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Source: Avian Diseases, 50(1):77-81. 2006.
Published By: American Association of Avian Pathologists
DOI: http://dx.doi.org/10.1637/7417.1
URL: http://www.bioone.org/doi/full/10.1637/7417.1

AVIAN DISEASES 50:77–81, 2006

Virulence Genotyping of *Salmonella* spp. with Multiplex PCR

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Received 2 August 2005; Accepted 30 October 2005

SUMMARY. The purpose of this study was to develop a multiplex polymerase chain reaction (PCR) protocol useful in the virulence genotyping of *Salmonella* spp. with the idea that genotyping could augment current *Salmonella* characterization and typing methods. Seventeen genes associated with *Salmonella* invasion, fimbrial production, toxin production, iron transport, and intramacrophage survival were targeted by three PCR reactions. Most of these genes are required for full *Salmonella* virulence in a murine model, and many are also located on *Salmonella* pathogenicity islands (PAIs) and are associated with type III secretion systems (TTSSs). Once the success of procedures that used positive and negative control strains was verified, the genotypes of 78 *Salmonella* isolates incriminated in avian salmonellosis (primarily from sick, commercially reared chickens and turkeys) and 80 *Salmonella* isolates from apparently healthy chickens or turkeys were compared. Eleven of the 17 genes tested (*invA, orgA, prgH, tolC, spaN* [*invJ*], *sipB, sitC, pagC, msgA, spiA,* and *iroN*) were found in all of the isolates. Another (*sopB*) was present in all isolates from sick birds and all but one isolate from healthy birds. The remaining five genes (*lpfC, cdtB, sifA, pefA,* and *spvB*) were found in 10%–90% of the isolates from sick birds and 3.75%–90% of the healthy birds. No significant differences in the occurrence of these genes between the two groups of isolates were detected. These results suggest that these virulence genes, and presumably the PAIs and TTSSs with which they are associated, are widely distributed among *Salmonella* isolates of birds, regardless of whether their hosts of origin have been identified as having salmonellosis.

RESUMEN. Genotipificación de la virulencia de *Salmonella* spp. mediante una prueba múltiple de reacción en cadena por la polimerasa.

El propósito del presente estudio fue desarrollar un protocolo para una prueba de reacción en cadena por la polimerasa múltiple (por sus siglas en Iñgles PCR), que pueda ser útil en la genotipificación de la virulencia de *Salmonella* spp., con la idea de que la genotipificación pueda unirse a los métodos de caracterización y tipificación actuales. Mediante tres reacciones de PCR se dirigió a diecisiete genes asociados con invasión de la *Salmonella*, producción de fimbria, producción de toxinas, transporte de hierro, y supervivencia dentro de macrófagos. En un modelo basado en ratón, la mayoría de estos genes son requeridos para una virulencia total en *Salmonella* y muchos de ellos además se ubican en las denominadas islas de patogenicidad de *Salmonella*, asociadas con los sistemas de secreción tipo III. Una vez verificado el éxito de los procedimientos utilizando cepas control positivas y negativas, se compararon los genotipos de 78 aislamientos de *Salmonella* incriminados en salmonelosis aviar (fundamentalmente de pollos y pavos enfermos criados comercialmente) y 80 aislamientos de *Salmonella* provenientes de pollos o pavos aparentemente sanos. Once de los 17 genes analizados (*invA, orgA, prgH, tolC, spaN* [*invJ*], *sipB, sitC, pagC, masgA, spiA*, e *iroN*) fueron identificados en todos los aislamientos. Otro gen (*sopB*) estuvo presente en todos los aislamientos provenientes de aves enfermas y en todos menos uno de los aislamientos provenientes de aves sanas. Los cinco genes restantes (*lpfC, cdtB, sifA* y *spvB*) se identificaron en el 10%–90% de las aves enfermas y en el 3.75%–90% de las aves sanas. No se detectaron diferencias significativas en la ocurrencia de estos genes entre los dos tipos de aislamientos. Estos resultados sugieren que estos genes de virulencia y presumiblemente las islas de patogenicidad de *Salmonella* y los sistemas de secreción tipo III a los cuales están asociados, se encuentran ampliamente distribuidos entre los aislamientos de *Salmonella* en las aves, independientemente de si el huésped originario ha sido identificado padeciendo salmonelosis.

