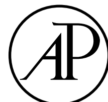

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Analytical Biochemistry 316 (2003) 259–269

ANALYTICAL BIOCHEMISTRY

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Quantitative intra-short interspersed element PCR for species-specific DNA identification

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Received 17 December 2002

Abstract

We have designed and evaluated four assays based upon PCR amplification of short interspersed elements (SINEs) for species-specific detection and quantitation of bovine, porcine, chicken, and ruminant DNA. The need for these types of approaches has increased drastically in response to the bovine spongiform encephalopathy epidemic. Using SYBR Green-based detection, the minimum effective quantitation levels were 0.1, 0.01, 5, and 1 pg of starting DNA template using our bovine, porcine, chicken, and ruminant species-specific SINE-based PCR assays, respectively. Background cross-amplification with DNA templates derived from 14 other species was negligible. Species specificity of the PCR amplicons was further demonstrated by the ability of the assays to accurately detect trace quantities of species-specific DNA from mixed (complex) sources. Bovine DNA was detected at 0.005% (0.5 pg), porcine DNA was detected at 0.0005% (0.05 pg), and chicken DNA was detected at 0.05% (5 pg) in a 10-ng mixture of bovine, porcine, and chicken DNA templates. We also tested six commercially purchased meat products using these assays. The SINE-based PCR methods we report here are species-specific, are highly sensitive, and will improve the detection limits for DNA sequences derived from these species.

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Bovine spongiform encephalopathy (BSE),[1] commonly referred to as “mad cow disease,” has a human form termed vCJD that is a variant of Creutzfeldt–Jakob disease, a fatal neurodegenerative disease that has caused many deaths in the United Kingdom [1]. In response to the BSE epidemic in Europe, the United States Food and Drug Administration (FDA) imposed strict guidelines in 1997, prohibiting the use of ruminant-derived protein in the manufacture of animal feed intended for cows or other ruminants (http://www.usda.gov). Ruminants are defined as a suborder of the *Artiodactyla* order of mammals and represent the “cud-chewing” families *Bovidae* (antelope, cattle, goats, sheep) and *Cervidae* (deer) [2]. It is widely believed that the practice of utilizing ruminant carcasses in animal feed for livestock is responsible for the spread of BSE to epidemic proportions [1]. As a result, the need for sensitive detection of ruminant species remains in animal feed is a paramount agricultural issue.

The risk associated with infectious transmissible spongiform encephalopathy in humans has discouraged many individuals around the globe from consuming beef. Hindu populations also choose not to eat beef, while Jewish and Muslim populations choose to avoid consumption of pork, even in minute quantities, due to their religious beliefs. Many consumers prefer to include more chicken in their diet instead of beef or pork. In addition to infectious disease and religious concerns, many individuals are altering their eating behavior to include more chicken simply to reduce dietary fat intake in accordance with health trends. Any conceivable ambiguity in the labeling practices of commercial suppliers or grocery stores is unacceptable to these populations. The need for sensitive detection and quantitation of

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[1] *Abbreviations used:* BSE, bovine spongiform encephalopthy; SINE, short interspersed element; FDA, Food and Drug Administration.

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doi:10.1016/S0003-2697(03)00095-2

bovine, porcine, and chicken species in food and mixed-food products is critical in response to this consumer demand.

The quantitative detection of meat species in mixed samples has been approached using a variety of different systems. Early approaches to identify species-specific components within mixed samples involved the use of high-performance liquid chromatography [3,4]. These methods have proven useful for the identification of many different animal species, but the detection limits using these approaches are restrictive. The detection of nuclear DNA sequences has also been useful in this regard, but is limited as a result of their generally low copy number [5]. Meat species identification using enzyme-linked immunosorbent assays [6] and protein profiles [7] have also been used, but polymerase chain reaction (PCR)-based assays are currently the method of choice for species identification [8]. PCR analysis of species-specific mitochondrial DNA sequences is the most common method currently used for identification of meat species in food [9–14] and animal feedstuffs [15–17]. The advantage of mitochondrial-based DNA analyses derives from the fact that there are many mitochondria per cell and many mitochondrial DNA molecules within each mitochondrion, making mitochondrial DNA a naturally amplified source of genetic variation. Recently, PCR-based methods using multicopy nuclear DNA sequences such as satellite DNA [18,19] and repetitive elements [8,20] have been introduced. Like mitochondrial-based systems, these nuclear PCR-based assays take advantage of multiple target amplification sites in the genome of interest. However, many of these systems require additional procedural steps and at least 1–250 pg of starting DNA template for species detection [8,19]. Tajima and co-workers [20] recently reported the development of PCR assays for the detection of ruminant-, pig-, and chicken-derived materials based on sequences of short and long interspersed repetitive elements. Although these assays exceed the detection limits of previously reported assays [10,12,16] there are several limitations to their methods. Primarily, the detection of PCR products is exclusively gel based. In addition, the size of the PCR amplicons for the assays (179–201 bp) reported by Tajima and co-workers [20] may limit their utility for testing trace forensic materials that contain degraded DNA. In order to overcome these and other shortcomings associated with previously reported methods for nuclear-based species-specific DNA detection and quantitation, we have designed and evaluated a series of assays based upon PCR amplification of short interspersed elements (SINEs).

Materials and methods

Primer design and PCR amplification

DNA sequences from bovine [21–23], porcine [24,25], and chicken [26,27] genomes were subjected to computational analysis using the RepeatMasker server at the University of Washington (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker) to identify SINEs contained within those genomes. Oligonucleotides were designed using either Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) or Primer Express software (Applied Biosystems, Inc.) and purchased from MWG Biotech, Inc., or Sigma–Genosys, Inc. Each primer pair was evaluated in our laboratory for species specificity and sensitivity using standard PCR and agarose gel electrophoresis. Only those oligonucleotide pairs meeting the project criteria were selected for further analysis (Tables 1 and 2). The SYBR Green PCR core reagent kit was purchased from Applied Biosystems, Inc. (SYBR is a registered trademark of Molecular Probes, Inc.).

