Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Evaluation of outer membrane vesicle conjugated with either Poly-(lactide-co-glycolide) Microparticles or Chitosan nanoparticles or polyanhydride nanoparticles as an oral vaccine against poultry salmonellosis.

Yasmin Begum
Assam Agricultural University
Jayanta Sarma Borua
Institute of Advanced Study in Science and Technology
Vivek Mondal
National Institute of Cholera and Enteric Diseases
Shantanu Tamuly
drsan100@gmail.com

Assam Agricultural University
Rajeev Sharma
Assam Agricultural University
Probodh Borah
Assam Agricultural University
Devashish Chowdhury
Institute of Advanced Study in Science and Technology
Hemanta Koley
National Institute of Cholera and Enteric Diseases
Biswajit Dutta
Assam Agricultural University
Dipankar Hazarika
Assam Agricultural University

Research Article

Keywords: Chitosan Nanoparticles, Poly(lactic-co-glycolic acid) (PLGA) Microparticles, Poly(anhydride) nanoparticles (Gantrez® nanoparticles), Salmonella Typhimurium

Posted Date: November 18th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11756163/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations:
The authors declare no competing interests.

All animal experiments conducted in the present study were approved by the Institutional Animal Ethics Committee (IAEC), Faculty of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India, vide Approval No. 770/ac/CPCSEA/FVSc/AAU/IAEC/17-18/605.

Evaluation of outer membrane vesicle conjugated with either Poly-(lactide-co-glycolide) Microparticles or Chitosan nanoparticles or polyanhydride nanoparticles as an oral vaccine against poultry salmonellosis.

Yasmin Begum[1], Jayanta Sarma Borua[2], Vivek Mondal[3], Shantanu Tamuly[1]*, Rajeev Sharma[4], Probodh Borah[5], Devashish Chowdhury[2], Hemanta Koley[3], Biswajit Dutta[6], Dipankar Hazarika[1]

Abstract

Salmonella infections are a major concern in poultry, impacting both animal health and food safety. Conventional vaccines, including live attenuated and inactivated bacterins, often exhibit limited efficacy and short-lived immunity. This study aims to develop and evaluate a novel oral vaccine formulation incorporating poly-lactide-co-glycolide (PLG) microparticles conjugated with *Salmonella Typhimurium* outer membrane vesicles (OMVs). PLG microparticles were synthesized using the solvent evaporation technique and characterized for size (3654 ± 914.6 nm) and zeta potential (-12.3 mV). Immunization trials in poultry birds revealed that the PLG-OMV vaccine elicited significantly higher IgG and sIgA responses compared to unadjuvanted OMVs, chitosan nanoparticle-OMVs, and Gantrez® nanoparticle-OMVs. The vaccine also induced robust cellular immunity, with elevated IFN-γ production and CD8+ T-cell activation. Protective challenge studies demonstrated reduced *Salmonella*

[1] Department of Veterinary Biochemistry,College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781022.

[2] Institute of Advanced Study in Science and Technology (IASST), Guwahati.
[3] National Institute of Cholera and Enteric Diseases (NICED), Kolkata
[4] Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781022
[5] Department of Animal Biotechnology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781022.
[6] Department of Veterinary Pathology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781022.

*Correspond author:
Email: drsan100@gmail.com

Typhimurium fecal shedding and 100% survival against *Salmonella Gallinarum* infection. These findings highlight PLG-OMV microparticles as a promising strategy for *Salmonella* control in poultry.

INTRODUCTION

Salmonella infections pose a significant threat to poultry health and food safety, leading to economic losses and public health concerns. Among the various strategies developed to mitigate *Salmonella* infections, vaccines play a crucial role in preventing and controlling outbreaks. Traditional vaccines, including live attenuated and inactivated bacterins, have limitations such as safety concerns, variable efficacy, and short-lived immunity. Therefore, innovative vaccine delivery systems, such as polymer-based microparticles, have gained attention for their ability to enhance antigen stability and controlled release, leading to prolonged immune stimulation.

Poly-lactide co-glycolide (PLG) is a biodegradable and biocompatible polymer widely used in drug delivery and vaccine development. PLG microparticles can encapsulate antigens, protect them from degradation, and enhance mucosal immune responses. Outer membrane vesicles (OMVs) derived from *Salmonella Typhimurium* serve as promising vaccine candidates due to their ability to present multiple antigenic components in a native conformation, eliciting strong humoral and cellular immune responses.

This study aims to formulate and evaluate PLG microparticle-OMV conjugates as a novel oral vaccine against *Salmonella* infections in poultry. By assessing immune responses and protective efficacy, this research seeks to establish the potential of PLG-OMV microparticles as an effective alternative to conventional poultry vaccines.

