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Invasion of Caco-2 Cells by Salmonella typhimurium (Copenhagen) Isolates from Healthy and Sick Chickens

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SUMMARY. In a previous study, *Salmonella* isolates of sick birds were distinguished from those of apparently healthy birds by their high degree of invasion of tissue culture cells. In this study, a single pair of *Salmonella* isolates was examined to determine the source of this observed difference in invasion. When isolates were allowed to invade Caco-2 cells for 8 hours, the isolate from the sick bird (S) appeared to invade in greater numbers than did the isolate from the healthy bird (H). However, when invasion was distinguished from intracellular growth/survival, it was found that H invaded in greater numbers than S, but once inside the cell, H declined in number, and S increased. Inhibition of RNA, protein, and DNA syntheses lessened the degree to which both invaded. The presence of mannose inhibited invasion by S but did not appear to inhibit invasion by H. Trypsin treatment of monolayers affected invasion of S and H, whereas neuraminidase treatment did not. There was no significant difference noted between S and H in ability to adhere to fixed monolayers. Therefore, the two isolates tested differ in their mechanisms of entry into Caco-2 cells, the efficiency with which they invade, and their ability to survive within Caco-2 cells.

RESUMEN. Invasión de las células Caco-2 por cepas de Salmonella typhimurium (Copenhage) procedentes de aves sanas y enfermas.

En un estudio previo, las cepas de *Salmonella* de aves enfermas se diferenciaron de aquellas de aves aparentemente sanas por su capacidad para invadir cultivos de células. En este estudio se evaluaron dos cepas de *Salmonella* para determinar el origen de esta capacidad invasora. Cuando las bacterias fueron puestas en contacto con los cultivos de células Caco-2 durante 8 horas, la cepa procedente de aves enfermas mostró mayor invasividad que la cepa procedente de las aves sanas. Sin embargo, cuando la invasión bacteriana fue diferenciada por la forma de crecimiento y sobrevivencia intracelular, se encontró que la cepa procedente de aves sanas invadió en mayor número que la cepa de las aves enfermas; pero una vez dentro de las células, la cepa de las aves sanas disminuyó en número y aumentó la de las aves enfermas. La inhibición del ácido ribonucléico, la proteína y la síntesis del ácido desoxiribonucléico disminuyeron a medida que ambas cepas invadían las células. La presencia de manosa inhibió la invasión de la cepa procedente de las aves enfermas pero no la de la cepa de las aves sanas. El tratamiento de las monocapas con tripsina afectó la invasión de ambas cepas, mientras que el tratamiento con la neuraminidasa no lo hizo. No hubo diferencia significativa entre ambas cepas en la capacidad de adherirse a las monocapas. Por lo tanto, las dos cepas en estudio difieren en el mecanismo de entrada a las células Caco-2, en la eficiencia invasora y en la capacidad de sobrevivencia dentro de ellas.

Abbreviations: FBS = fetal bovine serum; H = isolate from healthy bird; LB = Luria-Bertani; LPS = lipopolysaccharide; MDCK = Madin-Darby canine kidney; MEM = minimum essential medium; MSHA = mannose-sensitive hemagglutinins; NA = nutrient agar; PBS = phosphate-buffered saline; S = isolate from sick bird

Avian salmonellosis is a major concern of the poultry industry. Reports show that as many as 75% of the chicken and turkey flocks in the United States may be infected with one or more of the numerous *Salmonella* serotypes (13) and that this percentage has changed little since 1969 (13). From an economic standpoint salmonellosis is considered to be one of the most

important bacterial diseases of the poultry industry. Financial losses due to the disease in poultry have been estimated at $77 million per year (8).

Much of the past research on poultry salmonellosis has dealt with characterization of the numerous *Salmonella* isolates found in avian hosts, the prevalence of salmonellosis in certain geographical regions, the epidemiology of the disease, and its effects on the public health sector. Only limited work has been done on identifying the virulence factors enabling salmonellae to cause disease in birds. One study did show that salmonellae of chickens with salmonellosis were more invasive in cultured epithelial cells than salmonellae carried by healthy chickens (9).

