TITLE:

Towards a non-living vaccine against *Shigella flexneri*: from the inactivation procedure to protection studies

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Abstract

Shigellosis is one of the leading causes of diarrhea worldwide with more than 165 million cases annually. Hence, a vaccine against this disease is a priority, but no licensed vaccine is still available. Considering target population as well as intrinsic risks of live attenuated vaccines, non-living strategies appear as the most promising candidates. Remarkably, the preservation of antigenic properties is a major concern since inactivation methods of bacteria affect these qualities. We previously reported the use of a subcellular antigen complex for vaccination against Shigellosis, based on outer membrane vesicles (OMVs) released from *Shigella flexneri*. Now, we describe in more detail the employment of binary ethylenimine (BEI) for inactivation of *Shigella* and its subsequent effect on the antigenic conservation of the vaccinal product. Results demonstrate the effectiveness of BEI treatment to completely inactivate *Shigella* cells without disturbing the antigenicity and immunogenicity of the OMVs. Thus, OMVs harvested after BEI inactivation were able to protect mice against an experimental infection with *S. flexneri.*

Keywords: *Shigella;* subunit vaccine; safety; outer membrane vesicles

1. Introduction

Diarrheal diseases are the major causes of morbidity and mortality in the developing world [1]. Among them, shigellosis is one of the most contagious types of diarrhea caused by bacteria. *Shigella spp,* the causative agent*,* invades the intestinal mucosa, spreads to the adjacent epithelial cells and causes tissue damage, fluid secretion and inflammation, producing the clinical manifestations of dysentery, diarrhea with blood and mucus [2]. Shigellosis is endemic throughout the world where it is responsible for more than 165 million cases of severe dysentery annually, the majority of which occur in children under 5 years old in the developing world [3]. In addition, about 500 000 cases of shigellosis are reported each year among travellers from industrialized countries and military personnel [4]. In the absence of an existing effective vaccine and the ever-increasing frequency of antimicrobial-resistant *Shigella* strains worldwide, Shigellosis has become a major source of concern [5]. Several candidate shigellosis vaccines are currently in development including live attenuated, inactivated whole cell, or subunit vaccines [6-8]. Considering intrinsic risk of living vaccines, the non-living vaccine alternative, including either inactivated whole-cell or acellular approaches, results the safest direction. However, even being inactivated the final vaccine product, the production process managing alive *Shigella* cells is a risk that should be controlled, considering this pathogen is one of the most common causes of laboratory-acquired infections [9;10]. Besides, outbreaks of disease have occurred in the past due to improper inactivation [11;12]. Considerable knowledge has been accumulated regarding the efficiency of microbicidal treatments but, at the same time, the preservation of immunogenicity and native structure during production may limit their potential due to the alteration of epitopes that may be essential to confer protection. Thus, most used treatments, such as heat, acetone, phenol or formaldehyde, cause membrane damage or

induce cross-linking of proteins leading to antigenic alterations and consequent failure in the ability to elicit protective immunity [13-21]. Here, we evaluate binary ethylenimine (BEI) alone or in combination with formaldehyde (BEI-FA) as inactivating agent against *Shigella* cells viability. BEI is an alkylating substance specific for the nucleic acids widely used in virus vaccine production [22]. In contrast to some other biocides, BEI intercalates with nucleic acids and does not react with proteins, so it is not expected to alter their immunogenic properties.

In the context of subunit vaccines, OMV are attractive candidates by itself and for vaccine delivery platforms. OMVs are spherical lipid bilayer blebs with an average diameter of 50-250 nm that are naturally secreted by Gram-negative bacteria [23]. OMVs encase outer membrane and periplasmic constituents, and function in diverse roles such as biofilm formation and virulence. Thus, contain significant immunogens and appear to be safe when used as vaccines [24]. Currently, an OMV-vaccine is being successfully used against serogroup B meningococcal disease [25;26] and it also represents a promising vaccine candidate for several other pathogens like *Pseudomonas* [27], *Vibrio* [28], *Salmonella* [29] or *Acinetobacter baumannii* [30].

We have previously demonstrated the potential of outer membrane vesicles (OMVs) for vaccination against shigellosis [31]. Here, we go further into characterizing the protective ability of OMVs harvested from a BEI-inactivated *Shigella* culture. Results demonstrated that the combination of BEI-FA was efficient for inactivation of *Shigella*, did not affect its immunogenic properties and, what´s more, confirm the potential of the OMVs as a valuable strategy vaccination against Shigellosis.

