Stool serology: development of a non-invasive immunological method for the detection of Enterovirus-specific antibodies in Congo gorilla feces

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Abstract

The incidence of poliovirus has been significantly reduced by 99.9% globally meanwhile vaccine-associated paralytic poliomyelitis emerges. Recently, a new recombinant virus (Enterovirus C/Poliovirus) was identified in humans working as eco-guards and gorillas in Democratic Republic of Congo, including one gorilla with polio-like sequeler. A strain of this recombinant virus (Ibou002) was also isolated from feces. In order to assess the potential role of poliovirus infection, we have developed and optimized a protocol, based on the lyophilization and solubilization of stool in a small volume, for the detection of specific antibodies. A high level of total immunoglobulins was first detected in the concentrated stool extracts. Then, specific antibodies were detected in 4/16 gorilla samples and 2/3 human samples by western blot using both poliovaccine antigen and Ibou002 antigen and by ELISA using polio vaccine antigen. The humoral responses were greater with the Ibou002 antigen both in number and intensity of bands. Thus, we suggest that this recombinant virus could cause polio-like disease in the endangered western lowland gorilla. The development of a non-invasive method for the detection of microorganism-specific immunoglobulins from fecal samples opens up new perspectives for the exploration of humoral responses of pathogens in animals and knowledge in zoonotic infectious diseases.

INTRODUCTION

Poliovirus is a virus belonging to the Picornaviridae family and the *Enterovirus* genus (Enterovirus C), which causes poliomyelitis in humans[1]. The poliovirus genome is a 7.5 kilobase single-stranded RNA surrounded by a capsid, which is composed by four proteins (VP1, VP2, VP3, and VP4)[2]. There are three poliovirus types (1, 2, and 3); the immunity to one type would not protect against the two others[3].

As a result of the global polio eradication program, initiated in 1988 with the use of live-attenuated oral poliovirus vaccine, the number of worldwide wild poliovirus cases has declined by more than 99.9%, from an estimated 350,000 cases in more than 125 endemic countries to 175 wild cases in 2019 (https://www.who.int/health-topics/poliomyelitis#tab=tab_1). Wild poliovirus types 2 and 3 were certified eradicated in 2015 and 2019, respectively[4,5]. The lack of circulation of wild poliovirus type 1 in Nigeria has been reported since 2016[14,15]. On 25 August 2020, the WHO African region was certified as free of wild poliovirus, leaving only the Eastern Mediterranean Region with endemic circulation of wild poliovirus type 1 in 2 countries (https://www.who.int/wer/2020/wer9541/en/)[6,11–13]. In parallel, another challenge arose with the emergence of vaccine-associated paralytic poliomyelitis[6]. Indeed, in the past 12 months, a total of 132 wild virus cases were reported in Afghanistan (61) and Pakistan (142), (http://polioeradication.org/polio-today/polio-now/this-week), while 605 cases of vaccine-associated paralytic poliomyelitis were detected worldwide, including in 19 African countries (406 cases), Afghanistan (101), Pakistan (80), Malaysia (1), Philippines (1), and Yemen (16). To note, among these 605 vaccine-associated cases, 424 originated from non-endemic countries and 181 from known endemic countries[6–10]. It has been also reported that 5-10% of polio deaths worldwide are due to cVDPVP[6].

The transmission of Poliovirus is fecal-oral. Humans remain the natural host, but it can also infect primates and monkeys[1,3]. There is a positive correlation between zoonotic diseases

and the emergence of infectious diseases in humans[16]. It has been shown under experimental conditions that hominoids and monkeys can be infected with poliovirus[17,18]. A 2005 study in Madagascar demonstrated the circulation of vaccine-derived recombinant poliovirus strains, which are recombinant Enterovirus-C viruses, and their ability to give "polio-like" in humans[19].

A study conducted by Harvala *et al.* in Cameroon and the Democratic Republic of Congo (DRC) screening for enteroviruses by PCR on chimpanzee and gorilla fecal samples demonstrated both the circulation of genetically divergent variants of enteroviruses in apes and monkeys as well as a strain (EV-A89) that they share with local human populations[20]. In addition, *Enterovirus* species described in humans and other species as a potential source of emerging infectious diseases in humans have been detected in monkeys[21,22].

Previously, our team reported the presence of a new Enterovirus C with a high similarity to Coxsackievirus as well as similarity with poliovirus 1 and 2 and associated with flaccid paralysis in chimpanzee virus EV-C99 strains[21] in the 3C, 3D, and 5'UTR regions[23]. Thus, we hypothesized a suspicion of poliovirus infection in a gorilla male living alone in Lésio-Louna-Léfini Nature Reserve (Republic of the Congo) based on clinical observations including facial paralysis. Our aim was to develop and to optimize a non-invasive experimental method to assess our hypothesis by the use of gorilla fecal samples to identify specific antiviral antibodies.

MATERIALS AND METHODS

Collection of gorilla feces samples

Fresh fecal samples were collected from western lowland gorillas (*Gorilla gorilla gorilla*) in semi-captivity and in wild in the Lésio-Louna-Léfini Gorilla Nature Reserve, located in the northern Pool Department, Republic of Congo. A total of 16 gorilla fecal samples were collected. Samples were collected from Iboubikro (Lat: 03.27040; Long 15.47071) for semi-captive gorilla youngsters and from Abio2 (Lat 03.094; Long 15.52633) for wild gorillas. Samples were collected in individual pots (Labelians, Nemours, France) and 50 ml Falcon tubes

(Dutscher, Brumath, France). At each collection time and for each sample, information relating to the GPS position of the place, the estimated time of decomposition of the feces as well as the morphological and/or physical aspect was recorded. During sample collection, we observed a male named Sid living alone on an island in front of Abio resort of Lésio-Louna-Léfini Nature Reserve, where he was placed after being sick. We observed evident signs of facial myodystrophy and paresis and upper limbs paresis (**Figure 1**). Three fecal samples were collected from 3 people working on the site (eco-gardian); an anonymous identification code was given to each sample (Ibou1, Ibou2, and Ibou3). A poliovirus serology-positive control serum (Poliomyelitis virus kit, GenWay, San Diego, California, USA) was also used as a control. Research and administrative authorizations for the collection of samples were granted by the Congolese Ministries of Scientific Research and Technological Innovation (N°003/MRSIT/DGRST/DMAST), Forest Economy and Sustainable Development represented by the Congolese Agency for Wildlife and Protected Areas (N°0134/ACFAP-DTS) and (N°94/MEFDD/CAB/DGACFAP-DTS) as well as the Ministry of Health and Population (N°000220/MSP-CAB-17 and N°208/MSP/CAB.15).

Concept and optimization of the methodological approach

The principle of our approach consisted initially in developing a method of extracting immunoglobulins which makes it possible to obtain structural and functional proteins for humoral evaluation. The extraction buffer contained protease inhibitor cocktail to inhibit the general degradation of proteins from stool extracts, tissues or cells by endogenous or exogenous microorganisms' proteases and to study processes that involve blocking the activity of specific proteases. Three gorilla and 19 human stool samples were used to optimize the concept. Different stool treatment protocols have been tested for the detection of immunoglobulins (IgG and IgA), mainly: (1) Filtration of stools extract from 2 g of stools plus 2 ml of buffer; (2) Lyophilization of the filtered extract, reconstituted in 500 µl of buffer and then purification of

IgG and IgA in the reconstituted extract with protein G and pectin M; (3) Lyophilization of the filtered extract, reconstituted in a small volume of buffer (500 µl) then concentrated by the Amicon® Ultra-1. After the evaluation of the data, a final protocol was adopted (**Figure 2**).

