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Research Note

Cloth-Based Hybridization Array System for the Detection of *Clostridium botulinum* Type A, B, E, and F Neurotoxin Genes

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ABSTRACT

A simple cloth-based hybridization array system was developed for the characterization of *Clostridium botulinum* isolates based on the botulinum neurotoxin serotype. Bacterial isolates were subjected to a multiplex PCR incorporating digoxigenin-dUTP and primers targeting the four botulinum neurotoxin gene serotypes (A, B, E, and F) predominantly involved in human illness, followed by hybridization of the amplicons with an array of toxin gene-specific oligonucleotide probes immobilized on polyester cloth and subsequent immunoenzymatic assay of the bound digoxigenin label. This system provided sensitive and specific detection of the different botulinum neurotoxin gene markers in a variety of *C. botulinum* strains, exhibiting the expected patterns of reactivity with a panel of target and nontarget organisms.

Clostridium botulinum is an anaerobic spore-forming bacterium with the ability to produce highly potent botulinum neurotoxin (BoNT) responsible for the paralytic disease known as botulism. BoNTs are classified into seven different types (A through G) based on the serological neutralization of the toxin with specific antisera. Strains of *C. botulinum* are divided into four groups (designated groups I to IV) based on physiological differences. Group I includes the proteolytic strains of toxin types A, B, and F. Group II contains toxin type E strains and the nonproteolytic strains of toxin types B and F. Group III contains strains of toxin types C and D and is responsible for animal botulism. Group IV contains strains producing type G toxin and is considered a unique species, *Clostridium argnentinse.* All *C. botulinum* strains involved in human illness fall into groups I and II *(2, 10, 16).*

A foodborne source of botulism is confirmed for patients exhibiting the clinical syndrome of botulism when neurotoxin or *C. botulinum* cells or both are detected in suspect food samples or clinical samples *(3).* The standard method for detection and typing of viable *C. botulinum* involves growth in broth followed by detection and typing of the neurotoxins using the mouse bioassay *(3, 13).* The mouse bioassay requires specialized facilities and is costly, labor intensive, and time-consuming. There is a need for more rapid and practical methods for the characterization of bacterial isolates with respect to botulinum toxin production.

Molecular-based detection methods such as PCR have revolutionized the field of diagnostic microbiology. PCR offers high sensitivity and specificity in the detection of gene markers associated with a variety of pathogenic microorganisms. Several PCR-based methods have been reported for the detection and characterization of *C. botulinum* based on the amplification of BoNT gene sequences *(1, 5, 7, 8, 11, 18–20).* The use of multiplex PCR approaches in which several target DNA sequences are coamplified in a single reaction might increase the amount of information that can be rapidly generated from isolates in order to assist in traceback investigations of incidents of foodborne botulism. The analysis of amplicons generated in multiplex PCR systems is commonly achieved on the basis of their differential electrophoretic mobility by agarose gel electrophoresis, as previously described for a multiplex PCR system targeting botulinum toxin genes *(14).* Agarose gel electrophoresis analysis is time-consuming, and the complex electrophoretic patterns obtained with multiplex PCR can be difficult to interpret. Furthermore, the identity of individual amplicons can be difficult to confirm, especially if restriction endonuclease analysis is required. High-density microarray systems have been developed for the analysis of DNA fragments from bacteria *(6, 17),* but these rely on the use of highly sophisticated instruments and procedures to process the arrays and are not generally within the means of a basic food microbiology laboratory.

As a practical alternative for the analysis of multiplex PCR products, we propose a simple low-density array technique based on the use of macroporous, hydrophobic polyester cloth as a solid phase for the detection of amplicons from a multiplex PCR targeting BoNT gene serotypes A, B, E, and F by their hybridization with immobilized oligonucleotide probes. The advantages of polyester cloth as

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a DNA adsorbent for nucleic acid hybridization assays have been previously demonstrated *(4)* and were recently applied in the identification of antibiotic resistance and other genes associated with the multidrug-resistant food pathogen *Salmonella* Typhimurium DT104 *(9)*. Polyester cloth is a cost-effective solid support yielding improved reaction kinetics as a result of a large and readily accessible surface and is easy to wash between reaction steps to remove unbound reagents. In the proposed cloth-based hybridization array system (CHAS), an array of target-specific oligonucleotide probes is immobilized on a polyester cloth strip and hybridized with digoxigenin (DIG)-labeled amplicons generated in a multiplex PCR targeting multiple BoNT gene markers, followed by immunoenzymatic detection of the bound label. Here we demonstrate the applicability of the CHAS in the characterization of *C. botulinum* isolates based on their BoNT gene serotypes.

