SHORT RESEARCH ARTICLE

Role of bacteriophages in STEC infections: new implications for the design of prophylactic and treatment approaches

[version 1; peer review: 2 approved with reservations]

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Abstract

Shiga toxin (Stx) is considered the main virulence factor in Shiga toxin-producing *Escherichia coli* (STEC) infections. Previously we reported the expression of biologically active Stx by eukaryotic cells *in vitro* and *in vivo* following transfection with plasmids encoding Stx under control of the native bacterial promoter. Since *stx* genes are present in the genome of lysogenic bacteriophages, here we evaluated the relevance of bacteriophages during STEC infection. We used the non-pathogenic *E. coli* K12 strain carrying a lysogenic 933W mutant bacteriophage in which the *stx* operon was replaced by a gene encoding the green fluorescent protein (GFP). Tracking GFP expression using an *In Vivo* Imaging System (IVIS), we detected fluorescence in liver, kidney, and intestine of mice infected with the recombinant *E. coli* strain after treatment with ciprofloxacin, which induces the lytic replication and release of bacteriophages.

In addition, we showed that chitosan, a linear polysaccharide composed of D-glucosamine residues and with a number of commercial and biomedical uses, had strong anti-bacteriophage effects, as demonstrated *in vitro* and *in vivo*. These findings bring promising perspectives for the prevention and treatment of hemolytic uremic syndrome (HUS) cases.

Keywords

DNA, mice, solutions, eukaryotic cells

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Any reports and responses or comments on the article can be found at the end of the article.

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Introduction

Infections by Shiga toxin-producing *Escherichia coli* (STEC) strains are a serious public health concern, resulting in diarrhea, hemorrhagic colitis, and haemolytic uremic syndrome (HUS).

Stx is the main virulence factor in STEC strains. The *stx* gene is present in the genome of prophages, which are similar to the bacteriophage lambda found in the lysogenic form of various *E. coli* strains. Previously we reported that the native promoter of the Stx-encoding gene can drive expression of the toxin in eukaryotic cells in both *in vivo* and *in vitro* conditions^{1,2}.

Many questions remain unanswered with regard to the mechanism by which STEC infection causes HUS. In particular, we are interested in understanding how Stx enters the systemic circulation and why only very small numbers of bacteria are sufficient to induce HUS in humans.

Based on our previous observations that the native *stx* gene promoter is active in host cells, we seek to understand the role that bacteriophages play in the pathogenesis of STEC strains. Recently, it was reported that bacteriophages carrying the *stx* gene are required for the development of HUS in the murine model³. We hypothesise that eukaryotic host cells might be transduced with and/or infected by Stx-encoding bacteriophages, leading to Stx dissemination *in vivo* to enter the systemic circulation. This would also explain why very small numbers of bacteria are sufficient to develop HUS.

In order to test whether bacteriophages are responsible for the induction of HUS, we used an anti-bacteriophage agent to inactivate them. Chitosan, a linear polysaccharide polymer obtained after the deacetylation of chitin, the structural element in the exoskeleton of crustaceans, possesses strong antimicrobial activity against several pathogenic microorganisms⁴. Its antiviral activity was reported on the bacteriophage c2, which infects *Lactococcus* strains, and on bacteriophage MS2, which infects *E. coli*⁵ without affecting significantly the growth of the bacterial culture⁶. In order to test our hypothesis, which would make Stx-encoding bacteriophages a new target for preventing and treating STEC infections, we used chitosan as an anti-bacteriophage agent *in vitro* and *in vivo*.

Inactivation of bacteriophages was observed *in vitro* after incubation with chitosan, inhibiting both the infection of, and replication in bacterial cells, and the transduction of eukaryotic cells.

GFP dissemination was significantly reduced in mice treated with chitosan following infection with a non-pathogenic strain carrying a bacteriophage in which the *stx* gene was replaced by the GFP-encoding sequence. Last, preliminary results showed partial protection by chitosan *in vivo* of mice infected with STEC.

These results contribute to understanding STEC infections, posing implications for a similar scenario to occur in other infections caused by bacteria carrying lysogenic bacteriophages.

Materials and methods

Strains

C600ΔTOX:GFP, a lysogenized C600 strain carrying the 933W bacteriophage in which the *stx* gene was replaced by the *gfp* sequence,

was generously provided by Dr. Alison Weiss⁷. EDL933W, an enterohemorrhagic *E. coli* (EHEC) strain carrying the wild-type bacteriophage from which C600ΔTOX:GFP was obtained, was generously provided by Dr. Luis Carlos de Souza Ferreira, LDV-USP, Brazil.

Transduction of eukaryotic cells

Baby BHK-21 cells (Syrian hamster kidney fibroblasts from the American Type Culture Collection) cells were grown on 12-well plates (Nunc) in complete medium (10% fetal bovine serum in DMEM medium, Gibco, USA) for use in the transduction assay.

