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ORIGINAL PAPER

Isolation and purification of O and H antigens from *Salmonella* Enteritidis as diagnostic tool

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Abstract Lipopolysaccharide (LPS) and flagellin are the major antigenic structures of *Salmonella* Enteritidis. Purified antigens are essential components for the diagnosis of and research on *Salmonella* Enteritidis. In this study, LPS were isolated and purified with modified hot phenol–water procedure in a yield of 6%. The flagellin isolation and purification were done by strong shaking with glass pearl beads with 3.9 mg/ml protein content. Purity was demonstrated on SDS-PAGE, ELISA and with colorimetric assay for both isolated antigens. The antigen-mediated ELISA results exhibited immunoreactive properties for both LPS and flagellar antigen specific for *Salmonella* Enteritidis O:9 and H:g,m, respectively. The findings demonstrated low reactivity with Mab O:9 for isolated LPS due to their mono-epitopic specificity. However, isolated flagellin reactivity was high with Mab H:g,m related to their cross-reactivity potential between H:g and H:m. Additionally in this study. the results indicated that similar antigenic structures have alternative potential for antigenic isolation of *Salmonella* Enteritidis, such as *Salmonella* Gallinarum for LPS antigen and *Salmonella* Adeyo for flagellar antigen isolation.

Keywords *Salmonella* Enteritidis · LPS · Flagellin · Isolation · Purification

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Introduction

Salmonellas are widespread in the environment worldwide, resulting in human and animal disease and costing many billions of pounds each year. *Salmonella* Enteritidis, however, only rose to its predominant position in man and poultry in the mid-1980s as a result of vertical and horizontal transmission within and between large poultry organizations in many parts of the world (Calnek et al. 1996; Davision 1996; Davies et al. 1997; OIE 2008). The ability of bacterial cells to invade organs after oral inoculation of a host is an indication of virulence. Virulence is a complex phenomenon that is influenced by a number of molecules called virulence factors. Consequently, epidemics due to virulent organisms occur sporadically, i.e., when conditions that result in the expression of virulence factors occur. Due to the number of virulence factors and the complexity of their expression, it is unusual for a single factor on the outer membrane to be a reliable predictor of virulence. Recent investigations of *Salmonella* Enteritidis have indicated that the lipopolysaccharide (LPS) is a virulence factor, and have also indicated that a particular structure of the LPS molecule might be a reliable indicator of virulence potential (Barrow 1996; Rahman et al. 1997). LPS molecules consist of a bisphosphorylated lipid (lipid A) forming the matrix of the outermost membrane leaflet

