Clustered Regularly Interspaced Short Palindromic Repeats Genotyping of Multidrug-Resistant *Salmonella* Heidelberg Strains Isolated From the Poultry Production Chain Across Brazil

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Salmonella enterica subsp. enterica serovar Heidelberg has been associated with a broad host range, such as poultry, dairy calves, swine, wild birds, environment, and humans. The continuous evolution of *S*. Heidelberg raises a public health concern since there is a global dispersal of lineages harboring a wide resistome and virulome on a global scale. Here, we characterized the resistome, phylogenetic structure and clustered regularly interspaced short palindromic repeats (CRISPR) array composition of 81 *S*. Heidelberg strains isolated from broiler farms (*n* = 16), transport and lairage (*n* = 5), slaughterhouse (*n* = 22), and retail market (*n* = 38) of the poultry production chain in Brazil, between 2015 and 2016 using high-resolution approaches including whole-genome sequencing (WGS) and WGS-derived CRISPR genotyping. More than 91% of the *S*. Heidelberg strains were multidrug-resistant. The total antimicrobial resistance (AMR) gene abundances did not vary significantly across regions and sources suggesting the widespread distribution of antibiotic-resistant strains from farm to market. The highest AMR gene abundance was observed for *fosA7*, *aac(6')-Iaa*, *sul2*, *tet(A)*, *gyrA*, and *parC* for 100% of the isolates, followed by 88.8% for *blaCMY−2*. The β-lactam resistance was essentially driven by the presence of the plasmid-mediated AmpC (pAmpC) *blaCMY−2* gene, given the isolates which did not carry this gene were susceptible to cefoxitin (FOX). Most *S*. Heidelberg strains were classified within international lineages, which were phylogenetically nested with *Salmonella* strains from European countries; while CRISPR genotyping analysis revealed that the spacer content...
INTRODUCTION

Salmonella enterica subsp. enterica serovar Heidelberg is most often associated with eggs and poultry (Hennessy et al., 2004; Chittick et al., 2006; Foley et al., 2008; Folster et al., 2012; Jackson et al., 2013). However, this scenario has changed since Salmonella Heidelberg has been associated with other sources from a broad host range, such as dairy calves (Centers for Disease Control and Prevention [CDC], 2017), swine (Cabral et al., 2017), wild birds (Liakopoulos et al., 2016a), environmental sources (Antony et al., 2018), human-derived clinical specimens (Abdullah et al., 2021), and outbreaks (Antony et al., 2018), which denotes their importance as a high-priority pathogen.

One of the most important risk factors surrounding foodborne illness is the international food trade that has been circumstantially accompanied by Salmonellae dispersal beyond borders. In this context, there is a global dispersal of multidrug-resistant lineages of serovar S. Heidelberg, reaching various countries in North America (Andrysiak et al., 2008; Centers for Disease Control and Prevention, 2014; Public Health Agency of Canada, 2014; Deblais et al., 2018; Cox et al., 2021), South America (Kipper et al., 2021), Europe (Liakopoulos et al., 2016b; Campos et al., 2018), and Asia (Wu et al., 2013). Therefore, the simultaneous increase and extended protraction of S. Heidelberg in many parts of the world have favored their genetic acquisition of virulence and antimicrobial resistance (AMR) genes through horizontal gene transfer (HGT), which has ultimately led to one of the most pressing global concerns.

Owing to their importance as a key poultry producer globally, Brazil quickly became the hotspot of S. Heidelberg and urgent actions were needed from the food safety authorities to mitigate this pathogen in order to reduce the economic losses in the poultry sector. In this context, most investigations to detect Salmonella enterica serovars in the poultry sector still had important methodological gaps, since the food industry focuses especially on Salmonella Typhimurium and Salmonella Enteritidis, which demonstrate the need for a combined approach between classical microbiology and high-resolution methods such as whole-genome sequencing (WGS) and clustered regularly interspaced short palindromic repeats (CRISPR) genotyping (Barrangou and Dudley, 2016; Thompson et al., 2018; Youssi et al., 2020). Indeed, the use of these high-throughput sequencing analyses exemplifies a useful means, not only for identifying Salmonella serovars but also to trace back the origin of the contamination conferring a substantial aid in decision-making to the poultry sector. In this regard, we demonstrated the usefulness of WGS-based identification in our previous study for genotyping rare Salmonella enterica serovars isolated from food and related sources (Monte et al., 2021). This previous survey demonstrated that the CRISPR arrays were highly conserved, and this genomic inspection provides high-resolution genotyping of Salmonella serovars. Hence, we performed a genomic study by combining WGS and CRISPR genotyping to characterize S. Heidelberg isolates from different sources at broiler farms, slaughterhouses, transport, lairages, and retail markets in Brazil.

