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Microbiological assessment of chicken meat with focus on non-typhoidal *Salmonella* and *mcr*-mediated colistin resistance in *Enterobacteriaceae*

Sofia Gomes Ribeiro

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Abstract

Currently, poultry-meat industries face diverse challenges related to sustainable production, animal welfare and economic viability while maintaining food safety. The growing consumption of poultry meat demands better control practices of microbiological hazards and food safety risks for the consumer, including pathogenic bacteria and antimicrobial resistance, in a "farm to fork" strategy. Therefore, the general goal of this study was to investigate the occurrence of the foodborne pathogen *Salmonella* and other *Enterobacteriaceae* carrying mobile colistin resistance (MCR)-encoding genes among chicken meat supplied by Portuguese farms after recent voluntary colistin withdrawal. Fifty-three samples (neck skin pool of 10 carcases from the same batch; 29 intensive-based farms) were collected at the final processing level over six months during 2018. Sample (25g+25g) processing included cultural non-selective/selective pre-enrichment followed by selective plating and total DNA extraction for detection of *Salmonella* and *mcr*-carrying *Enterobacteriaceae*. Clinically-relevant *Salmonella* serotypes, their antibiotic resistance and metal tolerance genes were identified by PCR and clonality by MLST. MCR-producing species were identified by MALDI-TOF MS and/or PCR and *mcr-1* to *mcr-5* genes were searched by multiplex-PCR. Clonality was established by FT-IR spectroscopy, PFGE, and MLST plus PCR for capsular typing in *Klebsiella pneumoniae* or phylogenetic groups (PhG) in *E. coli.* Plasmid typing (PBRT/pMLST/I-*Ceu*I/S1-PFGE-hybridization) and WGS (Illumina-HiSeq 2x150bp) were done in representative *mcr*-positive isolates. Susceptibility to antibiotics was studied (disk diffusion; colistin-microdilution) according to international standards (EUCAST/CLSI) in all the isolates as well as to copper, in *Salmonella* (agar dilution). Only two samples (4%) contained *Salmonella* identified as *S*. Enteritidis/ST11 or atypical non-H_2S-producing *S*. 1,4,[5],12:i:-/ST3478. This phenotype was related to a nonsense *phsA* thiosulfate reductase mutation. In addition, ST3478 presented antibiotic resistance (*bla*$_{TEM}$+*strA*-*strB*+*sul2*±*tetB*) and metal tolerance (*pcoD*+*silA*+*arsB1*±*merA*) genes plus phenotypic copper tolerance typical of other emerging *S*. 1,4,[5],12:i:- clones. *mcr-1* was detected in 68% and 62% of samples by molecular and cultural approaches, respectively, but with a declining trend in the last sampling month (12% of batches). Ninety *E. coli* (30 samples/21 farms) and sixteen *K. pneumoniae* (7 samples/6 farms) colistin-resistant isolates (MICcolistin=4->16 mg/L) were recovered. *E. coli* was separated in six FT-IR/PFGE clones, corresponding to widespread ST117/ST297/ST533/ST602/ST6469 and PhG F/B1/G, with *mcr-1.1* chromosomal or plasmid (IncX4/IncHI2-ST2-ST4/IncI2) located. *K. pneumoniae* belonged to the emerging ST147 (2 PFGE profiles), K35 type, and carried *mcr-1.1* in IncHI2-ST2 plasmids. Persistence of 3 *E. coli* and 1 *K. pneumoniae* clones,

originated from the same or different farms, was observed. Co-resistance to several antibiotic classes (100%-amoxicillin, chloramphenicol, nalidixic acid and sulfamethoxazole; 96% to ciprofloxacin and tetracycline) were obtained. WGS in representative isolates revealed genes encoding resistance to diverse antibiotics [*aadA*/*aph*/*aac(3)*, bla_{TEM-1}, *catA*/*cmlA*/*tet(A)*/*sul*/*dfrA* in both species; in *K. pneumoniae* also *qnrB91* and/or*oqxAB* and in *E. coli gyrA*/*parC* mutations], as well as metal tolerance (*cus*/*pco*/*sil*, *ars, mer*, *ter*) in both species. This study shows current consumer exposure to poultry meat associated hazards, emerging *Salmonella* atypical strains and bacterial reservoirs of clinically-relevant antibiotic resistance genes (*mcr*-carrying *Enterobacteriaceae*) adapted to diverse poultry-production stresses. This scenario alerts for the importance of evaluating the impact/efficacy of food safety interventions at farm and processing level (e.g., biocides and metal as alternatives to antibiotics; antibiotics-colistin withdrawal) in retail poultry-meat to guide concerted decisions that ensure adequate food production and protect the consumer health from “farm to fork” under the “One-Health” perspective.

Keywords: poultry chain, chicken meat, *Salmonella*, *mcr-1*-carrying *Enterobacteriaceae*, antibiotics, colistin, metals, copper.

Resumo

Atualmente, as indústrias de carne de aves enfrentam diversos desafios relacionados com a produção sustentável, o bem-estar animal e a viabilidade económica, mantendo sempre o foco na segurança alimentar. O crescente consumo de carne de aves exige melhores medidas de controlo dos perigos microbiológicos e dos riscos de segurança alimentar para o consumidor, incluindo bactérias patogénicas e resistências aos antimicrobianos, numa estratégia "do prado ao prato". Assim, o objetivo geral deste estudo foi investigar a ocorrência do patogénico de origem alimentar *Salmonella* e de outras *Enterobacteriaceae* portadoras de genes mobilizáveis que codificam para a resistência à colistina (MCR), em carne de frango produzida em explorações avícolas portuguesas após uma recente retirada voluntária de colistina. Cinquenta e três amostras (pele do pescoço de 10 carcaças do mesmo lote; 29 explorações avícolas de produção intensiva) foram colhidas no final da etapa de processamento ao longo de seis meses durante o ano de 2018. O processamento das amostras incluiu um pré-enriquecimento não seletivo/seletivo seguido de espalhamento em meios seletivos e extração de DNA total para deteção de *Salmonella* e *Enterobacteriaceae* portadoras de genes *mcr.* Os serótipos clinicamente relevantes de *Salmonella*, os seus genes de resistência a antibióticos e tolerância a metais foram identificados por PCR e a clonalidade por MLST. Espécies produtoras de MCR foram identificadas por MALDI-TOF MS e/ou PCR e os genes *mcr-1* ao *mcr-5* foram pesquisados por PCR *multiplex*. A clonalidade foi estabelecida por espectroscopia de FT-IR, PFGE e MLST, para além de PCR para tipagem capsular para *Klebsiella pneumoniae* ou dos grupos filogenéticos para *E. coli*. A tipagem dos plasmídeos (PBRT/pMLST/I-*Ceu*I/S1-PFGE-hibridação) e a sequenciação genómica total (*WGS*; Illumina-HiSeq 2x150bp) foram realizadas em isolados representativos positivos para genes *mcr*. A suscetibilidade aos antibióticos foi avaliada (difusão em disco, colistina - microdiluição) de acordo com os critérios internacionais (EUCAST/CLSI) em todos os isolados e ao cobre apenas em *Salmonella* (diluição em agar). Apenas duas amostras (4%) continham *Salmonella*, identificada como *S*. Enteritidis/ST11 ou *S*. 1,4,[5],12:i:-/ST3478 não produtora de H_2S (perfil bioquímico atípico). Este fenótipo relaciona-se com a presença de uma mutação "*nonsense*" na redutase do tiossulfato (gene *phsA*). Adicionalmente, o ST3478 apresentava genes de resistência a antibióticos (*bla*$_{TEM}$+*strA-strB*+*sul2*±*tetB*) e de tolerância a metais (*pcoD*+*silA*+*arsB1*±*merA*), para além da tolerância fenotípica ao cobre, típica de outros clones emergentes de *S*. 1,4,[5],12:i:-. O gene *mcr-1* foi detetado em 68% e 62% das amostras utilizando a abordagem molecular e cultural, respetivamente, mas com uma tendência de diminuição no último mês de amostragem (12% dos lotes). Foram isolados

noventa *E. coli* (30 amostras/21 explorações) e dezasseis *K. pneumoniae* (7 amostras/6 explorações) resistentes à colistina (MICcolistina=4->16 mg/L). Os isolados de *E. coli* foram divididos em seis clones por FT-IR/PFGE, correspondendo aos ST117/ST297/ST533/ST602/ST6469 amplamente disseminados e aos grupos filogenéticos F/B1/G, contendo o gene *mcr-1.1* localizado no cromossoma ou em plasmídeos (IncX4/IncHI2-ST2-ST4/IncI2). Os isolados de *K. pneumoniae* pertenciam ao clone emergente ST147 (2 perfis de PFGE), correspondente ao tipo capsular K35, sendo portadores do gene *mcr-1.1* em plasmídeos IncHI2-ST2. Foi observada a persistência de 3 clones de *E. coli* e 1 de *K. pneumoniae*, provenientes da mesma ou de diferentes explorações. Verificou-se co-resistência a várias classes de antibióticos (100%-amoxicilina, cloranfenicol, ácido nalidíxico e sulfametoxazol; 96%-ciprofloxacina e tetraciclina). A análise da WGS em isolados representativos revelou a presença de genes que conferem resistência a diversos antibióticos [*aadA*/*aph*/*aac(3)*, bla_{TEM-1}, *catA*/*cmlA*/*tet(A)*/*sul*/*dfrA* nas duas espécies; em *K. pneumoniae* também *qnrB91* e/ou *oqxAB* e em *E. coli* mutações *gyrA*/*parC*] assim como tolerância a metais (*cus*/*pco*/*sil*, *ars, mer*, *ter*) em ambas as espécies. Este estudo mostra a atual exposição do consumidor a diversos riscos associados à carne de aves, presença de estirpes atípicas e emergentes de *Salmonella* e de reservatórios bacterianos de genes de resistência a antibióticos clinicamente relevantes (*Enterobacteriaceae* portadores de *mcr*), adaptados a diversos stresses existentes na produção de aves. Este cenário alerta para a importância de avaliar o impacto e a eficácia das intervenções de segurança alimentar ao nível das explorações e processamento (por exemplo, biocidas e metais como alternativas aos antibióticos; retirada de antibióticos/colistina) na carne de aves, de modo a orientar decisões concertadas que garantam uma produção alimentar adequada e protejam a saúde do consumidor desde o "prado ao prato" na perspetiva de "Uma só saúde".

Palavras-chave: produção de aves, carne de frango, *Salmonella, Enterobacteriaceae* portadoras de *mcr-1*, antibióticos, colistina, metais, cobre.

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Abbreviations

AMR – Antimicrobial Resistance
ATR – Attenuated Total Reflectance
BPW – Buffered Peptone Water
CDC – Centers for Disease Control and Prevention
CGE – Centre for Genomic Epidemiology
CLSI – Clinical and Laboratory Standards Institute
COL – Colistin
DNA – Deoxyribonucleic Acid
EC – European Commission
EFSA - European Food Safety Authority
EMA – European Medicines Agency
EU – European Union
EUCAST – European Committee of Antimicrobial Susceptibility Testing
FT-IR – Fourier Transform Infrared
HPCIA – Highest Priority Critically Important Antimicrobials
ISO – International Organization for Standardization
MDR – Multidrug Resistant
MIC - Minimum Inhibitory Concentration
MKTTn – Mueller-Kauffmann Tetrathionate-novobiocin
MLST – Multi-locus Sequence Typing
MS – Mass Spectrometry
NTS – Non-typhoidal *Salmonella*
PCR – Polymerase Chain Reaction
PFGE – Pulsed-Field Gel Electrophoresis
PhG – Phylogenetic Groups
pMLST – Plasmid Multi-locus Sequence Typing
RNA - Ribonucleic Acid
rRNA - Ribosomal Ribonucleic Acid
RVS – Rappaport-Vassiliadis Medium with Soya
SISTR – *Salmonella* In Silico Typing Resource
US – United States
WGS – Whole Genome Sequencing
WHO – World Health Organization
XDR – Extensively Drug Resistant
XLD – Xylose-Lysine Deoxycholate

1. INTRODUCTION

1.1. Poultry meat production chain

Poultry meat production, particularly of chickens/broilers, has been increasing steadily for many years in Europe and around the world, since poultry meat presents many advantages for consumers and poultry industry (e.g. cheap meat protein, absence of religious restrictions limiting consumption, association with healthy lifestyle, lower production costs due to short rearing time and lower required investments) compared with other types of meat (1). Europe produced around 15 million tonnes of poultry meat in 2018 (2), representing a cumulative increase of almost 3.3 million tonnes in 10 years, since 2008 (1). Broiler production (chickens raised for meat) is by far the largest sub-sector of the poultry meat production chain (representing about 81% of poultry production in 2018) followed by turkey (13% in 2018) and, to a lesser extent, duck (3% in 2018) (2). In fact, chicken is the second most consumed meat in Europe, with a consumption of 24,1 kg per capita in 2018 (compared with 32,5 kg for pig meat and 11,0 kg for beef) (1, 2). Moreover, according to the National Statistics Institute (INE), the consumption of poultry meat in Portugal, in 2018, was 43,0 kg per capita (3).

With the increase in consumption, poultry industry faces many challenges aiming to ensure animal welfare and be environmental-friendly, sustainable and safety. In Europe, the intensive poultry system represents around 90% of broiler production. In this system, poultry is intensively produced in very large indoor environments (poultry houses) under high stocking densities (maximum 33 kg/m^2) and at fast growth rates (chickens must be fed continuously), reaching market weight in five to six weeks (1). In poultry houses, lighting must be controlled, ventilation must avoid overheating and, when necessary, there must be heating systems designed to remove excess moisture (4). Despite these controlled conditions, the high stocking densities increase the risk of transmission of infectious diseases agents, including human zoonotic pathogens (1). To reduce chances of bacterial contamination and transmission, poultry farms should be located far from other farms or livestock companies and water treatment plants. The buildings should also be designed in order to reduce the contact between animals and contaminated matter like faeces and wastewater. Additionally, since the farm workers and the visitors present a major biosecurity risk it is important to have barriers for human access like footwear changing facilities, foot dips, washing facilities and locker rooms to change into protective clothing, for example (5, 6).

For maximum growth and good animal health, intensively reared poultry need a balanced array of nutrients in their diet. In addition to energy and protein, formulations contain supplements to provide minerals (e.g. calcium, sodium, iron, zinc and copper), vitamins and specific amino acids (7). Feed formulations can also contain a wide range of non-nutritive additives, which may not be essential, but have an important impact on poultry performance and health (7). In fact, feed additives, as defined in Regulation (EC) No 1831/2003 (8), are products intentionally used in animal nutrition (added to feed or water) to improve the quality of feed, the animals' welfare (e.g. providing enhanced digestibility of the feed materials) and performance (5).

At the end of the growing period and before the transport stage, broilers are subjected to fasting (a pre-slaughter feed withdrawal period) in order to empty the gut and reduce the risk of carcass contamination (9). Also, broilers fitness is assessed prior to loading it. Broilers with broken bones (wings, legs) and/or with severe difficulties to move are considered unfit animals for transport (10, 11). Good handling practices will reduce the incidence of undesirable consequences (e.g. injuries, broken bones and even death) and also benefit other aspects of animal production, such as the quality of the final meat product (10). During the transport stage, there are also some stress factors that can negatively affect animal welfare, so trucks and cages (floor area and height considering the poultry size) must be specially designed for the safe transport of poultry (10, 11). Good transport is important for poultry comfort and welfare, so the Regulation (EC) No. 1255/97 of 2004 (11) aimed for the protection of animals during transport and related operations (such as loading, unloading, transferring and resting, until the unloading of animals at the destination is completed).

Poultry can experience stress and pain when they are caught, put into cages and transported from the farm to the slaughterhouse (**Figure 1**) (1). The poultry slaughter begins with hanging the live birds on the overhead shackles followed by stunning and killing. After bleeding, the birds are scalded in hot water (50 to 60ºC) which promotes defeathering. Feathers are mechanically removed using rubber fingers and then the carcasses are eviscerated. Evisceration involves the opening of the body cavity and removal of internal organs, which can be mechanically performed. Following this, the carcasses are washed and inspected by a veterinary. In the end of the processing, the carcasses are chilled (4ºC) by immersion in cold water or air chilling and stored at refrigeration temperature before packaging and retail distribution (12, 13).