Table 1
Repetitive elements and amplicon sizes for intra-SINE PCR detection assays

Common name	Order	Family	Genus and species	Repeat element	PCR amplicon size (bp)
Cow	Artiodactyla	Bovidae	*Bos taurus*	1.711B bovine repeat	98
Pig	Artiodactyla	Suidae	*Sus scrofa*	PRE-1 SINE	134
Chicken	Galliformes	Phasianidae	*Gallus gallus*	CR1 SINE	169
Ruminants	Artiodactyla	N/A	N/A	Bov-tA2 SINE	100

Table 2
Oligonuclotide primers for intra-SINE-based PCR detection assays

	Forward primer	Reverse primer
Bovine	5′ TTTCTTGTTATAGCCCACCACAC 3′	5′ TTTCTCTAAAGGTGGTTGGTCAG 3′
Porcine	5′ GACTAGGAACCATGAGGTTGCG 3′	5′ AGCCTACACCACAGCCACAG 3′
Chicken	5′ CTGGGTTGAAAAGGACCACAGT 3′	5′ GTGACGCACTGAACAGGTTG 3′
Ruminants	5′ CAGTCGTGTCCGACTCTTTGT 3′	5′ AATGGCAACACGCTTCAGTATT 3′

PCR conditions were optimized for each assay with regard to annealing temperature and concentrations of $MgCl_2$ and oligonucleotide primers. Quantitative PCRs were carried out in 50 µl using 1X SYBR Green buffer, 1 mM dNTPs, 3.0 mM $MgCl_2$, and 1.25 units AmpliTaq Gold DNA polymerase as recommended by the supplier. The concentrations of oligonucleotide primers used were 0.3 µM for the bovine assay and 0.2 µM each for the porcine, chicken, and ruminant PCR-based assays. Each sample was subjected to an initial denaturation of 12 min at 95°C to activate the AmpliTaq Gold, followed by 40 amplification cycles of denaturation at 95°C for 20 s and either 55°C to anneal for 45 s and 30 s of extension at 60°C (bovine, chicken, and ruminant assays) or 63°C for 1 min to anneal and extend (porcine assay). Each reaction contained 49 µl of PCR master mix and 1 µl of DNA template. Quantitative PCR experiments were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems, Inc.).

Conventional PCRs for agarose gel detection were carried out in 25 µl using 2 ng of DNA template, 1X PCR buffer II (Applied Biosystems, Inc.), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 unit *Taq* DNA polymerase, and the same oligonucleotide concentrations as described above. Each sample was subjected to an initial denaturation of 1 min at 95°C, followed by 30 amplification cycles of denaturation at 95°C for 30 s and either 55°C to anneal for 30 s and 30 s of extension at 72°C (bovine, chicken, and ruminant assays) or 63°C for 1 min to anneal and extend (porcine assay). In the porcine assay, using "hot-start PCR" (automatic with AmpliTaq Gold) and an annealing/extension temperature of 63°C or higher was critical to assay specificity.

DNA samples

DNAs from cow (*Bos taurus*), horse (*Equus caballus*), sheep (*Ovis aries*), antelope (*Antilocapra americana*), dog (*Canis familiaris*), cat (*Felis catus*), and rabbit (*Oryctolagus cuniculus*) were obtained by tissue and blood extraction using the Wizard Genomic DNA Purification kit (Promega) and from samples provided by the Louisiana State University School of Veterinary Medicine. Chicken (*Gallus gallus*) DNA was extracted from blood using the QIAamp DNA Blood Mini Kit (Qiagen, Inc.). DNAs from pig (*Sus scrofa*), deer (*Odocoileus virginianus*), duck (*Anas discors*), rat (*Rattus norvegicus*), and mouse (*Mus musculus*) and from commercial food products were prepared from tissue with proteinase K digestion followed by phenol:chloroform extraction and ethanol precipitation [28]. Human DNA (HeLa cell line ATCC CCL2) isolations were performed using Wizard genomic DNA purification (Promega). Extracted DNA was stored in 10 mM Tris/0.1 mM EDTA (TLE), quantified spectrophotometrically, and then serially diluted 10-fold in TLE such that concentrations from 10 ng to 0.01 pg were assayed in triplicate using PCR.

Data analysis

Data from triplicate DNA standards were exported from the ABI Prism 7000 SDS software into a Microsoft Excel spreadsheet on which the mean value and standard deviation were calculated for each point on the standard curve. Using the Excel trendline option, a line of best fit was plotted with y error bars equal to 1 standard deviation to form a standard curve. Data from the negative control (no template) replicates and the composite mixed-DNA test samples (Table 3) (mean $\pm$ 1 standard deviation of three replicates) were then plotted on the graph for comparison to the standard curve. DNA samples from six different commercially purchased meat products were evaluated in duplicate for each species of interest. Mean quantitation values were calculated using the standard curve and plotted with x and y error bars equal to 1 standard deviation. Pairwise t tests were performed to determine if calculated values were statistically different from the no-template control ($p = 0.05$).

Data from the multispecies cross-amplification experiments were exported to Excel in a similar manner and the mean and standard deviation were calculated for each of three replicates. The Excel chart wizard was used to construct bar graphs with y error bars equal to 1 standard deviation.

