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Linden flower (*Tilia* spp.) as potential vehicle of *Clostridium botulinum* spores in the transmission of infant botulism

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ABSTRACT

Infant botulism is an intestinal toxemia caused principally by *Clostridium botulinum*. Since the infection occurs in the intestinal tract, numerous food products have been investigated for the presence of *C. botulinum* and its neurotoxins. In many countries, people use linden flower (*Tilia* spp) tea as a household remedy and give it to infants as a sedative. Therefore, to help provide a clear picture of this disease transmission, we investigated the presence of botulinum spores in linden flowers. In this study, we analyzed 100 samples of unwrapped linden flowers and 100 samples of linden flowers in tea bags to determine the prevalence and spore-load of *C. botulinum*. Results were analyzed by the Fisher test. We detected a prevalence of 3% of botulinum spores in the unwrapped linden flowers analyzed and a spore load of 30 spores per 100 grams. None of the industrialized linden flowers analyzed were contaminated with botulinum spores. *C. botulinum* type A was identified in two samples and type B in one sample. Linden flowers must be considered a potential vehicle of *C. botulinum,* and the ingestion of linden flower tea can represent a risk factor for infant botulism.

Key words: botulinum spores, linden flower tea, infant botulism

RESUMEN

El té de tilo como vehículo potencial de esporas de *Clostridium botulinum* en la transmisión del botulismo infantil. El botulismo del lactante es una toxiinfección causada, principalmente, por *Clostridium botulinum*. Debido a que esta infección ocurre en el tracto intestinal, la presencia de esta bacteria y sus neurotoxinas ha sido investigada en numerosos alimentos. En muchos países se utiliza el té de tilo (*Tilia* spp.) como sedante natural, el que se administra incluso a los lactantes. A fin de contribuir al esclarecimiento de la transmisión de esta enfermedad, se investigó la prevalencia y la carga de esporas botulínicas en esta hierba. Se analizaron 100 muestras de tilo comercializado a granel y 100 muestras de tilo industralizado en "saquitos". Los resultados de prevalencia fueron analizados por el test de Fisher y la carga de esporas por la técnica del número más probable. Se halló una prevalencia de esporas de *C. botulinum* del 3% en el tilo comercializado a granel, con una carga de 30 esporas/100 g de hierba. En tanto, ninguna de las muestras en saquitos acusó la presencia del patógeno. Se identificaron tres cepas de *C. botulinum*, dos tipo A y una tipo B. En virtud de estos resultados, el tilo podría considerarse un potencial vehículo de esporas de *C. botulinum* y la administración de sus infusiones a menores y lactantes, un riesgo para la transmisión de la enfermedad.

Palabras clave: esporas botulínicas, té de tilo, botulismo del lactante

INTRODUCTION

Infant botulism is an intestinal toxemia caused by botulinum neurotoxins (BoNT) mainly produced by *Clostridium botulinum.* Some unusual strains of *C. butyricum* and *C. baratii* that produce BoNT type E and F, respectively, were isolated from a few patients with infant botulism (2, 5, 7, 17, 23, 32). Most cases result from *C. botulinum* types A and B (2, 4); however, some rare bivalent strains, Ba and Bf, were identified in a few cases (6, 16, 18).

Swallowed botulinum spores germinate, multiply, and vegetative cells produce BoNT *in situ* (2, 32). Since these spores are ubiquitous and widely distributed in soil, environmental exposure has been identified as an important risk factor for infant botulism (13, 22, 27). Soil is the principal source of these spore-forming bacteria, and botulinum spores may be present in dust; therefore, they can contaminate agricultural products. Due to the fact that the infection occurs in the intestinal tract, numerous food products have been investigated for the presence of *C. botulinum* spores and BoNTs. Botulinum

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spores have been found in household dust (27), honey, corn syrup (25), and in some medicinal plants (8, 30). However, for most cases of infant botulism, the source of botulinum spores has not been identified (4, 13, 25, 32).