The purpose of the current study was to evaluate several factors involved in the process of invasion to determine whether the invasion of Caco-2 cells by an isolate from a chicken showing signs of salmonellosis and an isolate obtained from an apparently healthy chicken was influenced by these factors. The overall invasion processes of these two isolates, including adherence, actual invasion, and intracellular survival, were compared.

MATERIALS AND METHODS

Test organisms. The two *Salmonella typhimurium* (Copenhagen) isolates used in this study were from a bird with salmonellosis (S isolate) and from a healthy monitored bird (H isolate). Both have been characterized elsewhere and were chosen as representatives of the two groups of isolates studied by Nolan *et al.* (9). In the previous study by Nolan *et al.*, these two groups of isolates were distinguished by a difference in numbers invading primary chick kidney cells after a 6-hour invasion period, by their ability to use various carbon sources, and by their possession of mannose-sensitive hemagglutinins. Further, H contained a single 32 megadalton plasmid, and S contained no plasmids (9). Other organisms used in this study were *Escherichia coli* strain HB101 (6), used as a negative control in the adhesion experiments, and *S. typhimurium* strain SR11 (6), used as a positive control in the adhesion experiments.

Media. Bacterial isolates were maintained on Luria-Bertani (LB) agar (12) or in LB broth supplemented with antibiotics (12) as required. For adhesion and invasion assays, nutrient agar (NA) (12) was used to determine the number of colony-forming units (CFU).

Tissue culture. Caco-2 cells (HTB 37) (ATCC, Rockville, Md.), isolated from an adenocarcinoma from an adult human colon, were used between passages 18 and 30 (2). Cells were grown in minimum essential medium (MEM) with nonessential amino acids and 20% fetal bovine serum (FBS) without antibiotics in an atmosphere of 95% air–5% CO_2. Trypsinized Caco-2 cell suspension (1.5×10^5 cells) (2) in tissue culture medium with FBS was added to three wells of a 24-well microtiter plate (Becton Dickinson, Lincoln Park, N.J.). Media were changed every 3 days, and monolayers were used after 10–14 days of incubation. Prior to bacterial addition, monolayers were rinsed with MEM containing FBS.

Adhesion assay. The number of bacteria that adhered to glutaraldehyde-fixed cells was determined according to the procedure of Kusters *et al.* (6). The day preceding the assay, confluent monolayers were washed twice with cold (4 C) phosphate-buffered saline (PBS). Two milliliters of 2.5% glutaraldehyde (Sigma, St. Louis, Mo.) were added for 1 hour at 4 C. The glutaraldehyde solution was removed, and the cells were rinsed once with 4 ml of cold PBS. Then the cells were incubated with 4 ml of cold PBS per well for 30 minutes before that solution was discarded. This 30-minute wash was repeated three times. After the PBS washes, 4 ml of PBS were added to each well, and the fixed monolayers were stored at 4 C overnight. On the day of the assay, the cold PBS was removed, and the monolayers were rinsed once with warm (37 C) MEM. The bacterial inoculum was added to the monolayer, and the plates were placed in a centrifuge and spun at $600 \times g$ for 5 minutes. Bacteria were applied to the monolayer for 30 minutes to allow adhesion. The bacterial suspension was then removed, and the monolayers washed five times each with 4 ml of cold PBS to remove nonadherent bacteria. To release adherent bacteria, PBS containing 1% (vol/vol) Triton X-100 (1 ml per well) was applied to the monolayer and incubated at room temperature for 5 minutes or until tissue culture cells were ruptured. The bacteria were resuspended in this solution, diluted, and plated on NA plates, and the plates were incubated overnight to determine the number of viable cells.

