Protection against Clostridium difficile infection in a hamster model by oral vaccination using flagellin FliC-loaded pectin beads

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Abstract

Clostridium difficile flagellin FliC is a highly immunogenic pathogen-associated molecular pattern playing a key role in *C. difficile* pathogenesis and gut colonization. Here, we designed an oral vaccine against *C. difficile* with FliC encapsulated into pectin beads for colonic release. Bead stability and FliC retention was confirmed in vitro using simulated intestinal media (SIM), while bead degradation and FliC release was observed upon incubation in simulated colonic media (SCM). The importance of FliC encapsulation into pectin beads for protection against *C. difficile* was assessed in a vaccination assay using a lethal hamster model of *C. difficile* infection. Three groups of hamsters orally received either FliC-loaded beads or unloaded beads in gastro-resistant capsule to limit gastric degradation or free FliC. Two other groups were immunized with free FliC, one intra-rectally and the

other intra-peritoneally. Hamsters were then challenged with a lethal dose of *C. difficile* VPI 10463. Fifty percent of hamsters orally immunized with FliC-loaded beads survived whereas all hamsters orally immunized with free FliC died within 7 days post challenge. No significant protection was observed in the other groups. Only intra-peritoneally immunized hamsters presented anti-FliC IgG antibodies in sera after immunizations. These results suggest that an oral immunization with FliC-loaded beads probably induced a mucosal immune response, therefore providing a protective effect. This study confirms the importance of FliC encapsulation into pectin beads for a protective oral vaccine against *C. difficile*.

Keywords: *Clostridium difficile*; oral vaccination; flagellin; pectin beads; colonic delivery

1 Introduction

Clostridium difficile (Clostridioides) difficile [1] is a Gram-positive, anaerobic spore-forming bacterium and is the leading cause of antibiotic-associated diarrhea. Gut microbiota dysbiosis enables *C. difficile* colonization of the intestinal tract. After contamination, *C. difficile* spores germinate, vegetative forms multiply, and toxins are released, disrupting epithelium integrity and inducing an inflammatory response in the colon [2]. *C. difficile* is a non-invasive pathogen, thus, promoting local intestinal immunity could trigger early protection against *C. difficile* infection **(CDI)** [3]. **Even though the intra-rectal route has shown promising results previously by inducing a protective immune response directed to *C. difficile*** [4-6], developing the oral route of immunization represents a rational choice to induce a gut mucosal immune response with a better patient acceptance and comfort. To overcome the gastrointestinal barrier, antigen encapsulation is recommended to maintain its integrity and its immunogenicity. Biocompatible and biodegradable polymers are interesting materials for encapsulation. For instance, pectin, a non-toxic polysaccharide,

has previously shown promising capacity for protein encapsulation and colonic delivery [7, 8]. Indeed, pectin is not degraded by gastric or intestinal enzymes but is almost totally degraded by pectinolytic enzymes produced by the microbiota present in the colon [7, 9].

Current strategies for vaccine development against *C. difficile* target either toxins or colonization factors. Systemic vaccines targeting toxins are currently being tested in clinical trials (NCT01887912; NCT03090191; NCT02316470) and have shown efficacy in protecting against **CDI in animal models** [10, 11]. However, targeting colonization factors could prevent *C. difficile* colonization**, growth** and symptomatic infection therefore limiting dissemination of the bacteria in the environment. Several vaccine candidates targeting colonization factors showed promising results [4, 8, 12]. For instance, flagellin which is highly immunogenic, harbors a unique pathogen-associated molecular pattern implicated in toll-like receptor-5 (TLR-5) recognition. However, the central domain of the flagellin is highly variable among different species [13, 14]. Regarding *C. difficile* flagellin, FliC is a 39 kDa structural protein of the flagellum. The central domain, the N- and C-terminal domains of FliC are well conserved among the different strains of *C. difficile*. FliC antibodies have been detected in patient sera [15] and non-CDI patients presented significantly more anti-FliC serum antibodies than CDI patients [16]. Interestingly, the immunological properties of FliC enable it to act as adjuvant [17] and as antigen, so, FliC represents a promising vaccine candidate [12, 18]. Ghose *et al*. intraperitoneally immunized hamsters and mice with FliC and described the induction of a systemic IgG response protective against **CDI [12]**.

