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RESEARCH ARTICLE

Comparative dendrogram analysis of OMPs of *Salmonella enterica* serotype Enteritidis with Typhimurium, Braendurup and Lomita isolated from pigeons

***Kamelia M Osman and Sherif H Marouf**

Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt.

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Abstract

Outer membrane proteins (OMPs) is a distinguishing feature of Gram negative bacteria located at host–bacterial interface and are important for virulence, host immune responses and as targets for drug therapy. In the present study, Omps profiling of three *Salmonella enterica* serotypes (Typhimurium, Braendurup and Lomita) obtained from pigeons and implicated in human salmonellosis were differentiated by SDS-PAGE using a Triton X-100-lysozyme-EDTA extraction method. More than 21 protein bands could be resolved ranging in size from 61.0 kDa to 7.5 kDa as analyzed using Gel Compar II software of their approximate molecular masses. The *Salmonella* serotypes used in this study were un-related and could be differentiated depending on their OMPs profiles using SDS-PAGE. Dendrograms were constructed by the unweighted pair-group using the average linkage clustering method. The Dice index similarity coefficients between serotypes ranged from 45% to 100%.

Introduction

Salmonella infection is a serious medical and veterinary problem worldwide and causes great concern in the human health and food safety (Maripandi and Al-Salamah, 2010). Although there are few reports of disease transmission between pigeons and humans (Haag-Wackernagel and Moch, 2004), their close interaction, together with the observation that these birds are vectors for zoonotic agents (Hoelzer et al., 2011) may make them a public health risk.. The isolation of *S.* Typhimurium, *S.* Braenderup and *S*. Lomita has important implications because they are a significant cause of global food poisoning and enteric fever in humans (WHO, 2000; CDR, 2000; Rossier et al., 2000; Urfer et al., 2000; Gupta et al., 2007; IASR, 2009; Mizoguchi et al., 2011) which makes the spread of *S.* Braenderup and *S.* Lomita among pigeons a health concern for human health.

The outer membrane is a distinguishing feature of Gram negative bacteria. The outer membrane contains numerous proteins, referred to as Outer membrane proteins (OMPs) and the only known function to these OMPs is to serve as a protective barrier. These OMPs, function to allow the passive diffusion of small molecules. It is generally true that Gram-negative bacteria are more resistant to antibiotics than are their Gram-positive cousins (Silhavy et al., 2010). Because OMPs are also surface virulence factors and have a significant role in pathobiology and bacterial adaptation to environmental conditions, researchers have directed their investigations through the analysis of *Salmonella* OMPs patterns and have attempted to identify among them key molecular targets of the protective immune response against *Salmonella*.

Salmonella OMPs play an important role in the virulence and immunological properties of bacteria (Chooneea et al., 2010). These surface exposed proteins play a critical role in pathogenic processes such as motility, adherence and colonisation of the host cells, injection of toxins and cellular proteases, as well as the formation of channels for the removal of antibiotics (antibiotic resistance) (Bina et al., 2006; Cordwell, 2006). Therefore these functions make OMPs attractive targets for the development of antimicrobial drugs and vaccines (Grandi, 2001; Bernardini et al., 2007).

In addition to genomic-based methods, protein-based methods offer a different and complementary approach for the identification and characterization of *Salmonella* isolates, to reveal epidemic patterns, trace sources of infection and aid the development of reasonable intervention strategies to reduce the presence and spread of *Salmonella* infections in humans or/and animals. Previous epidemiological studies of the OMPs profiles using electrophoretic separation of OMPs by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of *Escherichia coli* (Achtman et al., 1983; Nart et al., 2008), *Chlamydia* spp (Hatch et al., 1981), *Haemophilus influenzae* (Barenkamp et al., 1981), *Neisseria* species (Buchanan et al., 1981), *Pasteurella* (Jain, 2004) and *Aeromonas* species (Sachan et al., 2012) isolated from different sources have been studied. However, the OMPs profile of *Salmonella* under the host conditions have not been specified in pigeons. Consequently, we carried out a detailed analysis of the OMPs of non-typhoidal *Salmonella* species (NTS) isolated from pigeons in our study. The study was to determine and compare the electrophoretic relatedness of a *S*. Enteritidis reference strain (Group D_1, ATCC4931) to three NTS representing two serologically different serogroups (Typhimurium, Group B; Braendurup, Group C_1; Lomita, Group C_1) field isolates based on their OMPs profiles and to determine if clustering was related to the serotype.