Materials and Methods

1. Bacterial Strain and Culture Conditions

Salmonella enterica serovar Typhimurium was used for the production of outer membrane vesicles (OMVs). The bacterial cultures were grown in Luria-Bertani (LB) broth and the OMVs were isolated following the protocol mention by (Solo et al., 2021). The protein concentration of OMVs was determined using Lowry's method **(**Lowry et al., 1951**).**

2. Preparation of Nanoparticle-Adjuvanted Vaccine Formulations

Three different nanoparticle-based formulations were used for vaccine development:

(i) Chitosan Nanoparticles (CNPs)

Chitosan nanoparticles were synthesized as per the method described by Chowdhury et al. (2012)**.** A 1% glacial acetic acid and glycerol solution (1:3 ratio) was prepared, and 100 mg of chitosan was dissolved in 10 mL of this solution. The mixture was stirred at room temperature for 1–2 hours until a pale yellow solution was formed. Neutralization was carried out using 5N NaOH, leading to the immediate formation of a clear gel. The gel was further treated with 0.1M acetic acid for 10–15 minutes, and the nanoparticles were stored at -20ºC for further use. The OMVs were conjugated to chitosan nanoparticles by incubating them together at room temperature.

(ii) Poly(lactic-co-glycolic acid) (PLGA) Microparticles

PLGA microparticles were prepared using the solvent evaporation technique, as described by Zhao and Rodgers (2006)**.** PLGA (50:50) was dissolved in methylene chloride, followed by emulsification in distilled water using sonication. The resulting emulsion was added to a polyvinyl alcohol (PVA) solution under continuous stirring to facilitate solvent removal. The microparticles were collected by centrifugation, washed with distilled water, lyophilized, and stored at 4°C. OMVs were conjugated to PLGA microparticles by mixing the aqueous OMV suspension with the microparticles, vortexing for 1 hour, followed by centrifugation and lyophilization. The loading efficiency was measured using Lowry's protein estimation method.

(iii) Poly(anhydride) nanoparticles (Gantrez® nanoparticles)

Poly(anhydride) nanoparticles were synthesized using a double emulsion method, as per the method described by Camacho et al. (2011). The Gantrez®AN copolymer was dissolved in acetone with continuous magnetic stirring at room temperature. Distilled water was then gradually added to this solution under vigorous stirring. Organic solvents were removed under reduced pressure, and the resultant nanoparticle suspension was centrifuged at 27,000 × g for 20 minutes at 4°C. The pellet was rinsed with distilled water and stored at -20°C. OMVs were conjugated to Gantrez® nanoparticles by incubating them together for 1 hour at room temperature, followed by centrifugation. The maximum OMV loading efficiency was determined using Lowry's method.

4. Immunization and Sample Collection

(i) Optimization of Dose of Vaccine in Poultry Birds

To determine the optimal dose for immunization, poultry birds were divided into groups and administered different doses (0.50 mg, 0.75 mg, and 1.00 mg per bird) of the PLG-OMV, Chitosan-OMV, Gantrez®-OMV, unadjuvanted OMV vaccines. The control group received PBS. Blood and fecal samples were collected at **0, 7, 14, 21, 28, 35, and 42 days post-primary immunization (dppi)**. The dose eliciting the highest immune response with minimal adverse effects was considered optimal.

(ii) Evaluation of Humoral and Cell-Mediated Immune Response in Poultry Birds

Humoral immunity was evaluated by measuring serum IgM and IgG titers using an indirect ELISA, allowing for a comparative analysis of antibody responses across different vaccine formulations. Mucosal immunity was assessed by quantifying secretory IgA (sIgA) levels in fecal samples through ELISA to determine the extent of mucosal immune activation. Cellular immunity was analyzed by measuring cytokine interferon-gamma (IFN-γ) production from spleenocytes, which were isolated and stimulated *in vitro* on day 42 post-vaccination. These

immunological assessments provided a comprehensive evaluation of the vaccine-induced immune responses.

5. Protective Challenge Experiment

Thirty-six birds were challenged with **10^8 CFU of *Salmonella Typhimurium*** on **day 35 post-immunization** to evaluate protective efficacy. Fecal shedding of *Salmonella* was monitored by culturing fecal samples on **brilliant green agar (BGA)**. A second heterologous challenge study with **Salmonella Gallinarum** was conducted using the best-performing vaccine formulation. The birds were monitored for survival rates and bacterial clearance.

6. Statistical Analysis

Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Differences were considered statistically significant at $p < 0.05$. Results were expressed as mean ± standard error (SE). all the data analysis was carried out in statistical software R.

Results and Discussion

Size Characterization of Delivery Systems

The physicochemical characteristics of vaccine delivery systems, including particle size and surface charge, play a crucial role in determining antigen stability, cellular uptake, and immune response induction. In this study, three different nanoparticle-based vaccine carriers—polyanhydride nanoparticles (Gantrez®), chitosan nanoparticles (ChNP), and poly(lactic-co-glycolic acid) (PLGA) microparticles—were evaluated for their size distribution and zeta potential to understand their potential impact on vaccine efficacy.

