



Optimization of Cultural Conditions for Maximum Production of Outer Membrane Vesicles from *Salmonella* Typhimurium

Menguzotunuo Solo, Shantanu Tamuly, Luit Moni Barkalita²,
Girin Kumar Saikia¹, Dhruba Jyoti Kalita²

10.18805/IJAR.B-4449

ABSTRACT

Background: The non-typhoidal *Salmonella* causes gastroenteritis in humans that makes its way to the food chain mainly through the animal products. The multiple drug resistance imposes one of the major hurdle in the treatment of the disease. The vaccination appears to be the most important method for prevention of the disease. Unfortunately, there is no licensed vaccine available against non-typhoidal *Salmonellae*. The use of outer membrane vesicles (OMVs) of *Salmonella* as a vaccine candidate has attained significant centre-stage in the recent years given to its protective immunogenicity. However, the large scale production of OMVs is difficult owing to low yield per liter of culture.

Methods: In the present study, we have optimized the culture conditions viz. pH, phase of growth and presence of oxidative stress for maximum production of OMVs from *Salmonella* Typhimurium. The OMVs were characterized based on yield based on protein concentration, lipopolysaccharide concentration and zeta size.

Result: In the present study, it was found that incubation of *Salmonella* Typhimurium up to peak of the growth phase at pH 7 in presence of oxidative stress was found to be the most suitable condition for maximum production of OMVs.

Key words: LPS, OMV, *Salmonella*, Zeta-size.

INTRODUCTION

Salmonellosis is one of the most common food borne diseases with reportedly 5.35 million burden of non-typhoidal *Salmonella* worldwide (Stanaway *et al.*, 2019). The *Salmonella* serovars have been reported by various workers in food and animal products posing a potential public health issues (Badhe *et al.*, 2013; Hassan *et al.*, 2020; Singh *et al.*, 2018). The antibiotic resistant *Salmonella* are increasingly being observed (Chakraborty *et al.*, 2019; Chiu *et al.*, 2002; Eng *et al.*, 2015) and so far, only two vaccines, Ty21a and Vi capsular polysaccharide, are available (MacLennan *et al.*, 2014; Xiong *et al.*, 2017). Gram negative bacteria, including *Salmonella* Typhimurium, secrete vesicles from their outer membrane which can generate immune and inflammatory responses inside the host cells (Alaniz *et al.*, 2007). These outer membrane vesicles possess range of intact outer-membrane proteins that are similar to the bacterium that has released it. The immunogenicity of these OMVs are similar to the parent bacterium in terms of uptake by macrophages. In addition, the vaccine based on OMVs do not require adjuvant as they have the potential of self-adjuvantation (van der Pol *et al.*, 2015). A vaccine based on outer membrane vesicles, Bexsero (Novartis), has already been approved and licensed against meningococcal serogroup B (McCarthy *et al.*, 2018). The *Salmonella* Typhimurium OMVs can produce cross-immunity among the various strains of *Salmonella* (Liu *et al.*, 2016). Presently, the production of OMVs poses an important challenge in terms of yield. The yield of OMVs is generally less making it commercially expansive. The yield of OMVs is generally

Department of Veterinary Biochemistry, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 022, Assam, India.

¹Department of Animal Biotechnology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 022, Assam, India.

²Department of Veterinary Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 022, Assam, India.

Corresponding Author: Menguzotunuo Solo, Department of Veterinary Biochemistry, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 022, Assam, India. Email: drsan100@gmail.com

How to cite this article: 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. DOI: 10.18805/IJAR.B-4449.

Submitted: 15-03-2021 **Accepted:** 14-06-2021 **Online:** 27-07-2021

influenced by great majority of factors such as pH of the medium, type of media, presence of oxidative stress, presence of antibiotics *etc.* (Balhuizen *et al.*, 2021; Klimentová and Stulík, 2015). Hence, it is important to standardize the cultural condition of growing *Salmonella* Typhimurium for obtaining maximum yield of OMVs. This study was conducted to optimize the conditions for *Salmonella* Typhimurium outer membrane vesicle production by altering pH of the medium, period of incubation of bacterial culture and induction of H₂O₂ mediated oxidative stress.

MATERIALS AND METHODS

Place and time of work

The work was carried out in the Department of Veterinary Biochemistry, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati in the year 2018.

Bacterial strains

For this study, the MTCC-98 strain of *Salmonella* Typhimurium was obtained from Department of Animal Biotechnology, College of Veterinary Science, AAU, Khanapara, Guwahati.

