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Anti-Paraflagellar Rodc Antibodies Inhibit the In-Vitro Growth of Trypanosoma Brucei Brucei

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Abstract

Paraflagellar rod (PFR), a conserved structure expressed in all lifecycle stages of the order kinetoplasida except in the amastigotes is vital for the parasites survival. In *T.b.brucei*, the PFR protein has two major components, PFRc and PFRa with molecular mass 73kDa and 68kDa respectively. Experimental evidences implicate the PFR protein as a highly immunogenic and protective antigen. However, its immunogenic properties underlying its suitability as vaccine candidate has not been adequately investigated *in-vitro*. This study aimed to demonstrate the growth inhibitory potential of PFR protein against *T.b.brucei* parasites *in–vitro*. Antibodies against a recombinant form of the PFRc protein were produced and used to generate immune response. A deoxyribonucleotide (DNA) segment of approximate 672bp encoding the PFRc protein component was amplified using polymerase chain reaction (PCR), cloned and expressed in *E.coli* (*BL21*) cells. A 200 µg portion of the purified PFRc protein mixed with 100µl Freund's complete adjuvant (FCA) was used to immunize rabbits. An antibody titre of 2.5 x 10^4 reciprocal dilutions was obtained following three immunisation boosts, spaced two weeks apart. Western blot analysis showed that rabbit anti-PFRc antibodies recognised specifically a 25kDa protein corresponding to the estimated size of the expressed PFRc protein. 25% of purified anti-rabbit IgG antibodies were able to inhibit ~70% *T.b.brucei* parasite i*n vitro.*

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This confirmed that the PFRc protein is immunogenic in rabbits and can elicit specific growth inhibitory antibodies. However, we recommend invivo studies in humans and domestic animals infected by trypanosomes to ascertain the vaccine potential of this candidate protein for trypanosomiasis.

Key words: Anti-Paraflagellar rod c; antibodies; *in-vitro* growth; *Trypanosoma Brucei Brucei*.

1. Introduction

Trypanosomes are uni-flagellate parasites of the genus trypanosoma [1]. They are transmitted by tsetse fly vector of the genus Glossina but also biting insects like, tabanids, chrysops and haemopota [2]. There are three sub-species of *Trypanosoma brucei*; *Trypanosoma brucei gambiense* which causes the slow onset and chronic trypanosomiasis in humans in Africa, most commonly in Central and West Africa, where humans are the primary reservoir. *Trypanosoma brucei rhodesiense* on the other hand causes a fast onset and acute type of trypanosomiasis in humans, commonly in Southern and Eastern Africa, where game animals and livestock are the primary reservoir. *Trypanosoma brucei brucei* along with several other species of trypanosomes cause African trypanosomiasis in animals [3, 4] and is not human infective due to its susceptibility to lysis by human serum apolipoprotein L1. Consequently, *T. brucei brucei* is only used as a model for human infections in laboratory and animal studies since it shares many features such as antigenic variation with *T. b. gambiense* and *T. b. rhodesiense* which infect human. In the mammalian host, the bloodstream form of the parasite concentrate within the blood in the early stage of infection, but may migrate to other areas of the host such as the brain in later stage of infection where they cause meningoencephalitis [5].

The main control measures of trypanosomiasis is by either vector eradication using dichlorodiphenyltrichloroethane (DDT) [6] or chemotherapy using suramin for the early stage of the disease and melasoprol for the late stage [7]. However, due to complications such as drug resistance, non-compliance due to the high drug toxicity and cost, accompanying treatment regimes, there is an urgent need for the development of new and probably safer drug therapies for the management of trypanosomiasis [8, 9]. Drug resistance to trypanosomiasis is mainly due to the parasites ability to evade the immune system of the host by a unique and notable variable surface glycoprotein (VSG) coat [10, 11, 12]. This VSG coat is believed to hide the antigens on the inside, which has for many years hindered the development of a vaccine against the deadly trypanosomiasis despite a lot of research been done [13].

