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Detection of Castor Contamination by Real-Time Polymerase Chain Reaction

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Due to the potential for intentional contamination of food with crude preparations containing ricin, a real-time PCR method was developed for the detection of castor plant material in ground beef. One primer pair was identified and confirmed to be castor-specific and efficient for amplification of ricin in DNA extracts from castor or beef matrices. Of three different DNA extraction protocols compared, the hexadecyltrimethylammonium bromide (CTAB) method yielded the highest quality of DNA for QPCR assay. The detection limit for castor contamination in ground beef samples was <0.001% (<10 μg of castor acetone powder per gram of beef, corresponding to 0.5 μg of ricin), indicating excellent sensitivity for the assay, well below the threshold for oral toxicity.

KEYWORDS: ***Ricinus communis*; ground beef; DNA extraction method; quantitative PCR; SYBR-Green I chemistry**

INTRODUCTION

Ricinus communis (castor) seeds are highly toxic to mammalian cells. Although the high toxicity of castor was originally attributed to the hemagglutinating activity of a seed protein termed ricin (*1*), we now know that the original preparations were mixtures of ricin and a related agglutinin termed *R. communis* agglutinin (RCA, often designated RCA_{120} or RCA-1). RCA is a weak cytotoxin and a powerful hemagglutinin, whereas ricin is a potent cytotoxin and a weak hemagglutinin (*2*). Ricin is synthesized as a single large preprotein, which is composed of two heterodimer chains (A and B), linked by a single disulfide bond. The A chain is an *N*-glycosidase that can remove a specific adenine corresponding to residue A4324 in rat 28S rRNA (*3*). The resulting ribosomal inactivation irreversibly disrupts protein synthesis. The B chain is a lectin that binds to galactosyl residues of cell membrane glycoproteins and glycolipids and helps the entire ricin molecule enter target cells. Ricin accounts for about 5% of the soluble protein (*4*) in mature seeds. Persons ingesting ricin exhibit symptoms including nausea, vomiting, diarrhea, gastric hemorrhaging, and shock. The lethal dosage of ricin administered intravenously is about 3−5 μg/kg of body weight (*5*). With a sufficient dose, death occurs within 3−5 days. At present, the worldwide production of castor beans exceeds 1 million tons annually. Because highly toxic crude ricin could be easily produced from this plentiful source and used as an intentional bioweapon, the development of a fast, sensitive, and reliable method for detecting the intentional contamination of food with ricin is of great importance.

The most common methods used to detect ricin are mouse bioassay (*6*), enzymatic activity assay (*7*), and immunobased assays (*8*−*10*). The first approach used to be the "gold standard" for the detection of ricin. However, it is expensive, time-consuming, and not suitable for analyzing crude extracts because other substances present could be toxic or have synergistic effects. The second approach is based on the measurement of *N*-glycosidase activity. It requires expensive equipment (LC-MS) and lengthy, complicated sample preparation steps. The third approach includes enzyme-linked immunosorbent assay (ELISA), immunochromatographic devices, and chip-based methods in which the sensitivity and specificity of the assay depend chiefly on the antibody.

Our goal was to develop an assay with improved efficiency and sensitivity for the detection of ricin in food. Because it seems likely that crude, rather than purified, ricin would be used as a bioweapon, it seemed feasible to employ PCR to detect the castor nucleic acid that remains associated with crude ricin preparations. Moreover, the recently developed quantitative PCR (QPCR) technique completely revolutionizes the detection of both RNA and DNA (*11*). This technique allows investigators to visualize the reaction as it takes place, instead of the amount of accumulated product at the end of the final PCR cycle. Therefore, it is a rapid, sensitive, and accurate system to utilize. It has been applied for the quantitative detection of pathogens (*12*, *13*), toxins (*14*), and genetically modified organisms in food (*15*, *16*). The specific objectives of this study were (1) to identify a primer set that is specific for the detection of castor genomic DNA; (2) to evaluate different DNA extraction protocols for