Key words: *Salmonella*, virulence, poultry, multiplex PCR, genotyping

Abbreviations: CFU = colony-forming unit; ddH_2O = double-distilled water; DNA = deoxyribonucleic acid; PAI = pathogenicity island; PCR = polymerase chain reaction; SPI = *Salmonella* pathogenicity island; TTSS = type III secretion system

In this study, salmonellae incriminated in avian salmonellosis and salmonellae isolated from clinically healthy birds were characterized by their possession of certain putative virulence traits in an effort to identify parameters that could be useful in *Salmonella* detection protocols and factors that are important to the virulence of these organisms. Three multiplex polymerase chain reaction (PCR) protocols targeting 17 putative virulence genes were developed. Previous studies demonstrated that *Salmonella* spp. isolated from birds with salmonellosis were different from the *Salmonella* isolates from healthy birds by their invasiveness in cell culture (13,17). Therefore, in an effort to genetically differentiate between *Salmonella* from sick and healthy birds, the majority of the genes targeted in this study—*invA, orgA, prgH, spaN* (*invJ*), *tolC, sipB, pagC, msgA, spiA, sopB, lpfC, pefA, spvB,* and *sifA*—encode proteins associated with invasiveness traits, such cellular invasion/survival and adhesin or pili production (2,3,4,5,7,8,12,16,18,20,26,27,28). Other targeted genes encoded other traits thought to be important to virulence, including *sitC* (11) and *iroN* (1), both involved in iron acquisition, and *cdtB*, a putative toxin-encoding gene (9). All of the genes except *pefA, iroN, cdtB, sipB*, and *spaN* (*invJ*) have been shown to be required for full *Salmonella* virulence in a murine model (2,3,5,7,8,11,12,16,18,20,26,27).

MATERIALS AND METHODS

Bacterial strains. Seventy-eight *Salmonella* isolates incriminated in avian salmonellosis and 80 *Salmonella* isolates obtained from apparently healthy birds (either fecal swabs or carcass swabs at a poultry processing plant) were used in this study. The *Salmonella* isolated from sick birds

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Table 1. *Salmonella* serovars used in virulence genotyping.

Serovar	Source	
	Sick Bird ($n = 78$)	Healthy Bird ($n = 80$)
Agona	12	1
Anatum	1	1
Binza	2	0
Braenderup	1	1
Brandenburg	7	0
Bredeny	5	0
Derby	1	0
Enteritidis	1	1
Hadar	3	48
Heidelberg	7	1
Infantis	1	1
Kentucky	3	1
Litchfield	1	1
London	1	0
Madelia	2	0
Mbandaka	1	1
Ohio	1	0
Schwarzengrund	0	7
Senftenberg	16	4
Typhimurium	11	4
Multiple serovars	1	0
Not typed	0	8
Total	78	80

were collected from various diagnostic laboratories throughout the United States and originated from different avian hosts diagnosed with salmonellosis, primarily commercially reared chickens and turkeys. The *Salmonella* strains tested include the serovars listed in Table 1. The following non-*Salmonella* strains were obtained from the North Dakota Veterinary Diagnostic Laboratory and were used to evaluate the specificity of the PCR protocol for *Salmonella*: *Bacillus cereus*, *Micrococcus luteus*, *Pseudomonas fluorescens*, *Rhodococcus equi*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Streptococcus canis*, *Staphylcoccus aureus*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Shigella sonnei*, *Streptococcus suis*, and several *Escherichia coli* strains, some of which were known to contain homologs of the *tolC, iroN, sitC*, and *cdtB* genes. In preparation for amplification, isolates were grown on MacConkey or LB agar (Difco Laboratories, Detroit, MI) overnight at 37 C. Organisms were stored frozen at −80 C in brain heart infusion broth (Difco) containing 10% glycerol until use (23).