Materials and methods

Bacterial isolates

The strains utilized have been previously characterized (Osman et al., 2012; 2013; 2014). Three serotypes of *S. enterica* (Typhimurium, Braendurup and Lomita) were culturally, biochemically and serologically identified (Osman et al., 2014) within the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, and the Department of Poultry Diseases, Animal Research Institute, Dokki, Egypt. The isolates originated from the Department of Poultry Diseases, Animal Research Institute, Dokki, Egypt. Establishing and maintaining bacterial isolates for further study was accomplished by following the isolation procedures practised by NARMS (Fedorka-Cray et al., 2002). In addition, *S*. Enteritidis standard strain ATCC 13076 was used as a positive control.

Bacteria and Growth Conditions

In order to avoid possible variation between isolates (Chen et al., 2011; Lagha et al., 2012), all *Salmonella* isolates were grown under the same culture conditions and procedures used for the isolation of major Omps. Bacterial culture conditions used for Omp isolation has been described by Chalghoumi et al. (2008). *Salmonella* cells were grown in nutrient broth (231000, Difco Laboratories, Detroit, MI) at 37°C for 16 to 18 h with agitation (130 rpm). Following incubation, cells were harvested by centrifugation at 7,000 × *g* for 30 min at 4°C. Cells were then washed 1 time with sterile double-distilled water and 2 times with sterile 10 mM Tris-HCl buffer (pH: 7.8; T5941, Sigma Chemical Co., St. Louis, MO), and subsequently resuspended in Tris-HCl buffer containing 10 mM EDTA (Sigma Chemical Co.; Tris-HCl/EDTA buffer).

Preparation of Outer Membrane Proteins

The OMPs from *Salmonella* were prepared as described by Nurminen (1985) with some modifications. The resupended bacteria were treated with lysozyme (L76R5, Sigma Chemical Co.; 0.5 mg/mL). After incubation for 1 h at 37°C, ribonuclease (R6513, Sigma Chemical Co.; 30 µg/mL) was added and the sample was allowed to stand for 10 min at room temperature. Then, 1 *M* $MgCl_2$ solution (40 µL/mL) and deoxyribonuclease (D5025, Sigma Chemical Co.; 30 µg/mL) were added. After incubation for 5 min at room temperature, the mixture was centrifuged at 1,600 × *g* for 10 min at 4°C. The pellet, corresponding to bacterial envelopes, was washed twice with Tris-HCl/ EDTA buffer and once with Tris-HCl buffer containing 5 mM $MgCl_2$ (Tris-HCl/$MgCl_2$ buffer). Cell pellets were disrupted by treatment with lysozyme. Lysozyme-EDTA-treated envelopes were suspended in 10 mM Tris-HCl (pH: 7.5) containing 10 mM EDTA and 2% Triton X-100 (T8787, Sigma Chemical Co.; TX-buffer) and incubated for 2 h at 37°C with trypsin (93615, Sigma Chemical Co.; 0.5 mg/mL). Then, a further 0.5 mg/mL of trypsin was added, and the mixture was incubated again for 2 h at 37°C. The resulting digested mixture was centrifuged at 10,000 × *g* for 10 min at 4°C, the supernatant was collected, and the pH was adjusted to 6.0 with acetic acid 10%. Two milliliters of 5 *M* NaCl solution was added to the supernatant, and the sample was leaved overnight at 42°C to precipitate the porin fraction. The precipitate was collected by centrifugation at 10,000 × *g* for 10 min at 4°C, dissolved in 10 m*M* Tris-HCl (pH: 7.5). The suspension was centrifuged at 100,000 × *g* for 60 min at 4°C. The precipitate was recovered and washed twice with the same buffer, then dissolved in 10 m*M* Tris-HCl (pH: 7.5) containing 0.03% thimerosal (T5125, Sigma Chemical Co.) as a preservative, and stored at −20°C. The protein content of the OMP extracts was determined by BCA assay system (BCA1-1KT, Sigma Chemical Co.) according to