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Table 1. Primers Used in This Study

primer name	oligonucleotide sequence (5′–3′)	location	product size (bp)	Genbank sequence
18S-F	GAGAAACGGCTACCACATCCA	362–82		AY674633
18S-R	CCGTGTCAGGATTGGGTAATTT	423–402	62	AY674633
18S-R[a]	GGGTCGGGAGTGGGTAATTT	503–484	60	DQ222453
ricin-F-1	GTGCGTATCGTAGGTCGAAATG	1283–1304		X52908
ricin-R-1	GCGTTTCCGTTGTGGAATCT	1350–1331	68	X52908
ricin-F-2	TCTATGTGTTGATGTTAGGGATGGA	1306–1330		X52908
ricin-R-2	CTTGCATGGCCACAACTGTATT	1372–1351	67	X52908
ricin-F-3	GACTGTAGCAGTGAAAAGGCTGAA	1739–1762		X52908
ricin-R-3	GCTGAGGACGTATTGAACCATCT	1805–1783	67	X52908
ricin-F-4	GCTGAACAACAGTGGGCTCTT	1757–1777		X52908
ricin-R-4	AAGGCAATTATCTCGGTTTTGC	1825–1804	69	X52908

[a] The origin of this primer is *Bos taurus*. All other primers are from *R. communis*. Target gene of primer pair: 18S-F/R, 18S-ribosomal RNA; ricin-F/R, ricin.

optimum assay sensitivity; and (3) to determine the limit of detection for castor DNA sequences in a ground beef matrix by QPCR.

MATERIALS AND METHODS

Preparation of Stable Acetone Powder from Castor Seeds. Castor beans, accession PI215769, were obtained from USDA-GRIN, Southern Regional Plant Introduction Station (Griffin, GA). Acetone powder was prepared as described by Tewfik and Stumpf (*17*) with slight modification. Dry seeds with shell removed were ground in liquid nitrogen to a fine powder with a mortar and pestle, and cold acetone (5−10 mL/g powder) was added to form a uniform homogenate. The resulting mixture was filtered by suction on a large Büchner funnel and washed three times with cold acetone and two times with small portions of dry ethyl ether. The freshly prepared powder was dried overnight in a vacuum desiccator over P_2O_5 and stored at −20 °C for future use.

DNA Extraction. Ground beef labeled as containing 20% fat was purchased at a local supermarket and stored at −20 °C until subsampled. The frozen sample was divided into subsamples weighing about 1 g (wet weight) and ground in liquid nitrogen with a mortar and pestle. The samples were then incubated in 4 mL of a solution containing 3.9 mL of CTAB extraction buffer [1.4 M NaCl, 2% CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris, 20 mM EDTA, pH 8.0, 1% polyvinylpyrrolidone-40], 2% of 2-mercaptoethanol, and 100 μg/mL of proteinase K for 2 h at 55 °C with shaking. After centrifugation for 5 min at 3000*g*, the supernatants were used immediately for DNA extraction by two methods.

In the phenol/chloroform/isoamyl alcohol (25:24:1, PCI) method, 2 mL of supernatant was extracted twice with equal volumes of PCI. To precipitate the DNA, two-thirds volume of cold 2-propanol was added to the upper aqueous phase and the samples were placed at −20 °C for at least 30 min before centrifugation. The DNA pellet was resuspended in 100 μL of buffer containing 10 mM Tris·Cl, pH 8.0, and 1 mM EDTA (TE buffer).

For the CTAB method, the extraction was performed as described by Nemeth et al. (*18*). Briefly, 2 mL of supernatant was precipitated with 2 volumes of CTAB precipitation buffer (40 mM NaCl, 0.5% CTAB) at room temperature for 60 min prior to centrifugation for 20 min at 15000*g*. The pellet was dissolved in 700 μL of 1.2 M NaCl and then extracted with an equal volume of chloroform. DNA in the aqueous phase was precipitated with 1 volume of 2-propanol and 1 μL of glycogen (5 mg/mL). The pellet was washed with 75% ethanol and dissolved in 100 μL of TE buffer.

For the silica gel purification method (QIA column), the DNeasy Tissue Kit (Qiagen, Valencia, CA) was used according to the manufacturer's instructions for the purification of DNA from animal tissues protocol.

Castor genomic DNA was isolated from seed acetone powder using the procedure developed for DNA extraction from plant tissues by Monsanto (*19*).

