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Human *Salmonella* Clinical Isolates Distinct from Those of Animal Origin▽

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The global trend toward intensive livestock production has led to significant public health risks and industry-associated losses due to an increased incidence of disease and contamination of livestock-derived food products. A potential factor contributing to these health concerns is the prospect that selective pressure within a particular host may give rise to bacterial strain variants that exhibit enhanced fitness in the present host relative to that in the parental host from which the strain was derived. Here, we assessed 184 *Salmonella enterica* human and animal clinical isolates for their virulence capacities in mice and for the presence of the *Salmonella* virulence plasmid encoding the SpvB actin cytotoxin required for systemic survival and Pef fimbriae, implicated in adherence to the murine intestinal epithelium. All (21 of 21) serovar Typhimurium clinical isolates derived from animals were virulent in mice, whereas many (16 of 41) serovar Typhimurium isolates derived from human salmonellosis patients lacked this capacity. Additionally, many (10 of 29) serovar Typhimurium isolates derived from gastroenteritis patients did not possess the *Salmonella* virulence plasmid, in contrast to all animal and human bacteremia isolates tested. Lastly, among serovar Typhimurium isolates that harbored the *Salmonella* virulence plasmid, 6 of 31 derived from human salmonellosis patients were avirulent in mice, which is in contrast to the virulent phenotype exhibited by all the animal isolates examined. These studies suggest that *Salmonella* isolates derived from human salmonellosis patients are distinct from those of animal origin. The characterization of these bacterial strain variants may provide insight into their relative pathogenicities as well as into the development of treatment and prophylactic strategies for salmonellosis.

Salmonella enterica is a significant food-borne pathogen of humans transmitted via the consumption of meat, animal products, and food products (e.g., fruits and vegetables) contaminated with animal waste (24, 41, 76). Clinical manifestations of human and animal salmonellosis range from self-limiting gastroenteritis to severe bacteremia and typhoid fever. More than 2,300 serovars of *S. enterica* have been identified and classified previously, typically by serotyping based on antigenic variation in the lipopolysaccharide (O-antigen) and phase 1 (H1) and phase 2 (H2) flagella (22, 45, 46). Although serotyping has been an epidemiologically useful resource for classifying isolates, it provides relatively limited information regarding bacterial diversity, evolutionary relatedness, and pathogenicity (11, 14, 18, 30, 57, 61).

There are six subspecies of *S. enterica*, and the vast majority of human and animal infections are caused by strains belonging to subspecies I. In spite of the close genetic relationship among serovars assigned to subspecies I, there are significant differences in virulence, host adaptation, and host specificity (70, 71), and accordingly, they have been categorized into three different groups: broad-host-range, host-adapted, and host-restricted serovars (8, 42, 64, 71). Serovars Typhimurium and Enteritidis are broad-host-range serovars capable of causing systemic disease in a wide range of animals but are usually associated with gastroenteritis in a broad range of phylogenetically unrelated host species (13, 44). Serovars Dublin and Choleraesuis are host-adapted serovars that are often associated with systemic disease in cattle and pigs, respectively, but can cause disease in other animals, including humans (54, 66, 78). Serovars Typhi, Gallinarum, and Abortusovis are host-restricted serovars that are associated nearly exclusively with systemic disease in human (21), fowl (5), and ovine (56) hosts, respectively.

However, the virulence determinants that define host range and the degree of pathogenicity in a particular animal host are not fully understood. First, an assessment of *Salmonella* pathogenicity and/or host range is complicated by the fact that some isolates are capable of asymptomatic colonization and/or persistence in a particular animal species (asymptomatic carriers) while causing acute disease in another animal species (4, 16, 29, 61, 80; reviewed in reference 71). Second, most infections of livestock are subclinical, as evidenced by the disparity among salmonella isolates from surveillance and clinical submissions (2). Third, some of the relative differences in pathogenicity and/or host range may be attributed to differential gene regulation (4, 14). Fourth, pathogenicity and shedding in livestock are dependent on management and environmental events that contribute to compromised host immunity and in-

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creased pathogen exposure. These events include an increase in herd size, adverse weather conditions, and surface water management, as well as numerous variables such as mechanical failures, variable feedstuff availability and quality, and labor compliance issues that impact the effectiveness of farm management (2, 25–27, 37, 38). Thus, an assessment of pathogenicity and risk to human and animal health depends on parameters such as the diversity of salmonellae present on farms and feedlots, clinical and subclinical infections, the potential for regulatory and virulence differences within and among serovars, and management and environmental events that increase pathogen exposure and/or compromise host immunity. In addition, lifestyle differences between humans and animals may lead to the selection of bacterial strain variants that exhibit enhanced fitness in a present host relative to that in a former host. Here, we show distinct differences in murine virulence levels between *Salmonella* isolates derived from animals and those derived from human salmonellosis patients. The characterization of such strain variations may have implications for prevention and treatment strategies for human and animal salmonellosis.

