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RESEARCH ARTICLE

The outer membrane proteins profile of *Salmonella enterica* serotypes Enteritidis, Muenster, Florian, Omuna and Noya and their dendrogram analysis

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Abstract

Non-typhoidal *Salmonella* is increasingly recognized as an important pathogen associated with gastroenteritis. However, there is limited data specifically describing the Outer membrane proteins (OMPs), 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. The present study is based on molecular characterization of four *Salmonella* serotypes representing three serologically different serogroups (Muenster, Florian, Omuna and Noya) and their relatedness to a *S*. Enteritidis reference strain. The OMPs profiling of the *S. enterica* serotypes were differentiated by SDS-PAGE using a Triton X-100-lysozyme-EDTA extraction method. Eighteen protein bands could be resolved ranging in size from 61.0 kDa to 14.2 kDa. The *Salmonella* serotypes used in this study were un-related and could be differentiated depending on their OMPs. The Dice index similarity coefficients between serotypes ranged from 52% to 85.7%. In conclusion, the identification of the low molecular weight fractions of *Salmonella* OMP in our study need to be correlated with the incidence of *Salmonella* induced reactive arthritis (ReA)/undifferentiated spondyloarthropathy (uSpA) in Egypt. This will help in identifying the T cell immunodominant antigens which can be used for vaccine or for developing a diagnostic test for ReA/uSpA.

Introduction

The World Health Organization (WHO) has estimated that annually 1.3 billion cases of acute gastroenteritis or diarrhea due to non typhoid salmonellosis occur with 3 million deaths. The recent CDC report attributes an estimated 1.0 million cases, 19,336 hospitalizations, and 378 deaths annually in United States to infections caused by NTS serotypes (Scallan et al., 2011). The predominant *Salmonella* serovars causing infections in humans in 2009 in the United States include *S*. Enteritidis (MMWR, 2010). However, other serotypes are often more prevalent in other parts of the world and result in more severe infections with higher morbidity (Hendriksen et al., 2011). In Africa (Feasey et al., 2012; Okoro et al., 2012), invasive strains of NTS have emerged as a prominent cause of bloodstream infection in African adults and children, with an associated case fatality of 20-25%.

The relatedness of *Salmonella* isolates outer membrane proteins (OMP) analyses (Davies, 1991) has proved to be a useful technique in the characterization of these bacteria. Gram-negative bacterial OMPs have an important role in pathogenesis and signal reception (Jang et al., 2011). Thus, OMPs represent important virulence factors and play essential roles in bacterial adaptation to host niches, which are usually hostile to invading pathogens (Lin et al., 2002). Understanding the structure and functions of bacterial OMPs will facilitate the design of antimicrobial drugs and vaccines. Isibasi et al. (1988) previously investigated the OMP of *Salmonella* as potential vaccine candidates, diagnostic antigens, and virulence factors. Some of the OMPs from bacteria induce DC maturation and regulate Th1/Th2 immune responses (Shaw et al., 2002; Jeannin et al., 2003; Lee et al., 2007). Several studies have been carried out on *Salmonella* serovars using electrophoretic separation of OMPs by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with special reference to serotypes Enteritidis, Typhimurium and

Typhi (Udhayakumar and Muthukkaruppan, 1987; Verdugo-Rodriguez et al., 1993; Singh et al., 2007; Hu et al., 2009; Bhat and Jain, 2010; Ho et al., 2010; Lee et al., 2010; Maripandi and Al-Salamah, 2010; Lagha et al., 2012) with a neglection to the rest of the 2600 serotypes.

Therefore, the objectives of this study were to determine and compare the electrophoretic relatedness of a *S*. Enteritidis reference strain (Group D_1, ATCC4931) and four *Salmonella* serotypes representing three serologically different serogroups (Muenster, Group E_1; Florian, Group, E_1; Omuna, Group C_1; Noya, Group C_2-C_3) field isolates from diarrhoeic patients based on their OMPs lysate profiles and to determine if clustering was related to the serotype.

