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(54) Title: A MULTI-SEROTYPE OUTER MEMBRANE VESICLES (MOMV) OF SHIGELLAE AS A NOVEL CANDIDATE VACCINE

(57) Abstract: This invention relates to a vaccine formulation against Shigellosis comprising the outer membrane vesicles (OMVs) of antigen from six serotypes of Shigella which includes- Shigella dysenteriae type 1 from sero-group A Shigella liexneri 2a from sero group B; Shigella fiexneri 3a and Shigella flexneri 6 from sero group B; Shigella boydii type **4** from sero group C and Shigella sonnei (phase -1) from sero group D.

FIELD OF THE INVENTION:

This invention relates to a novel vaccine formulation against shigellosis from the outer membrane vesicles (OMVS) antigen of Shigella.

This invention further relates to a novel vaccine formulation comprising a combination of OMVS antigen from different serotype of Shigellae and to the duration and efficacy of protection and unmunogenicity of OMV inmunogen against homologous as well as heterologous Shigella strains.

BACKGROUND OF THE INVENTION:

Shigella, the causative organism of shigellosis, is an antigenically diverse pathogen containing four species (or groups), 50 serotypes and subserotypes; that makes the development of a vaccine challenging. Oral vaccine is pursuing green promise to reduce the burden of disease and mortality caused by enteric pathogen like Shigella. It is generally acknowledged that the protection stimulated by a Shigella vaccine must be broad enough in spectrum to protect against 16 serotypes, including S. dysenteriae 1, all 14 S. flexneri types and S. sonnei. A pentavalent strategy developed at the CVD claimed that 5 Shigella strains (S.sonnei, S.dysenteriae i, and S. flexneri 2a, 3a, and 6) can collectively provide the necessary broad spectrum protection needed to achieve a vaccine of global utility. Epidemiologically across the world, these are the most important serotypes from the purview of prevalence and disease severity. This strategy is based on the assumption (from analysis of Shigella O antigens and animal cross protection studies) that inclusion of S. flexneri 2a, 3a, and 6 in the vaccine will provide cross protection against the other 11 S. flexneri serotypes because of shared group antigens.

OBJECTS OF THE INVENTION:

It is therefore an object of this invention to propose a vaccine formulation for Shigellosis, which gives broad spectrum protection against different types of Shigellae.

It is a further object of this invention to propose a vaccine formulation for Shigellosis, which offers a longer duration of protection from Shigellae, compared to conventional vaccines.

Another object of this invention is to propose a vaccine formulation for Shigellosis, which is a non-living vaccine formulation.

Yet another object of this invention is to propose a vaccine formulation for Shigellosis, which is easy to prepare and is cost effective.

These and other objects of this invention will be apparent from the ensuing description, when read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS:

Fig. 1 shows all strains were kept in 15% glycerol with Brain Heart Infusion Broth (BHIB, Difco, USA) at-70°C.

Fig. 2 shows electron micrograph of outer membrane vesicles attached to the bacteria (A) and purified (B) (Shigella boydii type 4 BCH612)

Fig. 3 Scheme for oral immunization with MOMV and subsequent challenge Studies

Fig. 4 shows the comparative analysis of outer membrane vesicles present in hexavalent vaccine preparation

Fig. 5 shows the comparative analysis of outer membrane vesicles present in hexavalent vaccine preparation

Fig. 6 shows serum immunoglobulin titers in immunized sera.

Fig. 7 shows the comparative data of protective efficacies between control and immunized neonates

Fig. 8 shows lgtiters in the stomach contents of sucklings from immunized dams and nonimmunized dams.

DETAILED DESCRIPTION OF THE INVENTION:

According to this invention, is provided a novel vaccine formulation against Shigellosis from the OMVs of antigen of Shigella.

In accordance of this invention, six serotypes were selected from the four sero-groups of Shigella sp. according to their different epidemic and endemic outbreak nature. S.

dysenteriae type 1 from sero-group A and S.flexneri 2a from sero-group B, were selected because S. dysenteriatype type 1 is the epidemic strain whereas, S. flexneri 2a are mainly dominant in endemic areas. Two more serotypes from sero group B i.e. S. flexneri 3a and S. flexneri 6 were included. These three S. flexneri serotypes i.e. S. flexneri 2a, S. flexneri 3a and S. flexneri 6 have O-antigen group determinants that are shared by the remaining 11 S. flexneri serotypes.

Shigella boydii type 4 from sero group C which is predominant in endemic areas and Shigella sonnei (phase 1) from sero group D, which is predominant in developed countries, were also included.

Among, 72 clinical isolates strains were collected and one strain selected from each group after screening according to their genotypic and phenotypic virulence properties **(Fig. 1).** All strains were kept in 15% glycerol with Brain Heart Infusion Broth (BHIB, Difco, USA) at-70°C.

Characterization of Shigella :

Shigella entry into susceptible host cells required the co-ordinated expression of numerous genes that are activated in response to environmental cues. The entire complement of genes critical for invasion of epithelial cells is contained on a large 220-kb plasmid, termed the virulence plasmid or the invasion plasmid, which is present in all pathogenic strains.

