This work was performed at the Microbiology Laboratory of the Faculty of Pharmacy, University of Porto (FFUP, Porto, Portugal). The author received financial support through an individual pre-doctoral fellowship funded by the Fundação para a Ciência e a Tecnologia (FCT) of Portugal with the reference SFRH/BD/93091/2013. The experimental research performed in this work was supported by FCT/Ministério da Educação e Ciência (MEC) through national funds and co-financed by Fundo Europeu de Desenvolvimento Regional (FEDER) under the Partnership Agreement PT2020 with the reference [UID/MULTI/04378/2013–POCI/01/0145/FERDER/007728] as well as through the projects PTDC/AAC-AMB/103386/2008 and from QREN by project NORTE-01-0145-FEDER-000011–Qualidade e Segurança Alimentar—uma abordagem (nano) tecnológica. Additionaly, this work was supported by a European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Food- and Water-borne Infection Study Group (EFWISG) Research Grant - ESCMID Executive Committee [Proposal #8168] attributed under the project entitled “Discrimination of *Salmonella* non-typhoid high-risk clones by highthroughput spectroscopic techniques (MALDI-TOF and FTIR-ATR)”.




**Joana Araújo Alves Campos**

# Tracking *Salmonella* Population Dynamics and Antimicrobial Resistance: local insights with a global impact

*Tese do 3.º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas na Especialidade de Microbiologia*

Trabalho realizado sob a orientação de:

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**July 2018**

**É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.**

**Joana Araújo Alves de Campos**

*À minha querida Família,*
*Amigos,*
*Equipa.*

## AGRADECIMENTOS (ACKNOWLEDGMENTS)

Ao concluir esta Tese de Doutoramento quero deixar expresso a minha gratidão e reconhecimento a instituições e acima de tudo a todas as pessoas que durante este difícil percurso, me acompanharam, auxiliaram e que, de uma forma decisiva, contribuíram para que a realização desta tese fosse possível. MUITO OBRIGADA!

Em primeiro lugar, à minha orientadora Prof. Doutora Patrícia Antunes, coorientadoras Prof. Doutora Luísa Peixe e Doutora Clara Sousa, quero deixar expresso o meu profundo agradecimento por terem aceite esta tarefa tão árdua que é a orientação de um aluno de Doutoramento. Agradeço todo o apoio durante estes quatro anos e a partilha de conhecimentos constante.

À Prof. Doutora Patrícia Antunes quero manifestar um agradecimento muito especial. A conclusão desta tese não teria sido possível sem o seu esforço e contributo. Muito obrigada do fundo do coração pela paciência, pelo estímulo diário, pelos conhecimentos transmitidos, pelas opiniões, críticas e motivação. Agradeço a sua naturalidade e espontaneidade, que faz de si a pessoa especial e única que é! Agradeço ainda a repreensão quando estava errada, bem como o reforço positivo quando estava certa, o que para mim é a verdadeira definição de Orientadora. Hoje, quero manifestar a minha gratidão, admiração e carinho. Nunca, mas nunca a esquecerei!

*"Feliz aquele que transfere o que sabe e aprende o que ensina."*

**Cora Coralina**

À Prof. Doutora Luísa Peixe, um muito obrigado por ter permitido realizar o meu trabalho de doutoramento na sua equipa, da qual me orgulho em pertencer. Obrigada por me proporcionar os meios para a realização do mesmo e pelas discussões de cariz construtivo que foram cruciais para minha formação e desenvolvimento de espírito crítico, de modo a alcançar o sucesso! Obrigada pelo grande incentivo e pelas oportunidades que me foram dadas para a realização de cursos e estágios no estrangeiro, o que contribuiu para a pessoa independente que sou hoje.

*"Nossas vidas começam a terminar no dia em que permanecemos em silêncio sobre as coisas que importam."*

**Martin Luther King Jr.**

Em segundo lugar quero agradecer todo o apoio financeiro concedido ao longo destes quatro anos de doutoramento: à Fundação para a Ciência e Tecnologia (FCT), Ministério da Ciência, Tecnologia e Ensino Superior de Portugal (MEC) (Bolsa SFRH/BD/93091/2013, QREN – POPH – Tipologia 4.1 – Formação Avançada, comparticipado pelo Fundo Social Europeu e por fundos sociais do MEC). Adicionalmente, agradeço à Unidade de Investigação/FCT - UCIBIO/REQUIMTE (através do projeto PEst-C/EQB/LA0006/2011 UID/MULTI/04378/2013–POCI/01/0145/FERDER/007728, PTDC/AAC-AMB/103386/2008, QREN - NORTE-01-0145-FEDER-000011–Qualidade e Segurança Alimentar—uma abordagem (nano) tecnológica e FCT/MEC ao abrigo do programa PT2020 UID/MULTI/04378/2013).

À Prof. Doutora São José Nascimento agradeço a oportunidade de realizar este trabalho no Laboratório de Microbiologia da Faculdade de Farmácia da Universidade do Porto. A todos os Prof. Doutores do Laboratório de Microbiologia agradeço todas as palavras de carinho, disponibilidade e a simpatia com que me receberam.

À Prof. Doutora Nazaré Pestana, que me fez "olhar" para a Microbiologia. Hoje digo orgulhosamente que já fui sua aluna. Agradeço toda a preocupação e palavras de conforto. Foi consigo que a "minha história" começou!

Ao Doutor Rafael Cantón por ter permitido o meu estágio no Hospital Universitário Ramon Cajal em Madrid, estágio esse que foi fundamental para obtenção de dados e para a inclusão neste trabalho. E um agradecimento muitíssimo especial ao Doutor Juan de Dios-Caballero, pela preocupação, apoio e acompanhamento diário, o que tornou muito mais fácil de ultrapassar as dificuldades. *"Muchas Gracias por todo!"*

Não posso deixar de agradecer à Doutora Margarida Saraiva, Teresa Lopes e à restante equipa do Departamento de Alimentação e Nutrição INSA-Porto, que para além da determinante contribuição na minha formação, me receberam com todo o carinho e simpatia. Obrigada! Agradeço também ao Doutor Jorge Machado e Leonor Silveira do Laboratório Nacional de Referência de Infeções Gastrintestinais, Departamento de Doenças Infeciosas INSA-Lisboa, por também contribuírem com todo o profissionalismo para a minha formação.

À Cristina Pinto da Costa um agradecimento muito especial, que para além de ser uma técnica indispensável no Laboratório de Microbiologia da FFUP, é uma pessoa extraordinária. Agradeço toda a ajuda e apoio técnico que me deu, crucial para o meu trabalho experimental, e sobretudo a amizade, gargalhadas e o carinho maternal nos momentos mais complicados. Algo difícil de explicar, impossível de esquecer, e que vou guardar eternamente no meu coração. À Doutora Cristina Réu por todas as palavras de apoio, incentivo e carinho. Ao Nuno Oliveira agradeço todo o apoio, risadas e incentivo ao longo destes anos, sem esquecer que foi

contigo que comecei a dar os meus “primeiros passos” no Laboratório de Microbiologia da FFUP.

**À minha EQUIPA, que tanto me orgulho em pertencer:**

Aos **companheiros, amigos** e **confidentes** de longa data, Doutora Joana Mourão, (feliz por já dizer) Prof. Doutora Liliana Silva e a incomparável Doutora Teresa Ribeiro, quero agradecer todos os momentos de companheirismo, amizade, risadas e conforto! Estes quatro anos não seriam tão especiais sem vocês! Ao João Botelho, que apesar de um começo atribulado, nunca vou esquecer das tuas palavras de força e carinho!

Às **“Séniores”** Prof. Doutora Carla Novais, Prof. Doutora Ana Freitas, Doutora Filipa, Doutora Ângela, que para além da partilha de conhecimentos, quero agradecer muitíssimo os conselhos, as palavras de motivação e incentivo. Nunca esquecerei!

Às “**Júniores**”, Magdalena Księżarek e Andreia Rebelo, e “**Séniores**” mais recentes, Doutora Joana Rocha e Doutora Svetlana Perovic, sem dúvida que também tornaram este caminho muito mais fácil de ultrapassar. Obrigada pelo carinho, simpatia e apoio.

Aos companheiros que um dia já fizeram parte desta Equipa**,** mas que nunca me esquecerei: Prof. Doutora Sandra Quinteira, agradeço o apoio e as gargalhadas que me arrancou; Prof. Doutora Eduarda Silveira (minha Edu), agradeço o companheirismo, conselhos e palavras de incentivo; Prof. Doutora Elisabete Machado agradeço o apoio e conhecimento transmitido e à Doutora Ana Sofia por toda a amizade e carinho. Finalmente, ao mais recente Doutor João Pires e, futuramente, Michael Brilhante, muito obrigada pela vossa AMIZADE!

A todos os alunos de projeto e de iniciação à investigação da Faculdade de Ciências da Nutrição e Alimentação e Faculdade de Ciências da UP, Sara Marçal, Joana Pereira, Joana Gil, Luís Cristino e Sofia Ribeiro, toda a ajuda prestada e simpatia. Foram todos profissionais e cruciais para que o meio trabalho se realizasse com qualidade.

Aos alunos estrangeiros, Houyem, Aymen e António. Obrigada por todos os momentos únicos que passei com vocês. Foi sem dúvida um prazer conhecer-vos.

*“A amizade, depois da sabedoria, é a mais bela dádiva feita aos homens.”*

**François La Rochefoucauld**

**Aos AMIGOS de uma vida:**

**Às minhas "Irmãs"**, Sofia e Carla, obrigada por todo o AMOR e APOIO incondicional, por me motivarem e terem acreditado em mim! Obrigada pelos dois irmãos que ganhei às vossas custas, Pedro e Luís, que de igual modo demonstraram sempre o carinho e apoio que precisava!

**À Sofia**, obrigada por estes 23 anos de uma verdadeira amizade. Por me conheceres tão bem, uma sintonia tal que até por um simples olhar sabes o que penso e sinto! Obrigada pelos abraços, risadas, carinho, pelo apoio e sermões, que me ajudaram ao longo destes anos a tornarem na pessoa que sou hoje! Obrigada pela minha querida e deliciosa Benedita, que em tão pouco tempo já mudou a minha vida!

**À Carla,** minha "*avec"* preferida, a nossa história começou em 2012, Londres. Das poucas certezas que tenho, é que esta amizade não terá um fim! Aliás, já faço parte da tua família, os teus amigos são meus amigos, e sinto Bragança como minha também! Apesar da distância, deixaste-me uma prendinha que me agarro quando a saudade mais aperta, e por momentos a distância Porto-Paris diminui. De ti ficam todas as gargalhadas, os aparatos, os dramas, as confidências. Obrigada pelo apoio (a dita "*conchinha*") que foi essencial para superar momentos muito complicados. O que mais espero é que brevemente me dês o prazer inexplicável de sentir que vou ser tia novamente (e não me refiro a outro cão, a Kira e Kika são únicas)!

Aos meus amigos, Joana Mercedes, Luís Xavier; aos meus amigos de há 30 anos: Iolanda Abreu (Paulo, Santiago, querido afilhado Salvador), Carla Sofia (Toni e o Gonçalinho), Tiago Gomes e Pedro Dieguez, ao Miguel (Daniela e o pequenote Elias); à Sandrinha, e por fim, aos meus antigos colegas e grandes amigos da FCUP, Joana Balça e João Carneiro (e Diana), a todos vocês agradeço a amizade, carinho, apoio e motivação ao longo destes anos. Obrigada por fazerem parte da minha vida e tornarem este percurso mais fácil! Aliás:

*"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis."*

**Fernando Pessoa**

**À minha Família:**

Aos meus queridos Padrinhos: Nininha, Ritinha, Chiquinho e Miguel! Obrigada pelo apoio incondicional e fazerem parte da minha vida.

À minha querida "**Madrinha Grande**"**,** Maria Teresa Garrett, obrigada por ter acreditado em mim, e juntamente com o "Padrinho Grande", me ter educado, dado oportunidades para uma infância feliz e inesquecível e que contribuiu para a pessoa que sou hoje!

*"Aurora da existência, infância amável, idade abençoada da mão que rege, que aviventa os dias, mimo da natureza, da cândida inocência bafejada, breve, mas linda flor sobre o gomo da vida despontada, infância, oh! meiga idade!"*

**Almeida Garrett**

Ao meu PAI, ao meu IRMÃO Francisco, à Lídia e ás minhas lindas sobrinhas, Lara e Natacha e aos meus queridos AVÓS. Obrigada por acreditarem em mim, pelos conselhos, pelo apoio e nunca me terem deixado desistir!

À Minha querida **MÃE**,

porque …

*… "A Mãe é a mais bela obra de Deus."*

**Almeida Garrett**

## ABSTRACT

Non-typhoidal *Salmonella* (NTS) is one of the leading causes of zoonotic foodborne diseases worldwide, with poultry and pork products being the most frequent sources of human infections. Several changes along the food-chain are leading to new problems and challenges regarding salmonellosis burden and control, namely involving the intensive food animal production, large-scale production/distribution, globalization of food supply as well as changes in the consumer demands and lifestyles (e.g. increase demand of poultry and pork meat products). Nevertheless, the extension of the impact of critical antibiotics usage in intensive food-animal production and of food trade globalization in the emergence and spread of antibiotic resistant NTS are insufficiently established. Additionally, the role of aquacultures, a food sector of growing relevance, and of several regions (e.g. African countries) as reservoirs and transmission routes of NTS has been scarcely addressed. The availability of rapid, low-cost and accurate typing methods is required for the clarification of those issues. Among the promising methodological alternatives are Fourier Transform Infrared Spectroscopy (FTIRS) and Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS), high throughput methodologies of potential interest, still barely explored for *S. enterica* discrimination at different infra-species levels.

The **goals** of this thesis were: i) to unveil the contribution of several food-chain drivers for the emergence and spread of antibiotic resistant NTS, including their role in the animal-food-human transmission of *Salmonella* in different epidemiological scenarios; and ii) to assess the suitability of FTIRS and MALDI-TOF MS for *S. enterica* discrimination at different infra-species levels. To accomplish these purposes, 1010 *S. enterica* isolates (2002-2015), selected by serogroups, serotypes, clones, sources (human clinical cases, food-animal production settings, national/imported food products) and regions were analysed. Additionally, 53 samples from national trout aquacultures (2010-2012) and 63 non-clinical samples from Benguela, Angola (2013) were studied. The potential of FTIRS and MALDI-TOF for *S. enterica* discrimination was assessed using a representative set of isolates from different serogroups, serotypes, and *S.* Typhimurium and *S.* 1,4,[5],12:i:- clones (2002-2015).

The **results** of this Thesis revealed the circulation of *mcr-1* gene, conferring colistin resistance, particularly among isolates from pork products and human infections, since 2011, reflecting the high use of polymyxins in Portuguese intensive pig food-production. The detection of *mcr-1* on broad-host range plasmids (IncX4/IncHI2) and on successful pig-associated MDR clones (*S.* 1,4,[5],12:i:-/ST34 and *S.* Rissen/ST469) alerts for the potential spread of colistin resistance by plasmid and clonal expansion. The detection of *mcr-1* in those copper-tolerant clones raises questions about the efficacy of copper-based interventions to reduce colistin use and contain *mcr-1* dissemination. Additionally, an IncHI2 plasmid-carrying *oqxAB* gene, conferring resistance to fluoroquinolones and to other antimicrobials widely used in animal-production, was detected in metal-tolerant *S.* Typhimurium/ST34 isolates. The similarity of the *S.* Typhimurium strain and *oqxAB* genetic platform with others found in human/animal settings in Asia, suggests international animal/food trade as a potential driver for their occurrence in our country. The relevance of food trade globalization was further corroborated with the characterization in imported poultry products of uncommon extended-spectrum cephalosporin-resistant *Salmonella* serotypes (*S.* Minnesota and *S.* Heidelberg) carrying $bla_{\text{CMY-2}}$ located on two

epidemic plasmids (IncA/C-ST2 or IncI1-ST12), including a strain similar to an American epidemic clone causing invasive human infections. These data raise questions about EU food regulations setting a food safety criterion for fresh poultry meat, which not takes into account emergent and uncommon poultry related-serotypes. In which concerns the contribution of aquacultures to NTS spread, our findings support the possibility of NTS transmission to humans associated with farmed trout consumption or contact with contaminated water from the trout farms. The river water supplying the trout farms was identified as the source of the NTS serotypes contamination detected in the summer season. Remarkably, colistin resistant *Salmonella* with new mutations in *pmrAB* genes was observed, possibly reflecting the high use of colistin in animal food-production in Portugal. A high occurrence and diversity of unusual NTS serotypes (healthy humans, aquatic environments, farm and wild animals), including putative new *S. enterica* subsp. *salamae* serotypes, was observed in Benguela (Angola). These data extended the knowledge about NTS serotypes reservoirs and possible transmission routes in a geographical region with commercial/travel relationships with Europe. Finally, this thesis contributed to elucidate the potential of FTIRS and MALDI-TOF MS on *S. enterica* discrimination at the infra-species level. FTIRS was able to discriminate isolates belonging to clinically-relevant serogroups (B, C, D and E), sub-serogroups (C1, C2 and C3, and E1-E2-E3 and E4), and particular serotypes (*S.* Rissen and *S.* Enteritidis). Moreover, we demonstrated the role of somatic O-antigen composition on the serogroup discrimination achieved by FTIRS. Conversely, MALDI-TOF MS showed a limited potential for serogroup discrimination, possibly due to a high conservation degree of ribosomal proteins. Nevertheless, good discrimination rates were observed for relevant serotypes (*S.* Enteritidis, *S.* 1,4,[5],12:i:-, *S.* Rissen), paving a way for further methodological improvements. Both techniques showed a limited discriminative potential for *S.* Typhimurium and *S.* 1,4,[5],12:i:- clones.

This Thesis provided significant advances on the knowledge and impact of several food-chain drivers, namely the use of human critical antibiotics (colistin, fluoroquinolones, extended-spectrum cephalosporins) in intensive food-animal production and the global food trade, for the emergence and spread of antibiotic resistant NTS transmitted to humans through the food-chain. This work also unveiled potential reservoirs and transmission routes of NTS serotypes from understudied food-producing systems (trout aquacultures) and regions (Benguela, Angola). Finally, our results showed that FTIRS is more reliable than MALDI-TOF MS to assist *Salmonella* typing at serogroup, sub-serogroup and serotype levels, with potential application on microbiology laboratories, as well as the first evidences of cellular components leading to FTIRS serogroup discrimination.

**Keywords:** Non-typhoidal *Salmonella*, food-chain drivers, critical antibiotics, typing, high-throughput methodologies

## RESUMO

*Salmonella* não tifóide (NTS) é um dos principais agentes zoonóticos causadores de doenças de origem alimentar a nível mundial, sendo os produtos derivados de aves e de porco as principais fontes de infeção humana. Alterações em diferentes fatores ao longo da cadeia alimentar (por exemplo, na produção animal intensiva e sua origem geográfica) podem influenciar a ocorrência de salmonelose assim como o sucesso de medidas estabelecidas para o seu controlo. Contudo, a extensão do impacto da utilização de antibióticos críticos na produção animal intensiva e da globalização do comércio de alimentos na emergência e disseminação de estirpes de NTS resistentes a antibióticos ainda se encontra insuficientemente estabelecida. Para além disso, o papel das aquaculturas (setor de produção alimentar de relevância crescente) e de várias regiões (ex. países de África), como reservatórios e vias de transmissão de NTS, tem sido pouco explorado. Métodos de tipagem rápidos, baratos e precisos são necessários para o esclarecimento destes problemas. Entre as alternativas metodológicas promissoras encontram-se a *Fourier Transform Infrared Spectroscopy* (FTIRS) e *Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry* (MALDI-TOF MS), metodologias de alto rendimento com potencial interesse, apesar de ainda pouco exploradas para a discriminação de *S. enterica* a diferentes níveis de infra-espécie.

Os **objetivos** deste trabalho consistiram em: i) elucidar a contribuição de vários fatores da cadeia alimentar na emergência e disseminação de NTS resistentes a antibióticos, incluindo do seu papel na transmissão animal-alimentos-Homem, em diferentes cenários epidemiológicos; e ii) explorar o potencial de FTIRS e MALDI-TOF MS na tipagem de *S. enterica*. Para o efeito, foram estudados 1010 isolados de *S. enterica* (2002-2015), selecionados por serogrupos, serótipos, clones, origens (clínicos humanos, ambientes de produção animal, produtos alimentares nacionais/importados) e regiões. Além disso, foram estudadas 53 amostras de aquaculturas nacionais de trutas (2010-2012) e 63 amostras não clínicas de Benguela, Angola (2013). O potencial de FTIRS e MALDI-TOF na discriminação de *S. enterica* foi avaliado utilizando um conjunto de isolados representativos de diferentes serogrupos, serótipos e clones de *S.* Typhimurium e *S.* 1,4,[5],12:i:- (2002-2015).

Os **resultados** obtidos no âmbito desta Tese demonstraram a circulação, desde 2011, do gene *mcr-1*, que confere resistência à colistina, em isolados associados a carne de porco e infeções humanas, refletindo o elevado uso de polimixinas na produção intensiva de suínos em Portugal. A deteção de *mcr-1* em plasmídeos de largo espectro (IncX4/IncHI2) e em clones bem sucedidos de *Salmonella* associados a suínos (*S.* 1,4,[5],12:i:-/ST34 e *S.* Rissen/ST469) e resistentes a múltiplos antibióticos, alerta para a potencial disseminação clonal ou por expansão plasmídica da resistência à colistina. A deteção de *mcr-1* nesses clones tolerantes ao cobre levanta questões sobre a eficácia das intervenções baseadas na aplicação deste metal para reduzir o uso de colistina e conter a disseminação de *mcr-1*. Além disso, foi detetado em isolados de *S.* Typhimurium/ST34 tolerantes a metais um plasmídeo IncHI2 contendo o gene *oqxAB*, o qual confere resistência a fluoroquinolonas e a outros agentes antimicrobianos amplamente utilizados na produção animal. As semelhanças encontradas entre a estirpe de *S.* Typhimurium e a plataforma genética contendo o gene *oqxAB* com isolados de humanos/animais na Ásia, sugere que o comércio internacional de animais/alimentos poderá ser um potencial fator para a sua ocorrência em Portugal. A relevância da globalização do comércio alimentar foi também corroborada com a caracterização em produtos avícolas importados de serótipos pouco comuns de *Salmonella* e resistentes a cefalosporinas de espectro alargado (*S.* Minnesota e *S.* Heidelberg) com

*bla*$_{CMY-2}$ localizado em dois plasmídeos epidémicos (IncA/C-ST2 ou IncI1-ST12 transferível), incluindo uma estirpe semelhante a um clone epidémico Americano a causar infeções humanas invasivas. Estes dados questionam os regulamentos alimentares da União Europeia que estabelecem um critério de segurança para carne fresca de aves de capoeira que não tem em conta serótipos emergentes e pouco comuns associados a aves. No que diz respeito à contribuição das aquaculturas para a disseminação de NTS, os nossos resultados suportam a possibilidade de transmissão para o Homem de NTS associada ao consumo de trutas de aquacultura ou através do contato com água contaminada. A água do rio que abastece estas aquaculturas foi identificada como a fonte de contaminação por NTS durante a estação de verão. De salientar, a deteção de isolados de *Salmonella* resistentes à colistina com novas mutações nos genes *pmrAB*, possivelmente refletindo o elevado uso de colistina na produção animal em Portugal. Em Benguela (Angola) foi também observada uma elevada ocorrência e diversidade de serótipos pouco comuns de NTS (humanos saudáveis, ambientes aquáticos, produção animal e animais selvagens), incluindo possíveis novos serótipos de *S. enterica* subsp. *salamae*. Estes dados vieram contribuir para avanços do conhecimento sobre os reservatórios de serótipos de NTS e possíveis vias de transmissão, numa região geográfica com relações comerciais estreitas com a Europa. Finalmente, esta tese contribuiu para elucidar o potencial de FTIRS e MALDI-TOF MS na discriminação de *S. enterica* a diferentes níveis de infra-espécie. O FTIRS mostrou ser capaz de discriminar isolados pertencentes a serogrupos clinicamente relevantes (B, C, D e E), sub-serogrupos (C1, C2 e C3, e E1-E2-E3 e E4), e serótipos particulares (*S.* Rissen e *S.* Enteritidis). Além disso, foi demonstrado o papel da composição da unidade somática antigénio-O na discriminação de serogrupos obtida pelo FTIRS. Em contraste, o MALDI-TOF MS mostrou ter um potencial limitado para a discriminação de serogrupos, possivelmente devido ao elevado grau de conservação das proteínas ribossomais. No entanto, mostrou ter um bom grau de discriminação de alguns serótipos clinicamente relevantes (*S.* Enteritidis, *S.* 1,4,[5],12:i:-, *S.* Rissen), abrindo caminho a posteriores melhorias metodológicas. Ambas as técnicas mostraram um potencial limitado para a discriminação de clones de *S.* Typhimurium e *S.* 1,4,[5],12:i:-.

Esta Tese contribuiu para produzir avanços significativos no conhecimento e impacto de vários fatores da cadeia alimentar, nomeadamente o uso de antibióticos críticos (colistina, fluoroquinolonas, cefalosporinas de espectro alargado) na produção animal intensiva e o comércio global de alimentos, para a emergência e disseminação de NTS resistentes a antibióticos transmitidos ao Homem através da cadeia alimentar. Este trabalho revelou também potenciais reservatórios e vias de transmissão de serótipos de NTS em sistemas de produção de alimentos (aquaculturas de truta) e regiões (Benguela, Angola) pouco estudados. Finalmente, o FTIRS revelou maior potencial para a tipagem de *Salmonella* ao nível de serogrupo, sub-serogrupo e serótipo do que o MALDI-TOF MS, com aplicação potencial em laboratórios de microbiologia, evidenciando-se pela primeira vez os componentes celulares que levam à discriminação de serogrupos.

**Palavras-chave:** *Salmonella* não tifóide, fatores da cadeia alimentar, antibióticos críticos, tipagem, metodologias de alto rendimento.

## CONTENTS

Chapter 1

# INTRODUCTION

## 1.1. Revision Article

## Salmonellosis: The role of poultry meat

REVIEW

# Salmonellosis: the role of poultry meat

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## Abstract

Salmonellosis remains one of the most frequent food-borne zoonoses, constituting a worldwide major public health concern. Currently, at a global level, the main sources of infection for humans include meat products, including the consumption of contaminated poultry meat, in spite of the success of *Salmonella* control measures implemented in food-animal production of industrialized countries. In recent years, a shift in *Salmonella* serotypes related to poultry and poultry production has been reported in diverse geographical regions, being particularly associated with the spread of certain well-adapted clones. Moreover, antimicrobial resistance in non-typhoidal *Salmonella* is considered one of the major public health threats related with food-animal production, including the poultry production chain and poultry meat, which is an additional concern in the management of salmonellosis. The circulation of the same multidrug-resistant *Salmonella* clones and/or identical mobile genetic elements encoding antibiotic resistance genes from poultry to humans highlights this scenario. The purpose of this review was to provide an overview of the role of poultry meat on salmonellosis at a global scale and the main problems that could hinder the success of *Salmonella* control measures at animal production level. With the increasing globalization of foodstuffs like poultry meat, new problems and challenges might arise regarding salmonellosis control, making new integrated intervention strategies necessary along the food chain.

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**Keywords:** AmpC, extended-spectrum β-lactamases, multidrug-resistance, plasmid-mediated quinolone resistance, *Salmonella*, Enteritidis, Infantis, Kentucky, Heidelberg, Typhimurium, Virchow

**Article published online:** 17 December 2015

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## Introduction

*Salmonella* infections are a worldwide major public health concern; Salmonellosis is caused by non-typhoidal *Salmonella enterica* serotypes (serotypes other than S. Typhi and S. Paratyphi) and is typically characterized by a self-limiting gastroenteritis syndrome (manifested as diarrhoea, fever and abdominal pain), with an incubation period between 4 and 72 h and mortality being rare [1–3]. In healthy humans, the infectious dose is generally $10^6$ to $10^8$, but lower bacterial counts can cause disease in certain conditions, as well as in infants and the elderly [2]. Although uncommon, life-threatening invasive infections with bacteraemia (5%–10% of infected persons) and/or other extra-intestinal infections may occur, affecting especially the risk groups (infants, young children, older people and immunocompromised patients) [1–3]. In severe cases, effective antimicrobial agents are essential, so the emergence of *Salmonella* that are resistant to critical antibiotics is of concern [1,2].

In industrialized countries, the main reservoir of non-typhoidal *Salmonella* is the intestinal tract of food-producing animals, which readily leads to contamination of diverse foodstuffs [3–5]. Therefore, despite other sources (e.g. contact with animals/reptiles, environment or person-to-person), food-borne salmonellosis is the most relevant source with a high

*Clin Microbiol Infect* 2016; **22:** 110–121
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http://dx.doi.org/10.1016/j.cmi.2015.12.004

global impact in human health. It was estimated that non-typhoidal *Salmonella* causes around 93.8 million illnesses and 155 000 deaths each year worldwide [6]. In the USA, more than 1 million annual cases of food-borne salmonellosis were estimated by the CDC; they were associated with the largest number of hospitalizations and deaths compared with other food-borne microbial agents [7]. In Europe, salmonellosis has been the second most common zoonosis (82 694 confirmed cases and 20.4 cases per 100 000 population in 2013) and the most frequent cause of food-borne outbreaks, in spite the reported decreasing trend that has resulted from *Salmonella* control programmes [8].

Although different serotypes have been associated with salmonellosis, a limited number are responsible for most human infections; *S. enterica* Enteritidis being the most frequent one in the EU (39.5% in 2013) and USA (14.5% in 2012) followed by *S. enterica* Typhimurium (including its monophasic variant) (28.8% in EU, 2013; 11.6% in USA) [8,9]. *Salmonella* Enteritidis is commonly associated with poultry and products thereof, whereas *S.* Typhimurium has a wider species range, including pigs and cattle as well as poultry [10]. Therefore, foods of animal origin, in particular contaminated poultry products (eggs and poultry meat) have been considered the main vehicles of *Salmonella* infection and clearly associated with the worldwide epidemic of *S.* Enteritidis [4,5,11,12]. Moreover, diverse epidemiological studies have supported the great contribution of poultry foodstuffs to the salmonellosis burden [4,5,12,13].

In recent years, with the implementation of *Salmonella* control programmes mainly in poultry production (e.g. in the EU and USA), changing trends in food-borne salmonellosis and associated serotypes were observed, with the expansion of previously less common serotypes, frequently resistant to antibiotics. With the increasing globalization of foodstuffs like poultry meat, which is one of the most consumed and increasingly globally traded meat products, new problems might arise regarding salmonellosis control. An overview of the role of poultry meat in salmonellosis at a global scale is provided in this review.

## Non-typhoidal *Salmonella* and poultry meat

Poultry populations, in particular chicken and turkey, are frequently colonized with *Salmonella* without detectable symptoms (sub-clinical infections/healthy carriers) by horizontal and vertical transmission at primary production level [4,5]. The presence of *Salmonella* in healthy poultry animals is suggested as the main risk factor, by allowing bacteria to easily transmit in table eggs and poultry meat to humans [10]. In fact, in Europe it is assumed that the observed reduction in salmonellosis cases (32% between 2008 and 2012) is mainly due to successful *Salmonella* control measures (involving surveillance, biosecurity and vaccination) implemented in poultry/egg production and focused on particular serotypes (e.g. *S.* Enteritidis and *S.* Typhimurium) that are considered of public health significance [4,8,10,12]. These measures led to the achievement of reduction targets for poultry populations in most EU countries and lower non-compliance regarding *Salmonella* in poultry products [4,8]. Moreover, decreasing contamination rates in raw poultry products are in agreement with those recently observed in industrialized countries from other geographical regions with pathogen reduction programmes, such as the USA [12,14,15]. It should be noted that by the 2000s a high incidence of *Salmonella* in poultry products was reported in the EU, with rates >50% for several countries [16]. In the 2013 zoonosis EFSA/ECDC report involving data from European countries, as in previous years, *Salmonella* was most frequently reported, although at low levels, in fresh turkey (5.4%) and fresh broiler meat (3.5%), in comparison with eggs (0.1%) or fresh pig meat (0.7%) [8]. Despite the highest incidence being detected in poultry meat, eggs still remain the most important source of food-borne *Salmonella* outbreaks [8]. In fact, using quantitative source attribution models the higher number of human salmonellosis cases in Europe was attributable to eggs (65% in 2011 and 17% in 2012) and pigs (28% in 2011 and 56.8% in 2012) compared with broilers (2.4% in 2011 and 10.6% in 2012) and turkey meat (2.6% in 2011 and 4.5% in 2012) [17,18]. However, diverse surveys targeted to detect *Salmonella* in poultry products in developing countries, some with expansion of the poultry industry, still detected high percentages of positive samples, ranging from ~13% to 39% in South America [19,20], ~35% in Africa [21,22] and ~35% to 50% in Asia [23–25]. Those differences possibly reflect diverse poultry production husbandry practices and absence of control measures along the food chain, highlighting the importance for *Salmonella* spread of the extensive international trade in animals and their products [4].

Worldwide data about *Salmonella* serotype prevalence in humans and in the diverse range of foodstuffs have contributed to establish an epidemiological link between salmonellosis and poultry products, with diverse serotypes overlapping between humans and poultry meat (chicken and turkey) (Fig. 1). In the EU, recent changes in the frequency of *Salmonella* serotypes causing human infections were reported, which in some cases were in line with those occurring in poultry (Fig. 1). Nevertheless, interpretation of these data should be cautious, owing to limitations in the number of poultry isolates serotyped each year. Of particular relevance is the decrease in *S.* Enteritidis human cases (19% reduction between 2011 and 2013 in the EU), a serotype typically associated with poultry meat and egg

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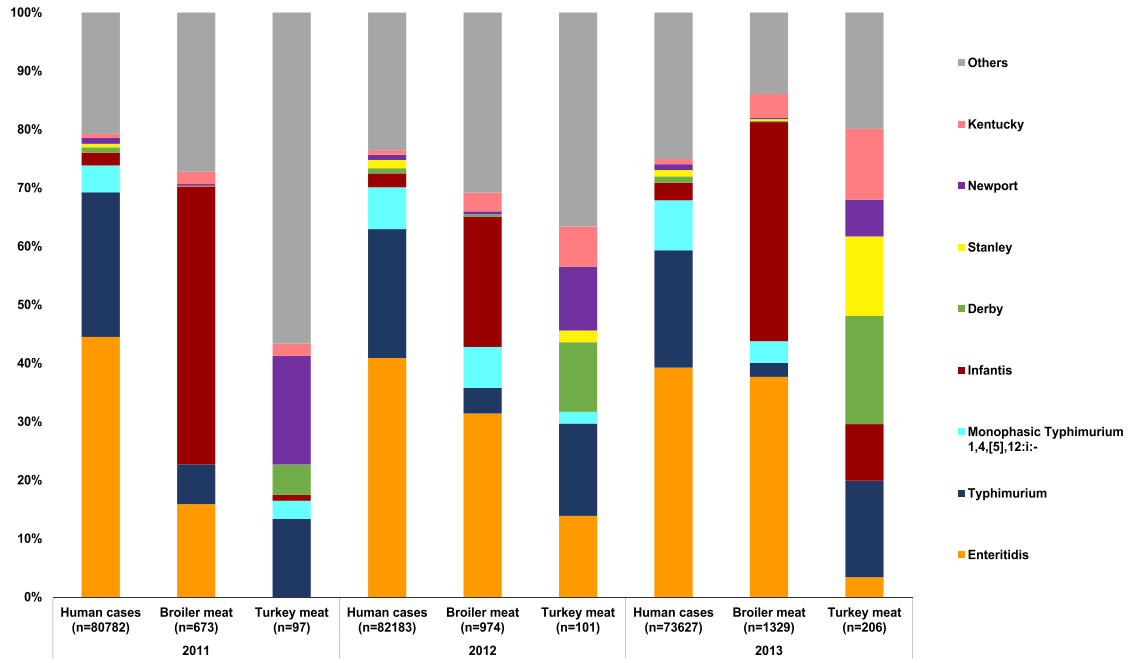


**FIG. 1.** Distribution of the major serotypes of non-typhoidal *Salmonella* associated with human cases (salmonellosis) and poultry meat in EU, 2011 to 2013. Data were obtained from EFSA reports: humans (2011–2013) and turkey/broiler meat (2013) [8]; turkey/broiler meat (2011–2012) (http://www.efsa.europa.eu/en/efsajournal/pub/3129 and http://www.efsa.europa.eu/en/efsajournal/pub/3547) (last accessed 1 September 2015). The percentages were calculated based on the total number of serotyped isolates per type of meat or human salmonellosis cases.

consumption, in line with a decreasing trend in poultry and poultry products pointed by different studies [8,12,26–29]. Increasing occurrences of other serotypes implicated in human infections (e.g. *S.* Infantis, *S.* Stanley, *S.* Kentucky) related with poultry meat (chicken and turkey) have been reported in the EU (Fig. 1). However, one major difference in the serotype pattern between humans and poultry is related to *S.* Typhimurium and its monophasic variant, both frequently associated with human salmonellosis, but less common in poultry meat, pigs and pig meat being the main source [8]. In USA and Canada, other serotypes, like *S.* Heidelberg and *S.* Kentucky, have emerged as predominant serotypes in poultry and have also been implicated in human salmonellosis, beyond *S.* Enteritidis [9,12,14,15,28,30–32].

The shift in *Salmonella* serotypes related to poultry and poultry production has been associated with the spread of certain clones. For instance, the *S.* Infantis increase (26.5% in human cases between 2011 and 2013 accounting for the fourth most common serotype and the most reported in broilers and broiler meat) [8], has been associated with the spread of several clones of broiler origin in diverse European countries, including the dominant Hungarian clone [33–35]. Also, *S.* Stanley showed an increase since 2011 with a peak in 2012, being the sixth most common human serotype and one of the three most common in turkey meat, together with *S.* Derby and *S.* Kentucky in 2013 [8]. A large *S.* Stanley outbreak caused by a new clone (novel Pulsed Field Gel Electrophoresis-type), was linked with the consumption of turkey meat, and is still circulating in the European food market (at least since 2011), with a considerable risk of becoming endemic in the poultry production chain in Europe [36–38]. In the USA, *S.* Heidelberg in particular has been identified as one of the top human and poultry serotypes, with several clones implicated in diverse large multistate outbreaks resulting from the consumption of contaminated chicken or turkey products [12,31]. The spread and the global persistence of serotype *S.* Kentucky reflect other particular situations related to the increased globalization of travel and the food/animal trade in different geographical regions. This serotype has been associated with a worldwide (Europe, Africa and Asia) spread of a particular epidemic clone (*S.* Kentucky ST198-X1), recovered from several livestock reservoirs, particularly poultry farms, with chicken and turkey implicated as the potential major human infection vehicles [39–43]. These and other examples of multi-country/multi-state outbreaks or clonal expansion of *Salmonella* infections linked to poultry meat (Table 1) serve as a reminder of the importance of acting upon any *Salmonella* contamination in the food chain and monitoring to detect the emergence of any serotype or new clone. This

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**TABLE 1. *Salmonella* outbreaks and emerging clones linked with poultry meat products (2002–ongoing)**

| Serotype (outbreak/clone designation)[a] | Source | Year(s)[b] | Geographical region | No. of cases[c] | Reference |
|---|---|---|---|---|---|
| Enteritidis (outbreak) | Chicken | 2015– | USA | 3 | CDC, 2015[d] |
| Enteritidis (outbreak) | Chicken | 2015– | USA | 9 | CDC, 2015[d] |
| Hadar (outbreak) | Turkey | 2010–2011 | USA | 12 | CDC, 2015[d] |
| Heidelberg (outbreak) | Chicken | 2013–2014 | USA, Puerto Rico | 634 | CDC, 2015[d] |
| Heidelberg (outbreak) | Chicken | 2013 | USA | 9 | CDC, 2015[d] |
| Heidelberg (outbreak) | Chicken | 2012–2013 | USA | 134 | CDC, 2015[d] |
| Heidelberg (outbreak) | Chicken | 2011 | USA | 190 | CDC, 2015[d] |
| Heidelberg (outbreak) | Turkey | 2011 | USA | 136 | CDC, 2015[d] |
| Infantis (Israel clone) | Broiler chickens | 2007–2009 | Israel | NA | [26] |
| Infantis (Hungarian clone) | Broilers and chicken | 2004–2009 | Europe | NA | [33–35] |
| Kentucky (ST198-X1) | Chicken and turkey | 2002–2013 | Europe, Africa, Asia | NA | [39,40,42] |
| Stanley (outbreak) | Turkey | 2011–2013 | Europe | 710 | [36,37] |

[a]ST, sequence type.
[b]'year-' include ongoing reported outbreaks.
[c]Estimated number of cases only when outbreaks were reported. NA, not applicable in emerging clones.
[d]CDC, 2015. *Salmonella*. Reports of selected *Salmonella* Outbreak Investigations. Available at: http://www.cdc.gov/salmonella/outbreaks.html [accessed September 2015].

scenario also remains alert for the need for inclusion of those serotypes, or particular clones, considered to be of public health significance, in control and surveillance programmes [10,12,38].

*Salmonella* serotypes and clones associated with human infections and with an enhanced ability to colonize several food animals, able to persist along the food chain (e.g. primary production on-farm, slaughter operations, equipment and meat handlers, retail meat) with efficient transmission and rapid spread are of public health relevance [11,34,43–45]. Although understanding the exact mechanisms of their persistence and spread in poultry production are still largely unknown, recent studies focusing on emergent poultry-associated *Salmonella* strains unveiled specific features that could provide a significant advantage both in the environment and in the host (poultry/human) [12]. For example, in Israel, an S. Infantis emergent clone possessed a megaplasmid, which increased its tolerance to stress factors (e.g. mercury and oxidative stress) and its virulence/pathogenicity (e.g. enhanced biofilm formation, adhesion and invasion into avian and mammalian host cells) [46]. Also, a genomic study of several predominant *Salmonella* serotypes from Canadian broiler chickens showed the presence of multiple features related with pathogenicity (e.g. genes encoding adhesins, flagellar proteins, iron acquisition systems, type III secretion system) and stress tolerance (e.g. metal and antiseptic tolerance genes; better acid-stress response) [32]. S. Heidelberg, including ground turkey outbreak isolates, carried phages and plasmids with diverse virulence factors (e.g. P2-like phage-*sopE1* gene, IncX-type IV secretion system), which could play a role in their virulence (a serotype highly related to invasive infections), colonization and persistence (a poultry-associated serotype) [12,31]. In S. Kentucky, the acquisition of an *E. coli* ColV virulence plasmid was also associated with enhanced colonization ability in chicken, particularly in a dominant avian clonal type [12]. These features can play a role in the successful spread of emergent and virulent serotypes/clones that could contribute in a short time to replacing other *Salmonella*. Moreover, those emergent *Salmonella* serotypes are usually enriched with antimicrobial resistance determinants conferring multidrug-resistance [12,31,32,46], which is currently one of the major public health concerns (discussed in the next section).