MATERIALS AND METHODS

Bacterial strains. *Salmonella* human clinical isolates were recovered from specimens submitted to diagnostic laboratories in different outbreaks; fecal isolates derived from patients with gastroenteritis were obtained from Utah Public Health Laboratories (UPHL) and Primary Children's Medical Center (PCMC), Salt Lake City, UT, and the Monterey County Public Health Laboratory (MCPHL), Monterey, CA. Blood isolates derived from patients with bacteremia were obtained from UPHL; the serotype Dublin Lane strain was obtained from Donald Guiney, School of Medicine, University of California, San Diego (23). *Salmonella* animal clinical isolates were recovered from specimens submitted to diagnostic laboratories in different outbreaks or individual cases and were obtained from the following sources: the Faculty of Veterinary Science, University of Sydney, Camden, NSW, Australia; the Department of Pathology, Microbiology, and Immunology, University of California, Davis; and the California Animal Health and Food Safety Laboratory System (CA AHFSL) (including serovar Typhimurium strain TY1212 and *S. enterica* O6,14,24:e,h-monophasic K00-670) (19, 20). Pathogenic serovar Typhimurium strains UK-1 and F98, serovar Enteritidis strain 4973 (6, 33), and serovar Choleraesuis strain χ3246 were obtained from Roy Curtiss III, Center for Infectious Diseases and Vaccinology, Arizona State University, Tempe, AZ. All *Salmonella* animal nonclinical isolates were derived from on-farm surveillance studies of healthy animals and were obtained from the USDA Agricultural Research Service (USDA-ARS) (4). The pathogenic *Salmonella* reference strain *S. enterica* serovar Typhimurium ATCC 14028 (CDC6516-60) was used in all studies.

Virulence assays. (i) CI. *Salmonella* test strains and the wild-type serovar Typhimurium reference strain MT2057 (a virulent derivative of strain 14028) were grown overnight in Luria-Bertani (LB) broth (17) with aeration at 37°C. Bacterial cells were resuspended in 0.15 M NaCl and used to intraperitoneally (i.p.) infect 6- to 8-week-old BALB/c mice with a 1:1 ratio of the test strain to the reference wild-type strain at a dose of ~500 cells each. Five days postinfection, the bacterial cells were recovered from the spleens. The competitive infectivity index (CI) is the ratio of test strain cells to reference wild-type strain cells recovered from the target tissue (spleen tissue) divided by the ratio of the input inoculum; the bacterial cell number was determined by direct colony counting. The wild-type serovar Typhimurium reference strain MT2057 used in the CI studies is a virulent derivative of strain 14028 containing a Lac^+ MudJ transcriptional fusion which is used to discern it from other *Salmonella* strains which are inherently Lac^-. Note that the oral and i.p. 50% lethal doses (LD_{50}; 10^5 and <10 organisms, respectively), as well as the i.p. CI, of strain MT2057 are indistinguishable from those of the parental wild-type strain, 14028 (15, 36).

(ii) Oral LD_{50}. An oral LD_{50} virulence assay was used to determine the lethal dose required to kill 50% of the animals. *Salmonella* test strains and wild-type serovar Typhimurium reference strain 14028 were grown overnight in LB medium with aeration at 37°C. Bacterial cells were resuspended in 0.2 ml of 0.2 M Na_2HPO_4, pH 8.1, and used to orally infect 6- to 8-week-old BALB/c mice at a dose of either 10^5 or 10^7 bacterial cells via gastrointubation. Mice were examined daily for morbidity and mortality for up to 3 weeks postinfection. The oral LD_{50} for the reference wild-type serovar Typhimurium strain 14028 is 10^5 organisms (36).

***Salmonella* virulence plasmid analysis.** The size of the *Salmonella* virulence plasmid is serovar dependent, ranging from ~50 to 100 kb; e.g., the virulence plasmid of serovar Typhimurium strains is typically ~90 kb, that of serovar Dublin strains is ~80 kb, that of serovar Enteritidis strains is ~60 kb, and that of serovar Choleraesuis strains is ~50 kb (62). *Salmonella* virulence plasmids of appropriate sizes were detected via the visual inspection of genomic DNA derived from *Salmonella* strains subjected to agarose gel electrophoresis (0.8 to 1% agarose) and stained with ethidium bromide (40). Strains containing *Salmonella* virulence plasmid candidates of the appropriate sizes were evaluated for the presence of virulence plasmid-associated markers *spvB* and *pef*, detected by PCR and confirmed by hybridization analysis.

DNA hybridization. Probes used for hybridization were generated by PCR amplification from serovar Typhimurium strain 14028 by using primer pairs that are complementary to internal gene sequences, as follows: *spvB* forward (5′ ATC TCT GCC TCT CCC TTA 3′) and *spvB* reverse (5′ TAT ACC GCT GCC TCT GCC 3′) and *pefA* forward (5′ TTG CAC TGG GTG TTC TGG 3′) and *pefA* reverse (5′ TGT AAG CCA CTG CGA AAG 3′). Genomic DNA (~250 ng) was spotted onto a nylon membrane (Amersham), and the membrane was incubated overnight with 50 ng of chemically labeled *spvB* or *pefA* probe DNA (ECL; Amersham). Membranes were washed at 42°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.4% sodium dodecyl sulfate and 6 M urea, and hybridization was detected by a chemiluminescence kit (Amersham).

Colony PCR. A single bacterial colony, derived from an individual cell incubated for 16 to 18 h at 37°C on solid LB agar, was suspended in sterile water and used for *Taq* polymerase (NEB)-based PCR amplification using the *spvB* and *pefA* primer pairs listed in "DNA hybridization" above. Colony PCR was performed according to methods adapted from reference 43 by using a PTC-200 thermocycler (MJ Research, Watertown, MA). In some cases, colony PCR results were confirmed via PCR amplification of purified bacterial genomic DNA.

Statistical analysis. Statistical significance for proportions was calculated using chi-square tests. For all statistical analyses, a significance level (P) of less than 0.05 was used to reject the null hypothesis.

RESULTS

Serovar Typhimurium isolates derived from animals are more likely to cause murine systemic disease than those derived from human salmonellosis patients. Selective pressure within a particular host may give rise to bacterial strain variants that are more adapted to the present host than to the parental host from which they were derived. Thus, we evaluated whether the virulence capacities of *Salmonella* human clinical isolates were distinct from those of animal isolates. It is well appreciated that the ability of a *Salmonella* isolate to cause systemic disease in mice has no bearing on whether the same isolate will cause intestinal or systemic disease in another host (animal or human) (70). However, the mouse model may serve as a useful tool to identify bacterial strain variants that may have important clinical applications regarding prophylactic strategies.