2. Material and Methods

2.1. Bacterial strains and culture conditions:

A clinical isolate of *Shigella flexneri* 2a ("Clínica Universidad de Navarra", Pamplona, Spain) was used along the study of inactivation and OMV production. On the day previous to each experiment, *S. flexneri* originally isolated in Tryptone-Soya-Agar (Biomerieux) was seeded into Tryptone-Soya-Broth and incubated for 16 h at 37 °C with agitation to reach stationary phase.

2.2 Inactivating agents:

Phenol and formaldehyde were purchased from Panreac (Barcelona, Spain). 2-bromoethylamine hydrobromide (BEA) from Sigma-Aldrich (St. Louis, MO USA) and Na-thiosulfate (Merck). All stock solutions of the chemicals were prepared by dissolving or diluting the chemicals to obtain the required concentrations using deionized water. Working solutions were freshly prepared and sterilized using 0.22 μm filters prior to each experiment

BEI was prepared by cyclization of 0.1 M BEA (Sigma) in 0.175 M NaOH solution for one hour at room temperature following the method of Bahnemann [32]. The cyclization of 0.1 M 2-bromoethylamine into the active BEI form was confirmed by the change in the pH, from 12.7 to 8.7. The solution was freshly prepared prior to each experiment. Controls were performed with other commonly used treatments (phenol 0. 5% v/v, 4 h) or heat (60 ºC, 30 min) and an untested culture was also included.

Bacteria in stationary phase culture were dispensed into 50 ml and 500 ml aliquots. Different volumes of BEI or a combination of BEI –FA were added to each aliquot containing bacteria to obtain final BEI concentrations of 2 mM, 4 mM, 6 mM (see table

2). These suspensions were incubated at 37 °C with continuous stirring up to 24 h. At different time points of 1 h intervals, aliquots of 1,000 µl were directly spread onto TSA plates, incubated at 37 °C and inspected for growth during 7 days. The colonies were then counted to evaluate bactericidal activity. Full bactericidal activity was considered when no colonies appeared after 7 days incubation; in contrast, above one colony was considered incomplete bactericidal activity.

The residual BEI was hydrolysed by the addition of 1 M sterile Na-thiosulfate solution at 10% of the volume of the BEI used before discarding.

2.3 Activation of Ipa proteins secretion by Congo Red.

Ipa proteins release was induced using a Congo Red secretion assay [33]. Exponential-phase bacteria were harvested, suspended in 10 µM Congo Red (Panreac)/PBS, and incubated at 37 ºC for 30 min. Then, cultures were treated with the inactivating agent. Following incubation, bacteria were pelleted by centrifugation at 14,000 × *g* for 15 min at 20 °C, and supernatants were collected, filtered using a 0.22 µm pore diameter filter and analysed by SDS-PAGE and immunoblotting [31] using monoclonal antibodies against IpaB and IpaC (kindly provided by A. *Phalipon*, Institut Pasteur).

2.4 Cell integrity assessment after inactivation.

Cells were grown to stationary phase in liquid Tryptic soy broth and inactivated with BEI-FA, phenol (0.5%, 4 h) or heat (60 ºC, 30 min). Then, cells were harvested by centrifugation (6,000 × g, 10 min). Proteins on supernatant were precipitated with tricholoroacetic acid and analysed by SDS-PAGE and silver staining for proteins, as

described elsewhere [31].

2.5 Preparation and characterization of OMV.

Cells were grown to stationary phase in tryptic soy broth, inactivated with BEI-FA, and after that, OMVs were recovered from supernatant as previously described [31]. Supernatant was purified by diafiltration via a 300-kDa tangential filtration concentration unit (Millipore). Then, the retentate was frozen. Final product was recovered by centrifugation at 40,000 × g, 2 h and finally liophylized. Total protein content was determined by the method of Lowry, with bovine serum albumin as standard. Lypopolysaccharide (LPS) content was determined by the Purpald assay [34;35]. Antigenic complexes were analyzed by SDS-PAGE and silver staining for LPS and immunoblotting, using polyclonal pool sera from rabbit hyperimmunized with *S. flexneri* [36] or monoclonal antibodies against IpaC (kindly provided by A. *Phalipon*, Institut Pasteur) or OmpA. OMVs release and antigenic complex were examined by transmission electron microscopy (Zeiss LIBRA® 120 EFTEM).