Developing a mucosal vaccine targeting *C. difficile* gut colonization could enable early protection against CDI. Oral vaccines can be expected to have much greater acceptability than injectable vaccines. Oral vaccine administration could also lead to simplified manufacturing vaccine, thereby increasing the potential for local vaccine production. Here we describe the development and the efficacy of a mucosal vaccine strategy with FliC against *C. difficile* virulence in

hamster. To this end, here we first assess the suitability of pectin beads to deliver FliC into the colon and we evaluate FliC-loaded pectin beads in gastro-resistant capsules as an oral vaccine candidate against *C. difficile* in a lethal hamster model of **CDI**.

2 Material and methods

2.1 Preparation of pectin beads

Recombinant FliC was obtained and purified as previously described [13] with exception that the purified protein was dialyzed against TRIS buffer (25 mM, pH 7.5). Pectin beads were prepared as previously detailed [8], pectin solution was obtained with Unipectine^{TM} OG175C (Cargill) dissolved in TRIS buffer (25 mM, pH 7.5) at the concentration of 6% (w/V). Unloaded beads were prepared with the pectin solution only and FliC-loaded beads with pectin solution and recombinant FliC. The pectin solution containing FliC or not was then dropped into a cross-linking solution of Zn acetate (12%, w/V). Beads, formed instantaneously by contact with zinc ions, were left in the cross-linking solution for 30 minutes at room temperature under magnetic stirring. Beads were washed three times with distilled water and then dried 3 hours at 37°C. To determine encapsulation **efficiency (EE)**, FliC-loaded beads were disintegrated in TRIS buffer (25 mM, pH 7.5) added with EDTA (50 mM). Then, proteins were dosed using Bradford dye-binding method. **EE(%) was determined by the following formula : (actual amount of FliC encapsulated / theoretical amount of FliC encapsulated) *100.**

2.2 Analysis of protein release from beads in simulated digestive media

Analysis of FliC release was carried out in two different simulated digestive media as previously described [8]: 5 h in Simulated Intestinal Medium (SIM, HEPES/NaCl buffer, pH 6.8) containing pancreatin (1% w/V) followed by 5 h in Simulated Colonic Medium (SCM, HEPES/NaCl buffer, pH 6) containing ≥760 U/mL of pectinase from *Aspergillus aculeatus* (Sigma). Presence of FliC and

pectinase in the same medium makes difficult to dose proteins by Bradford dye-binding method. Therefore, loaded beads were prepared using rhodamine-labelled FliC. Labeling and release protocol were performed as previously described [8] and the amount of FliC released was determined by spectrofluorometry in duplicate.

2.3 Gastro-resistant coating of capsules containing beads for oral immunization

To protect beads from the harsh gastric acid environment, loaded and unloaded beads were placed into gelatin capsules (size 9, Harvard Apparatus) coated with a hydroxypropyl methylcellulose phthalate (HPMCP 50) film. Coating was performed by dipping capsules into a 10% (w/V) solution of HPMCP 50 in acetone/ethanol (1:1 v:v) and drying them at room temperature [8]. This process was repeated six times. The efficacy of the gastro-resistant coating was checked by a disintegration test according to European pharmacopoeia (capsules undamaged after 2 h in HCl 0.1 M) [8].

2.4 Animals

The vaccination study was carried out in a model of infection using *Mesocricetus auratus* female hamsters (weight, 80–100 g, Janvier Laboratories). Protocols involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies. The protocol was approved by the Committee on the Ethics of Animal Experiments University of Paris-Sud and the French Minister of Research (APAFIS#4577-2016020913152994 v4). All efforts were made to minimize animal suffering.