The polyanhydride nanoparticles exhibited an average size of 247.4 ± 14.36 nm with a strongly negative zeta potential of -52.6 ± 4.38 mV, indicating high colloidal stability due to electrostatic repulsion. Nanoparticles within this size range have been shown to be efficiently taken up by antigen-presenting cells (APCs), leading to enhanced antigen processing and

presentation (Camacho et al., 2011). The highly negative surface charge suggests strong stability in suspension, which is beneficial for maintaining vaccine integrity.

The chitosan nanoparticle-OMV (ChNP-OMV) complex had a mean size of 286 ± 27.65 nm and a positive zeta potential of 12.8 mV, which is characteristic of chitosan-based formulations. The positive surface charge of chitosan nanoparticles enhances their interaction with negatively charged mucosal surfaces, thereby improving antigen adhesion, uptake, and prolonged residence time in mucosal-associated lymphoid tissues (Chowdhury et al., 2012). This property is particularly relevant for oral vaccine formulations where prolonged antigen exposure is beneficial for stimulating mucosal immunity.

In contrast, PLGA microparticles displayed a significantly larger average diameter of 3654 ± 914.6 nm and a moderately negative zeta potential of -12.3 mV. The larger particle size of PLGA microparticles is expected due to their microparticulate nature, which allows for sustained antigen release over an extended period (Makadia & Siegel, 2011). Although the surface charge is less negative than that of polyanhydride nanoparticles, it remains sufficient to maintain dispersion stability while allowing gradual antigen release. The larger size of PLGA microparticles suggests that their uptake by APCs may rely more on phagocytosis rather than pinocytosis, leading to prolonged immune stimulation.

The variations in particle size and surface charge among these three delivery systems significantly influence their interaction with immune cells, antigen uptake, and subsequent immune responses. While polyanhydride and chitosan nanoparticles fall within the ideal size range for efficient cellular uptake, PLGA microparticles provide the advantage of a sustained antigen depot effect, which is crucial for prolonged immune activation. These differences underscore the importance of selecting an appropriate delivery system based on the desired immunological outcome, with chitosan nanoparticles favoring mucosal immunity,

polyanhydride nanoparticles facilitating rapid antigen processing, and PLGA microparticles enabling long-term antigen release and immune stimulation.

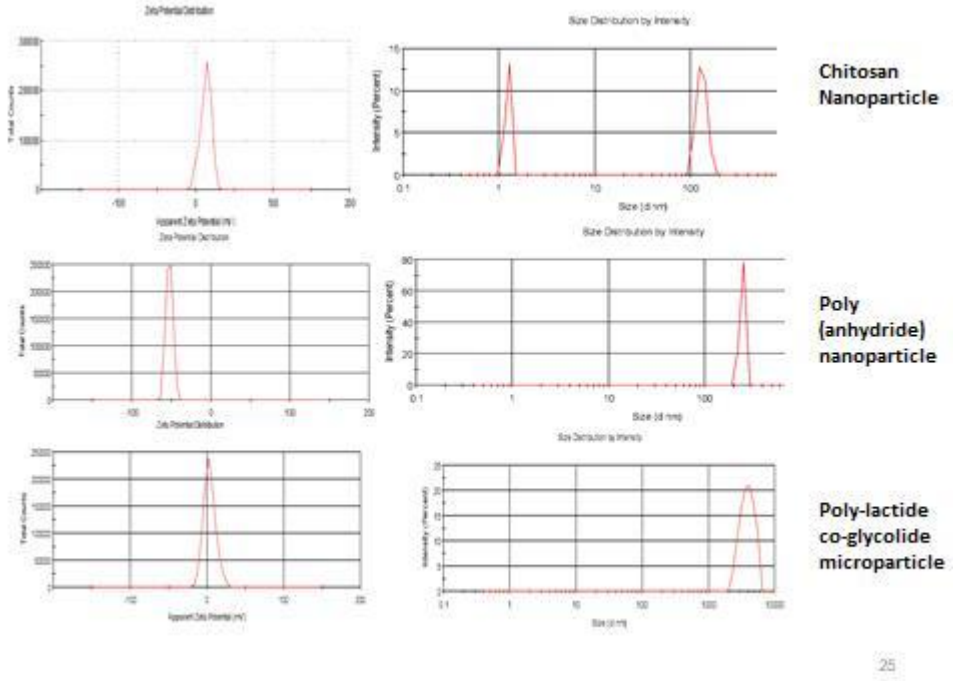


Fig. 1: Zeta size and zeta potential

Optimization of vaccine dose:

Determining the optimal vaccine dosage is crucial for eliciting a robust immune response while minimizing potential adverse effects. In this study, poultry birds were administered varying doses (0.50 mg, 0.75 mg, and 1.00 mg per bird) of different vaccine formulations: PLG-OMV, Chitosan-OMV, Gantrez®-OMV, and unadjuvanted OMV, with a PBS control group. Serological analyses revealed that birds receiving the 0.75 mg dose of the PLG-OMV vaccine exhibited significantly higher serum IgG and IgM titers compared to the 0.50 mg and 1.00 mg groups ($p < 0.05$), suggesting that this dose is optimal for inducing a strong humoral immune response with the PLG-OMV formulation. Interestingly, the 1.00 mg dose did not further enhance antibody levels, indicating a potential threshold beyond which no additional immunological benefit is observed, aligning with previous studies that suggest higher antigen doses may not proportionally increase immune responses and could potentially induce

immunotolerance (Liu et al., 2023). Similarly, for the Chitosan-OMV vaccine, birds administered the 0.75 mg dose demonstrated the highest serum IgG and IgM titers among the tested doses ($p < 0.05$), likely due to the mucoadhesive properties of chitosan nanoparticles that enhance antigen uptake and presentation, leading to a more robust humoral response at this dosage. Increasing the dose to 1.00 mg did not significantly enhance antibody levels, further supporting the notion that optimal dosing is critical for maximizing immune responses while avoiding potential adverse effects (Liu et al., 2023). The Gantrez®-OMV vaccine followed a similar trend, with the 0.75 mg dose group exhibiting superior serum IgG and IgM responses compared to the 0.50 mg and 1.00 mg groups ($p < 0.05$), highlighting that the 0.75 mg dose is effective in eliciting a strong humoral immune response with this formulation. The lack of additional benefit at the 1.00 mg dose suggests a plateau effect, emphasizing the importance of dose optimization in vaccine efficacy. Each vaccine formulation contained 0.75 mg of OMV per bird, with corresponding delivery system doses of 1.5 mg/kg body weight for Gantrez® nanoparticles, 3 mg/kg body weight for chitosan nanoparticles, and 15 mg/kg body weight for PLG microparticles.

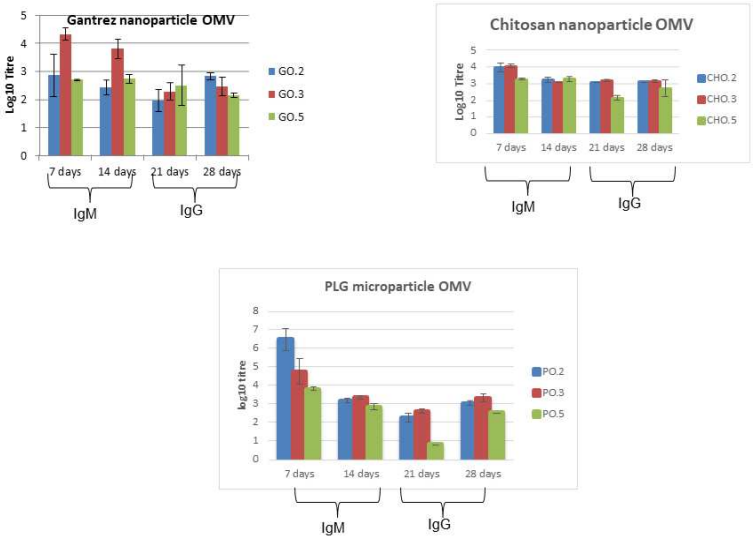


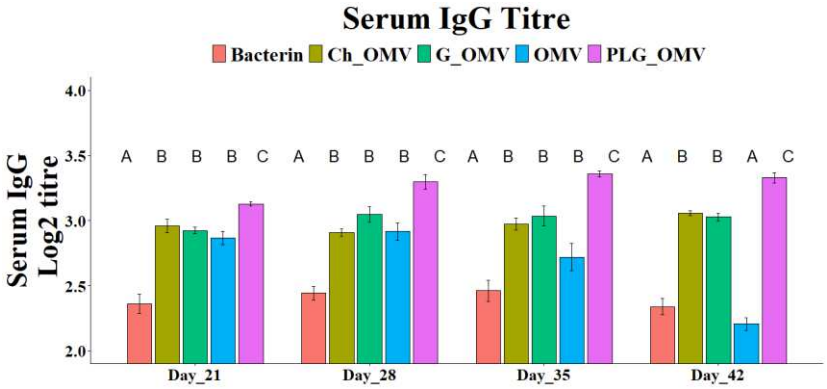
Fig.

2. Evaluation of Humoral and Cell-Mediated Immune Responses

2.1 Humoral Immune Response

Following the determination that the 0.75 mg dose was optimal, further evaluations of humoral and cell-mediated immune responses were conducted using this dosage. Salmonella-free birds were divided into six groups (n=6) and administered different vaccine formulations, with booster doses on days 14 and 28 post-primary immunization. Blood and fecal samples were collected at specified intervals up to 42 days post-primary immunization (dppi). The PLG-OMV and Gantrez® nanoparticle-OMV vaccine formulations elicited the highest antibody responses by 21 dppi. Notably, the PLG-OMV group maintained elevated IgG levels through 42 dppi, whereas the Gantrez® nanoparticle-OMV group experienced a slight, non-significant decline after 21 dppi. Statistical analysis revealed that the IgG response in the PLG-OMV group was significantly higher than in other vaccine groups on days 28, 35, and 42 dppi. In contrast, the unadjuvanted OMV group exhibited significantly lower IgG responses throughout the study period. These findings suggest that the PLG-OMV vaccine formulation induces a robust and sustained humoral immune response in poultry. The prolonged elevation of IgG levels indicates that PLG-OMV effectively enhances systemic immunity. This aligns with previous studies highlighting the potential of OMV-based vaccines to trigger comprehensive immune responses (Acevedo et al., 2014).