Results

Here, we report the development of three species-specific intra-SINE-based PCR assays for the identification and quantitation of bovine, porcine, and chicken DNA. We also report the development of a multispecies ruminant-specific intra-SINE-based PCR assay for the sensitive detection of common ruminant species (Tables 1 and 2). SINEs reside within almost every genome that has been studied to date [29,30]. Most SINEs have amplified in the past 65 million years and are thought to have been spread throughout each genome via an RNA-mediated duplication process termed retroposition [29]. Because each of the SINE families within the different genomes was derived independently, every mammalian order has a significant number (in excess of 100,000) of characteristic mobile elements. These large dispersed gene families serve as novel markers that identify the DNA from the species within that order, thus providing specific genomic tags that can be used in conjunction with PCR to amplify specific subsets of genomic sequences unique to the genome or species of interest from mixed-DNA sources. During intra-SINE PCR, primers are designed within the core body of the SINE

Table 3
Compositions of mixed-DNA test samples

Contents	Bovine		Porcine		Chicken		Total template	
	DNA ng	(%)	DNA ng	(%)	DNA ng	(%)	DNA ng	(%)
Bovine mix								
1	5	(50)	5	(50)	0	(0)	10	(100)
2	1	(10)	0[a]	(0)	0[a]	(0)	10[a]	(100)
3	0.05	(0.5)	7	(70)	2.95	(29.5)	10	(100)
4	0.005	(0.05)	7.2	(72)	2.795	(27.95)	10	(100)
5	0.0005	(0.005)	7.22	(72.2)	2.7795	(27.795)	10	(100)
Porcine mix								
1	5	(50)	5	(50)	0	(0)	10	(100)
2	4.5	(45)	1	(10)	4.5	(45)	10	(100)
3	4.5	(45)	0.5	(5)	5	(50)	10	(100)
4	4.95	(49.5)	0.1	(1)	4.95	(49.5)	10	(100)
5	4.95	(49.5)	0.05	(0.5)	5	(50)	10	(100)
6	4.995	(49.95)	0.005	(0.05)	5	(50)	10	(100)
7	4.9995	(49.995)	0.0005	(0.005)	5	(50)	10	(100)
8	4.99995	(49.9995)	0.00005	(0.0005)	5	(50)	10	(100)
Chicken mix								
1	2.5	(25)	2.5	(25)	5	(50)	10	(100)
2	4.5	(45)	4.5	(45)	1	(10)	10	(100)
3	4.75	(47.5)	4.75	(47.5)	0.5	(5)	10	(100)
4	4.95	(49.5)	4.95	(49.5)	0.1	(1)	10	(100)
5	4.9925	(49.925)	4.9925	(49.925)	0.015	(0.15)	10	(100)
6	4.9975	(49.975)	4.9975	(49.975)	0.005	(0.05)	10	(100)

[a] Ovine and deer DNA at 4.5 ng each (45% each).

to amplify multiple copies of the element and generate a homogeneous product composed entirely of the repeat core unit DNA sequences characteristic of the genome being amplified. In conjunction with SYBR Green fluorescence detection, species-specific intra-SINE PCR is also highly quantitative.

Bovine SINE families such as Bov-tA, Bov-A, and Bov-B are common to all ruminant members of the order *Artiodactyla* such as shown when using our Bov-tA2 ruminant assay (Fig. 1D) [21]. But these elements have also undergone recombination events throughout bovine evolution such that some sequence variants have formed satellites of the original SINE families. Some of these satellites, such as the 1.711B bovine repeat (GenBank No. V00116) used in our bovine assay, emerged after the radiation of the *Bovidae* approximately 5–15 million years ago and are absent from other ruminant species [21] (Fig. 1A). The 1.711B bovine repeat is thought to occupy 7.1% of the bovine genome [23].

The porcine SINE PRE-1 used in our porcine assay [24] (GenBank No. Y00104) is present in pig, and other members of the *Suidae* family, but is absent from other genomes (Fig. 1B). The PRE-1 SINE sequence reportedly diversified at least 43.2 million years ago and has about 100,000 copies per genome [25].

The CR1 family of SINEs reportedly has six subfamilies designated A through F [27]. Our chicken assay was designed in the CR1 SINE subfamily "C" (GenBank No. X03517) and, while present in the chicken genome, it is absent from other avian genomes such as duck (Fig. 1C) and dove (data not shown).

The bovine assay based on the 1.711B bovine repeat had a linear quantitation range of 10–0.0001 ng (0.1 pg), or 10^6, as shown by the standard curve (Fig. 2A). The mean value of the negative template control (NT) was 29.1 ± 0.1 and was not significantly different from the lowest value tested (0.00001 ng or 0.01 pg). This assay detected the known values of bovine DNA within mixed-DNA samples from 50% (5 ng) to 0.005% (0.5 pg) as indicated by the triangles on the standard curve. The compositions of the various DNA mixtures are shown in Table 3. A total of 10 ng of DNA template was used in each test. Background cross-amplification was detected in trace amounts only from rabbit (*Or. cuniculus*) and dog (*C. familiaris*) DNA templates following 26 cycles of PCR when tested with an equivalent amount of DNA (2 ng) (Fig. 3A). Therefore, cross-species amplification does not limit the effective quantitation range of this assay when testing DNA samples from complex (mixed) sources.