Experimental protocol adopted for stool protein extraction

Two grams of each sample were solubilized in 2 µl of an extraction buffer consisting of phosphate buffered saline solution supplemented with 4-(2-aminoethyl)-benzenesulfonyl fluoride 4 mM, bestatin 0.26 mM, E-64 28 µM, leupeptin 2 µM and aprotinin 0.6 µM, pH 7.4, and a protease inhibitor "SIGMAFAST™ Protease Inhibitor Tablets" (Sigma-Aldrich, Saint-Louis, Missouri, USA). The stool-buffer mixture was incubated for 15 min at room temperature, vortexed for 1 min, and then centrifuged at 1,500 rpm for 15 min at 4°C. The supernatant was lyophilized for 24 h and then re-solubilized in 1 ml of distilled water. Then, 500 µl of re-solubilized stool was concentrated using Amicon® Ultra-15 3k centrifugal filters (Merck, Saint-Romain, France) for further studies (**Figure 2**). Concentrations of total immunoglobulins (IgA and IgG) were evaluated by ELISA (Abcam, Paris, France) to validate the method of protein extraction from stools (**Figure 2**).

Quantification of anti-poliovirus immunoglobulins by ELISA

Determination of specific immunoglobulins was performed (IgG and IgA) by ELISA according the manufacturer's instructions (Poliomyelitis virus kit, GenWay, San Diego, California, USA). The polio antigen derived from the human pathogenic poliovirus types, specifically type 1 (Brunhilde), type 2 (Lansing), and type 3 (Leon) was bound in the wells of the plate. The diluted samples or standards were then deposited. After 1-hour incubation at room temperature, the plate was rinsed with wash buffer. The anti-human IgG peroxidase conjugate was then added and incubated for 30 min, followed by a wash step. Afterwards, the substrate solution was added and incubated for 20 min. The reaction was stopped by addition of a stop solution.

The resulting dye was measured using a Thermo Fisher spectrophotometer (Uppsala, Sweden) at a wavelength of 450 nm.

Characterization of enterovirus-specific antibodies by western blot from fecal samples

Preparation of antigen from Polio vaccine. Imovax Polio vaccine (Sanofi, Antony, France) was used as an antigen source for the characterization of poliovirus-specific immunoglobulins by western blot. The vaccine is a sterile suspension of polioviruses types 1 (Mahoney), 2 (MEF1), and 3 (Saukett), which are grown on Vero cells, purified, and then inactivated with formaldehyde. Four doses of Imovax Polio were treated with the TS lysis buffer and fractionated by sonication to release the antigen molecules. The antigen suspension was lyophilized and re-solubilized in 100 µl of distilled water (**Figure 3**). The concentrated antigen was measured by Bradford protein assay (Bio-Rad, Hemel Hempstead, UK), and a SDS-PAGE was performed with 50 µg of the purified antigen and 12% of polyacrylamide.

Preparation of antigen from enterovirus strain Ibou002. The strain Ibou002[23] of recombinant enterovirus C originally isolated from a eco-guardian of Lésio-Louna Nature Reserve was replicated during 7 days in MRC5 cells line (ATCC® CCL-171™). The virus infected MRC5 cells were collected and centrifuged at 700 × g for 15 min (Eppendorf Centrifuge 5810R). Supernatant was filtered by serial filtration steps to eliminate cells and cellular debris, going throw 0.8 µm, 0.45 µm, and 0.2 µm filters. Then, virus particles were precipitated in 10% PEG-8000 and 2.2% NaCl at 4ºC overnight, in agitation. The precipitates were centrifuged at 10,000 × g (Sorvall Evolution, SLA-3000 Recent-1 rotor, Thermo Fisher) for 30 min at 4ºC, and the virus pellet was resuspended in HEPES saline solution (9 g NaCl, 10 mL HEPES 1M, 990 mL distilled H_2O). The resuspended virus was purified by a 30% (wt/vol) sucrose cushion at 100,000 × g (Sorvall Discovery 90SE, Thermo Fisher) for 2 hours and 30 min at 4°C. The virus pellet was washed in HBSS (Thermo Fisher), and centrifuged at 100,000 × g (Beckman SW 32 Ti rotor) for 30 min at 4°C. The viral particles were finally resuspended in HEPES saline and

then fractionated with TS buffer (7 M Urea, 2 M Thiourea, 4% CHAPS) to release the antigen. The released antigen was then concentrated with the Amicon 3 kDa filter (Merck KGaA, Darmstadt, Germany) before being analyzed by SDS gel electrophoresis, and the western blot. The concentration of the antigen was measured. Then, 30 µg of purified were loaded onto 12% SDS-polyacrylamide gel for analysis.

Western blot. Western blot was performed using Jess™ Simple Western system (Protein Simple, San Jose, California, USA) according to the manufacturer's protocol[24]. This technique is an automated capillary size separation and nano-immunoassay system for performing gel-free, blot-free, and hands-free capillary-based immunoassay that integrates and automates the entire protein separation and detection process with home-made antigens. To quantify the absolute response to viral proteins from samples, we followed the manufacturer's standard method for 12-230 kDa Jess™ separation module (SM-W004). First, poliovirus antigen (1 µg/µL) was mixed with 5X master mix (Protein Simple) to achieve a final concentration of 0.2 µg/µL in the presence of fluorescent molecular weight markers and 400 mM dithiothreitol (DTT, Protein Simple). Then, this preparation was denatured at 95°C for 5 min. The ladder (12-230 kDa) and the viral proteins were separated in capillaries as they migrate through a separation matrix at 375 volts. A protein simple proprietary photoactivated capture chemistry was used to immobilize separated viral proteins on the capillaries. Patient feces extract was added and incubated for 30 min. After a wash step, goat HRP-conjugated anti-human IgG/IgM/IgA antibodies (Jackson ImmunoResearch, Ely, UK) diluted 1:250 was added for 30 min. Finally, the chemiluminescent revelations of ladder and samples were established with peroxide/luminol-S (Protein Simple). Digital image was analyzed with Compass for SW software (v 4.1.0, Protein Simple), and quantified data of the detected proteins was reported as the molecular weight, chemiluminescence intensity, and signal/noise ratio. Western blot

analyses were performed according this protocol using as antigens (1) the polio vaccine and (2) the purified Ibou002 virus strain.

RESULTS

Differentiation of the feces of gorilla individuals

A previous study by our team had confirmed that each feces sample corresponded to an individual gorilla[23]. A code was given to each of the 16 gorillas. Twelve had a G0 code followed by a numbering from 1 to 12 and 4 had a GNLL code followed by a numbering from 1 to 4.

