

## Immune response study of outer membrane vesicles from *Salmonella enterica* serovar Typhimurium var. Weltevreden (*S. Weltevreden*) associated with poultry enteritis

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### ABSTRACT

*Salmonella enterica* serovar Typhimurium var. Weltevreden (*S. Weltevreden*) is an important foodborne pathogen in South-East Asia and poses a growing public health risk. The present study evaluated the potential of an outer-membrane vesicle (OMV) based vaccine derived from poultry-associated isolates to stimulate immune protection in chickens. Young birds were immunized through different routes (intranasal (I.N.), intramuscular (I.M.), or subcutaneous (S.C.) routes), along with a non-vaccinated control. All vaccinated groups received a booster dose on (14<sup>th</sup> day post vaccination). Serum samples were collected at regular intervals (0, 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup>, and 28<sup>th</sup> day post-vaccination) to monitor IgG responses using indirect ELISA. Birds vaccinated through S.C. or I.N. route demonstrated an early (7<sup>th</sup> DPV) and significant rise in antibody levels after the primary dose ( $p \leq 0.01$ ), followed by stabilization until booster administration. A marked enhancement in IgG response was observed after the booster vaccination, which continued to strengthen over the remaining observation period. I.M. vaccination produced a more gradual initial response but showed clear improvement following the booster. Overall, the study indicated that OMV-based vaccination particularly via subcutaneous and intranasal routes can effectively stimulate humoral immunity in chickens. These findings highlighted the promise of OMV formulations as a control strategy for *Salmonella* infections in poultry, with potential benefits for reducing zoonotic transmission. Further clinical and immunological validation is warranted to determine their protective value under field conditions.

**Keywords:** Humoral response, Poultry Enteritis, Outer membrane vesicles, *Salmonella* Weltevreden

Poultry enteritis is a multifactorial illness produced by several pathogens, including bacteria, viruses, macro parasites, and other infectious and non-infectious factors (Sain *et al.* 2024). Salmonellosis, a zoonotic infection caused by *Salmonella* spp., is one of the most significant infections (Nazir *et al.* 2025, Afshari *et al.* 2018). *Salmonella* infections occurring by any serotype, except the typhoidal and paratyphoidal subgroups, are referred to as non-typhoidal salmonellosis. *Salmonella enterica* serovars, Enteritidis, and Typhimurium are the more prevalent serotypes that can infect people with non-typhoidal salmonella (NTS) (Abebe *et al.* 2020). There are several types of vaccines available in the market, including live-attenuated, inactivated, and subunit vaccines, giving a range of alternatives for disease prevention in poultry (Raut *et al.* 2023).

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Outer membrane's (OM) of bacteria is an asymmetric bilayer made up of lipopolysaccharides on the outside and phospholipids on the inside, both embedded with nonspecific porins and specialized channels (Kaur and Mingeot, 2024; Furuyama and Sircili, 2021). OMVs are spherical particles of approximately 10-300 nm diameter that pinch from the cell surface during bacterial development in both pathogenic and nonpathogenic Gram-negative bacteria including *Escherichia coli*, *Shigella* spp., *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Vibrio* spp., *Salmonella* spp. (Zhao *et al.* 2025, Schwechheimer and Kuehn, 2015, Chen *et al.* 2020). These vesicles are spontaneously released during both *in vitro* and *in vivo* growth (Raju *et al.* 2024, Avila Calderon *et al.* 2020, MacDonald and Kuehn, 2012).

OMVs can be a good vaccine option because they have many biologically active substances, including lipopolysaccharides (LPS), outer membrane proteins (OMPs), and phospholipids, as well as periplasmic components (Kashyap *et al.* 2022, Liu *et al.* 2018). OMVs possess the optimal size to be uptaken by immune cells, and present a range of surface-exposed antigens in native configuration and Toll-like receptor (TLR) activating

components, they represent an attractive and potent vaccine platform able to induce both humoral and cell-mediated immune responses (Micoli *et al.* 2024, Mancini *et al.* 2020). The present study aimed to report the humoral immune response of OMVs from *S. Weltevreden* in poultry.

## MATERIALS AND METHODS

**Place of work:** The work was carried out at the Department of Veterinary Microbiology, College of Veterinary and Animal Science (CVAS) Navania, Udaipur, Rajasthan.