C600ΔTOX:GFP was generously provided by Dr Alison Weiss⁷. This is a non-pathogenic phage resulting from purified 933W bacteriophage in which *stx* gene was replaced by *gfp* sequence (φ ΔTOX:GFP). Phages at a multiplicity of infection (M.O.I) equal to 1 were added to BHK-21 cells cultured the day before on 12 wells plate (Nunc). BHK-21 cells were counted with a Neubauer camera, and bacteriophage titer was measured by the titration assay as described below. Transduction of BHK-21 cells was enhanced by centrifugation at 1000 × g for 10 minutes at room temperature as previously reported¹. After incubation at 37°C for 3 hours, the phage-containing medium was removed. Cells were washed twice with phosphate buffered saline (PBS) and then incubated in complete DMEM medium (Gibco, USA). Twenty four hours post-transduction, cells were washed with PBS, harvested and centrifuged at 2655 × g for 15 minutes. DNA was harvested from pellets after incubation for 5 minutes at 98°C in lysis solution (Tris pH8 50mM, SDS 2%, Triton-X100 5%) and the harvested DNA was used for PCR. Primers: Up-R 5'CCGCTCGAGACTAGTGCAAAAGC-GAGCCTGGTAAATAAATATG3'; Up-D 5'GGAATTCCATAT-GCTCGTTGAGGCATATGAAAATCAGAC3'. The reaction was run in a Eppendorf Thermocycler at an initial 92°C for 120 seconds and then at 92°C for 20 seconds and 60°C for 20 seconds and 72°C for 120 seconds for 35 cycles using primers giving a fragment of 1310 bp on the upstream region of *gfp* gene into the bacteriophage genome.

Bacteriophage induction

The C600ΔTOX:GFP strain was grown in Luria Broth (LB) plus 10 mM CaCl₂ and chloramphenicol (Sigma) (15 µg/ml final concentration) overnight (ON) at 37°C under agitation. The ON culture was diluted to OD_{600nm} = 0.1 in LB plus 10 mM CaCl₂ and chloramphenicol (Sigma) (15 µg/ml final concentration). Induction was carried out by adding ciprofloxacin to a final concentration of 40 ng/ml⁸. Bacteria were incubated for 6 hours at 37°C under agitation. Cultures were then centrifuged at 5000 rpm for 15 minutes. The bacteriophage-containing supernatant was filtered with 0.2 µm filters and kept at 4°C until the titration assay was performed.

Titration assay

E. coli strain Y1090 (ATCC 37197) was grown in LB plus ampicillin ON at 37°C under agitation. The ON culture was diluted 1:100 in LB plus ampicillin and incubated for 2 hours at 37°C under agitation. At the end of the incubation, 500 µl samples of *E. coli* Y1090 were incubated with 5, 50 and 100 µl of a suspension containing bacteriophages for 30 minutes at room temperature. At the end of this incubation, 3 ml of Top Agar (Tryptone 1%; NaCl 0.5%;

Agar 0.7%) plus CaCl_2 (10 mM final concentration) was added, and plated on LB-Amp agar plates. Plates were incubated at 37°C and lysis plaques were visually counted.

Bacteriophage inactivation assay

$\phi\Delta\text{TOX}:\text{GFP}$ was incubated with 5 mg/ml of a chitosan (Sigma 448877) solution in phosphate buffer 10 mM, at pH = 7 for 10 minutes at room temperature, and bacteriophage titers were measured as described in titration assay section.

Chitosan was also used in the bacteriophage induction assay described above. Chitosan was added 2 and 4 hours post-induction and bacteriophage titers were analyzed at 6 hours post-induction.

Mice

BALB/c mice were bred in-house at the animal facility of the Microbiology Department of the São Paulo University, Brazil. The experimental protocol of this study followed the ethical principles for animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Ethics Committee on Animal Experiments of the Institute of Biomedical Sciences (Protocol number 106), University of São Paulo, in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1985).

Male mice aged 6 weeks (18 to 20 g) were used for the *In Vivo* Imaging System (IVIS). Immature male and female DBA-2 mice (17–21 days of age, approximately 8–11 g body weight) were used immediately after weaning for the infection assays with EDL933W strain ($n = 4$). Mice were maintained under a 12-h light-dark cycle at $22 \pm 2^\circ\text{C}$ and fed a standard diet and water *ad libitum*.

EHEC infection

Immature male and female DBA-2 mice (17–21 days of age, approximately 8–11 g body weight) were used immediately after weaning for the infection assays ($n = 4$).

E. coli EDL933W (ATCC 43895) was used for infection of mice following the protocol previously reported by Brando and collaborators⁸. Briefly, *E. coli* EDL933W was grown in Tryptic Soy Broth (TSB, DIFCO, BD) ON at 37°C. The ON culture was centrifuged at 14000 rpm for 15 minutes and the bacterial pellets were washed twice in PBS. Pellets were resuspended to have a final concentration of 3×10^{12} CFU/100 μl per mouse.

The bacterial suspension was delivered directly into the stomach of mice after 8 hours of food starvation, *via* a 5-French paediatric feeding tube. After 4 hours of ingesting the bacterial suspension, mice were given food and water. Control animals received 100 μl of sterile PBS. Survival was observed for one week. Both groups were composed by 4 animals.

Effect of chitosan *in vivo*

To analyze the effect of chitosan *in vivo*, immature male and female DBA-2 were infected as described previously and treated with 100 μl of a chitosan solution at a concentration of 5 mg/ml, orally administered 2 hours after infection. Survival was observed for one week.

