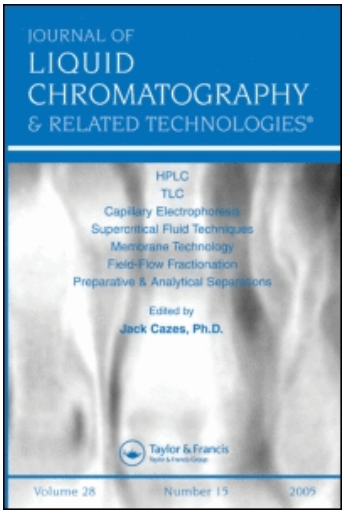

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Analysis of Polymerase Chain Reaction-Amplified DNA Fragments of Clostridium Botulinum Type E Neurotoxin Gene by High Performance Capillary Electrophoresis

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To cite this Article Sciacchitano, Carl J.(1996) 'Analysis of Polymerase Chain Reaction-Amplified DNA Fragments of Clostridium Botulinum Type E Neurotoxin Gene by High Performance Capillary Electrophoresis', Journal of Liquid Chromatography & Related Technologies, 19: 13, 2165 — 2178

To link to this Article: DOI: 10.1080/10826079608017149

URL: http://dx.doi.org/10.1080/10826079608017149

J. LIQ. CHROM. & REL. TECHNOL., 19(13), 2165–2178 (1996)

ANALYSIS OF POLYMERASE CHAIN REACTION-AMPLIFIED DNA FRAGMENTS OF CLOSTRIDIUM BOTULINUM TYPE E NEUROTOXIN GENE BY HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS

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ABSTRACT

Detection of *Clostridium botulinum* neurotoxin-producing strains is primarily accomplished using the mouse bioassay. The polymerase chain reaction (PCR) method has proven to be a rapid, sensitive technique for amplifying target DNA sequences of pathogenic microorganisms. Four PCR-amplified gene fragments derived from the *Clostridium botulinum* Type E neurotoxin gene, ranging from 410-630 bp, were analyzed by capillary gel electrophoresis (CGE). Sample preparation of PCR fragments required membrane dialysis to remove salt ions. PCR fragments were analyzed by CGE using both linear and covalently cross-linked polymers. Conditions for low-viscosity entangled polymer solutions were optimized to achieve the desired separation efficiency. Assessment of both types of polymer systems included resolution, reproducibility, and sizing accuracy of the PCR fragments. Advantages and limitations of each polymer system are discussed. Electropherograms were compared to results obtained from the agarose slab gel method. CGE afforded more rapid analytical times, automation, higher resolution, and increased DNA sizing accuracy in comparison to the agarose slab gels.

INTRODUCTION

Analysis of the neurotoxin-producing strain, *Clostridium botulinum* Type E, in fish, is usually accomplished by the mouse bioassay.[1] Alternative tests include the enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and reverse phase haemagglutination. Recently, the polymerase chain reaction (PCR) has gained significant importance in detecting pathogenic microorganisms including *Vibrio cholerae,*[2] *Listeria monocytogenes,*[3] *Salmonella,*[4] *and enterotoxigenic Escherichia coli.*[5] Traditionallly, the amplification products are resolved and detected on an agarose or polyacrylamide gel. Although gel electrophoresis has become the workhorse in molecular biology, it has its disadvantages. The slab gel method is time consuming, labor intensive, and requires a hazardous chemical, ethidium bromide, for DNA detection. Capillary gel electrophoresis (CGE) is an alternative approach to the slab gel method, offering automation, higher speed of separation, increased sizing accuracy, and enhanced resolution and sensitivity.[6,7] Size separation of PCR products is obtained by electrophoresis of the double-stranded DNA product through a suitable polymer which serves as a molecular sieve. Several types of polymers can be used, of which the mechanism of separation is identical. These include covalently cross-linked (bis-acrylamide, polyacrylamide), hydrogen bonded (agarose), and linear (hydroxyethyl methylcellulose) polymers. The use of polyacrylamide[8] and hydroxyalkyl cellulose polymers[9,10] for separation of DNA fragments have been previously reported.

This paper reports the analysis of four *C. botulinum* Type E neurotoxin-gene PCR products ranging from 410-630 base pairs by CGE using both polyacrylamide and low viscosity entangled polymer solutions. Resolution, sizing accuracy, and sensitivity were determined with respect to each type of polymer system.

