Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2021.046

RESEARCH ARTICLE

Purification and Antigenic Detection of Lipopolysaccharides of *Salmonella enterica* Serovar Typhimurium Isolate from Faisalabad, Pakistan

Mujtaba Hassan[1], Aamir Ali[1]*, Abrar Ahmad[1], Muhammad Kashif Saleemi[2], Muhammad Wajid[1], Yasra Sarwar[1] and Mazhar Iqbal[1]

[1]National Institute for Biotechnology and Genetic Engineering College, Pakistan Institute of Engineering and Applied Sciences (NIBGE-C, PIEAS), Faisalabad, Pakistan; [2]Department of Pathology, University of Agriculture, Faisalabad, Pakistan
*Corresponding author: myaamirali@yahoo.com

ARTICLE HISTORY (20-584)

Received: November 14, 2020
Revised: March 17, 2021
Accepted: March 26, 2021
Published online: June 10, 2021

Key words:
Conjugate vaccine
Lipopolysaccharides
Poultry
Salmonella Typhimurium

ABSTRACT

Genus *Salmonella* of the bacterial family Enterobacteriaceae contains clinically important *Salmonella enterica* species comprising more than 2600 serovars which cause infections in both humans and animals particularly in poultry, mounting a major public health concern. Due to fecal-oral route transmission of *Salmonella*, improving sanitation and hygienic conditions can control the disease. However, the target is difficult to achieve in densely populated developing countries like Pakistan, thus precise diagnosis and vaccination are essential. Eleven *Salmonella enterica* serovar Typhimurium were isolated from 26 *Salmonella* suspected poultry samples collected from local poultry farms. Different biochemical tests, genus and serovar-specific polymerase chain reactions lead to biochemical identification and molecular confirmation of the isolates. The nucleotide sequencing of the amplified products of 16S rRNA genes resulted in molecular confirmation and NCBI Genbank accession numbers MK041590 and MK041597. Large scale growth of the representative *S.* Typhimurium MAW1 isolate resulted in sufficient bacterial cell pellet. The purification of lipopolysaccharide (LPS) antigens of the local *S.* Typhimurium isolate yielded LPS as 53 mg/L of the culture broth. Hyper immune sera were raised in mice by formalin-killed whole-cell bacteria. The LPS endotoxicity was detected as >1000 EU/mL by Limulus Amebocyte Lysate assay and the antigenicity was found adequate by distinct precipitin line against the developed antisera using immuno-diffusion assay. The work will be helpful in making the first polysaccharide-protein based conjugate vaccine candidate against the local *S.* Typhimurium isolate.

To Cite This Article: Hassan M, Ali A, Wajid M, Ahmad A, Saleemi MK, Sarwar Y and Iqbal M, 2021. Purification and antigenic detection of lipopolysaccharides of *Salmonella enterica* serovar Typhimurium isolate from Faisalabad, Pakistan. Pak Vet J, 41(3): 434-438. http://dx.doi.org/10.29261/pakvetj/2021.046

INTRODUCTION

The *Salmonella* infections are responsible for substantial economic losses to the poultry industry (Dar *et al.*, 2017). The important zoonotic serovars infecting both humans and animals includes mainly *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) which is one of the most prevalent serotypes isolated from poultry meat (Hendriksen *et al.*, 2011). Fatal invasive diseases such as meningitis, sepsis and bacteremia have also been reported by *S.* Typhimurium particularly in countries with poor sanitation (Chiu *et al.*, 2020). Salmonellosis often occurs through contaminated food, especially food products with an animal origin and *Salmonella* are isolated more often from poultry and poultry products than from any other food animals (Menghistu *et al.*, 2011; Saha *et al.*, 2012). Although different vaccines have been tested against *S.* Typhimurium infections in model organisms still no effective prophylactic vaccine against invasive non-typhoidal *Salmonella* (NTS) is available (Park *et al.*, 2020). Though most of the developed countries have controlled it through improved sanitation and personal hygiene still it is a serious health concern in low and middle income countries. In developing countries, *S.* Typhimurium infection is mostly controlled through the extensive use of broad-spectrum antibiotics in poultry