Selection of stool preparation protocol

Using the protocol 1 based on raw stool extracts, very low concentrations of immunoglobulins were detected (**Supplementary figure 1**). Using the protocol 2 based on purification with protein G and peptin M, we also noticed a loss of immunoglobulin concentrations after purification (Data not shown). Protocol 3 based on the lyophilization of the filtered stool extract followed by its reconstitution in a small volume allowed to obtain the best results as shown in. In gorilla stool, the concentration mean with range of 222 (121.85-320.5) ng/ml was observed for IgA and 123.12 (25.87-208.9) ng/ml for IgG. In human feces, the concentration mean with range of 1600.55 (500-2978.8) ng/ml was observed for IgA and 619.7 (453.67-870.58) ng/ml for IgG (**Figure 4**). It is therefore the latter that was selected to continue analyzes on the stool.

Quantification and detection of poliovirus-specific immunoglobulins by ELISA and Western Blot

Quantification of poliovirus-specific immunoglobulins (IgA-IgG) by ELISA

The poliovirus-IgG/IgA evaluation of 16 gorilla and 3 human stool samples was carried out by ELISA; IgG/IgA level was represented by the OD at 450 nm. A sample was concluded as positive for IgG / IgA when the OD value was greater than 2 times the negative control value for the kit used (OD positive > 0.35). Specific antibodies were detected in 4 of 16 (25%) gorilla

samples (G01, G02, G03, and G07) and 2 of 3 human fecal samples (Ibou2 and Ibou3) (**Figure 5**). Besides the recombinant Enterovirus C (strain Ibou002) was isolated from one of the two eco-guardians (Ibou3) positive to poliovirus[22].

Detection of poliovirus- and enterovirus C-specific immunoglobulins by western blot

Protein profiles of both antigens (Imovax vaccine and strain Ibou002)

The protein profile was performed for both antigens (Imovax vaccine and strain Ibou002). Different protein profiles were identified for each antigen by SDS-PAGE. The vaccine displayed five bands possibly indicating five proteins with molecular weights ranging from 20-35 kDa. We found seven bands in the antigen purified from virus, which correspond to a molecular weight from 28 to 130 kDa (Figure 6A and B).

Western blot

The immunoglobulin evaluation by western blot showed positive samples for both antigens source (poliovirus vaccine and Ibou002 virus strain), but with different bands profiles between them. Bands were detected in 4 samples (G01, G02, G03, and G07) from 16 samples of gorilla feces, and 2 samples (Ibou2 and Ibou3) from 3 human feces, which were previously detected positive by ELISA. We also noted that all samples with bands from the vaccine antigen, also showed bands for the virus isolated, but at different molecular weight between antigen source (14 kDa for vaccine antigen, and 60 kDa and 116 kDa for antigen Ibou002 virus strain).

DISCUSSION

In this study, we developed a non-invasive method for screening animal immune responses using fresh stool samples. We also provided a proof of concept on the feasibility of exploring immunity from feces. Indeed, the detection of the total antibody levels (IgA / IgG) in concentrated stool extracts via the developed protocol confirms the possibility of using these samples for the detection, quantification and characterization of specific antibodies by ELISA

and western blot. The very high concentrations of IgA in feces can be explained by the microbial colonization of the gut that triggers the production of IgA by the gut associated lymphoid tissue (GALT)[25]. This supports our choice to use an anti-IgA, IgG mixture in our ELISA and western blot techniques to detect the maximum amount of specific antibodies. In addition, we did not look for specific IgM because the concentration of total IgM in the digestive mucosa is very low or even zero[26].

By testing a working hypothesis that circulating (probably vaccine) poliovirus[19] caused poliomyelitis-like disease in gorillas, we attempted to identify specific anti-poliovirus antibodies in the stools of 16 gorillas and 3 eco-guardians of the reserve. Using our original method of antibodies extraction, we detected antibodies binding to poliovirus antigen in fecal samples by ELISA[27], allowed us to identify positive samples among 4 of 16 gorillas and 2 of 3 eco-guardians. Such results in humans were not surprising as the majority of the population is vaccinated against poliomyelitis in this area. However, the positive results in 4 gorillas including Sid, the one with sequelae, were more surprising, indicating a potential circulation of the poliovirus or a close Enterovirus in this community. Then, we performed a western blot by Jess™ to characterize the specific antibodies. Western blot analysis of antibodies from gorilla feces detected a single band of around 14 kDa using the antigens extracted from poliovirus vaccine. These data do not allow confirmation of immunization of the gorilla with poliovirus due to the absence of antibodies against the main proteins of the poliovirus, namely VP1, VP2, and VP3. In addition, PCR testing for poliovirus RNA was negative[22].

We then based on the circulation of recombinant Enterovirus C[22] having at least 3 genes very close to the poliovirus in this geographical area to hypothesize that the gorilla Sid may suffer from the sequelae of polio-like illness caused by this virus. Indeed, these polio-like diseases can be caused in humans by chimeric/recombinant viruses[19–21]. These diseases often produce the typical sequelae in the form of flaccid paralysis and muscle atrophy. In order to

verify this hypothesis, we prepared the antigen from the recombinant Enterovirus C (Ibou002 strain) previously isolated from a stools sample of eco-guardian from the Lésio-Louna reserve. We noticed that the 4 samples of gorilla feces that showed bands with the vaccine antigen were the same samples that showed bands with the antigen from the recombinant Enterovirus C. This concordance was also observed with the two human feces samples. Western blot analysis allowed the detection of specific immunoglobulins to different viral proteins. Indeed, two 60 kDa and 110 kDa molecular weight bands were present in all positive samples from gorillas and humans with the Ibou002 strain antigen, and one 14 kDa band was also present in all positive samples with the vaccine antigen. Evaluation of the western blot results showed that the positive specimens for the two antigen sources (polio vaccine and Ibou002 virus strain) were the same but with different profiles. Thus, we hypothesize that the recombinant Enterovirus C that we recently isolated from an eco-guardian may have wide circulation and it was probably responsible for the immunization of humans and gorillas.

Given the presence of typical post-polio sequelae in the gorilla Sid, the presence of specific antibodies binding to the Ibou002 virus strain and the recombinant nature of this virus, we suggest that this new virus could cause polio-like disease in the endangered western lowland gorilla. We also believe that the detection of a band in western blot with the poliovirus antigen is the result of a non-specific cross-reaction between the antibodies induced by the recombinant Enterovirus C strain Ibou002 and the genetically related poliovirus. Another interesting finding is the detection of specific anti-recombinant Enterovirus C Ibou002 antibodies in the same stool sample from the person from which the virus was isolated. This may indicate a long persistence of the virus in the human organism and the absence of protective immunity.

CONCLUSION

Thanks to the non-invasive immunological developed method using feces, we were able to assess the presence of specific antibodies against a recombinant Enterovirus C in gorillas and

eco-guardians from the same reserve. This method, which does not require medical or invasive procedures for the collection of samples and allowing the detection of specific antibodies, opens numerous prospects for application in zoonotic infectious diseases and can revolutionize the screening of animals for emerging infections of importance, such as Ebola fever, rabies, and coronavirus infections.