Nonetheless in the recent years, the trypanosome flagellum has been discovered to have a compact and filamentous structure called the Paraflagellar rod (PFR) which runs alongside the conical 9 + 2 micro-tubular axoneme. This PFR structure is has been shown to be conserved and unique among trypanosomatids, euglenoids, and dinoflagellate [14, 15]. And is believed to be expressed in all lifecycle stages of kinetoplasida except in the amastigotes. PFR has a 3-dimensional entity composed of proximal, intermediate and distal domains [16]. It has two major components, PFRc and PFRa proteins of approximately similar molecular mass of 73kDa and 68kDa respectively [17] that are the classic defining features of PFR [18]. In terms of function, the PFR has been associated with flagella motility where they help in both stiffening the flagellum and other biochemical processes like regulation, metabolism and signaling. Recent studies *in vivo* indicate that interfering

with the expression of PFRc proteins in trypanosomes, causes cell paralysis and is lethal in the bloodstream forms of parasites [19, 20, 21]. It was further demonstrated that the PFR protein is a highly immunogenic molecule having reactivity to not only many pre-immune sera but also cross reacts with specific polyclonal antibodies. This therefore suggests that a vaccine derived from such proteins will be effective against trypanosomiasis. As a prerequisite for vaccine development, this study set out to assess the effect of anti-PFRc antibodies on growth of trypanosome parasites *in vitro*. Hence this paper reports results of a study that investigated the inhibitory properties of antibodies generated against a recombinant PFRc protein.

2. Methods

2.1. PCR amplification, expression and purification

The recombinant PFRc protein which was used in this study was expressed in BL21 bacteria cells and purified using Ni-NTA affinity column. Briefly the genomic DNA (gDNA) was extracted from *T. b. brucei* parasites using a DNA extraction kit (QIAGEN). A DNA segment of approximately 672bp encoding the PFRc protein was then successfully amplified by PCR using forward primers: 5'CAG ATC GAT TAA GCG CTC 3' and Reverse primers: 5'ATG GCC GCA GTT GAC GAT GCC 3'.The PCR reaction contained; 200mM dNTPs, 1X PCR buffer, 1.5mM $MgCl_2$, 1.25units/µl of Taq polymerase, 0.4mM each of the forward and reverse primers and 1µl of DNA template in a total volume of 25µl. The PCR reaction involved a hot start at 95^{o}C for 5 minutes, followed by 30 cycles of denaturation at 94^{o}C for 1 min, annealing at 50^{o}C for 30 seconds, extension at 72^{o}C for 1min and a final extension at 72^{o}C for 10 minutes. After amplification, the PCR products were resolved on a 1% agarose gel and the 672bp size amplicon estimated using a DNA ladder after staining with ethidium bromide. The amplified DNA was excised from the gel and purified using a DNA purification kit (QIAGEN). The purified DNA and the expression vector (pET28a+) (Fig.6) were both digested using restriction enzymes BamH I and HindIII at 37^{0}C for three hours in a water bath. The cut DNAs were ligated using DNA ligase at 16^{0}C for 16 hours. *E.coli (BL21)* cells were first made competent by treatment with ice-cold 50mM $CaCl_2$ as described by Dagert & Ehrlich, 1979. The competent cells were then transformed with the recombinant vector pET28a+ using a protocol by Froger and Hall, 2007 (appendix IV). Protein expression was induced by the addition of 0.2mM IPTG (Isopropyl thio β-galactoside) to the culture and incubating for 4 hours. The purity and molecular weight of the recombinant protein was determined by SDS-PAGE and Western blot as described by Eslami and Lujan, 2014 using antibody dilutions of 1:2000 as primary anti-histidine monoclonal antibody and 1:5000 horseradish peroxidase (HRP) conjugated secondary antibody. The protein was then purified using a Nickel Affinity chromatography (Ni-NTA agarose) column (Qiagen) (according to manufacturer's instructions) and eluate stored at -80^{o}C until use.

2.2. Immunisation and Antibody purification

Swiss rabbits were immunized subcutaneously with 200µg of purified recombinant PFRC protein from *T. brucei brucei* emulsified with an equal volume of Freund's complete adjuvant (FCA) and then boosted twice with 100 µg of the PFR_C protein emulsified with an equal volume of incomplete Freund's adjuvant (IFA) on day1, 14, 28 and 42 respectively. For the control group, mice were immunized with adjuvant emulsified only with

phosphate buffer saline (PBS). Antibody levels and response were determined by collecting blood from the marginal vein of the rabbits' ears, pre immunization and on days 13, 27 and 41. From the blood samples, sera were separated and stored at −20°C until use for enzyme-linked immunosorbent assay (ELISA), Western blot and *in vitro* growth inhibition assays. Immunoglobulin class G (IgG) from serum were purified using resin agarose beads conjugated 50% with protein G and their concentration determined using the Bradford protein quantification method.

