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Multiplex qPCR Assay for Identification and Differentiation of *Amblyomma americanum*, *Amblyomma cajennense*, and *Amblyomma maculatum* (Ixodida: Ixodidae) Tick Species in the Eastern United States

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Abstract

Many ticks of the genus *Amblyomma* are vectors of human pathogens, and the correct species identification is medically and epidemiologically important. Morphological identification is time-consuming and requires a high level of expertise. Identification of engorged, immature, or damaged ticks and the differentiation of closely related species remain problematic. Here, we report the development of a real-time TaqMan assay for the genomic identification and differentiation of *Amblyomma americanum* (L.), *Amblyomma cajennense* (F.), and *Amblyomma maculatum* (Koch), which are human-biting species found in the eastern United States. New species-specific sets of oligonucleotides for the multiplex reaction that detect and differentiate the ITS2 genomic regions of three target species were designed using Visual OMP; the previously published *A. americanum* oligonucleotide set was also incorporated into our assay. Specificity and sensitivity tests for two multiplex master mixes using different *A. americanum* sets were performed using individual and pooled samples of adult, nymphal, and larval ticks, and optimization procedures were applied. The multiplex assay successfully differentiates between genomes of three target species and does not cross-react with DNAs of ticks from other genera. Rare cases of nonspecific amplification occurred with DNAs of *A. imitator* and *Amblyomma triste* Koch misidentified as *A. americanum* and *A. maculatum*, respectively. However, this cross-reaction does not diminish the usefulness of the developed assay east of the 95th meridian, where neither *A. imitator* nor *A. triste* are found. Two master mixes incorporating the previously published or newly developed *A. americanum* sets are being recommended for identification of individual ticks or pooled samples, respectively.

Keywords

Amblyomma *spp*; Amblyomma americanum; Amblyomma cajennense; Amblyomma maculatum; *multiplex qPCR*

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Numerous species of ticks are vectors of human pathogens and their accurate identification is medically and epidemiologically important, “because errors would lead to reports of unusual associations of vertebrates, pathogens and ticks” (Estrada-Peña et al. 2013), and consequently to erroneous decisions regarding control and prevention of tick-borne diseases. Morphological keys have been traditionally used for identification purposes. Several guidelines for differentiation of North American tick species with pictorial keys are available (Sonenshine 1979, Keirans and Litwak 1989, Keirans and Durden 1998). However, morphological identification requires a high level of expertise and is time-consuming, especially for large-scale field collections when hundreds and thousands of ticks must be speciated. Identification of engorged ticks, immature stages, and damaged samples remains problematic. Misidentification of closely related species using morphological keys is a relatively common problem. Mertins et al. (2010), using molecular methods, found that some *Amblyomma triste* (Koch) ticks from different North American collections were misidentified as *Amblyomma maculatum* (Koch). Our own experience indicates that differentiating between look-alike *Amblyomma cajennense* (F.) and *Amblyomma imitator* (Kohls) may be a challenging task. Thus, more reliable, time-efficient, and standardized methods of tick identification are needed.

With advancement of molecular biological methods, assays are being developed for polymerase chain reaction (PCR)-based identification of epidemiologically important tick species. Conventional PCR was used to differentiate three *Ixodes* tick species: *Ixodes ricinus* L., *Ixodes persulcatus* (Schulze), and *Ixodes hexagonus* Leach, as well as *Dermacentor reticulatus* (F) (Rumer et al. 2011). Restriction fragment length polymorphism (RFLP) analysis was exploited to identify 17 *Ixodes* species of the United States (Poucher et al. 1999). Combined PCR-RFLP or PCR-SSCP (Single-stranded conformation polymorphism) assays were developed to distinguish between Metastriate genera (Anderson et al. 2004) and to differentiate between three closely related *Dermacentor* species (Dergousoff and Chilton 2007). A protocol capable of distinguishing between epidemiologically important *Ixodes scapularis* Say, *Ixodes pacificus* Cooley & Kohls, *Dermacentor variabilis* (Say), and *Amblyomma americanum* (L.) was based on the real-time PCR technique (Shone et al. 2006).

The aforementioned methods of tick identification either consist of multiple steps or allow identification of one tick species at a time, and thus remain time-consuming and relatively inefficient. Development of multiplex PCR assays simultaneously identifying and differentiating several species in a single step would be beneficial in projects requiring speciation of large numbers of ticks.

Ticks of the genus *Amblyomma* include major vectors of human and veterinary pathogens around the world. In North America, *A. americanum, A. cajennense* (proposed as *Amblyomma mixtum* by Nava et al., 2014), and *A. maculatum* attract major attention as prevalent and aggressive human-biting tick species, as well as known vectors of multiple human pathogens (Paddock and Yabsley 2007, Paddock et al. 2008, 2010). Their distributions overlap in some parts of the United States. The lone star tick, *A. americanum*, is widely distributed in eastern, southern, and part of central states of the United States (Keirans and Lacombe 1998, Hooker et al. 1912, Merten and Durden 2000). The Gulf Coast

tick, *A. maculatum*, is found along the Gulf of Mexico and the Atlantic coasts (Bishopp and Hixson 1936). The cayenne tick, *A. cajennense*, has limited distribution within the U.S. borders and is present mainly in the southern parts of Texas (Estrada-Peña et al. 2004). The aim of the current study is to develop a single-step multiplex assay in which *A. americanum, A. cajennense*, and *A. maculatum* are specifically detected and differentiated in the presence of other *Amblyomma* species and the ixodid ticks of other genera in both individual and pooled samples.

