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Experimental and Theoretical Approaches To Investigate the Immunogenicity of *Taenia solium*-Derived KE7 Antigen

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ABSTRACT *Taenia solium* cysticercosis, a parasitic disease that affects human health in various regions of the world, is preventable by vaccination. Both the 97-amino-acid-long KETc7 peptide and its carboxyl-terminal, 18-amino-acid-long sequence (GK-1) are found in *Taenia crassiceps*. Both peptides have proven protective capacity against cysticercosis and are part of the highly conserved, cestode-native, 264-amino-acid long protein KE7. KE7 belongs to a ubiquitously distributed family of proteins associated with membrane processes and may participate in several vital cell pathways. The aim of this study was to identify the *T. solium* KE7 (TsKE7) full-length protein and to determine its immunogenic properties. Recombinant TsKE7 (rTsKE7) was expressed in *Escherichia coli* Rosetta2 cells and used to obtain mouse polyclonal antibodies. Anti-rTsKE7 antibodies detected the expected native protein among the 350 spots developed from *T. solium* cyst vesicular fluid in a mass spectrometry-coupled immune proteomic analysis. These antibodies were then used to screen a phage-displayed 7-random-peptide library to map B-cell epitopes. The recognized phages displayed 9 peptides, with the consensus motif Y(F/Y)PS sequence, which includes YYYPS (named GK-1M, for being a GK-1 mimotope), exactly matching a part of GK-1. GK-1M was recognized by 58% of serum samples from cysticercotic pigs with 100% specificity but induced weak protection against murine cysticercosis. *In silico* analysis revealed a universal T-cell epitope(s) in native TsKE7 potentially capable of stimulating cytotoxic T lymphocytes and helper T lymphocytes under different major histocompatibility complex class I and class II mouse haplotypes. Altogether, these results provide a rationale for the efficacy of the KETc7, rTsKE7, and GK-1 peptides as vaccines.

KEYWORDS cysticercosis, vaccine, KETc7, S3Pvac, proteomics, *Taenia solium*, *Taenia crassiceps*

Taenia solium cysticercosis is a public health concern and the cause of significant economic losses among rural pig breeders from nondeveloped countries in Latin America, Asia, and Africa, where it is endemic (1–3). The persistence of this ancient

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disease, closely linked to human poverty, low hygienic standards, and poor government performance, justifies the efforts to control it (4).

Porcine cysticercosis, a step required for *T. solium* transmission, is preventable by vaccination. Thus, a country-wide immunization program relying on an effective vaccine and including a large number of pigs naturally exposed to *T. solium* cysticercosis in rural Mexico could be an affordable and reliable approach to control and curtail disease transmission. Thus, several effective vaccines have been developed (5–8). However, the immunological features that underlie vaccine efficacy have been much less well explored.

It is usually assumed that an effective anticysticercosis vaccine must elicit robust humoral and cellular immunity against targets crucially important for parasite survival (9–12). However, despite these theoretical considerations, our work has identified the most promising vaccine candidates by selecting those that induce higher levels of protection against experimental *Taenia crassiceps* murine cysticercosis, disregarding the immunity elicited or the target involved (13). This approach allowed us to identify four different protective clones in a *T. crassiceps* cDNA library (14). One of them was a 97-amino-acid (aa) polypeptide named KETc7. Structural analysis of its sequence led to the identification of three epitopes with antigenic potential: GK-1, GK-2, and GK-3 (15). Among these, GK-1 induced the highest protection levels, ranging from 89% to 94% in mice immunized with GK-1 synthetically produced in a linear, uncoupled form (16).

KETc7 and GK-1 are highly conserved parasite targets and are extensively expressed in the different development stages of the helminth, i.e., the oncosphere, adult tapeworm, and cysticercus (16–18). Additional experiments using intramuscular or intradermal DNA vaccination confirmed the high protective capacity of KETc7 against murine cysticercosis (19). Furthermore, KETc7 was expressed in different delivery systems: the surface of the M13 bacteriophage and transgenic papaya clones (20, 21). GK-1 was expressed in the M13 bacteriophage and lumazine synthase from a *Brucella* sp. (BLS) as a protein carrier (22). When they were expressed in these systems, KETc7 and GK-1 provided high levels of protection against murine cysticercosis. After these promising results were obtained, both peptides were expressed in the M13 bacteriophage and included in the S3Pvac-phage vaccine.

Some of the immunogenic properties of KETc7 and GK-1 have already been established. They elicit an antibody (Ab) response and increase $CD8^+$ and $CD4^+$ T-cell proliferation with the production of high levels of gamma interferon and interleukin-2 (16, 18, 19).

In this study, the native *T. solium* KE7 protein, named nTsKE7, which includes the protective peptides KETc7 and GK-1, was identified in the genome of *Taenia solium*. Its structural and immunogenic properties were experimentally and theoretically analyzed to understand why it may be a vaccine target in the parasite and to learn that vaccination with the full-length protein is not likely to be an improvement over that with the shorter peptide.

RESULTS

Sequence analysis of nTsKE7. The sequence of the complete native protein that includes the 97-aa protective peptide KETc7 and GK-1 was compared with the sequence of the complete *T. solium* genome (23). This search allowed us to identify a putative protein (TsM_000832200), localized in scaffold00063 29898-scaffold00063 32178, that comprised five introns and six exons, accounting for 795 nucleotides and a 264-aa final product (Fig. 1). According to the conserved domain prediction, the nTsKE7 protein contains a Pleckstrin homology (PH)-like domain from amino acids 1 to 124, which includes a GRAM domain (glucosyltransferases, Rab-like GTPase activators, and myotubularins) from aa 19 to 82, and the WW binding protein (WWbp) domain (a repeating proline-rich domain that binds repeated proteins with tryptophan residues) (6). The latter includes part of the KETc7 peptide but not GK-1, from aa 100 to 217 (Fig. 1). nTsKE7 has an isoelectric point (pI) of 7.77 and a hydrophobicity level of 0.42; it