Virulence was assessed according to the degree of pathogenicity in mice via the i.p. route of administration by using a CI assay. Briefly, BALB/c mice were infected i.p. with a 1:1 ratio of the *Salmonella* test strain to the wild-type reference serovar Typhimurium strain MT2057. At 5 days postinfection, bacterial cells were recovered from the spleens and the CI was calculated as the ratio of *Salmonella* test strain cells to wild-type reference strain cells recovered from splenic tissue. Our initial efforts were focused on serovar Typhimurium since surveillance studies in the United States have revealed that serovar Typhimurium is the most common serovar among human and

animal clinical specimen submissions and is the second most common isolate from nonclinical nonhuman specimen submissions (13).

All (21 of 21) animal serovar Typhimurium isolates were virulent in mice, regardless of whether they were derived from animals with gastrointestinal (GI) or nongastrointestinal (NGI) infections (Table 1). In contrast, many of the serovar Typhimurium isolates derived from patients exhibiting gastroenteritis (13 of 29) or bacteremia (3 of 12) exhibited 50- to >1,000-fold reductions in their abilities to cause systemic disease in mice. These data suggest that serovar Typhimurium animal clinical isolates are more likely to cause murine systemic disease than isolates derived from human salmonellosis patients considered together ($P = 0.003$) or individually (animal isolates versus gastroenteritis isolates, $P = 0.001$; animal isolates versus bacteremia isolates, $P = 0.04$).

Serovar Typhimurium isolates derived from human gastroenteritis patients often do not contain the *Salmonella* virulence plasmid. *Salmonella* virulence plasmids of several *Salmonella* serovars have been associated with virulence in many animal models of infection (32, 39, 55; reviewed in reference 64). Much of the virulence capacity of the *Salmonella* virulence plasmid has been attributed to *spvB* (31, 50, 63, 74), encoding a toxin that ADP-ribosylates actin in macrophages and destabilizes the cytoskeleton (47, 67). Depending on the serovar, the *Salmonella* virulence plasmids carry additional virulence-associated factors such as *pef* (for plasmid-encoded fimbriae), *rck* (for resistance to complement killing), *srgA* (an Sdi-regulated gene encoding a putative disulfide bond oxidoreductase), or *mig-5* (a macrophage-inducible gene encoding a putative carbonic anhydrase).

Here, we evaluated whether human isolates differed from animal isolates in their retention of the *Salmonella* virulence plasmid and associated virulence attributes. All (21 of 21) serovar Typhimurium animal isolates of GI and non-GI origins, as well as all (12 of 12) blood isolates derived from human bacteremic patients, contained the *Salmonella* virulence plasmid and the *spvB* gene (Table 1). In contrast, many (10 of 29) fecal isolates derived from gastroenteritis patients did not possess the virulence plasmid, providing an explanation for the avirulent phenotype of the plasmidless isolates in mice. These data suggest a difference in selective pressure for the maintenance of the *Salmonella* virulence plasmid in serovar Typhimurium isolates derived from human gastroenteritis patients relative to those derived from bacteremic patients ($P = 0.02$) or animals ($P = 0.003$).

Among serovar Typhimurium isolates that retain the *Salmonella* virulence plasmid, those derived from human salmonellosis patients are more likely than animal isolates to exhibit defects in murine virulence. *Salmonella* animal isolates are capable of asymptomatic colonization and/or persistence in a particular animal species while causing acute disease in another animal species (4, 16, 29, 61, 80; reviewed in reference 71). Since the *Salmonella* virulence plasmid has been associated with systemic disease in a variety of animal models of infection (32, 39, 55), we assessed whether there was a difference in murine virulence levels between human and animal isolates that contained the virulence plasmid. All animal serovar Typhimurium isolates (22 of 22; 21 of 21 clinical isolates [Table 1] and 1 of 1 subclinical isolate [Table 2]) that contained the virulence plasmid (and *spvB*) were proficient for murine systemic disease. In contrast, among serovar Typhimurium isolates derived from gastroenteritis and bacteremic patients, some (6 of 31; 3 of 19 gastroenteritis isolates and 3 of 12 bacteremia isolates) that retained the virulence plasmid (and *spvB*) were deficient in the ability to cause murine systemic disease via the i.p. route of administration (Table 1). These six serovar Typhimurium human isolates were subsequently examined for defects in virulence via the oral route of administration (in oral LD_{50} assays). Table 1 shows that, in each case, a ≥1,000-fold reduction in oral virulence relative to the virulence of the reference wild-type serovar Typhimurium strain 14028 was observed. These data suggest that *Salmonella* virulence plasmid-containing isolates derived from human salmonellosis patients are more likely than animal isolates to exhibit defects in murine virulence ($P < 0.04$).

***pef* is retained in virulence plasmid-harboring serovar Typhimurium isolates derived from gastroenteritis patients.** The *Salmonella* virulence plasmid-associated gene *pef* encodes Pef fimbriae that are involved in bacterial adherence to the murine intestinal epithelium (7). We assessed whether possible differences in selective pressure resulted in differences in the maintenance of *pef* in human and animal clinical isolates. Of the human fecal serovar Typhimurium isolates derived from gastroenteritis patients, all (19 of 19) that contained the *Salmonella* virulence plasmid (and *spvB*) also retained *pef* (Table 1). In contrast, among other serovar Typhimurium isolates that harbored the plasmid (and *spvB*), 4 of 21 animal isolates (3 of 14 GI isolates and 1 of 7 NGI isolates) and 2 of 12 human bacteremia isolates did not retain *pef* ($P = 0.07$). These data suggest that, among clinical serovar Typhimurium isolates that possess the virulence plasmid, there is a trend toward the maintenance of *pef* in isolates derived from gastroenteritis patients relative to isolates from bacteremic patients or animals. Moreover, *pef* is not required to cause systemic disease, as several human and animal isolates that harbored the virulence plasmid (and *spvB*) did not retain *pef*.