Design of experiment

For optimization, the full factorial design was utilized. The layout of the design is depicted in Table 1.

Culture and isolation of outer membrane vesicles

The 30 ml BHI (brain heart infusion broth) broth was inoculated with 0.6 ml overnight grown culture and incubated at 37°C up to either mid-log phase or peak of growth phase or mid-stationary phase. The culture was then centrifuged at 5000 ×g for 5 minutes at room temperature. The supernatant was discarded and the pellet was re-suspended in 30 ml of fresh BHI medium having pH 6 or 7 with or without oxidative stress. The oxidative stress was generated by

Table 1: Experimental layout for determination of maximum OMV yield.

pH	Oxidative stress	Growth	Designation of condition
6	Present	Pgp	A
6	Present	Mlp	B
6	Present	Msp	C
6	Absent	Pgp	D
6	Absent	Mlp	E
6	Absent	Msp	F
7	Present	Pgp	G
7	Present	Mlp	H
7	Present	Msp	I
7	Absent	Pgp	J
7	Absent	Mlp	K
7	Absent	Msp	L

NB: mlp= mid-log phase; pgp=peak of growth phase; msp=mid-stationary phase.

adding hydrogen peroxide to a final concentration of 250 iM. Incubation was done for 1 hour and the culture was centrifuged at 10,000 ×g for 10 minutes at room temperature. The OMVs were purified using the method described by MacDonald and Kuehn (2013). The protein concentration of OMVs was estimated using modified Lowry's method (Lowry *et al.*, 1951). The yield of OMV was expressed in terms of protein concentration. The three conditions were selected that showed the highest yield of OMVs. The OMVs from these three conditions were selected for further characterization in terms of protein profiling by SDS-PAGE (Sambrook and Russel, 2001) as recommended by Klimentová and Stulík, (2015) and lipopolysaccharide concentration and zeta size. The estimation of the lipopolysaccharide (LPS) content of OMVs was done by Kdo method (Sunayana and Reddy, 2015). The zeta size of the outer membrane vesicles were measured using the dynamic light scattering in Institute of Advanced Study in Science and Technology, Guwahati, Assam.

Statistical analysis

The three factor ANOVA was carried out to determine the effect of different factors on yield of OMVs. The multiple comparison of means was carried out by Tukey's HSD test at 5% significance level. Linear regression analysis was carried to assess the influence of different factors on LPS concentration and size of OMVs. All analysis was carried out using statistical software R version 4.0.3 (R Core Team, 2020).

RESULTS AND DISCUSSION

The conditions where the protein yield was found to be the highest are pH 7, peak of growth phase with oxidative stress; pH 7, peak of growth phase without oxidative stress and pH 7, mid-stationary phase with oxidative stress (Fig 1). The 3-factor ANOVA has indicated that all the factors that were taken has significant influence on the yield of OMVs in terms of protein concentration (Table 2). The interaction of factors were found to exist only between the pH of the medium and the phase of the growth. The pH was found to have the highest contribution in the yield of OMVs followed by phase of the growth and presence of oxidative stress. The protein profiling of the OMVs of the three conditions did not yield any significant difference (Fig 2).

Table 2: Three factor ANOVA depicting the influence of the three factors on the yield of outer membrane vesicles.

	Factors	Mean SS	F value	Significance	Contribution (%)
Main effects	pH	2774.268	30.56	*	26.09
	OS	1532.439	16.88	*	14.41
	Phase	1380.319	15.2	*	25.96
Interaction effects	pH×OS	33.68854	0.37	NS	0.32
	OS×Phase	149.3271	1.64	NS	2.81
	pH×Phase	513.5018	5.66	*	9.66
	pH×OS×Phase	14.60443	0.16	NS	0.27
	Residuals	90.79291			20.49

NB: OS=Oxidative stress; NS= Non-Significant ($p>0.05$); *=Significant ($p<0.05$).

The LPS was found to be lowest in the condition “G” followed by conditions “J” and “I”. The condition “G” where the media was grown up to peak of growth phase in presence of H_2O_2 mediated oxidative stress at pH 7, was found to yield minimum LPS concentration and maximum OMV yield (Fig 3). In addition, this condition appears to have yielded the OMVs of significantly lowest mean zeta size of $858.96 \pm$

83.29 nm (Fig 4). We observed that the oxidative stress and peak of growth phase has significantly negative influence on the LPS concentration and zeta size of OMVs (Table 3 and Table 4).