2.3. Evaluation of the level of antibody responses by ELISA

Each well of the 96-well microtitre plates (Greiner, Germany) were coated overnight at 4°C with 120 µL per well of 100ng recombinant PFRc protein in PBS buffer pH7.6. Then wells were then blocked with 200 µL of PBST (pH 7·2, 0·05% Tween 20) and 5% bovine serum albumin (BSA) at 37°C for 1 h. One hundred and sixty µL of each serum sample diluted 1 : 1000 in PBST was then added to each well and incubated at 37°C for 1 h. Bounded antibody was then detected by peroxidase-conjugated anti-rabbit IgG (Santa Cruz) diluted 1 : 10,000 in PBST. After three washes with 200ul of 1xPBST buffer, colour reaction was developed by addition of 100 µL (per well) of 2 µmol of deo-phenylenediamine (Sigma Fast) plus 0·003% H_2O_2 (hydrogen peroxide) at room temperature for 10 min. The reaction was stopped by addition of 100 µL of 1 mol/L sulphuric acid to each well. The plates were read at 490 nm in an ELISA plate reader (Biotek)

2.4. In vitro growth inhibition assay

Bloodstream *T. brucei brucei* parasites (strain GVR35) were used in the inhibition assay using protocols described by Lubega and his colleagues (2003) with modifications. Briefly, parasites were seeded in 96 well culture plates at a density of 1.32 x 10^5 parasites/ml in culture medium containing serial dilutions of anti- PFRc antiserum, pre-immune or adjuvant alone serum. Each sera sample collected from the rabbit on day 41 post immunisation were initially diluted (1:1) in 5% sterile BSA dissolved in PBS prior to making serial dilutions from 25% (highest) to 3.125% (lowest).

The experimental control in wells 10 and 11 contained the culture medium alone without antiserum. This was to cater for the normal growth of trypanosome parasites.

While wells 1 and 12 were also filled with culture medium to prevent evaporation during the incubation period. The plates in triplicates for each sera sample were then incubated at 37°C, 5% CO_2, in a humid incubator for 24 hours, after which viable parasites (i.e. parasites still actively moving) were counted using an improved Neuber hemocytometer at a magnification of X 200 [22].

3. Results

3.1. PFRC gene amplification and Ligation

The PFRc gene was amplified using specific primers (as indicated in methods) containing restriction sites (BamH I and Hind III) and targeting an amplicon of 672bp as observed on a 1% agarose gel (Fig1).

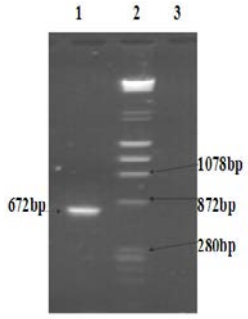

Figure 1: Agarose gel analysis showing a PCR product of PFRc gene.

The gene was amplified using specific primers containing restriction sites (BamH1 and Hind111) and targeting an amplicon of 672bp. Lane 1; a 672bp PCR product, Lane 2; Molecular marker, Lane 3; Negative control (PCR water).

Transformation of BL21 bacterial cells with recombinant pET expression vector and subsequently digesting the plasmid DNA with BamH I and Hind III revealed the presence of PCR amplicon of 672bp alongside a 43kb vector band (Fig.2), hence representing successful cloning.

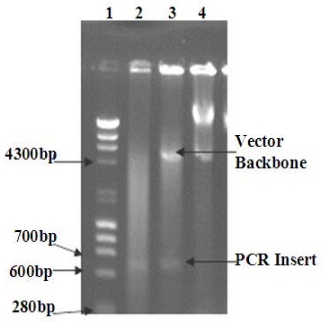

Figure 2: Agarose gel analysis of PFRc gene restriction digestion from pET28a+ vector

. Plasmid DNA was extracted from BL21 cells and digested with BamHl and Hindlll. An insert was released from the vector backbone that was of approximately the same size (672bp) as the PCR product that was cloned. Lane 1; Molecular Marker, Lane 2; Positive control (PFRc-PCR product), Lane 3; Digested plasmid (pET-28a [+]) with the PCR Product/insert, Lane 4; undigested plasmid.

PFRc recombinant protein of *T. brucei brucei* was expressed in the pET bacterial expression system as a fusion protein with six his-tag residues at the N-terminus.

SDS-PAGE analysis of the supernatant and pellet from the induced E. coli culture revealed that the recombinant PFRc protein was present in the soluble fraction (data not shown).

