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Virulence gene profiles and antimicrobial susceptibility of *Salmonella* Brancaster from chicken

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ABSTRACT

Background: The current conventional serotyping based on antigen-antisera agglutination could not provide a better understanding of the potential pathogenicity of *Salmonella enterica* subsp. *enterica* serovar Brancaster. Surveillance data from Malaysian poultry farms indicated an increase in its presence over the years.
Objective: This study aims to investigate the virulence determinants and antimicrobial resistance in *S.* Brancaster isolated from chickens in Malaysia.
Methods: One hundred strains of archived *S.* Brancaster isolated from chicken cloacal swabs and raw chicken meat from 2017 to 2022 were studied. Two sets of multiplex polymerase chain reaction (PCR) were conducted to identify eight virulence genes associated with pathogenicity in *Salmonella* (invasion protein gene [*invA*], *Salmonella* invasion protein gene [*sipB*], *Salmonella*-induced filament gene [*sifA*], cytolethal-distending toxin B gene [*cdtB*], *Salmonella* iron transporter gene [*sitC*], *Salmonella* pathogenicity islands gene [*spiA*], *Salmonella* plasmid virulence gene [*spvB*], and inositol phosphate phosphatase gene [*sopB*]). Antimicrobial susceptibility assessment was conducted by disc diffusion method on nine selected antibiotics for the *S.* Brancaster isolates. *S.* Brancaster, with the phenotypic ACSSuT-resistance pattern (ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracycline), was subjected to PCR to detect the corresponding resistance gene(s).
Results: Virulence genes detected in *S.* Brancaster in this study were *invA*, *sitC*, *spiA*, *sipB*, *sopB*, *sifA*, *cdtB*, and *spvB*. A total of 36 antibiogram patterns of *S.* Brancaster with a high level of multidrug resistance were observed, with ampicillin exhibiting the highest resistance. Over a third of the isolates displayed ACSSuT-resistance, and seven resistance genes (β-lactamase temoneira [bla_{TEM}], florfenicol/chloramphenicol resistance gene [*floR*], streptomycin resistance gene [*strA*], aminoglycoside nucleotidyltransferase gene [*ant(3'')-Ia*], sulfonamides resistance gene [*sul-1*, *sul-2*], and tetracycline resistance gene [*tetA*]) were detected.
Conclusion: Multidrug-resistant *S.* Brancaster from chickens harbored an array of virulence-associated genes similar to other clinically significant and invasive non-typhoidal *Salmonella* serovars, placing it as another significant foodborne zoonosis.

Keywords: *Salmonella*; chickens; drug resistance; bacterial; genes

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Conflict of Interest
The authors declare no conflicts of interest.

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INTRODUCTION

Salmonella is a foodborne pathogen of significant public and animal health concern worldwide. More than 2,500 serovars have been identified to date, and non-typhoidal *Salmonella* (NTS)-related invasive infections are progressively being reported in several countries [1]. NTS can cause self-limiting gastroenteritis in humans and animals. In certain individuals, severe complications may be life-threatening when the bacteria invade normally sterile sites, causing bacteremia and meningitis [2]. In recent years, the *Salmonella* National Surveillance Program conducted by the Department of Veterinary Services (DVS) Malaysia identified an increasing trend in the proportion of *Salmonella enterica* subsp. *enterica* serovar Brancaster isolated from chickens in the country. *Salmonella* Enteritidis was the dominant serovar in poultry throughout the 1990s, and it is listed as a notifiable serovar due to its economic impact and zoonotic potential [3], along with serovar Typhimurium, Pullorum, and Gallinarum. Since 2014, *S.* Brancaster has been the most commonly isolated serovar from chickens in Malaysia. It was isolated from every aspect of the processing line, including the floor, chopping board, wash water, and chicken cuts [4]. In Europe and West Africa, *S.* Brancaster was isolated from patients with diarrhea and implicated in fatal cases among infants and elderly patients [5,6]. Few reports indicate that the prevalence of *S.* Brancaster ranged from 5%–21% in the Asian region, suggesting that *S.* Brancaster could be an emerging serovar [7,8].

Salmonella infection in humans has been linked to poultry farms as the pathogen can colonize healthy, asymptomatic chickens [9]. The increasing multidrug-resistant (MDR) *Salmonella* isolated from foods of animal origins signifies a global public health concern [10]. In 2017, an MDR *S.* Brancaster isolated from chicken meat was reported, with a 5,036,442 bp genome containing various antimicrobial resistance genes towards aminoglycosides, fluoroquinolones, fosfomycin, chloramphenicols, sulphonamides, β-lactams, tetracycline, macrolides, and trimethoprim [11]. In the ongoing evolution of bacteria across various phyla and habitats, frequent gene transfer events and gene acquisition under selective pressures from the host, the environment, and antimicrobials represent the opportunities and challenges for the emergence of MDR pathogens.

