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Anti-SpCas9 IgY Polyclonal Antibodies Production for CRISPR Research Use

Esteban León, Valentina Ortiz, Alexander Pérez, Jair Téllez, Gonzalo J. Díaz, María H. Ramírez H, and Luis E. Contreras R*

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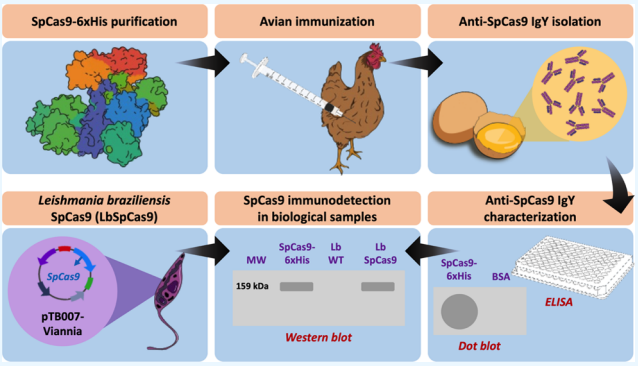
ABSTRACT: The CRISPR/Cas adaptative immune system has been harnessed as an RNA-guided, programmable genome editing tool, allowing for diverse biotechnological applications. The implementation of the system relies on the ability to detect the Cas9 protein in biological samples. This task is facilitated by employing antibodies, which exhibit several advantageous features and applications in the context of tropical neglected diseases. This study reports a one-month immunization scheme with the Cas9 protein from*Streptococcus pyogenes* to produce IgY polyclonal antibodies (anti-SpCas9), which can be rapidly isolated by combining yolk de-lipidation with protein salting out using pectin and ammonium sulfate, respectively. Immunodetection assays indicate that the antibodies are highly sensitive, specific, and useful for detecting the SpCas9 protein in promastigotes of*Leishmania braziliensis*expressing exogenous SpCas9. Thus, the simple method for producing anti-SpCas9 IgY antibodies will accelerate CRISPR/Cas-based studies in *Leishmania* spp. This approach serves as a valuable research tool in this parasite model and holds the potential for wide application in various other biological samples, promoting the implementation of the system. In fact, a bioinformatics approach based on the identification of antigenic determinants in the SpCas9 protein suggests the possibility of using the anti-SpCas9 IgY antibodies in applications such as Prime and Base editing.



1. INTRODUCTION

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and their associated sequences (Cas) constitute a programmable molecular system for precise genome editing. The system requires the assembly of a ribonucleoprotein complex composed of a guide RNA with complementation to the sequence of interest that directs the Cas9 protein to cut the target DNA on both strands.[1,2]

The CRISPR/Cas system has been adapted for *in vivo* genomic editing in unicellular and multicellular organisms, enabling a wide variety of genes to be edited. In addition to the classic use of active Cas9 protein for DNA cleavage, recent advancements led to the development of applications using the deactivated Cas9 protein (dCas9). The dCas9 variant is engineered with specific mutations in its HNH or RuvC domains, effectively disabling its nuclease activity. By utilizing dCas9, researchers can now manipulate gene expression by fusing dCas9 to transcriptional activators or repressors, effectively controlling the activity of specific genes.[3] The therapeutic potential of CRISPR/Cas and its next-generation derivatives such as the Prime Editing system is currently being studied in the context of various genetic diseases.[4]

The detection of Cas9 protein in biological samples is essential for optimizing experimental conditions and fine-tuning parameters to ensure the specific and effective implementation of these applications. In gene editing experiments, Cas9 is often delivered into cells using a variety of approaches, including viral vectors, nanoparticles, and synthetic peptides.[5,6] Detection of Cas9 helps to assess the success of the delivery process. In addition, verifying its expression levels ensures that there is enough active protein in the cells to perform the desired genome editing. Furthermore, Cas9 protein can be degraded or denatured in biological samples, affecting its activity. By detecting Cas9, researchers can monitor its stability and ensure its proper function during genome editing experiments in biological samples, including pathogens such as *Leishmania* spp.

IgY antibodies are a favorable alternative in this regard, as they offer numerous advantages such as high specificity and yield, low immunogenicity in humans, animal welfare, and ease of production.[7] In terms of cost and simplicity, polyclonal IgY

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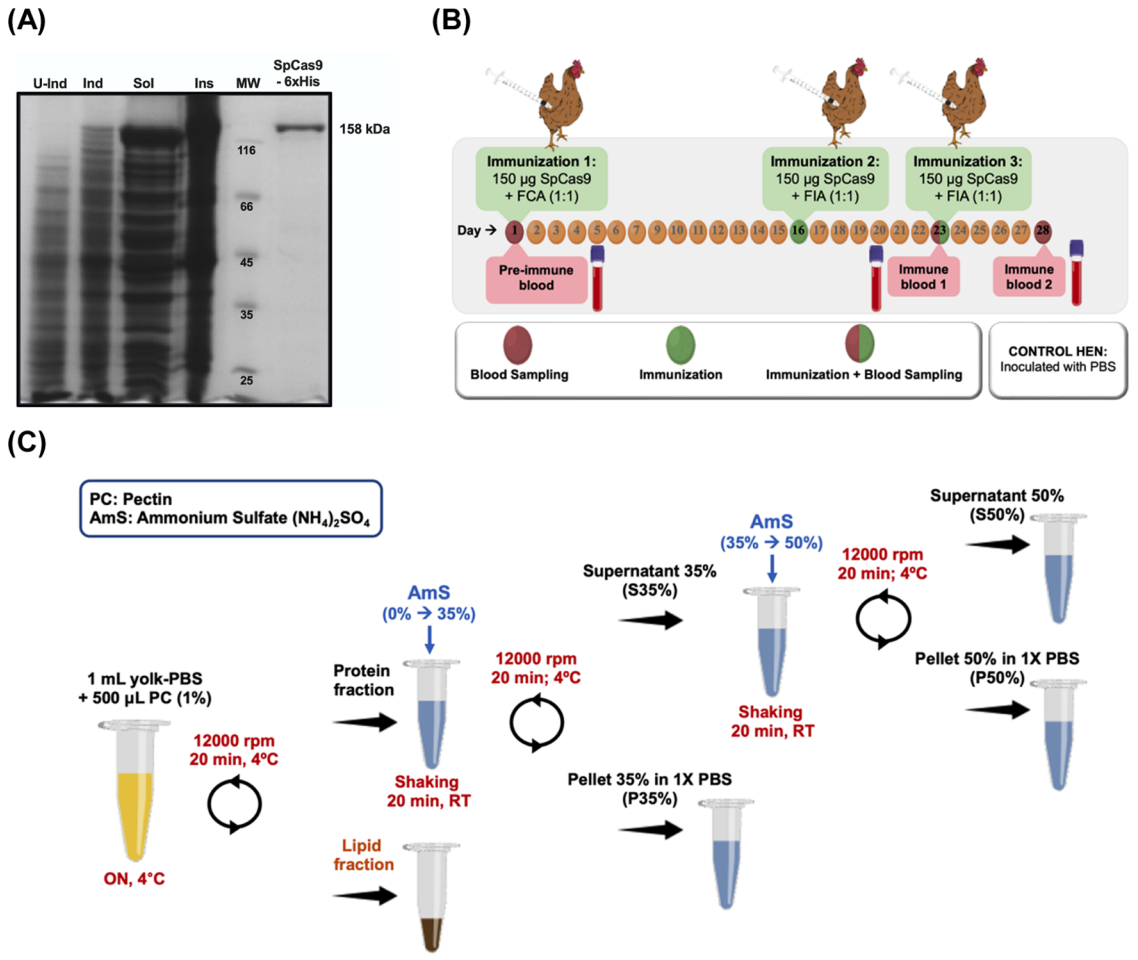