These virulent plasmids were isolated from six strains following the plasmid isolation method of Kado and Liu. The genes responsible for virulency were screened by standard polymerase chain reaction Invasive strains of S. dysenteriae 1 (NT 4907), S. flexneri 2a (B 294), S. flexneri 3a (C 519), S. flexneri 6 (C 347), S. sonnei phase I (IDH 00968) and S. boydii 4 (BCH 612) were selected among 72 clinical isolates of shigellae (12 isolates from each serotype) according to the presence of 212 kb plasmid as well as the presence of both ipaH and virF gene. Only six selected serotypes harboured 212 kb plasmid along with ipaH and virF gene among 72 clinical isolates (Table 1).

Table : 1 Strains used in our study after screening among 72 clinical isolates.

	Genotypic Character									Phenotypic Character		
	212kb virulent plasmid	virF	IpaH	IpaBCD	stx1	ial	Set1A	Set1B	sen	Congo Red Assay	Invasion assay Caca-2 cell	Sereni Test in Guinea pig
i	+	+	+	+	+	+	-	-	+	+	+	+
	+	+	+	+	-	+	+	+	+	+	+	+
	+	+	+	+	-	+	-	-	+	+	+	+
6	+	+	+	+	-	+	-	-	+	+	+	+
	+	+	+	+	-	+	-	-	+	+	+	+
3	+	+	+	+	-	+	-	-	+	+	+	+

The results of DNA amplification by the multiplex PCR method based on the primers used in this study showed the presence of a 618 bp fragment for the ipaH gene and a 933-bp fragment for the virF gene in the DNA preparations obtained from only six isolates. Selected Shigella isolates were positive for invasive genes and confirmed phenotypically Keratoconjivities in Guinea pig model.

Isolation of outer Membrane Vesicles :

OMVs were isolated from the six strains following the method of Balsalobre et al. (2006). Briefly, 1 L of Luria-Bertani broth (LB, Difco) was inoculated with lOmL of the stationary phase culture and grown for 8 h to the late exponential phase. Bacteria were removed by centrifugation (4500 g, 15 min, 4 1C), and the supernatant was filtered by passing it consecutively through 0.45-mm and 0.22mm pore size filters, respectively. **Fig. 2** shows the Electron micrograph of outer membrane vesicles attached to the bacteria (A) and purified (B) (Shigella boydii type 4 BCH612). Supernatant from overnight grown culture was negatively stained and observed under transmission electron microscope (Bio Twin Transmission electron Microscope), FEI, Netherland) operating at 80 KV) (x 20 magnification).

To confirm the absence of viable bacteria, lmL of the filtrate was plated on an LB agar plate, which was incubated overnight at 37°C. Protease inhibitors [Complete EDTA-free protease inhibitors cocktail (Roche), 1 tablet per 1L of filtrate] were added to the filtrate to prevent protein degradation. OMVs were subsequently purified from the filtrate by ultracentrifugation (4 h, 140 000 g, 41C) using a Beckman SW32Ti rotor, washing once with phosphate buffered saline (PBS; pH 7.4) and finally resuspended in 625 mL of PBS. Protein concentration was determined by the standard Bradford assay. OMVs from six strains were mixed and adjusted to a final concentration of 50microgram/200 microlitre using PBS. Purified OMVs were stored at -80°C until use.

Oral Immunization :

Swissmale and female mice, six to seven weeks old, were caged separately in a group of five and maintained at 25° C with 75 humidity and fed sterile food and water under the care of full time staff and in accordance with the rules of the institutional animal ethical committee (IAEC) (Apro/77/24/1 1/2010, Reg. No. NICED/CPCSEA (AW) 215/2009 - 2015).

Female mice were immunized orally at days 0,7,14, and 2 1 with 50µg per 100 µl of purified OMVs using the concentration. Fifteen minutes before the oral immunization; each mouse was anaesthetized by intramuscular injection of a mixture of ketamine (35 mg kg-1 body weight; Sterfil Laboratories Pvt. Ltd, India) and xylazine (5 mg kg-1 body weight, AstraZeneca Pharma India Ltd, India). Two boluses of sodium bicarbonate (500µ1of a 5% solution; SRL, India) at 5 min intervals were introduced directly into the stomach through a mouse feeding needle (Havard Instrument, USA). The second bolus was immediately followed by oral administration of MOMV for the experimental mice and the same volume of PBS, for the non-immunized group. All immunized and non-immunized group of mice were returned to their cages and given limited amounts of sterile food and water **(Fig. 3).**

MOMV Non-Reactogenic :

MOMV induced very low IL-8 secretion (Figure. 4) than live and heat killed Shigella

flexneri 2a (2457T) which supports the worthy of MOMV immumogen than the live or the heat-killed Shigella immunogens. **Fig.** 4 shows the comparative analysis of outer membrane vesicles present in hexavalent vaccine preparation. SDS-PAGE with silver staining of outer membrane vesicle extracted from six strains. Lane 1: S. dysenteriae 1 Astx (NT4907); Lane 2:S. flexneri 2a (B294); Lane 3: S. flexneri 3a (C519); Lane 4: S. flexneri 6 (C347); Lane 5:S. boydii 4(BCH612); Lane 7: S. sonnei (IDH00968); Lane M:protein molecular weight marker (Biorad).