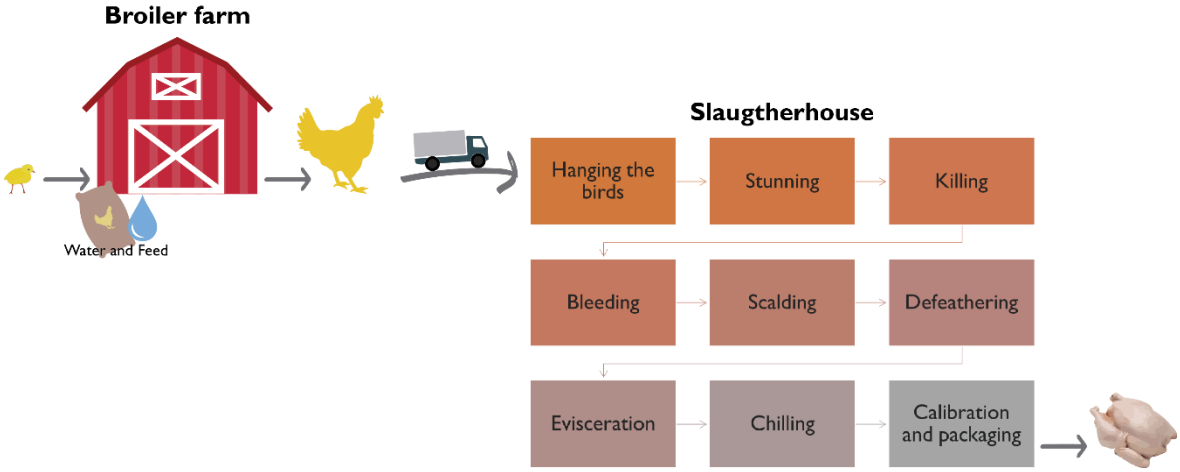


Figure 1 - Main steps of poultry production chain - from day-old chickens to poultry carcass ready for retail distribution. Adapted from (12)

1.1.1. Bacterial contamination in the poultry meat production chain

Poultry meat bacterial contamination is potentially associated with all the practices and events that occurred from farm and processing level to the consumption (from farm-to-fork). Thus, a critical issue for the animal host welfare is the broiler gut microbiota, known to be predominantly dominated by bacteria (14). The intestinal tract of poultry can be asymptomatically colonized as a result of horizontal (contamination from environmental sources - contaminated feed, water, bedding, soil, air; vectors - farm staff, insects, and rodents; and equipment) or vertical (transferred directly from colonized breeder flocks to their offspring) transmission at primary production stage (farms) (15). Some of the bacteria found in broiler gut microbiota (generally non-pathogenic to chickens) includes human zoonotic foodborne pathogens, such as *Salmonella*, intestinal pathogenic or diarrheic *Escherichia coli* and *Campylobacter* (14). The infections caused by these pathogenic bacteria are currently an important risk to public health, making poultry and derived products one of the most frequent reservoirs and transmission routes of foodborne diseases (16).

There are a number of stages in the poultry meat production chain, from farm (growing) and processing (slaughtering and transport) to retail that can contribute for the spread of zoonotic pathogens present in the poultry microbiota (1). The bacteria that inhabit the poultry's intestines are excreted in the faeces and are also detected in the poultry environment, such as in the litter (16, 17). The intensive production of broilers produces huge amounts of waste that are still widely used as a fertiliser in agriculture (1). Thus, poor

litter management can cause environmental contamination and risks for human health, such as the spread of pathogens in soil and water resources that could end in the food chain (1). In addition, during transport, birds can produce faeces in the cages/containers which, if not properly cleaned and disinfected after each transport, can help to spread pathogens among birds of different flocks (10). Although production of faeces during transport can be avoided with a pre-period of fasting, which should not exceed 24 hours (9, 10). During slaughter, evisceration is the critical step in which, even using sophisticated machines, the gut can be ruptured (9) generating cross-contamination events (for whole carcasses and also for equipment/surfaces) due to bacteria from gastrointestinal tract of birds (12, 13). Therefore, emptying the gut before slaughter is an important preventive measure. Nevertheless, cross-contamination of carcasses can occur from equipment surfaces (e.g. rubber fingers for defeathering), water baths, food handlers and air (12). Water baths are used with the aim of washing carcasses, diminishing the bacterial loads, but they can also promote cross-contamination between carcasses (12). So, the bacteria from poultry microbiota or slaughter environment can contaminate the carcasses and their derived meat products, thus reaching the consumer through the handling or consumption of contaminated food products (12, 13).

1.1.2. Antimicrobial resistance and poultry meat production chain

Antimicrobials are necessary for treating human and animal (livestock and pets) infectious diseases. Any overuse of antimicrobials, either in human or veterinary medicine, might result in the development of antimicrobial resistance. Combating antimicrobial resistance (AMR) is a global health priority of the 21st century (World Health Organization-WHO considered AMR one of the top ten global health threats) (https://www.who.int/news-room/spotlight/ten-threats-to-global-health-in-2019). WHO is working with human, animal and environmental sectors to implement a global action plan to tackle AMR by increasing awareness and knowledge, reducing infection, and encouraging prudent use of antimicrobials. Also, the European Commission (EC), have been implementing diverse integrated measures to combat AMR based on a holistic approach, in line with the 'One Health' approach (5).

Antimicrobial agents have been used for many years in animal husbandry mostly for treating infections in sick animals and also for other animal production purposes. Their use as feed additives for growth promotion was banned in the European Union (EU) since 1 January 2006 (18) and in the US in 2017 (19) (Editors, 2017, 9). However, intensive

production systems are still highly dependent of antimicrobials use to improve animal health, welfare and productivity (20). Although the use of antibiotics as growth promoters in animal feed was forbidden in Europe (18), in the subsequent years there was no significative decrease in the use of antimicrobials in food-producing animals (20). In fact, there was an increase in their use (20) for prevention of infections in a single animal (prophylaxis) when the risk of disease is very high and the consequences are likely to be severe or also for controlling the spread of infection in animals belonging to the same flock (metaphylaxis - administration to a group of animals) (21, 22).

In intensive production systems, due to the high stocking densities, there is a higher probability of dissemination of infectious bacteria through the entire flock, leading to huge economic losses (23). Thus, the treatment and prevention of diseases play a very important role in productivity. In poultry production, antibiotics are generally administered to the entire flock (by applying in drinking water or in feed) and are used for prevention of infections such as necrotic enteritis by *Clostridium perfringens*, and/or treatment of particular infections caused by avian pathogenic *Escherichia coli* (colibacillosis) and other diseases generally caused by *Salmonella* or *Clostridium* spp. (23, 24).

In Europe, the sales of tetracyclines (30,4%), penicillins (26,9%) and sulfonamides (9,2%) for veterinary use in food-producing animals, accounted for 66,55% of the total sales in 2017 (25). Also, sales of pharmaceutical forms suitable for group treatment accounted for 89,4% of the total sales: premixes (to apply in feed) accounted for 28,8%; oral powders (to apply in feed) for 9,9%; and oral solutions (to apply in water) for 50,7% (25). Regarding to Portugal, the most sold class of antibiotics for veterinary use in food-producing animals were tetracyclines (33,3%), penicillins (26,1%) and macrolides (13,2%), accounting for 72,6% of the total sales in 2017 (26). The top 5 of the most sold antibiotics are oxytetracycline (26,8%), amoxicillin (19,8%), tylosin (12,3%), colistin (8,1%) and doxycycline (6,11%). In poultry, amoxicillin (14-30 mg/Kg of body weight/day) can be used for the treatment of diverse infections caused by Gram positive and Gram negative bacteria, while tylosin (10-20 mg/Kg of body weight/day) and doxycycline (10-20 mg/Kg of body weight/day) for the treatment of necrotic enteritis caused by *Clostridium perfringens*, and colistin (75 000 UI/Kg of body weight/day) has been extensively used for the treatment of gastrointestinal infections caused by *E. coli* (26, 27).

Generally, any use of antibiotics (in human or veterinary medicine) allows the elimination of susceptible bacteria, contributing for the selection of those presenting antibiotic resistance mechanisms (28). The risk increases if these antimicrobial agents are used improperly, as in the case of mass medication or use in non-susceptible microorganisms, in sub therapeutic doses, repeatedly or for inappropriate periods (29). Therefore, the excessive and imprudent use of antibiotics in poultry production promotes

resistance by selective pressure, facilitating the spread and persistence of resistant bacteria from animals to the food chain (23, 27). The horizontal acquisition of genetic units (e.g. plasmids) carrying genes encoding for antibiotic resistance (some also carrying genes encoding metal/biocide tolerance or virulence) is one of the main mechanisms of dissemination of mobilized antimicrobial resistance genes among birds and their production environment (30) besides vertical spread due to clonal expansion. In fact, there is a correlation between the use of antibiotics in animals and the development of resistant bacteria (31, 32), being poultry recognised as an important reservoir and/or transmission route of antibiotic resistant bacteria, including clinically-relevant multidrug resistant (MDR) *Salmonella* strains/clones and other *Enterobacteriaceae* (28, 33). Resistant bacteria selected in animals/farms can spread through their meat products and can be transmitted to humans by ingestion or handling of contaminated meat (23, 28, 34). Those bacteria transmitted by foods include pathogenic zoonotic organisms (e.g. *Salmonella*) and commensal bacteria (e.g., *E. coli* and other *Enterobacteriaceae*), which could colonize humans and be reservoirs of clinically relevant antibiotic resistance genes mobilizable to pathogens (35). Then, the infections in humans caused by pathogenic or opportunistics MDR bacteria can be more severe and difficult to treat, resulting sometimes in treatment failures and additional costs for public health (23, 28, 36).

Antibiotics used in intensive poultry production also have an impact on the environment. It is estimated that about 75% of the antibiotics administered to animals are excreted in faeces or urine (37), as well as the resistant bacteria (commensal and zoonotic pathogens), contaminating the environment. The slow rate of degradation of some antibiotics and the use of poultry manure as fertilizer in agriculture can also lead to the accumulation of antimicrobials in diverse environments/hosts, including in food-producing animals and consequently in the food chain production (37). The diversity of interactions between poultry production and agriculture or poultry slaughter contributes to the spread of antibiotic resistance genes and the selection of antibiotic resistant bacteria elsewhere in the food chain (38) (**Figure 2**). The consequent spread of resistance to the community and to hospital environments makes antibiotic resistance a phenomenon that flows between humans, animals and the environment (20), thus AMR should be considered a poultry-associated hazard with impact in the public and animal health.

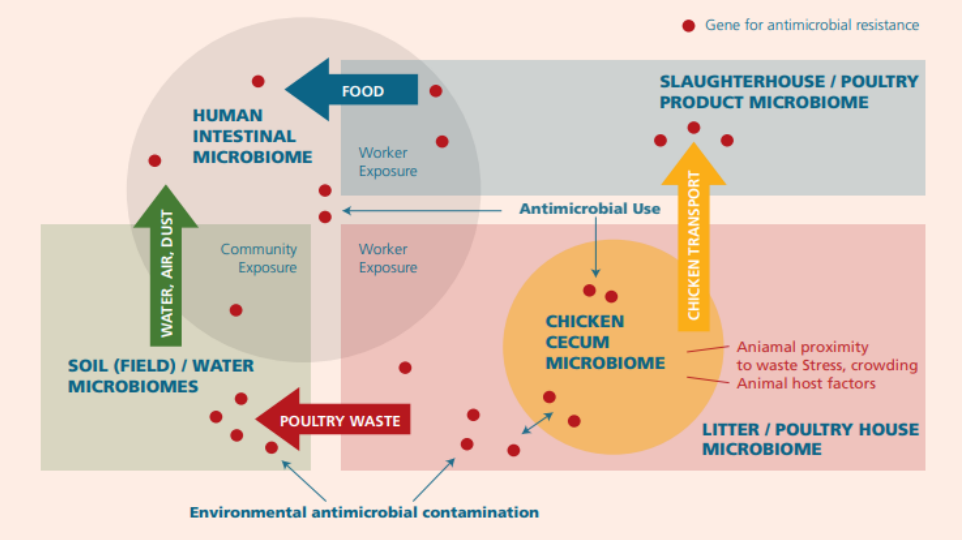


Figure 2 - Conceptual role of selective pressure of antimicrobial use in poultry production system and potential dissemination pathways of antimicrobial resistance genes. Adapted from (38).

Because of AMR concerns for public health there is an increasing focus on measures to reduce antimicrobial usage in animal husbandry by promoting prudent use initiatives, as well as exploring alternatives to their use in animal production systems (5). In the context of antibiotic reducing and replacing, non-antibiotic compounds with antimicrobial activity, such as metals and organic acids, are currently used in animal farming management (5, 39). Several metals can be incorporated into biocidal products, such as disinfectants, or in additives in animal feeds for growth promotion (e.g. Cu - Copper, Zn - zinc) (40-42). Consequently, metals can remain in the production environment (e.g. manure, waste lagoons, amended soils) and accumulate in toxic concentrations, representing a long-term selective pressure potentially contributing to the co-selection of metal-tolerant and antibiotic-resistant bacteria, such as *Salmonella* (5, 41). In fact, metal tolerance (e.g. to copper – *sil* operon) and antibiotic resistance genes [e.g. to tetracyclines - *tet(A)* and *tet(B)*; ampicillin - bla_{TEM} and sulphonamides - *sul1* and *sul3* class 1 integron related or *sul2*] were frequently co-located in the same genetic platforms (plasmids or chromosome) in *Salmonella* strains/clones (40, 42) as well as in other bacteria of the food-producing animal setting (43). Thus, the potential impact of metals usage in food-producing animals for the selection, dissemination and persistence of metal tolerant and antibiotic resistant (particularly to critically important antibiotics) bacteria should be more explored as antibiotic alternatives.

1.2. Major poultry-associated hazards

1.2.1. Non-typhoidal *Salmonella*

Salmonella infections are a global public health problem, being salmonellosis caused by non-typhoidal *Salmonella enterica* (NTS) serotypes (serotypes other than *S*. Typhi and *S*. Paratyphi). In industrialized countries, the main reservoir of NTS is the intestinal tract of food-producing animals, which readily lead to contamination of diverse foodstuffs (33). Poultry are among the animal populations most frequently colonized with *Salmonella* belonging to diverse serotypes (44). Diverse serotypes belonging to *S*. *enterica* subsp. *enterica* are responsible for most human infections worldwide, being classified according to their host specificity and the disease that they cause in their hosts (**Figure 3**). Some host-specific serotypes, such as *S*. Pullorum and *S*. Gallinarum, can cause systemic disease in broilers, being an animal health problem. However, poultry can be colonized with diverse human pathogenic *Salmonella* serotypes (e.g. *S*. Enteritidis, *S*. Typhimurium, *S*. 1,4,[5],12:i:- and *S*. Infantis) without manifestation of detectable symptoms (44). For some of those serotypes (e.g. *S*. Enteritidis and *S*. Infantis), the intestinal tract of poultry is the most significant reservoir (33). The presence of human pathogenic *Salmonella* serotypes in healthy animals poses a threat to food safety by allowing bacteria to be transmitted from food to humans (33). Therefore, contaminated foods of animal origin, including eggs and poultry meat have been considered the main vehicles of NTS infections in humans (33, 45). In the EU, salmonellosis remains the second most common zoonosis (91 857 confirmed cases and 20,1 cases per 100 000 population in 2018) and the first cause of foodborne outbreaks (1580 in 2018). In 2018, there were 151 strong-evidence foodborne outbreaks (51% of total strong-evidence foodborne outbreaks) caused by consumption of poultry products (eggs, meat and products thereof) (46), being *S*. Enteritidis responsible for causing 95 of the 151 strong-evidence foodborne outbreaks (46). Moreover, a huge percentage of *Salmonella* isolates recovered from poultry carcasses (broilers and turkeys) in 2018 showed resistance to at least one antibiotic. In fact, a high level (32,7%) of MDR among *Salmonella* isolates recovered from carcases of broilers was detected and a moderate level (15,1%) in *Salmonella* isolates recovered from turkey carcases (47).