Most non-serovar Typhimurium human and animal clinical and nonclinical isolates are deficient in the ability to cause murine systemic disease. *Salmonella* virulence plasmids of several *Salmonella* serovars have been associated with virulence in many animal models of infection (32, 39, 55). Consistent with these reports, all (46 of 46) human and animal serovar Typhimurium clinical isolates that were capable of murine systemic disease contained the *Salmonella* virulence plasmid and *spvB* (Table 1). Here, we tested whether the presence of the virulence plasmid and *spvB* and/or *pef* was required for murine virulence in several human clinical isolates as well as isolates from animals with clinical and subclinical infections. This survey comprised more than 50 representatives of non-Typhimurium serovars, including GI and NGI isolates derived from livestock and other animals (host sources were chickens, cows, dogs, horses, humans, lizards, pigs, sheep, a seal, a snake, and a turtle).

All three serovars that had the capacity to cause systemic disease in mice (Bovismorbificans, Dublin, and Enteritidis) contained the *Salmonella* virulence plasmid and *spvB* (Table 2). Note that the presence of the *Salmonella* virulence plasmid and *spvB* did not ensure murine virulence, as several clinical isolates that retained the plasmid exhibited an avir-

TABLE 1. Murine virulence of human and animal serotype Typhimurium isolates from clinical specimen submissions

Strain[a]	Serotype	Site[b]	Host	Yr of isolation	Location[c]	Virulence (CI) in i.p. infection[d]	Virulence plasmid[e]	*spvB*[e]	*pef*[e]	Source[a]
Serotype Typhimurium animal GI and extraintestinal isolates										
11	Typhimurium	GI	Chicken	2003	U.S.	0.95	+	+	+	CA AHFSL
UK-1	Typhimurium	NGI	Chicken	1988	NY	2.4	+	+	+	R. Curtiss III
F98	Typhimurium	GI	Chicken	1991	England	0.46	+	+	+	R. Curtiss III
TY1212	Typhimurium	NGI	Cow	2001	CA	0.44	+	+	−	CA AHFSL
(97)-1332	Typhimurium	NGI	Dog	1997	CA	41.2	+	+	+	UC—Davis
(05)-6192	Typhimurium	GI	Horse	2005	CA	2.2	+	+	+	UC—Davis
(05)-4360	Typhimurium	NGI	Horse	2005	CA	1.22	+	+	+	UC—Davis
(03)-1516	Typhimurium	NGI	Horse	2003	ID	0.02	+	+	+	UC—Davis
(04)-8740	Typhimurium	GI	Horse	2004	CA	3.01	+	+	+	UC—Davis
131	Typhimurium	GI	Sheep	2005	VIC	0.54	+	+	+	University of Sydney
146	Typhimurium	GI	Sheep	2005	SA	2.0	+	+	+	University of Sydney
148	Typhimurium	NGI	Sheep	2005	SA	0.33	+	+	+	University of Sydney
150	Typhimurium	NGI	Sheep	2005	VIC	0.95	+	+	+	University of Sydney
222	Typhimurium	GI	Sheep	2005	WA	0.73	+	+	+	University of Sydney
227	Typhimurium	GI	Sheep	2005	WA	0.32	+	+	+	University of Sydney
230	Typhimurium	GI	Sheep	2005	WA	2.32	+	+	+	University of Sydney
250	Typhimurium	GI	Sheep	2005	WA	0.23	+	+	+	University of Sydney
(04)-9639	Typhimurium var. 5−	GI	Cow	2004	CA	11.3	+	+	+	UC—Davis
(04)-8511	Typhimurium var. 5−	GI	Cow	2004	CA	0.68	+	+	−	UC—Davis
(04)-4985	Typhimurium var. 5−	GI	Cow	2004	CA	2.8	+	+	−	UC—Davis
12	Typhimurium var. 5−	GI	Turkey	2000	U.S.	1.8	+	+	−	CA AHFSL
Serotype Typhimurium human fecal isolates from gastroenteritis patients										
69	Typhimurium	Feces	Human	2005	CA	<0.0003	−	−	−	MCPHL
5	Typhimurium	Feces	Human	2005	CA	<0.0003	−	−	−	MCPHL
3h	Typhimurium	Feces	Human	2006	Western U.S.	0.24	+	+	+	PCMC
5h	Typhimurium	Feces	Human	2006	Western U.S.	22.2	+	+	+	PCMC
6h	Typhimurium	Feces	Human	2006	Western U.S.	0.82	+	+	+	PCMC
9h	Typhimurium	Feces	Human	2006	Western U.S.	<0.001	−	−	−	PCMC
10h	Typhimurium	Feces	Human	2006	Western U.S.	0.003*	+	+	+	PCMC
11h	Typhimurium	Feces	Human	2006	Western U.S.	<0.001	−	−	−	PCMC
12h	Typhimurium	Feces	Human	2006	Western U.S.	1.2	+	+	+	PCMC
13h	Typhimurium	Feces	Human	2006	Western U.S.	1.1	+	+	+	PCMC
F1	Typhimurium	Feces	Human	2007	Western U.S.	0.0009	−	−	−	UPHL
F2	Typhimurium	Feces	Human	2007	Western U.S.	0.072	+	+	+	UPHL
F3	Typhimurium	Feces	Human	2007	Western U.S.	0.1	+	+	+	UPHL
F4	Typhimurium	Feces	Human	2007	Western U.S.	0.36	+	+	+	UPHL
F5	Typhimurium	Feces	Human	2007	Western U.S.	0.004	−	−	−	UPHL
F6	Typhimurium	Feces	Human	2007	Western U.S.	0.0003	−	−	−	UPHL
F7	Typhimurium	Feces	Human	2007	Western U.S.	0.05	+	+	+	UPHL
F8	Typhimurium	Feces	Human	2007	Western U.S.	0.0006	−	−	−	UPHL
F9	Typhimurium	Feces	Human	2007	Western U.S.	5.2	+	+	+	UPHL
F10	Typhimurium	Feces	Human	2007	Western U.S.	0.008*	+	+	+	UPHL
F11	Typhimurium	Feces	Human	2007	Western U.S.	0.05	+	+	+	UPHL
F12	Typhimurium	Feces	Human	2007	Western U.S.	1.1	+	+	+	UPHL