The Gantrez® nanoparticle-OMV formulation also elicited a strong initial IgG response; however, the slight decline observed post-21 dppi may indicate a shorter duration of efficacy compared to PLG-OMV. This observation is consistent with studies where Gantrez®-based formulations enhanced serum antibody responses but did not sustain prolonged immunity (García et al., 2013).



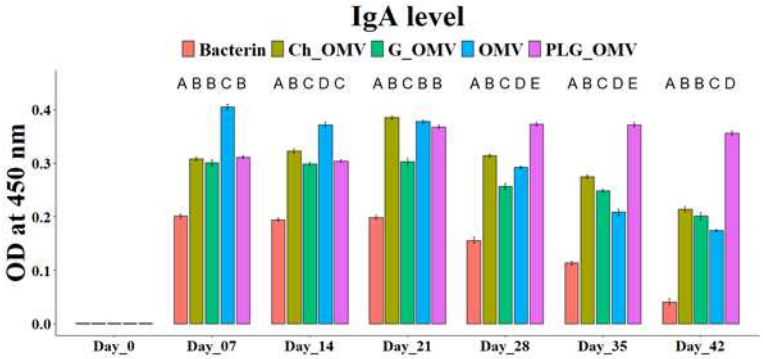
Secretory IgA Response

The unadjuvanted OMV group demonstrated a peak in secretory IgA (sIgA) levels at 7 dppi, which persisted until 21 dppi before significantly declining. The PLG-OMV group showed a gradual increase in sIgA, achieving significantly higher levels than other formulations by 28 dppi, with these levels sustained through 42 dppi. Other vaccine formulations did not maintain elevated sIgA levels beyond 21 dppi.

The unadjuvanted OMV group's early peak in sIgA suggests an initial mucosal immune activation without long-term persistence. This transient response may be due to the lack of an adjuvant to promote sustained antigen exposure and immune stimulation.

Secretory Immunoglobulin A (sIgA) plays a crucial role in mucosal immunity by serving as the first line of defense against pathogens at mucosal surfaces. It achieves this by neutralizing toxins and pathogens, thereby preventing their adherence and entry into epithelial cells (Mantis et al., 2011). Additionally, sIgA contributes to the maintenance of mucosal homeostasis by facilitating immune exclusion, a process that limits the access of antigens and pathogens to the mucosal surfaces without triggering inflammatory responses (Mantis et al., 2011). This selective defense mechanism is essential for protecting mucosal surfaces while preserving the integrity of the epithelial barrier.

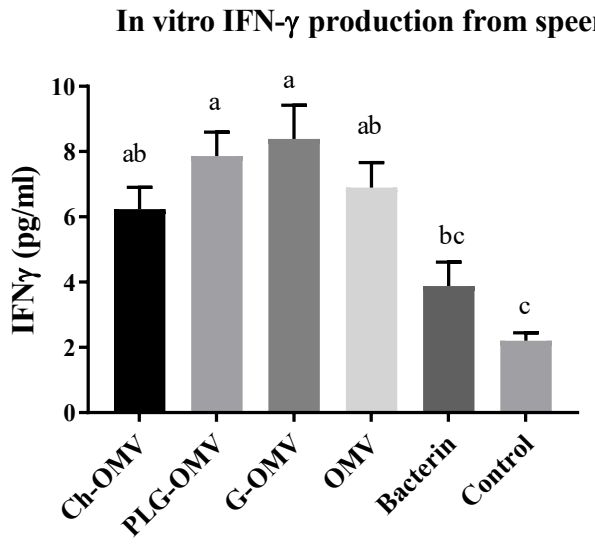
In summary, the PLG-OMV vaccine formulation demonstrates superior potential for inducing long-lasting humoral immunity in poultry, making it a promising candidate for protecting against Salmonella infections.



2.3 **Cell-Mediated Immune Response**

The observed elevation of interferon-gamma (IFN-γ) production in spleenocytes on day 42 post-primary immunization with poly-lactide co-glycolide (PLG)-OMV and Gantrez® nanoparticle OMV formulations highlights their potential in inducing strong cellular immune responses. IFN-γ is a key Th1 cytokine associated with cellular immunity, playing a critical role in activating macrophages, enhancing antigen presentation, and promoting resistance to intracellular pathogens. The high IFN-γ production induced by PLG microparticle (PLG-MP)-OMV and Gantrez® nanoparticle-OMV formulations suggests their ability to enhance antigen delivery and presentation effectively. PLG-MPs, due to their biodegradable and sustained-release properties, provide prolonged antigen exposure, leading to extended immune system activation (Makadia & Siegel, 2011). Gantrez® nanoparticles, derived from poly(anhydride) polymers, have been shown to improve antigen stability and uptake by antigen-presenting cells (APCs), enhancing the immune response (Pérez et al., 2007). The comparable levels of IFN-γ production among the chitosan nanoparticle-OMV, PLG-MP-OMV, and Gantrez® nanoparticle-OMV groups suggest that while all three formulations effectively stimulate Th1 responses, PLG-MP-OMV and Gantrez®-OMV exhibit the

