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Original Article

Comparison of immunoblot analysis of emergent multidrug-resistant *Salmonella typhimurium* definitive phage type 104 with a non-multidrug-resistant definitive phage type 104 strain

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Background and Purpose: The emergent multidrug-resistant (MDR) *Salmonella typhimurium* definitive phage type 104 (DT104) is a public and veterinary health problem not only due to its wide host range and potential for enhanced virulence, but also the difficulty associated with its control. There is thus a need to investigate possible antigens of MDR DT104.

Methods: Using standard protocols, whole cell lysates, outer membrane extracts and cell-free ultracentrifuge supernatants of selected isolates of MDR DT104 were prepared, electrophoretically separated and tested for their antigen-antibody reactivity in comparison with a non-MDR DT104 strain.

Results: Protein antigens of both strain types were recognized by antibodies in chick serum in a similar manner for all methods of antigen preparation used.

Conclusions: This study did not find differences between the antibody recognition of MDR DT104 and that of the non-MDR DT104 strain tested. This observation should strengthen the quest for the possible use of vaccines to control this emergent strain in poultry.

Key words: Antibody response, antigens, bacterial drug resistance, immunoblotting, proteome, *Salmonella typhimurium* DT104

Introduction

Salmonella typhimurium definitive phage type 104 (DT104) is notable for its chromosomally encoded multidrug resistance [1,2]. The overall gene complex in multidrug-resistant (MDR) DT104 comprises a sequence of approximately 1.4 kilo-base containing 2 integrons and intervening plasmid-derived sequences coding for resistance to chloramphenicol and tetracyclines [3]. Resistance genes are known to mediate changes in bacterial cell surface physicochemical properties, such as hydrophobicity and charge [4,5] that usually are determined by the components of the cell surface, such as proteins, lipopolysaccharides (LPS) and phospholipids, which interact with host cells [6].

A primary infection with salmonellae induces the production of antibodies in addition to T-cell responses [7,8]. Anti-*Salmonella* immunoglobulin M appears in serum early after infection followed by immunoglobulin G (IgG) [9,10]. Serum and mucosal immunoglobulin A has also been detected following immunization with live and some killed vaccines [11]. Antibody responses to predominantly LPS determinants (O-polysaccharide and core regions) and a large number of non-serotype–specific antigens including porins, outer membrane proteins (OMP), lipoproteins, heat-shock proteins, flagella and fimbriae have been reported [8,2,13].

Although seroconversion is considered a measure of active immunity to *Salmonella*, correlation between the presence of antibodies alone and resistance to reinfection cannot always be established [14]. Thus,

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Table 1. Relevant characteristics of test strains

Strain	Source	Country of isolation	Minimum inhibitory concentration[a] (μg/mL)										Resistance
			A	T	C	F	G	K	S	Su	TMP	NA	
306-98	Human	USA (CDC)	>512	128	256	64	1	2	512	>512	0.5	4	ACFSSuT
T980018	Human	USA (CDC)	>512	64	256	64	0.5	2	256	>512	0.5	4	ACFSSuT
T980021	Human	USA (CDC)	>512	256	256	128	1	2	>512	>512	1	4	ACFSSuT
T980042	Human	USA (CDC)	>512	64	256	64	1	2	128	>512	0.5	4	ACFSSuT
T980043	Human	USA (CDC)	>512	128	256	64	1	2	256	>512	0.5	4	ACFSSuT
ST	Chick	Japan	1	2	8	8	0.5	2	32	64	0.5	4	SSu

Abbreviations: A = ampicillin; T = tetracycline; C = chloramphenicol; F = florfenicol; G = gentamicin; K = kanamycin; S = streptomycin; Su = sulfamethoxazole; TMP = trimethoprim; NA = nalidixic acid; CDC = Centers for Disease Control; ST = *Salmonella typhimurium* L1388
[a]Mimimum inhibitory concentration was determined by the doubling agar dilution method of National Committee for Clinical Laboratory Standards, USA.

despite the broad antigen specificity of the immune responses against *Salmonella*, the nature of the protective antigens needs to be better defined. In addition, it is not known whether the antigen-antibody recognition of emergent MDR DT104 differs from that of non-MDR DT104 strains. This study examined the recognition of protein antigens of MDR DT104 strains by antibodies in chick and rabbit sera in comparison with a non-MDR DT104 strain.

