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The corn smut-made cholera oral vaccine is thermostable and induces long lasting immunity in mouse

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Highlights

- Stability and immunogenicity of a corn smut CTB-based cholera vaccine are reported
- The vaccine induced toxin neutralizing IgA responses in mice at 15 µg doses
- The vaccine is highly thermo stable and induced long lasting immunity

Abstract

The use of corn smut for the production of recombinant vaccines has been recently implemented by our group. In this study, the stability and immunogenic properties of the corn smut-based cholera vaccine, based on the cholera toxin B subunit (CTB), were determined in mouse. The immunogenic potential of distinct corn smut CTB doses ranging from 1 to 30 µg were assessed, with maximum humoral responses at both the systemic (IgG) and intestinal (IgA) levels at a dose of 15 µg. The humoral response last for up to 70 days after the third boost. Mice were fully protected against a challenge with cholera toxin after receiving three 15 µg-doses. Remarkably, the corn smut-made vaccine retained its immunogenic activity after storage at room temperature for a period of 1 year and no reduction on CTB was observed following exposure at 50°C for 2 h. These data support the use of the corn smut-made CTB vaccines as a highly stable and effective immunogen and justifies its evaluation in target animal models, such as piglet and sheep, as well as clinical evaluations in humans.

Abbreviations

CTB = Cholera Toxin B subunit

CT =Cholera Toxin

FA = Fluid Accumulation

Keywords: cholera; low-cost vaccine; oral immunization; cold chain; neutralizing antibodies; cholera toxin.

1. Introduction

Cholera is an acute intestinal infection derived from the ingestion of food or water contaminated with *Vibrio cholerae*, a gram negative bacteria, which produces the cholera toxin (CT) responsible for causing a copious, painless, watery diarrhea. Subsequently, patients can present severe dehydration and even death (WHO). CT consists of two subunits: the A subunit (CTA) and the B subunit (CTB). The latter forms a 55 kDa homopentameric non-toxic protein that binds the GM1 ganglioside on mammalian cells and induces a potent humoral immunity that leads to CT neutralization in the gut (Kopic and Geibel 2010). Cholera remains as a global threat, being poor countries the most affected since cholera transmission is associated to the lack of potable water sources and adequate sanitation of public services. In 2012, a WHO report estimated that 2.8 million cases of cholera (uncertainty range: 1.2–4.3 million) and about 91 000 deaths (uncertainty range: 28 000–142 000) occur in endemic countries every year. In contrast, 87 000 cases and 2500 deaths occur in non-endemic countries. The burden of cholera is greatest in Africa and southern Asia, where poor economies do not achieve adequate access and/or sanitization of fresh water (Mohamed et al. 2012).

To prevent cholera, safe and effective oral cholera vaccines have been licensed and used by affluent tourists for more than a decade. Thus far, oral vaccine consists on rCTB-alone or rCTB combined with two dominant domestic killed *V. cholerae* strains (O1 Ogawa El Tor and O1 Inaba El Tor) plus one standard *V. cholerae* strain (O1 Ogawa classic ATCC 14035). Both formulations have shown an acceptable protection against toxigenic *V. Cholera* (Boustanshenas and Bakhshi 2014). Currently, two oral cholera commercial vaccines are available. Dukoral is internationally licensed and prequalified by the WHO for purchase by United Nations agencies. Dukoral is formulated with inactivated *Vibrio cholerae* O1 whole cells plus recombinant cholera toxin B subunit (BS-WC). In the 1980's, this vaccine showed to be safe and highly protective (~85%) in a large-scale field trial in Bangladesh (Clemens et al. 1986, 1990). Moreover, Shanchol is a much less expensive killed oral cholera vaccine licensed in Vietnam and India and is undergoing the WHO prequalification. The Shanchol vaccine, consists of WC

without recombinant cholera toxin B subunit and proved to be safe and efficacious in a double-blind, placebo-controlled trial (Sridhar 2009; Sur et al. 2009).