Materials and Methods

Amblyomma Ticks: Target and Background Species

Human-biting ticks of North American *Amblyomma* species were considered for assay development. *A. americanum, A. cajennense*, and *A. maculatum* were considered as target species. *Amblyomma tuberculatum* Marx, *A. imitator, A. triste, Amblyomma ovale* Koch, and *Amblyomma rotundatum* Koch, which do have limited distribution within the United States but rarely bite people, were designated as background species.

DNA Extraction and Species Confirmation

DNAs were extracted from colony-derived adult ticks of three target *Amblyomma* species: *A. americanum, A. cajennense*, and *A. maculatum* maintained in the Medical Entomology Laboratory at Centers for Disease Control and Prevention (CDC). DNAs of background *Amblyomma* species were obtained from wild ticks collected in Georgia (*A. tuberculatum*), ticks from our laboratory collection (*A. imitator*) as well as from voucher specimens (*A. triste, A. ovale, and A. rotundatum*) kindly provided by Dr. M. Labruna from University of San Paulo, Brazil. Both target and background tick specimens were morphologically identified according to standard keys (Cooley and Kohls 1944, Hoskins 1988, Guerrero 1996, Faccioli 2011). DNA was extracted from each sample using the Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol of extracting DNA from tissues and eluted in 100 μl (final volume). Sample preparation included grinding ticks previously frozen in liquid nitrogen. No extraction controls were performed. The concentration of isolated DNA was determined using Qubit 2.0 fluorometer (Invitrogen Life Technologies, Grand Island, NY). Tick DNA extracts were stored at 4°C during the period of assay development. Morphological identification of each target tick species was additionally confirmed by partial sequencing of 12s mitochondrial RNA gene as described earlier (Beati and Keirans 2001). An ABI PRISM 3.0 BigDye Terminator Cycle Sequencing kit (Applied BioSystems, Foster City, CA) was used for performing sequence reactions as recommended by the manufacturer. The amplicons were purified using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI), and sequenced on an Applied BioSystems 3130*xl* genetic analyzer. Sequences were assembled using DNAStar Lasergene 9 (DNAStar Inc., Madison, WI). Tick species were confirmed using the National Center for Biotechnology Information by basic local alignment sequence tool application.

Primer and Probe Design

Originally, we designed and tested three multiplexing oligonucleotide sets targeting 12s, 16s, and ITS2 genes. Each multiplexing set included specific primer-probe combinations for each

target *Amblyomma* spp. The primer-probe sets were evaluated for their specificity in identifying DNA of respective target species before the assessment of their ability to work in combination with two other oligonucleotide sets and the sensitivity in detecting target DNA in pooled samples. The subsequent work was focused on the ITS2 gene as the most suitable target. Sequences of ITS2 regions of *A. americanum* (AF548538.1), *A. cajennense* (AF469605.1) from GenBank, and sequence of *A. maculatum* (KF373075) from the colony-derived ticks were used to design primers and probes. A primer-probe combination specifically amplifying *A. americanum* DNA was adopted from the previously published qPCR assay (Shone et al. 2006). Oligonucleotide sets for detection of *A. cajennense* and *A. maculatum* genomes, as well as an additional set for detection of *A. americanum* genome, were designed using the oligonucleotide modeling platform Visual OMP and ThermoBLAST (DNA Software Inc., Ann Arbor, MI). The following criteria were considered in developing primer and TaqMan probe sets for the three target multiplex: target genomic sequence alignments to identify islands of differentiation, target sequence secondary structure and G-C base composition, primer and probe length and unimolecular and bimolecular melting temperatures, primer and probe sensitivity, and predicted cross-reactivity between the three multiplexed ITS2 genomic target regions and closely related background genomic sequences, respectively, PCR annealing temperature and salt conditions, and the desired length of the ITS2 amplicons. Probes for *A. americanum, A. cajennense*, and *A. maculatum* were labeled with HEX, CalRed610, and Quasar670 on the 5′ end, and the appropriate black hole quencher on the 3′ end. The length of the oligonucleotides designed in this study varies from 20 to 24 bp and the melting temperatures are between 65 to 72°C with the only exception being the *A. maculatum* probe, where the melting temperature is 62°C (Table 1). Two locked nucleic acids were incorporated to the *A. maculatum* probe to increase its melting temperature and improve specificity (You et al. 2006).

Multiplex qPCR

Stratagene Brilliant Core reagent kit (Agilent Technologies, LA Jolla, CA) was used to set up a PCR mixture with a final volume of 20 μl using 2 μl of extracted DNA as a template. Oligonucleotides used in the study were synthesized by the Biotechnology Core Facility Branch at CDC. The qPCR assays and data analysis via CFX manager software (version 1.5) were performed on a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). The following amplification conditions were applied: denaturation at 95°C for 8 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 62°C for 30 s. The reaction was concluded with a final extension at 62°C for 10 min. The fluorescence threshold was automatically set by the CFX Manager software. Temperature gradient was con-ducted within the range from 50 to 60°C to determine the optimal annealing temperature for each oligonucleotide set tested. No positive control was used. Two negative controls (distilled water) were included in each PCR run. All samples were run in duplicate. Samples with the fluorescence level exceeding the threshold before cycle 40 were considered as positive.