The porcine intra-PRE-1 SINE-based PCR assay had a linear quantitation range of 10–0.00001 ng (0.01 pg), or 10^7, as shown by the standard curve (Fig. 2B). The mean value of the negative control was 34.2 ± 0.3 and was significantly different from 31.3 ± 0.6 at the 0.01 pg level ($p = 0.0037$). This assay detected the known values of porcine DNA within mixed-DNA samples from 50% (5 ng) to 0.0005% (0.05 pg) as indicated by the triangles

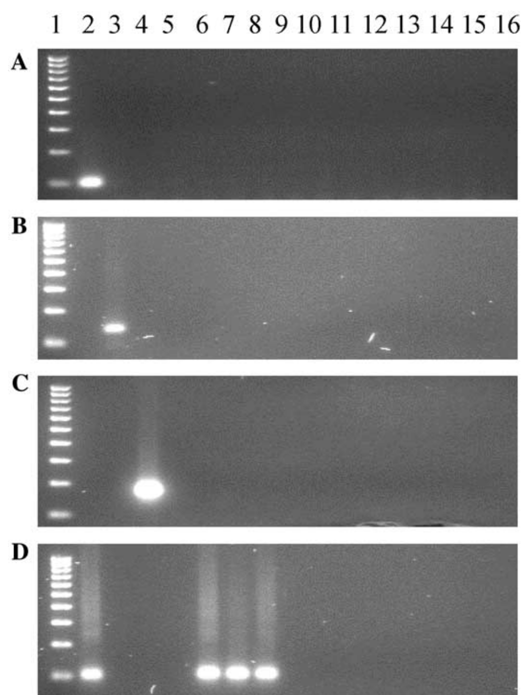

Fig. 1. Species-specific DNA detection using four SINE-based PCR assays. Following 30 cycles of conventional PCR using (A) the bovine-specific assay designed in the 1.711B bovine repeat, (B) the porcine-specific intra-PRE-1 SINE-based assay, (C) the chicken-specific intra-CR1 SINE-based assay, or (D) the intra-Bov-tA2 SINE-based assay for detection of ruminant species, amplicons were chromatographed on a 2% agarose gel that contained ethidium bromide and visualized using UV fluorescence. Lanes: (1) 100 bp ladder, (2) cow, (3) pig, (4) chicken, (5) horse, (6) sheep, (7) deer, (8) antelope, (9) rabbit, (10) duck, (11) dog, (12) cat, (13) rat, (14) mouse, (15) human, (16) NTC (no-template control).

on the standard curve. Background amplification was detected in trace amounts only from duck (*Anas discors*) and rat (*R. norvegicus*) following 29 cycles of PCR when tested with an equivalent amount of DNA template (2 ng) (Fig. 3B). Therefore, cross-species amplification limits the effective quantitation range of this porcine intra-SINE PCR assay to 0.1 pg when equivalent amounts of duck or rat DNA may be present in the samples. However, when DNA samples derived from most complex sources were tested the effective minimum quantitation range was 0.01 pg.

The chicken intra-CR1 SINE-based PCR assay had a linear quantitation range of 10–0.005 ng, or 2000-fold, as shown by the standard curve (Fig. 2C). The mean value of the negative control was 32.9 ± 0.5 and was not significantly different from the lowest value tested (0.001 ng). This assay detected the known values of chicken DNA within mixed-DNA samples from 50% (5 ng) to 0.05% (5 pg), as indicated by the triangles on the standard curve. No amplification was detected from any of the other species tested, making this assay absolutely chicken-specific within its quantitation range (Fig. 3C).

The intra-Bov-tA2 SINE-based PCR assay for detection of multiple ruminant species has a linear quantitation range of 10–0.001 ng (10^4) using bovine DNA as shown by the standard curve and also using ovine DNA shown by the triangles superimposed along the standard curve (Fig. 2D). The combined mean value of the negative control (not shown) was 34.8 ± 0.4 and was significantly different from 30.9 ± 0.5 at the 0.001 ng level ($p = 0.02$). PCR amplification was detected from all ruminant species tested (cow, sheep, deer, and antelope) and no signal was detected from other species (Fig. 3D). The intra-Bov-tA2 SINE-based PCR assay not only allows sensitive simultaneous quantitation (down to 1 pg of starting DNA template) of DNA derived from various ruminant species in a single assay but also permits detection (100 pg) and rough quantitative estimates to be performed by simple, inexpensive agarose gel electrophoresis as an initial screening tool (Fig. 4).

Once we verified the specificity of our four quantitative intra-SINE PCR techniques with the various multispecies DNA mixtures (Table 3 and Fig. 2), we then applied the assays to a series of six different meat products purchased at random from local grocery stores. A description of these samples, as taken directly from the product labels, is shown in Table 4. DNA from the meat samples was tested for content accuracy with our four assays. First, samples were screened for individual species-specific detection by traditional PCR for 30 cycles followed by agarose gel electrophoresis (Fig. 5). The same samples were then analyzed quantitatively with SYBR Green-based intra-SINE PCR for species-specific quantitation of sample components (Fig. 6).

The results from the gel-based assays indicated that the ground beef sample (Fig. 5, lane 4) contained only beef. The ground pork (Fig. 5, lane 5) sample that was analyzed contained only pork. The ground lamb (Fig. 5, lane 6) contained DNA from a ruminant species and did not contain beef, pork, or chicken. The pork sausage that was analyzed (Fig. 5, lane 7) contained only pork and no beef, chicken, or ruminant species. The chicken sausage (Fig. 5, lane 8) contained chicken, but also appeared to have some beef and pork components. The mixed sausage (lane 9) contained beef and pork as labeled and also trace amounts of chicken (Fig. 5). However, the findings from the gel-based assays were limited by the sensitivity of detection. When the same meat samples were analyzed using quantitative intra-SINE PCR, the results indicated that both the ground beef sample (a) and the ground lamb sample (c) contained trace amounts of pork, 0.17 ± 0.10 pg ($\sim$0.002%) and 0.40 ± 0.00 pg ($\sim$0.004%), respectively (Fig. 6B). These calculated values were both significantly different from the negative control ($p = 0.05$). The mixed sausage (f) contained beef and pork in almost equal amounts as indicated on the product label (Figs. 6A, 6B and 6D) and did not contain any chicken DNA within the