MATERIALS AND METHODS

Salmonella Heidelberg Strains and Antimicrobial Susceptibility Testing

A total of 79 non-duplicate Salmonella enterica subsp. enterica serovar Heidelberg from our collection that included isolates obtained from broiler farms (n = 16), transport and lairage (n = 5), slaughterhouses (n = 22), and retail markets (n = 38) in Brazil between 2015 and 2016 were used in this study (refer Table 1). We also included two S. Heidelberg strains (SH159 and SSc139) from our previous work (Monte et al., 2019) for comparative purposes, totaling eighty-one isolates. The Salmonella isolation was performed according to the International Organization for Standardization (Anonymous, 2007, 2017). These isolates were serotyped on the basis of somatic O, phase 1, and phase 2 of H flagellar antigens by agglutination tests with antisera as specified in the Kauffmann–White–Le Minor scheme (Grimont and Weil, 2007; Guibourdenche et al., 2010).

Minimum inhibitory concentrations (MICs) were determined by broth microdilution using Sensititre® Gram-Negative Plates (Trek Diagnostic Systems, OH), such as 14 antimicrobials: cefoxitin (FOX), ceftriaxone (AXO), amoxicillin/clavulanic acid 2:1 ratio (AUG2), ceftiofur (XNL), ampicillin (AMP), nalidixic acid (NAL), ciprofloxacin (CIP), chloramphenicol (CHL), tetracycline (TET), gentamicin (GEN), sulfisoxazole (FIS), trimethoprim/sulfamethoxazole (SXT), streptomycin (STR), and azithromycin (AZI). MIC values were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute [CLSI], 2021) and the National Antimicrobial Resistance Monitoring System (US Food and Drug Administration [FDA], 2015). Multidrug resistance was defined as resistant to three or more classes of antimicrobials (Magiorakos et al., 2012).

Genomic Analysis

All S. Heidelberg isolates (n = 81) underwent DNA extraction performed by using a commercial kit (QiAmp tissue, Qiagen,
TABLE 1  | Features of S. Heidelberg strains (n = 81) isolated from different sources.

| Strain ID | Location*/year | Origin | Source | Resistance profile | Resistance genes | Sequence type |
|-----------|----------------|--------|--------|-------------------|------------------|---------------|
| SH018     | SP/2016 Farm   | Broiler chicken | FOX-TET-AOX2-CIP-NAL-XNL-AMP | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_002270265.1 |             |         |        |                   |                  |               |
| SH019     | SP/2016 Farm   | Broiler chicken | FOX-TET-AOX2-CIP-NAL-XNL-AMP | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_002260805.1 |             |         |        |                   |                  |               |
| SH10211124 | SC/2016 Farm   | Broiler chicken | TET-CIP-NAL | fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_006332685.1 |             |         |        |                   |                  |               |
| SH10227492 | SC/2016 Farm   | Broiler chicken | TET-CIP-NAL-STR | fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_006291695.1 |             |         |        |                   |                  |               |
| SH10230633 | MS/2016 Farm   | Broiler chicken | FOX-TET-AOX2-CIP-NAL-XNL-AMP | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_004161895.1 |             |         |        |                   |                  |               |
| SH10190712 | PR/2016 Farm   | Broiler chicken | FOX-TET-AOX2-CIP-NAL-XNL-AMP | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_011157915.1 |             |         |        |                   |                  |               |
| SH10201911 | SC/2016 Farm   | Broiler chicken | TET-CIP-NAL | fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_011519745.1 |             |         |        |                   |                  |               |
| SH10225532 | SC/2016 Farm   | Broiler chicken | TET-CIP-NAL-STR | fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_007640935.1 |             |         |        |                   |                  |               |
| SH715     | SP/2016 Farm   | Chicken cage after cleaning | FOX-TET-AOX2-CIP-NAL-XNL-AMP | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_011598585.1 |             |         |        |                   |                  |               |
| SH134     | SP/2016 Farm   | Chicken cage after cleaning | FOX-TET-AOX2-CIP-NAL-XNL-AMP | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_011158435.1 |             |         |        |                   |                  |               |
| SH159     | MG/2016 Farm   | Chicken cage after cleaning | FOX-TET-AOX2-CIP-NAL-XNL-AMP | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_011157595.1 |             |         |        |                   |                  |               |
| SH415     | SC/2016 Farm   | Chicken cage after cleaning | FOX-TET-AOX2-CIP-NAL-XNL-AMP | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_006332505.1 |             |         |        |                   |                  |               |
| SH414     | SC/2016 Farm   | Chicken cage after cleaning | FOX-TET-AOX2-CIP-NAL-XNL-AMP | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_006291935.1 |             |         |        |                   |                  |               |
| SH715     | SP/2016 Farm   | Chicken cage after cleaning | TET-CIP-GÉN-NAL-STR | fosA7, aac(3)-Ia-Vla, aadA1, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_003874535.1 |             |         |        |                   |                  |               |
| SH264     | PR/2016 Transport and lairage | Truck after cleaning | FOX-TET-AOX2-CIP-NAL-XNL-AMP | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_01093975.1 |             |         |        |                   |                  |               |
| SH265     | PR/2016 Transport and lairage | Truck after cleaning | FOX-TET-AOX2-CIP-NAL-XNL-AMP | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_010884255.1 |             |         |        |                   |                  |               |
| SH415     | SC/2016 Transport and lairage | Truck after cleaning | FOX-TET-AOX2-CIP-NAL-XNL-AMP-STR | blao<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| GCA_003877275.1 |             |         |        |                   |                  |               |

