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HEMAGGLUTINATING PROPERTIES OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS ISOLATED FROM DIFFERENT SOURCES

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ABSTRACT

Twenty-five strains of *Salmonella enterica* serovar Enteritidis isolated from different sources were examined for hemagglutinating activity. Bacteria cultured in different media induced hemagglutination of human erythrocytes, but no reaction was observed with erythrocytes from other animal species. The hemagglutinating expression activity was better for cultures on CFA agar at 37ºC than other conditions examined. The hemagglutination was inhibited by D-mannose, D-mannitol, melibiose, D-raffinose, L-rhamnose and sucrose. The absence of cell-surface appendages in electron microscope examinations suggested a nonfimbrial hemagglutinin. The data suggest that *Salmonella* Enteritidis produces nonfimbrial mannose-sensitive hemagglutinin, specific for human erythrocytes, which could be extracted in soluble form.

Key words: *Salmonella* Enteritidis, hemagglutinating activity, mannose-sensitive hemagglutinin, nonfimbrial hemagglutinin

INTRODUCTION

Salmonella species infect a wide range of hosts including humans and can cause disease ranging from severe enteric fever to self-limiting gastroenteritis that, in some individuals, can become systemic and life-threatening (17). The incidence of food poisoning by *Salmonella* spp. has been increasing in various parts of the world and foods of animal origin continue to be the major factors responsible, among them chicken meat, eggs and derivatives. Since the mid 1980s, there was a dramatic increase of food-borne salmonellosis outbreaks caused by *Salmonella enterica* serovar Enteritidis (23,25). This disease has an important economic impact since it can affect human health and general food chain.

Relatively little information is available on the mechanisms of pathogenesis of *Salmonella* spp., although adherence to host intestinal surfaces is recognized as an important initial step in *Salmonella* infections. This interaction depends upon bacterial adhesins which recognize specific glycoconjugate receptors on the host cell surface (4,15). These bacterial structures may also cause agglutination of erythrocytes of different species of animals. Such hemagglutination reactions have been designated mannose-sensitive or resistant, depending whether D-mannose or its derivatives inhibit the hemagglutination reaction (13). Since the erythrocytes posses different receptors, the bacteria-erythrocyte interaction gives a clue as to the nature of the receptors for these pathogens in the intestinal mucosa (13). For many intestinal bacterial pathogens, a correlation between hemagglutinating ability and adhesiveness has been shown (11,13,15).

Fimbrial and nonfimbrial hemagglutinins have been described in *Salmonella* spp. *Salmonella enterica* serovar Enteritidis produces several types of fimbrial adhesins, including SEF14, SEF17, SEF21, long polar fimbriae (LPF) and plasmid-encoded fimbriae (PEF) (4). Their roles in infection have been studied, but their functions are still poorly understood. However, some

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evidence indicates that they may be involved in the colonization and adherence of the organism to specific host tissues in the early stages of infection (9).

Whereas several fimbrial hemagglutinins have been characterized and associated with adhesion and pathogenicity, little is known about nonfimbrial hemagglutinins in *Salmonella* spp. Jones and Richardson (14) described a nonfimbrial mannose-resistant hemagglutinin in *S.* Typhimurium that was responsible for adhesion to and invasion of HeLa cells. Nonfimbrial mannose-resistant hemagglutinins have also been described in *S.* Typhimurium and *S.* Dublin, however they were not involved in adhesion to either HeLa or HEp-2 cells (19,26).

In this study, we presented some hemagglutinating properties of *Salmonella enterica* serovar Enteritidis strains isolated from different sources.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The twenty five strains of *Salmonella enterica* serovar Enteritidis used in this study were obtained from food, animal feed, animals and humans (Table 1).

The bacteria were grown on various solid and liquid media at 16ºC and 37ºC to determine the conditions for optimal expression of hemagglutinating activity. These media included brain heart infusion (BHI), colonization factor antigen (CFA), casaminoacids yeast extract (CYE), Luria-Bertani (LB), minimal medium (MM), phosphate buffered agar (PBA) and phosphate buffered broth (PBB), trypticase soy agar (TSA) and trypticase soy broth (TSB). Growth on solid media involved incubation at 16ºC for 72 h and at 37ºC for 18 h. Cultures in liquid media were incubated at 16ºC and at 37ºC and subcultured every 24 h for four days, with hemagglutination activity being tested each day.

Strain SA 183, isolated from a human foodborne infection, was chosen for further studies because when grown on CFA agar, the bacteria consistently expressed hamagglutinating activity.