In many countries, teas prepared with medicinal plants are frequently given to infants as household remedy; therefore, ingestion of teas prepared with medicinal plants contaminated with botulinum spores could represent a risk for infant botulism. In Argentina, *Tilia* spp. (linden flower) tea is commonly given to infants and some physicians recommend this tea as a natural sedative. The US Food and Drug Administration (FDA) places linden flower on the generally-recognized-as-safe (GRAS) list based on the chemical composition of this herb. However, linden flowers could be contaminated with botulinum spores. These spores can resist high temperatures; therefore, boiling water to prepare linden flower tea does not destroy the spores, but, rather, activates them. For these reasons, our aim was to determine the prevalence and spore-load of botulinum spores in linden flowers. This information is important to help elucidate the transmission of infant botulism and prevent this illness.

MATERIALS AND METHODS

We examined 200 samples of linden flowers that were obtained from markets and herbal stores from Mendoza, Argentina. Two groups of 100 samples were analyzed: 1) unwrapped linden flowers, which are delivered in large amounts to the herbal store and sold by weight to the customer in individual paper or plastic bags from open containers; and 2) linden flowers in tea bags (industrialized linden flowers), which are industrially processed, packaged in tea bags, and sold in closed boxes.

The samples were transferred to sterile recipients and stored at room temperature until examination. Then, 4 g of each sample of linden flower was suspended in 40 ml of saline solution (0.15 M NaCl) in a sterile recipient with hermetic closing. Suspensions were vigorously shaken and filtered through sterile gauze; the filtrates were centrifuged at 12,000 x g for 10 min to concentrate the spores. The pellets were suspended in 4 ml of saline solution, and these suspensions were subjected to 10 min of heat shock at 80 °C. The suspensions were inoculated in a chopped-meat medium (CMM) (14) and incubated at 31 °C for 5 days. After incubation, broths were centrifuged at 12,000 x g for 20 min at 4 °C. Cultures without signs of proteolysis were treated by mixing equal volumes of the supernatant culture and 1% trypsin (1:250, Difco) and incubated at 31 °C for 1 h. We inoculated 0.5 ml of each supernatant (each sample), in duplicate, intraperitoneally in mice and observed the mice for 96 h for characteristic botulinal signs and/or death (28).

We cultivated each of the toxic cultures in each of the following three solid media by streaking the surface: 1) 1.5% agar, 2) 4% agar (9), and 3) egg yolk agar (11). The cultures were incubated at 31 ºC in BBL jars with an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. After incubation for 24 h in the 1.5% agar media, 48 h in the 4% agar media, and 72 h in the egg yolk media, suspected colonies were transferred to CMM and incubated at 31 ºC for 4 days. The presence of BoNT in each of these cultures (from isolated colonies) was investigated by inoculating broths in mice as previously described. To assure a pure culture, toxic broths were cultivated in solid media, and these cultures were incubated in aerobic and anaerobic conditions. We identified the genera *Clostridium* based on the following characteristics: gram positive, strict anaerobe, and spore-forming rods. The cell morphology of each pure culture was observed by using an optical microscope. Isolated strains were characterized by biochemical tests: acid production from sugars, reaction in milk, meat, and gelatine, nitrate reduction, indole, lecithinase, and lipase production; esculin hydrolysis, and volatile acids production in peptone-yeast extract-glucose medium by gas-liquid chromatography (10).

Serologic typing of the pure cultures was carried out by quantitative neutralization tests on toxic cultures with monovalent and polyvalent botulinum antitoxin (15). When the levels of BoNTs were not sufficient for a neutralization test, we cultivated strains by dialysis in a cellophane sack immersed in toxin-production medium (33). After incubation at 32 °C for 6 days, the contents of the cellophane sacks were centrifuged at 12,000 × g for 10 min at 4 °C and serially diluted twofold in buffered solution. Each dilution was inoculated intraperitoneally into six mice (0.5 ml per mouse). Deaths were recorded for 96 h, and the 50% lethal doses (LD_{50}) were calculated by the Reed and Muench method (29).

The Fisher test was used to compare the prevalence of *C. botulinum* spores in the two groups of linden flowers (unwrapped linden flowers and linden flowers in tea bags).