Amplification studies. *Multiplex procedures.* Test and control organisms were examined by multiplex PCR for the presence of several genes thought to be involved in *Salmonella* virulence. Targeted genes and their descriptions are summarized in Table 2, and the primer sequences used in the amplification studies are summarized in Table 3. Three reactions were used to amplify the seventeen genes. One reaction (set 1) amplified *spvB, spiA, pagC, cdtB,* and *msgA.* Another (set 2) amplified *invA, sipB, prgH, spaN, orgA,* and *tolC.* The third (set 3) amplified *iroN, sitC, lpfC, sifA, sopB,* and *pefA.* The cycling conditions and reaction mixtures were the same for each multiplex procedure used; only the primers differed among the three reactions. Appropriate primer sequences to be used in PCR amplification were selected with Lasergene software (DNAStar, Inc., Madison, WI) on the basis of published sequences for these genes (GenBank NC003197, NC003277, and AL627271). All primers used in the amplification studies were obtained from Integrated DNA Technologies (Coralville, IA).

To obtain the deoxyribonucleic acid (DNA) template, a single colony of the bacterium to be tested was transferred into a tube containing 40 µl of a lysing buffer (10 mM Tris-Cl, pH 7.5, 1 mM ethylenediaminetetraacetic acid with 50 µg/ml proteinase K [Amresco, Solon, OH] added the day of use). This mixture was heated in a thermocycler at 55 C for 10 min, followed by 10 min at 80 C. Then, 80 µl of double-distilled water (ddH_2O) was added, and the mixture was centrifuged for 30 sec at 10,000 × *g*.

Table 2. *Salmonella* genes used in virulence genotyping.

Gene	Virulence-related function	Reference
invA[A]	Host recognition/invasion	(5)
orgA[A]	Host recognition/invasion	(12)
prgH[A]	Host recognition/invasion	(3)
spaN	Entry into nonphagocytic cells, killing of macrophages	(4)
tolC[A]	Host recognition/invasion	(25)
sipB	Entry into nonphagocytic cells, killing of macrophages	(4)
sitC[A]	Iron acquisition	(11)
pagC[A]	Survival within macrophage	(16)
msgA[A]	Survival within macrophage	(8)
spiA[A]	Survival within macrophage	(18)
iroN	Iron acquisition	(1)
sopB[A]	Host recognition/invasion	(19)
lpfC[A]	Host recognition/invasion	(2)
cdtB	Host recognition/invasion	(9)
sifA[A]	Filamentous structure formation	(24)
pefA	Host recognition/invasion	(27)
spvB[A]	Growth within host	(7)

[A]Required for full virulence in a murine model.

Amplification was performed in a 25-µl reaction mixture that included 2.5 µl of template DNA, 15.75 µl of ddH_2O, 2.5 µl of 10× PCR buffer, 3.0 µl of 50 mM $MgCl_2$, 0.15 µl of *Taq* (5 U/µl; Invitrogen, Carlsbad, CA), 0.5 µl of 10 mM dNTPs mix (USB, Cleveland, OH), and 0.05 µl of 0.1 mM forward and reverse primers. Reaction mixtures were subjected to the following conditions in a Mastercycler Gradient (Perkin-Elmer, Boston, MA): 5 min at 95 C, 25 cycles of 30 sec at 94 C, 30 sec at 66.5 C, and 2 min at 72 C, with a final cycle of 10 min at 72 C, followed by a hold at 4 C. PCR products obtained were subjected to horizontal gel electrophoresis in 1.5% agarose, and the size of the amplicons was determined by comparison with the Hi-Lo DNA marker (Minnesota Molecular Inc., Minneapolis, MN). Strains known to possess or lack the genes of interest were examined with each amplification procedure. An isolate was considered to contain the gene of interest if it produced an amplicon of the expected size (Table 3).

Sensitivity and specificity of the multiplex PCR protocol. The sensitivity of the multiplex PCR protocol was determined in the following manner. The *Salmonella* strain to be used as template was incubated overnight in Luria-Bertani broth. (For sets 2 and 3, a single strain [15] was used as template; however, for set 1, two different strains and reactions were needed to assess its sensitivity because no strain was found to contain both *cdtB* and *spvB.*) A 1:2 dilution series was then made from this broth, and 50 µl of each dilution was placed in a 200-µl microfuge tube. These dilutions were then centrifuged at 10,000 × *g* for 5 min. The supernatant fluid from each tube was discarded, and the pellet was used to generate the template DNA. The number of colony-forming-units (CFUs) used as template was determined by viable plate counts performed in triplicate. Amplification was undertaken as described previously, and the success of the amplification was evaluated in relation to the number of bacterial cells serving as a source of template DNA. The specificity of the assay for *Salmonella* was determined by subjecting a number of non-*Salmonella* bacteria (see the Bacterial Strains section) to the multiplex PCR protocol.