MATERIALS AND METHODS

Instrumentation

A commercially available Capillary Electrophoresis Unit (270A-HT, Applied Biosystems, Inc., Foster City, CA) was used. Data acquisition was controlled by a Macintosh IIci (Apple, Cupertino, CA). Polyacrylamide gel columns (3%T, 3%C), 75μm I.D. x 75cm, 50cm effective length, were purchased from J&W Scientific, Folsom, CA. Capillaries used with low-viscosity entagled polymer solutions, 75μm I.D. x 60cm, (40cm effective length) were purchased from Applied Biosystems, Inc.

Analyses were carried out using Model 600 software (Applied Biosystems, Inc). Data integration was carried out using a Laser Jet III (Hewlett-Packard, Boise, ID).

Reagents and Materials

Buffer used in conjunction with polyarylamide gels (100mM Tris-borate, 7M urea, pH 8.3), was purchased from Applied Biosystems, Inc. DNA Fragment Analysis Reagent and Buffer (low viscosity entangled polymer) was also purchased from Applied Biosystems, Inc. DNA molecular weight markers (pUC18 HaeIII digest, ϕX174 HaeIII digest) were purchased from Sigma.

DNA Sample Preparation

PCR templates were prepared by boiling *C. botulinum* Type E cultures for 10 minutes followed by DNA purification using Instagene DNA Purification Matrix (Biorad Laboratories, Inc.). PCR conditions were as described previously.[13] Primers used in the PCR were derived from the nucleic acid sequence data of *Clostridium botulinum* Type E.[11,12] PCR products analyzed by capillary gel elctrophoresis required membrane dialysis using a 0.025µm filter (Millipore) to remove salt ions. Removal of spuriuos PCR products including primer-dimers, single-stranded primers, and dNTPs was accomplished using a PCR Prep DNA Purification System (Promega). PCR fragments were quantified using a DU 650 UV/VIS spectrophotometer (Beckman).

Capillary Gel Electrophoresis Conditions

Polyacrylamide gel system

Applied voltage: -15kV
Temperature: 25°C
Electrokinetic Injection: -5kV, 10 sec
UV Detection: 260nm

Low-viscosity entangled polymer system

Applied Voltage: -13kV
Temperature: 30°C
Electrokinetic Injection: -5kV, 10 sec
UV Detection: 260nm