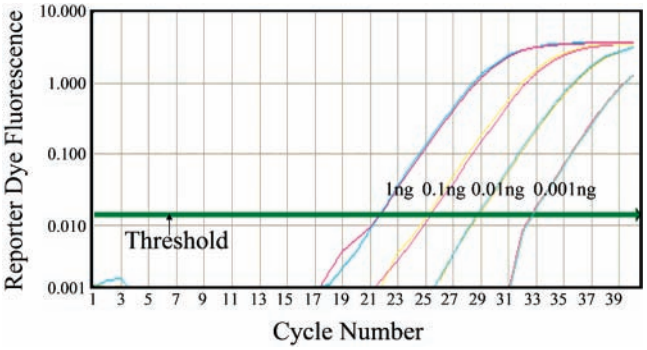

Figure 1. Representative QPCR amplification plots for castor genomic DNA serially diluted in water (0.001–1 ng). The threshold indicated was determined automatically by the Sequence Detection Systems software. For each curve, the fractional cycle number at which the fluorescence passes the threshold is defined as the cycle threshold (Ct) value for that curve.

Oligonucleotides. Oligonucleotide primers were designed using Primer Express, version 3.0 software (Applied Biosystems, Foster City, CA), or manually following the primer design guidelines (Applied Biosystems 7500 Real-Time PCR System, Chemistry Guide). Primers were based on the sequence of the *R. communis* ricin gene (X52908) and 18S-ribosomal RNA gene (AY674633) and the sequence of *Bos taurus* 18S-ribosomal RNA gene (DQ222453). Nucleotide sequences and location of primers used in this study are listed in **Table 1**. The target genes for primer pairs 18S-F/R and ricin-F/R are, respectively, 18S-ribosomal RNA and ricin. The oligonucleotides were synthesized by MWG-Biotech (Oaks Parkway, NC).

QPCR System. The Applied Biosystems 7500 Fast Real-Time PCR system and SYBR-Green I dye chemistry were employed. Amplification mixtures for QPCR contained 1× Power SYBR-Green PCR Master Mix (Applied Biosystems), forward and reverse primers (50 nM each), and variable amounts of template DNA in a final volume of 25 μL. Standard default thermal cycling conditions were chosen for all PCR amplifications [initial step, 95 °C, 10 min for polymerase activation; PCR (40 cycles), 95 °C, 15 s for melting, 60 °C, 1 min for annealing and extending; dissociation steps, 95 °C, 15 s; 60 °C, 1 min, and 95 °C, 15 s].

QPCR Quantification. Three replicate PCR measurements were carried out for each serial dilution of castor genomic DNA in water. A nontemplate control (NTC) was included in each experiment. Representative QPCR amplification plots obtained from castor DNA serially diluted in water are shown in **Figure 1**. A threshold indicated by an arrow in **Figure 1** was selected automatically by the software. For each curve, the fractional cycle number at which the fluorescence passes the threshold is defined as the cycle threshold (Ct) value. The Ct value is inversely proportional to the amount of castor DNA present in the PCR reaction. The amplification efficiency of a real-time PCR assay is estimated on the basis of the equation $E = (10^{-1/\text{slope}} - 1) \times 100$

Table 2. Cycle Threshold (Ct) Values for Castor Genomic DNA in Water, Using Different Primer Pairs[a]

primer pair	0.1 ng of DNA	1.00 ng of DNA	10 ng of DNA	100 ng of DNA
ricin-F1/R1	26.22 ± 0.15	22.80 ± 0.29	20.22 ± 0.12	17.75 ± 0.06
ricin-F2/R2	29.50 ± 0.31	25.80 ± 0.19	22.79 ± 0.41	19.51 ± 0.31
ricin-F3/R3	31.02 ± 0.15	26.51 ± 0.06	23.41 ± 0.33	19.60 ± 0.42
ricin-F4/R4	24.35 ± 0.33	21.45 ± 0.15	18.87 ± 0.05	14.16 ± 0.34

[a] Mean values of triplicate values ± SD.