Sequencing. To validate the multiplex PCR protocols to be used in this study to determine gene prevalence, the identity of the amplicons was confirmed by DNA sequencing. Amplicons were purified with the Promega Wizard PCR purification kit (Promega Inc., Madison, WI) and were then sent to the Iowa State Sequencing and Synthesis Facility for direct sequencing.

Biostatistics. The null hypothesis that the proportion of *Salmonella* isolated from sick birds exhibiting each factor was equal to the proportion of *Salmonella* isolated from healthy birds with that factor was tested by a *Z*-test on the difference between the proportions (25).

Table 3. Primers used for the amplification of virulence genes.

Gene	Primer sequence (5′→3′)[A]	Size (bp)	GenBank no.
spvB	F: CTATCAGCCCCGCACGGAGAGCAGTTTTTA R: GGAGGAGGCGGTGGCGGTGGCATCATA	717	NC003277
spiA	F: CCAGGGGTCGTTAGTGTATTGCGTGAGATG R: CGCGTAACAAAGAACCCGTAGTGATGGATT	550	NC003197
pagC	F: CGCCTTTTCCGTGGGGTATGC R: GAAGCCGTTTATTTTTGTAGAGGAGATGTT	454	NC003197
cdtB	F: ACAACTGTCGCATCTCGCCCCGTCATT R: CAATTTGCGTGGGTTCTGTAGGTGCGAGT	268	AL627271
msgA	F: GCCAGGCGCACGCGAAATCATCC R: GCGACCAGCCACATATCAGCCTCTTCAAAC	189	NC003197
invA	F: CTGGCGGTGGGTTTTGTTGTCTTCTCTATT R: AGTTTCTCCCCCTCTTCATGCGTTACCC	1070	NC003197
sipB	F: GGACGCCGCCCGGGAAAAACTCTC R: ACACTCCCGTCGCCGCCTTCACAA	875	NC003197
prgH	F: GCCCGAGCAGCCTGAGAAGTTAGAAA R: TGAAATGAGCGCCCCTTGAGCCAGTC	756	NC003197
spaN	F: AAAAGCCGTGGAATCCGTTAGTGAAGT R: CAGCGCTGGGGATTACCGTTTTG	504	NC003197
orgA	F: TTTTTGGCAATGCATCAGGGAACA R: GGCGAAAGCGGGGACGGTATT	255	NC003197
tolC	F: TACCCAGGCGCAAAAAGAGGCTATC R: CCGCGTTATCCAGGTTGTTGC	161	NC003197
iroN	F: ACTGGCACGGCTCGCTGTCGCTCTAT R: CGCTTTACCGCCGTTCTGCCACTGC	1205	NC003197
sitC	F: CAGTATATGCTCAACGCGATGTGGGTCTCC R: CGGGGCGAAAATAAAGGCTGTGATGAAC	768	NC003197
lpfC	F: GCCCCGCCTGAAGCCTGTGTTGC R: AGGTCGCCGCTGTTTGAGGTTGGATA	641	NC003197
sifA	F: TTTGCCGAACGCGCCCCCACACG R: GTTGCCTTTTCTTGCGCTTTCCACCCATCT	449	NC003197
sopB	F: CGGACCGGCCAGCAACAAAACAAGAAGAAG R: TAGTGATGCCCGTTATGCGTGAGTGTATT	220	NC003197
pefA	F: GCGCCGCTCAGCCGAACCAG R: GCAGCAGAAGCCCAGGAAACAGTG	157	NC003277

[A]F = forward; R = reverse.

RESULTS

None of the non-*Salmonella* strains (including *E. coli*, which is known to contain homologs of the *cdtB, iroN, sitC*, and *tolC* genes) produced amplicons by the multiplex PCR protocol described here. The sensitivity of this assay was also determined by relating the number of CFUs used to generate template with the ability to detect all amplicons in each protocol. For all three protocols, 1000 CFUs were sufficient to provide visible amplification of all genes possessed by an isolate in a 25-μl reaction. Also, the sequencing data obtained for each gene reflected its published sequence.