GPF dissemination assay (IVIS)

Two-month old BALB/c mice were used to infect orally with C600 $\phi\Delta\text{TOX}:\text{GFP}$. Bacteriophage induction *in vivo* was performed with ciprofloxacin as described immediately below. After 2 hours of induction with ciprofloxacin, 100 μl of chitosan solution at a concentration of 5 mg/ml was administered orally to the mice and GFP dissemination by IVIS was analyzed.

In Vivo Imaging System (IVIS)

This time, we used two-month old BALB/c mice. An ON culture of C600: $\phi\Delta\text{TOX}:\text{GFP}$ was used to infect them. The ON culture was centrifuged at 14000 rpm for 15 minutes at 4°C. The pellet was washed with PBS and centrifuged again at 14000 rpm for 15 minutes at 4°C. The pellet was resuspended in a solution of 20% sucrose to have a concentration of 1×10^9 CFU/mouse. Mice were inoculated orally with strain C600: $\phi\Delta\text{TOX}:\text{GFP}$ and *in vivo* bacteriophage excision was induced following the procedures described by Zhang and collaborators⁸. The mice were sacrificed with CO_2 inhalation 24 hours after bacterial inoculation. Blood, spleens, kidneys, lungs, brains, intestines, hearts and livers were harvested by surgical removal and kept in PBS solution and evaluated for GFP expression using the IVIS system. To determine the effects of chitosan *in vivo*, the mice received 100 μl of a chitosan solution at a concentration of 5 mg/ml.

Statistical analysis

Statistical significance between treatments and controls was analyzed using the Prism 5.0 software (GraphPad Software), and the *P* value is indicated by asterisks in the figures.

All other data correspond to the means $\pm$ standard errors of the means (SEM) for individual mice. Statistical differences were determined using the one-way analysis of variance (ANOVA).

Results

Induction of $\phi\Delta\text{TOX}:\text{GFP}$ by ciprofloxacin and anti-phage effects of chitosan

Bacteriophage lytic induction was triggered in *E. coli* C600 $\Delta\text{TOX}:\text{GFP}$ using ciprofloxacin⁸. We observed a significant decrease in the optical density of the bacterial culture after addition of the antibiotic and the release of phages into the culture supernatant (Figure 1, panel A and B). The bacteriophage titer was analyzed at different time points and a significant increase was observed after induction (Figure 1, panel B). The effect of chitosan as an anti-bacteriophage agent was also examined. To this aim, we added chitosan at a final concentration of 5 mg/ml to the bacterial culture 2 or 4 hours post-induction, and we observed the complete inactivation of the $\phi\Delta\text{TOX}:\text{GFP}$, without measurable toxic effects to the bacterial strain (Figure 1, panels A and B).

Transduction of mammalian cells with $\phi\Delta\text{TOX}:\text{GFP}$

We previously reported the capacity of $\phi\Delta\text{TOX}:\text{GFP}$ to transduce macrophages *in vitro*¹. To further evaluate the ability of chitosan to inhibit bacteriophage transduction of mammalian cells, BHK cells were transduced for 3 hours with $\phi\Delta\text{TOX}:\text{GFP}$, $\phi\Delta\text{TOX}:\text{GFP}$ plus chitosan or $\phi\Delta\text{TOX}:\text{GFP}$ treated with DNase. Addition of DNase to the bacteriophage sample would preclude any free DNA in the

Figure 1. Induction of $\phi\Delta\text{TOX:GFP}$ by ciprofloxacin and effect of chitosan *in vitro*. **A.** Growth curve: C600 $\Delta\text{TOX:GFP}$ was induced with ciprofloxacin and the optical density was measured at 600 nm at 0, 2, 4 and 6 hours after induction. Non-induced C600 $\Delta\text{TOX:GFP}$ was used as control. Chitosan was added at 2 or 4 hours after induction. **B.** Bacteriophage $\phi\Delta\text{TOX:GFP}$ titer: bacteriophage titers were analyzed at 0, 2, 4 and 6 hours post-induction. Chitosan was added at 2 or 4 h post-induction. Asterisks represent $P < 0.05$.

bacterial lysates prior to the transduction of cells. Untreated cells were used as a control. As shown in Figure 2, the bacteriophage DNA was detected by PCR in mammalian cells, showing the capacity of the virus to transduce this cell line. However, when BHK cells were transduced with bacteriophages pre-incubated with chitosan, no phage DNA was detected, confirming the inactivating

action of chitosan on bacteriophages. Bacteriophage DNA was also detected in cells transduced with $\phi\Delta\text{TOX:GFP}$ treated with DNase (Figure 2).

GFP detection *in vivo*

To demonstrate the *in vivo* behavior of bacteriophages, mice were infected with the lysogenic *E. coli* C600 $\Delta\text{TOX:GFP}$ strain, followed by oral administration of ciprofloxacin 1 hour or 2 hours later. In order to evaluate the effect of chitosan *in vivo*, a group of mice was administered with chitosan 2 hours post-induction and a control group of uninfected mice was evaluated for auto-fluorescence background control in each organ. Twenty four hours after infection, organs were harvested and examined using the IVIS. As shown in Figure 3, GFP was detected in the intestine, liver and, to a lesser extent, kidney of mice orally infected and treated with ciprofloxacin. Remarkably, the addition of chitosan 2 hours after infection caused a sharp decrease in GFP detection in organs of mice orally infected with the *E. coli* strain C600 $\Delta\text{TOX:GFP}$ (Figure 3, panels A and B), indirectly indicating reduction of bacteriophages in the cells, GFP release and dissemination. Moreover, viable phages were detected via the lysis plaque assay in intestine homogenates and blood samples of infected mice, in which bacteriophages were induced by ciprofloxacin (data not shown).

