2014

Molecular Epidemiology of Infant Botulism in California and Elsewhere, 1976–2010

Haydee A. Dabritz
Center for Infectious Diseases

Karen K. Hill
Center for Infectious Diseases

Jason R. Barash
Center for Infectious Diseases

Lawrence O. Ticknor
Los Alamos National Laboratory

Charles H. Helma
Center for Infectious Diseases

See next page for additional authors

Authors
Haydee A. Dabritz, Karen K. Hill, Jason R. Barash, Lawrence O. Ticknor, Charles H. Helma, Nir Dover, Jessica R. Payne, and Stephen S. Arnon

Journal of Infectious Diseases Advance Access published July 18, 2014

MAJOR ARTICLE

Molecular Epidemiology of Infant Botulism in California and Elsewhere, 1976–2010

Haydee A. Dabritz,[1,a] Karen K. Hill,[2] Jason R. Barash,[1] Lawrence O. Ticknor,[3] Charles H. Helma,[2] Nir Dover,[1] Jessica R. Payne,[1] and Stephen S. Arnon[1]

[1]Infant Botulism Treatment and Prevention Program, Division of Communicable Disease Control, Center for Infectious Diseases, California Department of Public Health, Richmond, California; [2]Bioscience Division, and [3]Computing, Computational and Statistical Sciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico

Background. Infant botulism (IB), first identified in California in 1976, results from *Clostridium botulinum* spores that germinate, multiply, and produce botulinum neurotoxin (BoNT) in the immature intestine. From 1976 to 2010 we created an archive of 1090 BoNT-producing isolates consisting of 1012 IB patient (10 outpatient, 985 hospitalized, 17 sudden death), 25 food, 18 dust/soils, and 35 other strains.

Methods. The mouse neutralization assay determined isolate toxin type (56% BoNT/A, 32% BoNT/B). Amplified fragment-length polymorphism (AFLP) analysis of the isolates was combined with epidemiologic information.

Results. The AFLP dendrogram, the largest to date, contained 154 clades; 52% of isolates clustered in just 2 clades, 1 BoNT/A (n = 418) and 1 BoNT/B (n = 145). These clades constituted an endemic *C. botulinum* population that produced the entire clinical spectrum of IB. Isolates from the patient's home environment (dust/soil, honey) usually located to the same AFLP clade as the patient's isolate, thereby identifying the likely source of infective spores. *C. botulinum* A(B) strains were identified in California for the first time.

Conclusions. Combining molecular methods and epidemiological data created an effective tool that yielded novel insights into the genetic diversity of *C. botulinum* and the clinical spectrum, occurrence, and distribution of IB in California.

Keywords. botulinum toxin; clinical spectrum; *Clostridium baratii*; *Clostridium botulinum*; *Clostridium butyricum*; honey; infant botulism; molecular epidemiology; sudden infant death.

Infant botulism (IB) results when swallowed spores of *Clostridium botulinum* (or rarely, neurotoxigenic *C. butyricum* or *C. baratii*) germinate, multiply, and produce botulinum neurotoxin (BoNT) in the large intestine. BoNT, the most poisonous substance known, causes constipation, bulbar palsies, and flaccid paralysis in infants by blocking acetylcholine release at the neuromuscular junction [1]. Approximately 20–50 IB cases occur annually in California, more than in any other state. Since initial identification of IB in California in 1976 [2–4], the California Department of Public Health Infant Botulism Treatment and Prevention Program has archived patient epidemiological information and *C. botulinum* strains isolated from patients' feces and their home environments.

Seven botulinum toxin types (A–G) are currently known (an eighth was recently recognized [5, 6]); *bont* gene sequencing has identified toxin subtypes or variants [7–10] (eg, A1, A2). Although most isolates produce a single toxin type, dual-toxin-producing strains (Ab, Af, Ba, Bf, Bh) [5, 11–14] and type A strains with an unexpressed *bont/B* gene, denoted A(B), exist [15–17]. Neurotoxigenic *C. butyricum* produces BoNT/E, while neurotoxigenic *C. baratii* produces BoNT/F [18, 19].

Because the BoNT gene (*bont*) cluster is associated with mobile genetic elements, the BoNT-producing clostridia exhibit substantial genomic diversity, identifiable by various molecular techniques (reviewed in [10]). We utilized amplified fragment-length polymorphism

Received 2 July 2013; accepted 3 June 2014.

[a]Present Address: Yolo County Health Department, Woodland, CA 95695.

Correspondence: Stephen S. Arnon, MD, Chief, Infant Botulism Treatment and Prevention Program, California Department of Public Health, 850 Marina Bay Pkwy E-361, Richmond, CA 94804 (stephen.arnon@cdph.ca.gov).

The Journal of Infectious Diseases

DOI: 10.1093/infdis/jiu331

(AFLP) analysis for this study because of its robustness and suitability for examining large collections of *C. botulinum* isolates [7, 20, 21], together with real-time polymerase chain reaction (RT-PCR) to identify *bont* subtypes, and combined the molecular results with epidemiologic and clinical information. This combination provided novel and unexpected insights into both the bacterium and the occurrence of IB in California and other parts of North America in the past 35 years.

METHODS

Case Definition, Case Identification, and Collection of Epidemiologic Data

An IB case was defined as an infant with symptoms consistent with the known paralyzing action of BoNT, including sudden death, in which BoNT or *C. botulinum* was identified in intestinal contents or feces. Cases outside of California were identified through distribution of Human Botulism Immune Globulin Intravenous (BIG-IV; BabyBIG [1]) and had *C. botulinum* freshly isolated from a second fecal specimen or were identified through the Centers for Disease Control and Prevention (CDC) national surveillance reports (http://www.cdc.gov/nationalsurveillance/botulism-surveillance.html). Cases were assigned to the state and county in which the infant lived for the 2 weeks immediately before onset (defined as the date on which the parent first contacted a medical provider about the symptoms of the illness). The incidence calculations included all US IB cases from 1976 to 2010 and live-births in California by county and nationwide by state, available from the California Department of Public Health (CDPH) at http://www.cdph.ca.gov/data/statistics/Pages/StatewideBirthStatisticalDataTables.aspx (accessed November 21, 2011) and from the National Center for Health Statistics, Hyattsville, MD, at http://www.cdc.gov/nchs/births.htm (accessed December 8, 2011), respectively. Epidemiological data for all California and BIG-IV-treated patients elsewhere were obtained by home visit and/or telephone interview.

Culture and DNA Isolation

Clostridium botulinum was originally isolated from patient and environmental specimens using standard methods [22], frozen at −70°C, and then freshly cultured to create cell pellets for DNA isolation (see online Supplementary Data) that followed the protocol for DNA extraction from Gram-positive bacteria using the Qiagen DNeasy Blood and Tissue Kit.

AFLP Analysis

For AFLP analysis, DNA preparations were digested with 2 restriction enzymes selected for A/T-rich genomes, linkers were ligated to the resulting fragments, and a series of PCR amplifications were performed as previously described [7]. Forty DNA fragment sizes were used to create a genetic fingerprint representing each isolate [23]. Clustering analysis of the fragment sizes for the 1091 isolates created a dendrogram with 154 clades. (The nontoxigenic *C. sporogenes* ATCC 11437 isolate was part of the collection.) Differences of >0.20 genetic distance units, the error inherent to the method, differentiated the clades from each other. (See online Supplementary Data for details.)

Toxin Gene Characterization by RT-PCR

Six hundred thirty-five type A and type B isolates, 58% of the collection, were characterized by RT-PCR to determine the subtype and presence of an unexpressed ("silent") *bont/B* gene (see online Supplementary Data for primers, probes, and conditions used).