The genes associated with survival fitness, antibiotic resistance, and virulence are located on the *Salmonella* pathogenicity island (SPI), encoding the type III secretion systems (T3SS) responsible for consequent pathological sequences in host phagocytes [12]. The conventional *Salmonella* serotyping using slide agglutination tests is based on the surface somatic (O) cell wall and flagellar (H) antigen recognition. In the interest of protecting public and animal health, such serotyping data based on the White-Kauffmann-Le Minor scheme was unlikely to identify invasive NTS and is insufficient to provide a deeper analysis in understanding the characteristics and potential pathogenicity of this emerging serovar [13]. Therefore, molecular virulence profiling using polymerase chain reaction (PCR) to detect virulence genes has been proposed to augment the conventional *Salmonella* serotyping method [14]. This study aims to investigate the virulence genes and antimicrobial resistance profiles of the *S.* Brancaster isolated from chickens in Malaysia.

MATERIALS AND METHODS

S. Brancaster isolates

This study used 100 *S.* Brancaster isolates archived at the Veterinary Research Institute

(VRI), Ipoh, Malaysia. These isolates originated from chicken cloacal swabs and raw chicken meat from farms and abattoirs sent to the DVS Malaysia regional laboratories as part of the routine national *Salmonella* surveillance program between 2017 and 2022. Isolates identified as *Salmonella* spp. through selective media and biochemical tests were submitted to VRI for serotyping. Serotyping was performed using the slide agglutination method with specific O and H antisera (SSI Diagnostica, Denmark), and *S.* Brancaster isolates were identified based on the antigenic formulae of 1,4,12,27:z_{29}:- [15].

DNA extraction

The bacterial DNA was extracted by heat lysis from an overnight culture at 37°C [16]. A single colony was transferred into 100 µL nuclease-free water and incubated at 95°C in a thermal block for 10 min. After cooling to room temperature and centrifuging for 10 min at 13,000 rpm, the supernatant was used as the DNA template.

Detection of virulence genes by PCR

Two PCR reactions were performed to examine for the presence of eight virulence genes using the primers listed in **Table 1** [17]. Reaction one amplified the invasion protein gene (*invA*), *Salmonella* iron transporter gene (*sitC*), *Salmonella* pathogenicity islands gene (*spiA*), and cytolethal-distending toxin B gene (*cdtB*), while reaction two amplified the *Salmonella* invasion protein gene (*sipB*), *Salmonella* plasmid virulence gene (*spvB*), *Salmonella*-induced filament gene (*sifA*), and inositol phosphate phosphatase gene (*sopB*). The reference strain used was *Salmonella* Typhimurium ATCC 14028. The amplifications were performed in a 50-µL reaction mixture containing 25 µL of 2× MyTaq Mix (Bioline, UK), 1 µL of each 10 µM forward and reverse primers (1st Base, Malaysia), 5 µL of DNA template, and topped up with 12 µL nuclease-free water. The PCR was performed using a thermal cycler (Eppendorf, Germany) as follows: 95°C for 5 min followed by 25 cycles of denaturation (94°C for 30 sec), annealing (66.5°C for 30 sec), and extension (72°C for 2 min). A final extension step was carried out at 72°C for 10 min. The amplified DNA was electrophoresed in a 1.5% agarose stained with SYBR Safe DNA Gel Stain (Invitrogen, USA) at 90 V for 50 min in 1× Tris-borate-EDTA buffer. The PCR products were visualized using a UV transilluminator (Uvitec, UK) and compared to the 100 bp DNA HyperLadder (Bioline).

Antimicrobial susceptibility test

The disk diffusion method using Muller-Hinton agar (Oxoid, UK) was used to determine the antimicrobial susceptibility of *S.* Brancaster towards ampicillin (10 µg), amoxicillin/clavulanic acid (30 µg), chloramphenicol (30 µg), streptomycin (10 µg), gentamicin (10 µg), sulfamethoxazole/trimethoprim (25 µg), tetracycline (30 µg), nalidixic acid (30 µg), and ciprofloxacin (5 µg; Oxoid). Results were obtained after incubating the samples for 16–18 h at 37°C, and the inhibition zones were measured and categorized as susceptible, intermediate, or resistant according to the Clinical and Laboratory Standards Institute guidelines [18]. *Escherichia coli* ATCC 25922 was used as the quality control reference strain.

Detection of antimicrobial resistance gene in selected *S.* Brancaster isolates by PCR

Twelve pairs of primers were used to target antimicrobial resistance genes (β-lactamase gene [bla_{PSE-1}], β-lactamase temoneira [bla_{TEM}], florfenicol/chloramphenicol resistance gene [*floR*], aminoglycoside adenyltransferase gene [*aadA2*], streptomycin resistance gene [*strA*], aminoglycoside nucleotidyltransferase gene [*ant(3″)-Ia*], sulfonamides resistance gene [*sul-1*, *sul-2*, *sulA*], tetracycline resistance gene [*tetA*, *tetB*, and *tetG*]) in *S.* Brancaster