**Chicken:** Day-old chickens were procured from a clean flock of local poultry farm for animal experiments. The experiment was carried out as per the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA), New Delhi, India. Permission had been obtained from the Institutional Animal Ethics Committee (IAEC), College of Veterinary and Animal Science, Navania, Vallabhnagar, Udaipur (Protocol No. IAEC/RES/03/12, Dated 10/08/2023).

**Bacterial strain, media, and growth conditions**

*S. Weltevreden* strains isolated in poultry enteritis from several poultry farms of Southern Rajasthan in previous work and stored in the repository of Department of Veterinary Microbiology, CVAS, Navania, Vallabhnagar, Udaipur, Rajasthan, were utilized in the study. All *S. Weltevreden* strains preserved in -80°C were recovered on Luria Bertani agar (LB agar) plates at 37°C after overnight incubation.

**Extraction of OMVs from *S. Weltevreden*:** Outer membrane vesicles (OMVs) were isolated from *S. Weltevreden* using the Exo Bacteria OMV Isolation Kit (System Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions with slight modifications (Jiang *et al.* 2022). Briefly, a single bacterial colony was cultured overnight in Luria-Bertani LB broth at 37°C until an optical density (OD<sub>600</sub>) of approximately 1.5 was achieved. The culture was centrifuged twice at 5,000 × g for 20 min at 4°C, and the supernatant was sequentially filtered through 0.45 µm and 0.22 µm membrane filters to remove residual cells. Thirty milliliters of clarified culture supernatant was incubated with OMV Binding Resin for 30 min at 4°C on a rotating platform. After washing the resin three times with Binding Buffer, the bound OMVs were eluted with 1.5 mL of OMV Elution Buffer and collected for further characterization.

**Characterization of OMVs:** The concentration of protein was estimated by UV absorbance biospectrophotometer (Eppendorf, Hamburg, Germany). The characterization of OMVs in terms of actual size was carried out by transmission electron microscopy (TEM) at the Sophisticated Analytical Instrumental Facilities Unit, All India Institute of Medical Sciences (AIIMS), New Delhi. For analysis, the OMVs samples were placed onto a 200-mesh copper grid, negatively stained with 4% uranyl acetate and visualized using a transmission electron microscope (Thermo Fisher Scientific, Mumbai, India) to determine the size of the OMVs.

**OMVs immunization protocol:** Four groups of 10-day-old chickens (six chickens per group) were used in the study. Each bird in the first three groups was immunized with 100µg OMVs in 100 µl Dulbecco's Phosphate – Buffered Saline (DPBS) (Wang *et al.* 2019, Wang *et al.* 2020), administered via different routes: the first group received the vaccine intra-nasally (I.N.), the second group intramuscularly (I.M.), the third group subcutaneously (S.C.). The fourth group served as a control and received 100 µl DPBS only. The booster dose, identical in composition and volume, was administered two weeks after the initial immunization (Wang *et al.* 2019, wang *et al.* 2020).

**Immune response study:** The day of first immunization was marked as day 0, and the booster dose administered was marked day 14. On 7<sup>th</sup>, 21<sup>st</sup>, and 28<sup>th</sup> day, 1–2 ml of blood was aseptically drawn from chicken wing vein after the first and second immunizations. Serum was separated and estimation of serum antibodies against *S. Weltevreden* OMVs was performed by indirect single serum dilution ELISA using polysorb 96-well U-bottomed micro titer plates (coated with 10 µg *S. Weltevreden* OMVs dissolved in coating buffer (carbonate buffer). The OMVs coated plates were kept at 4°C overnight. The plates were washed thrice with washing solution (PBST). Two hundred µl of blocking solution (Bovine serum albumin) was added to each well and the plates were kept at 37°C for one hour. The plates were washed thrice with washing solution. Two hundred µl of 1:100 diluted serum was added to each well in triplicates and the plates were incubated for one hour at 37°C. The plates were washed thrice with washing solution. Hundred µl of 1:5000 diluted rabbit antichick-HRP conjugated IgG (Sigma Aldrich Chemicals Pvt. Ltd., Bangaluru, India) was added to each well and the plates were kept for incubation for one hour at room temperature. Subsequently, 100 µl of TMB substrate was transferred to the wells and kept for 1 h at room temperature. Then, 100 µl of stop solution (1 N H<sub>2</sub>SO<sub>4</sub>) was added to terminate the reaction. The OD<sub>450</sub> was measured immediately using ELISA plate reader (Bio-Rad iMark Microplate Reader, Hercules, California, USA).