feed. The extensive use of antibiotics in animal feed to promote the growth of food animals not only results in the development of multi-drug-resistant (MDR) strains but also spread of the resistance genes among zoonotic *Salmonella* serovars (Eng *et al.,* 2015). Therefore, in developing countries the only option for its prevention is, effective vaccination. Instead of protein antigens, the major protective antigens of most Gram negative bacteria including *S*. Typhimurium are surface polysaccharides (Salman *et al.,* 2017). The surface lipopolysaccharides (LPS) constitute the outer membrane that covers ~40% of the bacterial surface and are the key antigenic tools used in the development of effective vaccines (Weintraub, 2003). Conjugate vaccine strategy is used by fusing these bacterial surface polysaccharides with a carrier protein resulting in increase in their molecular size with benefits of improved immunogenicity and induction of memory cell formation (Rondini *et al.,* 2015). The current study was designed to isolate and identify *S*. Typhimurium from local poultry farms using biochemical and molecular approaches, followed by purification and antigenic evaluation of surface LPS of the local *S*. Typhimurium isolate. These LPS has the potential to be used in preparation of conjugate vaccine candidates against *S*. Typhimurium infections.

MATERIALS AND METHODS

Clinical Isolates and Biochemical Identification: Poultry samples (n=26) including liver, intestine and ova, suspected for *Salmonella* infections based on postmortem lesions (abnormal enlargement of liver with necrotic foci, enflamed spleen and peritonitis and off-white diarrhea), belonging to different commercial poultry farms of Faisalabad (Samundri, Jaranwala and Faisalabad) were collected directly and from Diagnostic Laboratory, University of Agriculture, Faisalabad after approval from the Institutional Bioethical Committee. For isolation and identification of *Salmonella* from collected clinical samples, the standard procedure according to ISO-6579-2017 (ISO, 2017) was followed. Briefly, the 15 mg of each minced sample was pre-enriched as 1:10 in buffered peptone water (Cat # CM0509 Oxoid, UK) followed by selective enrichment of *Salmonella* as 1:100 in Rappaport-Vassiliadis Soya (RVS, Cat # CM0866 Oxoid, UK) broth and the bacterial growth was streaked on plates of Salmonella-Shigella (SS) agar (Cat # CM0099 Oxoid, UK). The well-isolated colonies were sub-cultured on xylose-lysine-deoxycholate (XLD) agar (Cat # CM0469 Oxoid, UK) plates and on CHROM agar Orientation™ (Cat# RT412 CHROMagar, France). Incubation in each of the media was done at 37°C for 16-20 hr except RVS for which the broth was kept at 42°C for 24 hrs. The colonies showing typical *Salmonella* characteristics on CHROM agar were biochemically identified using triple sugar iron (TSI) agar (Cat # CM0277 Oxoid, UK) slants and by remel RapID ONE biochemical identification kit (Cat R8311006, ThermoFisher Scientific). The small chambers of the kit containing different substrates were inoculated with bacterial cultures and incubated at 37°C for 4 hrs and the results were interpreted according to the manufacturer's instructions using ERIC software.