Figure 1. SpCas9-6xHis implementation to produce and isolate polyclonal IgY antibodies. (A) The SpCas9-6xHis protein (158 kDa) was purified by immobilized metal affinity chromatography (IMAC) and proteins were visualized in 10% SDS-PAGE stained with Coomassie R-250. (B) Immunization program scheme. The SpCas9-6xHis purified protein was inoculated into 36-week-old Hy-Line Brown hens. A total of 28 eggs were collected and 3 blood samples were taken at days 0, 23, and 28. **FCA:** Freund's Complete Adjuvant. **FIA:** Freund's Incomplete Adjuvant. (C) Isolation protocol of IgY antibodies from yolks achieved by combining de-lipidation with pectin and ammonium sulfate protein precipitation processes. **U-Ind:** Uninduced cells. **Ind:** Induced cells. **Sol:** Soluble fraction. **Ins:** Insoluble fraction. **SpCas9:** SpCas9-6xHis protein. **MW:** Molecular weight (kDa).

antibody production is a more cost-effective and relatively straightforward process compared to other methods such as the traditional hybridoma technology and phage display-derived monoclonal antibodies. These methods involve the fusion of antibody-producing cells with myeloma cells or the construction of combinatorial antibody libraries, respectively.[8,9] Regarding sensitivity, polyclonal IgY antibodies have been shown to exhibit specific binding affinity and sensitivity in immunochemical assays. For instance, in a study comparing the sensitivity of IgY antibodies to a widely used rabbit polyclonal antibody, IgY antibodies showed comparable sensitivity in Western blot analysis.[10] In addition, these IgY antibodies specifically bind to the target antigen without cross-reaction.[11]

Despite the availability of commercial anti-Cas9 antibodies for various applications, such as flow cytometry, Western blotting, ELISA, immunoprecipitation, and immunocytochemistry, the affordability and accessibility of these supplies pose significant challenges for the utilization of CRISPR/Cas-based tools in developing countries. Furthermore, challenges in the supply chain and delivery timelines, including import/export regulations, shipping logistics, and limited availability of specific reagents, can impact the timely delivery of resources. Therefore, the local production of anti-Cas9 antibodies represents a contribution to advancing basic and applied research in the field of genome editing in developing countries.

IgY antibody isolation requires the removal of lipids to form a water-soluble fraction (de-lipidation) and subsequent extraction of antibodies from this fraction using various approaches, including precipitation, chromatography, or filtration methods. Lipid removal can be accomplished using organic solvents, acidified (pH 5.0) water, and polysaccharides, whereas the extraction procedures typically involve ammonium sulfate and polyethylene glycol precipitation.[8]

In this study, the production of polyclonal IgY antibodies against the recombinant Cas9 protein from*Streptococcus pyogenes*(SpCas9-6xHis) is reported. Simple isolation from immunized eggs was achieved by combining de-lipidation with pectin and ammonium sulfate protein precipitation. Furthermore, the successful implementation of these antibodies in immunodetection assays in biological samples of*Leishmania braziliensis*promastigotes constitutively expressing the exogenous SpCas9 protein was accomplished. In addition, *in silico* identification of immunogenic epitopes suggests the possibility

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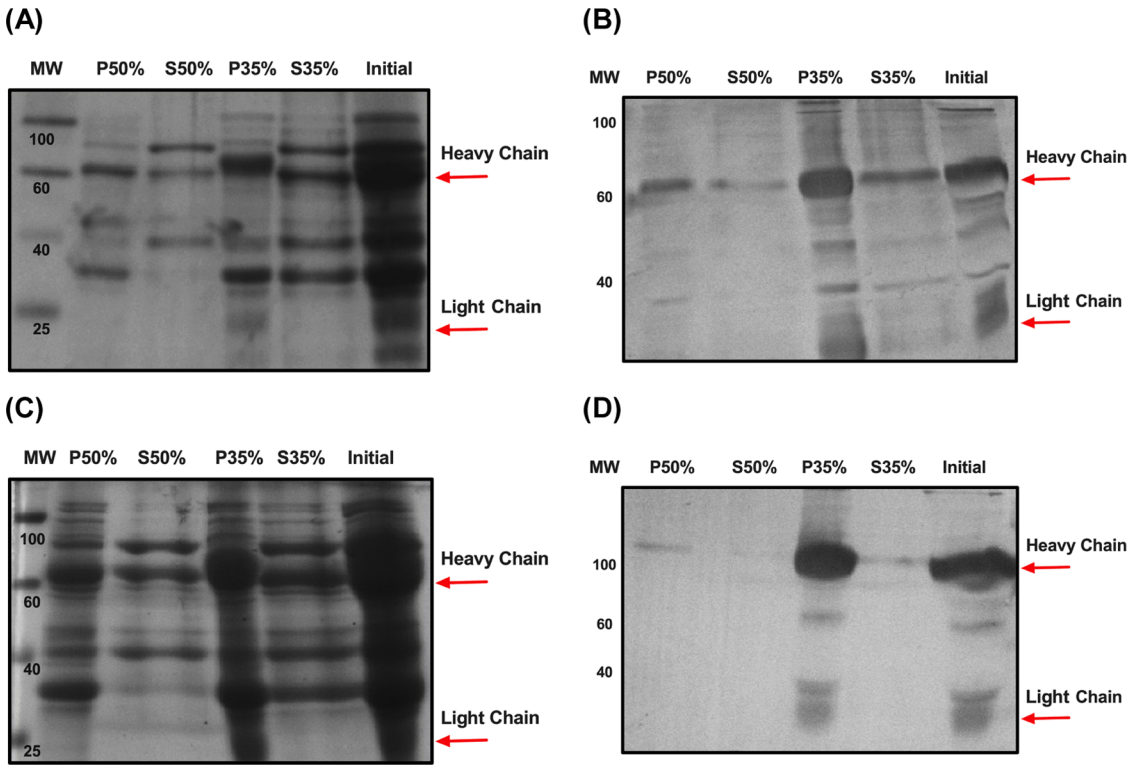

Figure 2. Simple isolation of IgY antibodies from yolks. (A) De-lipidation and fractional precipitation of IgY antibodies from the yolk of a control egg (immunized with PBS). 12% SDS-PAGE. Coomassie R-250. (B) Western blot of control egg samples. PVDF membrane. Antibody: anti-IgY-alkaline phosphatase (1:10,000). (C, D) De-lipidation and fractional precipitation of IgY antibodies from the yolk of egg number 28 (E28) from the hen immunized with SpCas9-6xHis and Western blot. **P50%**, **S50%**, **P35%**, **S35%:** Pellets and supernatants at 50 and 35% AmS, respectively. **Initial:** Initial fraction. **MW:** Molecular weight (kDa).