Spatial Analysis

Latitude and longitude coordinates were assigned to the patient's residence address at onset of illness, with ZIP code or city centroid coordinates assigned for nonmatching or missing addresses. For clades with ≥10 isolates, SaTScan (http://www.satscan.org) with a case-control model was used to search for geographic or geographic-temporal clusters within that clade by comparing its IB patient isolates to those of all other California IB patients [24]. Environmental, food, and sudden-death case isolates were excluded from this analysis. Possible geographic-temporal clusters were sought in circular geographic areas of radius ≤125 km and maximum time windows of ≤5 years (see online Supplementary Data for details).

RESULTS

Infant Botulism Occurrence in California and Elsewhere

From 1976 to 2010, 2803 IB cases were reported in the United States; 1093 (39%) occurred in California. Table 1 lists the origins of the 1091 isolates studied. These 1091 isolates included 1012 IB patient isolates: 948 California, 55 other US states, and 9 other countries. These 1012 IB patients included the entire clinical spectrum of IB (ie, 10 outpatient, 985 hospitalized, and 17 sudden-death cases). An additional 79 patient-associated environmental isolates and reference strains were also analyzed. By the mouse neutralization assay, the 1090 neurotoxigenic isolates consisted of 609 (56%) BoNT/A; 345 (32%) BoNT/B; 22 (2%) bivalent BoNT/Af, /Ba, or /Bf; 11 (1%) BoNT/E; 9 (1%) BoNT/F; and 8 (1%) BoNT/C, /D or /G. Eighty-six (8%) strains were A(B) (Table 1).

The distribution of IB cases in California reflected the underlying population, with IB cases generally occurring in or near large population centers in the Los Angeles basin, San Francisco Bay area, and metropolitan San Diego (Figure 1*A*–*D*). However, cases also occurred in less populated rural areas. More than half of California IB cases were caused by BoNT/A strains and approximately one-third by BoNT/B strains. In comparison, bivalent and *C. baratii* BoNT/F strains were rare. IB incidence from 1976 to 2010 was 6.2/100 000 live-births, almost 3-fold higher

Table 1. Source and Geographic Origin of Clostridial Isolates in the AFLP-Based Dendrogram (n = 1091)

Source	California	United States (Outside California)	International (Outside United States)	Unknown	Total
Infant botulism[a]	948	55	9	. . .	1012
Honey	13	. . .	. . .	. . .	13
Other foods	2	7	3	. . .	12
Environmental	14[b]	2	2	. . .	18
Foodborne botulism	2	. . .	. . .	. . .	2
Wound botulism	12	3	. . .	. . .	15
Intestinal toxemia	1	1	2	. . .	4
Reference strains[c]	. . .	5	6	4	15
Total	992	73	22	4	1091

Abbreviations: AFLP, amplified fragment-length polymorphism; BoNT, botulinum neurotoxin.

[a] Includes 10 outpatient and 17 sudden-death cases.

[b] n = 11 soil, n = 2 vacuum cleaner dust, and n = 1 ceiling fan dust; isolates collected from California IB patient homes.

[c] Includes reference strain *C. sporogenes* ATCC11 437. Note that the bottom 4 rows of the table consist of 35 BoNT-producing isolates and 1 nontoxigenic *C. sporogenes* strain.

than the nationwide incidence of 2.1/100 000 live-births in the same interval. The annual ratio of BoNT/A to BoNT/B cases in California over the 35-year study was 1.7 (range, 0.7–8.5).

AFLP Analysis and *bont* RT-PCR Characterization

The AFLP dendrogram differentiated the 1091 clostridial isolates into 154 clades, 64 with multiple isolates and 90 with only a single isolate (Figure 2; Supplementary Data Appendix I). A striking feature of the dendrogram was its 2 largest clades, 1 containing 418 BoNT/A isolates (n = 387 California IB patients, Figure 1*B*) and the other containing 145 BoNT/B isolates (n = 141 California IB patients, Figure 1*D*). Hence, these 2 clades contained more than half (563; 52%) of all the isolates in the collection and represented the 2 predominant genotypes of *C. botulinum* that have caused IB in California for the past 35 years. The other 528 isolates of the collection located to the 152 other clades within the dendrogram.

The *bont* gene RT-PCR characterization identified subtypes/variants *bont/A1, /A2, /A4*, and *bont/B1, /B2, /B3, /B4, /B5*, and silent B (Figure 2). The Loch Maree BoNT/A3 strain, the only BoNT/A3 isolate in the collection, was included for reference purposes [25, 26]. The 86 A(B) isolates containing the unexpressed *bont/(B)* gene located to 14 clades; 67 isolates were from California IB patients (Figure 3*A*). Also, a large number of bivalent BoNT/Ba (n = 17), and /Bf (n = 3) and *C. baratii* type F (n = 2) isolates were obtained from infant botulism cases that occurred in different years and geographic locations in California (Figure 3*C*).

Isolates From Outpatient and Sudden-Death Cases

The clinical spectrum of IB includes outpatient, hospitalized, and fulminant (ie, sudden-death) cases [27, 28]. Ten IB outpatient isolates located to 5 clades: 4 to the largest BoNT/A clade, 3 to a closely related BoNT/A clade, 1 to the largest BoNT/B clade, 1 to the largest A(B) clade, and 1 to a small BoNT/A clade. Twelve of the 17 sudden-death cases located to the largest BoNT/A clade and 1 to the largest BoNT/B clade (Figure 2; Supplementary Data Appendix I). Hence, both the largest BoNT/A and the largest BoNT/B clades contained *C. botulinum* isolates indistinguishable by AFLP analysis that produced the entire clinical spectrum of infant botulism.

Clades Associated With Honey Consumption

The AFLP analysis produced 2 clades that contained isolates both from honeys and from IB patients fed those honeys before onset of illness. The first clade contained 5 BoNT/A2 isolates from 4 patients (1982, 1995, 1997, 2001) and from 1 commercial honey consumed by the 1982 patient. The honeys fed to the other 3 patients were not available for testing. The second clade associated with honey consumption contained 18 patient and 8 honey type B isolates from 1983 to 1988 (Table 2). All 26 isolates were subtyped and determined to be *bont/B5*. Fourteen (78%) of the 18 patients had been fed honey in the month before onset of illness, and 7 of the 8 honey isolates in this clade were from honeys consumed by 6 of the patients. One patient without a history of honey consumption lived in the honey-producing area.

Clades Associated With Dust and Soils Collected at Patients' Homes

Clostridium botulinum was isolated from 14 environmental samples from the homes of 8 IB patients (Table 1, footnote b), of which 11 located to the same clade as the patient's isolate. The co-location of patient and environmental isolate(s) in the same clade included: 1 vacuum cleaner dust and 2 soil isolates from the home of 2 sibling type A patients in an Alameda County city; 1 vacuum cleaner dust and 2 soil isolates from

