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Non-infectious plasmid engineered to simulate multiple viral threat agents

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Crimean-Congo virus

A B S T R A C T

The aim of this study was to design and construct a non-virulent simulant to replace several pathogenic viruses in the development of detection and identification methods in biodefense. A non-infectious simulant was designed and engineered to include the nucleic acid signature of VEEV (Venezuelan Equine Encephalitis virus), Influenza virus, Rift Valley Fever virus, Machupo virus, Lassa virus, Yellow Fever virus, Ebola virus, Eastern Equine Encephalitis virus, Junin virus, Marburg virus, Dengue virus, and Crimean-Congo virus, all in a single construct. The nucleic acid sequences of all isolates available for each virus species were aligned using ClustalW software in order to obtain conserved regions of the viral genomes. Specific primers were designed to permit the identification and differentiation between viral threat agents. A chimera of 3143 base pairs was engineered to produce 13 PCR amplicons of different sizes. PCR amplification of the simulant with virus-specific primers revealed products of the predicted length, in bands of similar intensity, and without detectable unspecific products by electrophoresis analysis. The simulant described could reduce the need to use infectious viruses in the development of detection and diagnostic methods, and could also be useful as a non-virulent positive control in nucleic acid-based tests against biological threat agents.

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1. Introduction

Viruses and their diseases have been one of the greatest threats to mankind ever since the threat of large predators was mostly controlled several centuries ago and the risk of bacterial disease was reduced by antibiotics during the 20th century. As well as the mortality produced by naturally occurring viruses, there is the additional risk of the potential use of viral agents for warfare or terrorism. The viruses of particular interest to biodefense are those that cause acute diseases which can be generally transmitted from human to human, thus causing epidemics with high mortality rates (Eitzen et al., 1998). The etiological agent of these diseases include RNA viruses that produce either (i) hemorrhagic fever like Ebola [EBOV] and Marburg [MBGB] viruses (classified in the *Filoviridae*), Lassa (LASV), Junin and Machupo viruses (*Arenaviridae*), Crimean-Congo hemorrhagic fever (CCHFV) and Rift Valley Fever (RVFV) viruses (*Bunyaviridae*) and Yellow fever virus (YFV) and dengue virus (DENV) (viruses in the *Flaviviridae*), (ii) acute encephalitis (Eastern (EEEV) and Western (WEEV) equine encephalitis viruses), or (iii) endemic influenza (influenza A virus, a member of the Orthomyxoviridae family) (Knipe & Howley, 2001).

The definitive diagnosis of viral hemorrhagic fever, encephalitis or influenza relies mainly on laboratory testing. Several of the viruses listed above have been identified individually by PCR (Demby et al., 1994; Deubel et al., 1997; Ibrahim et al., 1997; Leroy et al., 2000) and their diagnosis has been expedited with the development of real time PCR (Drosten et al., 2002; Yapar et al., 2005; Bird et al., 2007). However, the majority of nucleic acid-based tests, including most PCR methods, only target individual viruses. The current need for batteries of different tests against each individual potential virus impede rapid detection and identification, particularly during the early stages of an epidemic (natural or man-made) or while the etiological agent of an outbreak is still unknown. Multiplex PCR has been developed in an attempt to detect several viruses simultaneously (Elnifro et al., 2000). Simultaneous detection of several (up to 14) respiratory viruses has been accomplished by multiplex PCR (Syrmis et al., 2004; Coiras et al., 2004). Another strategy that uses real time reverse transcription-PCR assay to detect up to six viral hemorrhagic fever viruses in parallel was described (Drosten et al., 2002). It was reported recently that multiplex real time PCR can detect simultaneously three influenza and four parainfluenza viruses in two different reactions (Templeton et al., 2004). A multiplex of conventional RT-PCR followed by agarose gel electrophoresis

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Table 1
Selected sequences and products of virus amplification.