MATERIALS AND METHODS

DNA extraction and primer design. Genomic DNA was extracted and purified from broth cultures of *C. botulinum* strains (strains 1, 4, 10, and 14; Table 1) as previously described *(12)*. Purified genomic DNA was quantified using a Hoefer DyNA Quant 200 Fluorometer (Amersham Biosciences, San Francisco, Calif.) and stored at −20°C until use.

The oligonucleotide primer sequences targeting the four BoNT gene serotypes (A, B, E, and F) used in the multiplex PCR were designed using DNAMAN software (Lynnon Corporation, Vaudreuil, Quebec, Canada) and are presented in Table 2. All primers were synthesized by contract with Sigma Genosys (The Woodlands, Tex.).

Bacteria. The performance characteristics of the combined multiplex PCR-CHAS procedure were assessed using a variety of reference strains listed in Table 1. Three strains of *C. botulinum* type A, three strains of group I *C. botulinum* type B, three strains of group II *C. botulinum* type B, three strains of *C. botulinum* type E, three strains of group II *C. botulinum* type F, and 12 other *Clostridium* species were used in this study (Table 1). *Clostridium* strains were routinely grown in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.) in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2, at their respective optimum temperatures (35°C for *C. botulinum* type A, group I *C. botulinum* type B, and other *Clostridium* species; 25°C for group II *C. botulinum* type B, *C. botulinum* type E, and *C. botulinum* type F) for 17 h in 8 ml of special peptone–peptone–glucose–yeast extract broth (5% [wt/wt] special peptone [Oxoid, Basingstoke, Hampshire, England], 0.5% [wt/wt] peptone [Becton Dickinson, Sparks, Md.], 0.4% [wt/wt] glucose [Difco, Becton Dickinson, Sparks, Md.], 2% [wt/wt] yeast extract [Difco], 0.1% [wt/wt] sodium thioglycollate [Sigma, St. Louis, Mo.], pH 7.2). In instances where tests were carried out on isolated colonies, *C. botulinum* strains were plated on McClung Toabe egg yolk agar plates supplemented with yeast extract (7.5% [wt/wt] McClung Toabe agar base [Difco], 0.5% [wt/wt] yeast extract [Difco], 5.0% [wt/wt] egg yolk in 0.85% [wt/vol] NaCl, pH 7.6 [Difco]) *(15)* and incubated anaerobically for 48 h at their respective optimum growth temperatures as indicated above.

A variety of non-*Clostridium* bacteria was also used in this study (Table 1). All *Vibrio* strains were routinely grown on marine agar (Difco) for 18 h at 37°C. *Listeria monocytogenes*, *Enterobacter cloacae*, and *Bacillus cereus* were grown on tryptone soya agar (TSA; Difco) for 18 h at 30°C. All other strains were cultured on TSA for 18 h at 37°C. Bacteria cell counts were determined by performing serial dilutions of an overnight culture of the target organism and performing a direct microscopic count using a Petroff-Hausser counting chamber.

Oligonucleotide probes. Oligonucleotide probe sequences for immobilization on the arrays were selected on the basis of nucleotide sequence information deposited in the National Center for Biotechnology Information database as indicated in Table 2. The probes were designed to target sequences located within the individual serotype-specific BoNT amplicons generated in the multiplex PCR (Table 2). Oligonucleotide probes were synthesized by Sigma Genosys and rehydrated at a concentration of 100 μM in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA; Sigma), then stored at −20°C until use.