Molecular confirmation of *S.* Typhimurium: For molecular confirmation of the isolates, the genomic DNA was extracted. For genomic DNA extraction, the biochemically identified isolated colonies of *Salmonella* were picked and inoculated in 3 mL of tryptic soya broth (TSB, Cat # CM0129 Oxoid, UK) and incubated at 37°C overnight. DNA was extracted from the overnight cultures by chloroform-isoamyl alcohol method (Sambrook *et al.,* 1989) and quantified using NanoDrop spectrophotometer (Thermo scientific). The polymerase chain reaction (PCR) was used to target *Salmonella* genus-specific *invA* gene fragment following already reported protocol (Wajid *et al.,* 2019a) followed by targeting serovar *S*. Typhimurium-specific *stm* gene fragment using PCR reaction mixture and thermal cycler conditions reported earlier (Liora *et al.,* 2013). The PCR amplified products were electrophoresed on 1.5% agarose, stained with ethidium bromide (0.5 µg/ml) and photographed under UV illumination (Eagle Eye, Strategene, USA). For further confirmation of the representative isolates, the 16S rRNA gene was targeted (1.5 kb) by PCR (Janda and Abbott, 2007) and the PCR amplicons were purified and shipped to Macrogen, Korea for commercial DNA sequencing. The received DNA sequences were compared with already submitted sequences using online NCBI BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and two representative sequences of 16S RNA gene were submitted to the GenBank database to get accession numbers. The sequences were subjected to online software Clustal W for sequence alignment and the aligned sequences were processed as the input for MEGA6 software to draw the phylogenetic tree.

Extraction and purification of LPS antigens: For LPS extraction, one representative *S*. Typhimurium isolate (MAW1) was grown in shake flasks. To prepare inoculum, the isolate was cultured in 200 mL of TSB in 1L flask and incubated at 37°C for 10 hrs with 180 rpm shaking. The 50 mL of the inoculum was transferred to each of the 2 L flasks containing 950 mL of TSB and allowed to grow for 12 hrs at 37°C and 180 rpm. The culture was inactivated with 1% formalin and kept stirring at 100 rpm overnight and harvested by centrifugation at 7000 rpm for 20 min and the pellet was stored at -20°C till further use. The cultures were grown in 16 flasks using 4 batches. The LPS was extracted from the harvested cell pellet by hot phenol method as previously described (Ali *et al.,* 2014). Briefly, the cell pellet (75 gram) was suspended in 750 mL of distilled water by stirring at room temperature for 3 hrs. Then 90% phenol was added in equal amount to cell suspension and vortexed vigorously at 68°C for 30 min and allowed to cool down by stirring on ice for 15 min. The mixture was centrifuged at 7000 rpm at 10°C for 30 min and water layer was carefully separated. Added an equal amount of distilled water (equal to separated water layer) to the remaining organic layer and repeated the step of vortex at 68°C, cooling and centrifugation two more times. The water layers of 3 batches were combined and centrifuged at 8000 rpm for 50 min at 10°C to separate the supernatant carefully. The supernatant was brought to 0.01 M sodium acetate, 0.002 M calcium chloride and 25% ethanol, mixed well and stored at 4°C overnight for nucleic acid precipitation.

The mixture was centrifuged at 10,000 rpm for 1 hr at 10°C and supernatant was taken carefully. The supernatant was brought to 75% ethanol (for LPS precipitation), mixed well and stored overnight at 4°C. The clear upper layer of the solution was aspirated carefully and the remaining layer containing LPS was centrifuged at 10,000 rpm for 1 hr at 10°C. The pellet was taken and dissolved in pyrogen free de-ionized water. The LPS solution was dialyzed (cut off 6000–8000 Daltons) against de-ionized distilled water for 3 days at 4°C with two water changes daily and lyophilized as crude LPS. The lyophilized LPS were dissolved in deionized water and ultracentrifuged at 35000 rpm for 4 hrs at 10°C. The pellet was dissolved in deionized water and freeze-dried as purified LPS.