(Continued)
| Strain ID | Location*/year | Origin | Source | Resistance profile | Resistance genes | Sequence type |
|----------|----------------|--------|--------|--------------------|------------------|---------------|
| SH433    | SC/2016        | Transport and lairage | Truck after cleaning | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_006332565.1 | | | | | | |
| SH435    | SC/2016        | Transport and lairage | Truck after cleaning | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_006291875.1 | | | | | | |
| SH122    | SP/2016        | Slaughterhouse | Chicken carcass | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_011616265.1 | | | | | | |
| SH125    | SP/2016        | Slaughterhouse | Chicken carcass | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_011544755.1 | | | | | | |
| SH128    | SP/2016        | Slaughterhouse | Chicken carcass | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_010956115.1 | | | | | | |
| SH129    | SP/2016        | Slaughterhouse | Chicken carcass | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_011591705.1 | | | | | | |
| SH258    | PR/2016        | Slaughterhouse | Chicken carcass | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_011537055.1 | | | | | | |
| SH283    | SP/2016        | Slaughterhouse | Chicken carcass | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_011517875.1 | | | | | | |
| SH284    | SP/2016        | Slaughterhouse | Chicken carcass | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_010005265.1 | | | | | | |
| SH285    | SP/2016        | Slaughterhouse | Chicken carcass | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_006291795.1 | | | | | | |
| SS.c148  | DF/2016        | Slaughterhouse | Chicken carcass | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_003877035.1 | | | | | | |
| SS.c155  | DF/2016        | Slaughterhouse | Chicken carcass | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_006209245.1 | | | | | | |
| SH268    | PR/2016        | Slaughterhouse | Chicken carcass after chiller | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_010979095.1 | | | | | | |
| SH269    | PR/2016        | Slaughterhouse | Chicken carcass after chiller | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_011157135.1 | | | | | | |
| SH270    | PR/2016        | Slaughterhouse | Chicken carcass after chiller | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_010978655.1 | | | | | | |
| SH28     | SP/2016        | Slaughterhouse | Mechanically recovered chicken meat | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_011492695.1 | | | | | | |
| SH1      | SP/2016        | Slaughterhouse | Mechanically recovered chicken meat | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_006211165.1 | | | | | | |
| SH296    | SP/2016        | Slaughterhouse | Mechanically recovered | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | blactm-2, fosA7, sul2, tet(A), aac(6')-Iaa, gyrAp.S89F, parCp.T75S | ST15 |
| GCA_006292135.1 | | | | | | |