Viruses[a] and isolations	Complete genome[b]	Primer sequences	Length[c] (bp)	Tm[d]	Quality[e]	PCR Product (bp)	
						Simulant[f]	Pathogen[g]
VEEV	6	GTTAGTTGCGACGGGTACGT GCAGCACAAGAATCCCTCGCG	20 21	58.1 62.3	99 113	108	245
Influenza, 8 segments	2000	ACCATTTGAATGGATGTCAATCCGAC GACTGTGTCCATGGTGTATCCTGTTC	26 26	58.4 59.9	108 68	138	256
RVFV	1	ATGAGTGCACTGCTCAGTACGCCA GCAAGGCTCAACTCTCTGGATGGGCT	24 26	63 65	144 130	170	280
Machupo, S segment (5), L segment (4)	9	CATTCATCATGTCTAAAGCAATGC GGCTGTGAAGCCTAAAGTGGTGAGA	24 25	54.1 61.4	106 96	200	292
Lassa, S segment (3), L segment (7)	10	ATCCTGGGTGACCACTTCAT GCAATAGAACTGGGTGCATGGGTCTT	20 26	56.8 61.9	73 95	245	118
Yellow Fever	18	CTAAGCTGTGAGGCAGTGCAGGCTGG AGGTCTGCTTATTCTTGAGCAAACTG	26 26	66.0 58.1	126 116	268	128
Ebola Zaire	5	GCAATTGCACTCGGAGTCGCCACAGC CACTTGTTTTGGGCAGTGACGCAGCA	26 26	67 65	150 138	304	149
EEEV	6	TACTTGTCTGCGGCGCCTTGGGCG CGCAGAAGCAGTAGGCTCCTCC	24 22	68.2 62.7	104 116	321	160
Junin, S segment (2), L segment (5)	7	ACCTCTGATCCAGACATGCAGTCGA AAACAGGTGAGAAGTCAAGGTGCT	25 24	62 59	144 93	355	180
Marburg	6	GAAGTTGCTAGTTTCAAGCAGGCGT TTTCTCGTTTCTGGCTGAGGACGGC	25 25	60.5 63.6	128 111	376	190
Actin (+)[h]		CGCGAGCACAGAGCCTCGCCTTTGCC GAGAAGATGACCCAGATCATGTTTG	26 25	70.0 56.6	122 86	435	435
Dengue	190	TCAATATGCTGAAACGCGAGAGAAACCG TTGCACCAACAGTCAATGTCTTCAGGTTC	28 29	61.3 61.6	88 86	511	210
Crimean Congo, S segment (29), M segment (24), L segment (10)	63	TTGATGATGAGCATGTCAGGCAT AATTCCCTCATAAGGTGTGCCAAT	23 24	57.7 57.5	106 105	549	220

[a] All genomes used in this work were downloaded from the respective viral sequences in Genbank on April 2007 [NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov)]. In parenthesis are the genomic segments in each virus, corresponding to (1) mono-segmented genomes, or (S, M, L) to small, medium, and large segments, respectively. Numbers in parenthesis indicate the number of virus segment's sequences included in this study.

[b] Number of complete genome sequences reported in NCBI. The number of complete genomes corresponded to the sum of all the segment sequences in those cases in which the virus has more than one segment.

[c] Primer lengths as number of nucleotides.

[d] Primers were selected with similar annealing temperature (Ta) and melting temperature (Tm) to improve the amplification efficiency in multiplex PCR assays.

[e] The efficiency of primers was predicted by using the FAST PCR software (see Methods and results). High quality values correlate with high amplification efficiency. Maximal quality values for primers 24–26 nucleotides in length are near 150.

[f] In silico PCR prediction for amplicon size amplified from the simulant.

[g] In silico PCR prediction for amplicon size resulting from the viral pathogen.

[h] The chimerical simulant included as a control a 450 bp sequence from the actin gene that is frequently used as a positive control of reverse transcriptase activity in commercial kits.

was described also as a method for typing and subtyping influenza viruses (Boonsuk et al., 2008). The development of methods to detect simultaneously threat viruses has been delayed by the nearly insurmountable logistic and economic difficulties that are involved in producing a number of different highly pathogenic viruses, isolating and characterizing them under adequate bio-containment, and preparing a representative control of each agent for evaluation of the testing method.

The use of nucleic acids from infectious bio-threat viruses as positive controls for testing is demanding also due to restrictions in handling and producing these organisms. Method development using heat or radiation-inactivated organisms (or their nucleic acids) instead of viable viruses still involves the production of virulent viruses at high risk and cost before inactivation. Threat viruses have been replaced occasionally in methods testing by the phage MS2, which is the viral simulant generally used during the development of countermeasures in biodefense (O'Connell et al., 2006). However, MS2 is particularly inadequate for evaluating the specificity and sensitivity of nucleic acid-based technologies because the phage does not share nucleic acid targets with any threat virus. Thus, the identification of viral threat agents is hindered by the lack of an adequate simulant to be used in the development of advanced detection systems and then used as a non-infectious positive control during testing.