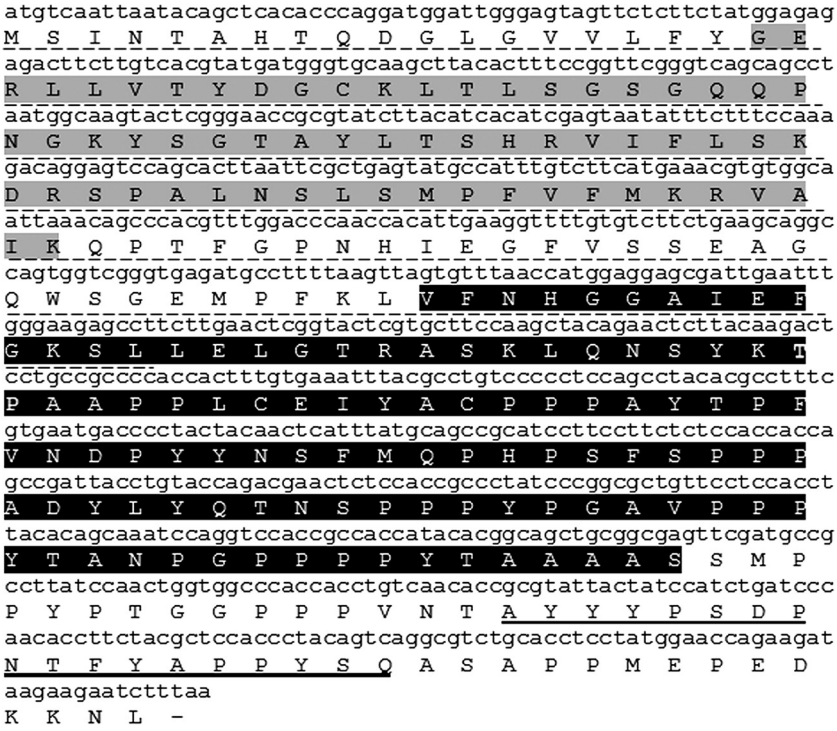

FIG 1 Nucleotide and amino acid sequences of the native *Taenia solium* full-length KE7 protein (nTsKE7). The GK-1 (232 to 250 aa) sequence and the PH-like domain (1 to 124 aa) are underlined with a solid line and a dashed line, respectively. The GRAM domain (aa 19 to 82) and the WWbp domain (aa 100 to 217) are depicted with gray and black backgrounds, respectively.

includes 30 predicted glycosylation sites and no evidence of transmembrane regions. A total of 20 phosphorylation sites were identified, and these were two times more frequent in the carboxyl terminus than in the rest of the protein.

KE7 is highly conserved in other helminths. The *T. solium* KE7 sequence was found to be identical in both Mexican and Chinese *T. solium* genomes (see Fig. S1 in the supplemental material). A neighbor-joining tree (Fig. 2) was inferred with the sequences of 28 taxa (11 Platyhelminthes, 4 Nematoda, 1 Brachiopoda, 2 Annelida, 3 Mollusca, 3 Arthropoda, and 4 Mammalia). The tree yielded one big cluster consisting of the sequences of the 11 flatworms and received strong bootstrap support of 99%. However, two subclusters were found; the first included three species of the class Trematoda, and the second consisted of six species of the class Cestoda (including TsKE7 from *T. solium*) with 100% bootstrap support. The genetic divergence among the six species of taeniids was very low, ranging from 0.6% to 9.8%, whereas the genetic divergence of the sequence of *T. solium* from the sequences of *T. saginata* and *T. crassiceps* ranged from 0.6% to 3.7%.

Experimental search for B-cell epitopes in nTsKE7. Figure 3 shows two-dimensional (2D) maps of vesicular fluid (VF) and soluble protein extracts from *T. solium* cysts obtained from the skeletal muscle of naturally cysticercotic pigs. A complex group of approximately 350 spots with a wide range of molecular masses and isoelectric points was found. An anti-recombinant TsKE7 (anti-rTsKE7) recognized the respective recombinant protein employed for its production (experimental molecular mass, 31 kDa; experimental pI, 7.2 to 7.4). It also recognized nTsKE7 in vesicular fluid but not in soluble extracts from *T. solium* cysticerci. On the basis of the reactivity of the anti-rTsKE7 Abs, these polyclonal antibodies were then used for epitope mapping by screening a phage-displayed random peptide library. Table 1 shows the sequences of nine peptides independently isolated after three panning rounds. The recognition frequency and the consensus amino acid sequence are also shown.