The H_2O_2 mediated oxidative stress mimics the reactive oxygen burst in intracellular environment of neutrophils in response to bacterial infection that eventually stimulates the

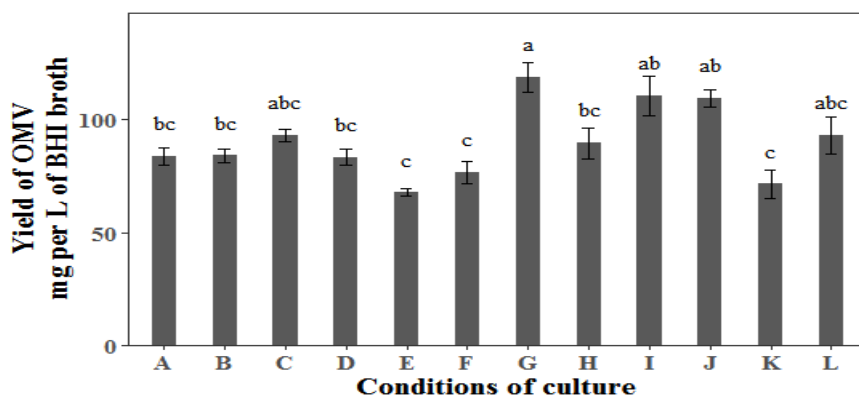


Fig 1: Mean ($\pm$ SEM) yield of outer membrane vesicles per liter of BHI broth in different cultural conditions (as depicted in Table 1). The different letters designation over the bars indicates the statistically significant difference ($p < 0.05$).

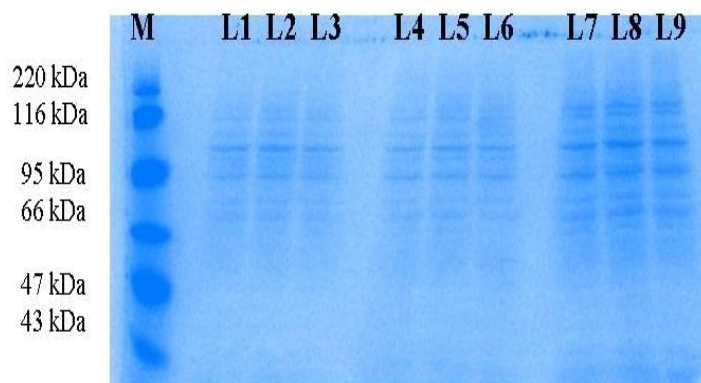


Fig 2: Protein profile of OMV OF *Salmonella* Typhimurium by SDS-PAGE.

Lane M – protein marker, L1, L2, L3 – OMV (pH 7, peak, oxidative stress), L4, L5, L6 – OMV (pH7, peak, without oxidative stress) L7, L8, L9 – OMV (pH7, mid-stationary, oxidative stress).

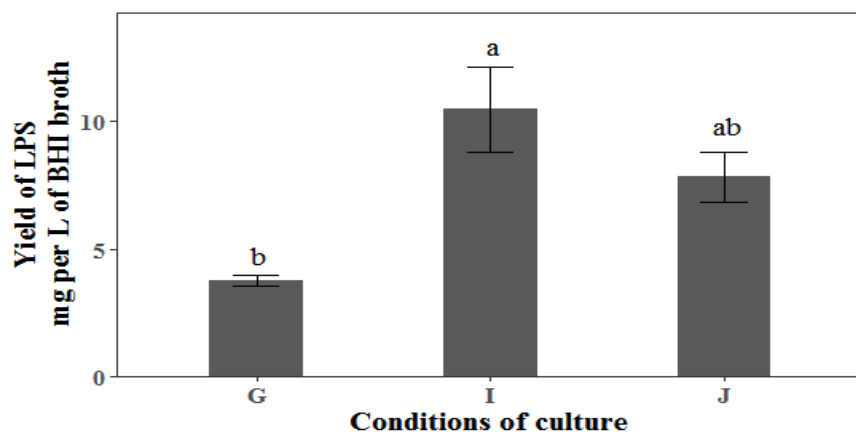


Fig 3: Graphical representation of mean ($\pm$ SEM) LPS yield from OMV of *Salmonella* Typhimurium (grown in pH 7.00). The different letters designation over the bars indicates the statistically significant difference ($p < 0.05$).

bacteria to overproduce the OMVs through quorum sensing (Gerritzen *et al.*, 2018; MacDonald and Kuehn, 2013).