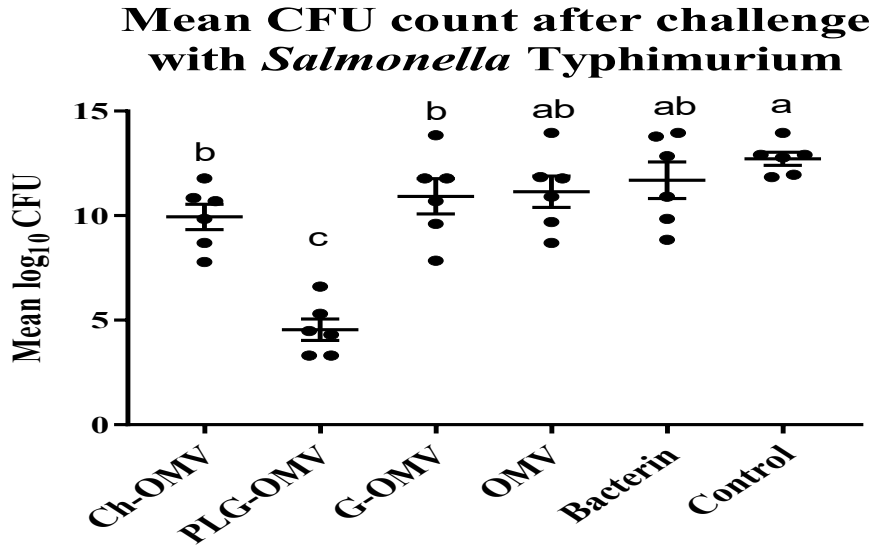
strongest induction of IFN-γ, likely due to their efficient antigen depot effects and uptake mechanisms. Chitosan nanoparticles, known for their mucoadhesive properties, also facilitate antigen presentation but may differ in their intracellular trafficking pathways, leading to a slightly different cytokine profile. These findings underscore the importance of selecting the appropriate delivery system to optimize both humoral and cellular immune responses in vaccine development.

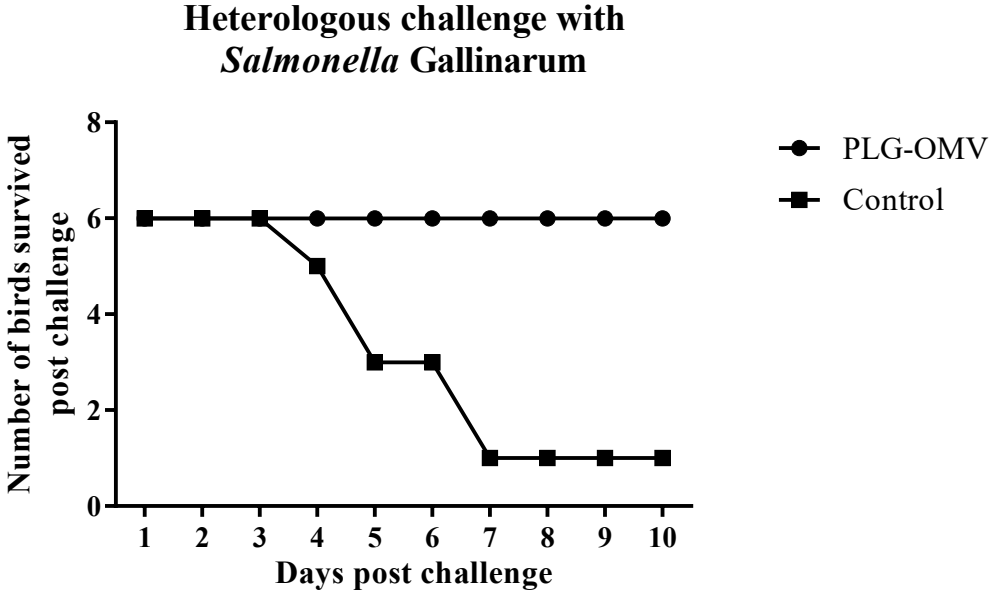


3. Protective Efficacy Against *Salmonella* Challenge

The study's findings demonstrate that immunization with poly-lactide co-glycolide microparticle-outer membrane vesicle (PLG-MP-OMV) formulations provides significant protection against both homologous and heterologous *Salmonella* challenges in poultry. Specifically, birds vaccinated with the PLG-MP-OMV formulation exhibited a substantial reduction in *Salmonella* Typhimurium fecal counts post-challenge, indicating robust protection against the homologous strain. Notably, this formulation also conferred complete protection against a heterologous challenge with *Salmonella Gallinarum*, with all vaccinated

birds surviving up to day 10 post-challenge, whereas the control group experienced high mortality, with only one survivor by day 10.