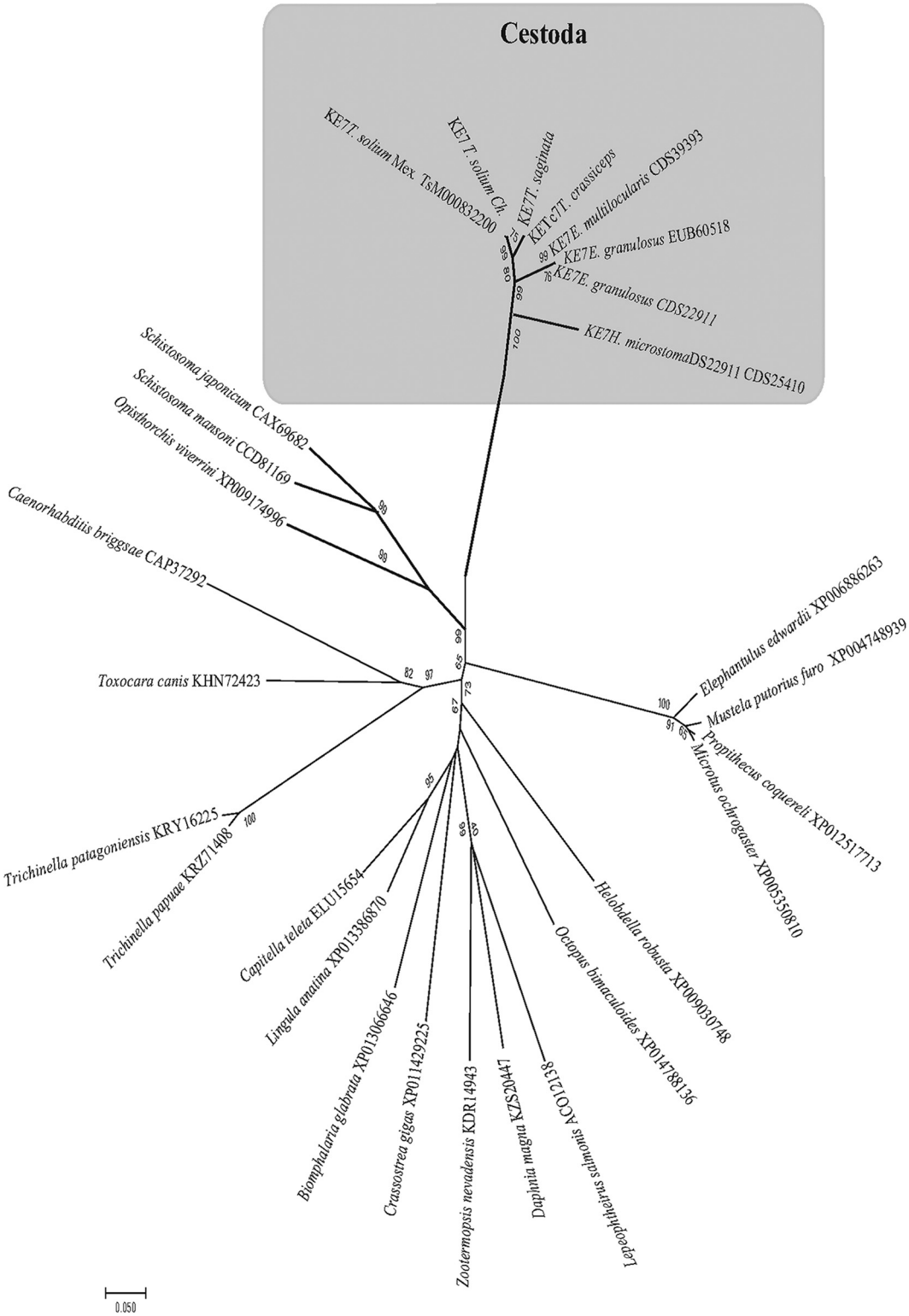

FIG 2 Neighbor-joining tree, inferred from a data set consisting of the sequences of 28 taxa with 216 characters. Numbers near the nodes indicate the percentage values of the bootstrap replicates. *H. microstoma*, *Hymenolepis microstoma*.

Evaluating the antigenic density using the AAR value. The abundance of antigenic regions (AAR) value determines the number of amino acids that are needed to find one antigenic region in the corresponding sequence (see Materials and Methods). Hence, a low AAR value means that a protein has more antigenic regions (i.e., a higher

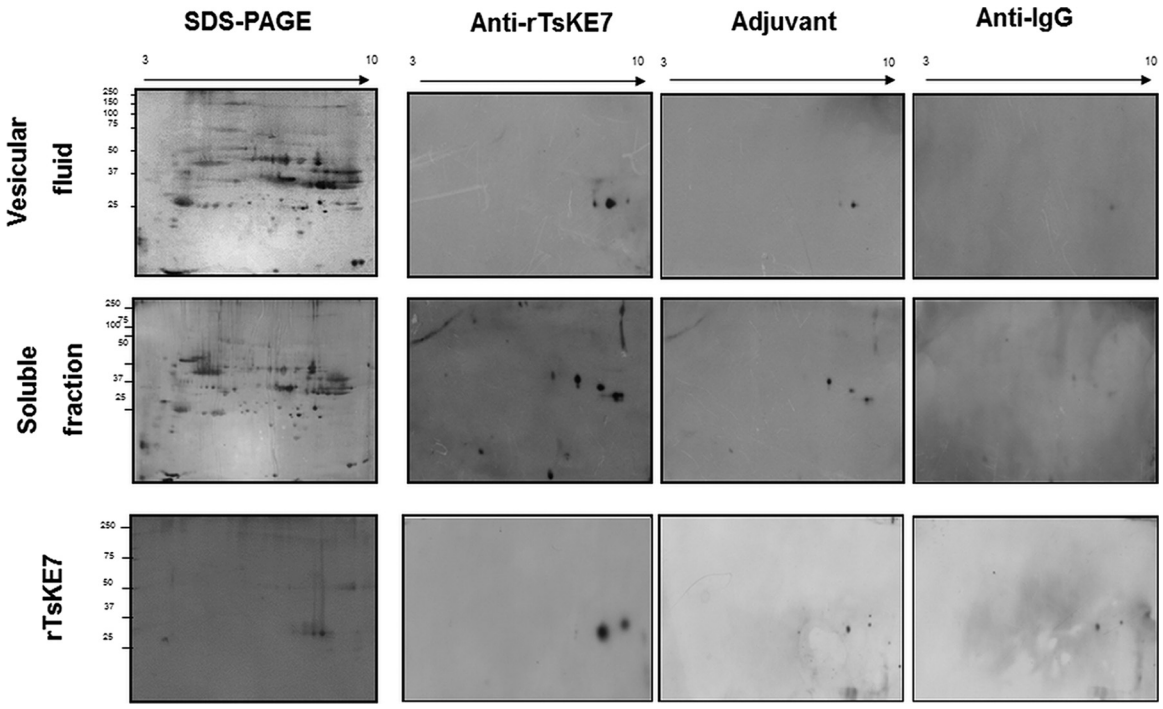

FIG 3 Representative two-dimensional gel electrophoresis analysis (SDS-PAGE) of cysticercus antigens recognized by anti-rTsKE7 mouse antibodies in vesicular fluid and soluble protein extract of *Taenia solium* cysticerci. Three independent experiments were performed. Numbers to the left of the gels are molecular masses (in kilodaltons), and the numbers at the top indicate the isoelectric point gradient.

epitope density). The AAR value for the nTsKE7 protein was 44.2, as estimated by BepiPred analysis. This value is lower than the previously established AAR value for the antigenic experimental *T. solium* secretome, 81.3 (24), which suggests a higher density of antigenic regions in the nTsKE7 protein. This result is consistent with the immunogenic properties reported herein for the rTsKE7 protein.

