Campylobacter spp. and *Salmonella* spp. strains from meat matrices: resistance genes involved and molecular methods to detect

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Antimicrobial resistance is a growing concern worldwide and has been extensively studied in the emergence of *Salmonella* spp. and *Campylobacter* spp. multidrug-resistant isolated from meat matrices (poultry, pork, and beef). Salmonellosis and campylobacteriosis are among the most frequently reported foodborne diseases worldwide. Most strains that have been isolated from meat matrices are resistant to various antimicrobials. Infection by multi-drug resistant (MDR) strains through the ingestion of meat may hinder treatment. Thus, it is important to identify the mechanism of resistance of these microorganisms. It varies according to the class of antimicrobial and includes the presence of specific genes or mutations in genes sequence. This review discusses resistance genes and mutations in genes that characterize antimicrobial resistance in *Salmonella* spp. and *Campylobacter* spp. strains isolated from meat matrices, and survey of the most used methods for detection of these genetic strategies.

Keywords: antimicrobial resistance, poultry, pork, beef, polymerase chain reaction (PCR), sequencing

1. Introduction

Salmonella spp. and *Campylobacter* spp. are considered the major foodborne bacterial pathogens, have persisted and been emerging foodborne pathogens [1–4]. Salmonellosis and campylobacteriosis are the most commonly reported cases worldwide, with *Campylobacter* spp. infections being the most common among *Salmonella* spp. infections. These bacteria require great attention from the collective health services since they are pathogenic to humans and are commonly found in the gastrointestinal tract of animals. Poultry, pig, and cattle are main reservoirs of these zoonotic bacteria, including multi-resistant microorganisms, being transmitted through the food chain [5]. The concern with this fact lies in the scope of collective health since some antimicrobials of choice for the treatment of this foodborne disease are no longer effective [6]. The expected effect of antimicrobial action not achieved is due to infection by multiresistant strains. Infections with resistant *Salmonella* spp. and *Campylobacter* spp. results in higher mortality compared to that causes by susceptible strains [7].

The antimicrobial resistance concern has been growing around the world and has been extensively studied. The cause of a large number of multi-resistant microorganisms has been researching. However, many factors can interfere when dealing with *Salmonella* spp. and *Campylobacter* spp. Some studies have correlated the use of antimicrobials as growth promoters, once permitted to the fact that multi-drug resistant microorganisms are present in the gastrointestinal tract of production animals [2,8]. In addition, others correlate with the appropriate therapeutic use of these antimicrobials, and also the possibility of resistant strains remaining in the environment and colonize the animals [8,9]. Several studies have been carried out to verify the presence of strains resistant to quinolones, fluoroquinolones, macrolides and tetracyclines in *Salmonella* spp. and *Campylobacter* spp. strains isolated from meat matrices [10–14].

Determination of the sensitivity of these microorganisms to antimicrobials can be measured by conventional techniques, such as determination of minimum inhibitory concentration (MIC) [11,12,15], or disk diffusion test [11,12,16], or minimum duration for killing (MDK) [17]. However, these detections only allow the determination of the degree of sensitivity of these microorganisms. Thus, more elaborate techniques are implemented to verify the possible mechanisms that led the strain to be resistant. New resistance mechanics are constantly described and new genes and vectors are discovered responsible for transmitting this resistance [18]. Molecular techniques are widely used in this case, especially polymerase chain reaction (PCR) and sequencing. The mechanisms of resistance most described in *Salmonella* spp. and *Campylobacter* spp. are the presence of resistance genes, or genes coding for efflux pump, and also a mutation in certain specific regions in specific genes. PCR is widely used when the objective is to identify and determine the presence of genes, while sequencing tools for the verification of mutations are applied [10,11,19,20].

For *Salmonella* are reported genes coding for aminoglycosides the alleles of aac, aad, aph, strA/B, chloramphenicol as cat, flo, cmAA, beta-lactams as blaTEM, blaCMY, blaPSE-1, tetracyclines as tet (A, B, C, D), to sulfamethoxazole as “sull”, and to trimethoprim as dFRA; besides these other genes may also be associated with resistance or multi-drug resistance as class-I integrons, calss-II integrons, *bla*TEM genes and *bla*CMY genes [19,20]. In *Campylobacter,* the genes related to antimicrobial resistance for fluoroquinolones the main mechanism is the mutation in the gene *gyr*A

(Thr-86-Ile, Asp-90-Asn, and Ala-70-Thr), besides the efflux by the pump CmeABC, due to mutation in cmeR gene or in the sequence of repetition; for macrolides, mutations in 23S rRNA and L4/L22 ribosomal proteins are observed; while for tetracyclines the modification of the ribosomal target by TetO protein binding occurs and EF-G gen; for aminoglycosides is the presence of Aph 3=-III, Aad A2, and Sat1 genes; for beta-lactams the presence of blaOXA-61: beta-lactamase EC 3.5.2.6 or Cam-1 genes; for lincosamides InuC gen [10,11,21].