Specificity Test and Reportable Range

The specificity of oligonucleotide sets in the multiplex real-time PCR assay was tested with DNA extracted from tick species representing five genera of Ixodidae including

Rhipicephalus sanguineus (Latreille), *I. scapularis, D. variabilis*, and *Haemaphysalis leporispalustris* Packard, as well as the "background" *Amblyomma* species: *A. imitator, A. triste, A. tuberculatum, A. ovale*, and *A. rotundatum* (five samples representing each species). To determine the reportable range, a linearity test was performed using indicated PCR parameters (Burd 2010). Serial 10-fold dilutions of whole tick DNA from each target species ranging from 100 ng/μl to 0.1 pg/μl were prepared and 2 ml of these extracts were added to reaction. The lowest concentration of DNA with detectable fluorescence above the threshold before cycle 40 was considered as the limit of linearity or the lower limit of quantification (LLOQ). Reaction efficiency (E) and coefficient of determination or goodness-of-fit (R^2) were determined using the CFX Manager software.

The assay was evaluated for its ability to detect and identify all developmental stages of the target tick species at different stages of engorgement by testing DNA samples extracted from flat and engorged males, females, nymphs, and larvae of each target *Amblyomma* species. Considering a possibility of genetic variability between ticks throughout the geographic range of a species, the universality of the designed assay was evaluated by testing morphologically identified tick samples from different territories: Georgia, Florida, Virginia, and Missouri for *A. americanum*, and Georgia and Virginia for *A. maculatum.* At least 10 flat adults from each location were tested.

Verification of Multiplexing

To test the assay's capability for simultaneous detection of several tick species in mixed samples, we prepared and tested pools, which included combinations of nymphs or larvae of two and three target species. Pooled samples were prepared to include species in either equal proportion (one nymph or larvae of each species) or unequal proportion (one tick of a species and four ticks of another species) of ticks. Pools consisting of three different species of ticks included a single tick of each of species.

Results

Partial sequencing of the 12s gene of *A. americanum, A. cajennense*, and *A. maculatum* individual tick samples used for testing purposes was 100% identical to AF150050.1, EU791613.1, and U95854 from GenBank, respectively, and thus confirmed the results of morphological identification.

Overall, original multiplexing oligonucleotide sets, each consisting of 3 primers-probe combinations, for 12s, 16s, and ITS2 genes of *Amblyomma* spp. ticks were rejected in the process of the initial testing because of their insufficient specificity in identifying individual ticks, or inefficiency in the presence of other primers and probes in a multiplex setting (data not shown). Nucleotide sets listed in Table 1 showed the best results in the initial assessment of specificity and were included for further evaluation and optimization of multiplex reaction.

An *A. americanum*-specific primers-probe set designed by Shone et al. (2006) was adopted and validated for conditions of the designed multiplex assay (Table 1, set no.1). Three additional species-specific oligonucleotide sets, each including a forward and reverse primer

and a TaqMan probe, were designed for each of the three target *Amblyomma* species (Table 1; sets no.2, no.3 and no.4). PCR conditions were optimized for better multiplexing results using a temperature gradient and the optimum annealing temperature was found to be 55°C. Extension temperature has been set at 62°C to adjust PCR parameters for the properties of *A. maculatum* probe.

Different concentrations of primers and probes were tested to balance the efficiency of composing oligonucleotides. The reaction concentrations of oligonucleotides resulting in the best multiplexing performance vary from 100 to 500 nMol (Table 1). The lengths of amplified fragments for sets no. 2, no. 3, and no. 4 are 253, 119 and 193 bp, respectively.

Multiplex master mix containing oligonucleotide sets no. 1, no. 3, and no. 4 (MMix I) successfully amplified DNAs of target species under the standard conditions established for this assay in DNA extracts containing single ticks (Fig. 1). When individual *A. americanum* ticks were tested using MMix I, multiplex assay consistently identified *A. americanum* DNA regardless of whether flat or engorged ticks were tested, and was sufficiently sensitive to correctly identify a single unfed larva (Table 2). DNA of neither *A. cajennense* and *A. maculatum* nor nontarget tick species was misidentified as *A. americanum*. Tick samples from Florida, Virginia, and Missouri, morphologically identified as *A. americanum*, were correctly recognized using MMix I (Table 2). However, amplification of *A. americanum* DNA was inhibited in mixed pooled samples in combination with *A. cajennense* DNA; and no *A. americanum* DNA amplification was recorded in any pooled samples containing *A. maculatum* DNA or both *A. cajennense* and *A. maculatum* DNAs (Table 3).

When individual *A. cajennense* ticks were tested using MMix I, the assay consistently identified either flat or engorged ticks in all stages using the aforementioned PCR conditions. Oligonucleotide set no. 3 developed for specific identification of *A. cajennense* did not cross-react with DNA of any other tested tick species (Table 2). Set no. 3 also differentiated *A. cajennense* in all mixed samples containing either two or three target species regardless of pool complexity (Table 3).