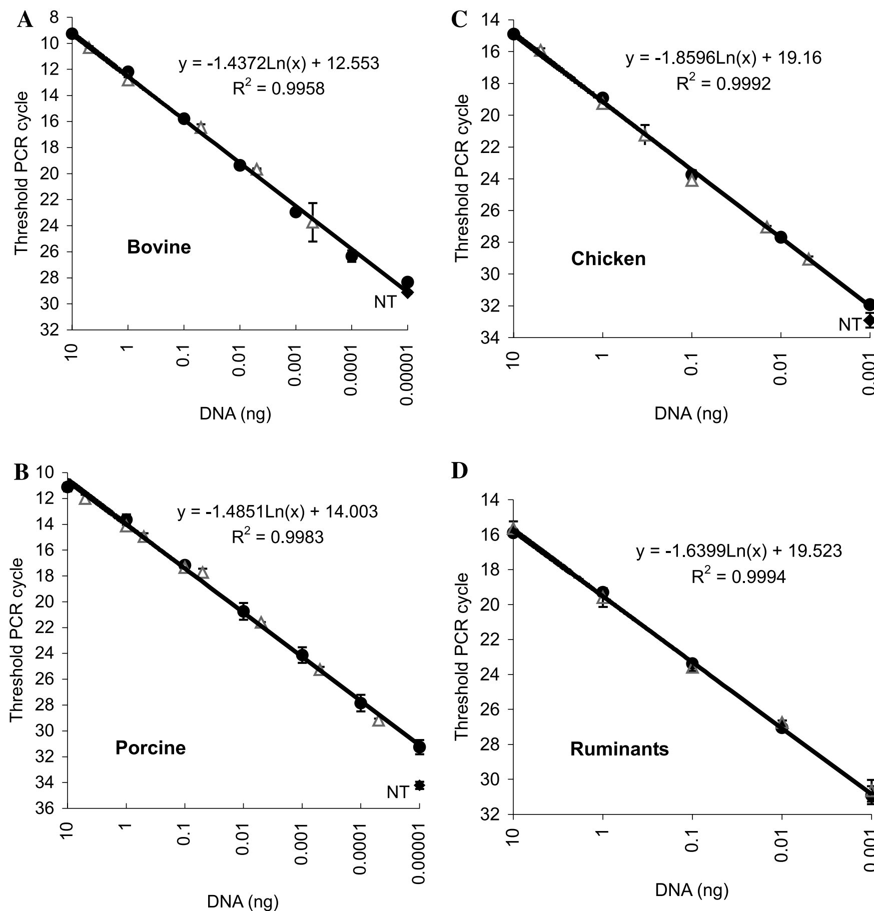

Fig. 2. Quantitation range for four SINE-based PCR assays. The effective ranges for (A) the bovine-specific assay designed in the 1.711B bovine repeat, (B) the porcine-specific intra-PRE-1 SINE-based assay, (C) the chicken-specific intra-CR1 SINE-based assay, and (D) the intra-Bov-tA2 SINE-based assay for detection of ruminant species using SYBR Green fluorescence detection are shown. The PCR cycle at which the fluorescent signal crosses baseline is considered to be the threshold cycle, plotted on the *y* axis. The fluorescent signal produced by a 10-fold dilution series of (A) bovine, (B) porcine, (C) chicken, or (D) bovine and ovine DNA is plotted as the mean of three replicates ± 1 standard deviation. The R^2 value is 99–100% for all four standard curves. Analyses of the various species in DNA mixtures outlined in Table 3 are plotted as open triangles along the appropriate standard curve as the mean of three replicates ± 1 standard deviation. This demonstrates the specificity of these four quantitative PCR techniques.

quantitative range of the assay (Fig. 6C), contrary to the indications of the initial gel-based screening. The chicken sausage (e) contained significant levels of both beef and pork, 5.6 ± 0.0 pg (~0.06%) and 0.77 ± 0.09 ng (~7.7%), respectively, as suggested by the gel-based assay (Figs. 6A and 6B).

Discussion

In this study we have designed and tested four quantitative intra-SINE-based PCR assays, optimized for detection and quantitation of bovine, porcine, chicken, and ruminant species DNA from complex (mixed) sources. The consumer demand for sensitive and reliable techniques such as those we report has increased dramatically in response to the BSE epidemic in Europe. In addition to the risk associated with infectious transmissible spongiform encephalopathy, other health concerns or religious affiliations dictate the necessity for these highly sensitive assays. Several researchers have previously reported species-specific PCR-based assays for this purpose [5,7–20]. However, there are several advantages to our intra-SINE-based PCR methods over previously reported approaches. First, these simple PCR assays do not require any additional processing steps such as restriction endonuclease digestion or hybridization for scoring [11,15,16]. In addition, no special expertise or unique equipment, such as an automated DNA sequencer, is required [13,15]. Species-specific DNA detection and quantitative estimates can be performed by simple agarose gel analysis as an initial screening tool using our intra-SINE-based PCR assays. This assay format minimizes the cost of performing these analyses on a large scale and gives most laboratories with average resources the ability to perform these assays.

The addition of SYBR Green-based detection to the amplification protocol facilitates accurate quantitation