2.5 Vaccination protocol

Five groups of 6 animals were used. Each vaccinated animals received a total of 300 µg of FliC in three administrations of 100 µg of FliC every fifteen days **(at day 0, day 15 and day 30)**. One group was immunized with FliC-loaded pectin beads given orally and a group orally received unloaded

beads. Three other groups were immunized with recombinant free FliC by oral, intra-rectal or intra-peritoneal route. **Fifteen days after the last immunization (at day 45) and b**efore *C. difficile* challenge, hamsters were orally given clindamycin at a single dose of 50 mg/kg and gentamicin twice a day during 5 days **(from day 45 to day 49)** at a dose of 2.5 mg/kg to disrupt the intestinal microbiota. Then **at day 50**, hamsters were orally challenged with $7{,}5x10^4$ spores of *C. difficile* strain VPI 10463 (Fig. 1). Two days after challenge, ***C. difficile* colonization** was checked by detection of *C. difficile* vegetative cells in the feces **by plating adequate dilutions on Columbia agar containing 5% of horse blood, 25% (w/v) of D-cycloserine, and 0.8% (w/v) of cefoxitin and taurocholate**.

2.6 Evaluation of specific antibody level in sera after immunization

To evaluate the antibody response in sera, blood samples were withdrawn before the first immunization and 15 days after the last immunization, before *C. difficile* challenge. Indirect ELISA was used to detect antibodies in the sera as previously described [6]. Briefly, wells of 96-well microtitre plates (MaxiSorp, Nunc) were coated with 100 µL of a 5 µg/mL solution of recombinant purified FliC. Sera were tested in duplicate at dilution 1:500 in 100 µL final volume. After five washings with phosphate buffer (PBS) and Tween-20 (0.1%), an aliquot of 100 µL per well of a rabbit anti-hamster IgG conjugated with biotin (1:8,000 dilution; Biovalley) was added and plates were incubated for 30 min at 37°C. Then, after five washings, 100 µL per well of streptavidin–HRP (1:10,000 dilution; ThermoScientific) were added and plates were incubated for 30 min at 37°C. Assays with antigen in the absence of sera served as negative controls. Immunoglobulin levels are expressed as OD units at 450 nm.

2.7 Statistical analysis

Animal surviving rate was analyzed using Kaplan–Meier estimates. Survival rates between groups were compared using log rank test, p-values < 0.05 were considered as statistically significant. Mann-Withney U-test was performed to analyzed specific anti-FliC antibody levels in sera after immunizations, p-values < 0.05 were considered as statistically significant.

3 Results

3.1 Flagellin encapsulation in pectin beads and *in vitro* release

In order to perform oral vaccination, we encapsulated the recombinant *C. difficile* flagellin FliC into pectin beads. About 40 to 45 beads were obtained per milliliter of pectin solution. After drying, beads had an ellipsoid shape with a 1.2-1.5 mm diameter and weighed between 1.5 and 2 mg (Fig. 2). The total amount of encapsulated FliC was dosed after total disintegration of beads. FliC-loaded bead contained a total amount of 3 ± 1 µg of FliC by bead, this corresponds to an encapsulation **efficiency** of about 30 ± 10% (n=5). In vitro characterization confirms beads stability in SIM and protein release in SCM containing pectinase. Less than 10% of FliC was released after 5 h of incubation in SIM and dried beads swelled keeping their shape. After being placed in SCM, beads started to disintegrate leading to the release of FliC. Between 2 and 3 h were sufficient to release more than 80% of entrapped FliC (Fig. 3). This *in vitro* characterization of FliC-loaded beads confirms the interest of using this encapsulation strategy for colonic delivery of an antigen after oral administration.

3.2 Vaccination with flagellin against **CDI** in the hamster model

C. difficile flagellin FliC is a promising vaccine candidate. To evaluate the efficacy of mucosal vaccination with FliC against *C. difficile* virulence, we immunized hamsters with the same amount of FliC via different mucosal routes and compare them with the intra-peritoneal parenteral route. We showed here that encapsulation of FliC into pectin beads for oral administration significantly

protected hamsters against *C. difficile* lethal challenge (Fig. 4). Indeed, at day 17 after challenge, 50% of hamsters survived in the group orally vaccinated with FliC-loaded beads, whereas no survival was observed after oral vaccination with free FliC (p-value=0.041). Only 17% of hamsters (n=1) survived in the unloaded bead group. The same percentage of surviving animals was observed for the intra-rectally vaccinated group. After immunization by intra-peritoneal route, 33% of hamsters survived. Interestingly, analysis of *C. difficile* fecal shedding showed that, whereas all 30 animals were infected by *C. difficile* two days post-challenge, all surviving animals in the different groups were no more colonized at the end of the assay at 17 days post challenge. These results showed that oral vaccination with FliC-loaded pectin beads led to the best protection against *C. difficile* virulence in the hamster model.