Likewise, MMix I efficiently identified DNAs of *A. maculatum* ticks from our laboratory colony in different life stages, both flat and engorged. All 10 *A. maculatum* tick samples obtained from Virginia were correctly identified as well (Table 2). No cross-reaction was observed between oligonucleotide set no. 4 and DNAs of ticks from other genera or most of nontarget *Amblyomma* species, with a notable exception of *A. triste* (Table 2). In all five samples containing individual *A. triste*, MMix I misidentified these as *A. maculatum*. DNA of *A. maculatum* was successfully identified and differentiated by MMix I from either *A. americanum* or *A. cajennense* in all types of mixed-species pooled samples that included immature ticks of either two or three target tick species simultaneously (Table 3). In combination with *A. americanum* set no. 1, the LLOQ for *A. cajennense* (set no. 3) was determined to be 1 pg/μl with R^2 = 0.999 and E = 95.7% (Fig. 2); LLOQ for *A. maculatum* (set no. 4) is also 1 pg/μl with R^2 = 0.999 and E = 97.6% (Fig. 3); and for *A. americanum* set no. 1 it is 10 pg/μl with R^2 =0.995 and E = 73.6% (Fig. 4).

The multiplex master mix II (MMix II) included the same oligonucleotide sets for detection of *A. cajennense* DNA (set no. 3) and *A. maculatum* DNA (set no. 4) as the MMix I, but used a different oligonucleotide set for detection of *A. americanum* DNA (set no. 2) designed specifically for this assay. This master mix also amplified DNAs of intended species using established PCR conditions. MMix II successfully detected presence of the target DNA in samples consisting of either flat or engorged individual *A. americanum* ticks from Georgia in all life stages. This set, however, gave inconsistent results when testing samples of individual adult ticks field-collected in other states and morphologically identified as *A. americanum:* 2 of 10 and 3 of 10 samples obtained from Missouri and Virginia, respectively, were not amplified. Concentration of nonamplified samples ranged from 50 to 85 pg/μl. No cross-reaction was observed with DNAs of ticks from other genera or most of nontarget *Amblyomma* species, with a notable exception of *A. imitator* (Table 2). Amplification of *A. imitator* DNA was nonspecific with low (up to 100 RFU-relative fluorescence units) level of fluorescence (data not shown). Presence or absence of *A. americanum* DNA was correctly detected in all pooled samples containing combinations of either pooled larval or nymphal ticks of any two of the three target species with no misidentification detected (Table 3). Tick species were correctly identified in 5 of 10 and 7 of 10 pools containing all three target species in larval and nymphal stages, respectively (Table 3). LLOQ for *A. americanum* set no. 2 was 100 pg/μl with R^2 = 1.000 and E = 70.2%. Performance of oligonucleotide sets no. 3 and no. 4 in MMix II was similar to their performance in MMix I, although with higher LLOQ: 10 pg/μl (R^2 = 0.980 and E = 69.3%) and 10 pg/μl (R^2 = 0.993 and E = 95.3%) for *A. cajennense* and *A. maculatum* sets, respectively (data not shown).

Discussion

Here, we present development of a multiplex assay capable of simultaneous detection of and discrimination between three species of human-biting ticks—*A. americanum, A. cajennense*, and *A. maculatum*. Multiplexing is a challenging but attractive molecular tool because of its ability to identify a great number of samples quickly and easily (Gurvich and Skoblov 2011). The ability of the designed assay to discriminate among species regardless of the tick's life stage and feeding status makes this test useful in every day practice, especially as an alternative to the time and labor-consuming morphological identification technique as well as to an expensive sequencing application.

The greatest challenge for the developed molecular assay is the high level of DNA homology between target and "background" *Amblyomma* species, resulting in certain specificity limitations. Indeed, the developed assay does not distinguish between *A. maculatum* and *A. triste* genomes within confines of the current multiplex design. These species belong to the *A. maculatum* group (Estrada-Peña et al. 2005); their morphological identification is difficult (Cooley and Kohls 1944, Guglielmone et al. 2003). The degree of homology is extremely high even within their respective variable ITS2 gene regions. The possibility of developing a complimentary assay to specifically discriminate between these two species should be considered. Meanwhile, this limitation of the developed assay can be mitigated by its use in collection sites where *A. triste* is not likely present. The known distribution of *A. triste* within the United States is confined to the arid areas of Chihuahuan

Desert and its fringes west of the 100th meridian (Mertins et al. 2010). *A. maculatum*, however, is restricted to more humid habitats east of the 100th meridian. Thus, we recommend using this multiplex assay for identification of *Amblyomma* ticks collected in the eastern United States—east of the 100th meridian.