Poly-lactide-co-glycolide (PLG) microparticles have been extensively studied as delivery vehicles for outer membrane vesicle (OMV)-based vaccines, demonstrating significant immunoprotective effects in poultry. The integration of OMVs with PLG microparticles enhances antigen stability and facilitates sustained release, thereby promoting robust immune

responses. Studies have shown that PLG-OMV formulations can effectively stimulate both humoral and cellular immune responses. For instance, Hu et al. (2020) reported that an OMV-based nanovaccine conferred protection against pathogenic *Escherichia coli* in poultry by eliciting strong humoral and predominantly Th1 immune responses. The sustained antigen release from PLG microparticles ensures prolonged exposure to the immune system, leading to enhanced antibody production and T-cell activation. This dual activation is crucial for comprehensive protection against infections. The biocompatibility and biodegradability of PLG make it a favorable candidate for vaccine delivery. Vaccination with PLG-OMV formulations has been associated with minimal adverse effects while providing substantial protective efficacy. Hu et al. (2020) demonstrated that chicks immunized with an OMV-based nanovaccine exhibited no significant side effects and showed improved survival rates upon challenge with pathogenic *E. coli*. The mechanism underlying the immunoprotection conferred by PLG-OMV vaccines involves the activation of both innate and adaptive immunity. OMVs possess intrinsic adjuvant properties due to their pathogen-associated molecular patterns (PAMPs), which can trigger Toll-like receptors (TLRs) on immune cells, leading to the activation of innate immune responses (Acevedo et al., 2014). The encapsulation within PLG microparticles facilitates the uptake by antigen-presenting cells and ensures a sustained release of antigens, thereby enhancing the adaptive immune response (Hu et al., 2020). This combined activation results in a comprehensive immune defense against pathogens. In summary, PLG-OMV vaccines represent a promising strategy for inducing robust and long-lasting immunity in poultry. The combination of PLG microparticles and OMVs leverages the advantages of sustained antigen release and intrinsic adjuvant properties, leading to enhanced humoral and cellular immune responses. Ongoing research and development in this field hold the potential to improve vaccine efficacy and safety, contributing significantly to poultry health management.

Conclusion

The findings of this study highlight the superior efficacy of poly-lactide co-glycolide (PLG)-OMV microparticles as a vaccine formulation against *Salmonella* infections in poultry compared to other formulations, including unadjuvanted OMV, chitosan nanoparticle-OMV, and Gantrez® nanoparticle-OMV. The PLG-OMV vaccine induced significantly higher and sustained IgG and sIgA titers, enhanced CD8+ T cell activation, and robust IFN-γ production, demonstrating its ability to elicit both strong humoral and cell-mediated immune responses. Unlike the unadjuvanted OMV formulation, which exhibited only transient immune activation, and the chitosan-OMV formulation, which showed moderate but short-lived immune responses, the PLG-OMV vaccine provided prolonged antigen release, leading to sustained immune stimulation. Similarly, while the Gantrez® nanoparticle-OMV formulation elicited an initial strong IgG response, its efficacy declined after 21 days post-immunization, indicating a shorter duration of immunity compared to the PLG-OMV vaccine. Most importantly, in protective challenge studies, birds vaccinated with PLG-OMV microparticles exhibited significantly lower *Salmonella Typhimurium* fecal shedding and achieved a 100% survival rate against a heterologous *Salmonella Gallinarum* challenge, whereas birds vaccinated with other formulations did not show comparable levels of protection. These results emphasize the importance of selecting an optimal delivery system to maximize vaccine efficacy, with PLG-OMV emerging as the most promising candidate for oral *Salmonella* vaccination in poultry. Further studies focusing on large-scale production, field trials, and applications in other livestock species are warranted to support the commercial viability of this vaccine strategy.

Acknowledgement

The authors gratefully acknowledge the Department of Biotechnology (DBT), Government of India for providing the financial support to carry out this research work. The authors also extend sincere thanks to the Director of Research (Veterinary), Assam Agricultural University for providing the necessary laboratory and infrastructural facilities. The technical assistance received from the Institute of Advanced Study in Science and Technology (IASST), Guwahati and the National Institute of Cholera and Enteric Diseases (NICED), Kolkata is duly acknowledged.