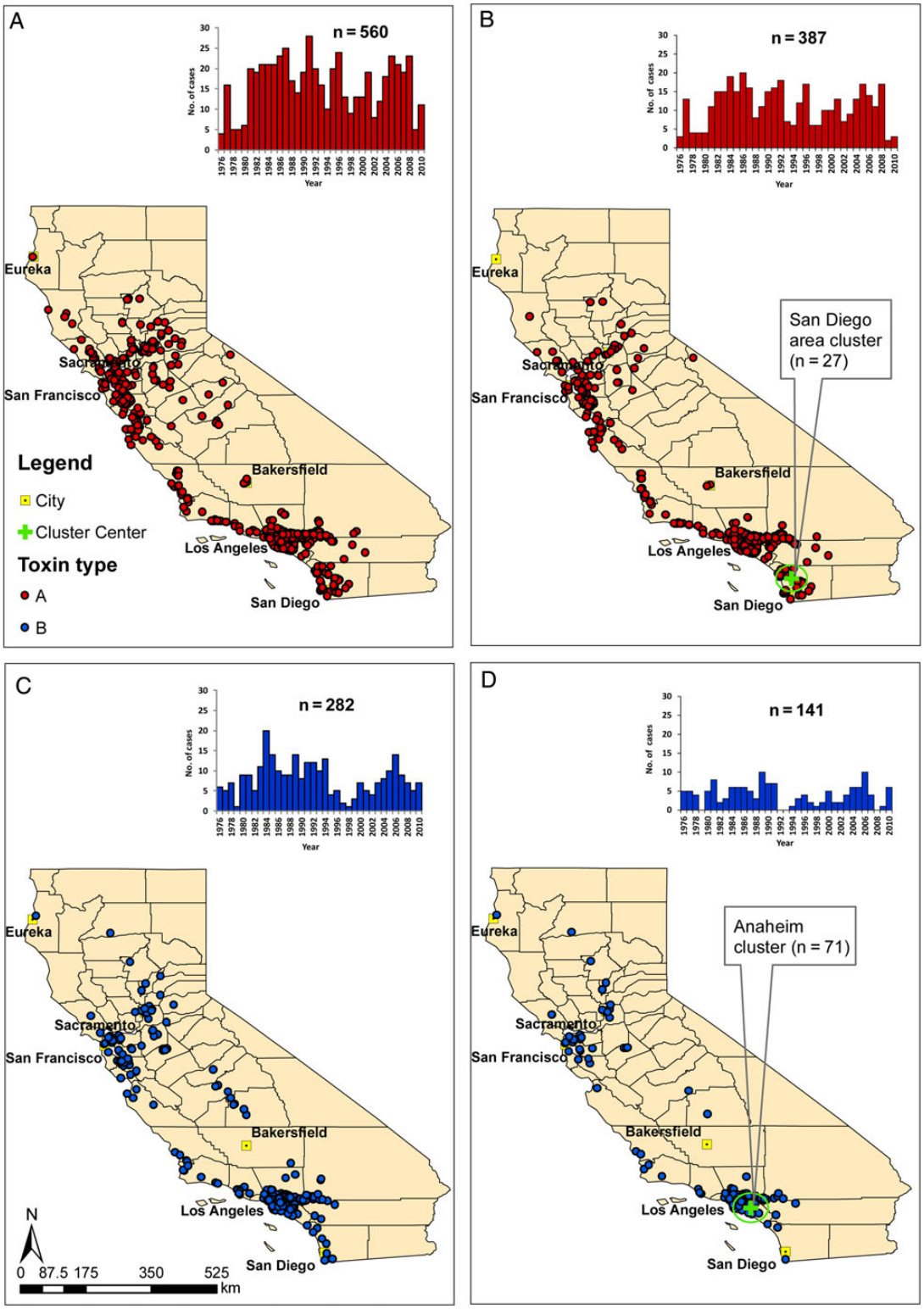

Figure 1. Infant botulism cases caused by *C. botulinum* types A and B in California, 1976–2010. *A*, All BoNT/A IB patient isolates (n = 560), excluding A(B) isolates. *B*, Isolates in the largest AFLP BoNT/A clade, which contained a type A Hall strain, 383 California hospitalized IB cases, 4 outpatient cases, and 12 sudden-death cases (not included on map). Note that cases occurred in all 35 years of the study period. A geographic but not temporal cluster of radius 35 km was detected near Escondido (San Diego County). *C*, All BoNT/B IB patient isolates (n = 282). *D*, The largest AFLP BoNT/B clade (n = 141). Note that cases occurred in all years of the study period except for 1979, 1992, 1993, and 2008. One geographic but not temporal cluster was identified in the Los Angeles area near Anaheim (radius 39 km). Geographic-temporal clustering was determined using the Kulldorff spatial-scan statistic with a case-control model [24]. Abbreviations: AFLP, amplified fragment-length polymorphism; BoNT, botulinum neurotoxin; IB, infant botulism.

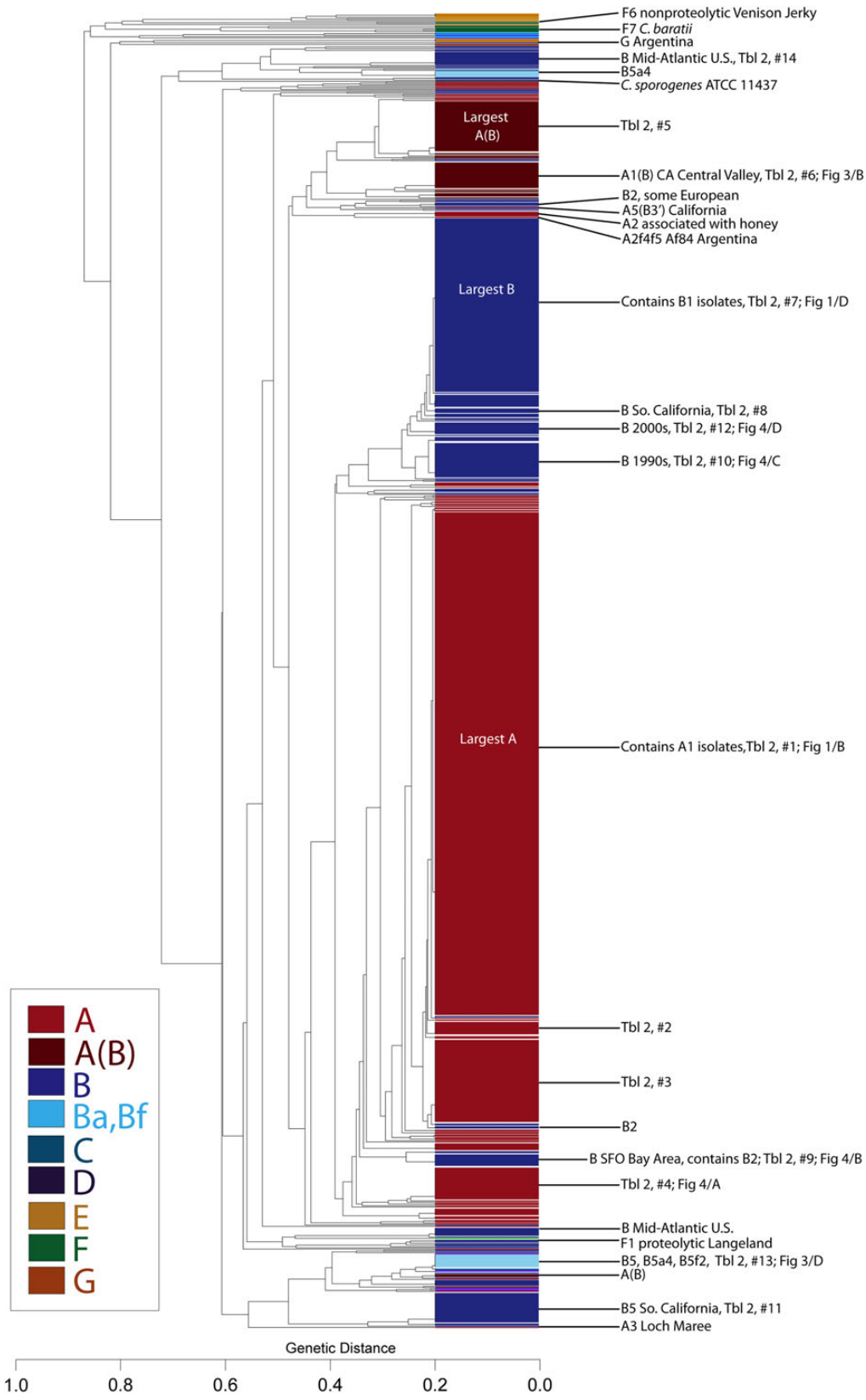

Figure 2. Schematic diagram of the AFLP dendrogram of 1091 clostridial isolates. Note that more than half the isolates (52%) located to just 2 large clades, 1 of toxin type A and the other of toxin type B. Note also the wide separation of the 2 major A(B)-containing clades, the bivalent (BoNT/Ba- and /Bf-containing) clades and other BoNT/A- and /B-containing clades. Dendrogram constructed using 40 DNA fragments and a cluster algorithm with the Jaccard distance measure [23]. The full Figure 2 in pdf format may be found in Supplementary Data Appendix I; when the isolates in it are displayed in an 8-point font, the figure measures approximately 4.5 m in length. Abbreviations: AFLP, amplified fragment-length polymorphism; BoNT, botulinum neurotoxin.