The OMV production occurs in all stages of growth of bacteria but the maximum production of OMV occurs in later part of log phase that is at the peak of growth phase (Chatterjee and Das, 1967; Gamazo and Moriyón, 1987; Hoekstra *et al.*, 1976; McCaig *et al.*, 2013). The higher yield of OMV may facilitate the cell to cell communication in the cells in the later stage of growth. The large amount of cellular materials are deposited during the log phase in the OMVs that continues till the peak of growth phase thereafter the cell death ensues leading to protease mediated degradation of OMVs (Gamazo and Moriyón, 1987). The low pH causes damage to the membrane due to alteration in membrane fluidity, integrity and lipid composition (Guan and Liu, 2020), which could be the possible reason of lower yield of OMVs in acidic pH.

The genetic control of LPS synthesis occurs through the environmental stress (Klein and Raina, 2019) leading to reduction in LPS synthesis as observed in our study. The LPS is responsible for febrile and allergic response (Steiner *et al.*, 2006; Williams *et al.*, 2005). It may be assumed that

lower concentration of LPS in the OMV-based vaccines may be beneficial in terms of febrile and possible allergic response.

The oxidative stress has significant influence on the size of OMVs produced by the Gram negative bacteria (Mozaheb and Mingeot-Leclercq, 2020). Though the exact mechanism of the influence on stress is not understood however as it is evident in the present study, the size of OMVs are significantly negatively influenced by the presence of oxidative stress which could probably be due to the fact that reduction in size of OMVs invariably increase surface to volume ratio, hence greater surface area of OMVs would probably be helpful to bacteria to capture the larger amount of free radicals. Similar findings were also reported by Baumgarten *et al.* (2012) in case of *Pseudomonas putida*. The small size of antigens are known to increase their immunogenicity that could be due to ease in uptake by macrophages (Jia *et al.*, 2018; Jia *et al.*, 2012) and would be a useful candidate for vaccine development. The three best conditions recognized in the present study did not show any difference in protein profile in SDS-PAGE indicating the change in conditions within pH 7 did not have significant

Table 3: Linear regression model showing the effect of oxidative stress and peak of growth phase on LPS concentration of OMVs (at pH 7.0).

Term	Estimate	t-statistic	p value	Significance
(Intercept)	14.559	7.5434	0.00028	***
Presence of oxidative stress	-4.0851	-2.5923	0.04108	*
Peak of growth phase	-6.7203	-4.2645	0.00529	**

Signif. codes: '***' p<0.001 '**' p<0.01 '*' p<0.05.

Table 4: Linear regression model showing the effect of oxidative stress and peak of growth phase on zeta size of OMVs (at pH 7.0).

Term	Estimate	t-statistic	p value	Significance
(Intercept)	1623.0	13.120	>0.001	***
Presence of oxidative stress	-262.7	-2.601	0.04063	*
Peak of growth phase	-501.4	-4.964	0.00254	**

Signif. codes: '***' p<0.001 '**' p<0.01 '*' p<0.05.

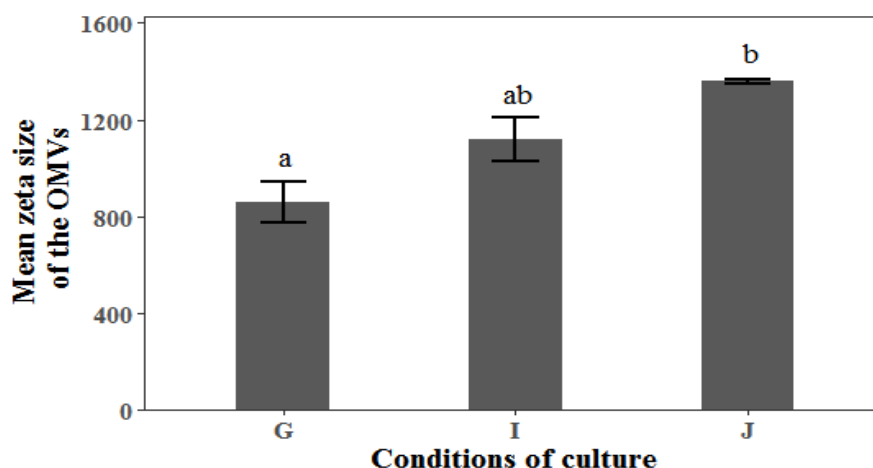


Fig 4: Graphical representation of mean ($\pm$ SEM) zeta size of OMVs of *Salmonella* Typhimurium (grown in pH 7.00). The different letters designation over the bars indicates the statistically significant difference (p<0.05).

change in the protein contents. Based on the protein profile of the SDS-PAGE, we may conclude that the optimized conditions may not be having any significant alteration in the immune-potential.