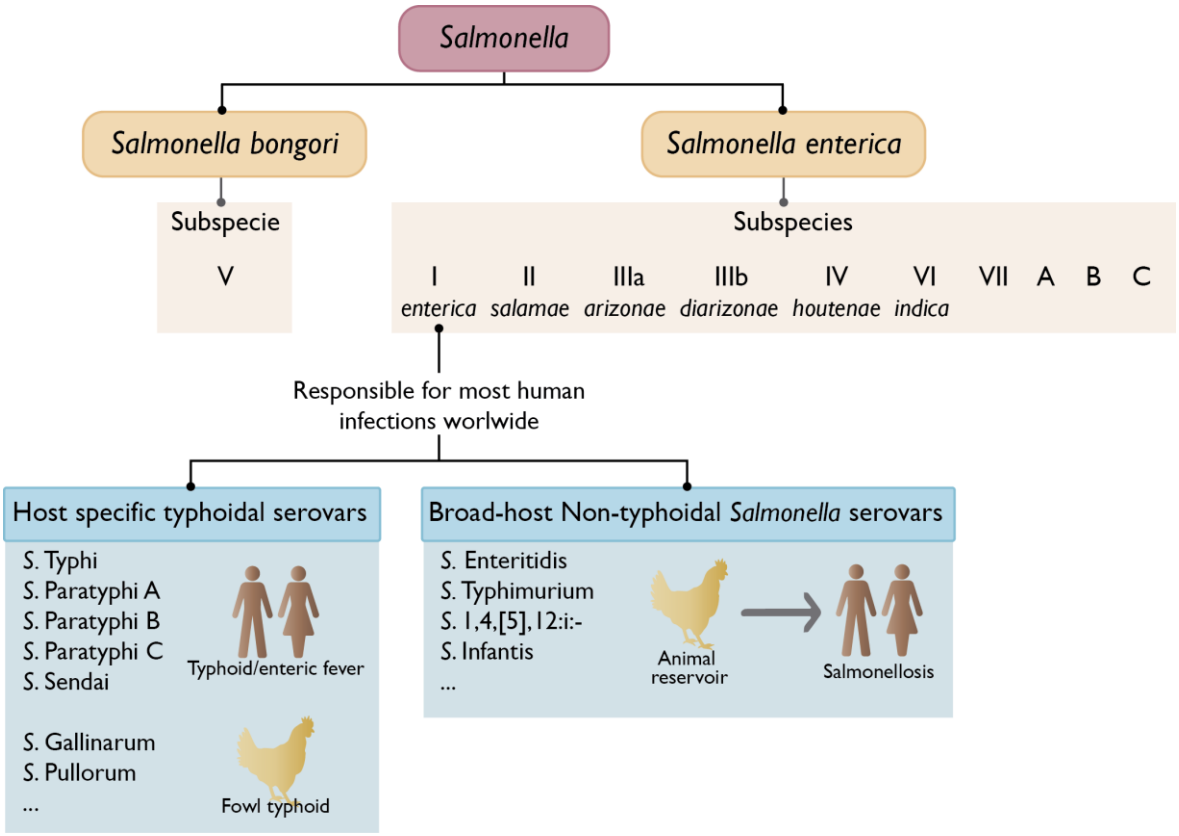


Figure 3 - Classification of *Salmonella* by classic methods and according to their host specificity and the disease that they cause in their hosts. Adapted from (44, 48).

NTS is a worldwide major cause of foodborne salmonellosis with a high global impact in human health (33, 44, 45, 49). In healthy patients, salmonellosis is usually a self-limited disease (gastroenteritis) without the need for medical intervention (44). However, in susceptible patients (infants, young children, older people and immunocompromised patients), life-threatening invasive infections with bacteraemia (5 – 10% of infected people) and/or other extra-intestinal infections may occur (33, 49). Thus, in severe cases, effective antibiotic therapy (fluoroquinolones or 3rd generation cephalosporins) is essential (44), being the emergence of *Salmonella* resistant to diverse antibiotics a worldwide public health concern (33).

In the EU, to reduce the prevalence of *Salmonella* and their risk to public health, control measures (involving surveillance, biosecurity and vaccination) were implemented since 2006 with focus in poultry/egg production chain (50) and on particular serotypes (for broiler flocks: *S*. Enteritidis, *S*. Typhimurium, *S*. 1,4,[5],12:i:-, *S*. Infantis, *S*. Hadar and *S*. Virchow) that were considered of public health significance (51). Tracking of *Salmonella* along the food chain is performed during primary production stage (in farm animals and their feed),

processing (at slaughterhouses) and shelf-life of products placed on the market (46). In the EU, the regulatory limits (microbiological criteria) (52) indicate that *Salmonella* spp. should be “not detected in 25 g or 10 g” for different products when they are on the market, during their shelf life. For fresh poultry meat (53), only the target serotypes (*S*. Enteritidis, *S*. Typhimurium and *S*. 1,4,[5],12:i:-) cannot be detected in 25 g. Although, a recent scientific opinion from the European Food Safety Authority (EFSA) proposes the enlargement of target serotypes for broiler flocks in order to be more effective in reducing the risk of introduction of newly emerging or re-emerging strains with epidemic potential (54).

The success of *Salmonella* control programmes at poultry level led to an initial long-term decrease in human salmonellosis (particularly associated with *S*. Enteritidis). However, according to the last EFSA annual zoonosis report, a stabilization trend was observed during the years 2014–2018 in the overall incidence of salmonellosis considering all reported cases of the participating countries (46). Nevertheless, expansion of newly emerging strains with epidemic potential or with atypical biochemical features (e.g., hydrogen sulfide-H_2S negative), and frequently MDR strains, have been reported in poultry production over diverse geographical regions (33, 46, 47). Therefore, it is essential to monitor poultry meat contamination rates by NTS, currently performed through ISO standard cultural methods, as well as to characterize the adaptive features contributing to their survival in poultry production.

1.2.2. Colistin-resistant *Enterobacteriaceae* carrying *mcr* genes

The emergence and spread of MDR *Enterobacteriaceae* to last-resort antibiotics, like colistin, is a global multi-factorial phenomenon transversal to animal, food, environmental and human sectors that should be contained. Colistin (also known as polymyxin E) belongs to the class of polymyxins, a family of cationic antimicrobial peptides (CAMPs), which has activity against most Gram-negative bacteria (27, 55). In the last decades the emergence of MDR and extensively drug-resistant (XDR) Gram-negative bacteria (specially *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*), as well as the lack of novel antimicrobial agents against these pathogens have led to colistin reintroduction as a valuable therapeutic option (56). Therefore, colistin is now considered as a last-resort antibiotic used for the treatment of severe infections caused by MDR Gram-negative bacteria in humans (27, 56). To preserve colistin, WHO (57) updated the polymyxins-colistin category to the highest priority critically important antimicrobials (HPCIA) and the European Medicines Agency

(EMA) (58) reclassified them to the higher risk category, both due to the risk for public health resulting from non-human use.

Colistin has been extensively used in veterinary medicine, including in poultry production, for prevention (prophylaxis and metaphylaxis) or treatment of bacterial infections, since 1950 (56). In Europe, between 2011-2017, the sales of polymyxins for use in food-producing animals decreased by 66,4%, being, in 2017, the seventh most sold class of antimicrobials (3,6%) (25). Most of polymyxins consumption is through oral route, being used different pharmaceutical formulations (oral solutions, powder or premix) (25). Regarding Portugal, data from 2017 revealed that colistin was the fourth most sold active substance (accounting for 8,1% of overall sales) for use in food-producing animals (26). In fact, the widespread use of colistin in animal farming can lead to the emergence and dissemination of colistin resistance, with potential transmission from animals/foods to humans (31, 32, 59).

The concern related to the animal-human transmission of colistin-resistant bacteria/genes increased with the discovery and global dissemination of the horizontally transferable colistin resistance genes, the *mcr* (mobilized colistin resistance) family. These genes (until now from *mcr-1* to *mcr-10*) and respective variants encoded for phosphoethanolamine transferases, which add a phosphoethanolamine group to the lipid A of the lipopolysaccharide, reducing the negative charge of the Gram-negative outer membrane and attenuating its affinity for colistin (31, 55, 60, 61). Global epidemiological data strongly links *mcr* expansion to food-producing animals, particularly pigs, which together with extensive colistin use in veterinary medicine sustain the food chain as a potential origin and transmission route of *mcr* genes (31, 62).

Since *mcr* genes, encoding colistin resistance, are dispersed at global level (61, 63) (**Figure 4**) within animals, foods, humans and their environments, the issue of colistin resistance needs to be addressed holistically by adopting the One Health concept. The One Health approach focuses on the role of interconnected ecosystems, where the health of any of them may affect the health of the others, including the human health (64). The global One Health aim is to implement integrated measures to combat antimicrobial resistance in a geographically close location (64). In Europe, measures (Directive 2003/99/EC and Decision 2013/652/EU) have been taken with the aim to ensure that zoonotic bacteria isolated from food-producing animals (e.g. broilers and fattening turkeys) or derived food products (e.g. meat) and related antimicrobial resistance are properly monitored (65, 66). Moreover, the veterinary use of colistin is being re-evaluated, since colistin was considered by WHO a higher priority antibiotic among those critically important for human medicine (57). More recently, EMA proposed a new categorization of antimicrobials (58), including polymyxins-colistin in the Category B-“Restrict”, antibiotics that should be restricted in

animals to mitigate the risk to public health. In Portugal, the National Plan for the Reduction of Antibiotics Usage in Animals was implemented in 2014 (67) followed by a new Strategic National Plan established for 2019-2023 under the 'One Health' approach (68). Meanwhile, new national initiatives have already been taken, namely voluntary programmes in animal-farming industries for the reduction of antimicrobials use, including colistin in chicken production. However, effects of these changes are still largely unknown, particularly at the poultry production chain. Therefore, it is essential to monitor poultry meat (just before consumer distribution) contamination rates by *mcr*-carrying *Enterobacteriaceae* after colistin withdraw at chicken farm level, as well as to characterize their drivers (bacterial species and plasmid genetic background).

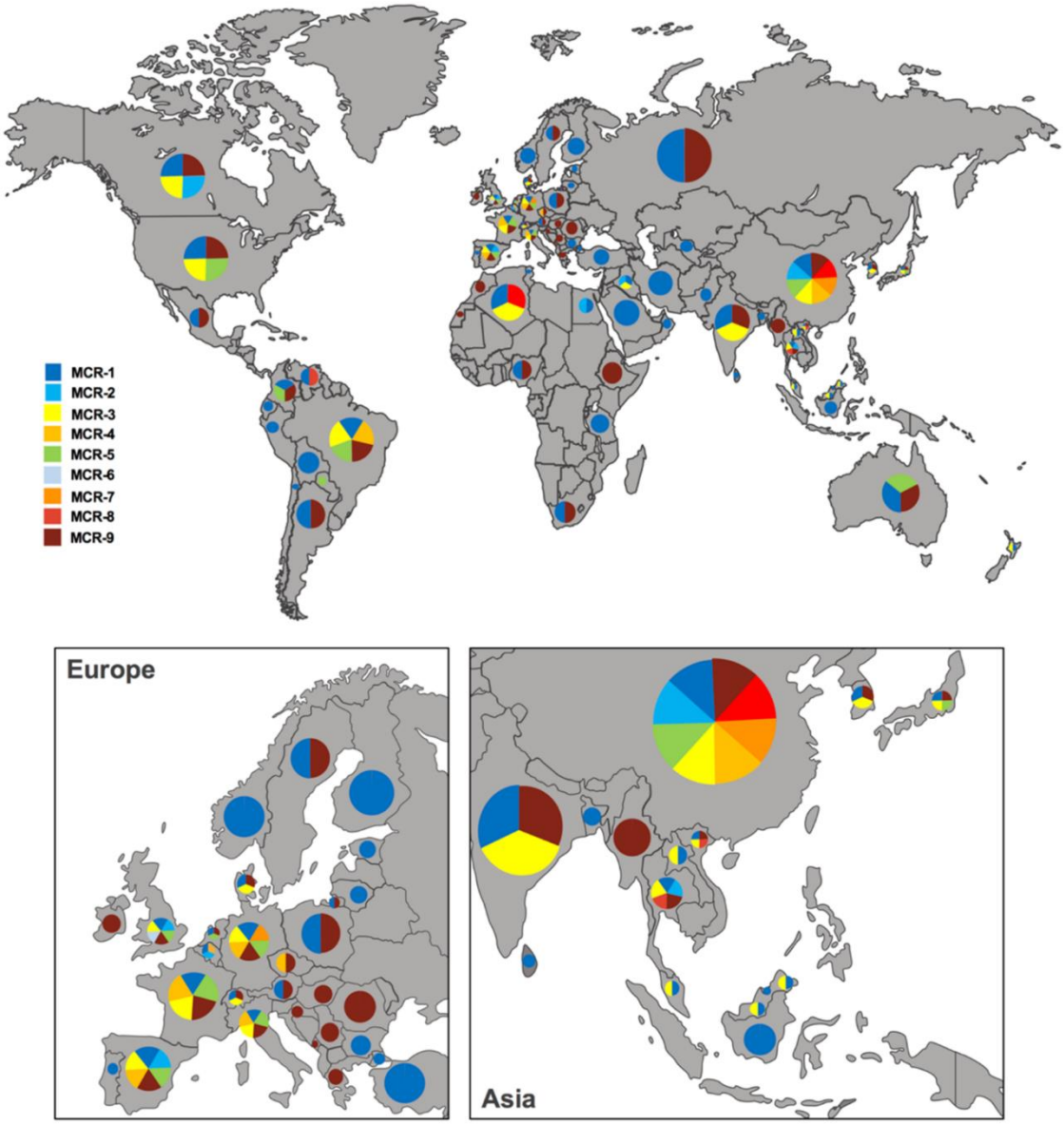


Figure 4 - Dispersion of *mcr* genes (*mcr-1* to *mcr-9*) across different countries. Adapted from (61).

2. OBJECTIVES

Currently, poultry-meat production faces diverse challenges related to the improvement of sustainable production growth, warrant animal welfare, economic viability, and minimize the environmental impact while maintaining food safety. Besides, poultry meat is consumed on a large scale worldwide, thus demanding greater control of biological hazards and food safety risks for the consumer. Microbiological food safety is an important concern in poultry meat production, being *Salmonella* still one of the leading causes of zoonotic foodborne diseases worldwide and currently targeted by EU actions, including a safety criterion in poultry meat. Moreover, the spread of multidrug-resistant bacteria, including to last-resort antibiotics like colistin, is also a global and multi-factorial phenomenon transversal to animal-food-environmental-human sectors. Colistin has been extensively used as feed-additive (e.g. prophylaxis, metaphylaxis and growth-promotion in some countries) in diverse poultry intensive farms, however, more recently some animal farming industries voluntary banned their use at the farm level. However, the efficacy/optimization of those control practices on the reduction of foodborne pathogenic bacteria (*Salmonella*) and colistin-resistant *Enterobacteriaceae*, in order to obtain poultry meat with better microbiological quality and safety, remain scarcely explored.

Thus, the global objective of this study was to investigate the occurrence of the foodborne pathogen *Salmonella* and other *Enterobacteriaceae* carrying mobile colistin resistance (MCR)-encoding genes among chicken meat supplied by Portuguese farms after the recent voluntary colistin withdraw. Specific objectives included:

› To investigate the presence of clinically-relevant *Salmonella* serotypes by a cultural and molecular approach as well as to characterize their ability to grow under antibiotics and metals stress factors;

› To investigate the occurrence of *mcr*-carrying *Enterobacteriaceae* by a cultural and molecular approach over six-months as well as to characterize bacterial and plasmid genetic background.

The assessment of consumer exposure by chicken meat to clinically relevant strains/clones adapted to poultry production-related stresses and/or carrying mobile genetic elements associated with *mcr* expansion can help to establish effective strategies to limit human health risks associated with the food-chain.

3. MATERIAL AND METHODS

3.1. Sampling strategy in the slaughterhouse and processing plant

Raw chicken meat samples (n=53) recovered after slaughter and air chilling were collected over six months during 2018 (including Spring and Summer seasons) in a Portuguese poultry-production slaughterhouse, immediately before distribution for retail sale. Each sample consisted of a pool of neck skin from 10 carcasses of the same batch (each batch corresponded to one flock from the same house and farm). All samples were collected in sterile plastic bags, transported at 4ºC and processed on the same day at the laboratory. Subsequent sample processing was performed by cultural and molecular approaches as described in the next sections.

The broilers were from 29 intensive-based farms located in rural areas of Portugal, with a similar conventional indoor and floor-raised production system (broiler flocks ranged from 2500 to 8000 per house with age at slaughter from 28 to 42 days). Concerning practices at farm level, since January 2018 colistin was voluntary banned for any purpose in all the chicken farms that supplied the slaughterhouse where sampling was carried out. Moreover, copper and organic acids (unknown composition) were routinely used as additives in the poultry feed. At slaughter, the birds were hung upside down, stunned and killed. After bleeding, the birds were scalded in hot water (50 to 60ºC) and mechanically defeathered using a rubber finger system. The opening of the body cavity and the removal of internal organs (evisceration) was also performed mechanically. At the end of processing, the carcasses were chilled (4ºC) by air chilling and stored at refrigeration temperature before retail distribution. Concerning biosecurity measures, peracetic acid with hydrogen peroxide (between 0,5–3%) was used at the processing plant as a biocide for disinfection.