Methods

Bacterial isolates and culture media

Five isolates of MDR DT104 (306-98, T980018, T980021, T980042, T980043) from human sources and a non-multi-resistant strain *Salmonella typhimurium* L1388 (ST) were used in this study. Relevant characteristics of the DT104 isolates are given in Table 1. ST is a chick isolate having resistance to streptomycin (minimum inhibitory concentration [MIC] = 32 μg/mL) and sulfamethoxazole (MIC = 64 μg/mL). Both isolate types were kindly provided by Dr. Kazumitsu Tamura (formerly of the National Institute of Infectious Disease, Tokyo, Japan). All the isolates were propagated in trypticase soy broth (TSB; BBL, USA) or as otherwise indicated. Unless otherwise indicated, all chemicals used were from Wako Chemical Company, Japan.

Chick and rabbit sera

The chick serum used in this study was raised to whole cell (WC) of ST by the method of Nakamura et al [15], and represented nonspecific serum. Briefly, white leghorn chicks were randomly allocated to groups and infected once orally with approximately 5×10^5 colony-forming units of bacteria in phosphate-buffered saline (pH 7.2). Twenty two days later, blood was collected from the chicks [16] and left to stand overnight at room temperature. The resulting serum (titer of above 1:15,000) was recovered by centrifugation (3000 rpm × 30 min) and stored at 5°C until needed.

Rabbit serum (titer of above 1:15,000) raised to a specific (36.5-kDa) protein of ST was a kind gift from Mr. Torima Itoh (formerly a postgraduate student at the Animal Health Laboratory, Ibaraki University, Japan). The pure fraction of 36.5-kDa protein was obtained by purification of excised sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separated protein using ÄKTA™ FPLC™ (Amersham Biosciences Ltd., Piscataway, NJ, USA), a biocompatible, high-performance liquid chromatography system for purification of proteins.

Preparation of protein samples and SDS-PAGE

WC lysates, crude OMP extracts, and cell-free ultracentrifuge supernatant were prepared based on the methods adapted from Hitchcock and Brown [17], Filip et al [18] and Chopra et al [19], respectively. Samples were separated by discontinuous (denaturing) SDS-PAGE based on the method of Laemmli [20].

Immunoblot analysis

SDS-PAGE separated samples were transferred onto nitrocellulose membrane (Hybond-C; Amersham Pharmacia Biotech, Buckinghamshire, UK) at constant 20 V for 30 min with a semi-dry blotter (TransBlot; BioRad, CA, USA) based on the method of Towbin et al [21] using a transfer buffer (20 mM Tris-160 mM glycine-20% methanol). The membranes containing proteins were rinsed in distilled water and then immersed for 1 h in blocking solution (5% skimmed milk in

phosphate-buffered saline-0.05% Tween 20) at room temperature to block unbound membrane sites. Chick serum or rabbit serum specific to 36.5 kDa protein of ST were diluted 1 in 50 with PBS-0.05% Tween 20, then poured over the membranes in sufficient quantity and left for another 1 h at room temperature. Thereafter, the membranes (blots) were washed with distilled water for 10 min each a total of 3 times, and then overlaid with anti-chicken IgG-alkaline phosphatase conjugate (Sigma Chemicals, St Louis, MO, USA) or anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) diluted 1 in 10,000 with PBS-0.05% Tween 20 for 1 h at room temperature. The blots were again washed with distilled water for 10 min each a total of 3 times, and antigen-antibody reactions were detected when the blots were immersed in the enzyme's substrate mixture containing 100 mg magnesium sulfate heptahydrate, 50 mg Fast Blue BB salt (Sigma), 50 mg 1-naphthyldisodium phosphate, 90 mL distilled water, and 10 mL of 10X boric acid solution (3.7 g boric acid, 1.8 g sodium hydroxide, distilled water to 100 mL) until reactions became sufficiently visible. The blots were then washed in sufficient quantity of distilled water and air dried at room temperature.

Results

WC antigenicity

Antibody recognition among the MDR DT104 strains was similar to that of the non-MDR DT104 strain ST, both producing sharp reactions with antibodies in chick serum by the 36.5-kDa and 65-kDa proteins in WC extracts (Fig. 1).

OMP reactivity

Only 1 major protein (65-kDa) in the OMP extracts from all the isolates reacted with antibodies in the chick serum (Fig. 2).

Ultracentrifuged filtrate reactivity

The 65-kDa proteins in the ultracentrifuge supernatants of all the isolates also reacted with antibodies in the chick serum (Fig. 3).