*(Continued)*

**Note:** The table continues with additional entries, each representing a strain with its unique characteristics and resistance profiles.
### TABLE 1 (Continued)

| Strain ID | Location*/year | Origin | Source | Resistance profile | Resistance genes | Sequence type |
|-----------|----------------|--------|--------|-------------------|------------------|---------------|
| SH297     | GCA_003877075.1 SP/2016 Slaughterhouse | Mechanically recovered chicken meat | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH697     | GCA_003874475.1 SC/2016 Slaughterhouse | Mechanically recovered chicken meat | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH700     | GCA_006291975.1 SC/2016 Slaughterhouse | Mechanically recovered chicken meat | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH712     | GCA_006210745.1 SP/2016 Slaughterhouse | Mechanically recovered chicken meat | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH164     | GCA_010875785.1 SP/2016 Slaughterhouse | Viscera | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH118     | GCA_011163895.1 SP/2016 Retail market | Chicken breast | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH276     | GCA_011571185.1 SP/2016 Retail market | Salted chicken breast | TET-CIP-NAL | fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH405     | GCA_006332425.1 SC/2016 Retail market | Chicken breast fillet | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH410     | GCA_006209405.1 SC/2016 Retail market | Chicken breast fillet | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH694     | GCA_006291675.1 SC/2016 Retail market | Chicken breast fillet | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH120     | GCA_011590585.1 SP/2016 Retail market | Chicken thigh and drumstick | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH286     | GCA_006291895.1 SP/2016 Retail market | Chicken thigh and drumstick | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH411     | GCA_006209285.1 SC/2016 Retail market | Chicken thigh and drumstick | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH692     | GCA_006211665.1 SC/2016 Retail market | Chicken thigh and drumstick | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH121     | GCA_010946195.1 SP/2016 Retail market | Chicken fillet sasami | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, lnu(O), gyrA:p.S83F, parC:p.T57S | ST15 |
| SH127     | GCA_011146095.1 SP/2016 Retail market | Chicken fillet sasami | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, lnu(O), gyrA:p.S83F, parC:p.T57S | ST15 |
| SH135     | GCA_011146615.1 SP/2016 Retail market | Whole chicken | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH427     | GCA_003877155.1 SC/2016 Retail market | Whole chicken | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| SH138     | GCA_010980075.1 SC/2016 Retail market | Leg quarter | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR | bla<sub>CMY</sub>-2, fosA7, sul2, tet(A), aac(6'')-Iaa, gyrA:p.S83F, parC:p.T57S | ST15 |
| Strain ID | Location*/year | Origin | Source | Resistance profile | Resistance genes | Sequence type |
|----------|----------------|--------|--------|--------------------|------------------|---------------|
| SH158    | MG/2016        | Retail market | Fiesta boneless | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *aadA<sub>1</sub>, *aadA<sub>2</sub>, *aac(6<sup>-I</sup>)<sub>a</sub>, *cmiA<sub>1</sub>, *dfrA<sub>12</sub>, *sul<sub>2</sub>, *tet(A), *qacL, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_010902135.1 |             |        |        |                    |                  |               |
| SH287    | SP/2016        | Retail market | Chicken skin | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP-STR | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_004158845.1 |             |        |        |                    |                  |               |
| SH289    | SP/2016        | Retail market | Seasoned chicken fillet | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_004159315.1 |             |        |        |                    |                  |               |
| SH403    | SC/2016        | Retail market | Seasoned chicken fillet | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_006292115.1 |             |        |        |                    |                  |               |
| SH290    | SP/2016        | Retail market | Chicken liver | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_006332625.1 |             |        |        |                    |                  |               |
| SH402    | SC/2016        | Retail market | Chicken liver | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_006332585.1 |             |        |        |                    |                  |               |
| SH408    | SC/2016        | Retail market | Chicken liver | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_006291855.1 |             |        |        |                    |                  |               |
| SH422    | SC/2016        | Retail market | Chicken liver | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_006291955.1 |             |        |        |                    |                  |               |
| SH423    | SC/2016        | Retail market | Chicken liver | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_006209445.1 |             |        |        |                    |                  |               |
| SH429    | SC/2016        | Retail market | Chicken liver | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_004160665.1 |             |        |        |                    |                  |               |
| SH430    | SC/2016        | Retail market | Chicken liver | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_006291835.1 |             |        |        |                    |                  |               |
| SH423    | SC/2016        | Retail market | Chicken liver | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_006209445.1 |             |        |        |                    |                  |               |
| SH674    | SC/2016        | Retail market | Chicken liver | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_006332645.1 |             |        |        |                    |                  |               |
| SH680    | SC/2016        | Retail market | Retail meat | FOX-TET-AXO-AUG2-CIP-NAL-XNL-AMP | *bla<sub>CMY-2</sub>, *fosA<sub>7</sub>, *tet(A), *aac(6<sup>-I</sup>)<sub>a</sub>, *gyrA,p.S83F, *parC,p.T57S | ST15 |
| GCA_003877135.1 |             |        |        |                    |                  |               |