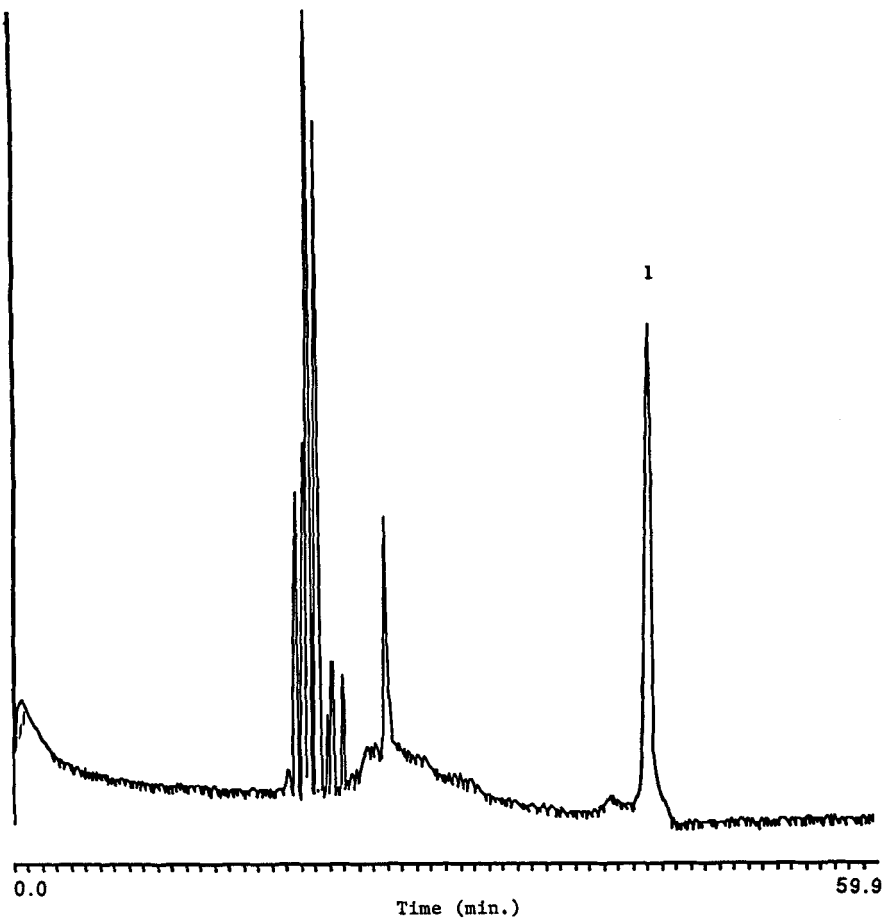

Figure 1. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragment, 410 bp (1), using the polyacrylamide polymer system. CGE conditons: 100mM Tris-borate, 7M urea, pH 8.3, electrokinetic injection, -5kV/10sec., -15kV, 25^{0}C, and UV detection at 260nm.

DNA Fragment Size Determination

Migration times of four pUC18 HaeIII digest fragments (102, 298, 434, 587 bp) were used to produce a size calibration curve. Migration times of *C. botulinum* Type E PCR amplified DNA fragments were used to calculate the size of each fragment resolved by either polymer system. DNA fragment size accuracy using low-viscosity entangled polymer solutions was also determined

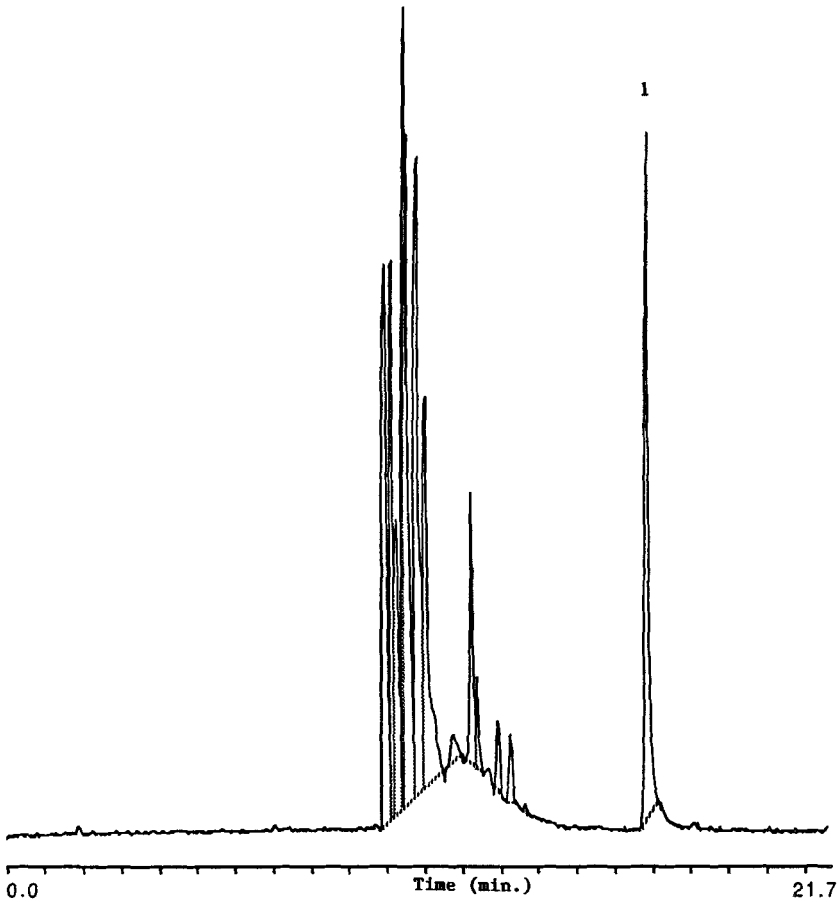

Figure 2. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragment, 410 bp (1), using the low-viscosity entangled polymer system. CGE conditions: DNA Fragment Analysis Reagent and Buffer, electrokinetic injection, -5kV/10 sec., -13kV, 30°C, and UV detection at 260nm.

by the following equation as per manufacturer's instructions (Applied Biosystems , Inc.):

$$\text{slope} = (\text{Sz DNA2-Sz DNA 1})/(\text{DNA 2 - DNA 1})$$

where DNA 1 and DNA 2 are migration times of two DNA standard (STD) peaks and Sz DNA 1 and Sz DNA 2 are the sizes of two DNA STD peaks in base pairs.