the protocol of standard analysis of the manufacturer. Each sample was tested in duplicate. The purity of each OMP extract was analyzed by SDS-PAGE by the method of Laemmli (1972) in 5 to 15% gradient acrylamide gels.

Computer-aided analysis of the gels

Gels were photographed, scanned (Kodak Image Station, Rochester, NY) and the image was digitized (Kodak Molecular Imaging Software, New Haven, CT). Protein profiles were analyzed using Gel Compar II software (Applied Maths, Austin, TX). Bands were coded as binary data (absent = 0 or present =1), regardless of band intensity. The optimal position tolerance value gives the highest group contrast: selected scores are as high as possible within groups and as low as possible between groups. Since a band matching algorithm (Dice) was used, both tolerance and optimization were calculated. Similarity matrices were obtained from single SDS-PAGE data using the Dice similarity coefficient: $F = 2nxy/(nx + ny)$, where nx is the total number of fragments from isolate X, ny is the total number of fragments from isolate Y, and nxy is the number of fragments shared by the two isolates. Neighbor joining (NJ) dendrograms were constructed with 1000 bootstrap values. The similarity-derived dissimilarity matrix was used in the cluster analysis by using the unweighted pair-group method with arithmetic averages (UPGMA).

Results

Table 1 shows details of the OMPs analysis revealed that the different isolates of *S*. Enteritidis (Reference strain), Typhimurium, Braendurup and Lomita isolates did not show a uniform pattern and intensity of protein bands expression and the OMPs differ in different isolates and the seven isolates were clearly distinct from each other. The OMPs profiles of the *Salmonella* serotypes were determined using SDS-PAGE using 12% (w/v) separating and 4% (w/v) stacking gels. More than 21 protein bands could be resolved ranging in size from 61.0 kDa to 7.5 kDa as analyzed using Gel Compar II software of their approximate molecular masses. *Salmonella* Enteritidis reference strain (Group D_1, ATCC4931) isolate served as control as described for Table 1. The *S*. Enteritidis homogenous protein bands appeared as intense protein bands in the range from 14.5 and 61.0 kDa. Twenty one points 61.0, 46.8, 43.0, 38.8, 36.0, 34.9, 31.6, 30.9, 30.1, 29.0, 28.6, 27.7, 27.2, 25.2, 24.8, 17.1, 14.2, 14.5, 14.4, 10.7 and 7.5 kDa protein bands were detected in all *Salmonella* serotypes. There were no common protein bands to appear as major bands in all serotypes. Each local field serotype possessed OMPs with molecular weights ranging from 7.5 kDa to 43.0 kDa.

Salmonella Enteritidis reference strain (Group D_1, ATCC4931) (lane 1) isolate served as control as described for Figure 1. Using dendrogram analyses Fig. 1 shows the constructed dendrogram from gel analysis Gel Compar II software (Applied Maths, Austin, TX) from scanned photographs of SDS-PAGE gels. Using the software, lanes were identified, the background subtracted and bands detected automatically. Matching was achieved by construction of a synthetic lane and comparing relatedness between bands in lanes. The results in Fig. 1 show a high degree of dissimilarity between *S*. Enteritidis (Reference strain), Typhimurium, Braendurup and Lomita isolates. Nine of these isolates were _-lactamase positive and the other 6 BLNAI. In total, the four serotypes analysed indicated a high level of heterogeneity. Two of the *S*. Typhimurium isolates were genetically identical to one another (exhibited 100% similarity). The other isolates were genetically unrelated.