Immunogenicity Of MOMV In Adult Mice :

Each component of MOMV preparation were found to be adequately immunogenic in adult mice and thus proved to be an important part of MOMV. All the component OMVs showed substantial antibody response during the course of immunization as well as during the post immunization period till 120 days. IgM response was noticed just after the first dose on day 7 but satisfactory levels of IgA, IgGl, IgG2a and IgG3 responses were noted from day 14 onwards, i.e. after the second booster dose. The overall anti-body titers increased during the oral immunization period, with a peak at day 28 and decreased gradually with time. But anti-OMV IgA, IgGl, IgG2a, IgG3 responses were found above the level of detection till 120 days when compared with control mice. Above all, IgG2a and IgG3 response were higher than IgA and IgGl which is indicative of higher Thl cell mediated immune response and S.flexneri 2a (B294) and S.flexneri 6 (C347) OMVs were more immunogenic.

Opsonization Assay :

The ability of immunized sera to opsonize bacteria and to enhance phagocytosis as evaluated in an in vitro model with isolated macrophages from mice. Virulent Shigella strains were mixed with both immunized and non-immunized sera and then incubated with mouse peritoneal macrophages.

Immunized sera enhanced bacterial internalization by 10 fold, after one hour incubation (Table 2). .Specificity of the antibody response.

Table 2: Opsonization activity of the immunized and nonimmunized sera

Bacteria	Mean CFU×10^4after 1 hrincubation[a]	
	Incubation condition	
	Bacteria incubated with nonimmune sera	Bacteria incubated with immune sera
*S. dysenteriae*1 (NT4907)	0.6±0.5	18 ± 3.6
*S. flexneri*2a (B294)	0.5±0.7	16 ± 1.8
*S. flexneri*3a (C519)	0.25±0.12	20 ± 4.1
*S. flexneri*6 (C347)	0.55±0.5	13 ± 2.7
*S. boydii*type 4 (BCH612)	0.27±0.17	25 ± 4.9
S. sonnei(IDH00968)	0.45±0.62	21±3.1

[a]Values are means ± SD of triplicate samples

The specificity of the anti-MOMV response was verified against whole cell lysates of seven strains (Table 1; Serial no. 7-13) using sera, collected on day 28, from immunized mice. No bands were detected on the immunoblots where non-immune sera were used. Multiple bands were generated by anti-MOMV sera as seen in the representative figure **(Fig. 5),** demonstrating that MOMV contained numerous proteins that had served as antigens. Fig 5 shows a comparative analysis of outer membrane vesicles present in hexavalent vaccine preparation. SDS-PAGE with silver staining of outer membrane vesicle extracted from six strains. Lane 1: S. dysenteriae 1 Astx (NT4907); Lane 2:S. flexneri 2a (B294); Lane 3: S. flexneri 3a (C519); Lane 4: S. flexneri 6 (C347); Lane 5:S. boydii 4(BCH612); Lane 7: S. sonnei (IDH00968); Lane M:protein molecular weight marker (Biorad). Representative immunoblot against whole cell lysates of seven Shigella strains probed with 28 day's anti-OMVS serum from a single mouse. Lane M:prestain molecular weight marker (Pierce) Lane 1: S. dysenteriae 1(NT4907); Lane 2:S. flexneri 2a (B294); Lane 3: S. flexneri 3a(C519): Lane 4:S. flexneri 6 (C347); Lane 5:S. boydii type4 (BCH 612); Lane 6:S. sonnei (IDH00968); lane 7: non invasive strain S. flexneri 1a (NK4238).

The most-reactive bands were in between the region of 80 and 32 kDa, correlated with the area of the most abundant proteins found in OMVs; VirG, (120 kDa); IpaB

(62 kDa); IpaC (42 kDa) IpaD (38 kDa); OmpA (34 kDa). None of these bands were visible in non-invasive control strains S.flexneri 1a (NK4238), lacking the virulent plasmid and thus plasmid encoded genes VirG, IpaB, IpaC and IpaD. However a very distinct band at ~34KDa position was noticed for this strain which corresponded to chromosomally encoded OmpA protein. The intensities of the detected bands confirmed that vaccinated mice have induced comparable levels of serum IgG response against ipa-encoded membrane proteins. Moreover, the comparable banding patterns indicated that, at least for the IgGisotype, there were no qualitative differences in antigen target.

Protective efficacy of MOMV:

The infectious dose and the challenge dose of each strain were listed in table 3. The ID50 was considered to be the dose which ensured -10^6 - 10^7 bacteria per gram of intestine of the challenged neonates, after six hour incubation in 3 to 4 days old suckling mice.