Unless otherwise stated, the test strain was cultivated on CFA agar and incubated at 37ºC for 18 h prior to use.

Hemagglutination assays

Hemagglutination (HA) assays were carried out with erythrocytes of horse, sheep, cow, guinea pig, chicken and human phenotype O^+ D^+ C^{w-} C^+ c^+ E^- e^+ K^+ k^+ $Kp(a^-b^+)$ $Jk(a^+b^-)$ P_1+ $Le(a^-b^-)$ $Lu(a^-b^+)$ M+ N+ S+ s+ $Fy(a^+b^+)$ or phenotype A^+ D^- C^{w-} C^+ c^+ E^- e^+ K^- k^+ $Kp(a^-b^+)$ $Jk(a^+b^+)$ P_1+ $Le(a^-b^+)$ $L(a^-b^+)$ M+ N- S+ s- $Fy(a^+b^+)$. Bacteria were harvested and suspended in 10 mM phosphate buffered saline (PBS) pH 7.2 to yield approximately 10^9 bacteria per ml. The bacterial suspension and the crude hemagglutinin (100 ml) (see below) was serially diluted twofold with PBS in a 96-well U-bottomed microtiter plate, followed by incubation with an equal volume of a 1% suspension of erythrocytes in PBS for 2 h at 4ºC. The HA titers were recorded as the reciprocal of the highest dilution of bacteria or crude hemagglutinin yielding visible agglutination. Titers higher than four were considered positive.

Carbohydrate inhibition of hemagglutination

The inhibitory effect of sugars on hemagglutinating activity was determined using human erythrocytes as described above. Fifty microliters of a 1% erythrocyte suspension containing 1% sugar was added to 50 ml of serially diluted crude hemagglutinin. In another test crude hemagglutinin was added to 10 mM PBS pH 7.2, containing 1% sugar followed by incubation at room temperature for 30 min. After the addition of erythrocytes (1% suspension), the plates were again incubated and the hemagglutination recorded as described above. The sugars tested were adonitol, D-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, D-mannitol, D-mannose, maltose, L-rhamnose, D-raffinose, D-sorbitol, sucrose, D-trehalose and D-xylose.

Preparation of cell-free hemagglutinating supernatant

S. Enteritidis (strain SA 183) grown on CFA agar at 37ºC for 18 h were harvested in 10 mM PBS, pH 7.2, and crude hemagglutinin was obtained by gentle shaking according to Camargo *et al*. (5). The supernatant and the pellet obtained by centrifugation (10,000 x g, 30 min) were tested for HA by the microtiter assay. The supernatant with hemagglutinating activity was filtered through a 0.45 mm membrane, dialyzed against deionized water for 48 h and designated crude hemagglutinin.

Electron microscopy

S. Enteritidis (strain SA 183) grown on CFA agar at 37ºC for 18 h was deposited on Formvar-coated copper grids (400 mesh), then negatively stained with 1% ammonium molybdate and examined with a LEO-EM906 transmission electron microscope operated at 60 kV.

RESULTS AND DISCUSSION

Adherence is an important initial event in bacterial pathogenicity. Pathogenic bacteria usually develop surface structures whose primary function is interaction with receptors in the membranes of target cells, but may also cause agglutination of erythrocytes of particular animal species. This interaction with erythrocytes reflects the presence of surface structures with a role in adherence to epithelial cells that may be essential to the progress of an infection (11,13,15).

The 25 *S.* Enteritidis strains tested were able to agglutinate human erythrocytes and this effect was not observed with erythrocytes from other species. Hemagglutinins specific for human erythrocytes have also been described previously in *Escherichia coli* (32,34). In addition, *Salmonella* strains able

to agglutinate erythrocytes from a range of animals, or which show only weak hemagglutination of human erythrocytes have been reported (2,3).

The optimum culture medium for the expression of hemagglutinating activity was CFA agar, while hemagglutinin were poorly expressed in MM and CYE agar. Most strains showed hemagglutinating activity when cultivated in BHI, LB, PBA and TSA (Table 1). These results agree with previous studies on the expression of *Salmonella* adhesins which concluded that CFA was the most suitable medium for expression of colonization factors (7,24,33).

When *Salmonella* strains were grown on several solid media they agglutinated human red blood cells, but no reaction was observed when the bacteria were grown in liquid media, even after serial subculturing. Growth on solid media promotes the expression of mannose-resistant hemagglutinin but diminishes the expression of mannose-sensitive hemagglutinin, whereas cultures in broth produce mannose-sensitive hemagglutinins (13). In the present study, the best production of mannose-sensitive hemagglutinin was obtained when the strains were cultivated on solid media.