Figure 2. A. PCR on DNA extracted from eukaryotic cells: 24 hours after transduction, BHK cells were washed and treated with Trypsin-EDTA solution. DNA was extracted and PCR was performed. Line 1: Cells transduced with $\phi\Delta\text{TOX:GFP}$. Line 2: Cells transduced with $\phi\Delta\text{TOX:GFP}$ plus chitosan. Line 3: Cells transduced with $\phi\Delta\text{TOX:GFP}$ previously treated with DNase. Line 4: Untreated cells. Line 5: Positive control ($\phi\Delta\text{TOX:GFP}$ DNA). Line 6: Negative control. Line 7: 1 kb ladder (Invitrogen).

Effect of chitosan on the mortality of mice orally inoculated with the EDL933W strain

In order to evaluate the *in vivo* effect of chitosan during the infection process, mice were orally challenged with a wild-type EDL933W strain, based on the model described by Brando and collaborators⁹. Another mouse group was also treated with chitosan, administered orally 2 hours post-infection, and survival was followed for one

Figure 3. GFP expression *in vivo* in mice infected with C600ΔTOX:GFP using *In Vivo* Imaging System (IVIS). **A.** IVIS Representative image: ciprofloxacin was administered 2 hours post-infection to induce $\phi\Delta\text{TOX:GFP}$ *in vivo*. A group of mice was treated with chitosan 2 hours after bacteriophage induction. All mice were sacrificed 24 hours post-infection and brains, hearts, lungs, livers, spleens, kidneys and intestines were harvested and analyzed by IVIS. Fluorescence intensity was recorded as photons/sec/cm², and the signal intensity represents the amount of GFP present. **B.** Graphic of fluorescence intensity on GFP-positive organs. Four animals per group were analyzed and the fluorescence intensity was quantified using Living Imaging 4.3.1 in Caliper Life Sciences.

week. In this preliminary study, partial protection was observed in mice treated with chitosan, resulting in a delay in the death time (Figure 4). Mice infected with EDL933W strain died at 72 hours post-infection, and mice infected followed by treatment with one dose of chitosan died at 168 hours after infection.

Data sets for bacteriophage induction and effect of chitosan

3 Data Files

<http://dx.doi.org/10.6084/m9.figshare.960794>

Discussion

Lambda bacteriophages are used in gene transfer and vaccine delivery because of their capacity to transduce mammalian cells *in vivo*¹⁰. Tyler and collaborators recently showed that prophage induction is required for renal disease and lethality in the EHEC mouse model, suggesting that free bacteriophages encoding Stx may play a direct role in the disease³.

In previous reports, we have showed that the native phage promoter controlling Stx expression is active in eukaryotic cells as demonstrated both *in vitro*¹ and *in vivo*². Based on these results and

Figure 4. Treatment with chitosan in mice after EDL933W infection. Mice were infected orally with EDL933W strain. Controls did not receive chitosan (dots and broken line) and the experimental group received chitosan 2 hours post-infection (square and fill line). Survival rates were observed for one week: two mice infected with EDL933W died 72 hours post-infection, while two mice infected with EDL933W plus chitosan died 168 hours post-infection. The remaining two mice of each group survived 192 hours post-infection.

the reports previously described, we sought to evaluate whether bacteriophages could be considered a target for treating STEC infections. To this aim, we measured GFP by the strain C600 Δ TOX:GFP and the mortality of infected mice following bacteriophage induction, and *in vivo* inactivation upon chitosan treatment positive expression was analyzed. GFP was observed in liver, intestine and kidney by IVIS on mice in which the bacteriophage lytic phase was induced by ciprofloxacin following infection. Of particular relevance was the observation that chitosan exerted a direct inactivation effect on $\phi\Delta$ TOX:GFP *in vitro* and drastically reduced the detection of fluorescence in mice orally infected with the C600 Δ TOX:GFP strain. Bacteriophage transduction of mammalian cells was also inhibited after incubation with chitosan.

Our findings indicate that chitosan possesses strong anti-bacteriophage properties *in vitro* and *in vivo*. This positively charged polymeric polysaccharide has been reported to inhibit other bacteriophages and probably acts through electrostatic interactions with negatively charged capsid proteins⁵. Based on these effects we propose that chitosan may be a viable alternative for the treatment of STEC infections. Chitosan is already used in food and medicine, and it is harmless to humans, making it a cheap and safe option for this application.

The fact that only partial protection was observed *in vivo* using chitosan may be due to its short half-life¹¹. Our results may contribute to understand why only small numbers of bacteria are sufficient to induce HUS in humans. If bacteriophages are induced in the gastrointestinal tract, then replicate, infect bacteria in the intestine and transduce host cells, small numbers of bacteria should be enough to produce a Stx concentration sufficient to cause significant damage.