Materials and Methods

Bacterial isolates

The strains utilized were from diarrhoeic patients and have been previously characterized. Four serotypes of *S. enterica* (Muenster, Floria, Omuna and Noya) were culturally, biochemically and serologically identified (Osman et al., 2013) within the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University. The isolates originated from the Department of Clinical and Chemical Pathology, Faculty of Medicine, Kasr ElAini, Cairo University, Cairo, 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, all *Salmonella* isolates were grown under the same culture conditions and procedures used for the isolation of major OMPs (Lagha et al., 2012). The method used for OMP isolation has been previously described (Chalghoumi et al., 2008; Nurminen, 1985). *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 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 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. Arbitrary subdivision, clades and subclades, were derived for OMP lysate SDS-PAGE dendrograms by examining the clades as a function of percent similarity. The similarity-derived dissimilarity matrix was used in the cluster analysis by using the unweighted pair-group method with arithmetic averages (UPGMA).

Results

SDS-polyacrylamide gel electrophoresis (PAGE) clearly showed the presence of different OMP profiles between the serotypes recovered from human diarrhoeic patients. These bands had molecular masses ranging from 61 to 14.2 kDa. These serotypes were characterized by eighteen very heterogeneous patterns protein bands of different molecular sizes, with the following molecular masses: two bands between two bands with a molecular mass between 61 and 50 kDa, two bands of 46.8 kDa, one band of approximately 41.5 kDa, four bands between 38.8 and 33.9 kDa, four bands between 22.3 and 20.5 kDa and six bands between 14.2 and 15.1 kDa.

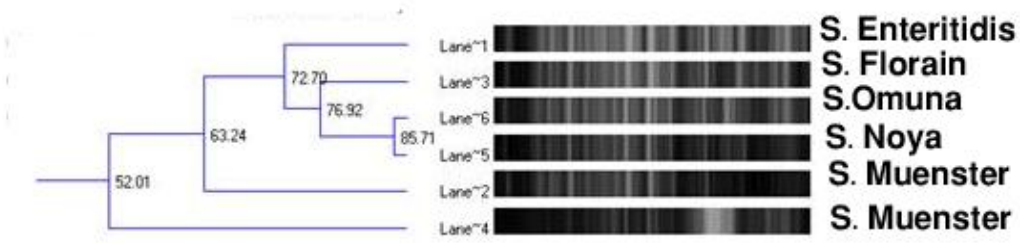
Salmonella Enteritidis reference strain (Group D_1, ATCC4931) (lane 1) isolate served as control as described for Figure 1. Using dendrogram analyses Fig. 1 shows the constructed dendrogram from gel analysis Gel Compar II software (Applied Maths, Austin, TX) from scanned photographs of SDS-PAGE gels. 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), Muenster, Florian, Omuna and Noya isolates. In total, the four serotypes analysed indicated a high level of heterogeneity and similarity ranged between 85.7-52.0%.

Table 1. SDS-PAGE profiles of isolated *Salmonella* serotypes Outer Membrane Proteins of *S. enterica* (Muenster, Floria, Omuna and Noya)

Marker (kDa)	***Salmonella* serotypes**					
	Enteritidis*	Florian	Omuna	Noya	Muenster	Muenster
66.0	61.0	22.3	50.8	41.5	22.0	21.3
55.0	46.8	20.5	46.8	37.5	14.9	15.1
45.0	38.8	14.2	35.3	14.8		
36.0	14.5		33.9			
29.0			14.4			
24.0						
20.0						
14.2						
6.5						

* Reference strain (Group D_1, ATCC4931)

Figure 1. Dendrogram grouping highlighting the Outer Membrane Proteins of *S. enterica* (Muenster, Floria, Omuna and Noya) based on the SDS-PAGE gel electrophoresis profiles



Discussion

The present study is based on analysis of the outer membrane protein profiles of clinical isolates of four serotypes of *S. enterica* (*S*. Muenster, *S*. Floria, *S*. Omuna and *S*. Noya) originated from the Department of Clinical and Chemical Pathology, Faculty of Medicine, Kasr ElAini, Cairo University, Cairo, Egypt in addition to the reference *S*. Enteritidis strain.