Multiplex PCR and CHAS procedure. Samples were subjected to a multiplex PCR incorporating four primer pairs targeting BoNT gene serotypes A, B, E, and F (Table 2). In instances where samples were composed of whole cell suspensions (e.g., enrichment broth cultures), the cells were lysed by mixing with an equal volume of 2% (wt/vol) Triton X-100 (Sigma) in water and heating at 100°C for 10 min. Purified genomic DNA was used directly in the PCR. For the PCR, 10 μl of test sample was added to 90 μl of multiplex PCR mixture (2.5 units of HotStar *Taq* and 1× PCR buffer containing 1.5 mM $MgCl_2$, plus 200 μM each deoxynucleotide triphosphate [dNTP], 5 μM DIG-11-dUTP [Roche, Indianapolis, Ind.], 0.5 μM each of primers BoNT A-1, BoNT A-2, BoNT B-1, and BoNT B-2; 0.2 μM each of primers BoNT E-1, BoNT E-2, BoNT F-1, and BoNT F-2 [Table 2]; and 2 μg of bovine serum albumin per ml). The PCR was carried out in a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) using the following program: initial heating at 94°C for 15.5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 52°C for 30 s, and primer extension at 72°C for 1.5 min, with an additional 2 min at 72°C following the last cycle.

For the CHAS, polyester cloth (Sontara 8100, Dupont, Mississauga, Ontario, Canada) was cut into strips measuring 1.5 by 5 cm and washed with 95% (vol/vol) ethanol, followed by rinsing with deionized distilled water on a filter with vacuum suction. The strips were then soaked in coating buffer (0.1 M Tris-HCl [pH 8.0], 0.01 M $MgCl_2$, and 0.15 M NaCl) and lightly blotted immediately prior to use for oligonucleotide probe immobilization. The oligonucleotide probes were diluted in coating buffer (10 μM) and heated at 100°C for 10 min (to disrupt any possible secondary structures), then placed on ice. Probes (5 μl) were pipetted in discrete spots on a cloth strip, followed by incubation at 37°C for 30 min. Probes were cross-linked to the cloth by exposing the strips to UV light for 1 min (254 nm, 100 mJ/cm^2) using a UV crosslinker (Stratalinker 1800, Stratagene, La Jolla, Calif.). The strips were blocked by incubating for 1 h at 37°C with hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.02% [wt/vol] sodium dodecyl sufate, 0.1% [wt/vol] *N*-lauroyl sarcosine, and 1% [wt/vol] protein blocking reagent [Bio-Rad, Hercules, Calif.]), after which they were washed five times by saturating strips with 0.01 M phosphate-buffered saline (PBS; pH 7.2, 0.15 M NaCl) containing 0.05% (vol/vol) Tween 20 (PBST) on a filter under vacuum suction. The resulting probe-coated cloth strips were air dried and stored at 4°C until use.

For the assay of DIG-labeled multiplex PCR products, amplicons were denatured by heating at 100°C for 10 min and placed on ice. The PCR product (100 μl) was mixed with 900 μl of ice-cold hybridization solution containing 50% (vol/vol) formamide.