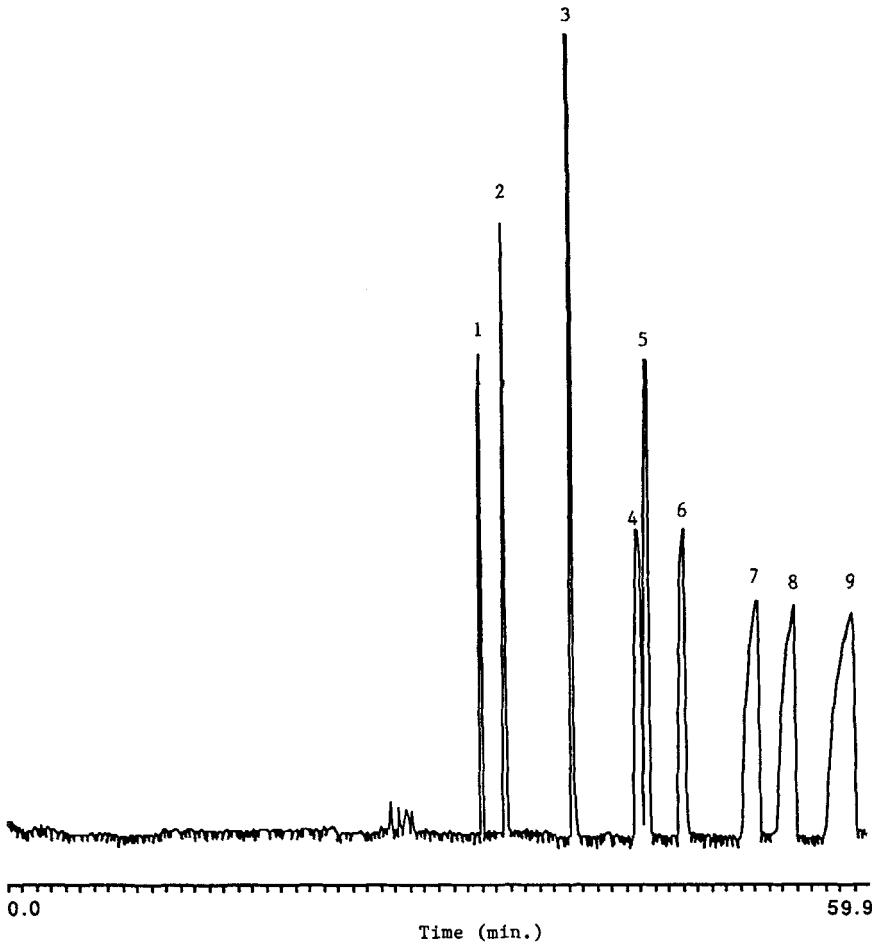

Figure 3. Separation of plasmid pUC18 HaeIII restriction digest using the polyacrylamide polymer system. The fragments identified are: (1) 80, (2) 102, (3) 174, (4) 257, (5) 267, (6) 298, (7) 434, (8) 458, and (9) 587 base pairs. CGE conditions: see Figure 1.

DNA Unknown Size = X +(DNA Unknown - DNA 1)*Slope

where DNA 1 and DNA Unknown are migration times of the smaller DNA STD peak used in the slope calculation and of unknown DNA, and X is the size of the DNA 1 STD peak in base pairs.