(Continued)
TABLE 1 (Continued)

| Strain ID | Location*/year | Origin | Resistance profile | Resistance genes | Sequence type |
|-----------|----------------|--------|--------------------|------------------|---------------|
| SH681     | SC/2016 Retail market Chicken wing | FOX-TET-AXO-AUG2-CIP-NAL- | bla, fosA7, sul2, tet(A), aac(6\text{-I}_{aa}), CMY\text{-2} | GCA_006292015.1 | ST15 |
| SH685     | SC/2016 Retail market Chicken neck | FOX-TET-AXO-AUG2-CIP-NAL- | bla, fosA7, sul2, tet(A), aac(6\text{-I}_{aa}), CMY\text{-2} | GCA_004161515.1 | XNL-AMP-STR |
| SH691     | SC/2016 Retail market Chicken wing | FOX-TET-AXO-AUG2-CIP-NAL- | bla, fosA7, sul2, tet(A), aac(6\text{-I}_{aa}), CMY\text{-2} | GCA_006291915.1 | XNL-AMP |
| SH693     | SC/2016 Retail market Chicken wing paddle | FOX-TET-AXO-AUG2-CIP-NAL- | bla, fosA7, sul2, tet(A), aac(6\text{-I}_{aa}), CMY\text{-2} | GCA_006210725.1 | XNL-AMP |
| SSC139    | SP/2016 Retail market Chicken wing | FOX-TET-AXO-AUG2-CIP-NAL- | bla, fosA7, sul2, tet(A), aac(6\text{-I}_{aa}), CMY\text{-2} | GCA_011578645.1 | XNL-AMP-STR |
| SH716     | SP/2016 Retail market Chicken wing | FOX-TET-AXO-AUG2-CIP-NAL- | bla, fosA7, sul2, tet(A), aac(6\text{-I}_{aa}), CMY\text{-2} | GCA_010932755.1 | XNL-AMP |

Results of the antimicrobial susceptibility testing are presented in Table 2. MICs vary among S. Heidelberg strains.

1http://www.genomicepidemiology.org/
2https://github.com/CRISPRlab/CRISPRviz
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Minimum inhibitory concentration values for Salmonella Heidelberg strains (n = 81).

TABLE 2

| Antimicrobial | S. Antimicrobials Resistance (%) | Intermediate resistance (%) |
|---------------|---------------------------------|-----------------------------|
| Cefoxitin     | 88.8                            | 0                           |
| Azithromycin  | 12.3                            | 22                          |
| Chloramphenicol | 1.23                          | 62                          |
| Tetracycline  | 100                             | 0                           |
| Ciprofloxacin | 100                             | 0                           |
| Ceftiofur     | 88.8                            | 1                           |
| Sulfisoxazole | 100                             | 0                           |
| Trimethoprim/sulfamethoxazole | 1.23          | 72                          |
| Ampicillin    | 88.8                            | 2                           |
| Streptomycin  | 16.0                            | 68                          |
| Blue MIC values indicate intermediate resistance, while red MIC values in gray squares indicate resistance profiles, which were determined by broth microdilution method using CLSI interpretative breakpoints (Clinical and Laboratory Standards Institute [CLSI], 2021).

The total AMR gene abundances did not vary significantly across regions and sources suggesting pervasive distribution of antibiotic resistant strains from farm to market in six different States of Brazil (Figure 1). The highest AMR gene abundances were observed for fosfomycin (fosA7; 100%), sulfonamide (sul2; 100%), tetracycline [tet(A); 100%], and aminoglycoside [aac(6′)-Iaa; 100%]. Seventy-two (88.8%) S. Heidelberg strains harbored the plasmid-mediated AmpC β-lactamase (blaCMY-2), encoding resistance to third-generation cephalosporin (3GC). Unlike, Int(G) (n = 2), aadA1 (n = 2), aph(3′)-Ia (n = 1), aac(3)-VIA (n = 1), aadA2 (n = 1), cmlA1 (n = 1), dfrA12 (n = 1), sul3 (n = 1), and qacr (n = 1) AMR genes were detected at very low levels (Table 1). On the other hand, chromosomal point mutations in gyrA [p. Ser83Phe (tcc→tcc)] and parC [p. Thr57Ser (acc→agc)] were identified in 100% of the strains. This quinolone resistance-determining region (QRDR) among S. Heidelberg strains was sufficient to promote high-level resistance at > 32 μg/ml for NAL.