## Antimicrobial-resistant non-typhoidal *Salmonella* and the poultry linkage

The emergence and spread of *Salmonella* isolates presenting resistance to several antibiotics is of concern because these medicines are crucial to the successful treatment of invasive infections [1,11]. Since resistance to older antibiotics (e.g. ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole) has been increasing for many years, recommended treatment options for salmonellosis included fluoroquinolones (ciprofloxacin) and extended-spectrum cephalosporins [1,2]. However, resistance to those 'critically important antibiotics for human health' is emerging, leading to increased severity, morbidity and mortality of diseases and the need for the use of last-line antimicrobials (e.g. carbapenems) in therapy [47].

For several decades, the contribution of the food-animal as a reservoir of antimicrobial resistance with impacts on human health has been controversial, but accumulating evidence linking particularly poultry production with human disease have been reported, namely involving non-typhoidal *Salmonella* [48–50]. The first evidence is the close association between the use of antimicrobial agents and the occurrence of resistance. In fact, regular use of antibiotics (mainly for animal health and in some countries as growth promoters), a practice associated with modern intensive food-animal/poultry production, has been

considered the main driver for the development of antibiotic resistance in zoonotic bacteria, such as *Salmonella* [48,50,51]. For example, licensing of the fluoroquinolone enrofloxacin for animal use, especially in poultry, in the 1990s led to increased rates of decreased susceptibility to ciprofloxacin in *S.* Typhimurium DT104 recovered from animal/food (particularly poultry) and humans [5]. Other findings points to a link between resistance to nitrofurans in human *Salmonella* and the food chain, suggesting that its illegal use in the poultry industry might have contributed to the selection and persistence of *S.* Enteritidis in poultry, and consequently to human salmonellosis in Portugal [52]. More recently, a voluntary withdrawal of ceftiofur by the poultry producers in Canada was correlated with a decreasing occurrence of ceftiofur-resistant *S.* Heidelberg (one of the most common serotypes associated with salmonellosis in this country) from both human infections and retail poultry, with an increase of the resistance levels after reintroduction of use [30].

Further evidence for the impact of poultry production on human health problems associated with antimicrobial resistance in *Salmonella* is the correlation between different reservoirs (humans and food-animals) obtained from systematic surveillance data. In the last EU report, *Salmonella* resistant (R) or multidrug-resistant (MDR) to commonly used antimicrobials was frequently detected in humans (R = 50%; MDR = 31.8%) and animals, especially broilers (MDR = 56%) and turkeys (MDR = 73%), but also in derived meat products [43]. Also, in the USA, high levels of resistance and MDR in chicken (R = 60%; MDR = 26%) and turkey (R = 77%; MDR = 39.6%) seems to greatly contribute to levels in humans (R = 19%; MDR = 10%) [53]. Those data about MDR are extremely worrying because of the possible role of diverse antibiotics in the co-selection of *Salmonella* strains resistant to clinically relevant antibiotics, such as fluoroquinolones and extended-spectrum cephalosporins. In the EU, relatively low levels of *Salmonella* non-susceptible ('clinically' resistant and 'intermediate' resistant categories combined) to ciprofloxacin (3.8%) and 'microbiological' resistant (non-wild type by epidemiological cut-off values) to cefotaxime (1.4%) were observed in humans. Moreover, the highest levels of 'microbiological' resistance to these critically important antimicrobials were detected in broiler meat (68% ciprofloxacin and 10.1% cefotaxime) and turkey meat (73.4% ciprofloxacin and 4.7% cefotaxime), suggesting an important contribution of the poultry production chain to the human burden [43]. In addition, it was reported that the highest levels of resistance to ciprofloxacin were more common in *S.* Enteritidis, *S.* Infantis and *S.* Kentuchy, three serotypes commonly associated with poultry meat [43]. Also, in the USA, in spite of a decreasing trend since 2009, cephalosporin's resistance levels are low in humans (2.5% ceftriaxone), but still high in turkey (9%) and retail chicken (20%), especially in *S.* Heidelberg a poultry-associated serotype [53].

In middle-income countries such as in the Asiatic continent, resistance to ciprofloxacin and extended-spectrum cephalosporins is currently a growing problem, with poultry products playing a potential role in its emergence [2,3]. High levels of *Salmonella* non-susceptible to ciprofloxacin (15%–48%) and cephalosporins (38% ceftriaxone) were observed in humans in Asian countries [54,55]. These data are in agreement with several studies that documented a high prevalence of resistance to fluoroquinolones (>22.5% ciprofloxacin) and cephalosporins (12.5% to >23.4% ceftriaxone and 26.6% ceftazidime) in poultry meat from South Korea and China [24,25]. In these countries the high rates of antimicrobial resistance detected reflect the levels of antimicrobial consumption in livestock, including for growth promotion, an area that remains largely unregulated [25,55,56]. Recent global estimates indicate that consumption of antibiotics in livestock (especially in poultry), which outranks the consumption by humans, will rise especially in middle-income countries. In these countries extensive farming systems will be replaced by large-scale intensive husbandry systems that routinely use antibiotics, to respond to the increasing demand for animal protein [56]. Therefore, international trade of contaminated breeding animals (poultry production depends on a pyramid-like breeding system), feed and poultry products with antibiotic-resistant *Salmonella* may contribute to the rapid worldwide spread of these bacteria, with impacts on human health.

A third indication of the significant impact on human health of antibiotic-resistant *Salmonella* contaminating these foodstuffs, is provided by diverse reports demonstrating their association with plasmid-mediated resistance determinants to cephalosporins and fluoroquinolones [49,51,57]. Moreover, several studies provide further evidence of transmission from poultry to humans of those clinically relevant MDR *Salmonella* clones [11,49,51,57]. A wide range of *Salmonella* serotypes (e.g. Enteritidis, Heidelberg, Infantis, Kentucky, Typhimurium, Virchow) frequently recovered from poultry (animal and food) have been associated with the worldwide dissemination of extended-spectrum β-lactamases, plasmid-encoded AmpC β-lactamases (AmpC), or plasmid-mediated quinolone resistance genes (Tables 2 and 3). By far the most common genes, found in poultry and/or poultry meat samples, associated with resistance to extended-spectrum cephalosporins, were those coding for extended-spectrum β-lactamases, CTX-M (e.g. CTX-M-1, -2, -9 and -15) and TEM-52 enzymes followed by AmpC-type CMY-2 (Table 2). For example, in several European countries, spread of *S.* Virchow producing CTX-M-2 or CTX-M-9 and *S.* Infantis producing TEM-52 has been found among poultry and humans [58–66]. Instead, *S.* Heidelberg, one of the

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**TABLE 2.** ***Salmonella*** **serotypes/clones carrying extended-spectrum and AmpC β-lactamases recovered from poultry and poultry products**

| Salmonella serotype | ESBL or AmpC[a] enzymes (no. of isolates) | Source | Concomitant presence in humans[b] | Country(ies)/ year(s) | Non-β-lactam resistance phenotype[c] | Genetic element PL – Inc group | Reference(s) |
|---|---|---|---|---|---|---|---|
| Agona | CTX-M-1 (n=1) | Poultry (caecum) | | The Netherlands/2006 | SUL-TET | PL – I1 | [65,66] |
| | CTX-M-2 (n=2) | Poultry | | Brazil/2011 | NR | PL – NR | [71] |
| | | Turkey carcass | | Brazil/2008 | CIP-NAL-STR-SXT-TET | PL – P | [72] |
| | CTX-M-8 (n=3) | Poultry | | Brazil/2008, 2010 | NR | PL – NR | [71] |
| | TEM-52 (n=3) | Poultry | | Belgium/2001, 2004 | (–) | PL– I1 | [62] |
| | CMY-2 (n=5) | Turkey | | Germany/2005 | (–) or NAL | PL – I1 | [73] |
| Bareilly | ACC-1 (n=6) | Poultry | Yes | The Netherlands/ 2001–02 | (–) | PL – NR | [74] |
| Blockley | TEM-52 (n=6) | Poultry, poultry meat | Yes | The Netherlands/ 2001–02 | (–) or SPT-SUL-TMP | PL – NR | [74] |
| Brackenridge | CTX-M-14 (n=1) | Poultry | | Brazil/2011 | NR | PL – NR | [71] |
| Braenderup | ACC-1 (n=2) | Broilers | | The Netherlands/2006 | (–) | PL– NT | [65] |
| | | Broiler | | The Netherlands/2001 | (–) | PL – NR | [74] |
| | SHV-12 (n=1) | Poultry | | Italy/2006 | (–) | PL – NR | [75] |
| Bredeney | CTX-M-1 (n=1) | Turkey | | USA/2010 | NR | PL – N | [76] |
| | CMY-2 (n=1) | Turkey | | Canada/1999 | SUL-TET | PL – A/C | [77] |
| Derby | TEM-52 (n=1) | Poultry | | Belgium/2001 | (–) | PL – I1 | [62] |
| Enteritidis | CTX-M-2 (n=1) | Poultry | | Brazil/2004 | NR | NR | [71] |
| | CTX-M-9 (n=1) | Broiler | | Spain/2000–04 | STR-SUL-SXT-NAL-TET | NR | [60] |
| | CTX-M-14 (n=5) | Imported chicken meat | | China/2004 | NAL | PL – NR | [78] |
| | | Poultry | | China/2012–13 | CHL-CIP-ENR-FFC-NAL-OLA- SXT-(AMK-GEN-LVX-TET) | PL – HI2, F, N or B/O | [70] |
| | CTX-M-15 (n=34) | Chicken meat, faeces or disease | Yes | Korea/2009 | STR-SUL-TET-(GEN-NEO) | PL – FII | [79] |
| | | Chicken | | South Korea/2009–10 | NR or GEN-STR-SUL-TET | PL – FII | [80] |
| | SHV-12 (n=2) | Broiler | | Portugal/2012–13 | CHL-CIP-SUL-NAL-TET | NR | [81] |
| | | Poultry | | Italy/2006 | TET | NR | [75] |
| Emek | CMY-2 (n=1) | Poultry | | Brazil/2011 | NR | NR | [71] |
| Essen | CTX-M-15 (n=1) | Diseased chicken | | Korea/2009 | GEN-STR-SUL-TET | PL – FII | [79] |
| Gaminara | CTX-M-14 (n=1) | Poultry | | Brazil/2009 | NR | NR | [71] |
| Give | CTX-M-2 (n=1) | Poultry | | Brazil/2009 | NR | NR | [71] |
| Hadar | CTX-M-14 (n=1) | Poultry | | Brazil/2005 | NR | NR | [71] |
| Havana | CTX-M-1 (n=1) | Broiler | | Portugal/2012–13 | SUL-TET | NR | [81] |
| | CMY-2 (n=2) | Broiler | | Portugal/2012–13 | (–) | NR | [81] |
| Heidelberg | CTX-M-1 (n=1) | Broiler carcass | | Portugal/2012–13 | SUL-TMP | NR | [81] |
| | CTX-M-2 (n=14) | Poultry | | Brazil/2009, 2011 | NR | NR | [71] |
| | | Chicken | | Venezuela/2005–07, 2008 | CIP-GEN | PL – NR | [82] |
| | CTX-M-14 (n=1) | Poultry | | Brazil/2011 | NR | NR | [71] |
| | SHV-2 (n=1) | Retail chicken meat | | Canada/2007 | GEN-STR-SUL | PL – I1 | [83] |
| | CMY-2 (n=25) | Chicken or chicken retail | Yes | Canada/2001–04 | CHL-GEN-KAN-STR-SUL-SXT-TET | PL – A/C, I1 | [67] |
| | | Chicken food | | USA/2002, 2004, 2010 | (CHL-GEN-KAN-STR-SUL-TET) | PL – A/C, I1 | [31] |
| | | Poultry | | Brazil/2011 | NR | NR | [71] |
| Indiana | CTX-M-14 (n=21) | Poultry | | China/2012–13 | ENR-NAL-SXT-(AMK-CHL-CIP-FFC-GEN-LVX-OLA-TET) | PL – HI2, A/C, F, N, P-1α, B/O | [70] |
| | CTX-M-24 (n=29) | Chicken | | China/2008–09 | AMK-CHL-FFC-GEN-NAL-OLA-RIF-STR-SXT-(TET) | PL – HI2 | [84] |
| | CTX-M-65 (n=3) | Poultry | | China/2012–13 | CHL-NAL-OLA-SXT-(CIP-ENR-FFC-GEN-LVX-TET) | PL – HI2, F, N | [70] |
| | TEM-52 (n=1) | Broilers | | The Netherlands/2006 | (–) | PL – NT | [65] |
| | DHA-1 (n=3) | Chicken faeces | | South Korea/2006–07 | SUL-TMP-(APM-NAL-NEO-STR-TET) | PL – NR | [85] |
| Infantis | CTX-M-1 (n=2) | Poultry (caecum or unknown) | | The Netherlands/2006 | SUL-TMP | PL – I1 | [65,66] |
| | CTX-M-2 (n=3) | Poultry | | Brazil/2005 | NR | NR | [71] |
| | | Retail Chicken Products | | Japan/2002-03 | (–) | NR | [86] |
| | TEM-20 (n=2) | Retail Chicken Products | | Japan/1997, 2003 | (–) | NR | [86] |
| | TEM-52 (n=14) | Poultry | Yes | Belgium/2001–05 | (–) | PL – I1 | [62] |
| | | Poultry (caecum or unknown) | | The Netherlands/2006 | (–) | PL – I1 | [65,66] |
| | | Broiler | | Belgium/2004 | (–) | PL – I1 | [63] |
| | | Chicken | | Japan/2004–06 | (–) | PL – NR | [87] |
| | | Retail Chicken Products | | Japan/2000, 2003 | (–) | NR | [86] |
| | CMY-2 (n=48) | Retail Chicken Products | | Japan/1997, 1999–2003 | (–) | PL – NR | [86] |
| Kentucky | CTX-M-25/OXA-21 (n=1) | Turkey | | Poland/2009 | CIP-NAL | PL – A/C | [42] |
| | SHV-12 (n=3) | Whole chicken and chicken neck skin | | Ireland/2008–09 | CHL-SUL-TET | PL – NR | [88] |
| | CMY-2 (n=3) | Whole chicken | | Ireland/2009 | (–) | PL – NR | [88] |
| | | Chicken meat | | Germany/2005 | STR | PL – I1 | [73] |
| Kiambu | SHV-2 (n=1) | Abattoir chicken cecum | | Canada/2006 | STR-SUL-SXT | PL – I1 | [83] |
| Livingstone | SHV-12 (n=9) | Poultry or turkey carcasse, poultry faeces, broiler faeces | | Italy/2005-06 | GEN-NAL-(STR-SUL) | NR | [75] |
| Llandoff | CTX-M-1 (n=1) | Poultry | | France/2006 | SUL-TET | PL – I1 | [89] |

***Continued***

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**TABLE 2. Continued**

| Salmonella serotype | ESBL or AmpC[a] enzymes (no. of isolates) | Source | Concomitant presence in humans[b] | Country(ies)/ year(s) | Non-β-lactam resistance phenotype[c] | Genetic element PL – Inc group | Reference(s) |
|---|---|---|---|---|---|---|---|
| Manhattan | CTX-M-2 (n=2) | Retail Chicken Products | | Japan/2000, 2003 | (–) | NR | [86] |
| | CTX-M-15 (n=4) | Retail Chicken Products | | Japan/2002, 2003 | (–) | NR | [86] |
| | TEM-52 (n=4) | Retail Chicken Products | | Japan/2000, 2002–03 | (–) | NR | [86] |
| | SHV-12 (n=2) | Retail Chicken Products | | Japan/2002, 2003 | (–) | NR | [86] |
| | CMY-2 (n=2) | Retail Chicken Products | | Japan/2002 | (–) | PL – NR | [86] |
| Minnesota | CTX-M-2 (n=1) | Poultry | | Brazil/2009 | NR | NR | [71] |
| | CTX-M-8 (n=1) | Poultry | | Brazil/2008 | NR | NR | [71] |
| | CTX-M-14 (n=1) | Poultry | | Brazil/2010 | NR | NR | [71] |
| | CMY-2 (n=2) | Poultry | | Brazil/2011 | NR | NR | [71] |
| Newport | CTX-M-2 (n=1) | Poultry | | Brazil/2008 | NR | NR | [71] |
| Ouakam | CTX-M-1 (n=6) | Turkey | | USA/2011 | NR | PL – N | [76] |
| Panama | CTX-M-1 (n=1) | Poultry | | Brazil/2009 | NR | NR | [71] |
| Poona | CTX-M-2 (n=1) | Poultry | | Brazil/2009 | NR | NR | [71] |
| | CTX-M-8+CTX-M-14 (n=1) | Poultry | | Brazil/2005 | NR | NR | [71] |
| Rissen | CMY-2 (n=1) | Poultry | | Brazil/2005 | NR | NR | [71] |
| Saintpaul | CMY-2 (n=1) | Poultry | | Brazil/2011 | NR | NR | [71] |
| Schwarzengrund | CTX-M-2 (n=10) | Turkey, chicken, chicken carcass | | Brazil/2008 | CIP-NAL-STR-TET | PL – P | [72] |
| Senftenberg | CTX-M-15 (n=1) | Chicken | | South Korea/2009 | NR | PL – FII | [80] |
| Typhimurium | CTX-M-1 (n=1) | Poultry meat | | Germany/2006 | KAN-NEO-STR-SUL-SXT-TET-TMP | PL – I1 | [73] |
| | CTX-M-2 (n=10) | Poultry | | Brazil/2004–05, 2007 | NR | NR | [71] |
| | | Poultry | Yes | Brazil/2003–04 | SUL-SXT-TET | PL – NR | [90] |
| | CTX-M-8 (n=1) | Poultry | | Brazil/2011 | NR | NR | [71] |
| | CTX-M-14 (n=1) | Poultry | | Brazil/2005 | NR | NR | [71] |
| | CTX-M-15 (n=1) | Broiler | | Belgium/2007 | (–) | PL – I1 | [63] |
| | TEM-52 (n=4) | Poultry | Yes | Belgium/2002, France/2004 | (–) | PL – I1 | [62] |
| | | Poultry | | The Netherlands/2001–02 | SPT-SUL-TMP-(NEO-TET) | NR | [74] |
| | TEM-52+SHV-12 (n=1) | Poultry meat | | The Netherlands/2002 | (–) | NR | [74] |
| Virchow | CTX-M-2 (n=12) | Poultry or poultry product | | Belgium/2000–01, 2003 | NAL-SUL-TET-TMP-(STR) | PL – HI2 | [61] |
| | | Poultry | | Ireland/UN | SUL-TET-TMP | PL – P | [77] |
| | | Broiler | | Belgium/2001, 2004 | STR-SUL-TET-TMP | PL – HI2 | [63] |
| | | Poultry | Yes | Belgium-France/2000–03 | NAL-SUL-TMP-(STR-TET) | PL – NR | [59] |
| | CTX-M-2+SHV-2+TEM-1 (n=1) | Broiler | | The Netherlands/2002 | NAL-SPT-SUL-TET- TMP | NR | [74] |
| | CTX-M-9 (n=8) | Chicken faeces | | France/2002–03 | KAN-NAL-SPT-STR-SUL-TET-TMP | PL – NR | [58] |
| | | Chicken faeces and retail chicken meat | Yes | France/2002–03, Spain/2000 | NAL-SPT-STR-SUL-TET-TMP-(KAN) | PL – HI2 | [61] |
| | | Broiler | Yes | Spain/2000–04 | STR-SUL-SXT-NAL-(TET) | NR | [60,64] |
| | CTX-M-32 (n=2) | Poultry | | Greece/2001 | CHL-KAN-STR-SUL-TET-TMP | PL – NR | [91] |
| | TEM-52 (n=1) | Poultry | | The Netherlands/2002 | SPT-STR-SUL-TMP | NR | [74] |
| Weslaco | CTX-M-8 (n=1) | Poultry | | Brazil/2009 | NR | NR | [71] |
| 35:c:1,2 | SHV-12 (n=5) | Poultry | Yes | Senegal/2000 | CHL-GEN-TET-TOB | NR | [92] |

Antimicrobial abbreviations: AMK, amikacin; APM, apramycin; CHL, chloramphenicol; CIP, ciprofloxacin; ENR, enrofloxacin; FFC, florfenicol; GEN, gentamicin; KAN, kanamycin; LVX, levofloxacin; NAL, nalidixic acid; NEO, neomycin; OLA, olaquindox; RIF, rifampicin; SPT, spectinomycin; STR, streptomycin; SUL, sulphonamides compound; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; TOB, tobramycin; NR, not reported; NT, plasmids that were not typable with the scheme used; PL, plasmid.
[a]Only references with full-characterized extended-spectrum β-lactamases /AmpC genes were considered.
[b]'Yes', clones or serotypes simultaneoulsy detected in poultry and humans in the same study.
[c](–), Non-β-lactam resistance phenotype was not detected using the tested antibiotics and according to the susceptibility criteria adopted. Variable phenotypes were present between curved brackets.

top MDR serotypes in both poultry and humans (frequently associated with invasive infections) in North America, presented increasing resistance to cephalosporins related with CMY-2 [31,67]. In fact, transmission of these most reported genes in both human and poultry has been associated with diverse plasmid families, such as IncI1 (e.g. $bla_{CTX\text{-}M\text{-}1}$, $bla_{TEM\text{-}52}$ and $bla_{CMY\text{-}2}$), IncA/C (e.g. $bla_{CMY\text{-}2}$) or IncHI2 (e.g. $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}9}$) (Table 2). Recently, plasmid-mediated carbapenem-resistant bacteria from food-animals have been reported, alerting us to a new public health problem with indefinable risks [51]. One of the first reports was on a poultry farm in Germany where a $bla_{VIM\text{-}1}$ gene was detected on *S.* Infantis [68]. Besides the worldwide resistance to clinically important β-lactams, plasmid-mediated quinolone resistance mechanisms, which typically confer decreased susceptibility to ciprofloxacin, have been widely reported in *Salmonella* [57]. Among them, Qnr proteins (e.g. QnrB2, QnrB19, QnrS1) have been commonly described in different serotypes and geographic locations, with the aminoglycoside acetyltransferase AAC(6′)-Ib-cr and the efflux pump OqxAB associated with *Salmonella* serotypes recovered from Asian poultry and poultry meat (Table 3). In fact, poultry seems to be an important vehicle of non-typhoidal *Salmonella* carrying plasmid-mediated quinolone resistance genes, highlighting the role of food-producing

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**TABLE 3.** ***Salmonella*** **serotypes/clones carrying plasmid-mediated quinolone resistance genes recovered from poultry and poultry products**

| Salmonella serotype | PMRQ[a] mechanism (no. of Isolates) | Source | Country(ies)/year(s) | Antibiotic resistance phenotype[b] | Genetic element PL – Inc group | Reference(s) |
|---|---|---|---|---|---|---|
| Agona | QnrB2 (n=1) | Turkeys | Germany/NR | NR | NR | [69] |
| Braenderup | QnrD (n=2) | Fowls | Spain/NR | NR | NR | [69] |
| Dabou | QnrD (n=1) | Fowls | Spain/NR | NR | NR | [69] |
| Derby | QnrB2 (n=56) | Fowls or turkeys | Spain/NR | NR | NR | [69] |
| Enteritidis | QnrB2 (n=1) | Fowls or turkeys | Spain/NR | NR | NR | [69] |
| | QnrB10/B19 (n=1) | Laying hen flock | Poland/2009 | CIP-NAL | NR | [93] |
| | QnrD (n=3) | Fowls | Spain/NR | NR | NR | [69] |
| | QnrS1/S3 (n=2) | Broiler meat, broiler flock – faeces | Poland/2008–09 | AMP-CIP-(STR-TET) | NR | [93] |
| | OqxAB (n=3) | Poultry | China/2012–13 | AMP-CHL-CIF-CIP-ENR-FFC-NAL-OLA-SXT-(GEN-LVX-TET) | PL – HI2, F, N, B/O | [70] |
| | OqxAB + AAC(6′)-Ib-cr (n=1) | Poultry | China/2012–13 | AMK-AMP-CHL-CIF-CIP-CTX-ENR-FFC-GEN-LVX-NAL-OLA-SXT-TET | PL – HI2 | [70] |
| Give | QnrB19 (n=1) | Imported turkey meat from Brazil | Finland/NR | NR | NR | [69] |
| Hadar | QnrB2 (n=2) | Fowls or turkeys | Spain/NR | NR | NR | [69] |
| | QnrB5 (n=4) | Imported turkey meat | Germany/2007 | STR-TET | NR | [94] |
| | QnrB19 (n=15) | Fowls, turkeys | Germany, Denmark/NR | NR | NR | [69] |
| Havana | QnrB2 (n=3) | Poultry | Portugal/2009–10 | (–) or NAL | PL – L/M | [95] |
| | QnrB19 (n=1) | Poultry | Portugal/2009–10 | (–) | PL – HI2 | [95] |
| Heidelberg | QnrB19 (n=2) | Chicken | Venezuela/2005–07, 2008 | CIP-GEN | PL – NR | [82] |
| Indiana | OqxAB (n=50) | Poultry | China/2012–13 | AMP-CIF-NAL-SXT-(AMK-CAZ-CHL-CIP-CTX-ENR-FFC-GEN-LVX-OLA-TET) | PL – HI2, F, N, P-1α, B/O | [70] |
| | | Chicken | China/2008–09 | AMK-CHL-FFC-GEN-NAL-OLA-RIF-STR-SXT-(TET) | PL – HI2 | [84] |
| | OqxAB + AAC(6′)-Ib-cr (n=3) | Poultry | China/2012–13 | AMP-CHL-CIF-NAL-SXT-(CIP-CTX-ENR-FFC-GEN-LVX-OLA-TET) | PL – HI2, A/C, N | [70] |
| Infantis | QnrS1 (n=1) | Chicken | Germany/2004 | AMP | NR | [96] |
| | QnrB19 (n=1) | Retail chicken | Colombia/2004 | KAN-NAL-NEO-STR-TET | PL – ColE like | [97] |
| Kentucky | QnrS1 (n=1) | Chicken | The Netherlands/NR | NR | PL – N | [98] |
| London | QnrB2 (n=22) | Fowls or turkeys | Spain/NR | NR | NR | [69] |
| Mbandaka | QnrB19 (n=1) | Poultry | Portugal/2009–10 | NAL | PL – HI2 | [95] |
| Montevideo | QnrB2 (n=3) | Fowls or turkeys | Spain/NR | NR | NR | [69] |
| | QnrD (n=6) | Fowls, turkeys | Italy/NR | NR | NR | [69] |
| Newport | QnrB5 (n=3) | Imported turkey meat | Poland/2007 | (–) | NR | [94] |
| | QnrS1/S3 (n=12) | Broiler (meat, flock), turkey meat, goose flock – faeces, duck flock – faeces | Poland/2008–11 | CIP-(AMP-CHL-FFC-KAN-STR-SUL-TET-NAL) | NR | [93] |
| | QnrB19 (n=3) | Turkeys | Denmark/NR | NR | NR | [69] |
| Ohio | QnrD (n=5) | Fowls | Spain/NR | NR | NR | [69] |
| Saintpaul | QnrS1 (n=15) | Imported turkey meat | Germany/2007–08 Poland/2008 | AMC-AMP-STR-TET-(CPD-CHL) | NR | [94] |
| | | Turkeys | Germany, Denmark/NR | NR | NR | [69] |
| Typhimurium | QnrA1 (n=1) | Turkeys | Germany/NR | NR | NR | [69] |
| | QnrD (n=2) | Fowls | Spain/NR | NR | NR | [69] |
| | OqxAB (n=2) | Chicken | China/2007–09 | OLA-FFC-SMX-(AMP-CHL-CIF-GEN-NAL-TET) | PL – HI2, F | [99] |
| | OqxAB + AAC(6′)-Ib-cr (n=8) | Chicken, duck | China/2009–10 | NAL-OLA-SMX-(AMP-CHL-CIF-ENR-FFC-GEN-TET) | PL – HI2 | [99] |
| | OqxAB + AAC(6′)-Ib-cr + QnrS1 (n=1) | Duck | China/2010 | AMP-CHL-CIF-FFC-GEN-NAL-OLA-SMX-TET | PL – HI2 | [99] |
| Virchow | QnrS1 | Chicken carcass | Korea/2002 | AMP-CEF-STR-SUL- TET-TMP | N | [100] |
| | | Chicken | UK/2004-05 | AMP-CIP | N | [101] |
| | | Chicken | Turkey/2005 | AMP-NAL | PL – NT | [102] |

Antimicrobial abbreviations: AMC, amoxicillin/clavulanate; AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CEF, cefalotin; CHL, chloramphenicol; CIF, ceftiofur; CIP, ciprofloxacin; CPD, cefpodoxime; CTX, cefotaxime; ENR, enrofloxacin; FFC, florfenicol; GEN, gentamicin; KAN, kanamycin; LVX, levofloxacin; NAL, nalidixic acid; NEO, neomycin; OLA, olaquindox; RIF, rifampicin; STR, streptomycin; SUL, sulphonamides compound; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; NR, not reported; NT, plasmids that were not typeable with the scheme used; PL, plasmid.

[a]Only references with full-characterized plasmid-mediated quinolone resistance genes were considered.

[b](–), Antibiotic resistance phenotype was not detected using the tested antibiotics and according to the susceptibility criteria adopted. Variable phenotypes were present between curved brackets.

animals, including the animal/food trade, in its dissemination [69,70].

In addition to the therapeutic consequences, antimicrobial resistance acquisition to agents commonly used in livestock production has been associated with the spread of particular *Salmonella* clones in the poultry production environment, which might contribute to their association with human diseases. Interestingly, the high-level resistance to ciprofloxacin (due to

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*gyrA*/*par*C mutations) and other antibiotics (amoxicillin, streptomycin, spectinomycin, gentamicin, sulfamethoxazole and tetracycline) [39–43] of the previously mentioned *S.* Kentucky ST198-X1-SGI1 strain might also have contributed to its clonal expansion. In the EU, several *S.* Stanley outbreaks linked with the consumption of turkey meat products have been reported, with the clone presenting resistance to nalidixic acid and decreased susceptibility to ciprofloxacin. Additionally, in the recent outbreak in Austria three strains also presented resistance to gentamicin and cephalosporins (CTX-M-15) [36,38]. Several successful *S.* Infantis clones of broiler origin are also characterized by multidrug resistance profiles, including nalidixic acid, tetracyclines, sulphonamides or nitrofurans [26,35,46], antibiotics frequently used in livestock production, which could contribute to their co-selection in the poultry industry.

Data compiled here, highlight the role of the poultry production chain (including poultry meat) as a reservoir of epidemic MDR clones or genes conferring resistance to critical antibiotics, which might spread to humans through the food chain. Globalization of food-animal production is currently posing a major challenge in antimicrobial resistance of zoonotic bacteria like non-typhoidal *Salmonella* with a consequent involvement of human health. In particular in the poultry production pyramid it is crucial to restrict the global use of antimicrobials to minimize the selection of resistant *Salmonella* (from the top of the poultry production pyramid and within flocks), and also to control the spread of MDR epidemic clones by improving biosecurity measures from the farm (e.g. hygiene high standards, vaccination) throughout the food chain (e.g. slaughtering and processing of poultry meat) and control of animal/food trade.

## Conclusions

In the last decade, *Salmonella* control programmes, mainly targeting poultry production, have led to a significant reduction in salmonellosis in different countries, including in the EU. This positive effect has been associated with a shift in *Salmonella* serotypes of poultry and human disease, associated with a marked reduction on *S.* Enteritidis and the increase of less common serotypes, driven by the dissemination of particular clones carrying features favouring host (poultry/human) adaptability and frequently MDR.

Poultry has been reported as a source of non-typhoidal *Salmonella* resistant to clinically relevant antibiotics with a remarkable higher incidence reported for middle-income countries, which due to food trade globalization can dramatically challenge worldwide the treatment of severe salmonellosis cases.

Integrated surveillance (collaboration between human health, food safety and animal health—the 'One Health' approach) and containment strategies (including farms, retail, catering and consumers) to minimize contamination and reduce the transmission of *Salmonella* (including epidemic clones) along the food chain (from primary production to consumption) are mandatory on a global scale, in particular in the poultry production chain. In addition, continuous surveillance of *Salmonella* resistance levels globally is critical for clinicians to support the best salmonellosis treatment choices, avoid treatment failures, particularly for patients that would benefit from empirical antibiotic treatment.

Continuous monitoring to detect the emergence of any serotype or new clone along the food chain is of critical importance for public health, warning of the emergence of new *Salmonella* food safety risks, involving foodstuffs like poultry meat, which is one of the most consumed and increasing globally traded meat products.

## Transparency Declaration

The authors declare no conflict of interest.

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## 1.2. Revision Article

### Non-typhoidal *Salmonella* and the pig production chain: a comprehensive analysis of its impact on human health

# Non-typhoidal *Salmonella* in the pig production chain: a comprehensive analysis of its impact on human health

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**Abstract**

Salmonellosis remains one of the most frequent foodborne zoonosis, constituting a worldwide major public health concern. The most frequent sources of human infections are food products of animal origin, being pork meat consumption one of the most relevant. Currently, particular well-adapted and persistent *Salmonella enterica* serotypes (e.g. *Salmonella* Typhimurium, *Salmonella* 1,4,[5],12:i:-, *Salmonella* Derby and *Salmonella* Rissen) associated with pig food production and causing human infections have been reported in diverse industrialized countries. The dissemination of those clinically-relevant *Salmonella* serotypes/clones has been related to the intensification of pork production chain and to an increased international trade of pigs and pork meat. Those changes occurring over the years along the food-chain may act as drivers leading to new problems and challenges, compromising the successful control of *Salmonella*. Moreover, antibiotic resistance in non-typhoidal *Salmonella* is considered one of the major public health threats related to food-animal production, including pork production chain, leading to concerns in the management of salmonellosis. The transmission of successful pig-related multidrug-resistant *Salmonella* clones and/or genetic elements carrying clinically-relevant antibiotic resistance genes, frequently associated with metal tolerance genes, from pigs and pork meat to humans highlights this scenario. These data enhance the need for global mandatory interventions and strategies for effective *Salmonella* control and surveillance across pork production chain. The purpose of this review is to provide an overview of the role of pig and pork meat on human salmonellosis at a global scale, highlighting the main factors contributing for the successful persistence and dissemination of clinically-relevant pig-related *Salmonella* serotypes and clones.

**Keywords:** *Salmonella,* pig production, pork meat, antimicrobial resistance, clones, *S.* Typhimurium, *S.* 1,4,[5],12:i:-, *S.* Derby, *S.* Rissen

## 1. Introduction

*Salmonella enterica* infections are a worldwide major public health concern, being human salmonellosis caused by non-typhoidal *Salmonella* (NTS) (1,2). Salmonellosis is typically characterized by a self-limiting gastroenteritis syndrome, being diarrhoea the main symptom, but fever, vomiting and abdominal pain can also occur (1,2). Despite uncommon, more severe invasive *Salmonella* infections, as bacteraemia, and/or other extra-intestinal infections can occur affecting in particular high-risk groups (infants, young children, older people or immunocompromised patients) (1,2). In these cases, the use of antimicrobial agents is required, being of concern the emergence of *Salmonella* resistant to antibiotics, especially to those considered by World Health Organization (WHO) as "critically antibiotics of highest priority" such as fluoroquinolones and extended-spectrum cephalosporins (3), which can compromise the effective treatment of infections (1,2).

In industrialized countries, the predominant reservoir of NTS is the gastrointestinal tract of warm-blooded animals, in particular food-producing animals, which lead to foodstuffs contamination (1,2). Therefore, the ingestion of contaminated food, particularly foods of animal origin, is recognized as the most relevant source of transmission of NTS to humans, with a high global impact in human health (2). Actually, NTS causes an estimated 93.8 million cases of human illnesses and 155.000 deaths each year worldwide (1,2). In the United States of America (USA), it was estimated by the Centers for Disease Control and Prevention (CDC) more than 1 million annual cases of foodborne salmonellosis and 450 deaths each year, being NTS associated with the largest number of hospitalizations (35%) and deaths (28%) compared with other foodborne microorganisms (4). Furthermore, the last report from USA (2015) showed that *Salmonella* was the second foodborne pathogen responsible for outbreaks (34%), being the first associated with outbreak illnesses (39%), hospitalizations (64%) and deaths (60%) (5). Also, in the European Union (EU), salmonellosis has been the second most common zoonosis (94,530 confirmed cases and 20,400 cases per 100,000 populations in 2016) and the most frequent cause of foodborne outbreaks (26.5% of all cases in 2016) (6), in spite of a decreasing trend of human salmonellosis cases since 2008, with a slowing trend in the years 2012–2016 (6). Despite salmonellosis has been mostly associated with the consumption of poultry products, including eggs and eggs products, at a global level (2,7,8), pork meat has been considered one of the major food products of animal origin responsible for *Salmonella* transmission to humans in diverse countries (2,9–11). In the USA, data from 2015 showed that pork meat was the second cause of *Salmonella* outbreaks, including the meat product mostly associated with the largest number of illnesses, hospitalizations and deaths cases (5).

Although different serotypes have been associated with salmonellosis, the major ones responsible for human infections in diverse industrialized countries include

*Salmonella* Enteritidis, *Salmonella* Typhimurium, and its monophasic variant – *Salmonella* 1,4,[5],12:i:- (5,6,12–15). At a global level, *S.* Enteritidis is commonly associated with poultry and products thereof, whereby is considered a poultry-related serotype (2,6,7). In contrast, *S.* Typhimurium and *S.* 1,4,[5],12:i:- have a wider host range, including pigs (1,2,6). Nevertheless, in the last years, a changing trend in *Salmonella* serotypes associated with foodborne salmonellosis has been observed, with the worldwide expansion of previously less common pig-related serotypes (e.g. *S.* 1,4,[5],12:i:-, *Salmonella* Derby and *Salmonella* Rissen) and particular clones, frequently multidrug-resistant, with a growing association with pig sources (including pork meat) (6,9,16,14,17–27). In EU, the successful implementation of mandatory *Salmonella* control programmes along poultry/egg production chain was responsible for the reduction of the prevalence of *Salmonella* serotypes considered relevant for public health, particularly *S.* Enteritidis (6,28). In contrast with poultry production, where *Salmonella* control programmes are harmonized, for pig production each EU member state applies a specific national monitoring programme (6,11). In addition, the intensive food production/farming and increased globalization of food supply (live animals and foodstuffs), with pork meat being one of the most consumed and traded meat product (pork exports increased in value by 18.2% from 2015 to 2016) (6,29) (www.worldstopexports.com/pork-exports-by-country), may trigger new problems regarding salmonellosis control. This review will provide evidences of the relevant role of pig production and pork meat in salmonellosis on a global scale.

## 2. Non-typhoidal *Salmonella* in pig populations

The colonization of pig populations with NTS is frequent and normally results in asymptomatic healthy carriers. This pig colonization can occur throughout all stages of pork production chain by horizontal (through external agents in the environment, e.g. rodents, birds, people, trucks, pets, other foodstuffs) and vertical transmission (e.g. from sow to piglet and from pig to pig at herd to slaughter). Also, a designated circular transmission (combination of vertical and horizontal transmission), which is a permanent cycle of contamination on a farm (e.g. environmental contamination through pig shedding and pig contamination through farm environment) can also determine pig colonization (9,11,30–32). The presence of *Salmonella* in those healthy pig carriers (e.g. tonsils, gut and gut-associated lymphoid tissue) is suggested to be the main risk factor for the spread and transmission of these bacteria across pork production chain to humans: in pre-harvest (during farming food-producing animals), in the harvest stage (during slaughter and further processing of meat carcasses) and in the post-harvest stage (during final preparation of pork meat and products thereof) (9,11,30,31,33,34).

Since 2006, in all EU member states the report of *Salmonella* monitoring and surveillance data is mandatory under the Commission Regulation (EC) No 2073/2005 on microbiological criteria in foodstuffs, including for *Salmonella* in pig carcasses at dressing and before chilling stage. Furthermore, competent authorities must verify the correct implementation of the process hygiene criteria for *Salmonella* on pig carcasses by food business operators and, if is not complied, business operators might implement corrective actions (e.g. improvements of hygiene slaughter, biosecurity measures in the farms and revision of process controls) with the specific instructions of authorities (35). In 2014 legislation were revised, with Regulation (EC) No 217/2014 proposing the reduction of *Salmonella* acceptable number in pig carcasses, from "c=10 out of n=50" - 10% to "c=3 out of n=50" - 6% (n-number of units comprising the sample; c- detection number of samples with *Salmonella*), in order to strengthen the process hygiene criterion (36).

In most of the industrialized countries, pork meat has been the second main source of human cases of salmonellosis, after poultry products (2,5,9,11,23,37–39). In fact, in the EU, it was observed an increase of human salmonellosis cases associated to pork meat (9% in 2014 to 13% in 2015), the second source after eggs and egg products (44% in 2014 and 21% in 2015) (37,38). Also, in the USA (2015 data), pork meat was the second major source attributed to *Salmonella* outbreaks (5%) and the meat product mostly associated with the largest number of illnesses (16%), hospitalizations (2%) and deaths (11%) (5).