CONCLUSION

The present study has indicated that the optimal conditions for OMV production from *Salmonella* Typhimurium is neutral pH of growth media, presence of oxidative stress mediated by H₂O₂ and growth of bacteria up to peak of growth phase. The further studies are needed to assess the immune response of the OMV produced by the optimized condition in a laboratory animal model.

ACKNOWLEDGEMENT

The authors are grateful to the DBT, New Delhi for providing financial support to carry out the work. The authors are also grateful to the Dean, Faculty of Veterinary Science, Assam Agricultural University, Khanapara for providing the laboratory facilities, Guwahati Biotech Park for helping in ultracentrifugation and CIF of IASST for the works related to dynamic light scattering.

REFERENCES

- Alaniz, R.C., Deatherage, B.L., Lara, J.C. and Cookson, B.T. (2007). Membrane vesicles are immunogenic facsimiles of *Salmonella* typhimurium that potently activate dendritic cells, prime B and T cell responses and stimulate protective immunity *in vivo*. *Journal of Immunology (Baltimore, Md.: 1950)*. 179(11): 7692-7701.
- Badhe, S.R., Fairuze, M.N. and Sudarshan, S. (2013). Prevalence of food borne pathogens in market samples of chicken meat in Bangalore, India. *Indian Journal of Animal Research*. 47(3): 262-264.
- Balhuizen, M.D., Veldhuizen, E.J.A. and Haagsman, H.P. (2021). Outer membrane vesicle induction and isolation for vaccine development. *Frontiers in Microbiology*. 12: 629090.
- Baumgarten, T., Sperling, S., Seifert, J., Bergen, M. von, Steiniger, F., Wick, L.Y. and Heipieper, H.J. (2012). Membrane vesicle formation as a multiple-stress response mechanism enhances *Pseudomonas putida* DOT-T1E cell surface hydrophobicity and biofilm formation. *Applied and Environmental Microbiology*. 78(17): 6217-6224.
- Chakraborty, S., Roychoudhury, P., Samanta, I., Subudhi, P.K., Lahraipuii, Das, M., *et al.* (2019). Molecular detection of biofilm, virulence and antimicrobial resistance associated genes of *Salmonella* serovars isolated from pig and chicken of Mizoram, India. *Indian Journal of Animal Research*. 54(5): 608-613.
- Chatterjee, S.N. and Das, J. (1967). Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. *Journal of General Microbiology*. 49(1): 1-11.
- Chiu, C.-H., Wu, T.-L., Su, L.-H., Chu, C., Chia, J.-H., Kuo, A.-J., Chien, M.-S. and Lin, T.-Y. (2002). The emergence in Taiwan of fluoroquinolone resistance in *Salmonella* enterica serotype choleraesuis. *The New England Journal of Medicine*. 346(6): 413-419.
- Eng, S.-K., Pusparajah, P., Ab Mutalib, N.-S., Ser, H.-L., Chan, K.-G. and Lee, L.-H. (2015). *Salmonella*: A review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in Life Science*. 8(3): 284-293.
- Gamazo, C. and Moriyón, I. (1987). Release of outer membrane fragments by exponentially growing *Brucella melitensis* cells. *Infection and Immunity*. 55(3): 609-615.
- Gerritzen, M.J.H., Maas, R.H.W., van den Ijssel, J., van Keulen, L., Martens, D.E., Wijffels, R.H. and Stork, M. (2018). High dissolved oxygen tension triggers outer membrane vesicle formation by *Neisseria meningitidis*. *Microbial Cell Factories*. 17(1): 157.
- Guan, N. and Liu, L. (2020). Microbial response to acid stress: mechanisms and applications. *Applied Microbiology and Biotechnology*. 104(1): 51-65.
- Hassan, N., Randhawa, C.S., Kumar, A., Chandra, M., Sood, N.K. and Gupta, K. (2020). *Salmonella enterica* Subsp. enterica serovar reading infection in dairy cattle and buffaloes suffering from chronic diarrhea. *Indian Journal of Animal Research*. 54(8): 1029-1033.
- Hoekstra, D., van der Laan, J. W., de Leij, L. and Witholt, B. (1976). Release of outer membrane fragments from normally growing *Escherichia coli*. *Biochimica Et Biophysica Acta*. 455(3): 889-899.
- Jia, J., Zhang, Y., Xin, Y., Jiang, C., Yan, B. and Zhai, S. (2018). Interactions between nanoparticles and dendritic cells: from the perspective of cancer immunotherapy. *Frontiers in Oncology*. 8: 404.
- Jia, R., Guo, J.H. and Fan, M.W. (2012). The effect of antigen size on the immunogenicity of antigen presenting cell targeted DNA vaccine. *International Immunopharmacology*. 12(1): 21-25.
- Klein, G. and Raina, S. (2019). Regulated assembly of LPS, its structural alterations and cellular response to LPS defects. *International Journal of Molecular Sciences*. 20(2): 356.
- Klimentová, J. and Stulík, J. (2015). Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiological Research*. 170: 1-9.
- Liu, Q., Liu, Q., Yi, J., Liang, K., Hu, B., Zhang, X., Curtiss, R. and Kong, Q. (2016). Outer membrane vesicles from flagellin-deficient *Salmonella enterica* serovar *Typhimurium* induce cross-reactive immunity and provide cross-protection against heterologous *Salmonella* challenge. *Scientific Reports*. 6: 34776.
- Lowry, O.H., Rosebrough, N.J. and Randall, R.J. (1951). Protein estimation with the folin phenol reagent. *Journal of Biological Chemistry*. 193: 265-275.
- MacDonald, I.A. and Kuehn, M.J. (2013). Stress-induced outer membrane vesicle production by *Pseudomonas aeruginosa*. *Journal of Bacteriology*. 195(13): 2971-2981. <https://doi.org/10.1128/JB.02267-12>.
- MacLennan, C.A., Martin, L.B. and Micoli, F. (2014). Vaccines against invasive salmonella disease: Current status and future directions. *Human Vaccines and Immunotherapeutics*. 10(6): 1478-1493.
- McCaig, W.D., Koller, A. and Thanassi, D.G. (2013). Production of outer membrane vesicles and outer membrane tubes by *Francisella novicida*. *Journal of Bacteriology*. 195(6): 1120-1132.