3.2. Detection and characterization of *Salmonella*

3.2.1. Detection of *Salmonella* by a cultural and molecular approach

Detection of *Salmonella* was performed based on the standard cultural method ISO 6579-1:2017 (69), including a pre-enrichment step of 25 g of sample into 225 mL of Buffered Peptone Water (BPW; 18h/34-38ºC) (Liofilchem, Italy), followed by a selective enrichment in Rappaport-Vassiliadis medium with soya (RVS; 24h/41,5ºC±1) and Mueller-Kauffmann

tetrathionate-novobiocin (MKTTn; 24h/34-38ºC) broths (Biogerm, Portugal). Then, both selective broths were streak-plated on Xylose-Lysine Deoxycholate (XLD) agar (Liofilchem, Italy) and CHROMagar™ *Salmonella* Plus (Biogerm, Portugal). Presumptive *Salmonella* colonies recovered from both selective media (up to five colonies per plate) were confirmed by biochemical tests (e.g. API-20 E - bioMérieux, France) and agglutination assays with *Salmonella* O poly antisera and serogroup-specific antisera (BD Difco™, USA) (**Figure 5**). Additionally, *Salmonella* isolates were confirmed by a molecular approach using a standard PCR for the amplification of *invA* gene (70) (**Figure 5, Table AI** – see in Annex I).

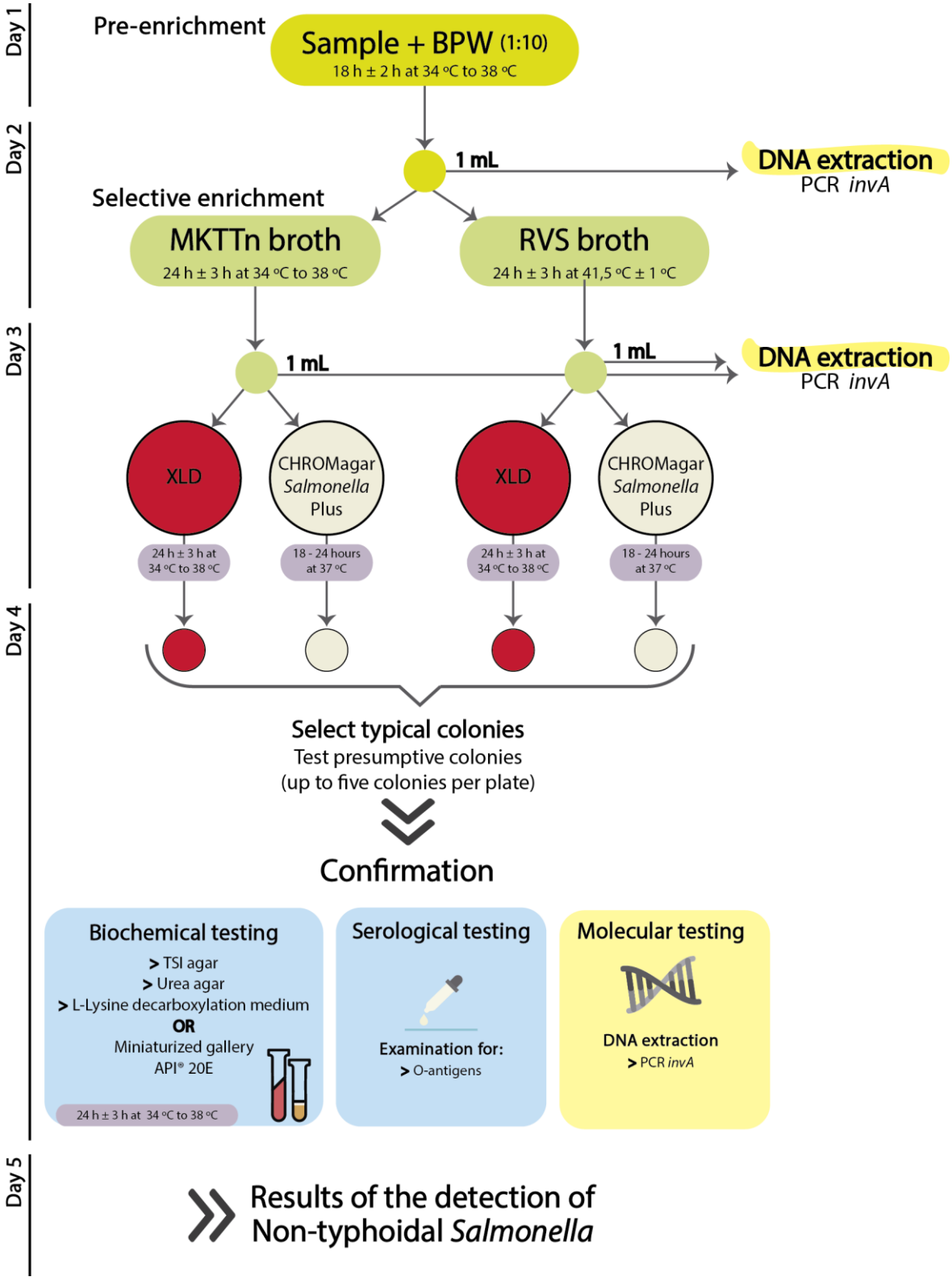


Figure 5 - Workflow for the detection and confirmation of *Salmonella* isolates using the standard cultural method (ISO 6579-1:2017) and the additional molecular approach.

Salmonella detection in raw poultry samples was also performed by a molecular approach using a standard PCR targeting *Salmonella invA* gene applied directly to DNA extracted from the pre-enriched and enriched broths (BPW and RVS/MKTTn) (**Figure 5**). DNA was extracted by a boiling-based protocol optimized in this study for poultry high-fat samples. Briefly, 1 mL of enriched cultures (BPW, RVS or MKTTn) was added to Eppendorf tubes. Then, the suspension was centrifuged (13000 g, 5 min), the resulting supernatant was rejected, and the pellet washed with 200 µL of saline. This step was repeated two more times, first washing the pellet with 200 µL of Triton X-100 (1%) and then washing again with 200 µL of saline. After that, other centrifugation was done, the supernatant was rejected, and the pellet was resuspended in 100 µL of sterilized ultrapure water. After boiling (100ºC, 20 min) a final centrifugation (13000 g, 5 min) was performed and the supernatant containing the total DNA was recovered and stored at -20ºC. The efficiency of the bacterial DNA extraction was evaluated by a standard PCR targeting the 16S rRNA gene (**Table AI**) (71).

3.2.2. Genotypic and phenotypic characterization of *Salmonella* isolates recovered from positive samples

The search of EU-targeted *Salmonella* serotypes (Enteritidis, Typhimurium and 4,5,12:i:-) (72, 73), their antibiotic resistance (amoxicillin - bla_{TEM}; chloramphenicol - *cmlA1-catA-floR*, aminoglycosides - *strA-strB-aadA-aac(3)*-IV-*aphA1*, Sulfonamides - *sul1-sul2-sul3*, tetracycline - *tet(A)-tet(B)* and trimethoprim - *dfrA1-dfrA12-dfrA17*) and metal (*pcoD*, *silA*, *merA*, *arsB* and *terF*) tolerance markers was performed by PCR (**Table AI**) in all isolates recovered from positive chicken meat samples (40-42).

Antibiotic susceptibility profiles of *Salmonella* isolates were determined by disc diffusion for 16 antibiotics (amikacin-30 µg, amoxicillin-10 µg, amoxicillin + clavulanic acid-30 µg, cefotaxime-5 µg, ceftazidime-10 µg, chloramphenicol-30 µg, gentamicin-10 µg, kanamycin-30 µg, meropenem-10 µg, nalidixic acid-30 µg, pefloxacin-5 µg, streptomycin-10 µg, sulfamethoxazole-300 µg, tetracycline-30 µg, tobramycin-10 µg and trimethoprim-5 µg) (Oxoid, Thermo Fisher Scientific, USA) following European Committee of Antimicrobial Susceptibility Testing (EUCAST) guidelines (74) and, when it was not possible, Clinical and Laboratory Standards Institute (CLSI) guidelines (75). Minimum Inhibitory Concentration (MIC) for colistin was performed by the reference broth microdilution method (76). MDR

was considered when the isolates were resistant to three or more antibiotics of different families.

MIC to copper sulphate ($CuSO_4$) were determined in aerobic and anaerobic atmospheres (GENbox jar with GENbox anaer and an anaerobic indicator – bioMérieux, France) by the agar dilution method. The Mueller-Hinton II agar plates (bioMérieux, France) were supplemented with different concentrations of $CuSO_4$ (0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32 and 36 mM; adjusted to pH 7.2) (40-42). The results were taken after 18-20 hours of incubation at 37ºC and the first concentration of $CuSO_4$ without visible bacterial growth was considered the MIC. To assess the growth ability of all isolates in different atmospheres, we inoculated a first and last plate of Mueller-Hinton II agar without $CuSO_4$. *Enterococcus faecium* BM4105RF (negative for copper tolerance genes) and *Escherichia coli* ED8739 (plasmid pRJ1004 with *pco*+*sil* genes) were used as controls in this assay.

3.3. Detection and characterization of *mcr*-carrying *Enterobacteriaceae*

3.3.1. Detection of *mcr*-positive *Enterobacteriaceae* by a cultural and molecular approach

Detection of *Enterobacteriaceae* was carried out using molecular and cultural approaches, with an initial common step of selective pre-enrichment of 25 g of sample (pooled raw chicken meat) into 225 mL of BPW supplemented with colistin (3,5 mg/L; BPW+COL; 16-18h/37 ºC) yielding a tenfold dilution (**Figure 6**).

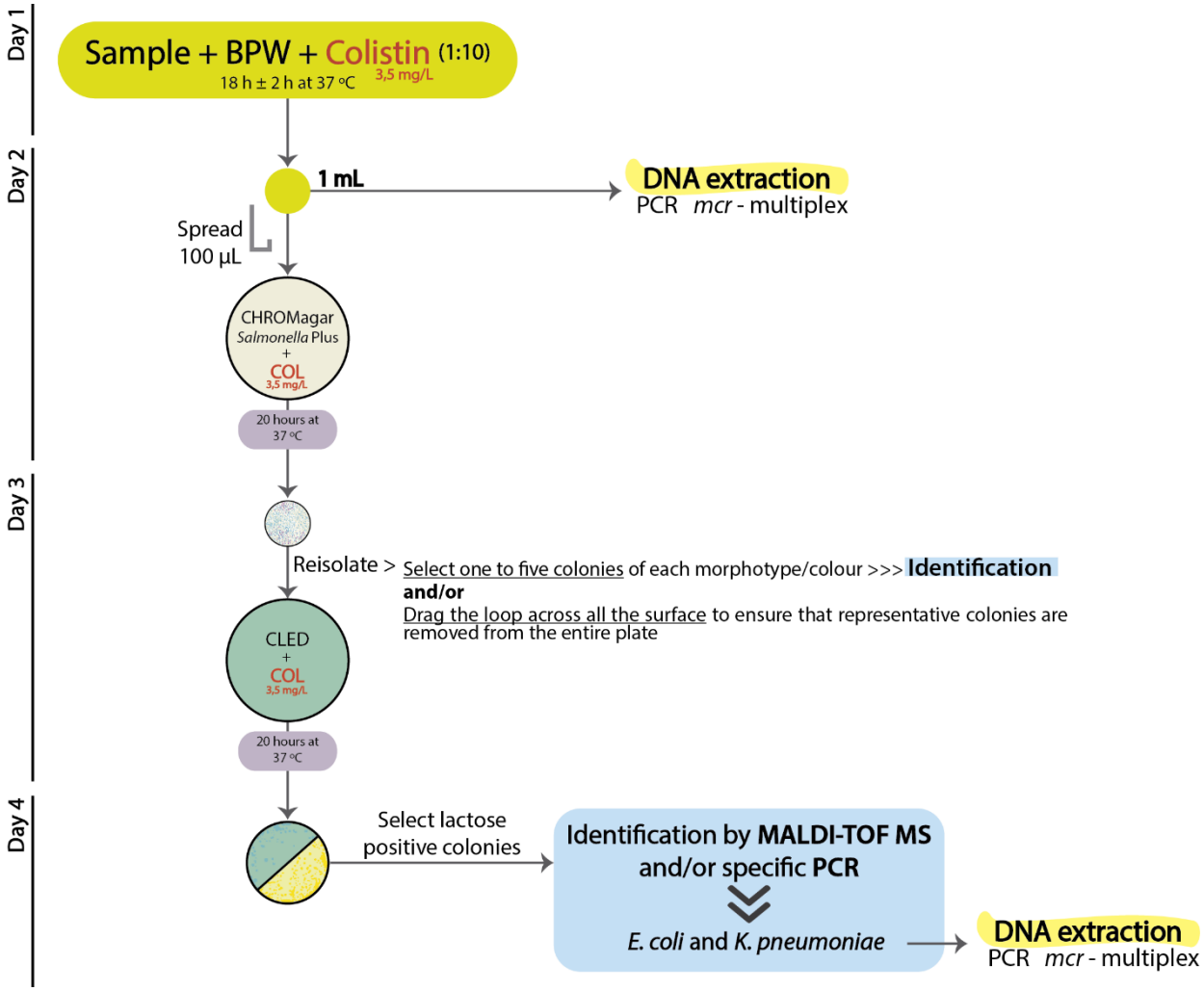


Figure 6 - Workflow for the detection of colistin-resistant *Enterobacteriaceae* and confirmation of *mcr* genes.

After pre-enrichment, 1 mL was collected from each sample and DNA was extracted by the boiling-based protocol optimized for high-fat samples describe in the section 3.2.1. The screening of colistin resistance genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5*) was verified by PCR (77). The efficiency of the bacterial DNA extraction was also evaluated by PCR targeting 16S rRNA gene (**Table AI**) (71).

In the cultural method, an aliquot (0,1 mL) of the pre-enriched BPW+COL was spread on CHROMagar™ *Salmonella* Plus supplemented with colistin (3,5 mg/L; 140 mm plates; 24h/37ºC). CHROMagar™ *Salmonella* Plus is a chromogenic medium that allows the detection of diverse coliforms/*Enterobacteriaceae*, while inhibiting *Proteus* (78) (a bacteria intrinsically resistant to colistin (75)). One to five colonies of each morphotype/colour were selected for identification and/or a loopful from the chromogenic selective plate was spread

on CLED agar (Liofilchem, Italy) supplemented with colistin (3,5 mg/L; 20h/37ºC) for selecting lactose positive colonies. The selected colonies were identified by Matrix-Assisted Laser Desorption-Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) using the MALDI-TOF VITEK MS (bioMérieux, France) and/or by PCR for *E. coli* (79) and *K. pneumoniae* (80) (**Figure 6**, **Table AI**). Colistin resistance genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5*) were searched in *E. coli* and *K. pneumoniae* isolates by a multiplex PCR (77) (**Table AI**). Amplified simplex PCR products were purified using the NZYGelpure kit (NZYTech, Portugal) and sequenced at Eurofins Genomics (https://www.eurofinsgenomics.eu/).

3.3.2. Genotypic and phenotypic characterization of *mcr*-carrying *Enterobacteriaceae* isolates

Isolates' relatedness was firstly investigated by Fourier Transform Infrared (FT-IR) spectroscopy with attenuated total reflectance (ATR) (Perkin Elmer Spectrum BX FT-IR System spectrophotometer equipped with a PIKE Technologies Gladi ATR), using a workflow for spectra acquisition and data analysis (chemometric data analysis-PLSDA-Partial Least Square Discriminant Analysis and/or PCA-Principal Component Analysis) previously described (81, 82). Briefly, three independent bacterial cultures (in triplicate) of *E. coli* and *K. pneumoniae* were directly applied in the crystal, spectra were acquired in standardized conditions (4000-400 cm^{-1}, $4cm^{-1}$ resolution and 32 scan co-additions) and compared between each other to assess clonal relatedness among isolates. Those belonging to *K. pneumoniae* were further compared with an in-house database (including 19 international clones/K-types) for identification of capsular types and presumptive clonal identification (81). These FT-IR-based assignments were confirmed for *E.coli* and *K. pneumoniae* by Pulsed-Field Gel Electrophoresis (PFGE) using the total genomic DNA of the isolates digested with the restriction endonuclease *XbaI* (New England Biolabs, USA), according to optimized protocol of Centers for Disease Control and Prevention (CDC) (83). Furthermore, multi-locus sequence typing (MLST) for *K. pneumoniae* was performed using the seven housekeeping genes (*gapA*, *infB*, *pgi*, *mdh*, *phoE*, *rpoB*, *tonB*) proposed in Pasteur MLST scheme (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) (84) and determination of capsular type was performed by PCR and sequencing of *wzi* gene (**Table AI**) (81, 85). The MLST Atchman scheme was used for *E. coli* (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search) (86), while phylogenetic

groups (PhG) were inferred by the multiplex PCR proposed by Clermont et al. (87, 88) (**Table AI**).