Endotoxin testing and antigenicity evaluation of the purified LPS: The endotoxicity of the purified *S.* Typhimurium LPS was tested by Limulus Amebocyte Lysate (LAL) assay using gel-clot method (Levine and Bang 1964). The control standard endotoxin (CSE 4037-5006, lot # G-78USA) and the LAL reagent PYROSTAR™ ES-F (lot # 11910) were obtained from FUJIFILM, Wako Chemicals, USA and assay was performed according to the manufacturer's instructions. Briefly, reconstituted the CSE in 4.5 ml of sterile water to get 1000 endotoxin units per mL (EU/mL) solution and further diluted in sterile water to get 100, 10, 1, 0.5, 0.25 and 0.125 EU/mL concentrations in duplicate glass tubes. Two-fold dilutions of the purified LPS were made in duplicate using sterile water. A 100 µL volume of each standard and sample dilution was mixed with 100 µL of the LAL reagent in a glass test tube and placed at 37°C for 1 hr. The tubes were observed and the formation of gel-clot that remained intact after inversion to 180 degree was considered as positive (United States Pharmacopeia, 2019).

For antigenic evaluation of the purified LPS using immunodiffusion assay, we needed standard hyper-immune sera. The standard hyper-immune sera were raised in mice following reported protocol. Briefly, the formalin-killed whole cell culture of *S.* Typhimurium was injected to 8-week-old mice for three consecutive weeks with 3 doses/week on alternate days. Blood was drawn by euthanizing the mice, one week after final injection and the serum was collected and stored at -20°C in eppendorf tubes. The serum was examined for the presence of antibodies against *S.* Typhimurium purified LPS through immunodiffusion assay. For immunodiffusion assay, petri plate coated with agarose (1% in normal saline) was used (Ouchterlony and Nilson). After agarose solidification, circular wells were created and the raised antiserum was added (20 µL) in the central well. Different concentrations (125, 250, 500, 750 and 1000 µg/mL) of the purified LPS were added (20 µL each) in the wells surrounding the central antisera well. The petri plate was kept overnight at 4°C under moist conditions. The plates were stained with Coomassie blue to see the precipitin lines to ensure the antigenicity of the purified LPS (Ali *et al.*, 2014).

RESULTS

Bacterial isolates: From the 26 collected samples, 11 were suspected as *Salmonella* based on biochemical tests including distinctive colony morphologies on different agar media i.e, colorless colonies with black center on SS agar (Fig. 1-A); pink colonies with black center on XLD agar (Fig. 1-B) and whitish colonies on CHROM agar plates (Fig. 1-C). The inoculated TSI agar slants showed characteristic *Salmonella*-specific reactions as yellow butt, pinkish slant with central blackening due to H_2S production (Fig. 1-D). The typical reaction on remel RapID ONE identification system resulted in detection microcode 0320011, identifying the isolate as *Salmonella* (Fig. 1-E).

Molecular confirmation of *S.* Typhimurium isolates: The PCR targeting *Salmonella* genus-specific gene identified these isolates as belonging to genus *Salmonella* (Fig. 2-A) by amplification of 284 bp fragment of *invA* gene. The PCR for identification of *S.* Typhimurium targeting serovar Typhimurium-specific *stm* gene fragment (401 bp) identified 4 isolates as *S.* Typhimurium (Fig. 2-B). The PCR targeting 16S rRNA gene resulted in amplification of 1500 bp fragment (Fig. 2-C). The DNA sequencing results of the 16S rRNA gene amplicons from two representative *S.* Typhimurium isolates confirmed their identity with MK041590 and MK041597 accession numbers and the phylogenetic tree is shown in Fig. 3.