Antigenic capacity. As shown in Fig. 4, all serum samples from *T. solium* cysticercotic pigs recognized the cysticercal vesicular fluid antigens, as well as the rTsKE7 protein and the GK-1 peptide, while 58% (7/12) recognized GK-1M (the GK-1 mimotope) with 100% specificity. None of the infected serum samples recognized the nonrelated RV1818C recombinant protein (PE.6His) or the nonrelated 16-aa peptide.

***In silico* search for T-cell epitopes on nTsKE7.** Seven and three peptides with the highest scores for an association with major histocompatibility complex class I (MHC-I) and MHC-II, respectively, were identified in the nTsKE7 sequence. The one that exhibited the highest score for an association with MHC-II (TFYAPPYSQASAPPM) included the last 9 amino acids of the GK-1 sequence (Fig. 5). The GK-1 peptide was then searched for sequences with prediction results for MHC classes I and II. As shown in Table 2, GK-1 contains conspicuous peptides that bind well to the *H-2D*, *H-2K*, and *H-2L* genes in the *d* and *b* haplotypes. It can also bind MHC class II molecules (H-2A and H-2E), although with a lower strength score.

TABLE 1 Peptide sequences displayed by the selected phages that bind anti-rTsKE7

Amino acid sequence[a]	Frequency[b]
Y**Y**F**P**S	5
F**Y**F**P**S	2
Y**YYP**S	1
W**Y**Y**P**T	1

[a]Consensus amino acids are highlighted in boldface type.
[b]Frequency indicates the number of times that each sequence was independently isolated after three biopanning rounds.

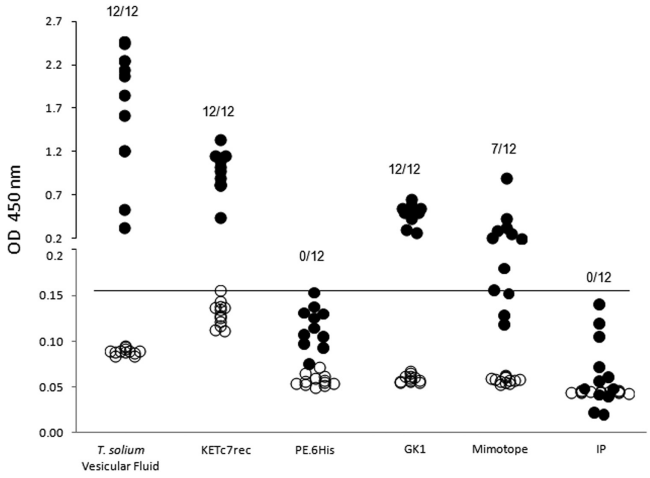

FIG 4 Recognition of *T. solium* vesicular fluid antigens, rTsKE7, the nonrelevant protein PE.6His (RV1818C recombinant protein with a tail of histidines), the GK-1 and GK-1M peptides, and an irrelevant peptide (IP) by sera from 12 noninfected pigs (○) and 12 pigs infected with *Taenia solium* cysticerci (●), as determined by ELISA. A sample was considered positive when the value for the sample was higher than the corresponding cutoff value, indicated by a horizontal line. The number of positive serum samples/number of negative serum samples is indicated for each antigen. All serum samples from cysticercotic pigs were recognized by GK-1 and rTsKE7. The ROC curve was employed to determine the optimal cutoff value. A statistically significantly ($P < 0.03$) lower percentage of serum samples (58%) was recognized by GK-1M according to Fisher's exact test.

Protective capacity. Figure 6 shows that immunization with rTsKE7 and GK-1 emulsified in saponin significantly reduced the parasite load by 59.3% and 56.7%, respectively, compared with that for animals that received saponin alone. A nonstatistically significant reduction of 47% was observed in mice immunized with recombinantly expressed GK-1M compared with that for animals immunized with M13 alone ($P = 0.10$).

DISCUSSION

In this study, the 97-aa KETc7 peptide (which contains the protective peptide GK-1) was found to be part of a 264-amino acid, cestode-conserved, native protein named KE7. This protein includes one GRAM domain and one WWbp domain; the former is totally included in the PH domain, and the latter includes only the carboxyl terminus of the KETc7 peptide. The 70-aa length of the GRAM domain has been reported to be conserved in many different membrane proteins from yeasts to higher eukaryotes. The family of proteins that include this domain is likely to be involved in membrane-

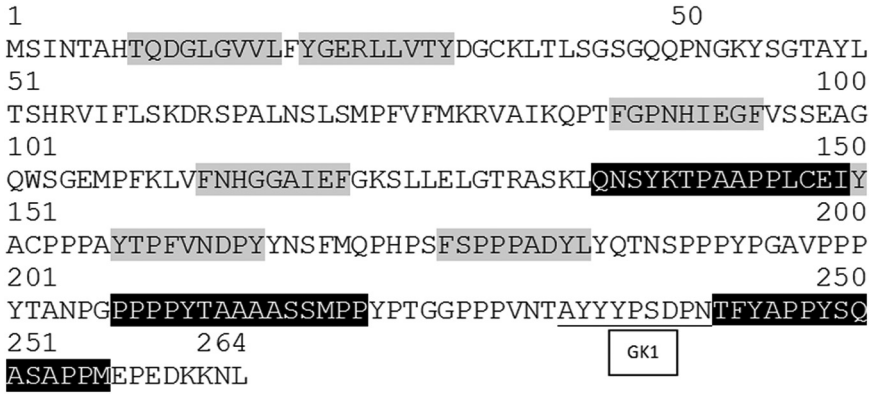

FIG 5 Epitope prediction for MHC class I and II in the native KETc7 protein sequence using the IEDB and SYFPEITHI web servers. Sequences with higher score for class I (gray) and class II (black) epitopes are indicated. The underlined sequence corresponds to that of the GK-1 peptide.