In this context, this review discusses resistance genes and mutations in genes that characterize antimicrobial resistance found in *Campylobacter* spp. and *Salmonella* spp. strains isolated from meat matrices (poultry, pork, and beef). At the same time, a survey of the most commonly used molecular methods for detecting resistance genes and mutations in genes sequences are also reported.

2. Main Points on *Salmonella* spp. and *Campylobacter* spp.

2.1 *Salmonella* spp. and public health

Salmonella was first listed in the 'Approved List of Bacterial Names' in the 1900s [22]. Currently, 9 different species and 14 subspecies are recorded [23]. The principal specie is *Salmonella enterica*, over 2500 serovars of this species have been identified belonging to six subspecies. The subspecies are subdivided into serovars by their flagellar, carbohydrate and lipopolysaccharide (LPS) structure. In human infections, the principal serovars are Typhi, Paratyphi and Sendai [24]. Although, in infections with food of animal origin, the serovar *S.* Enteretidis presents the main importance. Some serovars, initially, had their name attributed to the place where they were discovered, *S*. Dublin and *S*. Heidelberg. While others have their name related to the diseases they caused and the animals that they affected. For example, *S.* Typhi which causes typhoid fever or type typhoid fever in humans, and *S*. Thyphimurium which causes typhoid fever in mice [25]. Salmonella serovars differentiate through O, H and Vi antigens using the Kayfmann-White model [26–28]. Serovars are divided into serogroups, being those that present a common antigenic factor. O antigen is derived from a complex structure of lipopolysaccharides (LPS) [25].

This foodborne pathogen is phenotypically characterized as short gram-negative bacilli, with 1 to 2 μm, facultative anaerobe microorganism, not sporulated, with peritracheal flagellum with allows mobility (excepted *S.* Galinnarum and *S.* Pullorum). It obtains the energy for its development through fermentation of glucose, producing acid and gas. However, its cannot metabolize sucrose and lactose [25,29,30]. In addition to these characteristics, its development occurs at 35 to 39ºC, under aerophilic conditions. Although, some authors specify that present development at 5 to 7ºC [25,29,31].

CDC [32] estimate that *Salmonella* causes one million foodborne disease in the United States, with 380 deaths and 19,000 hospitalizations. Salmonellosis causes multiple clinical syndromes, high morbidity, mortality and burden of disease [33]. The main transmission mode is consumption of contaminated food of animal origin, meat, and dairy products. Main foods that are involved are handled improperly or undercooked to, especially poultry, eggs and meat, and also dehydrated foods. In general, the following diseases are observed, gastroenteritis in the case of *S.* Enteretidis and *S*. Typhimurium, enteric fever when *S.* Typhi and *S*. Paratyphi were observed, and invasive systemic disease when *S*. Cholerasuis is present. The characteristic symptoms of salmonellosis are diarrhea, abdominal pain, nausea and sometimes vomiting, headache and weakness, and hyperthermia. Complications, such as chronic disease, can develop reactive arthritis and Reiter's syndrome, characterized as rheumatic diseases. *Salmonella* infection is usually a self-limiting disease. However, severe cases may occur, where there is a need for rehydration and administration of antimicrobial [3], and it is necessary to perform an antimicrobial susceptibility test due to the existence of multidrug resistant strains. An increase of a number of antimicrobial resistant *Salmonella* has been observed. In the United Kingdom, in 1969 was reported that use of antimicrobials as growth promoters was contributing to the rise in multidrug-resistant *Salmonella* spp. [2]. *Salmonella* quinolone resistance isolated from animals and foods of animal origin has increased worldwide in the last years [34,35].