### 2.1. Occurrence of *Salmonella* in the pig production chain

Diverse studies aiming to detect *Salmonella* in the pig production chain, including pigs and pork meat, have been performed in high or low-income countries. *Salmonella*-positive samples in fresh pork meat (2.4%) and products thereof (1.9%) were reported in the EU in 2016, with an overall *Salmonella* prevalence in pigs of 6.7% at the herd (ranging from 0%-63% between the different member states) and of 3.5% at slaughter (0%-10.6%) (6). Noteworthy, a high prevalence of *Salmonella* in faecal samples (30.5%), rectal swabs (24%) and carcass swabs (9.6%) of slaughter pigs (40) was reported by an United Kingdom (UK) study. In contrast, other EU countries (Finland, Sweden and Norway), with special guarantees concerning *Salmonella* on pig carcasses [according to Regulation (EC) No 853/2004)] reported a lower incidence of *Salmonella* in pig carcasses samples (0.02%) (6,41). In fact, with the objective to establish the main targets for *Salmonella* reduction in breeding herds of pigs [in line with the Regulation (EC) No 2160/2003], a baseline survey was performed in EU (2008) due to the lack of information about *Salmonella* control in holdings of breeding pig. In this survey, high levels of *Salmonella*-positive holdings with breeding pigs (31.8%) and breeding holdings (28.7%) were observed. These data have shown that breeding pigs may be a relevant source of *Salmonella* dissemination along the

pig-production chain (e.g. to slaughter pigs through trade and movement of live animals and contamination of holding, transport, lairage and slaughter facilities), therefore leading to pork meat contamination and consequently to human infections (30). Other EU survey, based on the analysis of quantitative microbiological risk assessment of *Salmonella* in slaughter and breeder pigs showed that 80%-90% reduction of lymph node prevalence should result in a comparable reduction in the number of human cases attributable to pork meat products (42). Consequently, several interventions have been proposed in order to prevent or reduce *Salmonella* contamination, persistence and dissemination across pig production (at farm and slaughterhouse). These included the use of uncontaminated feed, isolation of newly purchased animals before introducing them into herd, regular veterinary checks, vaccination, prevention of environmental contamination at the farm, transport, lairage and slaughter, and implementation of high standards of hygiene (cleaning and disinfection). In addition, the need for a continuously *Salmonella* monitoring/control to identify more possible improvements has been highlighted by several entities (30,32,42). In the USA, data from 2015 showed a remarkable higher prevalence of *Salmonella* in pig caeca samples (35%-market swine and 50%-sows) than in other animal samples (25%-chickens, 9%-turkeys, 22%-dairy and 8%-beef) (www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/ucm059103.htm). In other regions, including in developing countries (some with an expansion of food-animal industry), *Salmonella* was detected at high levels in pig samples (animal, carcasses and meat), ranging from 17%-39% in South America (43,44), 14%-40% in Africa (45,46), and 29%-100% in Asia (47,48). Those high levels possibly reflect the different pig production practices and the absence of control measures. Overall, these data point to the need of implementing effective global measures for *Salmonella* control, highlighting the need for its detection at all stages of pig production chain, including in primary production (11,30,31). This is particularly urgent in developing countries, which currently seem to present a severely underserved monitoring surveillance programmes (49).

### 2.2. Major pig-related *Salmonella* serotypes associated with human infections

In the last years, *Salmonella* transmission from pigs to humans through the pork-food-chain has been evidenced, namely through the study of serotypes prevalence in different matrices as well as food-borne outbreaks associated with consumption of pork products.

Worldwide data concerning the prevalence of *Salmonella* serotypes in humans, pigs and products thereof have contributed to establish their epidemiological correlation, with particular serotypes overlapping between humans, pig and pork meat

(6,14,12,15,38,50–53). For instance, in EU, an association between *Salmonella* serotypes causing human infections and those occurring in pig and pork meat was observed (**Figure 1**), reinforcing the major role of pork meat in the transmission of *Salmonella* to humans. In fact, the most frequent serotypes in pig and pork meat have been *S.* Typhimurium (pig: 54.7%-2014, 56.9%-2015 and 29.5%-2016; pork meat: 27.8%-2014, 23%-2015 and 30.7%-2016), *S.* 1,4,[5],12:i:- (pig: 8.4%-2014, 8.6%-2015 and 34.1%-2016; pork meat: 18%-2014, 22.3%-2015 and 24.3%-2016), and *S.* Derby (pigs: 17.5%-2014, 13.7%-2015 and 19.2%-2016; pork meat: 24.4%-2014, 22.9%-2015 and 17%-2016) (**Figure 1**). These serotypes are also among the major serotypes associated with human salmonellosis (second-, third- and fifth-ranked, respectively) (6,38,53). In fact, from the 2008 EU baseline survey, *S.* Derby was the most frequent serotype found in both breeding (29.6%) and production holdings (28.5%), with *S.* Typhimurium the second most detected (breeding holdings-25.4% and production holdings-20.1%). Moreover, it is also of note the emergence of *S.* Rissen in pig sources (pigs: 1.5%-2014, 2.8%-2015 and 1.2%-2016; pork meat: 4.9%-2014, 5.1%-2015 and 5.9%-2016) in EU (since 2014 is the fifth most common serotype) as well as its association with human infections (**Figure 1**) (6,38,53). The same survey reported a high incidence of *S.* Rissen in breeding and production holdings, particularly in Portugal (40% and 22.4%, respectively) and Spain (25% and 29.7%, respectively), being the first or second most frequently reported serotype in those settings (30,31). Despite *S.* Enteritidis (top 1 in human infections) being typically associated with eggs and poultry meat, it is important to point out that in the last years in EU this serotype was also common (top 6) in both pig and pork meat samples, with an increase of its prevalence between 2015 and 2016 (1.3%-2015 and 3.5%-2016) (**Figure 1**). Moreover, *Salmonella* Infantis, another typically poultry-related serotype causing human infections (top 4), was also detected in pigs (1.7%-2014, 2.2%-2015 and 0.4%-2016), and particularly in pork meat (8.8%-2014, 4.1%-2015 and 5.6%-2016) (**Figure 1**). Therewithal, both *S.* Enteritidis and *S.* Infantis serotypes have also been recovered from pigs/pork and products thereof in other non-EU regions, and associated with human salmonellosis (54–56) (www.health.gov.au/) (www.cdc.gov/salmonella/outbreaks.html).

### 2.3. Dissemination of pig-associated *Salmonella* serotypes and clones

Besides worldwide data concerning *Salmonella* serotype prevalence in humans and pig sources, the contribution of pork meat for human salmonellosis has been also evidenced throughout the spread of certain pig-associated strains and clones. Several examples of outbreaks associated with pig-related *Salmonella* serotypes have been described involving diverse countries (**Table 1**). For instance, *S.* 1,4,[5],12:i:- strains causing human infections, including some particular major clones, were identified in diverse European countries and

associated with the consumption of different pork products (**Table 1**). Moreover, since 2015, several notifications were reported by the Rapid Alert System for Food and Feed (RASFF), due to the presence of *Salmonella* in pork products from several European countries, including alerts of suspected multi-country foodborne outbreaks (e.g. *S.* Typhimurium ST19 in Denmark associated with chilled sliced salami from Spain) (**Table 1**). This scenario alerts for the relevant role of pig/pork meat international trade on the dissemination of clinically-relevant pig-related *Salmonella* serotypes/clones, highlighting the need for a global effective surveillance and detection programmes at all stages of pork production (9,11).

Regarding *S.* Typhimurium, diverse examples of pork products-related outbreaks have been reported in the last years, highlighting the importance of this serotype in the pork production chain (**Table 1**). Moreover, during the last decade, *S.* Typhimurium has been associated with clinically-relevant multi-drug resistant (MDR) clones, being of note the globally disseminated "*S.* Typhimurium DT104 clone"/ST19, already reported associated with pig production (18,57–61). In the same way, MDR "*S.* Typhimurium OXA30-producer"/ST19 associated with swine production samples was also reported in Portugal and other European countries (58,62–64). More recently, MDR "*S.* Typhimurium European clone"/ST34 with PFGE-types profiles similar to *S.* 1,4,[5],12:i:- "European clone", was reported in Europe, particularly among piggeries, abattoirs, pork meat as well as human infections (18,57,60,65).

In EU, *S.* 1,4,[5],12:i:- has been considered an emerging serotype causing human infections (66–68), with a remarkable increase in pig and pork meat, surpassing even in the last years *S.* Typhimurium (6,38,53). In fact, recent European surveys have demonstrated a high prevalence of *S.* 1,4,[5],12:i:- in pigs, carcass and environmental samples (14% to 43%) (9,69). Furthermore, several studies have already demonstrated the same clonal relatedness between *S.* 1,4,[5],12:i:- isolates from human clinical and pigs and/or products thereof (17,23,57,67,70), which corroborates previous data evidencing that pigs are the main animal reservoir of this emerging serotype in European countries (6,57,67). Interestingly, a remarkable increase of this serotype in human clinical cases was observed in Portugal, from third- (4.5%, between 2000 and 2012) (71) to first-ranked serotype (36.6%, in 2014 to 2016) (72), surpassing *S.* Enteritidis and *S.* Typhimurium. In addition to *S.* Typhimurium, *S.* 1,4,[5],12:i:- was in the last years the other major serotype responsible for large outbreaks associated with diverse pork products (**Table 1**). Moreover, two predominant MDR clones of *S.* 1,4,[5],12:i:-, the "European clone"/ST34 and the "Spanish clone"/ST19, have been recognized as responsible for most human infections associated with this serotype through

pork products. The "European clone", frequently belonging to DT120 and DT193 phage type has been circulating in several regions of Europe (65,66,73,74) and more recently in America (27,75), Asia (22), and even Australia (21). Meanwhile, the "Spanish clone", with most of the isolates belonging to DT104/U302 phage type, was originally identified in Spain and further reported since 2002 in the Iberian Peninsula (57,76–78). The maintenance and dissemination of these MDR clones in Europe could be explained by common pig breeding lines and by the intense commercial trade of pigs and products thereof between countries (31). Additionally, a third less frequent MDR clone of *S.* 1,4,[5],12:i:-, "Southern-European clone"/ST19, was reported in Portugal (17,78) and sporadically in Italy and Spain (79). Noteworthy, these three *S.* 1,4,[5],12:i:- clones have specific phenotypic and genotypic MDR profiles, being suggested that those features may constitute specific biomarkers for clone detection (17).

*S.* Derby has been one of the predominant serotypes in both pig and pork meat in Europe (6,38,53), and in USA (www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/UCM498134.pdf), despite being less implicated in human salmonellosis. Nevertheless, *S.* Derby has been reported with identical MDR and/or PFGE profiles in isolates from human clinical cases, pigs and products thereof, demonstrating their potential role in human infections (23,24,57,58,60,80–84).

Finally, *S.* Rissen has been one of the most frequently detected serotype in pigs and pork meat in the last years despite being less frequently associated with human clinical cases (6,38,53). In Southern European countries, *S.* Rissen is considered a clinically-relevant serotype, being frequently detected the same strains in humans, pigs and products thereof (17,23,25,26,57,58,60,69,85,86). Indeed, *S.* Rissen was the fourth most frequently serotype detected in human clinical isolates in Portugal, between 2002 and 2016 (71,72), being demonstrated its association with pig sources (18,58). Moreover, *S.* Rissen strains detected among humans and across pig production chain, particularly belonging to the successful MDR clone ST469, have been reported in geographic distant countries (14,20,23,25,26,57,58,60,87,88). In particular, the circulation of a specific MDR *S.* Rissen clone between the Iberian countries can be explained by the intensive trade of pigs and products thereof (25). Additionally, some *S.* Rissen isolates recovered from human, pig and pork isolates in Denmark showed similar PFGE profiles with isolates from imported pigs or pork meat from Spain and Germany, as well as with isolates from human clinical cases of people who travelled to Thailand (20) (**Table 1**). These data enhance the contribution of live animals and international food trade to the spread of this *S.* Rissen clone, besides human travel to developing countries.

### 2.4. Colonization and persistence features of pig-related *Salmonella* serotypes and clones

The enhanced ability to colonize food animals and to persist along the food chain of pig-related *Salmonella* serotypes and clones associated with human infections is a topic of great concern (19,59,60). Specific adaptive features, such as colonization/virulence determinants, have been pointed out as an advantage for the maintenance and spread of these serotypes/clones in diverse environments and hosts (pig/human) (89). Recent studies have found the presence of several virulence genes, associated with an enhanced adaptation to the food-animal host, in *S.* Typhimurium (89,90), in specific clones of *S.* 1,4,[5],12:i:- strains circulating in Europe (67,68,77,91) and in *S.* Rissen, including in isolates belonging to the ST469 (92,93). Those virulence genes encode for proteins that improve colonization (e.g. *clpB*), adhesion (e.g. *csgA*, *fimA/C*, *pefA*, *stbD, marT*), intestinal invasion (e.g. *invA, invE, spvC*), survival in host tissues (e.g. *sopA*, *avrA*, *sseI, mig5*) and biofilm formation (e.g. *bss*) (79,90,92,94,95). Interestingly, *S.* Typhimurium DT193 and *S.* 1,4,[5],12:i:- were associated with a long-term survival in pig faeces comparing with other serotypes (*S.* Derby and *S.* Bredeney), due to their increased adaptation to acid faecal pH and organic acid supplementation of feed (96). Additionally, an UK study demonstrated that *S.* Derby strains with SPI-23, which contain genes (e.g. *potR*) that encode Type III effector proteins, contributes for the host intestinal cells invasion in pigs (97).

Moreover, those emergent pig-related *Salmonella* serotypes/clones are usually enriched with antimicrobial resistance genes, in most cases located in the same genetic mobile elements also carrying virulence genes. For example, resistance plasmids of *S.* 1,4,[5],12:i:- isolates (from pigs and humans) circulating in Europe, carried several virulence genes, namely *spvC*±*mig5* genes in IncA/C and IncR plasmids, associated with the "Spanish" and "Southern European" clones, respectively (79). Furthermore, several genes associated with tolerance to metals and/or biocides, widely used in food production, were found in pig-related *Salmonella* serotypes/clones (e.g. *S.* Rissen MDR clone, "European clone" of *S.* 1,4,[5],12:i:- and *S.* Typhimurium), which might also be an additional advantage for their maintenance and spread in the food production environment and host (pig/human) (18,98,99).

## 3. Antimicrobial-resistant in *Salmonella* and the pork linkage

Antibiotic resistance is considered by several relevant Public Health entities one of the major threats to human health and a relevant concern for food safety, particularly if involves pathogenic bacteria transmitted to humans through food-chain (3,19). The emergence and spread of *Salmonella* isolates presenting resistance to several antibiotics, especially to "critically important" ones (fluoroquinolones and 3rd and higher generations

cephalosporins) (3) is of concern since they are crucial to the successful treatment of NTS invasive infections (1,2). The adverse consequences of resistance to critically important antibiotics in humans include an increase in severity of infections and in frequency of treatment failures, as well as the requirement of last-line antibiotics use (e.g. carbapenems, colistin) (3,19).

The common practice of antibiotic use in intensive food-animal production has been considered the main driver for the selection and transmission of antibiotic-resistant foodborne bacteria, including *Salmonella,* to humans (1,19,100–104). This scenario is aggravated particularly in pork production, which has been associated with a higher annual antimicrobial consumption, comparing with other animal-food production systems, at global level (105), including in the EU (106). In 2010, the annual average of antimicrobial consumption per kilogram of animal produced was 172 $mg \cdot kg^{-1}$ in pigs, higher than the 148 $mg \cdot kg^{-1}$ and 45 $mg \cdot kg^{-1}$ consumption in chicken and cattle, respectively (105). Although there is still controversy about the contribution of food-animal reservoirs and food vehicles in the transmission of antibiotic-resistant bacteria with an impact in human health, there is accumulating evidence linking the pork production with antimicrobial resistance in NTS that will be discussed in the next sections.

### 3.1. Association between antibiotic use in pig production and resistance in *Salmonella*

The first evidence of this linkage is the association between the amount and pattern of pork production usage of antimicrobial agents and the occurrence of resistant NTS in pigs, pork meat and/or humans. One illustrative case in pork production includes a study showing that the administration of tetracycline to pigs colonized with tetracycline-resistant *S.* Typhimurium DT104 was associated with higher pig shedding of this resistant strain compared with untreated pigs (107). Other study reported that enrofloxacin treatment of pigs induced the selection of *S.* Typhimurium with decreased susceptibility to ciprofloxacin (108). Furthermore, a Danish surveillance study performed after the ban of antibiotics as growth promoters showed higher levels of tetracycline resistance in *S.* Typhimurium isolates recovered from pig and human clinical cases, potentially caused by an increased usage of tetracycline in pigs (109).

### 3.2. Correlation between rates of antimicrobial resistance among *Salmonella* from pigs and humans

The correlation between antibiotic resistance rates among *Salmonella* from pigs, pork meat and humans obtained from systematic surveillance data evidences the impact of pork production practices on NTS antibiotic resistance. The 2016 European Food Safety

Authority (EFSA) report showed a high prevalence of MDR *Salmonella* in humans (29.3%), pigs (58.7%) and pork meat (40.4%), including to the most used antibiotics (tetracyclines, penicillins, sulphonamides and colistin) in pork production (19,106). In fact, high levels of resistance to ampicillin (A-27.8%), sulphonamides (Su-32.4%) and tetracycline (T-28.1%) and MDR (29.3%) were observed in *Salmonella* from human isolates, as well as from pigs (A-45.3%; Su-52.6%; T-53.5%; MDR-43.9%) and pork meat (A-44.7%; Su-48.5%; T-49.1%; MDR-40.4%). Furthermore, the ASuT phenotype was the most frequent MDR profile observed in pig and pork meat (82.3% and 80%, respectively), with the majority of the isolates belonging to the pig-related serotype *S.* 1,4,[5],12:i:- (66.4%-pig and 69.6%-pork meat) (19). Indeed, high levels of MDR were observed in pig-related serotypes, namely *S.* 1,4,[5],12:i:- (MDR=81.1%-humans; 82.3%-pigs; 73.8%-pork meat), *S.* Typhimurium (44.4%-humans; 52.4%-pigs; 54.5%-pork meat), *S.* Derby (23.8%-humans; 20.3%-pigs; 10.4%-pork meat) and *S.* Rissen (33.3%-pigs; 52.8%-pork meat). Also, this report highlighted that those pig-related serotypes were the major contributors to the observed prevalence of resistance in *Salmonella* in both pig and pork meat samples. Indeed, from 2014 to 2015 the MDR in humans has increased more than 10% in both *S.* Typhimurium and *S.* 1,4,[5],12:i:- serotypes (19). Data from the National Antimicrobial Resistance Monitoring System (NARMS) in the USA, showed an increase of the ASSuT (streptomycin-S) phenotype in *S.* 1,4,[5],12:i:- from human (from 43% in 2014 to 60% in 2015), and swine isolates (65% in 2015) (110).

Data about MDR phenotypes are of concern due to the possible role of diverse antibiotics in the co-selection of *Salmonella* strains resistant to clinically-relevant antibiotics, such as fluoroquinolones (e.g. ciprofloxacin-CIP), extended-spectrum cephalosporins (e.g. ceftazidime-CAZ, cefotaxime-CTX) and colistin (COL) (3,19,104,106). In the last EU report, relatively low levels of *Salmonella* resistance to CIP (13.3%), CAZ (0.9%), CTX (0.9%), and COL (11.4%) were observed (19) (https://www.efsa.europa.eu/en/interactive_pages/AMR_Report_2015). Moreover, the highest levels of COL resistance in humans (excluding the *S.* Enteritidis serotype which presents intrinsic resistance) were more common in the pig-related serotypes *S.* 1,4,[5],12:i:- (2.4%) and *S.* Typhimurium (1.5%) (19). Also, data from 2015 in USA revealed low levels of decreased susceptibility to CIP (5.8%) among humans, being the highest levels detected in retail pork samples (5.3%) comparing with other retail meat (0% in poultry and beef)
(https://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/ucm416741.htm), which suggest an important contribution of pork production chain to the human disease burden.

In contrast, high rates of antibiotic resistance were reported among humans and pig/pork products in middle-income countries. For instance, high rates of *Salmonella* with resistance to CIP (15-48%), ceftriaxone-CRO (38%) and COL (36%) were observed in humans (111–113). High levels of resistance to clinically-relevant antibiotics were also observed in pigs or pork meat , in particular resistance to CIP (10%-49.4%) (114–116) and COL (7%-21%) (112,117), being in most cases higher than those detected in poultry samples (CIP-0%-29%, COL-3.8%) (112,114,116). Moreover, different Chinese studies reported higher rates of acquired COL resistance mechanism (MCR) in pigs (10%-14.8%) than in poultry samples (0.8%-7.5%) (118–120). Indeed, it was already suggested that pork production can have the highest impact on colistin resistance in humans because of its extensive use in this food-animal production systems contrasting with others food animals (121,122). Overall, the high incidence of resistance to clinically-relevant antibiotics observed in pork production highlights the potential role of pork products in its spread and may be the consequence of the high, inappropriate or uncontrolled use of antibiotic in farming practices in those middle-income countries (105,113,117,120,121,123,124). In fact, it was estimated that the global consumption of antimicrobials in livestock will increase by 67% between 2010 and 2030, with an expected increase in pigs of 124%, particularly in Asia, in order to respond to the increasing demand of pork meat, one of the most consumed and traded meat product (29,105,125). Hence, these data demonstrate the potential contribution of the international trade of live pigs (piglets, weaner, grower or breeder pigs) and pork meat for the worldwide spread of antibiotic-resistant *Salmonella,* with a consequent impact on human health.

### 3.3. Transmission of antimicrobial resistant *Salmonella* from pork meat to humans

The presence of the same mobile genetic elements carrying clinically-relevant antibiotic resistance genes and/or antibiotic-resistant *Salmonella* serotypes/clones in pork production and humans is an additional evidence of transmission of antimicrobial resistant *Salmonella* from pork meat to humans. In the last decades, clinically-relevant antibiotic resistance genes have been globally reported in a wide range of *Salmonella* serotypes associated with pig and pork products, such as those coding for extended-spectrum β-lactamases (ESBLs), acquired AmpC β-lactamases (qAmpC), plasmid-mediated quinolone resistance (PMQR) and more recently plasmid-mediated colistin resistance (MCR) (**Table 2**). Additionally, some examples that illustrate the linkage and transmission of MDR *Salmonella* from pigs/pork products to humans are shown in **Table 2**. For instance, as examples of strains/clones shared between pork and human were *S.* Typhimurium with

*bla*$_{CTX-M-1}$, *bla*$_{CMY-2}$ or *oqxAB±aac(6´)-Ib-cr*, *Salmonella* Virchow with *bla*$_{CTX-M-15}$, and *S.* Typhimurium, *S.* 1,4,[5],12:i:- or *S.* Bovismorbificans carrying *mcr-1* gene (**Table 2**).

The most prevalent genes coding for resistance to extended-spectrum cephalosporins were those coding for CTX-M enzymes (e.g. CTX-M-1, CTX-M-14, CTX-M-15) followed by qAmpC CMY-2, both reported in pigs/pork products and humans, and increasingly associated with pig-related serotypes (e.g. *S.* Typhimurium and *S.* 1,4,[5],12:i:-) (**Table 2**) (104,126). Plasmid-mediated quinolone resistance (PMQR) mechanisms have been also widely reported in diverse NTS serotypes from both pig/pork products and humans (126–128). The most frequent PMQR were QNR proteins (e.g. QnrB19, QnrS1/S2) in diverse regions as well as the aminoglycoside acetyltransferase AAC(6´)-Ib-cr, commonly associated with the efflux pump OqxAB, and widely found in Asian countries among relevant pig-related serotypes (*S.* Typhimurium, *S.* Derby and *S.* Rissen) (**Table 2**). Finally, pig/pork seems to be an important vehicle of NTS carrying the emergent plasmid-mediated colistin resistance mechanism, (mostly MCR-1) (**Table 2**), also predominantly in pig-related NTS serotypes (123,126).

All these data evidenced that pig/pork products might be an important vehicle for the transmission and dissemination of NTS carrying acquired clinically-relevant resistance genes to humans through the food-chain. Besides, the most frequent clinically-relevant antibiotic resistance genes reported in pigs/pork products, also genetic elements (such as specific plasmids) were also shared among pork and humans (**Table 2**). In fact, transmission of the most commonly reported genes in both sources, has been associated with several epidemic plasmid families, such as IncN (*bla*$_{CTX-M-1, -27, -65}$), IncFIB (*bla*$_{CTX-M-14, -27, -65}$, *bla*$_{CMY-2}$), IncA/C (*bla*$_{CTX-M-27}$, *bla*$_{CMY-2}$), IncI1 (*bla*$_{CTX-M-1}$, *bla*$_{CMY-2}$), IncHI2 (*bla*$_{CTX-M-1, -14, -15}$, *oqxAB±aac(6´)-Ib-cr*, *mcr-1*), IncI2 or IncX4 (*mcr-1*) (**Table 2**) (104,123,126,127). More worrying is the presence of different clinically-relevant antibiotic resistance genes (e.g. *mcr-1+bla*$_{CTX-M-1}$, *mcr-1+oqxAB+aac(6´)-Ib-cr*) co-located in the same genetic elements and identified in the same strain (120,129) (**Table 2**), worsening the possibility of clinical treatment failure of invasive NTS infections.

In addition to those therapeutic consequences, the acquisition of resistance mechanisms to antibiotics commonly used in food-animal production (e.g. ampicillin, sulphonamides, tetracyclines), is also relevant for the successful persistence and dissemination of pig-related MDR *Salmonella* clones in pork production and further transmission to humans. Several studies provide evidence of transmission from pork to humans of successful MDR *Salmonella* clones from diverse serotypes (17,18,23,24,130,131). During the last decade, *S.* Typhimurium has been associated with clinically-relevant MDR clones, being of note the globally disseminated "*S.* Typhimurium DT104 clone"/ST19, typically presenting resistance to ACSSuT (C-chloramphenicol, S-streptomycin) (18,57–61)

and "*S.* Typhimurium OXA30-producer clone"/ST19 with ACSSuT phenotype (58,62–64). More recently, the worldwide dissemination of *S.* 1,4,[5],12:i:- and *S.* Typhimurium "European clone"/ST34 presenting the ASSuT MDR profile, was also linked with the pork production chain (17,21,22,27,65,66,68,73–75), as well as *S.* 1,4,[5],12:i:- "Spanish clone"/ST19 with ACGSSuTTm profile (G-gentamycin, Tm-trimethoprim) (17,57,76–78). The dissemination of other pig-related MDR *Salmonella* clones through pork production to humans has been evidenced by other less common serotypes. Specific clones of *S.* Derby, with similar MDR profiles (mainly SSuT), have been described mostly in European countries (23,24,57,58,60,80–84). In the EU, *S.* Rissen/ST469 clone has been also frequently associated with a typical MDR profile, namely ASSuTTm (14,18,20,23,25,26,57,58,60,88), including with origin in Thailand (87). Moreover, some of these successful clones presented acquired metal tolerance genes, an additional feature that might be contributing for the survival and persistence of these strains in metal contaminated environments, such as the pig production setting (18,98,132). For example, *sil*±*pco* genes encoding for copper/silver tolerance were often found in the emergent "European clone" of *S.* 1,4,[5],12:i:- and *S.* Typhimurium, and *S.* Rissen/ST469 MDR clone (18,98,99). In fact, copper is one of the most used metal compounds in pig settings (e.g. as supplements in animal feed), suggesting that in these environments higher selective pressures could contribute for the co-selection of MDR NTS clones (18,98,99,132–134), with consequences for food safety and human health.

The data presented here, show that pig production setting can be a relevant reservoir of successful and worldwide emergent MDR pig-related *Salmonella* serotypes/clones, enriched with different adaptive features (e.g. metal/biocides tolerance genes) besides genes conferring resistance to critical antibiotics, which might spread to humans through the food chain. Moreover, the increasing trend of intensive pig production systems and the globalization of pork products poses a major challenge for the spread of antimicrobial resistance in zoonotic bacteria like NTS with a consequent impact on human health. Therefore, is crucial to restrain the use of antimicrobial agents in pork production, in order to minimize the selection of resistant *Salmonella*, but also to improve biosecurity measures (e.g. high standards of hygiene, regular veterinary checks, vaccination) to control the introduction and spread of successful pig-related MDR clones along all stages of pork production chain.

## 4. Conclusions

Pork products are among the most frequent foodstuffs implicated in human salmonellosis, with pig and pork meat reported worldwide as important sources of NTS resistant to clinically-relevant antibiotics, representing a major threat to the treatment of

invasive infections. Furthermore, the high incidence of resistance to clinically-relevant antibiotics reported in diverse countries together with the increasing demand for pork meat and the global trade of pig/pork products enhanced the current public health concern.

Hence, the continuous control and monitoring of *Salmonella*, particularly targeting specific successful MDR pig-related serotypes/clones, along the food chain (from primary production to consumption) is critical to minimize their introduction in the food-animal production and further transmission to humans. Therefore, a global integrated surveillance ("One Health" approach), and the implementation of more effective measures are critically needed, including the improvement of biosecurity measures at farm (e.g. providing uncontaminated feed, isolation of new purchased animals, high standards of hygiene, regular veterinary checks, vaccination), slaughtering/processing (e.g. prevent external sources of contamination during transport, lairage and slaughter, and cross-contamination with equipment and workers, hygiene practices, as cleaning and disinfection) and retail/consumer level (e.g. avoiding cross-contamination, using safe cooking temperature). Surveillance of antibiotic resistance levels in NTS throughout the pig food chain is crucial to ensure public health, not only through the detection of new food safety risks involving foodstuffs like pork meat but also to avoid salmonellosis treatment failures.

**Transparency Declaration**

The authors declare no conflict of interest.

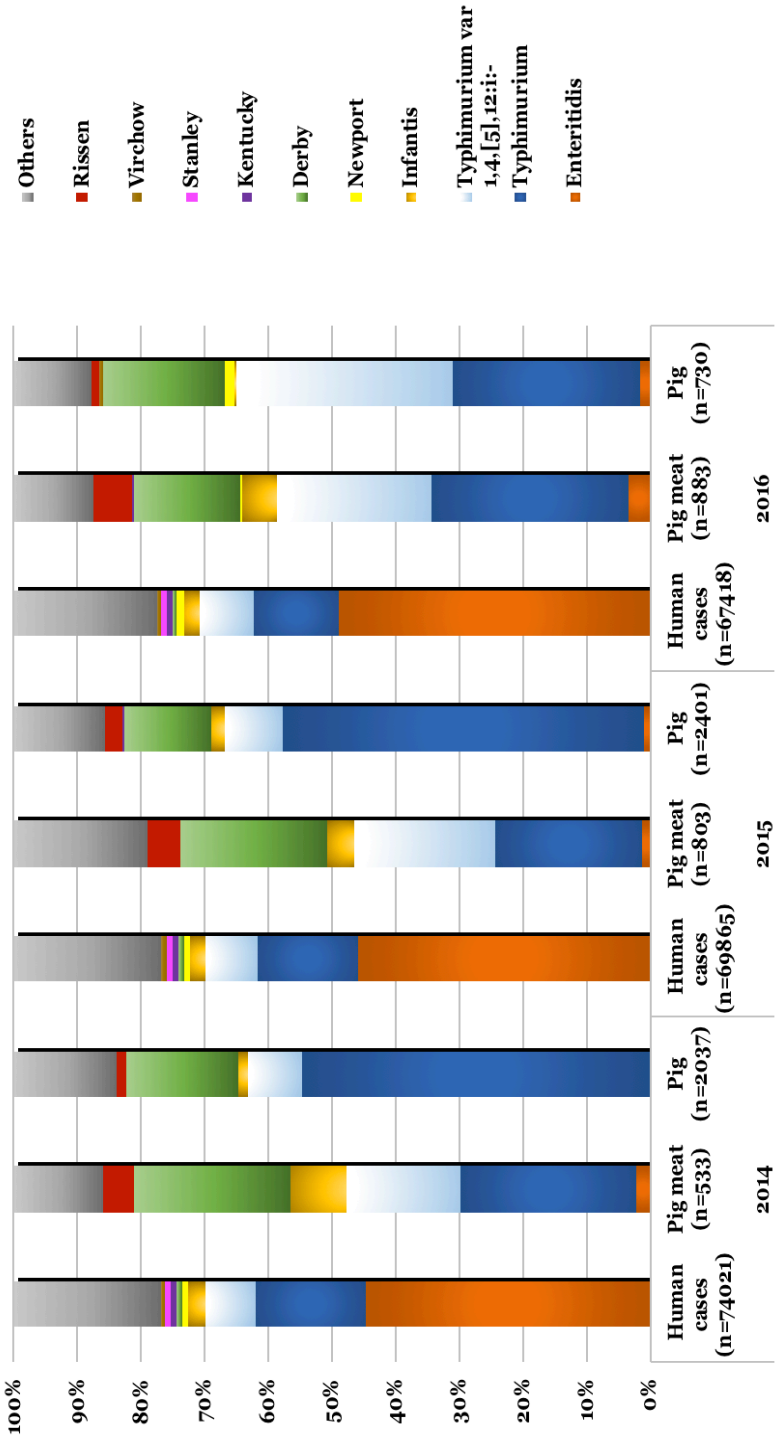


**FIGURE 1.** Distribution of the major serotypes of non-typhoidal *Salmonella* associated with human cases (salmonellosis), pig and pig meat in European Union (EU) from 2014 to 2016. The data were obtained from EFSA reports: humans, pig and pig meat (2014 to 2016) (last accessed February 2018) (6,38,53). *S.* Rissen serotype was included for being one of the five most frequent *Salmonella* serotypes recovered from pig meat and pig animal in EU from 2014 to 2016 (6,38,53). The percentages were calculated based on the total number of serotyped isolates per human salmonellosis cases, pig meat or pig animal.

**TABLE 1.** *Salmonella* outbreaks linked to pork meat products (2004-2018).

| **Serotype** (Subtyping features/molecular markers, when available)[a] | **Country(ies)** | **Source** | **Year(s)** | **No. of[b]** | | **Reference** |
|---|---|---|---|---|---|---|
| | | | | **Cases** | **Death(s)** | |
| **Typhimurium** | | | | | | |
| (DT104A) | Italy | Pork salami | 2004 | 63 | 0 | (135) |
| (DT12) | Denmark | Pork products | 2005 | 26 | 0 | (136) |
| (MLVA-type 3-12-4-13-2) | Multi-country outbreak: Denmark/Norway/Sweden | Danish pork meat/minced meat | 2008 | 37/10/4 | 4/0/0 | (137) |
| (DT193; MLVA-type 3-14-12-NA-211) | Denmark | Pork salami | 2010 | 20 | NS | (138) |
| (DT120; MLVA-type 3-11-14-NA-211) | Denmark | Imported smoked pork tenderloin | 2011 | 22 | 0 | (139) |
| (DT193) | Spain | Dried pork sausage | 2011 | 8 | 0 | (140) |
| | Australia | Barbecued pork | 2010 | 4 | NS | AGDH[d], 2010 |
| | | Pork spit roast | 2011 | 5 | NS | AGDH[d], 2011 |
| | | Cooked pork hock | 2014 | 4 | NS | AGDH[d], 2014 |
| | | Pork dish | 2015 | 10 | NS | AGDH[d], 2015 |
| (ST19) | Denmark[c] | Spanish chilled sliced salami | 2017 | NS | NS | RASFF[e], 2017 |
| | Sweden | Spanish salami | 2017 | NS | NS | RASFF[e], 2017 |
| **1,4,[5],12:i:-** | | | | | | |
| (DT193; PFGE-type STYMXB.0131; ASSuT) | Luxemburg | Pork meat | 2006 | 133 | 1 | (141) |
| (MLVA-Type 3-13-15-NA-211) | France | Dried pork sausage | 2010 | 69 | 0 | (142) |
| (PFGE-type XTYM-159; MLVA-type 3-13-9-NA-211/ASSuT) | | Dried pork sausage | 2011 | 337 | 0 | (143) |
| (PFGE-type STYMXB.0131/ STYMXB.0083; MLVA-type 3–13–9-NA-211; ASSuT) | Italy | Pork salami | 2012-5 | NS | NS | (144) |
| (DT138; PFGE-type XbaI.0005/BlnI.00X; ASSuT) | Spain | Pork product chorizo | 2014 | 6 | 0 | (146) |
| (DT138) | Spain | Dried pork sausage | 2011 | 38 | 0 | (140) |

| **Serotype** (Subtyping features/molecular markers, when available)[a] | **Country(ies)** | **Source** | **Year(s)** | **No. of[b]** | | **Reference** |
|---|---|---|---|---|---|---|
| | | | | **Cases** | **Death(s)** | |
| **1,4,[5],12:i:- (*cont.*)** | USA | Pork meat | 2015 | 188 | 0 | CDC[f], 2015 |
| | Sweden[c] | Italian chilled truffle salami | 2018 | NS | NS | RASFF[e], 2018 |
| **Derby** | | | | | | |
| | Spain | Dried pork sausage | 2011 | 3 | 0 | (140) |
| **Rissen** | | | | | | |
| (PFGE-type TEEX01.0017.DK) | Denmark[c] | Danish/imported pork products | 2000-5 | NS | NS | (20) |
| (PFGE-type TEEX01.0017.DK) | Thailand[c] | Pork products | 2004 | NS | NS | (20) |
| **Others:** | | | | | | |
| **Bovismorbificans** | | | | | | |
| (PT24) | Germany | Pork minced meat | 2004-05 | 525 | 1 | (147) |
| (ST142) | Multi-country outbreak: The Netherlands/Belgium/France | Pork ham products | 2016-17 | 54/NS/NS | 0/NS/NS | (148) |
| **Give** | | | | | | |
| | Germany | Minced pork meat | 2004 | 115 | 1 | (149) |
| **Goldcoast** | | | | | | |
| (PFGE-type SCG Xba3) | Hungary | Pork | 2009-10 | 44 | 0 | (150) |
| | Italy | Pork salami | 2009-10 | 79 | 0 | (151) |

| Serotype (Subtyping features/molecular markers, when available)[a] | Country(ies) | Source | Year(s) | No. of[b] | | Reference |
|---|---|---|---|---|---|---|
| | | | | Cases | Death(s) | |
| **Infantis** | | | | | | |
| (PT29; PFGE-type XB27) | Germany | Raw pork products | 2013 | 267 | 1 | (152) |
| | Australia | Pork rools | 2013 | 2 | NS | AGDH[c], 2013 |
| | USA | Pork meat | 2015 | 5 | 0 | CDC[d], 2015 |
| **Manhattan** | | | | | | |
| | France | Pork products | 2005-06 | 69 | 0 | (153) |
| **Muenchen** | | | | | | |
| (PFGE-type XbaI.1056) | Germany | Raw pork sausage | 2013-14 | 203 | 4 | (154) |
| **Ohio** | | | | | | |
| (PFGE-type *Bln*I-A) | Belgium | Pork meat | 2005 | 60 | 0 | (155) |

[a]Antimicrobial compounds: A, ampicillin; S, streptomycin; Su, sulphonamides compound; T, tetracycline. DT/PT, Phage type; MLVA, Multiple Locus Variable-number Tandem Repeat Analysis; NA, locus not present; NS, not specified; PFGE, Pulsed-field Gel Electrophoresis; ST, Sequence type.

[b]Estimated number of cases only when outbreaks were reported.

[c]Suspected outbreak.

[d]AGDH, Australian Government Department of Health. OzFoodNet: Foodborne disease in Australia Annual reports of the OzFoodNet network. Available at: http://www.health.gov.au/ [accessed February 2018].

[e]RASFF, Rapid Alert System for Food and Feed. Available at: https://ec.europa.eu/food/safety/rasff_en [accessed February 2018].

[f]CDC, Centers for Disease Control and Prevention. Reports of selected *Salmonella* Outbreak Investigations. Available at: http://www.cdc.gov/salmonella/outbreaks.html [accessed February 2018].