Table 1. PCR primers used in this study

Gene target	Primer sequence (5′–3′)	Amplicon size (bp)	Reference
Virulence-associated gene (multiplex PCR reaction 1)			
invA	CTG GCG GTG GGT TTT GTT GTC TTC TCT ATT	1,070	[17]
	AGT TTC TCC CCC TCT TCA TGC GTT ACC C		
sitC	CAG TAT ATG CTC AAC GCG ATG TGG GTC TCC	768	
	CGG GGC GAA AAT AAA GGC TGT GAT GAA C		
spiA	CCA GGG GTC GTT AGT GTA TTG CGT GAG ATG	550	
	CGC GTA ACA AAG AAC CCG TAG TGA TGG ATT		
cdtB	ACA ACT GTC GCA TCT CGC CCC GTC ATT	268	
	CAA TTT GCG TGG GTT CTG TAG GTG CGA GT		
Virulence-associated gene (multiplex PCR reaction 2)			
sipB	GGA CGC CGC CCG GGA AAA ACT CTC	875	[17]
	ACA CTC CCG TCG CCG CCT TCA CAA		
spvB	CTA TCA GCC CCG CAC GGA GAG CAG TTT TTA	717	
	GGA GGA GGC GGT GGC GGT GGC ATC ATA		
sifA	TTT GCC GAA CGC GCC CCC ACA CG	449	
	GTT GCC TTT TCT TGC GCT TTC CAC CCA TCT		
sopB	CGG ACC GGC CAG CAA CAA AAC AAG AAG AAG	220	
	TAG TGA TGC CCG TTA TGC GTG AGT GTA TT		
Antimicrobial resistance gene (singleplex PCR)			
bla_{PSE-1}	CGC TTC CCG TTA ACA AGT AC	419	[19]
	CTG GTT CAT TTC AGA TAG CG		
bla_{TEM}	GCA CGA GTG GGT TAC ATC GA	310	[20]
	GGT CCT CCG ATC GTT GTC AG		
floR	CTG AGG GTG TCG TCA TCT AC	673	[16]
	GCT CCG ACA ATG CTG ACT AT		
aadA2	CGG TGA CCA TCG AAA TTT CG	249	[20]
	CTA TAG CGC GGA GCG TCT CGC		
strA	CTT GGT GAT AAC GGC AAT TC	548	[20]
	CCA ATC GCA GAT AGA AGG C		
ant(3")-Ia	GTG GAT GGC GGC CTG AAG CC	526	[19]
	ATT GCC CAG TCG GCA GCG		
sul-1	ATC GCA ATA GTT GGC GAA GT	797	[19]
	GCA AGG CGG AAA CCC GCG CC		
sul-2	AGG GGG CAG ATG TGA TCG AC	249	[20]
	GCA GAT GAT TTC GCC AAT TG		
sulA	CAC TGC CAC AAG CCG TAA	360	[20]
	GTC CGC CTC AGC AAT ATC		
Antimicrobial resistance gene (multiplex PCR)			
tetA	GCT ACA TCC TGC TTG CCT TC	210	[21]
	CAT AGA TCG CCG TGA AGA GG		
tetB	TTG GTT AGG GGC AAG TTT TG	659	
	GTA ATG GGC CAA TAA CAC CG		
tetG	CAG CTT TCG GAT TCT TAC GG	468	
	GAT TGG TGA GGC TCG TTA GC		

PCR, polymerase chain reaction; *invA*, invasion protein gene; *sitC*, *Salmonella* iron transporter gene; *spiA*, *Salmonella* pathogenicity islands gene; *cdtB*, cytolethal-distending toxin B gene; *sipB*, *Salmonella* invasion protein gene; *spvB*, *Salmonella* plasmid virulence gene; *sifA*, *Salmonella*-induced filament gene; *sopB*, inositol phosphate phosphatase gene; bla_{PSE-1}, β-lactamase gene; bla_{TEM}, β-lactamase temoneira; *floR*, florfenicol/chloramphenicol resistance gene; *aadA2*, aminoglycoside adenyltransferase gene; *strA*, streptomycin resistance gene; *ant(3")-Ia*, aminoglycoside nucleotidyltransferase gene; *sul-1*, *sul-2*, *sulA*, sulfonamides resistance gene; *tetA*, *tetB*, *tetG*, tetracycline resistance gene.

isolates that confer phenotypic ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT)-resistance pattern [16,19-21]. Amplifications were conducted in a 50-μL reaction mixture containing 25 μL of 2× MyTaq™ Mix (Bioline), 1 μL of each 10 μM forward and reverse primers (1st Base), 5 μL of DNA template, and topped up with the appropriate volume of nuclease-free water. All PCR were optimized and performed as follows: 95°C for 5 min followed by 30 cycles of denaturation (95°C for 1 min), annealing (56°C for 1 min), and extension (72°C for 1 min). A final extension step was carried out at 72°C for 7 min. *S.* Typhimurium strain DT104 (NCTC 13348) with ACSSuT-resistance pattern was used as the positive control. Amplified PCR products (*bla_{TEM}*, *strA*, and *sul-2*) without the positive control were verified by DNA sequencing and sent to a commercial sequencing laboratory (Apical Scientific Sdn Bhd, Malaysia). The resulting sequences were assembled using SeqMan Pro software (DNAStar Lasergene, USA) and confirmed using the National Centre for Biotechnology Information database and BLAST program. Singleplex and multiplex PCR primer sequences and the expected amplicon sizes of the amplified products are listed in **Table 1**.

RESULTS

Virulence genes of S. Brancaster

All *S.* Brancaster isolates carried the *invA*, *sitC*, *spiA*, and *sipB*. Almost all isolates are positive for *sopB* (99%), while *sifA* (59%) and *cdtB* (26%) are variably present in the tested isolates. The *spvB* is present in just one isolate (1%). All *S.* Brancaster tested in this study had at least four virulence genes, as presented in **Table 2**.