Antibiotic susceptibility profiles of representative *mcr-1* positive isolates were determined for 20 antibiotics (amoxicillin-10 µg, amoxicillin + clavulanic acid-30 µg, amikacin-30 µg, aztreonam-30 µg, cefepime-30 µg, cefotaxime-5 µg, cefoxitin-30 µg, ceftazidime-10 µg, chloramphenicol-30 µg, ciprofloxacin-5 µg, fosfomycin-200 µg-*E.coli*, gentamicin-10 µg, imipenem-10 µg, kanamycin-30 µg, nalidixic acid-30 µg, streptomycin-10 µg, sulfamethoxazole-300 µg, tetracycline-30 µg, tobramycin-10 µg and trimethoprim-5 µg) (Oxoid, Thermo Fisher Scientific, USA) by disc diffusion following EUCAST guidelines (74) and, when it was not possible, CLSI guidelines (75). MIC for colistin was performed by the reference broth microdilution method (76). MDR was considered when the isolates were resistant to three or more antibiotics of different families.

Classification of plasmids into incompatibility (Inc) groups by PCR-based replicon typing (PBRT; IncHI2, IncX4 and IncI2) (89, 90) and for IncHI2 by plasmid MLST (pMLST) was performed (**Table AI**). The amplified PCR products for pMLST (locus *smr0018* and *smr0199*) were purified using the NZYGelpure kit (NZYTech, Portugal), sequenced at Eurofins Genomics (https://www.eurofinsgenomics.eu/) and queried at plasmid typing database (http://pubmlst.org/plasmid/). The location of *mcr-1* gene in plasmids or chromosome was accomplished by digesting total genomic DNA with *S1* nuclease (Takara Bio Inc., Japan) and *I-CeuI* (New England Biolabs, USA), followed by PFGE and Southern blot hybridization using specific probes (*mcr*-1, IncX4, InHI2, IncI2). The probes were prepared according to the manufacturer's instructions (Gene Images AlkPhos Direct™ Labelling System; Amersham GB/GE Healthcare Life Sciences UK Ltd., UK) using the amplification products (after purification) of the genes of interest. *S.* Braenderup H9812 (CDC) was digested with the endonuclease *XbaI* (New England Biolabs, USA) and used as a molecular weight marker.

3.4. Whole-genome sequencing (WGS) for characterization of representative isolates

One representative isolate from each *Salmonella* serotype (n=2) and from each clone among *mcr*-positive isolates (n=6 *E. coli* and n=2 *K. pneumoniae*) were selected for WGS sequencing. The DNA was extracted using the Wizard® Genomic DNA purification kit (Promega Corporation, Madison, WI), according to manufacturer's instructions, and

quantified with Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, USA). DNA was then sequenced with a standard HiSeq (2x150bp) Illumina platform (Illumina, San Diego, CA) at Eurofins Genomics (https://www.eurofinsgenomics.eu/). The FastQC software v0.11.8 was used to evaluate the quality of the raw reads after sequencing (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). High-quality raw reads were then de novo assembled using SPAdes v3.14.0 (91), and the final quality was assessed by QUAST (http://quast.bioinf.spbau.ru). The assembled draft genomes were annotated for metal tolerance genes using RAST genome annotation server (92) and manually curated within the Geneious Software v2020.1.2 (https://www.geneious.com/). Tools from the Centre for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org) were used to assess the content in antibiotic resistance genes (ResFinder and PointFinder) (93, 94), plasmid replicons (PlasmidFinder) and typing (pMLST) (89) and Multilocus Sequence Typing (MLST) (86, 95). Confirmation of *Salmonella* serotypes was performed with *Salmonella* In Silico Typing Resource (SISTR) (96). BLASTn alignment and annotation of the *phs* operon, coding for thiosulfate reductase, was performed using Geneious Software v2020.1.2 (https://www.geneious.com/).

4. RESULTS AND DISCUSSION

4.1. Detection and characterization of *Salmonella* in poultry meat

4.1.1. Detection of *Salmonella* in raw chicken meat

Human salmonellosis cases have been stabilizing in most EU countries since 2014, but with significant increasing trends in Portugal (46). The importance of poultry meat analysis just before distribution for retail sale remains critical, as it is a major source of *Salmonella* infections. In this study, *Salmonella* was detected in 4% (n=2 out of 53 analysed batches) of the fresh chicken meat samples studied over six months (Spring and Summer seasons) (**Figure 7**).

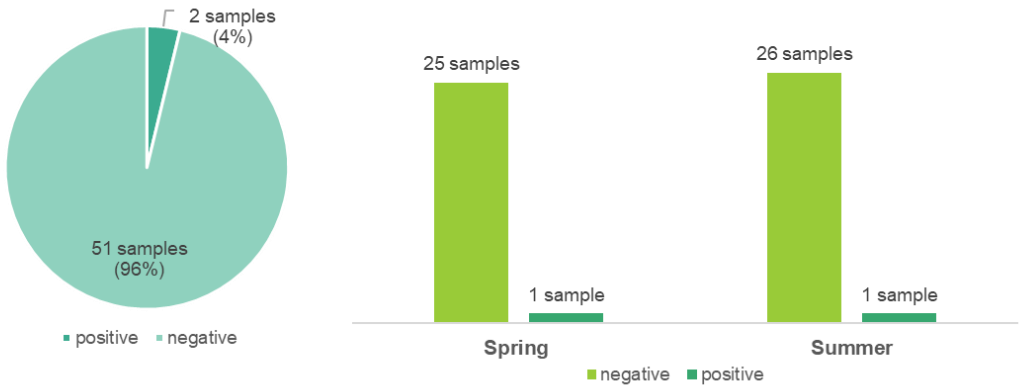


Figure 7 - Occurrence of *Salmonella* in the analysed fresh chicken meat samples and dispersion by season.

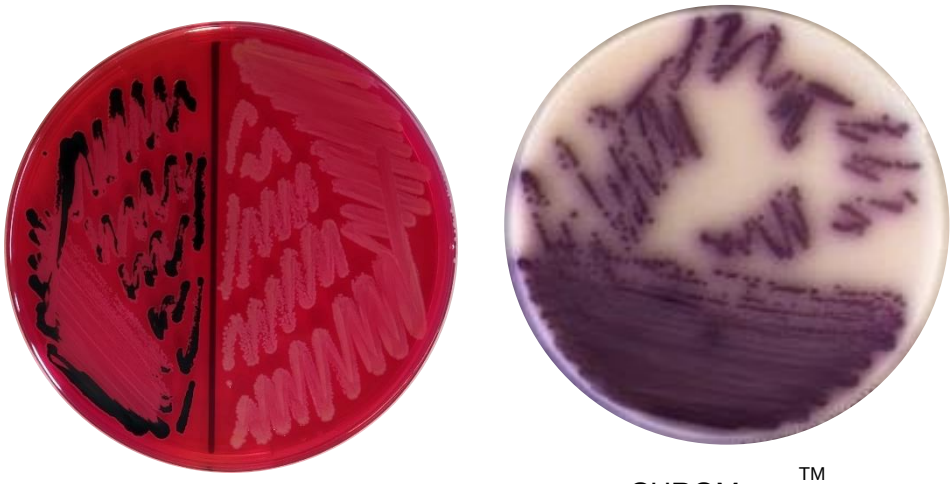
This low occurrence of *Salmonella* in raw poultry products is in accordance with data recently published from other industrialized countries with pathogen reduction programmes (46, 54). The presence of *Salmonella* was confirmed in the same samples by the standard cultural approach (ISO 6579), as proposed in the Microbiological Criteria Regulation in Europe (52), and the PCR assay in total sample DNA obtained from the selective enrichments (RVS/MKTTn), showing that molecular detection is a good alternative to laborious and time-consuming conventional approaches (97). Moreover, molecular-based methods (such as PCR or real-time PCR) have been pointed out as potential rapid

alternative methods for detecting *Salmonella* because they offer advantages of high sensitivity and specificity as well as the ability of discriminating *Salmonella* from competitive microorganisms, targeting DNA or RNA sequences that only exist in *Salmonella* cells (97), thus allowing easier detection of atypical *Salmonella* strains (98) as well as viable non-cultivable cells, which may not be detected with the classical methodology (99, 100). In general, these methods have been combined with cultural steps (non-selective and selective enrichments) to improve the efficiency and time for detecting *Salmonella* among food chain samples (98, 100, 101). Nevertheless, molecular methods still would benefit from further improvements in terms of sensitivity at the pre-enrichment step (99).

4.1.2. Characterization of *Salmonella* recovered from raw chicken meat

Among *Salmonella* recovered isolates (n=9), we identified the monophasic variant of *S*. Typhimurium (*S*. 1,4,[5],12:i:-/ST3478, a Single Locus Variant of the epidemic ST34) (Spring sample) and *S*. Enteritidis/ST11 (Summer sample), both serotypes currently covered by EU Regulations, including as a food safety microbiological criterion for fresh poultry meat (53, 102). Both serotypes have been reported by EFSA to be among the most frequent serotypes causing human infections in the EU in the last years (46, 54) as well as by the Portuguese authorities (103), justifying the relevance of surveillance studies. Moreover, several *S*. 1,4,[5],12:i:- belonging to ST3478 have been recently described in Europe, including associated with human infections, as available from Enterobase data (http://enterobase.warwick.ac.uk/species/index/senterica), which justify their close tracking.

In this study, all *S*. Enteritidis isolates presented the typical biochemical *Salmonella* profiles, whereas all *S*. 1,4,[5],12:i:- isolates showed to be non-H_2S-producers, and thus lacked the typical black colour on XLD agar plates (**Figure 8**). Thus, *S*. 1,4,[5],12:i:- isolates were only detected in CHROMagar™ *Salmonella* Plus (mauve colour). This atypical *Salmonella* phenotype is especially worrisome since these strains can escape detection (conducting to low *Salmonella* detection rates) on the traditional medium, supporting the utility of the chromogenic media and a further combination with molecular-based methods, as performed here. See more details about this atypical *Salmonella* in the chapter 4.1.3.



XLD agar

CHROMagar™ *Salmonella* Plus

Figure 8 – Growth of *Salmonella* recovered from the two positive poultry samples on XLD agar and CHROMagar™ *Salmonella* Plus. On the left side of XLD agar, hydrogen sulphide-(H_2S)-producing *S*. Enteritidis strain, presenting the typical biochemical *Salmonella* profile. On the right side, atypical non-H_2S-producing *S*. 1,4,[5],12:i:- .

The *S*. 1,4,[5],12:i:- poultry isolates presented the typical antibiotic resistance [*bla*$_{TEM}$+*strA-strB*+*sul2*±*tet(B)*] genes and the integrative and conjugative element carrying metal tolerance (*pcoD*+*silA*+*arsB*±*merA*) features of the widespread clinically-relevant European clone (ST34) (41, 42). Resistance to the tested antibiotics and tolerance to copper (MICs=32 mM) was restricted to *S*. 1,4,[5],12:i:- isolates and absent in the *S*. Enteritidis (**Table 1**), as described previously (40, 41). The frequent use of copper as a feed additive in food-animal production, as occurred in the Portuguese poultry farms studied, alerts for the potential co-selection of MDR (emerging) clonal lineages, as suggested for other *S*. 1,4,[5],12:i:- clones (41). Acquired resistance to critically antibiotics like colistin, fluoroquinolones and extended-spectrum beta-lactams was not observed in any isolate from both serotypes (**Table 1**).

Table 1 - Characterization of *Salmonella* isolates recovered from chicken meat samples.

Serotype	**No. Isolates / ST**	**No. Samples (farm / Season)**	**Antibiotic resistance phenotype / genotype** [a]	**Metal tolerance genes** [b]	**MIC Copper anaerobiosis (mM)**
1,4,[5],12:i:-	n=6 / ST3478	1 sample (farm A / Spring)	ASSu[T] / *bla*$_{TEM}$, *strA-strB*, *sul2*, [*tet(B)*]	*pcoD*, *silA*, *arsB*, [*merA*]	32
Enteritidis	n=3 / ST11	1 sample (farm B / Summer)	-	-	4

Abbreviations: A: Ampicillin; S: Streptomycin; Su: Sulfamethoxazole; T: Tetracycline; MIC, Minimum Inhibitory Concentration; ST, Sequence Type.

[a] Variable antibiotic resistance phenotypes and genotypes are presented between brackets.

[b] Metal tolerance genes that were not observed in all the isolates are presented between brackets.

4.1.3. Characterization of the non-H_2S-producing atypical *S*. 1,4,[5],12:i:- isolate

All the *S*. 1,4,[5],12:i:- isolates recovered in the present study showed absence of H_2S production, which is a rare phenotypic feature among *Salmonella*, regardless of the serotype. However, non-H_2S-producing *Salmonella* has been reported in the last years in emerging or outbreak-associated strains recovered from diverse food and human sources worldwide (104-107), including in poultry meat from diverse geographical regions (108, 109).

Until now, two molecular mechanisms were reported as responsible for the inability to produce H_2S in diverse *Salmonella* serotypes (e.g. Aberdeen, Havana, Infantis, Senftenberg, Typhimurium and 1,4,[5],12:i:-) and sources (e.g. human-clinical, poultry meat, vegetables, surface water and seafood products) (105, 106, 108-111). The most frequent one is associated with mutations in *phsA* gene - belonging to *phsABC* operon - encoding the precursor of thiosulfate reductase (106, 111). The other one is related to mutations in *moaC* gene – belonging to *moaABCDE* - affecting the activity of a thiosulfate reductase cofactor (110). Our non-H_2S-producing *S*. 1,4,[5],12:i:- isolates, when compared with the H_2S-producing *S*. Typhimurium LT-2 strain (where this operon was initially described) (112), had a mutation at position 1669 of *phsA* consisting of a single-nucleotide substitution of C to T, resulting in a codon change from CAG (Glutamine-Q) to UAG a Stop codon (**Figure 9**). This mutation resulted in the premature termination of *phsA* translation; hence the non-H_2S-producing *S*. 1,4,[5],12:i:- isolates were not able to produce the integral PhsA protein.

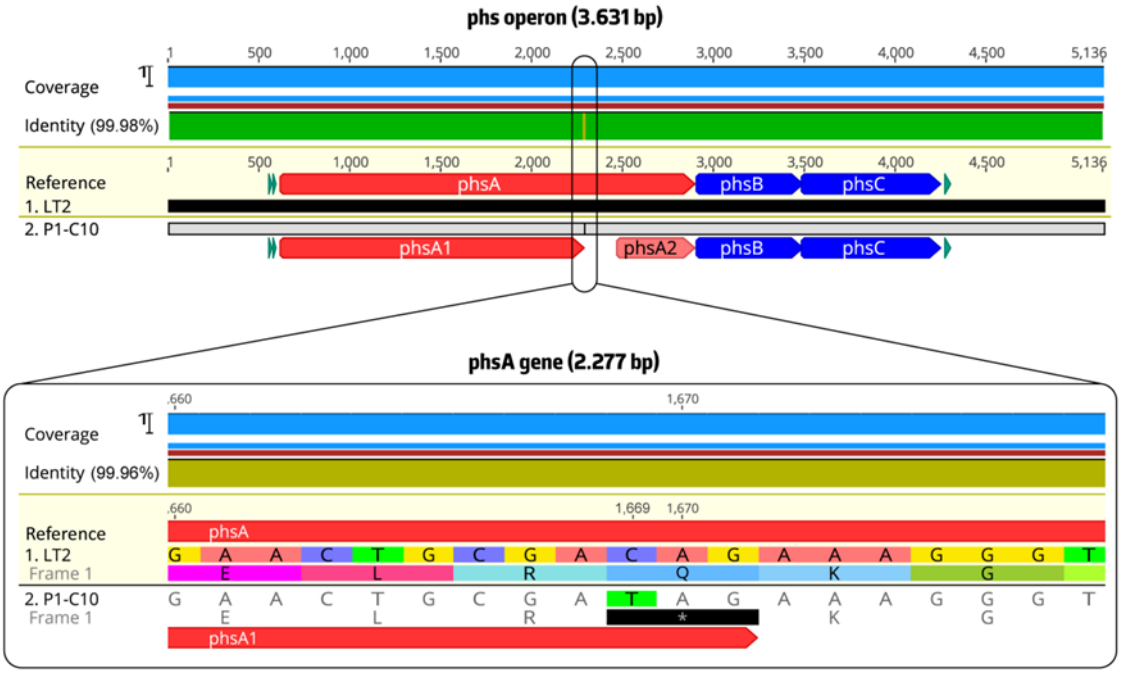


Figure 9 – Analysis of mutations in *phsA* gene belonging to *phsABC* operon encoding the precursor of thiosulfate reductase. Upper panel - nucleotide alignment and gene synteny of the *phs* operon between *S*. 1,4,[5],12:i:- (P1-C10) and the reference strain *S*. Typhimurium LT-2 (accession no. L32188.1). The light blue and green bars represent coverage and sequence identity, respectively. Lower panel – exact location of the nonsense mutation in the *phsA* gene encoding the thiosulfate reductase subunit. Filled arrows indicate the position and transcriptional direction of open reading frames (red – *phsA* gene and blue – *phsB* and *phsC* genes). The black square with the asterisk represents the stop codon.