The emergence of large and prolonged outbreaks, particularly in sub-Saharan Africa, make WHO to reconsider the recommendation of not oral cholera vaccination once an outbreak had started (Bhattacharya et al. 2009). Therefore, the limited economic resources in the affected countries limits the current supply of cholera vaccines to implement national vaccination campaigns and is not sufficient to meet endemic and epidemic worldwide needs (Deen et al. 2015). Thus, the research and development of new affordable vaccines is crucial to prevent cholera outbreaks. Given the relevance of CTB as protective immunogen, it has been expressed in several recombinant systems, including plant species, such as rice (Yuki et al. 2013), yeast (Arzanlou et al. 2005; Jung-Gu and Hyo-Sang 2008) and silkworm (Li et al. 2014). We have recently reported on the expression of CTB using corn smut or 'huitlacoche' as a new platform observing competitive yields (Juárez-Montiel et al. 2015) The present study aimed at characterizing the immunogenic properties of the corn-smut CTB vaccine, which induced long lasting immunoprotective responses at low doses.

2. Materials and methods

2.1. Corn smut production

Corn smut expressing CTB was produced as previously described (Juárez-Montiel et al. 2015). Briefly, FB1 WT and FB2-CTB3 *Ustilago maydis* strains were grown in either liquid YPD (2% yeast extract, 1% peptone, and 1% glucose) or YEPSL (0.4% yeast extract, 0.4% peptone, and 2% sucrose) medium at 28°C and shaking at 250 *g*. Crosses of paired strains were performed as previously reported (Holliday 1974). 4-6 days after silking, a 10 mL volume of each strain mixture was injected into silk channel of primary ears in order to induce maize ear galls. Inoculated maize plants were maintained under greenhouse conditions. 'Huitlacoche' was harvested 18 days post inoculation and subjected to lyophilization. Samples were processed in a LABCONCO freeze-dry system (FreeZone 6 Liter) during 48 h at a -50°C collector temperature. Dry material was subsequently milled and stored at room temperature until further use.

2.2. Thermostability assessment

Freeze-dried corn smut samples, which were maintained at approximately 25°C during one year, were subjected to 37°C, 50°C, 60°C, and 80°C treatments for 2 h. CTB levels and integrity were assessed using Western blot assays and ELISA. Protein extracts of corn smut galls were obtained by resuspending 30 mg of freeze-dried tissue in 300 µL of the extraction buffer consisting of 750 mM Tris-HCl pH 8.0, 15% (w/v) sucrose, 100 mM β-Mercaptoethanol and 1 mM PMSF (Franklin et al. 2002). Then, protein extracts were centrifugated at 16,000 *g* for 15 min at 4^{o}C. Supernatants were separated and 30 µL aliquots were mixed with reducing loading buffer. Samples were denatured at 95^{o}C for 5 min and a SDS-PAGE was performed in 4-12% polyacrylamide gels. The gel was blotted onto PVDF membranes (Pall Corporation, http://www.pall.com), which were blocked with a 5% fat-free milk (Carnation, www.nestle.com) solution prepared in phosphate saline buffer (PBS) plus 0.01% Tween 20 (PBST). Primary labelling was performed overnight using a mouse anti-CTB antiserum (1:200 dilution) followed by labelling with a horseradish peroxidase-conjugated secondary anti-mouse antibody 1:2000 dilution, (Sigma, http://www.sigmaaldrich.com) during 2 h at room temperature. Immunodetection was completed by using the SuperSignal West Dura solution following the manufacturer's instructions (Thermo Scientific, http://www.thermoscientific.com), pure CTB was included as positive control (Sigma).

For ELISA analysis, 50 mg of lyophilized corn smut subjected to thermal treatments were resuspended in 500 µl of protein extraction buffer (50 mM Tris pH 8, 40 mM NaCl, 0.1% Tween 20, 1 mm PMSF). Samples were centrifuged at 16,000 *g* for 15 min at 4°C and supernatants were diluted 1:2 in carbonate buffer (0.2 M, pH 9.6) and used for coating GM1-ELISA plates by an overnight incubation at 4°C. After washing, plates were blocked for 2 h at room temperature with a 5% fat-free dry milk solution. Plates were washed and primary labelling was conducted by adding an anti-CTB mouse serum diluted 1:800 in PBS and incubating overnight at 4°C. The secondary labelling was conducted by incubating 2h at 25°C a goat anti-mouse horseradish peroxidase-conjugated antibody diluted 1:2000 (Sigma).

After washing, a substrate solution of 0.3 mg/mL 2-20-azino-bis-3 etilbenztiasoline-6-sulphuric acid (ABTS; Sigma) and 0.1 mM H_2O_2, was added for 30 min at 25°C. Optical density (OD) was read in an iMark^{TM} microplate reader (BIO-RAD, Hercules, CA, USA) at 410 nm.