which is stabilized by divalent cations, and a hydrophilic polysaccharide (PS) extending outward from the bacterium. The PS consists generally of two distinct regions, a core oligosaccharide containing 10–12 sugars, and a polysaccharide chain of repeating units, the O-specific chain. The core is covalently bound through an acidic sugar, usually 3-deoxy-D-manno-octulopyranosonic acid (Kdo), to the lipid A. Although also present in some capsular polysaccharide structures, Kdo seems to be a characteristic and essential sugar for the great majority of endotoxins. The proximal "inner core" region also contains heptoseresidues which are often substituted by phosphate, pyrophosphate or diphosphoethanolamine. The "outer core" region usually consists of neutral or amino hexoses such as D-glucose, D-galactose, D-glucosamine, D-galactosamine or *N*-acetyl derivatives. Wild-type enterobacterial species with O-chains are termed "smooth" (S) because of the morphology of their colonies. Enterobacterial mutants producing rough-looking colonies and lacking LPS O-chains are accordingly termed "rough" (R), and their LPSs designated Ra, Rb… Re, in the order of decreasing core length (Caroff and Karibian 2003; Caroff et al. 2002; Mitov et al. 2003; Rahman et al. 1997; Brooks et al. 2008). Salmonella serotypes A, B, and Dl, share a common trisaccharide repeating unit α-D-mannose-(1,4)-α-L-rhamnose-(1,3)-α-D-galactose for the 0-antigen but differ in their branching carbohydrate moieties,α-(3,6)-dideoxyhexose α-(1,3)-linked to D-mannose. Dideoxyhexoses are paratose (serogroup A), abequose (serotype B), or tyvelose (serotype D1), and reflect the correlation of serotype classification and chemical structure of the 0-antigenic repeats of *Salmonella* (Steinbacher et al. 1996). Many diagnostic tests have been developed for the detection of salmonella infections in poultry. Conventional methodology for identifying *Salmonella* is based on the isolation in pure cultures, and biochemical and serological tests (Fitzgerald et al. 2003; Schneid et al. 2005; Sonne-Hansen and Jenabian 2005). However, culture is expensive and time-consuming and also suffers because individual birds excrete *Salmonella* Enteritidis intermittently or may eliminate the infection altogether. Serology is the other component of field and laboratory testing of flocks to establish their *Salmonella* Enteritidis status. Because of decreased costs and rapid turnaround time, serology has developed into a promising screening tool for flocks. *Salmonella* Enteritidis is an invasive serotype, and immunoglobulin G (IgG) responses persist in birds that have been infected with *Salmonella* Enteritidis. Therefore, serology would be a superior method for screening birds that are intermittently culture-positive or that have eliminated *Salmonella* Enteritidis infection (McDonough et al. 1998; Schneid et al. 2005). The most used diagnostic tests are based on the detection of antibodies against the "O" chain of the S-LPS and/or flagellin (Barrow 1994; Ochoa-Reparaz et al. 2005). Serological identification and classification techniques of *Salmonella* are based on O and H antigens according to the Kauffmann–White scheme (Fitzgerald et al. 2003; Iankov et al. 2002). Today, the development of novel reagents for diagnostics has become economically important for the evaluation of more reliable diagnostic kits.

The objectives of the present study were to isolation, identification and purification of *Salmonella* Enteritidis O and H antigens for the various applications of diagnostic tools and to examine the use of these antigens to define the serological specificities.

Materials and methods

Bacterial strains and growth conditions

Salmonella Enteritidis 64 K (1, 9, 12: g,m) strain, which was used for extracting LPS antigen, was kindly provided by Dr K. Tayfun Carli from Uludag University, Faculty of Veterinary Medicine, Bursa, Turkey. *Salmonella* Adeyo (16:g,m), *Salmonella* Oranienburg (6,7:m,t), *Salmonella* Typhimurium (1,4,5,12:-) and *Salmonella* Senftenberg [1,3,19:g,(s),t] strains were kindly provided by the National Center of Infectious and Parasitic Diseases (NCIPD), Bulgaria, and were used for the preparation of flagellar antigens. *Salmonella* Adeyo and *Salmonella* Oranienburg were used for the isolation of flagella and *Salmonella* Typhimurium and *Salmonella* Senftenberg were used for the isolation of LPS in the confirmation studies of the isolated antigens. Cultures were maintained by three replicating subcultures through a single colony to enhance the purity, identity and homogeneity of the culture. After that, a streaked single colony checked by Gram stain technique was used in stock culture preparation. *Salmonella* Enteritidis was grown in Brain Heart Infusion broth (Becton Dickinson, USA) and a bioreactor was used for the LPS isolation as described by Nalbantsoy et al. (2007). *Salmonella* Adeyo, *Salmonella* Oranienburg, *Salmonella* Typhimurium and *Salmonella* Senftenberg were grown overnight on semi-solid beef extract agar (Difco, USA) used for the isolation of flagellar antigens. Flagella isolation was done as described briefly: bacteria inoculated into the center of Petri dishes (diameter 9 cm) and incubated at 37°C for 18–24 h in order to obtain the motility. If it is motile, the strain must cover the whole petri. Then, the samples were collected in 500 ml Nutrient broth and used as inoculums for larger Petri dishes (diameter ~15 cm). Next, 90–100 ml of 0.7% of semi-solid beef extract agar were dispersed for each larger Petri dish and inoculated with 5 ml prepared inoculums. After incubation at 37°C for 18–24 h, the samples were collected with physiologic saline, and 3–4 ml formalin (37-38%) was added

to kill the bacteria. Following the formalin treatment, the flagellin extract was obtained after checking for viability.