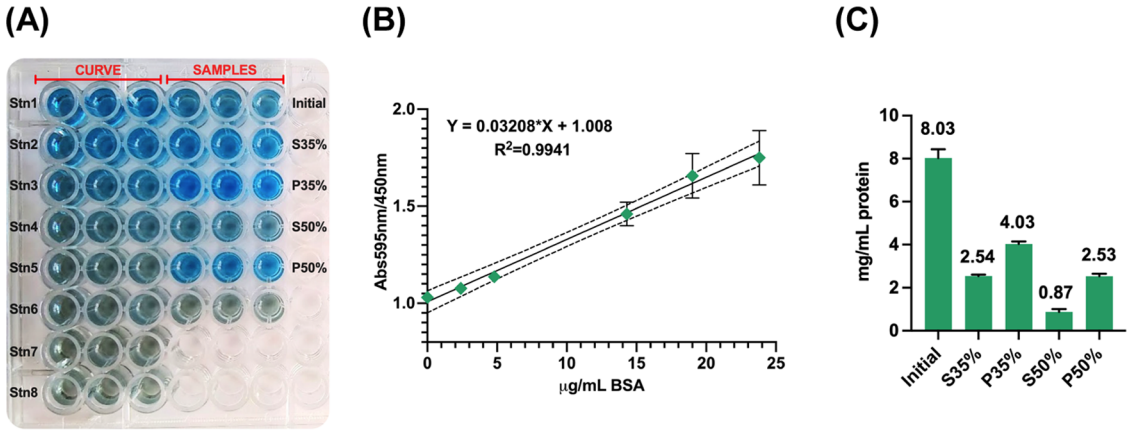

Figure 3. IgY quantification of fractions from immune egg number 28. (A) Bradford assay to determine the total protein in the collected fractions from immune egg number 28 (E28). (B) Calibration curve. Standards 5 and 7 were excluded. Values correspond to the mean ± SD from three technical replicates. (C) Protein concentration in immune E28 fractions. **Stn1** to **Stn 8:** Standards. **Initial**, **P50%**, **S50%**, **P35%**, **S35%:** Initial fraction, pellets, and supernatants at AmS 50 and 35%, respectively.

of immunodetection of SpCas9 protein variants used in currently relevant applications such as Prime and Base editing.

2. RESULTS

2.1. SpCas9-6xHis Antigen Induces the Production of Polyclonal IgY Antibodies. The SpCas9-6xHis protein was purified from the soluble fraction of*Escherichia coli*BL21(DE3) bacteria by one-step salting out with ammonium sulfate (AmS) using immobilized metal affinity chromatography (IMAC) (Figure 1A). This strategy allowed obtaining a pure protein useful as an antigen to produce polyclonal antibodies of the Y class (IgY) through a one-month immunization scheme in an avian model (Figure 1B). Considering the use of laying hens, it was possible to daily collect immunized eggs, which permitted the standardization and optimization of a simple protocol to extract IgYs from yolks. The protocol was conducted using a control egg (CE) (Figure 1C).

Lipid removal was carried out using pectin treatment followed by fractional precipitation of proteins with AmS. This led to fractions enriched for IgY light and heavy chains at 35% and 50% both in the supernatant and precipitates for the

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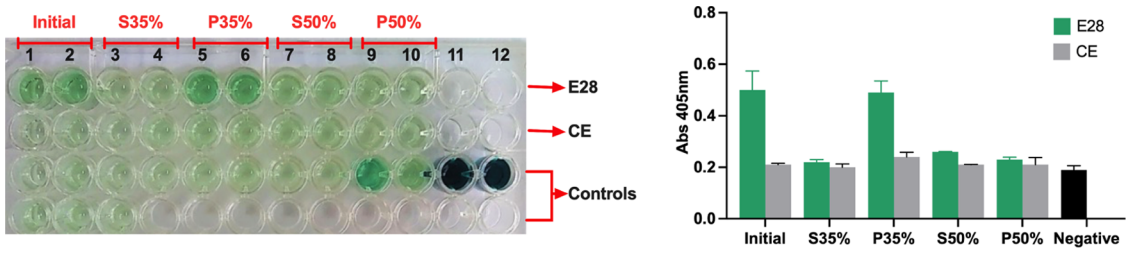

Figure 4. P35% fraction exhibited strong antigen recognition. (A) Indirect ELISA to test fractions from the E28 immune and control egg (CE) on the SpCas9-6xHis antigen. Primary antibody: anti-SpCas9 IgY (1:250). Secondary antibody: anti-IgY-HRP (1:10,000). (B) Absorbance values of tested samples at 405 nm. Values correspond to mean ± SD from two technical replicates. The negative control corresponds to duplicate wells without antigens or primary or secondary antibodies.

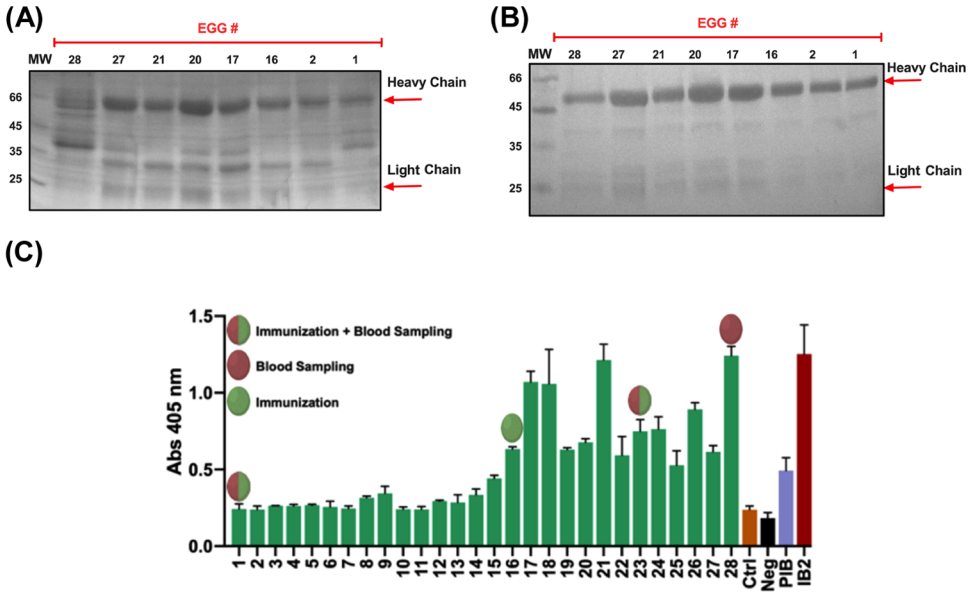

Figure 5. Isolation and activity of anti-SpCas9 antibodies from several eggs in the P35% fraction. (A) Evaluation of P35% fractions from different eggs by 12% SDS-PAGE. Coomassie R-250. (B) Western blot of P35% fractions. PVDF membrane. Antibody: anti-IgY-alkaline phosphatase (1:10,000). (C) Indirect ELISA to test the P35% fractions. Primary antibody: anti-SpCas9 IgY (1:250). Secondary antibody: anti-IgY-HRP (1:10,000). Control egg (**Ctrl**), Negative detection control (**Neg**), Pre-immune (**PIB**), and immune blood number 2 (**IB2**) were included. Values correspond to mean ± SD from two technical replicates.