The non-H_2S-producing strains, which are unable to convert thiosulfate to H_2S, may increase the tetrathionate-dependent respiration, representing a potential competitive advantage over other bacteria in the gut (**Figure 10**) (109, 111). *Salmonella* inflammation generates reactive oxygen species (ROS) leading to the conversion of thiosulfate to tetrathionate (113), which is used as an electron acceptor in anaerobic respiration through tetrathionate reductase (encoded by *ttrABCRS* cluster located on a *Salmonella* pathogenicity island - SPI2) (113). Normally, the tetrathionate is reduced to thiosulfate which can be further reduced to H_2S by thiosulfate reductase (encoded by *phsABC* operon). However, when the strains are unable to convert thiosulfate to H_2S, there will be increased availability of thiosulfate substrate for *Salmonella* tetrathionate anaerobic respiration, representing a competitive advantage over other bacteria due to growth and colonization promotion (111).

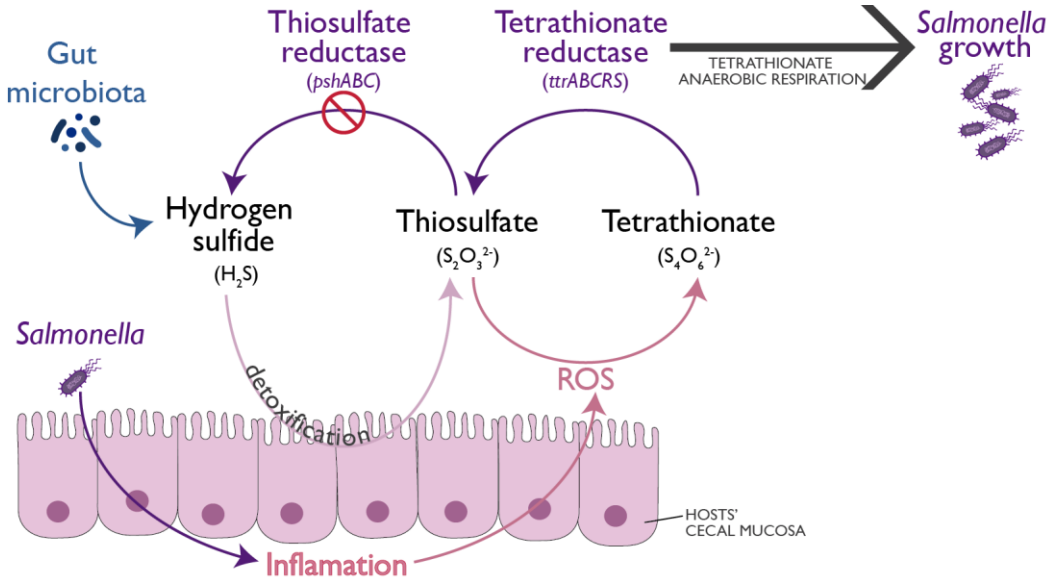


Figure 10 – The *Salmonella*'s selective advantage to survive in the host's inflamed gut. Adapted from (113, 114).

4.2. Detection and characterization of *mcr*-carrying *Enterobacteriaceae* in poultry meat

4.2.1. Detection of *mcr*-positive *Enterobacteriaceae* in raw chicken meat

Portugal is still one of the European countries with extensive colistin use in food-producing animals (25), with MCR-1-producing *Enterobacteriaceae* detected in few studies on animals or foodstuffs (e.g. pigs, rabbits and turkey) and humans (115-120). The recent recommendations for colistin reduction/ban at the farm level have been voluntarily implemented by some poultry production industries in Portugal. Due to the short life span of birds (around 40 days), sampling at slaughterhouse might reflect the current global farm practices, including antibiotic usage, allowing studies combining antibiotic consumption and resistance in a farm-to-fork perspective.

In the present study, the *mcr-1* gene was the only acquired plasmid-borne gene conferring resistance to colistin detected in chicken meat samples by the molecular and cultural approaches. Among the *mcr*-family genes, *mcr-1* is one of the most widely disseminated, being the only one that was detected in Portugal until now (61). The *mcr*-1

gene was detected in 68% of poultry meat batches (n=36 out of 53) by our molecular approach applied directly on the enriched samples (DNA extracted from BPW+COL) (**Figure 11**). These *mcr* positive-samples were from 79% (n=23/29) of poultry farms, without colistin use, who supplied chickens for the slaughterhouse where poultry meat was collected. In fact, our sampling was initiated after 3 months of the complete voluntary withdrawal of colistin at the farm level, which might justify the high rates of colistin resistance and *mcr* still founded (above 60%) in poultry meat. Although the occurrence rates of *mcr* among food-animal samples cannot be directly compared across studies due to sampling or methodological differences, in other European countries were described MCR-1 producers in poultry meat (chicken and/or turkey) ranging between 25% to 67% of tested samples (121-123). Nevertheless, in these few studies there was no information regarding colistin consumption at the flock level. In other countries such as China and Brazil (two of the largest poultry meat producers), where heavy colistin usage was still widespread as a feed additive for growth promotion, *mcr* detection among poultry samples ranged from 20% to 90% (124-128), suggesting a potential role for direct selective pressure on *mcr*-carrying bacteria persistence as well as for other underestimated factors (e.g. biocides, metals-Cu) contributing for their co-selection.

Molecular-based assays (such as PCR and real-time-PCR) represent a powerful approach for screening a large number of samples as well as to overcome the failure in detection of low counts or viable non-cultivable cells, avoiding *mcr* genes underestimation, as suggested by others (126-128). However, in our study, using a selective cultural method, the presence of *mcr-1* was confirmed in 62% of poultry meat batches (n=33 out of 53) and 72% of farms (n=21/29) (**Figure 11**).The culture-based method using a selective pre-enrichment with BPW+COL and subsequent culturing on the CHROMagar™ *Salmonella* Plus medium supplemented with colistin revealed a high sensitivity and specificity to detect MCR-1 producers, with only 3 samples positive by PCR and negative by the cultural approach, contrasting with other studies (121, 122). In addition, our strategy allowed the identification of diverse genetic backgrounds (species+clones and plasmids), impossible with only a direct PCR approach (126).

Interestingly, a declining trend in the last sampling month (8 months after the colistin voluntary withdrawal) was observed by cultural and molecular approaches, with only 12% (n=2 out of 17) of chicken meat batches carrying *mcr-1*-positive isolates (**Figure 11**). Also, on a Portuguese pig farm, a significant decrease in the presence of colistin-resistant *E. coli* in faeces, from 98% in 2016 to 27,5% in 2018, was detected after a two-year ban on colistin use (118, 119). Similar results were reported in a Great Britain pig farm where no detection of *mcr* among faeces samples occurred only after 20 months of colistin withdrawal,

suggesting that those control measures can successfully mitigate long-term on-farm *mcr* persistence (129).

Globally, colistin withdrawal has also led to promising results, as demonstrated by the reduction on the occurrence of colistin resistance and/or *mcr-1* in both animals (rates below 5% in pigs and chickens) and humans until after two years of colistin ban as a feed additive for growth promotion in China in 2017 (130, 131). Although an association between animal polymyxin consumption and resistance in *E. coli* from poultry and swine have also been suggested in an EU report (132), studies evaluating the impact on food safety (e.g. poultry meat and other foodstuffs) of the ongoing colistin reduction measures in food-producing animal samples are missing. Moreover, the persistence of colistin resistance and *mcr* genes, including in not treated poultry flocks (Poland) (133) or pigs (Great Britain) (129), suggests that other factors (e.g. metals-copper and other antibiotics) beyond colistin consumption might contribute to their selection, but this needs to be better explored and understood.

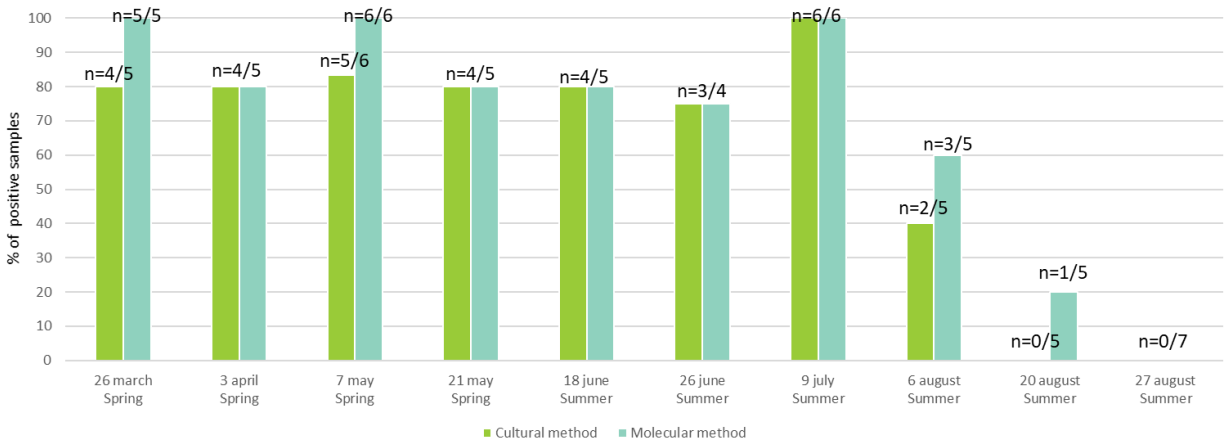


Figure 11 - Occurrence of *mcr-1* among chicken meat, obtained by cultural (green bars) and molecular (blue bars) approaches, dispersed across all Spring and Sumer sampling period. (n=number of positive samples/number of total samples).

4.2.2. Characterization of *mcr*-positive *Enterobacteriaceae*

We recovered 106 *Enterobacteriaceae* isolates with *mcr-1* gene, mostly *E. coli* (n=90), but also *K. pneumoniae* (n=16) among the 53 poultry meat batches. All MCR-1 producers

were colistin-resistant, with MICs varying between 4 and >16 mg/L, and presented MDR profiles (**Table 2**). In fact, MDR in colistin-resistant *mcr*-positive isolates is a constant feature observed in diverse studies (121, 123, 134, 135)), highlighting the complex co-selection mechanisms (other than colistin) triggered by the overall high use of several antibiotic classes in the intensive poultry production chain. For instance, Portugal is one of the EU countries with higher fluoroquinolones, tetracyclines and penicillins sales (136), antibiotics frequently used for therapeutics at poultry farms. All the *mcr-1*-isolates presented co-resistance to several classes of antimicrobial agents including amoxicillin, chloramphenicol, nalidixic acid and sulfamethoxazole and 96% to ciprofloxacin and tetracycline (**Table 2**). All of them were also susceptible to extended-spectrum cephalosporins and carbapenems. The high level of resistance to beta-lactams, quinolones and tetracyclines in *Enterobacteriaceae* carrying *mcr-1* from chicken meat found in this study is consistent with the results of antibiotic resistance in food-producing animals in other EU countries (121, 134). In fact, broiler chicken production is characterized by a short fattening period (about 40 days), with antibiotic therapy frequently needed at farm level to control bacterial infections (56) (133), leading to potential diverse co-selection events that could be aggravated in the contexts of antibiotic (colistin) reducing and replacing. In addition, the next steps during poultry slaughter (e.g. evisceration) and processing can also increase the risk of meat contamination with MDR bacteria by diverse cross-contamination events.

Table 2 - Characterization of the *mcr*-positive clones recovered from chicken meat batches in a Portuguese slaughterhouse over six-months.

FT-IR (PFGE-type)[a], no. Isolates	PhG / MLST-ST[b]	Farm supplier (batch number/collection visit)[c]	*mcr-1* location [d] Chr/PL-size (kb)/pMLST	Colistin MIC (mg/L)	Antibiotic resistance phenotype other than colistin [e]
E. coli					
E-I (A), n=1	B1 / ST533	B (**37/C7**)	PL-HI2-260/ST4	4	AMX, AMC, CHL, CIP, NAL, STR, SUL, TET
E-II (C), n=10	B1 / ST602	B (21/C4), D (5/C1, 19/C4), L (18/C4), M (22/C4)	PL-X4-33	4	AMX, CHL, CIP, NAL, SUL, TET
E-III (E), n=7	F / ST6469	A (1/C1), J (15/C3), H (**17/C3**), K (**16/C3**)	PL-HI2-260/ST2 or PL-X4-33	4	AMX, CHL, CIP, KAN, NAL, STR, SUL, TET, TMP (GEN, TOB)
E-IV (D), n=65	B1 / ST297	B (**37/C7**), C (4/C1), D (10/C2), E (6/C2), F (7/C2), G (8/C2), H (11/C3, **17/C3**, **32/C7**), I (12/C3, 13/C3, **30/C6**), K (**16/C3**, **33/C7**), N (23/C5), O (24/C5), Q (27/C5, 31/C6), R (35/C7), S (**36/C7**), T (38/C8), U (41/C8)	Chr	4 - >16	AMX, AMC, CHL, CIP, NAL, SUL, TET
E-V (non-typable), n=3	G / ST117	B (2/C1)	PL-I2-nd	8	AMX, AMC, CHL, CIP, NAL, SUL
E-VI (B), n=4	B1 / ST533	P (26/C5)	PL-HI2-260/ST4	4	AMX, AMC, CHL, CIP, KAN, NAL, STR, SUL, TET, TMP

FT-IR (PFGE-type)[a], no. Isolates	PhG / MLST-ST[b]	Farm supplier (batch number/collection visit)[c]	*mcr-1* location [d] Chr/PL-size (kb)/pMLST	Colistin MIC (mg/L)	Antibiotic resistance phenotype other than colistin [e]
K. pneumoniae					
K-I (1 and 2); n=16	ST147	B (**37/C7**), H (**32/C7**), I (**30/C6**), K (29/C6, **33/C7**), L (34/C7), S (**36/C7**)	PL-HI2-250-280/ST2	>16	AMC, CHL, CIP, NAL, STR, SUL, TET (FOX, GEN, TMP, TOB)

[a] FT-IR-types, *Escherichia coli*: E-I to EVI; *Klebsiella penumoniae*: K-I; PFGE-types, *E. coli*: A to E; *K. penumoniae*: 1 and 2.

[b] PhG, Phylogenetic Group; MLST, Multilocus Sequence Typing

[c] Farm supplier is designated by capital letters (A to U); Batches are designated by numbers (1 to 41); Batches with more than one clone are presented in bold; Collection visits are designated by a capital letter and a number: C1 to C5 were collected in Spring and C5 to C8 in Summer.

[d] Chromosomal (Chr) and/or plasmid (PL) location of *mcr-1* gene. pMLST, plasmid MLST.

[e] AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; CHL, chloramphenicol; CIP, ciprofloxacin; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SUL, sulphonamides; TET, tetracycline; TMP, trimethoprim; TOB, tobramycin.