Death or visible side effects due to toxicity (such as ruffled fur or lethargy or diarrhea or weight loss) did not occur in mice after four successive oral immunizations with 5(^g of MOMV. A significant level of protection after both first and second challenge studies were achieved in newborn mice of immunized dams. Most of the suckling mice from non-immunized mother became sick and eventually died between 10 and 16 hr of incubation (Table 3). More or less same results were obtained after both challenge studies. Control mice from non-immunized groups showed higher intestinal colonization (-107 CFU/gm of intestine) leading to shigellosis **(Fig.** 7). Dead mice from immunized groups showed colonization -105 CFU/gm of intestine which as 100 fold lower than the rate of intestinal colonization in control mice (-107 CFU/gm of intestine) and alive neonates showed even lesser intestinal colonization (-102 CFU/gm of intestine). MOMV conferred 100% protection against S.flexneri 2a and S.flexneri 6 after both challenge studies. The OMVs of these two strains were more immunogenic than others **(Fig.** 6). Fig. 6 shows serum immunoglobulin titers in immunized sera separately measured against outer membrane vesicles secreted by

each strain at preimmunization, immunization and post immunization periods on the days indicated along the horizontal axis. Data are mean values ±SD. (A) S. dysenteriae 1 (NT4907 Astx); (B) S. flexneri 2a (B294); (C) S. flexeri 3a (C347); (D) S. flexneri 6 (C519); (E) S. boydii 4 (BCH612); (F) S. sonnei (IDH00968).

Protective efficacies against S.dysenteriae 1, S.flexneri 3a, S.boydii 4 and S.sonnei (Table 3) were above- 83%. The above results suggest MOMV immunization could confer 83-100% passive protection against shigellosis in neonatal mice model. Immunoglobulins found in the stomach content of neonates.

IgA, IgGl, IgG2a and IgG3 and little IgM response were noticed in the stomach contents of the neonatal mice from immunized dams, after the first mating period, against each of the six component OMVs present in the MOMV preparation. Control group did not show such anti-MOMV response. Same result was observed after the second mating period. To avoid duplication of data, only the observation after the second mating was represented in Figure 8.

Fig. 8 shows the lgliters in the stomach contents of sucklings from immunized dams and also from the nonimmunized dams. Stomach contents of ten suckling mice were examined individually for every immunoglobulins. Each circle represents the data obtained from a single mouse.

IgG3 was the most abundant and IgM was least abundant isotype in neonatal stomach. IgA response was next to IgG3. This observation again enlightened the predominance of Thl cell mediated immune response by MOMV in adult mice and the neonatal protection against shigellosis was mainly achieved by the anti-MOMV IgG3, IgG2a and IgA, present in mother's milk.

The protective nature of the Ipa proteins along with the natural adjuvant, lipopolysaccharide, present in the outer membrane vesicles have made the newly developed multiserotype hexavalent outer membrane vesicles formulation an ultimate

broad spectrum non-living vaccine candidate that conferred passive protection to neonatal mice against shigellosis. Thus, the antigens present in MOMV will also elicit considerable protective passive immune response against shigellosis in human.

Table 3. First and second challenge study in suckling mice from immunized mother

Challenged strain	**Experimental group**	**No. of neonates in each group**	**% of survival after first challenge**	**Protective efficacy [a](%) after first challenge**	**% of survival after second challenge**	**Protective efficacy [a] (%) after second challenge**
*S.dysenteriae*1 (A1)	Control	10	10 (1/10)	88.88	20 (2/10)	87.5
	Immunized	20	90 (18/20)		90 (18/20)	
S. flexneri 2a (NK3809)	Control	10	20 (2/10)	100	20 (2/10)	100
	Immunized	20	100 (20/20)		100 (20/20)	
S. flexneri 3a (NK3758)	Control	10	10 (1/10)	88.88	10 (1/10)	83.33
	Immunized	20	90 (18/20)		85 (17/20)	
S. flexneri 6 (NK4025)	Control	10	30 (3/10)	100	30 (3/10)	100
	Immunized	20	100 (20/20)		100 (20/20)	
S. boydii 2 (NK4023)	Control	10	20 (2/10)	93.75	20 (2/10)	87.5
	Immunized	20	95 (19/20)		90 (18/20)	
S. sonnei (NK3918)	Control	10	20 (2/10)	93.75	20 (2/10)	93.75
	Immunized	20	95 (19/20)		95 (18/20)	

"Protective efficacy was calculated as {[(percent deaths of nonimmunized mice)-(percent deaths of immunized mice)] ÷[percent deaths of nonimmunized mice]} × 100 [56].

Six novel serotypes from four sero-groups were selected for development of multiuvalent vaccine. The selected serotypes are S. dysenteriae 1, S. flexneri 2a, S.

flexneri 3a, S. flexineri 6, S. sonnei and S. boydii 4. All selected serotypes were virulent and the sereny positive derivative of each serotype was developed for challenged experiment. Female mice model was used to measure the protective efficacy and to study the immune responses that are elicited following disease or single serotype immunization. Two groups of mice (Immunized and Control, weighing between 25 g) were selected for oral immunization OMVs single serotype immunogen. Each group contained 10 mice. The immunization experiment was done according to the method of Sack et al (1988) and the challenge experiment was done according to the method of Fernandex et al **(Fig.** 7).