TABLE 2 Epitope prediction of the GK-1 peptide

Gene	Haplotype	Sequence	Score
MHC-I			
H-2D	*b*	YYPSDPNTF	14
		PSDPNTFYA	13
		PSDPNTFYAP	11
		YYYPSDPNTF	10
H-2K	*d*	TFYAPPYSA	16
		YYYPSDPNT	15
		YYPSDPNTFY	14
		YYPSDPNTF	12
		GYYYPSDPNT	10
		YYYPSDPNTF	10
		PSDPNTFYA	10
	b	PNTFYAPP	11
		DPNTFYAP	10
	d	YPSDPNTFY	13
		PSDPNTFYA	13
		YYPSDPNTF	12
		DPNTFYAPP	10
MHC-II			
H-2IA	*d*	YYYPSDPNTFYAPPY	8
		YYYPSDPNTFYAPPY	10
	k	YPSDPNTFYAPPYSA	10
H-2IE	*d*	GYYYPSDPNTFYAPP	10
	k	GYYYPSDPNTFYAPP	10

associated processes, such as intracellular protein or lipid binding (25, 26). In KE7, it is accompanied by the WWbp domain. The latter is characterized by several short PY- and PT-like motifs of the PPPPY form (Fig. 1). This domain was found in different structural and signaling proteins that are involved in a variety of cellular processes (27–30). The C terminus of nTsKE7 shows other PPY motifs, not included in the WWbp domain, that may promote protein-protein interactions, as reported in other proline-rich proteins (31). The phosphorylation status of the tyrosine in the PPY motifs may mediate many biological signaling outcomes critical for parasite survival (23, 32).

Alignment of the amino acid sequence of KE7 with the sequences of other reported cestode genomes and phylogenetic analysis showed that KE7 is highly conserved among different cestodes (see Fig. S1 in the supplemental material). Indeed, the KE7 sequence is identical in Mexican and Chinese *T. solium* cysticerci. Furthermore, GK-1 and the native KE7 protein were previously identified in the *Echinococcus multilocularis*

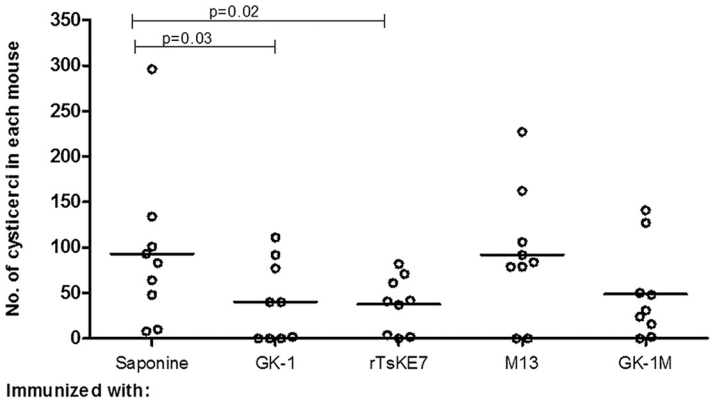

FIG 6 Protection induced by subcutaneous immunization with saponin, rTsKE7 (1 μg per mice), synthetic GK-1 (10 μg per mouse), M13, and GK-1M expressed in M13 at 1×10^{10} PFU two times every 10 days. Mice were challenged 15 days after the second immunization and sacrificed 30 days later. Each circle corresponds to the number of cysticerci recovered in each mouse. The number of parasites was compared using the nonparametric Mann-Whitney test.

genome (33). The presence of this conserved sequence among various cestodes indicates its possible important role in the biology and pathogenesis of *T. solium*, even though its biological function remains unknown; this issue merits further studies.

Once evidence pointed out that nTsKE7 may be critically involved in functions vital for parasite survival, it is important to identify the protein epitopes involved in its capacity to induce the robust humoral and cellular immunity previously reported (16, 18).

To identify possible B-cell epitopes, specific antibodies against rTsKE7 were produced. An anti-rTsKE7 Ab specifically recognized the 31-kDa native protein among 304 spots detected in *T. solium* cysticercus protein extracts, as shown by proteomic assays. Thus, this antibody was employed to search for KE7 epitopes. The most remarkable finding was that all antibody-binding phages contained the motif Y-F/Y-P-S/T (Table 1). Interestingly, all of them were included in the GK-1 sequence, a finding that suggests the relevance of linear B-cell epitopes in the KE7 protein. Indeed, the sequence of one of the motifs, named GK-1M (YYYPS), exactly fit the sequence of GK-1 and the C terminus of the KE7 protein. However, GK-1M exhibited a lower antigenicity and immunogenicity than the other motifs (Fig. 4 and 6), probably due to the presence of additional motifs in GK-1, like FY, also detected among those selected (Table 1). The similar antigenicity and immunogenicity observed between GK-1 and rTsKE7 (Fig. 4 and 6) is a remarkable result that points to GK-1 possibly being the most relevant peptide for recognition and protection in the entire protein. It is important to note that a much lower reactivity against GK-1 was previously reported, probably due to the presence of lower levels of anti-*T. solium* antibodies in the serum samples employed (15).

In addition, the sequence of GK-1 was almost identical in the different *Taenia* species searched and in *Echinococcus granulosus* and *E. multilocularis* (Fig. 2). Thus, GK-1 may be used to formulate a vaccine against the different cestodes in which this peptide is conserved.