2.3. Immunogenicity assay

Experimental procedures in test mice were approved by the Institutional Animal Care and Use Committee (Protocol number: CEID-2013-004). Five groups (n = 4) of 12 week-old female BALB/c mice were established, and received by the oral route one of the following treatments: 1, 8.5, 10 or 25 mg of freeze-dried FB2-CTB3 galls containing approximately 1, 10, 15, and 30 µg of CTB, respectively; or 25 mg of freeze-dried WT galls. The corn smut used in this experiment was previously maintained at 25°C during one year period before conducting this experiment. The vaccine consisted of the corresponding amount of corn smut resuspended in 200 µL PBS, and administered to mice on days 0, 7, 14, and 21. Mice were bled on days 21, 61 and 91 to conduct ELISA analysis for determining anti-CTB IgG levels.

In order to determine IgG, IgG1, IgG2a, IgA and IgM antibody levels by ELISA analysis, two groups of immunized mice as aforementioned, one with 10 mg CTB corn smut (15 µg of CTB) and another with 10 mg WT corn smut, were bled at days 21, 61 and 91 after the first immunization. For IgA determination feces were collected at the same time points (Rosales-Mendoza et al. 2008).

ELISA assay was conducted using ninety six-well polystyrene plates coated overnight with CTB at 0.25 µg/well at 4°C. After blocking with 5% fat-free milk for 2 h, plates were incubated overnight incubation at 4°C with serial dilutions of mice sera (1:20 to 1:160). Anti-IgG, -IgG1, -IgG2a, -IgA and -IgM horseradish peroxidase-conjugated secondary anti-mouse antibodies (1:2000 dilution, Sigma) were applied for 2 h at room temperature, and after washing, signals were detected following incubation with an ABTS substrate and 0.1 mM H_2O_2 for 15 min (Sigma). Optical density values were measured at 405 nm using a Microplate reader (Thermo). Antibody titers were determined as the reciprocal of the higher serum

dilution with an OD value above the mean OD value of the WT group plus 2 times its standard deviation.

2.4. CT challenge

Cholera toxin (CT) challenge experiment was performed according to a previously described method (Richardson et al. 1984; Rosales-Mendoza et al. 2008). The following test mice groups (n=5) were set up: mice treated with WT 'huitlacoche' (challenged); mice treated with transgenic 'huitlacoche' (challenged); mice treated with the vehicle alone (naïve/unchallenged). 'Huitlacoche' samples used for immunization was previously maintained at 25°C during one year. Food was withheld for a period of 16 h and mice were subsequently subjected to oral administration of either 10 µg of CT diluted in 10% $NaHCO_3$ or the vehicle alone. Mice diet was restricted to only water and sacrificed by cervical dislocation at 6 h post-challenge. The entire small intestine, from the pyloric valve to the ileal-cecal junction, was dissected, and weighed. The volume of fluid accumulation (FA) was calculated with the formula FA = (G/B-G) x 1000; wherein B is body weight in grams and G is total gut weight expressed in grams (Richardson et al. 1984).

2.5. Statistical analysis

Significant differences in antibody levels and fluid accumulation values between pairs of groups were assessed using one-way analysis of variance followed by mean comparisons using Tukey's test ($P < 0.05$).

3. Results

3.1. Corn smut-CTB vaccine is thermostable

We previously demonstrated that recombinat 'huitlacoche' FB2-CTB3 galls expressed high levels of CTB. The milled freeze-dried corn smut material was stored at room temperature, and no considerable changes in CTB levels a year after the freeze-dried process was observed (Fig. 1). Thereafter, we carry out the stability experiments in an exposure to high temperatures. Western blot and ELISA analysis revealed that no important changes in CTB levels occurred after exposing

the sample at temperatures up to 50°C during 2 h. A notorious decrease in CTB content was observed after treatment at 60°C, whereas 80°C treatment resulted in a total degradation of the recombinant protein (Fig. 2). Thus, this thermostable recombinant protein was used in the present study to characterize in detail its immunogenic properties. Freeze-dried corn smut that was maintained at room temperature for one year and subsequently it was used in all immunization assays.