**TABLE 2.** *Salmonella* serotypes/clones carrying clinically-relevant antibiotic resistance genes recovered from pig and products thereof.

| ***Salmonella* serotype** | **Antibiotic resistance gene(s)[a] (no. isolates)** | **Source** | **Human concomitant presence[b]** | **Country/ year(s)** | **Antibiotic resistance phenotype[c]** | **Gene location (PL-Inc group/Chr)** | **Reference** |
|---|---|---|---|---|---|---|---|
| **Typhimurium** | *bla*$_{TEM-52}$ (n=2) | Pigs | | Belgium/2009 | NR | NR | (156) |
| | *bla*$_{CTX-M-1}$ (n=1) | Pork meat | | Germany/2007 | KAN-NEO-STR-SUL-TET-TMP-SXT | PL-N | (157) |
| | *bla*$_{CTX-M-14}$ + *bla*$_{SHV-1}$ + *oqxAB* + *aac(6')-Ib-cr* (n=2) | Pigs | Yes | China/2014 | NS | PL-FIB, N | (158) |
| | *bla*$_{CTX-M-27}$ + *bla*$_{SHV-1}$ + oqxAB + *aac(6')-Ib-cr* (n=1) | Pig | | China/2014 | NS | PL-P | (158) |
| | *bla*$_{CTX-M-27}$ + *qnrB* + *oqxAB* + *aac(6')-Ib-cr* (n=1) | Pig | | China/2014 | NS | PL-NT | (158) |
| | *bla*$_{CTX-M-55}$ + *qnrA* + *qnrB* (n=4) | Pigs | | China/2016 | AMP-(CIP-ENO-FFC-LVX- NAL-NEO-TET) | NR | (55) |
| | *bla*$_{CTX-M-65}$ + *oqxAB* (n=1) | Pig | | China/2014 | NS | PL-FIB | (158) |
| | *bla*$_{CMY-2}$ (n=52) | Pork | | Colombia/UN | AMP-TET | NR | (159) |
| | | Pork and pig | Yes | Mexico/2002-05 | CHL-STR-SUL-TET-(GEN-KAN-NAL-SXT) | NR | (160) |

**TABLE 2.** *Continued*

| ***Salmonella* serotype** | **Antibiotic resistance gene(s)[a] (no. isolates)** | **Source** | **Human concomitant presence[b]** | **Country/ year(s)** | **Antibiotic resistance phenotype[c]** | **Gene location (PL-Inc group/Chr)** | **Reference** |
|---|---|---|---|---|---|---|---|
| | | Pigs | | Mexico/NR | AMP-CAZ-CTX-FOX-TET-(GEN) | NR | (44) |
| | | Pigs | | USA/2007 | AMP-CHL-CRO-FOX-GEN-KAN-STR-SUL-SXT-TET-TIO | PL-A/C, FIB, I1 | (161) |
| | | Pigs | | Belgium/2009 | NR | NR | (156) |
| | | Diarrheic pigs | | South Korea/ 2011-2012 | AMP-CEF-CHL-FFC-FOX-GEN-NAL-SXT-TET-TIO | PL-A/C, FIB | (162) |
| | *qnrS1* + *oqxAB* + *aac(6')-Ib-cr* (n=4) | Pork | | China/2012-13 | AMP-CHL-CIP-GEN-KAN-NAL-OLA-STR-SUL-TET | NR | (163) |
| | *oqxAB* (n=3) | Pigs | | China/2010 | AMP-CHL-FFC-NAL-OLA-SUL-TET-(GEN) | PL-F | (164) |
| | | Pork | | China/2012-13 | AMP-CHL-CIP-KAN-NAL-OLA-STR-SUL-TET-(GEN) | NR | (163) |
| | *oqxAB* + *aac(6')-Ib-cr* (n=6) | Pigs | | China/2008-10 | AMP-NAL-OLA-SMX-TET-(CHL-CIF-FFC-GEN) | PL-HI2 | (164) |
| | | Pork | | China/2012 | AMP-CHL-CIP-GEN-KAN-NAL-OLA-STR-SUL-TET | NR | (163) |
| | *mcr-1* (n=10) | Pigs | | Spain/2009-11 | COL | PL-NR | (165) |
| | | Ready-to-eat pork | | China/2014 | CHL-CIP-COL-FFC-GEN-NAL-STR-SXT-TET | PL-HI2 | (166) |

**TABLE 2.** *Continued*

| ***Salmonella* serotype** | **Antibiotic resistance gene(s)[a] (no. isolates)** | **Source** | **Human concomitant presence[b]** | **Country/ year(s)** | **Antibiotic resistance phenotype[c]** | **Gene location (PL-Inc group/Chr)** | **Reference** |
|---|---|---|---|---|---|---|---|
| | | Ready-to-eat pork | | China/2014 | CHL-CIP-COL-FFC-GEN-NAL-STR-SXT-TET | PL-HI2 | (166) |
| | *mcr-1* + $bla_{CTX-M-1}$ (n=1) | Pig | | Portugal/2011 | AMP-ATM-CAZ-CHL-COL-CTX-FEP-FOX-GEN-TET-TOB | PL-HI2 | (129) |
| | *mcr-1*+ $bla_{CTX-M-14}$ (n=2) | Pork | | China/2015 | AMP-CAZ-CHL-COL-CTX-FOS-GEN-SUL-TET | PL-HI2 | (167) |
| | *mcr-1* + *oqxAB* (n=17) | Pigs | | China/2013-14 | CIP-COL-FFC-OLA-SXT-TET-(AMP-GEN-STR) | PL-HI2-F4:A-:B5[d] | (120) |
| | *mcr-1* + *oqxAB* + *aac(6')-Ib-cr* (n=4) | Pigs | | China/2008-09 | AMP-COL-FFC-GEN-NAL-OLA-STR-SXT-TET-(CIP) | PL-I2, HI2 | (119) |
| | *mcr-1* + *aac(6')-Ib-cr* (n=1) | Pig | | China/2008-09 | AMP-COL-FFC-GEN-NAL-OLA-STR-SXT-TET | PL-I2 | (119) |
| | | Pigs | | China/2015 | AMP-CIP-COL-GEN-(CHL-NAL-SXT) | PL-HI2 | (167) |
| **1,4,[5],12:i:-** | $bla_{CTX-M-1}$ (n=15) | Pigs | | UK/2009 | AMP-CTX-SUL-(CHL) | PL-I1-γ | (168) |
| | | Pigs | | Germany/ 2007, 09-10 | AMP-ATM-CRO-CTX-CEF-CXM-PIP-TIC-TIO-(FEP-STR) | PL-N, I1 | (169) |
| | $bla_{CTX-M-14}$ (n=1) | Pork | | Portugal/2010 | AMP-CHL-CTX-TET-TMP | NR | (86) |

**TABLE 2.** *Continued*

| ***Salmonella* serotype** | **Antibiotic resistance gene(s)[a] (no. isolates)** | **Source** | **Human concomitant presence[b]** | **Country/ year(s)** | **Antibiotic resistance phenotype[c]** | **Gene location (PL-Inc group/Chr)** | **Reference** |
|---|---|---|---|---|---|---|---|
| **1,4,[5],12:i:-** | *bla*$_{\text{CTX-M-15}}$ + *bla*$_{\text{SHV-12}}$ (n=1) | Pork | | Portugal/2011 | AMP-CHL-CTX-STR-SUL | NR | (86) |
| | *bla*$_{\text{CTX-M-32}}$ (n=1) | Pork | | Portugal/2011 | AMP-CHL-CTX-GEN-STR-SUL-TET | NR | (86) |
| | *qnrB19* (n=1) | Pig | | USA/2014 | CIP | PL-NR | (170) |
| | *mcr-1* (n=16) | Pigs | Yes | Italy/2012-15 | AMP- COL-STR-TET-(CIP-CHL-FFC) | NR | (70) |
| | | Pork | Yes | Italy/2013-15 | AMP- COL-STR-TET-(CHL-FFC) | NR | (70) |
| | | Pork carcass | | France/2016 | COL | PL-NR | (171) |
| | | Pork carcass | | Belgium/2012 | AMP-COL-STR-SUL-TET | PL-X4 | (172) |
| | *mcr-4* (n=1) | Pigs | | Italy/2014 | AMP-COL-STR-SUL-TET | PL-ColE | (173) |
| **Derby** | *bla*$_{\text{CTX-M-1}}$ (n=2) | Pigs | | Belgium/2009 | NR | NR | (156) |
| | *qnrA* (n=2) | Pig | | China/2016 | AMP | NR | (55) |
| | *qnrB19* (n=3) | Pigs | | USA/2014 | CIP | PL-NR | (170) |
| | | Pork chops | | USA/2014-15 | CIP | PL-NR | (170) |
| | *qnrB* + *qnrS1* + *oqxAB* (n=1) | Pork | | China/2013 | AMP-CHL-CIP-GEN-KAN-NAL-OLA-STR-SUL-TET-(AZI) | NR | (163) |
| | *qnB8* + *qnrS2* + *oqxAB* + *aac(6')-Ib*-cr (n=1) | Pork | | China/2012 | AMP-CHL-CIP-GEN-KAN-NAL-OLA-STR-SUL-TET | NR | (163) |

**TABLE 2.** *Continued*

| ***Salmonella* serotype** | **Antibiotic resistance gene(s)[a] (no. isolates)** | **Source** | **Human concomitant presence[b]** | **Country/ year(s)** | **Antibiotic resistance phenotype[c]** | **Gene location (PL-Inc group/Chr)** | **Reference** |
|---|---|---|---|---|---|---|---|
| | *qnrS2* (n=1) | Pork chops | | USA/2014 | CIP | NR | (170) |
| | *qnrS2 +oqxAB + aac(6')-Ib-cr* (n=11) | Pork | | China/2012-13 | AMP-CHL-CIP-GEN-KAN-NAL-OLA-STR-SUL-TET-(AZI) | NR | (163) |
| | *oqxAB* (n=3) | Pork | | China/NR | CHL-NAL-OLA-TET | Chr | (174) |
| | | Pork | | China/2013 | AMP-CHL-CIP-GEN-KAN-NAL-OLA-STR-SUL-TET | NR | (163) |
| | *mcr-1* (n=13) | Pigs | | Italy/2012-15 | CHL-COL-STR-SUL-SXT-TET | NR | (70) |
| | | Pork sausage | | France/2013 | COL-(AMP-STR-TET) | PL-P | (175) |
| | | Pork | | China/2015 | AMP-CHL-COL-TET | PL-X4 | (167) |
| | *mcr-2* (n=1) | Pork carcass | | Belgium/2012 | CHL-COL-SUL-TMT | PL-X4 | (172) |
| **Rissen** | $bla_{\text{CTX-M-1}}$ (n=1) | Pig | | Belgium/2009 | NR | NR | (156) |
| | $bla_{\text{CTX-M-55}}$ (n=1) | Pork carcass | | Thailand/2014-15 | AMP-CAZ-CTX-CHL-CPD-GEN-STR-SUL-TET | PL-NR | (48) |
| | $bla_{\text{SHV-12}}$ (n=1) | Pig | | Spain/1999 | AMP-ATM-CAZ-CEF-CTX-STR-SUL-TET | NR | (176) |
| | *qnrB19* (n=1) | Pig | | USA/2013 | CIP | PL-NR | (170) |
| | *qnrS1* (n=1) | Pig | | Korea/2012-13 | CIP-NAL | NR | (54) |
| | *qnrVC4* (n=1) | Pig | | Thailand/2007 | AMP-CHL-CIP-NAL-STR | PL-Q1 | (177) |
| | *oqxAB* (n=1) | Pork | | China/2013 | AMP-CHL-CIP-KAN-NAL-OLA-STR-SUL-TET | NR | (163) |

**TABLE 2.** *Continued*

| ***Salmonella* serotype** | **Antibiotic resistance gene(s)[a] (no. isolates)** | **Source** | **Human concomitant presence[b]** | **Country/ year(s)** | **Antibiotic resistance phenotype[c]** | **Gene location (PL-Inc group/Chr)** | **Reference** |
|---|---|---|---|---|---|---|---|
| | *mcr-1* (n=1) | Pig | | Spain/2009-11 | COL | PL-NR | (165) |
| **Others:** | | | | | | | |
| **Anatum** | $bla_{CMY-2}$ (n=4) | Pigs | | USA/2008-2011 | AMP-CRO-FOX-SUL-TET-TIO | PL-I1-γ | (178) |
| | *qnrB19* (n=5) | Pigs | | USA/2014 | CIP | PL-NR | (170) |
| | *mcr-1* + $bla_{CMY-2}$ (n=3) | Pigs | | Tawain/2013 | AMP-CAZ-CHL-CIP-COL-CTX-FOX-NAL-SUL-STR-SXT-TET | PL-NR | (112) |
| **Adelaide** | *qnrB19* (n=1) | Pig | | USA/2014 | CIP | PL-NR | (170) |
| **Bovismorbificans** | $bla_{CTX-M-1}$ (n=1) | Pig | | UK/2009 | AMP-CTX-SUL | PL-I1-γ | (168) |
| | *mcr-1* (n=1) | Pork | Yes | Italy/2013-15 | AMP-CHL-CIP-COL-STR-TET | NR | (70) |
| **Brandenburg** | *qnrB19* (n=1) | Pigs | | USA/2014 | CIP | PL-NR | (170) |
| **Concord** | *qnrB2* (n=1) | Pig | | Czech Republic/NR | NR | NR | (128) |
| **Dublin** | *mcr-1-like* (n=1) | Pig | | France/NS | NS | NR | (179) |
| **Enteritidis** | $bla_{CTX-M-15}$ (n=2) | Pigs | | Korea/2012-13 | AMP-CEP-GEN-NAL-NEO-STR-TET-TIO | PL-HI2 | (54) |
| | $bla_{CTX-M-55}$ + *qnrA* + *qnrB* (n=2) | Pigs | | China/2016 | AMP-(CIP-FFC-FOS-NAL-TET-TIO) | NR | (55) |
| | *qnrA*+ *qnrB* (n=1) | Pig | | China/2016 | AMP-CIP-FFC-NAL | NR | (55) |
| | *qnrS1/S3* (n=1) | Pig | | Poland/2008 | AMP-CIP | NR | (180) |

**TABLE 2.** *Continued*

| ***Salmonella* serotype** | **Antibiotic resistance gene(s)[a] (no. isolates)** | **Source** | **Human concomitant presence[b]** | **Country/ year(s)** | **Antibiotic resistance phenotype[c]** | **Gene location (PL-Inc group/Chr)** | **Reference** |
|---|---|---|---|---|---|---|---|
| **Give** | *bla*$_{CMY-2}$ (n=1) | Pig | | USA/1998-99 | AMP-AZT-CAZ-CHL-CTX-FOX-GEN-PIP-STR-SUL-TET-TIC-TIO | PL-NR | (181) |
| **Goldcoast** | *qnrS1* (n=1) | Pig | | Belgium/NR | NR | NR | (128) |
| **Heidelberg** | *bla*$_{CMY-2}$ (n=3) | Pigs | | Canada/2004 | AMP-CEF-FOX-TIO | PL-NR | (182) |
| | | Pigs | | USA/1998-99 | AMP-AZT-CAZ-CHL-CTX-FOX-GEN-PIP-STR-SUL-SXT-TET-TIC-TIO | PL-NR | (181) |
| | *mcr-1* + *oqxAB* (n=1) | Pig | | China/2013-14 | CIP-COL-FFC-OLA-STR-SXT | PL-HI2-F4:A-:B5[d] | (120) |
| **Hinsingen** | *qnrD* + *aac(6')-Ib-cr* (n=1) | Pig | | China/2009-10 | CIP-NAL | PL-NR | (183) |
| **Indiana** | *bla*$_{CTX-M-27}$ + *bla*$_{SHV-1}$ + *oqxAB* (n=2) | Pigs | | China/2014 | NS | PL-N, P | (158) |
| | *bla*$_{CTX-M-65}$ + *bla*$_{SHV-1}$ + *oqxAB* + *aac(6')-Ib-cr* (n=1) | Pig | | China/2014 | NS | PL-NT | (158) |
| | *bla*$_{CTX-M-65}$ + *oqxAB* + *aac(6')-Ib-cr* (n=7) | Pig | | China/2011 | AMP-CAZ-CHL-CIP-CTX-GEN-NAL-SXT-TET | PL-NR | (114) |
| | | Pork | | China/2012 | AMP-AZI-CHL-CIP-CRO-GEN-KAN-NAL-OLA-STR-SUL-TET | NR | (163) |

**TABLE 2.** *Continued*

| ***Salmonella* serotype** | **Antibiotic resistance gene(s)[a] (no. isolates)** | **Source** | **Human concomitant presence[b]** | **Country/ year(s)** | **Antibiotic resistance phenotype[c]** | **Gene location (PL-Inc group/Chr)** | **Reference** |
|---|---|---|---|---|---|---|---|
| | | Pig | | China/2014 | NS | PL-N | (158) |
| | *qnrA* + *oqxA* + *aac(6')-Ib-cr* (n=1) | Pig | | China/2009-10 | CIP-OLA-NAL | PL-NR | (183) |
| | *oqxA* (n=2) | Pig | | China/2009-10 | CIP-OLA-NAL | PL-NR | (183) |
| | *aac(6')-Ib-cr* (n=1) | Pig | | China/2009-10 | CIP-NAL | PL-NR | (183) |
| **Infantis** | $bla_{CMY-2}$ (n=2) | Pigs | | Japan/2007-08 | AMP-CAZ -CEF-CHL-FOX-STR-SUL-TET | PL-NR | (56) |
| | $bla_{VIM-1}$ + $bla_{AAC-1}$ (n=4) | Pigs | | Germany/2011 | AMP-CAZ-CEF-CHL-CRO-CTX-CXM-FEP-FOX-PIP-STR-SUL-TIC-TIO-TMP | PL-HI2 | (184) |
| **Kedougou** | *mcr-4.1* (n=1) | Pig carcass | | Spain/2016 | COL | PL-NR | (171) |
| **London** | $bla_{CTX-M-14}$ (n=1) | Pork | | Portugal/2012-13 | AMP-CTX | NR | (185) |
| | *qnrB19* (n=1) | Pig | | USA/2014 | CIP | PL-NR | (170) |
| | *mcr-1* + *oqxAB* (n=1) | Pig | | China/2013-14 | AMP-CIP-COL-FFC-GEN-OLA-STR-SXT | PL-HI2-F4:A-:B5[d] | (120) |
| **Miami** | $bla_{CMY-2}$ (n=2) | Diarrheic piglets | | India/2014 | AMP-CFL-CTX-ENO-FIX-PIP | NR | (186) |
| **Muenchen** | *qnrB19* (n=8) | Pigs | | USA/2013-14 | CIP | PL-NR | (170) |
| **Newport** | *mcr-1* + $bla_{TEM-135}$ (n=5) | Pigs | | China/2015 | AMP-CIP-CHL-COL-GEN | PL-HI2 | (167) |

**TABLE 2.** *Continued*

| ***Salmonella* serotype** | **Clinical relevant gene(s)[a] (no. isolates)** | **Source** | **Human concomitant presence[b]** | **Country/ year(s)** | **Antibiotic resistance phenotype[c]** | **Gene location (PL-Inc group/Chr)** | **Reference** |
|---|---|---|---|---|---|---|---|
| **Senftenberg** | *qnrB6* + *aac(6')-Ib-cr* (n=1) | Pig | | USA/2013 | CIP | NR | (170) |
| **Typhimurium var Copenhagen** | *mcr-1* (n=1) | Pig | Yes | Great Britain/NR | AMP-CHL-COL-FOX-SUL-TET-TMP-TYG | PL-I2 | (187) |
| **Virchow** | $bla_{\text{CTX-M-15}}$ (n=5) | Pigs | Yes | Korea/2012-13 | AMP-CEP-GEN-NAL-NEO-STR-TET-TIO | PL-HI2 | (54) |
| **Weltevreden** | *mcr-1* (n=1) | Pork | | China/2015 | COL-TET | PL-X4 | (167) |
| ***Salmonella* spp.** | *mcr-1* (n=3) | Pigs | | Italy/2010-11 | NR | NR | (179) |

Antimicrobial abbreviations: AMP, ampicillin; ATM, aztreonam; AZI, azithromycin; CAZ, ceftazidime; CPD, cefpodoxime: CEF, cephalotin, CFL, cephalexin; CHL, chloramphenicol; CIP, ciprofloxacin; COL, colistin; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; ENO, Enrofloxacin; FEP, cefepime; FFC, florfenicol; FIX, Cefixime; FOS, fosfomycin; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; LVX, levofloxacin; NAL, nalidixic acid; NEO, neomycin; OLA, olaquindox; PIP, piperacillin; STR, streptomycin; SUL, sulphonamides compound; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TIC, ticarcillin; TIO, ceftiofur; TMP, trimethoprim; TOB, tobramycin; TYG, tigecycline. Chr, chromosome; NS, not specified; NR, not reported; NT, not typable; PL, plasmid.

[a] Only references with full-characterized clinical relevant antibiotic resistance genes were considered.

[b] "Yes", clones or serotypes detected both in pigs/products thereof and humans in the same study.

[c] Variable phenotypes were present between curved brackets

[d] IncF replicon sequence typing.

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## 1.3. Mini Review Article:

## Non-typhoidal *Salmonella* typing: from classical to high-throughput techniques

# Non-typhoidal *Salmonella* typing: from classical to high-throughput techniques

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**Abstract**

Non-typhoidal *Salmonella* is one of the leading causes of foodborne diseases worldwide, with food-producing animals as the main reservoirs, with poultry and pork products the most frequent sources of human infections. Subtyping methodologies are crucial to accurately track *Salmonella* in epidemiological surveillance and investigation contexts (e.g. detection and control of foodborne outbreaks, source attribution).

Nowadays, several typing techniques are used for the differentiation of *S. enterica* at different infra-species levels, being currently required simpler, quick and low-cost high-throughput approaches for their identification and discrimination in routine and public health surveillance laboratories. Throughout this review, phenotypic, DNA-based and non-DNA molecular typing approaches will be addressed, providing an overview of the evolution, limitations and applications of the several techniques proposed for *S. enterica* typing.

**Keywords:** Non-typhoidal *Salmonella*, Typing, infra-species level, Serotyping, Phage typing, PFGE, MLVA, MLST, WGS, FTIR, MALDI-TOF MS.

## 1. Introduction

Non-typhoidal *Salmonella* (NTS) *enterica* serotypes are one of the leading causes of foodborne diseases worldwide, representing a major public health burden (1,2). Furthermore, *S. enterica*, which comprises a quite diverse population, is transmitted to humans by a wide range of foodstuffs, mainly of animal origin, with most infections being associated with particular serotypes (commonly *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* 1,4,[5],12:i:-) (3–6). Therefore, the identification and discrimination of *S. enterica* strains at the infra-species level is crucial for the control of endemic or epidemic scenarios (e.g. foodborne outbreaks) and for epidemiological surveillance and investigation (e.g. source attribution) (7–9).

Infra-species discrimination of *S. enterica* at different levels has been widely attempted by different typing techniques, such as serotyping, phage typing, Pulsed-Field Gel Electrophoresis (PFGE), Multiple Locus Variable-number Tandem Repeat Analysis (MLVA), Multilocus Sequence Typing (MLST), and more recently Whole-Genome Sequencing (WGS) (7,9–11) (Figure 1). However, due to the current demand of more quick, low-cost and user-friendly typing techniques, but keeping accuracy for discrimination and reproducibility, new non-DNA molecular approaches, such as the high-throughput techniques Fourier Transform Infrared Spectroscopy (FTIRS) and Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS), have been explored with different degrees of success (12,13) (Figure 1).

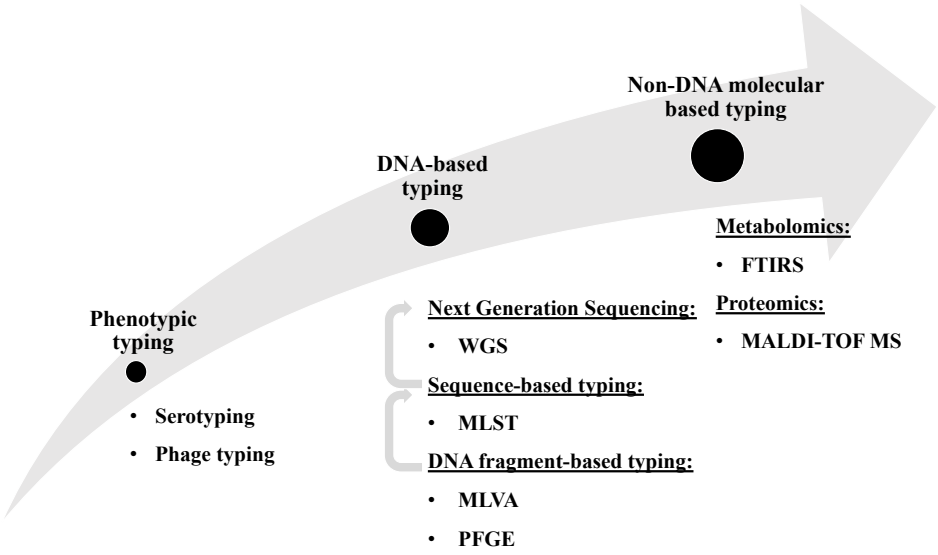


**Figure 1.** Evolution of *Salmonella enterica* typing techniques.
FTIRS, Fourier Transform Infrared Spectroscopy; MALDI-TOF MS, Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry; MLST, Multilocus Sequence Typing; MLVA, Multiple Locus Variable-number Tandem Repeat Analysis; PFGE, Pulsed-Field Gel Electrophoresis; WGS, Whole-Genome Sequencing.

## 2. Phenotypic Typing

Phenotyping techniques were critical between 1940 and the 1980s to aid the understanding of bacterial infra-species diversity and the epidemiology of bacterial food-borne infections. Some of them were historically important or are still useful nowadays in *Salmonella* typing, with most of them restricted to reference centres, as serotyping and phage typing. However, presenting known limitations in *Salmonella* discrimination (7,9,14).

### 2.1. Serotyping

*Salmonella* serotyping is a classical technique based on the Kauffmann–White-Le Minor scheme. This technique consists of agglutination reactions with specific antisera and surface bacterial antigens, the somatic O-antigen, the flagellar H-antigens (two alternatively expressed phases, phases 1 and 2) and in some cases the capsular Vi-antigens (e.g. *Salmonella* Typhi, *Salmonella* Paratyphi C or *Salmonella* Dublin) (15). The serogroup determination depends on the polysaccharide composition of O-antigen (outermost component of the cell surface lipopolysaccharide), which diversity is associated with the monosaccharides composition (3- to 5-sugars) and the structure of O-units (covalent bond nature and monosaccharides linkage), besides the polymerization linkage between the repeated units (2 to 8 O-units repetitions) (15–17). The final antigenic formula of most *Salmonella enterica* serotypes is based on a specific combination of O- and H-antigens, written as O:H1:H2 (e.g. antigenic formulae 1,4,[5],12:i:1,2 defines *S.* Typhimurium serotype) (15). Currently, there are about 46 known serogroups and over 2,600 *Salmonella* serotypes (with 1547 serotypes as part of subspecies I - *enterica*) (15,18).

Nevertheless, serotyping could be labour an intensive technique, time-consuming, subject to variations in interpretation (due to auto-agglutination and cross-reactions), and associated with high cost and universally unavailable sera, being therefore restricted to reference laboratories (19). In addition, given the diversity of clonal lineages belonging to the different serotypes currently known, this method is insufficient for *Salmonella* typing.

Despite those limitations, the serotyping technique is still globally recognized and used to firstly discriminate *S. enterica* isolates (19). For that, new approaches for *Salmonella* serotyping as PCR-based or WGS-based techniques (20–23) have been proposed as potential alternatives to the classical serotyping method (see next sections).

### 2.2. Phage typing

Phage typing is another subtyping technique beyond the serotype level and carried out to discriminate *Salmonella* strains belonging to the same serotype. In phage typing, the *Salmonella* strains are differentiated into different phage types according to the lysis

susceptibility pattern obtained from a highly specific phage's collection for a particular serotype (24,25). In fact, the usage of phage typing is now being restricted to the most common serotypes *S.* Enteritidis and *S.* Typhimurium (26–28). Nevertheless, phage typing presents some limitations, including phage cultures not universally available, lacks reproducible results and requires expertise for interpretation, which consequently restrict this technique to surveillance reference centres (24,29,30). Hence, phage typing has limited application in surveillance and epidemiological studies nowadays, being usually performed in association with other typing tools (26–28,31).

## 3. DNA-based Typing

The rapid advances in molecular biology tools during the 1970s have changed the course of bacterial strain typing (14). DNA-based typing techniques have progressively replaced phenotyping techniques in microbiology laboratories, mainly due to their higher discriminatory power, but also because they are generally less laborious and time-consuming. The genotypic methods can be classified as DNA fragment-based and DNA sequencing-based techniques (30,32,33).

### 3.1. DNA fragment-based Typing

#### 3.1.1. Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is a DNA-based strain typing technique developed in the 1990s and was considered a gold standard technique for many years (34,35). It is based on the analysis of restriction fragments after digestion of genomic DNA with a macro-restriction enzyme (*Xba*I is used as a primary enzyme for *Salmonella*) according to a standardized protocol (https://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c.pdf) (7,9,36). The DNA fragments produce a specific fingerprint pattern, which analysis was initially performed using Tenover' criteria (34), but later simplified by the use of commercially available software (e.g. InfoQuest) that allowed an analysis based on similarity coefficients (www.bio-rad.com/webroot/web/pdf/lsr/literature/10013994.pdf). Also, the inter-laboratory comparisons were facilitated by the development of the worldwide disseminated PulseNet network database (https://www.cdc.gov/pulsenet/) (37,38). The DNA fingerprint produced by PFGE allows a higher discriminatory power (comparing with the phenotypic typing tools), at the clonal level, being therefore commonly used for outbreak investigations and short-term epidemiological surveys (37,38).

Regarding *S. enterica*, the PFGE pattern analysis has to take into account the high clonality of some *Salmonella* serotypes (7,39). Consequently, in order to improve *Salmonella* strains discrimination in widespread outbreak investigations and long-term epidemiological studies, additional typing tools were normally required (7,9,11,37,39).

Although PFGE is still widely used, is technically demanding, time-consuming, associated with subjectivity in the analysis of results and operator-dependent, thus hindering the inter-laboratory results reproducibility and comparability (7,40).

### 3.1.2. Multiple Locus Variable-number Tandem Repeat Analysis (MLVA)

The MLVA is another DNA-based typing technique based on the amplification of nucleotides repeats in coding and non-coding DNA sequences, the variable-number tandem repeat arrays (VNTR) loci, by polymerase chain reaction (PCR). The analysis of multiple VNTR loci is based on the determination of the sizes of the PCR products by capillary electrophoresis. From the variation in sizes of the PCR products, the number of repeat units at each locus can be deduced using specific software (e.g. BioNumerics), and easily compared to references database, allowing to determine the relatedness between *Salmonella* strains (7,9,41) (https://www.cdc.gov/pulsenet/pathogens/mlva.html). Therefore, MLVA data allow to create a genotype able to distinguish very closely related *Salmonella* strains belonging to the same serotype being, in general, more discriminatory than PFGE (9,41–43). MLVA is commonly used by some reference centres in outbreak investigations as a complementary technique of PFGE, allowing to achieve more detailed differences between strains that have similar PFGE patterns, which is particularly relevant for highly clonal *Salmonella* serotypes (7,9,41) (https://www.cdc.gov/pulsenet/pathogens/mlva.html). This technique presents several advantages particularly when comparing with PFGE: it is cheaper and less time consuming, has a higher repeatability and reproducibility, it is easy to analyse and to share inter-laboratories data and there are databases for some *Salmonella* serotypes (e.g. http://www.mlva.eu/recherche.php?type=senterica) (41,43). However, this genotyping tool also presents some limitations: requires trained and skilled technicians, and data analysis can get problematic due to the rapid evolution of certain loci (7,9) (https://www.cdc.gov/pulsenet/pathogens/mlva.html). Besides, it is not a practical routine typing technique as only a few standardized protocols are available, including for specific *S. enterica* serotypes (e.g. *S.* Enteritidis, *S.* Typhimurium, *S.* Dublin and *Salmonella* Infantis) (www.pulsenetinternational.org/protocols/mlva) (44–46).

## 3.2. Sequence-based Typing

### 3.2.1. Multilocus Sequence Typing (MLST)

The first MLST scheme for *Salmonella* was created in the middle of 2000s (47). After that, several schemes were developed based on housekeeping genes or a combination of those with virulence or flagellin genes (48,49). However, the most currently used and standardized MLST scheme for *Salmonella* is the one from Achtman and colleagues, which

is based on PCR amplification and sequencing of internal fragments of seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA*) distributed along the chromosome (http://enterobase.warwick.ac.uk/). After sequencing, the numerical designation for each allele and for the final Sequence Type (ST) is obtained by depositing each housekeeping gene internal sequence on the *Salmonella* online database (http://enterobase.warwick.ac.uk/species/senterica/allele_st_search). Additionally, several STs can be genetically related, where we can have single locus variants (SLV; differing in only one of the seven alleles) or double locus variants (DLV; differing in two of the seven alleles) of other STs. The primary founder ST is the one that has the greatest number of SLVs.

Although this genomic technique has a lower discriminatory power than PFGE, the MLST scheme has been used to investigate the population structure of *Salmonella*, including to give evolutionary insights of *Salmonella* populations or specific clonal lineages, such as those that are clinically-relevant, disseminated worldwide and/or involved in outbreaks (20,50–55).

Moreover, this MLST scheme has shown that most of *Salmonella* STs are grouped into several genetically closely related clusters, called eBurstGroups-eBGs (two SLV STs can be in a common eBG and some DLV STs can be affiliated with an existing eBG if they belong to the same serotype). The same serotype can be associated with the same eBG (e.g. *S.* Typhimurium ST19 or ST34 belong to the same eBG1 cluster). Less frequent is the case of unrelated eBGs corresponding to the same serotype, as occurs in polyphyletic serotypes (e.g. *Salmonella* Newport isolates can belong to several STs associated with eBG2, 3, 7 or 35). Even more rare is the case of the same eBG comprising multiple serotypes (e.g. eBG4 includes *S.* Enteritidis, *Salmonella* Galinarum and other 6 serotypes) (20) (https://enterobase.warwick.ac.uk/species/index/senterica). In fact, some of the discrepancies between eBGs and serotyping have inclusively driven to new serotype designations (20).

Currently, due to the amount of available information (giving the genetic relatedness between serotypes, STs and eBGs) in *Salmonella* database (https://enterobase.warwick.ac.uk/species/index/senterica), MLST has been recommended for the replacement of the traditional *Salmonella* serotyping (20). In fact, diverse studies, including the ones using other MLST target genes, reported the ability of this technique to correctly predict *Salmonella* serotypes (91%-100%), with the major inconsistencies associated with closely related (e.g. *S.* Typhimurium and *S.* 1,4,[5],12:i:-) and polyphyletic serotypes (e.g. *S.* Newport, *Salmonella* Saintpaul) (21,56–59).

Nevertheless, the MLST is still laborious, expensive and a slow technique if many isolates are tested (32,60). Besides, its discriminatory power is questionable due to the low

number of housekeeping genes used, which does not provide the desired resolution needed for outbreak analysis and short-term epidemiological studies (20,60).

### 3.3. Whole-Genome Sequencing (WGS)

WGS is a genomic high-throughput technique based on the DNA sequence of a full bacterial genome. WGS provides a higher resolution/sensitivity and discriminatory power comparing with the previous DNA-based typing tools (PFGE, MLVA and MLST) (7,9,30,50,60,61). In fact, due to its high discriminatory power, WGS has been recognized since 2011 as the gold standard methodology for typing proposes (62). Different typing schemes have been used to study bacterial isolates by WGS, the ones based on single nucleotide polymorphisms (SNPs), or in a gene-by-gene approach such as the ones that use a set of ribosomal genes (rMLST), genes of a core genome (cgMLST) or the whole genome (wgMLST) (30,50,60). Currently, several online database platforms containing *S. enterica* genomes are available (e.g. www.applied-maths.com/applications/wgmlst, https://enterobase.warwick.ac.uk/, www.genomicepidemiology.org), which facilitate the genetic comparisons between *Salmonella* strains (61,63).

WGS has been also used as a new reference technique for outbreak investigations, gradually replacing PFGE technique in diverse public health reference laboratories/centres (e.g. CDC, EFSA, HPA, Statens Serum Institut) (9–11,61,64–69). Additionally, the accessibility of WGS genomic data available in public databases has allowed a deeper characterization and understanding of evolutionary insights of *Salmonella* populations. For example, WGS is of great importance to explore diverse important features (e.g. virulence markers, antimicrobial resistance genes and other stress tolerance markers) for maintenance and expansion of *Salmonella* populations, including clinically-relevant multidrug-resistant *S. enterica* strains/clones (10,50,70–73). Moreover, due to the classical limitations of *Salmonella* serotyping, WGS has also been widely used as a complement or as a substitute for this agglutination technique. In fact, several studies have obtained high rates (88%-99%) of successful serotype prediction using WGS (23,74–77). Different applications with web user interfaces, such as SeqSero (23) and the *Salmonella* in silico Typing Resource (SISTR) (77), have used gene sequences encoding individual somatic (e.g. *rfb* region containing *wzx* and *wzy* genes) and flagellar antigens (e.g. *fliC* and *fljB* genes), aligned against curated databases to determine *Salmonella* serotypes (23,74–77). Yet, ambiguous serotype designations can occur as a result of serotype versus individual antigen determination, such as similar antigenic formula (differing mainly on minor O-antigenic factors) and the occurrence of serotypes and their monophasic variants (e.g. *S.* Typhimurium and its monophasic variant *S.* 1,4,[5],12:i:-). In these cases, additional core genome MLST genes (cgMLST) can be used to provide a phylogenetic context and to choose

the most likely serotype (77). Also, the ability of WGS to predict the presence of antibiotic resistance genes in *Salmonella* serotypes, from human and food isolates, was also possible, with an overall correlation between the resistance genotypes and phenotypes of 99% (78). Despite these promising results, the genotype and phenotype correlation are still controversial, being required more detailed data, the use of more proper surveillance breakpoints, single public databases regularly updated and increased knowledge of new resistance genes to improve those correlations and correctly conclude about the real potential of WGS on antimicrobial resistance prediction in *Salmonella* (78,79).

Besides all advantages, WGS is still laborious, time-consuming and expensive in order to obtain useful data in routine surveillance. Additionally, requires workflow improvements, quality controls, expertise skills for data processing, automated data analysis, annotation harmonization and the problems in storage huge amount of obtained information (30,60,61,63,80). Besides, most of the several proposed WGS typing schemes available online (for cgMLST, wgMLST or SNPs) for epidemiological data interpretation have not yet been completely validated, including in *S. enterica* (63). Despite the promising application of WGS to improve *Salmonella* surveillance, the use of methodologies that can be practised among routine diagnostic laboratories remains a current challenge, namely the selection of standardized analytical tools as well as the epidemiologic interpretation of WGS data (30,80).

## 4. Non-DNA Molecular Typing Approaches

Nowadays, rapid, simple low-cost, user-friendly and high-throughput techniques based on non-DNA approaches, such as the proteomics MALDI-TOF MS and the metabolomics FTIRS, have been applied for bacteria identification (since the 1990s) and typing (since 2000s and 2010s, respectively), including in *Salmonella*, with potential for routine implementation (12,81,82).

### 4.1. Fourier Transform Infrared Spectroscopy (FITRS)

FTIRS is a simple, quick and low-cost technique that allows the discrimination of intact cells in a non-destructive manner through infrared spectra reflecting the vibrational modes of the microbial cell molecules (e.g. nucleic acids, proteins, carbohydrates, membrane and cell wall components) (Figure 2) (12,83). Therefore, this spectroscopic technique provides a specific fingerprint that reflects the structure and composition of the whole cell (12,84). The spectra corresponding to phospholipids/DNA/RNA and polysaccharides regions (1500-900 $cm^{-1}$) is the most discriminatory, being widely and successfully used to discriminate diverse pathogenic bacteria causing foodborne or nosocomial infections at different infra-species levels (e.g. serogroups, serotypes, clones)

(85–89), including in *Salmonella* (90–94). In fact, in the few *S. enterica* studies reported, FTIRS was already explored for *S.* Enteritidis phage type discrimination (95) as well as for serotype discrimination, some with good rates of correct assignments in particular cases (e.g. *S.* Typhimurium, *S.* Enteritidis, *Salmonella* Kentucky, *Salmonella* Senftenberg) (90–94,96). Nevertheless, those studies presented important limitations, such as lack of robustness, mainly due to poor diversity (serogroups/serotypes tested) and the number of isolates on the bacterial collection, the variability of sampling procedures or bioinformatics analysis tools. In addition, the development of standardized protocols (e.g. isolates culture, sample preparation and spectra acquisition), until now lacking, is crucial to obtain highly reproducible spectra and results (97). Remarkably, this quick and low-cost high-throughput technique has currently gained more interest with the recent (2016) available system "IR Biotyper" (www.bruker.com/applications/microbiology/strain-typing-with-ir-biotyper/features). This system allows bacterial identification and typing (e.g. *Streptococcus pneumoniae* subtypes), which facilitates data analysis, automation of the whole process and interlaboratory comparisons, representing a potential and attractive alternative to the DNA-based typing techniques for routine implementation.

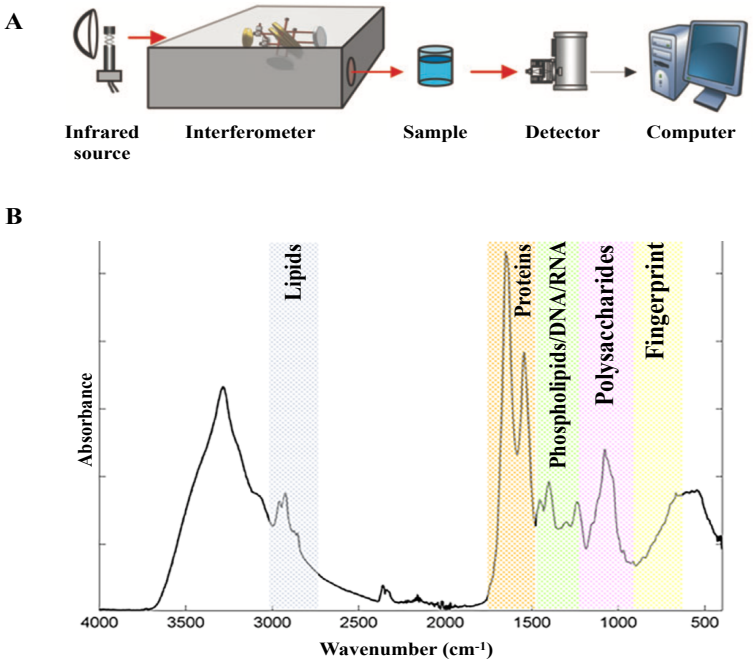


**Figure 2. A)** Components of FTIR spectrometer. **B)** FTIRS bacterial characteristic spectrum: lipids (3000–2800 $cm^{-1}$), proteins/amides I and II (1700–1500 $cm^{-1}$), phospholipids/DNA/RNA (1500–1185 $cm^{-1}$), polysaccharides (1185–900 $cm^{-1}$) and fingerprint region (900–600 $cm^{-1}$) [Adapted from (12,98)].

### 4.2. Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

The proteomic MALDI-TOF MS is a simple, quick and low-cost spectrometric high-throughput technique available since 1990s and used in routine diagnostic laboratories for microorganism's identification (13,81,82). However, only in 2010s, this technique started to present an increased attention as a bacterial typing tool (13,81,82).

MALDI-TOF MS is based on the ionization and separation of ions according to mass-to-charge (m/z) ratio, given a characteristic fingerprint of a specific microbial cell that reflects mostly peptides and small proteins content, mainly ribosomal (Figure 3) (13,82,99). Moreover, the microbial identification is established by specific biomarker peaks (universally conserved within species), in the mass range of 2,000 to 20,000 Da, and comparing the mass spectra obtained with those to other known species included in a database (13,81,82). In fact, MALDI-TOF MS has been successfully explored as an alternative technique to discriminate several pathogenic bacteria causing foodborne or nosocomial infections at different taxonomic levels (100–103).

Regarding *S. enterica*, the potential of MALDI-TOF MS has been barely explored and limited to the subspecies level (104,105), as well as to the discrimination of a few serotypes (e.g. *S.* Typhimurium, *S.* Enteritidis, *S.* Infantis, *Salmonella* Rissen, *Salmonella* Virchow) (106–109), with different degrees of success. Globally, incongruent results were observed between different studies since the peaks identified as presumptive biomarkers were not always present or, when present, were not specific or exclusive of a particular serotype (106–109). It should be noted that this technique is still controversial, predominantly due to the wide diversity of workflows (e.g. variability of bacterial collections, growth conditions, extraction methods, matrix, mass spectrometers or bioinformatics tools used for data analysis) or due to closely related strains expressing similar or the same proteins, compromising their desired reproducibility, discriminatory power and robustness (13,81,82,99). Therefore, improvements in MALDI-TOF MS technique are required, such as the development/implementation of standardized protocols and workflows for bacterial typing to be used in routine diagnostic laboratories and/or reference surveillance centres (99). However, the recognized MALDI-TOF MS advantages (simplicity, rapidity and low-cost) are a current challenge for next-generation sequencing technologies as WGS to be implemented for routine use in a very near future (82,110).