Table 1. SDS-PAGE profiles of isolated pigeon *Salmonella* serotypes Enteritidis (Reference strain), Typhimurium, Braendurup and Lomita Outer Membrane Proteins. Gradient SDS-PAGE gels were stained with Coomassie Brilliant Blue R250. Each lane was loaded with 10 µg of protein. Molecular weights are indicated in kiloDaltons (kDa)

Marker (kDa)	***Salmonella* serotypes**						
	Enteritidis*	Typhimurium	Typhimurium	Typhimurium	Typhimurium	Braendurup	Lomita
66.0	61.0	28.6	30.1	29.0	36.0	34.9	43.0
55.0	46.8	25.2	27.7	27.2	31.6	30.9	17.1
45.0	38.8	10.7	10.7	24.8	14.4	7.5	
36.0	14.5			14.2			

29.0							
24.0							
20.0							
14.2							
6.5							

- Reference strain (Group D_1, ATCC4931)

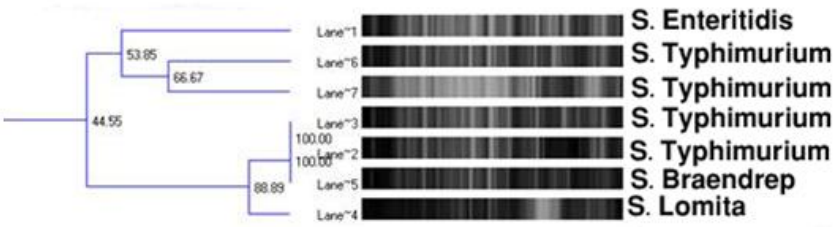

Figure 1. Dendrogram grouping highlighting the pigeon *Salmonella* serotypes Enteritidis (Reference strain), Typhimurium, Braendurup and Lomita Outer membrane proteins based on the SDS-PAGE gel electrophoresis profiles

Discussion

Non-typhoid *Salmonella* (NTS) are broadly dispersed in the environment as well as in the gastrointestinal tracts of both domesticated and wild animals. The OMP of Gram negative bacteria has a role in disease processes as it acts at an interface between the host and pathogen (Lin et al., 2002). Thus OMP variation among the isolates may help in epidemiological survey by assessing their inter strain heterogeneity and can be used to assess intra species diversity (Davies et al., 2003).

For *S*. Enteritidis (Reference strain), Typhimurium, Braendurup and Lomita, it turned out that within the serotypes the major OMP pattern was not uniform for all strains tested in contrast to the uniform findings of Helmuth et al. (1985) in *S*. Typhimurium, Choleraesuis, Infantis, Panama and Heidelberg. The SDS–PAGE analysis revealed heterogeneity in the OMPs profiles within each of the four serotypes. The comparison of membrane patterns between the four serotypes did not reveal any striking similarities in contrast to the findings of Helmuth et al. (1985). Similar variability in OMP profiles has been described by several authors (Santos et al., 1996; Sachan et al., 2011) who observed that *Aeromonas* strains belonging to different serogroups and as well as to the same serogroup exhibited differences in their protein banding pattern.