Isolation of the LPS

The LPS isolation was performed by modified Westpal procedure (Toman and Skultety 1996). The lyophilized *Salmonella* cells (1 g) were solubilized in 50 mM Tris-HCl buffer (100 ml, pH 7.5) and treated with 0.2 g $MgCl_2$, $6H_2O$, 0.5 mg bovine serum albumin (BSA; Sigma, USA), 100 μl RNase (100 μg/ml, EC 3.1.27.5) and 100 μl DNase (100 μg/ml, EC 3.1.21.1) both from bovine pancreas (Boehringer Mannheim, Germany) shaking at 37°C, then enzymes were inactivated for 10 min at 75°C and then treated with 100 μl trypsin (300 μg/ml EC 3.4.21.4, from bovine pancreas; Serva, Germany) for 90 min at 37°C. Enzyme inactivation has been repeated as described above, and 100 μl proteinase K (EC 3.4.21.14, from *Tritirachium album*; Sigma, USA) was added and incubated shaking overnight at 37°C. Enzyme inactivation was repeated and the suspension was centrifuged at 30,000 × *g* for 1 h. After the pellet was incubated in 100 ml acetone at room temperature, the cell suspension was centrifuged at 1,500 × *g* for 15 min. The cells were further extracted with chloroform–methanol (Sigma), (2:1, v/v) at 20°C overnight. The cell suspension was centrifuged at 1,500 × *g* for 20 min. Then, cells were suspended in preheated distilled water (50 ml, 68°C) and extracted with an equal volume of aq. 90% phenol for 20 min. at 68°C by shaking. The extracts were cooled on ice and the aqueous phase was collected after centrifugation at 1,500 × *g* for 20 min. The phenol phase was re-extracted with water twice and the combined aqueous phases were evaporated and dialyzed against distilled water for 3 days and freeze-dried.

Isolation of flagellin

Bacteria were grown on 0.7% semi-solid beef extract agar at 37°C for 24 h. Expression of H antigen was confirmed by routine serological methods which were used for flagellin isolation. After harvesting the bacteria in aq. 0.85% NaCl, the H:g,m antigen was prepared as described by Ivanova (1986). Briefly, *Salmonella* cell suspension in aq. 0.85% NaCl was centrifuged under refrigeration at 12,500 × *g* for 40 min. The sediment was resuspended in aq. 0.85% NaCl and shaken at high rpm vigorously with glass pearls beads for 1 h to get the material ready for centrifugation to separate the antigens in the supernatant from the cells. The suspension was centrifuged under refrigeration at 3,000 × *g* for 40 min. After centrifugation, sediment was used for isolation of LPS. Supernatant was concentrated with polyethylene glycol (PEG 40000 MW, Sigma) and then dialyzed (8,000–15,000 MW; Serva) against 0.05 M sodium acetic acid for 1–2 days. After coating the 96-well plate with the crude flagellin, the antigenic properties were checked by ELISA against flagella-specific antisera, and the final purity was obtained after column chromatography (Sephadex G 200; Pharmacia). Column length ~40 cm, diameter 3.8 cm, flow rate 0.2 ml/min provided by peristaltic pump, and 0.05 M sodium acetic acid (pH 5.8) buffer was supplied. Fractions were collected every 20 min and the fractions between 10 and 35 were combined. Combined fractions were concentrated with PEG. The protein content was determined by Bradford assay. The pure flagellins were kept at +4°C or freeze-dried.

Gel electrophoresis for LPS

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slabs containing 18% of polyacrylamide and the gels were silver-stained for LPS as described by Toman and Skultety (1992), and 5 μg of LPS was loaded to the gel. The SDS Molecular Weight Markers kit was purchased from Sigma.