3.2. Corn smut-CTB vaccine induces dose-dependent, long-lasting humoral responses

At first, to evaluate the minimum effective dose to induce IgG systemic immune responses, doses of 1, 10, 15 and 30 µg of CTB were evaluated in corn smut-CTB immunized mice groups sampling blood for antibody measurements on days 21, 61, and 91. Overall a dose dependent response was observed. On day 21 (three doses received) mice group treated with 15 µg of CTB showed significant IgG systemic response ($P < 0.05$), whereas no significant increases in IgG titers were observed in the mice group treated with WT 'huitlacoche' (Fig. 3). Although mice group treated with 30 µg of corn smut CTB showed a higher response after two immunizations, at the following time points the response tends to be lower than the response observed with 15 µg doses (Fig. 3).

Further experiments were conducted to characterize the humoral response induced in mice immunized with selected dose of 15 µg CTB or 10 mg WT galls. IgG and IgM levels were monitored on time points 14, 21, 61 and 91. IgM levels reached a maximum response in the group immunized with corn smut-CTB one week after the first immunization with subsequent decreases in the further time points. IgG levels gradually increased among the time points with a maximum response in the 61 days timepoint (1:40). IgG titer at 91 time point were 1:20 (Fig. 4A). On the other hand, IgG subclasses were determined at time point 61 days. IgG1 levels predominated over the IgG2a subclass with an IgG1/IgG2a ratio of 2. This finding suggests that the immune response induced by the corn smut-CTB is Th2 polarized (Fig. 4B).

3.2. Corn smut-CTB induces long-lasting intestinal IgA immunoprotective responses

In terms of mucosal immune responses, IgA detection in feces from mice immunized with selected dose of 15 µg CTB revealed the presence of anti-CTB IgA levels in the corn smut-CTB-treated group after receiving two immunizations (Fig. 4A). Interestingly, sustained IgA levels were measured 10 weeks (day 90) after the last boost (fourth immunization).

3*.3. Protective capacity of the corn smut-CTB-based vaccine*

The protective capacity of the corn smut-CTB-based vaccine was evaluated by challenging mice with CT. Mice groups were immunized at weekly intervals with two, three or four 15 µg-doses of corn smut-CTB or fed with WT corn smut, and subsequently challenged with 10 µg CT at 7 days after receiving the last immunization. Remarkably, a reduction on FA values were observed in all tested time points in the case of the corn smut-CTB vaccinated group. For instance, for the group receiving three immunizations, the FA mean value for the corn smut-CTB vaccinated group was 69.2, whereas mice group treated with WT 'huitlacoche' had a mean FA value of 98.2 ($P < 0.05$). Unchallenged individuals have a FA mean value of 54.3 ± 3.9. Remarkably, the groups receiving three or four doses showed no statistical differences ($P < 0.05$) between FA values of unchallenged and corn smut-CTB-treated groups, indicating a high degree of toxin neutralization (Fig. 5).

4. Discussion

Developing low-cost vaccines against cholera is a needed, especially in endemic countries. Towards this goal, we have previously produced CTB in corn smut with attractive productivity, providing evidence on its immunoprotective effect in mouse following administration of three 60 µg oral doses of CTB administered at one-week intervals (Juárez-Montiel et al. 2015). Herein, we have characterized in detailed the immunogenic properties of the CTB corn smut-made antigen. We have made a number of important observations, which constitute a step forward in the development of a low-cost vaccine against cholera. First, different doses were assessed ranging from 1 to 30 µg to determine the optimal scheme for inducing protective anti-CTB antibody titers. A dose dependent response was observed in

terms of serum antibody levels. IgM levels were associated to a primary immune response while IgG responses were predominant in further time points, when secondary immune response is induced by subsequent boosts. In this sense, other studies support the production of CTB vaccine in recombinant systems including yeast and plants. For instance, Yuki *et al.* (2013) reported a CTB accumulation in rice at 2.35 mg of CTB/g of seed. An immunization scheme of four doses administered at 2-week intervals conferred immunoprotection in mice. Thus, the rice-made CTB provided immunoprotection at 240 and 360 µg CTB doses (Yuki et al. 2013). Furthermore, CTB has been expressed in chloroplasts as a chimeric protein along with malaria antigens. In this case, long term immunity against CT was achieved when orally administered to mice using leaf extracts at doses of 500 mg of plant material expressing CTB at 10-13% TSP (Davoodi-Semiromi et al. 2010). On the other hand, the yeast CTB has no further characterization of its immunogenic properties (Arzanlou et al. 2005; Jung-Gu and Hyo-Sang 2008).