The PFRc protein of approximately 25kDa size was observed by SDS-PAGE in BL21 cells at an optimum time of 3 hours post-induction (Fig3A).

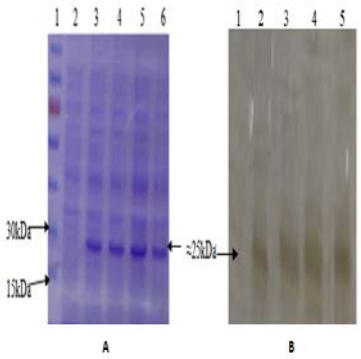

Figure 3: A). A 15% SDS-PAGE gel and Western blot analysis of the bacteria expression of PFR

c protein Culture aliquots of bacteria culture were collected after every hour of post induction for 4hours and prepared for SDS-PAGE. Following SDS-PAGE electrophoresis, a protein band of 25kDa was observed as expected with optimal expression occurring after 3hours post induction.A1= protein ladder, A 2=pre induction, A3=after an hour, A4= 2hours, A5 =3hours, A6= 4hours (B).Western blot analysis of PFRc identity. A membrane loaded with bacterial whole cell lysates at different expression times was probed with anti-His, followed by HRP-anti–Rabbit IgG, 1= pre induction, 2=1hour post induction, 3= 2hour, 4=3hour, 5= 4hour

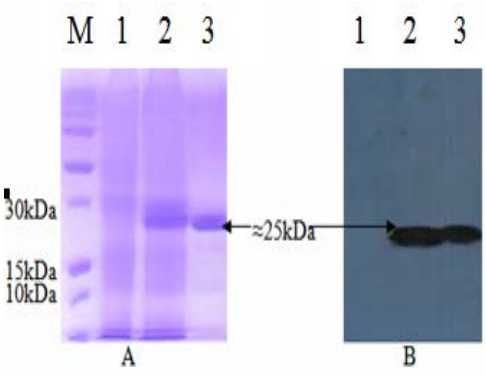

Figure 4

Western blot analysis showed that the expressed protein was recognized by anti-histidine-tag monoclonal antibodies (Fig3b). Analysis of the Nickel affinity chromatography (Ni-NTA agarose column) purification of PFRc protein on SDS-PAGE also showed a single protein band of approximately 25kDa (Fig4a Lane3). The purified protein was also recognized by anti-His-tag monoclonal antibodies (Fig4b Lane3).

Figure 4: A= SDS-PAGE and B=Western blot analysis of the purified PFRc protein expressed in BL21 bacterial cells was purified on a Nickel affinity chromatograph column under denaturing conditions and analyzed by SDS PAGE followed by western blot (A). M=Protein marker, Lane 1: Pre induction sample, Lane2: Crude protein after 3hours post induction, Lane3 purified protein after 3hours of post induction. (B) Lane 1=pre induction sample 2=crude protein sample, lane 3 = purified protein sample.

Immunization of rabbits with recombinant PFRc protein induced production of antibodies which reached the highest level on day27post immunization (Fig5).

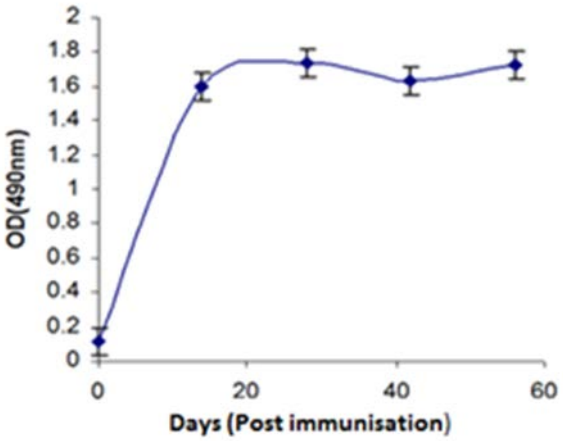

Figure 5: Antibody responses in vaccinated rabbit

The rabbits were immunized subcutaneously with 200 µg of recombinant PFRc protein and booster doses given every fortnight for 56 days**.** When used in Western blot analysis, the antibodies in serum from immunised animals specifically recognised the recombinant PFRc protein (Fig6).