Altogether, these findings suggest a paradigm change on the role of bacteriophages in STEC infections, indicating they may be responsible for the development of disease rather than their bacterial host. Thus, prophylaxis and treatment of human bacterial infections carrying virulence factors on lysogenic bacteriophages may require targeting of the bacteriophages instead of, or as well as, the bacteria and toxins involved.

Data availability

Figshare: Data sets for bacteriophage induction and effect of chitosan. <http://dx.doi.10.6084/m9.figshare.960794>¹²

Author contributions

LVB designed and performed experiments, analyzed the data and wrote the manuscript; LCSF provided advice on experimental design, data interpretation, obtained funding and critical reading of the manuscript; JHA performed experiments and provided advice on experimental design, MJRR-WBL-BFMMP, PDG and RCCF provided advice on experimental design. EGS provided critical feed-back and editing on the manuscript.

Competing interests

No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Raúl Raya

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The article written by Amorim *et al.* describes the anti-phage activity of chitosan on two variants (wild type and a derivative where the *stx* gene was replaced by the *gfp* gene) of the temperate Shiga-toxin producing phage EDL933W. The anti-phage activity was evaluated both *in vivo* and *in vitro*. The authors suggest that chitosan could be a viable alternative for the treatment of STEC infections.

Major:

Phage Induction/anti-phage effects of chitosan/Figure 1:

It seems that chitosan not only sequesters free-phage particles, but also stimulates the growth of uninduced cells (see induced cells treated with chitosan 2 hours post-induction reached higher final OD values). So, does chitosan inhibit the induction process of the temperate phage? Or, does chitosan also adsorb/inactivate ciprofloxacin? Even though in the Materials and Methods a "Bacteriophage inactivation assay" is described, no data is presented. A dose-response curve should be presented, to determine the phage binding (inactivation) capacity of chitosan.

In vivo experiments:

If the authors suggest that Stx phages, rather than bacterial cells, may be responsible for the development of the STEC infections, why they did not use purified phage in *the vivo* experiments? Does chitosan adsorb/inactivate the Shiga-toxins? If so, may it explain the delayed response observed in Figure 4 ("EDL933W plus chitosan")?

Minors:

Abstract:

- Provide a reference after "... plasmids encoding Stx under control of the native bacterial

promoter.”

- Change “*E. coli* K12 strain” to “*E. coli* C600 strain”.

Materials and Methods:

- Delete “... was generously provided by Dr. Luis Carlos de Souza Ferreira, LDV-USP, Brazil.”, since Dr. Ferreira is one of the authors of the manuscript.
- Check the sentence “...was generously provided by Dr. Alison Weiss”; it is repeated twice in the Materials and Methods, and also in the Acknowledgments.
- Change “This is a non-pathogenic phage...” to “This is a non-pathogenic cell...”
- Change “...complete DMEM medium” to “DMEM medium”. Or, if the “complete DMEM medium” contains 10% fetal bovine serum, change “...complete DMEM medium” for “...complete medium”.
- Please, indicate how DNA was harvested.
- Change “Tris pH8 50mM” to “Tris pH8 50 mM”.
- Change “BALB/c mice were bred...” to “BALB/c and DBA-2 mice were bred...”
- Change “...under a 12-h light-dark...” to “...under a 12-hour light-dark...”.
- Delete the sentence “Two-month old BALB/c mice ... and GFP dissemination by IVIS was analyzed”. It is redundant.

Results:

- Figure 1B: Should “Bacteriophage/ml” be “PFU/ml”? Why phage titers are so low?
- Figure 1A: Change “hs” for “hours” or “h”.
- Figure 2: Lanes 5 and 6 should read: “positive PCR control” and “negative PCR control”, respectively. In lane 78, indicate in the figure the kb values of the ladder.
- Delete “viable” in “viable phages”. Were phages transduced or adsorbed to mammalian cells?
- The sentence “Mice were orally challenged with a wild-type EDL933W” is not correct, since there was a direct delivery of bacterial cells into the stomach of mice.

Discussion:

- Check “deliv-ery”

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 01 Aug 2014

Leticia Bentancor, Universidad Nacional de Quilmes, Buenos Aires, Argentina

Phage Induction/anti-phage effects of chitosan/Figure 1:

The higher final OD values determined on the cells treated with chitosan 2 hours post-induction, versus the OD value determined on un-induced cells, is not statistically significant. However, we tested if chitosan is capable of inactivating ciprofloxacin. Ciprofloxacin and chitosan were incubated for 10 minutes at room temperature with chitosan at 5mg/ml. After pre-incubation, the mix was used to induce bacteriophage excision. To see a more significant effect on bacteriophage excision, the induction was incubated overnight. The OD value measured showed a non-significant difference between non-induced culture and induced culture with the antibiotic pre-incubated with chitosan (the experiment was performed in triplicate).

On the other hand, purified bacteriophages were incubated with chitosan, and bacteriophage inactivation was observed with a lysis plaques assay. Bacteriophages were incubated for 10 minutes at room temperature with chitosan at 5mg/ml. After incubation, bacteriophage inactivation was evaluated. The bacteriophage solution containing a titer of 4×10^3 pfu/ml was 100% inactivated after chitosan incubation. The assay was performed in triplicate. This result showed the capacity of chitosan to inactivate bacteriophage *in vitro*.