The differences in hemagglutinin production between broth- and agar-grown cultures suggested that surface contact was important in regulating of the expression of hemagglutinins. Other studies have shown that the expression of *S*. Enteritidis adhesins is enhanced by growth on agar surfaces, indicating

Table 1. Mannose-sensitive hemagglutination (MSHA) of human erythrocytes by *Salmonella* Enteritidis cultured in different solid media.

Strain Designation	Origin	MSHA*/ culture medium /temperature													
		BHI		CFA		CYE		LB		TSA		MM		PBA	
		16ºC	37ºC	16ºC	37ºC	16ºC	37ºC	16ºC	37ºC	16ºC	37ºC	16ºC	37ºC	16ºC	37ºC
SA 101	Chicken	-	-	-	512	-	16	-	32	-	16	-	-	-	256
SA 109	Chicken	-	16	-	256	-	32	-	256	-	64	-	-	-	128
SA 163	Chicken	-	32	-	512	-	-	-	16	-	32	-	-	-	512
SA 164	Chicken	-	32	-	128	-	-	64	64	-	8	-	-	-	-
SA 166	Chicken	16	32	-	512	-	-	-	64	16	32	-	-	-	512
SA 177	Chicken	8	64	-	256	-	32	128	256	-	32	-	-	-	256
SA 186	Chicken	-	-	-	32	-	-	16	32	-	-	-	-	-	32
SA 189	Chicken	-	-	-	256	-	-	32	128	-	32	-	64	-	512
SA 200	Chicken	-	-	-	256	-	-	32	32	8	16	-	-	-	32
SA 212	Chicken	-	8	-	256	-	-	128	256	8	128	8	64	-	256
SA 213	Chicken	-	64	-	256	-	-	8	16	8	16	-	16	-	32
SA 242	Chicken	8	32	8	512	-	-	64	64	-	16	-	-	-	256
SA 436	Chicken	-	128	16	256	-	-	16	32	8	16	16	64	8	256
SA 054	Chicken	32	64	8	256	32	64	8	256	-	8	-	-	-	32
SA 232	Egg	8	16	-	32	-	-	16	16	-	16	-	-	-	32
SA 108	Feed	-	8	-	256	-	32	32	32	-	64	-	-	-	256
SA 150	Feed	-	128	-	256	-	-	8	8	-	16	-	-	-	32
SA 145	Food	-	-	32	64	-	-	16	16	8	16	-	-	8	32
SA 091	Human	16	32	32	256	-	32	128	256	8	64	-	-	-	32
SA 103	Human	-	32	-	256	-	256	32	256	-	16	-	-	-	128
SA 104	Human	-	32	-	256	-	256	32	256	-	32	-	-	-	128
SA 183	Human	16	128	-	1024	-	-	16	256	8	16	8	64	-	32
SA 153	Pig	-	16	-	256	-	32	32	64	-	64	-	-	-	64
SA 155	Pig	-	-	-	512	-	-	-	256	-	32	-	64	-	256
SA 235	Pig	-	32	-	512	32	64	64	128	-	16	-	-	-	-
Total positive		7	19	5	25	2	10	20	25	8	24	3	6	2	23
Total negative		18	6	20	0	23	15	5	0	17	1	22	19	23	2

*Values are the reciprocals of the highest dilutions at which hemagglutination was detectable.

that surface contact is an environmental signal for fimbrial expression (29,31). Contact between *Salmonella* spp. and host cells appears to be important in salmonellosis. Many of invasion-associated genes of *S.* Typhimurium are expressed only after the bacteria has already established intimate contact with the host cell (27). Type III secretion system, important in the invasion process, is triggered when the pathogen comes in close contact with host cells (6). Inv J protein and surface appendages (invasomes), required for bacterial internalization, are induced upon contact with epithelial cells (12,35).

The expression of hemagglutinating activity was better for cultures at 37ºC than those at 16ºC. Strains grown at 16ºC showed decreased mannose-sensitive hemagglutination compared with those grown at 37ºC (Table 1). These results agree with previous reports on the expression of *Salmonella* hemagglutinins (7,14,24,31).

The expression of hemagglutinating activity by *S.* Enteritidis was highly dependent on the culture conditions, already observed for *Salmonella* serotypes and other gram-negative bacteria (11,24,29,31). Thus, the correct choice of conditions for bacterial growth is important when evaluating the capacity for hemagglutination.