control egg, as indicated by SDS-PAGE (Figure 2A). Immunodetection analysis indicated a robust signal for the light and heavy chains mainly in the 35% AmS precipitated fraction (P35%) (Figure 2B). Similar results were observed for the egg collected on day 28 from the immunized hen with the SpCas9-6xHis antigen (E28) (Figure 2C,D).

Based on the SDS-PAGE and Western blot analysis, the Bradford determination assay for E28 confirmed the protein accumulation in the P35% fraction. This fraction exhibited the highest protein concentration among all of the collected fractions (Figure 3). Therefore, the simple method used in this study was effective in obtaining protein fractions enriched in IgYs, such as the P35%.

2.2. SpCas9-6xHis Antigen Detection by the P35% Fraction. Fractions from the IgY antibodies extraction protocol were evaluated by indirect ELISA to identify those with higher immunodetection activity against the SpCas9-6xHis antigen. Similar antigen detection values were observed for the initial and P35% fractions from the E28 immunized hen. The remaining fractions showed similar values to the CE and the negative detection controls, indicating the absence of anti-SpCas9 IgY antibodies in these samples (Figure 4A,B). The results suggest that combining de-lipidation with pectin and AmS precipitation could be a suitable approach to increase the concentration of anti-SpCas9 IgY antibodies in the P35% fraction.

Other yolks collected at various points during the immunization scheme were processed using the combined method for extracting IgY. The reproducibility of the combined extraction method was again demonstrated by the consistent enrichment of both light and heavy chain antibodies in the P35% fractions (Figure 5A,B).

The detection of the SpCas9-6xHis antigen by indirect ELISA showed that eggs 17, 18, 21, and 28 contained anti-SpCas9 IgY antibodies in the P35% fractions. This was indicated by their higher detection values compared to the CE and negative detection controls (Figure 5C). Thus, from the first immunization booster on day 16, a notable increase in the accumulation of anti-SpCas9 IgY antibodies in the yolks was noticed. Moreover, the immunodetection assay revealed the presence of anti-SpCas9 antibodies in the immune blood (IB2), with detection values that were twice higher than those registered in the pre-immune blood (PIB) (Figure 5C).

2.3. Anti-SpCas9 Antibodies Are Useful to Detect SpCas9 Protein in Biological Samples. To evaluate the sensitivity and specificity of the anti-SpCas9 antibodies, we

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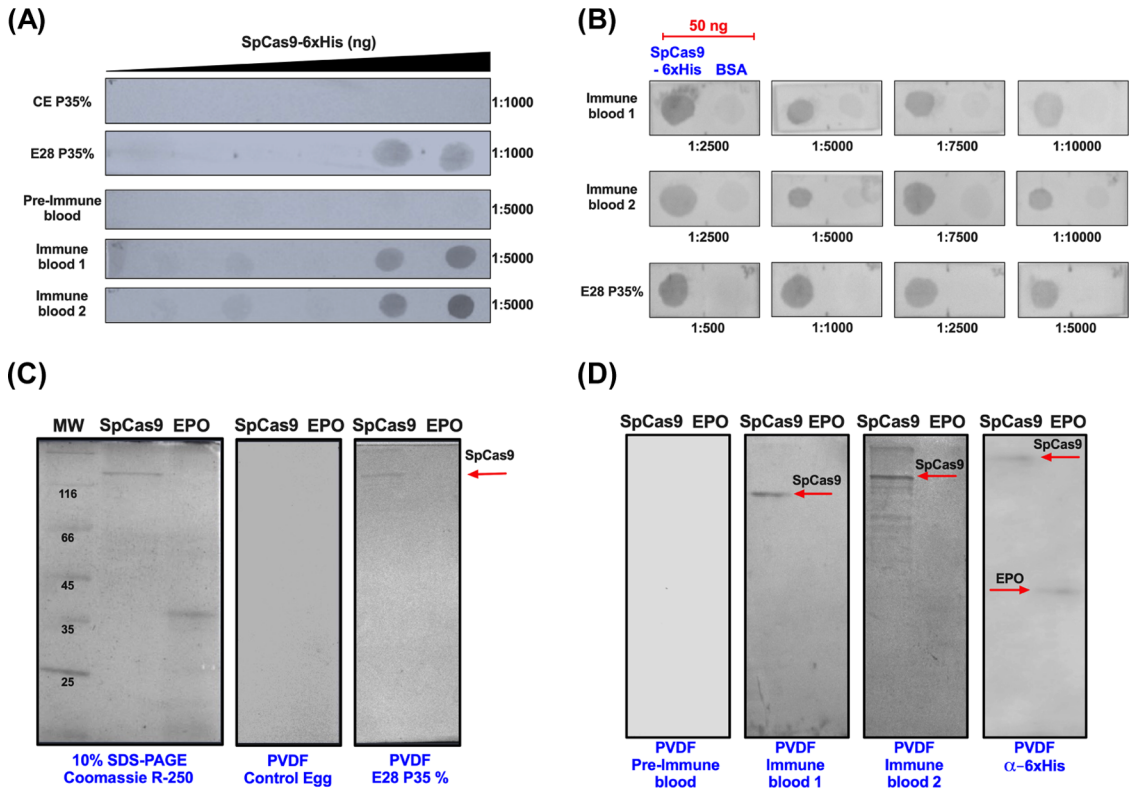

Figure 6. Anti-SpCas9 antibodies are sensitive and specific. (A) Detection of increasing amounts of antigen at a fixed dilution of antibodies and (B) different dilutions of antibody at a fixed amount of antigen were evaluated by Dot blot assays. PVDF membranes. Secondary antibody: anti-IgY-alkaline phosphatase (1:10,000). (C) Specific antigen detection by the anti-SpCas9 antibodies from eggs and (D) blood was evaluated by Western blot. Primary antibodies: anti-SpCas9 IgY from eggs (1:1000), immune blood (1:5000), and anti-6xHis IgG (1:5000). Secondary antibodies: anti-IgY-alkaline phosphatase (1:10,000) and anti-IgG-alkaline phosphatase (1:10,000, for the anti-6xHis membrane). P35% fractions from Control Egg (**CE P35%**) and immune egg 28 (**E28 P35%**). Recombinant SpCas9-6xHis (**SpCas9**) and Erythropoietin-6xHis (**EPO**).