3.3 Antibody response induced after vaccination

To correlate the observed protection with the systemic immune response induced by vaccination, we evaluated the anti-FliC response in sera. We observed an increased level of FliC-specific IgG antibodies in sera after intra-peritoneal immunizations of hamsters. In contrast, in the other groups, no significant increase of anti-FliC-specific serum IgG level was observed (Fig. 5).

4 Discussion

As *C. difficile* is a non-invasive enteropathogen, the induction of a mucosal immune response close to the site of infection appears to be a relevant choice for inducing protection. Vaccine development against non-invasive gastrointestinal infections such as enterotoxigenic *Vibrio cholerae* or *Escherichia coli* showed that the protection is conferred mainly by specific secretory IgA (sIgA) and by the induction of a memory immune response [19]. A mucosal vaccine strategy, which aims to prevent alteration of the intestinal epithelium by targeting the early stages of the *C. difficile* infectious process, could benefit **from** further attention.

Furthermore, one of the vaccine strategies against *C. difficile* is to target surface proteins involved in intestinal colonization. The flagellin FliC appears a promising vaccine candidate, since Ghose *et al.* reported that intra-peritoneal immunization with FliC and alum led to 43 to 64% of protection in a hamster model and 40 to 100% protection in a mouse model in a dose-dependent manner [12]. In our assay, intra-peritoneal immunization of hamsters with FliC led to 33% of survival against *C. difficile*. Compared to Ghose *et al* results [12], this difference of protection is probably due to the adjuvant used for immunization. Indeed, as already demonstrated, *C. difficile* flagellin FliC has adjuvant properties, avoiding alum use as adjuvant [12, 17]. Consequently in our study, immunizations were performed without additional adjuvant. In addition, *C. difficile* strain used for challenge was not the same, with probably a difference of virulence between the VPI 10463 strain used here and the 630Δ*erm* strain used in Ghose *et al* study, as described elsewhere [20]. Of note here, we demonstrated that the high immunogenicity of FliC enables to induce a strong systemic IgG antibody response after intra-peritoneal immunization without supplementary adjuvant.

Interestingly, here we obtained a better protection with an oral vaccine consisting of FliC encapsulated into pectin beads to target colonic release than after intra-peritoneal immunization with purified FliC alone. We showed that this strategy can significantly protect 50% of hamsters from **CDI (3 out of 6)**. **However, these results should be confirmed in a larger study**. **This significant but partial protection confirms the multifactorial aspect of colonization, suggesting that a combination of several proteins will be necessary to trigger an efficient immune response against *C. difficile* colonization factors, and consequently to prevent the colonization process. In addition, according to animal variability to *C. difficile* infection, especially regarding immune response and microbiota, a combined vaccine could be necessary**.

Ghose *et al.* demonstrated that the protection induced by FliC immunization by intra-peritoneal route was anti-FliC IgG-mediated. In our study, the immune response induced after oral immunization with

FliC-loaded pectin beads is able to partially protect animal from death. However, no specific IgG antibody response was detected in sera. This was previously observed in another assay of oral vaccination of hamsters with the Cwp84 protease encapsulated in pectin beads. Although vaccinated hamsters were partially protected (40%) against CDI, they did not develop a systemic anti-Cwp84-IgG antibody response [8]. This suggests that after vaccination by mucosal route, beside a systemic immune response, a local immune response with sIgA production could be the key factor of protection. It has been previously shown in a mouse model that parenteral immunization with flagellin can activate mucosal dendritic cells and induce an isotype switch to IgA [18]. Unfortunately secondary antibodies are not commercially available to detect specific IgA in hamsters.

Here, we used the hamster model of CDI, which is highly sensitive to this infection and reflects more severe infection in human than mild infection. Our results indicate that in this model, protection could probably be related to neutralizing sIgA but other factors may play an important role in the host immune response against **CDI**. In particular, our immunization strategy might have generated a wider cell-based immunity that could have induced partial protection. Regarding *Streptococcus pneumoniae,* it has been demonstrated that multiple immune cell types are required for the induction of a protective immunity in a murine model which lacks mature B cells and fails to produce antibody [21]. Further studies are needed to specify the immune effectors induced by immunization.