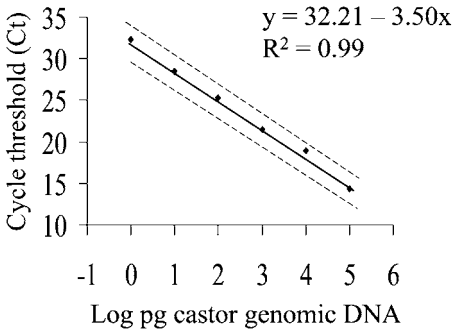

Figure 2. Cycle threshold value versus the log of castor genomic DNA (picograms) by QPCR (averages of three replicate determinations). The linear regression and 95% confidence limits are plotted using JMPIN software, version 3.2.1 (SAS Institute Inc., Cary, NC).

(*Applied Biosystems: Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR*).

Sequencing. To confirm positive PCR results, PCR products from a regular PCR method were purified on gels using a QIAquick Gel Extraction Kit (Qiagen) and sequenced using a TOPO TA Cloning Kit for Sequencing (Qiagen) and an ABI PRISM BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems).

Spiking Castor Materials in Ground Beef Samples. Ground beef samples (1 g each) were spiked with various amounts of castor acetone powder (0.001% to 1.0% by weight). DNA extraction was performed as described above, using the CTAB method after spiking. Three replicate QPCR measurements were carried out for each of four serial dilutions of castor acetone powder in the beef specimen plus an unspiked beef blank, using 100 ng of total DNA from beef matrix diluted in 10 μL of water as template.

RESULTS

Selection of Primers for Sensitive Detection of Castor DNA. Because SYBR-Green I dye chemistry will detect all double-stranded DNA, including nonspecific reaction products and primer dimers, selection of ideal primers is essential to minimize false-positive signals. Before testing spiked food, we selected optimal primers for amplification of a ricin gene fragment using castor genomic DNA as templates. Four primer pairs derived from the ricin gene, ricin-F1/R1, F2/R2, F3/R3, and F4/R4 (**Table 1**), were used for quantitative detection of the ricin DNA fragment by QPCR. The primer pair ricin-F4/R4 consistently gave the lowest Ct value for each of four serial dilutions of castor genomic DNA in water (range from 0.1 to 100 ng) among the four primer pairs tested (**Table 2**), and the amplification efficiency estimated from the slope reached 100% when using this primer pair, indicating that the ricin-F4/R4 was the best primer pair. For the primer pai ricin-F4/R4, a plot of the average serial dilution Ct values versus the log of the amount of castor genomic DNA ($1-10^5$ pg) for the average of three replicate measurements of ricin is shown in **Figure 2**. The assay is linear over 5 orders of magnitude.

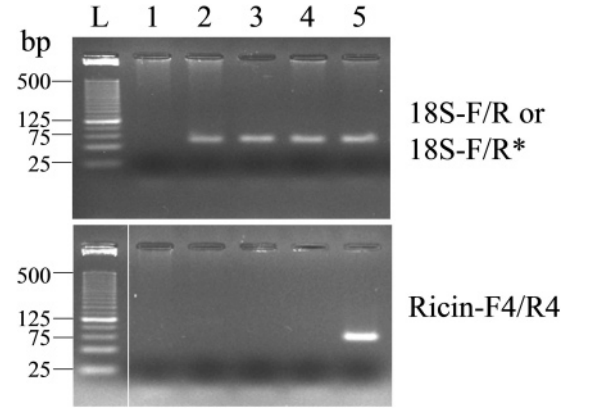

Figure 3. Specificity test of the primer pair ricin-F4/R4. QPCR was performed using ricin-F4/R4 (bottom panel) and 18S-F/R (top panel, 5) or 18S-F/R* (top panel, 1–4) as primer and DNA (100 ng) extracted from castor or beef as template. Three extraction methods (PCI, CTAB, and QIA column) were used for preparation of DNA from beef. The ethidium bromide stained agarose gel with 10 μL of QPCR end products is shown. L, 25-bp DNA ladder; 1, nontemplate control (NTC); 2, beef DNA, PCI method; 3, beef DNA, CTAB method; 4, beef DNA, QIA column; 5, castor DNA, PCI method.