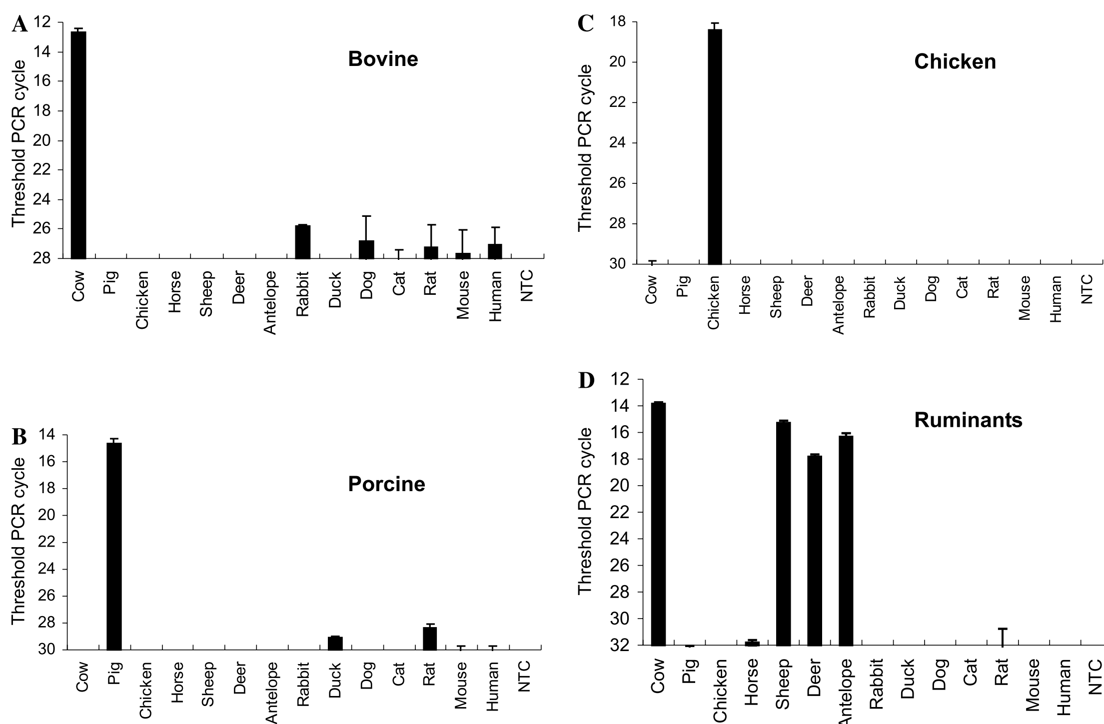

Fig. 3. Background PCR amplification using DNA templates from 14 species. The cross-amplification of DNA templates derived from various species is shown for the: (A) bovine-specific assay, (B) porcine-specific intra-PRE-1 SINE-based assay, (C) chicken-specific intra-CR1 SINE-based assay, and (D) intra-Bov-tA2 SINE-based assay for detection of ruminant species using SYBR Green fluorescence detection. The PCR cycle at which the fluorescent signal crosses baseline is considered to be the threshold cycle, plotted on the *y* axis (mean of three replicates $\pm$ 1 standard deviation). Using DNA (2 ng) from 14 different species as template, background amplification was detected in trace amounts from rabbit (*Or. cuniculus*) and dog (*C. familiaris*) following 26 cycles of PCR in the bovine assay and from duck (*Anas discors*) and rat (*R. norvegicus*) following 29 cycles of PCR in the pork assay, and no background signal was detected with the chicken assay or the ruminant species assay. This demonstrates that cross-species amplification does not restrict the effective quantitation range of these assays when testing DNA samples from most complex sources.

using any quantitative PCR system. The high copy number of SINEs in various genomes makes these assays ideal for species-specific DNA detection and quantitation. The quantitation range of our bovine detection assay is approximately 10^6, with a minimum effective quantitation level of 0.1 pg of DNA. The detection limits using previously reported methods range from 2.5 [19] to 250 pg [10,16,17] of bovine DNA. In other words, the low range detection limit of the intra-SINE-based quantitative bovine PCR assay we report here exceeds the previously reported assays by a minimum of 25-fold.

Our intra-SINE-based porcine quantitative PCR assay proved to be even more sensitive than the bovine assay, with a quantitative range of 10^7 and a minimum effective quantitation level of 0.01 pg. The detection limits previously reported using other methods ranged from 1 pg (0.005% in 20 ng) [8,20] to 250 pg [10]. Thus, the low range detection limit of our porcine intra-SINE-based quantitative PCR assay exceeds the currently available methods by a minimum of 100-fold.

A comparison of our chicken intra-SINE-based quantitative PCR assay to previously reported methods was less clear than the comparisons for the bovine and porcine assays because the previous studies describing chicken-specific PCR-based quantification assays either did not report a detection limit for poultry [12] or reported a minimum detection limit of 250 pg [10]. The quantitative range of our chicken intra-SINE PCR assay was approximately 2000-fold, with a minimum effective quantitation level of 5 pg of template. The quantitative range of our ruminant species intra-SINE detection assay was approximately 10^4, with a minimum effective quantitation level of 1 pg. The detection limits of previously reported assays for ruminant species detection are similar to those reported for bovine detection assays.

The detection limits of our intra-SINE-based quantitative PCR assays should be directly comparable to that reported by Tajima and co-workers [20] using quantitative PCR. The results for the assays would vary depending on the actual copy number of repeats amplified with the respective primer pairs. The new assays reported here also include a bovine-specific quantitative PCR assay in addition to a ruminant-specific assay. In addition, our PCR assays involve the amplification of smaller PCR products. The size of the PCR amplicons used to detect ruminant, porcine, and chicken DNA in our intra-SINE-based quantitative PCR assays are 81

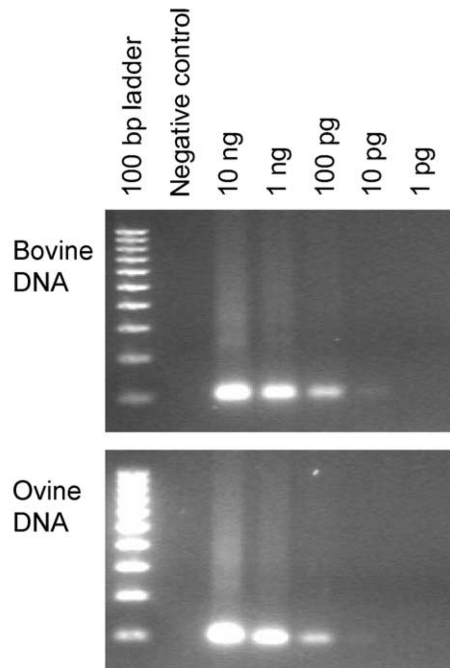

Fig. 4. DNA detection using the intra-Bov-tA2 SINE-based PCR assay. Following 30 cycles of conventional PCR using the intra-Bov-tA2 SINE oligonucleotide primers, amplicons were chromatographed on a 2% agarose gel stained with ethidium bromide. Using DNA standards from both bovine and ovine genomes, this assay easily detects 100 pg of ruminant DNA, corresponding to 1% in a 10-ng mixed-DNA sample of starting template. This demonstrates that this assay is a simple, rapid, and inexpensive means of ruminant species DNA detection that also provides quantitative estimates of template DNA.