Within the OMP complex, certain types of proteins were shared by five serotypes (14.2, 14.4, 14.5, 14.8 and 14.9); following the arbitrarily classification into four electrophoretic regions proposed by Kudrna et al. (1985) three serotypes (Florian, Omuna and Muenster) from the isolated field strains could be characterized as possessing a unique complement of proteins in regions III (19-34 kDa) and IV (12-19 kDa). Similar protein constituents in these electrophoretic regions have been reported for other members of the *Enterobacteriaceae* (Hofstra and Dankert, 1979; Kudrna et al., 1985). It is worthnoting however, that possible differences in OMP profiles may be due to either strain or serotype differences (Kudrna et al., 1985). Region IV (12-19 kDa) was a common feature between the isolated field strains and the reference *S.* Enteritidis. The two isolted strains of *S.* Muenster were very closely related in their OMP profiles in that the OMP were in the region III (19-34 kDa) and IV (12-19 kDa). The polyacrylamide gel electrophoresis of extracted OMs from each serotype (*Salmonella* Enteritidis, Muenster, Florian, Omuna and Noya) did not reveal vast differences in their protein profiles as previously revealed (Ames et al., 1974; Kudrna et al., 1985) and identified by other investigators (Kamio and Nikaido, 1977; Lugtenberg et al., 1975, 1977; Nurminen, 1978; Verstreate et al., 1982; Odumeru et al., 1983) in a variety of Gram-negative bacteria.

The interesting feature observed in the present investigation is the dominance of the low molecular weight proteins in all of the investigated serotypes. Singh et al. (2007) showed that low molecular weight fractions of OMP (≤ 40 kDa) contain T cell immunodominant antigens that stimulate T cells in *Salmonella* induced reactive arthritis (ReA)/undifferentiated spondyloarthropathy (uSpA) which is similar to earlier reports in *Yersinia* and *Chlamydia* induced ReA, which have shown low molecular weight OMPs to be immunogenic (Mertz et al., 1998; Thiel et al., 2000).

Emergence of resistance to a variety of antibiotics in *Salmonella* has been found in many areas of the world and is a potentially serious public health problem. It has been shown that OMPs participated in antibiotic resistance either by decreasing permeability or by increasing export of drug. A reduction in the permeation of antibiotics is generally related to a decrease in porin expression or an alteration in the porin structure (Pages et al., 2008). Previous reports have shown OMP alterations to be associated with quinolone and β-lactam resistance in *Klebsiella*, *Enterobacter* and *Serratia* species (Sanders et al., 1984; Gutmann et al., 1985). A lack of porin OmpK35 is found in most extended-spectrum beta-lactamase-expressing *K. pneumoniae* clinical isolates (Domenech-Sanchez et al., 2003). In the present study *S*. Omuna was the only serotype that expressed OMP 35 kDa and was resistant to ampicillin (Osman et al., 2012). On the other hand, all the *Salmonella* serotypes were resistant to streptomycin, lincomycin and were susceptible to and the fluoroquinolones ciprofloxacin and norfloxacin, and amoxicillin (Osman et al., 2012). None of the isolates expressed the OmpK36 porin recorded by Ardanuy et al. (1998) in *Klebsiella*. However, a diminution or loss of a 31-32 kDa OMP has been correlated with quinolone resistance in *P. aeruginosa* (Dalkos et al., 1988). The assigned role of the 54-kilodalton OM protein in the impaired cellular permeability to ciprofloxacin as has been shown for *E. coli* norfloxacin-resistant mutants (Hirai et al., 1986; Hooper et al., 1986; Aoyama et al., 1987; Legakis et al., 1989) was not evident in our results. Interestingly, this protein was not expressed in our isolates. It appears that quinolone resistance involving OM permeability in *Salmonella* may differ from the one mentioned for *E. coli* (Hirai et al., 1986, 1987).

The identification of the low molecular weight fractions of *Salmonella* OMP in our study need to be correlated with the incidence of *Salmonella* induced ReA/uSpA in Egypt. This will help in identifying the T cell immunodominant antigens (Lee et al., 2010) which can be used for vaccine or for developing a diagnostic test for *Salmonella* induced reactive arthritis (ReA)/undifferentiated spondyloarthropathy (uSpA)**.**

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