2.2 *Campylobacter* spp. and public health

Previously classified as *Vibrio* spp., after applying Hugh and Leifson's tests for the fermentative metabolism and composition of DNA bases, *Campylobacter* was first proposed by Sébald and Véron in 1963. *Campylobacter fetus* and '*Campylobacter bubulus'* (now classified as *Campylobacter sputorum*) were included in this genus [36]. By comparing the components of the microorganisms, it was possible to obtain different results in biochemical and serological tests, which allowed the designation of the genus *Campylobacter* [37]. The genus was divided into three groups, *Campylobacter* catalase and production of hydrogen sulphide (H_2S) positive, in this group are included the species *C. jejuni* and *C. coli*; *Campylobacter* catalase positive and production of H_2S negative, in this group are classified the species *C. fetus* subp. *fetus* and *C. fetus* subsp. *veneralis*; and the latter group is *Campylobacter* catalase negative, including species *C. sputorum* subsp. *bubulus* and *C. sputorum* subsp. *sputorum* [36]. These species were first listed in the 'Approved List of Bacterial Names' in the 1980s [38]. With the increase of studies due to the importance of this

microorganism, there was an increase in the number of described species. In 2010, the number of species increased to 32 and subspecies remained at 13. Currently, 34 different species and 14 subspecies are recorded [23].

Phenotypically they are gram-negative bacilli, in the majority they present in the form of a wing of gull or comma, with 0.5 to 5mm of length and 0.2 to 0.9mm of width, presenting a polar flagellum, such writing allows move in "corkscrews" [39]. It obtains the energy for its development through amino acids intermediates of the cycle of Krebs with four or six carbons, are oxidase positive and catalase variable (positive or negative), do not produce spores, do not promote hemolysis, do not oxidize or ferment carbohydrates and do not hydrolyze gelatin nor urea, except some atypical strains of *C. lari* and *C. sputorum* sorovar Paraureolyticus [25,29,30,39,40]. In addition to these characteristics, its development occurs at 42 to 47ºC, under microaerophilic conditions, that is, at low oxygen concentrations. Some authors specify that the ideal atmosphere for growth and development should contain oxygen concentrations between three and five percent and carbon dioxide between five and ten [25,29,31].

Campylobacteriosis is sporadic in human and in many cases, are sub notified, expected per year more than 2.4 million people are infected [4,41]. *Campylobacter* was the most isolated gastrointestinal pathogen of humans in the European Union since 2005. It is considered the major cause of foodborne disease in developed countries [42,43]. For humans, *Campylobacter jejuni* is more pathogenic than being more frequent than *C. coli*, although coinfections may also occur [44,45]. Usually, *Campylobacter* infections are self-limiting but can persist and be serious consequences as Miller Fisher syndrome, irritable bowel syndrome and Guillain-Barre Syndrome, this last is the principal sequelae characterized by demyelinating polyneuropathy [4].

The main foods that are the source of *Campylobacter* infections are pork, beef and, especially poultry. Most of the infections by this microorganism are associated with the consumption of chicken meat and its by-products, which can be contaminated during processing [46,47]. Studies determine the prevalence in bovine carcass by 2.7% of *Campylobacter* [48]. Each chicken carcass can be carried 100 to 100 mil *Campylobacter* cells, its know is necessary only 500 cells to cause infections, so present a high risk to public health, the principle of the undercooked meat or cross-contamination [31]. Zhao et al [49], find highest numbers in breast chicken (49.9%) followed by turkey (1.6%), pork and beef, in the USA. Tests made by National Antimicrobial Resistance Monitoring System (NARMS), found the prevalence of *Campylobacter* in 47% of undercook poultry samples [50]. In broiler and chicken, the most isolated specie is *C. jejuni* [11,51], although Miller et al [52], in Caribe, found *C. coli* as most isolated. In addition, pork present most *C. coli* [4], Qin et al. [53] found 98.9%. In 2013, in Lower Saxony, 76.2% of pork livers samples was positive for *C. coli* and 21.1% of *C. jejuni* [54].

With the increase in cases of campylobacteriosis, there also was an increase in the number of *Campylobacter* isolated from humans and resistant to ciprofloxacin, ampicillin, tetracyclines, and nalidixic acid. The same was observed for resistance to nalidixic acid and tetracycline in strains isolated from chicken meat [7].

