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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:z₂₉- [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 (*sjfA*), 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-I}], β-lactamase temoneira [*bla*_{TEM}], florfenicol/chloramphenicol resistance gene [*floR*], aminoglycoside adenylyltransferase 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 *tetC*]) 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)			
<i>invA</i>	CTG GCG GTG GGT TTT GTT GTC TTC TCT ATT AGT TTC TCC CCC TCT TCA TGC GTT ACC C	1,070	[17]
<i>sitC</i>	CAG TAT ATG CTC AAC GCG ATG TGG GTC TCC CGG GGC GAA AAT AAA GGC TGT GAT GAA C	768	
<i>spiA</i>	CCA GGG GTC GTT AGT GTA TTG CGT GAG ATG CGC GTA ACA AAG AAC CCG TAG TGA TGG ATT	550	
<i>cdtB</i>	ACA ACT GTC GCA TCT CGC CCC GTC ATT CAA TTT GCG TGG GTT CTG TAG GTG CGA GT	268	
Virulence-associated gene (multiplex PCR reaction 2)			
<i>sipB</i>	GGA CGC CGC CCG GGA AAA ACT CTC ACA CTC CCG TCG CCG CCT TCA CAA	875	[17]
<i>spvB</i>	CTA TCA GCC CCG CAC GGA GAG CAG TTT TTA GGA GGA GGC GGT GGC GGT GGC ATC ATA	717	
<i>sifA</i>	TTT GCC GAA CGC GCC CCC ACA CG GTT GCC TTT TCT TGC GCT TTC CAC CCA TCT	449	
<i>sopB</i>	CGG ACC GGC CAG CAA CAA AAC AAG AAG AAG TAG TGA TGC CCG TTA TGC GTG AGT GTA TT	220	
Antimicrobial resistance gene (singleplex PCR)			
<i>bla_{PSE-1}</i>	CGC TTC CCG TTA ACA AGT AC CTG GTT CAT TTC AGA TAG CG	419	[19]
<i>bla_{TEM}</i>	GCA CGA GTG GGT TAC ATC GA GGT CCT CCG ATC GTT GTC AG	310	[20]
<i>floR</i>	CTG AGG GTG TCG TCA TCT AC GCT CCG ACA ATG CTG ACT AT	673	[16]
<i>aadA2</i>	CGG TGA CCA TCG AAA TTT CG CTA TAG CGC GGA GCG TCT CGC	249	[20]
<i>strA</i>	CTT GGT GAT AAC GGC AAT TC CCA ATC GCA GAT AGA AGG C	548	[20]
<i>ant(3'')-Ia</i>	GTG GAT GGC GGC CTG AAG CC ATT GCC CAG TCG GCA GCG	526	[19]
<i>sul-1</i>	ATC GCA ATA GTT GGC GAA GT GCA AGG CGG AAA CCC GCG CC	797	[19]
<i>sul-2</i>	AGG GGG CAG ATG TGA TCG AC GCA GAT GAT TTC GCC AAT TG	249	[20]
<i>sulA</i>	CAC TGC CAC AAG CCG TAA GTC CGC CTC AGC AAT ATC	360	[20]
Antimicrobial resistance gene (multiplex PCR)			
<i>tetA</i>	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210	[21]
<i>tetB</i>	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	659	
<i>tetG</i>	CAG CTT TCG GAT TCT TAC GG GAT TGG TGA GGC TCG TTA GC	468	

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 adenytransferase 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							
	<i>invA</i>	<i>sitC</i>	<i>spiA</i>	<i>cdtB</i>	<i>sipB</i>	<i>spvB</i>	<i>sifA</i>	<i>sopB</i>
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
Phenicol	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	-
4	AMP-S-CN-SXT-TE-NAL	1	-
	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		
	<i>bla_{PSE-1}</i>	<i>bla_{TEM}</i>	<i>floR</i>	<i>aadA2</i>	<i>strA</i>	<i>ant(3^{II})-Ia</i>	<i>sul-1</i>	<i>sul-2</i>	<i>suIA</i>	<i>tetA</i>	<i>tetB</i>	<i>tetG</i>
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^{II})-Ia*, aminoglycoside nucleotidyltransferase gene; *sul-1*, *sul-2*, *suIA*, 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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