Invasion assays. Overnight cultures of bacteria to be tested were washed three times in PBS and resuspended to their original volumes. This suspension was diluted to obtain approximately 1×10^7 bacteria in an inoculum that was applied to the MEM-washed monolayers. The infected monolayers were incubated at 37 C for 30 minutes in an atmosphere of 95% air–5% CO_2. Monolayers were washed with PBS, and fresh, warm MEM containing 125 μg/ml gentamicin was added for 90 minutes to kill extracellular bacteria (2). After this incubation period, monolayers were rinsed with PBS, bacteria were released by lysis

of the tissue culture cells, and viable counts were performed as described previously.

Numerous agents were used to alter the surface of tissue culture cells or to interrupt bacterial metabolism to further test for differences in invasion between the test isolates (3,7). These invasion experiments were conducted with a 30-minute invasion time period. Invasion of monolayers treated with trypsin (0.025%) at room temperature for 4 minutes was used to study the role that protein receptors on the Caco-2 cells might play in invasion. Tissue culture cells were also treated with neuraminidase (1 U/ml) at 37 C for 15 minutes to study the effect that destruction of sialic acid residues might have on invasion. Pretreatment of bacterial isolates with rifampin, chloramphenicol, or naladixic acid (all antibiotics at 30 μg/ml for 15 minutes) (7) was also done in order to determine the effect of inhibiting bacterial RNA transcription, peptidyl transfer, and DNA replication, respectively, on invasion. Treatment of bacteria with mannose (17 mg/ml in MEM for 15 minutes) prior to invasion was used to determine if mannose-sensitive hemagglutinins were used in the invasion process (7).

Intracellular survival assay. To measure intracellular survival the cells were infected as described in the invasion assay, but after the gentamicin was removed, fresh MEM was added to the cells and these were allowed to incubate for 6 and 8 hours before viable counts were performed.

Lipopolysaccharides (LPS). Overnight cultures, in an amount sufficient to yield 1 g of wet pellet each when centrifuged, were pelleted by centrifugation (4000 × *g* for 15 minutes), and the pellets subjected to the Darveau and Hancock method of LPS isolation (1).

Biostatistics. The data were analyzed using an analysis of variance and a follow-up Tukey multiple comparison test (14).

RESULTS

Adhesion. There was a significant difference in adhesion among the isolates tested ($F = 40.7$, df = 3,29, $P < 0.0001$) (Table 1). This difference was due to the low numbers of the control *E. coli* that adhered to the cultured cells as compared to the salmonellae tested. There was no significant difference between the mean adhesion of the S and H isolates.

Invasion and intracellular survival. After a 30-minute period of exposure to cultured cells, extracellular bacteria were killed with gentamicin. Then, aliquots of cultured cells were lysed to release intracellular bacteria (at the 30-minute time period) as an indication of initial invasion. Aliquots of cultured cells were also lysed following a period of intracellular existence at 6 and 8 hours following gentamicin treatment as an indication of intracellular survival and growth. Numbers of bacteria invading or surviving were determined by viable counts. All assays were performed in triplicate. At 30 minutes, H invaded the Caco-2 cells at significantly greater numbers than did S ($F = 40.1$, df = 5,45, $P < 0.0001$) (Table 2). At the 6-hour interval, there was no significant difference in bacteria within the cells. At the 8-hour interval, S was present inside the cells in significantly greater numbers than was H ($F = 40.1$, df = 5,45, $P < 0.05$). To test the need for *de novo* RNA transcription, protein synthesis, or DNA replication for invasion of Caco-2 cells by these isolates, invasion assays were performed following pretreatment of bacteria with rifampin, chloramphenicol, or nalidixic acid, respectively. The addition of these three antibiotics significantly decreased invasion by the bacteria. Pretreatment of the Caco-2 cells with trypsin decreased invasion significantly by both S and H (Table 3), whereas neuraminidase treatment did not. The presence of mannose significantly decreased invasion by S but not by H.