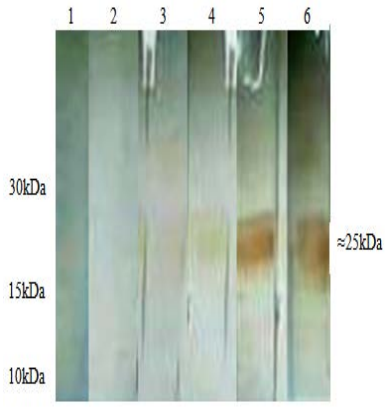

Figure 6: Western blot analysis for the specificity of anti-PFRc antibodies

Nitrocellulose membranes embedded with recombinant PFRc protein were probed with sera from the four different boosts followed by anti-rabbit IgG-HRP before visualization using DAB. Lane 1= Protein marker, Lane 2=pre-immune, Lane 3=1st boost, Lane 4=2nd boost, Lane 5=3rd boost and Lane 6=4th boost.

Addition of serum collected from immunised rabbits at day 27post immunisation inhibited *T.brucei brucei* parasite *in-vitro* growth in dose dependent way such that the numbers of parasites increased gradually with decreasing dilutions (Fig7).

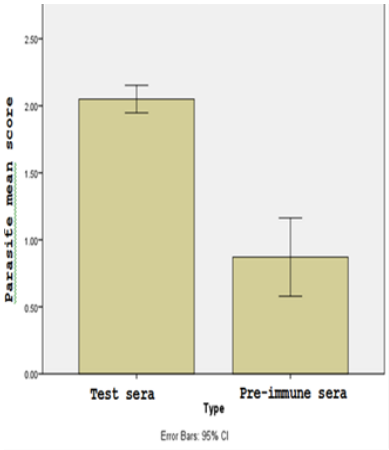

Figure 7: Growth inhibitions of parasites by different anti-sera groups

. Showing parasite mean scores (parasites death score) from test and pre-immune sera. 13.2×10^4 trypanosome parasites seeded in 96 well ELISA plate with HM1-9 medium were subjected to different dilutions (ranging from 25% - 0.4% and maximum parasite death score was observed at the highest concentration ie 25%) of anti-PFRc antibodies for 24 hrs in a 5% CO2 incubator, with $P<0.0001$

4. Discussion

In effort to further investigate the immunological and vaccine potential of the PFRc antigen, a 672 bp segment of the PFRc gene from the *T. brucei* parasites (GVR35 strain) was successfully amplified using PCR and cloned into pET-28a [+] vector backbone as observed in Fig.1 which was restrict digested with Bam HI and Hind III enzymes (Figure 2). The result of protein expression experiment in *E. coli BL21* cells (Fig.3A Lane 3,4,5 and 6) shows that cloning and expression of the recombinant protein was successful since the expected protein size of 25 kDa was obtained after induction.

No corresponding protein was expressed in the non-induced bacterial culture (Fig.3A Lane 2). This implies that the observed recombinant protein band was a result of induction of protein production by the inducer agent IPTG. In a related expression studies for PFRc in *T. cruzi* [14] proteins of different sizes were obtained. Suggesting that the PFRc gene is readily expressible in any species of trypanosome; this could be attributed to the lack of introns in the parasite genome, allowing a continuous gene expression without interruption. The expression of recombinant PFRc protein in *T. brucei* was confirmed by Western blot analysis using anti-histidine antibodies which preferentially bind the six histidine residues tagged to the expressed protein. The Histidine tag (His-Tag) is usually placed at the amino, and carboxyl-terminals or inside the region of the fusion protein expression in bacteria, yeast and mammalian cells which easies effective purification of recombinant proteins [23]. The six His-Tag can subsequently be cleaved-off from the expressed protein using enzymes thrombin and enterokinase, but due to its small size and poor immunogenicity, the six His-Tag) could also be used as protein conjugates in immunization without cleavage and insignificantly alters the biochemical properties of the recombinant protein. Although, conjugation has been reported to alter the finest specificity of humoral and cellular immune responses , Histidine-tagged PFRc protein was used for the immunization