We reported previously a chimerical molecule engineered to simulate nine bacterial threat agents and variola major (Carrera and Sagripanti, 2009) but this simulant did not contain any sequence corresponding to RNA viruses. A nucleic acid simulant consisting of sequences corresponding to herpes virus, varicella zoster, rickettsia akari, variola and other orthopox virus sequences distributed among three engineered plasmids was previously reported (Charrel et al., 2004). These plasmids were constructed by successive PCR amplifications of the desired sequences followed by T4 ligation. The goal of the present study was to develop a simulant that included specific sequences of the main viral threat agents to be represented in only one plasmid molecule. Here it is reported an approach that permitted the production of a single chimerical construct that included the nucleic acid signature of 12 viral agents, without requiring access to these viruses at any stage of the work.

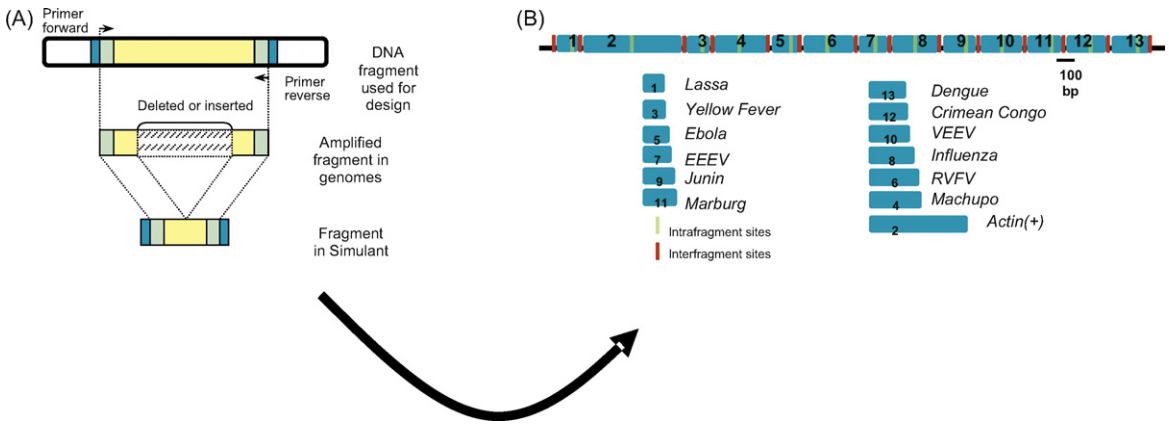

Fig. 1. Scheme of the designed chimerical molecule. Panel A: Yellow segments represent the genomic sequence of each threat agent. Specific sequences within the yellow segment that were selected for primer design appear in green. The deletion or insertion of nucleotides in the simulant (transversal lines) gives a smaller or larger amplified fragment depending on the threat agent. Panel B shows the organization of specific sequences (blue segments) corresponding to 12 threat viruses. Interfragment sites in red contain the specific sites for the enzymes BamHI (–GGATCC–) and HindIII (–AAGCTT–) and Intrafragment sites in yellow contain the specific sites for enzymes (EcoRI –GAATTC– and SmaI –CCCGGG–). The labels and abbreviations correspond to each of the virus described in the text. Actin (+) corresponds to a 450 bp sequence from the actin gene that is frequently used as a positive control of reverse transcriptase activity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2. Methods and results

2.1. Primer selection

A database was built with all the sequences available in the public domain for the viruses listed in Table 1 (the viral strains and accession number of each virus included in the study can be found in the Supplementary data #1). The Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/BLAST) was used to find regions of local similarity between sequences of different viral strains and isolates from the same species. The alignments of sequences from different strains were performed by ClustalX software (a windows interface to ClustalW multiple sequence alignment software) (Thompson et al., 1997) and the conserved regions in each viral species were identified. Further analysis of these conserved sequences was performed with Bioedit software [http://www.mbio.ncsu.edu/BioEdit/bioedit.html] to identify short sequences of at least 15 bases and up to 25–30 bases that were conserved in all isolates of each virus species (listed in Supplementary data #1).

All potential primers were generated by FastPCR, a program developed by Kalendar (2008) to design primers. This software predicted the amplification efficiency of each potential primer by assigning a relative quality value. Among those primers with higher quality values for each virus, specific primers were selected so that (i) were between 22 and 26 nucleotides long, (ii) produced a fragment between 200 and 500 bp long, and (iii) had a theoretical annealing temperature above 55 °C. Each potential primer was then compared against all the sequences in GenBank using BLAST and those (unspecific) primers that showed homology to any other genome were discarded. The primers that fulfilled all criteria indicated above were ultimately selected and the sizes of the amplified fragments produced by them in each target virus genome are indicated in Table 1.