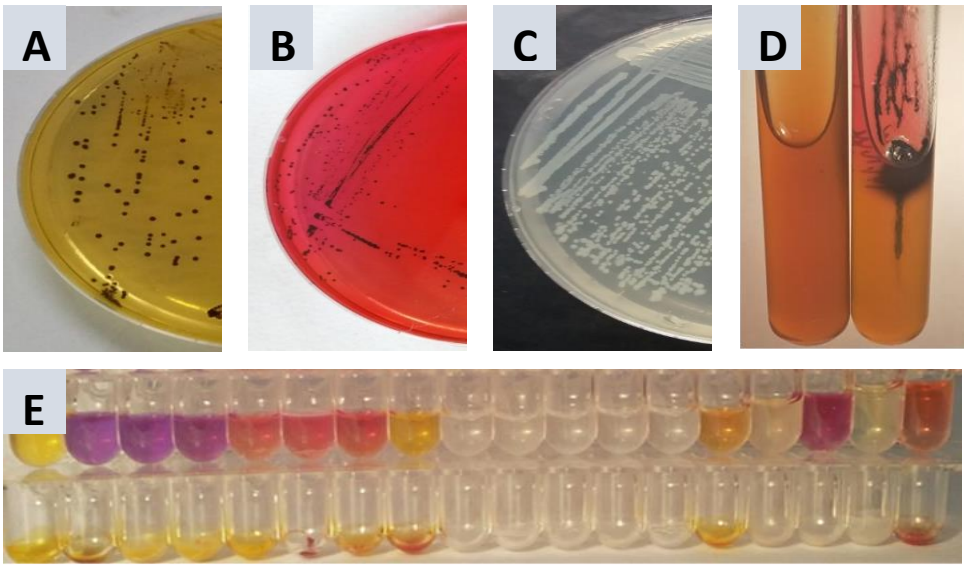

Fig. 1: Biochemical identification of *Salmonella* isolates. Typical growth of *Salmonella* colonies on SS agar plate (A), XLD agar plate (B) and CHROMagar plate (C). Typical reaction of *Salmonella* in TSI agar (D) slant (acidic yellow butt, alkaline pink slant, central blackening) as compared to the adjacent negative control tube. Typical reaction of *Salmonella* in remel RapID ONE kit upper wells panel while the lower row panel is showing the wells without bacterial culture.

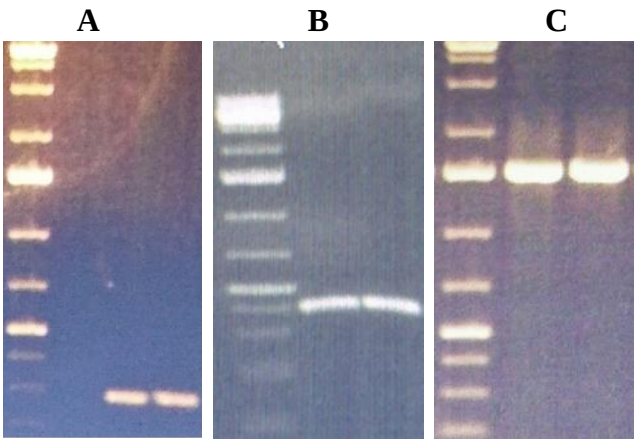

Fig. 2: Molecular confirmation of *S*. Typhimurium isolates by PCR. (A) Amplified product (284 bp) of *Salmonella* genus-specific gene (*invA*), (B) Amplified product (401 bp) of *S*. Typhimurium serovar-specific gene (*stm*) and (C) Amplified product (1500 bp) of *S*. Typhimurium 16S rRNA gene for DNA sequencing.

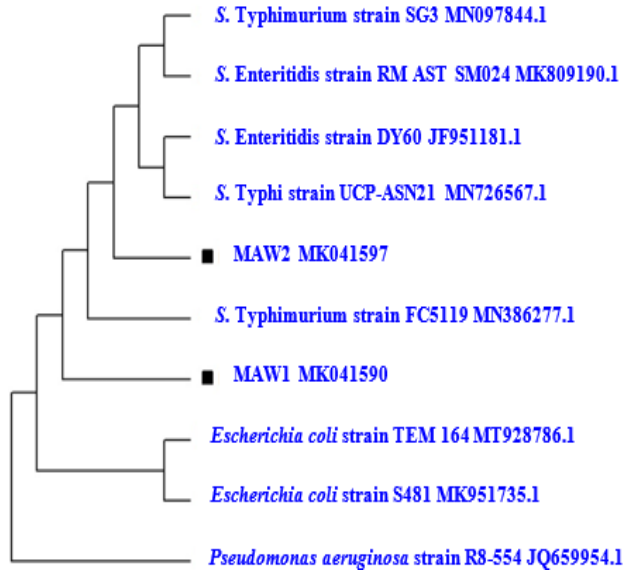

Fig. 3: Phylogenic tree of the *S*. Typhimurium isolates. The tree showed the genetic relatedness of the local isolates MAW1 and MAW2 with *S*. Typhimurium and other bacteria.