Antimicrobial resistance profiles of *S.* Brancaster

In the current study, *S.* Brancaster indicated the highest resistance rates to ampicillin (91%), followed by tetracycline (86%), sulfamethoxazole/trimethoprim (62%), chloramphenicol (61%), gentamicin (45%), nalidixic acid (43%), streptomycin (33%), ciprofloxacin (9%), and amoxicillin/clavulanic acid (4%), as shown in **Table 3**.

Overall, 85% (85/100) of *S.* Brancaster isolates were identified as MDR, which confers resistance to at least three classes of antimicrobials tested [22]. The *S.* Brancaster antimicrobial resistance profiles are presented in **Table 4**. They displayed 36 antibiogram

Table 2. Detection of virulence genes by polymerase chain reaction

No. of isolates	Virulence gene							
	invA	*sitC*	*spiA*	*cdtB*	*sipB*	*spvB*	*sifA*	*sopB*
24	+	+	+	+	+	−	+	+
38	+	+	+	−	+	−	−	+
2	+	+	+	+	+	−	−	+
34	+	+	+	−	+	−	+	+
1	+	+	+	−	+	−	−	−
1	+	+	+	−	+	+	+	+
Prevalence (%)	100	100	100	26	100	1	59	99

invA, invasion protein gene; *sitC*, *Salmonella* iron transporter gene; *spiA*, *Salmonella* pathogenicity islands gene; *cdtB*, cytolethal-distending toxin B gene; *sipB*, *Salmonella* invasion protein gene; *spvB*, *Salmonella* plasmid virulence gene; *sifA*, *Salmonella*-induced filament gene; *sopB*, inositol phosphate phosphatase gene.

Table 3. Antimicrobial resistance of *Salmonella* Brancaster isolates detected from cloacal swab and raw chicken meat

Group of antimicrobials	Antimicrobial disc	Isolate with resistance		Frequency (%)
		Cloacal swab (n = 68)	Raw chicken meat (n = 32)	
β-lactams	Ampicillin	60	31	91
	Amoxicillin/Clavulanic acid	3	1	4
Phenicols	Chloramphenicol	42	19	61
Aminoglycosides	Streptomycin	22	11	33
	Gentamicin	34	11	45
Sulfonamides	Sulfamethoxazole/ Trimethoprim	45	17	62
Tetracycline	Tetracycline	59	27	86
Quinolones	Nalidixic acid	30	13	43
Fluoroquinolones	Ciprofloxacin	4	5	9

Table 4. Antimicrobial resistance patterns of *Salmonella* Brancaster isolates

No. of antibiotic classes	Resistance phenotype	No. of isolates	ACSSuT-resistance type
7	AMP-AMC-C-S-SXT-TE-NAL-CIP	3	Yes
	AMP-C-S-SXT-TE-NAL-CIP	1	Yes
6	AMP-C-S-CN-SXT-TE-NAL	5	Yes
	AMP-C-S-SXT-TE-NAL	4	Yes
	AMP-C-CN-SXT-TE-NAL	7	Yes
	AMP-C-S-SXT-TE-CIP	1	Yes
	AMP-C-SXT-TE-NAL-CIP	2	-
5	AMP-C-S-CN-SXT-TE	4	Yes
	AMP-C-S-SXT-TE	2	Yes
	AMP-C-CN-SXT-TE	6	Yes
	AMP-C-SXT-TE-NAL	12	-
	AMP-S-CN-SXT-TE-NAL	1	-
4	AMP-S-CN-SXT-TE	1	-
	S-CN-SXT-TE-NAL	1	-
	AMP-C-SXT-TE	3	-
	AMP-C-CN-TE	2	-
	AMP-C-TE-NAL	2	-
	AMP-CN-TE-NAL	3	-
	AMP-CN-SXT-TE	2	-
	AMP-S-SXT-CIP	1	-
	C-SXT-TE-NAL	1	-
3	AMP-S-CN-TE	4	-
	AMP-AMC-SXT-TE	1	-
	AMP-C-TE	4	-
	AMP-CN-TE	7	-
	AMP-TE-NAL	1	-
	AMP-S-SXT	2	-
	C-SXT-TE	1	-
2	S-CN-TE	1	-
	AMP-TE	4	-
	AMP-C	1	-
	AMP-SXT	1	-
	AMP-CIP	1	-
1	S-CN	1	-
	AMP	3	-
	S	1	-
0	Susceptible	3	-

ACSSuT, ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; C, chloramphenicol; S, streptomycin; CN, gentamicin; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin.

patterns, with AMP-C-SXT-TE-NAL (12 isolates) being the most frequent resistance type. Three isolates of *S.* Brancaster were found to be susceptible to all antimicrobials tested, while four isolates were found resistant to all seven antimicrobial classes.

About 33% (33/100) of the *S.* Brancaster isolates exhibited the phenotypic ACSSuT-resistance pattern. Among them, seven resistance genes to ampicillin (*bla*$_{TEM}$), chloramphenicol (*floR*), streptomycin (*strA*), gentamicin (*ant(3″)-Ia*), sulphonamides (*sul-1* and *sul-2*), and tetracycline (*tetA*) were detected at the rates of 93.9%, 69.7%, 6.1%, 57.6%, 3.0%, 12.1%, and 72.7%, respectively (**Table 5**).