Figure 7 show the comparative data of protective efficacies between control and immunized neonates. Immubized sucklings showed long-term protection and less intestinal colonization than control sucklings, against wild type invasive Shigella strains. Intestinal colonization of each suckling was expressed as log 10 of recovered Colony Forming Unit (CFU)/gm of intestine, as presented on vertical axis. Pups were challenged according to the challenge dose mentioned in table 2, 100 fold higher ID50. Each circle represents the colonization data obtained from a single mouse. The numbers, given in each graph on 'Control' and 'Immunized' data, are as follows: number of mice alive/total number of challenged mice. 100% homologous protection was observed against all six selected virulent wild serotypes. High reciprocal increase of serum lgG antibody titer was observed during the period of immunization against heat killed single serotype immunogen. Immunoblot data of whole cell lysate (WCL) and outer membrane protein (OMP) also supported strong homologuous protection against single serotype immunization.

Now, the inventors have improved from single serotype to a cocktailed multiserotype outer membrane vesicles (MOMV) vaccine candidate and studied its protective efficacy against different serotypes of Shigella. Adult mice were immunized orally with 50μg of MOMV, four times at weekly intervals. Immunological parameters were observed at various time points before, during and after immunization in immunized adult mice and passive immunity was examined in their offspring.

Immunogenicity studies in adult mice exhibited induction of a consistent broad spectrum antibody response. Significant long term protection in 3-4 day old sucklings from the immunized dams was noticed against diverse Shigellae challenge.

Stomach contents of the neonates also revealed significant amounts of anti-MOMV immunoglobulins, might have been transferred to them postnatally by suckling milk. MOMV formulation constitutes an easy, safe immunization strategy, passively protective against all four serogroups of Shigellae and can thus be exploited for the development of a novel non-living vaccine against human shigellosis.

The individual serotype of the hexavalent formulation is not efficient to provide cross protection against different Shigella strains. Besides, single serotype is only able to provide homologous protection. Hexavalent OMVs vaccine formulation provides protection against various strains (encompassing the four serogroups of Shigella).

OMVs from single strain was not sufficient to provide necessary cross protection amongst various Shigellae. The immunogenicity of the OMVs were varying for different Shigella strains due to their difference in O-antigenic structure, which has been taken care of by taking OMVs from six different strains.

So, the hexavalent formulation has greater efficacy than any single serotype Shigella vaccine.

WE CLAIM:

1. A vaccine formulation against Shigellosis comprising the outer membrane vesicles (OMVs) of antigen from six serotypes of Shigella which includes-

Shigella dysenteriae type 1 from sero-group A

Shigella flexneri 2a from sero group B;

Shigella flexneri 3a and Shigella flexneri 6 from sero group B;

Shigella boydii type 4 from sero group C and

Shigella sonnei (phase -1) from sero group D.

2. The vaccine formulation against Shigellosis as claimed in claim 1, wherein the six serotypes of Shigella harboured 212kb plasmid alongwith a 618 bp fragment for the ipaH gene and 933-bp fragment for the vir F gene in the DNA preparations obtained from only six isolates of Shigella.

3. A process for the preparation of the vaccine formulation against Shigellosis as claimed in claim 1 comprising the steps of

(i) isolation of OMVs from the six strains

(ii) purification of OMVs from the filtrate obtained by ultracentrifugation (4h, 1400g, 41°C) and washing with Phosphate Buffered Saline (PBS) once and finally resuspension in PBS;

(iii) mixing of OMVs from six strains and adjusting to a final concentration of 50 mg/200 ml using PBS; and

(iv) storage of purified OMVs at -80°C until use.

4. The process as claimed in claim 3, wherein the phosphate buffered saline (PBS) used is of pH 7.4.

5. The process as claimed in claim 3, wherein the OMVs are resuspended in 625 ml of PBS.

6. Use of formulation comprising the outer membrane vesicles (OMVs) of antigen from six serotypes of Shigella, for the preparation of a vaccine for the treatment of Shigellosis which includes-

Shigella dysenteriae type 1 from sero-group A

Shigella flexneri 2a from sero group B;

Shigella flexneri 3a and Shigella flexneri 6 from sero group B;

Shigella boydii type 4 from sero group C and

Shigella sonnei (phase -1) from sero group D.

7. A method of treatment of Shigellosis comprising administering to the subject of formulation comprising the outer membrane vesicles (OMVs) of antigen from six serotypes of Shigella which includes-

Shigella dysenteriae type 1 from sero-group A

Shigella flexneri 2a from sero group B;

Shigella flexneri 3a and Shigella flexneri 6 from sero group B;

Shigella boydiitype 4 from sero group C and

Shigella sonnei (phase -1) from sero group D.