The characterization of the corn smut-CTB indicates that strong immune responses were attained with 15 µg doses and highlights the potency of this vaccine. Importantly, 15 µg dose-scheme conferred protection against cholera toxin challenge, pointing out a similar immunoprotective potential as other CTB production systems. A scheme comprising two immunizations with corn smut-CTB were sufficient to provide immunoprotective effects. Particularly, the three-dose scheme was required to provide full protection. The oral immunogenic activity observed for the corn smut-made CTB could be attributed in part to the bioencapsulation effect provided by the fungi biomass, particularly by the spores structure, which are abundant in corn smut (Banuett and Herskowitz 1996). Similar effects have been proposed when plants are used as vaccine delivery vehicle of oral vaccines (Rosales-Mendoza and Salazar-González 2014).

The typical humoral response induced by CTB when orally administered is Th2 polarized (Hamorsky et al. 2014). This property was preserved in the case of the corn-smut CTB as IgG1 was predominant over IgG2a subclass. Remarkably, evaluation of long-term persistence of anti-CTB IgG in immunized mice revealed the induction of long lasting responses as antibodies levels, IgA in feces and IgG in serum, were maintained for up to 90 days after the last immunization. Interestingly,

our CTB vaccine was highly stable as it preserves the immunogenic activity after storage the freeze-dried vaccine at room temperature (around 25°C) over a one year period. Similar observations were reported in a previous study on a CTB rice-based vaccine, where no changes in the CTB content were found after several years of storage at room temperature (Yuki et al. 2013). Moreover, no important changes in CTB content were caused by exposing the corn smut vaccine at 37 and 50°C treatments. This stability is considered promising since a previous study with a vaccine formulated with whole inactivated *V. cholerae* cells along with recombinant CTB reported a reduction of 50% in the CTB content after storage at 42°C for 6 months (Ahmed et al. 1994).

Despite the benefit of reactive cholera vaccinations in endemic countries, costs are crucial to extend their use and prevent epidemics or at least to quickly control outbreaks by vaccination campaigns. Current low coverage is due CTB production is conventionally performed in recombinant *E. coli* cultures. This process requires extensive purification and refolding steps to eliminate endotoxins and obtain an acceptable product with pentameric structure (Rodrigues et al. 2014). Additionally, production of whole cell vaccines involves handling the pathogen, the use of bioreactors and vaccine distribution under refrigeration. Our CTB vaccine produced in corn smut is proposed as a safe and low-cost vaccine for the implementation of large scale vaccination campaigns since is produced in an edible tissue and avoids expensive upstream and downstream processing, cold chain and parenteral administration. Further research will pursue the evaluation of this vaccine candidate in a phase I clinical trial.

Plants and algae have been explored over the last decades as hosts for the production and even delivery of vaccines and other biopharmaceuticals, leading to the implementation of attractive platforms for vaccine production (Wardemann et al. 2003; Scheid et al. 2009; Bonsignori et al. 2011; Gray et al. 2011; Walker et al. 2011; Gaebler et al. 2013). In this context, the corn smut platform has substantial advantages as leads to high yields and shorter time length for generating transformed clones than that required for transgenic plant development, especially grains or transplastomic lines. Therefore, corn smut platform provides a robust and fast platform for cholera vaccine production.

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Conflicts of interest

There are no conflicts of interest between the authors

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Figure Captions

Fig. 1 Long-term stability of the corn smut CTB vaccine. The corn smut CTB vaccine was maintained at room temperature (average 25°C) during one year. A Western blot analysis was subsequently performed by using an anti-CTB mouse antiserum. Lanes: 1, purified recombinant CTB (positive control, 500 ng); lines 2, protein extracts from corn smut-CTB. Samples were immediately analyzed after freeze-drying and milling the material (A) or after one year storage at 25°C (B).

Fig. 2 Thermostability assessment of the corn smut CTB vaccine. The corn smut CTB vaccine was incubated at 37°C, 50°C, 60°C, and 80°C during 2 h. **a** Levels and Integrity of the corn smut-CTB were measured by immunodetection. ELISA analysis was conducted using a GM1 dependent binding modality labelling with an anti-CTB mice antiserum. **b** A Western blot analysis was performed by using an anti-CTB mouse antiserum. Lanes: 1, purified recombinant CTB (positive control, 500 ng); lines 3, 5, 7, and 9, protein extracts from corn smut-CTB incubated at 37°C, 50°C, 60°C, and 80°C, respectively; lanes 2, 4, 6, and 8: WT 'huitlacoche' samples from the vaccine incubated at 37°C, 50°C, 60°C, and 80°C, respectively.