For the three positive samples, the spore-load was estimated by the most probable number method (1). We use three dilutions (1:1, 1:5, and 1:25) and three tubes for each dilution. After 10 min of heat shock at 80 °C, 1 ml of each dilution was inoculated in each tube with CMM, and these cultures were incubated for 5 days at 31 °C. The presence of *C. botulinum* was detected by bioassay as described previously.

RESULTS

Prevalence of botulinum spores in linden flower. We detected *C. botulinum* spores in 3% (3/100) of unwrapped linden flower samples analyzed, but none of the industrialized linden flower samples appeared to be contaminated with these spores (0/100). The difference in the occurrence of botulinum spores between both types of samples was analyzed by the Fisher test, and results were not significant ($p = 0.2462$).

Spore-load of *C. botulinum* in linden flower. We detected 30 spores per 100 grams of linden flower in each of the three positive samples (95% confidence limits: 9-103 spores per 100 grams).

Phenotypic characteristics of strains of *C. botulinum* isolated from linden flower. We isolated three toxigenic strains of *C. botulinum* from positive samples of linden flower. The results of serological and biochemical test were the following:

1) Serological test. Botulinum neurotoxin type A was identified in two of the three positive samples and type B in only one sample.

2) Biochemical tests. All isolated strains were gram-positive rods with oval and subterminal spores. The three strains isolated from linden flower produced acid from glucose. Mannitol, maltose, lactose, mannose, fructose, and sucrose were not fermented and nitrate was not reduced. Gelatine was liquefied. Indole and lecithinase were not produced. Esculin was hydrolyzed and lipase was produced. Milk and meat were digested. Acetic, propionic, butyric, isobutyric, valeric, isovaleric, and isocaproic acids were detected in peptone-yeast extract-glucose medium cultures by gas-liquid chromatography.

According to biochemical test results, the three strains corresponded to metabolic group I.

DISCUSSION

Prevalence of botulinum spores in linden flower. The prevalence of botulinum spores found in unwrapped linden flower (3%) was lower than that detected in the following medicinal plants: *Matricaria* spp. (chamomile) (9), *Lippia turbinata* (penny royal), *Alternanthera pungens* (khakiweed), *Pimpinella anisum* (anise), and *Senna acutifolia* (senna) (30) (Table 1). These medicinal plants can be contaminated with botulinum spores present in the environment (i.e. soil, dust). The soil is the principal source of botulinum spores and the height of the medicinal plants can be an important factor in the contamination with these spores. The linden tree is several feet high; therefore, linden flower is less easily contaminated with botulinum spores than other plants of minor height. On the contrary, chamomile, khakiweed, anise, penny royal, and senna are small shrubs growing close to the ground (Table 1); therefore, these plants are easily contaminated with botulinum spores. On the other hand, the high prevalence of *C. botulinum* found by Satorres *et al.* (1999) in penny royal, khakiweed, anise, and senna could not be real because of the few samples analyzed for each one of these species (Table 1).

The prevalence of botulinum spores in linden flower was also lower than the 6-10% detected in honey (3, 13, 19, 20, 24, 31), but higher than that found in corn syrup (0.5%) (4). These spores have been detected in honey from various countries (3, 12, 19, 20, 24, 26, 34) and some studies have also considered honey consumption a risk factor for this illness. For these reasons, and because honey is not nutritionally essential, the United States public health agencies, all major pediatricians, and the honey industry have recommended not to feed infants younger than one year old with honey (4). Moreover, *C. botulinum* type B spores were found in approximately 0.5% (5 of 961) of light and dark corn syrup samples (20). However, in 1991 a FDA market survey of 738 corn syrup samples concluded that none contained *C. botulinum* spores (21). Therefore, although the possible role of corn syrup in infant botulism has been proposed, it is not a recognized source of botulinum spores or a risk factor for infant botulism (4).