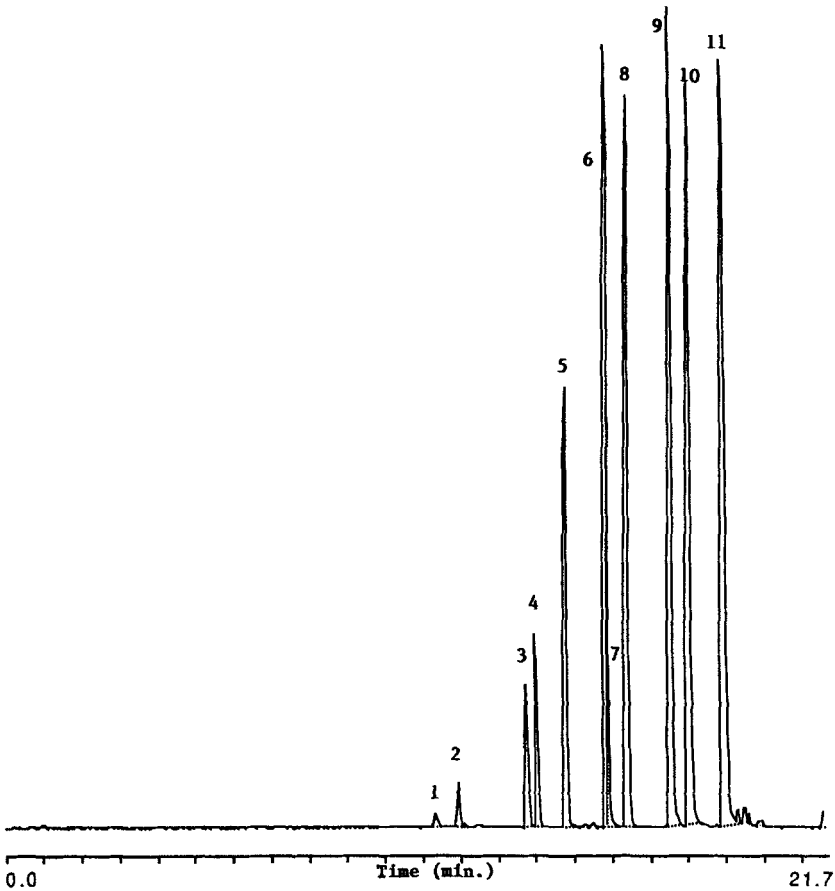

Figure 4. Separation of plasmid pUC18 HaeIII restriction digest using the low viscosity entangled polymer system. The fragments identified are: (1) 11, (2) 18, (3) 80, (4) 102, (5) 174, (6) 257, (7) 267, (8) 298, (9) 434, (10) 458, and (11) 587 base pairs. CGE conditions: see Figure 2.

Gel Electrophoresis

A 20μl aliquot of each PCR product was resolved on a 1.8% agarose slab gel in 1X TBE (Tris-borate, EDTA), electrophoresed at 1V/cm, and visualized by UV-induced fluorescence after staining with 1μg/mL ethidium bromide.

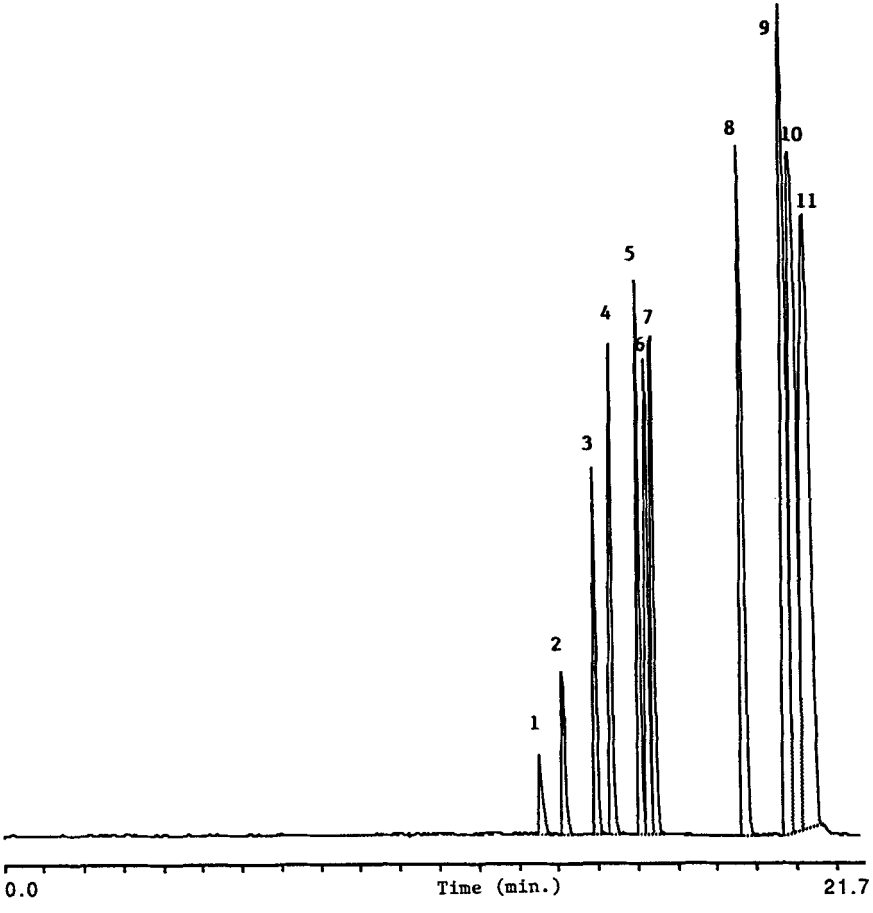

Figure 5. Separation of fX174 HaeIII restriction digest using the low viscosity entangled polymer system. The fragments detected are:(1) 72, (2) 118, (3) 194, (4) 234, (5) 271, (6) 281, (7) 310, (8) 603, (9) 872, (10) 1078, and (11) 1353 base pairs. CGE conditons: see Figure 2.