The ninety *mcr*-carrying *E. coli* isolates were recovered from 30 samples (57%) corresponding to 21 out of 29 poultry farm suppliers, during most of the Spring and Summer samplings (8 out of 10 sampling dates). The sixteen *mcr*-carrying *K. pneumoniae* isolates were from 7 samples (13%) corresponding to 6 poultry farm suppliers, only in Summer samplings. In 5 samples (9%) from 5 poultry farm suppliers those species were found in co-occurrence (**Table 2**). Both species have been reported as the most frequent carrying *mcr* genes among animal or animal-derived foods (118, 119, 122, 123, 128, 134), suggesting that meat contamination might occur at slaughter/processing from the animal gastrointestinal tract. Although our cultural approach was able to detect *K. pneumoniae* in 13% of poultry meat samples, this is a less frequent recovered species (in comparison with *E. coli*) from animal and food samples, as occurred in other studies (118, 119, 122, 123). The use of other more accurate and sensitive medium (e.g. SCAi agar) for *K. pneumoniae* detection is critical for a comprehensive understanding of their reservoirs and transmission routes at food chain level.

Evaluation of clonal diversity among *mcr-1*-positive *E. coli* identified six clones by FT-IR that were corroborated by PFGE (an example in the **Annexes**). The application of FT-IR spectroscopy in a study with a high number of samples/isolates proved to be useful since it allowed a fast, accurate and inexpensive evaluation of the clonal relationship of isolates from the same or between different samples. However, in-house databases, including the most clinically-relevant food-associated strains/clones (EPEC and ExPEC) for presumptive clonal identification of *E. coli* are still needed in the food safety context (82). Our six clones corresponded to PhG B1, F and G (PhGs most associated with commensal *E. coli* population and not to extraintestinal pathogenic strains) and five Sequence Types-STs (ST117, ST297, ST533-ST40 Cplx, ST602-ST446 Cplx and ST6469-ST648 Cplx) (**Table 2**). According to Enterobase (http://enterobase.warwick.ac.uk/species/index/ecoli), four of them (ST117, ST297, ST533 and ST602) were identified globally in diverse animal, food and human sources as well as have been associated with diverse human infections, suggesting that they have potential to spread, including from food chain to humans. Moreover, several STs of *E. coli* identified in this study (e.g. ST117 and ST533) were previously reported as MCR-1–producing isolates among poultry (137, 138).

These diverse *E. coli* clonal backgrounds contributing for colistin resistance, as previously reported among pig farms in Portugal (118, 119) or poultry production from other EU countries (122, 123) suggests diverse sources/routes of contamination. In this study, we were able to detect three *E. coli* colistin-resistant clones persisting over time in different chicken meat batches supplied from the same (clone E-II/ST602 and E-IV/ST297) or different (clones E-II/ST602, E-III/6469 and E-IV/ST297) farms (**Table 2**), suggesting introduction/persistence at farm level (e.g. by one-day-old chickens, feed, poultry houses

environment) and/or eventual cross-contamination during slaughter/processing (e.g. evisceration step).

The *K. pneumoniae* isolates detected in our study during the Summer period belonged to one single clone - ST147 - which clustered by FT-IR spectroscopy, and corresponded to 2 PFGE-profiles. Those ST147 isolates corresponded to capsular (K)-type K35 by the genotypic method that was not presumptively identified by the FT-IR-based spectroscopy approach used (81). The lack of clonal/ST identification by FT-IR could be related with the FT-IR database used, currently focused on clinical isolates (e.g. the K-type most common among ST147 isolates is K14 and K64), and to the diversity of strains/K-types founded in non-clinical sources (81, 139). The identification of ST147 (with a different K-type) carrying *mcr-1* gene in diverse samples of chicken meat deserves to be further studied as it is an emerging MDR high-risk clone frequently related to healthcare-associated infections/outbreaks (139), with food transmission sources still not well defined.

All the colistin-resistant isolates identified in this study carried the variant *mcr-1.1*, which is still the main colistin resistance variant detected among diverse sources (61, 118, 119, 123). Among *E. coli*, the *mcr-1* gene was located either in the chromosome (22 batches/16 farms, clone E-IV) or carried on diverse plasmid families such as IncX4 (6 batches, 5 farms, clones E-II and E-III), IncHI2-ST2/ST4 (5 batches, 5 farms, clones E-I, E-III and E-VI) or IncI2 (1 batch-farm, clone E-V), dispersed in different samples and clones (**Table 2**). In the *K. pneumoniae* clone, the *mcr-1* gene was always identified on IncHI2-ST2 plasmid (7 batches/6 farms) (**Table 2**). Our data is in accordance with previous findings showing that the IncHI2-type plasmid (here detected in 3 clones recovered from 11 poultry meat batches and 9 farms) was the most common plasmid associated with *mcr-1* among *E. coli* and *K. pneumoniae* isolates recovered in Portugal (116, 118, 119). Although less frequent *mcr-1* genes were also located within other plasmids (IncX4 and IncI2), both frequently associated with dissemination of those genes in poultry meat in different countries and circulating among diverse hosts (55, 123). The presence of *mcr-1* gene in diverse genetic contexts (i.e. different plasmid types) may favour their dissemination among different bacterial species/strains, playing an important role in the spread of *mcr* genes in the poultry production. Nevertheless, the occurrence of the *mcr-1* gene on the chromosome of the predominant E-IV clone also alerts for the possible fixation of plasmid-mediated colistin resistance genes into specific *E. coli* populations driving vertical spread.

Based on the analysis of whole-genome sequencing data from the representative isolates (one from each clone: 6 *E.coli* and 2 *K. pneumoniae*), diverse genes encoding resistance to different antibiotic classes: aminoglycosides [*aadA*/*aph*/*aac(3)*], beta-lactams (bla_{TEM-1}), phenicols (*catA*/*cmlA*), tetracyclines [*tet(A)*], sulfonamides and trimethoprim (*sul*/*dfrA*) were detected. In *K. pneumoniae* were also detected the acquired *qnrB91* and/or

oqxAB genes and in *E.coli gyrA*/*parC* mutations, conferring resistance to ciprofloxacin and nalidixic acid. Moreover, diverse metal tolerance gene clusters encoding for copper/silver (*cus*/*pco*/*sil*), arsenic (*ars*), mercury (*mer*) and/or tellurite (*ter*) tolerance were detected (**Figure 12**). The high occurrence of genes encoding tolerance to diverse metals, some of them frequently used in food-producing animals like copper, alerts for the potential co-selection of *mcr*-1-carrying strains/clones, as suggested for diverse *Salmonella* serotypes (40-42). In the context of antibiotic reducing, replacing and farming rethinking, as described with the voluntary withdraw of colistin, metal stress factors must be seen as potential factors contributing for the persistence of *mcr*-1-carrying strains/clones and further studied. Even more when recent studies showed that *mcr-1* negatively impacts the biological fitness of *E. coli* and *K. pneumoniae* (140, 141) , which opens the possibility of limiting *mcr*-1 spread by reducing/banning colistin in both food-producing animals and healthcare sectors.

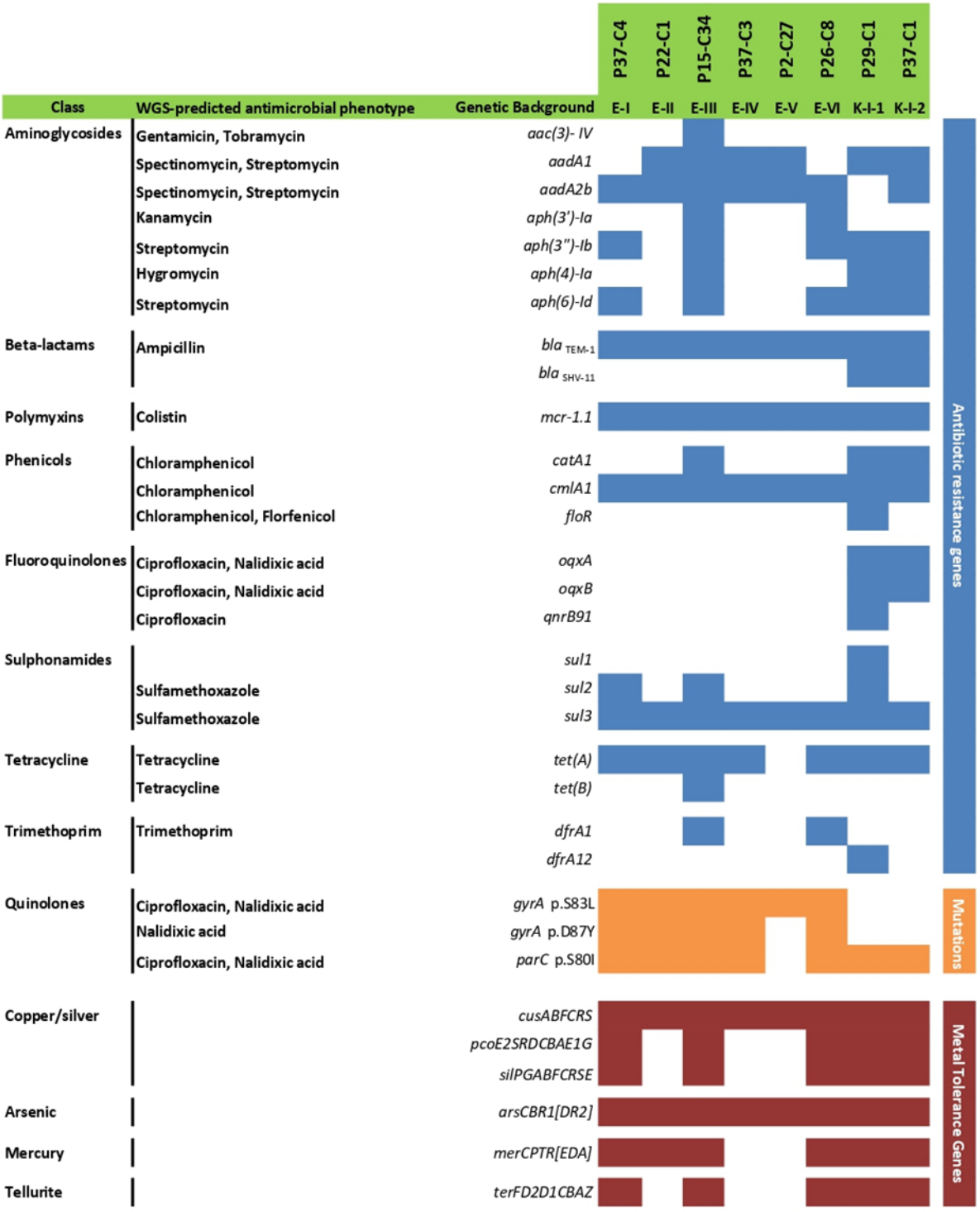


Figure 12 - Heatmap representing the distribution of antibiotic resistance genes and metal tolerance genes (red) from the sequenced *Escherichia coli* and *Klebsiella pneumoniae* genomes. Only known mutations conferring fluoroquinolone resistance are presented. Coloured squares indicate the presence of genes (blue) or mutations (orange).

5. CONCLUSIONS

Control measures for reducing foodborne pathogenic bacteria and antimicrobial resistance to produce poultry meat with microbiological quality and safety for the consumer are a current challenge for poultry-meat industries, public health and food safety authorities. In this study, we report a low occurrence of *Salmonella* serotypes of public health significance in raw chicken meat produced in Portugal, indicating the successful implementation of avian control practices. However, food safety authorities and public health laboratories should be aware of unusual non-H_2S-producing *Salmonella* strains, currently circulating in diverse sources worldwide. This phenotype is especially worrisome since these strains may go undetected on the traditional medium because of the lack of black colour, supporting the utility of chromogenic media and combination between cultural and molecular-based methods. Moreover, non-H_2S-production in combination with the ability of these strains to grow under diverse stresses (antibiotics/copper) could anticipate a future expansion of *S*. 1,4,[5],12:i:- ST3478 clonal lineage, due to an increased probability of selection throughout the food chain, thereby leading to a high risk of infection.

Moreover, we report high rates of chicken meat contaminated with diverse *mcr-1*-carrying *Enterobacteriaceae* short time after colistin withdrawal in the farms from which the samples were originated. The persistence over time of multiple MDR widespread *E. coli and K. pneumoniae* clones and common plasmids carrying *mcr-1* highlights poultry-production chain as a major source and supports foodborne transmission of *mcr-1* drivers to human consumers (e.g., by raw poultry meat handling, ingestion of undercooked chicken meat, or cross-contaminated ready-to-eat foods). The *mcr-1*-carrying strains enriched in diverse antibiotic resistance and metal tolerance genes suggest other potential factors for their co-selection. Nevertheless, a decreasing trend was observed in *mcr-1* occurrence eight months after voluntary colistin withdraw, indicating a positive outcome of the lack of selective pressure with colistin at farm level. We showed that CHROMagar™ *Salmonella* Plus supplemented with colistin after selective pre-enrichment (BPW+COL) is an efficient cultural strategy for screening *mcr*-carrying *Enterobacteriaceae* and that FT-IR spectroscopy allowed fast, accurate and inexpensive evaluation of the clonal relationship in non-clinical isolates.

In summary, this study shows current consumer exposure to poultry meat associated hazards, emerging *Salmonella* atypical strains and *mcr*-carrying *Enterobacteriaceae*, adapted to diverse poultry-production stresses. This scenario alerts for the importance of evaluating the impact/efficacy of food safety interventions at farm and processing level (e.g. biocides and metal-alternatives; antibiotics-colistin withdrawal) in retail poultry-meat to

guide concerted decisions that ensure adequate food production and protect the consumer health “from farm-to-fork” under the “One-Health” strategy.

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ANNEXES

Annex I – **Table AI -** List of primers and conditions used in the PCR assays.

Target gene/region [a]	Gene product or region description	Primer name	Primers sequences (5'-3') [b]	Amplicon size (bp)	Annealing temperature (°C)	Primers reference
Bacterial identification						
16S rRNA	16S ribosomal RNA	SEQA	AGAGTTTGATC**H**TGG**Y**T**Y**AGA	Variable	55	(71)
		SEQB	ACG**Y**TACCTTGTTACGACTTC			
***Salmonella* identification and characterization**						
invA	Invasion gene	invA_1	ACAGTGCTCGTTTACGACCTGAAT	243	60	(70)
		invA_2	AGACGACTGGTACTGATCGATAAT			
Sdf I region	Region for *Salmonella* Enteritidis identification	sdf_F	CGTTCTTCTGGTACTTACGATGAC	333	65	(73)
		sdf_R	TGTGTTTTATCTGATGCAAGAGG			
fljB	Flagellar gene of phase II	Sense-59	CAACAACAACCTGCAGCGTGTGCG	1389	64	(72)
		Antisense-83	GCCATATTTCAGCCTCTCGCCCG			
***fliB-fliA* intergenic region**	Intergenic region for identification of *Salmonella* serotypes	FFLIB	CTGGCGACGATCTGTCGATG	250 or 1000	64	(72)
		RFLIA	GCGGTATACAGTGAATTCAC			
Antibiotic Resistance						
***bla*$_{TEM}$**	Beta-lactamase	TEM-F	ATGAGTATTCAACATTTCCG	900	58	(142)
		TEM-R	CTGACAGTTACCAATGCTTA			

Target gene/region [a]	Gene product or region description	Primer name	Primers sequences (5'-3') [b]	Amplicon size (bp)	Annealing temperature (°C)	Primers reference
cmlA1-like	Phenicol resistant antibiotic efflux pump	cmlA_F	TGTCATTTACGGCATACTCG	435	55	(143)
		cmlA_R	ATCAGGCATCCCATTCCCAT			
catA	Chloramphenicol acetyltransferase	catA_F	CCACCGTTGATATATCCC	623	55	(143)
		catA_R	CCTGCCACTCATCGCAGT			
floR	Phenicol resistant antibiotic efflux pump	floR_F	CACGTTGAGCCTCTATAT	868	55	(143)
		floR_R	ATGCAGAAGTAGAACGCG			
strA	Aminoglycoside 3'-phosphotransferase	strA_F1	GCAGGAGGAACAGGAGGGTGC	587	68	This study
		strA_R1	CCCAAGTCAGAGGGTCCAATCG			
strB	Streptomycin 3''-kinase	strB_F1	TCCAGCCTCGTTTGGAAAGT	597	58	This study
		strB_R1	TGCAATGCGTCTAGGATCGA			
aadA	Aminoglycoside (3'') (9) adenylyltransferase	ant(3'')-LA_F	GTGGATGGCGGCCTGAAGCC	526	58	(143)
		ant(3'')-LA_B	ATTGCCCAGTCGGCAGCG			
aac(3)-IV	Aminoglycoside N(3)-acetyltransferase	aac(3)-IV_F	GTTACACCGGACCTTGGA	674	55	(143)
		aac(3)-IV_R	AACGGCATTGAGCGTCAG			
aphA1	Aminoglycoside phosphotransferase	aphA_F	AAACGTCTTGCTCGAGGC	461	55	(143)
		aphA_R	CAAACCGTTATTCATTCGTGA			
sul1	Sulfonamide resistant dihydropteroate synthase	sul1_F	CGGCGTGGGCTACCTGAACG	433	69	(144)
		sul1_R	GCCGATCGCGTGAAGTTCCG			