Also, we did a dose-response curve of chitosan. We used 5mg/ml, 2.5mg/ml, and 1mg/ml of chitosan on purified bacteriophage solution. To evaluate it, the bacteriophage solution was incubated for 10 minutes at room temperature with the different doses of Chitosan and the bacteriophage inactivation was evaluated with a lysis plaques assay. Chitosan at 1mg/ml lost the inactivation activity on the bacteriophages. Chitosan at 5mg/ml and 2.5 mg/ml showed a 100% efficiency on bacteriophage inactivation, however, 1mg/ml of chitosan showed a loss of inactivation, showing between 5-10% of bacteriophage inactivation. This experiment was performed in triplicate.

***In vivo* experiments:**

Chitosan was analyzed *in vitro* and *in vivo* on fD_{TOX}:GFP. Inactivation of fD_{TOX}:GFP was observed *in vitro* with a lysis plaques assay. On the other hand, a decrease of GFP was observed *in vivo* by IVIS. These results shown that chitosan has the capacity to inactivate bacteriophages in absence of Shiga-toxins. A direct action of chitosan on Shiga toxin was not evaluated in this work since we do not have purified Stx2 for such experiments. The authors are working on murine infection with fStx2, but the results obtained will be part of a new publication.

Abstract:

- Provide a reference after "... plasmids encoding Stx under control of the native bacterial

promoter."

The reference was provided.

- Change "*E. coli* K12 strain" to "*E. coli* C600 strain".

The change was made.

Materials and Methods:

- Delete "... was generously provided by Dr. Luis Carlos de Souza Ferreira, LDV-USP, Brazil.", since Dr. Ferreira is one of the authors of the manuscript.

Answer: We deleted "... was generously provided by Dr. Luis Carlos de Souza Ferreira, LDV-USP, Brazil."

- Check the sentence "...was generously provided by Dr. Alison Weiss"; it is repeated twice in the Materials and Methods, and also in the Acknowledgments.

We deleted the sentence in the sub-section "*Transduction of eukaryotic cells*" of material and methods section.

- Change "*This is a non-pathogenic phage...*" to "*This is a non-pathogenic cell...*"

We changed "*This is a non-pathogenic phage...*" to "*This is a non-pathogenic cell...*". However, this non-pathogenic cell produces the excision of a non-pathogenic phage.

- Change "...complete DMEM medium" to "DMEM medium". Or, if the "complete DMEM medium" contains 10% fetal bovine serum, change "...complete DMEM medium" for "...complete medium".

Complete DMEM medium contains 10% fetal bovine serum, so, we changed for the second option "...complete medium".

- Please, indicate how DNA was harvested.

Cells were harvested using Trypsin-EDTA solution. After that, DNA was harvested from pellets by incubation with lysis solution described in material and methods. We included "by Trypsin-EDTA incubation" to clarify the procedure.

- Change "*Tris pH8 50mM*" to "*Tris pH8 50 mM*".

The change was made.

- Change "*BALB/c mice were bred...*" to "*BALB/c and DBA-2 mice were bred...*"

We changed "*BALB/c mice were bred...*" for "*BALB/c and DBA-2 mice were bred...*"

- Change "...under a 12-h light-dark..." to "...under a 12-hour light-dark...".

The change was made.

- *Delete the sentence "Two-month old BALB/c mice ... and GFP dissemination by IVIS was analyzed". It is redundant.*

We have two different mouse models. First, we have the model used to analyze GFP dissemination in which we used two months old mice. Second, we have the model used to analyze protection effect in which we used immature mice. For this reason we clarify the model every time. Let me know if you consider that we need to delete the sentence "Two-month old BALB/c mice ... and GFP dissemination by IVIS was analyzed".

Results:

- *Figure 1B: Should "Bacteriophage/ml" be "PFU/ml"? Why phage titers are so low?*

Bacteriophage/ml was changed to PFU/ml as reviewer suggested. See below. It is true that bacteriophage titers are low. An optimization for bacteriophage purification was done to obtain a higher titer of bacteriophage. The antibiotics used to induce C600D_{TOX}:GFP was selected as an alternative for mitomycin C. The efficiency of bacteriophage induction is strain dependent. Zhang and collaborators reported a titer equal to 1.3×10^5 pfu/ml using ciprofloxacin but they used pathogenic strain *E. coli* O157:H7. We also observed a higher titer inducing the EDL933W strain, for this reason we suppose that the low titer observed is dependent on the strain used.

- *Figure 1A: Change "hs" for "hours" or "h".*

The change was made.

- *Figure 2: Lanes 5 and 6 should read: "positive PCR control" and "negative PCR control", respectively. In lane 78, indicate in the figure the kb values of the ladder.*

The changes were made.

- *Delete "viable" in "viable phages". Were phages transduced or adsorbed to mammalian cells?*

We deleted "viable" in "viable phages". In this context, phages purified from tissue were detected by lysis plaque assay.

- *The sentence "Mice were orally challenged with a wild-type EDL933W" is not correct, since there was a direct delivery of bacterial cells into the stomach of mice.*

The sentence "Mice were orally challenged with a wild-type EDL933W" was change by "Mice were intragastrically infected with a wild-type EDL933W".