F13	Typhimurium	Feces	Human	2007	Western U.S.	0.001	−	−	−	UPHL
F14	Typhimurium	Feces	Human	2007	Western U.S.	0.05	+	+	+	UPHL
1h	Typhimurium var. 5−	Feces	Human	2006	Western U.S.	<0.001*	+	+	+	PCMC
4h	Typhimurium var. 5−	Feces	Human	2006	Western U.S.	0.007	−	−	−	PCMC
7h	Typhimurium var. 5−	Feces	Human	2006	Western U.S.	3.9	+	+	+	PCMC
8h	Typhimurium var. 5−	Feces	Human	2006	Western U.S.	1.3	+	+	+	PCMC
630	Typhimurium var. 5−	Feces	Human	2005	CA	0.05	+	+	+	MCPHL
Serotype Typhimurium human blood isolates from bacteremic patients										
B1	Typhimurium	Blood	Human	2000	Western U.S.	0.001*	+	+	+	UPHL
B2	Typhimurium	Blood	Human	2001	Western U.S.	3.2	+	+	+	UPHL
B3	Typhimurium	Blood	Human	2001	Western U.S.	4	+	+	+	UPHL
B4	Typhimurium	Blood	Human	2001	Western U.S.	15.5	+	+	+	UPHL
B5	Typhimurium	Blood	Human	2001	Western U.S.	0.003*	+	+	+	UPHL
B6	Typhimurium	Blood	Human	2002	Western U.S.	3.3	+	+	+	UPHL
B7	Typhimurium	Blood	Human	2002	Western U.S.	0.49	+	+	−	UPHL
B8	Typhimurium	Blood	Human	2002	Western U.S.	0.50	+	+	+	UPHL
B9	Typhimurium	Blood	Human	2003	Western U.S.	5.2	+	+	+	UPHL
B10	Typhimurium	Blood	Human	2003	Western U.S.	0.003*	+	+	+	UPHL
B11	Typhimurium	Blood	Human	2003	Western U.S.	1.1	+	+	+	UPHL
B12	Typhimurium	Blood	Human	2004	Western U.S.	0.33	+	+	−	UPHL

[a] *Salmonella* human clinical isolates were recovered from specimens submitted to diagnostic laboratories in different outbreaks; fecal isolates derived from patients with gastroenteritis were obtained from UPHL, PCMC, and the MCPHL. Blood isolates derived from patients with bacteremia were obtained from UPHL. *Salmonella* animal clinical isolates were recovered from specimens submitted to diagnostic laboratories in different outbreaks or individual cases and were obtained from the following sources: the University of Sydney; the University of California, Davis (UC—Davis); and the CA AHFSL. Pathogenic serotype Typhimurium strains UK-1 and F98 (6, 33) were obtained from Roy Curtiss III, Arizona State University, Tempe, AZ. The pathogenic reference strain *S. enterica* serovar Typhimurium ATCC 14028 (CDC6516-60) was used in all studies.

[b] Host site from which the specimen containing bacteria was obtained. Animal isolates were obtained from specimens from GI sites (e.g., intestinal specimens and feces) and NGI sites (e.g., spleen, liver, and urine specimens, etc.); human isolates were obtained from the feces and blood of gastroenteritis and bacteremic patients, respectively.

[c] U.S., United States; VIC, Victoria, Australia; WA, Western Australia; SA, South Australia.

[d] CIs for *Salmonella* test strains relative to the reference wild-type serotype Typhimurium strain 14028. BALB/c mice were i.p. infected with a 1:1 ratio of the test strain to the reference wild-type strain (MT2057, a virulent derivative of strain 14028) at a dose of ~500 cells each. Five days postinfection, the bacterial cells were recovered from the spleens; the CI is the ratio of test strain cells to reference wild-type strain cells recovered. Three to five mice per challenge strain were tested. Isolates with a CI of <0.02 were designated as avirulent and were subsequently evaluated for virulence via the oral route of administration (in the oral LD_{50} assay). All six virulence plasmid-containing *Salmonella* serotype Typhimurium human isolates that were avirulent via the i.p. route (in the CI assay) exhibited a reduction in virulence via the oral route of ≥1,000-fold relative to the reference wild-type strain 14028 (values for these isolates are indicated by an asterisk).