Probably, the absence of these spores in industrialized linden flowers is due to the fact that the industrialization process reduces the contamination. Linden flowers in tea bags are usually dried in closed furnaces (25-30 °C), while unwrapped linden flowers are often dried in the open air or in sheds. Therefore, industrialized linden flowers are less exposed to contamination with spores present in environmental dust. In a previous study, we found similar results when comparing the prevalence of botulinum spores in unwrapped chamomile and chamomile in tea bags. Unwrapped chamomile showed a prevalence of 13% whereas chamomile in tea bags of only 2% (9). Because botulinum spores can be present in environmental dust, an important way to prevent contamination with *C. botulinum* of "unwrapped" herbs is to optimize the hygiene conditions in herbal stores and to keep these herbs in closed bags.

Spore-load of *C. botulinum* in linden flower. The minimum infective dose of *C. botulinum* spores for human

Table 1. Prevalence of *C. botulinum* in medicinal plants

Medicinal plant	Approximate height (f)	Number of samples studied (f)	Number of positive samples	Prevalence of botulinum spores (%)	Reference
Linden flower (*Tilia* spp.)					
- Unwrapped	15-20	100	3	3	This study
- In tea bags	15-20	100	0	0	This study
Chamomile (*Matricaria* spp.)					
- Unwrapped	0.50	100	13	13	9
- In tea bags	0.50	100	2	2	9
Penny royal (*Lippia turbinata*)	1.50	9	1	11.1	30
Anise (*Pimpinella anisum*)	1.00	9	1	11.1	30
Khakiweed (*Alternathera pungens*)	0.15	7	1	14.9	30
Senna (*Senna acutifolia*)	0.10	3	1	33.3	30

infants is unknown; however, from estimates from exposure to spore-containing honey, this dose may be as low as 10 to 100 (3, 4). This value is higher than the spore-load detected in the three positive samples of linden flowers (30 spores per 100 grams); however, a cup of linden flower tea is prepared with several grams of this herb, and linden flower tea may be ingested by an infant several times a day, for many days. Therefore, repetitive doses of this tea could accumulate the minimun infective dose of *C. botulinum* necessary for infant botulism. This spore-load is similar to that detected in chamomile (30-40 spores/100g) (9) and smaller than that detected in honey (5-80 spores/g) (3). However, infants may be more frequently given herbal teas than honey. In Mendoza, in western Argentina, epidemiological data of patients with infant botulism showed that 9.6% (10/104) had ingested herbal teas, while 4.8% (5/104) of patients were honey-fed (8). Moreover, a study about the use of alternative medicine in Mendoza showed that children are commonly given herbal teas (Femenía, Guida, Azcurra *et al*., personal communication). An 18.92% of infants had ingested some kind of herbal teas, and linden flower tea was one of the most common teas given to infants. This 18.9% of infants (younger than one year old) had ingested teas prepared with the following medicinal plants: *Matricaria* spp. (64.3%), *Tilia* spp. (14.3%), *Chenopodium ambrosioides* (14.3%), *Peumus boldus* (7.1%), *Eucalyptus* spp. (7.1%), *Pimpinella anisum* (7.1%), *Artemisia douglasiana* (7.1%), and a mixture of *Chamomilla recutita, A. pungens, Mentha viridis, L. turbinata,* and *Faeniculum vulgare* ("té del niño") (7.1%) (Femenía, Guida, Azcurra *et al.,* personal communication).

Phenotypic characteristics of strains of *C. botulinum* isolated from linden flower. Serotypes A and B are the most frequently identified in cases of infant botulism around the world. In Argentina, between the years 1982 and 2006, *C. botulinum* type A has been identified in 99.75% (409/410) of patients with infant botulism whereas *C. botulinum* type B was detected in one case (9). Moreover, we observed that strains of *C. botulinum* isolated from linden flower and from infant botulism cases showed similar biochemical characteristics. These results suggest that *C. botulinum* strains present in linden flower could collaborate to produce infant botulism.

An important way to prevent the occurrence of infant botulism results from not giving infants food products in which the presence of botulinum spores has been reported. Results presented in this study suggest that unwrapped linden flowers are a potential vehicle of *C. botulinum* spores. Therefore, to minimize the risk of acquisition of infant botulism, we recommend that linden flower tea prepared with unwrapped linden flowers should not be given to infants under one year of age.

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