TABLE 1. *Bacteria examined in this study*

Organism	Group	Strain	Serotype[a]: BoNT A	BoNT B	BoNT E	BoNT F
Clostridia						
1. *C. botulinum*	I	62A[b]	+	−	−	−
2. *C. botulinum*	I	17A[b]	+	−	−	−
3. *C. botulinum*	I	A6[b]	+	−	−	−
4. *C. botulinum*	I	13983IIB[b]	−	+	−	−
5. *C. botulinum*	I	368B[b]	−	+	−	−
6. *C. botulinum*	I	IB1-B[b]	−	+	−	−
7. *C. botulinum*	II	17B[b]	−	+	−	−
8. *C. botulinum*	II	2B[b]	−	+	−	−
9. *C. botulinum*	II	DB-2[b]	−	+	−	−
10. *C. botulinum*	II	Russ[b]	−	−	+	−
11. *C. botulinum*	II	Bennett[b]	−	−	+	−
12. *C. botulinum*	II	Gordon[b]	−	−	+	−
13. *C. botulinum*	II	70F[b]	−	−	−	+
14. *C. botulinum*	II	610F[b]	−	−	−	+
15. *C. botulinum*	II	190F[b]	−	−	−	+
16. *C. aurantibutyricum*	NA	ATCC 17777[c]	−	−	−	−
17. *C. baratii*		4624[d]	−	−	−	−
18. *C. beijenrinckii*		A401[e]	−	−	−	−
19. *C. bifermentans*		ATCC 638[c]	−	−	−	−
20. *C. butyricum*		ATCC 19398[c]	−	−	−	−
21. *C. difficile*		ATCC 9689[c]	−	−	−	−
22. *C. hastiforme*		ATCC 33268[c]	−	−	−	−
23. *C. novyi* B		ATCC 27606[c]	−	−	−	−
24. *C. perfringens*		ATCC 13124[c]	−	−	−	−
25. *C. sordellii*		ATCC 9714[c]	−	−	−	−
26. *C. sporogenes*		ATCC 3584[c]	−	−	−	−
27. *C. tetani*		A064[e]	−	−	−	−
Salmonella						
28. *Salmonella* Typhimurium		ATCC 14028[c]	−	−	−	−
29. *Salmonella* Montevideo		ATCC 8387[c]	−	−	−	−
30. *Salmonella* Enteritidis		ATCC 13076[c]	−	−	−	−
31. *Salmonella* Senftenberg		ATCC 8400[c]	−	−	−	−
32. *Salmonella* Alachua		OLC57[f]	−	−	−	−
33. *Salmonella* Johannesburg		OLC58[f]	−	−	−	−
Escherichia coli						
34. *E. coli*		ATCC 11775[c]	−	−	−	−
35. *E. coli* O157:H7		ATCC 35150[c]	−	−	−	−
36. *E. coli* O111:H11		910093[g]	−	−	−	−
37. *E. coli* O111:H8		910115[g]	−	−	−	−
38. *E. coli* O26:H11		910114[g]	−	−	−	−
39. *E. coli* O157:H7		920026[g]	−	−	−	−
40. *E. coli* O157:H7		930413[g]	−	−	−	−
41. *E. coli* O159:H4		99-4701-1[h]	−	−	−	−
42. *E. coli*		TD427C2[h]	−	−	−	−
43. *E. coli*		TD231C2[h]	−	−	−	−
44. *E. coli*		ATCC 35401[c]	−	−	−	−
45. *E. coli*		ATCC 43886[c]	−	−	−	−
Other spp.						
46. *Listeria monocytogenes*		ATCC 15313[c]	−	−	−	−
47. *Shigella sonnei*		ATCC 29938[c]	−	−	−	−
48. *Enterobacter cloacae*		ATCC 13047[c]	−	−	−	−
49. *Klebsiella pneumoniae*		ATCC 13883[c]	−	−	−	−
50. *Proteus vulgaris*		ATCC 13315[c]	−	−	−	−
51. *Vibrio parahaemolyticus*		ATCC 17802[c]	−	−	−	−
52. *Staphylococcus aureus*		ATCC 12600[c]	−	−	−	−
53. *Citrobacter freundii*		ATCC 8090[c]	−	−	−	−

TABLE 1. *Continued*

Organism	Group	Strain	Serotype[a]: BoNT A	BoNT B	BoNT E	BoNT F
54. *Bacillus cereus*		ATCC 14579[c]	−	−	−	−
55. *Vibrio cholerae*		ATCC 9459[c]	−	−	−	−
56. *Shigella flexneri*		HC25[i]	−	−	−	−
57. *Shigella dysenteriae*		HC8[i]	−	−	−	−

[a] Gene markers are as follows: BoNT A, botulinum neurotoxin type A; BoNT B, botulinum neurotoxin type B; BoNT E, botulinum neurotoxin type E; and BoNT F, botulinum neurotoxin type F; +, positive; −, negative.
[b] Botulism Reference Service, Bureau of Microbial Hazards, Health Products and Food Branch, Health Canada, Ottawa, Ontario, Canada.
[c] American Type Culture Collection, Manassas, Va.
[d] Dr. L.V. Holdeman, Virginia Polytechnic Institute and State University, Blacksburg.
[e] Laboratory Centre for Disease Control, Population and Public Health Branch, Health Canada, Ottawa, Ontario, Canada.
[f] Ottawa Laboratory (Carling) Culture Collection, Canadian Food Inspection Agency, Ottawa, Ontario, Canada.
[g] S. Read, Laboratory for Foodborne Zoonoses, Health Canada, Guelph, Ontario, Canada.
[h] T. Kruk, National Laboratory for Enteric Pathogens, Health Canada, Winnipeg, Manitoba, Canada.
[i] C. Bin-Kingombe, Bureau of Microbial Hazards, Health Products and Food Branch, Health Canada, Ottawa, Ontario, Canada.