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AUTHOR CONTRIBUTIONS

YS conducted the experiments, analyzed the data and wrote the paper; SMZ, HM, JV, NO and SM helped conduct the experiments and participated in the writing of the paper; JA, BD and IA contributed to the collection of samples; OM, DR, FF designed the project, participated in the writing of the paper and provided great support for the realization of the experiments.

ADDITIONAL INFORMATION

Competing interest statement

The authors declare no competing interests in relation to this study.

Figure legends.

Figure 1. Geographic map of the collection site and the gorilla named "Sid" with myodystrophy and paresis on the face that lives alone on an island in front of the Abio resort of the Lesio-Louna nature reserve (Republic of Congo).

Figure 2. Schematic representation of the three protocols evaluated for protein extraction from stools up to evaluation of immunoglobulins by ELISA and western blot. The protocol 3, written in black, is the one that was retained.

Figure 3. Protocol for the extraction of antigens from the polio vaccine (Imovax®) for analysis by western blot.

Figure 4. Evaluation of total IgA (A) and IgG (B) concentrations (ng/mL) in stools of 3 gorillas and 2 humans by ELISA.

Figure 5. Evaluation of poliovirus IgG/IgA by ELISA. Graph representing the specific IgG/IgA (OD) level of all samples, measured at a wavelength of 450 nm. A sample was concluded as positive for IgG / IgA when the OD value was greater than 2 times the negative control value for the kit used (OD positive > 0.35).

Figure 6. Silver stain SDS-PAGE analyses for poliovirus vaccine (A) and antigen of Ibou002 virus strain (B) proteins. In both profiles, the left lane corresponds to the molecular weight standard and the right lane to the antigen; 50 µg of poliovirus vaccine and 30 µg of antigen of Ibou004 virus strain were running in a 12% polyacrylamide gel.

Figure 7. Western blot Jess^{TM} using two different antigens, poliovirus vaccine (A) and Ibou002 virus strain (B). Anti-human IgG/IgM/IgA antibodies detection was done for 16 gorilla stools, 3 human stools, and 1 control positive serum (non-diluted and diluted 1:100).

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Figure 1. Geographic map of the collection site and the gorilla named "Sid" with myodystrophy and paresis on the face that lives alone on an island in front of the Abio resort of the Lesio-Louna nature reserve (Republic of Congo).

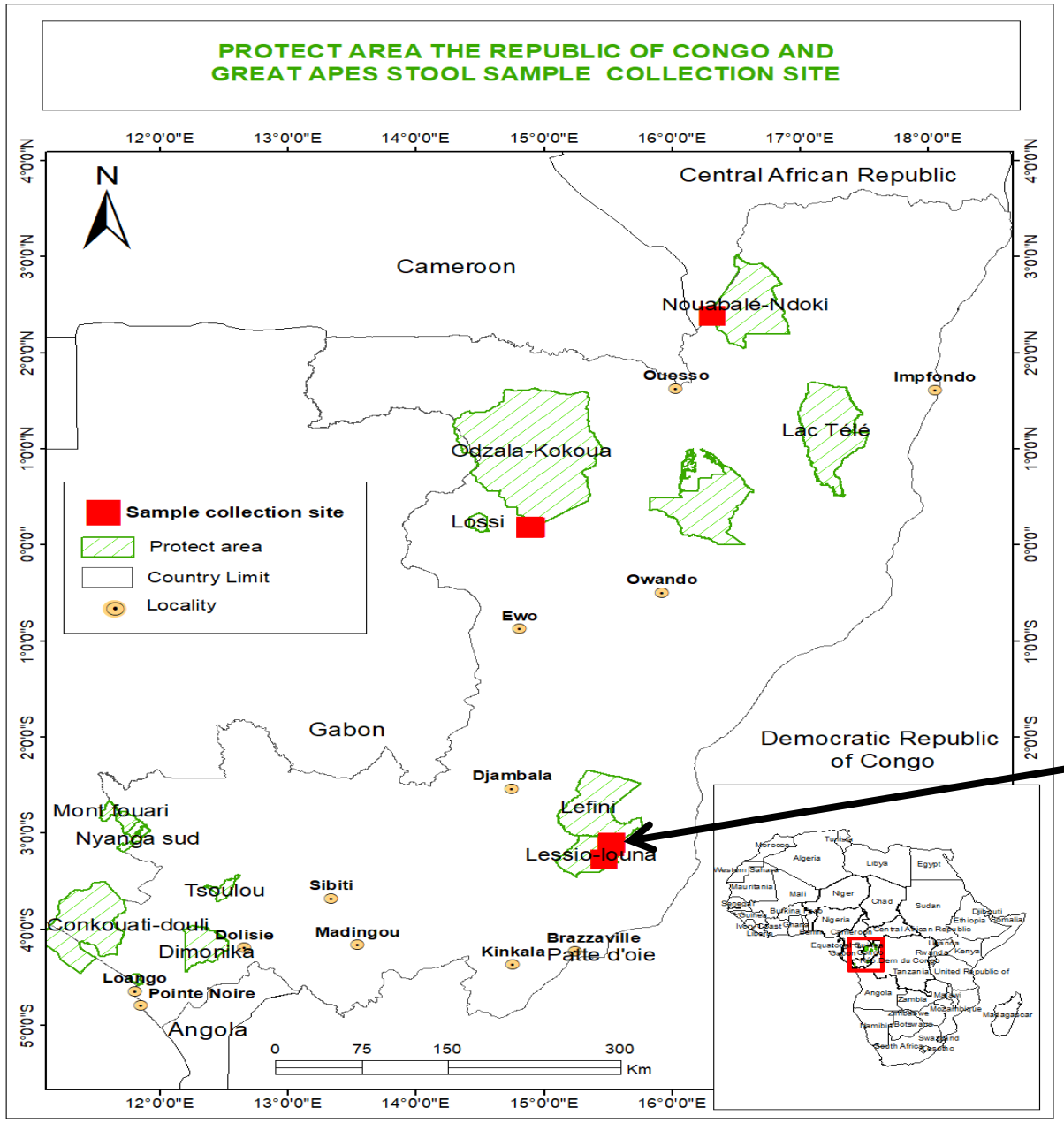


Figure 2. Schematic representation of the three protocols evaluated for protein extraction from stools up to evaluation of immunoglobulins by ELISA and western blot.