In this study, the least protection of hamsters observed by intra-rectal administration of FliC compared to the oral administration of FliC-loaded beads and the absence of protection for the free FliC orally treated group, is presumably due to the degradation of the free antigen by gut enzymes before it reaches the colon. This further confirms the importance of the administration route and the use of pectin beads as a delivery system for FliC.

To conclude, we showed that oral vaccination with *C. difficile* FliC-loaded pectin beads is partially protective against a virulent strain of *C. difficile* in a hamster model. **In order to assess in further depth the mechanisms of protection, further studies in mouse model could inform on the protection against *C. difficile* colonization and a better understanding of the immune response elicited with this vaccine.** This study confirms the importance of the adequacy between the administration route, the delivery system and the vaccine candidate in the design of a mucosal protective immunization strategy targeting *C. difficile*.

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Conflict of interest

No conflicts of interest to declare.

Figures

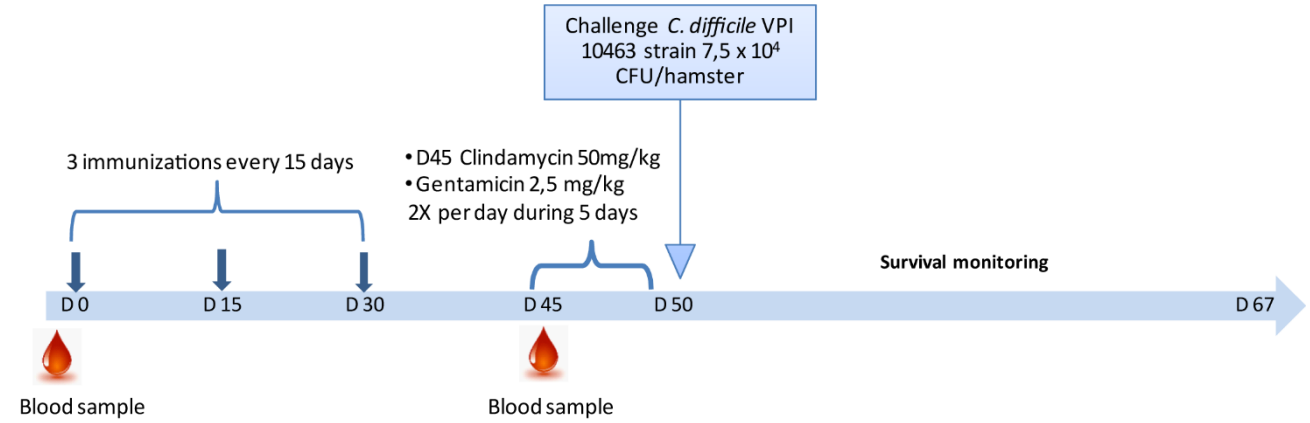

Figure 1. Vaccination protocol.

Animals received a total of 300 µg of FliC in three administrations of 100 µg of FliC on days 0, 15 and 30. Before challenge, hamsters received clindamycin and gentamicin to disrupt the intestinal microbiota. Then, hamsters were orally challenged by $7{,}5 \times 10^4$ spores of *C. difficile* strain VPI 10463. Two days after challenge **CDI** was checked by detection of *C. difficile* in the feces. Blood samples were withdrawn before the first immunization and 15 days after the last immunization to evaluate the antibody response in sera.

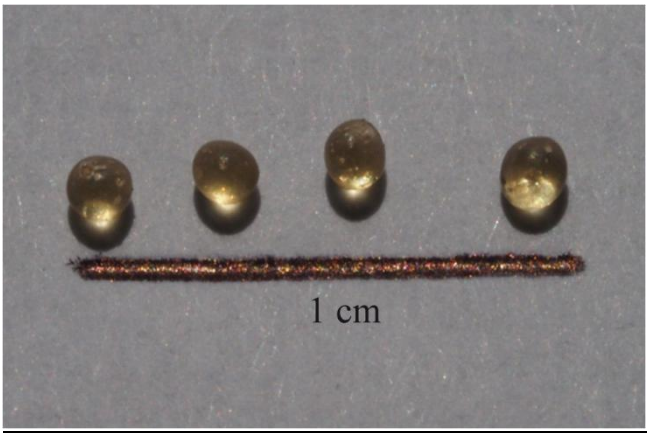

Figure 2. Dried pectin beads (scale bar represents 1 cm).