DISCUSSION

S. Brancaster is an emerging serovar that has been isolated frequently from chicken processing environments, wet markets, and chicken meat in recent years but has been

Table 5. Antimicrobial resistance genes identified from *Salmonella* Brancaster isolates with ACSSuT-resistance pattern

ACSSuT-resistance type isolate number	PCR result for											
	Ampicillin		Chloramphenicol	Streptomycin		Gentamicin	Sulfonamides			Tetracycline		
	bla$_{PSE-1}$	*bla*$_{TEM}$	*floR*	*aadA2*	*strA*	*ant(3")-Ia*	*sul-1*	*sul-2*	*sulA*	*tetA*	*tetB*	*tetG*
2	−	+	+	−	−	+	−	−	−	+	−	−
6	−	−	−	−	−	−	−	−	−	−	−	−
8	−	+	+	−	−	+	−	−	−	+	−	−
10	−	+	+	−	−	−	−	−	−	+	−	−
18	−	+	+	−	−	+	−	−	−	+	−	−
23	−	+	+	−	−	−	−	−	−	+	−	−
24	−	+	+	−	−	−	−	−	−	+	−	−
34	−	+	+	−	−	+	−	−	−	+	−	−
37	−	+	+	−	−	+	−	−	−	+	−	−
38	−	−	−	−	−	−	−	−	−	−	−	−
39	−	+	−	−	−	−	−	−	−	−	−	−
42	−	+	+	−	−	−	−	−	−	+	−	−
52	−	+	−	−	−	+	−	−	−	−	−	−
54	−	+	+	−	−	+	−	−	−	+	−	−
55	−	+	+	−	−	−	−	−	−	+	−	−
56	−	+	+	−	−	+	−	−	−	+	−	−
59	−	+	−	−	−	−	−	+	−	−	−	−
61	−	+	−	−	−	−	−	−	−	−	−	−
62	−	+	+	−	−	−	−	−	−	+	−	−
63	−	+	−	−	−	−	−	−	−	−	−	−
65	−	+	+	−	+	+	−	+	−	+	−	−
69	−	+	+	−	−	+	−	−	−	+	−	−
70	−	+	+	−	−	+	−	−	−	+	−	−
72	−	+	+	−	+	+	−	+	−	+	−	−
74	−	+	+	−	−	+	−	−	−	+	−	−
79	−	+	+	−	−	−	−	−	−	+	−	−
80	−	+	−	−	−	+	+	−	−	+	−	−
82	−	+	+	−	−	+	−	−	−	+	−	−
91	−	+	−	−	−	−	−	+	−	−	−	−
94	−	+	+	−	−	+	−	−	−	+	−	−
96	−	+	−	−	−	+	−	−	−	−	−	−
97	−	+	+	−	−	+	−	−	−	+	−	−
98	−	+	+	−	−	+	−	−	−	+	−	−
Rate (%)	0/33	31/33 (93.9%)	23/33 (69.7%)	0/33	2/33 (6.1%)	19/33 (57.6%)	1/33 (3.0%)	4/33 (12.1%)	0/33	24/33 (72.7%)	0/33	0/33

ACSSuT, ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline; PCR, polymerase chain reaction; *bla*$_{PSE-1}$, β-lactamase gene; *bla*$_{TEM}$, β-lactamase temoneira; *floR*, florfenicol/chloramphenicol resistance gene; *aadA2*, aminoglycoside adenyltransferase gene; *strA*, streptomycin resistance gene; *ant(3")-Ia*, aminoglycoside nucleotidyltransferase gene; *sul-1*, *sul-2*, *sulA*, sulfonamides resistance gene; *tetA*, *tetB*, *tetG*, tetracycline resistance gene.

reported infrequently in humans [6]. For several decades, *Salmonella* Pullorum and *S.* Enteritidis are typically the most common serovars with economic impact responsible for *Salmonella* infection in poultry and humans. However, this predominance has decreased in many regions of the world, where other examples of NTS, such as *Salmonella* Infantis, *Salmonella* Derby, and *Salmonella* Anatum, have become the dominant serovars, reflecting the current situation in many countries [8,23]. The *S.* Brancaster in this study were all isolated from healthy chicken flocks with no reported clinical disease or mortality. The persistence of *S.* Brancaster in poultry farms has been related to the viability adaptive mechanism of biofilm formation on contact surfaces to ensure persistent colonization [4]. Besides, the widespread dissemination of *S.* Brancaster in poultry farms has been related to the occurrence of genes at the SPI and plasmid associated with fitness, virulence, and antimicrobial resistance. In this study, *S.* Brancaster exhibits six combinations of virulence genes tested. This serovar carries multiple virulence genes, including *invA*, *sopB*, *spvB*, and *cdtB*, that have epidemiological association with other clinically significant and invasive serovars to cause human salmonellosis, such as *S.* Enteritidis and *S.* Typhimurium [24].

The presence of the *cdtB* in *S.* Brancaster is of major concern as the toxin is associated with severe human bloodstream infection, as it has been discovered in human clinical isolates [25]. Previously, the *cdtB* was only reported in the *Salmonella* Typhi typhoidal strain. However, studies indicated that the *cdtB* is becoming more prevalent in NTS serovars of human and chicken origin that are capable of causing invasive *Salmonella* infection, including serovars Javiana, Montevideo, Schwarzengrund, Indiana, Enteritidis, and Agona [26]. Previous research has shown that identifying virulence genes that play an important role in the invasive mechanism can predict disease-inducing ability and improve understanding of the potential risks of human infection [22].