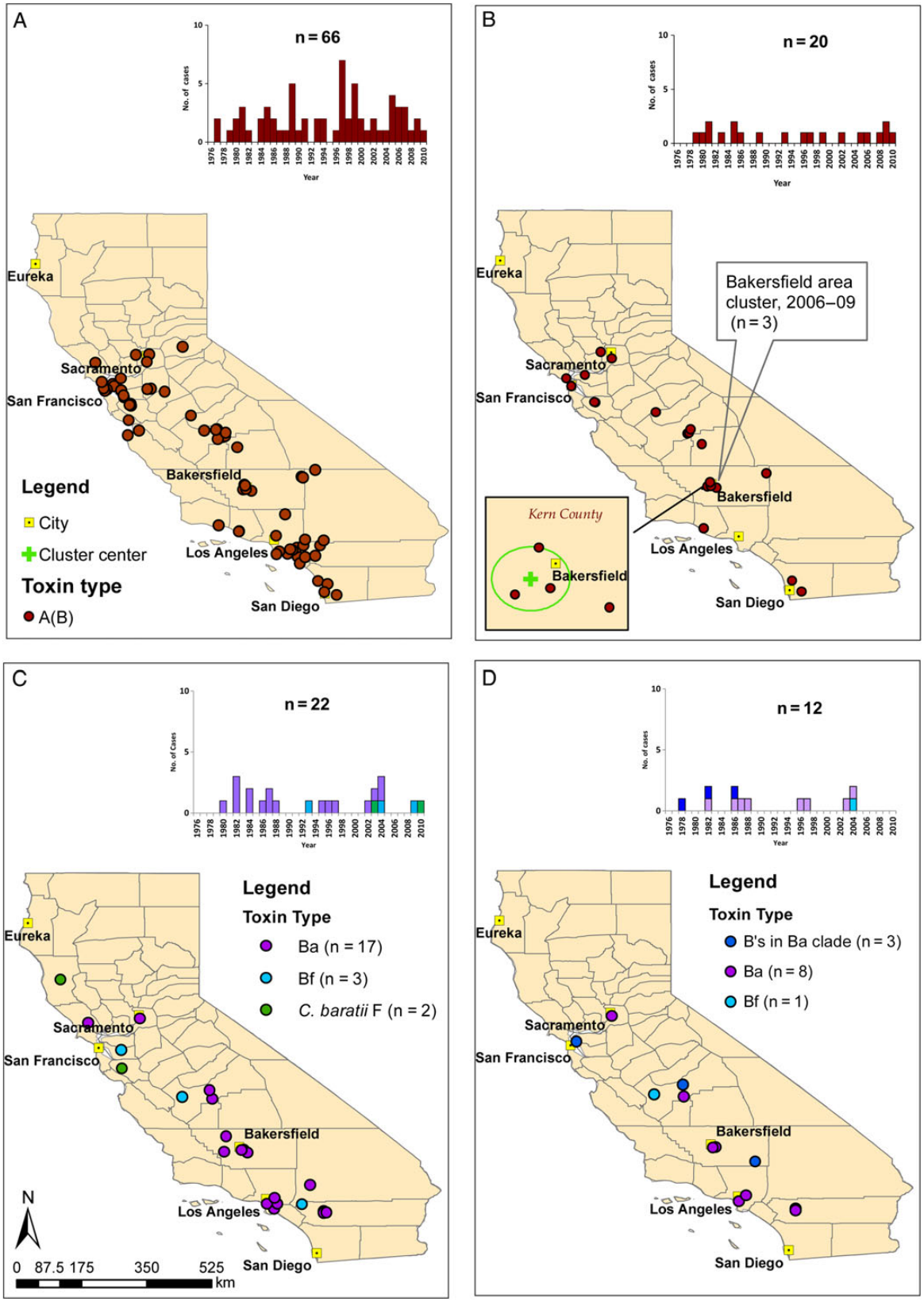

Figure 3. Occurrence and distribution of A(B), bivalent (Ba, Bf) and rare strains of *C. botulinum*. *A*, All A(B) California IB patient isolates (n = 66) located to 6 multimember and 5 singleton clades. One California 3-year-old intestinal toxemia patient isolate and 2 WB isolates not depicted. *B*, The second-largest A(B) clade (n = 23) included 20 California patients and contained a geographic-temporal cluster of 3 patients near Bakersfield. *C*, BoNT/Ba (n = 17), BoNT/Bf (n = 3) and *C. baratii* type F (n = 2) California IB patient isolates. *D*, Clade of 12 BoNT/B, /Ba and /Bf patient isolates. Geographic-temporal clusters determined using the Kulldorff spatial-scan statistic [24]. Abbreviations: BoNT, botulinum neurotoxin; IB, infant botulism; WB, wound botulism.

Table 2. Descriptive Features of the Largest Clades (≥10 Isolates) Identified by AFLP Analysis

AFLP Clade in Figure 2[a]	Figure/Panel Where Mapped	Years Isolated	Number and Source of Isolates					
			CA IB Isolates	IB Isolates From Outside CA	Environmental[b]	Food/Honey[b]	WB	Reference Strains[c]
1. Largest BoNT/A	1/B	1976–2010	399[d]	5	3	3	5	3
2. Southern California A closely related to above largest BoNT/A	N/A	1987–2007	12	. . .	. . .	. . .	. . .	. . .
3. Northern California A closely related to above largest BoNT/A	N/A	1977–2010	66[e]	1	3	. . .	. . .	. . .
4. Northern California BoNT/A	4/A	1977–2010	23	. . .	5	. . .	. . .	. . .
5. Largest A(B)	N/A	1977–2010	31	9	. . .	. . .	2	1
6. Central Valley A(B)	3/B	1979–2010	20	3	. . .	. . .	. . .	. . .
7. Largest BoNT/B	1/D	1976–2010	142[f]	1	2	. . .	. . .	. . .
8. Primarily Southern California BoNT/B closely related to the above largest BoNT/B clade	N/A	1980–2001	12	. . .	. . .	. . .	. . .	. . .
9. East San Francisco Bay area BoNT/B	4/B	1978–2009	10	. . .	1	. . .	. . .	. . .
10. Primarily 1992–1994 BoNT/B	4/C	1992–1999	30[g]	. . .	. . .	. . .	. . .	. . .
11. Southern California BoNT/B	N/A	1983–1988	18	. . .	. . .	8[h]	. . .	. . .
12. Primarily 2000s BoNT/B	4/D	2008–2009[i]	10	. . .	. . .	. . .	1	. . .
13. BoNT/B, BoNT/Ba, BoNT/Bf	3/D	1978–2004	12	. . .	. . .	. . .	. . .	. . .
14. Primarily Mid-Atlantic BoNT/B	N/A	2005–2010	1	10	. . .	. . .	. . .	. . .

Abbreviations: AFLP, amplified fragment-length polymorphism; BoNT, botulinum neurotoxin; CA, California; IB, infant botulism; N/A, not applicable; WB, wound botulism.