RESULTS AND DISCUSSION

Analysis of the PCR products by CGE required membrane dialysis to remove salt ions incurred in the PCR preparations. Salt ions compete with the PCR products resulting in less DNA electrophoresed onto either the polyacrylamide or coated capillaries. Specifically, membrane dialysis requires floating a Millipore 0.025μm filter on top of deionized water and pipeting

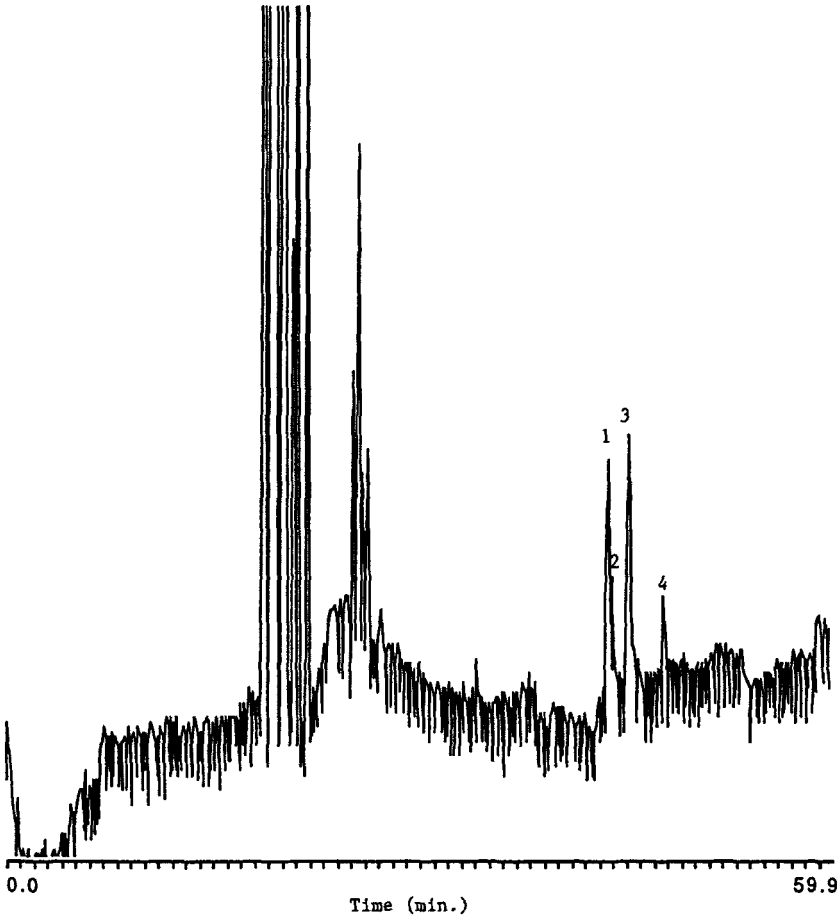

Figure 6. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragments using the polyacrylamide polymer system. The amplified DNA fragments detected are: (1) 410, (2) 471, (3) 513, and (4) 630 base pairs. CGE conditions: see Figure 1.

approximately 25μL of the PCR preparation onto the hydrophobic side of the filter. Removal of salt ions significantly increases the sensitivity of CGE (data not shown).

Figure 1 and 2 represent typical electropherograms demonstrating the separation of PCR products using both polymer systems. Identification of the 410 bp fragment using the low-viscosity polymer solution required approximately half the analytical time (30 minutes) needed for the