Target gene/region [a]	Gene product or region description	Primer name	Primers sequences (5'-3') [b]	Amplicon size (bp)	Annealing temperature (°C)	Primers reference
sul2	Sulfonamide resistant dihydropteroate synthase	sul2_F	GCGCTCAAGGCAGATGGCATT	293	69	(144)
		sul2_R	GCGTTTGATACCGGCACCCGT			
sul3	Sulfonamide resistant dihydropteroate synthase	sul3_R	CATCTGCAGCTAACCTAGGGCTTTGGA	789	52	(145)
		sul3_F	GAGCAAGATTTTTGGAATCG			
tetA	Tetracycline efflux pump	tetA_F	GCTACATCCTGCTTGCCT	210	55	(143)
		tetA_R	CATAGATCGCCGTGAAGA			
tetB	Tetracycline efflux pump	tetB_F	TTGGTTAGGGGCAAGTTTTG	600	55	(143)
		tetB_R	GTAATGGGCCAATAACACCG			
dfrA1-like	Trimethoprim resistant dihydrofolate reductase	dfrA1_F	GTGAAACTATCACTAATGG	473	53	(143)
		dfrA1_R	CCCTTTTGCCAGATTTGG			
dfrA12	Trimethoprim resistant dihydrofolate reductase	dfrA12_F	ACTCGGAATCAGTACGCA	462	58	(143)
		dfrA12_R	GTGTACGGAATTACAGCT			
dfrA17	Trimethoprim resistant dihydrofolate reductase	dfrA17_F	GATTTCTGCAGTGTCAGA	384	40	(143)
		dfrA17_R	CTCAGGCATTATAGGGAA			
mcr-1	Phosphoethanolamine transferase	MCR-1_320bp_fw	AGTCCGTTTGTTCTTGTGGC	320	60-62	(77)
		MCR-1_320bp_rv	AGATCCTTGGTCTCGGCTTG			

Target gene/region [a]	Gene product or region description	Primer name	Primers sequences (5'-3') [b]	Amplicon size (bp)	Annealing temperature (°C)	Primers reference
mcr-2	Phosphoethanolamine transferase	MCR-2_700bp_fw	CAAGTGTGTTGGTCGCAGTT	700	60-62	(77)
		MCR-2_700bp_rv	TCTAGCCCGACAAGCATACC			
mcr-3	Phosphoethanolamine transferase	MCR-3_900bp_fw	AAATAAAAATTGTTCCGCTTATG	900	60-62	(77)
		MCR-3_900bp_rv	AATGGAGATCCCCGTTTTT			
mcr-4	Phosphoethanolamine transferase	MCR-4_1100bp_fw	TCACTTTCATCACTGCGTTG	1100	60-62	(77)
		MCR-4_1100bp_rv	TTGGTCCATGACTACCAATG			
mcr-5	Phosphoethanolamine transferase	MCR-5_F	ATG CGG TTG TCT GCA TTT ATC	1644	60-62	(77, 146)
		MCR-5_R	TCA TTG TGG TTG TCC TTT TCT G			
Metal Tolerance						
pcoD	Copper inner membrane pump	pcoD_F	CTGGCCACACTTGCCTGGGG	500	55	(41)
		pcoD_R	CACGCTACGGCGCCCAGAAT			
silA	Silver inner-membrane proton/cation antiporter	silA_Fw	GCAAGACCGGTAAAGCAGAG	936	62	(41)
		silA_Rv	CCTGCCAGTACAGGAACCAT			
merA	Mercuric reductase	merA_1F	ACCATCGGCGGCACCTGCGT	1238	65	(147)
		merA_5R	ACCATCGTCAGGTAGGGGAAC			
arsB	As[III] efflux antiporter	arsB_1F	CGTTACAAACAGCACAGG**Y**A	833	56	(42)
		arsB_1R	T**S**ATGGC**N**GC**N**GG**K**TTTATT			

Target gene/region [a]	Gene product or region description	Primer name	Primers sequences (5'-3') [b]	Amplicon size (bp)	Annealing temperature (°C)	Primers reference
terF	Tellurite resistance protein	terF_Fw1	ATAGCACTGGATCGTGTTCC	990	60	(42)
		terF_Rv	TTCATCGATCCACGGTCTG			
***Escherichia coli* identification and characterization**						
E. coli malB	Maltoporin	ECO1	GACCTCGGTTTAGTTCACAGA	585	59	(79)
		ECO2	CACACGCTGACGCTGACCA			
Phylogenetic groups for *Escherichia coli*						
arpA	Ankyrin repeat protein A	AceK.f	AACGCTATTCGCCAGCTTGC	400	60	(87)
		ArpA1.r	TCTCCCCATACCGTACGCTA			
chuA	Outer membrane heme/hemoglobin receptor	chuA.1b	ATGGTACCGGACGAACCAAC	288	60	(87)
		chuA.2	TGCCGCCAGTACCAAAGACA			
yjaA	Uncharacterized protein	yjaA.1b	CAAACGTGAAGTGTCAGGAG	211	60	(87)
		yjaA.2b	AATGCGTTCCTCAACCTGTG			
TspE4.C2	DNA fragment from putative lipase esterase	TspE4C2.1b	CACTATTCGTAAGGTCATCC	152	60	(87)
		TspE4C2.2b	AGTTTATCGCTGCGGGTCGC			
ybgD	Uncharacterized fimbrial-like protein	ybgD.1	TATGCGGCTGATGAAGGATC	177	59	(88)
		ybgD.2	GTTGACTAAGCGCAGGTCGA			

Target gene/region [a]	Gene product or region description	Primer name	Primers sequences (5'-3') [b]	Amplicon size (bp)	Annealing temperature (°C)	Primers reference
cfaB	CFA/I fimbrial subunit B	cfaB.1	CTAACGTTGATGCTGCTCTG	384	59	(88)
		cfaB.2	TGCTAACTACGCCACGGTAG			
MLST scheme for *Escherichia coli*						
adk	Adenylate kinase	adk_F	ATTCTGCTTGGCGCTCCGGG	583	62	(86)
		adk_R	CCGTCAACTTTCGCGTATTT			
fumC	Fumarate hydratase	fumC_F	TCACAGGTCGCCAGCGCTTC	806	60	(86)
		fumC_R	GTACGCAGCGAAAAAGATTC			
gyrB	DNA gyrase	gyrB_F	TCGGCGACACGGATGACGGC	911	62	(86)
		gyrB_R	ATCAGGCCTTCACGCGCATC			
icd	Isocitrate/isopropylmalate dehydrogenase	icd F	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	878	54	(86)
		icd R	GGACGCAGCAGGATCTGTT			
mdh	Malate dehydrogenase	mdh_F	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	932	68	(86)
		mdh_R	TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT			
purA	Adenylosuccinate dehydrogenase	purA_F	CGCGCTGATGAAAGAGATGA	816	54	(86)
		purA_R	CATACGGTAAGCCACGCAGA			
recA	ATP/GTP binding motif	recA_F	CGCATTCGCTTTACCCTGACC	780	60	(86)
		recA_R	TCGTCGAAATCTACGGACCGGA			

Target gene/region [a]	Gene product or region description	Primer name	Primers sequences (5'-3') [b]	Amplicon size (bp)	Annealing temperature (°C)	Primers reference
***Klebsiella pneumoniae* identification and characterization**						
Kp50233	Putative acyltransferase	50233F	GCTCTGGGAGATAGACCGCA	484	63	(80)
		50233R	GCGATSGCAGACCAGATGAAT			
wzi	Outer membrane protein involved in capsule attachment to the cell surface	wzi_for2	GTGCCGCGAGCGCTTTCTATCTTGGTATTCC	580	55	(85)
		wzi_rev	GAGAGCCACTGGTTCCAGAA**Y**TT**S**ACCGC			
MLST scheme for *Klebsiella pneumoniae*						
rpoB	Beta-subunit of RNA polymerase	Vic3	GGCGAAATGGCWGAGAACCA	1075	52	(84)
		Vic2	GAGTCTTCGAAGTTGTAACC			
gapA	Glyceraldehyde 3-phosphate dehydrogenase	gapA173	TGAAATATGACTCCACTCACGG	662	50	(84)
		gapA181	CTTCAGAAGCGGCTTTGATGGCTT			
mdh	Malate dehydrogenase	mdh130	CCCAACTCGCTTCAGGTTCAG	756	52	(84)
		mdh867	CCGTTTTTCCCCAGCAGCAG			
pgi	Phosphoglucose isomerase	pgi1F	GAGAAAAACCTGCCTGTACTGCTGGC	566	50	(84)
		pgi1R	CGCGCCACGCTTTATAGCGGTTAAT			
phoE	Phosphorine E	phoE604.1	ACCTACCGCAACACCGACTTCTTCGG	602	50	(84)
		phoE604.2	TGATCAGAACTGGTAGGTGAT			
infB	Translation initiation factor 2	infB1F	CTCGCTGCTGGACTATATTCG	462	50	(84)
		infB1R	CGCTTTCAGCTCAAGAACTTC			

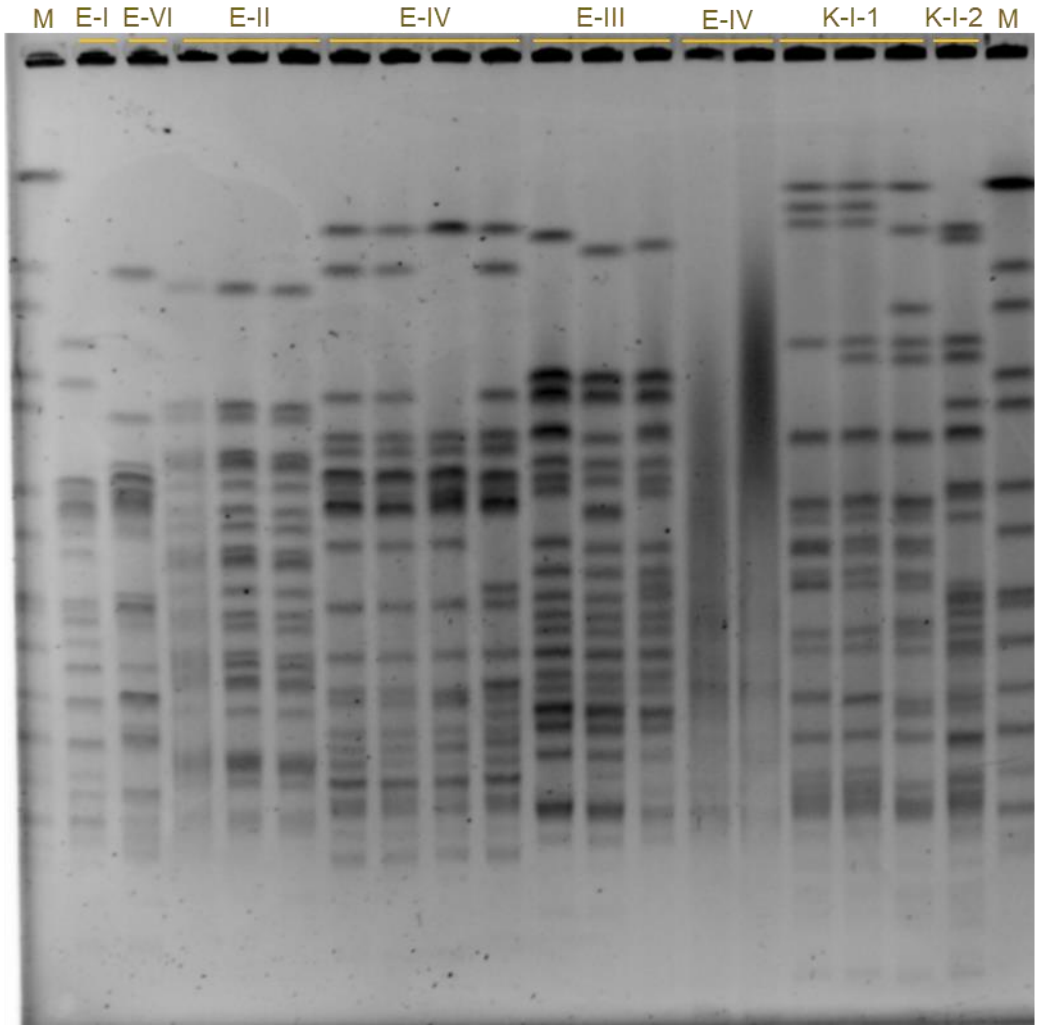
Target gene/region [a]	Gene product or region description	Primer name	Primers sequences (5'-3') [b]	Amplicon size (bp)	Annealing temperature (°C)	Primers reference
tonB	Periplasmic energy transducer	tonB1F	CTTTATACCTCGGTACATCAGGTT	539	50	(84)
		tonB2R	ATTCGCCGGCTGRGCRGAGAG			
Plasmids characterization						
IncHI2 (Iterons)	Plasmid replication protein	HI2_FV	TTTCTCCTGAGTCACCTGTTAACAC	644	60	(89)
		HI2_RW	GGCTCACTACCGTTGTCATCCT			
IncX4 (Specific *taxC* gene)	Plasmid mobilization protein	X4_Fw	AGCAAACAGGGAAAGGAGAAGACT	569	56	(90)
		X4_Rv	TACCCCAAATCGTAACCTG			
IncI2 (Plasmid-specific replication genes *repA* and *repR*)	Replication proteins	I2repR_Fw	TTACAGTGCAAGCTAAGTGCAG	615	58	(148)
		I2repA_Rv	GATTCACG**R**TCCCATATCGT			
pMLST scheme for IncHI2						
smr0018	Open reading frame similar to the *Salmonella* typhi putative surface exclusion protein	smr0018 Fw	ATAATGATTCACCGGGGTAG	364	56	(149)
		smr0018 Rv	CTTCAGGCTATCGTTTCG			
smr0199	Bundle-forming pilus biogenesis protein	smr0199 Fw	TGTTTACACCACCAGCAG	536	58	(149)
		smr0199 Rv	TTTAACAACAGGAGTCGGG			

[a] All the PCR assays were conducted in a Bio-Rad T100TM Thermo Cycler using the NZYTaq II 2x Green Master Mix (*invA*, 16S rRNA, bla_{TEM}, *cmlA-catA-floR*, *strA-strB-aadA-aac(3)-IV-aphA1*, *sul1-sul2-sul3*, *tetA-tetB*, *dfrA1-dfrA12-dfrA17*, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5* *pcoD*,

arsB, *terF*, *E. coli malB*, *Kp50233*, *wzi*, *IncHI2*, *IncX4*, *IncI2*, *smr0018* and *smr0199* genes), Supreme NZYTaq II 2x Colourless Master Mix (*invA*, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5* from total DNA, *merA*, *arpA*, *chuA*, *yjaA*, *TspE4.C2*, *ybgD*, *cfaB*, adk, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*, *rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB* genes) or KAPA Taq Ready Mix (*silA*, *fljB* genes and *fliB-fliA* intergenic region).

[b] Degenerated bases: **H** = A, C or T; **K** = G or T; **N** = A, T, C, G; **S** = G or C; **Y** = C or T, **R** = A or G

Annex II - Example of Pulsed-Field Gel Electrophoresis (PFGE) applied to the diverse clones detected in this study.



E. coli (E-I, E-II, E-III, E-IV, E-V and E-VI) and *K. pneumoniae* (K-I-1 and K-I-2) clones identified by FT-IR. Total genomic DNA of the isolates was digested with the restriction endonuclease *XbaI* (New England Biolabs, USA), according to optimized protocol of Centers for Disease Control and Prevention (CDC) (83). The DNA fragments were separated in a 1% agarose gel (Seakem® Gold Agarose, Lonza) using the equipment CHEF-DR® III Variable Angle System (BIO-RAD, USA). The electrophoresis was performed under the following conditions: 5–20 s for 4 h and 25–50 s for 18 h, 14°C, 6 V/cm^2. *Salmonella enterica* subsp. *enterica* Braenderup H9812 (control strain provided by the CDC) was also digested with the endonuclease *XbaI* (New England Biolabs, USA) and used as a molecular weight marker (M). The gels were stained with ethidium bromide and the images were obtained using the Molecular Image Gel Doc™ XR+ device and the Image Lab™ software (BIO-RAD, USA).