Identification of larval and nymphal ticks poses a special challenge. Not only are immature stages usually the hardest to identify morphologically requiring higher levels of expertise, but also usually hundreds and thousands of nymphs and larvae are needed to be processed during either ecological or epizootiological studies. Together, these factors often make speciation of every individual immature tick impractical, if not virtually impossible. Individual identification, extraction, and testing are recommended for adult ticks, while larvae and nymphs are processed in polls assembled by life stages much more frequently. Differentiation between closely related immature ticks is important, especially in regions of overlapping distribution where either larvae or nymphs of several species may end up in mixed pools. Thus, the demonstrated ability of the developed multiplex assay to differentiate immature stages of target species in species mixed samples is essential. Identification of *A. cajennense* and *A. maculatum* species is consistently adequate in mixed samples, regardless of which *A. americanum* oligonucleotide set (no. 1 or no. 2) was included into the master mix for a multiplex reaction. Unexpectedly, the multiplex MMix I containing oligonucleotide set no. 1 failed to detect the presence of *A. americanum* DNA in mixed pools, even though its performance was excellent for individual tick samples. Apparently, the presence of either *A. maculatum* or *A. cajennense* DNAs in the sample negatively impacted performance of this primer-probe set, although to a different extent with *A. maculatum* DNA having a greater adverse effect than *A. cajennense* DNA. To overcome this issue, we used a newly designed oligonucleotide set (no. 2) targeting *A. americanum* in MMix II. The identification of *A. americanum* DNA in mixed pools of immature ticks was significantly better using the oligonucleotide set no. 2. Even then, inconsistent results were observed when we pooled together larvae or nymphs of all three target species. The inconsistency might be attributed to the sensitivity of *A. americanum* set no. 2, which has a higher limit of quantification. In addition, MMix II recognized *A. imitator* DNA as *A. americanum*. As a result, use of this assay for identification of pooled samples in southern Texas, where both *A. imitator* and *A. americanum* may be present can result in misidentification. Therefore, we recommend applying the presented multiplex assay to pools of *Amblyomma* ticks only east of the 95th meridian, outside of the known *A. imitator* distribution. Overall, we recommend using the Master Mix I, containing *A. americanum* set no. 1, for any individually extracted tick samples and the Master Mix II, containing *A. americanum* set no. 2, for pooled samples in eastern United States, east of the 95th meridian. To account for the somewhat limited sensitivity of the *A. americanum* set no. 2, measuring DNA concentration in extracted samples may be advisable.

A minor inconvenience of the designed assay is the relative complexity of the master mix containing nine oligonucleotides, which might be a predisposing factor for procedural mistakes. In cases where large numbers of DNA extracts need to be tested, we suggest preparing mixtures (with exclusion of probes and *Taq*) in advance.

The current study represents an effort to develop an assay that has the advantages of having high capacity, specificity, and sensitivity, and would be applicable to a wide range of ecological and population studies. Further development of high capacity multiplex PCR assays is worth an investment, as they will eventually simplify and standardize the identification process.

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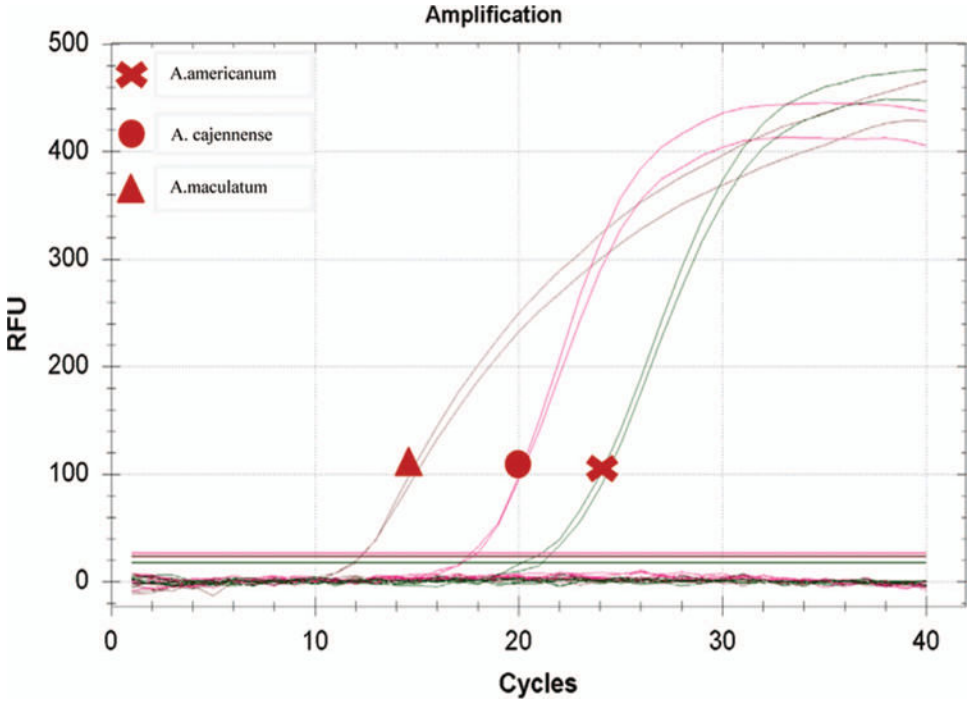

Fig. 1.

Amplification of *A. americanum*, *A. cajennense*, and *A. maculatum* DNAs with MMixI. (Online figure in color.)