In creating the multiplex PCR protocols, care was taken to ensure that the individual amplicons could be distinguished from each other and were easily measured when compared with a standard DNA size marker (Fig. 1). To test the reproducibility of these assays, the multiplex procedures were repeated three times, each with 10 of the isolates assayed. In no case did the results differ among trials, indicating that these assays were reproducible.

A total of 80 isolates obtained from apparently healthy birds and 78 isolates from clinically ill birds were examined in this study (Table 1). Eleven of the genes tested—*invA, orgA, prgH, spaN, tolC, sipB, sitC, pagC, msgA, spiA,* and *iron*—were found in all of the isolates from both sick and healthy birds, whereas *sopB* was found in all of the isolates obtained from clinically ill birds, and all but one of the isolates was obtained from healthy birds. The other five genes were found in 10%–90% of the isolates (Table 4). Genes *lpfC* and *sifA* were found more frequently in isolates from healthy birds than in isolates from ill birds (90% *vs.* 79.49% and 90% *vs.* 83.33%, respectively); however, these differences were not statistically significant. Genes *cdtB, pefA,* and *spvB* were present less frequently than the other genes targeted in this study and also occurred in a limited number of serovars (Table 5). These three genes were found in a higher percentage of isolates from sick, rather than healthy, birds (16.67% *vs.* 10% for *cdtB*, 10.26% *vs.* 3.75% for *pefA*, and 10.26% *vs.* 5% for *spvB*, respectively). Again, however, the differences observed were not statistically significant.

DISCUSSION

In many enteric bacteria, virulence can be conferred by a single region of the genome (6). For example, the large virulence plasmid of *Shigella flexneri* can render a laboratory strain of *E. coli* invasive on transfer (19). However, the facultative intracellular lifestyle of *Salmonella* places demands on a large number of genes distributed around the chromosome (6). Many of these genes are also found in nonpathogenic or pathogenic strains of *E. coli*, indicating that they might not be useful in the specific detection of virulent salmonellae. Therefore, in this study, genes thought to contribute to *Salmonella* virulence were targeted. These virulence-specific *Salmonella* genes were the focus of this study because it was felt that they had the most promise in augmenting current detection protocols. A few of the

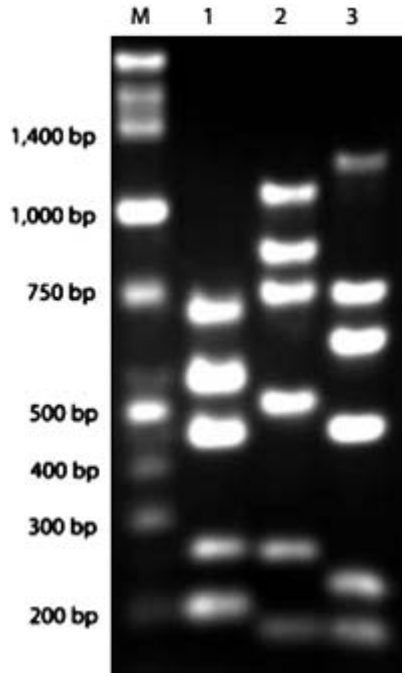

Fig. 1. Agarose gel of amplicons generated in the multiplex PCR protocols with the use of positive control *Salmonella* strains as a source of template DNA. Lane M contains a Hi-Lo DNA marker (Minnesota Molecular). Lane 1 is the result of the PCR reaction (in this case, template was pooled from two strains because no isolate was found to contain both *cdtB* and *spvB*) amplifying (from top to bottom) *spvB, spiA, pagC, cdtB,* and *msgA*. Lane 2 is the result of the PCR reaction amplifying (from top to bottom) *invA, sipB, prgH, spaN, orgA,* and *tolC*. Lane 3 is the result of the PCR reaction amplifying (from top to bottom) *iroN, sitC, lpfC, sifA, sopB,* and *pefA*.

genes targeted (*iroN, sitC, tolC*, and *cdtB*) have homologs in other enteric bacteria. However, the DNA homology between these genes is not 100%, providing differences we could exploit in designing PCR primers unique to *Salmonella*. Specifically, the *cdtB* and *tolC* forward primers, along with the *iroN* and *sitC* reverse primers, have no significant homology with any other bacteria, as determined by GenBank BLAST searches. The specificity of this multiplex PCR assay for *Salmonella* appeared to be confirmed because none of the non-*Salmonella* strains produced amplicons when tested.