Endotoxin testing and antigenic evaluation of the purified *S*. Typhimurium LPS: The harvesting of bacterial cell pellet from growth in shake flasks (16 L) yielded wet bacterial pellet of 75 gram. Hot phenol LPS extraction from all of the wet bacterial cell pellet, yielded 1.2 gram of the crude LPS. After purification steps of ultracentrifugation and freeze drying, the yield of the purified *S*. Typhimurium LPS was 850 mg. The endotoxin testing of the LPS detected >1000 EU/mL in the purified *S*. Typhimurium LPS. For antigenic evaluation of the purified LPS, the required standard hyper-immune sera were developed in mice at "NIBGE Animal House Facility" as described earlier. The developed standard hyper immune sera, when coated in central well surrounded by wells containing different concentrations of the purified LPS, resulted in clear precipitation lines particularly against the 250, 500 and 1000 µg/mL concentrations of the purified LPS ensuring the intact antigenicity of the LPS (Fig. 4).

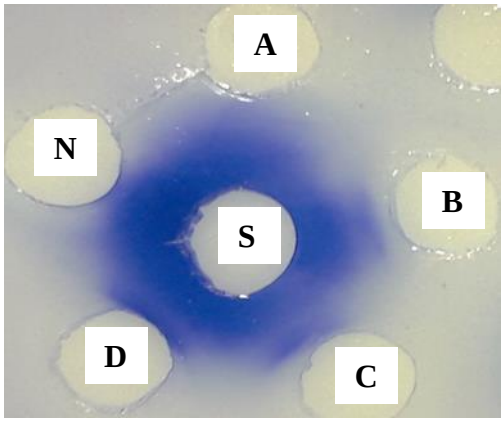

Fig. 4: Antigenicity evaluation of the purified LPS of *S*. Typhimurium. Central well S contained the developed hyper immune sera from mice; the well N is negative control contained only saline; the wells A, B, C and D contained 125, 250, 500 and 1000 µg/mL of the purified LPS of *S*. Typhimurium showing clear precipitin lines against the central well.

DISCUSSION

Salmonella is an important foodborne pathogen causing widespread contaminations and *S*. Typhimurium is among the leading *Salmonella* serovars responsible for animal and human salmonellosis worldwide (Wang *et al.*, 2019). *S*. Typhimurium infects both broiler and laying chicken farms of all ages, thereby contaminating the food chain through various infectious routes, mainly eggs and meat (Dar *et al*., 2017). Therefore, an efficient control involving precise detection of *S*. Typhimurium infections in poultry is necessary to make a healthy environment for the birds, ensure food safety to build the confidence of consumers and to meet the international regulations for trade of poultry products. Early and precise diagnosis of any infection plays a key role in its effective control and the molecular detection methods including PCR ensure the rapid molecular confirmation of the prevailing infectious agents with high accuracy. The *invA* gene has been targeted by various researchers for identification of bacterial genus *Salmonella* (Yanestria *et al.*, 2019) and *stm* gene for serovar *S*. Typhimurium (Liora *et al.*, 2013; Wajid *et al.*, 2019b). The PCRs used in current study targeting *invA*, *stm* and 16S rRNA genes not only confirmed the *S*. Typhimurium identity but also described it phylogeny. No effective prophylactic vaccine against invasive NTS is available (Park *et al*., 2020) particularly against local isolates of *S*. Typhimurium. Traditional vaccine strategies do not work as the surface polysaccharides, instead of proteins, are major antigenic determinants of *S*. Typhimurium (Micoli *et al.*, 2014). These polysaccharide antigens when used alone are very poor immunogens because of their T-cell independent nature, therefore conjugate vaccines are synthesized in a way that involves attachment of these polysaccharides with a carrier protein, making the host immune system to recognize the polysaccharide-protein conjugate as a T-lymphocyte dependent antigen. This induces the involvement of specific T-lymphocytes resulting in production of memory cells leading a long lasting immune response (Weintraub, 2003; Ali *et al.*, 2014). Recently, a biologically-conjugated polysaccharide vaccine candidate has shown the enhanced homologous protection against *S*.