All 81 Salmonella genomes were analyzed for the content of plasmid replicons by using the Center for Genomic Epidemiology (CGE) web-tool PlasmidFinder 2.1, with 100% of the genomes containing at least two replicons, like ColpVC and IncA/C2. The remaining plasmids replicons such as IncX1 (n = 80; 98.7%), IncI1 (n = 56; 69.1%), IncFII (n = 3; 3.7%), IncFII(29) (n = 2; 2.4%), IncY (n = 2; 2.4%), Col156 (n = 1; 1.2%), IncFIA (n = 1; 1.2%), IncQ1 (n = 1; 1.2%), and IncX4 (n = 1; 1.2%) were identified within S. Heidelberg genomes (Figure 1).

Spacer Composition and Sequence Type Were Highly Conserved Within Salmonella Heidelberg Strains

Next, we visualized CRISPR loci extracted from WGS data to analyze the pattern of repeats and spacers distributed among S. Heidelberg strains (n = 81). In doing so, we observed 13 unique CRISPR array patterns [P1 (n = 13), P2 (n = 3), P3 (n = 1), P4 (n = 3), P5 (n = 26), P6 (n = 1), P7 (n = 16), P8 (n = 1), P9 (n = 1), P10 (n = 8), P11 (n = 5), P12 (n = 2), and P13 (n = 1)] as shown in Table 3. Overall, we observed a maximum of 44 spacers across S. Heidelberg strains (P7), spread across two loci. SH265 and SH268, belonging to profile P12, contained 37 spacers, the lowest number presented here. Spacer composition was highly conserved across strains, which shared 43 (P1, P2, P3, P4, P5, and P6), 42 (P7 and P8), 40 (P9 and P10), 39 (P11), and 36 (P12) identical spacers, reflecting a common ancestral origin (Figure 2). Next, we performed a comparative analysis of the architecture of the type I-E CRISPR-Cas system present in these strains and observed 100% amino acid identity across all strains—further evidence of shared ancestral origin (Figure 3).
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We further evaluated the multi-locus sequence typing by *in silico* prediction, which revealed that all S. Heidelberg strains matched the international sequence type (ST15) (Table 1).

**Salmonella Heidelberg Strains Isolated From Brazil Are Genetically Related to South American, European, and Asian Isolates**

While assessing the phylogenetics of S. Heidelberg strains (*n* = 81) sequenced in this study, we noticed that the core genome, calculated from WGS data, represented 74% of the pan-genome (4,139 out of 5,582 total genes). Little genomic variation was present among the core genome, as only 704 SNP sites were detected. Bootstrap values varied across the phylogeny, likely attributed to the small genomic variation among strains. S. Heidelberg strains did not cluster by year, source, or geographic location across the phylogeny suggesting the widespread distribution [regions (*n* = 6), sources (*n* = 4), years of isolation (*n* = 2)] and persistence of *Salmonella* strains in Brazil (Figure 1), which validate the previous surveys (Monte et al., 2019). Furthermore, 11 different clusters were identified as shown in Figure 1. Of these, 14 strains appear to be from independent lineages, given that they nested out of the main branches.
TABLE 3 | Clustered regularly interspaced short palindromic repeats (CRISPR) patterns obtained from 81 Salmonella Heidelberg strains.