2.6. Active immunization and experimental infection in mice:

All mice were treated in accordance with institutional guidelines for treatment of animals (Protocol 081-11 approved December 16, 2011 by the Ethical Comittee for the Animal Experimentation, CEEA, of the University of Navarra). Nine-week-old BALB/c mice [37] were separated in randomized groups of 10 animals and immunized with one dose of free OMVs (20 µg) by the nasal route (3 µl per nostril). Experimental infection was performed on day 35 post immunization intranasally with a lethal dose of 1×10^7 UFCs of *Shigella flexneri* 2a grown to logarithmic phase and suspended in 20 µL of prewarmed PBS. The number of dead mice after challenge was recorded daily. Mice

weight was determined at different time points post-challenge.

2.7. Statistics

Statistical analyses were performed using GraphPad Prism 5 for Mac OS X. The Kaplan-Meyer curves were used for analysis of the protection experiment.

3. RESULTS

3.1 Effect of binary ethylenimine as bactericidal agent.

The inactivating effect of binary ethylenimine alone (BEI) or in combination with formaldehyde (BEI-FA) was studied in vitro against stationary phase cultures of *S. flexneri* (Table 1). Results indicate that BEI was effective against *Shigella* in small volume cultures (50 mL). For larger volumes (500 mL) in which BEI alone was not as efficient, the combination with a small amount of formaldehyde (0.06% v/v) led to the complete inactivation of the *Shigella* inocula.

3.2. Cell integrity assessment after inactivation:

The efficiency of the chemical agent was rated not only for its bactericidal activity, but also for the lack of effects on the structural integrity on the bacterial cell. For this purpose, a batch of *Shigella* culture was distributed in aliquots in different flasks and treated with BEI-FA reagent (BEI 6mM-FA 0.06%, 4h). Controls performed with other commonly used treatments (phenol 0.5% v/v, 4 h) or heat (60 ºC, 30 min) and an untreated culture, were also included. After inactivation, supernatants were analyzed by SDS-PAGE and silver staining, suggesting that BEI-FA did not affect the cellular integrity since no proteins were found in BEI-FA supernatant culture (Fig. 1). In

contrast, phenol and heat had a marked disrupting effect referring to the untreated control according to the wide range of proteins observed in the supernatants (Fig. 1).

3.3. Ipa proteins antigenic conservation after inactivation.

In order to evaluate the potential value of BEI-FA inactivation method, the effect on the Ipa proteins, important virulence factors of *Shigella*, was studied. After induction of the secretion of Ipa proteins with Congo red, bacteria were treated with BEI-FA. Ipa proteins were identified by SDS-PAGE and immunoblotting in the supernatant of the inactivated bacterial culture (Fig. 2A). As shown in Figure 2, BEI-FA did not affect the antigenicity of Ipa proteins. Two bands corresponding to IpaB and IpaC were identified using monoclonal antibodies against these proteins (Fig. 2B).

3.4. Isolation of OMVs after BEI-FA inactivation.

OMVs were harvested from supernatants of stationary phase *S. flexneri* culture inactivated by BEI-FA. OMV released from the outer surface of *S. flexneri* (Fig. 3A) appeared as bilayer spherical structures with an average diameter of 52.0 ± 17.6 nm (Figure 3B). SDS-PAGE and immunoblotting analysis indicated that major immunodominant proteins such as OmpA [38], porins (37-38 kDa) [39], IpaB and Ipa C (42 KDa) were present in the extract (Fig. 4). Immunoblotting using Mab against IpaC and OmpA confirmed their identity (data not shown).

In order to evaluate the immunogenicity and protection conferred by OMVs vaccine, mice were immunized with OMV (20 μg) by nasal route. On day 35 after immunization, mice were challenged with a lethal dose of *S. flexneri* via intranasal route and monitored for survival over 15 days. Immunization with OMVs isolated after BEI-FA inactivation

of *S. flexneri* culture conferred full protection (Fig. 5) and no clinical manifestations or changes in body weight were observed after challenge (data non shown). Furthermore, no symptoms were observed after immunization.