**Statistical analysis:** The statistical significance of differences in indirect ELISA was assessed by two way ANOVA test using SPSS 16.0 software. Differences were considered significant if P<0.05. All data were statistically analyzed using GraphPad Prism version 8.0.2 (GraphPad Software package, San Diego, USA). The data were expressed as mean ± standard deviation (SD). The differences between the immunized group and the PBS control group were compared using Student's t-test. P values of < 0.05 were considered significant.

## RESULTS AND DISCUSSION

Outer membrane vesicles (OMVs) are naturally shed from the surface of many Gram-negative bacteria and carry a repertoire of native surface antigens and pathogen-associated molecular patterns (PAMPs). Because OMVs present proteins and lipids in their natural membrane

context and contain intrinsic adjuvating molecules (e.g., LPS, lipoproteins), they efficiently engage innate sensing pathways and deliver antigenic cargo to antigen-presenting cells. (Velimirov and Velimirov, 2024, Lei *et al.* 2024, Liu *et al.* 2018). Host immune responses act as a critical barrier against infections. In contrast to whole pathogens, the nanoscale size of outer membrane vesicles (OMVs) allows efficient uptake by antigen-presenting cells (APCs). Once internalized, OMVs stimulate the innate immune system by engaging Toll-like receptors (TLRs) through diverse pathogen-associated molecular patterns (PAMPs), including lipoproteins, lipopolysaccharides (LPS), and nucleic acid fragments (Lu, *et al.* 2025). The primary constituent of OMVs is LPS, which plays a crucial role in determining the safety profile of OMVs when used as vaccines (Wang *et al.* 2020). OMVs hold promise as native vaccine due to their multiple antigens, reducing the chance of pathogen mutations and vaccine escape variants. Their cost-effective isolation makes them particularly attractive for vaccine development (Zahid *et al.* 2025 Van der Pol *et al.* 2015).

OMVs are highly immunogenic and act as natural adjuvants due to their composition of immunogenic elements like LPS and OMPs (Lei *et al.* 2024, Schwechheimer and Kuehn, 2015). A study on *Salmonella* OMVs highlighted their ability to stimulate B cells through LPS and porins (OmpC, OmpF, and OmpD), with the O antigen in LPS enhancing their immunogenicity as an adjuvant (Cui, *et al.* 2022, Schager *et al.* 2018). *Salmonella* OMVs exhibit key characteristics of an effective vaccine, including safety, accessibility, stability, efficacy, and immunogenicity (Lu *et al.* 2025).

**OMVs yield from *S. Weltevreden*:** The average protein concentration in OMVs from the *S. Weltevreden* strain was approximately 5 mg/ml of culture. Developing OMVs based vaccine is largely dependent on the high yield of OMVs with consistent composition. It is well recognized that some bacterial genera like *Pseudomonas* naturally produce larger amounts of OMVs than others (Zavan *et al.* 2023, Furuyama and Sircili, 2021). As long as the antigen is sorted into the OMVs, it may be beneficial to express target antigenic proteins in a species that produces more OMVs. Due to their representation of the makeup of the OM, the OMVs naturally include some of the most immunogenic and significant antigens, making them essential components of a successful vaccination. In contrast to the present findings, a lower protein concentration in OMVs of *S. Weltevreden* was detected by Sharmah (2017) as 0.045 mg per 1 ml of culture through ultracentrifugation. Further, the lower protein concentration in OMVs in *S. Enteritidis* strains as observed by Jiang *et al.* (2022) as 0.206 mg/ml, 0.182 mg/ml and 0.836 mg/ml in wild type strain, DrfaQ strain and DtolR strain, respectively might be due to the method difference.

In order to visualization and estimation of diameter of the OMVs from *S. Weltevreden* strains, transmission electron micrographs were performed using negative