[e] *Salmonella* virulence plasmids were detected via visual inspection of genomic DNA derived from *Salmonella* strains subjected to agarose gel electrophoresis (40). Strains containing *Salmonella* virulence plasmid candidates of the appropriate sizes were evaluated for the presence of virulence plasmid-associated markers *spvB* and *pef*, detected by PCR and hybridization analysis. + denotes a strong signal via hybridization and/or PCR; − denotes no detectable signal via PCR and hybridization analysis.

TABLE 2. Murine virulence of *Salmonella* human and animal isolates from clinical and nonclinical specimen submissions

Serotype[a]	Serogroup	Site(s)[b]	Host(s)	No. of isolates tested	Virulence (CI) in i.p. infection[c]	No. of isolates[c] with:			Source(s)[d]
						Virulence plasmid	*spvB*	*pef*	
Serotypes of *Salmonella* isolates obtained from animal clinical specimen submissions									
Adelaide	O	GI	Sheep	1	0	0	0	0	*a*
Agona	B	GI	Dog	1	0	0	0	0	*b*
Anatum	E1	GI, NGI	Cow, horse	3	0	0	0	0	*b*, *c*
Arizonae	*S. enterica* subsp. IIIa	NGI	Horse	1	0	0	1	0	*b*
Bergedorf	D2	NGI	Snake	0	0	0	0	0	*a*
Bovismorbificans	C2-C3	GI, NGI	Cow, sheep	11	3	11	11	11	*a*
Braenderup	C1	GI	Horse	1	0	0	0	0	*b*
Bredeney	B	GI	Sheep	2	0	0	0	0	*a*
Cannstatt	E4	GI	Horse	1	0	0	0	0	*b*
Cerro	K	GI	Cow, pig	2	0	0	0	0	*b*, *c*
Choleraesuis	C1	GI, NGI, −	Dog, pig	3	0	3	3	3	*b*, *c*, *d*
Derby	B	GI	Pig	2	0	0	0	0	*a*, *c*
Dublin	D1	GI, NGI	Cow, sheep	7	5	7	7	0	*a*, *b*, *c*
Enteritidis	D1	GI, −	Chicken, dog	2	2	2	2	2	*b*, *d*
Give	E1	GI	Cow, horse	5	0	0	0	0	*b*, *a*
Havana	G	GI	Sheep	3	0	0	0	0	*a*
Heidelberg	B	GI, NGI	Horse	1	0	0	0	0	*b*
Infantis	C1	GI	Chicken, cow, horse, sheep	6	0	0	0	0	*a*, *b*
Kentucky	C2-C3	GI	Chicken	1	0	0	0	0	*c*
Kottbus	C2-C3	GI	Sheep	1	0	0	0	0	*a*
Krefeld	E4	GI	Horse	1	0	0	0	0	*b*
Litchfield	C2-C3	GI	Dog	1	0	0	0	0	*b*
Matadi	J	GI	Lizard	1	0	0	0	0	*b*
Mbandaka	C1	GI	Chicken, cow	2	0	0	0	0	*b*, *c*
Meleagridis	E1	GI	Horse	1	0	0	0	0	*a*
Minnesota	L	GI	Seal	1	0	0	0	0	*b*
Montevideo	C1	GI, NGI	Cow, dog, pig	6	0	0	0	0	*b*, *c*
Mountpleasant	X	GI	Turtle	1	0	1	1	1	*b*
Muenchen	E2	GI	Cow, horse	3	0	0	0	0	*b*
Muenster	E1	GI	Cow	2	0	0	0	0	*a*, *b*
Newport	C2-C3	GI	Cow, horse	9	0	0	0	0	*b*, *c*
O6,14,24:e,h-monophasic	H	NGI	Chicken	1	0	0	0	0	*c*
Ohio	C1	GI	Cow, dog	2	0	0	0	0	*a*, *b*
Oranienburg	C1	GI	Horse	1	0	0	0	0	*b*
Panama	D1	GI	Horse	1	0	0	0	0	*b*
Pomona	M	GI	Horse	1	0	0	0	0	*b*
Reading	B	GI	Cow	2	0	0	0	0	*a*, *b*
Rubislaw	F	GI	Lizard	1	0	0	0	0	*b*
S. enterica subsp. I serotype 4,12:d	*S. enterica* subsp. I	GI	Cow	1	0	0	0	0	*a*
Senftenberg	E4	GI	Cow	1	0	0	0	0	*b*
Singapore	C1	GI	Sheep	1	0	0	0	0	*a*
Tennessee	C1	GI	Cow, sheep	2	0	0	0	0	*b*
Thompson	C1	GI	Horse	1	0	0	0	0	*b*
Welikade	I	GI	Cow	2	0	0	0	0	*a*
Worthington	G	GI	Horse	1	0	0	0	0	*b*
Serotypes of *Salmonella* isolates obtained from surveillance studies of on-farm healthy animals									
Anatum	E1	GI	Cow	1	0	0	0	0	*e*
Cerro	K	GI	Cow	1	0	0	0	0	*e*
Enteritidis	D1	GI	Chicken	1	1	1	1	1	*e*
Istanbul	C2-C3	−	Chicken	1	0	0	0	0	*e*
Kentucky	C2-C3	GI	Cow	1	0	0	0	0	*e*
Meleagridis	E1	GI	Cow	1	0	0	0	0	*e*
Montevideo	C1	GI	Chicken, cow	2	0	0	0	0	*e*
Paratyphi B var. Java	B	GI	Cow	1	0	0	0	0	*e*
Sandiego	B	GI	Cow	1	0	0	0	0	*e*
Thompson	C1	GI	Chicken	1	0	0	0	0	*e*
Typhimurium var. 5−	B	−	Chicken	1	1	1	1	1	*e*
Serotypes of *Salmonella* isolates from human clinical specimen submissions									
Agona	B	Feces	Human	1	0	0	0	0	*f*
Brandenburg	B	Feces	Human	1	0	0	0	0	*f*
Cerro	K	Feces	Human	1	0	0	0	0	*f*
Dublin	D1	Blood	Human	1	1	1	1	0	*g*