4. DISCUSSION

Current vaccines strategies against *Shigella* can be grouped into two fundamental approaches: live-attenuated vaccines and nonliving vaccines [6-8]. While the essential concern regarding live attenuated candidates is to find the appropriate balance between immunogenicity and acceptable side effects, different scenery appears for inactivated whole cell or subunit vaccines. The success of these kinds of vaccines depends, in part, on preparing the bacteria in a manner that, after inactivation, the immunogenicity and native structural epitopes essential for protection are preserved. Several methods have been used to inactivate *Shigella* cultures for eventual vaccine evaluation. Usually, methods include the use of formaldehyde, heat or acetone. However, most of these agents cause membrane damage and may lead to antigenic denaturising [40-42]. In line with this, we have previously described the use of binary ethylenimine for inactivation of *Shigella* cells [31]. In the former study, we used an acellular vaccine against shigellosis based on outer membrane vesicles (OMVs) obtained from supernatants of BEI-inactivated *Shigella* cultures. BEI is an aziridine compound reacting with nucleic acids, which has been shown elsewhere to efficiently inactivate several RNA and DNA viruses in vaccine production [22]. For example, BEI is widely used in the preparation of the Foot and Mouth virus vaccine, with about 500 million doses administered in cattle annually without any described drawbacks [22;32]. Given that BEI reacts by intercalation on nucleic acids, it was expected that this compound would not disrupt

membrane surface [16;43]. In the present study, we make an in-depth understanding of the potential of BEI to inactivate *Shigella* cells in order to offer an achievable implement into non-living vaccine production.

Our results demonstrate that the combination of BEI-FA (BEI, 6 mM containing formaldehyde, 0.06 %, 6 h) inactivated completely the *Shigella* inocula used (Table 1). The combination with formaldehyde resulted in a synergistic effect between the two agents previously described by Bahneman [22]. Moreover, it is described that the stability of vaccines increased significantly when, after inactivation with aziridine, the antigen was treated with formaldehyde [44]. At this point, it is important to highlight that the concentration of formaldehyde used was 0.06%, a much lower concentration than the generally used of 1-3% [19;20]. This low concentration of formaldehyde implies a negligible disturbing effect on membrane surface or antigenicity. In fact, as Figure 1 shows, the absence of new released proteins in culture supernatant after BEI-FA treatment suggests that the membrane structure is maintained. Moreover, antigenic properties were also preserved. To this respect, of particular interest are the Ipa (Invasion antigen plasmid) proteins. They are relevant effectors on the pathogenesis and are highly conserved among *Shigella* species. Four types, named Ipa (A-D), have being described as main immunogenic proteins of *Shigella* [45;46]. After the induction of the secretion of Ipa proteins by Congo red, cultures were treated with BEI-FA. Results showed that BEI-FA did not have denaturing effects on Ipa proteins antigenicity (Fig. 2). This is an important aspect since Ipa proteins are very labile to antigenic conformation changes [47]. In view of these results, BEI-FA method fulfilled the two basic seek requirements: firstly, the complete, fast and reliable inactivation of *Shigella*; secondly, the lack of disruption of the membrane surface or any denaturing effect. To this respect, it is worthy to mention that to our knowledge, although some studies have

been carried out using BEI [22;48], this is the first report describing its use against *Shigella*.

Finally, in order to study the protective immunogenic of an antigenic complex obtained from *Shigella* BEI-inactivated cells, we focused on OMVs. *Shigella* releases OMVs as spherical lipid bilayer conformed by LPS, OMP and Ipa proteins [49]. These are considered as main protective antigens against shigellosis [6;8]. Accordingly, current subcellular strategies include these antigens in their composition. For instance, LPS, IpaB and IpaC are contained in the "Invaplex 50", a subcellular vaccine candidate against shigellosis that is already under phase II of clinical trials [8]. Other approaches include IpaB and IpaD obtained by molecular recombinant techniques [50]. Results demonstrated that BEI treatment did not affect immunogenic of OMV antigens. In fact, one single nasal dose of OMV was able to fully protect mice against a lethal dose of *S. flexneri* 2a, confirming previous results [31]. It is important to highlight that our proposal aims the utilization of these antigens maintaining their membrane environment and native conformation and hence, the original epitopes repertoire. Furthermore, it has been described that the "OMV delivery system" may act as a protective environment for delivery of soluble antigens [51] and besides, that the presentation of antigens under this OMV-context is expected to be more efficient [52]. To sum up, BEI inactivation comes into sight as a useful method for inactivation of bacteria with no alteration of antigenic properties as well as leading to optimal immunogenic condition. From this valuable role regarding vaccination, inactivation with BEI-FA emerges as a good safety implement for non-living *Shigella* vaccines.