Typhimurium and *E. coli* (Han *et al.*, 2021). The advantage of conjugate vaccine is that it mediates opsonophagocytic antibody production which can kill complement-sensitive as well as complement-resistant *Salmonella* strains including *S*. Typhimurium (Gondwe *et al.*, 2010). The efficacy of the conjugate vaccine candidate against *S*. Typhimurium can also be enhanced using a self-adjuvanting carrier protein such as bacterial flagellin (Chiu *et al*., 2020).

S. Typhimurium is prevalent among local poultry farms of Faisalabad region and has been reported earlier (Wajid *et al.*, 2019b), however, purification and antigenic evaluation of LPS antigens of local *S*. Typhimurium from any host has not been explored before. The isolation, identification and molecular confirmation of *S*. Typhimurium isolates were successfully executed followed by extraction and purification of the LPS antigens. The antigenicity of the purified LPS was found adequate as evident precipitin lines were detected between the purified LPS and the raised hyper immune sera, thus promising their suitability for preparation of conjugate vaccine candidates using the local poultry isolate of *S*. Typhimurium.

Acknowledgments: The research work was supported by Higher Education Commission (HEC), Islamabad, Pakistan under National Research Program for Universities, Project # 6111.

Authors contribution: MH and AA performed the fermentation, LPS purification and antigenicity experiments, MW and ABA performed biochemical and molecular identification, MKS performed clinical diagnosis and sample collection, YS performed the microbiology and data analysis. AA and MI also conceived, designed and supervised the overall study. All of the authors have contributed in writing and refining the manuscript with intellectual inputs and accepted the final version.

REFERENCES

Ali A, An SJ, Cui C, *et al.,* 2014. Synthesis and immunogenicity evaluation of *Salmonella enterica* serovar Paratyphi A O-specific polysaccharide conjugated to diphtheria toxoid. Hum Vaccines Immunother 10:1494-8.

Chiu TW, Peng CJ, Chen MC, *et al.,* 2020. Constructing conjugate vaccine against *Salmonella* Typhimurium using lipid-A free lipopolysaccharide. J Biomed Sci 27:1-14.

Dar M, Ahmad S, Bhat S, *et al.,* 2017. *Salmonella* Typhimurium in poultry: a review. World Poultry Sci J 73:345-54.

Eng SK, Pusparajah P, Ab Mutalib NS, *et al.,* 2015. *Salmonella*: a review on pathogenesis, epidemiology and antibiotic resistance. Front Life Sci 8:284-93.

Gondwe EN, Molyneux ME, Goodall M, *et al.,* 2010. Importance of antibody and complement for oxidative burst and killing of invasive nontyphoidal *Salmonella* by blood cells in Africans. Proc Natl Acad Sci 107:3070-5.

Han Y, Luo P, Chen Y, *et al.,* 2021. Regulated delayed attenuation improves vaccine efficacy in preventing infection from avian pathogenic *Escherichia coli* O78 and *Salmonella* Typhimurium. Vet Microbiol 254:109012.

Hendriksen RS, Vieira AR, Karlsmose S, *et al.,* 2011. Global monitoring of *Salmonella* serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: results of quality assured laboratories from 2001 to 2007. Foodborne Pathog Dis 8:887-900.