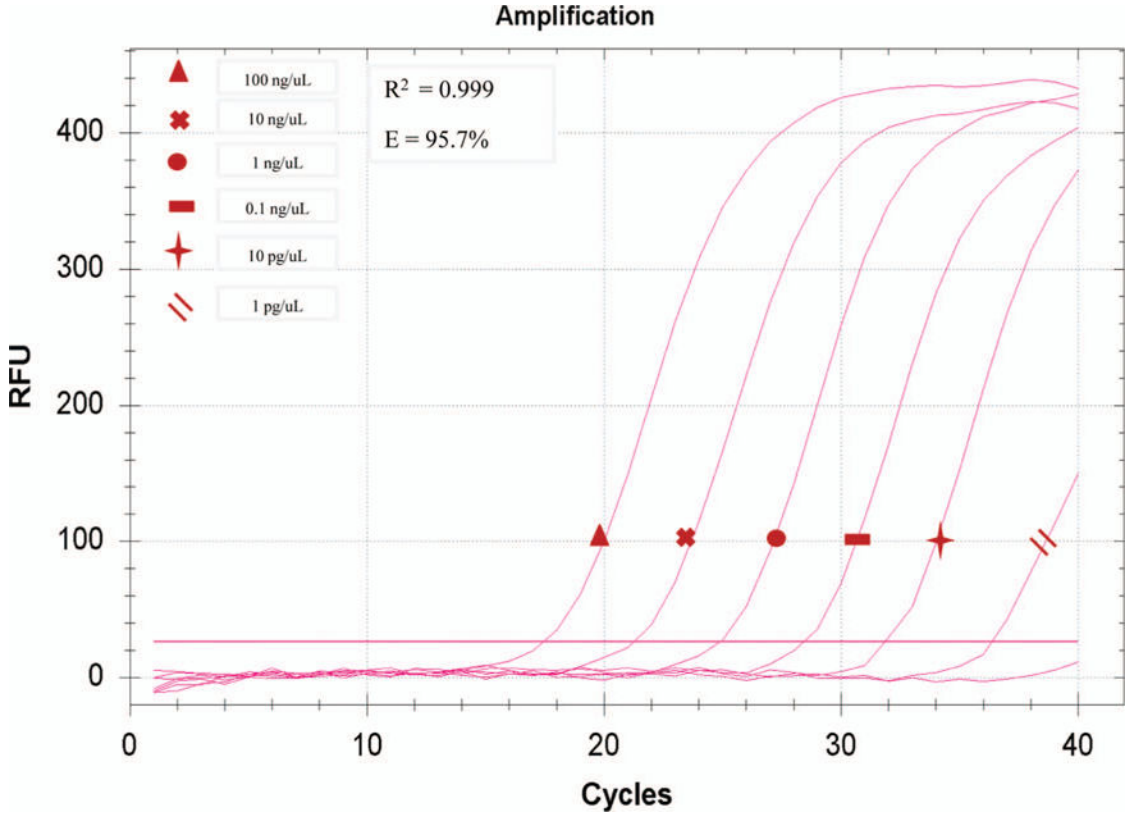

Fig. 2.

Reportable range of set no. 3 in multiplex reaction for detection of DNA isolated from *A. cajennense* adult tick using 10-fold serial dilution. (Online figure in color.)

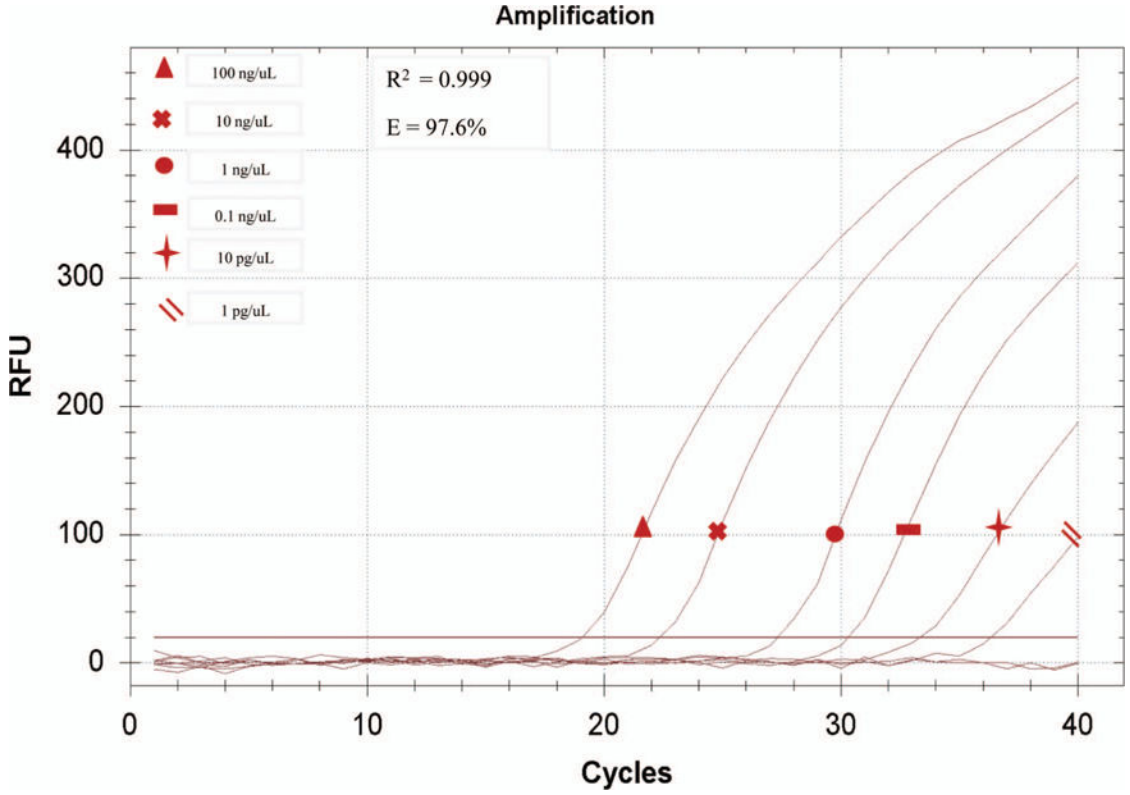

Fig. 3.