With respect to T-cell epitopes, several different possible MHC class I and class II epitopes were theoretically predicted in nTsKE7. These epitopes may be recognized by multiple haplotypes, which would explain its immunogenic capacity among such different species, like mice, rabbits, and pigs. Indeed, some of the predicted H-2 class I and II mouse epitopes were found in human MHC (data not shown). Epitopes with the highest scores for an MHC-I/H-2 association were broadly distributed in the entire sequence, while those for an MHC class II association were found at the carboxyl terminus of the protein. Class I and II epitopes are included in the GK-1 sequence, and these may also be relevant for protection.

The results reported herein deepen our knowledge of the possible biological roles of nTsKE7. Moreover, the results reported herein let us conclude that GK-1 includes a B-cell epitope in the N terminus (YYYPS) (Table 1) and a class II T-cell epitope in the C terminus (Fig. 5). This could explain the ubiquitous expression of GK-1 in cestodes and its high antigenicity and protective capacity, along with the B- and T-cell epitopes included in this peptide. Altogether, these results support the testing of GK-1 for its potential as a vaccine against multiple cestodes.

MATERIALS AND METHODS

Mice. Six-week-old BALB/cAnN mice, bred and maintained in microisolators in the animal facilities at the Biomedical Research Institute, Universidad Nacional Autónoma de México (UNAM), were employed for KETc7 immunization. All experiments reported herein were conducted in accordance with the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (34). All protocols used in this study were approved by the Ethics Committee of the Biomedical Research Institute of UNAM.

Sequence analysis and phylogenetic analysis. The nTsKE7 sequence obtained here was compared with the already published sequence of the *T. solium* complete genome (23). Additionally, all KE7 orthologue protein sequences were identified in other helminth genomes available in the data set (http://www.genedb.org/Homepage). The *T. crassiceps* and *Taenia saginata* sequences were reported in personal communications from J. P. Laclette and C. Maroni, respectively, and were based on unpublished results. Sequences were aligned by use of the ClustalW program (35). The alignment included the sequences of 28 taxa with 216 characters. A neighbor-joining tree was inferred with the software MEGA (v.6) (36) by the use of *p* distances and with the complete deletion option enabled. Tree nodes were supported with 10,000 bootstrap replicates.

Automated determination of protein features. Glycosylation patterns were predicted using the GPP prediction server (37). The TMHMM (v.2.0) server (38) was employed to search for transmembrane domains, and NetPhos (v.2) software was used to search for phosphorylation sites (39). Hydrophobicity and isoelectric points were predicted using the GPMAw-Lite server (http://www.alphalyse.com/gpmaw_lite.html).

Protein domain automatic prediction. On the basis of the previously published nTsKE7 sequence from the *T. solium* genome (23), an automated protein BLAST search was performed, and the predicted conserved domains were confirmed by use of the NCBI Conserved Domain Database (40). Information regarding the predicted domains was manually retrieved (Fig. 1).

Cloning and expression of rTsKE7. The *T. solium* KE7 gene was synthesized by GenScript, optimizing the DNA sequence for its expression in *Escherichia coli*. Then, it was subcloned from pUC57-kan into the pET28b(+) plasmid and sequenced to confirm the identity of the construct. To this end, genes were amplified by PCR using the oligonucleotides 5′-GCCATACCATGGCAATCAATACGGCTCATACGCAAGAT-3′ with a NcoI restriction site (underlined) as a 5′ end primer and 5′-CTGCGAAAGCTTTTAGTGGTGGTGGTGGTGGTGCAGGTTCTTTTTGTCTTCCGGTTCCATC-3′ with an HindIII restriction site (underlined) as a 3′ end primer. The amplified product was purified and ligated into pET28b(+). The construct was sequenced entirely to exclude the possibility of the presence of inadvertent mutations. Overexpression was performed in *E. coli* Rosetta2 cells (Novagen) after transforming the pET28b(+) plasmid carrying the nTsKE7-encoding sequence. One liter of kanamycin-supplemented (50 μg/ml) LB medium was inoculated with a 5-ml preculture and incubated at 37°C. After an optical density at 600 nm (OD_{600}) value of 0.5 was reached, gene expression was induced by adding 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and growth was continued for another 16 h at 30°C. Cells were harvested by centrifugation at 4,000 rpm for 5 min at 4°C. Cell pellets were resuspended in 20 ml of 20 mM sodium phosphate buffer (pH 8), 150 mM NaCl, 0.5 mM imidazole, 0.1 mM phenylmethylsulfonyl fluoride (PMSF); then, the cells were lysed by sonication in a Branson Sonifier 450, applying six 50% pulses for 20 s at 30-s intervals at 4°C. The cells were centrifuged again (for 30 min at 10,000 rpm and 4°C) to separate the soluble cell extract from the insoluble fraction. nTsKE7 was purified from the soluble fraction in a nickel-Sepharose column (5 ml; HisTrap FF crude; GE Healthcare) that had previously been equilibrated with 20 mM sodium phosphate buffer (pH 8), 150 mM NaCl, 0.5 mM imidazole. The bound His6-tagged protein was eluted by applying a linear gradient from 1 mM to 500 mM imidazole. Fractions with the pure protein were loaded into a Superdex 200 column (GE Healthcare) that had previously been equilibrated with 20 mM sodium phosphate buffer (pH 8), 150 mM NaCl. Fractions with the pure protein were pooled, concentrated in an Amicon Ultra-15 system, and stored at 4°C until they were used.

AAR value for the nTsKE7 protein. The number of antigenic regions in the nTsKE7 protein was calculated by the BepiPred method (24, 41). The BepiPred (v.1.0) method predicts linear epitopes in a protein on the basis of the physicochemical profile of each amino acid (41). The threshold score was set to 0.35, and only those antigenic regions with 6 or more amino acids in length were selected. Then, an abundance of antigenic regions (AAR) value was calculated to normalize the number of antigenic regions according to the protein length, as previously reported (24).

Mouse antibodies. Anti-rTsKE7 antiserum was obtained by subcutaneously immunizing female BALB/cAnN mice with two 10-μg doses of rTsKE7 plus saponin (20 μg per mouse) every 10 days. Ten days after the last immunization, the mice were bled and serum was collected. The presence of specific antibodies was monitored by enzyme-linked immunosorbent assay (ELISA) during the immunization schedule, using the synthetic GK-1 peptide as the antigen source, according to a previously reported procedure with minor modifications (16, 42).