3. Characteristics of antimicrobial resistance

3.1 Main antimicrobials and resistance in meat matrix

Antimicrobials used in human medicine is classified by World Health Organization (WHO) in categories 1 and 2, based on a level of critical importance of antimicrobial resistance. On this includes aminoglycosides, carbapenems, and 3rd and 4th generation of cephalosporin, macrolides, and quinolones [55]. Furthermore, the priority of the CIAs group is based on two criteria in three applications (1.1, 1.2 and 2.1) [56], with antimicrobials meet both criteria and all three applications are considered of the highest priority (Table 1). Campylobacter and Salmonella, foodborne pathogens resistant to antimicrobials, are mostly of animal origin [35]. Based on the WHO critically important antibiotics, quinolone and cephalosporin (3ª and 4ª generation) resistance in *Salmonella* spp. [12,20,55,57], and quinolone and macrolide resistance in *Campylobacter* spp. [10–12,35,55,57]. The main antimicrobial resistance-related strains of *Campylobacter* spp. and *Salmonella* spp. isolates from meat are quinolones more specifically fluoroquinolones and macrolides resistant.

Table 1 Categorization, prioritization and important antimicrobials of highest priority (meet both criteria and all three applications) by WHO [55–57]

Criterion	**1 - Antimicrobial agent is used as sole therapy or one of limited available therapy, to treat human disease**		**2- Antimicrobial agent is used to treat diseases caused by either organisms that may be transmitted via non-human sources or human diseases caused by organisms that may acquire resistance genes from non-human sources**
Application	1.1	1.2	2.1
Description	high absolute number of people affected by diseases for which the antimicrobial is the sole or one of few alternatives to treat serious human disease	high frequency of use of the antimicrobial for any indication in human medicine, since usage may promote selection of resistance	greater degree of confidence that there are non-human sources that result in transmission of bacteria (*Campylobacter* spp.) or their resistance genes to humans (high for *Salmonella* spp., *Escherichia coli* and *Enterococcus* spp.)

Drugs	Fluoroquinolones	Macrolides and ketolides	3rd and 4th generation cephalosporins
Description	Quinolones are widely used in food animal production and are known to select for fluoroquinolone-resistant *Salmonella* spp. and *Escherichia coli* in animals. At the same time, fluoroquinolones are one of few available therapies for serious *Salmonella* infections, particularly in adults.	Macrolides are widely used in food animal production and are known to select for macrolide-resistant *Campylobacter* spp. in animals. At the same time, macrolides are one of few available therapies for serious *Campylobacter* infections, particularly in children, in whom quinolones are not recommended for treatment. Given the high incidence of human disease due to *Campylobacter* spp., the absolute number of serious cases is substantial.	3rd and 4th generation cephalosporins are widely used in food animal production and are known to select for cephalosporin resistant *Salmonella* spp. and *E. coli* in animals. Additionally, 3rd and 4th generation cephalosporins are one of few available therapies for serious *Salmonella* infections, particularly in children. Given the high incidence of human disease due to *Salmonella* spp. and *E. coli* the absolute number of serious cases is substantial.

In humans, erythromycin is the drug of choice for treatment of campylobacteriosis. It is bacteriostatic from the macrolide group [2]. Macrolides action on bacterial cells occurs by interruption of protein synthesis, by binding to the 50S P-site of the ribosomal subunit and thereby blocking the activity of the peptidyl transferase enzyme [58]. Important antimicrobial in the *Salmonella* resistance chain is cephalosporins, it is β-lactam antimicrobial age by the inhibition of cell wall biosynthesis [59]. A mechanism of resistance presented by this microorganism to the β-lactams constitutes in the presence of the enzyme β-lactamase. In addition, most of the producing strains of extended-spectrum β-lactamases (ESBL)-AmpC were identified with resistance genes [5].

C. coli isolated from broiler meat and broilers present extremely resistance to ciprofloxacin (82.7 and 78.4%, respectively), with some lower levels in *C. jejuni* (59.5 and 44.1%) can be observed [7]. In *Salmonella* isolated from bovine and porcine, only 6.1 and 4.0% present resistance to ciprofloxacin, although chicken isolates present 47.8% [60]. Maka et al [61], reported *Salmonella* strains from poultry products with a large spectrum of resistance by antimicrobials. EFSA and ECDC [7], report a higher occurrence of *Salmonella* spp. ciprofloxacin resistant from fattening turkey, broiler meat, and fowl (37.3 to 86.2%). In addition, in Poland, turkey isolates present 89 of resistance to nalidixic acid and ciprofloxacin [7]. In the USA, Mc Dermott [13], verify the presence of resistant strains of *Salmonella* in retail meat, about 55% to tetracycline, 41% to streptomycin, 38% to sulfisoxazole, and 38% to ampicillin as most common resistance. Therefore, in *Campylobacter*, the principle antimicrobial resistance observed is by quinolone (principle fluoroquinolone) and macrolides. In *Salmonella* is in fluoroquinolones and cephalosporin.