- McCarthy, P.C., Sharyan, A. and Sheikhi Moghaddam, L. (2018). Meningococcal vaccines: Current status and emerging strategies. *Vaccines*. 6(1): 12.
- Mozaheb, N. and Mingeot-Leclercq, M.-P. (2020). Membrane vesicle production as a bacterial defense against stress. *Frontiers in Microbiology*. 11: 600221.
- R Core Team. (2020). A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.
- Sambrook, J. and Russel, D.W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
- Singh, P., Singh, R.V., Gupta, B., Tripathi, S.S., Tomar, K.S., Jain, S. and Sahni, Y. P. (2018). Prevalence study of *Salmonella* spp. in milk and milk products. *Asian Journal of Dairy and Food Research*. 37(1): 7-12.
- Stanaway, J.D., Parisi, A., Sarkar, K., Blacker, B.F., Reiner, R.C., *et al.* (2019). The global burden of non-typhoidal *Salmonella* invasive disease: A systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Infectious Diseases*. 19(12): 1312-1324.
- Steiner, A.A., Chakravarty, S., Rudaya, A.Y., Herkenham, M. and Romanovsky, A.A. (2006). Bacterial lipopolysaccharide fever is initiated via Toll-like receptor 4 on hematopoietic cells. *Blood*. 107(10): 4000-4002.
- Sunayana, M.R. and Reddy, M. (2015). Determination of Keto-deoxy-d-manno-8-octanoic acid (KDO). *Bio-protocol*. 5(24): e1688. <https://doi.org/10.21769/BioProtoc.1688>.
- van der Pol, L., Stork, M. and van der Ley, P. (2015). Outer membrane vesicles as platform vaccine technology. *Biotechnology Journal*. 10(11): 1689-1706.
- Williams, L.K., Ownby, D.R., Maliarik, M.J. and Johnson, C.C. (2005). The role of endotoxin and its receptors in allergic disease. *Annals of Allergy, Asthma and Immunology* : Official Publication of the American College of Allergy, Asthma and Immunology. 94(3): 323-332.
- Xiong, K., Zhu, C., Chen, Z., Zheng, C., Tan, Y., Rao, X. and Cong, Y. (2017). Vi capsular polysaccharide produced by recombinant *Salmonella* enterica serovar paratyphi a confers immunoprotection against Infection by *Salmonella* enterica serovar typhi. *Frontiers in Cellular and Infection Microbiology*. 7: 135.