[a] Geographical coordinates of patient isolate clusters within these clades may be found in Table 3.

[b] Isolates associated with CA IB patients.

[c] Reference strains that locate to the largest BoNT/A clade: Hall strain; 62A (Nevada cow liver 1921 [29]); California carrot 2006 [30]. Reference strains that located to the largest A(B) clade: NCTC 2916 Colorado yellow corn 1929 [16].

[d] Includes 4 outpatient (shown) and 12 sudden-death cases (not shown) in Figure 1*B*.

[e] Includes 3 outpatient and 3 sudden-death cases.

[f] Includes 1 sudden-death case not shown in Figure 1*D*.

[g] All but 1 case occurred in 1992–1994.

[h] Includes 1 isolate from a honey producer in Tulare County.

[i] Nine of 10 IB cases occurred in 2008–2009. In 1986, the tenth case traveled from San Diego County to the San Francisco Bay area through the Los Angeles basin (center of the 2008–2009 geographic cluster) within 2 weeks of onset of illness. The WB case (not shown in Figure 4*D*) occurred in 1999 in Santa Clara County.

the Sierra Nevada foothills home of a Madera County type A patient; type A soil isolates from the homes of type A patients in Ontario, California, and Reno, Nevada; and 1 type B soil isolate and 1 ceiling fan dust type B isolate from the home of 2 type B twin patients in Stanislaus County. The 3 exceptions to the clade co-location of patient and environmental isolates consisted of a type A soil isolate and a type B soil isolate from the Alameda County home of the 2 type A sibling patients and 2 type A soil isolates from the homes of 2 Yolo County type A patients.

The Largest BoNT/A Clade

This clade contained 418 isolates from 399 California IB patients (Table 2, #1; Figure 1*B*), 1 IB patient from Mexico, 4 IB patients from neighboring western states (Idaho, Nevada, Utah, Washington), 5 wound botulism (WB) patients (n = 4 California, n = 1 Utah), 3 honeys, 2 food items (carrots, cow's liver), 3 soil isolates associated with patients (n = 2 California, n = 1 Nevada), and a Hall strain. All 12 isolates subtyped were *bont/A1*. Notably, no isolates in this clade were from states east of the Rocky Mountains.

Geospatial analysis of the 387 California IB patient isolates (excluding the 12 sudden-death cases) in this clade identified a geographic cluster centered near Escondido (San Diego County) that contained 27 IB cases (radius approximately 35 km; $P = .005$) (Figure 1*B*). Two other closely related BoNT/A clades (Table 2, #2–3) also contained geographic clusters: 1 clade (n = 12) contained a cluster of 7 isolates centered near Rialto (San Bernardino County) (radius approximately 15 km; $P < .001$), and the other clade (n = 70) contained a cluster of 48 isolates (76% of the clade's IB cases) centered near Napa, north of San Francisco Bay (radius approximately 124 km; $P < .001$).

Table 3. Geographical Coordinates of Patient Isolate Clusters

AFLP Clade in Figure 2	Figure/Panel Where Mapped	Cluster Center, Lat/Long
1. Largest BoNT/A	1/B	33.12, −117.06
2. Southern California A closely related to above largest BoNT/A	N/A	34.11, −117.38
3. Northern California A closely related to above largest BoNT/A	N/A	38.31, −122.32
4. Northern California BoNT/A	4/A	38.63, −121.22
5. Largest A(B)	N/A	None
6. Central Valley A(B)	3/B	35.32, −119.09
7. Largest BoNT/B	1/D	33.83, −117.98
8. Primarily Southern California BoNT/B closely related to the largest BoNT/B clade	N/A	34.16, −118.13
9. East San Francisco Bay area BoNT/B	4/B	37.73, −121.44
10. Primarily 1992–1994 BoNT/B	4/C	34.29, −188.49
11. Southern California BoNT/B	N/A	34.06, −118.43
12. Primarily 2000s BoNT/B	4/D	34.43, −118.47
13. BoNT/B, BoNT/Ba, BoNT/Bf	3/D	None
14. Primarily Mid-Atlantic BoNT/B	N/A	ND

Abbreviations: AFLP, amplified fragment-length polymorphism; BoNT, botulinum neurotoxin; Lat/Long, latitude/longitude; N/A, not applicable; ND, not determined.

The Largest BoNT/B Clade

This clade (Table 2, #7; Figure 1*D*) contained 145 isolates, 142 of which were from California IB patients, including 1 outpatient and 1 sudden-death case. The single strain in the clade not from California was isolated in 1979 from the first-recognized IB patient in the Czech Republic [31]. All 6 isolates subtyped were *bont/B1*. This clade contained a geographic cluster in Southern California centered near Anaheim (Los Angeles County) (radius approximately 39 km; $P = .001$) that contained 71 (50%) of the clade's California IB patients.

A(B) Strain Clades

The analysis identified 86 *C. botulinum* A(B) isolates (66 from California IB patients and 14 from IB patients in Alaska, Arkansas, Colorado, Georgia, Illinois, Nevada, New Mexico, Oklahoma, Texas, Washington, and Wyoming). Such A(B) strains were not previously known to exist in California (Table 2, #6; Figure 3*A*). The A(B) isolates located to 14 clades (7 multimember clades and 7 singleton clades); 5 clades contained non-California A(B) strains, and 2 contained both A(B) and type B strains. A(B) strains were isolated in California in all years of the study period except 1976, 1978, 1983, and 1995 (Figure 3*A*). The largest A(B) clade (n = 43) contained isolates from 31 California IB patients, 8 other US IB patients, 1 Canada IB patient, 2 California WB patients and the National Collection of Type Cultures reference strain NCTC 2916 (Table 2, #5). This clade had no geographic clusters in California, whereas the second-largest A(B) clade (Table 2, #6) contained a geographic-temporal cluster from 2006 to 2009 centered in California's Central Valley near Bakersfield (Kern County) (radius approximately 8 km; $P = .047$) that contained 3 IB cases (Figure 3*B*).

Bivalent (Ba, Bf) Clades

The molecular analysis identified 17 bivalent B5a4 isolates that located to 5 clades and 4 B5f isolates, 3 from California and 1 from Texas, that located to 3 clades. The largest of the bivalent clades (Table 2, #13) notably contained several B5 and B5a4 isolates and 1 B5f isolate (Figure 3*D*). A single unique strain that contained a *bont/A5* gene and a partial *bont/B* gene [the *bont/A5(B3′)* gene arrangement [9]] that was isolated from a San Diego County IB patient located to a singleton clade.

Mid-Atlantic US Type B Clades

Two clades of BoNT/B patient isolates mapped mainly to certain areas in the eastern Mid-Atlantic United States. The first clade (n = 8) included the 1939 Tennessee BoNT/B1 Okra reference strain [32], 3 patients from Maryland, and 1 patient each from New Jersey, Pennsylvania, Alabama, and California. The California IB patient was born prematurely and had not been discharged from her birth hospitalization before onset of illness. However, some of the specialty formulas fed to her were produced in the Mid-Atlantic region; although these formulas tested negative for *C. botulinum,* 1 contained *C. sporogenes*. The second Mid-Atlantic BoNT/B clade (Table 2, #14) contained 11 *bont/B1* isolates: 8 IB patients from either New Jersey, New York, or Pennsylvania; 1 patient from Colorado; and 2 patients from California and Oregon who had traveled to New York City and Pennsylvania, respectively, in the month before onset of illness. Geographical clustering in these clades could not be evaluated because the reference set of East Coast patient isolates was too small.

Other Noteworthy California Clades

Five additional California clades demonstrated either geographic or geographic-temporal clustering. A BoNT/A clade with 23 California IB patient and 5 environmental isolates (Table 2, #4; Figure 4*A*) had a geographic-temporal cluster of 7 patients from 2005 to 2008 centered northeast of Sacramento (radius approximately 101 km; $P = .003$).