References

1. Acevedo, R., Fernández, S., Zayas, C., Acosta, A., Sarmiento, M. E., Ferro, V. A., García, L., Pérez, J. L., & Cardoso, D. (2014). Bacterial outer membrane vesicles and vaccine applications. *Frontiers in Immunology, 5*, 121. https://doi.org/10.3389/fimmu.2014.00121
2. Hu, R., Liu, H., Wang, M., Li, J., Lin, H., Liang, M., Gao, Y., & Yang, M. (2020). An OMV-based nanovaccine confers safety and protection against pathogenic *Escherichia coli* via both humoral and predominantly Th1 immune responses in poultry. *Nanomaterials, 10*(11), 2293. https://doi.org/10.3390/nano10112293
3. Makadia, H. K., & Siegel, S. J. (2011). Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers, 3*(3), 1377–1397. https://doi.org/10.3390/polym3031377
4. Camacho, A. I., et al. (2011). *Poly(methyl vinyl ether-co-maleic anhydride) nanoparticles as innate immune system activators.* Vaccine, **29(41), 7130–7135**. https://doi.org/10.1016/j.vaccine.2011.05.072
5. Chowdhury, D., et al. (2012). *Fluorescent carbon dots obtained from chitosan gel.* RSC Advances, **2(32), 12156**. https://doi.org/10.1039/c2ra21705h

6. Klimentová, J., & Stulík, J. (2015). *Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria.* Microbiological Research, **170, 1–9**. https://doi.org/10.1016/j.micres.2014.09.006
7. Lowry, O. H., et al. (1951). *Protein Measurement with the Folin Phenol Reagent.* Journal of Biological Chemistry, **193(1), 265–275**.
8. Zhao, A., & Rodgers, V. G. J. (2006). *Using TEM to couple transient protein distribution and release for PLGA microparticles for potential use as vaccine delivery vehicles.* Journal of Controlled Release, **113(1), 15–22**. https://doi.org/10.1016/j.jconrel.2006.03.018
9. Hu, R., Liu, H., Wang, M., Li, J., Lin, H., Liang, M., Gao, Y., & Yang, M. (2020). An OMV-based nanovaccine confers safety and protection against pathogenic *Escherichia coli* via both humoral and predominantly Th1 immune responses in poultry. *Nanomaterials*, 10(11), 2293. https://doi.org/10.3390/nano10112293
10. Jiang, W., Gupta, R. K., Deshpande, M. C., & Schwendeman, S. P. (2005). Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. *Advanced Drug Delivery Reviews*, 57(3), 391–410. https://doi.org/10.1016/j.addr.2004.09.003
11. Salvador, A., Igartua, M., Hernández, R. M., & Pedraz, J. L. (2012). Combination of immune stimulating adjuvants with poly(lactide-co-glycolide) microspheres enhances the immune response of vaccines. *Vaccine*, 30(3), 589–596. https://doi.org/10.1016/j.vaccine.2011.11.057
12. Waeckerle-Men, Y., Allmen, E. U., Gander, B., Scandella, E., Schlosser, E., Schmidtke, G., Merkle, H. P., & Groettrup, M. (2006). Encapsulation of proteins and peptides into biodegradable poly(D,L-lactide-co-glycolide) microspheres prolongs

and enhances antigen presentation by human dendritic cells. *Vaccine*, 24(15), 1847–1857. https://doi.org/10.1016/j.vaccine.2005.10.032

13. Acevedo, R., Fernández, S., Zayas, C., Acosta, A., Sarmiento, M. E., Ferro, V. A., & García, L. (2014). Bacterial outer membrane vesicles and vaccine applications. *Frontiers in Immunology*, 5, 121. https://doi.org/10.3389/fimmu.2014.00121
14. García, A., Espinosa, M., Delgado, D., & Genta, I. (2013). Gantrez® AN nanoparticles as a new adjuvant for vaccines. *Journal of Drug Delivery Science and Technology*, 23(3), 255-260. https://doi.org/10.1016/S1773-2247(13)50036-9
15. Mantis, N. J., Rol, N., & Corthésy, B. (2011). Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunology*, 4(6), 603–611. https://doi.org/10.1038/mi.2011.41
16. Makadia, H. K., & Siegel, S. J. (2011). Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers, 3*(3), 1377–1397. https://doi.org/10.3390/polym3031377
17. Pérez, O., Lastre, M., Cabrera, O., del Campo, J., Bracho, G., Cuello, M., Zayas, C., Acevedo, R., & Sierra, G. (2007). New vaccines require potent adjuvants like AFPL1 and AFCo1. *Scandinavian Journal of Immunology, 66*(2–3), 271–277. https://doi.org/10.1111/j.1365-3083.2007.01974.x
18. Solo, M., Tamuly, S., Barkalita, L. M., Saikia, G. K., & Kalita, D. J. (2021). Optimization of Cultural Conditions for Maximum Production of Outer Membrane Vesicles from Salmonella Typhimurium. *Indian Journal of Animal Research*, *Of*. https://doi.org/10.18805/IJAR.B-4449
19. Camacho, A. I., et al. (2011). Poly(methyl vinyl ether-co-maleic anhydride) nanoparticles as innate immune system activators. *Vaccine, 29*(41), 7130–7135. https://doi.org/10.1016/j.vaccine.2011.05.072

20. Chowdhury, D., et al. (2012). Fluorescent carbon dots obtained from chitosan gel. *RSC Advances, 2*(32), 12156. https://doi.org/10.1039/c2ra21705h

21. Makadia, H. K., & Siegel, S. J. (2011). Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers, 3*(3), 1377–1397. https://doi.org/10.3390/polym3031377