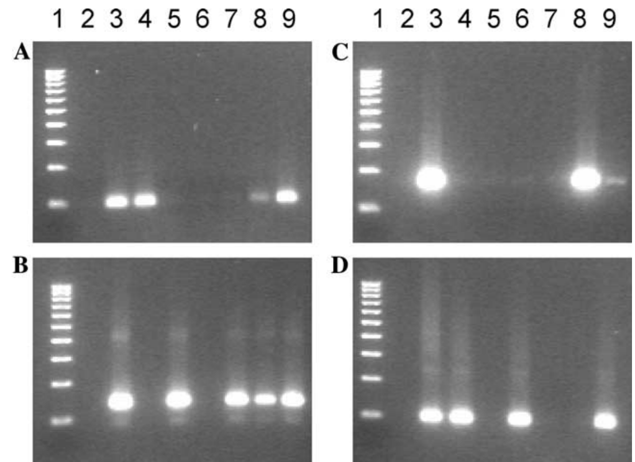

Fig. 5. Analysis of complex mixtures derived from meat food products by PCR. Detection of: (A) bovine DNA using the 1.711B bovine repeat assay, (B) porcine DNA using the intra-PRE-1 SINE assay, (C) chicken DNA using the intra-CR1 SINE assay, and (D) ruminant species using the intra-Bov-tA2 SINE assay following 30 cycles of conventional PCR using 2 ng of template DNA from six different commercially purchased meat products, chromatographed on a 2% agarose gel stained with ethidium bromide. Lanes: (1) 100 bp DNA ladder; (2) negative control; (3) positive control DNA (A, bovine; B, porcine; C, chicken; and D, ovine); (4) ground beef; (5) ground pork; (6) ground lamb; (7) pork sausage; (8) chicken sausage; (9) mixed beef and pork sausage. This suggests that the chicken sausage sampled in this experiment (lane 8) also contains beef and pork; whereas the other products appear to be labeled accurately.

Table 4
Contents of six commercially purchased meat samples

Meat product	Ingredients
a. Ground beef	73% ground beef; 27% fat
b. Ground pork	Fresh ground pork; 28% fat
c. Ground lamb	Fresh ground lamb; 28% fat
d. Pork sausage	Pork, water, green onions, salt, sugar, spices, paprika, granulated garlic, natural flavors
e. Chicken sausage	Chicken, green onions, salt, red pepper, black pepper, garlic powder, sugar, paprika
f. Mixed pork and beef sausage	Pork, beef, salt, red pepper, black pepper, garlic powder, sugar, paprika

(45%), 45 (25%), and 32 bp (16%) shorter than those reported by Tajima and co-workers [20] and should be more useful for the analysis of samples that contain degraded DNA templates.

We also systematically evaluated each of our four intra-SINE quantitative PCR assays for species specificity. In addition to agarose gel-based analysis and SYBR Green fluorescence detection of amplified DNA from 14 different species, assay specificity was evaluated by the ability of the assays to accurately detect known trace quantities of species-specific DNA from complex (mixed) templates. Bovine DNA was detected at 0.005% (0.5 pg), porcine DNA was detected at 0.0005% (0.05 pg), and chicken DNA was detected at 0.05% (5 pg) in a 10-ng mixture of bovine, porcine, and chicken DNA. This highly sensitive species specificity makes these assays ideal for the identification of beef, pork, and chicken DNA contained in complex sources. By contrast, other previously reported methods may be limited by background amplification of templates derived from other species.

We have utilized the specificity and sensitivity of these assays in the analysis of commercially purchased meat products. These tests resulted in some rather interesting findings. The trace amounts of pork found in both the ground beef and ground lamb samples were not detectable using agarose gel-based analysis, but were detected at significant levels using quantitative PCR. The most likely explanation for these trace amounts of pork within the samples comes from their place of origin, the meat department of the local grocery store. Apparently, it is common practice to process all the ground meat for a particular day without thoroughly cleaning the grinder between meat samples. This practice could easily account for the trace pork found in the samples we have analyzed by intra-SINE-based PCR. Although the level of pork contamination in both these products was considerably less than 0.01%, it remains a matter of concern for many population groups.

