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Outer membrane proteins (OMPs) profiling of a field isolate of *Salmonella* Typhimurium: Isolation and its characterization

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Abstract
Salmonella Typhimurium is a significant zoonotic pathogen affecting human and animal health. In the present study, a *S.* Typhimurium isolate, previously isolated from poultry samples was revived and re-confirmed as *S.* Typhimurium by isolation in Hektoen enteric agar followed by molecular detection. PCR with the *stn* gene, *his J* gene and *Typh* gene primers revealed the specific amplicon of size 617 bp, 496 bp and 402 bp respectively. Antibiotic sensitivity pattern of the isolate was also determined. Isolate was then cultured in LB broth in bulk and outer membrane proteins (OMPs) were isolated by ultracentrifugation. The OMPs were successfully isolated and confirmed by SDS-PAGE analysis. The band pattern revealed nine major bands ranging from 11-90 kDa.

Keywords: Outer membrane proteins (OMPs), *Salmonella* Typhimurium

Introduction
One of the leading cause for the zoonotic, foodborne illness occurring worldwide is *Salmonella enterica*. As per WHO, *Salmonella* is one of the major bacterial candidates of prime concern for AMR monitoring (Brunelle *et al*., 2017) [3]. *Salmonella* has been one of the most important zoonotic pathogens infecting diverse group of animal species. *Salmonella* Typhimurium and *Salmonella* Enteritidis are the major cause of food-borne human illnesses. *Salmonella* resides efficiently in the intestinal tract of many animal species. It is a Gram negative, non-endospore forming, motile bacilli which belongs to family *Enterobacteriaceae*.

OMPs of *S.* Typhimurium have been demonstrated to be effective in providing protection against lethal *Salmonella* infection in mice, which indicates that OMPs are good immunogens (Qiong *et al.*, 2016) [15]. OMPs produce comparatively higher degree of protection than that induced by lipopolysaccharides (LPS) (Isibasi *et al*., 1988) [9]. The outer membrane of the bacteria is unique in its composition and asymmetrical distribution of lipids, with the inner leaflet containing phospholipids, whereas the outer leaflet is composed of LPS which is a highly negatively charged molecule that protrudes into the external environment (Gan *et al*., 2008) [6]. The outer membrane is a home to lipoproteins and integral membrane proteins called outer membrane proteins (OMPs) (Rollauer *et al*., 2015) [16]. It is estimated that about 3% of the Gram-negative bacterial genome codes for OMPs (Wimley, 2003) [18]. OMPs are integral membrane proteins which adopt a β-barrel architecture in the membrane with short loops between strands on the periplasmic side and large, extended loops on the extracellular side. Several *Salmonella* OMPs have been researched and documented as potential candidates for development of vaccines, virulence factors, and antigens utilized for diagnostics (Isibasi *et al*., 1988) [9]. OMPs are valuable immunogens in the protection against *Salmonella* infection and thus have the potential for developing universal sub-unit vaccines to prevent homologous and heterologous *Salmonella* infections (Qiong *et al*., 2016) [15].

Materials and Methods
Bacterial isolate: A field isolate of *Salmonella* Typhimurium, previously isolated from poultry was procured from Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar was used for the present study.

Characterization of *S*. Typhimurium culture: The isolate was reconfirmed as *S*. Typhimurium by first culturing the glycerol stock of the culture in LB broth, then its enrichment in Tetrathionate broth and identification by its colony characteristics on Brilliant Green Agar (BGA) and Hektoen Enteric Agar (HEA). The isolate was further confirmed by gene specific PCR. The genomic DNA was isolated from the culture grown overnight at 37 ºC in orbital shaker and used as a template for PCR amplification of *stn* gene (Murugkar *et al*., 2003) [13], *hisJ* gene (Cohen *et al*., 1993) [5] and *Typh* gene (Alvarez *et al*., 2004) [1]. PCR amplicons were analyzed on 1.5% agarose gel.

Antibiotic sensitivity pattern of *S*. Typhimurium isolate: The disk diffusion method was conducted to test the drug sensitivity and the resistance patterns of different antibiotics against the bacteria as per the Clinical Laboratory and Standard Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines and recommendations. The culture similar to the O.D. of 0.5 McFarland standard was taken for the ABST test. The antibiotic sensitivity test of the bacteria was carried out on Mueller Hinton agar (MHA) plates. The culture was inoculated on the agar ensuring that the culture is spreaded evenly on whole of the agar surface and allowed to get absorbed and dry. Then the discs of different antibiotics were on the agar plate by gently pressing on the agar surface and incubated for overnight at 37 ℃ (Jorgensen and Ferraro, 2009). Eighteen antibiotic discs were used: Norfloxacin, Gentamicin, Cotrimoxazole, Ampicillin, Tetracycline, Streptomycin, Amikacin, Cefixime, Ceftriaxone, Chloramphenicol, Azithromycin, Ciprofloxacin, Tobramycin, Carbenicillin, Cefotaxime, Cephalothin, Cefuroxime, Cefoxitin.