The entire mixture (1 ml) was pipetted onto a strip of probe-coated cloth and incubated for 10 min at 37°C, followed by washing with PBST on a filter under vacuum suction. All subsequent incubations were carried out at room temperature. Strips were saturated with 1 ml of anti-DIG–peroxidase conjugate (Roche) diluted 1:2,000 in PBST and 0.5% (wt/vol) protein blocking reagent (Bio-Rad) and incubated for 10 min. After washing with PBST, the strips were saturated with 1 ml of tetramethylbenzidine membrane peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) and incubated for 10 min. Reactions were graded qualitatively as positive (blue spot) or negative (no spot).

RESULTS

The proposed method involves a two-phase process in which test samples (e.g., a colony from a plate) are first subjected to a multiplex PCR amplifying DNA sequences unique to the type A, B, E, and F BoNT genes, followed by hybridization of the PCR products with an array of oligonucleotide probes specific for each toxin gene immobilized on a polyester cloth strip. To assure its reliability as a tool for the characterization of *C. botulinum* toxin types in colonial isolates, the combined multiplex PCR-CHAS procedure must exhibit suitable performance characteristics with respect to its detectability and spectrum of reactivity with various clostridia and nonclostridial strains.

Detectability of the multiplex PCR-CHAS procedure. The minimum quantity of genomic DNA input into the PCR required to obtain positive reactions with the immobilized probes on the cloth array was determined using various amounts of genomic DNA extracted from a representative strain of each of the four *C. botulinum* serotypes (Table 3). The detectability of the combined multiplex PCR-CHAS for genomic DNA varied with the serotype (Table 3). For instance, at least 0.5 pg of *C. botulinum* serotype F genomic DNA was required to obtain a positive reaction with the BoNT F gene probe on the array, with no reactions occurring with any of the other BoNT gene probes at any DNA level tested, whereas a minimum of 0.015 pg

TABLE 2. *Oligonucleotide primers and probes used in the combined multiplex PCR-CHAS procedure*

Primer or probe[a]	Sequence (5′ to 3′)	Amplicon size (bp)	NCBI accession no.[b]
BoNT A-1	CAT GGT AAA TCT AGG ATT GC	125	AF461540
BoNT A-2	CAT AGC TGC CTC CGT AGC TT		
Probe A	CGT GTT TAT ACA TTT TTT TCT TCA GAC TAT GTA AA	35	AF461540
BoNT B-1	CAA GAT CCC AGC ATC ATA AGT	122	AF295926
BoNT B-2	AAT GTT AGG ATC TGA TAT GC		
Probe B	TAG AGG GAT AGT TGA TAG ACT TAA CAA GGT TTT AG	35	AF295926
BoNT E-1	GAC AGG TTC TTA ACT GAA AG	144	X62089
BoNT E-2	TCT CCC AAG ATT GAT CCA TG		
Probe E	GAG AAT ATG ATG AGA ATG TCA AAA CGT ATT TAT TG	35	X62089
BoNT F-1	CAT GCA GAT ACC ATA TGA AG	113	X81714
BoNT F-2	AAA TCA CTA GGA TCC GTT CC		
Probe F	GAG ATT ATG CGT AAT GTT TGG ATA ATT CCT GAG AG	35	X81714

[a] BoNT A, botulinum neurotoxin type A; BoNT B, botulinum neurotoxin type B; BoNT E, botulinum neurotoxin type E; BoNT F, botulinum neurotoxin type F.
[b] NCBI, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/.

TABLE 3. *Detectability of the combined multiplex PCR-CHAS for different* Clostridium botulinum *serotypes*[a]

Organism (strain)[b]	Sample	Minimum detected[c]			
		BoNT A	BoNT B	BoNT E	BoNT F
C. botulinum type A (strain 1)	DNA (pg)	0.06	—	—	—
	Whole cells (CFU)	4	—	—	—
C. botulinum type B (strain 4)	DNA (pg)	—	0.03	—	—
	Whole cells (CFU)	—	4	—	—
C. botulinum type E (strain 10)	DNA (pg)	—	—	0.015	—
	Whole cells (CFU)	—	—	4	—
C. botulinum type F (strain 14)	DNA (pg)	—	—	—	0.5
	Whole cells (CFU)	—	—	—	420

[a] Serial doubling dilutions of genomic DNA (range of 0.01 to 1.0 pg) and tenfold dilutions of whole cells (range of ca. 0.4 to 4 $\times$ 10^6 CFU) from different serotypes of *C. botulinum* were subjected to the combined multiplex PCR-CHAS procedure as described in ''Materials and Methods.''

[b] Strain number designated in Table 1.