Table 4. Prevalence of virulence genes in avian *Salmonella* spp.

Gene	Prevalence (%)		*Z*	*P*
	Healthy Bird ($n = 80$)	Sick Bird ($n = 78$)		
spvB[A]	5.0	10.26	−1.25	0.2124
spiA[BC]	100	100	—	—
pagC[B]	100	100	—	—
cdtB	10.0	16.67	−1.23	0.2172
msgA[B]	100	100	—	—
invA[BC]	100	100	—	—
sipB[BC]	100	100	—	—
prgH[BC]	100	100	—	—
spaN[BC]	100	100	—	—
orgA[B]	100	100	—	—
tolC	100	100	—	—
iroN	100	100	—	—
sitC[B]	100	100	—	—
lpfC[B]	90.0	79.49	1.84	0.0657
sifA[BC]	90.0	83.33	1.23	0.2172
sopB[B]	98.75	100	−0.991	0.3219
pefA[A]	3.75	10.26	−1.61	0.1081

[A] Plasmid encoded (7,27).
[B] Located on a *Salmonella* PAI or islet (6,11,14).
[C] Associated with a TTSS (18,26,28).

Table 5. Avian *Salmonella* serovars containing rarely found genes.

Serovar	No. of birds		*spvB*	*pefA*	*cdtB*
	Sick	Healthy			
Typhimurium	7	2	+	+	−
Enteritidis	1	1	+	+	−
Bredeny	5	0	−	−	+
Brandenburg	7	0	−	−	+
Schwarzengrund	0	7	−	−	+
Hadar	0	1	+	−	−
Untyped	0	1	−	−	+
Multiple	1	0	−	−	+

Of the 17 *Salmonella* genes targeted in this study, 12 (*invA, orgA, prgH, spaN* [*invJ*], *sipB, sitC, pagC, msgA, spiA, sopB, lpfC*, and *sifA*) are known to reside on pathogenicity islands (PAIs) or pathogenicity islets (6,11,14), which are distinct pieces of DNA containing blocks of virulence-associated genes. Common features of most, if not all PAIs, include that they encode one or more virulence factors and are likely introduced into the genome via horizontal transfer, resulting in G–C ratios and codon usage that might deviate from the organism's typical pattern (6). An additional two genes, *pefA* and *spvB,* are carried on plasmids (7,29), whereas the remaining genes (*iroN, tolC,* and *cdtB*) are located elsewhere in the *Salmonella* genome (1,9,27).

Of particular interest in this study were genes involved in intracellular survival, adhesion, and invasiveness because these traits were previously shown to discriminate between *Salmonella* obtained from clinically ill chickens and their healthy counterparts (13,17). Fourteen genes—*invA, orgA, prgH, spaN* (*invJ*), *tolC, sipB, pagC, msgA, spiA, sopB, lpfC, pefA, spvB,* and *sifA*—assayed in this study encode products that are associated with cellular invasion, survival within a cell, and adhesin or pili production (2,3,4,5,7,8,12,16,18,19,26,27,29). The *invA, prgH, spaN* (*invJ*), *sipB, spiA*, and *sifA* genes are also associated with type III secretion systems (TTSSs) (18,28,30), complex structures of more than 20 proteins that are used in protein delivery (10). The remaining genes targeted in this study were associated with other traits thought to be important in *Salmonella* pathogenesis, such as iron acquisition (*iroN* and *sitC* [1,11]) and toxin biosynthesis (*cdtB* [9]).