5. Conclusions

A combination of binary ethylenimine and formaldehyde (BEI-FA) demonstrated to be effective for the inactivation of *Shigella* cells. BEI-FA did also maintain the antigenic properties of Ipa proteins and OMV complex. Results showed that OMVs obtained after BEI-FA treatment kept antigenicity and immunogenicity, confirming their potential for nasal vaccination against *S. flexneri.*

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Table 1. Bactericidal effect of binary ethylenimine (BEI) against *Shigella flexneri.* Aliquots of stationary phase cultures of *S. flexneri* were treated with BEI alone or in combination with formaldehyde (BEI-FA)

Inactivating agent		Chemical treatment time (h)	Culture Volume (mL)	bactericidal activity[1] (minimal treatment time)
BEI	4 mM	2-24	50	Complete (6 h)
		2-24	500	Incomplete
BEI	6 mM	2-24	50	Complete (6 h)
		2-24	500	Incomplete
BEI -FA	4 mM (0.04% FA)	2-24	50	Complete (4 h)
		2-24	500	Complete (16 h)
BEI -FA	6 mM (0.06% FA)	2-24	50	Complete (4 h)
		2-24	500	Complete (6 h)

[1] Complete: full bactericidal activity (no growth after 7 days incubation on TSA plates); Incomplete: null or partial bactericidal activity at the indicated incubation time.

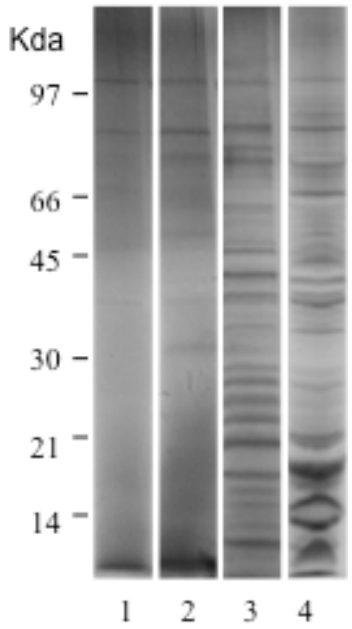

Figure 1. Comparative analysis of *Shigella flexneri* supernatants after inactivation. SDS-PAGE with silver staining for proteins of *Shigella flexneri* culture supernantant after (1) no treatment or (2) binary ethylenimine-formaldehyde (BEI-FA): [BEI (6mM)-FA (0.06%)], (3) Phenol 0.5% or (4) heat (60 ºC, 15 min) treatments. Standard molecular weight markers are indicated.

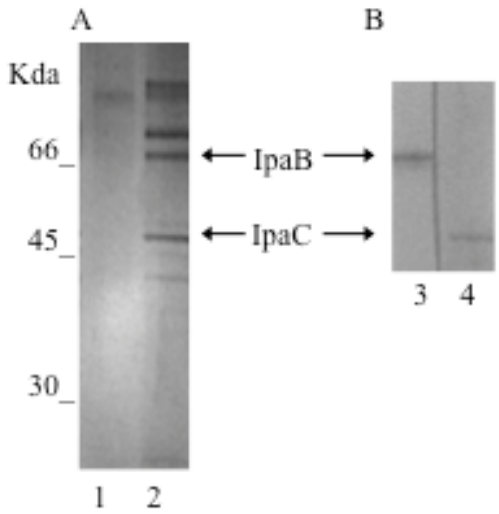

Figure 2. Antigen conservation of Ipa proteins after inactivation. Bacteria were suspended in PBS at a concentration of 1.0×10^8 CFU/ml and incubated at 37°C in the absence (lane 1) or presence (lane 2) of 10 μM Congo red. After 30 min of incubation, the inactivating agent [BEI (6mM)- FA (0.06%)] was added. Samples were centrifuged and supernatant extracts were subjected to SDS-PAGE with silver staining (A) and immunoblotting (B) with monoclonal antibodies against IpaB (lane 3) or IpaC (lane 4). Molecular weight markers and the identity of Ipa proteins are indicated.

