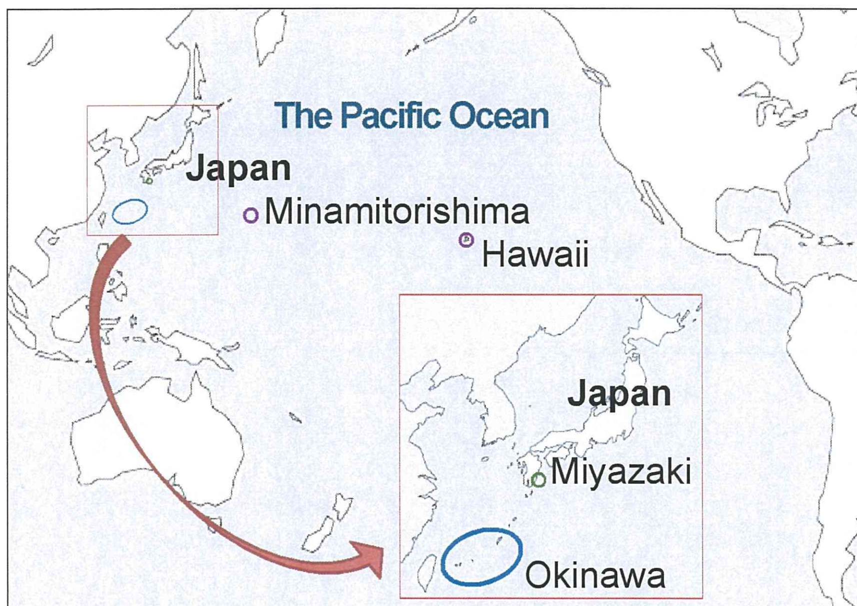


Fig. 4

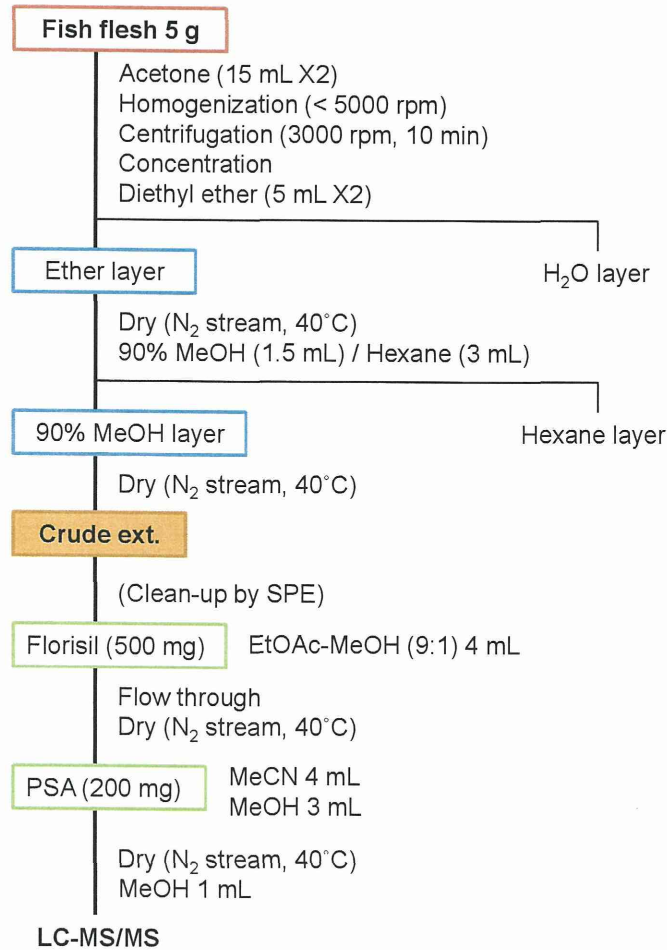


Fig. 5

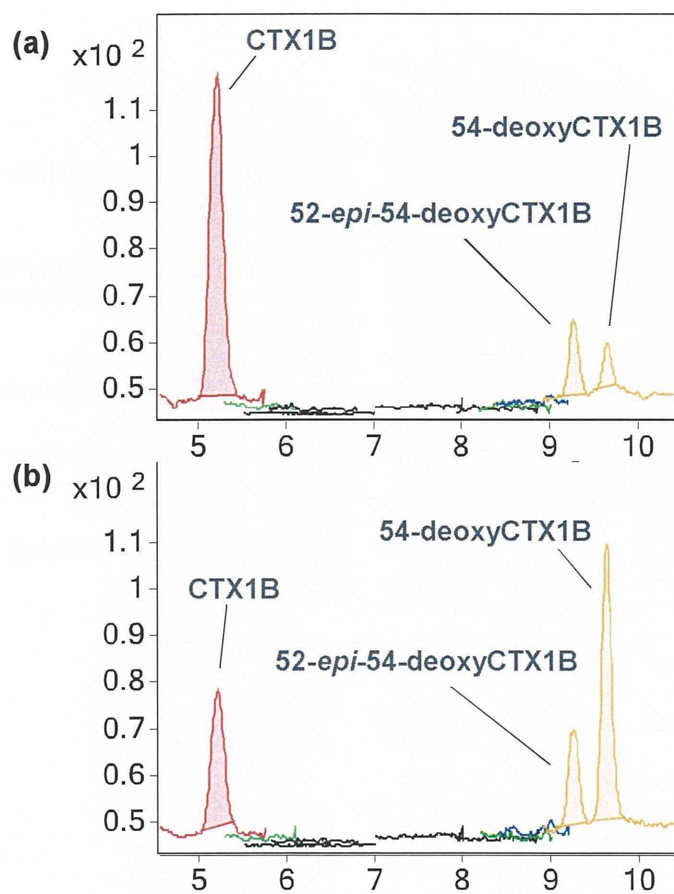
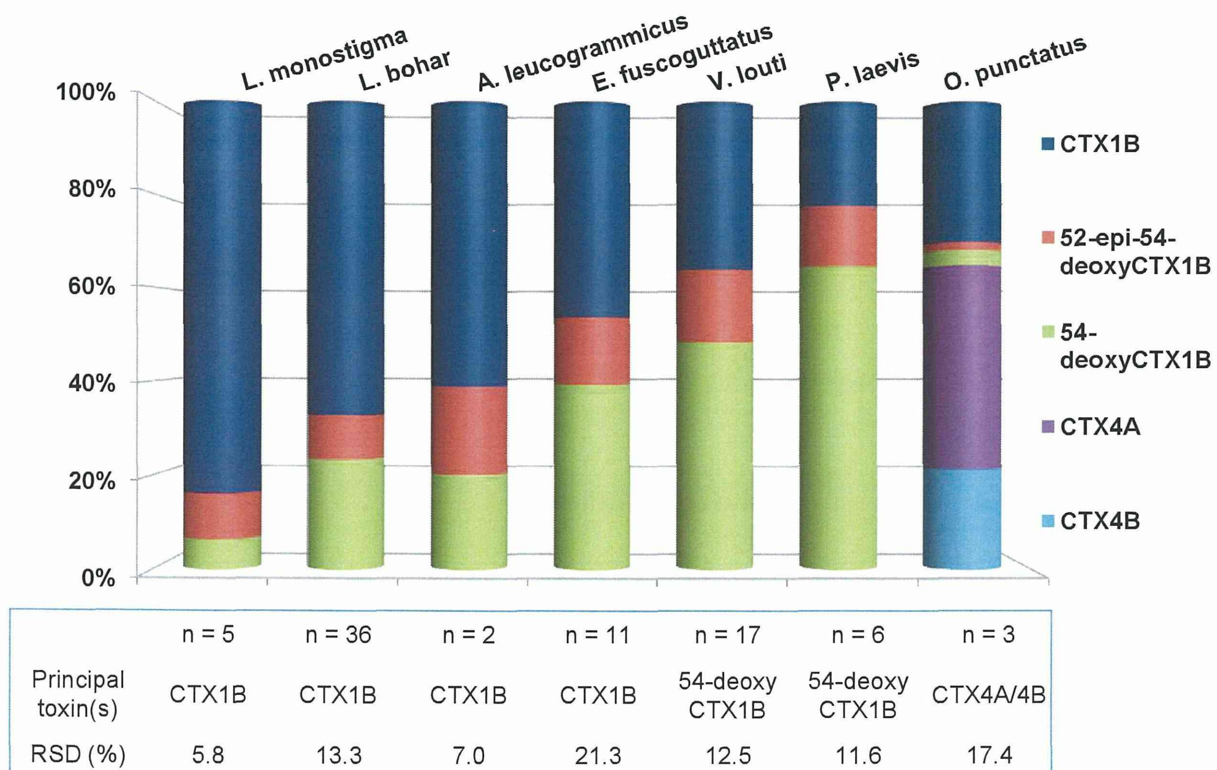


Fig. 6



	n = 5	n = 36	n = 2	n = 11	n = 17	n = 6	n = 3
Principal toxin(s)	CTX1B	CTX1B	CTX1B	CTX1B	54-deoxy CTX1B	54-deoxy CTX1B	CTX4A/4B
RSD (%)	5.8	13.3	7.0	21.3	12.5	11.6	17.4

Fig. 7