Discussion:

- *Check "deliv-ery"*

We did not find delivery in the Discussion section.

Competing Interests: No competing interests were disclosed.

Reviewer Report 31 March 2014

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Mikael Skurnik

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The paper by Amorim *et al.* deals with the role of bacteriophages in STEC-infections. The authors have earlier demonstrated that the *stx* genes can be expressed within eukaryotic cells, provided the prophage-carried *stx*-DNA is introduced there in naked form, i.e., in transfected plasmids. In the present work, the authors wanted to test/prove the hypothesis that the *stx*-carrying prophage upon induction *in vivo* could contribute to the toxin production. They also tested whether the polysaccharide chitosan has anti-*stx* phage effect. I have some major and minor points:

Major

1. **Bacteriophage inactivation assay:** The experimental design of the bacteriophage inactivation assay uses only one concentration of chitosan. To demonstrate specificity, dose dependence should be demonstrated. In addition, the *in vivo* dose of chitosan was not indicated in the methods section (100 µl/mouse of 5 ml/ml chitosan was given orally to mice as indicated in the **Effect of chitosan *in vivo*** section).
2. The mouse experiments were performed with too small a number of mice.
3. Figure 1A of growth curves is missing a crucial control. What happens to E. coli C600 under the ciprofloxacin treatment?
4. Figure 1B: The lack of the 4 hr column in chitosan 4h post-induction does not seem logical to me. There should be a ca 6000 PFU/ml column similar to that one in the induced 4hr sample. This discrepancy should be explained.
5. Figure 2: the PCR experiment does not provide evidence of transduction. The definition of transduction is that DNA moves from one cell to another. PCR detects the phage DNA either free in the cell cytoplasm or packed in endocytosed phage particles. Therefore, the authors need to demonstrate that infective phage particles disappear from infected cells. The experiment also does not exclude the possibility that phage particles are just adsorbed on the cell surface.

6. The experiment reported in figure 3 should also include bacterial counts from the organs as it is very likely that live *E. coli* bacteria, after a massive dose of 10^{13} bacteria per mouse, end up in the organs. Therefore the authors should demonstrate that the GFP response is not from bacteria infected by the GFP-phages.
7. The Figure 4 experiment was performed with only 4 mice. Such an experiment should not be shown in a publication. In addition, different chitosan doses should be tested here also.

Minor

1. **Introduction, paragraph 3:** This statement on the low number of bacteria during infection should be backed up with a reference.
2. **Materials and Methods:** Dr Alison Weiss is thanked twice for same strain. One time should be enough. In addition, in the acknowledgements she is thanked a third time. The bacterial strain designation in the latter is given differently than elsewhere in the text.
3. **Transduction of Eukaryotic Cells:** C600ΔTOX:GFP is a bacterial strain, not a non-pathogenic phage.
4. **EHEC infection:** The final concentration of CFU/100μl/mouse needs revision.
5. **Figure 2 legend:** The path the sample takes in the gel is called the lane, not line.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 01 Aug 2014

Leticia Bentancor, Universidad Nacional de Quilmes, Buenos Aires, Argentina

Major:

1. A dose-response curve of chitosan was done. We used 5mg/ml, 2.5mg/ml and 1mg/ml of chitosan on purified bacteriophage. To evaluate it, the bacteriophage solution was incubated for 10 minutes at room temperature with the different doses of chitosan and the bacteriophage inactivation was evaluated with a lysis plaques assay. Chitosan at 1mg/ml lost the inactivation activity on the bacteriophages. Chitosan at 5mg/ml and 2.5 mg/ml showed a 100% efficiency on bacteriophage inactivation, however, 1mg/ml of chitosan showed a loss of inactivation, showing between 5-10% bacteriophage inactivation. This experiment was performed in triplicate.

The effect of chitosan *in vivo* was evaluated using a final concentration of 5mg/ml of chitosan solution. Each mouse received 100ml, so, the dose used was 500mg/mouse.

The Material and Methods section was changed as follows:

“Immature male and female DBA-2 infected, as described previously, were treated with 100ml of a chitosan solution at a concentration of 5 mg/ml (500 mg of chitosan per mouse) was orally administrated 2 hours after infection and survival was observed.”

2. The experiment was shown as a preliminary result and this work it is a short communication. The model used has some experimental problems for the ages of mice used. The experiment was started using 6 mice per group, but some mice died after inoculation and not for the infection. For this reason, we had shown only 4 mice per group. We repeat the experiment, and again we have the same problem, however, we can see the same partial effect of chitosan *in vivo*. To further analyze the effect observed, we will report the results on a new publication with more details.
3. The controls we used were:
 1. Non-induced *E. coli* C600D_{TOX}:GFP (as a negative control) in which we can observe the normal growth of bacteria without bacteriophage induction
 2. Induced *E. coli* C600D_{TOX}:GFP (as a positive control) in which we can observe how bacteriophage induction affect the growth of bacteria.

If you are thinking about *E. coli* C600 in absence of *l*isogenic *f*D_{TOX}:GFP, we do not have access to this strain. But, we think that the controls used are well done. As an observation, we can said that no significant change in the growth was observed on *E. coli* Y1090 used for bacteriophage titration assay.