The second clade, BoNT/B with 10 IB patient and 1 soil isolate (Table 2, #9; Figure 4*B*), had a geographical cluster of 9 patient isolates that centered east of San Francisco Bay (radius of approximately 69 km; $P = .002$). The third clade, BoNT/B with 30 California IB patient isolates, contained a geographic-temporal cluster from 1992 to 1994 in southern California with 18 patient isolates centered in the San Fernando Valley (Los Angeles County) (radius approximately 97 km; $P < .001$) (Table 2, #10; Figure 4*C*). The fourth clade of 12 BoNT/B California patients

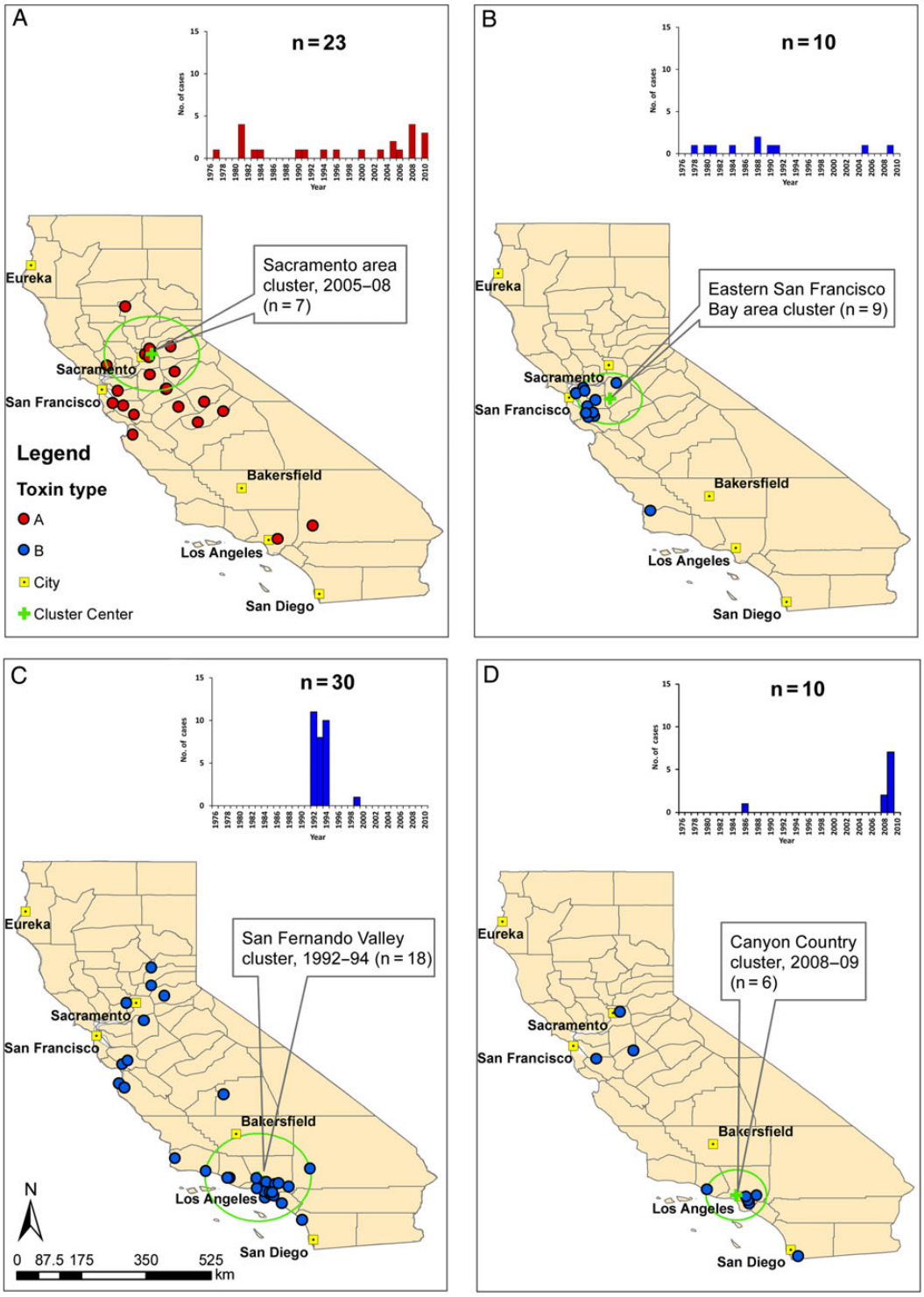

Figure 4. Geographic and geographic-temporal clusters of California IB cases between 1976 and 2010. *A*, BoNT/A clade of 23 patient isolates with a geographic-temporal cluster centered near Sacramento. This clade also contained 1 vacuum cleaner dust and 2 soil isolates (not shown). *B*, BoNT/B clade of 10 California patient isolates that clustered in the east San Francisco Bay area. The sole outlier patient lived in a central coastal county, had not traveled, and had been fed nonsterile solid foods. This BoNT/B clade also contained 1 soil isolate (not shown) from a patient home in an eastern San Francisco Bay city. *C*, BoNT/B clade of 30 California patient isolates with a geographic-temporal cluster that occurred in southern California, 1992–1994. Note the absence of this particular *C. botulinum* type B strain in California patients before 1992 and after 1999, with 97% of the cases in the clade having occurred in the 4 years 1992–1994. *D*, BoNT/B clade of 10 California IB patient isolates with a geographic-temporal cluster in southern California (1 WB patient from 1999 not shown). All IB cases occurred in 2008–2009 except for the 1986 case, who was driven through the Los Angeles basin (center of cluster) 2 weeks before onset of illness when the family moved from San Diego County to the San Francisco Bay area. Geographic-temporal clusters were determined using the Kulldorff spatial-scan statistic [24]. Abbreviations: BoNT, botulinum neurotoxin; IB, infant botulism; WB, wound botulism.

that also clustered in southern California both temporally (1980–1983) and geographically was centered near Pasadena (radius approximately 30 km; $P = .015$) (Table 2, #8).

The fifth clade, BoNT/B with 10 IB and 1 WB patient isolates (Table 2, #12; Figure 4*D*), contained a 2008–2009 geographic-temporal cluster of 6 patient isolates centered in the Santa Clarita Valley (Los Angeles County) (radius approximately 68 km; $P < .001$). Nine of 10 IB patients in this clade became ill in 2008–2009; in 1986, the tenth patient became ill 2 weeks after traveling through the Los Angeles basin (the center of the cluster).

DISCUSSION

The Infant Botulism Treatment and Prevention Program (IBTPP) collection of neurotoxigenic clostridial strains provided a unique opportunity to combine molecular and epidemiological information to better understand IB in California. Significant novel findings of this endeavor included: (1) the identification of 2 large clades that contained endemic strains that caused more than half of the BoNT/A and more than one-third of the BoNT/B IB cases in California in the 35-year study period; (2) the co-location of patient isolates from all 3 segments of the IB clinical spectrum (outpatient, hospitalized, sudden death) to the same BoNT/A or BoNT/B clade; (3) the identification of *C. botulinum* A(B) isolates in IB patients from California and 11 other US states that would not have been identified by the mouse neutralization assay; (4) the identification of rare strains isolated from 25 IB and 2 adult botulism cases that include BoNT/B5a4 (n = 17), BoNT/Bf (n = 4), and *C. baratii* BoNT/F (n = 6) isolates; (5) the recognition of clades whose isolates share a common-source origin (eg, honey); (6) the identification of clades whose isolates are restricted geographically to limited areas of California; and (7) the co-location to the same clade of *C. botulinum* patient and associated dust, soil, and honey isolates that could cause IB when swallowed by susceptible infants. The extensive branching of the dendrogram displays and emphasizes the different genetic backgrounds of *C. botulinum* strains that express the same toxin types.