[c] Minimum amount of genomic DNA (pg) or whole cells (CFU) into the PCR producing positive reactions with the immobilized probes on the cloth array. BoNT A, botulinum neurotoxin type A; BoNT B, botulinum neurotoxin type B; BoNT E, botulinum neurotoxin type E; BoNT F, botulinum neurotoxin type F; —, no reaction.

of *C. botulinum* serotype E genomic DNA was required to obtain a positive reaction with the BoNT E gene probe (Table 3). The two other *C. botulinum* serotypes tested (A and B) exhibited detectabilities with their respective relevant probes on the array within the 0.015 to 0.5 pg DNA range, and no heterologous probe reactions were observed for the different bacteria at any DNA level tested.

The detectability of the assay for whole cells was determined by preparing serial dilutions of the cells of the various strain serotypes (Table 3) in special peptone–peptone–glucose–yeast extract broth (as described above), which were then lysed and subjected to the multiplex PCR-CHAS. For the panel of bacteria tested, lysates derived from at least 0.4 to 4 CFU (or 42 to 420 CFU for serotype F) input into the PCR produced positive reactions with the relevant probes on the array, with no heterologous probe reactions occurring for the different serotypes at any cell level tested (Table 3). These experiments were repeated, and the results were reproducible.

Specificity of the combined multiplex PCR-CHAS procedure. The specificity of the combined multiplex PCR-CHAS for the target gene markers was ascertained by subjecting lysates from individual colonies of various target and nontarget bacteria to the procedure. The bacterial strains used in this study are detailed in Table 1. All *C. botulinum* strains displayed the expected reactivity patterns on the array, with the different *C. botulinum* BoNT serotypes producing positive reactions with their corresponding probes on the array (Fig. 1). No heterologous hybridization reactions were observed. Thus, the inclusivity of the multiplex PCR-CHAS is demonstrated by the homologous reactions of the different *C. botulinum* strains with their respective probes on the array, and its exclusivity is suggested by the lack of heterologous reactions between amplicons from target bacteria and the probes on the array. None of the PCR products obtained with lysates from the various other nontarget bacteria tested (other clostridia and non-*Clostridium* strains) produced positive reactions with any of the toxin gene probes on the array, further demonstrating the exclusivity of this procedure. These experiments were repeated, and the results were reproducible.

DISCUSSION

The CHAS used in conjunction with a multiplex PCR targeting multiple BoNT gene serotypes is designed to provide a tool for the characterization of *C. botulinum* isolates based on their toxin gene profiles. The system described here exhibited adequate detectability characteristics for all the BoNT gene markers tested to enable its reliable application in the characterization of colonies isolated on plating media. Variability in the detectability of the procedure with different strains of a given serotype (e.g., proteolytic versus nonproteolytic) was not examined in this study. The detectabilities reported for other PCR systems targeting *C. botulinum* BoNT genes ranged from 10 to 100 CFU per reaction *(14)* to 0.34 to 5,160 ng per reaction *(7)*, depending on the serotype, which is more or less comparable with the detectability characteristics observed for the present system. The relatively poorer detectability for the BoNT serotype F gene does not preclude the use of this method for the characterization of colonial isolates since the level of cells present in a colony would not be a limiting factor. However, the applicability of this method to the detection of BoNT serotype F strains in enrichment broth cultures from foods will need to be investigated. This method was highly specific for the BoNT toxin gene serotypes A, B, E, and F, with the combined multiplex PCR-CHAS correctly identifying the different *C. botulinum* BoNT toxin genes in the various serotypes tested. The combined multiplex PCR and CHAS procedure could be completed within a single day, thus providing information regarding the serotype of the isolates at an earlier juncture than the conventional mouse bioassay, enabling its use for the rapid and specific characterization of *C. botulinum* strains isolated from food samples or clinical specimens using suitable enrichment culture techniques. The CHAS facilitates the detection of different