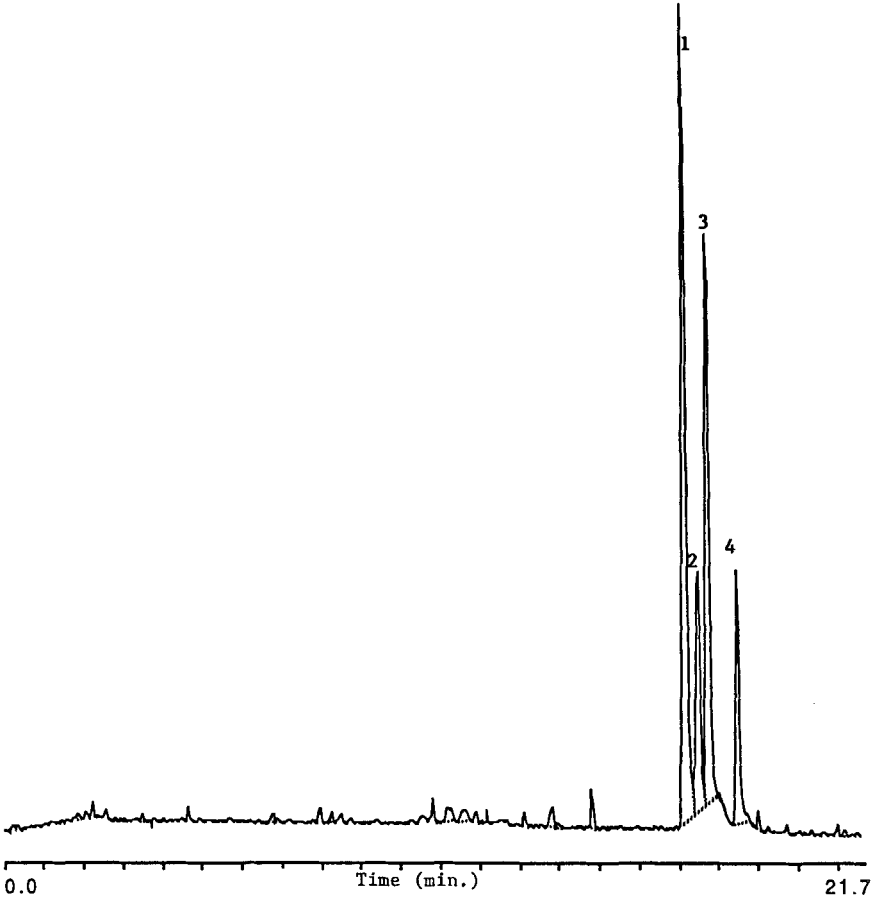

Figure 7. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragments using the low-viscosity entangled polymer system. The amplified DNA fragments are: (1) 410, (2) 471, (3) 513, and (4) 630 base pairs. CGE conditions: see Figure 2.

polyacrylamide polymer system. DNA molecular weight markers, pUC18 HaeIII and ϕX174 HaeIII restriction digests, were used to determine DNA size range capabilities with respect to both polymer systems.

DNA fragments in the size range of 434 - 587 base pairs appear to be the upper limit for 3% T, 3% C polyacrylamide gel caplillaries (Fig. 3). The low-viscosity entangled polymer system produced sharp peaks in the same DNA size range, and could even separate DNA fragments as large as 1000 base pairs (Fig. 4, 5).