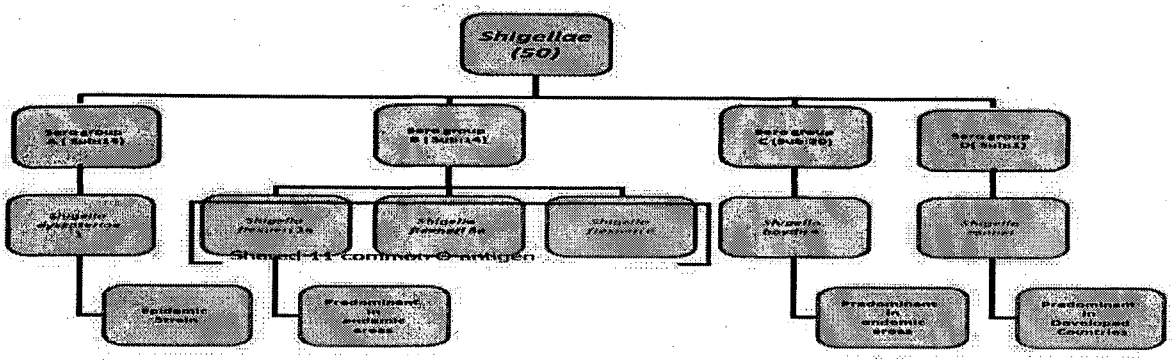

Figure : 1

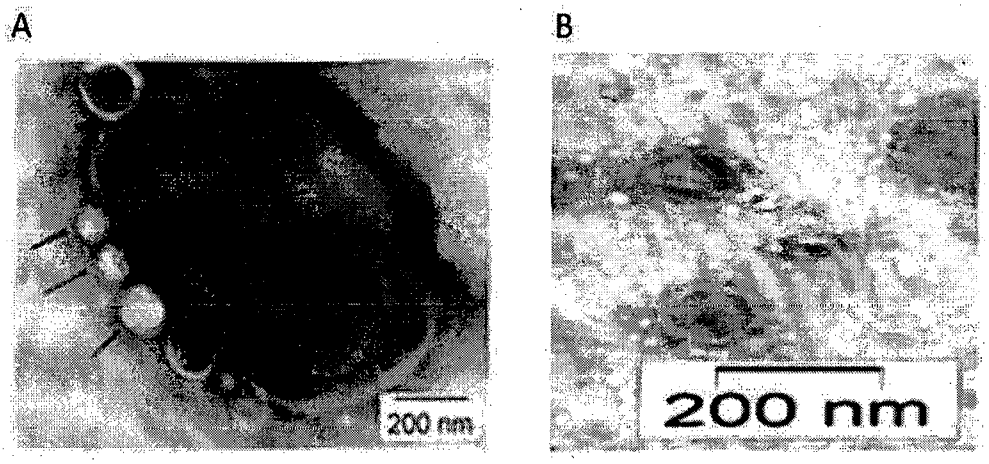

Figure : 2

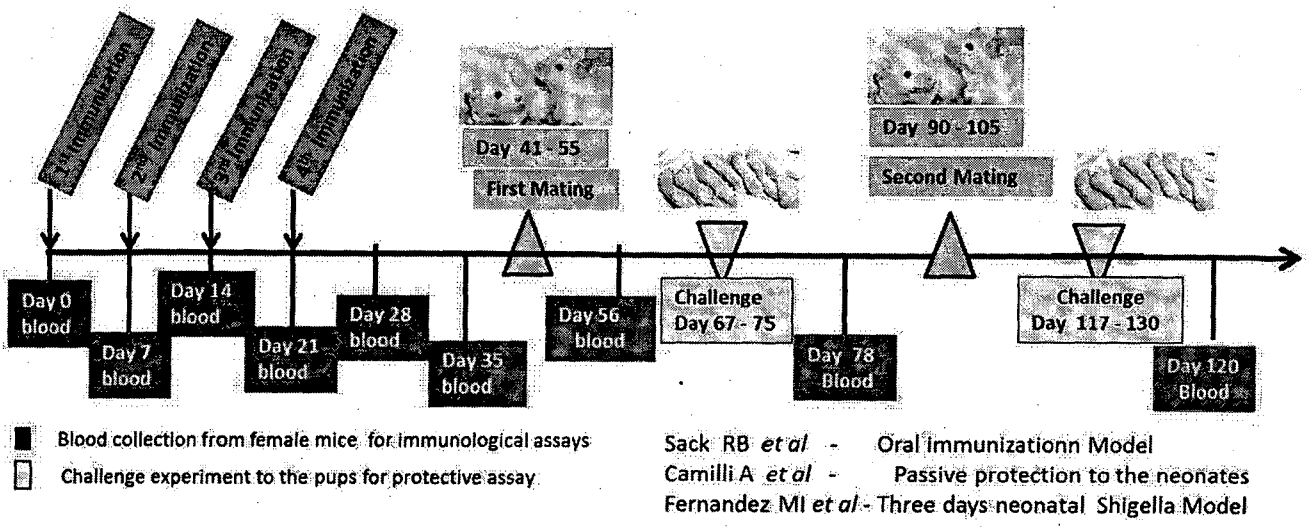

Figure : 3

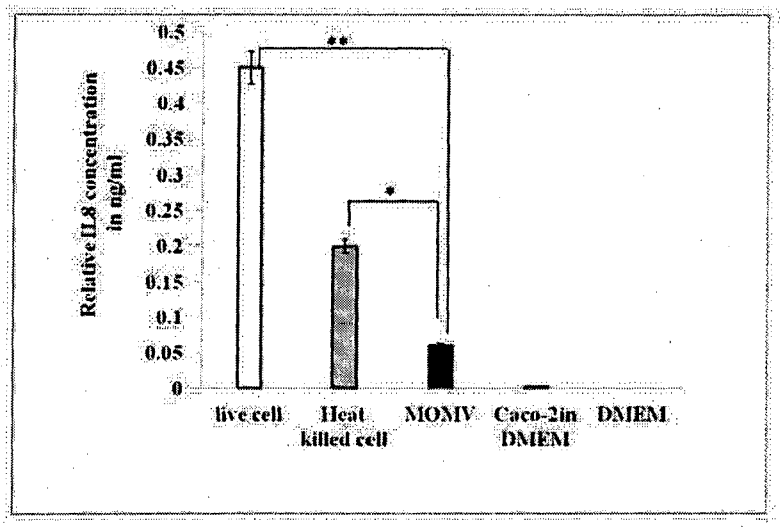

Figure : 4

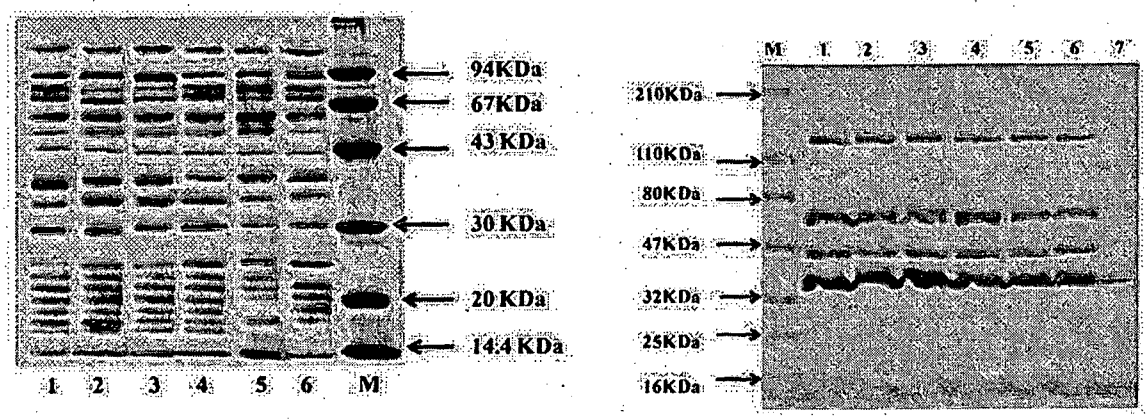

Figure : 5

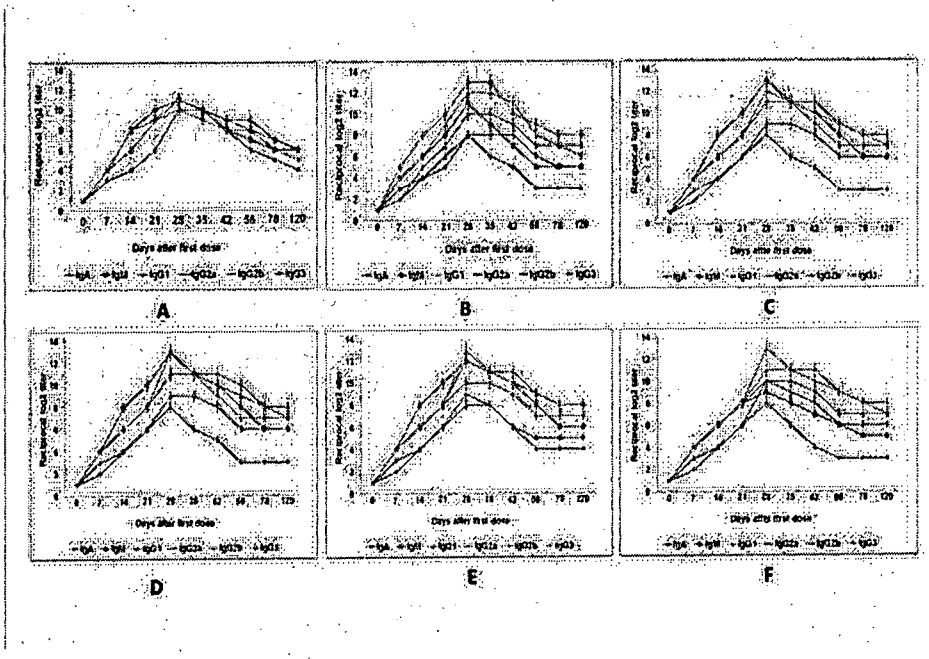

Figure : 6

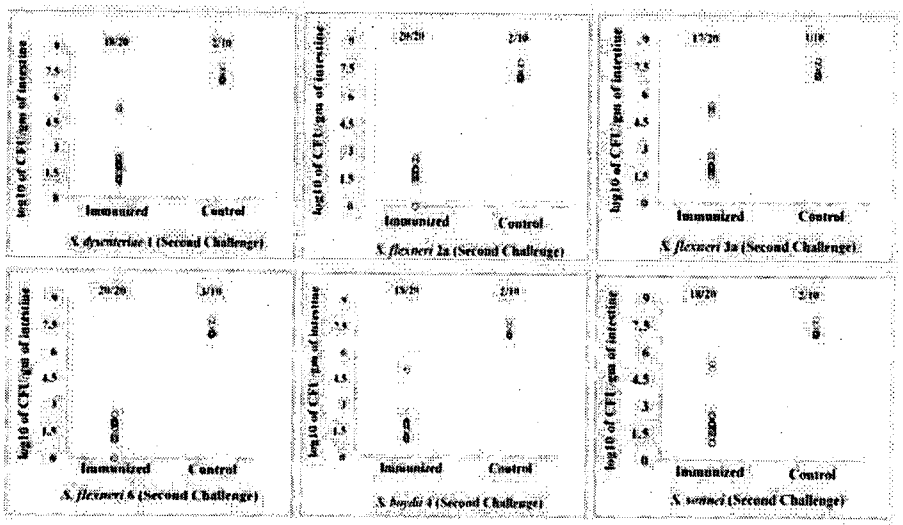

Figure : 7

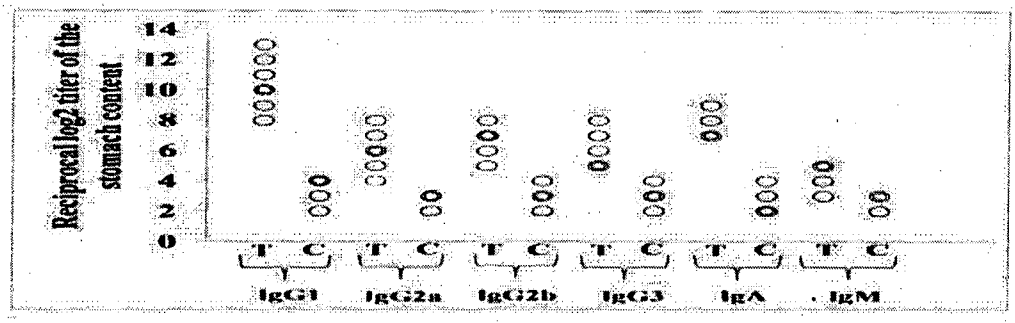

Figure : 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2014/000369

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 A61K39/112 A61P31/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MITRA SOMA ET AL: "Multi-serotype outer membrane vesicles of Shigella aeconfer passive protection to the neonatal mice against shigellosis", VACCINE, ELSEVIER LTD, GB, vol. 31, no. 31, 15 May 2013 (2013-05-15), pages 3163-3173, XP028568394, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2013.05.001	1,2,6,7