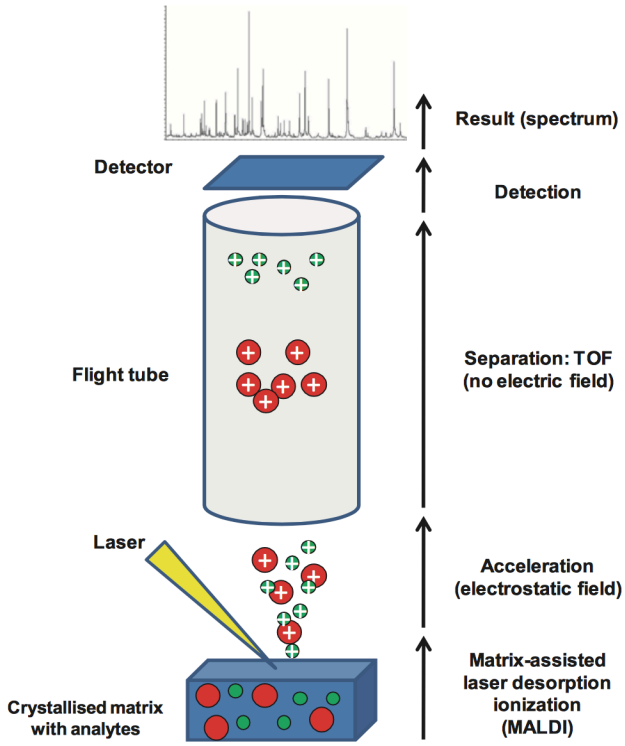


**Figure 3.** Schematic representation showing the technical fundament of MALDI-TOF MS [Reprinted from (111)].

## 5. Conclusions

NTS is one of the leading causes of foodborne diseases worldwide, being human salmonellosis associated with specific *Salmonella enterica* serotypes and related clones. Hence, the identification and differentiation at the infra-species level, particularly targeting clinically-relevant serotypes/clones, is crucial for outbreak investigation and epidemiological studies. Several typing techniques were introduced and developed across the last decades, being some of them gradually replaced by others with a higher discriminatory power, faster, cheaper and less laborious.

Nowadays, an ideal typing technique should be accurate for discrimination, quick, low-cost and reproducible, allowing its use in routine diagnostic laboratories. In fact, classical phenotyping techniques such as serotyping and phage typing were historically important as *Salmonella* typing tools, but DNA-based typing techniques (such as PFGE, MLVA and MLST) were crucial for defining population structure of *S. enterica*, due to their higher discriminatory potential. However, those techniques are still laborious, time-consuming, expensive or present a questionable discriminatory power.

The implementation of high-throughput techniques, as the genomic WGS, has revolutionized bacterial typing, including increasing resolution of *S. enterica* typing. Nevertheless, its massive use as a typing tool is being restricted to surveillance centres, because is expensive, time-consuming, requires bioinformatic expertise and completely

validated criteria for data interpretation, currently in progress in most bacteria, including *Salmonella*. Alternative non-DNA molecular high-throughput techniques, such as FTIRS or MALDI-TOF, could answer to some of the requirements of need for quick and low-cost techniques for bacterial typing, but until now with limited ability for discrimination in *Salmonella* at serotype level. However, the achievements obtained with those techniques open the possibility for further methodological improvements, particularly regarding the standardization of protocols, automatization of the analytical process and creation of robust reference spectrum libraries, allowing their implementation at routine diagnostic and public health surveillance laboratories.

**Transparency Declaration**

The authors declare no conflict of interest.

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Chapter 2

# OBJECTIVES AND OUTLINE OF THE STUDY

## 2.1. Statement of objectives

Non-typhoidal *Salmonella* (NTS) is one of the leading causes of zoonotic foodborne diseases worldwide, with poultry and pork products being the most frequent sources of human infections. A number of changes occurring over the last years along the food-chain may act as drivers leading to new problems and challenges regarding salmonellosis burden and control, including intensive food animal production (frequently associated with antimicrobials overuse), large-scale production/distribution and globalization of food supply (food and live animals), and changes in consumer demands and lifestyles (e.g. increase demand of meat products, mainly poultry and pork). Nevertheless, the impact of **critical antibiotics usage in intensive food-animal production** and of **food trade globalization** in the emergence of antibiotic resistant NTS and in their spread (clonal vs horizontal dissemination) have been scarcely addressed. The lack of comprehensive studies supported on detailed molecular characterization (including antimicrobial resistance and its genetic background) of robust collections, including a variety of sources and geographical regions, hinders the elucidation of food-chain drivers' role in the NTS animal-food-human transmission. Moreover, the study of underexplored food-producing systems (e.g. non-terrestrial farm producing animals) or geographical regions (e.g. African countries), which may constitute global potential reservoirs and transmission routes of NTS, is scarce.

To clarify the source attribution and tracking of multidrug-resistant (MDR) *Salmonella* serotypes/clones transmission along the food chain, is crucial the development of rapid and accurate typing methods. The increasing use of DNA-based typing tools, including more recently Whole Genome Sequencing, has contributed to better elucidate the NTS population structure, the transmission linkage between animal-food-humans as well as to explore relevant features responsible for the maintenance and expansion of particular NTS clones. However, these techniques are too expensive, time-consuming and/or require specialized skills, hampering their implementation in the routine of microbiology laboratories. Therefore, quicker, low-cost, user-friendly and high-throughput alternative methods are required in order to characterize epidemic scenarios and to prevent or minimize NTS-food-human transmission. Among those potential alternatives are Fourier Transform Infrared Spectroscopy (FITRS) and Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS), two non-DNA molecular approaches that have been explored in diverse bacterial species with different degrees of success. Since these techniques are based on targeting different bacterial cell biomolecules, they offer an opportunity to provide new insights regarding the elucidation of the role of the biomolecular composition on the discrimination of NTS at different infra-species levels.

The **goals of this Thesis are:**

1. **To unveil NTS serotypes, clones and genetic backgrounds associated with the emergence and dissemination of resistance to critical antibiotics, contributing to elucidate the role of food-chain drivers for animal-food-human transmission in different epidemiological scenarios:**

   - National human clinical and food-producing settings, and imported food products;

   - Understudied intensive trout-producing systems and a region of Angola.

2. **To assess the suitability of alternative, quick and low-cost non-DNA molecular based high-throughput techniques for discrimination of *S. enterica* at different infra-species levels (serogroups, serotypes and clones):**

   - FTIRS, using whole cell analysis, to routinely discriminate clinically-relevant *S. enterica* with exploitation of somatic O-antigen composition for the discrimination achieved;

   - MALDI-TOF MS, using intact bacterial cells and routine equipment, for the discrimination of clinically-relevant *S. enterica*.

To accomplish the first objective 1010 isolates were selected, based on the diversity of serogroups, serotypes, clones, sources (human clinical cases, food-animal production settings and food products of national and imported origin) and geographical regions from a collection of ~3000 NTS (2002-2015). Additionally, 53 samples from national fish aquacultures (trout farms, environment surroundings and marketed trout; 2010-2012) and 63 samples from Benguela, Angola (healthy volunteers, food-producing animals and their environments, wild animals and aquatic environments; 2013) were studied. Finally, the potential of FTIRS and MALDI-TOF MS for *S. enterica* discrimination at different infra-species levels was assessed using part of the well-characterized collection comprising different serogroups, serotypes and clones and obtained from diverse sources, countries and years (FTIRS-n=325, 2002-2015; MALDI-TOF MS-n= 183; 2002-2015).

## 2.2. Outline of the thesis

This Thesis is organized in four chapters as follows:

**Chapter 1** presents an overview of the state of the art regarding the topics of this Thesis and is subdivided in three sections. **Sections 1.1.** and **1.2.**, include Review Articles that provide an overview of the role of poultry and pig production chain on human salmonellosis, highlighting antimicrobial resistance as a relevant factor for the success of poultry and pig-related *Salmonella* serotypes and clones causing human infections, respectively. **Section 1.3.** presents a Mini Review Article which includes an overview of the evolution, limitations and applications of the several techniques proposed for *S. enterica* typing.

**Section 1.1.** Review Article: Antunes P, Mourão J, **Campos J**, Peixe L. 2016. Salmonellosis: the role of poultry meat. Review. *Clin Microbiol Infect.* 22:110-121. DOI: http://dx.doi.org/10.1016/j.cmi.2015.12.004.

**Section 1.2.** Review Article: **Campos J**, Mourão J, Peixe L, Antunes P. 2018. Non-typhoidal *Salmonella* and the pig production chain: a comprehensive analysis of its impact on human health *(manuscript final draft)*.

**Section 1.3.** Mini Review Article: **Campos J**, Mourão J, Peixe L, Antunes P. 2018. Non-typhoidal *Salmonella* typing: from classical to high-throughput techniques *(manuscript final draft)*.

**Chapter 2** includes the **objectives and outline** of the Thesis.

**Chapter 3** presents the **findings** that answer to the goals of the Thesis, which yielded different research articles (n=7; 6 publications in peer review journals and 1 manuscript in final draft). Such results have been organized throughout the manuscripts, according to the rationale proposed as follows:

### 3.1. Insights on the food-chain driver's contribution for the emergence and spread of antibiotic resistant NTS serotypes from clinical and non-clinical settings.

**3.1.1.** **Campos J**, Cristino L, Peixe L, Antunes P. MCR-1 in multidrug-resistant and copper-tolerant clinically relevant *Salmonella* 1,4,[5],12:i:- and *S.* Rissen clones in Portugal, 2011 to 2015. Euro Surveill. 2016;21:pii=30270.

**3.1.2.** **Campos J**, Mourão J, Marçal S, Machado J, Novais C, Peixe L, Antunes P. Clinical *Salmonella* Typhimurium ST34 with metal tolerance genes and an

IncHI2 plasmid carrying *oqxAB-aac(6')-Ib-cr* from Europe. J Antimicrob Chemother. 2016;7:843-845.

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**Chapter 4** presents the **General Conclusions,** highlighting the main results from these research studies.

Chapter 3

# RESULTS AND DISCUSSION

## 3.1. Insights on the food-chain driver's contribution for the emergence and spread of antibiotic resistant NTS serotypes from clinical and non-clinical settings

3.1.1. MCR-1 in multidrug-resistant and copper-tolerant clinically relevant *Salmonella* 1,4,[5],12:i:- and *S.* Rissen clones in Portugal, 2011 to 2015

3.1.2. Clinical *Salmonella* Typhimurium ST34 with metal tolerance genes and an IncHI2 plasmid carrying *oqxAB-aac(6')-Ib-cr* from Europe

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3.1.5. High occurrence and unusual serotype diversity of non-typhoidal *Salmonella* in non-clinical niches, Angola

### 3.1.1. MCR-1 in multidrug-resistant and copper-tolerant clinically relevant *Salmonella* 1,4,[5],12:i:- and *S.* Rissen clones in Portugal, 2011 to 2015

RAPID COMMUNICATION

# MCR-1 in multidrug-resistant and copper-tolerant clinically relevant *Salmonella* 1,4,[5],12:i:- and *S.* Rissen clones in Portugal, 2011 to 2015

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**Citation style for this article:**
Campos J, Cristino L, Peixe L, Antunes P. MCR-1 in multidrug-resistant and copper-tolerant clinically relevant Salmonella 1,4,[5],12:i:- and S. Rissen clones in Portugal, 2011 to 2015. Euro Surveill. 2016;21(26):pii=30270. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.26.30270

Article submitted on 17 June 2016 / accepted on 30 June 2016 / published on 30 June 2016

**The *mcr-1* gene was found in 11 isolates from a Portuguese *Salmonella* collection (n = 1,010; 58 serotypes; 2002–15) of clinical samples, foodstuff, food-animals and water. *Mcr-1* has been located on different plasmids (IncX4/IncHI2) in pig-associated multidrug-resistant, copper-tolerant S.1,4,[5],12:i:-/ST34 and *S.* Rissen/ST469 clones from human and pork products since at least 2011. Our data highlight dissemination of *mcr-1* by successful resistant clones in Europe and raise questions about the efficacy of copper-based interventions to reduce colistin use.**

Since the description of plasmid-mediated colistin resistance encoded by the *mcr-1* gene in *Enterobacteriaceae* from multiple sources in China [1] and its worldwide dissemination mostly in animal sources [2], the use of polymyxins (colistin) in food-producing animals has been questioned in Europe because it may have an impact on human health [3]. Nevertheless, data on the transmission of *mcr-1*-mediated colistin resistance particularly by clonal expansion are lacking [3,4]. In fact, the *mcr-1* gene has been found in zoonotic food-borne bacteria such as *Salmonella* [2] but the presence of this gene in particular successful resistant clones has not been demonstrated [3]. In this study, we report the presence of the *mcr-1* gene in pig-associated clinically relevant *Salmonella* serotypes and clones recovered from human clinical samples and pork products in Portugal, collected as early as 2011.

## Laboratory investigation

We analysed a total of 1,010 *Salmonella* isolates of 58 serotypes from several sources (human clinical cases, food products, food-animal production settings and aquatic environments) and regions of Portugal, collected between 2002 and 2015 (Table 1). The isolates were screened for the *mcr-1* gene by PCR and sequencing, using primers CLR5-F (5´-CGGTCAGTCCGTTTGTTC-3´) [1] and Mcr1-Rv2 (5´-CCAGCGTATCCAGCACATTT-3´) [this study].

The 1,010 isolates comprised the most frequent worldwide *Salmonella* serotypes (n = 256 *S.* Typhimurium and n = 34 *S.* Enteritidis), but also emerging serotypes (n = 436 *S.* 1,4,[5],12:i:- and n = 93 *S.* Rissen) or serotypes less frequently detected in European surveillance studies (n = 191 isolates from 54 different serotypes). They included all isolates previously characterised [5,6] and recent ones from ongoing surveillance studies (data not shown) covering all serotypes, sampling dates, sources, regions, antibiotic susceptibility phenotypes/genotypes and PFGE types. The isolates positive for *mcr-1* by PCR were further tested for susceptibility to colistin by the proposed broth microdilution method [7] and interpreted according to the European Committee on Antimicrobial Susceptibility Testing [8]. Isolates were also subjected to standard conjugation assays using the recipient strain *Escherichia coli* HB101 [6]. Replicon typing, pMLST, hybridisation experiments (I-CeuI/S1-PFGE nuclease) [5,9] and detection of the insertion sequence element IS*Apl1* was performed in *Salmonella* strains and transconjugants. The presence and location of IS*Apl1* was determined using primers ISApl1-Fw (5´-GTCGCTTTGGACATTGGGAA-3´) and ISApl1-Rv (5´-GATTGATGTCTTGGTGCTTCGG-3´) designed as part of this study, and CLR5-R (5´-CTTGGTCGGTCTGTAGGG-3´) [1]. Clonal relatedness of *Salmonella* strains was assessed by *Xba*I PFGE [5,6] and MLST [10].

## Detection of *mcr-1* gene in pig-associated clinically-relevant clones

The *mcr-1* gene was detected in 11 (1.1%) of the 1,010 Portuguese *Salmonella* isolates, recovered from human clinical sources and pork food products from across the country (Table 1, Table 2). This gene had 100% homology with the first published *mcr-1* sequence in an

**Table 1**

*Salmonella* isolates from different sources by year and presence of the *mcr-1* gene, Portugal, 2002–2015 (n = 1,010)

| Source (number of isolates) | Years | Isolates tested for *mcr-1* (serotype/ number of isolates)a | *mcr-1*-positive isolates (serotype/number of isolates)[a] |
|---|---|---|---|
| Human clinical cases (n = 522) | 2002–10 | 258 | 0 |
| | 2011–12 | 155 (*S.* 1,4,[5],12:i:-/n = 75) | 4 (*S.* 1,4,[5],12:i:-) |
| | 2013–15 | 109 | 0 |
| Food products (n = 413) | | | |
| Pork (n = 296) | 2002–13 | 44 | 0 |
| | 2014–15 | 252 (*S.* 1,4,[5],12:i:-/n = 130; *S.* Rissen/n = 23) | 7 (*S.* 1,4,[5],12:i:-/n = 5; *S.* Rissen/n = 2) |
| Other[b] (n = 117) | 2002–15 | 117 | 0 |
| Food production animals (n = 58) | | | |
| Pigs/piggeries (n = 54) | 2006–08 | 54 | 0 |
| Aquacultures (n = 4) | 2010–12 | 4 | 0 |
| Aquatic environment (n = 17) | 2002–11 | 17 | 0 |

[a] The serotypes of *Salmonella* isolates are presented only for those among which *mcr-1*-positive ones were detected.
[b] Other studied food products comprised: poultry, beef, cow, quail, clam and cooked meals.

*Escherichia coli* strain from China (GenBank accession number: KP347127) [1], which was further described in diverse other *Enterobacteriaceae* including sporadic *Salmonella* isolates from European countries (France, the Netherlands, Spain, the United Kingdom) [2,11-14]. In most of these studies, detection of *mcr-1* gene was only performed in colistin-resistant isolates. This impairs the determination of its real prevalence because the gene may be silent, as described in one *E. coli* strain [15]. All our isolates carrying the *mcr-1* gene presented a minimum inhibitory concentration (MIC) of 4–8 mg/L for resistance to colistin (Table 2).

During the study period (2002 to 2015), *Salmonella* isolates harbouring the *mcr-1* gene were only recovered between 2011 and 2015 and originated from human clinical sources (0.8%, n = 4/522) and pork products, mostly from slaughterhouses, (2.4%, n = 7/296) (Table 1). Colistin has been widely used in veterinary medicine, particularly in food-producing animals, primarily in pigs [16,17]. The available data from 2004 to 2006 had already shown high use of colistin for food-producing animals in Portugal [18], which is one of the European countries with highest consumption of polymyxins that has been increasing in the last years (2011–13) [3,19]. Taking into account the current picture of colistin use in Portugal, the detection of *mcr-1* in the most recent collections and in pork products is of concern. Nevertheless, data on chronology, current prevalence of the *mcr-1* gene and its evolution in bacteria from animals, food and humans are lacking [3].

The 11 *mcr-1*-positive *Salmonella* isolates belonged to the serotypes *S.* 1,4,[5],12:i:- and *S.* Rissen (Table 2), which have been strongly associated with pig production and caused human infections in Europe [5,6,20-22] including in Portugal [23]. In both cases, we found them associated with particular successful multidrug-resistant (MDR) clonal lineages, either of the *S.* Rissen/ST469 clone or the *S.* 1,4,[5],12:i:-/ST34 European clone that is currently spreading epidemically in European countries [5,20,22] and has been dominant in our *Salmonella* collection for the last years [5,6,20; unpublished data]. A previous report on *mcr-1* in the clinically relevant *Salmonella* serotype *S.* Typhimurium/ST34 was associated with travel to South-East Asia [13].

Of note, all *S.* 1,4,[5],12:i:- and *S.* Rissen *mcr-1*-carrying isolates were co-resistant to antibiotics used in a human and/or veterinary context and carried diverse metal tolerance genes, remarkably those conferring tolerance to copper (all carrying *pcoD+silA* on the chromosome) (Table 2), a feed additive mostly used for pigs or piglets in Europe. The fact that these successful clones presented higher tolerance to copper, as previously demonstrated [6,20], can contribute to their selection and wider expansion with potential repercussions for *mcr-1* transmission.

## Location of *mcr-1* gene in diverse plasmid backbones

The *mcr-1* gene was located on two plasmid types, IncX4 (n = 5; 35 kb; 4 transferable) and IncHI2 (n = 6), either of ST4 subtype (n = 3; 200–300 kb; all transferable) or non-typeable (n = 3; 120–125 kb; all non-transferable) and mostly associated with the IS*Apl1* transposable element (Table 2). IncHI2/ST4 and IncX4 plasmids have been widely implicated in the spread of *mcr-1* gene in diverse *Salmonella* serotypes and other *Enterobacteriaceae* in European and non-European countries, both from human and animal sources [2,12-14]. Transferability of the *mcr-1* gene was achieved from *S.* Rissen (n = 1) and *S.* 1,4,[5],12:i:- (n = 6) isolates and was associated with a 32–64-fold increase in the colistin MIC and, in some isolates, with acquisition of

**Table 2**

Characterisation of *Salmonella* isolates recovered from clinical and food samples and carrying the *mcr-1* gene, Portugal, 2011–2015 (n = 11)

| Serotype[a] (number of isolates) | Source-origin (number of isolates) | Clone designation; ST(eBG); PFGE-type[b] (number of isolates, source) | Year/ Regions | Antibiotic resistance phenotype/genotype[c] (number of isolates) | Metal tolerance genes (number of isolates)[d] | Plasmid-mediated colistin resistance *mcr-1* | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Colistin MIC-mg/L, donor (transconjugant/ number of isolates)[e] | Plasmid type carrying *mcr-1* gene (pMLST, Kb)[f] (number of isolates) | IS*Apl1* upstream of *mcr-1* (number of isolates) |
| 1,4,[5],12:i:- (n = 9) | Clinical faeces/blood (n = 4) | European clone; ST34(eBG1); C (n = 1, 1 hospital), E (n = 3, 2 hospitals) | 2011–12 North | AMP, (GEN), STR, SUL, TET/ $bla_{TEM}$, [*aac(3)-IV*], strA-*strB*, *sul2*, *tet*(B) (n = 4) | *pcoD* + *silA* + *merA* + (*terF*) (n = 4) | 4–8 (4–8/n = 1) | X4 (35) (n = 1) HI2 (NT, 120–125) (n = 3) | No (n = 1) Yes (n = 3) |
| | Pork carcass (n = 4) | European clone; ST34(eBG1); A (n = 1, 1 slaughterhouse), B (n = 2, 2 slaughterhouses), F (n = 1, 1 slaughterhouse) | 2014–15 North, Centre | AMP, (CLO), (CIP, PEF), STR, SUL, TET, (TMP)/ $bla_{TEM}$, (*floR*)/(*catA-cmlA*), (*aadA1/aadA2*) –*strA-strB*, *sul1-sul3/sul2*, *tet*(A)/*tet*(B), (*dfrA1/dfrA12*) (n = 4) | *pcoD* + *silA* + (*merA*) + (*terF*) (n = 4) | 4–8 (4–8/n = 4) | X4 (35) (n = 2) HI2 (ST4, 230–300) (n = 2) | No (n = 2) Yes (n = 2) |
| | Pork meat (n = 1) | European clone; STNew/ Single locus variant of ST34; B (n = 1, 1 meat production unit) | 2015 South | AMP, STR, SUL, TET/ $bla_{TEM}$, *strA-strB*, *sul2*, *tetB* (n = 1) | *pcoD* + *silA* + *merA* + *terF* (n = 1) | 4 (4/n = 1) | HI2 (ST4, 200) (n = 1) | No (n = 1) |
| Rissen (n = 2) | Pork carcass (n = 2) | ST469(eBG66); N (n = 2, 2 slaughterhouses) | 2014–15 North | AMP, CLO, STR, SUL, (TET), TMP/ $bla_{TEM}$, *cmlA*, *aadA1*, *sul1-sul3*, [*tet*(A)], *dfrA1* (n = 2) | *pcoD* + *silA* + *merA* (n = 2) | 4 (4/n = 1) | X4 (35) (n = 2) | No (n = 2) |

AMP: ampicillin; CIP: ciprofloxacin; CLO: chloramphenicol; GEN: gentamicin; MIC: minimum inhibitory concentration; PEF: pefloxacin; pMLST: plasmid multilocus sequence type; STR: streptomycin; SUL: sulfamethoxazole; TET: tetracycline; TMP: trimethoprim.

[a] The serotypes of *Salmonella* isolates were determined by classical serotyping, performed at the National Centre of Salmonella (INSA, Lisbon, Portugal) and/or PCR assay for determination of *S.* 4,[5],12:i:- [5].

[b] PFGE types are designated by capital letters and include previously described types [5,6] and types described for the first time in this study. The human clinical isolates (n = 4 from four patients) were recovered from three hospitals, and pork products (n = 7) were recovered from six slaughterhouses and one meat production unit.

[c] Antimicrobial susceptibility was evaluated by disc diffusion assay. Variable antibiotic resistance phenotypes and genotypes are presented between brackets; Antibiotic resistance patterns and genes transferred by conjugation are underlined; In two *S.* 1,4,[5],12:i:- isolates, transfer of genes *strA-strB* and/or $bla_{TEM}$ was observed; Some genes were included on class 1 integrons (1,700bp (*dfrA1-aadA1*) or 2,000bp (*dfrA12-orfF-aadA2*)); Integrons were located on the chromosome in *S.* Rissen (n = 2) and on the IncHI2/ST4 plasmid in *S.* 1,4,[5],12:i:- isolates (n = 2).

[d] Screening for genes encoding tolerance to metals were done by PCR [20]. Metal tolerance genes that were not observed in all the isolates are presented between brackets; Metal tolerance genes transferred by conjugation are underlined. All *pcoD* + *silA* genes were chromosomally located.

[e] Recipient strain used in conjugation assays: *Escherichia coli* HB101 (azide sodium, resistant to streptomycin and kanamycin); colistin MIC = 0.125 mg/L).

[f] Plasmid types carrying the *mcr-1* gene transferred by conjugation are underlined.

resistance to other antibiotics and metals tolerance genes (Table 2). The fact that successful MDR *S.* 1,4,[5],12:i:- and *S.* Rissen clones have the ability to acquire plasmids carrying the *mcr-1* gene is of concern because colistin resistance may contribute to their further expansion, particularly in the pig reservoir. In addition, those strains could act as reservoir of *mcr-1*-carrying plasmids with a broad host range enhancing colistin resistance transmission for other clinically relevant bacteria sharing the same ecological niche.

## Conclusions

This study has evidenced the acquisition of *mcr-1*-carrying plasmids by two clinically relevant MDR and copper-tolerant clones of *S.* 1,4,[5],12:i:- and *S.* Rissen, strongly associated with pork food products and which were dominant in the collection studied. The detection of *S.* 1,4,[5],12:i:- from human infections, already in 2011, is also of note, suggesting long-term dissemination of this resistance gene in humans in Portugal. Finally, the detection of *mcr-1* in copper-tolerant clones raises questions about the efficacy of recently suggested metal-based interventions (e.g. copper) to reduce the use of colistin and contain *mcr-1* dissemination [3].

### Acknowledgements

This work was supported by FCT/MEC (Fundação para a Ciência e a Tecnologia / Ministério da Educação e Ciência) through national funds and co-financed by FEDER, under the Partnership Agreement PT2020 [grant number UID/MULTI/04378/2013 – POCI/01/0145/FERDER/007728]. JC is supported by a Ph.D. fellowship from Fundação para a Ciência e a Tecnologia (grant number SFRH/BD/93091/2013).

The authors would like to thank to Linda Cavaco for providing the *mcr-1*-positive control DNA. We are also very grateful to Jorge Machado (INSA, Lisbon, Portugal) for the Salmonella serotyping.

### Conflict of interest

None declared.

### Authors' contributions

JC, LP and PA designed the study and analysed epidemiological, microbiological and molecular data, JC and LC performed the phenotypic and molecular assays, JC and PA wrote the first draft of the manuscript, PA and LP participated in the coordination and concept of the manuscript and revised the final version.

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### 3.1.2. Clinical *Salmonella* Typhimurium ST34 with metal tolerance genes and an IncHI2 plasmid carrying *oqxAB-aac(6')-Ib-cr* from Europe

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*J Antimicrob Chemother* 2016
doi:10.1093/jac/dkv409
Advance Access publication 17 December 2015

# Clinical *Salmonella* Typhimurium ST34 with metal tolerance genes and an IncHI2 plasmid carrying *oqxAB-aac(6′)-Ib-cr* from Europe

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Sir,

Fluoroquinolones are critical antibiotics for treating severe *Salmonella* infections, and the widespread of resistant isolates included in diverse epidemiological scenarios and carrying plasmid-mediated quinolone resistance (PMQR) is a global threat.[1,2] Among PMQR mechanisms, those encoded by *oqxAB* and *aac(6′)-Ib-cr* genes are of special concern as they also confer reduced susceptibility to other antibiotics (*oqxAB*: chloramphenicol, trimethoprim, olaquindox; *aac(6′)-Ib-cr*: aminoglycosides) and biocides [*oqxAB*: quaternary ammonium compounds (QACs)].[2,3] Although *oqxAB*± *aac(6′)-Ib-cr* are prevalent and widespread in Asia, where olaquindox is still widely used in animal production, they remain scarce in Europe.[1,2,4–6] Here we describe the molecular characterization of clinical ciprofloxacin-resistant *Salmonella enterica* Typhimurium with concomitant presence of *oqxAB* and *aac(6′)-Ib-cr* recovered for the first time in Europe.

Two ciprofloxacin-resistant (MIC 2 mg/L) *S. enterica* Typhimurium isolates carrying *oqxAB* and *aac(6′)-Ib-cr* genes were isolated from faeces of two young children (1 and 2 years old) admitted to a Portuguese hospital in September 2012. Further characterization included the study of susceptibility to 11 other antimicrobial agents and β-lactamase and carbapenemase production by disc diffusion, Etest and/or microdilution methods (EUCAST/CLSI guidelines) and the Blue-Carba test. Screening of genes encoding resistance to antibiotics (including quinolone resistance-determining region mutations and PMQR) or tolerance to biocides/metals found in the animal setting (e.g. feed/disinfectants) was performed by PCR and/or sequencing.[7–9] Determination of transferability of PMQR genes (conjugation at 25/30/37°C and transformation) and characterization of integrons and plasmid backbones were performed by PCR, plasmid-based replicon typing, plasmid MLST (pMLST; http://pubmlst.org/plasmid/), hybridization (I-CeuI/S1-PFGE nuclease) and/or sequencing.[1,7,8] The *oqxAB-aac(6′)-Ib-cr* genetic environment was characterized by PCR mapping based on known sequences.[1] Clonal analysis was assessed by XbaI-PFGE[8] and MLST (http://mlst.warwick.ac.uk/mlst/dbs/Senterica).

The two *Salmonella* Typhimurium isolates were assigned to the widespread ST34 (http://mlst.warwick.ac.uk/mlst/dbs/Senterica) and presented indistinguishable PFGE patterns (Figure S1, available as Supplementary data at *JAC* Online). Clonal expansion of *Salmonella* Typhimurium, including the ST34 clone, with PFGE profiles closely related to those of our isolates, has been associated with the spread of *oqxAB-aac(6′)-Ib-cr* genes in Asian human clinical and food-producing animal isolates.[1,6] However, the isolates reported here could not be related to any outbreak of foodborne infection or to particular food products/animal contact and foreign travel. Additionally, a single *gyrA* mutation (D87N) found in our *Salmonella* Typhimurium strain was identical to that detected in most *oqxAB*-positive *Salmonella* Typhimurium isolates from Asia.[1,6] The strain was co-resistant to amoxicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline and trimethoprim (Table 1), and carried diverse metal tolerance genes (*merA*, mercury; *silA*, silver/copper; *pcoD*, copper; *terF*, tellurite). Transferability of *oqxAB-aac(6′)-Ib-cr* was achieved by transformation, with an 8-fold increase in ciprofloxacin MIC and acquisition of resistance to other antibiotics (amoxicillin, chloramphenicol, gentamicin, kanamycin, sulfamethoxazole and trimethoprim) (Table 1). A 2-fold increase in benzalkonium chloride MIC was observed, highlighting the role of the OqxAB efflux pump in the decreasing susceptibility to diverse compounds with antimicrobial activity, including QACs.[3] The concomitant presence of genes encoding resistance to antibiotics (e.g. quinolones, quinoxalines, florfenicol) and biocides/metals (e.g. QACs, copper) widely used in farm animals could contribute to the successful spread of *Salmonella* carrying *oqxAB-aac(6′)-Ib-cr* genes by different events of co-selection.

The *oqxAB* genes were linked to an incomplete class 1 integron containing the *aac(6′)-Ib-cr-bla*$_{OXA-1}$*-catB3-arr3-qacEΔ1-sul1* gene cassette array and flanked by two IS*26* (Figure 1), widely spread mobilizing elements responsible for the dissemination of *oqxAB*[9] and other clinically relevant antibiotic resistance genes.[2] The *oqxAB* genetic environment was identical to that found in Asian *Salmonella* Typhimurium isolates from food-producing animals.[1] In other bacteria (including different *Salmonella* serotypes) similar genetic platforms were observed, although differing in the linkage of *oqxAB* with *bla*$_{CTX-M}$ alleles instead of the gene cassette array.[2,4,5] This IS*26-oqxAB*-IS*26-aac(6′)-Ib-cr-bla*$_{OXA-1}$*-catB3-arr3-qacEΔ1-sul1*-IS*26* was located on a 180 kb IncHI2 plasmid

Research letter

**Table 1.** Antimicrobial susceptibility phenotypes and genotypes of *Salmonella* Typhimurium isolates (234 and 248), the selected transformant (248_T) and the recipient (*Escherichia coli* DH5α)

| | MIC (mg/L) | | | | |
|---|---|---|---|---|---|
| Isolate | CIP[a] | NAL[b] | BZK | Resistance phenotype[c]/genotype to other antimicrobial agents | Metal tolerance genes |
| 234 | 2 | >256 | 32 | AMX, CHL, GEN, KAN, STR, SUL, TET, TMP/$bla_{OXA-1}$-$bla_{TEM}$, *catB3-cmlA-floR*, *aac(3)-IV*, *aphA1*, *aadA-strA-strB*, *sul1-sul2-sul3*, *tet*(B), *dfrA12* | *merA*, *silA*, *pcoD*, *terF* |
| 248 | 2 | >256 | 32 | AMX, CHL, GEN, KAN, STR, SUL, TET, TMP/$bla_{OXA-1}$-$bla_{TEM}$, *catB3-cmlA-floR*, *aac(3)-IV*, *aphA1*, *aadA-strA-strB*, *sul1-sul2-sul3*, *tet*(B), *dfrA12* | *merA*, *silA*, *pcoD*, *terF* |
| 248_T | 0.25 | 128 | 16 | AMX, CHL, GEN, KAN, SUL, TMP/$bla_{OXA-1}$, *catB3-cmlA-floR*, *aac(3)-IV*, *aphA1*, *aadA*, *sul1-sul2-sul3*, *dfrA12* | *terF* |
| *E. coli* DH5α | 0.03 | 64 | 8 | | |

[a]PMQR genes screened: *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *aac(6′)-Ib-cr* and *oqxAB*.
[b]*Salmonella* isolates presented a single *gyrA* mutation D87N.
[c]Susceptibility to amoxicillin (AMX), benzalkonium chloride (BZK), ciprofloxacin (CIP), chloramphenicol (CHL), gentamicin (GEN), imipenem, kanamycin (KAN), nalidixic acid (NAL), streptomycin (STR), sulfamethoxazole (SUL), tetracycline (TET) and trimethoprim (TMP) was determined.

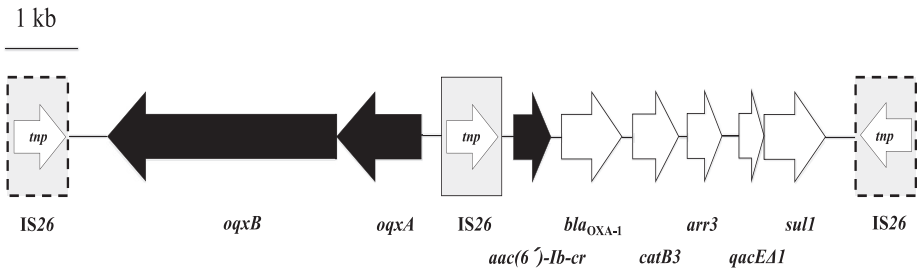


**Figure 1.** Genetic environment (12 110 bp) of *oqxAB-aac(6′)-Ib-cr* genes in an IncHI2 plasmid from an ST34 *Salmonella* Typhimurium strain. Horizontal arrows represent the positions and transcriptional directions of the ORFs. The IS*26* elements are shown as light grey boxes and dotted outlines indicate incomplete sequences.

carrying a *rep* gene 100% identical to Asian *Salmonella* Typhimurium pHXY0908 bearing *oqxAB-aac(6′)-Ib-cr* genes (GenBank accession number KM877269), but with only 95% identity to that of the prototype R478 (GenBank accession number BX664015) and prototype pAPEC-01-R (GenBank accession number DQ517526) plasmids. Also, among the metal tolerance genes found, our IncHI2 plasmid and pHXY0908 carried only *terF*, contrasting with the prototypes R478 and pAPEC-01-R with multiple metal tolerance genes (e.g. *merA*, *arsB*, *silA* or *pcoD*). The spread of *oqxAB*±*aac(6′)-Ib-cr*-producing bacteria of both human and animal origin in Asia has been linked mostly to IncHI2 MDR plasmids[1,4–6] belonging to ST1/ST2 by pMLST,[4] the two major plasmid variants associated with the dissemination of diverse clinically relevant genes (e.g. $bla_{ESBL}$).[10] Our MDR IncHI2 plasmid was non-typeable by pMLST (lacking allele smr0199/smr0018=allele number 3), suggesting the occurrence of multiple recombination events and of a new variant, considering those circulating in Europe (http://pubmlst.org/plasmid/).[10]

To the best of our knowledge, we describe for the first time in Europe an MDR *Salmonella* strain harbouring both *oqxAB* and *aac(6′)-Ib-cr* PMQR genes within an IncHI2 plasmid. The spread of these bacteria and/or MDR plasmids, as is occurring in Asia in human/animal settings, is of concern since they may contribute to amplification of *oqxAB-aac(6′)-Ib-cr* among Enterobacteriaceae in Europe, probably under different selective pressures (e.g. antibiotics/biocides/metals). Continuous surveillance to contain further transmission of these PMQR genes for new hosts or different settings, facilitated by international human and animal/food travel, is urgently required to preserve critical compounds with antimicrobial activity, including fluoroquinolones.

## Acknowledgements

We wish to thank to Dr Lina Cavaco (Division of Microbiology and Risk Assessment, DTU Food, Technical University of Denmark, National Food Institute, Denmark) who kindly provided PMQR control strains.

## Funding

This work was supported by a research grant from the Fundação para a Ciência e a Tecnologia (grant number UID/Multi/04378/2013). J. Campos and J. Mourão were supported by PhD fellowships from Fundação para a Ciência e a Tecnologia (grant numbers SFRH/BD/93091/2013 and SFRH/BD/77518/2011, respectively).

## Transparency declarations

None to declare.

## Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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*J Antimicrob Chemother* 2016
doi:10.1093/jac/dkv392
Advance Access publication 26 November 2015

# Assessment of a phenotypic algorithm to detect plasmid-mediated quinolone resistance in Enterobacteriaceae

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Sir,

Quinolone resistance in Gram-negative bacteria is the result of mutations in the quinolone resistance-determining region (QRDR) in chromosomally located genes encoding type II topoisomerases and, to a lesser extent, altered permeability.[1,2] Plasmid-mediated quinolone resistance (PMQR) has also been reported as encoding different proteins: Qnr proteins, acetyltransferase AAC(6′)-Ib-cr variant and the QepA and OqxAB active efflux pumps.[2]

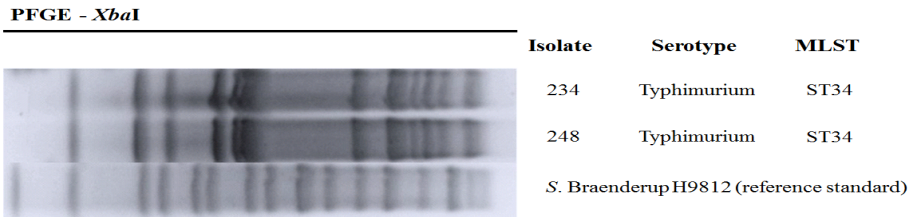
In Enterobacteriaceae, a single mutation in the QRDR of GyrA (frequently at Ser83) results in high-level resistance to nalidixic acid (MIC >32 mg/L with absence of inhibition zone) and is easily detected. Clinical resistance to fluoroquinolones (ciprofloxacin MIC >2 mg/L according to CLSI)[3] is attained with additional chromosomal mutations or the acquisition of PMQR. In the case of isolates harbouring PMQR, but lacking QRDR mutations, these determinants confer low-level resistance to quinolones and/or fluoroquinolones and molecular tests are needed to detect it. The detection of PMQR usually takes up to six PCRs. There are to date no specific phenotypic tests for the detection of PMQR mechanisms. The current prevalence of PMQR seems to be increasing.[2] The aim of this study was to determine the capacity of phenotypic detection of PMQR in Enterobacteriaceae with low-level quinolone resistance (LLQR) without modifications at the QRDR. This phenotype has been reported mainly in *Salmonella*.[4]

To assess this issue, a sample size of 49 positive cases and 49 negative cases was estimated necessary for a 95% CI, proportion expected of 85%, and estimation accuracy of 0.1, using NQuery Advisor. In our study, 99 (57 *Klebsiella pneumoniae*, 27 *Escherichia coli*, 7 *Enterobacter cloacae*, 2 *Klebsiella oxytoca*, 1 *Enterobacter aerogenes*, 1 *Citrobacter koseri*, 1 *Citrobacter freundii*, 1 *Morganella morganii*, 1 *Proteus mirabilis* and 1 *Serratia marcescens*) isolates (of 10 874 clinical isolates of Enterobacteriaceae) were selected during October 2012 to June 2014. They were susceptible to nalidixic acid, MIC ≤16 mg/L, and showed reduced susceptibility to ciprofloxacin, MIC=1–2 mg/L, on a Wider C095-31WREV1 panel (Soria Melguizo). The panel included the following quinolones and concentrations: nalidixic acid 16 mg/L and ciprofloxacin 0.12–1–2 mg/L. Ten additional enterobacterial isolates (3 *K. pneumoniae* and 7 *E. coli*) fully susceptible to quinolones were included as controls.

Antibiotic susceptibility testing was performed by both microdilution and disc diffusion tests as follows: susceptibility to ciprofloxacin and nalidixic acid was determined by disc diffusion (http://www.eucast.org/) and microdilution;[3] and susceptibility to norfloxacin, ofloxacin, moxifloxacin and levofloxacin was determined by microdilution. After microdilution (Table S1, available as Supplementary data at *JAC* Online), MIC values ranged between 2 and 64 mg/L for nalidixic acid and between 0.03 and 4 mg/L for ciprofloxacin, so that 93% of isolates could be defined as included in this low-level ciprofloxacin-resistant phenotype.

## Supplementary data

**Figure S1.** PFGE patterns of clinical ciprofloxacin-resistant *S. enterica* isolates carrying *oqxAB* and *aac-6'-Ib-cr* genes recovered from two young children admitted in a Portuguese hospital (2012). The *Salmonella* serotype and multilocus sequence type (MLST) are also indicated.