Fig. 3 Dose dependent IgG humoral responses in BALB/c mice immunized with corn smut CTB. Mice were orally immunized four times at weekly intervals with 10 mg of WT galls or FB1 x FB2-CTB 3 galls corresponding to different CTB doses (1, 10, 15 and 30 µg of CTB). Serum samples were taken at days 21, 61, and 91 and IgG levels were determined by ELISA (dilution 1:40). Statistical differences between the treated group and the WT corn smut group are indicated by an asterisk ($P < 0.05$).

Fig. 4 Long-lasting immune responses in mice immunized with corn smut CTB. a Anti-CTB IgM, IgG and IgA antibody levels of BALB/c mice immunized with corn smut CTB. Mice were orally immunized four times at weekly intervals with 10 mg of WT galls or 15 µg of CTB (FB1 x FB2-CTB 3 galls). Feces and blood samples were taken at 21, 60 and 90 days after the first immunization. Anti-CTB

antibody titers were determined by ELISA using serial serum dilutions. **b** Anti-CTB IgG subclass antibody levels of BALB/c mice immunized with corn smut-CTB. Mice were orally immunized four times at weekly intervals with 10 mg of WT galls or FB1 x FB2-CTB 3 galls (15 µg CTB). Serum samples were taken at day 60 and anti-CTB antibody levels were determined by ELISA using serial serum dilutions. Statistical differences ($P < 0.05$) are indicated by an asterisk (versus the group treated with WT corn smut).

Fig. 5 Immunoprotection against CT challenge of BALB/c mice immunized with corn smut-CTB. Mice were orally immunized two, three or four times at weekly intervals with 10 mg of WT galls or FB1 x FB2-CTB 3 galls (15 µg CTB) and subsequently challenged with CT (10 µg). Immunized WT corn smut group challenged with CT served as a control group. Mice were challenged at 7 days after the last immunization. After 6 h of CT toxin administration, mice were sacrificed, and fluid accumulation (FA) was estimated by weighing carcass and small intestines. Statistical differences ($P < 0.05$) are indicated by an asterisk (versus the group treated with WT corn smut and challenged with CT) or a cross (versus the unchallenged group).

Fig. 1

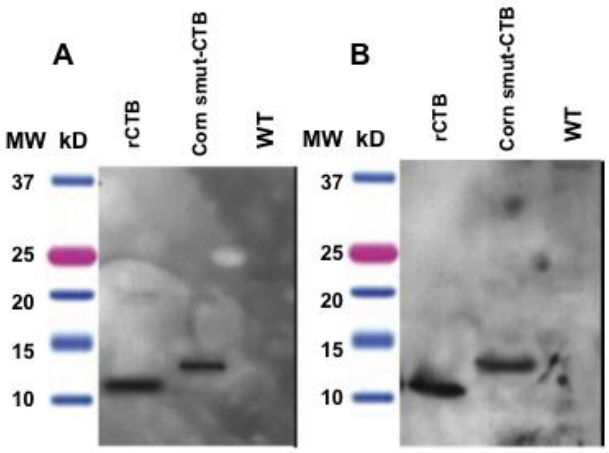
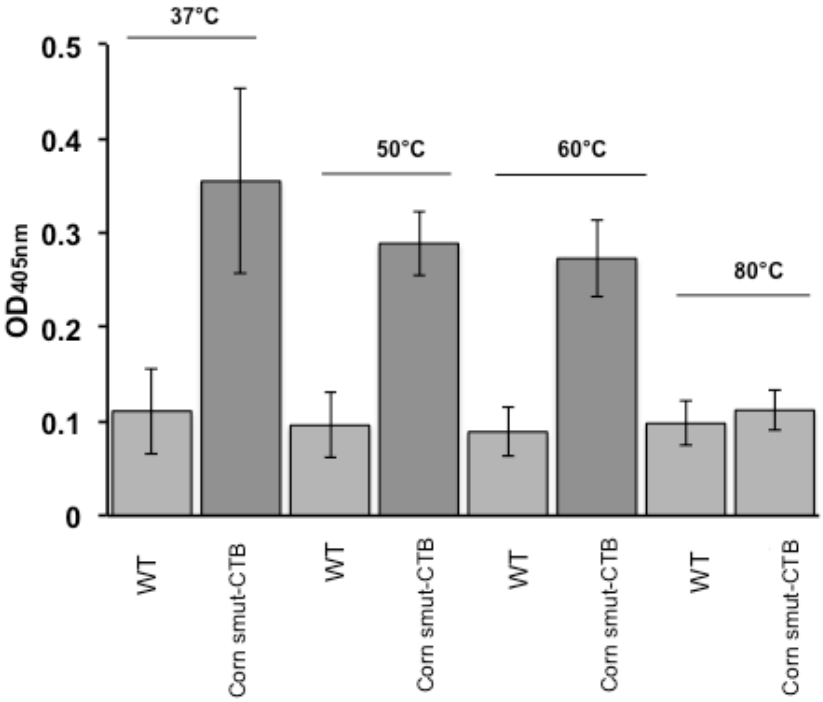