3.2 Mechanisms of resistance

In response to antibiotic pressure, bacteria optimize their resistance mechanism towards multiple drugs to survive [62]. Antimicrobial resistance in bacteria can be intrinsically, but can also acquire this by horizontal gene transfer or mutation in chromosomal genes. For β-lactams, aminoglycosides and fluoroquinolones intrinsic resistance are linked to many genes. A joint work between the industry and the academic institution is of great importance, aiming studies on the development of resistance and mechanisms of resistance of microorganisms, not only as a research, but also an initial phase in the development of drugs [18].

The mechanism resistance varies according to antimicrobial class. In aminoglicosides occurs the changes in antimicrobial by aminoglycosides modified enzymes, denominate phosphotransferase aminoglycoside (*aphA-3, aphA-1 e aphA-7*), 6'-adeniltransferase (*aadE*), and acetyl transferase (*sat*) [6]. In beta-lactamase, the beta-lactamase (penicillinase, OXA-61) made an inactivation and the porine of external membrane decrease the permeability of antimicrobial (Major Outer Membrane Porin-MOMP). In addition, the efflux pump CmeABC increase the elimination of antimicrobial out of intracellular space. This efflux pump mechanism can be a correlation to quinolones resistance to by the presence of mutation on cmeR or in sequence repletion and occurs a super expression of this pump, but the principle and primarily developed is the presence of the mutation (Table 3) in QRDR in *gyr*A gene [10,11]. In macrolides, the mutation is on rRNA23S, in ribosomal proteins L4/L22, and efflux pump CmeABC and reduce of membrane permeability by MOMP. In addition, on tetracilcins present efflux pump action and the principle mechanism is the modification of ribosomal point by ligation of TetO protein [6,63,64]. Macrolides mechanism resistance in *Campylobacter* spp. can be observed, as mutation A2075G in the 23S ribosomal RNA mutation in the 50S ribosomal subunit codified protein L4 and L22 gene [65]. For fluoroquinolones, presence of activated efflux pump was reported by Iovine [6].

Salmonella resistance to fluoroquinolones may be explained by two major mechanisms in combination, active efflux mediated by AcrAB-TolC and multiple target gene mutations in QRDR region of *gyrA*, *gyrB*, *parC* and *parE* genes [34]. Intrinsic resistance in *S. enterica* was attributed to AcrB multidrug efflux-pump and bulkiness of

lipopolysaccharides to oligosaccharides [66]. The mechanism of active efflux pump is an intrinsic resistance present in *Salmonella* spp. for erythromycin [2].

Several genes encoded antibiotic resistance (Table2), many of it can be transferred between bacteria [18]. The main genes related to the strains of *Salmonella* spp. and *Campylobacter* spp. multidrug resistant are listed in Table 2. Ahmed et al [20] identify β-lactamase-encoding genes in 75.5% of meat products by PCR being the most identified bla_{TEM-1} (41.5%). The plasmid-mediated quinolone resistance genes were identified in 28.3% of *S. enterica* isolated from meat sample [20].

Table 2 Drug resistance genes reported in Salmonella and Campylobacter and the related antimicrobials class.