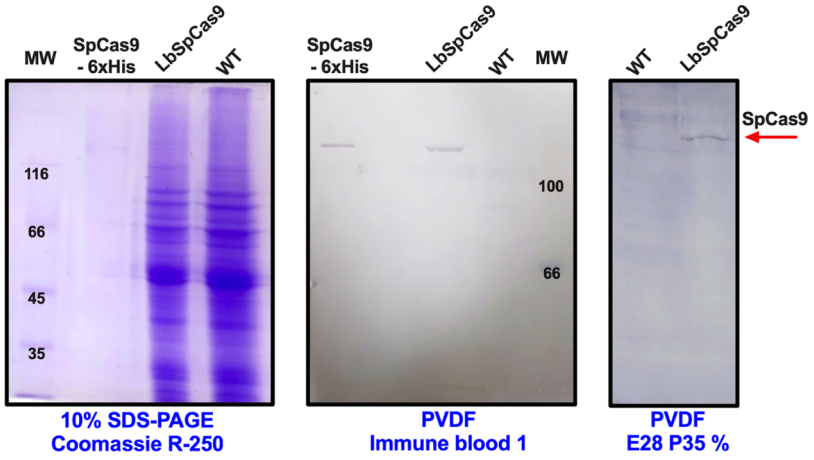

Figure 7. Immunodetection of SpCas9 protein in biological samples. Total protein extracts from*L. braziliensis* promastigotes were tested with anti-SpCas9 antibodies from blood (1:5000) and immune egg 28 (1:1000) by Western blot. Wild-type parasites (**WT**). Transfectant parasites expressing the exogenous SpCas9 protein from the episomal pTB007-Viannia plasmid (**LbSpCas9**). Recombinant SpCas9-6xHis (**SpCas9-6xHis**). Primary antibodies: anti-SpCas9 IgY from egg 28 (1:1000) and immune blood 1 (1:5000). Secondary antibody: anti-IgY-alkaline phosphatase (1:10,000).

analyzed the P35% fraction of the immune E28, as well as the pre-immune and immune blood samples. Initially, we tested the minimum amount of SpCas9 antigen recognized by a fixed dilution of the antibody using a Dot blot assay. The P35% fraction of immune E28 was able to detect 10 ng of antigen, while immune blood samples 1 and 2 were an order of magnitude more sensitive, detecting 1 ng of the SpCas9-6xHis antigen. The P35% fraction for CE and the pre-immune blood did not detect the antigen (Figure 6A).

Then, the maximum dilution of anti-SpCas9 antibodies capable of detecting a fixed amount of SpCas9 antigen (50 ng) was tested. For the P35% fraction for E28, all dilutions exhibited specific detection of the SpCas9-6xHis antigen (up to a 1:5000), while no detection was observed for the BSA sample

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Table 1. *In Silico* Identification of Antigenic Determinants in the SpCas9 Protein[a]

antigenic peptide in SpCas9	start	end	residues	score
1 - RIDLS	1359	1363	5	0.813
2 - EEFYKFIKPILEKMDGTEELLVKLNREDLLR	370	400	31	0.798
3 - IKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGA	322	367	46	0.792
4 - SFEKNPIDFLEAKGKDLII	1173	1196	19	0.776
5 - SVLVVSVKELLGIT	1142	1167	14	0.757

[a]The epitope identification was conducted with ElliPro.

used as a negative control. For immune blood 1 and 2, the antigen could be detected up to a 1:10,000 dilution (Figure 6B).

To assess whether anti-SpCas9 antibodies are specific against the SpCas9 antigen but not the 6xHis tag of the recombinant protein, we compared the immunodetection of 400 ng of SpCas9-6xHis (159 kDa) and EPO-6xHis (35 kDa, erythropoietin) by Western blot. The P35% fraction for immune E28 exclusively detected the SpCas9-6xHis antigen, whereas antibodies from CE did not detect any of the analyzed proteins (Figure 6C). Similar results were obtained with immune blood 1 and 2, which only detected the SpCas9-6xHis protein. Control detection with anti-6xHis antibodies revealed the expected bands for both SpCas9-6xHis and EPO-6xHis proteins (Figure 6D). These results indicate the specificity of the anti-SpCas9 antibodies to detect unique epitopes to the SpCas9 antigen.

The applicability of the anti-SpCas9 antibodies to detect SpCas9 protein in biological samples was evaluated by Western blotting. The P35% fraction from E28 and the immune blood 1 were tested on total protein extracts of*L. braziliensis* WT and LbSpCas9 promastigotes, constitutively expressing the exogenous SpCas9 protein from the episomal pTB007-Viannia plasmid, as well as on the SpCas9-6xHis antigen. These assays showed the immunodetection of SpCas9 protein using both yolk and blood antibodies (Figure 7). Furthermore, considering that the SpCas9 protein expressed from the pTB007-Viannia plasmid is not fused to the 6xHis tag, the generated anti-SpCas9 IgY antibodies do not require the presence of this tag to recognize the SpCas9 protein.

Our results indicate the effectiveness of combining simple de-lipidation and protein precipitation methods from eggs of immunized hens with the SpCas9-6xHis antigen to produce anti-SpCas9 IgY polyclonal antibodies. These antibodies are also present in immune blood and are capable of specifically detecting low amounts of antigen at high antibody dilutions, constituting a suitable research tool to detect SpCas9 protein in biological samples of interest.

2.4. Anti-SpCas9 Antibodies Could Be Used for Prime and Base Editing. To investigate the possibility of immunodetection of Cas9 proteins from other species currently used in genome editing research, a bioinformatics approach based on identifying antigenic epitopes likely to be recognized by the anti-SpCas9 antibodies was performed.

First, the solved three-dimensional structure of the SpCas9 protein (PDB ID: 4CMP) used as antigen in this study was loaded into the *ElliPro* continuous epitope prediction tool.[12] This predictor identified variable length antigenic determinants in regions protruding from the globular surface of the protein. The top five antigenic determinants are shown in Table 1.

These five most antigenic peptides were then searched for in the sequences of Cas9 proteins from various CRISPR/Cas systems, including*Staphylococcus aureus*(SaCas9),*Francisella novicida*(FnCas9),*Neisseria meningitidis*(NmCas9), and*Campylobacter jejuni*(CjCas9). No matches were found in these proteins. This finding could be explained by the low percentage of identity among the sequences (<20%) (Table 2), which are evolutionarily divergent and belong to different subtypes of nucleases in the current CRISPR/Cas classification system[13] (Figure 8A).