Clostridium botulinum type A(B) isolates have been reported from Alaska, Arizona, Colorado, Georgia, Illinois, Louisiana, Nebraska, New Mexico, Pennsylvania, Puerto Rico, Wisconsin, and West Virginia [29, 33–35]. In this study, A(B) isolates were newly identified in IB patients from Arkansas, California, Nevada, Oklahoma, Texas, Washington, and Wyoming, further suggesting that A(B) strains are widespread throughout the United States. The molecular analysis and mouse neutralization assay also identified 17 bivalent BoNT/B5a4 and 3 BoNT/Bf isolates that caused IB in California. Recognition of BoNT/B5a4 strains outside of California that cause IB has occurred infrequently; only the 1976 Texas [36–38] and 1999 Texas and 2006 Nevada patients reported in the CDC annual national botulism surveillance summaries were previously known.

The AFLP analysis also identified clades that contained isolates associated with honey exposure or that contained geographic-temporal clustering. The 4 patients in the only *bont/A2* clade were fed honey; the clade also contained a *bont/A2* isolate from a honey consumed by 1 of the patients. The BoNT/A2 subtype had previously been isolated only from Argentinian soils and from IB patients in Argentina [39], Italy [33], and Japan who were fed honey [40], as well as from a Brazilian honey [40] and from honey [41] fed to a New Jersey patient that may have contained blended imported Argentinian honey. Because honey may harbor *C. botulinum* spores, all major public health, pediatric, and honey-producing institutions have recommended that honey not be fed to infants [42].

Three clusters within California BoNT/B clades occurred in limited time spans: 1 in 1992–1994 (Figure 4*C*), 1 in 2008–2009 (Figure 4*D*), and 1 in 1983–1985 (not shown). The latter occurred in a clade of monovalent BoNT/B5 isolates associated with honey exposure; interestingly, no monovalent BoNT/B5-producing isolates have been identified in California since 1988. No common-source exposures to a food or environmental source were identified for the temporal clusters in the 2 other BoNT/B clades. Possible sources of exposure to *C. botulinum* spores include nonsterile infant foods (eg, powdered infant formulas, dry cereals, herbal teas [43–45]), and anthropomorphic activities or weather phenomena that disrupt surface soil [46, 47].

Geographical localization of isolates in some clades (Figures 1, 3, and 4) suggests that local soil and/or climatic conditions may contribute to multiplication and subsequent dissemination of *C. botulinum* spores. Notably, isolates from several patients and their home environments (dust/soil, honey) located to the same clade. Soil surveys in Argentina [48] and the United States [49] have reported differing prevalences and BoNT types in different geographic regions. In the United States, BoNT/A strains predominate in soils west of the Mississippi River, while BoNT/B strains predominate in soils east of the Mississippi River; this regionalization matches the nationwide distribution of IB patient toxin types [22]. Our geographic-temporal analyses found that IB isolates from certain clades concentrated in specific geographical areas; for example, a BoNT/A clade northeast of Sacramento (Figure 4*A*), a BoNT/B clade in the San Francisco Bay area (Figure 4*B*), and a BoNT/B clade in the Santa Clarita Valley (Figure 4*D*). Two clades of BoNT/B isolates from patients who resided primarily in the Mid-Atlantic United States or had recently traveled there were also identified. These examples suggest that IB may be acquired from the local environment or while traveling in endemic areas.

In conclusion, the molecular analyses provided new and useful information for elucidating epidemiological relationships and for differentiating *C. botulinum* isolates. The study resulted

in an AFLP dendrogram of 1091 isolates that displays the genetic and epidemiological complexity of neurotoxigenic clostridia (Figure 2 and Supplementary Data Appendix I). Importantly, the geographical clustering of isolates was identified by use of patient epidemiological information. The combination of molecular methods and epidemiological data created an effective tool that yielded novel insights into the genetic diversity of *C. botulinum* and the clinical spectrum, occurrence, and distribution of IB in California.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We especially thank the many former IBTPP epidemiologists for carefully collecting IB patient information. We also thank Theresa Smith, US Army Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, MD, for assistance in identifying the origins of certain reference strains and USAMRIID for permission to compare the AFLP patterns of its *C. botulinum* strains to the AFLP patterns of IBTPP strains. The Microbial Disease Laboratory, CDPH, isolated and generously provided the 12 California WB isolates. The type E reference strain K112 published by Hyytiä et al in 1999 [50] was an earlier gift to CDPH from the Food Research Institute, University of Wisconsin-Madison.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Los Alamos National Laboratory or the California Department of Public Health.

Financial support. The RT-PCR assay development work was supported by the US Department of Homeland Security Science and Technology Directorate. This work was otherwise supported by the Infant Botulism Treatment and Prevention Fund of the California Department of Public Health.