Isolation of Outer membrane proteins (OMPs): Isolation of the OMPs was done by ultracentrifugation method with some modifications (Choi-Kim *et al*., 1991) [4]. The bacterial culture was inoculated in 1 liter of LB broth and incubated overnight in a shaker-incubator. After this the overnight grown culture was pelleted by centrifugation at 8000 rpm for 10 min and then washed twice with PBS. The final pellet was then resuspended in 20 ml of HEPES buffer. The solution was then sonicated in ice (20 cycles-7.0 µ, 60 sec on and 30 sec off). The sonicated mixture was centrifuged at 1700 x g for 20 min. The supernatant was collected in a fresh tube and was then again centrifuged at 100,000 x g for 60 min at 4 ℃. Pellet thus obtained was resuspended in 4 ml of freshly prepared 2% sodium-lauryl sarcosinate (prepared in 10mM HEPES buffer-pH=7.4). This was then incubated at 37 ℃ for an hour. After incubation it was again centrifuged at 100,000 x g for 60 min at 4 ℃. Finally pellet was resuspended in 0.1 M PBS, lyophilized and stored at-80℃.

SDS-PAGE analysis and of determination of OMP concentration: The isolated OMPs were analysed on 10% resolving and 4% separating gels (Laemmli, 1970) [12] by using a vertical gel electrophoresis apparatus (Atto, Japan). Concentration of the isolated OMPs was determined by Lowry method of protein quantification, taking BSA of different concentration as the standard. The O.D. was taken at 660 nm.

Results and discussion

Salmonellosis is considered as a major food-borne illness, the outer as well as the inner egg contamination by *Salmonella* is a major problem thereby making its control a lot more difficult (Whiley and Ross, 2015) [17]. OMPs isolated from *S*. Typhimurium have proven to be capable of giving protection against challenge with lethal dose of homologous *Salmonella* in mice, which indicates that OMPs are good protective antigens (Kusii *et al*., 1979) [11].

Characterization of *S*. Typhimurium isolate: The colonies obtained on HEA plate were dark-green coloured with a black centre, which is specific to *Salmonella*. Further, PCR amplification of the *stn*, *hisJ* and *Typh* genes resulted in amplicons of 617 bp, 496 bp and 402 bp, respectively (Fig. 1). This confirms that the culture used was of *Salmonella* belonging to species Typhimurium.

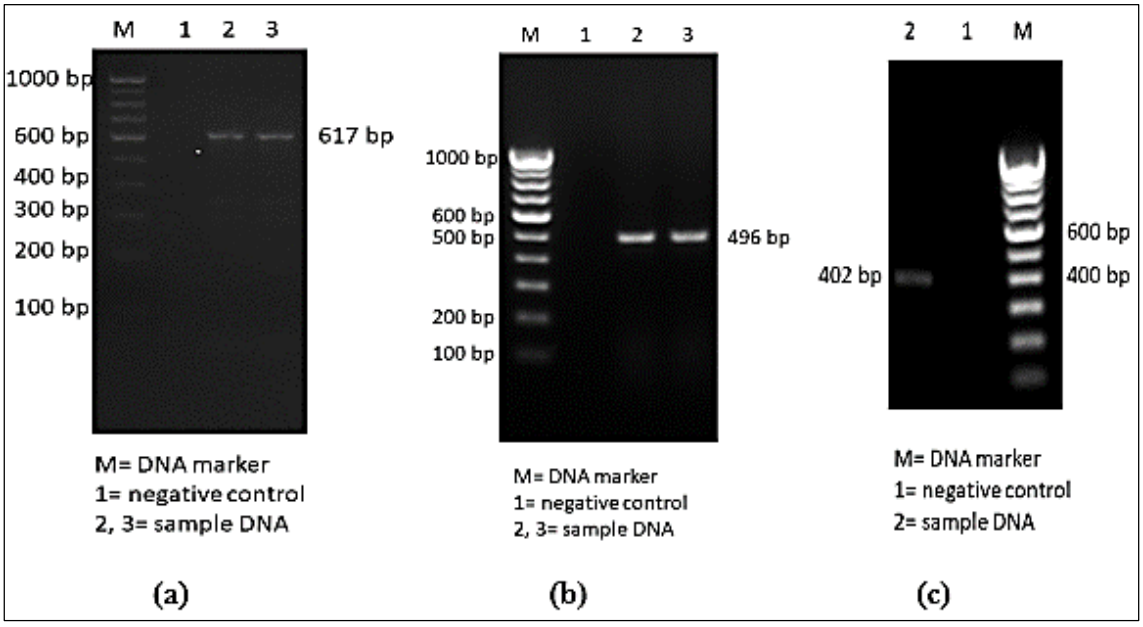

Fig 1: PCR amplification of different *S*. Typhimurium genes: a.) PCR amplification of *stn* gene; b.) PCR amplification of *hisJ* gene; c.) PCR amplification of *Typh* gene