Table 2. Identity Percentage Obtained from Multiple Alignments[a]

protein aligned with SpCas9	alignment length	identical residues	identity percentage
Staphylococcus aureus Cas9 (PDB ID: 5CZZ)	1459	259	17.75
Neisseria meningitidis Cas9 (PDB ID: 6JDQ)	1579	208	13.17
Campylobacter jejuni Cas9 (PDB ID: 5X2H)	1505	151	10.03
Francisella novicida Cas9 (PDB ID: 5B2O)	2028	211	10.40
SpCas9 deactivated (dSpCas9) (PDB ID: 6K57)	1372	1366	99.56
SpCas9 adenine base editor (dSpCas9-ABE8e) (PDB ID: 6VPC)	1372	1359	99.05

[a]The corresponding alignments were constructed with T-Coffee using the Clustal-O algorithm. Identity percentages were obtained with The Sequence Manipulation Suite (Supporting Material).

Although the in silico analysis indicates the absence of the antigenic peptides likely to be recognized by the anti-SpCas9 antibodies in the sequences analyzed, experimental verification such as that reported in this work could be performed using recombinant SaCas9, FnCas9, NMeCas9, and CjCas9 proteins in immunodetection assays, which is beyond the scope of this paper.

Finally, the presence of the five most antigenic peptides was confirmed in the inactivated version of SpCas9 (dSpCas9), which is currently the most widely used variant for applications such as Prime editing[4] and Base editing[14] (Identity percentage >95%) (Table 2 and Figure 8C). Therefore, the generated anti-SpCas9 antibodies could be used not only to detect the SpCas9 protein but also in other relevant biotechnological applications.

3. DISCUSSION

The discovery, characterization, and harnessing of the CRISPR/Cas system as a programmable tool for genome editing in cells and living organisms have revolutionized basic and applied science across various fields, including biochemistry, genetics, agriculture, and human health.[16] The development of research tools such as antibodies to simplify the use of the CRISPR/Cas system for detecting the Cas9 protein in different experimental approaches and applications is essential for the implementation of the system. In this regard, we

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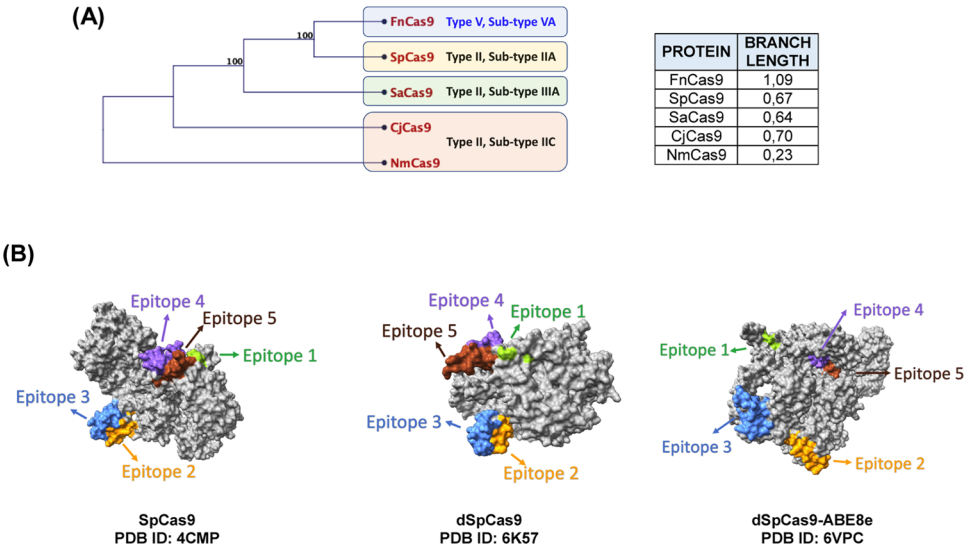

Figure 8. Evolutive relationships and antigenic epitopes in Class II Cas9 proteins. (A) The phylogenetic tree was generated based on the sequences from Table 2 using the nearest neighbor-joining method and 1000 iterations in CLC Sequence viewer (version 7.5). (B) The top five antigenic determinants predicted in the SpCas9 protein are shared with the deactivated SpCas9 variant (dSpCas9), which is used in the Prime and Base editing systems. Image generated with UCSF Chimera X (version 1.6.1).[15]

introduce a simple method for producing and isolating anti-SpCas9 IgY polyclonal antibodies from immunized eggs.

Through the implementation of a one-month avian immunization scheme and the combination of de-lipidation with pectin and protein salting out with AmS, we successfully obtained fractions enriched in functional, sensitive, and specific antibodies against the SpCas9 protein. The usefulness of this research tool in detecting SpCas9 in biological samples was validated using total protein extracts derived from promastigotes of*L. braziliensis*that express the exogenous SpCas9 protein from the episomal pTB007-Viannia plasmid.[17] Therefore, we consider that this immunological tool will facilitate studies based on CRISPR/Cas in this research parasitic model and various other biological samples.

To produce IgY antibodies, it is recommended to use polysaccharides such as carrageenan, carboxymethyl cellulose, and/or pectin during the de-lipidation phase. These polysaccharides aid in the removal of lipids and impurities, resulting in higher purity and yield of IgY antibodies.[8] These polymers can form hydrogen bonds with the polar heads of lipids and establish hydrophobic interactions through the formation of helical structures. The hydrophobic interior of these structures provides a suitable environment for accommodating lipids during the de-lipidation process.[18] These properties could positively impact the isolation processes to obtain the anti-SpCas9 IgY polyclonal antibodies in the present study.

In the extraction and purification of IgY antibodies, the use of AmS is advantageous due to its cost-effectiveness and simplicity, requiring only a few steps for implementation. Furthermore, studies have reported that the combined use of pectin and AmS does not have any detrimental effects on the immunoreactivity of IgY polyclonal antibodies.[19] Similarly, when compared to commercial kits, the combined method is more efficient in terms of yield and purity for obtaining IgY antibodies, making it a favorable choice for antibody production.[20]

In contrast to the production of IgG polyclonal antibodies in mammalian models, the production and isolation of IgY antibodies from the yolk are advantageous because this noninvasive method eliminates the need for sacrificing the animal, making it a more ethical approach for antibody production. In addition, the quantity of antibodies obtained from the yolk is generally greater than other sources and can be stored for extended periods without significant loss of their immunoreactivity. Additionally, these IgY antibodies exhibit minimal cross-reactivity with human proteins, making them highly suitable for a wide range of applications including therapeutic use.[21]

Other studies have reported the production of anti-SpCas9 monoclonal antibodies using hybridomas,[22] which is a time-consuming and expensive process that requires expertise in cell culture. In contrast, our methodology for obtaining anti-SpCas9 IgY polyclonal antibodies is simple and cost-effective, making it more accessible for implementation in research focused on the CRISPR/Cas system in developing countries.

While the applications evaluated in this study were limited to indirect ELISA and Western blot assays, it is important to note that the utility of anti-SpCas9 antibodies can be further expanded to include other applications such as immunofluorescence and immunoprecipitation. These additional techniques would enhance the versatility and potential applications of the IgY anti-SpCas9 antibodies.