experiments in this study and produced highly specific antibodies (Fig6). Furthermore, although the present study used unfolded protein immunogen that has been purified under denaturing conditions, the rabbit antibody titre produced were high as shown in (Fig5) and sufficient for subsequent experiments although [22], suggested and demonstrated the need for using a re-folded protein in protecting mice against challenge with *T. brucei*, *T. b. rhodesiense* and *T. congolese.* To determine whether the 25kDa size recombinant *T. brucei* PFRc protein could induce antibody production, rabbits immunized with 200µg of the purified protein emulsified in Freund's complete adjuvant produced an antibody titer of 25000 following 4 booster injections. This demonstrates successful production of an immunogenic recombinant molecule capable of stimulating antibody production in experimental animals. The high levels of parasite inhibition exhibited by these antibodies suggests that they are predominantly produced and hence less influence from non-specific components that might present. . Parasite growth inhibitory effect of rabbit antibody against the recombinant PFRc protein alongside three experimental controls: pre-immune rabbit sera (negative), Freunds adjuvant and culture medium, showed no significant difference ($p < 0.22$) (Fig.7A).One way ANOVA suggested that the three controls had a similar effect on parasite growth and hence using any of them as negative control against the rabbit anti-sera in subsequent experiment did not alter the outcome data analyzed using paired t-test between the pre-immune and the immune or antisera showed significant difference ($p < 0.0001$) in the parasite growth patterns (Fig.7B). The percentage of growth inhibition between the immune sera (test) and pre-immune sera (control) was 71.6%, which is significantly higher than ($p < 0.0001$) that of adjuvant against the culture medium (14.3%) measured at 25% antibody concentration (the highest IgG dilution). A parasite growth inhibition of more than 50% was observed with up to 1.56% dilution of antibody (IgG) beyond which the inhibitory power of the antisera gradually decreased with increasing dilution. In general, the results of this study show that anti-*T. Brucei* PFRc antibodies produced in rabbits significantly inhibited parasite growth *in-vitro* ($p<0.001$) implying that it has great potential as a vaccine candidate for trypanosomiasis if further characterized. However, this study did not establish the exact mechanisms and mode of action of anti-PFRc antibodies in inhibiting parasite growth. Although previous reports suggested that for antibodies to be able to recognize an internal antigen such as the PFRc protein, a portion of the parasite could have been lysed before to expose the target antigen [15]. It is also believed that antibodies could access the PFRc antigen through pockets within the flagellar protein by endocytosis [24]. Since endocytosis is the main process by which trypanosomes take in nutrients from the environment including antibodies bound on cell surfaces. This possibly explains the strong and specific interaction between the PFRc-IgG amid other non-specific co-factors. Thus, in addition to several successful experiments *in-vivo* involving PFR antigen from different sources [25], this study provides useful information and contributes to unraveling of the full vaccine potential of this unique and highly conserved antigen as the first vaccine against trypanosomiasis.

Due to limitation of infrastructure, this study did not investigate the inhibitory mechanisms of the antibodies

5. Conclusion and recommendations

Results of this study demonstrate the immunogenic and inhibitory potential of a recombinant version of the PFRc molecule deserving more studies to unravel its full potential for development into a stand-alone or a component of a future multi-component vaccine against all forms of trypanosomiasis.

We recommend further studies to elucidate the mechanism by which the paraflagellar antibodies inhibit the growth of the *T. Brucei* parasites

6. Ethical considerations

This study did not require the use of human and or human specimens. This study employed the use of animals (rabbits) which didn't require the ethical approval basically because the law against animal rights has not been established in Uganda. Therefore there were no informed consents required.

7. List abbreviations

PFRc	Paraflagellar rodc
DNA	deoxynucleotide
kDA	kilo Dalton
IgG	Immunoglobulin G
FCA	Freunds complete adjuvant
DDT	dichlorodiphenyltrichoro ethane
VSG	Variant surface glycoprotein
dNTP	deoxynucleotide triphosphate
gDNA	genomic DNA
$Mgcl_2$	Magnesium chloride
$Cacl_2$	Calcium chloride
IPTG	Isoproyl-thio-beta-galactoside
SDS-PAGE	Sodium deodecyl sulphate –Polyacrylamide gel electrophoresis.
HRP	Horseradish peroxidase
IFCA	Incomplete freunds complete adjuvant
PBS	Phosphate buffer saline
ELISA	Enzyme linked immunosorbent assay

Competing Interest

The authors declare that there are no competing interests

Authors' contributions

1. Mukisa Ambrose: helped in the conceptualization of the idea , performed the experiments and Drafted the manuscript
2. Claire Aguttu: helped in the conceptualization of the idea, provided technical advice and helped in supervision of experiments
3. G.W. Lubega: Provided Technical advice, supervision, proof reading of manuscript
4. Joseph Kyambadde: Provided Technical advice, supervision, proof reading of manuscript
5. Pius Alibu: Provided advise, supervision, and proof reading of manuscript
6. Peter Vuzi: Helped in, supervision of experiment and editing of manuscript

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