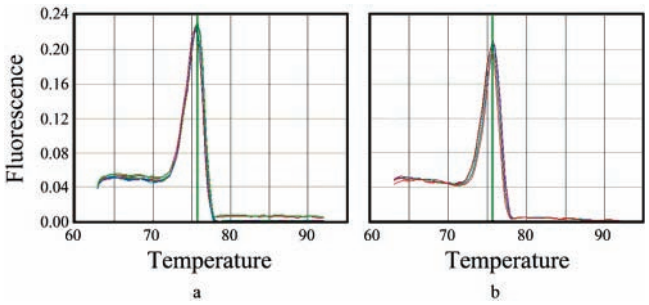

Figure 4. Representative melting curves from QPCR using SYBR-Green I dye chemistry: (**a**) castor genomic DNA serially diluted from 0.1 to 100 ng in water; (**b**) DNA from beef samples spiked with 0.001–1% of castor acetone powder.

Specificity of the Ricin Gene Targeted Primer Pair, Ricin-F4/R4. The QPCR was carried out using the primer pair ricin-F4/R4 for the amplification of ricin DNA fragment in the presence of castor or beef genomic DNA. An amplicon of the expected size, 69 bp, was detected when using DNA sample extracted from castor as template in the assay, and the amplicon sequences were confirmed to be identical to the reported ricin gene sequence. There was no PCR product observed when using the same primer pair and beef genomic DNA as template in the assay, using any of three different extraction methods to prepare DNA from beef (**Figure 3**, bottom panel). However, the 18S-ribosomal RNA gene was amplified using DNA templates from both castor and ground beef. The primer pair 18S-F/R was used for castor 18S-ribosomal RNA, and the primer pair 18S-F/R* was used for beef 18S-ribosomal RNA amplification (**Figure 3**, top panel). The sequences of the region for primer 18S-F are identical in castor and cow, and there is only a 2-base difference between 18S-R and 18S-R*. These results indicated that the DNA samples used were all amplifiable by PCR and that the primer pair ricin-F4/R4 was castor-specific among the samples we tested.

Figure 4 shows the melting curves obtained for ricin-F4/R4 PCR products by QPCR. The curves obtained from PCR assay using DNA from beef samples spiked with various amounts of castor acetone powder (**Figure 4b**) were similar to those

Table 3. Comparison of DNA Quality Using Different Extraction Protocols[a]

extraction method	Ct for unspiked sample		Ct for spiked sample	
	18S-F/R*	ricin-F4/R4	18S-F/R*	ricin-F4/R4
PCI	17.7 ± 0.1	undet[b]	19.0 ± 0.4	undet
CTAB	14.1 ± 0.1	undet	14.1 ± 0.1	26.4 ± 0.1
QIA column	14.6 ± 0.1	undet	14.6 ± 0.1	29.5 ± 0.2

[a] Mean values of triplicate Ct values ± SD, using either of two primer pairs with 100 ng of DNA template. [b] Undet, below detection level.

obtained using serial dilutions of castor genomic DNA as template (**Figure 4a**). A single T_M value, 75.7 °C, was obtained, demonstrating that there were no primer dimers or additional or nonspecific products present in the PCR reactions. This result also demonstrated that there was no matrix effect on the specificity of PCR amplification of ricin using ricin-F4/R4 as primers and DNA extracted by CTAB method as template.

Effect of DNA Extraction Method on Sensitivity of Detection by QPCR. Because food samples are highly heterogeneous and contain many compounds, including fats, proteins, enzymes, fiber, and polysaccharides, DNA extraction can be variable (*20*). To identify an efficient extraction protocol that provides consistently good recovery rates for low-copy genomic DNA fragments from ground beef, three different methods (PCI, CTAB, and QIA column) were compared. Using ground beef spiked with 0.1% castor acetone powder, all three methods yielded 220−240 μg of DNA/g of beef, determined by UV absorbance. The recovery rates for castor genomic DNA and the quality of the DNA purified from ground beef were determined by QPCR using 100 ng of DNA as template. **Table 3** shows the Ct values obtained for 18S-ribosomal RNA and ricin DNAs by QPCR assay for samples extracted according to the three methods. No ricin was detected in the unspiked beef samples. In samples spiked with castor acetone powder, the Ct value for ricin DNA corresponding to the PCI method was >40, whereas for 18S-ribosomal RNA, the PCI method yielded Ct values of about 18 in both unspiked and spiked beef samples, consistently higher than Ct values from CTAB and QIA column methods (about 14−15), indicating that the DNA quality from the PCI method was poor. Although the Ct values for 18S-ribosomal RNA corresponding to the CTAB and QIA column methods were close, the value for ricin DNA obtained with the CTAB method (about 26) was clearly lower than that obtained with the QIA column method (about 30), suggesting that the CTAB method is more effective than the QIA column method for the extraction of castor genomic DNA from beef matrix. Therefore, the CTAB method is the most suitable of the three methods tested for the detection of castor contamination in ground beef.