| CRISPR profile | Location* | Source | Year of isolation |
|----------------|-----------|--------|------------------|
| P1 | SC (n = 7), PR (n = 2), SP (n = 4) | Retail market (n = 5), transport and lairage (n = 1), slaughterhouse (n = 4), broiler farm (n = 3), transport and lairage (n = 1) | 2015 (n = 1), 2016 (n = 12) |
| P2 | SC (n = 2), MS (n = 1) | Retail market (n = 2), broiler farm (n = 1) | 2016 (n = 3) |
| P3 | SC (n = 1) | Slaughterhouse (n = 1) | 2016 (n = 1) |
| P4 | SC (n = 1), SP (n = 2) | Retail market (n = 2), slaughterhouse (n = 1) | 2016 (n = 3) |
| P5 | SC (n = 15), SP (n = 8), PR (n = 2), DF (n = 1) | Retail market (n = 13), transport and lairage (n = 3), slaughterhouse (n = 5), broiler farm (n = 5) | 2016 (n = 26) |
| P6 | SP (n = 1) | Broiler farm (n = 1) | 2016 (n = 1) |
| P7 | SC (n = 8), SP (n = 7), DF (n = 1) | Retail market (n = 9), slaughterhouse (n = 4), broiler farm (n = 3) | 2015 (n = 1), 2016 (n = 15) |
| P8 | SP (n = 1) | Retail market (n = 1) | 2016 (n = 1) |
| P9 | PR (n = 1) | Slaughterhouse (n = 1) | 2016 (n = 1) |
| P10 | SP (n = 6), MG (n = 1), PR (n = 1) | Retail market (n = 3), slaughterhouse (n = 3), broiler farm (n = 2) | 2016 (n = 8) |
| P11 | SP (n = 4), MG (n = 1) | Retail market (n = 3), slaughterhouse (n = 2) | 2016 (n = 5) |
| P12 | PR (n = 2) | Transport and lairage (n = 1), slaughterhouse (n = 1) | 2016 (n = 2) |
| P13 | SC (n = 1) | Broiler farm (n = 1) | 2016 (n = 1) |

*Brazilian States: PR, Paraná; SC, Santa Catarina; SP, São Paulo; MG, Minas Gerais; DF, Distrito Federal; MS, Mato Grosso do Sul.

We also observed through SNP clustering (PDS000037185.127; n = 765 isolates; NCBI pathogen detection tool), cases of international clustering of S. Heidelberg from our collection (n = 77) with strains isolated from a variety of sources (food, human, and environment) from Brazil (n = 201), Chile (n = 9), the United Kingdom (n = 444), Germany (n = 1), the Netherlands (n = 2), South Korea (n = 1), and China (n = 1), which suggest a common ancestor origin (Supplementary Figure 1). Another two strains (SH265 and SH268) from this study nested in the SNP cluster (PDS000029160.10) with strains isolated from Brazil (n = 11), the United Kingdom (n = 4), and Chile (n = 1) (Supplementary Figure 2).

DISCUSSION

There has been a great interest in surveying the adaptation of Salmonella serovars to the poultry production chain because of their extensive persistence in the past, notably with S. Typhimurium and S. Enteritidis, which have caused significant economic losses to this sector. Furthermore, the prevalence of S. Heidelberg shown in this study is not the only issue, but the fact that highly drug-resistant and/or MDR isolates are being recovered in most steps of the poultry production chain, particularly in Brazil could be considered a public health threat, as there is a risk of it becoming globalized.

Based on AMR results, the β-lactam resistance was essentially driven by the presence of plasmid-mediated AmpC (pAmpC) blacMY−2 gene, given the isolates which did not carry this gene were susceptible to FOX, while QRDR such as gyrA and parC genes drove quinolone resistance (Table 1). Indeed, the presence of strains harboring blacMY−2 gene could have implications on a one health interface, since this plasmid is more likely to persist (Teunis et al., 2018). Besides that, all strains harbored chromosomal mutations in gyrA and parC genes promoting high-level resistance against quinolones that could have implications on human health as treatment options become limited. Disturbingly, this result corroborates the findings by van den Berg et al. (2019) that found 98.4% of the S. Heidelberg isolates resistant to fluoroquinolones. On the other hand, all S. Heidelberg strains from our collection, displayed susceptibility to azithromycin, which could be considered a promising agent against Salmonellae infections (Crump et al., 2015; Wen et al., 2017). Azithromycin has been used as an alternative treatment option for enteric fever even when the guidelines on susceptibility testing were not available. Like fluoroquinolones, azithromycin is an antimicrobial agent with efficient intracellular penetration (Crump et al., 2015; Wen et al., 2017).

The total AMR load also included encoding resistance genes for aminoglycoside [aac(3)-Vla, aph(3′)-Ic, aadA1, aadA2], chloramphenicol (cmIa1), macrolides [Inu(G)], trimethoprim (dfrA12), and ammonium quaternary compounds (qacL) (Figure 1). Interestingly, the unique strain (SSc139) that carried the qacL gene was isolated from the retail market, which makes large use of such compounds to disinfect surfaces. Moreover, this strain nested in the same cluster with five strains isolated from broiler farms and slaughterhouse sources, in different states such as São Paulo, Santa Catarina, and Paraná, denoting the successful establishment of this lineage (ST15) in the Southern and South regions of Brazil. In this regard, we visualized the co-occurrence of AMR genes in all S. Heidelberg strains.