Parasite and protein extracts. The cyst tissue soluble fraction and vesicular fluid (VF) were obtained as previously described (43). *T. solium* cysticerci were excised from the skeletal muscle of naturally infected pigs and extensively washed with sterile 0.15 M phosphate-buffered saline (PBS; pH 7.3). Cysts were sectioned by using a scalpel, and the released VF was collected and diluted 1:2 using 50 mM Tris, pH 7.3, supplemented with protease inhibitors (0.5 M EDTA, 200 mM PMSF, 10 mM leupeptin, 1 mM pepstatin); VF was precipitated with acetone, and the pellet was solubilized in a conventional buffer (7 M urea, 2 M thiourea, 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}). Cyst tissue was washed several times using PBS supplemented with protease inhibitors and homogenized in 50 mM Tris, pH 7.3, with protease inhibitors using a Teflon pestle; tissue samples were subjected to three cycles of thaw-freezing. Then, the homogenized material was centrifuged at 14,000 $\times$ *g* for 60 min at 4°C. Soluble antigens in the supernatant were recovered and precipitated, and the pellets were solubilized in the same buffer. Protein fractions were quantified with a noninterfering (NI) protein assay kit with bovine serum albumin (BSA) as the standard (GBiosciences), aliquoted, and frozen at −70°C until use.

Two-dimensional polyacrylamide gel electrophoresis (PAGE). Isoelectric focusing (IEF) was performed using 7-cm immobilized pH gradient (IPG) strips (Bio-Rad) with a nonlinear 3 to 10 pH gradient and a Protean IEF cell (Bio-Rad) at a constant surface temperature of 20°C and a maximum current of 50 μA per strip. Samples of the protein extracts (150 μg) were diluted to a final volume of 125 μl using rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.2% IPG buffer for the respective pH gradient, bromophenol blue). These mixtures were used to rehydrate several IPG strips for at least 12 h at 20°C. IEF was performed for a total of 10,000 V-h. Then, the strips were reduced for 15 min in equilibration buffer (6 M urea, 0.05 M Tris pH 8.8, 2% SDS, 30% glycerol) containing 2% dithiothreitol and then alkylated using the same buffer supplemented with 2.5% iodoacetamide for 15 min. The second dimension was performed on 12% SDS-polyacrylamide precast gels (Bio-Rad) using a mini-Protean cell (Bio-Rad). After the gels were run at 200 V for about 60 min, the 2D gels were either silver stained or electroblotted onto nitrocellulose membranes for immunoblot analysis. The reproducibility of the

proteomic pattern was verified by running the 2D gels in triplicate at different times. 2D gels were routinely stained with Coomassie brilliant blue R-250 (Bio-Rad) when used for mass spectrometry (MS) analysis. 2D gels were analyzed with PDQuest 2D analysis software (Bio-Rad).

Immunoblot analysis. Proteins were electrotransferred from the 2D gels to nitrocellulose membranes at 120 V for 70 min. The membranes were initially blocked by overnight incubation at 4°C with 10% dried skimmed milk in PBS buffer and then incubated for 2 h at room temperature with one of the pooled mouse serum samples obtained before and after immunization with the rTsKE7 protein (diluted 1:200 in the same buffer). The membranes were washed three times with PBS containing 0.1% Tween 20 (PBST) and incubated again for 2 h at room temperature with horseradish peroxidase-conjugated anti-mouse IgG antibody (Sigma) diluted 1:60,000 in PBST. After the immunoblots were washed as described above, they were developed by standard procedures using a West Femto chemiluminescence kit (Thermo). The reproducibility of immune recognition was verified by replicating the immunoblot at least three times.

Mass spectrometry (MALDI-TOF-TOF). Protein spots were cut from the gel and digested using proteomics-grade trypsin. Tryptic peptides were desalted and analyzed using a matrix-assisted laser desorption ionization–time of flight–time of flight (MALDI-TOF-TOF) 4800 instrument (AB Sciex). The instrument was operated in the positive-ion mode and externally calibrated using a mixture of different mass calibrators ranging from 900 to 3,600 Da (AB Sciex TOF/TOF). The laser source was set to 2,500 to 2,800 for MS spectrum acquisition and 3,500 to 3,800 for MS/MS spectrum acquisition. Readings in the MS-positive reflector mode were performed using 25 laser shots; the fragmentation of selected precursors was performed at a collision energy of 2 kV, using air as the collision gas (pressure, 2×10^{-6} torr), with the accumulation of 400 shots for each spectrum. MS spectra were acquired at *m/z* 800 to 4,000. The parental ion of Glu1-fibrino-peptide B, diluted in the matrix (1.3 pmol/μl/spot), was used for internal calibration at *m/z* 1,570.690 Da. Up to 16 of the most intense spot signals with a signal-to-noise ratio of >20 were selected as precursors for MS/MS spectrum acquisition. Database searches were performed using Protein Pilot (v.2.0) software (AB Sciex) and the Paragon algorithm as the search engine. Each MS/MS spectrum was searched against the spectra for the *T. solium* and *Sus scrofa* proteins in protein databases. Searches were run using the cysteine-carbamidomethyl modification as a fixed modification and the methionine oxidation as a variable modification. The detected protein threshold unused postscore (confidence) was set to 0.47 to achieve a 66% confidence rate.