Table 1. Adhesion to glutaraldehyde-fixed Caco-2 cells by S and H isolates.

Isolate	Log CFU/ml at 30 minutes[A]
Salmonella typhimurium	
S	4.50[a]
H	4.44[a]
SR11	4.44[a]
Escherichia coli	
HB101	3.90[b]

[A]Means with different superscripts are significantly different from each other at the 5% level of probability.

LPS. No apparent differences were noted in the LPS patterns of S and H. Both isolates appear to exhibit smooth LPS patterns (Fig. 1).

DISCUSSION

The results presented here indicate that the H and S isolates, which were chosen as representatives of the larger groups of isolates (9), do differ in their mechanisms of entry into cells, in the efficiency with which they invade, and in their ability to survive within cells. In the pre-

Table 2. Invasion of/survival within Caco-2 cells by S and H isolates.

Isolate	Log CFU/ml[A]		
	30 minutes	6 hours	8 hours
S	3.67[a]	4.19[a]	4.66[a]
H	4.30[b]	4.31[a]	3.82[b]

[A]Within a column means with the same superscript are not significantly different at the 5% level of probability.

vious study, in which these and other isolates were allowed to invade primary chick kidney cells for 6 hours (9), isolates of sick and healthy birds were found to differ by their degree of invasiveness. In this study, the invasion of Caco-2 cells by a pair of these isolates was examined in greater detail, with invasion considered as a process involving the three discrete events of adhesion, invasion, and intracellular survival.

The isolates appeared to have equal abilities to adhere to fixed monolayers, although their mechanisms of adherence appeared to differ. In the previous study (9), it was found that S had the ability to hemagglutinate erythrocytes. This ability was inhibited in the presence of mannose, suggesting that S possessed type-I fimbriae, also known as mannose-sensitive hemagglutinins (MSHA) (9). H was negative for this trait. In this study, S demonstrated reduced invasion of tissue culture cells in the presence of mannose, suggesting that MSHA do play an important role in the invasion process by this isolate. Lack of MSHA, however, did not hinder adhesion or invasion by H, suggesting that H employs something other than MSHA in its adherence and invasion of cultured cells. Finlay *et al.* (4) also found that type-I fimbriae were not required for *Salmonella* to adhere to eukaryotic cells.

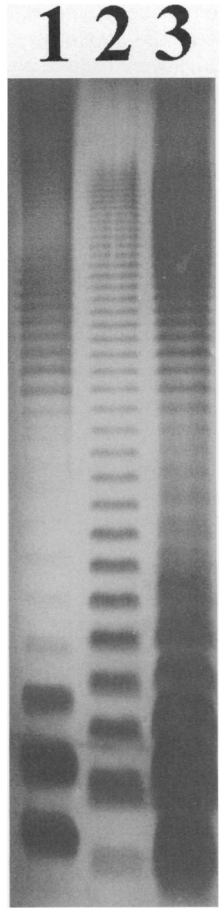

Fig. 1. LPS comparison. SDS-PAGE of S and H LPS. (1) S LPS, (2) H LPS, (3) commercially obtained LPS derived from the smooth strain, *E. coli* (0111:B4). S is the isolate from the bird with salmonellosis, and H is the isolate from the apparently healthy bird.

H entered tissue culture cells in significantly greater numbers than did S after a 30-minute exposure. Because this result did not mirror the greater invasion by S after the 6-hour invasion

Table 3. Invasion of Caco-2 cells by the S and H isolates following pretreatment of bacteria or monolayers.