1. The observation is right; we made an error in the graph. The values were checked and the correct value was added to the new graph.
2. We agree with the definition of transduction, it is the process by which DNA is transferred from one cell to another by a virus. In our previous paper, we used the same definition to evaluate the capacity of *f*D_{TOX}:GFP to transduce macrophages. In this case, we observed GFP expression and we concluded that macrophages were transduced by *f*D_{TOX}:GFP. In this report, we did a different approach and we use PCR to detect bacteriophage DNA inside the cell.

After your opinion, we did two assays. First, bacteriophages inside the cell were analyzed for titration assay. Second, as a preliminary data, *f*Stx2 was used to transduce Vero cells, as a representative Stx2-susceptible cell line.

Infective bacteriophage particles were not detected on transduced cells. The assay was made using lysis plaque assay of cellular extracts.

Results:

In order to analyze the transduction by an additional method, Vero cells were transduced with *f*Stx2 and cytotoxicity induced by Stx2 was evaluated by microscopy. Vero cells transduced with *f*Stx2 (panel A) showed a similar cytotoxicity to that of cells

incubated with 1 CD50 of purified Stx2 (panel B). No cytotoxic effects were observed in non-treated Vero cells (panel D). Vero cells transduced with a M.O.I. = 0,0625 did not shown cytotoxic effect, demonstrating the specificity of the effect observed by fStx2 (panel D).

Materials and Methods:

In vitro evaluation of the capacity of Bacteriophage 933W to transduce Vero cells.

E. coli EDL933W (ATCC 43895) was used to purify fStx2. *E. coli* EDL933W strain was grown in Luria Broth (LB) overnight (ON) at 37°C under agitation. The ON culture was diluted to OD600nm = 0.1 in LB. Induction was carried out by adding ciprofloxacin to a final concentration of 40 ng/ml⁸ in main text. Bacteria were incubated for 6 hours at 37°C under agitation. Cultures were then centrifuged at 5000 rpm for 15 minutes. The bacteriophage-containing supernatant was filtered with 0.2 mm filters, precipitated and purified. Briefly, supernatant was incubated on ice with a PEG-8000/NaCl solution for 30 minutes. After that, the solution containing bacteriophages was centrifugated and washed. The pellet was resuspended in STE buffer (1ml of Tris pH8, 0,2ml of 0,5M EDTA, 2ml of 5M NaCl, water up to 100ml). Phages at a multiplicity of infection (M.O.I) equal to 1 were added to Vero cells. Transduction of Vero cells was enhanced by centrifugation at 1000 x g for 10 min at room temperature. After 24 hours post transduction, cells were examined by microscopy using Nikon Eclipse TE2000 (NIS-Elements imaging software) equipped with a CCD camera. Dilutions of fStx2 were made to demonstrate specificity. Vero cells were incubated with purified Stx2 as positive control.

Additional figure. *In vitro evaluation of the capacity of Bacteriophage 933W to transduce Vero cells.* **A.** Vero cells transduced with fStx2 (M.O.I. = 1). **B.** Vero cells incubated with purified Stx2. **C.** Vero cells transduced with a M.O.I. = 0,0625. **D.** Vero cells with not treatment.

3. *E. coli* C600DTOX:GFP is not an invasive bacteria. Also, *E. coli* O157:H7 is a non invasive strain; for this reason we do not check for bacteria in organs. Bacteria were checked only on lungs samples, just to see if the inoculation was right. Bacteria were not detected in lungs. The dose used was selected after a previous experiment in which we evaluated the sensibility of IVIS in our system. GFP is not the best fluorescent protein for IVIS system; so, we needed to use a high dose of bacteria. As we described in this work, bacteriophages were detected by lysis plaques assay in intestine homogenates and blood samples of infected mice. It is important to do a highlight in the case of intestine sample, as it is very difficult to find *E. coli* C600DTOX:GFP. First, because the huge amount of bacteria present in the sample, and also, because the bacteria lysis induced by bacteriophage excision.
4. The experiment was shown as a preliminary result and this work it is a short communication. The model used has some experimental problems for the ages of mice used. The experiment was started using 6 mice per group, but some mice died after inoculation and not for the infection. For this reason, we had shown only 4 mice per group. We repeat the experiment, and again we have the same problem,

however, we can see the same partial effect of chitosan *in vivo*. To further analyze the effect observed, we will report the results on a new publication with more details.

Minor:

1. The statement "...very small numbers of bacteria are sufficient to induce HUS in humans..." is taking the data published recently, in which the authors demonstrated that a concentration of Stx2 as low as 10 fM is able to induce ribosome damage and to modulate selected cell signaling pathways that change cellular functions. If 10 fM of Stx2 is enough, very small numbers of bacteria should be sufficient to induce HUS (Petruzziello-Pellegrini & Marsden, 2012).
2. We deleted the sentence "...was generously provided by Dr. Alison Weiss" in the subsection "Transduction of eukaryotic cells" of material and methods section.
3. We changed "This is a non-pathogenic phage..." to "This is a non-pathogenic cell...". However, this non-pathogenic cell produces the excision of a non-pathogenic phage.
1. The dose is correct. We used a dose of 3×10^{12} CFU/mice in a volume of 100 ml.
2. Line was changed for Lane.

Competing Interests: No competing interests were disclosed.

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