Potential conflicts of interest. All authors: No potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Arnon SS, Schechter R, Maslanka SE, Jewell NP, Hatheway CL. Human Botulism Immune Globulin for the treatment of infant botulism. N Engl J Med **2006**; 354:462–71.
2. Pickett J, Berg B, Chaplin E, Brunstetter-Shafer MA. Syndrome of botulism in infancy: clinical and electrophysiologic study. N Engl J Med **1976**; 295:770–2.
3. Midura TF, Arnon SS. Infant botulism: identification of *Clostridium botulinum* and its toxins in faeces. Lancet **1976**; 2:934–6.
4. Arnon SS, Midura T, Clay S, Wood R, Chin J. Infant botulism: epidemiological, clinical, and laboratory aspects. JAMA **1977**; 237:1946–51.
5. Barash JR, Arnon SS. A novel strain of *Clostridium botulinum* that produces type B and type H botulinum toxins. J Infect Dis **2014**; 209:183–91.
6. Dover N, Barash JR, Hill KK, Xie G, Arnon SS. Molecular characterization of a novel botulinum neurotoxin type H gene. J Infect Dis **2014**; 209:192–202.
7. Hill KK, Smith TJ, Helma CJ, et al. Genetic diversity among botulinum neurotoxin–producing clostridial strains. J Bacteriol **2007**; 189:818–32.
8. Jacobson MJ, Lin G, Raphael B, Andreadis J, Johnson EA. Analysis of neurotoxin cluster genes in *Clostridium botulinum* strains producing botulinum neurotoxin serotype A subtypes. Appl Environ Microbiol **2008**; 74:2778–86.
9. Dover N, Barash JR, Arnon SS. Novel *Clostridium botulinum* toxin gene arrangement with subtype A5 and partial subtype B3 botulinum neurotoxin genes. J Clin Microbiol **2009**; 47:2349–50.
10. Hill KK, Smith TJ. Genetic diversity within *Clostridium botulinum* serotypes, botulinum neurotoxin gene clusters and toxin subtypes. Curr Top Microbiol Immunol **2013**; 364:1–20.
11. Lúquez C, Raphael BH, Maslanka SE. Neurotoxin gene clusters in *Clostridium botulinum* type Ab strains. Appl Environ Microbiol **2009**; 75:6094–101.
12. Fernández RA, Ciccarelli AS, Arenas GN, Giménez DF. First outbreak of botulism caused by *Clostridium botulinum* subtype Af. [Spanish]. Rev Argent Microbiol **1986**; 18:29–31.
13. Giménez DF. *Clostridium botulinum* subtype Ba. Zentralbl Bakteriol Mikrobiol Hyg A **1984**; 257:68–72.
14. Hatheway CL, McCroskey LM. Examination of feces and serum for diagnosis of infant botulism in 336 patients. J Clin Microbiol **1987**; 25:2334–8.
15. Franciosa G, Ferreira JL, Hatheway CL. Detection of type A, B, and E botulism neurotoxin genes in *Clostridium botulinum* and other *Clostridium* species by PCR: evidence of unexpressed type B toxin genes in type A toxigenic organisms. J Clin Microbiol **1994**; 32:1911–7.
16. Henderson I, Whelan SM, Davis TO, Minton NP. Genetic characterisation of the botulinum toxin complex of *Clostridium botulinum* strain NCTC 2916. FEMS Microbiol Lett **1996**; 140:151–8.
17. Hutson RA, Zhou Y, Collins MD, Johnson EA, Hatheway CL, Sugiyama H. Genetic characterization of *Clostridium botulinum* type A containing silent type B neurotoxin gene sequences. J Biol Chem **1996**; 271: 10786–92.
18. Aureli P, Fenicia L, Pasolini B, Gianfranceschi M, McCroskey LM, Hatheway CL. Two cases of type E infant botulism caused by neurotoxigenic *Clostridium butyricum* in Italy. J Infect Dis **1986**; 154:207–11.
19. Hall JD, McCroskey LM, Pincomb BJ, Hatheway CL. Isolation of an organism resembling *Clostridium barati* which produces type F botulinal toxin from an infant with botulism. J Clin Microbiol **1985**; 21:654–5.
20. Keto-Timonen R, Nevas M, Korkeala H. Efficient DNA fingerprinting of *Clostridium botulinum* types A, B, E, and F by amplified fragment length polymorphism analysis. Appl Environ Microbiol **2005**; 71:1148–54.
21. Macdonald TE, Helma CH, Shou Y, et al. Analysis of *Clostridium botulinum* serotype E strains using multilocus sequence typing, amplified fragment length polymorphism, and variable-number tandem-repeat analysis and botulinum neurotoxin gene sequencing. Appl Environ Microbiol **2011**; 77:8625–34.
22. Centers for Disease Control and Prevention. Botulism in the United States, 1899–1996: handbook for epidemiologists, clinicians and laboratory workers. Atlanta, GA: U.S. Dept. of Health and Human Services, **1998**.
23. Ticknor LO, Kolsto AB, Hill KK, et al. Fluorescent amplified fragment length polymorphism analysis of Norwegian *Bacillus cereus* and *Bacillus thuringiensis* soil isolates. Appl Environ Microbiol **2007**; 67:4863–73.
24. Kulldorff M. Bernoulli, discrete Poisson and continuous Poisson models: a spatial scan statistic. Comms Stat Theory Methods **1997**; 26:1481–96.
25. Monro TK, Knox WWN. Remarks on botulism as seen in Scotland in 1922. Br Med J **1923**; 1:279–81.
26. Leighton GR. Botulism and food preservation (the Loch Maree tragedy). London: W. Collins and Sons, **1923**.
27. Nevas M, Lindstrom M, Virtanen A, et al. Infant botulism acquired from household dust presenting as sudden infant death syndrome. J Clin Microbiol **2005**; 43:511–3.

28. Arnon SS, Midura TF, Damus K, Wood RM, Chin J. Intestinal infection and toxin production by *Clostridium botulinum* as one cause of sudden infant death syndrome. Lancet **1978**; 1:1273–7.
29. Raphael BH, Joseph LA, McCroskey LM, Lúquez C, Maslanka SE. Detection and differentiation of *Clostridium botulinum* type A strains using a focused DNA microarray. Mol Cell Probes **2010**; 24:146–53.
30. Sheth AN, Wiersma P, Atrubin D, et al. International outbreak of severe botulism with prolonged toxemia caused by commercial carrot juice. Clin Infect Dis **2008**; 47:1245–51.
31. Neubauer M, Milácek V. Infant botulism type B in central Europe. Zentralbl Bakteriol Mikrobiol Hyg [A] **1981**; 250:540–7.
32. Tucker CB, Swanson H. Outbreak of botulism in Tennessee due to Type B *Cl. botulinum*. Public Health Rep **1939**; 54:1556–60.
33. Franciosa G, Floridi F, Maugliani A, Aureli P. Differentiation of the gene clusters encoding botulinum neurotoxin type A complexes in *Clostridium botulinum* type A, Ab, and A(B) strains. Appl Environ Microbiol **2004**; 70:7192–9.
34. Kirma N, Ferreira JL, Baumstark BR. Characterization of six type A strains of *Clostridium botulinum* that contain type B toxin gene sequences. FEMS Microbiol Lett **2004**; 231:159–64.
35. Macdonald TE, Helma CH, Ticknor LO, et al. Differentiation of *Clostridium botulinum* serotype A strains using multiple-locus variable-number tandem-repeat analysis. Appl Environ Microbiol **2008**; 74:875–82.
36. Hatheway CL, McCroskey LM, Lombard GL, Dowell VR Jr. Atypical toxin variant of *Clostridium botulinum* type B associated with infant botulism. J Clin Microbiol **1981**; 14:607–11.
37. Giménez DF, Giménez JA. Identification of strain B 657 of *Clostridium botulinum*. [in Spanish]. Rev Argent Microbiol **1983**; 15:51–5.
38. Edmond BJ, Guerra FA, Blake J, Hempler S. Case of infant botulism in Texas. Tex Med **1977**; 73:85–8.
39. Sagua MD, Lúquez C, Barzola CP, Bianco MI, Fernández RA. Phenotypic characterization of *Clostridium botulinum* strains isolated from infant botulism cases in Argentina. Rev Argent Microbiol **2009**; 41:141–7.
40. Umeda K, Seto Y, Kohda T, Mukamoto M, Kozaki S. A novel multiplex PCR method for *Clostridium botulinum* neurotoxin type A gene cluster typing. Microbiol Immunol **2010**; 54:308–12.
41. Johnson EA, Tepp WH, Bradshaw M, Gilbert RJ, Cook PE, McIntosh ED. Characterization of *Clostridium botulinum* strains associated with an infant botulism case in the United Kingdom. J Clin Microbiol **2005**; 43:2602–7.
42. Arnon SS, Midura TF, Damus K, Thompson B, Wood RM, Chin J. Honey and other environmental risk factors for infant botulism. J Pediatr **1979**; 94:331–6.
43. Kautter DA, Lilly T Jr, Solomon HM, Lynt RK. *Clostridium botulinum* spores in infant foods: a survey. J Food Prot **1982**; 45:1028–9.
44. Bianco MI, Lúquez C, de Jong LI, Fernández RA. Presence of *Clostridium botulinum* spores in *Matricaria chamomilla* (chamomile) and its relationship with infant botulism. Int J Food Microbiol **2007**; 121:357–60.
45. Brett MM, McLauchlin J, Harris A, et al. A case of infant botulism with a possible link to infant formula milk powder: evidence for the presence of more than one strain of *Clostridium botulinum* in clinical specimens and food. J Med Microbiol **2005**; 54(Pt 8):769–76.
46. Long SS. Epidemiologic study of infant botulism in Pennsylvania: report of the Infant Botulism Study Group. Pediatrics **1985**; 75:928–34.
47. Flynn NM, Hoeprich PD, Kawachi MM, et al. An unusual outbreak of windborne coccidioidomycosis. N Engl J Med **1979**; 301:358–61.
48. Lúquez C, Bianco MI, de Jong LI, et al. Distribution of botulinum toxin-producing clostridia in soils of Argentina. Appl Environ Microbiol **2005**; 71:4137–9.
49. Smith LDS. The occurrence of *Clostridium botulinum* and *Clostridium tetani* in the soil of the United States. Health Lab Sci **1978**; 15:74–80.
50. Hyytiä E, Hielm S, Bjorkroth J, Korkeala H. Biodiversity of *Clostridium botulinum* type E strains isolated from fish and fishery products. Appl Environ Microbiol **1999**; 65:2057–64.