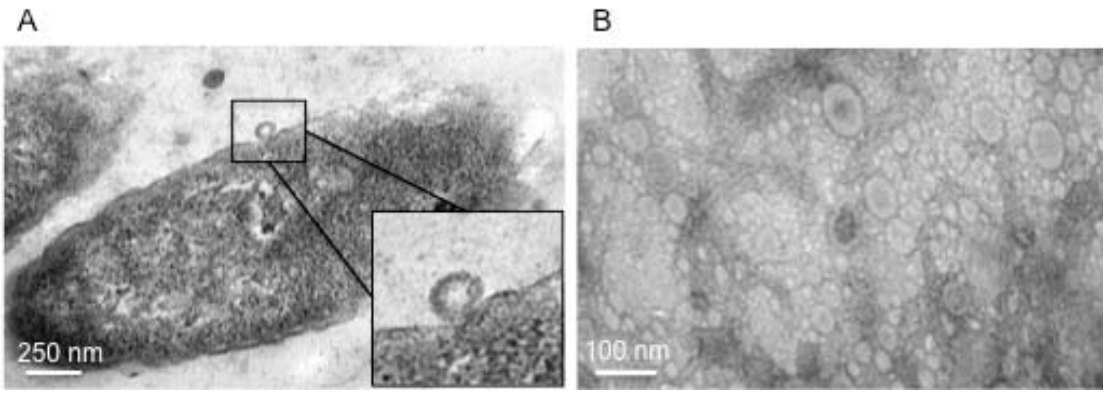

Figure 3: Transmission electron microscopy showing outer membrane vesicles (OMVs) released from *Shigella flexneri* 2a (A). Panel B shows negative staining of OMVs collected from BEI-FA inactivated *Shigella* culture followed by diafiltration as purification process.

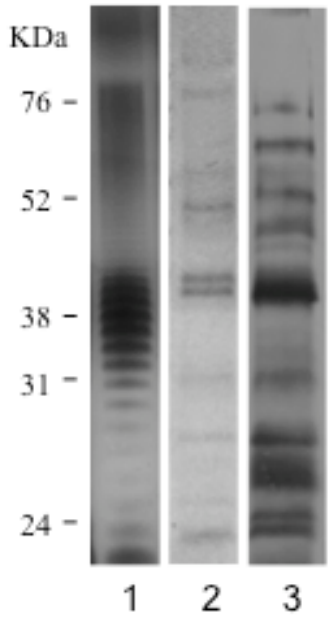

Figure 4. Analysis of *Shigella flexneri* outer membrane vesicles (OMV) collected from BEI treated cells. SDS-PAGE with silver staining of LPS form outer membrane vesicles (OMVs) (lane 1). SDS-PAGE with Coomassie Blue staining of OMVs (lane 2). SDS-PAGE and immunoblotting of OMVs using a pool of sera from rabbit hyperimmunized with whole cells from S. *flexneri* (lane 3) Molecular weight markers and identity of some bands are indicated on the left.

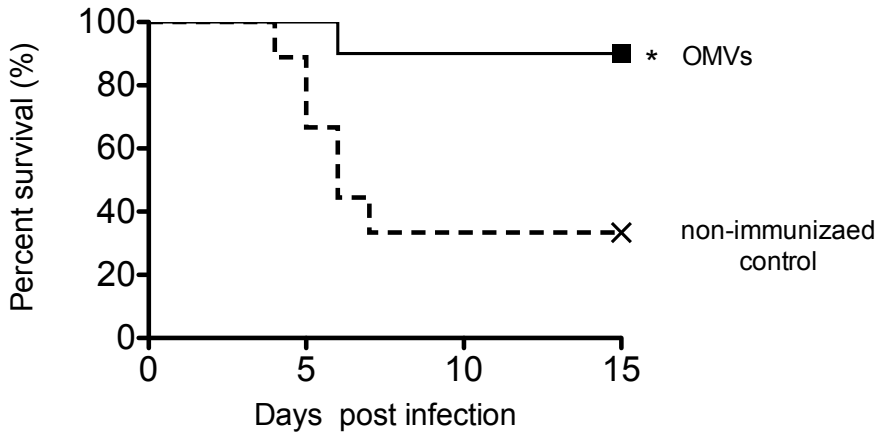

Figure 5. Protection study against *Shigella flexneri* experimental infection. BALB/c mice (20 ±1 g) were immunized oncewith 20 μg of outer membrane vesicles (OMVs) by nasal route. An extra group was included as non-immunized control. At day 35 after immunization, all groups received an intranasal lethal challenge of 1.0×10^7 UFC/mouse of *S. flexneri 2a* (clinical isolate). Graphs indicate the percentage of mice that survived the infective challenge at the indicated days after immunization (*, $P<0.01$, Logrank test)