Recently, the CRISPR/Cas system has been adapted for point-of-care (POC) diagnostic purposes, enabling the detection of pathogens during infections by analyzing their DNA or RNA.[23] In this context, anti-Cas9 antibodies can be employed in POC CRISPR-based diagnostics. For instance, by incorporating anti-Cas9 antibodies into the diagnostic assay, any unbound Cas9 molecule can be detected and neutralized, reducing the likelihood of off-target interactions and improving specificity to avoid false positives. Additionally, the incorporation of anti-Cas9 antibodies offers a secondary validation step in the diagnostic process. The presence of the anti-Cas9 antibody confirms the detection of the target DNA by the Cas9-guide-RNA complex. This dual control mechanism enhances reliability and confidence in the diagnostic results. Overall, the use of anti-Cas9 antibodies in POC CRISPR-

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based diagnostics can improve the assay, making it a promising tool for rapid and reliable detection of diseases, including neglected tropical diseases such as leishmaniasis.[24]

This work represents a significant advancement in the production technology of anti-SpCas9 IgY antibodies by establishing a standardized and simplified method based on lipid depletion with pectin and salting out with AmS. Furthermore, it holds great potential for gene editing research, as the application of anti-SpCas9 antibodies could be extended to immunodetection in diverse samples and applications such as Prime and Base editing, as well as studies focused on regulating Cas9 activity or its implementation in POC diagnostic purposes.

4. CONCLUSIONS

In this study, we successfully produced anti-SpCas9 IgY polyclonal antibodies for CRISPR research by using a one-month immunization scheme and the subsequent isolation of antibodies from immune yolks using a simple method involving de-lipidation with pectin and protein salting out with AmS. The IgY antibodies were successfully employed to detect the presence of the SpCas9 protein in*L. braziliensis*promastigotes expressing this exogenous protein. Therefore, our developed method for producing anti-SpCas9 IgY antibodies holds great promise in facilitating studies based on the CRISPR/Cas system across diverse biological samples, including protozoan parasites. The plausible cross-reactivity of anti-SpCas9 IgY polyclonal antibodies to detect widely used dSpCas9 protein in Prime and Base editing, as well as their use in CRISPR-based POC diagnostics, would increase the utility of this research tool.

5. EXPERIMENTAL PROCEDURES

5.1. Expression and Purification of the SpCas9-6xHis Recombinant Protein. Chemically competent*E. coli*BL21-(DE3) cells were transformed with the plasmid pET-NLS-Cas9-6xHis (Addgene #62934) by thermal shock and selected on LB solid media supplemented with 100 μg/mL ampicillin. A single clone was inoculated in the bacterial medium overnight (ON), at 37 °C, 180 rpm, diluted at 1:50, and incubated until the optical density (OD) was 0.6−0.8. The overexpression was induced by 0.5 mM IPTG at 25 °C for 6 h; the cells were collected by centrifugation at 7000 rpm for 10 min at 4 °C and resuspended in 5 mL of lysis buffer (20 mM Tris−HCl pH 8.07, 500 mM NaCl, and 20 mM imidazole). The suspension was supplemented with 1 mg/mL lysozyme and 25 μL of protease inhibitor cocktail (Thermo #87786) and then incubated with constant shaking at 4 °C for 40 min. The sample was sonicated (15s pulse, 45s repose, 60% amplitude, 12 pulses) and the soluble fraction was separated by centrifugation at 12,000 rpm for 20 min at 4 °C. This fraction was salted out with ammonium sulfate (AmS) at 35% with constant shaking at 4 °C for 30 min. The sample was centrifugated at 10,000 rpm for 15 min at 4 °C and the supernatant was subjected to immobilized metal affinity chromatography (IMAC) with the Co-IDA resin, which was equilibrated in lysis buffer. The sample was incubated with constant shaking at 4 °C for 45 min and gravity packaged in a chromatography column, which was washed once with 5 mL of washing buffer with 10 mM imidazole. The eluates were obtained with 400 μL of the elution buffer with 300 mM imidazole. The fractions were stored at −20 °C and analyzed by SDS-PAGE.[25,26]

5.2. Production of Anti-SpCas9 IgY Polyclonal Antibodies. Hy-Line Brown hens (36 weeks old) were used to produce anti-SpCas9 antibodies. The animal experiment was authorized by the Ethics Committee of the Faculty of Sciences of the Universidad Nacional de Colombia (Record Number 05-2022). A 28-day scheme was implemented as follows: on day one, 3 mL of a pre-immune blood sample was taken and 150 μg of pure SpCas9-6xHis antigen was mixed with Freund's complete adjuvant (1:1) and intramuscularly inoculated in the chest. On day 16, a boost was carried out by inoculating 150 μg of pure SpCas9-6xHis antigen prepared with Freund's incomplete adjuvant (1:1) (without heat-killed*Mycobacterium tuberculosis*). On day 23, 3 mL of an immune blood sample was taken, and another boost was administered as mentioned. Finally, 3 mL of a second immune blood sample was taken on day 28. Blood samples were incubated for 30 min at 37 °C, then the sera were collected by centrifugation at 3000*g* for 5 min at 4 °C and then stored at −20 °C. Along with the inoculation scheme, a daily collection of eggs was carried out. A hen inoculated with 1× PBS was used as a negative control.[27]

5.3. Extraction of Anti-SpCas9 IgY Polyclonal Antibodies. The yolks of the eggs collected from control and immunized hens on day 28 were used for standardization of the extraction process. Initially, the yolks were resuspended in 2 volumes of 1× PBS. Then, 1 mL of the sample was mixed with 500 μL of 1% (w/v) pectin and incubated ON at 4 °C. The lipid fraction was precipitated by centrifugation at 12,000 rpm for 20 min at 4 °C, and the protein fraction was salted out with AmS at 35% and incubated with constant shaking at room temperature for 20 min. The sample was centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant was used to repeat the salting out with AmS at 50%.[19] The enriched IgYs fractions were stored at −20 °C and further analyzed by SDS-PAGE, Western blot, and ELISA.

5.4. Protein Determination by Multiwell Plate Bradford Assay. Fractions from the IgY extraction protocol were diluted in 1× PBS (1:5). Then, 10 μL of these samples and BSA standards ranging from 25 to 500 μg/mL were loaded in the plate and mixed with 200 μL of the Bradford reagent (0.1 mg/mL Coomassie Brilliant Blue G-250, 5% ethanol and 8.5% H_3PO_4). The reaction was incubated for 10 min at room temperature and analyzed at 595 nm and 450 nm (iMark Microplate Reader, BioRad). A calibration curve was plotted (595 nm/450 nm vs concentration) and the fractions were interpolated.[28] Three technical replicates were made for all of the experiments.