### 3.1.3. Imported poultry meat as a source of extended-spectrum cephalosporin-resistant CMY-2-producing *Salmonella* Heidelberg and *S.* Minnesota in European Union, 2014-2015

International Journal of Antimicrobial Agents 51 (2018) 151–154

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Short Communication

# Imported poultry meat as a source of extended-spectrum cephalosporin-resistant CMY-2-producing *Salmonella* Heidelberg and *Salmonella* Minnesota in the European Union, 2014–2015

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ARTICLE INFO

*Article history:*
Received 14 June 2017
Accepted 10 September 2017
Editor: Professor A Tsakris

*Keywords:*
*Salmonella enterica*
Imported poultry meat
*Salmonella* Heidelberg
*Salmonella* Minnesota
$bla_{CMY-2}$
Extended-spectrum cephalosporin resistance

ABSTRACT

Extended-spectrum cephalosporin (ESC)-resistant *Salmonella* have been described at a low level in the EU, nevertheless the increasing importation of poultry meat could be an important source of epidemic strains carrying ESC resistance genes. This study evaluated ESC resistance and its genetic platform among *Salmonella* isolates from poultry meat products imported into Portugal as well as clonal relatedness of the isolates. All *Salmonella* isolates recovered from samples of fresh meat destined for import into the EU in the scope of Portuguese official border control (2014–2015) were studied. Antibiotic susceptibility and β-lactamase production was determined by disk diffusion/microdilution. Molecular studies included detection of genes encoding acquired AmpC and extended-spectrum β-lactamases, plasmid-mediated quinolone resistance and other antibiotic resistance genes by PCR/sequencing, and clonality by MLST and *Xba*I-PFGE. Plasmid characterisation was assessed by conjugation assays, replicon typing (PCR-PBRT/pMLST) and hybridisation experiments (I-*Ceu*I/S1-PFGE nuclease). Isolates belonged to *Salmonella* Heidelberg ($n = 6$; ST15/eBG26) and *Salmonella* Minnesota ($n = 1$; ST548/eBG77) and presented multidrug-resistant profiles, including to ESCs and/or fluoroquinolones. All but one carried $bla_{CMY-2}$, located on two epidemic plasmids, IncA/C (ST2, $n = 5$) or transferable IncI1 (ST12, $n = 1$). *Salmonella* Heidelberg was associated with five PFGE types, including one similar to an American epidemic clone. This study reveals imported poultry products as a source of uncommon and/or invasive ESC-resistant *Salmonella* strains in the EU. The increase of clinically relevant poultry-related serotypes in Europe must be taken into account in the current monitoring of antibiotic resistance trends and in re-evaluation of food regulations.
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## 1. Introduction

Salmonellosis remains one of the most frequent foodborne infections worldwide, being the second most commonly reported gastrointestinal infection and an important cause of foodborne outbreaks in the European Union (EU). The main source of infection for humans is the consumption of contaminated foods of animal origin, including poultry meat [1,2]. Since the implementation of successful *Salmonella* control measures in Europe, the most commonly detected poultry-associated clinically-relevant *Salmonella* serotype (*Salmonella enterica* serotype Enteritidis) has declined substantially [1–3]. This trend was accompanied by the expansion of previously less common serotypes, frequently resistant to antibiotics (e.g. *S.* Infantis, *S.* Stanley, *S.* Kentucky and *S.* Heidelberg) [1,2,4]. Importation of poultry meat has been pointed out as a possible source for the dissemination of *Salmonella* serotypes infrequently reported in Europe and/or of clinically-relevant multidrug-resistant strains [2,5]. In this study, we report for the first time the presence of extended-spectrum cephalosporin (ESC)-resistant CMY-2-producing *S.* Heidelberg and *S.* Minnesota strains in poultry meat imported into Portugal during 2014–2015.

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https://doi.org/10.1016/j.ijantimicag.2017.09.006
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## 2. Materials and methods

### 2.1. Samples and strains: epidemiological background

In the scope of official border control of poultry meat destined to be imported into the EU, during 2014 and 2015, respectively, 18 and 13 samples of fresh meat (gizzards) from *Gallus gallus* imported from Brazil were collected by the Directorate-General for Food and Veterinary (DGAV, Portugal) and were analysed in the National Institute of Health Doutor Ricardo Jorge (INSA, Portugal). In total, 155 units (5 units per sample) were tested for the presence of *Salmonella* according to VIDAS® SLM (bioMérieux, Marcy-l'Étoile, France) and positive results were confirmed by ISO 6579 method [6]. Of the 31 samples, 7 samples (22.6%) (4 in 2014 and 3 in 2015) of frozen poultry gizzards were positive for *Salmonella*. *Salmonella* serotypes were determined by classical serotyping performed at the National Reference Laboratory of Gastrintestinal Infections (INSA).

### 2.2. Study of antibiotic resistance and acquired genes

Susceptibility to diverse antimicrobial agents, including clinically relevant antimicrobials (ESCs and fluoroquinolones), as well as β-lactamase production was studied according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (http://www.eucast.org/clinical_breakpoints/). Molecular studies included the detection of genes coding for acquired AmpC β-lactamases ($bla_{CMY}$, $bla_{MOX}$, $bla_{FOX}$, $bla_{LAT}$, $bla_{ACT}$, $bla_{MIR}$, $bla_{DHA}$ and $bla_{ACC}$), extended-spectrum β-lactamases (ESBLs) ($bla_{TEM}$, $bla_{SHV}$ and $bla_{CTX-M}$), plasmid-mediated quinolone resistance [*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *aac(6')-Ib*-cr and *oqxAB*] and other antibiotic resistance genes by PCR and/or sequencing [7–10].

### 2.3. Plasmid characterisation and clonality study

Standard conjugation assays were performed by the filter mating method with log-phase cells at a 1:1 donor/recipient ratio at 30 °C and 37 °C to the recipient strain *Escherichia coli* HB101 (antibiotic susceptibility shown in Table 1). Transconjugants obtained from isolates with the $bla_{CMY-2}$ gene were recovered following incubation at 37 °C for 24 h from MacConkey agar plates supplemented with cefotaxime (1 mg/L) plus kanamycin (50 mg/L) or sodium azide (130 mg/L). In *Salmonella* strains and transconjugants, plasmid replicon typing, plasmid multilocus sequence typing (pMLST) (http://pubmlst.org/plasmid/) and hybridisation experiments (I-*Ceu*I/S1-PFGE nuclease) were performed. Clonal relatedness of *Salmonella* strains was assessed by *Xba*I-PFGE (https://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c.pdf) and MLST (http://mlst.warwick.ac.uk/mlst/dbs/Senterica), including a comparison with other clinical and foodborne strains.

## 3. Results and discussion

### 3.1. Detection of CMY-2-producing Salmonella Heidelberg and Salmonella Minnesota strains

In this study, all of the *Salmonella* isolates were resistant to ESCs and/or fluoroquinolones, with minimum inhibitory concentrations (MICs) of 8–16 mg/L for cefotaxime and/or 0.25–0.5 mg/L for ciprofloxacin (Table 1). Resistance to these 'critically important antibiotics for human health' (common first-line treatment for invasive salmonellosis in humans) has been reported at relatively low levels in humans, food and food-producing animals in Europe, although it was markedly higher in some serotypes commonly associated with poultry [4].

All of the ESC-resistant isolates presented an AmpC phenotype (Table 1) associated with a $bla_{CMY-2}$ gene, with one isolate also car-

**Table 1**
Characterisation of *Salmonella enterica* isolates ($n = 7$) recovered from imported poultry meat, Portugal, 2014–2015.

| *Salmonella* serotype (no. of isolates) | ST (eBG); PFGE type (no. of isolates) [a] | Year (no. of isolates) | Antibiotic resistance phenotype/genotype (no. of isolates) [b] | Clinically relevant antibiotic resistance | | | Location of *bla* and *qnr* genes-plasmid type (pMLST, Kb; no. of isolates) [d] |
|---|---|---|---|---|---|---|---|
| | | | | Cefotaxime MIC (mg/L) donor (transconjugants) [c] | Cefoxitin MIC (mg/L) donor (transconjugants) [c] | Ciprofloxacin MIC (mg/L) donor (transconjugants) [c] | |
| S. Heidelberg ($n = 6$) | ST15 (eBG26); H1 ($n = 1$), H2 ($n = 1$), H3 ($n = 1$), H4 ($n = 2$), H5 ($n = 1$) | 2014 ($n = 3$); 2015 ($n = 3$) | AMP, AMC, CAZ, CTX, FOX, CIP, PEF, NAL, SMX, TET/***bla*CMY-2***, *sul2*, *tet*(A) ($n = 4$); | 8 to 16 | 32 to >256 | 0.25 to 0.5 | *bla*CMY-2-A/C (ST2, 190–230; $n = 4$); |
| | | | AMP, AMC, CAZ, CTX, FOX, CIP, PEF, NAL, SMX, TET/***bla*CMY-2***, *sul2*, *tet*(A) ($n = 1$); | 16 (4) | 96 (32) | 0.25 | *bla*CMY-2-I1 (ST12, 140; $n = 1$) |
| | | | AMP, CIP, PEF, NAL, GEN, STR, SMX, TET/$bla_{TEM}$, *strA–strB–aadA1*, *sul1–sul2*, *tet*(A) ($n = 1$) | N/D | N/D | 0.5 | |
| S. Minnesota ($n = 1$) | ST548 (eBG77); M ($n = 1$) | 2014 ($n = 1$) | AMP, AMC, CAZ, CTX, FOX, KAN, CIP, PEF, NAL, SMX, TET/***bla*CMY-2***, *aphA1*, ***qnrB5***, *sul2*, *tet*(A) ($n = 1$) | 8 | 64 | 0.25 | *bla*CMY-2-A/C (ST2, 210; $n = 1$); *qnrB5*-NT (<20; $n = 1$) |

[a] ST, sequence type; eBG, eBURST group; PFGE, pulsed-field gel electrophoresis. PFGE types are designated by capital letters and numbers.
[b] Antimicrobial susceptibility was evaluated by disk diffusion assay and/or microdilution methods. AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CAZ, ceftazidime; CTX, cefotaxime; FOX, cefoxitin; CIP, ciprofloxacin; PEF, pefloxacin; NAL, nalidixic acid; SMX, sulfamethoxazole; TET, tetracycline; GEN, gentamicin; STR, streptomycin; KAN, kanamycin. Antibiotic resistance patterns and genes transferred by conjugation are underlined. Genes encoding resistance to clinically relevant antibiotics are presented in bold.
[c] MIC, minimum inhibitory concentration; N/D, not determined. Recipient strain used in conjugation assays was *Escherichia coli* HB101 (sodium azide-, streptomycin- and kanamycin-resistant); cefotaxime MIC = 0.03 mg/L; cefoxitin MIC = 4 mg/L and ciprofloxacin MIC ≤ 0.002 mg/L).
[d] pMLST, plasmid multilocus sequence type; NT, non-typeable. Plasmid type carrying clinically relevant genes transferred by conjugation are underlined.

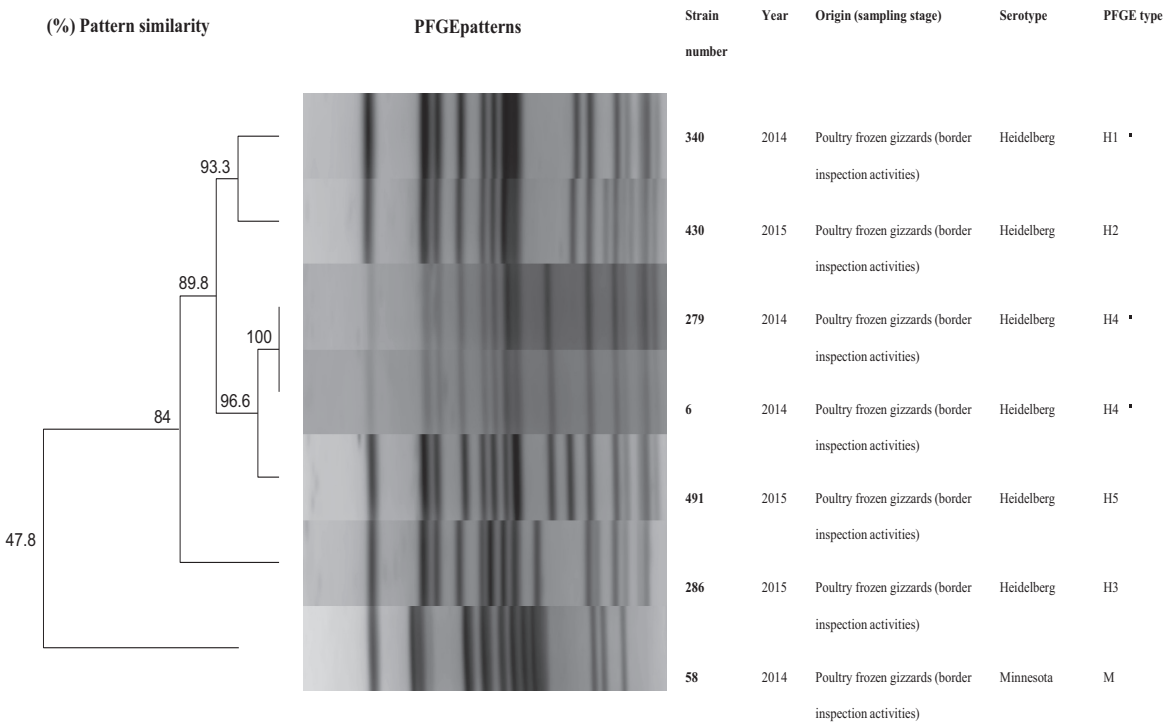


**Fig. 1.** Pulsed-field gel electrophoresis using the restriction enzyme *Xba*I (*Xba*I-PFGE) profiles of *Salmonella* Heidelberg and *Salmonella* Minnesota isolates. PFGE types were analysed by InfoQuest FP v.5.4 software (Bio-Rad Laboratories) using *Salmonella* Braenderup PulseNet Universal Control Strain (H9812) as molecular reference marker (https://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c.pdf). The percentages of similarity were calculated by applying the unweighted pair-group method using average linkages (UPGMA) algorithm based on the Dice coefficient (1.0% band tolerance; 1.0% optimisation). PFGE profiles marked with a bullet point present similar profiles with *Xba*I.1968/JF6X01.0258 (H1) and *Xba*I.1965 (H4) from The Netherlands study [14].

rying a *qnrB5* gene. CMY-2 is the most common acquired AmpC β-lactamase found in *Salmonella*, being those producers mostly reported in American countries [2,11,12] where they have been detected in poultry and/or poultry meat samples [2,11–13]. A recent study in The Netherlands reported $bla_{CMY-2}$ in almost all of the ESC-resistant *Salmonella* strains studied, mostly recovered from imported poultry meat [14].

The 6 CMY-2-producing *Salmonella* isolates belonged to *S.* Heidelberg ($n = 5$; ST15/eBG26) and *S.* Minnesota ($n = 1$; ST548/eBG77) serotypes (Fig. 1; Table 1). In this study, *S.* Heidelberg ST15 isolates were associated with five pulsed-field gel electrophoresis (PFGE) types, including similar ones shared between two isolates (designated H4) (Fig. 1). A profile comparison between these and the ESC-resistant *S.* Heidelberg strains from poultry products imported to The Netherlands [14] showed a high similarity with PFGE types *Xba*I.1965 and the predominant *Xba*I.1968/JF6X01.0258, the latter being indistinguishable from an epidemic type with invasive potential that caused outbreaks in the USA [14] (Fig. 1).

*Salmonella* Heidelberg and *S.* Minnesota serotypes are infrequently reported in human and animal sources from Portugal and other European countries. In North America, *S.* Heidelberg has become one of the top multidrug-resistant serotypes both in poultry and humans, being frequently associated with outbreaks and invasive infections [2,15,16]. In Brazil, both *Salmonella* serotypes are disseminated in poultry production and are among the serotypes frequently presenting ESC resistance associated with $bla_{CMY-2}$ or ESBL genes [11,13,17]. The presence of *S.* Heidelberg and/or *S.* Minnesota in poultry products from Brazil has been notified by several European countries through the Rapid Alert System for Food and Feed [18]. These data reveal imported poultry products as a source of uncommon ESC-resistant *Salmonella* serotypes in Europe, some associated with invasive infections (*S.* Heidelberg) in which antibiotics are critical for their treatment.

### 3.2. Location of the blaCMY-2 gene in diverse plasmid backbones

The $bla_{CMY-2}$ gene was located on two epidemic plasmid types: IncA/C ($n = 5$; ST2), associated with *S.* Minnesota and different PFGE types of *S.* Heidelberg; and less frequently IncI1 ($n = 1$; ST12) (Table 1). The latter was associated with our PFGE profile (designated H1) similar to *Xba*I.1968/JF6X01.0258 epidemic clone, as described in The Netherlands [14] (Table 1; Fig. 1). Transferability of the $bla_{CMY-2}$ gene was only achieved for one *S.* Heidelberg carrying an IncI1 plasmid encoding resistance solely to ESCs (Table 1). Plasmids belonging to IncI1-ST12 and IncA/C incompatibility groups have contributed to the worldwide spread of the $bla_{CMY-2}$ gene among *S.* Heidelberg and other *Salmonella* serotypes both from human and animal sources [14,16,19,20]. However, all of the IncA/C plasmids in the current study belonged to ST2 by pMLST, a less common $bla_{CMY-2}$-carrying plasmid [21], extending our knowledge on current $bla_{CMY-2}$-carrying plasmid types circulating in the EU.

## 4. Conclusions

In summary, this study reinforces previous evidence of the introduction into Europe of uncommon and/or invasive poultry-related *Salmonella* serotypes (*S.* Heidelberg and *S.* Minnesota) resistant to clinically relevant antibiotics and carrying $bla_{CMY-2}$ through imported poultry meat, including one PFGE profile identical to an epidemic clone. Furthermore, these data raise question about the Regulation (EU) N°1086/2011 [22] setting a food safety microbiological criterion for fresh poultry meat covering only *S.* Enteritidis

and *S.* Typhimurium (including the monophasic variant). The increase of other less common poultry-related serotypes in Europe, as described here, must be taken into account in the monitoring of antibiotic resistance trends and in the re-evaluation of food regulations.

**Acknowledgments**

The authors would like to thank to Apostolos Liakopoulos (Department of Bacteriology and Epidemiology, Central Veterinary Institute, Wageningen University & Research, Wageningen, The Netherlands) for providing the PFGE profiles of the *Salmonella* Heidelberg isolates from The Netherlands.

*Funding*: This work was supported by FCT/MEC (Fundação para a Ciência e a Tecnologia/Ministério da Educação e Ciência) through national funds and co-financed by FEDER, under the Partnership Agreement PT2020 [grant no. UID/MULTI/04378/2013 – POCI/01/0145/FERDER/007728]; the project NORTE-01-0145-FEDER-000011 – Qualidade e Segurança Alimentar—uma abordagem (nano)tecnológica. JC is supported by a PhD fellowship from FCT [grant no. SFRH/BD/93091/2013] and JM is supported by a post-doctoral fellowship from project NORTE-01-0145-FEDER-000024. The authors are greatly indebted to all of the funding sources.

*Competing interests*: None declared.

*Ethical approval*: Not required.

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### 3.1.4. Inflow water is a major source of trout farming contamination with *Salmonella* and multi-drug resistant bacteria

Science of the Total Environment 642 (2018) 1163–1171

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

# Inflow water is a major source of trout farming contamination with *Salmonella* and multidrug resistant bacteria

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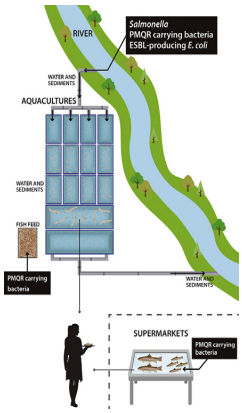
## HIGHLIGHTS

- Occurrence of resistant bacteria and *Salmonella* in trout farms and surroundings.
- Quinolone resistance genes were frequently found in trout farms and environment.
- Trout farms were contaminated with low-level ciprofloxacin resistant Enterobacteria.
- Inflow water in trout farms was a source of SHV-12-producing *E. coli*.
- Inflow water contaminated trout farms with *Salmonella* serotypes of diverse origin.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 31 March 2018
Received in revised form 11 June 2018
Accepted 11 June 2018
Available online xxxx

Editor: D. Barcelo

*Keywords:*
Antibiotic resistance
Food-chain
Aquatic environment
*Salmonella*
Rainbow trout aquacultures
Ciprofloxacin

## ABSTRACT

The impact of European aquaculture, namely trout farms, in the spread of antibiotic resistance and/or zoonotic pathogens has been scarcely addressed. Moreover, aquaculture contamination sources and bacterial dissemination routes have been barely explored. In this study, we assessed the contribution of Portuguese land-based intensive rainbow trout farms and retailed market trout to the spread of *Salmonella* and bacteria carrying clinically-relevant antibiotic resistance genes (ARGs) as well as inflow water and feed as possible sources of those contaminants. Cultural and molecular methods were used to analyse 53 fish farm samples (upstream/downstream water and sediments, tanks and trout) and 25 marketed trout. Plasmid-mediated quinolone resistance (PMQR) genes were found in 21% ($n = 11/53$) of samples (water/sediment/feed/trout), from all collection points (upstream/within/downstream tanks) and seasons, as well as in 12% ($n = 3/25$) of marketed trout (3 supermarkets). PMQR genes (*qnrS1-S2-S3*, *qnrB7-B19*, *qnrD1*, *oqxAB*) were detected in *Enterobacteriaceae* or *Aeromonas hydrophila*. An *E. coli* strain producing extended-spectrum-beta-lactamase SHV-12 was detected in all sampled points of a fish farm. *Salmonella* (4 serotypes, including *S.* Newport-ST118) was detected in 26% ($n = 14/53$) of the samples from both farms (water/sediment upstream/within tanks). The clinically-relevant plasmid-mediated colistin resistance *mcr* genes were not detected. However, colistin resistant *S.* Abony with new mutations in the chromosomal *pmrA* and *pmrB* genes was observed. Identical *Salmonella* and SHV-12-producing *E. coli* strains (by PFGE/MLST) in water upstream and within trout tanks points to inflow-water of trout farms as an important source of pathogenic bacteria and ARG contamination. These results highlight the need to

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https://doi.org/10.1016/j.scitotenv.2018.06.143
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define microbiological standards for water supplying fish farms in the EU and to establish surveillance and control strategies to limit bacterial transmission associated with this fastest growing food sector worldwide.

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## 1. Introduction

Aquaculture is currently the fastest growing food production sector, responsible for ~50% of the fish produced worldwide for human consumption, as a consequence of the global increase demand for fish products and decline in wild fish stocks (FAO, 2016, 2017). The intensification of fish farming has been accompanied by increasing environmental impacts, questioning the aquaculture sustainability, the quality of water resources and raising food safety issues (e.g. antibiotic resistant bacteria, residues of veterinary medicines, disinfectants and zoonotic pathogens) (EFSA, 2008; Cabello et al., 2016; Watts et al., 2017). In Europe, where the fish aquaculture industry has been substantially growing in the last years (estimates predict a production of 4 million tonnes by 2030) (FAO, 2016, 2017), the legal requirements include the use of safe water (Directive 2000/60/EC). This Directive quality standards (based on water chemical and ecological status) stimulates the development of environmentally-friendly aquaculture production systems, contributing to the maintenance of high water quality in the surrounding water bodies (Science for Environment Policy, 2015; European Commission, 2016). In fact, the aquaculture industry has been identified in diverse geographical regions as exerting a negative impact on water resources downstream of production, but the quality of fish farmed can also be affected by the quality of the water supplied to the farm (Science for Environment Policy, 2015; European Commission, 2016).

Nevertheless, few studies assessing sources of European fish farms contamination have been conducted (Topp et al., 2018; Watts et al., 2017), including in trout production, the second major farmed species in EU (FAO, 2017). Moreover, there is a lack of data on the impact of European fish farms in the spread of antibiotic resistance and/or zoonotic pathogens (standards not specified in the EWFD2000/60/EC) (Schmidt et al., 2000; Alcaide et al., 2005; EFSA, 2008; Muziasari et al., 2016; Novais et al., 2018). Specifically, the impact of freshwater fish farms on the dispersion of bacteria with resistance to antibiotics critical to treat human infections (such as fluoroquinolones, extended-spectrum β-lactams and colistin) through the environment and the food chain. We hypothesize that land-based intensive rainbow trout farms and retailed market trout could contribute to the spread of *Salmonella* and bacteria carrying clinically-relevant antibiotic resistance genes (ARGs), being inflow water and feed possible contamination sources. To confirm this hypothesis we assessed the occurrence of gram-negative bacteria carrying extended-spectrum beta-lactamase (ESBL), plasmid-mediated quinolone resistance (PMQR), mobile colistin resistance (MCR)-encoding genes and the pathogenic bacteria *Salmonella* in diverse trout farm samples (upstream and downstream water and sediments, tanks and trout). The identification of pathogens and antibiotic resistance sources in fish farms, as well as their dissemination routes, can help to establish effective strategies to develop sustainable aquaculture in Europe and to limit environmental bacterial contamination and human health risks associated with the food-chain.

## 2. Material and methods

### 2.1. Sampling strategy in fish farms, environment and supermarkets

Two land-based intensive aquacultures (AQ-A and AQ-B) with flow-through systems producing rainbow trout *Oncorhynchus mykiss* located in rural areas in the north and the centre of Portugal were studied. They were located in the course of secondary rivers (which were the only water source used) and presented apparently satisfactory hygienic conditions. Trout production was around 5 and 40 tons per year in AQ-A and AQ-B, respectively, mainly for human consumption. Trout were fed by commercialized feed (comprising 3 mg/kg of copper, fish flour, soy and genetically modified corn among other components) without antibiotics. Two (AQ-A) to three (AQ-B) visits were performed to the aquacultures facilities and included cold (winter) and warm (spring and summer) seasons of the year (between 2010 and 2012). Samples ($n = 53$) were collected from both river water ($n = 17$) and superficial sediments ($n = 11$) located upstream ($n = 13$) and downstream ($n = 15$) of the trout tanks, water ($n = 10$) and superficial/wall sediments ($n = 3$) from juvenile/adults trout tanks ($n = 13$), feed ($n = 6$) and trout (n = 6; muscle and viscera; 1 trout per sample). Sampling points were randomly selected in each farm and water and sediment were collected from the same selected points. Also, marketed rainbow trout ($n = 25$ samples of muscle and viscera trout; 3 trout per

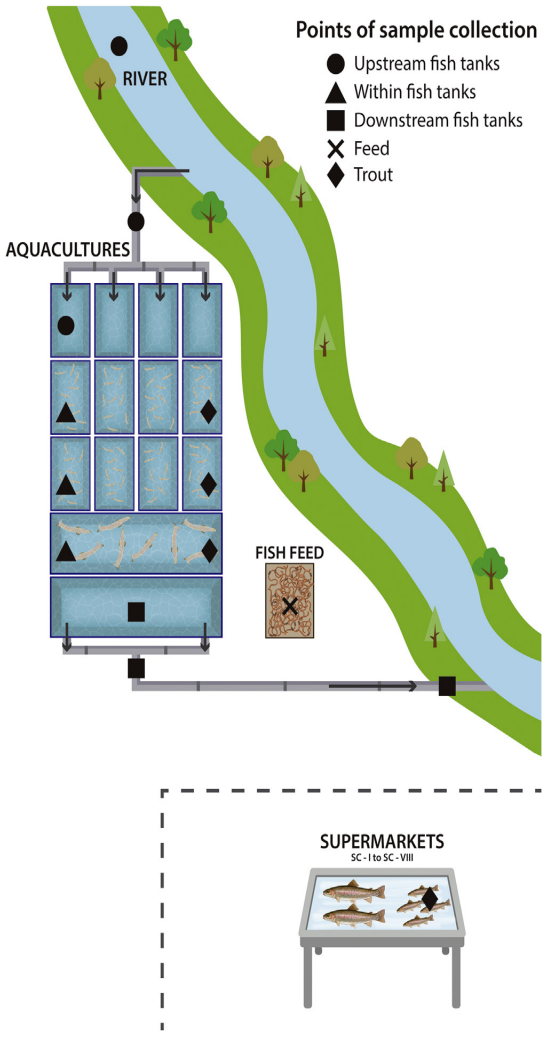


**Fig. 1.** Sampling strategy in trout farms, environment and supermarkets.

sample per brand) of national aquaculture origin purchased in the most popular supermarkets ($n = 8$; SC-I to SC-VIII) in the Porto region were included in this study (Fig. 1). All samples were collected in sterile plastic containers/bags (of 1 L for liquid samples and of 1 kg for solid samples), transported at 4 °C and processed in the same day at the laboratory. The procedure started with the inoculation of the test portion (25 g for solid samples or the filter used to analyse 1 L of water) in non-selective pre-enrichment medium buffered peptone water (BPW) yielding a tenfold dilution (37 °C for 16–18 h). Subsequent sample processing was performed as described in the next sections and Fig. 2.

*2.2. Study of gram-negative bacilli presenting clinically-relevant antibiotic resistance*

*2.2.1. Bacteria isolation and search of ARGs*

An aliquot of 0.1 mL of BPW was plated on MacConkey agar without antibiotics and supplemented with ciprofloxacin (0.125 Mg $L^{-1}$), ceftazidime (1 Mg $L^{-1}$) and cefotaxime (1 Mg $L^{-1}$), corresponding to concentrations allowing the detection of bacteria with low-levels of antibiotic resistance. Plates were incubated at 37 °C for 24 h. One to five colonies of each morphotype were selected from each antibiotic selective plate for further characterization. DNA of each isolate was

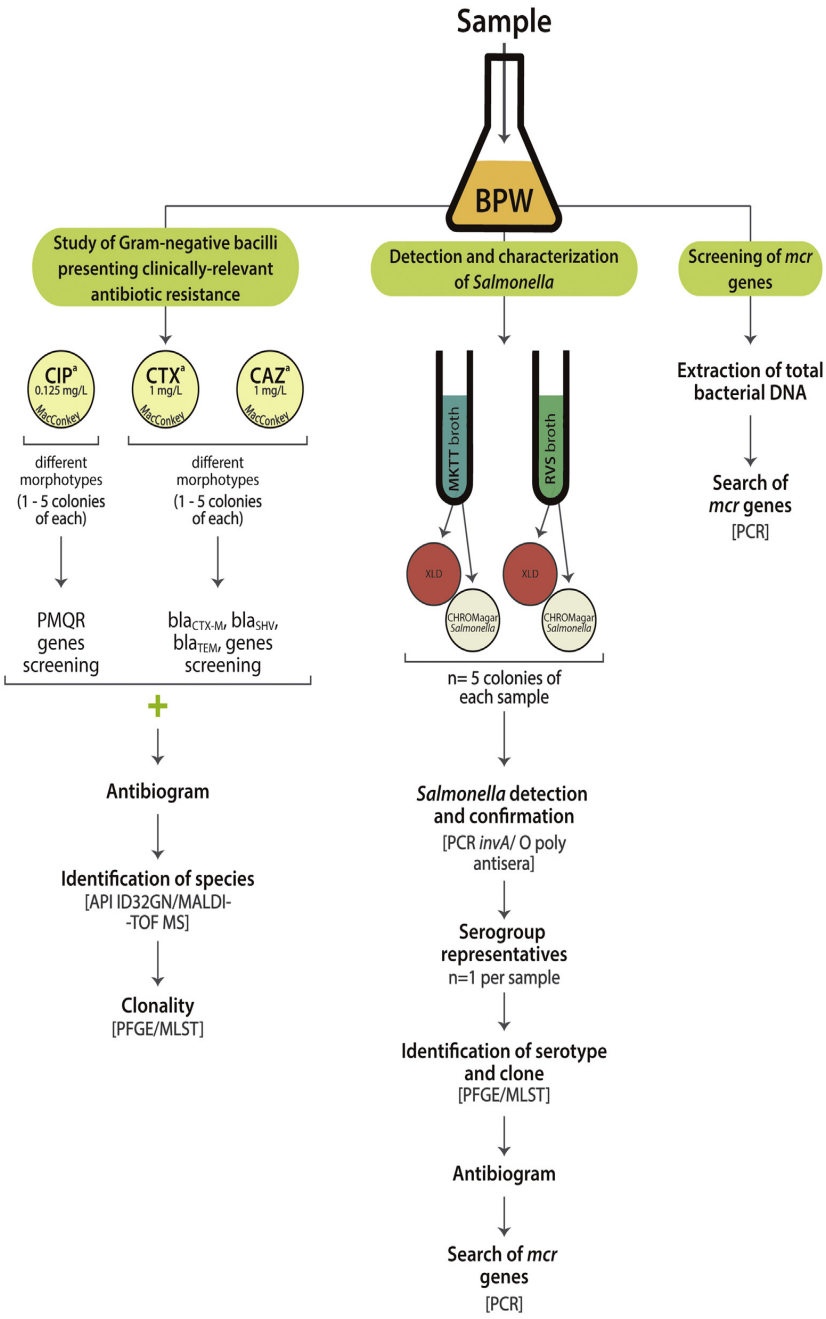


**Fig. 2.** Schematic representation of sample processing and bacterial characterization.

extracted by the microbiological standard boiling method (using lysis solution with 0.05 M NaOH + 0.25% SDS). Search of clinically-relevant acquired genes conferring resistance to β-lactams (ESBL-$bla_{TEM}$, $bla_{CTX-M}$, $bla_{SHV}$) and fluoroquinolones [PMQR-*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6′)-Ib-cr*, *qepA*, *oqxAB*] was performed by PCR, using previously described or newly designed primers (Table S1, available as Supplementary data), with further sequencing. Colistin resistance genes (*mcr*-1, *mcr*-2, *mcr*-3, *mcr-4* and *mcr-5*) were searched in isolates carrying PMQR or ESBL genes by PCR, using previously described primers and conditions (Table S1). Briefly, PCR assays reaction mixtures were prepared for a final volume of 25 μl containing 1× gel load reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.3 μM of each primer, 2.5 units of Taq DNA polymerase and 2 μl of total bacterial DNA. Amplified PCR products were purified using the NZYGelpure kit (NZYTech, Portugal) and sequenced at GATC Biotech (https://www.gatc-biotech.com/en/index.html). Previously reported isolates from our collections carrying ESBL and PMQR genes (Machado et al., 2006; Campos et al., 2016a) as well as *mcr*-carrying isolates provided by other authors (Campos et al., 2016a; Xavier et al., 2016; Borowiak et al., 2017; Carattoli et al., 2017; Yin et al., 2017) were included as positive controls and *Escherichia coli* ATCC 25922 as negative control in all PCR assays.

Due to the recent recognition of the clinically-relevant *mcr* genes, which confer resistance to colistin (Liu et al., 2016), screening of those genes, not included in the initial study design, was performed directly in BPW stored (−80 °C) enrichments of aquacultures and supermarket samples. Bacterial DNA was extracted using 1 mL of BPW unfreeze enrichments by a boiling protocol, as reported (Mainar-Jaime et al., 2013). Briefly, it consisted of the following steps: centrifugation (13,000 rpm, 10 min), resuspension of the pellet in 100 μL of ultrapure water, boiling (100 °C, 20 min) and centrifugation (4000 rpm, 4 min). The DNA in supernatant was used for *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* genes amplification, using conditions and primers, as described above. The efficiency of the bacterial DNA extraction was firstly evaluated by PCR targeting 16S rDNA gene (Héritier et al., 2003).

*2.2.2. Antibiotic susceptibility study*

Antibiotic susceptibility tests to 16 antibiotics (amoxicillin-10 μg, amoxicillin+clavulanic acid-30 μg, aztreonam-30 μg, cefepime-30 μg, cefotaxime-30 μg, cefoxitin-30 μg, ceftazidime-30 μg, chloramphenicol-30 μg, ciprofloxacin-5 μg, gentamicin-10 μg, kanamycin-30 μg, nalidixic acid-30 μg, streptomycin-10 μg, sulfamethoxazole-300 μg, tetracycline-30 μg and trimethoprim-5 μg) were performed by disc diffusion following EUCAST (2017) and when it was not possible by CLSI guidelines (CLSI, 2016). Minimum Inhibitory Concentration (MIC) determination for ciprofloxacin, ceftazidime and cefotaxime was performed by *E*-test method and for colistin by the broth microdilution method (EUCAST, 2016). Multidrug resistance (MDR) was considered when the isolates were resistant to three or more antibiotics of different families. Those isolates were also categorized as wild-type and non-wild-type accordingly to the epidemiological cut-offs (ECOFFs) preconized by EUCAST guidelines (http://www.eucast.org/mic_distributions_and_ecoffs/) for *Enterobacteriaceae* species. Production of ESBL was investigated by the standard double-disk synergy test (DDST) (EUCAST, 2017).

*2.2.3. Species identification and clonality study*

Isolates carrying PMQR or ESBL genes were identified at species level by API ID32GN (bioMérieux, Marcy l'E'toile, France) and/or Matrix-Assisted Laser Desorption-Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) using the MALDI Biotyper system (Microflex III spectrometer controlled by FlexControl version 3.3 data acquisition software, Bruker Daltonics, Bremen, Germany). Clonal relatedness was assessed by Pulsed-Field Gel Electrophoresis (PFGE) (CDC, 2002) and also by Multilocus Sequence Typing (MLST) for *Escherichia coli* (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search) and *Klebsiella pneumoniae* (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

*2.2.4. Conjugation assays and plasmid characterization*

Standard conjugation assays were performed by the filter-mating method with log-phase growth cells at a 1:1 donor/recipient ratio, at 30 °C and 37 °C to the recipient strain *E. coli* HB101 and/or *E. coli* K802N (antibiotic susceptibility shown in Table 1). Transconjugants obtained from isolates with PMQR or ESBL genes were recovered after incubation at 37 °C/24 h from MacConkey agar plates supplemented with ciprofloxacin (0.06–0.5 Mg $L^{-1}$) plus kanamycin (50 Mg $L^{-1}$) or nalidixic acid (100 Mg $L^{-1}$) and cefotaxime (1 Mg $L^{-1}$) plus nalidixic acid (100 Mg $L^{-1}$), respectively. In donors and transconjugants, class 1 integron characterization (Antunes et al., 2006), classification of plasmids by PCR-based replicon typing (PBRT scheme plus IncU, IncR, colE, IncX, IncL, IncM and Col3M) (Table S1), plasmid MLST (pMLST) (http://pubmlst.org/plasmid/) and location of antibiotic resistance genes by hybridization experiments (I-*Ceu*I/S1-PFGE nuclease) using specific probes ($bla_{SHV}$, *qnrB*, *qnrD*, *qnrS*, *oqxAB*, IncI1, IncX1, IncN, IncFIA, IncFII_K, IncU, Col3M) were performed.

*2.3. Detection and characterization of Salmonella*

Detection of *Salmonella* was performed based on standard methods (ISO 6579:2002), after one stage of pre-enrichment in BPW and enrichment in Rappaport-Vassiliadis medium with Soya (RVS) and Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broths. Then, both selective broths were streaked on Xylose-Lysine Deoxycholate (XLD) agar and CHROMagar™ *Salmonella* Plus. Presumptive *Salmonella* colonies recovered from both selective medium (up to five colonies per plate) were confirmed by agglutination with *Salmonella* O poly antisera and serogroup-specific antisera (BD, USA) and by PCR for *invA* gene (Pritchett et al., 2000). Clonal relatedness was assessed by PFGE (CDC, 2002) and MLST (http://enterobase.warwick.ac.uk/species/senterica/allele_st_search) in isolates representative of different serogroups obtained from each sample. Serotyping was done at *National Center of Salmonella* (INSA, Lisboa, Portugal). Antibiotic susceptibility tests, including to colistin, were performed as described above (see Section 2.2.2). Colistin resistance genes (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4 and mcr-5*) were also identified by PCR in all *Salmonella* isolates (see Section 2.2.1).

Colistin-resistant isolates were selected for Whole Genome Sequencing. Bacterial genomic DNA was extracted using the Wizard® Genomic DNA purification kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. Genome sequencing was accomplished by an Illumina HiSeq platform, according to standard Illumina protocols performed at the GATC Biotech (Konstanz, Germany). Data were analysed using: FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to test the quality of the raw data; SPAdes (v.3.9.1) for de novo assembly of the the paired-end reads; and QUAST (http://quast.bioinf.spbau.ru) for evaluating the quality of genome assembly. The assembled genomes were then screened for genes encoding colistin resistance using in silico genomic tools (ResFinder and PointFinder) available at the Center for Genomic Epidemiology (http://www. genomicepidemiology.org).