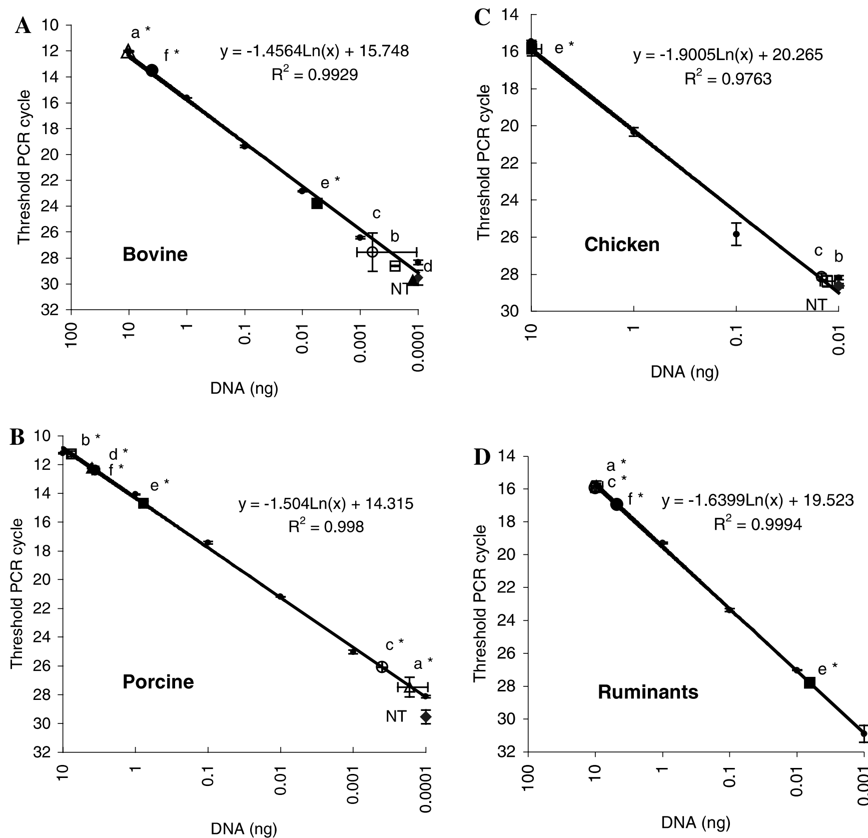

Fig. 6. Quantitative PCR analysis of complex mixtures derived from meat food products. SYBR Green fluorescence detection of (A) bovine, (B) porcine, (C) chicken, or (D) ruminant DNA from six different meat products: (a) ground beef—open triangles; (b) ground pork—open squares; (c) ground lamb—open circles; (d) pork sausage—filled triangles; (e) chicken sausage—filled squares; (f) mixed beef and pork sausage—filled circles. The PCR cycle at which the fluorescent signal crosses baseline is considered to be the threshold cycle, plotted on the *y* axis. The fluorescent signal produced by a 10-fold dilution series of (A) bovine, (B) porcine, (C) chicken, or (D) bovine and ovine DNA is plotted as the mean of duplicates $\pm$ 1 standard deviation, to form a standard curve. DNA (10 ng) from each meat sample was analyzed in duplicate using each of the four quantitative assays. Values were calculated using the standard curves and plotted as the mean with *x* and *y* error bars equal to 1 standard deviation. Values significantly different from the no template control are marked with an asterisk ($*p = 0.05$).

The most surprising finding of our meat analyses was that the chicken sausage we selected for testing contained significant amounts of both beef (~0.06%) and pork (~7.7%). The trace quantities of beef may be explained as outlined above. However, the amount of pork in the sample, nearly 8%, would appear to be more than trace quantities. Since only a single sample of chicken sausage from a single grocery store appeared to be mislabeled these findings may not be indicative of a widespread problem with regard to ambiguous meat labeling practices. However, if an individual consumer had a strong objection to pork consumption, or was allergic to pork, the consumption of this mislabeled meat product could potentially have devastating consequences.

An additional advantage of our intra-SINE-based PCR assays over many previously reported methods is that these assays employ a nuclear sequence with a high copy number for amplification, while simultaneously maintaining some of the same advantages of single locus PCR. For example, these amplicons are relatively short (Table 1) to minimize sensitivity to degraded DNA templates. In addition, the products of each assay are uniform size amplicons, making them amenable to multiple visualization and detection schemes such as ethidium bromide and UV fluorescence and SYBR Green or TaqMan chemistry for quantitative PCR.

We have designed a TaqMan probe for the Pre-1 intra-SINE porcine detection assay (5′-FAM-TTTGAT CCCTGGCCTTGCTCAGTGG-TAMRA-3′) and compared the results to SYBR Green-based detection with respect to sensitivity. No significant increase in assay sensitivity was observed with the TaqMan-based detection compared to SYBR Green (data not shown). Although we did not evaluate the use of TaqMan-based chemistry with every intra-SINE-based PCR assay, we have designed TaqMan probe/primer sets for some of the other intra-SINE quantitative PCR assays in our laboratory and have not found any significant differences in the sensitivities of the various assays. The

reason for no difference in sensitivity is unclear. Perhaps the sensitivity of SYBR is not significantly different from the 5′-FAM reporter typically used with TaqMan-based detection chemistry, while other reporter molecules may be more sensitive. Regardless, the uniformity of each species-specific amplicon in conjunction with fluorophor-specific TaqMan probes would make these assays amenable to multicolor multiplex detection, whereas SYBR Green-based detection would not. This multiple detection format could prove particularly useful in large-scale assay applications.

We have demonstrated that these assays are highly species-specific and highly sensitive. Following September 11, 2001, the United States Department of Agriculture formed the Food Biosecurity Act Team to increase emergency preparedness and biosecurity concerning food safety (http://www.usda.gov). The FDA has also implemented controls to minimize tampering in our food production cycle [31]. In light of the new technology concerning infectious prion-based diseases such as BSE in cows, scrapie in sheep, and variant Creutzfeldt–Jakob disease in humans, highly sensitive PCR-based detection methods are essential to disease prevention. Undoubtedly, the intra-SINE-based detection methods we have reported here will increase the detection sensitivity for these important agricultural species.

Acknowledgments

The authors thank Dr. Stephen Gaunt at the LSU Veterinary Pathology Laboratory, Laurie Henderson from the LSU Animal Science Department, and the Babineaux Slaughter House in Breaux Bridge, Louisiana, and Tony Latiolais for their generous donation of samples. This research was supported by the Louisiana Board of Regents Governor's Biotechnology Initiative GBI (2002-005) to M.A.B.

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