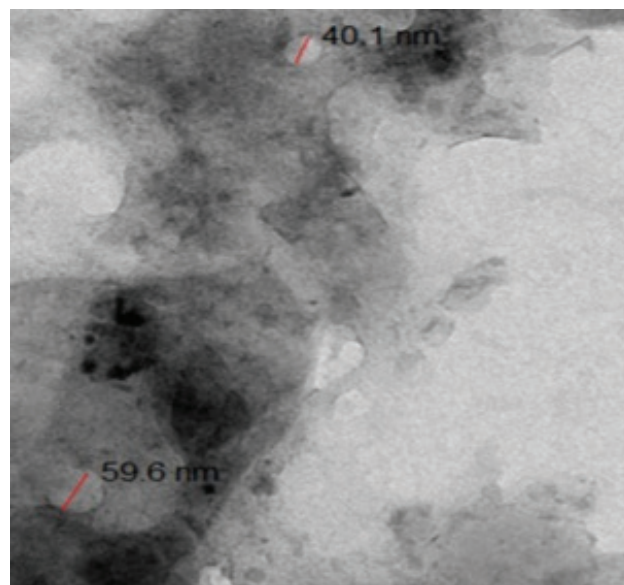


Fig. 1. Transmission Electron Microscopic image of OMVs

staining with 4% uranyl acetate. In the micrographs (Fig. 1), spherical morphology (average diameter 40–200 nm) of the purified OMVs was observed.

Elhenawy *et al.* (2016) employed transmission electron microscopy (TEM) to visualize the OMVs secreted by *S. Typhimurium*, *in vitro*. These OMVs were remarkably homogeneous in size, approximately 50 to 120 nm. Sharmah (2017) observed the mean size of ChNP-OMVs to be  $286 \pm 27.65$  nm in the transmission electron microscopy. Cui *et al.* (2022) showed that OMVs were spherical particles of diverse sizes, with diameter of about 100 nm in a scanning electron microscope. Jiang *et al.* (2022) revealed that the diameter of OMVs from WT and DrfaQ strains was  $72.99 \pm 30.74$  nm and  $60.78 \pm 25.27$  nm, respectively. OMVs from the DtolR strain had a diameter of  $85.31 \pm 32.93$  nm and contained electron-dense structures with various shapes and sizes.

**Antibody responses induced by OMVs:** The S.C. group showed significant rise of mean anti-OMVs IgG response (Fig. 2) from 0<sup>th</sup> day to 7<sup>th</sup> day (Post Primary Immunization (PPI), followed by a non-significant decline in IgG response after 7<sup>th</sup> days PPI to 14<sup>th</sup> day PPI which is after

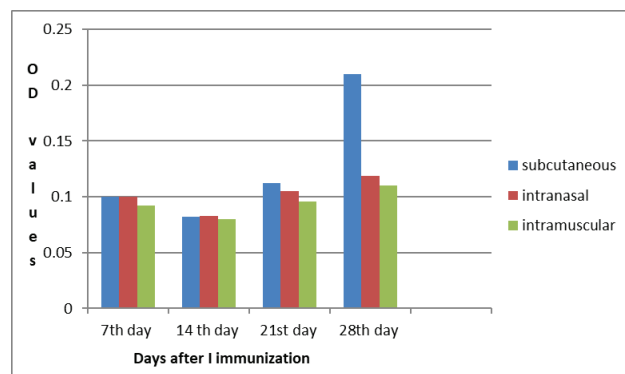


Fig. 2. IgG response in different group of immunized chickens

booster dose on 14<sup>th</sup> day the IgG response significantly rise after 14<sup>th</sup> day to 28<sup>th</sup> day ( $p \leq .01$ ). The I.M. group showed non-significant increase from 0<sup>th</sup> day to 7<sup>th</sup> day followed by a non-significant decline in IgG response up-to 14<sup>th</sup> day and thereafter, IgG response non-significantly rise after 14<sup>th</sup> day and a significant increase after 21<sup>st</sup> day ( $p \leq .01$ ). The I.N. group showed significant rise of IgG response from 0<sup>th</sup> day to 7<sup>th</sup> day followed by non-significant decline during 7<sup>th</sup> - 14<sup>th</sup> day PPI and then significant increase during 14<sup>th</sup> - 28<sup>th</sup> day ( $p \leq .01$ ) (Table 1 and 2). In the present study, it was observed that a significantly higher IgG response in subcutaneous group on 28<sup>th</sup> day PPI compared to the all-

other groups on the same day.

One study in chicken model by Maiti *et al.* (2022) isolated OMVs of *S. Typhimurium* (ST), *S. Enteritidis* (SE) and *S. Gallinarum* (SG) to formulate a trivalent immunogen by mixing them in 1:1:1 ratio. At two-week intervals, one day old chicks were vaccinated three times by oral route. After inoculation, serovar specific antibodies were detected up to 180 days post immunization. These results could not be compared with our data because of different route of inoculation. Further, in mice model, Maiti *et al.* (2021) formulated the immunogen by mixing both OMVs isolated from ST and SE (1:1 ratio). In adult mice, three doses of bivalent immunogen administered intraperitoneally (I.P.) generated strong humoral immune responses to LPSs and OMPs, as well as a cell-mediated immune response. I.P. vaccination can create an adaptive immune response and promote both humoral and cell-mediated immune responses, providing long-term protection against ST and SE infection.