Reportable range of set no. 4 in multiplex reaction for detection of DNA isolated from *A. maculatum* adult tick using 10-fold serial dilution. (Online figure in color.)

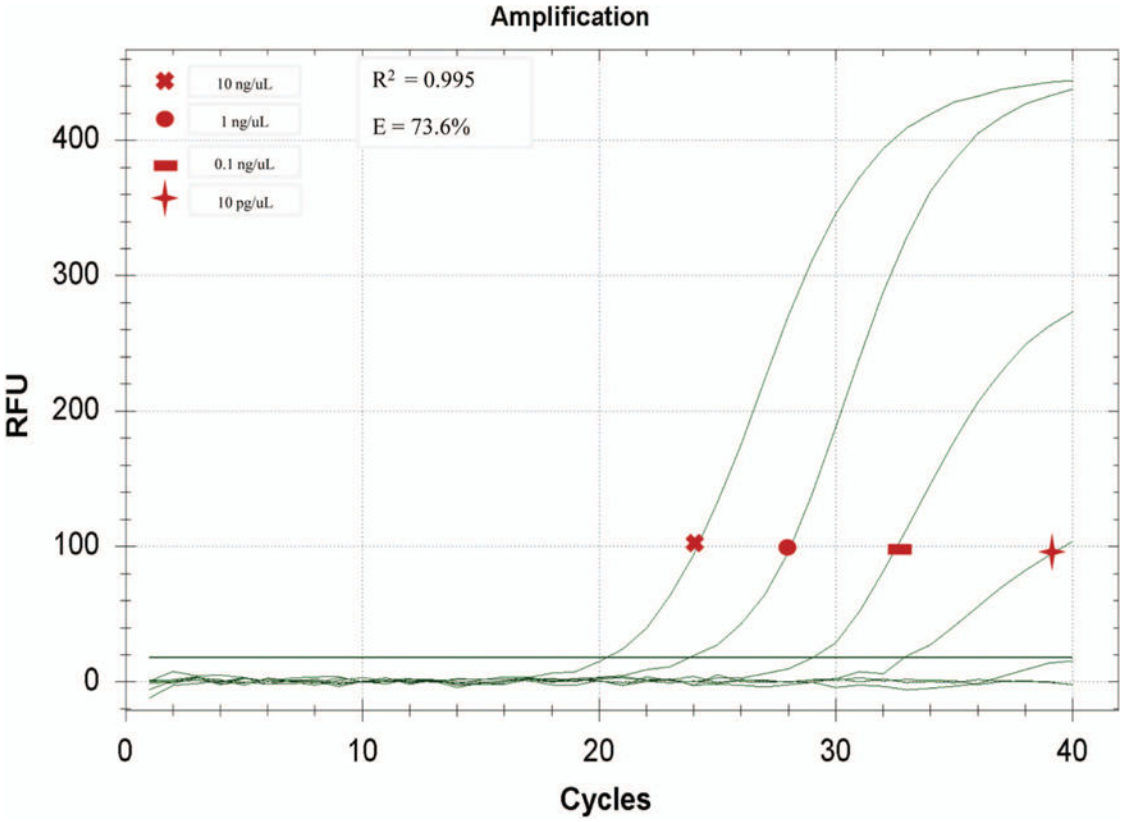

Fig. 4.

Reportable range of set no. 1 in multiplex reaction for detection of DNA isolated from *A. americanum* adult tick using 10-fold serial dilution. (Online figure in color.)

Table 1

Primer pairs and probes for multiplex qPCR assay discriminating *A. americanum*, *A. cajennense*, and *A. maculatum*

Set no.	Target species	Name	Sequence 5′–3′	Length	Tm (°C)	Final concn	Amplicon length (bp)	Reference
1	*A. americanum*	AamITS2ShF	AAGCCCGCGCTCCAAGC	17	62	100 nMol	214	Shone et al. 2006
		AamITS2ShR	GCAGCAGTTCGGCTACACGTA	21	60	100 nMol		
		AamITS2ShHEX	ACGACGTAACGCGGGACGGC	20	65	50 nMol		
2	*A. americanum*	AamITS2VF	CCTCCTCGAACGGGCGCAAAGTCG	24	72	100 nMol	253	This study
		AamITS2VR	TAACGCAGAGAGTTTCGAGCCC	22	65	100 nMol		
		AamITS2VHEX	ATTTTTCCCTGCCGCACCTCTC	22	70	300 nMol		
3	*A. cajennense*	AcajITS2F	CGAATGCGGTGTGCGCAGCACAGT	24	70	100 nMol	119	This study
		AcajITS2R	ACTTCGCGCCCACTCGAGGAG	21	72	100 nMol		
		AcajITS2CR610	TCCCTTTCCGCAGGGTCCGG	20	72	100 nMol		
4	*A. maculatum*	AmacITS2F	TTGTGCGGGAAACGACCGGGTGT	23	65	500 nMol	193	This study
		AmacITS2R	AACGCTCGTAACGAGATACGCG	22	65	500 nMol		
		AmacITS2Q670[a]	ACAATGCTTGAGCAGA + G +AGAC	21	62	500 nMol		

[a] + N, locked nucleic acid.