Microorganism	Meat matrices	Antimicrobial	Resistance gen	Reference
S. Typhimurium	Beef	β-Lactamics	bla_{TEM-1}	[20]
			bla_{CMY-2}	[20]
			$bla_{CTX-M-3}$	[20]
			bla_{SHV-12}	[20]
			$bla_{CTX-M-15}$	[20]
		Quinolones	*qnr*A	[20]
			*qnr*B	[20]
			*qnr*S	[20]
			aac(6')-Ib-cr	[20]
	Chicken	β-Lactamics	bla_{TEM-1}	[20]
			bla_{OXA-1}	[20]
	Retail Meat	Tetracycline	*tet*A	[13]
			*tet*B	[13]
			*tet*C	[13]
			*tet*D	[13]
			*tet*G	[13]
S. Enteretidis	Beef	β-Lactamics	bla_{TEM-1}	[20]
			bla_{SHV-12}	[20]
			bla_{CMY-2}	[20]
			bla_{OXA-1}	[20]
	Chicken	β-Lactamics	bla_{TEM-1}	[20]
			$bla_{CTX-M-3}$	[20]
		Quinolones	*qnr*S	[20]
			aac(6')-Ib-cr	[20]
	Poultry	Quinolones	*qnr*A1	[14]
S. Corvallis	Poultry	Quinolones	*qnr*B19	[14]
S. enterica	Retail meat	Aminoglycoside	*aac(3)*	[6]
			aac(6')-Ib	[13,19,67,68]
			*aad*A	[19]
			*aad*B	[13,19]
			*aad*E	[19]
			aph	[13,19]
			*str*AB	[13,19]
		β-lactam	bla_{TEM}	[13,19]
			bla_{CMY-2}	[13,19,67]
			bla_{PSE-1}	[19]
			bla_{SHV-2}	[13]
			$bla_{CTX-M-1}$	[13]
			*amp*R	[19]
		Quinolone	*qnr*A	[13,68]
			*qnr*B	[68]
			*qnr*B19	[13]
			*qnr*S	[13,67,68]
			*oqx*AB	[67]
		Chloramphenicol	*cat*	[19]
			*flo*R	[19,20]
			*cml*A	[19]
		Sulfamethoxazole	*sul*I	[13,19]
			*sul*II	[13,19]
			*sul*III	[13]
		Tetracycline	*tet*A	[19]
			*tet*B	[19]
			*tet*C	[19]
			*tet*D	[19]
			*tet*R	[19]
		Trimethoprim	*dfr*A	[19]

Campylobacter coli	Food	Gentamicin	*aph*A-3	[69]
C. jejuni *C. coli*	Broiler	Tetracycline	*tet*O	[70,71]
Campylobacter spp.	Retail meat	Gentamicin	*aph (2'')*-Ib	[71,72]
			aph (2'')-Ic	[71,72]
			aph (2'')-Ig	[71,72]
			aph (2'')-If	[71,72]
			aph (2'')-If1	[72]
			aph (2'')-If3	[72]
			aph (2'')-Ih	[71,72]
			aph (3')-Ic	[71]
			aph (3')-IIIa	[71,72]
			aac (6')-Ie	[71]
			aac (6')-Im	[71]
		Chloramphenicol	*cat*A	[71]
		Lincomycin	*lnu*C	[71]
		β-lactam	bla_{OXA-61}	[71]

Bacteria have a target in which the antibiotics bind with specificity and high affinity, so their replication activity is disrupted. Any change that occurs in the target structure and prevents the specific binding and high affinity of the antibiotics, but which does not interfere with the development of the bacteria, may confer antimicrobial resistance [18]. Examples are mutations in specific gene sequences or genome.

It is known that *qnr* genes confer a low level of resistance to fluoroquinolones in *Salmonella* spp., and there is an additional mechanism of resistance, which is determined by the presence of mutations in the quinolone resistance-determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes [73]. Ferrari et al. [14] identify 57.9% of mutation in *gyr*A gene in *Salmonella enterica* from Brazilian poultry. In *Salmonella*, mutations alter the DNA-gyrase binding sites of these antibiotics resulting in resistance to quinolones (Table 3). The mutation in the quinolone resistance determinant region (QRDR) is the main mechanisms of resistance in *Campylobacter* for fluoroquinolones to, this region is on *gyr*A gene, its code for the subunit 'A' of the DNA gyrase enzyme, conferring a decreased sensitivity to this antibiotic (Table 3).

Table 3 Resistance mutations in Salmonella and Campylobacter genes

Microorganism	Meat Matrices	Antimicrobial	Resistance gen	Mutation	Reference
S. Enteritidis	Poultry	Quinolone	*gyr*A	Ser 83 - Tyr	[14]
				Ser 83 - Phe	[14]
				Asp 87 - Asn	[14]
S. Heidelberg	Poultry	Quinolone	*gyr*A	Ser 83 - Phe	[14]
				Ser 83 - Tyr	[14]
				Asp 87 - Asn	[14]
Salmonella enterica	Retail meat	Quinolone	*gyr*A	Asp87 - Gly	[68]
				Asp87 - Asn	[68,74]
				Asp87 - Tyr	[68]
				Ser83 - Phe	[68,74]
			*par*C	Ser80 - Arg	[68]
Campylobacter spp.	Food	Quinolone	*gyr*A	Thr86-Ile	[69,71]
		Erythromycin	23rRNA	A2074T	[71]
				A2075G	[71]
		Erythromycin	23rRNA	A2075G	[70]
				Thr86-Ile	[10,11,70]
C. jejuni *C. coli*	Broiler	Quinolone	*gyr*A	Val-73-Glu	[11]
				Ser-114-Leu	[11]
				Val-88-Asp	[11]
				Ala-75- Asp	[11]
				Gly-119-Ser	[11]
				Arg-79-Lys	[11]