Continued on facing page

TABLE 2—*Continued*

Serotype[a]	Serogroup	Site(s)[b]	Host(s)	No. of isolates tested	Virulence (CI) in i.p. infection[c]	No. of isolates[c] with:			Source(s)[d]
						Virulence plasmid	*spvB*	*pef*	
Enteritidis	D1	Blood	Human	4	4	4	4	4	*h*
Heidelberg	B	Feces	Human	1	0	0	0	0	*f*
Infantis	C1	Feces	Human	2	0	0	0	0	*f*
Minnesota	L	Blood	Human	1	0	0	0	0	*h*
Muenchen	C2-C3	Feces	Human	1	0	0	0	0	*f*
Oranienburg	C1	Blood	Human	1	0	0	0	0	*h*
Panama	D1	Feces, urine	Human	2	0	0	0	0	*f*
Saintpaul	B	Feces	Human	2	0	0	0	0	*f*
S. enterica subsp. IV serotype 48:g,z51	*S. enterica* subsp. IV	Blood	Human	1	0	0	0	0	*h*
Stanley	B	Feces	Human	1	0	0	0	0	*f*
Telekebir	G	Blood	Human	1	0	0	0	0	*h*
Thompson	C1	Feces	Human	1	0	0	0	0	*f*
Uganda	E1	Blood, urine	Human	2	0	0	0	0	*f*, *h*

[a] *Salmonella* human clinical isolates were recovered from specimens submitted to diagnostic laboratories in different outbreaks; fecal and blood isolates were obtained from patients with gastroenteritis and bacteremia, respectively. *Salmonella* animal clinical isolates were recovered from specimens submitted to diagnostic laboratories in different outbreaks or individual cases. *Salmonella* field isolates recovered from surveillance studies of on-farm healthy animals were obtained from the USDA-ARS.

[b] Host site from which the specimen containing bacteria was recovered. Animal isolates were obtained from specimens from GI sites (e.g., intestinal specimens and feces) and NGI sites (e.g., spleen, liver, lung, and urine specimens, etc.); human isolates were obtained from the feces and blood of gastroenteritis and bacteremic patients, respectively; − denotes that the host site is unknown.

[c] Virulence assays and the detection of the *Salmonella* virulence plasmid, *spvB*, and *pef* were carried out as described in Table 1, footnotes *d* and *e*.

[d] Bacterial strain sources are as follows: *a*, University of Sydney; *b*, University of California, Davis; *c*, CA AHFSL; *d*, Roy Curtiss III, Arizona State University; *e*, USDA-ARS; *f*, MCPHL; *g*, D. Guiney, University of California, San Diego; *h*, UPHL.

ulent phenotype in mice; e.g., 8 of 11 animal clinical isolates of serovar Bovismorbificans did not have the capacity to cause murine systemic disease. Additionally, consistent with previous reports, the plasmid-associated *pef* gene was serovar specific, i.e., common to members of some serovars (e.g., Enteritidis and Choleraesuis [28]) but absent in other serovars (e.g., Dublin [77]). (Serovar Arizonae contains *spvB* in the chromosome but does not contain the virulence plasmid [or *pef*] [10, 48].)

Taken together, these data are consistent with earlier reports that the *Salmonella* virulence plasmid is common to a limited set of *Salmonella* serovars (reviewed in reference 64), the *Salmonella* virulence plasmid (and *spvB*) is necessary but not sufficient for murine systemic disease (12, 31, 74), *spvB* is common to all plasmid-containing serovars but *pef* is serovar specific (reviewed in reference 64), and representative strains among plasmid-containing clinical isolates exhibit a wide range of proficiency for murine virulence (49).

***Salmonella* strains that are associated with asymptomatic colonization and/or persistence in livestock are capable of causing acute systemic disease in mice.** Since all (21 of 21) serovar Typhimurium animal clinical isolates had the capacity to cause disease (Table 1), we examined whether *Salmonella* field isolates derived from surveillance studies of on-farm healthy animals had the capacity to cause murine typhoid fever. Serovar Typhimurium and Enteritidis strains were isolated from healthy chickens with no clinical disease manifestations in USDA-ARS on-farm surveillance studies (Table 2). Both of these strains maintained the virulence plasmid and associated *spvB* and *pef* genes and were virulent in an i.p. murine model of typhoid fever. Thus, although these *Salmonella* strains exhibited asymptomatic colonization and/or persistence in chickens, they contained the *Salmonella* virulence plasmid and associated virulence functions and had the capacity to cause fulminate systemic disease in mice.

DISCUSSION

The endemic prevalence of salmonellae in intensive commercial livestock production systems presents an explicit risk to the human food supply since human salmonellosis is often due to the ingestion of contaminated meat and animal products or fruits and vegetables contaminated by animal waste. A potential factor contributing to this public health risk is the prospective emergence of bacterial strain variants that are more suited to the present host than to a former host. Here, we show that clinical isolates derived from human salmonellosis patients exhibit distinct differences in virulence attributes relative to isolates derived from animals. The molecular characterization of these bacterial strain variants may yield insights into the disparities between human and animal pathogenesis processes and into the development of intervention strategies for human and animal salmonellosis.