Analytical methods for LPS

Estimation of 3-deoxy-D-manno-2-octulosonic acid (Kdo) residues was performed by a modified thiobarbiuric acid (TBA) assay, and protein content was estimated by a modified Lowry assay as reported by Toman and Skultety 1996. The LPS (500 μg) was hydrolyzed in 0.1 ml 2 M trifluoroacetic acid (Sigma-Aldrich, USA) at 100°C for 2 h. The residue was dissolved in 0.1 ml 1 M ammonium hydroxide (Sigma-Aldrich) solution containing 1 mg sodium borodeuteride and incubated at room temperature overnight. The dried residues were acetylated with acetic anhydride (50 μl)–pyridine (50 μl) mixture at 100°C for 1 h. Then water was added and solvents were removed by evaporation. The residues were extracted with chloroform–water (Merck Germany) (140 μl, 1:1, v/v) three times, the organic layers were pooled, dried (Na_2SO_4) and evaporated. The acetylated samples were finally dissolved in chloroform and immediately analyzed as follows: gas chromatography-mass spectrometry (GC-MS) of aditol acetates was performed on a Finnigan MAT SSQ 710 mass spectrometer using a fused-silica capillary column SP-2330 (30 m × 0.25 mm; Supelco, Bellefonte, USA). The column temperature programs were 80°C (2 min) to 245°C at 30°C/min with a 36 min hold at a helium gas flow rate of 25 cm s^{-1}. Electron impact mass spectra were recorded at electron energy of 70 eV, and an ion source temperature of 150°C.

Gel electrophoresis for flagellins

Purified flagellins were separated by SDS-PAGE in a 12% polyacrylamide gel as reported by Iankov et al. 2002. The bands were visualized using Commasie Brilliant blue dye.

ELISA

Indirect ELISA was used to confirm purity of antigens with LPS O:9 and H:g, H:m-specific monoclonal and polyclonal antisera which were obtained from NCIPD, Bulgaria. Briefly, 96-well polystyrene plates (Nunc, Denmark) were coated with purified LPS (2 μg per well diluted in 100 μl 0.05 M carbonate/bicarbonate buffer, pH 9.6) or flagellin (2 μg per well diluted in 100 μl 0.05 M acetate buffer). After overnight incubation at 37°C, the plates were washed three times. The diluted polyclonal and monoclonal antibody supernatants specific for O:9 and H:g, H:m (produced by Dr. Ivanova) were added to the coated LPS or flagellin wells. The plates were incubated at 37°C for 1 h, washed, and were incubated for 1 h after the addition of secondary anti-mouse IgG peroxidase (Sigma) and anti-rabbit IgG peroxidase (Sigma) conjugates. The enzyme reaction was developed with H_2O_2 (Merck, Germany) and *O*-phenylenediamine (Sigma) and stopped after 30 min by addition of 4 M H_2SO_4 (Riedel-de Haën, Seelze, Germany) per well. The optical density was measured at 492 nm.

Results and discussion

The incidence of human disease associated with food contaminated with *Salmonella* Enteritidis has increased over the last 20 years (Davies et al. 1997; OIE 2008). Traditional detection of *Salmonella* Enteritidis is labor-intensive, difficult and expensive as the culturing and identification of these bacterial isolates requires up to 1 week. These methods may differentiate among *Salmonella* species using specific antiserum at the confirmation stage (Brooks et al. 2008; Jaradat et al. 2004). *Salmonella* species are actually quite similar genetically, with the serotype differences based on surface antigen differences such as LPS and flagella (Kaufmann et al. 2001). Moreover, it must be taken into account that in vaccinology the golden standard should be one that does not interfere with serodiagnosis, and that in salmonellosis the most used diagnosis tests are based on detection of antibodies against the "O" chain of the S-LPS and/or flagellin (Ochoa-Reparaz et al. 2005). For this reason, many serological techniques for the rapid detection of *Salmonella* antigens have been evaluated with different antigens such as lipopolysaccharide (LPS), flagellin (Brooks et al. 2008; Fitzgerald et al. 2003; Schneid et al. 2005; Solano et al. 2000; Sonne-Hansen and Jenabian 2005).