2.2. Simulant design

The specific primers identified in each of the viral genomes were used to design a simulant that included as much of each viral sequence as possible. This provided the flexibility required to

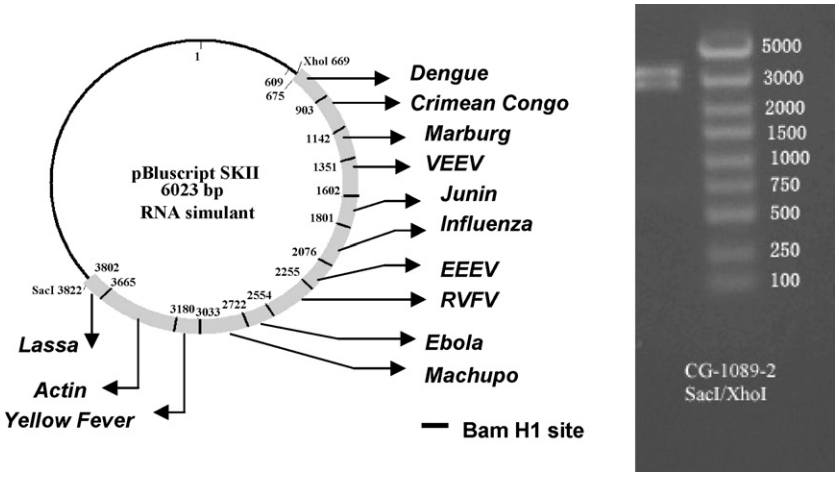

Fig. 2. Plasmid simulant construction. Panel A: Plasmid design indicating the relative position of sequences corresponding to each of the selected viruses. The drawing also indicates the relative location of sequences inserted to allow digestion with restriction enzyme Bam H1. Panel B: Quality control of the simulant inserted in plasmid pBluescript SK. Digital image of Bluscript II plasmid XhoI/SacI restriction digests analysed by gel electrophoresis on a 1% (w/v) agarose gel (at a potential of 3 V/cm and stained with ethidium bromide). The DNA size marker on the right lane is a commercial 1 kB ladder. The first lane represents two bands 3143 and 2880 bp, which correspond to the liberated simulant insert and (b) to the plasmid vector sequence, respectively.

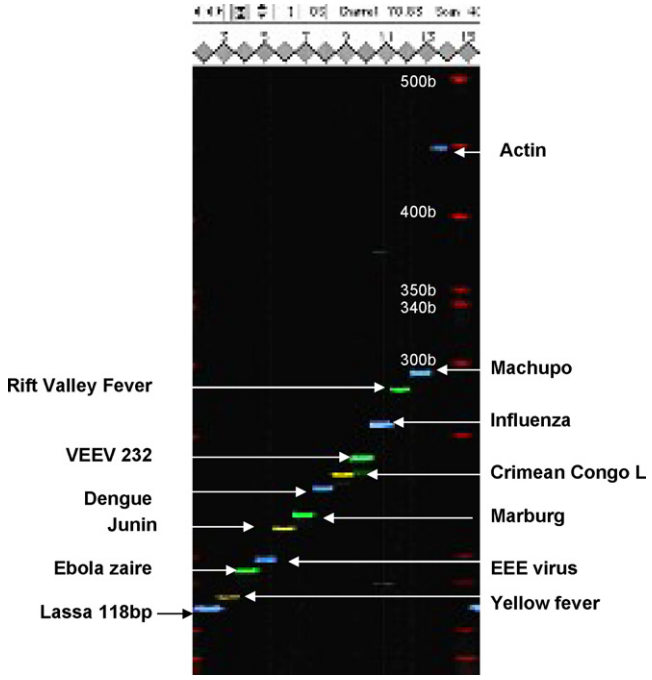

Fig. 3. Amplification of individual fragments. Electrophoresis of individual amplified fragments performed on an ABI 377 sequencer analyzer. The figure represents the reconstructed gel image showing the amplified fragments at the corresponding size and color given for the fluorescent label primer used. Fragments amplified using HEX (yellow) label primers, TET (green) label primers and FAM (blue) label primers. DNA fragments of known molecular weight labeled with TAMRA (red) were included as internal size standard. The fragment sizes were estimated by measuring their electrophoretic mobility through the gel relative to the internal size standard, as indicated by GeneScan 2.1 analysis software (PerkinElmer Corp., Foster City, CA). The accuracy of the size estimates, expressed to a fraction of a nucleotide unit, is specific to the electrophoretic separation conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

design capture or detection probes from the simulant. The size of fragments to be amplified using the simulant as a template were designed to be of different lengths than those fragments amplified by the same primers from the actual viral genomes (see sizes in Table 1). This difference in size between amplicons from simulant or viral origins should avoid false positive results due to any potential contamination with simulant sequences after repeated assaying.