5.5. Immunodetection of Anti-SpCas9 IgY Polyclonal Antibodies. The fractions from the IgY extraction protocol were analyzed by 12% SDS-PAGE and Western blot using PVDF membranes (Thermo #88518). The blocking step was completed ON at 4 °C with skim milk at 5% (w/v) in TBST buffer (20 mM Tris−HCl pH 7.5, 150 mM NaCl, and 0.1% (v/v) Tween). The membranes were washed three times for 5 min with TBST buffer and incubated with the anti-IgY coupled with alkaline phosphatase secondary antibody (Sigma #A9171) diluted in TBST buffer (1:10,000) for 90 min at room temperature. The membranes were washed as indicated and revealed with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium) in substrate buffer (100 mM Tris−HCl pH 9.0, 10 mM NaCl, 5 mM $MgCl_2$).[29]

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5.6. Antigen Detection Capability of Anti-SpCas9 IgY Polyclonal Antibodies. Indirect ELISA was carried out by fixing 1000 ng/well of the SpCas9-6xHis antigen ON at 4 °C, which was prepared at 10 ng/μL in carbonate solution (50 mM Na_2CO_3, 250 mM $NaHCO_3$, pH 9.6). The plate was rigorously washed with 1× PBS and blocked for 2 h at room temperature with 5% (w/v) of skim milk in 1× PBS. The plate was washed and incubated with 100 μL of the enriched IgY fraction from the extraction protocol and diluted 1:250 in 1× PBS for 2 h at room temperature. The plate was washed and incubated with 100 μL of anti-IgY coupled with horseradish peroxidase antibody (Sigma #A9046), diluted 1:10,000 in 1× PBS for 2 h at room temperature. The plate was washed and incubated with 150 μL of substrate solution (10 mL citrate buffer pH 9.5, 75 μL of H_2O_2, and 10 mg ABTS) for 1 h at room temperature and analyzed at 405 nm (iMark Microplate Reader, BioRad). Two technical replicates were made. Negative controls: two wells without antigens or antibodies. Positive reaction control: well with secondary antibody and substrate solution.[30]

5.7. Characterization of Anti-SpCas9 IgY Polyclonal Antibodies: Minimum Antigen, Maximum Dilution, and Antibody Specificity Assays. The minimum quantity of antigen detected by the anti-SpCas9 IgY polyclonal antibodies at a fixed dilution of 1:1000 for eggs and 1:5000 for blood was evaluated by Dot blot. Increasing amounts of 1, 5, 10, 25, and 50 ng of SpCas9-6xHis antigen were transferred to PVDF membranes. A similar procedure was applied to identify the maximum dilution of antibodies to detect a fixed amount of the antigen (50 ng), using different dilutions of the antibodies extracted from eggs (1:500, 1:1000, 1:2500, 1:5000) and blood (1:2500, 1:5000, 1:7500, 1:10,000). Blocking, washing, antibodies incubation, and the revealing steps were completed as mentioned.[29] Specific immunodetection assays were analyzed by Western blotting on PVDF membranes using 400 ng of the SpCas9-6xHis antigen and the recombinant erythropoietin (EPO-6xHis) as a nonrelated control. The anti-SpCas9 IgY from eggs (1:1000) and blood (1:5000), as well as the anti-6xHis (IgG) (1:5000) (Invitrogen #MA1-21315), were used as primary antibodies. Anti-IgY (1:10,000) and anti-mouse IgG (1:10,000) coupled with alkaline phosphatase were used as secondary antibodies.

5.8. Immunodetection of SpCas9 in Biological Samples. Exponential phase promastigotes of wild-type*L. braziliensis MHOM/BR/75/M2903* (**WT**) and*L. braziliensis* transfected with the episomal pTB007-Viannia plasmid that constitutively expresses the SpCas9 protein (referred to as **LbSpCas9** promastigotes),[17] were used to prepare total protein extracts. 10 mL of exponential phase parasites were collected by centrifugation at 6000 rpm for 5 min at 4 °C and washed with 10 mL 1× PBS. Then, a second wash was repeated with 1 mL of 1× PBS, and the precipitate was resuspended with 1× Laemmli loading buffer to 1×10^6 parasites/μL. The samples were incubated in boiling water, loaded (12 μL) in 10% SDS-PAGE, and transferred to PVDF membranes. Anti-SpCas9 IgY from eggs (1:1000) and immune blood (1:5000) were used as primary antibodies. Anti-IgY (1:10,000) coupled with alkaline phosphatase was used as the secondary antibody. Blocking, washing, antibody incubation, and the revealing steps were completed as mentioned.[29]

5.9. Bioinformatics Approach. The PDB file for the solved three-dimensional structure of the SpCas9 protein (PDB ID: 4CMP) was loaded into the *ElliPro Antibody Epitope Prediction* at the IEDB analysis resource (http://tools.iedb.org/ellipro/) using the default prediction parameters. The five most antigenic peptides were then searched for in the sequences of Cas9 proteins from different CRISPR/Cas systems, including*S. aureus*(SaCas9),*F. novicida*(FnCas9),*N. meningitidis*(NmCas9), and*C. jejuni*(CjCas9), as well as the inactivated version of SpCas9 (dSpCas9) and the adenine base editor ABE8e (dSpCas9-ABE8e). Multiple alignments were constructed with *T-Coffee*[31] using the Clustal-O algorithm and the identity percentages were obtained with *The Sequence Manipulation Suite*[32] Evolutionary relationships were analyzed by phylogenetic tree using the nearest Neighbor-Joining method with 1000 iterations in CLC Sequence viewer (version 7.5).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c04273.

Multiple alignment: Cas proteins from various CRISPR/Cas systems (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Luis E. Contreras R − *Facultad de Ciencias, Universidad Nacional de Colombia, 111311 Bogotá, Colombia;* orcid.org/0000-0002-3448-3640; Email: lecontrerasr@unal.edu.co

Authors

Esteban León − *Facultad de Ciencias, Universidad Nacional de Colombia, 111311 Bogotá, Colombia*

Valentina Ortiz − *Facultad de Ciencias, Universidad Nacional de Colombia, 111311 Bogotá, Colombia*

Alexander Pérez − *Facultad de Ciencias, Universidad Nacional de Colombia, 111311 Bogotá, Colombia*

Jair Téllez − *Escuela de Pregrado, Dirección Académica, Universidad Nacional de Colombia, 202017 sede La Paz, Colombia;* orcid.org/0000-0001-6646-8069

Gonzalo J. Díaz − *Facultad de Medicina Veterinaria y de Zootecnia, Laboratorio de Toxicología, Universidad Nacional de Colombia, 111311 Bogotá, Colombia*

María H. Ramírez H − *Facultad de Ciencias, Universidad Nacional de Colombia, 111311 Bogotá, Colombia*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c04273

Author Contributions

E.L. conceived, designed, and performed experiments; analyzed and interpreted the data; and wrote the paper. V.O. conceived, designed, and performed experiments. A.P. conceived, designed, and performed experiments. J.T. conceived and designed the experiments; analyzed and interpreted the data; and wrote the paper. G.J.D conceived and designed experiments, and contributed reagents and materials. M.H.R.H. conceived and designed the experiments; analyzed and interpreted the data; and contributed reagents and materials. L.E.C.R. conceived and designed the experiments; analyzed and interpreted the data; contributed reagents and materials; and wrote the paper.

Notes

The authors declare no competing financial interest.

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