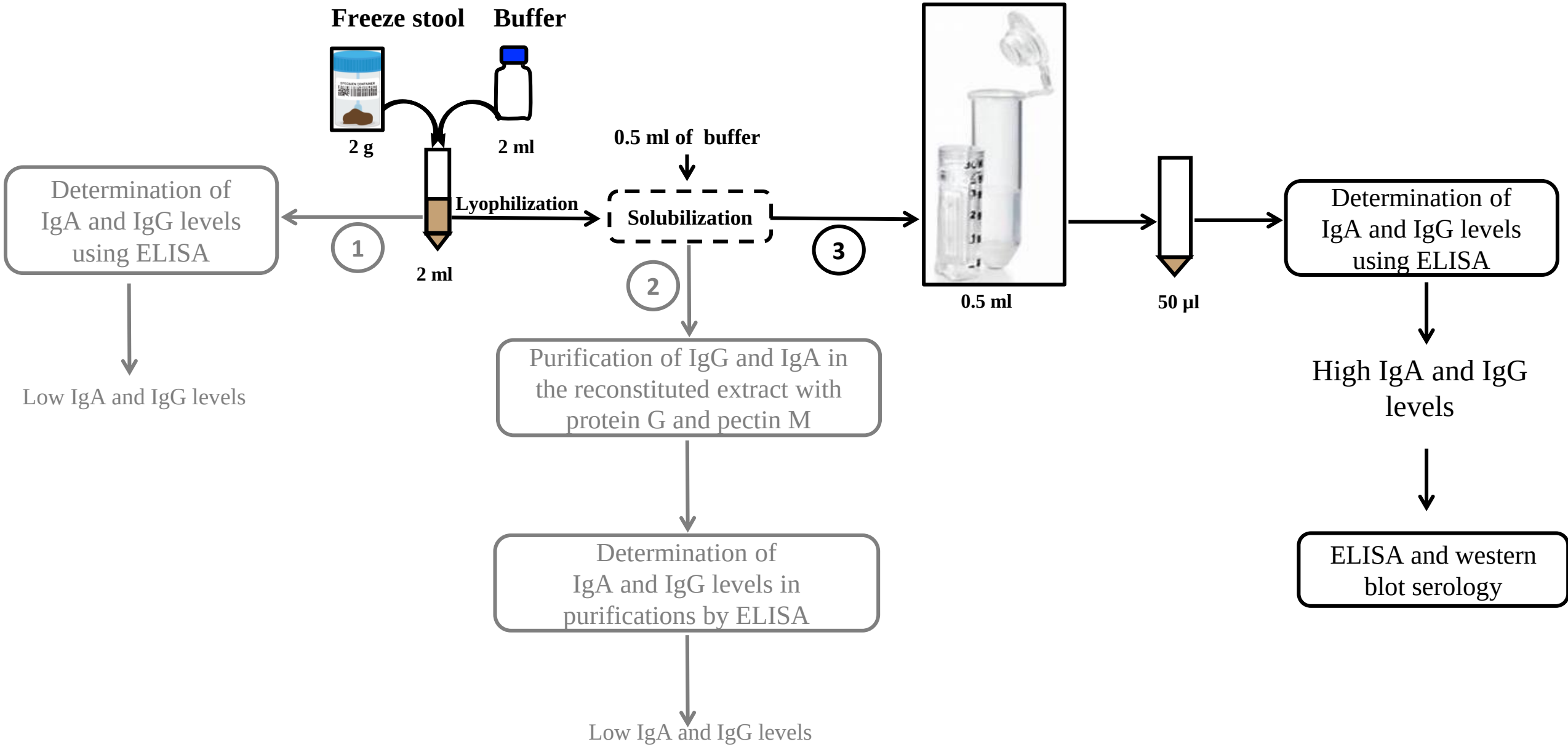


Figure 3. Protocol for the extraction of antigens from the polio vaccine (Imovax®) for analysis by western blot.

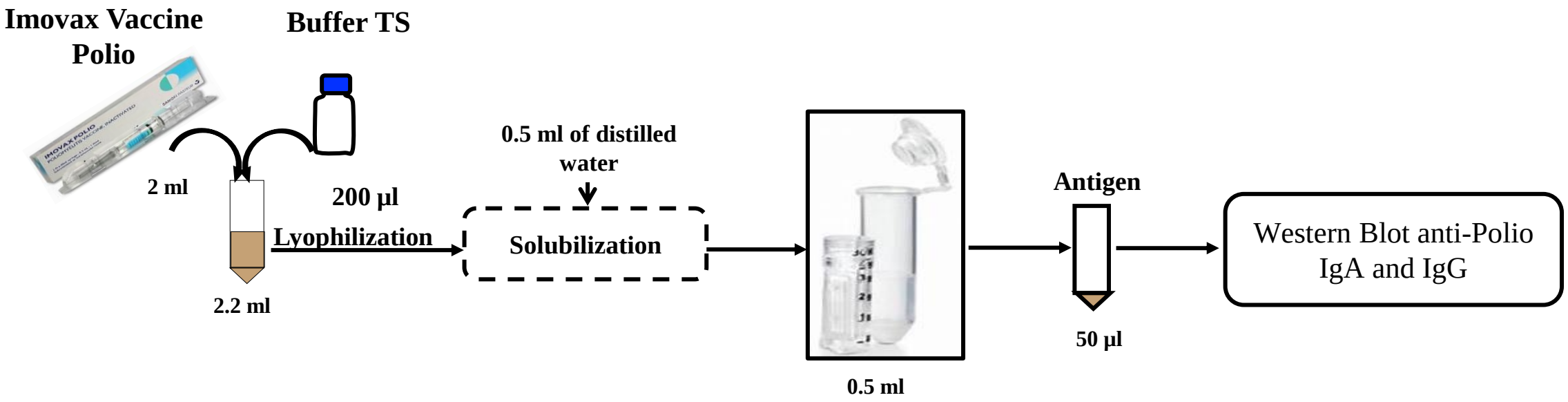


Figure 4. Evaluation of total IgA (A) and IgG (B) concentrations (ng/mL) in stools of 3 gorillas and 19 humans by ELISA.