Several studies have assessed the distribution of specific virulence-associated genes that can be proposed to distinguish enteric and invasive strains, where the *invA*, *sipB*, and *spvB* have been regarded as key markers for virulotyping studies [24]. One *S.* Brancaster in the current study tested positive for the *spvB* linked to a medically important NTS that causes enteric infection and non-typhoid bacteremia [27]. There have been varying reports on virulence gene profiling from different geographical locations. A virulence genotyping study of *Salmonella* in South Korea reported seven virulence profiles across eight serovars, including Albany, Montevideo, Virchow, Typhimurium, and Senftenberg [22]. Meanwhile, Kuang *et al.* [28] reported nine virulence profiles of *Salmonella* Newport in China. All the virulence factors are found with varying frequencies across serovars, making it difficult to set standard essential virulence factors in virulotyping profiling studies. These virulence genes or plasmids, which are discovered to be conjugative and contain genetic information, managed to spread genes throughout bacterial populations, responsible for *S.* Brancaster's capacity to induce salmonellosis in humans. However, it is noteworthy that the presence of these virulence genes is not a definitive determinant of their virulence, as the degree of expression also contributed to the pathogen's invasive properties [29].

The present study indicated that MDR *S.* Brancaster is widely distributed in chickens at two important points along the food production chain: the farm and poultry meat products. One-third (33%) of them were found to exhibit phenotypic ACSSuT-resistant type similar to the MDR *S.* Typhimurium DT104 strain. Besides, they also exhibit resistance to other classes of antimicrobials, including quinolones and fluoroquinolones. The resistance to ciprofloxacin, a critically important antimicrobial in treating *Salmonella* infection or typhoid fever in humans, was at 9%, i.e., much higher than those reported by others ranging from 0%–3.8% [16]. Invasive infections caused by ciprofloxacin-resistant *Salmonella* have been reported to cause pneumonia, gastroenteritis, fever, septic arthritis, and meningitis in children or immunocompromised adults [8]. MDR *Salmonella* has been reported to be more virulent and cause a direct threat to human health when treatment is hampered by resistance [30]. Bacteria can share their genetic material and spread resistance through horizontal gene transfer across commensal bacteria and pathogens. If the resistance genes were encoded by a transferable plasmid, they could spread horizontally to other human pathogens, causing an indirect threat, and this phenomenon has been noticed in *Salmonella* [31]. In this study, the phenotypic and genotypic MDR profiles of the *S.* Brancaster with ACSSuT-resistant pattern studied only partially matched, and they diverged in some antimicrobial classes more strongly than in other classes. They were mainly positive for the bla_{TEM} (93.9%), *floR* (69.7%), *ant(3″)-Ia* (57.6%), and *tetA* (72.7%) resistance genes. These findings agree with previous studies that reported these resistance genes in all *Salmonella* samples isolated from poultry and processing facilities, including serovars Brancaster, Albany, Corvallis, and Enteritidis [4,16].

In comparison, phenotypic resistance to streptomycin and sulfonamides corresponding to resistance genes *strA* (6.1%), *sul-1* (3.0%), and *sul-2* (12.1%) were found at a much lower frequency. A study reported that the bla_{PSE-1} and *sul-1* resistance genes were primarily found in *S.* Typhimurium [19]. Phenotypic resistance is the interplay between genotype and environmental conditions. The inconsistency between MDR phenotypes and antimicrobial resistant (AMR) genes may be related to the resistance genes located at mobilizable or conjugative plasmids that may disappear when there is a change in ecological settings, such as time of storage and subculturing that changes the plasmid's fitness [32,33]. Whole genome sequencing (WGS)-based AMR prediction is currently an alternative to conventional molecular PCR and phenotypic AMR characterization studies. Campioni *et al.* [34] who employed WGS analysis to predict AMR in *S.* Enteritidis, also only found partial matches between phenotypic and genotypic AMR among the isolates, with tetracycline being the strongest correlation found. There are limitations to these WGS prediction approaches, including challenges for plasmid detection in short read length, high error rate, and detection relies much on the database. Therefore, the AMR genomic database requires ongoing data input, validation, maintenance, and curation to strengthen the pipeline's robustness to become a powerful tool for AMR prediction.

The emergence of AMR *Salmonella* in poultry may be a result of the widespread use of antibiotics commonly used in veterinary medicine. It is a common practice to administer small doses via drinking water in integrated chicken farming to reduce stress, prevent diseases, and enhance poultry growth and production [35]. According to a recent survey conducted on poultry farms in Malaysia, the most commonly used antibiotics are enrofloxacin and amoxicillin, which belong to the same class of antibiotics as ciprofloxacin and ampicillin [36]. Therefore, the Malaysian government is taking proactive measures to phase out and even banned a few medically important antibiotics as antimicrobial growth promoters in livestock animals' production, including colistin, erythromycin, tilcomycin, tylosin, neomycin, and fosfomycin [37]. Consequently, alternatives such as essential oils, organic acids, and natural olive oil by-products to promote intestinal health and mucosal integrity can be used to reduce salmonellosis in poultry farms [30].