Phage typing, biotyping, and molecular fingerprinting are based on bacterial susceptibility to bacteriophages, fermentation, and molecular characteristics, respectively, and are epidemiologically useful tools to distinguish serotype Typhimurium variants (reviewed in reference 59). Epidemiological studies of livestock indicate that the persistence of serovar Typhimurium is characterized by a succession of relatively small epidemics in which one dominant clone of a distinct phage type is successively replaced by another dominant clone of a new phage type (reviewed in reference 60). The successive phage types appear to maintain a broad host range, as those that were dominant in cattle also circulated in other livestock and humans (3, 69), and some of these phage types, e.g., serovar Typhimurium-definitive phage type 104 (DT104) (68), have the capacity for pandemic spread (58). Additionally, selective pressure may give rise to variants that are more suited for colonization, persistence, and/or infection of a particular host or a limited set of hosts. For example, serovar Typhi-

murium variants from pigeons differ in phage type, biotype, and ribotype from those isolated from humans or animals, suggesting that pigeons are not a source of infection for other host reservoirs (53, 65, 73, 79). Thus, the broad-host-range serovar Typhimurium may comprise variants that differ significantly with regard to host range and their degrees of host adaptation (59).

There are likely to be significant differences in the selective pressures imposed on strains that colonize, persist in, and/or infect humans and those that colonize, persist in, and/or infect livestock due to marked disparities in host anatomy, physiology, and lifestyle. Here, we show that serovar Typhimurium isolates derived from human gastroenteritis patients often lose the *Salmonella* virulence plasmid and, accordingly, lack the capacity to cause systemic disease in mice. This characteristic is in contrast to all serovar Typhimurium isolates derived from human bacteremic patients or animal clinical isolates, which retained the *Salmonella* virulence plasmid. These data suggest that plasmidless strains of serovar Typhimurium exhibit an enhanced degree of fitness in human patients with gastroenteritis. A possible explanation for the source of the plasmidless strains is that they are selected among the diversity of salmonellae that colonize livestock and other animals. However, the epidemiologic path followed in animal-to-human salmonella transmission may often be complicated by exposure to environmental factors (contaminating fruits and vegetables), wherein selective pressures, for example, may not favor the retention of the plasmid. An alternative but non-mutually exclusive explanation is that plasmidless strains arise and/or are selected for within the human GI tract and are propagated via the human-to-human transmission of gastroenteritis.

Another distinction between isolates derived from humans and those derived from animals is that many human gastroenteritis and bacteremic isolates that retained the *Salmonella* virulence plasmid were not virulent in a mouse model either by the oral or by the i.p. route of administration. This finding is consistent with the results in an earlier report regarding the murine virulence of human gastroenteritis-associated strains of serovar Enteritidis, representing phage type 4 and phage type 8, which have been the most common subtypes in the United Kingdom and the United States, respectively (1, 9, 51, 75). These serovar Enteritidis strains were highly variable in their capacities to cause murine disease and in their in vitro phenotypes associated with pathogenicity (49). Such virulence variability highlights the complex and unique nature of specific host-pathogen interactions and the resultant selection for variants that are best suited to a particular host.

To identify and understand the molecular basis of the proposed variants characterized in this study, we analyzed human and animal serovar Typhimurium isolates for the presence of the *Salmonella* virulence plasmid-associated gene *pef*, encoding Pef fimbriae that are implicated in adherence to the murine intestinal epithelium (7). Of the isolates derived from human gastroenteritis patients, all those that maintained the virulence plasmid also retained *pef*, whereas many clinical isolates derived from human bacteremic patients or animals did not. These data suggest a selective pressure for the maintenance of *pef* among GI isolates that contain the virulence plasmid, possibly reflecting a need for adherence to the human mucosal epithelium. The selection for *pef* may not be as acute for isolates derived from human bacteremic patients or animals due to, for example, receptor specificity differences in human versus animal epithelia. Another possibility is that animal isolates may frequently transition to and from the GI and systemic phases within and among animals due to management and environmental events that contribute to compromised host immunity and increased pathogen exposure (2, 25–27, 37, 38). Indeed, serovar Typhimurium is usually associated with gastroenteritis in cattle but often leads to bacteremia in infected neonates. Such frequent transitioning between GI and systemic phases may reduce the selective pressure for variants that favor a particular phase in animals. In contrast, phase transitioning in humans is rare due to general hygiene, water sanitation, reduced animal exposure, and medical or physical interventions that may impose a selective bias for variants that are more suited to cause human gastroenteritis or bacteremia.

The broad-host-range serovar Typhimurium may comprise a spectrum of variants that differ with regard to virulence, reflecting a summation of the spatial and/or temporal selective pressures within a particular host(s) (59). Thus, such variant studies may provide insights into important differences between human and animal pathogenesis processes that may impact the design of prophylactic strategies that are logically targeted to distinct phage types that cause epidemics in livestock and also circulate in humans. An ideal vaccine would engender protection against a diverse array of *Salmonella* strains. Promising *Salmonella* live vaccine candidates that confer cross-protective immune responses include strains harboring mutations in regulatory genes that control the expression of a number of potential antigens, such as regulatory genes involved in catabolite repression (*cya* and *crp*) (33, 34), DNA methylation (*dam*) (35, 36), and lipopolysaccharide core and O-antigen synthesis (*rfaH*) (52). The multiple antigens ectopically expressed by these modified live vaccines may be shared among heterologous serotypes, and the expression of these shared diverse antigens may lead to cross-protective responses. Additionally, the characterization of strain variants may have implications regarding antimicrobial use in livestock (72), as clinical outcomes of human salmonellosis (gastroenteritis) are often different from those of livestock salmonellosis (e.g., bacteremia in calves) and, thus, antimicrobials that are not recommended for self-limiting human gastroenteritis may be necessary for a favorable clinical outcome in calves.

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