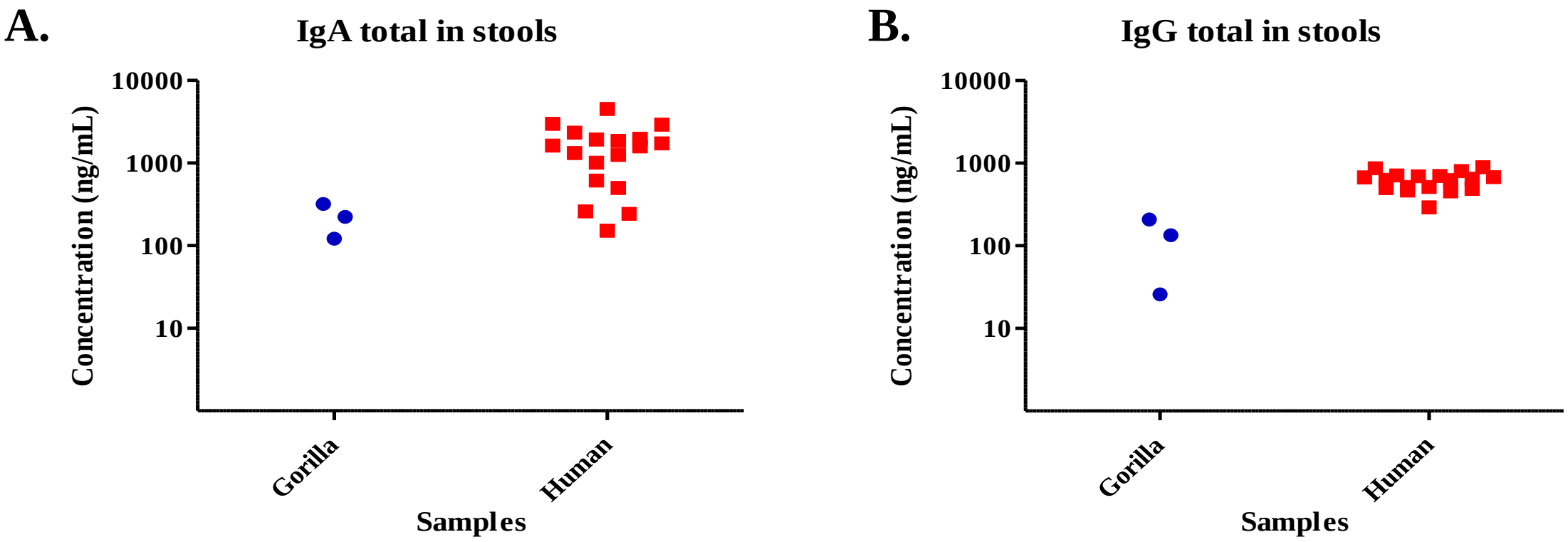


Figure 5. Evaluation of poliovirus IgG/IgA by ELISA. Graph representing the specific IgG/IgA (OD) level of all samples, measured at a wavelength of 450 nm. A sample was concluded as positive for IgG / IgA when the OD value was greater than 2 times the negative control value for the kit used (OD positive > 0.35).

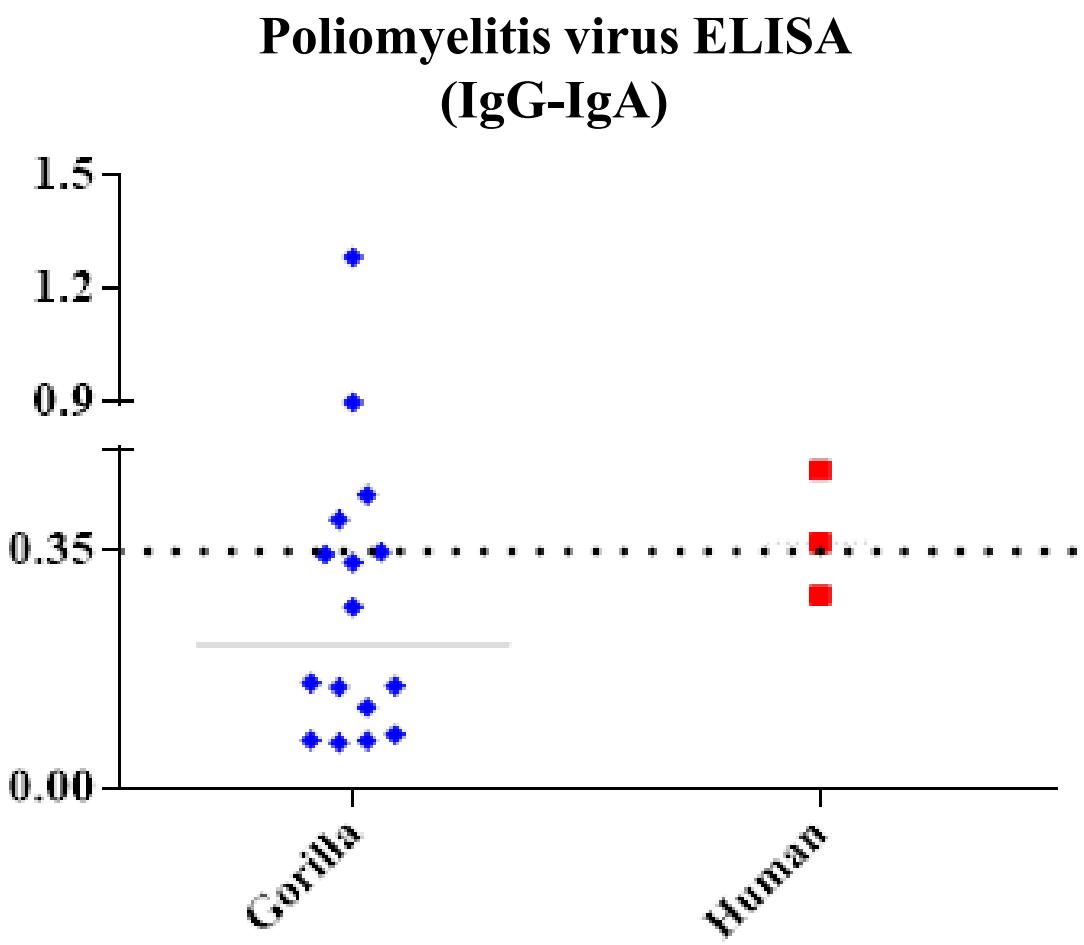


Figure 6. Silver stain SDS-PAGE analyses for poliovirus vaccine (A) and antigen of Ibou004 virus strain (B) proteins. In both profiles, the left lane corresponds to the molecular weight standard and the right lane to the antigen; 50 µg of poliovirus vaccine and 30 µg of antigen of Ibou004 virus strain were running in a 12% polyacrylamide gel.