In this paper, we describe the preparation of *Salmonella* Enteritidis major antigens, LPS and flagella for the various applications. LPS is an integral component of the outer membrane of Gram-negative bacteria and a major virulence determinant of most Gram-negative bacterial pathogens (Brooks et al. 2008). In this study, the LPS was isolated from the purified cells by a conventional hot phenol–water procedure in a yield of 6%. The purified LPS was obtained by treatment of RNase, DNase, trypsin and proteinase K and the LPS was found in the aqueous layer after hot phenol–water procedure due to the hydrophilic polysaccharide chain content. Then, after dialysis and lyophilization, white opaque LPS were obtained (Korhonen et al. 1985), and the isolated LPS protein content was calculated at 3.6%. Protein contamination was typically found between 1 and 3% per mg LPS by the hot phenol–water procedure examined previously (Korhonen et al. 1985). The LPS gave a single band (14 kDA) characteristic for the semi-smooth LPS on SDS-PAGE (Fig. 1). The LPS molecules had masses in the range of 2,000–20,000 Da and not in millions, as was estimated for their aggregates (Caroff et al. 2002). The SDS-PAGE profile of the LPS was low molecular weight (LMW) which is characteristic for the repeated few sugar units as described previously by Peter et al. (1999) and Brooks et al. (2008). In addition, Cox et al. (2005) prepared LPS from *Neisseria meningitidis* strain L3 *galE* (NRCC 4720) by the hot water–phenol method and mentioned that the method is efficient for the isolation of Ra LPS as well as smooth LPS.

The quantitative determination of Kdo residues by the TBA assay was calculated as 83 nmol/mg of the LPS. Determination of Kdo may, however, be useful to control the recovery of LPS at various stages of its isolation. Table 1 presents GC-MS analyses of the neutral sugar content of purified LPS.

The neutral sugar contents of isolated O-polysaccharide (O-PS), rhamnose, mannose, galactose and glucose were found to share a common structural oligosaccharide repeating unit in agreement with that previously described (Brooks et al. 2008; Hitchcock and Brown 1983; Nıkaıdo 1962; Steinbacher et al. 1996; Lüderitz et al. 1971) as the *Salmonella* serogroup D1 O-PS antigen. On the other hand, ribose sugar residues were slightly contaminated with proteins which belong to the nucleic acid which can be removed by repeating RNase treatment of LPS. Besides, the

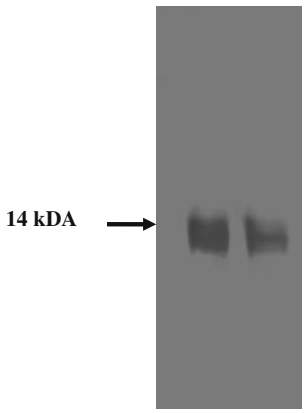

Fig. 1 SDS-PAGE silver stains of the LPS

Table 1 Neutral sugar composition of the LPS obtained from *Salmonella* Enteritidis 64 K

Composition in μg/mg LPS					
Rhamnose	Mannose	Galactose	Glucose	Heptose	Ribose
24.74	59.61	251.88	309.66	7.38	4.34

immunodominant sugar tyvelose that determines the serovar designation for group D1 *Salmonella* Enteritidis upon derivatization was not used to measure GC in this study as reported by Peter et al. (1999). Moreover, the ELISA analysis (Table 2) also confirmed the higher antibody level than the result given by Lüderitz et al. (1971) which related to the isolated semi-smooth or LMW LPS containing repeated and few sugar units (Brooks et al. 2008; Peter et al. 1995). These results reveal that the cultivation conditions affect the microorganisms phenotype performing as rough (R), smooth (S), rough-like or semi-smooth. Semi-smooth LPS were pathogenic unlike rough LPS or non-pathogenic strains LPS due to the LMW LPS structure content (Peter et al. 1999). Those observations indicate that the hot phenol–water procedure is suitable for the isolation of LPS from *Salmonella* Enteritidis. Furthermore, the investigators were used to isolate LPS successfully in Mab production. This part of study has also been submitted for publication (Fig. 2).