Detection Limits of QPCR for Castor Contamination in Spiked Beef Samples. We first examined the effect of beef matrix on the amplification efficiency of ricin DNA. Standard curves obtained from PCR assays of serially diluted castor genomic DNA in 10 μL of water versus in 10 μL of beef extract containing 100 ng of beef DNA were compared (**Figure 5**). The slopes indicate that amplification efficiencies for castor DNA in pure water and beef matrix were 95 and 82%, respectively, indicating a small matrix effect on the amplification efficiency for ricin DNA. When DNA template was diluted in water, the average Ct values ranged from 18.88 (10 ng of castor DNA) to 32.27 (0.001 ng of castor DNA), whereas the average

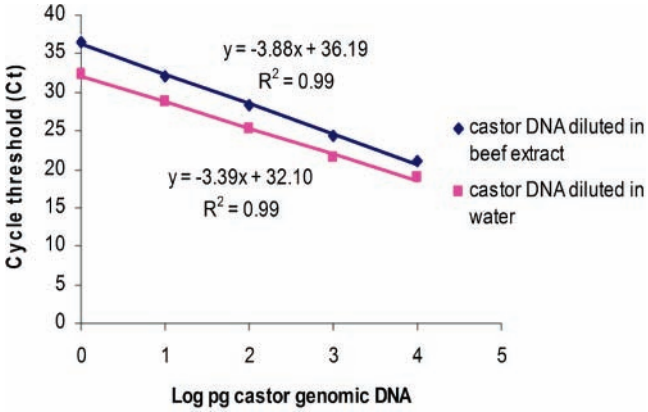

Figure 5. Cycle threshold values from three replicates for the measurements of ricin by QPCR versus the log of castor genomic DNA (picograms) diluted in water (■) or beef extract (◆).

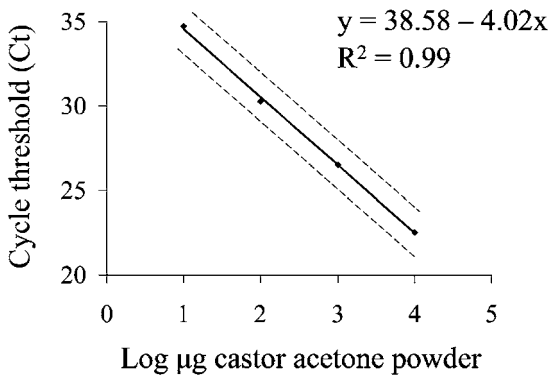

Figure 6. Cycle threshold values from three replicates for the measurements of ricin by QPCR versus the log of castor acetone powder (micrograms) spiked in beef. The linear regression and 95% confidence limits are plotted using JMPIN software, version 3.2.1 (SAS Institute Inc.).

Ct values for DNA template diluted in beef extract ranged from 21 (10 ng of castor DNA) to 36.57 (0.001 ng of castor DNA) (**Figure 5**).

The detection limit for castor contamination in beef was determined using the CTAB extraction method, ricin-F4/R4 as primer, and SYBR-Green I dye chemistry. **Figure 6** shows the average Ct values obtained by QPCR versus the log concentration of castor acetone powder in beef (micrograms per gram) for three replicate PCR measurements. The assay is linear within the range tested (3 orders of magnitude). The average Ct value of the lowest concentration tested (10 μg/g) was 34.71. Using DNA templates from unspiked beef in the PCR assay, no detectable reporter amplification was observed. Thus, the specificity of the assay for ricin DNA was confirmed.