Though this is a preliminary study, it needs to be validated in more number of birds, yet our finding suggest that OMVs from *S. Weltevreden* strains can elicit better immune response by subcutaneous route.

Although *Salmonella enterica* serovar Weltevreden is primarily acquired through the oral route, oral immunization was not evaluated in the present study because unformulated OMVs are prone to degradation in the gastrointestinal tract, which can markedly reduce antigen delivery and immunogenicity. Evaluation of oral OMV vaccines using gastro-resistant or nanoparticle-based delivery systems represents an important area for future investigation.

Further, another limitation of the present study was non evaluation of cell-mediated immune responses. Since *Salmonella enterica* serovar Weltevreden is a facultative intracellular pathogen, protective immunity depends not only on humoral antibodies but also on T-cell-mediated responses, particularly Th1-associated immunity characterized by IFN- $\gamma$  production and macrophage activation. The present investigation was designed as an initial assessment of the immunogenicity and protective efficacy of the OMV vaccine and therefore, focused only on antibody responses and challenge protection. Future studies need to include lymphocyte proliferation assays, cytokine profiling (e.g., IFN- $\gamma$ , IL-2, TNF- $\alpha$ ), ELISpot, or flow

Table 1. IgG response (MEAN $\pm$  SE) of different groups of chicken injected with different routes in different days post primary immunization

|                         | Subcutaneous | Intramuscular | Intranasal | Control  |
|-------------------------|--------------|---------------|------------|----------|
| 0 <sup>th</sup><br>DAY  |              |               |            |          |
| Mean                    | 0.071Aa      | 0.089Ba       | 0.085Aba   | 0.073Aa  |
| SE                      | 0.019        | 0.024         | 0.022      | 0.022    |
| 7 <sup>th</sup><br>DAY  |              |               |            |          |
| Mean                    | 0.10Bb       | 0.0919Ba      | 0.10Bb     | 0.074Aa  |
| SE                      | 0.037        | 0.018         | 0.034      | 0.02     |
| 14 <sup>th</sup><br>DAY |              |               |            |          |
| Mean                    | 0.082Aa      | 0.079Aa       | 0.093Aab   | 0.089Aab |
| SE                      | 0.034        | 0.04          | 0.04       | 0.042    |
| 21 <sup>st</sup><br>DAY |              |               |            |          |
| Mean                    | 0.115Bb      | 0.0957Aa      | 0.10Bb     | 0.087Aa  |
| SE                      | 0.043        | 0.034         | 0.041      | 0.016    |
| 28 <sup>th</sup><br>DAY |              |               |            |          |
| Mean                    | 0.213Cc      | 0.11Bb        | 0.117Bb    | 0.0935Ab |
| SE                      | 0.028        | 0.046         | 0.042      | 0.03     |

Means having different superscript in a column capital letter (A,B,C) and in a row small letter(a, b, c) differ significantly ( $p \leq .01$ )

Table 2. Two Way ANOVA with interaction between day and groups of chicken injected with different routes on different days post primary immunization

| Source                   | Type III Sum of Squares | df  | Mean Square | F        | Sig.  |
|--------------------------|-------------------------|-----|-------------|----------|-------|
| Treatment group          | .056                    | 3   | .019        | 13.862** | 0.000 |
| Period                   | 0.141                   | 4   | 0.035       | 26.200** | 0.000 |
| Treatment group * period | 0.123                   | 12  | 0.010       | 7.660**  | 0.000 |
| Error                    | 0.403                   | 300 | .001        |          |       |
| TOTAL                    | 4.132                   | 320 |             |          |       |

a. R Squared = .443 (Adjusted R Squared = .407)

cytometric analysis of T-cell subsets to comprehensively evaluate the cellular immune mechanisms elicited by the vaccine.

The present work provided an alternative way for developing a promising vaccine based on OMVs for prevention and control of *S. Weltevreden* infections in chickens. Further it is needed to be validated and more extensive studies are needed to ascertain CMI response against the OMVs vaccine.

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