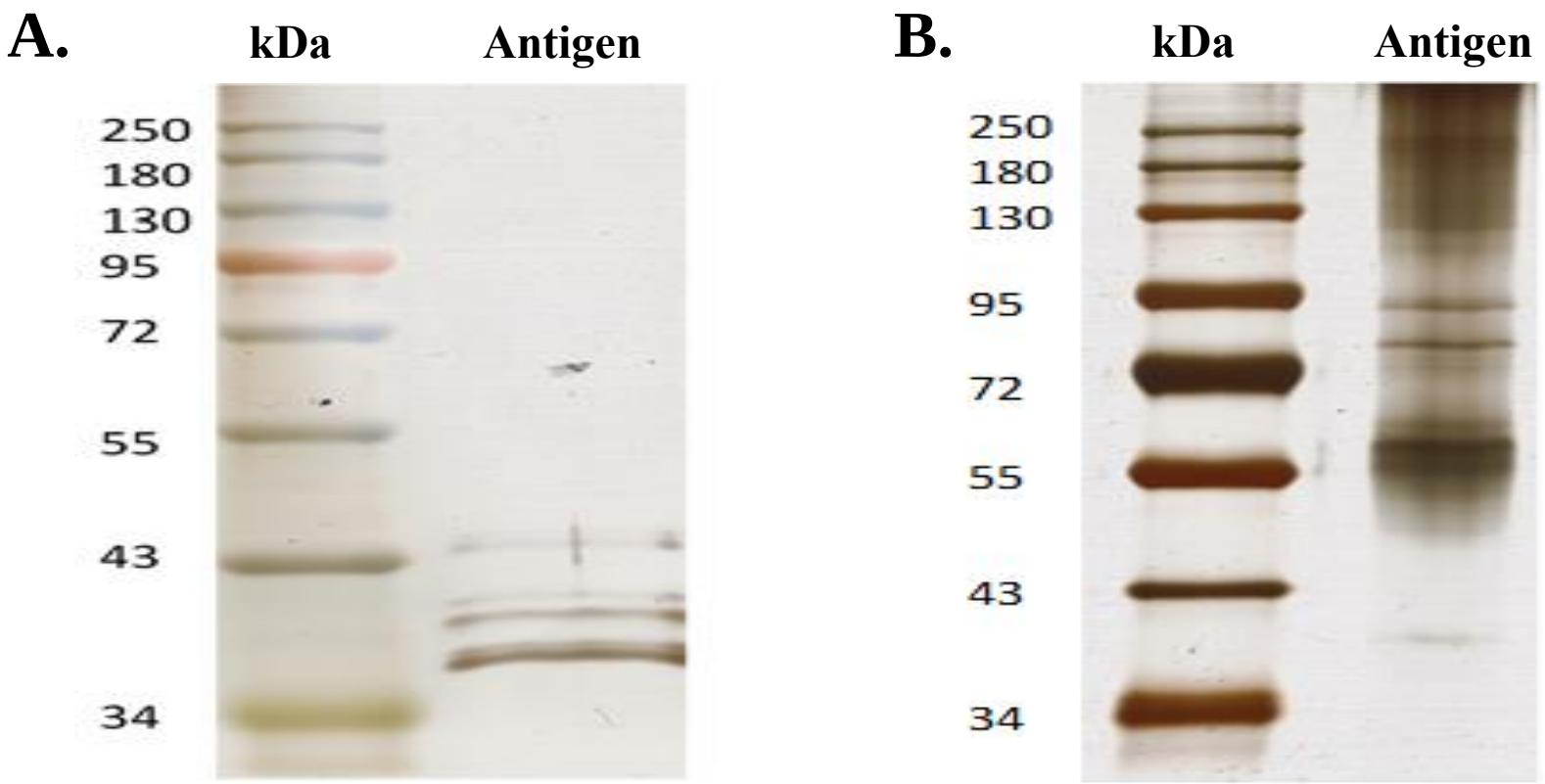
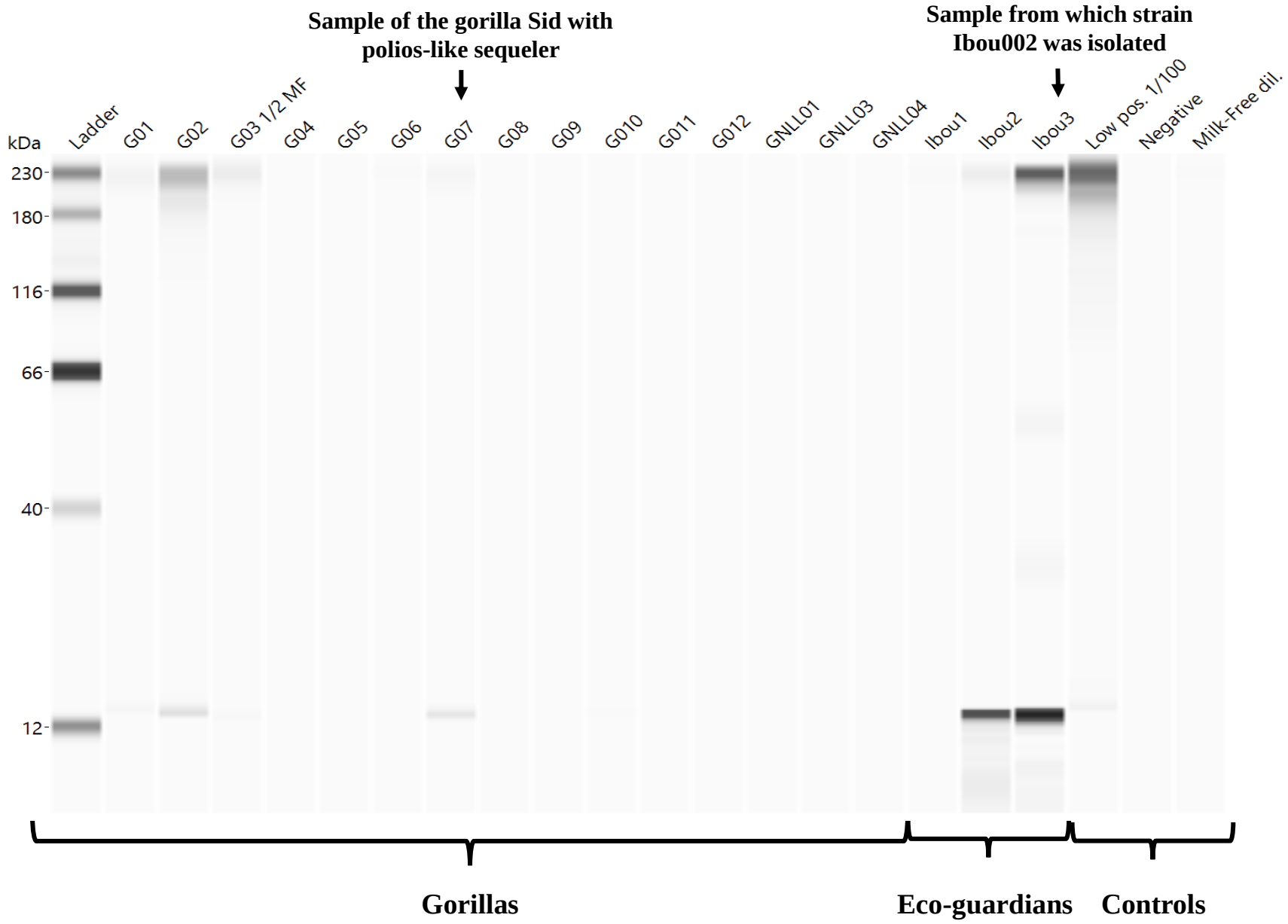
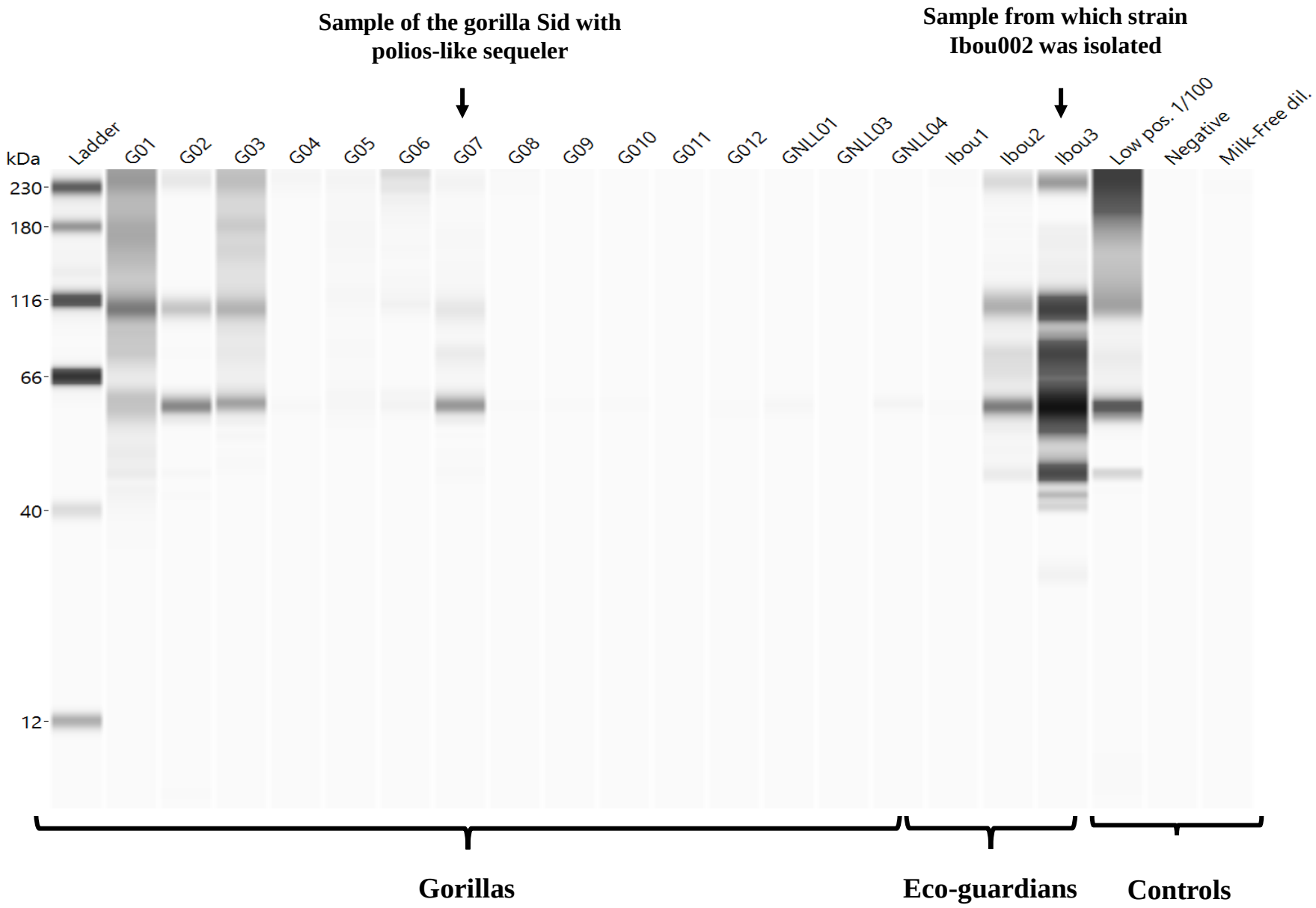
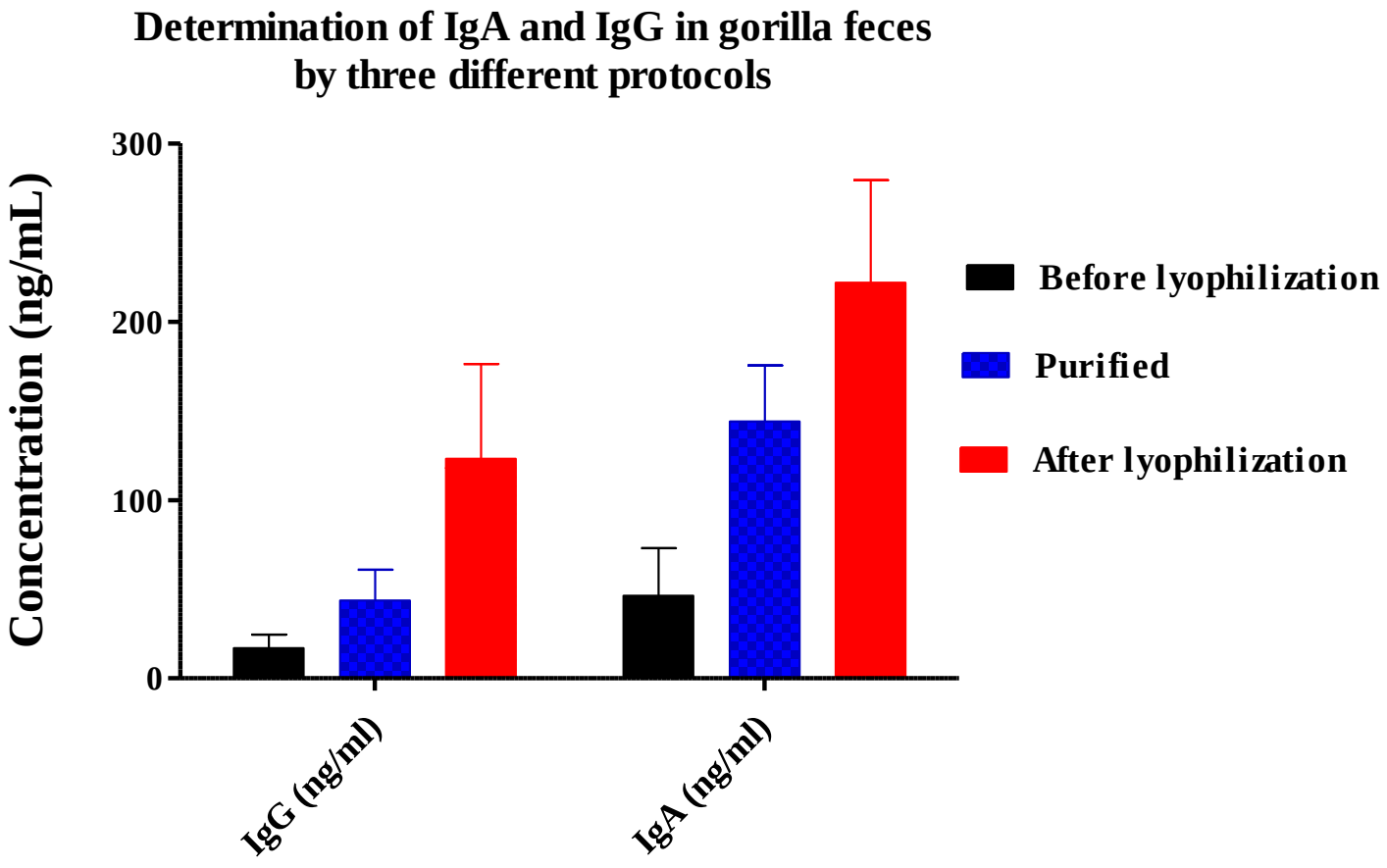


Figure 7. Western blot JessTM using two different antigens, poliovirus vaccine (A) and Ibou004 virus strain (B). Anti-human IgG/IgM/IgA antibodies detection was done for 16 gorilla stools, 3 human stools, and 1 control positive serum (non-diluted and diluted 1:100).

A.



B.


Sample of the gorilla Sid with polios-like sequeler
Sample from which strain Ibou002 was isolated
kDa
230
180
116
66
40
12
Ladder
G01
G02
G03
G04
G05
G06
G07
G08
G09
G010
G011
G012
GNLL01
GNLL03
GNLL04
Ibou1
Ibou2
Ibou3
Low pos. 1/100
Negative
Milk-Free dil.
Gorillas
Eco-guardians
Controls

Supplementary figure 1. Quantification of immunoglobulins (IgG and IgA) in gorilla feces with three different fecal treatment protocols (1) Filtration of stools extract from 2 g of stools plus 2 ml of buffer; (2) Lyophilization of the filtered extract, reconstituted in 500 µl of buffer and then purification of IgG and IgA in the reconstituted extract with protein G and pectin M; (3) Lyophilization of the filtered extract, reconstituted in a small volume of buffer (500 µl) then concentrated by the Amicon® Ultra-1