DISCUSSION

The development of QPCR for the detection, identification, and quantification of food contamination is often made difficult by insufficient DNA sequence information for the target agent and interference from the food matrix that is being sampled. The complete sequence information of the castor genome is still not available. However, sequences of castor ricin and its homologue RCA are published (*21*) and shown to be unique to the castor plant by nucleotide−nucleotide BLAST. In addition, Southern blot analyses using a ricin cDNA probe demonstrated that the ricin gene family was composed of at least eight members (*21*). Thus, ricin DNA is an appropriate surrogate analyte for crude ricin because of its uniqueness and abundance

in the genome. Our results showed that the primer pair ricin-F4/R4 was best in the PCR assay among the four primer pairs tested. Even though they may be amplified from the different genes, the amplicon sequences of ricin-F4/R4 were all identical. This is probably explained by the highly conserved sequence of the DNA fragment between primers ricin-F4 and ricin-R4 among the members of the ricin gene family (*21*). This study also demonstrated that ricin-F4/R4 provided a compromise between specificity and sensitivity and proved to be ideal for assay development.

Another challenge was to isolate plant DNA from a complex food matrix. Recently, many publications have reported the rapid, sensitive, and specific detection of foodborne pathogens using PCR, with preliminary enrichment culture to increase sensitivity (*22*, *23*). Because plant DNA cannot be similarly enriched, identification of an efficient DNA extraction method is critical. To simulate actual demands on assay performance, we performed the assay using ground beef spiked with castor acetone powder before extraction. Although some assays for toxins have provided data for only a buffer matrix (*24*) or food extract matrices (*25*), such data do not test the critical extraction step. In this study, the three extraction protocols yielded different results, apparently due to variation in both the quantity and quality of castor DNA recovered from beef.

The advantage of our method is the use of an 18S-ribosomal RNA gene as an amplification control. This control assists interpretation of the results. It is well-known that the ribosomal RNA genes are present in high copy numbers in eukaryotes, but their DNA sequence is highly conserved among organisms. The primer pair we used (18S-F/R*) amplifies both castor and beef 18S-ribosomal RNA genes. When using DNA template extracted by the PCI method, no ricin-PCR product was detected. For 18S-ribosomal RNA, the PCR sensitivity measured by Ct value was >1 order of magnitude less than that of the CTAB and QIA column methods (based on 100% PCR efficiency, every 3.32 cycles will result in a 10-fold increase in PCR amplicon). These results suggested that the amount of castor DNA recovered from ground beef by the PCI method was very low and that the quality of the DNA was poor. Alternatively, the PCI method may have coextracted components that bind or degrade the polymerase or the DNA template, or there may be PCR inhibitors in the beef matrix that are removed by the CTAB and QIA column methods, but not the PCI method. The Ct values for 18S-ribosomal RNA in unspiked or spiked beef samples corresponding to the CTAB and QIA column methods were almost identical, but the values for ricin gene differed significantly. The CTAB method yielded a higher quantity of castor DNA from the spiked beef matrix.

Using the CTAB method, we were able to detect and identify castor acetone powder spiked in ground beef down to 0.001% or 10 μg/g. The linear regression predicted a Ct value of 38.58 at a spike level of 0.0001% (1 μg/g). Because no reporter amplification was detected above background levels at cycle 40 for the blank, we conclude that the detection limit is 1−10 μg/g for this assay. This corresponds to 0.1−1 mg of castor acetone powder (about 5−50 μg of ricin) in a typical 100 g serving of ground beef. The presence of castor material in beef samples did not affect the Ct value of 18S-ribosomal RNA amplification (data not shown). Within the range tested, the slope of the curve (**Figure 6**) is close to that seen with the standard curve from serial dilutions of castor DNA spiked in beef DNA extract, indicating that the ratio of castor DNA to beef DNA recovered with the CTAB method was proportional to the initial ratio of castor acetone powder spiked in beef and that the amplification efficiency was reproducible for the same matrix. Because the lethal oral dose of ricin is estimated as 2 mg for an adult human (*26*), this PCR assay meets the need to detect the presence of a crude ricin contaminating ground beef at levels well below the lethal dose.

ABBREVIATIONS USED

Ct, threshold cycle; CTAB, hexadecyltrimethylammonium bromide; ELISA, enzyme-linked immunosorbent assay; NTC, nontemplate control; PCI, phenol/chloroform/isoamyl alcohol; QPCR, real-time quantitative Polymerase Chain Reaction; R^2, square of regression coefficient; RCA, *Ricinus communis* agglutinin; SD, standard deviation; TE, Tris-EDTA.

ACKNOWLEDGMENT

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