This study provides initial evidence for the occurrence of MDR *S.* Brancaster in Malaysian chicken farms. Furthermore, the isolates were found to co-harbor various combinations of virulence and resistance genes, indicating a risk of salmonellosis infection among consumers through chicken products and personnel involved in the pre and harvesting phases due to contaminated environments. Salmonellosis control management in farms needs to be improved by including the currently dominant serovar as a possible pathogen of public health concern in Malaysia. Likewise, appropriate husbandry practices and the prudent use of therapeutic antimicrobials are key veterinary practices for enhancing farm biosecurity management to control salmonellosis in chicken production. The limitation of this study is its inability to detect a wide array of resistance genes using conventional PCR. Further study into the plasmid-mediated quinolones resistance genes that impede the treatment of *Salmonella* infections in humans should be encouraged in surveillance studies so that they can be used to guide public health measures and treatments.

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REFERENCES

1. Popa GL, Papa MI. *Salmonella* spp. infection - a continuous threat worldwide. Germs. 2021;11(1):88-96.
   PUBMED | CROSSREF
2. Mohan A, Munusamy C, Tan YC, Muthuvelu S, Hashim R, Chien SL, et al. Invasive *Salmonella* infections among children in Bintulu, Sarawak, Malaysian Borneo: a 6-year retrospective review. BMC Infect Dis. 2019;19(1):330.
   PUBMED | CROSSREF
3. Modarressi S, Thong KL. Isolation and molecular subtyping of *Salmonella* enterica from chicken, beef and street foods in Malaysia. Sci Res Essays. 2010;5(18):2713-2720.
4. Chuah LO, Shamila Syuhada AK, Mohamad Suhaimi I, Farah Hanim T, Rusul G. Genetic relatedness, antimicrobial resistance and biofilm formation of *Salmonella* isolated from naturally contaminated poultry and their processing environment in northern Malaysia. Food Res Int. 2018;105:743-751.
   PUBMED | CROSSREF
5. Hinden E, Taylor J, Walther WW. An outbreak due to *Salmonella* brancaster. Lancet. 1952;260(6724):64-66.
   PUBMED | CROSSREF
6. Dieye Y, Hull DM, Wane AA, Harden L, Fall C, Sambe-Ba B, et al. Genomics of human and chicken *Salmonella* isolates in Senegal: broilers as a source of antimicrobial resistance and potentially invasive nontyphoidal salmonellosis infections. PLoS One. 2022;17(3):e0266025.
   PUBMED | CROSSREF
7. Zwe YH, Tang VC, Aung KT, Gutiérrez RA, Ng LC, Yuk HG. Prevalence, sequence types, antibiotic resistance and, *gyrA* mutations of *Salmonella* isolated from retail fresh chicken meat in Singapore. Food Control. 2018;90:233-240.
   CROSSREF
8. Chang YJ, Chen CL, Yang HP, Chiu CH. Prevalence, serotypes, and antimicrobial resistance patterns of non-typhoid *Salmonella* in food in northern Taiwan. Pathogens. 2022;11(6):705.
   PUBMED | CROSSREF
9. Anderson TC, Nguyen TA, Adams JK, Garrett NM, Bopp CA, Baker JB, et al. Multistate outbreak of human *Salmonella* Typhimurium infections linked to live poultry from agricultural feed stores and mail-order hatcheries, United States 2013. One Health. 2016;2:144-149.
   PUBMED | CROSSREF
10. Antimicrobial Resistance Collaborators. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 2022;399(10325):629-655.
    PUBMED | CROSSREF
11. Chin PS, Yu CY, Ang GY, Yin WF, Chan KG. Draft genome sequence of multidrug-resistant *Salmonella* enterica serovar Brancaster strain PS01 isolated from chicken meat, Malaysia. J Glob Antimicrob Resist. 2017;9:41-42.
    PUBMED | CROSSREF
12. Wang M, Qazi IH, Wang L, Zhou G, Han H. *Salmonella* virulence and immune escape. Microorganisms. 2020;8(3):407.
    PUBMED | CROSSREF
13. Chattaway MA, Langridge GC, Wain J. *Salmonella* nomenclature in the genomic era: a time for change. Sci Rep. 2021;11(1):7494.
    PUBMED | CROSSREF
14. Kim JE, Lee YJ. Molecular characterization of antimicrobial resistant non-typhoidal *Salmonella* from poultry industries in Korea. Ir Vet J. 2017;70(1):20.
    PUBMED | CROSSREF
15. Grimont PAD, Weill FX. *Antigenic Formulae of the Salmonella Serovars.* 9th ed. Geneva: World Health Organization; 2007.

16. Abatcha MG, Effarizah ME, Rusul G. Prevalence, antimicrobial resistance, resistance genes and class 1 integrons of *Salmonella* serovars in leafy vegetables, chicken carcasses and related processing environments in Malaysian fresh food markets. Food Control. 2018;91:170-180.
CROSSREF

17. Skyberg JA, Logue CM, Nolan LK. Virulence genotyping of *Salmonella* spp. with multiplex PCR. Avian Dis. 2006;50(1):77-81.
PUBMED | CROSSREF

18. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing. CLSI supplement M100*. 30th ed. Wayne: Clinical and Laboratory Standards Institute; 2020.