Identification of B-cell epitopes/mimotopes recognized by anti-rTsKE7 antibodies. Phages were selected by biopanning as previously described (44). A library of linear heptapeptides fused to the minor coat protein (pIII) of M13 bacteriophage was purchased from New England BioLabs (Beverly, MA). Polyclonal anti-rTsKE7 mouse hyperimmune serum was used for affinity selection. Microplate wells (MaxiSorp; Nunc, Roskilde, Denmark) were coated with 500 ng of mouse anti-IgG (Zymed, San Francisco, CA) diluted in 100 μl of PBS for 1 h at 37°C. After washing with PBS and blocking with 200 μl of 3.0% (wt/vol) BSA in PBS, 100 μl of anti-rTsKE7 antibody (1:100) was added, and the plates were incubated for 1 h at 37°C. After washing with 0.1% (vol/vol) Tween 20 in PBS, 1,011 phage particles from the library were added and the plates were incubated for 2 h at 4°C and for 1 h at 37°C. Then, nonbound phages were discarded and the wells were washed with PBS. Bound phages were eluted with glycine-HCl (pH 3). Eluates were transferred to microcentrifuge tubes, neutralized with 35 μl of 2 M Tris base, and amplified by infection of *E. coli* ER2537 cells in 2× YT medium (Gibco BRL, Gaithersburg, MD). Phages were purified by double precipitation with polyethylene glycol (USB, Cleveland, OH), and 1,011 PFU was used for the next biopanning round. After three rounds, individual phage clones were isolated. Phage single-stranded DNA was purified as described by Sambrook et al. (45) and used for DNA sequencing, performed with a T7 Sequenase (v.2.0) quick-denature plasmid sequencing kit (Amersham Biosciences, Cleveland, OH), the 96gIII sequencing primer (New England BioLabs), and [α-^{35}S]dATP (Amersham Biosciences) following the manufacturers' instructions. Both the DNA and the amino acid sequences of the inserts were analyzed with the ExPASy molecular biology server (https://www.expasy.org/tools/). Consensus sequences were determined by multiple-sequence alignment with the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Reactivity of phage-displayed epitopes/mimotopes by ELISA. To assess the binding of anti-rTsKE7 antibodies to the selected phage-displayed peptides, MaxiSorp multiwell plates (Nunc) were coated with 100 μl of a 5-μg/ml solution of an anti-mouse IgG anti-Fc monoclonal antibody (MAb) (Zymed) in PBS for 1 h at 37°C. After washing with 0.1% Tween 20 in PBS and blocking the wells with 200 μl of 3% BSA in PBS for 1 h at 37°C, anti-rTsKE7 antiserum was added and the plates were incubated for 1 h at 37°C. Then, phage particles, resuspended in 1% BSA in PBS, were added to the wells (1,010 PFU/ml) and the plates were incubated for 3 h at 4°C and for 30 min at room temperature (23°C). Positive phages were detected using anti-M13 MAb conjugated to horseradish peroxidase (Zymed) and ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] solution (Life Technologies, Frederick, MD, USA). The reaction was read at 405 nm.

Antigenic capacity. Twelve serum samples from *T. solium* cysticercotic pigs and 12 samples from noncysticercotic pigs diluted 1:100 were assayed by ELISA to test their reactivity against rTsKE7, GK-1, and GK-1M following a procedure previously described (15). A nonrelated recombinant protein originally isolated from *Mycobacterium tuberculosis* (RV1818C) with a His tag (kindly donated by Clara Espitia) and a nonrelated 17-amino-acid peptide (AALSPGSSAYPSATVLA) were used as negative controls. *T. solium* cyst vesicular fluid was employed as a source of antigen for the positive control. The accuracy of the antigen ELISA was evaluated by receiver operating characteristic (ROC) analysis. This was done by comparing GK-1-immunized pigs with nonimmunized animals. The optimal cutoff ratio value was selected as the point on the ROC curve (which displays the estimated percentages of sensitivity and

specificity at a selected cutoff value) with the minimum distance to the (0, 1) coordinate. ROC curves were generated using the R software package (46). All sampled animals were previously evaluated by necropsy to ascertain their infection status, following the rules approved by the Ethical Committee of the Veterinary Medicine School (UNAM).

***In silico* prediction of an nTsKE7 cell epitope(s).** To determine potential T-cell epitopes, the nTsKE7 protein sequence was submitted to the IEDB and SYFPEITHI web servers (http://tools.immuneepitope.org/main/ and http://www.syfpeithi.de/0-Home.htm, respectively), which measure the ligation strength of an amino acid sequence to a defined MHC haplotype. Analyses were run for MHC class I and II H-2 molecules of different haplotypes using the canonical peptide lengths (9 and 15 amino acids, respectively).

Protective capacity. The murine model of cysticercosis caused by *T. crassiceps* was employed to evaluate the protective capacity of rTsKE7, GK-1, and GK-1M. This experimental model has been extensively employed for this purpose and is based on the cross-reaction and cross-protection found between *T. solium* and *T. crassiceps* antigens (47, 48).

Mice were immunized with the recombinant rTsKE7 and synthetic GK-1 peptides, with saponin being used as the adjuvant. GK-1M was expressed in the filamentous phage M13. Mice were infected as previously reported (18) and sacrificed 30 days after infection; the parasites in the peritoneal cavity of control and vaccinated mice were counted. The protection level was estimated by the parasite load reduction in immunized mice with respect to the load in the controls, which received either saponin or M13 alone.

Statistical analysis. Data were collected in Excel (v.7.0) software (Microsoft, Redmond, WA) and analyzed with InStat software (GraphPad, La Jolla, CA).

The sensitivity and specificity of the assay for each tested antigen were compared using Fisher's exact test. Parasite loads between mouse groups were compared using the Mann-Whitney nonparametric test. A P value of <0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00395-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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R.J.B., E.S., and G.F. conceived of and designed the study. R.J.B., J.N.-P., A.O.-L., V.H.A., K.E., and M.G.-V. performed data acquisition, interpretation, and analysis. R.J.B., E.S., and G.F. drafted the manuscript. X.S., J.P.L., G.R., and C.M.N. critically revised the manuscript. R.J.B., J.N.-P., M.H., J.C.-T., F.S.-L., G.G., G.A., A.A.L.-Z., M.G.-V., and R.R.S.-M. performed the experiments and prepared the drawings.

We declare that were have no competing interests, either financial or of any other kind.

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