## 3. Results

*3.1. Study of gram-negative bacilli species carrying clinically-relevant ARGs*

*3.1.1. Plasmid-mediated quinolone resistance*

From plates supplemented with ciprofloxacin we recovered 133 isolates ($n = 82$ from aquacultures and $n = 51$ from supermarkets). They were obtained from samples collected upstream, within and downstream of the trout tanks (n=38 out of 41 samples), feed (n=3/6), trout (n=5/6) and trout marketed in supermarkets (n=23/25). The PMQR genes were detected in 11% (14 out of 133) of the isolates tested, not including the eight *Citrobacter* spp. isolates, in which *qnrB* is recognized as an intrinsic gene and considered the origin of the plasmid-

**Table 1**
Bacterial species carrying PMQR or ESBL genes in marketed trout, trout farms and environmental surroundings.

| Point of collection | Sample type (sample designation) | PMQR or ESBL genes | Species (no. isolates) | PFGE[a]/MLST[b] | Season-aquaculture/supermarket[c] | Resistance phenotype/resistance genotype[d] | MIC μg/ml - Donor (transconjugant)[e] CIP | CAZ | CTX | PMQR or ESBL location - Chr or PL (Kb; Inc)[h] |
|---|---|---|---|---|---|---|---|---|---|---|
| Upstream trout tanks | Water (46) | *qnrS1* | *Escherichia coli* (*n* = 1) | Ec1/ST2739 | Spring-AQ-B | AML/$bla_{TEM}$ | 0.25 (0.125[f]) | nd | nd | *qnrS1*/PL (~50Kb; X1) |
| | Water (3, 7) | $bla_{SHV-12}$ | *Escherichia coli* (*n* = 3) | E/ST3595 | Winter-AQ-B | AML-AZT-CAZ-CTX, STR, TET/$bla_{SHV-12}$-*strA-strB-tetB* | nd | **32** (>256[f]) | **8** (8[f]) | $bla_{SHV-12}$/PL (~65 Kb; I1) |
| Within trout tanks | Water (1, 2) | $bla_{SHV-12}$ | *Escherichia coli* (n = 5) | E/ST3595 | Winter-AQ-B | AML-AZT-CAZ-CTX, STR, TET/$bla_{SHV-12}$-*strA-strB-tetB* | nd | **32** (>256[f]) | **8** (8[f]) | $bla_{SHV-12}$/PL (~65 Kb; I1) |
| | Water (25) | *qnrS2* | *Aeromonas hydrophila* (n = 1) | Ah1/ND | Summer-AQ-B | TET | 0.064 | nd | nd | *qnrS2*/PL (~30Kb; NT) |
| | Water (15) | *qnrS3* | *Escherichia coli* (n = 1) | Ec4/ST641 (CC86) | Winter-AQ-A | AML, CLO, STR, SUL, TET, TRI/$bla_{TEM}$, *cmlA*, *aadA*, ***sul3***, *tetA*, *dfrA12* | 0.25 (0.25[g]) | nd | nd | *qnrS3*[i]/PL (~40Kb; N) |
| | Sediment (20) | *qnrS3* | *Escherichia coli* (n = 1) | Ec3/ST641 (CC86) | Winter-AQ-A | AML, KAN, STR, TET/$bla_{TEM}$, *aphA1*, ***aadA***, *tetB* | 0.25 (1[f]) | nd | nd | *qnrS3*[i]/PL (~50Kb; NT) |
| | Sediment (20) | *qnrS3* | *Citrobacter gillenii* (*n* = 1) | Cf1/ND | Winter-AQ-A | KAN, STR, TET/*strA-strB* | **0.5** | nd | nd | *qnrS3*[i]/PL (~110Kb; N-FIA) |
| Downstream trout tanks | Water (4, 5, 6) | $bla_{SHV-12}$ | *Escherichia coli* (*n* = 7) | E/ST3595 | Winter-AQ-B | AML-AZT-CAZ-CTX, STR, TET/$bla_{SHV-12}$-*strA-strB-tetB* | nd | **32** (>256[f]) | **8** (8[f]) | $bla_{SHV-12}$/PL (~65Kb; I1) |
| | Water (50) | *qnrS1* | *Escherichia coli* (n = 1) | Ec2/ST423 (CC23) | Spring-AQ-B | AML, NAL, SUL, TET, TRI/$bla_{TEM}$, ***aadA1***, ***sul1***, *tetA*, *dfrA1* | 0.25 (0.125[g]) | nd | nd | *qnrS1*/PL (~50Kb; X1) |
| | Water (28) | *qnrS2* | *Aeromonas hydrophila* (n = 1) | Ah1/ND | Summer-AQ-B | TET | 0.064 | nd | nd | *qnrS2*/PL (~30Kb; NT) |
| | Water (28) | *qnrS3* | *Escherichia coli* (n = 1) | Ec5/ST661 | Summer-AQ-B | AML/$bla_{TEM}$ | 0.25 | nd | nd | *qnrS3*/PL (~50Kb; X1) |
| | Water (6) | *qnrB19* | *Escherichia coli* (n = 1) | Ec6/ST1049 | Winter-AQ-B | AML, STR, SUL, TET, TRI/$bla_{TEM}$, *strA-strB*, ***aadA1***, ***sul1***, *sul2*, *tetA*, ***dfrA1*** | 0.25 | nd | nd | *qnrB19*[i]/Chr |
| | Sediment (31) | *qnrB7* + *oqxA* + *oqxB* | *Klebsiella pneumoniae* (n = 1) | Kp1/ST14 | Summer-AQ-B | CLO, STR, SUL, TET, TRI/*catA*, ***aadA***, ***sul1***, ***dfrA1*** | **1** | nd | nd | *qnrB7*/Chr<br>*oqxAB*[i]/PL (~240 kb; N-FII_K) |
| Feed | Feed (41) | *oqxA* + *oqxB* | *Klebsiella pneumoniae* (n = 1) | Kp2/ST new | Summer-AQ-A | CLO, KAN, NAL, SUL, TET, TRI/*cmlA*, *aphA1*, *aadA*, ***sul3***, *tetB*, *dfrA12* | **0.5** | nd | nd | *oqxAB*[i]/PL (~260Kb; NT) |
| Trout | Trout (65, 70) | *qnrS2* | *Hafnia alvei* (n = 2) | Ha1/ND | Spring-SM-I, SM-VII | CLO, NAL STR, SUL/*floR*, *strA-strB*, ***sul1*** | 0.25 | nd | nd | *qnrS2*[i]/PL (~20Kb; U) |
| | Trout (63) | *qnrD1* | *Proteus vulgaris* (n = 1) | Pv1/ND | Spring-SM-IV | TET | 0.25 | nd | nd | *qnrD1*/PL (<20Kb; Col3M) |

All the features underlined were transferred by conjugation.
[a] PFGE types: *Escherichia coli*: Ec1-Ec6; *Aeromonas hydrophila*: Ah1; *Klebsiella penumoniae*: Kp1-Kp2; *Hafnia alvei*: Ha1; *Proteus vulgaris*: Pv1.
[b] ST, sequence type; CC, clonal complex; ND, not determined.
[c] AQ-A/AQ-B, Aquacultures; SM, Supermarkets (I to VIII).
[d] AML, amoxicillin; AZT, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; CLO, chloramphenicol; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SUL, sulphonamides; TET, tetracycline; TRI, trimethoprim. Genes belonging to class 1 integrons are in bold. All the isolates were susceptible to colistin, with exception of the species intrinsically resistant (*Aeromonas hydrophila*, *Proteus vulgaris*, *Hafnia alvei*).
[e] MICs in bold represented isolates classified as resistant or with intermediate level of resistance according to EUCAST or CLSI guidelines.
[f] Positive conjugation with recipient *E. coli* K802 N (nalidixic acid-resistant; $MIC_{CIP}$ = 0.25 mg/L; $MIC_{CAZ}$ = 0.5 mg/L; $MIC_{CTX}$ = 0.125 mg/L).
[g] Positive conjugation with recipient *E. coli* HB101 (sodium azide-, streptomycin- and kanamycin-resistant; $MIC_{CIP}$ = 0.004 mg/L).
[h] Chromosomal (Chr) and/or plasmid (PL) location of clinically relevant resistance genes. NT, non-typable.
[i] IS*26* located in the same plasmid or chromossome as PMQR genes.

mediated *qnrB* (Ribeiro et al., 2015). The analysis of the PMQR genes and species distribution by point of collection is presented in Table 1. They were recovered from 21% of aquaculture samples (11 out of 53), including all types (water, sediment, feed and trout), collection points (upstream, within and downstream) and seasons in both fish farms (all 5 visits), and from 12% (3 out of 25) of supermarket samples (3 different brands).

The PMQR genes found encoded two different fluoroquinolone resistance acquired mechanisms, comprising three gyrase-protection proteins (*qnrS*, $n = 10$ isolates; *qnrB*, $n = 2$ isolates and *qnrD*, $n = 1$ isolate) and a MDR efflux pump (*oqxAB*, $n = 2$ isolates). They were found alone ($n = 13$) or in combination ($n = 1$; *qnrB7* + *oqxAB*) in isolates from *Enterobacteriaceae* ($n = 12$) or *Aeromonas hydrophila* ($n = 2$). No *qnrC*, *qepA* or *aac(6′)-Ib-cr* genes were detected. Diverse *E. coli* clones ($n = 5$; ST423, ST2739, ST641, ST661 and ST1049), *Klebsiella pneumoniae* ($n = 2$; ST14 and STnew), *Hafnia alvei* ($n = 2$), *Proteus vulgaris* ($n = 1$) and *Citrobacter gilleni* ($n = 1$) were detected as carrying PMQR genes. Two samples (water and sediment) from the same fish farm presented different species carrying diverse *qnrS* genes. All *Enterobacteriaceae* PMQR-positive isolates presented MIC to ciprofloxacin (0.25–1 Mg $L^{-1}$) above ECOFF for the species (EUCAST, 2017). The highest ciprofloxacin MIC value (above clinical breakpoint) was observed in a *K. pneumoniae* isolate carrying *qnrB7* and *oqxAB* genes. Most isolates carried additional ARGs, class 1 integrons and/or IS*26* often co-located with the PMQR genes (Table 1).

The *qnrS* family, involving three known variants (*qnrS1*, *qnrS2* and *qnrS3*), was the predominant PMQR genes (10 out of 14). They were particularly observed in *E. coli* belonging to worldwide described clonal lineages. In two cases, we observed the same strain in different samples of the same aquaculture (*E. coli* ST641 in water or sediment of juvenile and adult trout tanks of AQ-A and *A. hydrophila* in water within and downstream trout tanks of AQ-B). In one case, trouts of different supermarkets shared the same strain (*Hafnia alvei* from SC-I and SC-VII). Those *qnrS* genes were located in diverse ~30–50 kB plasmid families (IncX1, IncU, IncN and untypable), with transferability achieved for *E. coli* carrying *qnrS1* (IncX1) or *qnrS3* (IncN and untypable). Two variants of *qnrB* were identified among *E. coli* (*qnrB19*) and *K. pneumoniae* (*qnrB7*) isolates, both located in the chromosome. The recently described *oqxAB* gene was detected in large plasmids (~240–260 kB) in two different MDR *K. pneumoniae* (ST14-often associated with human infections and STnew). Finally, the *qnrD1* gene was detected in a small non-transmissible plasmid (family Col3M) of one *Proteus vulgaris* isolate (Table 1).

#### 3.1.2. Extended-spectrum β-lactamases

From plates supplemented with cefotaxime or ceftazidime we recovered 253 isolates ($n = 142$ from aquacultures and $n = 111$ from supermarkets). They were obtained from samples collected upstream, within and downstream of the trout tanks ($n = 39/41$), feed ($n = 3/6$), trout ($n = 5/6$) and trout marketed in supermarkets ($n = 25/25$). Only the ESBL encoding gene $bla_{SHV-12}$ was detected in 19 isolates (8% - $n = 19$ out of 253 isolates tested). They were all recovered from aquaculture AQ-B in the winter season visit, including 7 water samples (river and juvenile/adults fish tanks) in all collection points (upstream, within and downstream). All those isolates belonged to an *E. coli* (ST3595) clone carrying $bla_{SHV-12}$ in a transferable IncI1 plasmid (~65 kB) and exhibiting a MDR pattern (Table 1). No ESBL genes belonging to $bla_{TEM}$ or $bla_{CTX-M}$ were detected.

#### 3.1.3. MCR-encoding genes directly in enrichment samples

The recently identified acquired plasmid-borne genes conferring resistance to colistin (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4 and mcr-5*) were not detected in DNA extracted from the non-selective pre-enrichment medium BPW.

### 3.2. Detection of Salmonella in trout farms

*Salmonella* was detected in 26% ($n = 14/53$) of the samples studied from both trout aquacultures, restricted to the summer season. They were identified among samples collected upstream ($n = 4$) and downstream ($n = 6$) of aquacultures, within fish tanks ($n = 3$) and in the fish viscera ($n = 1$). *Salmonella* was not detected in any of the feed samples analysed from both aquacultures or from trout samples obtained from supermarkets.

Among *Salmonella* selected isolates ($n = 18$; one from each serogroup per positive sample) we identified *S.* Newport-ST118, *S.* Linguere-ST508, *S.* Guerin-ST508 and *S.* Abony-ST1672 (Table 2). All these serotypes were detected in diverse samples (water, sediment, and/or fish) upstream and inside aquacultures, with three out of the four also identified downstream trout tanks. Two water or sediment samples in each aquaculture were simultaneously contaminated by two different *Salmonella* serotypes. Acquired resistance to fluoroquinolones and beta-lactams were not observed in any isolate. Resistance to aminoglycosides [streptomycin ($n = 6$) and kanamycin ($n = 1$)] was observed in few isolates and resistance to colistin (MIC = 4 Mg $L^{-1}$) was restricted to S. Abony isolates (Table 2). Whole genome analysis of *S.* Abony revealed previously not recognized mutations in *pmrA* (A20V, I66V, T89S) and *pmrB* (M15 T, G73S, V74I, I83A, A111T, G272E, D282A, K354E) genes.

## 4. Discussion

A frequent occurrence of *Salmonella* and other gram-negative species carrying multiple clinically-relevant ARGs, including PMQR genes, was observed in the sampled aquaculture trout farms and in their aquatic environment surroundings. Noteworthy, we frequently detected *Enterobacteriaceae* with decreased susceptibility to ciprofloxacin in different samples of fish farms and trout's from supermarkets and the same strains of *Salmonella* and ESBL-producing *E. coli* in samples upstream and within trout tanks. These data pointed river water supplying the aquaculture systems as a source of bacterial and antibiotic resistance contamination, possibly due to a wastewater discharge, animal farms or recreational water activities.

A high occurrence of *Salmonella* was detected in the two flow-through trout aquaculture systems in the summer (a season which may have favored its multiplication or concentration in the environment), with its presence in water, sediment and/or fish trout. The diverse serotypes found in our trout aquaculture farms might reflect diverse human/animal contamination occurring in the surrounding water sources, since all belonged to *S. enterica* subsp. *enterica*, with some isolates belonging to *S.* Newport-ST118, a serotype/clonal lineage causing human infections and circulating among diverse hosts (humans, pigs, poultry, bovines, reptiles) and in the environment of different countries (Sangal et al., 2010; http://enterobase.warwick.ac.uk/species/senterica/search_strains?query=st_search). Our data also points to fresh water or sediment as important routes of transmission of the rare serotypes *S.* Abony, *S.* Linguere and *S.* Guerin, with possible transmission to humans by trout consumption or recreational water activities. Interestingly, we found the same strain of *S.* Guerin entering fish farms through water, spreading in trout tanks and colonizing trout's gut, providing further evidences of the relevance of influx water for fish contamination by an important human pathogen.

Our study demonstrates a frequent occurrence of PMQR genes among diverse gram-negative bacilli (*Enterobacteriaceae* and *A. hydrophila*) from aquacultures and aquatic surrounding environment, as well as in trout at commercial level. The frequent occurrence of PMQR genes was similar to that reported in few studies in fish farming or surrounding environment from China or Egypt (Ishida et al., 2010; Jiang et al., 2012; Wen et al., 2016), but not previously addressed in recent European studies (Muziasari et al., 2016; Muziasari et al., 2017). Aquatic environment (including rivers) have been pointed out as

**Table 2**
*Salmonella* serotypes in trout farms and environmental surroundings.

| Point of collection | Sample type (sample designation) | Serotype-MLST/PFGE-type[a] | Season-aquaculture[b] | Antibiotic resistance phenotype[c] (no. isolates; sample) |
|---|---|---|---|---|
| Upstream trout tanks | Water (23) | Newport-ST118/A | Summer-AQ-B | STR |
| | Sediment (32) | Linguere-ST508/B | Summer-AQ-B | |
| | Water (33), sediment (37) | Guerin-ST508/C | Summer-AQ-A | |
| | Sediment (37) | Abony-ST1672/D | Summer-AQ-A | COL + STR |
| Within trout tanks | Water (24) | Newport-ST118/A | Summer-AQ-B | STR |
| | Water (24, 25) | Linguere-ST508/B | Summer-AQ-B | |
| | Sediment (38) | Guerin-ST508/C | Summer-AQ-A | |
| | Trout (44) | Guerin-ST508/C | Summer-AQ-A | |
| | Sediment (38) | Abony-ST1672/D | Summer-AQ-A | COL + STR |
| Downstream trout tanks | Sediment (30) | Newport-ST118/A | Summer-AQ-B | STR, KAN |
| | Water (26, 27, 28) | Linguere-ST508/B | Summer-AQ-B | STR (n = 1; 28) |
| | Sediment (30, 31) | Linguere-ST508/B | Summer-AQ-B | |
| | Sediment (39) | Guerin-ST508/C | Summer-AQ-A | |

[a] PFGE-types are designated by capital letters. ST, sequence type.
[b] AQ-A/AQ-B, Aquacultures. *Salmonella* isolates were obtained in one visit to each aquaculture.
[c] COL, colistin; KAN, kanamycin; STR, streptomycin.

frequently contaminated with sub-inihibitory concentration of quinolones (known to accumulate in soils and sediments), which could exert selective pressure on environmental bacteria (Buschmann et al., 2012; Wen et al., 2016). In fact, parallel studies detected low concentrations of fluoroquinolones in almost all samples (including upstream aquaculture production and within fish tanks) of AQ-B (Pereira et al., 2015) as well as frequent occurrence of *Enterococcus* spp. with decreased susceptibility to ciprofloxacin in both aquacultures (Novais et al., 2018). It is currently known that even very low concentration of antibiotics (e.g. ciprofloxacin) can be effective to select and maintain resistance traits in bacteria (Gullberg et al., 2011). Thus, our data suggest that the selective pressure exerted by fluoroquinolone residues, possibly due to a wastewater discharge or an animal farm, could explain the high levels of PMQR genes within the trout aquacultures and surroundings studied, as also reported in a region of intensive aquaculture production outside EU (Tomova et al., 2015). In addition, those PMQR-carrying isolates were mostly MDR, including to tetracycline, an antibiotic also found in low levels in water samples of AQ-B (Pereira et al., 2015), suggesting its possible contribution for the co-selection of the MDR bacteria detected. The identification of an environmental source of bacteria presenting low levels of ciprofloxacin resistance (EUCAST, 2017), due to the presence of PMQR genes, is of concern since they could evolve for a high-level resistance and/or be mobilized from those bacteria to clinically-relevant counterparts (Poirel et al., 2012; Ribeiro et al., 2015), within aquaculture ecosystem or in humans.

In this study, we found a diversity of PMQR genes, particularly within and downstream aquacultures, with a predominance of *qnrS*, similarly to other European studies comprising mostly terrestrial farm producing animals (Veldman et al., 2011; Poirel et al., 2012). The prevalence of the three *qnrS* variants was not related to source or point of collection, being those genes identified in diverse species/strains of *Enterobacteriaceae* and *A. hydrophila*. In fact, *E. coli* and *Aeromonas* and the genetic determinant *qnrS* have been suggested as possible indicators to assess the antibiotic resistance status in environmental settings that are subjected to human activities and/or to evaluate water quality (Berendonk et al., 2015). However, more studies are needed to define the maximum admissible levels of those contaminants, critical for risk assessment analysis.

The presence of *qnrS* variants, *qnrD1* and *oqxAB* in diverse genetic contexts (i.e. different plasmid types) may favor the dissemination of these ARGs among different bacterial species/strains. In fact, most PMQR genes were located within plasmids frequently associated with dissemination of those genes in different countries and circulating among diverse hosts (Jacoby et al., 2014). However, IncX1 plasmids carrying *qnrS1* or *qnrS3* among *E. coli*, including in those belonging to widespread clonal lineages circulating among different sources, was here firstly associated with aquatic environments in Europe. Those data reinforce European aquaculture and environmental surroundings as potential hotspots for diverse genetic events associated with dissemination of ARGs (including multiple opportunities for genetic exchange between aquatic bacteria and human pathogens), as previously suggested (Tomova et al., 2015; Cabello et al., 2016; Muziasari et al., 2016; Cabello et al., 2017; Novais et al., 2018). However, our results prevent to clarify if the sources of all PMQR contamination were located upstream aquacultures or if genetic transfer and recombination occurred in trout tanks (e.g. feed) or supermarkets due to other contamination sources (e.g. humans).

In contrast, the detection of the same SHV-12-producing *E. coli* clone in water samples collected upstream and within aquaculture points to the river water supplying the studied fish farms as the main source of contamination. The detection of an *E. coli* clone carrying the $bla_{SHV-12}$ gene in trout farms was not surprising given that it was one of the most common ESBLs in the human community or farm environment in Portugal (Alves et al., 2014; Manageiro et al., 2017). Other studies have reported the presence of ESBL-producing bacteria in fish or fish farms suggesting external animal/human sources of contamination of aquatic environment (Ishida et al., 2010; Sousa et al., 2011; Jiang et al., 2012).

The emergence of resistance to colistin is nowadays a public health concern as this antimicrobial is the last resort therapeutic for infections by MDR bacteria (Campos et al., 2016b). The role of fish farms for the spread of colistin resistant bacteria was not previously addressed. Nevertheless, natural aquatic environment in China are contaminated with *mcr* genes (Yang et al., 2017; Lv et al., 2018), and genes similar to *mcr* are present in the chromosome of diverse bacteria of aquatic origin (Cabello et al., 2017; Yin et al., 2017). In the trout farms sampled in this study, the clinically-relevant plasmid-mediated colistin resistance *mcr* genes were not detected. However, colistin resistant *S.* Abony with new mutations in the chromosomal *pmrA* and *pmrB* genes was observed. Mutations in *pmrAB* genes have been described as conferring colistin resistance in *Salmonella* and other *Enterobacteriaceae* by modification of lipid A of the lipopolysaccharide (LPS) (Olaitan et al., 2014). The high consumption of colistin in the animal production setting (mainly in pig and poultry production), as occurred in Portugal, is consider the major driver for the emergence of colistin-resistant bacteria (Campos et al., 2016b; Liu et al., 2016), which could justify the presence of the colistin resistant zoonotic pathogen in the inflow water of fish farm, and subsequently in other fish farm samples. The continuous monitoring of colistin-resistant gram-negative bacteria is imperative for understanding and tackling the dissemination of those threats in aquatic environment worldwide.

## 5. Conclusions

We demonstrated that there is a potential risk of transmission of *Salmonella* and clinically-relevant antibiotic resistant bacteria/genes

(PMQR/ESBL) to humans associated with farmed trout consumption or contact with trout farms contaminated waters. Our data suggests, fish farm inflow water as an important contamination source of those threats, with a possible role also for feed. A continuous microbiological monitoring of the fish farms water resources, particularly in the summer (where recreational water activities could also be more frequent) is required, since they might represent an underreported threat to food safety and human health. As other authors suggested (Berendonk et al., 2015; Novais et al., 2018) is critical to define microbiological standards (e.g. pathogens, bio indicators and ARGs/bacteria) for evaluation of the water resources status related to aquacultures in the EU, currently absent from the EWFD (Directive 2000/60/EC). The presence of pathogenic bacteria and/or bacteria with decreased susceptibility to clinically-relevant antibiotics constitute hazards potentially associated with the aquaculture fish production, the fastest growing food sector worldwide, that highlights the need to establish control policies and surveillance systems within the One Health perspective.

## Conflict of interest statement

None to declare for all authors.

## Acknowledgements

The authors would like to thank to Linda Cavaco, Yang Wang, Alessandra Carattoli and Maria Borowiak for providing the *mcr*-positive controls and to Sofia Ribeiro (Faculty of Sciences, Porto University) for the Figures design.

## Funding

This work was supported by Fundação para a Ciência e a Tecnologia (FCT)/Ministério da Educação e Ciência (MEC) through national funds and co-financed by Fundo Europeu de Desenvolvimento Regional (FEDER) under the Partnership Agreement PT2020 with the reference UID/MULTI/04378/2013–POCI/01/0145/FEDER/007728 as well as through the projects PTDC/AAC-AMB/103386/2008 and from QREN by project NORTE-01-0145-FEDER-000011–Qualidade e Segurança Alimentar—uma abordagem (nano) tecnológica. It was also funded by Investigação Jovem da Universidade do Porto/Santander Totta (PP_IJUP2010–107). JC was supported by a Ph.D. fellowship from Fundação para a Ciência e a Tecnologia (Grant SFRH/BD/93091/2013). JM was supported by a post-doc fellowship from project NORTE-01-0145-FEDER-000024.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.06.143.

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**Inflow water is a major source of trout farming contamination with *Salmonella* and multi-drug resistant bacteria**

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**Table S1.** Primers and PCR conditions used in this study

| Target/ gene | Primer name | Primer sequence (5'-3')[a] | Amplicon size (bp) | Annealing temperature (ºC) | Primers reference |
|---|---|---|---|---|---|
| **Antibiotic resistance genes** | | | | | |
| *bla*-TEM | TEM-F | ATGAGTATTCAACATTTCCG | 900 | 58 | Rasheed et al., 1997 |
| | TEM-R | CTGACAGTTACCAATGCTTA | | | |
| *bla*-CTX-M-Group | CTX-M-F Group | TTTGCGATGTGCAGTACCAGTAA | 544 | 51 | Edelstein et al., 2003 |
| | CTX-M-R Group | CGATATCGTTGGTGGTGCCATA | | | |
| *bla*-SHV | SHV-1 | GGGTTATTCTTATTTGTCGC | 930 | 56 | Rasheed et al., 1997 |
| | SHV-2 | TTAGCGTTGCCAGTGCTC | | | |
| *qnrA* | qnrAm-F | AGAGGATTTCTCACGCCAGG | 580 | 54 | Cattoir et al., 2007 |
| | qnrAm-R | TGCCAGGCACAGATCTTGAC | | | |
| *qnrB* | qnrBnew-F | ATGACGCCATTACTGTATAAA | 681 | 51 | This study |
| | qnrBnew-R3 | GCATCGCGGTGATTGGTTAG | | | This study |
| *qnrB+psp2* | psp2-Fw | AAATTTAAYCAGAAAAAAGC | Variable | 52 | Jacoby et al., 2011 |
| *qnrB*+IS26 | IS26-Rv | ACCTTTGATGGTGGGGTAAG | Variable | 56 | This study |
| *qnrC* | qnrC-F | GGGTTGTACATTTATTGAATC | 447 | 52 | Wang et al., 2009 |
| | qnrC-R | TCCACTTTACGAGGTTCT | | | |
| *qnrD* | qnrD-Fw | CGAGATCAATTTACGGGGAATA | 600 | 64 | Cavaco et al, 2009 |
| | qnrD-Rv | AACAAGCTGAAGCGCCTG | | | |
| *qnrS* | qnrS-Fc | TGGAAACCTACAATCATACATATCG | 656 | 64 | Hopkins et al., 2007 |
| | qnrS-Rc | TTAGTCAGGATAAACAACAATACCC | | | |
| *aac(6')-Ib-cr* | AAC6´-A | TTGCGATGCTCTATGAGTGGCTA | 482 | 64 | Minarini et al., 2008 |
| | AAC6´-B | CTCGAATGCCTGGCGTGTTT | | | |
| *qepA* | qepA-F | GTAGATCGTCAGCAGCAC | 502 | 54 | Veldman et al., 2011 |
| | qepA-R | TCTCTGGATCCTGGACAT | | | |
| *oqxA* | oqxA-F | CTCGGCGCGATGATGCT | 392 | 62 | Kim et al., 2009 |

| Target/ gene | Primer name | Primer sequence (5'-3')[a] | Amplicon size (bp) | Annealing temperature (ºC) | Primers reference |
|---|---|---|---|---|---|
| | oqxA-R | CCACTCTTCACGGGAGACGA | | | |
| *oqxB* | oqxBs | TTCTCCCCCGGCGGGAAGTAC | 512 | 64 | Kim et al., 2009 |
| | oqxBa2 | CTCGGCCATTTTGGCGCGTA | | | |
| *mcr-1* | MCR-1_FW | CGGTCAGTCCGTTTGTTC | 915 | 63 | Liu et al., 2015 |
| | MCR-1_RV2 | CCAGCGTATCCAGCACATTT | | | |
| *mcr-2* | MCR-2_IF | TGTTGCTTGTGCCGATTGGA | 567 | 65 | Xavier et al., 2016 |
| | MCR-2_IR | AGATGGTATTGTTGGTTGCTG | | | |
| *mcr-3* | MCR-3_FW | TTGGCACTGTATTTTGCATTT | 542 | 56 | Yin et al., 2017 |
| | MCR-3_RV | TTAACGAAATTGGCTGGAACA | | | |
| *mcr-4* | MCR-4_FW | ATTGGGATAGTCGCCTTTTT | 487 | 58 | Carattoli et al., 2017 |
| | MCR-4_RV | TTACAGCCAGAATCATTATCA | | | |
| *mcr-5* | MCR-5-intern_FW | TATCTCGACAAGGCCATGCTG | 613 | 60 | Borowiak et al., 2017 |
| | MCR-5-intern_RV | GAATCTGGCGTTCGTCGTAGT | | | |
| **Plasmid typing** | | | | | |
| HI1 | HI1_Fw | GGAGCGATGGATTACTTCAGTAC | 471 | 58 | Carattoli et al., 2005 |
| | HI1_Rv | TGCCGTTTCACCTCGTGAGTA | | | |
| HI2 | HI2_Fw | TTTCTCCTGAGTCACCTGTTAACAC | 644 | 60 | Carattoli et al., 2005 |
| | HI2_Rv | GGCTCACTACCGTTGTCATCCT | | | |
| I1 | I1_Fw | CGAAAGCCGGACGGCAGAA | 139 | 60 | Carattoli et al., 2005 |
| | I1_Rv | TCGTCGTTCCGCCAAGTTCGT | | | |
| X | X_Fw | AACCTTAGAGGCTATTTAAGTTGCTGAT | 376 | 60 | Carattoli et al., 2005 |
| | X_Rv | TGAGAGTCAATTTTTATCTCATGTTTTAGC | | | |
| L/M | L/M_Fw | GGATGAAAACTATCAGCATCTGAAG | 785 | 60 | Carattoli et al., 2005 |
| | L/M_Rv | CTGCAGGGGCGATTCTTTAGG | | | |
| N | N_Fw | GTCTAACGAGCTTACCGAAG | 559 | 58 | Carattoli et al., 2005 |
| | N_Rv | GTTTCAACTCTGCCAAGTTC | | | |
| FIA | FIA_Fw | CCATGCTGGTTCTAGAGAAGGTG | 462 | 60 | Carattoli et al., 2005 |

| Target/ gene | Primer name | Primer sequence (5'-3')[a] | Amplicon size (bp) | Annealing temperature (ºC) | Primers reference |
|---|---|---|---|---|---|
| | FIA_Rv | GTATATCCTTACTGGCTTCCGCAG | | | |
| FIB | FIB_Fw | GGAGTTCTGACACACGATTTTCTG | 702 | 60 | Carattoli et al., 2005 |
| | FIB_Rv | CTCCCGTCGCTTCAGGGCATT | | | |
| W | W_FW | CCTAAGAACAACAAAGCCCCCG | 242 | 60 | Carattoli et al., 2005 |
| | W_RV | GGTGCGCGGCATAGAACCGT | | | |
| Y | Y_FW | AATTCAAACAACACTGTGCAGCCTG | 765 | 60 | Carattoli et al., 2005 |
| | Y_RV | GCGAGAATGGACGATTACAAAACTTT | | | |
| P | P_FW | CTATGGCCCTGCAAACGCGCCAGAAA | 534 | 62 | Carattoli et al., 2005 |
| | P_RV | TCACGCGCCAGGGCGCAGCC | | | |
| FIC | FIC_FW | GTGAACTGGCAGATGAGGAAGG | 262 | 60 | Carattoli et al., 2005 |
| | FIC_RV | TTCTCCTCGTCGCCAAACTAGAT | | | |
| A/C | A/C_FW | GAGAACCAAAGACAAAGACCTGGA | 465 | 60 | Carattoli et al., 2005 |
| | A/C_RV | ACGACAAACCTGAATTGCCTCCTT | | | |
| T | T_FW | TTGGCCTGTTTGTGCCTAAACCAT | 750 | 58 | Carattoli et al., 2005 |
| | T_RV | CGTTGATTACACTTAGCTTTGGAC | | | |
| FIIs | FIIs_FW | CTGTCGTAAGCTGATGGC | 270 | 58 | Carattoli et al., 2005 |
| | FIIs_RV | CTCTGCCACAAACTTCAGC | | | |
| FrepB | FrepB_FW | TGATCGTTTAAGGAATTTTG | 270 | 52 | Carattoli et al., 2005 |
| | FrepB_RV | GAAGATCAGTCACACCATCC | | | |
| K | K/B_FW | GCGGTCCGGAAAGCCAGAAAAC | 160 | 62 | Carattoli et al., 2005 |
| | K_RV | TCTTTCACGAGCCCGCCAAA | | | |
| B/O | K/B_FW | GCGGTCCGGAAAGCCAGAAAAC | 159 | 62 | Carattoli et al., 2005 |
| | B/O_RV | TCTGCGTTCCGCCAAGTTCGA | | | |
| ColE | oricolE_FW | GTTCGTGCATACAGTCCA | 187 | 60 | García-Fernández et al., 2009 |
| | oricolE_RV | GGCGAAACCCGACAGGACT | | | |
| R | IncR_FW | TCGCTTCATTCCTGCTTCAGC | 251 | 60 | García-Fernández et al., 2009 |
| | IncR_RV | GTGTGCTGTGGTTATGCCTCA | | | |
| U | IncU_FW | TCACGACACAAGCGCAAGGG | 843 | 60 | García-Fernández et al., |

| Target/ gene | Primer name | Primer sequence (5'-3')[a] | Amplicon size (bp) | Annealing temperature (ºC) | Primers reference |
|---|---|---|---|---|---|
| | IncU_RV | TCATGGTACATCTGGGCGC | | | 2009 |
| $FII_K$ | $FII_K$ FW | TCTTCTTCAATCTTGGCGGA | 142-148 | 60 | Villa et al., 2010 |
| | $FII_K$ RV | GCTTATGTTGCACRGAAGGA | | | |
| X1 | X1_FW | GCTTAGACTTTGTTTTATCGTT | 461 | 56 | Johnson et al., 2012 |
| | X1_RV | TAATGATCCTCAGCATGTGAT | | | |
| X2 | X2_FW | GCGAAGAAATCAAAGAAGCTA | 678 | 56 | Johnson et al., 2012 |
| | X2_RV | TGTTGAATGCCGTTCTTGTCCAG | | | |
| X3 | X3_FW | GTTTTCTCCACGCCCTTGTTCA | 351 | 56 | Johnson et al., 2012 |
| | X3_RV | CTTTGTGCTTGGCTATCATAA | | | |
| X4 | X4_FR | AGCAAACAGGGAAAGGAGAAGACT | 569 | 56 | Johnson et al., 2012 |
| | X4_RV | TACCCCAAATCGTAACCTG | | | |
| L | L_FW | CGGAACCGACATGTGCCTACT | 854 | 60 | Carattoli et al., 2015 |
| | L/M_RV | GAACTCCGGCGAAAGACCTTC | | | |
| M | M_FW | GGATGAAAACTATCAGCATCTGAA | 738 | 60 | Carattoli et al., 2015 |
| | L/M_RV | GAACTCCGGCGAAAGACCTTC | | | |
| Col3M | col3M_4F | GTTAAAAACGCACTCA**K**AAGC | 153 | 56 | This study |
| | col3M_156R | TATGTTAAACGGGCGAGTTAG | | | |
| **Bacterial identification** | | | | | |
| 16S rRNA | SEQA | AGAGTTTGATC**H**TGG**Y**T**Y**AGA | Variable | 54 | Héritier et al., 2003 |
| | SEQB | ACG**Y**TACCTTGTTACGACTTC | | | |

[a] Degenerated bases: K = G or T; Y = C or T; H = A, C or T.

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### 3.1.5. High occurrence and unusual serotype diversity of non-typhoidal *Salmonella* in non-clinical niches, Angola

*Epidemiol. Infect.* (2017), **145**, 883–886. © Cambridge University Press 2016
doi:10.1017/S095026881600296X

# SHORT REPORT
# High occurrence and unusual serotype diversity of non-typhoidal *Salmonella* in non-clinical niches, Angola

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*Received 19 August 2016; Final revision 14 October 2016; Accepted 14 November 2016; first published online 22 December 2016*

## SUMMARY

Non-typhoidal *Salmonella* is an important burden, particularly in developing countries of the African region. We report for the first time in Angola, a sub-Saharan African country with commercial/travel relationships with Europe, an unexpectedly high occurrence of *Salmonella* ($n = 12/63$, 19%) from a high diversity of sources, particularly farm and wild animals. The detection of diverse serotypes ($n = 12$), involving putative new *S.* enterica subsp. *salamae* serotypes, is also of note, reinforcing the need for a comprehensive surveillance in Angola critical to identify animal/food/environmental sources of salmonellosis with impact on animal health, local people, tourists and exported products.

**Key words**: Africa, Angola, non-clinical sources, non-typhoidal *Salmonella*, *Salmonella enterica* subsp. *salamae* serotypes.

Non-typhoidal *Salmonella* (NTS) is an important pathogen in developing countries of the African region, as was recently highlighted by the first WHO Initiative to Estimate the Global Burden of Foodborne Disease. In this report, 59 000 global deaths due to NTS were estimated (2010), with 32 000 deaths in the African region, and 22 000 deaths due to invasive disease, mostly in children [1]. Nevertheless, data to understand NTS epidemiology in most sub-Saharan African countries, namely related to the disease burden, serotype distribution, diversity of reservoirs and transmission routes are lacking, as recently highlighted [2, 3]. Among those countries with no NTS data is the former Portuguese African colony of Angola, a country with a high burden of severe infectious diseases (e.g. malaria, diarrhoeal diseases) associated with an extended vulnerable population (e.g. HIV-infected adults, young children) and poor hygiene, which facilitates bacterial transmission and infection spread [4]. Of note was the last year's economic growth and close demographic, commercial or travel relationships of Angola with European countries (http://eeas.europa.eu/delegations/angola/175/angola-and-the-eu_en#Economic+%26+trade+relations). Food and live animals are among the goods traded between the European Union (EU) and Angola, with an increased trade flow in the last years (2012–2015) (http://trade.ec.europa.eu/doclib/docs/2006/september/tradoc_122456.pdf). Here we report a high occurrence and wide spread of unusual or putative new serotypes of antibiotic susceptible *Salmonella* in non-clinical sources of Angola and discuss the potential impact of globalization for their spread to other geographical regions.

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Table 1. *Characterization of Salmonella isolates recovered from diverse sources in Angola*

| *Salmonella enterica* and subspecies Serotypes/PFGE type* (no. isolates)† | ST/eBG (no. isolates)‡ | Source (sample number/origin)§ | Regions (communes)¶ |
|---|---|---|---|
| *Salmonella enterica* subsp. *enterica* (subsp. I) | | | |
| Sundsvall/S2 (*n* = 2) | ST488 (*n* = 1) | Springboks (1/wild animal park) | Dombe Grande |
| Chester/S3 (*n* = 7) | ST411/eBG49 (*n* = 1) | Springboks (2/wild animal park) | Dombe Grande |
| | | Grey monkeys (12/wild animal park) | Dombe Grande |
| | | Cows (13/farm C) | Benguela |
| | | Pigs (16/farm D) | Benguela |
| | | Healthy person (20, 21) | Benguela |
| Poona/S4 (*n* = 2) | ST608 (*n* = 1) | Grey monkeys (11/wild animal park) | Dombe Grande |
| Muenster/S5 (*n* = 2) | ST321/eBG36 (*n* = 1) | Grey monkeys (11/wild animal park) | Dombe Grande |
| Braenderup/S9 (*n* = 2) | ST22/eBG24 (*n* = 1) | Avian feed (47/farm B) | Benguela |
| Cerro/S12 (*n* = 2) | ST367/eBG37 (*n* = 1) | Wastewater (59) | Benguela |
| *Salmonella enterica* subsp. *salamae* (subsp. II) | | | |
| **9,46:z:-**/S11 (*n* = 1) | **ST1840** (*n* = 1) | Grey monkeys (11/wild animal park) | Dombe Grande |
| **30:z29:z6**/S7 (*n* = 1) | **ST1841** (*n* = 1) | River (38) | Catumbela |
| **4,12:l,w:z39**/S8 (*n* = 1) | **ST1842** (*n* = 1) | River (38) | Catumbela |
| **50:b:z39**/S6 (*n* = 1) | **ST1839** (*n* = 1) | Grey monkeys (11/wild animal park) | Dombe Grande |
| **35:z39:1,7**/S1 (*n* = 1) | **ST1888** (*n* = 1) | Springboks (1/wild animal park) | Dombe Grande |
| 43:z29:e,n,x/S10 (n = 3) | **ST1887** (*n* = 1) | Avian feed (65/farm A) | Benguela |

* PFGE types are designated by 'S and number'.

† All the isolates were susceptible to ampicillin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline and trimethoprim.

‡ ST, Sequence type; eBG, eBurstGroup (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). Number of isolates analysed by MLST. Serotypes and STs described for the first time in this study are presented in bold.

§ Details of the type and number of samples for each source collected: healthy volunteers (*n* = 18/rectal swabs); food-producing animals and their environments [rectal swab pools of healthy chickens (*n* = 5/13 animals), cows (*n* = 3/9 animals) and pigs (*n* = 2/6 animals); corn feed (*n* = 3); floor/walls swabs (*n* = 2); water (*n* = 3)]; wild animal park [grey monkeys (*n* = 2/fresh faeces); springboks (*n* = 3/fresh faeces); water (*n* = 2)]; aquatic environments [rivers/lagoon (*n* = 3); drinking water (*n* = 7 treated and *n* = 5 untreated)]; and waste water [urban sewer lines (*n* = 3); treatment station (*n* = 2)].

¶ Samples were collected from Benguela Province, which comprises nine municipalities (Benguela, Lobito, Bocoio, Balombo, Ganda, Cubal, Caimbambo, Baía Farta, Chongorói), divided into >30 communes [6].

As part of a surveillance project on the occurrence of clinically relevant Gram-negative and Gram-positive bacteria [5, 6], we screened for the presence of *Salmonella* by standard methods [7] in a total of 63 samples recovered from different communes and sources from Benguela Province (including the second biggest city of Angola, Benguela): healthy volunteers, food-producing animals and their environments, wild animals and aquatic environments (Table 1). *Salmonella*-suspected colonies were identified by biochemical tests, slide agglutination and *invA* gene-PCR [7]. Antibiotic susceptibility was determined following European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org/clinical_breakpoints/) and clonal relatedness by *Xba*I PFGE [7] and MLST (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). Serotyping was performed at the National Centre of *Salmonella* (INSA, Lisbon) or the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* (Institute Pasteur, Paris).

A high percentage [19%, 95% confidence interval (CI) 9·3–28·7, *n* = 12/63] of samples from different origins carried *Salmonella*. NTS occurrence in healthy humans (11%, 95% CI 0·0–25·5, *n* = 2/18, non-related individuals) was higher than other studies (e.g. 1%, Senegal; 2·4%, Guinea–Bissau) [8]. Despite differences in methodology and different sample sizes, these results suggest that asymptomatic carriers may be an important source of NTS in Angola. The dispersion of NTS in aquatic environments (10%, 95% CI 0·0–23·2, *n* = 2/20), farm (22%, 95% CI 2·9–41·1, *n* = 4/18) and wild animals (57%, 95% CI 20·3–93·7, *n* = 4/7) (Table 1) also suggest an extended diversity of NTS reservoirs and possible transmission routes in this region, as described in a few studies performed in African countries [9, 10]. This previously unreported epidemiological NTS scenario is of relevance for local

and international salmonellosis control strategies. In fact, Angola is currently an important economic and trade partner for the EU, benefiting from free access to EU markets for all Angolan products, and an emigration and tourism destination for Europeans (http://eeas.europa.eu/delegations/angola/175/angola-and-the-eu_en#Economic+%26+trade+relations; http://trade.ec.europa.eu/doclib/docs/2006/september/tradoc_122456.pdf). Recent studies showed that illegal importation of food animal products (including bushmeat – meat of wild animals and livestock meat) from Africa to Europe through airport passenger's luggage, including from Angola, was commonly verified [11–13]. In addition, some of those illegal food items were contaminated with foodborne pathogens such as NTS, suggesting a potential public health risk by a neglected route of transmission [12, 13]. International travel to countries in the region of sub-Saharan Africa was also considered one of the most relevant risk factors for Danish travellers being hospitalized with NTS bacteraemia [14]. Based on these findings it is of utmost importance the implementation in Angola of an integrated surveillance system of NTS in non-clinical contexts (including farm-to-fork) and clinical settings, complemented by effective strategies for the control of foodborne pathogens.