Antigen cross-reaction assay

Antiserum produced in rabbits to the 36.5-kDa protein of ST cross-reacted with 19.8-kDa proteins in the ultracentrifuge supernatants of all the isolates but not the corresponding proteins in the WC or OMP extracts (Fig. 4).

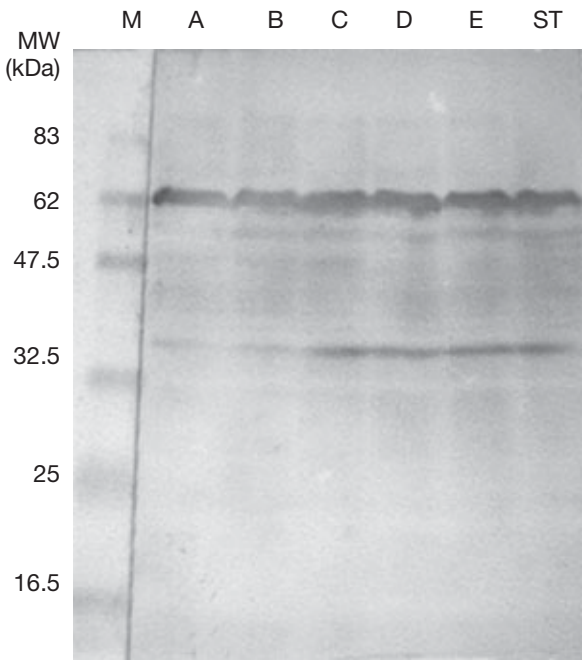

Fig. 1. Immunoblot analysis of whole cell (WC) protein of multidrug-resistant (MDR) *Salmonella typhimurium* definitive phage type 104 strains and non-MDR *S. typhimurium* L1388 (ST). WCs were prepared by boiling (100°C, 10 min) phosphate-buffered saline washed cells in sodium dodecyl sulfate (SDS)-sample buffer and recovering the filtrates after centrifugation. Samples were separated on SDS-10% polyacrylamide gel electrophoresis, electroblot-transferred to nitrocellulose membrane (0.45 μm), probed with 1:50 dilution of antiserum raised to WC of ST in chickens, and detected with alkaline phosphatase-conjugated anti-chicken immunoglobulin G. M = prestained broad range molecular weight marker (New England BioLabs, MA, USA); A = 306-98; B = T980018; C = T980021; D = T980042; E = T980043.

Discussion

Salmonella possess different surface components such as LPS, OMPs, fimbriae and flagellin, that can induce protective humoral and cellular immune responses in experimentally infected chickens [22,23]. The observation that only the 36.5-kDa and 65-kDa proteins in the WC extracts reacted with chick serum antibodies can be explained in 2 ways. First, host defense against salmonellae is thought to be directed mainly against the LPS [24]; and second, the presence of other possible antigens in the microbial cells in very low undetectable quantity [25]. The similarity in the antibody recognition of the protein antigens from isolates of MDR DT104 and the non-MDR DT104 strain ST separated by denaturing (SDS) PAGE suggests possible lack of

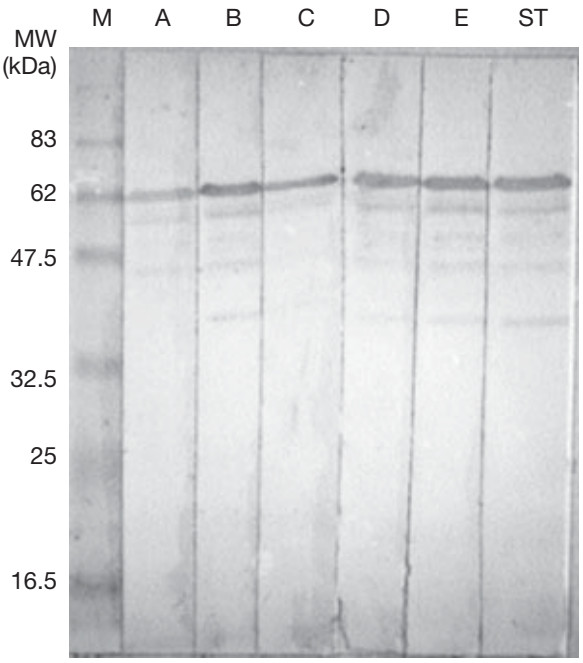

Fig. 2. Immunoblot analysis of outer membrane protein (OMP) of multidrug-resistant (MDR) *Salmonella typhimurium* definitive phage type 104 strains and non-MDR *S. typhimurium* L1388 (ST). OMP was prepared by the Sarkosyl method of Filip et al [18]. Samples were separated on sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis, electroblot-transferred to nitrocellulose membrane (0.45 μm), probed with 1:50 dilution of antiserum raised to whole cell of ST in chickens, and detected with alkaline phosphatase-conjugated anti-chicken immunoglobulin G. M = prestained broad range molecular weight marker (New England BioLabs); A = 306-98; B = T980018; C = T980021; D = T980042; E = T980043.

difference between the reactive protein antigens in the 2 strain types.