On the basis of the results of this study (Table 4), it would appear that these virulence genes, and presumably the *Salmonella* PAIs (SPIs), and their associated TTSSs are widespread in many *Salmonella* serovars isolated from both sick and healthy birds. All of the genes localized to SPI1 (*invA, orgA, prgH, sitC, sipB* and *spaN* [*invJ*]) and SPI2 (*spiA*), along with the *pagC-msgA* pathogenicity islet were found in 100% of the isolates tested, suggesting a ubiquity of these PAIs and the associated TTSSs of SPI1 and SPI2. In addition, *sopB* of SPI5 was present in all isolates from healthy birds and in all but one of the isolates incriminated in salmonellosis, whereas the *lpfC* gene, found on a pathogenicity islet, was detected in 90% of isolates from healthy birds and 79.49% of isolates from sick birds. The *sifA* gene, also found on a pathogenicity islet, was found in 90% of strains from healthy birds and 83.33% of strains from sick birds, whereas the *tolC* and *iroN* genes were both found in 100% of all isolates. In contrast to these highly prevalent genes, *cdtB, pefA*, and *spvB* were found in <17% of isolates from both sick and healthy birds. The *spvB* and *pefA* genes are both encoded by virulence

plasmids (7,29), which can be serovar specific (22). In addition, not all isolates of a plasmid-bearing serovar contain these virulence plasmids (22), which could explain why *spvB* and *pefA* are found in such a low proportion of isolates compared with the chromosomal genes. We also found that in all but one isolate (which contained *spvB* but not *pefA*) that *pefA* and *spvB* were either both present or not present at all. This observation was not entirely surprising because *spvB* and *pefA* are often (GenBank AY509003, NC002638, NC003277, AE006471, AB040415) but not always (GenBank NC007208) encoded by the same plasmid in *Salmonella* spp. The low prevalence of *cdtB* could reflect the possibility that it is limited to certain *Salmonella* serovars. Haghjoo and Galan (9) initially reported that Typhi was the only *Salmonella* serovar that encodes *CdtB,* but *cdtB* has also been found recently in the genome of *Salmonella* serovar Paratyphi A (GenBank CP000026), which along with Typhi are both exclusively human serovars. However, in this study, we detected *cdtB* in all isolates of serovars Bredeny, Brandenburg, and Schwarzengrund, along with one isolate assigned to multiple serovars and one isolate for which the serovar was not determined, making this the first report of the presence of *cdtB* in *Salmonella* serovars not exclusive to humans.

These results suggest these virulence genes, and presumably the PAIs and TTSSs with which they are associated, are widely distributed among *Salmonella* isolates of birds, regardless of whether their hosts of origin have salmonellosis, which is in contrast to similar studies with avian *E. coli* (a closely related enteric bacterium), showing that *E. coli* are quite heterogeneous in their virulence gene content, with the *E. coli* incriminated in colibacillosis more likely to contain certain virulence genes than the *E. coli* isolated from healthy birds (21,24). Furthermore, the strong similarities in virulence genotypes between isolates of sick and healthy birds might indicate that the salmonellae of healthy birds are capable of causing salmonellosis under conditions conducive to illness, and that virulence genotyping, at least with the genes studied here, might have marginal utility in enhancing existing *Salmonella* typing protocols.