ISO, 2017. International Organization for Standardization. Microbiology of the food chain-horizontal method for the detection, enumeration and serotyping of *Salmonella*-part 1:detection of *Salmonella* spp. ISO, Geneva, Switzerland pp:6579-1.

Janda JM and Abbott SL, 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J Clin Microbiol 45:2761-4.

Levine J and Bang FB, 1964. The role of endotoxin in the extracellular coagulation in the Limulus. Bull Johns Hopkins Hosp 115:265-74.

Liora M, Mihaiu M, Tabaran A, *et al*., 2013. Antimicrobial resistance evaluation of pathogen *Salmonella* strains isolated in pork and poultry meat. Bulletin UASVM Vet Med 70:266-70.

Menghistu HT, Rathore R, Dhama K, *et al.,* 2011. Isolation, identification and polymerase chain reaction (PCR) detection of *Salmonella* species from field materials of poultry origin. Int J Microbiol Res 2:135-42.

Micoli F, Ravenscroft N, Cescutti P, *et al.,* 2014. Structural analysis of O-polysaccharide chains extracted from different *Salmonella* Typhimurium strains. Carbohydr Res 385:1-8.

Ouchterlony O and Nilson L, 1986. Immunodiffusion and Immunoelectrophoresis. In: Weir DM, Herzenberg LA, Blackwell C, Herzenberg LA, eds Handbook of Experimental Immunology ed. Oxford: Blackwell 32.1-32.50.

Park S, Jung B, Kim E, *et al.,* 2020. *Salmonella* Typhimurium lacking YjeK as a candidate live attenuated vaccine against invasive *Salmonella* infection. Front Immunol 11:1277.

Rondini S, Micoli F, Lanzilao L, *et al.,* 2015. Design of glycoconjugate vaccines against invasive African *Salmonella enterica* serovar Typhimurium. Infect Immun 83:996-1007.

Saha A, Sufian M, Hossain M, *et al.,* 2012. Salmonellosis in layer chickens: pathological features and isolation of bacteria from ovaries and inner content of laid eggs. J Bangladesh Agric Univ 10:61.

Salman M, Michael FS, Ali A, *et al.,* 2017. First characterization of immunogenic conjugates of Vi negative *Salmonella* Typhi O-specific polysaccharides with rEPA protein for vaccine development. J Immunol Methods 450:27-33.

Sambrook J, Fritsch EF and Maniatis T, 1989. Molecular cloning: a laboratory manual: Cold Spring Harbor Laboratory Press.

United States Pharmacopeia, 2019. General Chapter 85, Bacterial Endotoxins Test (BET).

Wajid M, Awan AB, Saleemi MK, *et al.,* 2019b. Multiple drug resistance and virulence profiling of *Salmonella enterica* serovars Typhimurium and Enteritidis from poultry farms of Faisalabad, Pakistan. Microb Drug Resist 25:133-42.

Wajid M, Saleemi MK, Sarwar Y, *et al.,* 2019a. Detection and characterization of multidrug-resistant *Salmonella enterica* serovar Infantis as an emerging threat in poultry farms of Faisalabad, Pakistan. J Appl Microbiol 127:248-61.

Wang X, Biswas S, Paudyal N, *et al.,* 2019. Antibiotic resistance in *Salmonella* Typhimurium isolates recovered from the food chain through national antimicrobial resistance monitoring system between 1996 and 2016. Front Microbiol 10:985.

Weintraub A, 2003. Immunology of bacterial polysaccharide antigens. Carbohydr Res 338:2539-47.

Yanestria SM, Rahmaniar RP, Wibisono FJ, *et al.,* 2019. Detection of *invA* gene of *Salmonella* from milkfish (*Chanos chanos*) at Sidoarjo wet fish market, Indonesia, using polymerase chain reaction technique. Vet World 12:170-5.