The observed dependence of the reactions on the type of protein sample provides additional evidence that sample preparation could significantly impact the outcome of antigen-antibody reactions. The WC protein extract was prepared by solubilization of WC pellets at 100°C in a sample buffer containing 2% SDS, a high-powered detergent, capable of solubilizing even the frequently insoluble hydrophobic membrane proteins. Even though the high detergent action of SDS is desirable in producing a more representative cell protein fraction, it is also highly denaturing and thus capable of interfering with the antigen-antibody reactions by altering the native conformation of the protein antigen epitopes required for optimal binding to antibodies [26, 27]. The reasons given above could be responsible for the few reactions observed with antibodies in the chick serum. The OMP extract was prepared by mechanical cell disruption and subsequent solubilization in N-lauryl sarcosinate, a less denaturing detergent that selectively removes the cytoplasmic membrane leaving the outer membrane and peptidoglycan intact as insoluble pellet when centrifuged [28]. Consequently, limited antigens are present for the reaction with the antibodies contained in the serum. On the other hand, the ultracentrifuge supernatants prepared by mechanical cell disruption and subsequent ultracentrifugation offer more limited antigens for reaction with the serum. Hence, the observation that for all the isolates, only 1 (65-kDa) protein reacted with the chick antiserum both in the OMP extracts and ultracentrifuge filtrates.

Antiserum to the 36.5-kDa protein of ST produced in rabbits cross-reacted with 19.8-kDa proteins in the ultracentrifuge supernatants of all the isolates but not the corresponding proteins in the WC or OMP extracts

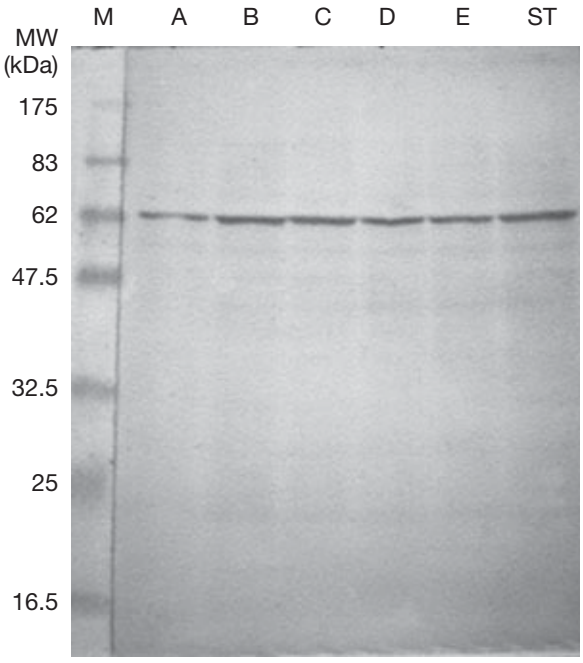

Fig. 3. Immunoblot analysis of ultracentrifuge filtrate (UF) of multidrug-resistant (MDR) *Salmonella typhimurium* definitive phage type 104 strains and non-MDR *S. typhimurium* L1388 (ST). UFs were prepared by sonically disrupting bacterial cells suspended in phosphate-buffered saline and ultra-centrifugation to recover the filtrate. Samples were separated on sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis, electroblot-transferred to nitrocellulose membrane (0.45 μm), probed with 1:50 dilution of antiserum raised to whole cell of ST in chickens, and detected with alkaline phosphatase-conjugated anti-chicken immuno-globulin G. M = prestained broad range molecular weight marker (New England BioLabs); A = 306-98; B = T980018; C = T980021; D = T980042; E = T980043.