Regarding inhibition of hemagglutinating activity by sugars, the activity was observed only in the absence of D-mannose and this effect was not observed in the presence of this sugar, suggesting the sole presence of mannose-sensitive hemagglutinins. D-mannitol, melibiose, D-raffinose, L-rhamnose and sucrose (1%) also inhibited the hemagglutination, whereas adonitol, D-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-sorbitol, D-trehalose and D-xylose did not. These observations confirmed some of the previous findings reported for *Salmonella*, although the inhibition by certain sugars differed from that reported in other studies (10,16,18). Presumably, the sugars that inhibited hemagglutination resemble or are identical to residues available for binding to adhesins on mammalian cell membranes.

In the present study hemagglutination activity produced by *S*. Enteritidis strain SA 183 was detected in cell-free culture supernatant after gentle agitation and centrifugation. Moreover, it was observed that washing bacteria with PBS inhibited hemagglutinating activity (data not shown). These findings indicate either that the mannose-sensitive hemagglutinin produced by *S.* Enteritidis is not firmly bound to the cell, or that an unidentified secretion mechanism may be involved. Tavendale *et al*. (26) and Old and Tavendale (19) described hemaglutinins produced by *S*. Typhimurium and *S*. Dublin that were not associated with bacterial cells, but could be detected in the supernatant.

Ultrastructural analysis of the hemagglutinating strain SA 183 by negative staining revealed no fimbria-like filamentous structures on the bacterial surface, suggesting that the hemagglutinin produced by this strain is not related to a fimbrial structure, which are usually characterized by a filamentous structure (11,15). Nonfimbrial soluble hemagglutinins have been described in *Salmonella* spp. (14,19,26), but they are mannose-resistant and hence different from the mannose-sensitive hemagglutinin identified here.

Many enteropathogenic bacteria have well-characterized hemagglutinating properties which are indicative of an ability to adhere to intestinal mucosal surfaces (11,13,15). Correlation between hemagglutination, adhesion and bacterial pathogenicity have been demonstrated (2,3,8,9). In *S.* Enteritidis, several fimbrial hemagglutinins have been implicated in bacterial attachment, colonization and pathogenicity. SEF14 has been shown to contribute to bacterial adherence to mouse epithelial cells and to chicken ovarian granulosa cells (20,28). In addition, SEF 14 may be required for systemic infections (8,21). Aslanzadeh and Paulissen (2,3) demonstrated a role for SEF21 fimbriae (type 1 fimbriae) in the *in vitro* adhesion of *S.* Enteritidis to mouse intestinal epithelial cells. Jones and Richardson (14) associated a nonfimbrial mannose-resistant hemagglutinin with adhesion of *S*. Typhimurium to HeLa cells. In contrast, other studies have failed to demonstrate a role for fimbrial and nonfimbrial hemagglutinins in the adherence of *S.* Enteritidis and *S*. Typhimurium, respectively (1,21,22,26,30).

Our findings indicate that *S*. Enteritidis produces a nonfimbrial mannose-sensitive hemagglutinin. Further studies of this hemagglutinin, as its purification and characterization and studies to correlate it with attachment to epithelial cell lines are being carried out to determine the possible role of this putative adhesin in salmonellosis.

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RESUMO

Propriedades hemaglutinantes de *Salmonella enterica* sorotipo Enteritidis isoladas de diferentes fontes

Foram estudadas 25 amostras de *Salmonella enterica* sorotipo Enteritidis isoladas de diferentes fontes, em testes de hemaglutinação. Amostras bacterianas cultivadas em diferentes meios de cultura causavam hemaglutinação na presença de hemácias humanas, entretanto, não foi observada reação com hemácias de outras espécies. A expressão da atividade hemaglutinante foi melhor em ágar CFA a 37ºC. A hemaglutinação foi inibida por D-manose, D-manitol, melibiose, D-rafinose, L-ramnose e sacarose. A análise ultraestrutural não revelou a presença de estruturas filamentosas na superfície bacteriana, sugerindo que a hemaglutinina de *Salmonella* Enteritidis seja de natureza não fimbrial. Os dados sugerem que *Salmonella*

Enteritidis produz uma hemaglutinina não fimbrial manose-sensível, específica para hemácias humanas, que pode ser extraída na forma solúvel.

Palavras-chave: *Salmonella* Enteritidis, atividade hemaglutinante, hemaglutinina manose-sensível, hemaglutinina não fimbrial

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