Y	abstract page 3164, right-hand column, lines 3-10; figures 1-6; tables 1-4	3-5

-/- .

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 October 2014	27/10/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cilensek, Zoran

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2014/000369

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SOMA MITRA ET AL: "Outer membrane vesicles of Shigella boydii type 4 induce passive immunity in neonatal mice", FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY, vol. 66, no. 2, 1 November 2012 (2012-11-01), pages 240-250, XP055145614, ISSN: 0928-8244, DOI: 10.1111/J.1574-695X.2012.01004.x	3-5
A	page 241, right-hand column, lines 8-30; figures 1-4; tables 1-5	1,2,6,7

A	CARLOS BALSALOBRE ET AL: "Release of the type I secreted alpha-haemolysin via outer membrane vesicles from Escherichia coli", MOLECULAR MICROBIOLOGY, vol. 59, no. 1, 1 January 2006 (2006-01-01), pages 99-112, XP055145611, ISSN: 0950-382X, DOI: 10.1111/j.1365-2958.2005.04938.x page 108, right-hand column, lines 3-24	1-7

A	FRANCESCO BERLANDA SCORZA ET AL: "High Yield Production Process for Shigella Outer Membrane Particles", PLOS ONE, vol. 7, no. 6, 6 June 2012 (2012-06-06), page e35616, XP055145618, DOI: 10.1371/journal.pone.0035616 the whole document	1-7

A	CAMACHO A I ET AL: "Mucosal immunization with outer membrane vesicles induced protection in mice", VACCINE, ELSEVIER LTD, GB, vol. 29, no. 46, 30 August 2011 (2011-08-30), pages 8222-8229, XP028314367, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2011.08.121 [retrieved on 2011-09-02] the whole document	1-7

A	MYRON M. LEVINE ET AL: "Clinical trials of Shigella vaccines: two steps forward and one step back on a long, hard road", NATURE REVIEWS MICROBIOLOGY, vol. 5, no. 7, 1 July 2007 (2007-07-01), pages 540-553, XP055145547, ISSN: 1740-1526, DOI: 10.1038/nrmicrol662 the whole document	1-7