4. Molecular tools to determine antimicrobial resistance

4.1 Polymerase chain reaction (PCR)

Kary Mullis describe PCR in 1980. Its principle is based on nucleic acid enzymatic replication for fast and easy amplification of DNA. This molecular method has an irreplaceable role as one of the basic methods for DNA analysis

[75]. This method is a rapid and specific nucleic acid amplification method for detection presence of antimicrobial resistance genes of foodborne pathogens, including *Salmonella* and *Campylobacter* [10,11].

For the development of the technique, a mixture of some reagents is prepared with reaction buffer, magnesium chloride, and deoxynucleotides triphosphates (dNTPs), which are the bases for guanine, adenine, thymine and cytosine. In addition to oligonucleotides complementary to the gene of interest and primers, the Taq polymerase, and the DNA sample of interest. Primers may be between 18 and 35 base pairs (bp) and normally amplify segments of DNA between 200 and 800 bp. This mixture is placed in an end-cycler device, which is programmed for a series of cycles depending on the microorganism and the primers used. At this stage, the DNA is denatured, followed by cooling for annealing the primer to the DNA strand. It is then submitted to a high temperature for the synthesis of DNA [38].

The food supply is enriched when PCR technology is used to detect pathogenic agents in foods. This step can reduce limitations on PCR inhibition by the composition of the food matrix and the detection of dead cells. However, this enrichment excludes the possibility of quantification [77]. With PCR, it is possible to synthesize specific DNA fragments using DNA polymerase with specific primers used for the antimicrobial resistance genes in *Salmonella* spp. and *Campylobacter* spp. Virulence and toxin genes, such as *cad*F and *cdt*B, are used [78] to verify the importance of the strains to public health.

Conventional PCR requires amplification in a thermocycler, followed by product separation using gel electrophoresis. The timeframe required to analyze the gel of PCR products, and the inability to verify large numbers of strains are major impediments to this approach. In addition, while ethidium bromide is a relatively inexpensive reagent for DNA gel staining, it poses human and environmental safety concerns [79]. To overcome these limitations, real-time PCR (qPCR) allows quantification and eliminates the need for gel electrophoresis.

Real-time PCR (qPCR) is presented as a tool that offers speed, robustness, high sensitivity and specificity for analysis. Accurate quantification of DNA and RNA can be performed, yet this method enables online detection of the PCR product. The method eliminates the need to manipulate PCR products after amplification, reducing the risk of false-positive results through cross-contamination between amplification products and subsequent test samples [80,81]. Quantitative PCR methods with endpoint detection utilize an internal or external control with known concentrations that are amplified in parallel with analyzed samples. The results are given from the control data with a sample. However, this methodology has limitations in its accuracy because the final quantity of the product that is accumulated in the final PCR process is very susceptible to small variation. The TaqMan 5 nuclease PCR method uses a fluorogenically labeled probe, which when hybridized and cleaved allows the detection of the PCR product accumulated during the amplification reaction. The fluorescence emission is directly the result of the specific PCR product of that probe. This increase can be monitored in real time, allowing accurate quantification of the DNA or RNA sequence [82].

This method has the ability to quantify the target organisms in complex matrices and is, therefore, a promising tool to improve the safety and quality of food. Food science and technology has been using real-time PCR for security management and food quality [83]. The possible sequence of specific pathogens is a promising technique and more convenient than conventional PCR for rapid and reliable detection and quantification [84]. Because the presence of labeled DNA is indicated by an increase in fluorescence, there is less risk of contamination compared to conventional PCR. Additionally, it is possible to quantify specific microorganism genes [85]. This methodology is attractive because it is faster and more robust, as there is no need for processing to detect the amplified product after PCR [79].