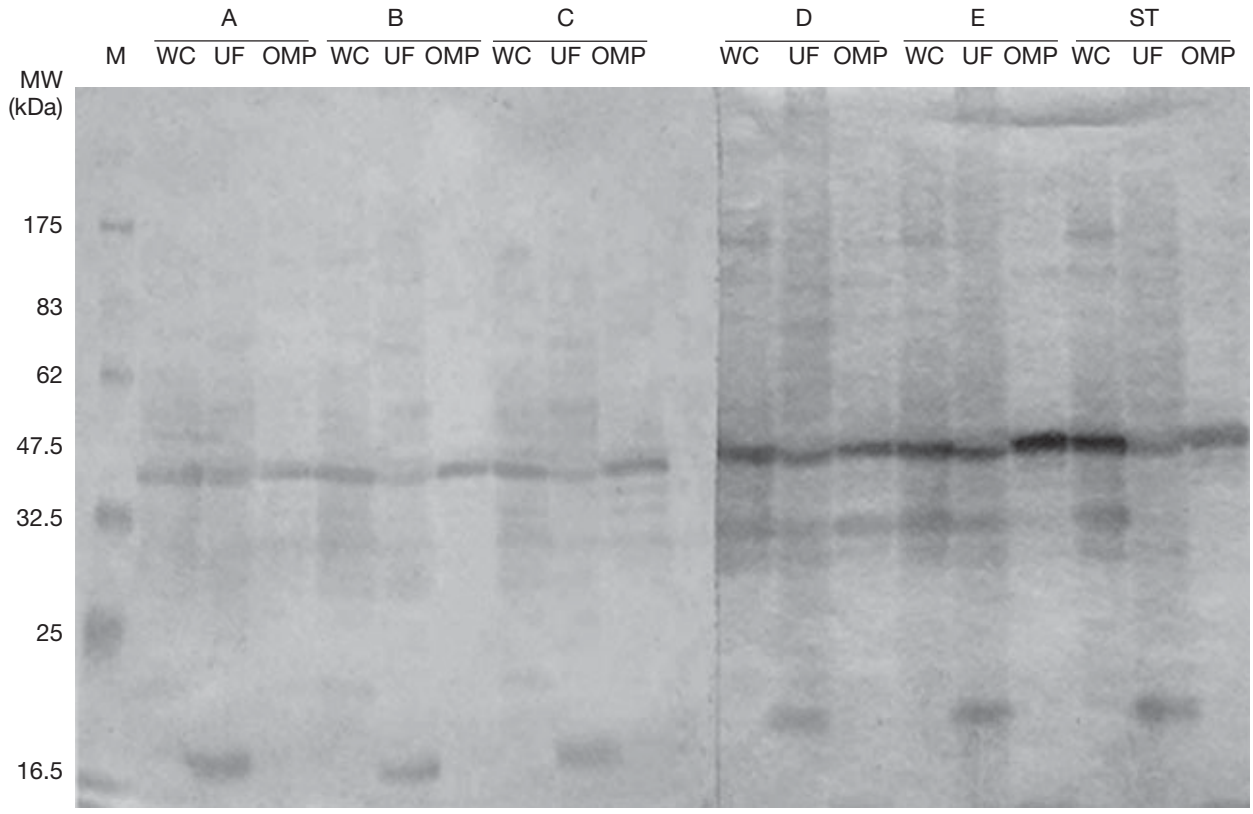

Fig. 4. Immunoblot analysis of whole cell (WC), outer membrane protein (OMP) and ultracentrifuge filtrate (UF) of multidrug-resistant (MDR) *Salmonella typhimurium* DT104 and non-MDR *S. typhimurium* L1388 (ST) strains with rabbit serum specific to 36.5-kDa protein of ST. Samples were separated on sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis, electroblot-transferred to nitrocellulose membrane (0.45 μm), probed with 1:50 dilution of the antiserum, and detected with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G. M = molecular weight marker (prestained broad range, New England BioLabs); A = 306-98; B = T980018; C = T980021; D = T980042; E = T980043.

(Fig. 4). There is thus the possibility that the 2 proteins share a common epitope, which was perhaps lost during preparation of the WC and OMP samples. It is known that naturally occurring innate immune reactions are based on the recognition of preserved homologous epitopes (homotopes) usually associated with microbes, cancer cells, degenerated cells, and with other (self) cell components [29]. The antibody non-specificity of the 36.5-kDa protein of ST will need to be re-evaluated using rabbit serum raised to 19.8-kDa, and chicken sera separately raised to the protein antigens.

Evidence from this study suggests that there may be no differences between the antibody recognition of *Salmonella typhimurium* MDR DT104 and that of the non-MDR DT104 strain tested.

Acknowledgments

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