Fig. 2

A



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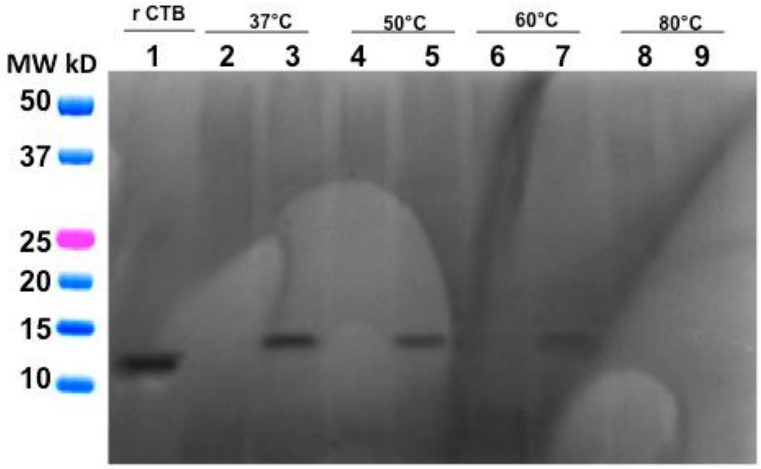


Fig. 3

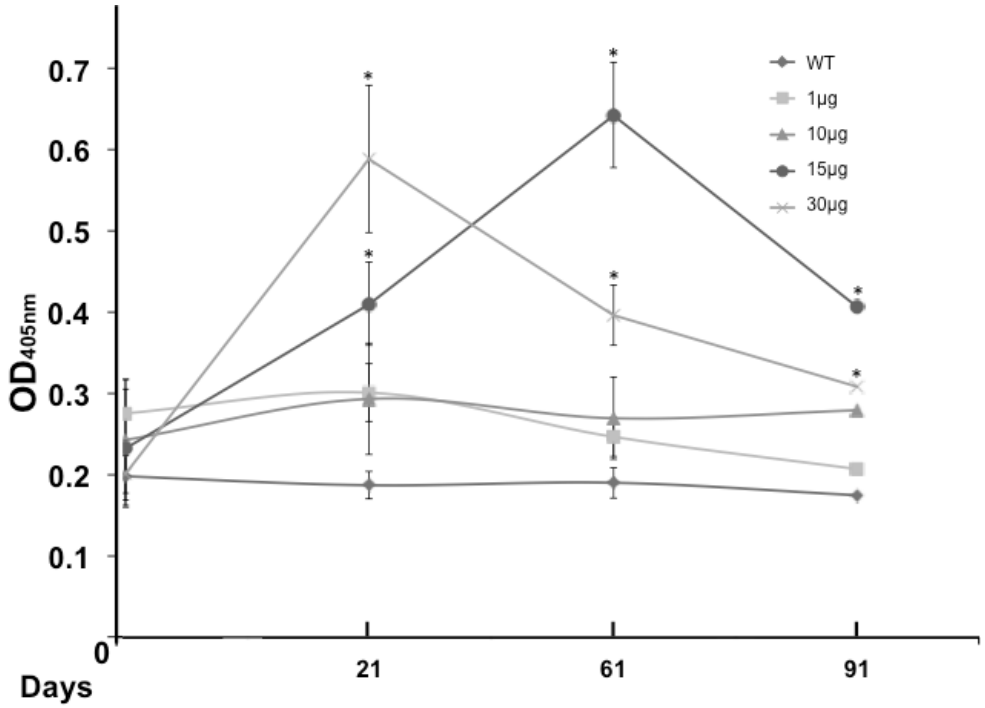