Table 2

Identification of individual ticks by MMix I and MMix II in a multiplex qPCR assay

Tick species (origin)	Life stage	Engorgement status	Species identification by MMix I			Species identification by MMix II		
			***A. americanum* set no. 1**	***A. cajennense* set no. 3**	***A. maculatum* set no. 4**	***A. americanum* set no. 2**	***A. cajennense* set no. 3**	***A. maculatum* set no. 4**
A. americanum (GA)	Larva	Flat	5/5	–	–	5/5	–	–
		Engorged	5/5	–	–	5/5	–	–
	Nymph	Flat	5/5	–	–	5/5	–	–
		Engorged	5/5	–	–	5/5	–	–
	Adult	Flat	5/5	–	–	5/5	–	–
		Engorged	5/5	–	–	5/5	–	–
A. americanum (FL)	Adult	Flat	10/10	–	–	8/10	–	–
A. americanum (VA)	Adult	Flat	10/10	–	–	7/10	–	–
A. americanum (MO)	Adult	Flat	10/10	–	–	8/10	–	–
A. cajennense (TX)	Larva	Flat	–	5/5	–	–	5/5	–
		Engorged	–	5/5	–	–	5/5	–
	Nymph	Flat	–	5/5	–	–	5/5	–
		Engorged	–	5/5	–	–	5/5	–
	Adult	Flat	–	5/5	–	–	5/5	–
		Engorged	–	5/5	–	–	5/5	–
A. maculatum (GA)	Larva	Flat	–	–	5/5	–	–	5/5
		Engorged	–	–	5/5	–	–	5/5
	Nymph	Flat	–	–	5/5	–	–	5/5
		Engorged	–	–	5/5	–	–	5/5
	Adult	Flat	–	–	5/5	–	–	5/5
		Engorged	–	–	5/5	–	–	5/5
A. maculatum (VA)	Adult	Flat	–	–	10/10	–	–	10/10
A. tuberculatum (GA)	Adult	Flat	–	–	–	–	–	–
A. ovale (Brazil)	Adult	Flat	–	–	–	–	–	–
A. rotundatum (Brazil)	Adult	Flat	–	–	–	–	–	–
A. imitator (TX)	Adult	Flat	–	–	–	4/5[a]	–	–

Tick species (origin)	Life stage	Engorgement status	Species identification by MMix I			Species identification by MMix II		
			***A. americanum* set no. 1**	***A. cajennense* set no. 3**	***A. maculatum* set no. 4**	***A. americanum* set no. 2**	***A. cajennense* set no. 3**	***A. maculatum* set no. 4**
A. triste (Brazil)	Adult	Flat	–	–	5/5[a]	–	–	5/5[a]

[a]Misidentification.

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Table 3

Identification of mixed pools by Master Mix I and II in a multiplex qPCR assay

Species combination	Life stage	No. of ticks in pool	No. of samples correctly identified by					
			MMix I			MMix II		
			A. americanum set no. 1	*A. cajennense* set no. 3	*A. maculatum* set no. 4	*A. americanum* set no. 2	*A. cajennense* set no. 3	*A. maculatum* set no. 4
Aa + Ac	Larva	1Aa + 1Ac	_[a]	10/10	–	10/10	10/10	–
		1Aa + 4Ac	–	10/10	–	10/10	10/10	–
		4Aa + 1Ac	_[a]	10/10	–	10/10	10/10	–
	Nymph	1Aa + 1Ac	_[a]	10/10	–	10/10	10/10	–
		1Aa + 4Ac	–	10/10	–	10/10	10/10	–
		4Aa + 1Ac	_[a]	10/10	–	10/10	10/10	–
Aa + Am	Larva	1Aa + 1Am	–	–	10/10	10/10	–	10/10
		1Aa + 4Am	–	–	10/10	10/10	–	10/10
		4Aa + 1Am	–	–	10/10	10/10	–	10/10
	Nymph	1Aa + 1Am	–	–	10/10	10/10	–	10/10
		1Aa + 4Am	–	–	10/10	10/10	–	10/10
		4Aa + 1Am	–	–	10/10	10/10	–	10/10
Am + Ac	Larva	1Am + 1Ac	–	10/10	10/10	–	10/10	10/10
		1Am + 4Ac	–	10/10	10/10	–	10/10	10/10
		4Am + 1Ac	–	10/10	10/10	–	10/10	10/10
	Nymph	1Am + 1Ac	–	10/10	10/10	–	10/10	10/10
		1Am + 4Ac	–	10/10	10/10	–	10/10	10/10
		4Am + 1Ac	–	10/10	10/10	–	10/10	10/10
Aa + Ac + Am	Larva	1Aa + 1Ac + Am	–	10/10	10/10	5/10	10/10	10/10
	Nymph	1Aa + 1Ac + Am	–	10/10	10/10	7/10	10/10	10/10

Aa, A. americanum; Ac, A. cajennense; Am, A. maculatum.

[a] suboptimal amplification.