Beyond the limitations previously mentioned regarding these techniques, it allows verification of the presence of a unique gene in each amplification reaction with a spent reagent and takes more time than multiplex PCR, which enables the determination of the presence of more than one genes in a single amplification reaction [75,86]. Multiplex PCR (mPCR) has been described in recent years and allows the detection of two or more species in the same sample [86]. In experiments where there is a need to identify any different antimicrobial genes in different species of *Salmonella* and *Campylobacter*, or of different genus, the application of the multiplex reaction is a tool or technique that has great potential. However, this technique has some limiting factors, such as the identification or obtaining of primers that have close annealing temperatures [87]. Although, segments of DNA can be amplified in a single reaction. This reaction can detect and differentiate various antimicrobial resistance genes species and strains of bacteria simultaneously.

4.2 Sequencing

In 1977, Maxam and Gilbert discovered the sequencing technique by chemical degradation, which comprises treatment with chemicals that cut the DNA molecule at specific nucleotides. In the same year, Frederick Sanger published two papers that described effective methodologies for determining the DNA sequence of organisms, enabling a complete revolution in biology with the unveiling of the complete sequence of genes and genomes [88]. According to the Sanger method, the base read by fluorescent labeling was observed together with the molecular weight of the compound containing the partial sequence of the DNA through an electrophoretic analysis. Its application is important for the sequencing of PCR products in studies of molecular diagnostics, such as the search for mutations. PCR is necessary before sequencing, so it is necessary to use specific primers depending on the purpose of the study [89]. The Sanger methodology already described can provide resistance identification by investigating the host using specificity determination for a specific gene [70,90,91]. Pyrosequencing is another method that has been widely used. Classified as second-generation sequencing, it consists of a technology involving DNA sequencing using enzymes to accurately

detect specific nucleic acid sequences. This technique is fast and accurate and can be automated. Additionally, it eliminates the need for primers, marked nucleotides, and gel electrophoresis. This method has been used for detect point mutations [92,93].

Next-generation sequencing was developed to overcome the limitations of the Sanger methodology with high speed, less labor, and lowered cost. Platforms were developed, such as the Ion Torrent semiconductor sequencing technologies, sequencing-by-synthesis 454 Life Sciences, Illumina (Solexa) sequencing, and SOLiD sequencing that use different detection principles. In addition to this technology, third-generation sequencing technologies were developed that include Nanopore Sequencing and real-time monitoring of PCR activity through fluorescent resonant energy transfer [93]. These technologies bring simplicity, scalability, increased DNA polymerase performance and yields, lower error rates, and economically feasible results in real time. The sequence in this tool can be applied for the determination of genes and plasmid presence [89].

Some types of sequencing have been reported to address *Salmonella* and *Campylobacter* for determining antibiotic resistance, such as for the identification of species or genus or genome and deleted or inserted genes [91,94]. This technique can be helpful and used to identify antimicrobial resistance genes, such as *tet*(O), aph-3-1, cmeB, and blaOXA-61 for *Salmonella*. Kashoma and others [95]presented highly prevalent $bla_{\text{OXA-61}}$ (52.6% and 28.1%), cmeB (26.3% and 31.3%), *tet*(O) (26.3% and 31.3%), and aph-3-1 (5.3% and 3.0%) in *C. coli* and *C. jejuni*), along with mutations in the *gyr*A gene [10,11]. Sequencing has great potential and interest for *Salmonella* and *Campylobacter* antimicrobial resistance genes.

5. Main Considerations

Isolation of multiresistant strains of *Salmonella* spp. and *Campylobacter* spp. has increased over the years. The main mechanisms of resistance are the presence of genes or mutations in specific segments of certain genes. For resistance to quinolones, the main mechanism in *Campylobacter* consists of the presence of mutations in the QRDR of the gene *gyr*A, as well as in *Salmonella,* but it also has related genes. Resistance to tetracyclines is related to the presence of the *tet* gene, in *Campylobacter tet*O, in *Salmonella tet* variations. Regarding β-lactam resistance, *Salmonella* shows the presence of the *bla* gene in its variations, whereas in *Campylobacter* it is observed mainly $bla_{\text{OXA-61}}$. Therefore, the main molecular techniques used for the detection of these mechanisms are PCR and sequencing.

However, it is of paramount importance to investigate strains isolated from food matrices, especially meat in the face of antimicrobial susceptibility and the presence of resistance mechanisms, in order to understand the behavior to eliminate this mechanism.

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