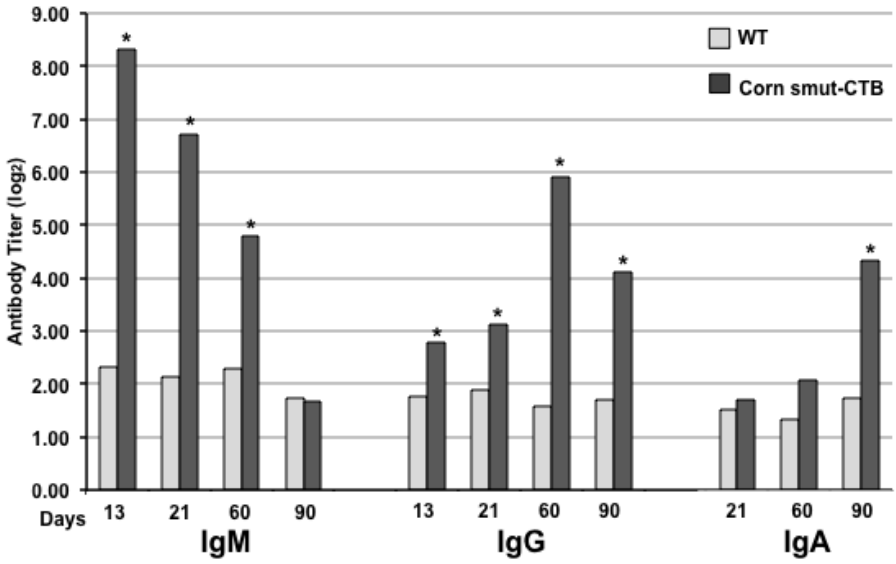


Fig. 4

A



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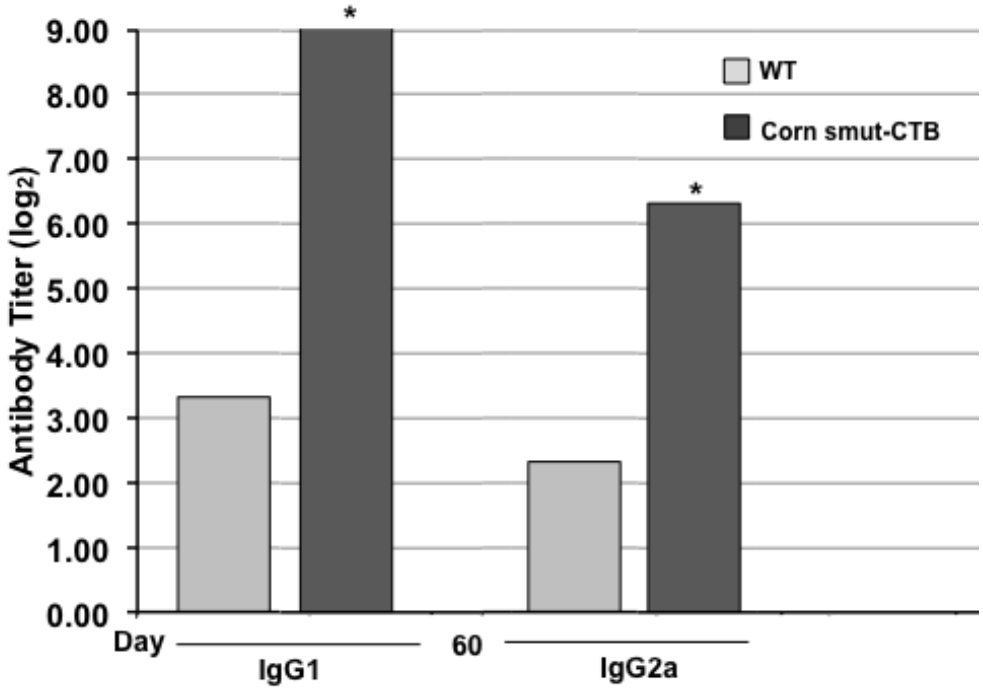

Fig. 5