REFERENCES

1. Baumler, A. J., T. L. Norris, T. Lasco, W. Voight, R. Reissbrodt, W. Rabsch, and F. Heffron. IroN, a novel outer membrane siderophore receptor characteristic of *Salmonella enterica*. J. Bacteriol. 180:1446–1453. 1998.
2. Baumler, A. J., R. M. Tsolis, and F. Heffron. The *lpf* fimbrial operon mediates adhesion of *Salmonella typhimurium* to murine Peyer's patches. Proc. Natl. Acad. Sci. U.S.A. 93:279–283. 1996.
3. Behlau, I., and S. I. Miller. A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. J. Bacteriol. 175: 4475–4484. 1993.
4. Chen, L. M., K. Kaniga, and J. E. Galan. *Salmonella* spp. are cytotoxic for cultured macrophages. Mol. Microbiol. 21:1101–1115. 1996.
5. Galan, J. E., and R. Curtiss III. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. U.S.A. 86:6383–6387. 1989.
6. Groisman, E. A., and H. Ochman. How *Salmonella* became a pathogen. Trends Microbiol. 5:343–349. 1997.
7. Gulig, P. A., H. Danbara, D. G. Guiney, A. J. Lax, F. Norel, and M. Rhen. Molecular analysis of *spv* virulence genes of the *Salmonella* virulence plasmids. Mol. Microbiol. 7:825–830. 1993.
8. Gunn, J. S., C. M. Alpuche-Aranda, W. P. Loomis, W. J. Belden, and S. I. Miller. Characterization of the *Salmonella typhimurium pagC/pagD* chromosomal region. J. Bacteriol. 177:5040–5047. 1995.
9. Haghjoo, E., and J. E. Galan. *Salmonella typhi* encodes a functional cytolethal distending toxin that is delivered into host cells by a bacterial-internalization pathway. Proc. Natl. Acad. Sci. U.S.A. 101:4614–4619. 2004.
10. Hueck, C. J. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. 62:379–433. 1998.
11. Janakiraman, A., and J. M. Slauch. The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of *Salmonella typhimurium*. Mol. Microbiol. 35:1146–1155. 2000.
12. Jones, B. D., and S. Falkow. Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. Infect. Immunol. 62:3745–3752. 1994.
13. Kottom, T. J., L. K. Nolan, and J. Brown. Invasion of Caco-2 cells by *Salmonella typhimurium* (Copenhagen) isolates from healthy and sick chickens. Avian Dis. 39:867–872. 1995.
14. Marcus, S. L., J. H. Brumell, C. G. Pfeifer, and B. B. Finlay. *Salmonella* pathogenicity islands: big virulence in small packages. Microbes Infect. 2:145–156. 2000.
15. McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 413:852–856. 2001.
16. Miller, S. I., A. M. Kukral, and J. J. Mekalanos. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. U.S.A. 86:5054–5058. 1989.
17. Nolan, L. K., R. E. Wooley, J. Brown, and J. B. Payeur. Comparison of phenotypic characteristics of *Salmonella* spp isolated from healthy and ill (infected) chickens. Am. J. Vet. Res. 52:1512–1517. 1991.
18. Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman. Identification of a pathogenicity island required for *Salmonella* survival in host cells. Proc. Natl. Acad. Sci. U.S.A. 93:7800–7804. 1996.
19. Parsot, C. *Shigella flexneri*: genetics of entry and intercellular dissemination in epithelial cells. Curr. Top. Microbiol. Immunol. 192: 217–241. 1994.
20. Pfeifer, C. G., S. L. Marcus, O. Steele-Mortimer, L. A. Knodler, and B. B. Finlay. *Salmonella typhimurium* virulence genes are induced upon bacterial invasion into phagocytic and nonphagocytic cells. Infect. Immunol. 67:5690–5698. 1999.
21. Rodriguez-Siek, K. E., C. W. Giddings, C. Doetkott, T. J. Johnson, and L. K. Nolan. Characterizing the APEC pathotype. Vet. Res. 36: 241–256. 2005.
22. Rotger, R., and J. Casadesus. The virulence plasmids of *Salmonella*. Int. Microbiol. 2:177–184. 1999.
23. Sanderson, K. E., and D. R. Zeigler. Storing, shipping, and maintaining records on bacterial strains. Methods Enzymol. 204:248–264. 1991.
24. Skyberg, J. A., S. M. Horne, C. W. Giddings, R. E. Wooley, P. S. Gibbs, and L. K. Nolan. Characterizing avian *Escherichia coli* isolates with multiplex polymerase chain reaction. Avian Dis. 47: 1441–1447. 2003.
25. Snedecor, G. W., and W. G. Cochran. Statistical methods. Iowa State University Press, Ames. 1980.
26. Stein, M. A., K. Y. Leung, M. Zwick, F. Garcia-del Portillo, and B. B. Finlay. Identification of a *Salmonella* virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. Mol. Microbiol. 20:151–164. 1996.
27. Stone, B. J., and V. L. Miller. *Salmonella enteritidis* has a homologue of *tolC* that is required for virulence in BALB/c mice. Mol. Microbiol. 17: 701–712. 1995.
28. Suarez, M., and H. Russmann. Molecular mechanisms of *Salmonella* invasion: the type III secretion system of the pathogenicity island 1. Int. Microbiol. 1:197–204. 1998.
29. van der Velden, A. W., A. J. Baumler, R. M. Tsolis, and F. Heffron. Multiple fimbrial adhesins are required for full virulence of *Salmonella typhimurium* in mice. Infect. Immunol. 66:2803–2808. 1998.
30. Waterman, S. R., and D. W. Holden. Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. Cell Microbiol. 5:501–511. 2003.