19. Bacci C, Boni E, Alpigiani I, Lanzoni E, Bonardi S, Brindani F. Phenotypic and genotypic features of antibiotic resistance in *Salmonella* enterica isolated from chicken meat and chicken and quail carcasses. Int J Food Microbiol. 2012;160(1):16-23.
PUBMED | CROSSREF

20. Hur J, Kim JH, Park JH, Lee YJ, Lee JH. Molecular and virulence characteristics of multi-drug resistant *Salmonella* Enteritidis strains isolated from poultry. Vet J. 2011;189(3):306-311.
PUBMED | CROSSREF

21. Ng LK, Martin I, Alfa M, Mulvey M. Multiplex PCR for the detection of tetracycline resistant genes. Mol Cell Probes. 2001;15(4):209-215.
PUBMED | CROSSREF

22. Shang K, Wei B, Jang HK, Kang M. Phenotypic characteristics and genotypic correlation of antimicrobial resistant (AMR) *Salmonella* isolates from a poultry slaughterhouse and its downstream retail markets. Food Control. 2019;100:35-45.
CROSSREF

23. Mughini-Gras L, van Hoek AH, Cuperus T, Dam-Deisz C, van Overbeek W, van den Beld M, et al. Prevalence, risk factors and genetic traits of *Salmonella* Infantis in Dutch broiler flocks. Vet Microbiol. 2021;258(109120):109120.
PUBMED | CROSSREF

24. Suez J, Porwollik S, Dagan A, Marzel A, Schorr YI, Desai PT, et al. Virulence gene profiling and pathogenicity characterization of non-typhoidal *Salmonella* accounted for invasive disease in humans. PLoS One. 2013;8(3):e58449.
PUBMED | CROSSREF

25. Liu Y, Jiang J, Ed-Dra A, Li X, Peng X, Xia L, et al. Prevalence and genomic investigation of *Salmonella* isolates recovered from animal food-chain in Xinjiang, China. Food Res Int. 2021;142(110198):110198.
PUBMED | CROSSREF

26. Rodriguez-Rivera LD, Bowen BM, den Bakker HC, Duhamel GE, Wiedmann M. Characterization of the cytolethal distending toxin (typhoid toxin) in non-typhoidal *Salmonella* serovars. Gut Pathog. 2015;7(1):19.
PUBMED | CROSSREF

27. Guiney DG, Fierer J. The role of the *spv* genes in *Salmonella* pathogenesis. Front Microbiol. 2011;2:129.
PUBMED | CROSSREF

28. Kuang D, Xu X, Meng J, Yang X, Jin H, Shi W, et al. Antimicrobial susceptibility, virulence gene profiles and molecular subtypes of *Salmonella* Newport isolated from humans and other sources. Infect Genet Evol. 2015;36:294-299.
PUBMED | CROSSREF

29. García-Pastor L, Puerta-Fernández E, Casadesús J. Bistability and phase variation in *Salmonella* enterica. Biochim Biophys Acta Gene Regul Mech. 2019;1862(7):752-758.
PUBMED | CROSSREF

30. V T Nair D, Venkitanarayanan K, Kollanoor Johny A. Antibiotic-resistant *Salmonella* in the food supply and the potential role of antibiotic alternatives for control. Foods. 2018;7(10):1-24.
PUBMED | CROSSREF

31. Ifeanyi CI, Bassey BE, Ikeneche NF, Al-Gallas N. Molecular characterization and antibiotic resistance of *Salmonella* in children with acute gastroenteritis in Abuja, Nigeria. J Infect Dev Ctries. 2014;8(6):712-719.
PUBMED | CROSSREF

32. McMillan EA, Gupta SK, Williams LE, Jové T, Hiott LM, Woodley TA, et al. Antimicrobial resistance genes, cassettes, and plasmids present in *Salmonella* enterica associated with United States food animals. Front Microbiol. 2019;10:832.
PUBMED | CROSSREF

33. Given C, Penttinen R, Jalasvuori M. Plasmid viability depends on the ecological setting of hosts within a multiplasmid community. Microbiol Spectr. 2022;10(2):e0013322.
PUBMED | CROSSREF

34. Campioni F, Gomes CN, Bergamini AM, Rodrigues DP, Falcão JP. Partial correlation between phenotypic and genotypic antimicrobial resistance of *Salmonella* enterica serovar Enteritidis strains from Brazil. Microb Drug Resist. 2020;26(12):1466-1471.
PUBMED | CROSSREF

35. Manyi-Loh C, Mamphweli S, Meyer E, Okoh A. Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. Molecules. 2018;23(4):795.
PUBMED | CROSSREF

36. Rachel WJ, Marni S, Zurina R, Ani Y, Rozanah Asmah A. Antibiotic usage in myGAP poultry farms in Malaysia. Malays J Vet Res. 2020;11(2):22-31.

37. World Organisation for Animal Health. Malaysia country report on the current situations of the use of antimicrobial agents as growth promoter in 2019 OIE Regional Workshop on Animal Feed Safety [Internet]. Paris: World Organisation for Animal Health; https://rr-asia.woah.org/wp-content/uploads/2020/01/malaysia.pdf. Updated 2020. Accessed 2023 May 31.