The characteristic migration patterns of OMPs during SDS-PAGE have been used for subdividing *Salmonella* serotypes (Helmuth et al., 1985; Poppe et al., 1993). In the present study, *S*. Typhimurium isolates contained OMPs with the molecular masses ranging from 10.7 kDa to 30.1 kDa. This contradicts the results of Helmuth et al. (1985) who found that their *S*. Typhimurium strains generally contained OMPs of 37 and 40 and 41.7 kDa and to Chart and Rowe (1991) who demonstrated three major OMPs of 33, 35 and 36 kDa and Elpek et al. (2002) who revealed that the majority of *S*. Typhimurium isolates contained two OMPs of 30.6 and 34.6 kDa, six isolates carried three OMPs of 27.2, 30.6 and 34.6 kDa, and three isolates contained only a 30.6 kDa OMP. Hamid and. Jain (2008) calculated the molecular masses of OMPs from *Salmonella* serovar Typhimurium to be 100, 78, 75, 70, 68, 65, 55, 49, 43, 37, 35, 32, 30, 25, and 15 kDa. Kim et al. (2006) found that the OMP profiles on SDS-PAGE of *S*. serotypes Typhimurium, Enteritidis, Thomasville, Hadar, Seftenberg and Brandenburg exhibited two or three bands between 48 and 54 kDa. Even Maripandi et al. (2010) found his isolated *S*. Enteritidis strain with different OMPs bands which exhibited with molecular weights ranging from 5-90 kDa in a striking contrast to our reference *S*. Enteritidis strain. These variations are expected due to the complex and diverse nature of *Salmonella*. In contrast to the other three serotypes, we recorded two OMPs bands in the medium molecular weight (43.04 kDa) and in the low molecular weight region (17.12 kDa) from extracts of *S*. Lomita. It is interesting to observe that *S*.

Typhimurium is not endowed with the same array of OMPs as seen in the other three serotypes investigated in this study.

Eight outer membrane proteins with apparent molecular mass of <25 kDa could be detected in the profile of each serotype, while no individual dominating protein peaks appeared in the OMP profiles. Low molecular weight fractions of *Salmonella* OMP with molecular weights of <25 kDa are involved in major biological processes such as ribosome formation, stress adaption, cell cycle control (Müller et al., 2010) stimulatory and immunogenic (Mertz et al., 1998; Thiel et al., 2000; Singh et al., 2007), adherence to cultured human epithelial cells and chicken caeca (Zhao et al., 1996) and in effective biofilm formation (Yonezawa et al.. 2011). The altered OMP profile is accompanied by significant changes in several virulence properties, including an increase in the ability to autoagglutinate, increased susceptibility to several antimicrobial agents increased biofilm formation (Rathbun and Thompson, 2009) and antibiotic sensitivity (Kustos et al., 2007).

The loss of porins OmpK36 (the homolog of OmpC) and OmpK35 (the homolog of OmpF) as a cause of antibiotic resistance has been noted in several reports, especially for *K. pneumoniae*, *E. coli* and *Salmonella* Typhimurium (Nikaido, 1989) and has been shown to probably contribute to ciprofloxacin, gentamicin, and fluoroquinolones resistance (Hirai, et al., 1986; van der Klundert et al., 1988; Chen et al., 1993; Martínez-Martínez et al., 1996; Ardanuy et al., 1998). Accordingly, the absence of OmpK36 and OmpK35 in this study could be correlated with the decrease in susceptibility to nalidixic acid previously recorded by Osman et al. (2012; 2013). These results may be interpreted to mean that quinolones penetrate the outer membrane through OmpF porin pores very efficiently, like aminoglycoside antibiotics (Nakae and Nakae, 1982), or through another specific site(s). As mentioned above, one of the other possible pathways for hydrophobic quinolones such as nalidixic acid is the phospholipids bilayer, but hydrophilic quinolones such as norfioxacin and ciprofloxacin seemed to be unable to permeate this bilayer.

The overall conclusion taking these observations together, it becomes clear that the low molecular weight fractions of the isolated field strains of *Salmonella* OMP with molecular weights of <25 kDa have a real potential as a candidate vaccine against *Salmonella* (Isibasi et al., 1988; Meenakshi et al., 1999; Fadl et al., 2002; Khan et al., 2003). Also, the results indicated a possible association between antibiotic resistance patterns and OMPs. The major outer membrane protein profiles are, within a given serotype, heterogenous and thus could be useful epidemiological markers.

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