Toxicon 56 (2010) 804–812

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Establishing a public health analytical service based on chemical methods for detecting and quantifying Pacific ciguatoxin in fish samples

Ian Stewart [a,b,*], Geoffrey K. Eaglesham [a], Sue Poole [c], Glenn Graham [a], Carl Paulo [c], Wasantha Wickramasinghe [d], Ross Sadler [b], Glen R. Shaw [e,f]

[a] Queensland Health Forensic and Scientific Services, 39 Kessels Road, Coopers Plains, Queensland 4108, Australia
[b] School of Public Health, Griffith University, Logan Campus, University Drive, Meadowbrook, Queensland 4131, Australia
[c] Queensland Primary Industries and Fisheries, Department of Employment, Economic Development and Innovation, 19 Hercules Street, Hamilton, Queensland 4007, Australia
[d] The University of Queensland, National Research Centre for Environmental Toxicology, 39 Kessels Road, Coopers Plains, Queensland 4108, Australia
[e] School of Public Health, Griffith University, Gold Coast Campus, Parklands Drive, Southport, Queensland 4222, Australia
[f] Australian Rivers Institute, Griffith University, Gold Coast Campus, Parklands Drive, Southport, Queensland 4222, Australia

ARTICLE INFO

Article history:
Received 15 January 2009
Received in revised form 13 July 2009
Accepted 21 July 2009
Available online 30 July 2009

Keywords:
Ciguatera
Ciguatoxin
HPLC-MS/MS
DNA sequence analysis
Pacific
Tropical reef fish

ABSTRACT

A referee analysis method for the detection and quantification of Pacific ciguatoxins in fish flesh has recently been established by the public health analytical laboratory for the State of Queensland, Australia. Fifty-six fish samples were analysed, which included 10 fillets purchased as negative controls. P-CTX-1 was identified in 27 samples, and P-CTX-2 and P-CTX-3 were found in 26 of those samples. The range of P-CTX-1 concentrations was 0.04–11.4 μg/kg fish flesh; coefficient of variation from 90 replicate analyses was 7.4%. A liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) method utilising a rapid methanol extraction and clean-up is reliable and reproducible, with the detection limit at 0.03 μg/kg fish flesh. Some matrix effects are evident, with fish oil content a likely signal suppression factor. Species identification of samples by DNA sequence analysis revealed some evidence of fish substitution or inadvertent misidentification, which may have implications for the management and prevention of ciguatera poisoning. Blinded inspection of case notes from suspect ciguatera poisoning cases showed that reporting of ciguatera-related paraesthesias was highly predictable for the presence of ciguatoxins in analysed fish, with 13 of 14 expected cases having consumed fish that contained P-CTX-1 ($p < 0.001$, Fishers Exact Test).

1. Introduction

Ciguatera is a food poisoning syndrome historically restricted to tropical and sub-tropical regions, though with an escalating global trade and movement of seafood products the phenomenon is increasingly seen in higher latitudes (Kipping et al., 2006). However, Queensland remains one of the world's high-risk regions for ciguatera poisoning, along with many South Pacific island nations; other geographically discrete (and less extensive) hot-spots are in the Caribbean islands and south Florida, and the western Indian Ocean.

Tropical finfish caught in Queensland waters have poisoned local residents (Fenner et al., 1997; Tonge et al., 1967) as well as individuals from southern Australian states who have eaten fish sourced from Queensland (Karalis et al., 2000; Kraa et al., 1994; Ng and Gregory, 2000). Hence ciguatera poisoning poses a significant threat to public health in Australia as well as to Queensland's export fish trade.

* Corresponding author at: School of Public Health, Griffith University, Logan Campus, University Drive, Meadowbrook, Queensland 4131, Australia. Fax: +61 7 3274 9186.
E-mail address: ian.stewart@griffith.edu.au (I. Stewart).

0041-0101/$ – see front matter Crown Copyright © 2009 Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2009.07.028

Some 40 ciguatera poisoning cases are reported annually to public heath authorities in Queensland. Ciguatera fish poisoning is a notifiable disease under the *Health Act 1937,* but significant under-reporting and misdiagnosis are thought likely (Neville and Warren, 2003). Ciguatera poisoning is a clinical diagnosis because there is as yet no confirmatory diagnostic test. However, the ability to detect and quantify ciguatoxins in suspect fish will support the diagnosis of ciguatera, and will lead to a better understanding of the epidemiology of this disease. Public health investigators have not systematically collected fish samples from victims of suspected ciguatera poisoning because of the absence of a routine analytical capability. Yet a stored backlog of frozen fish is available for analysis; these samples were acquired by scientists with a research interest in ciguatera, working at two Queensland Government agencies: Queensland Primary Industries and Fisheries at Hamilton, and Queensland Health Forensic and Scientific Services at Coopers Plains.