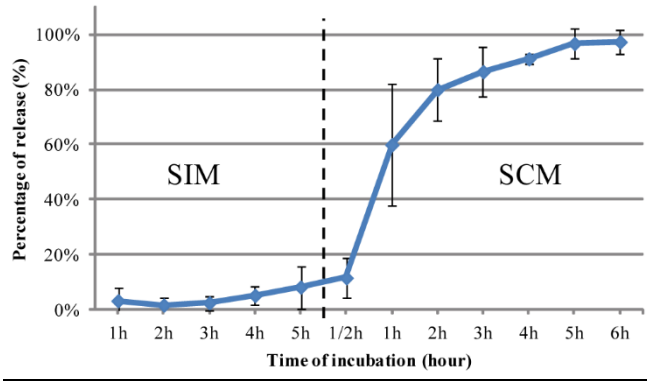

Figure 3. In vitro FliC release from pectin beads in Simulated Intestinal Medium (SIM) for 5h and Simulated Colonic Medium (SCM) for other 5h (n=2).

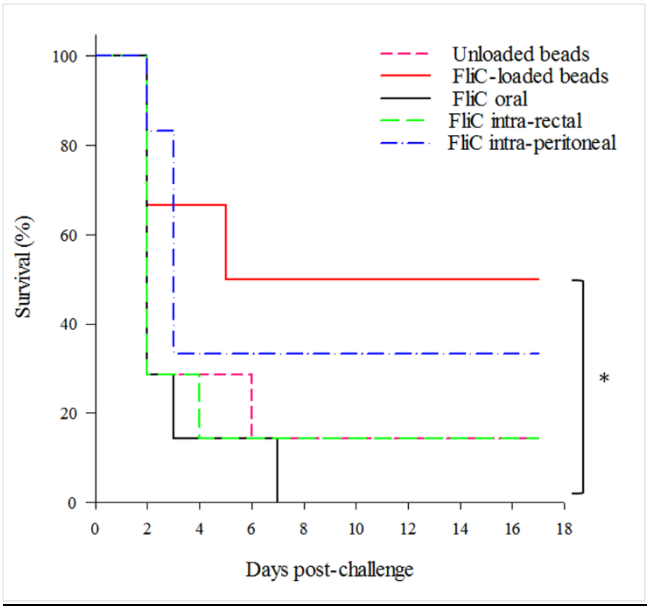

Figure 4. Kaplan-Meier survival estimates after immunizations. One group was immunized with FliC-loaded beads given orally (FliC-loaded beads), one group orally received unloaded pectin beads (Unloaded beads) and three other groups were immunized with recombinant free FliC by oral (FliC oral), intra-rectal (FliC intra-rectal) or intra-peritoneal route (FliC intra-peritoneal). After challenge with spores of *C. difficile* VPI 10463 strain, animals were monitored for 17 days. * p-value < 0.05

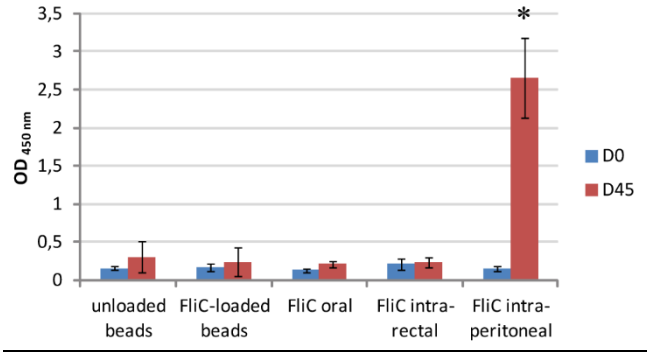

Figure 5. **Detection of specific IgG in animal sera by ELISA.** Mean of anti-FliC-specific IgG in serum of immunized hamsters (diluted 1:500) before (D0) and after vaccination (D45). Sera of hamsters were analysed by ELISA. *: statistically significant difference p-value < 0.05 (Mann-Withney U-test).

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