Treatment	Mean log CFU/ml[A]			
	S	S (treated)	H	H (treated)
Trypsin	4.36[b]	3.84[a]	4.38[b]	4.05[a]
Neuraminidase	4.36[ab]	4.24[a]	4.38[b]	4.33[ab]
Rifampin	4.36[b]	4.14[a]	4.38[b]	3.97[a]
Chloramphenicol	4.36[b]	2.30[a]	4.38[b]	2.00[a]
Nalidixic acid	4.36[b]	4.03[a]	4.38[b]	4.12[a]
Mannose	4.36[a]	4.00[b]	4.38[a]	4.47[a]

[A]Within a row means with different superscripts are significantly different at the 5% level of probability.

period used in the previous study (9), it was thought that the difference between the two isolates might lie in their abilities to survive once inside the cells. When intracellular survival of S and H was compared, it was found that although H enters cells with greater efficiency than S, it does not fare as well as S once inside the cell. Such results suggest that, at least for these isolates, the association of *Salmonella* isolates with disease may be due more to their intracellular survival ability than to their invasiveness.

Invasion by salmonellae involves interactions between bacteria and host cells, with the host cell playing an active role in the uptake of salmonellae (4,5). When trypsin was used to disrupt the surface proteins of the Caco-2 cells, a significant decline in the number of internalized bacteria occurred with both isolates. Pace *et al.* (10) demonstrated that *Salmonella* invasion activates a protein hormone receptor on the surface of tissue culture cells, which may trigger actin rearrangement in the plasma membrane to promote internalization.

When monolayers were treated with neuraminidase there was no significant decline in invasion by the isolates. This result indicates that sialic acid receptors were not important in this invasion model using these bacterial isolates. Finlay *et al.* (3), however, found that when Madin-Darby canine kidney (MDCK) cells were treated with neuraminidase prior to bacterial inoculation, significant decreases in *Salmonella* invasion occurred. *Salmonella* isolates from chickens may differ from mammalian isolates in host receptors used, or the invasion of MDCK and Caco-2 cells by salmonellae may be different due to the presence of different cell surface receptors on the cultured cells.

When the isolates were treated with bacteriostatic levels of antibiotics before invasion, both isolates showed significant decreases in invasion, indicating the need for DNA, protein, and RNA syntheses for invasion. Finlay *et al.* (3) found that *Salmonella choleraesuis* required protein and RNA syntheses for invasion but that DNA synthesis was not needed. Results from this study indicate that DNA replication may be necessary for invasion by some salmonellae. That is, different *Salmonella* isolates may use different processes for invasion.

Both isolates in this study exhibited smooth LPS profiles. Smooth LPS patterns are associated with virulence (11). Based on the results of this study, a smooth LPS does not appear to ensure the survival of salmonellae within the intracellular environment. Of course, H and S could vary in LPS composition in a way not detected with the LPS analysis technique used in this study.

Finally, the choice of cells for this comparison was complicated. The previous study used primary chick kidney cells for the invasion assay. In this study, a continuous cell line was sought to ensure that other researchers had access to similar cells and to eliminate any variability associated with primary cells. The Caco-2 cell line was chosen because it is frequently used in *Salmonella* invasion assays (2) and because it is morphologically similar to intestinal tract epithelium (2). However, it is a human-derived cell line. Although measurable and repeatable differences were observed using the Caco-2 cell line with these avian *Salmonella* isolates, repetition of the present study with a continuous avian-derived cell line may shed further light on the overall invasion process of salmonellae in birds. Further, study of intracellular survival of salmonellae in macrophage-like cell lines rather than in an epithelial-like line may be more reflective of virulence mechanisms important to the pathogenesis of salmonellosis in the host. Invasion and survival using these isolates in cultured macrophages must be completed before it can be determined if intracellular survival in a nonmacrophage cell line correlates with survival in macrophages.

In summary, the two isolates tested differ in their mechanisms of entry into Caco-2 cells, in the efficiency with which they invade, and in their ability to survive in Caco-2 cells. To further characterize these differences, future research may include an examination of the intravacuolar environment that *Salmonella* encounter in the host cell. Experiments mimicking the vacuole microenvironment might lead to the discovery of factors that further differentiate isolates from sick and apparently healthy chickens.

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