Antibiotic sensitivity pattern of *S.* Typhimurium isolate: The bacteria was observed to be resistant against 6 groups of antibiotics: I gen. cephalosporin (Cephalothin), II gen. cephalosporin (Cefuroxime), III gen. cephalosporin (Cefixime, Ceftriaxone, Cefotaxime), Penicillin (Ampicillin, Carbenicillin), Aminoglycoside (Gentamicin), II gen. fluoroquinolone (Ciprofloxacin). Antibiotics to which isolates were sensitive included: Cotrimoxazole, Azithromycin, Streptomycin, Norfloxacin and antibiotics showing intermediate action included: Chloramphenicol, Amikacin, Tetracycline, Cefoxitin, Tobramycin (Fig. 2). The results obtained confirm that the bacterial isolate is multi drug resistant as it is showing resistance to more than three group of antibiotics. This problem of AMR is a matter of great concern and have been reported very frequently in the last few years which may be due to the excessive use of antimicrobials as a therapeutic or a feed additive. According to the European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) scientific opinion, *Salmonella* has been considered as a priority microorganism for monitoring AMR (Argüello *et al.*, 2018) [2].

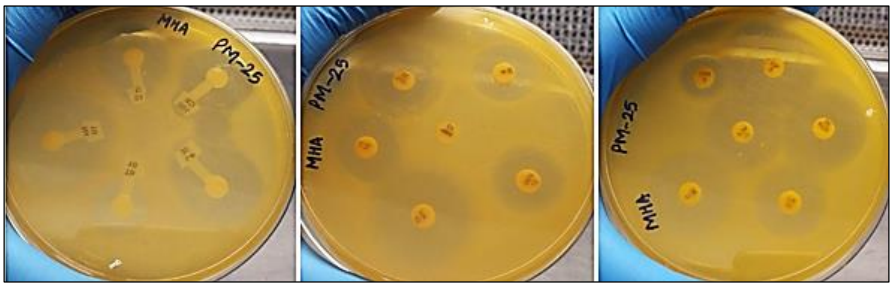

Fig 2: Antibiotic sensitivity pattern for the *S.* Typhimurium field isolate

Isolation and characterization of the isolated OMPs: The OMPs were successfully isolated and characterized by SDS-PAGE analysis. The band pattern of the isolated OMPs revealed nine bands ranging from 11-90 kDa (Fig. 3). The major bands observed were of the following sizes: 11 kDa, 15 kDa, 19 kDa, 33 kDa, 37 kDa, 49 kDa, 56 kDa, 66 kDa and 90 kDa. The concentration of the OMPs obtained as determined by Lowry method came out to be 7.2 mg/ml. Hamid and Jain (2008) [7] reported that they could isolate more than 15 OMPs of *S.* Typhimurium with sizes ranging from 15-100 kDa. In a study conducted, SDS-PAGE profile of the OMPs isolated from *S.* Typhi revealed nine protein bands of approximately 20, 26, 30, 32, 36, 39, 42, 47, 49 KDa molecular weight (Singh *et al.*, 2018) [14]. Husna *et al.* (2016) [8] reported that the OMP band sizes of *S.* Gallinarum ranged from 45-200 kDa.

Several studies indicate that OMPs from *Salmonella* have the potential for developing universal sub-unit vaccines to prevent homologous and heterologous *Salmonella* infections (Qiong *et al*., 2016, Hamid and Jain, 2008) [15, 7]. These OMPs can be utilized for various different purposes such as development of a vaccine, or a diagnostic or a therapeutic.

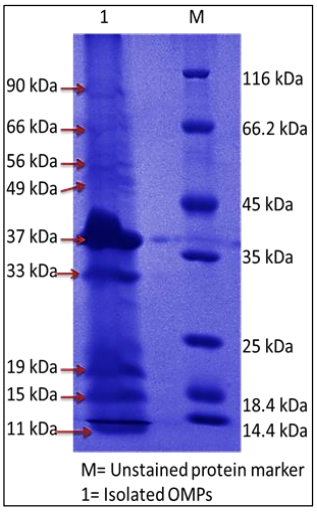

Fig 3: SDS-PAGE analysis of the isolated OMPs

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