The presence of AMR genes and transmissible plasmids demonstrated little variation across the strains. The broad distribution and abundance of S. Heidelberg in broiler farms, slaughterhouses, transport, lairages, and retail markets suggest the high adaptability of this serovar in the poultry production chain in Brazil. Similarly, a study by Edirmanasinghe et al. (2017) examining FOX-resistant S. Heidelberg strains isolated
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FIGURE 2 | Reconstructed phylogeny based on the core genome, distribution of spacers composition, clustered regularly interspaced short palindromic repeats (CRISPR) loci, and CRISPR profiles among Salmonella Heidelberg strains. The location of isolation of each strain is labeled on its respective branch. Color strips depict the year (Y) and source (S) of isolation, respectively. Brazilian States: PR, Paraná; SC, Santa Catarina; SP, São Paulo; MG, Minas Gerais; DF, Distrito Federal; MS, Mato Grosso do Sul.

FIGURE 3 | Salmonella enterica subsp. enterica serovar Heidelberg type I-E CRISPR locus architecture. This system contains two distinct CRISPR arrays—one associated with the cas genes and one disparate locus upstream (5') from the cas genes.
from human, abattoir poultry, and retail poultry sources in Canada revealed a potential common source among strains, which suggest the simultaneous dispersal of S. Heidelberg strains carrying CMY-2 gene in several sources and different geographical locations. In convergence with our results, another study revealed a high occurrence of S. Heidelberg in imported poultry meat in the Netherlands containing blaCMY-2 gene (vanden Berg et al., 2019). Although we could not find colistin-resistant strains, it is worthwhile to note the presence of the IncX4 plasmid since it is a promiscuous plasmid with a high capacity of self-transmissibility that is commonly associated with the presence of plasmid-mediated colistin-resistance (mcr-1) in Brazil (Moreno et al., 2019).

Consistent with observations obtained in our study, recent surveys strongly support that S. Heidelberg may originate from a common ancestor, which circulates and persists in the Brazilian poultry production chain since at least 2004 (Kipper et al., 2021). More importantly, these strains are also nested with strains isolated from several sources and countries around the world supporting the hypotheses of intercontinental spread, which demonstrate that probably the common ancestor underwent diversification through genetic changes over time (Supplementary Material).

Protracted dissemination of S. Heidelberg via poultry might be a risk for a globalized food trade era. The introduction and clonal expansion of Salmonella strains across borders remain challenging due to the difficulties of identifying the origins of contamination. In light of this, the continued need for combined approaches between classical microbiology and high-resolution methods such as WGS and CRISPR genotyping truly illustrate to us what is hidden in plain sight.

For the purpose of discussion, other studies provide compelling validation data to support the usefulness of high-resolution methods for genotyping rare Salmonella enterica serovars (Monte et al., 2021) and/or to resolve S. Heidelberg isolates involved in foodborne outbreaks (Vincent et al., 2018). Of the latter, while assessing the CRISPR array of 145 S. Heidelberg isolates, Vincent et al. (2018) found 15 different CRISPR profiles endorsing our results.

This study further illustrates the potential of CRISPR for the tracking of variable genotypes in diverse Salmonella strains, as previously determined (DiMarzio et al., 2013; Shariat et al., 2013a,b, 2015; Monte et al., 2021), with noteworthy methodological convenience. Indeed, CRISPR-based analyses have proven relevant for subtyping of Salmonella enterica serovars Typhimurium and Heidelberg strains involved in outbreaks (Shariat et al., 2013b) and occasionally associated with antibiotic resistance (DiMarzio et al., 2013). Our findings underscore the potential role of S. Heidelberg as a key pathogen in the poultry production chain, particularly in Brazil.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

DM, RB, MN, PF-C, and ML designed the study. RB, PF-C, NL, and ML supervised the work. DM, MN, HB, SK, NL, PF-C, RB, and ML participated, coordinated, and analyzed the data. DM, MN, and RB wrote the original draft. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.867278/full#supplementary-material

Supplementary Figures 1, 2 | SNP-based phylogram of S. Heidelberg isolates from various isolation sources and locations. Strain ID, isolate source, location, SNP cluster, and collection date were retrieved from Genbank. Red color indicates isolates from this study.

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