The selected representative sequence was repeated partially in those particular viruses (VEEV, RVEV, Machupo, and Influenza) where our size specifications required a larger amplified product in the simulant than the amplicons obtained from the pathogen. Partially repeating viral sequences also increased the number of sites in the simulant that potentially could be used to produce detection probes. The sequences ultimately selected for each virus were combined into a single chimerical molecule design (Fig. 1). Two restriction enzyme sites were added between the fragments corresponding to each virus in the chimera to allow a digestion step to be performed before the amplification process. This digestion step was necessary to prevent the amplification of two consecutives fragments by primers at the farthest extremes which would result in amplicons longer than expected and therefore confusing results. Thus, restriction specific sites for the enzymes BamHI (–GGATCC–) and HindIII (–AAGCTT–) were introduced between each virus-specific fragment and also at the beginning and end of the chimerical molecule as shown schematically in Fig. 1B. In addition to the restriction sites for BamHI and HindIII described above, two other restriction sites (for EcoRI –GAATTC– and SmaI –CCCGGG–) were added inside the sequence corresponding to each viral fragment present in the chimerical simulant. EcoRI and SmaI are able to cut every amplicon derived from the simulant but these two enzymes do not cut any of the fragments amplified directly from the virus genome (Fig. 1B). A DNA simulant 3143 bp long (see Supplementary data #2 for the complete sequence) was synthesized and cloned in the SacI/XhoI site of the plasmid vector pBluescript II SK (+) by Celtek Bioscience (Nashville, TN). The construct map in Fig. 2B shows for orientation purposes one of the restriction enzyme sites (Bam H1) introduced in the molecule to allow multiplex amplification. The digestion of the constructed plasmid with two restrictions enzymes Xho1 and SacI liberated the synthetic simulant of the expected size as depicted in Fig. 2A.

2.3. Amplification of individual fragments from the simulant

Sequences corresponding to each biological agent were amplified independently by PCR reactions using the conditions described previously (Carrera and Sagripanti, 2009). Each product corresponding to each one of the viruses under study was amplified with the expected length (as indicated in Table 1) and without any detectable unspecific amplification products as shown in Fig. 3. The similar intensity of the bands indicate that the relative amplification rate, and hence the representation of sequences, of every threat virus in the synthetic simulant are comparable.

3. Discussion

The increasing interest in developing methods to detect and identify viruses of interest in biodefense is hindered by the difficulty involved in working with highly virulent organisms. This study describes the design and engineering of a novel chimerical molecule, and present experimental data to demonstrate that signatures from 12 viral threat agents can be amplified from the chimerical simulant.

In order to construct the proposed simulant, computational analysis was used to identify conserved sequences from which to select potential primers to amplify portions of each viral genome. After designing the chimera with specific sequences from multiple viruses, a relatively straightforward strategy involving the *di novo* synthesis of the selected sequence followed by cloning into a cloning vector, (Blue script II SK+) was then employed. This combination of bioinfomatic tools, chemical synthesis, and cloning avoided the bio-hazard involved in the manipulation of threat viruses and resulted in a considerable reduction in the cost of this study.

The analysis of restriction enzyme digested products and the PCR amplification of virus-specific sequences (Fig. 3) confirmed the design and synthesis of the intended simulant. This engineered multiplex simulant molecule could be used to replace pathogenic viruses in the performance evaluation of nucleic acid-based biodetectors and diagnostic products intended for biodefense. A single simulant could reduce the need to prepare and use individual virulent viruses or their RNA as targets during method development or as positive controls during the actual analysis of clinical specimens or environmental samples. The simulant described here could be used to evaluate or compare a variety of diagnostics and biological detectors without fear of exposing testers or trainees to pathogenic viruses. Thus, using the simulant described above should lead to a reduction in both the cost and risk of testing and developing detection and diagnostic technology for the viruses included in this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2009.02.021. Sequences are protected by intellectual property law, patent pending.

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