Aims:

1. To develop a definitive referee analytical service for the detection and quantification of Pacific ciguatoxin in suspect fish caught in Queensland waters.
2. To identify fish analysed for ciguatera to species level by DNA sequence analysis and gene library affinity.
3. To investigate the reliability of clinical diagnosis for ciguatera poisoning by retrospective analysis of suspect fish samples.

2. Methods

2.1. Ciguatera-suspect fish

Forty-six frozen fish samples were collected from both formal and informal sources; the common feature linking the samples was a suspicion or query that the fish may have contained ciguatera toxins. Formal sources involved investigation of suspect ciguatera cases by Queensland public health authorities. These investigations produced an interview questionnaire form that elicited information about the onset of the illness and symptoms experienced by the victim, as well as an accompanying fish specimen. Informal sources were more diffuse. A number of the fish samples tested had been sent to one author (SP), with accompanying information of varying quality regarding the particular symptoms reported by victims of the associated food poisoning incident. Also included in the study were other samples such as a Spanish mackerel (*Scomberomorus commerson*) – a species known in Queensland as being high-risk for ciguatera – that was associated with a severe acute poisoning incident in a pet dog.

2.2. Control fish

Ten fish fillets that were anticipated to be free of ciguatera toxin were purchased from retail outlets to serve as negative controls. The control samples included both oily and non-oily fish species (to match an anticipated profile across suspect ciguatera fish). Control samples comprised species that have not been associated with ciguatera poisoning, e.g. non-reef fish such as sea mullet (*Mugil cephalus*), and fillets from small sized fish of species that are known to be capable of accumulating ciguatera toxins. Regarding the latter group, smaller sized fish are likely to be younger and therefore less likely to have toxic concentrations of ciguatera in their flesh than older, large fish (Lehane and Lewis, 2000).

2.3. Extraction and clean-up

Ciguatoxins were extracted by modifying the rapid extraction method of Lewis et al. (2009). Fish samples were cooked at 60–70 °C for 20 min, then ground in a blender to make a coarse mince before being re-frozen at −20 °C until required for analysis. 2 g of fish flesh were weighed in duplicate, 4 mL of 3:1 methanol in *n*-hexane were added and samples were homogenised using an Ultra Turrax T25 macerator. After centrifuging (3500 rpm for 10 min), the hexane layer was aspirated and discarded, the methanol layer was decanted and the sample was re-extracted with 3:1 methanol in hexane. The combined methanol layers were syringe filtered (0.45 μm), and water was added to produce a 55% methanol/water extract. Sample extracts were loaded onto reversed-phase solid-phase extraction cartridges (Alltech Prevail C_{18} 500 mg/4 mL); cartridges were washed with 65% aqueous methanol and eluted with 8 mL of 80% methanol. A total of 7 mL chloroform and 4 mL 1 M NaCl was added to each eluate, and after centrifuging at 2000 rpm for 20 min the methanol/saline layer was aspirated and discarded. The chloroform layer was transferred in aliquots of approximately 2 mL to a 5 mL tapered glass evaporating vial (Reacti-Vial; Thermo Fisher Scientific, Rockford, IL) and evaporated under a stream of N_2. The sample residue was reconstituted by twice washing the lower walls of the Reacti-Vial with 100 μL methanol. One sample was not run in duplicate because of insufficient quantity; 1.0 g of this sample was extracted as above and taken up in 200 μL methanol.

We modified the extraction method of Lewis et al. (2009) by employing a second methanol:hexane extraction and omitting the final normal-phase SPE step; although silica gel SPE reduced matrix effects we found the extra signal suppression experienced without this process was not severe enough to significantly affect results in our subsequent HPLC-MS/MS analysis.

To assess recoveries, 10 fish samples were spiked in duplicate with Pacific ciguatoxin-1 (P-CTX-1) standard, which was obtained from A/Prof. Richard Lewis, Institute of Molecular Biology, University of Queensland. Each duplicate was spiked with 3 ng P-CTX-1 when the initial solvents (methanol:hexane) were added prior to maceration of the sample. Unspiked duplicates of each spiked sample were concurrently analysed. A team member (author SP) not involved in the analytical work was asked to select a cross section of fish samples for spiking to include suspected ciguateric and control fish, both from oily and non-oily species. Team members doing the ciguatoxin extraction and analysis (authors IS, GKE and CP) were presented with 4-digit ID codes so that they maintained their investigator