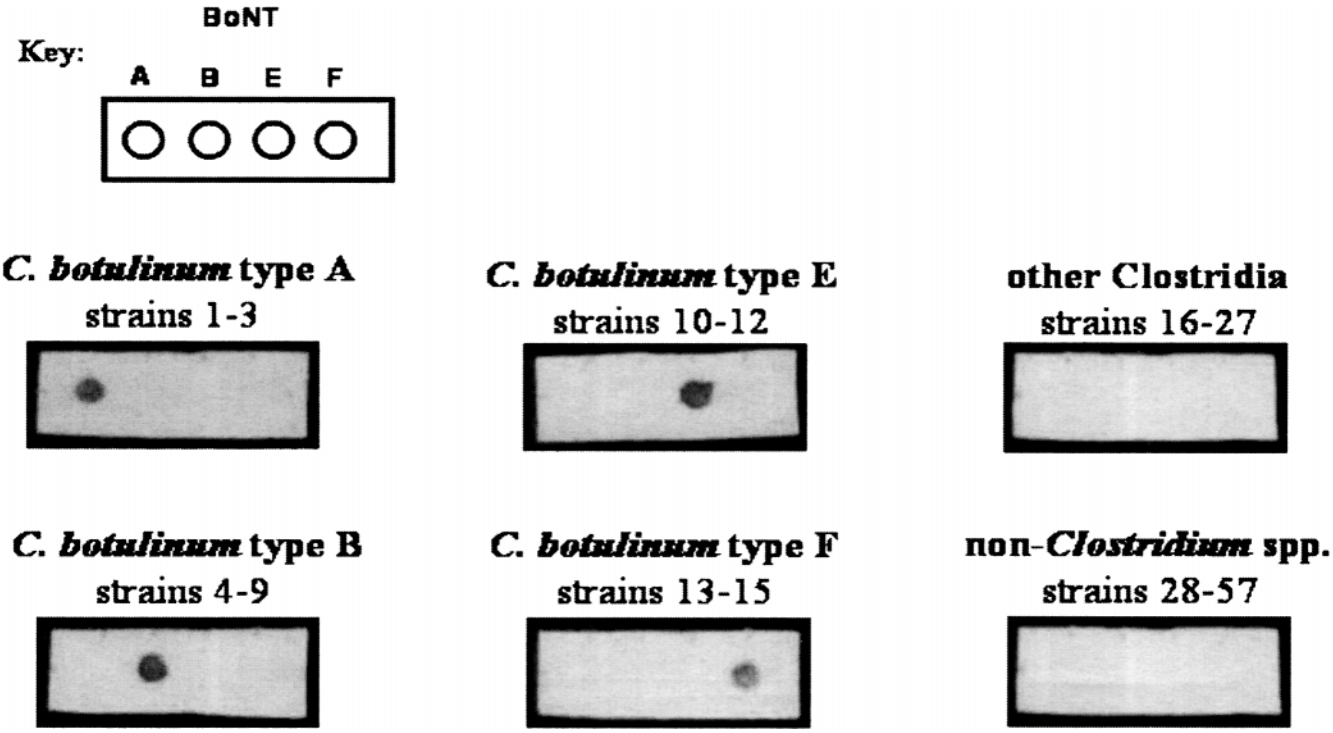

FIGURE 1. *Specificity of the combined multiplex PCR-CHAS. A variety of target (*Clostridium botulinum *type A, B, E, and F) and nontarget (other Clostridia and non-*Clostridium *spp.) bacteria were subjected to the combined multiplex PCR-CHAS procedure. Typical results obtained with the different bacteria are shown. For each isolate tested, a single colony was suspended in 50 μl of 1 × PCR buffer and then subjected to the lysis procedure described in ''Materials and Methods.'' BoNT A, botulinum neurotoxin type A; BoNT B, botulinum neurotoxin type B; BoNT E, botulinum neurotoxin type E; BoNT F, botulinum neurotoxin type F.*

amplicons generated in the multiplex PCR and provides confirmation of their identity by hybridization with immobilized probes, obviating the need for time-consuming restriction endonuclease analysis used with conventional agarose gel electrophoresis techniques. Thus, the specificity of the present system is doubly assured through the use of BoNT gene-specific PCR primers and probes. The detection of amplicons by hybridization with an array of immobilized probes has the further advantage of enabling the use of primers designed to generate amplicons of similar size (ca. 150 bp in the present study) in a multiplex PCR system, increasing the likelihood that all amplicons will be produced with near-equal efficiency in the amplification process. The differences in amplification efficiency for some of the amplicons are likely a function of the individual primer sequences and could be addressed by redesigning the primers. While in this study we focused on the identification of the four serotypes known to be involved in outbreaks of human illness, the multiplex PCR and CHAS procedures can be expanded to include the detection of all seven BoNT gene serotypes.

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