The development of immunoassay systems for the detection of S*almonella* requires specific high titers of flagellar antisera. Highly purified flagellin preparations are a prerequisite for such antiserum production. Procedures generally involved in bacteria propagation in complex media are followed by detachment of flagella by mechanical means with omni-mixers, blenders, and other apparatus that produce violent agitation (Ibrahim et al. 1985). In this study, the flagella were detached by shaking at high speeds with glass pearl beads, opposing the dissociation of flagella from *Salmonella* cells by reducing the pH to 2 with HCl as described by other investigators (Alexan et al. 2009; Baay and Veld 1993). Although the common method is to reduce the pH, it was not preferred for the isolation in this study because low pH could denaturize the structure of the flagellin. Moreover, the flagellin preparation contained O-antigen contamination, and purified flagellin showed with major and minor bands on SDS-PAGE when examined previously (Ibrahim et al. 1985). Herein, after isolating the purified flagellin, the protein content was determined to be 3.9 mg/ml by Bradford assay. The purified flagellin gave a single band (55 kDA) on SDS-PAGE which is different from the other investigators (Alexan et al. 2009; Ibrahim et al. 1985). Additionally, the purified flagellin did not contain *Salmonella* Enteritidis O-specific residue related to the strain used for isolation. Furthermore, the purified LPS and flagellin were characterized by antigen-mediated ELISA using specific antibodies against LPS and flagellin. The reactivity of antigens with antibodies is given in Table 2.

Table 2 Reactivity of the purified LPS and flagellin antigens with *Salmonella* Enteritidis O and H antigen-specific hyperimmune rabbit sera and monoclonal antibody supernatants by ELISA

	ELISA coated with purified LPS[a]		ELISA coated with purified flagellin	
Antibody	Pab[b], O:9	Mab[c], O:9	Pab[d], H gms	Mab[e], H g,m
Reactivity	2.762±0.056	0.610±0.049	2.594±0.035	1.743±0.023

[a] $O.D._{492\ nm}$

[b] H: g,m specific Rabbit polyclonal antibody provided from NCIPD, Bulgaria

[c] H: g,m specific monoclonal antibody supernatants provided from NCIPD, Bulgaria

[d] O:9 specific Rabbit polyclonal antibody provided from NCIPD, Bulgaria

[e] O:9 specific monoclonal antibody supernatants provided from NCIPD, Bulgaria

The antigen-mediated ELISA results exhibited immunoreactive properties for both LPS and flagellar antigen specific for *Salmonella* Enteritidis O:9 and H: g,m respectively. The findings demonstrated low reactivity with Mab O:9 for isolated LPS due to their mono-epitopic specificity. However, isolated flagellin reactivity was high with Mab H: g,m related to the cross-reactivity potential between H:g and H:m as described by Ivanova 1986. In addition, monoclonal antibody (Mab) was evaluated with prepared *Salmonella* Enteritidis flagella as described above using the conventional hybridoma method by Nalbantsoy et al. (2010). The ELISA analyses of developed Mab D7 clone was found to react with H:m in respect to their possible applications as diagnostic reagents. This result indicated that the isolation method as described above is very effective for the isolation of flagellar antigen and for further applications.

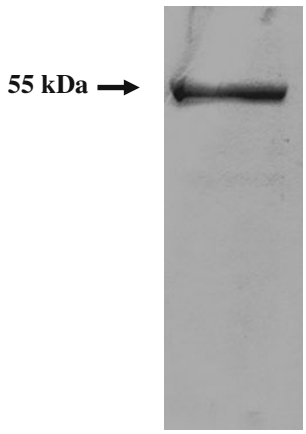

Fig. 2 SDS-PAGE commasie blue stains of the flagellin

In conclusion, methods were described for the preparation of purified O and H antigen against *Salmonella* Enteritidis which can be useful for the development of immunoassay or diagnostic reagents. The study demonstrates that the modified method for the isolation and purification of LPS and flagellin is beneficial for further research such as Mab production, development of diagnostic kit, etc. Additionally, since *Salmonella* Enteritidis is a pathogenic microorganism, some other strains having the similar antigenic structure also have alternative potential for antigen isolation, such as *Salmonella* Gallinarum for LPS and *Salmonella* Adeyo for flagellar antigen isolation.

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