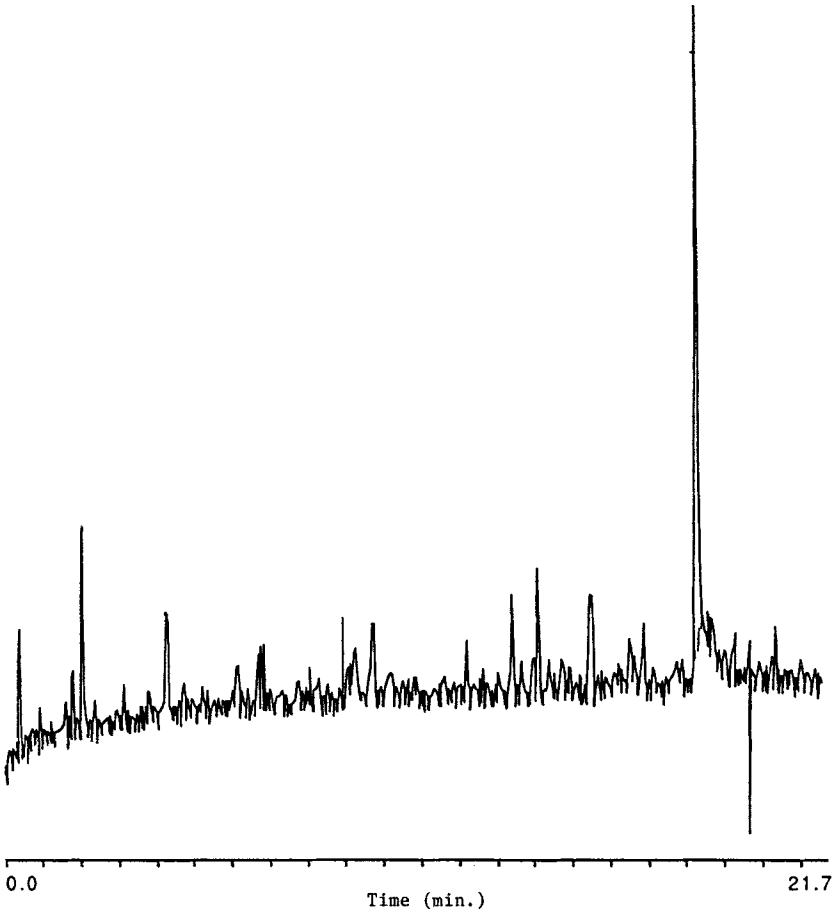

Figure 8. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragment, 410 bp, 35ng/mL, using the low-viscosity entangled polymer system. Primers and primer-dimers were eliminated using the PCR Prep DNA Purification System. CGE conditions: see Figure 2.

Resolution of four PCR products ranging from 410 - 630 base pairs by the polyacylamide and the low-viscosity entangled polymers is demonstrated in Fig. 6 and 7, respectively. The low-viscosity entangled polymer system resolved the 410 and 471 bp fragments, whereas the polyacylamide polymer could not. Base-line separation was not achieved in the polyacrylamide polymer system. The sensitivity of CGE using the low-viscosity entangled polymer solution approached picogram levels (20μL of a 35ng/mL 410 bp PCR product analyzed) (Fig. 8).

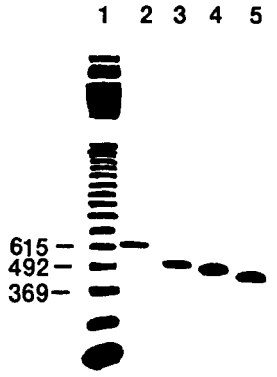

Figure 9. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragments using 1.8% agarose slab gel. Lane (1) 123 bp DNA ladder, (2) 630, (3) 513, (4) 471, (5) 410 bp. Electrophoretic conditions: see text.

The polyacrylamide polymer system also provided sensitivities in the picogram range (data not shown). Sensitivity may further be enhanced by increasing the time of electrokinetic injection or voltage using both polymer systems. However, it appears that longer electrokinetic injection times can be achieved with the low-viscosity entangled polymer systems. DNA sizing accuracy was also determined using both polymer systems. For a 513 base pair PCR product, the percent error in accurately sizing the DNA fragment in the polyacrylamide and low-viscosity entagled polymers was 1.6 (505 bp) and 4.3 (535 bp) percent, respectively. However, if the DNA sizing equation was used in lieu of the linearity, the sizing accuracy was increased (1.8 percent, 522 bp).

Determining the DNA size of PCR products resolved on a agarose slab gel required a visual comparison of band migration to respective molecular weight markers (Fig. 9). Gel preparation, loading samples, and electrophoresis, required approximately three hours to complete.

PCR product analysis was less cumbersome using the low-viscosity entangled polymer system. The polymer solution can be flushed out of the capillary after every run, ensuring no DNA frament carryover into the next analysis. This is in contrast to the polyacrylamide gel capillaries which cannot be flushed and require each analytical run to go to completion.

CONCLUSION

Analysis of PCR products by CGE using polyacrylamide or low-viscosity entangled polymers provides an alternative molecular detection method to

agarose slab gels. CGE affords rapid analytical times, reproducibility, and greater DNA sizing accuracy and sensitivity in comparison to the slab gel method. It appears that low-viscosity entangled polymers offer greater flexibility in the analysis of PCR products in comparison to polyacylamide; this includes faster analytical times, greater range in DNA size separation, and convenience. CGE, used in conjunction with the PCR technique, may prove to be a novel method for identifying pathogenic bacteria including *C. botulinum* neurotoxin-producing strains.

ACKNOWLEDGEMENTS

The author would like to thank Dr. Irvin Hirshfield, St. Johns University, Science Advisor, Northeast Regional laboratory, for his help in preparation of this manuscript, and Timothy Hawryluk, Microbiologist, Northeast Regional Laboratory, for his technical assistance.

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Received July 16, 1995
Accepted February 3, 1996
Manuscript 3937