In this study, a high diversity of NTS serotypes ($n$ = 12, 12 PFGE types/12 STs) was observed. Of note, was the unusually high prevalence of *Salmonella enterica* subsp. *salamae* serotypes ($n$ = 6), corresponding to potentially new ones and/or assigned to new STs (Table 1). In developed European countries such as the UK some serotypes of this subspecies have been recognized to cause disease, occasionally severe infections [15]. Some cases have been associated with reptile meat consumption and/or travel to African countries, but the sources and transmission routes of those serotypes are still not well-understood worldwide [15, 16]. The lack of global data together with our identification of diverse potential sources (e.g. wildlife and farm animals) of *S. enterica* subsp. *salamae* spread in Benguela province (Table 1) emphasize the need to clarify the role of this subspecies as a cause of human salmonellosis in Angola as well as in other countries, including developed ones to which Angola has commercial or travel relationships. Moreover, less-frequent human-related *S. enterica* subsp. *enterica* serotypes distributed in different non-clinical sources, including wildlife and farm animals, are here described for the first time for Angola. Of note is a *S.* Chester clone disseminated in diverse animal species and healthy persons from distant Benguela communes (Table 1), suggesting the occurrence of multi-niche adaptive features in these strains that might facilitate their association with salmonellosis in this region. The NTS serotypes detected in this study are not commonly associated with antibiotic resistance which might explain the antibiotic susceptibility of all isolates, despite the presence of multidrug-resistant bacteria of other species in the same samples [5, 6].

In conclusion, this study evidenced a high occurrence of NTS in Angola involving multiple non-clinical sources, including healthy humans, aquatic environments, farm and wild animals. Our data also suggest that Angola seems to be a hotspot of *Salmonella enterica* diversity involving potentially new serotypes. The detection of *Salmonella enterica* subsp. *salamae* serotypes from a high diversity of sources is also of note, suggesting potential reservoirs and transmission routes globally unknown for this subspecies. Insights on NTS epidemiology in Angola provided by this study reinforces the need for a comprehensive NTS surveillance in this country critical for developing public health strategies to prevent salmonellosis at both local and international levels.

## ACKNOWLEDGEMENTS

The authors are grateful for financial support from FCT/MEC (Fundação para a Ciência e a Tecnologia/Ministério da Educação e Ciência) through national funds and co-financed by FEDER, under the Partnership Agreement PT2020 (grant no. UID/MULTI/04378/2013 – POCI/01/0145/FERDER/007728) and from project NORTE-01-0145-FEDER-000011 – Qualidade e Segurança Alimentar — uma abordagem (nano) tecnológica. J.C. is supported by a Ph.D. fellowship from Fundação para a Ciência e a Tecnologia (grant no. SFRH/BD/93091/2013). J.M. was supported by a Ph.D. fellowship from Fundação para a Ciência e a Tecnologia (grant no. SFRH/BD/77518/2011). T.G.R. is supported by a Ph.D. fellowship from Fundação para a Ciência e a Tecnologia (grant no. SFRH/BD/75752/2011).

The authors thank Hélder Gonçalves, Erineia Gonçalves and Walter Bonfim, for support in sample recruitment and Larissa Margalho for laboratory technical support. We are also very grateful to Jorge Machado (INSA, Lisbon, Portugal) and to François-Xavier Weill (Institute Pasteur, Paris, France) for *Salmonella* serotyping.

## DECLARATION OF INTEREST

None.

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## 3.2. Developments on *Salmonella enterica* discrimination at infra-species levels by high-throughput typing approaches

3.2.1. Discrimination of non-typhoid *Salmonella* serogroups and serotypes by Fourier Transform Infrared Spectroscopy: a comprehensive analysis

3.2.2. Assessing the potential of Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry for discrimination of clinically relevant *Salmonella* serogroups and serotypes

### 3.2.1. Discrimination of non-typhoid *Salmonella* serogroups and serotypes by Fourier Transform Infrared Spectroscopy: a comprehensive analysis

International Journal of Food Microbiology 285 (2018) 34–41

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

# Discrimination of non-typhoid *Salmonella* serogroups and serotypes by Fourier Transform Infrared Spectroscopy: A comprehensive analysis

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ARTICLE INFO

*Keywords:*
*Salmonella enterica*
Typing
Fourier Transform Infrared Spectroscopy-Attenuated Total Reflectance
Multivariate data analysis
Bacterial identification methods
O-antigen

ABSTRACT

Simpler, quick and low-cost methods for routine *Salmonella enterica* typing are required for epidemiologic surveillance of this important zoonotic pathogen. In this study, using a comprehensive isolate collection, we investigated the potential of Fourier transform infrared spectroscopy (FTIRS) to discriminate the most clinically-relevant serogroups and serotypes of non-typhoid *Salmonella*. Moreover, the role of O-units composition on the FTIRS *Salmonella* discrimination was also explored.

*S. enterica* isolates (n = 325; 2002–2015; different sources and countries), of 57 serotypes and 15 serogroups [including the most frequent ones, B-n = 122; C-n = 108; D-n = 43 and E-n = 33)] were analysed by FTIRS. Infrared spectra were analysed by Partial Least Square Discriminant Analysis (PLSDA) and/or Principal Component Analysis (PCA).

The polysaccharides region provided the spectral sharpest differences being used in the subsequent *Salmonella* typing. Serogroups (B, C, D and E) discrimination was achieved with high accuracy (99.6% of correct assignments; PLSDA model). Differences in the O-unit structures composition of those serogroups are likely justifying the discrimination achieved. Other serogroups (G, H, K, L, M, N, O, T, U, Y, Z) were correctly predicted as not belonging to serogroups B, C, D nor E, except for 3 isolates of serogroups H (*S.* Sundsvall, n = 1) and K (*S.* Cerro, n = 2). In fact, O-unit structure of serogroup H and K shows some similarity with sub-serogroup C1 with the remaining serogroups presenting marked differences in this cellular component. The sub-serogroups discrimination was successfully achieved for C1, C2 and C3 (using PCA), and for E1-E2-E3 and E4 (by PLSDA). Appropriate serotype discrimination was obtained for most of *S.* Rissen from the remaining C1 serotypes (91.5%-PLSDA), and *S.* Enteritidis (D1) from the remaining D1/D2 serotypes (93.4%-PLSDA). The lack of available O-unit composition for particular serotypes prevents the elucidation of the role of this cellular component on the discrimination at serotype level obtained.

FTIRS was able to discriminate relevant serogroups (B, C, D and E), sub-serogroups (C1, C2 and C3; E1-E2-E3 and E4) and particular important serotypes (*S.* Enteritidis, *S.* Rissen and *S.* Senftenberg). Further studies on O-antigen composition would clarify the fundaments of discrimination obtained by FTIRS.

## 1. Introduction

*Salmonella enterica* is one of the leading causes of foodborne diseases worldwide, representing a major public health burden, even for industrialized countries of the European Union (EU) and North America (CDC, 2017; EFSA and ECDC, 2017). *S. enterica*, which comprises a quite diverse population, is transmitted to humans by a wide range of foodstuffs, mainly of animal origin, and is frequently involved in endemic or epidemic scenarios worldwide (Antunes et al., 2016; Antunes et al., 2017; Mourão et al., 2014, 2016; EFSA and ECDC, 2017). Differentiation of *S. enterica* at infra-species level is crucial, e.g. for the epidemiological investigation and control of foodborne outbreaks. Serotyping, phage typing, Multilocus Sequence Typing (MLST), Pulsed-Field Gel Electrophoresis (PFGE), Multiple Locus Variable-number

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https://doi.org/10.1016/j.ijfoodmicro.2018.07.005
Received 19 March 2018; Received in revised form 30 May 2018; Accepted 3 July 2018
Available online 10 July 2018
0168-1605/ © 2018 Published by Elsevier B.V.

Tandem Repeat Analysis (MLVA), and more recently Whole-Genome Sequence (WGS) are methods widely used in *S. enterica* typing. These methods provide infra-species discrimination at different levels, although time-consuming, laborious, expensive, and may require technical expertise (Abatcha et al., 2014; Sabat et al., 2013). Remarkably, serotyping is still the globally recognized and used approach to firstly discriminate *S. enterica* isolates. This classical methodology is based on agglutination reactions with specific antisera and surface bacterial antigens, the somatic O-antigen and flagellar H-antigens (phases 1 and 2) (Grimont and Weill, 2007). The serogroup determination depends on the polysaccharide composition of O-antigen (outermost component of the cell surface lipopolysaccharide) (Grimont and Weill, 2007), which diversity is associated with the monosaccharides composition (3- to 5-sugars) and the structure of O-units (covalent bond nature and monosaccharides linkage), besides the polymerization linkage between the repeated units (2 to 8 O-units repetitions) (Davis and Mauer, 2010; Knirel, 2011; Liu et al., 2014). Additionally, the serotypes are determined based on specific combinations of O-antigen and H-antigens, being more than 2600 serotypes described (Issenhuth-Jeanjean et al., 2014). Nevertheless, few serotypes (belonging to serogroups D, B, C and E) are causing infections worldwide, namely the common *S.* Enteritidis, *S.* Typhimurium and its monophasic variant (*S.* 1,4,[5],12:i:-), and the emerging *S.* Stanley, *S.* Infantis, *S.* Rissen, *S.* Newport and *S.* Kentucky (CDC, 2017; ; EFSA and ECDC, 2017; Grimont and Weill, 2007). Despite the relevance of serotype identification, it is usually lengthy to obtain, relying on high cost and universally unavailable sera, being therefore restricted to reference laboratories. Additionally, incorrect serotypes assignments by classical approach as well as by WGS were already recognized (Abatcha et al., 2014; Sabat et al., 2013; Yachison et al., 2017).

Fourier transform infrared spectroscopy (FTIRS) is a simpler, quick and low-cost technique, which allows the discrimination of intact cells in a non-destructive manner through infrared spectra reflecting the vibrational modes of the microbial cell molecules (e.g. nucleic acids, proteins, carbohydrates, membrane and cell wall components) (Davis and Mauer, 2010). Moreover, this quick and low-cost spectroscopic technique gained more interest with the recent available system, IR Biotyper, which allows bacterial typing at routine bacteriology laboratories (www.bruker.com/applications/microbiology/strain-typing-with-ir-biotyper/features). In fact, FTIRS has been successfully explored as an alternative to discriminate diverse pathogenic bacteria causing foodborne or nosocomial infections at different infra-species level (e.g. serogroups, serotypes, clones) (Johler et al., 2016; Nyarko et al., 2014; Quintelas et al., 2018; Rebuffo-Scheer et al., 2007; Sousa et al., 2013, 2014a, 2014b; Vaz et al., 2013). Nonetheless, a comprehensive and robust assessment of the suitability of FTIRS for *S. enterica* typing by whole cell approach is lacking.

In this study, we investigated in a comprehensive and robust *Salmonella* collection, the potential of FTIRS coupled with multivariate data analysis, using whole cell analysis, to discriminate the most frequent and clinically relevant serogroups and serotypes, correlating the discrimination obtained with the O-unit composition of somatic antigen.

## 2. Material and methods

### 2.1. Bacterial collection

A total of 325 *Salmonella enterica* isolates, most belonging to *S. enterica* subsp. *enterica* (subsp. I) (n = 316) were analysed by FTIRS with Attenuated Total Reflectance (FTIRS-ATR). This collection comprises isolates from 15 serogroups, including major [B-Group O:4), n = 122; C (C1-Group O:6,7; C2-Group O:6,8; C3-Group O:8); n = 108, D (D1-Group O:9; D2-Group O:9,46), n = 43; E (E1-E2-E3-Group O:3,10, E4-Group O:1,3,19), n = 33] and less frequent ones (G-Group O:13, H-Group O:6,14, K-Group O:18, L-Group O:21, M-Group O:18, N-Group O:30, O-Group O:35, T-Group O:42, U-Group O:43, Y-Group O:48, Z-Group O:50), n = 19 (Supplementary Table S1). These serogroups encompass 57 serotypes, including the three most frequent worldwide (*S.* Enteritidis, *S.* Typhimurium and its monophasic variant, *S.* 1,4,[5],12:i:-) and others that are emergent and have clinical relevance (e.g. *S.* Rissen, *S.* Heidelberg, *S.* Infantis, *S.* Newport, *S.* Mbandaka, *S.* Stanley and *S.* Senftenberg). Forty-five less frequently detected serotypes (including ones of subspecies II-*salamae*, IIIa-*arizonae* and IIIb-*diarizonae*) in surveillance studies were also included (Supplementary Table S1). The isolates were collected between 2002 and 2015 in several countries (Portugal, Angola and Austria), being the serotypes determined by classical serotyping (Grimont and Weill, 2007) in the National Reference Centre (INSA, Lisbon, Portugal) or Austrian Agency for Health and Food Safety (AGES, Vienna, Austria). *S.* Typhimurium, *S.* 1,4,[5],12:i:- and *S.* Enteritidis were also confirmed by a PCR assay (Tennant et al., 2010). The isolates were selected from a well-characterized collection, based on clonal lineages (MLST and *Xba*I-PFGE), phenotypic/genotypic antibiotic resistance profiles and sources (human clinical cases, food products, food-animal production settings and the environment).

### 2.2. Spectral acquisition

The infrared spectra of studied isolates were acquired from an overnight subculture on Mueller-Hinton agar after 18 h of grow at 37 °C. The colonies were directly transferred from the agar plates to the ATR crystal (without cell component extractions), followed by air-drying on the optical surface until obtain a thin film. Spectra were acquired using a Perkin Elmer Spectrum BX FTIR System spectrophotometer equipped with a PIKE Technologies Gladi ATR accessory from 4000 to 600 $cm^{-1}$ with a resolution of 4 $cm^{-1}$ and 32 scan co-additions. From each isolate, nine consistent spectra were acquired, corresponding to three biological replicates (obtained in three independent days and fresh cultures) and three instrumental replicates (obtained on the same day from the same agar plate). Between each isolate measurement, a background was acquired. Due to the large amount of data generated, a mean spectrum (obtained from the nine consistent replicates) was considered in all chemometric analysis.

### 2.3. Spectra pre-processing and modelling

The infrared spectra were firstly processed with standard normal variate (SNV) (Naes et al., 2002), followed by the application of a Savitzky-Golay filter (15 smoothing points, $2^{nd}$ order polynomial and $2^{nd}$ derivate) (Savitzky and Golay, 1964). After pre-processing, infrared data were analysed by chemometric methods: non-supervised Principal Component Analysis (PCA) and the supervised Partial Least Square Discriminant Analysis (PLSDA) (Geladi and Kowalsky, 1986; Naes et al., 2002). Before modelling with PCA or PLSDA the spectra dataset was mean-centred. PLSDA modelling was preferred to PCA. PCA was used in situations where the number of isolates belonging to each class was insufficient (less than 10) to develop a robust supervised method. All multivariate chemometric data analysis were performed with Matlab software version R2016a (MathWorks, Natick, MA) and the PLS Toolbox version 8.2.1 for Matlab (Eigenvector Research, Manson, WA).

### 2.4. Isolates discrimination work-flow

The rational work-flow of this study consisted firstly in the development of a PLSDA model to discriminate *S. enterica* belonging to the most frequent serogroups (B, C, D and E). The developed model was further tested with additional 29 isolates ("test" isolates) belonging to 15 serogroups, modelled (B, C, D and E; n = 10) and non-modelled (G, H, K, L, M, N, O, T, U, Y, Z; n = 19) serogroups and 23 serotypes. The "test" isolates were chosen to provide a maximal diversity (Supplementary Table S1). Subsequently, the discrimination within

each serogroup was attempted through a PCA or a PLSDA in order to discriminate the isolates according: I) their sub-serogroup (C1, C2 and C3; D1 and D2; E1-E2-E3 and E4) except for serogroup B; II) their serotype (for all serogroups); and III) their clone (only for *S.* Typhimurium and *S.* 1,4,[5],12:i:- serotypes of serogroup B).

## 3. Results and discussion

In this work the suitability of FTIRS coupled with multivariate data analysis for *S. enterica* typing as an alternative to classical serotyping was explored in a wide and diverse (serogroups, serotypes, clones and origin) collection. The few available studies demonstrating the potential of FTIRS for *S. enterica* typing presented important limitations, such as lack of robustness, namely due to insufficiencies on the bacterial collection (number and diversity of serogroups/serotypes tested), and/or requires complex bacterial cells processing (Baldauf et al., 2006, 2007; Kim et al., 2005, 2006; Männig et al., 2008; Preisner et al., 2012).

### 3.1. Spectra overview

Infrared spectra of *S. enterica* isolates exhibited the typical shape of intact bacterial spectra, presenting the absorbance bands associated with lipids (3000–2800 $cm^{-1}$), proteins/amides I and II (1700–1500 $cm^{-1}$), phospholipids/DNA/RNA (1500–1185 $cm^{-1}$), polysaccharides (1185–900 $cm^{-1}$) and the fingerprint region (900–600 $cm^{-1}$) (Fig. 1A) (Davis and Mauer, 2010). The spectral differences among the isolates were observed in the phospholipids/DNA/RNA (1500–1185 $cm^{-1}$) and polysaccharides (1185–900 $cm^{-1}$) regions, with the sharpest differences observed in the polysaccharide region (Fig. 1B), which was chosen for the multivariate analyses performed. Such wavelengths range has been successfully used in the typing of different species (Johler et al., 2016; Rebuffo-Scheer et al., 2007; Sousa et al., 2013, 2014a, 2014b; Vaz et al., 2013), and considered to be also of interest for *S. enterica* (Baldauf et al., 2006, 2007; Kim et al., 2005; Männig et al., 2008; Preisner et al., 2012). In fact, due to the somatic antigen length and carbohydrate composition diversity of O-units, a great impact of this surface cell structure is expected on the *S. enterica* differentiation at different levels. Therefore, using the available data on O-unit structures we attempted to correlate with the *S. enterica* isolates discrimination here obtained (Supplementary Fig. S1) (Knirel, 2011; Liu et al., 2014).

### 3.2. Discrimination between serogroups of S. enterica

The discrimination of isolates belonging to serogroup B, C, D and E was achieved with a corresponding confusion matrix of 99.6% (Supplementary Table S2). In fact, the scores map considering the first 3 latent variables (LVs), which encompasses 83.5% of the spectral variability, evidenced four well-defined clusters, each containing isolates belonging to a single serogroup (Fig. 2A). The O-unit of these serogroups, except for C1, comprises mannose, rhamnose and galactose in the backbone, and variable presence of side chain molecules, as abequose, tyvelose or *O*-acetyl groups (Supplementary Fig. S1) (Knirel, 2011; Liu et al., 2014), which might justify the high level of discrimination achieved. The few isolates incorrectly classified (0.4%) (Supplementary Table S2) belonged to serogroup D (sub-serogroup D2), which shows a similar O-unit composition, including in the initiating carbohydrates, with serogroup E isolates (Supplementary Fig. S1), which might prevent their discrimination. Actually, it has been suggested that D2 O-antigen gene cluster has arisen by reassortment of the serogroup D1 and E (Knirel, 2011; Liu et al., 2014; Reeves et al., 2013; Xiang et al., 1994).

Moreover, when the "test" isolates of modelled serogroups (B, C, D and E) were projected on the developed PLSDA model, all of them were correctly predicted. Furthermore, isolates of non-modelled serogroups (G, H, K, L, M, N, O, T, U, Y, Z) were correctly predicted as not belonging to serogroups B, C, D nor E, except for 3 isolates of serogroups H (*S.* Sundsvall, n = 1) and K (*S.* Cerro, n = 2) (Fig. 2B). Marked differences on sugar composition are observed between O-units of serogroups B, C, D and E isolates and the ones of non-modelled serogroups, except for members of serogroup H and K, for which the O-unit structure shows some similarity with sub-serogroup C1, possibly justifying their location in the border area of the serogroup C cluster (Supplementary Fig. S1; Fig. 2B) (Fitzgerald et al., 2003; Knirel, 2011; Liu et al., 2014).

### 3.3. Discrimination within S. enterica serogroup B isolates

The scores map of the PCA model obtained with spectra from serogroup B isolates (11 distinct serotypes) showed the isolates quite disperse across the map (data not shown) with two barely defined clusters, one containing *S.* Typhimurium, its monophasic variant *S.* 1,4,[5],12:i:- and *S.* Stanley isolates and another cluster with isolates belonging to remaining serotypes. In this context, a PLSDA model was performed in order to access the potential for discrimination between these two clusters. The scores map (3 LVs encompassing 87.2% of the spectral variability) revealed the same two clusters with some overlap (Fig. 3A) and the corresponding confusion matrix lead to 93.3% of correct assignments (Supplementary Table S3). We further attempted to discriminate *S.* Typhimurium and *S.* 1,4,[5],12:i:- from *S.* Stanley through another PLSDA model (3 LVs encompassing 87.6% of the spectral variability, Fig. 3B) being the percentage of correct assignments of 78.6% (Supplementary Table S4). Available O-unit structures of serogroup B isolates, suggests a common backbone comprising abequose(α1–3)-2)mannose(α1–4)rhamnose(α1-3)galactose(α1- (bold in the Supplementary Fig. S1), being the variations, between and within serotypes (including for *S.* Typhimurium), related with additional glucose and/or *O*-acetyl groups (Supplementary Fig. S1) (Knirel, 2011; Liu et al., 2014; Micoli et al., 2014). Absence of O-unit structure composition namely for *S.* Stanley, and tested isolates of *S.* Typhimurium and *S.* 1,4,[5],12:i:, prevents to assess the contribution of this structure on the clusterization obtained.

Subsequently, a third PLSDA model was developed in order to discriminate *S.* Typhimurium and *S.* 1,4,[5],12:i:-. The scores map, considering the first 3 LVs, which encompasses 84.9% of the spectral variability, does not provided defined clusters (Fig. 3C) with a corresponding confusion matrix leading to a low level of correct serotype assignment (77.4%) (Supplementary Table S5). Absence of differentiation at clonal level for those serotypes was also observed, being the percentage of correct predictions below 50% (data not shown). In fact, alignment of O-antigen biosynthesis gene cluster of isolates representing the main clones of *S.* Typhimurium and *S.* 1,4,[5],12:i:- demonstrated an identical composition (100% of similarity, data not shown), which also support the contribution of O-antigen polysaccharides for the FTIRS discrimination obtained in this study.

### 3.4. Discrimination within S. enterica serogroup C isolates

Infrared spectra of serogroup C isolates were modelled through a PCA according to their sub-serogroup (C1, C2 and C3). Three well-defined clusters were observed each containing the corresponding isolates (Fig. 4A). An additional PLSDA model was developed only with isolates from C1 and C2 (due to the small number of C3-group isolates) and a clear separation between the two sub-serogroups (Fig. 4B) was observed in the scores map (2 LVs encompassing 81.7% of the spectral variability). The corresponding confusion matrix leads to 100% of correct assignments (Supplementary Table S6). Actually, differences in the C1, C2 and C3 sub-serogroups O-unit composition was described involving the backbone and side chain groups that might justify the discriminations achieved (Supplementary Fig. S1) (Knirel, 2011; Liu et al., 2014). Further, a PCA model was developed with all isolates (C1, C2 and C3) aiming their discrimination at the serotype level, but no

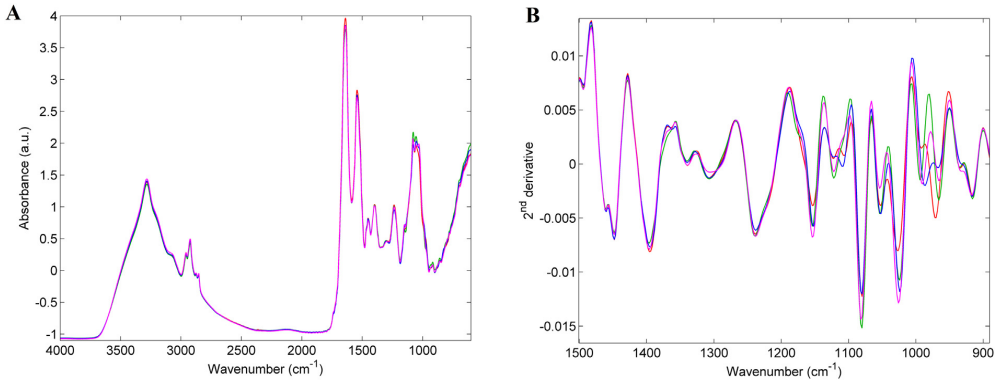


**Fig. 1.** A) Typical *Salmonella enterica* infrared spectra in the region 4000–600 $cm^{-1}$ of each of the four serogroups B, C, D and E. B) *Salmonella enterica* infrared spectra processed with standard normal variate (SNV) and Savitzky-Golay (15 points filter size, 2nd degree polynomial, 2nd derivative) in the region 1500–900 $cm^{-1}$ of each of the four serogroups. Legend: B, C (C1-C2-C3), D (D1-D2) and E (E1-E2-E3-E4).

defined clusters were observed (data not shown). Additional PCA models were developed for serotype discrimination including only isolates of the same sub-serogroup (C1 or C2), but no discrimination was achieved (data not shown). Nevertheless, the PCA model developed with C1-group isolates revealed two clusters, one containing the isolates of *S.* Rissen and other containing the isolates of the remaining 4 serotypes. In this context, a PLSDA model was developed aiming the discrimination of *S.* Rissen from the remaining serotypes. The scores map of the first three LVs, encompassing 90.8% of the spectral variability, exhibited two clusters (Fig. 5) each containing the corresponding isolates. The percentage of correct predictions obtained from the PLSDA confusion matrix was 91.5% (Supplementary Table S7). Variable O-unit structures, involving additional glucose linked to the backbone throughout mannose, were described for different C1 serotypes (*S.* Ohio, *S.* Thompson and *S.* Livingstone) (Supplementary Fig. S1) (Knirel, 2011; Liu et al., 2014; Reeves et al., 2013). Nevertheless, there is no available O-unit structure for *S.* Rissen preventing any relationship with the discrimination here obtained.

### 3.5. Discrimination within S. enterica serogroup D isolates

The PCA model to attempt the discrimination of D1 and D2 sub-serogroups did not provided defined clusters (data not shown). An additional PCA model was developed for serotypes discrimination and two clusters were observed, one containing *S.* Enteritidis isolates and other containing the isolates from the remaining six serotypes (data not shown). Due to such findings, a PLSDA model was further developed in order to globally discriminate *S.* Enteritidis isolates (D1) from the remaining serotypes from D1 and D2 sub-serogroups. The scores map of the first three LVs of the PLSDA model (Fig. 6), encompassing 94.1% of the spectral variability, shown two individualized clusters, one containing *S.* Enteritidis and other with the remaining isolates. The corresponding confusion matrix leads to 93.4% of correct assignments (Supplementary Table S8). The high rates of correct assignments for *S.* Enteritidis could be related with particular O-unit composition of these isolates, as *O*-acetylation of glucose attached to galactose of the backbone structure previously reported for this serotype (Supplementary Fig. S1) (Knirel, 2011; Liu et al., 2014; Rahman et al., 1997; Reeves et al., 2013). Nevertheless, molecular structure characterization of D1 and D2 serotypes including in the analysis would be required to clarify this possible relationship.

### 3.6. Discrimination within S. enterica serogroup E isolates

Serogroup E isolates infrared spectra were modelled by a PLSDA according to their groups, E1-E2-E3 and E4. The PLSDA scores map of the first three LVs, encompassing 78.5% of the spectral variability, evidenced two perfectly individualized clusters, each containing the corresponding E1-E2-E3 and E4 isolates (Fig. 7). The confusion matrix leads to 100% of correct assignments for both groups (Supplementary

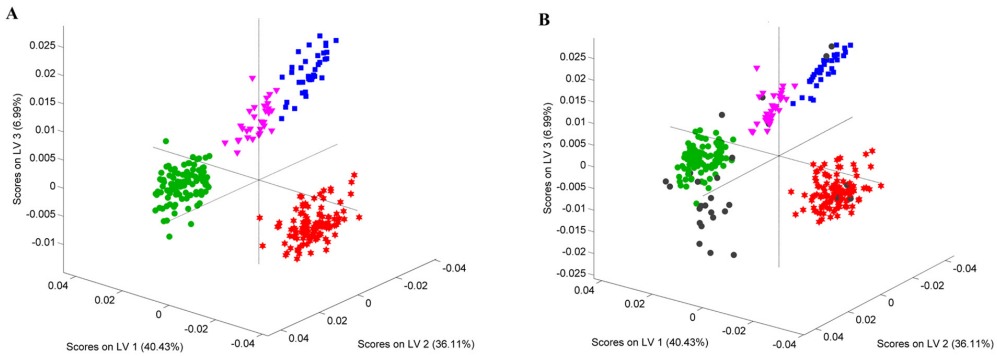


**Fig. 2.** A) Scores plot corresponding to the first three LVs of the PLSDA regression model using the spectral region 1500–900 $cm^{-1}$ B) Scores plot corresponding to the three first LVs of the PLSDA regression model with the projected test isolates. Legend: B, C (C1-C2-C3), D (D1-D2), E (E1-E2-E3-E4) and test isolates.

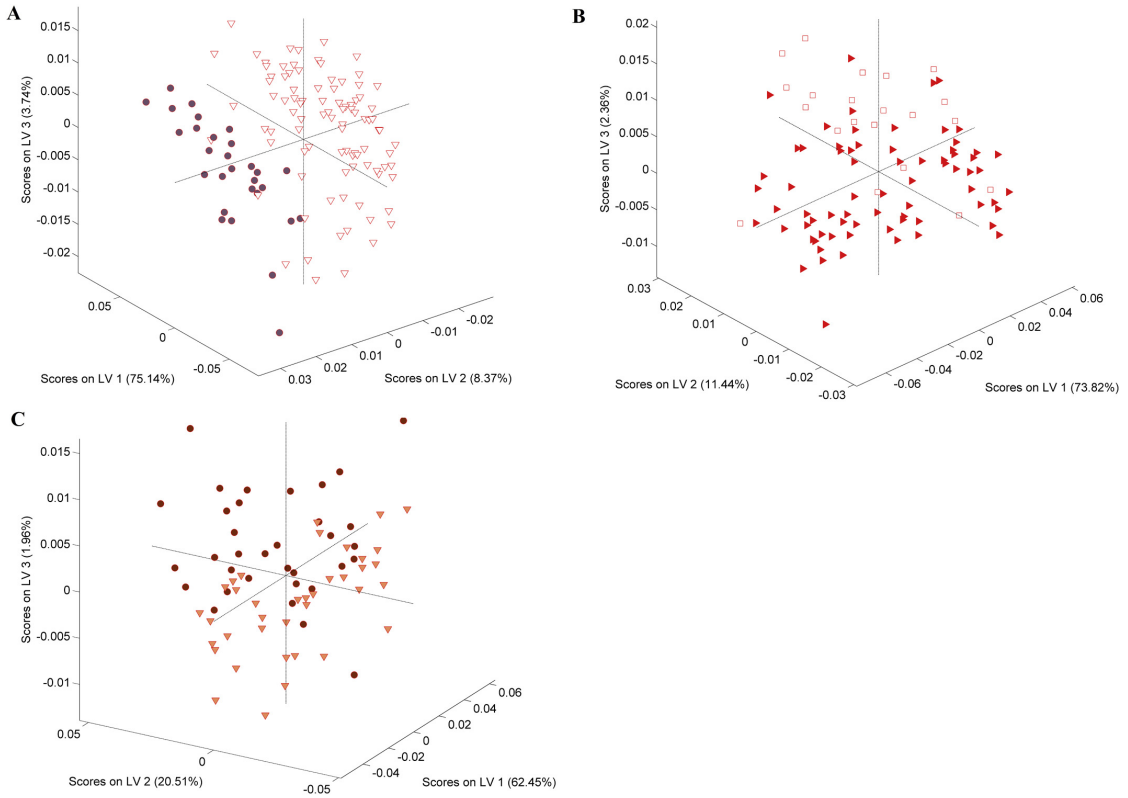


**Fig. 3.** A) Scores plot corresponding to the first three LVs of the PLSDA regression model of B serogroup isolates using the 1500–900 $cm^{-1}$ spectral region. Legend: ● *S.* Typhimurium, *S.* 1,4,[5],12:i:-, *S.* Stanley and ▽ other B serotypes B) Scores plot corresponding to the first three LVs of the PLSDA regression model of B serogroup isolates using the 1500–900 $cm^{-1}$ spectral region. Legend: ▶ *S.* Typhimurium, *S.* 1,4,[5],12:i:-, and □ *S.* Stanley. C) Scores plot corresponding to the first three LVs of the PLSDA regression model of B serogroup isolates using the 1500–900 $cm^{-1}$ spectral region. Legend: ● *S.* Typhimurium and ▼ *S.* 1,4,[5],12:i:-.

Table S9). Additionally, a PCA was attempted for serotype discrimination. The scores map shown two clusters, one containing E4 serogroup isolates, all belonging to *S.* Senftenberg serotype, and other containing the remaining seven serotypes (data not shown). Differences in the O-unit structure involving side chain groups have been reported for serogroup E isolates (e.g. glucose linked to galactose of the backbone in *S.* Senftenberg) (Supplementary Fig. S1) (Garegg et al., 1983; Knirel, 2011; Kochetkov et al., 1977; Liu et al., 2014). Nevertheless, O-unit

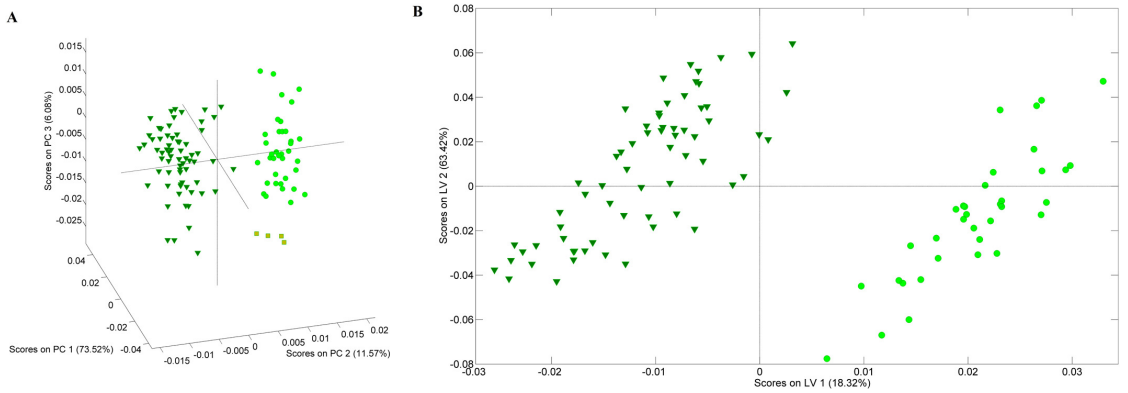


**Fig. 4.** A) Scores plot corresponding to the first three PCs of the PCA model using the 1500–900 $cm^{-1}$ spectral region with isolates belonging C1, C2, and C3 serogroups. B) Scores plot corresponding to the first two LVs of the PLSDA regression model using the 1500–900 $cm^{-1}$ spectral region with isolates belonging to C1 and C2 serogroups. Legend: ▼ C1, ● C2 and ■ C3 serogroups.

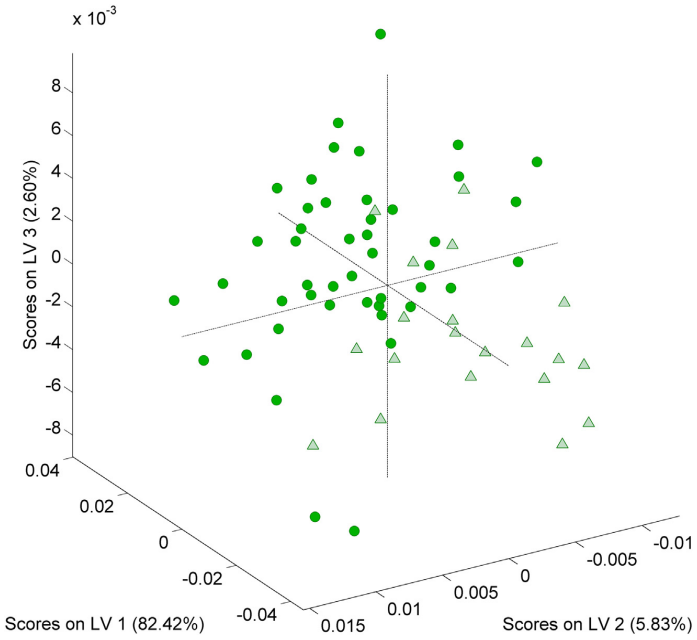


**Fig. 5.** Scores plot corresponding to the first three LVs of the PLSDA regression model of C serogroup isolates using the 1500–900 $cm^{-1}$ spectral region. Legend: △ *S.* Rissen and ● other C1 serotypes.

structure composition of other serotypes included in the study (e.g. *S.* Give and *S.* London) will be required for elucidating a possible role of this structure on the very good discrimination of *S.* Senftenberg isolates.

## 4. Conclusions

We demonstrated that FTIRS coupled with multivariate analysis is a reliable and alternative technique to accurately discriminate *Salmonella* isolates belonging to B, C, D and E serogroups, sub-serogroups C1, C2 and C3, and E1-E2-E3 and E4, as well as particular relevant serotypes (*S.* Rissen, *S.* Enteritidis and *S.* Senftenberg).

Differences in the O-unit structure might justify the very good rates of discrimination obtained for *Salmonella enterica* isolates belonging to B, C, D and E serogroups, the most frequent and clinically-relevant *Salmonella* ones. Also, backbone and side chain groups of O-units might also contribute for the differentiation obtained for C1, C2 and C3. The lack of available O-unit structures composition for several serotypes did not allow to suggest a possible role of this structure on the serotypes discrimination achieved. Further studies describing O-antigen composition of relevant serotypes and clarifying its impact on *S. enterica* discrimination by FTIRS are required for a molecular-based knowledge on the limitations of this high throughput and low-cost methodology.

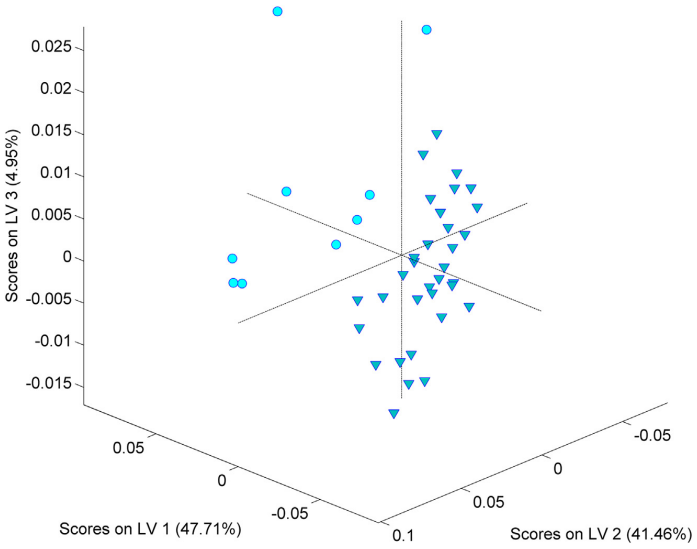


**Fig. 6.** Scores plot corresponding to the first three LVs of the PLSDA regression model of D serogroup isolates using the 1500–900 $cm^{-1}$ spectral region. Legend: ▽ *S.* Enteritidis and ○ D non-*S.* Enteritidis.

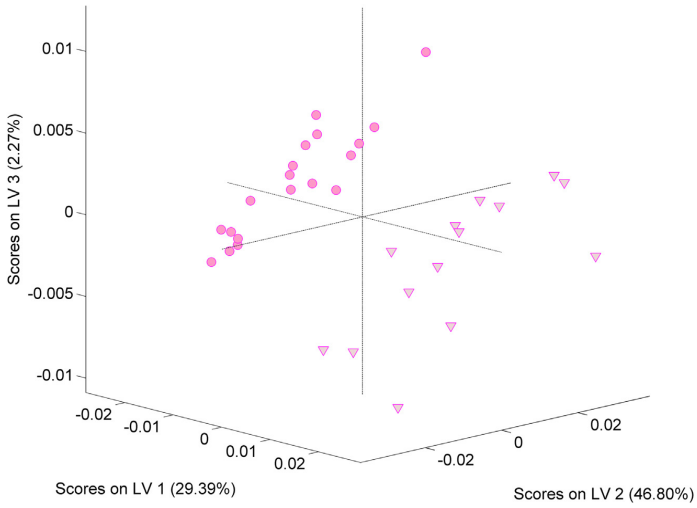


**Fig. 7.** Scores plot corresponding to the first three LVs of the PLSDA regression model of E serogroup isolates using the 1500–900 $cm^{-1}$ spectral region. Legend: ▽ E1-E2-E3 and ● E4.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.07.005.

**Acknowledgements**

The authors would like to thank to Franz Allerberger and Christian Kornschober from Austrian Agency for Health and Food Safety (AGES) for providing the *Salmonella* isolates from Austria and to the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Food- and Water-borne Infections Study Group (EFWISG).

**Funding**

This work was supported by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Food- and Water-borne Infection Study Group (EFWISG) Study Group Research Grant - ESCMID Executive Committee [Proposal #8168 - "Discrimination of *Salmonella* non-typhoid high-risk clones by high-throughput spectroscopic techniques (MALDI-TOF and FTIR-ATR)"]; the European Union FEDER funds (POCI/01/0145/FEDER/007728) and National Funds (FCT/MEC, Fundação para a Ciência e Tecnologia and Ministério da Educação e Ciência) under the Partnership Agreement PT2020 [UID/MULTI/04378/2013]. Joana Campos is supported by a Ph.D. fellowship from FCT [grant number SFRH/BD/93091/2013)]. Clara Sousa was funded through the project NORTE-01-0145-FEDER-000024. Joana Mourão is supported by a post-doc fellowship from project NORTE-01-0145-FEDER-000024.

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**Supplementary Information**

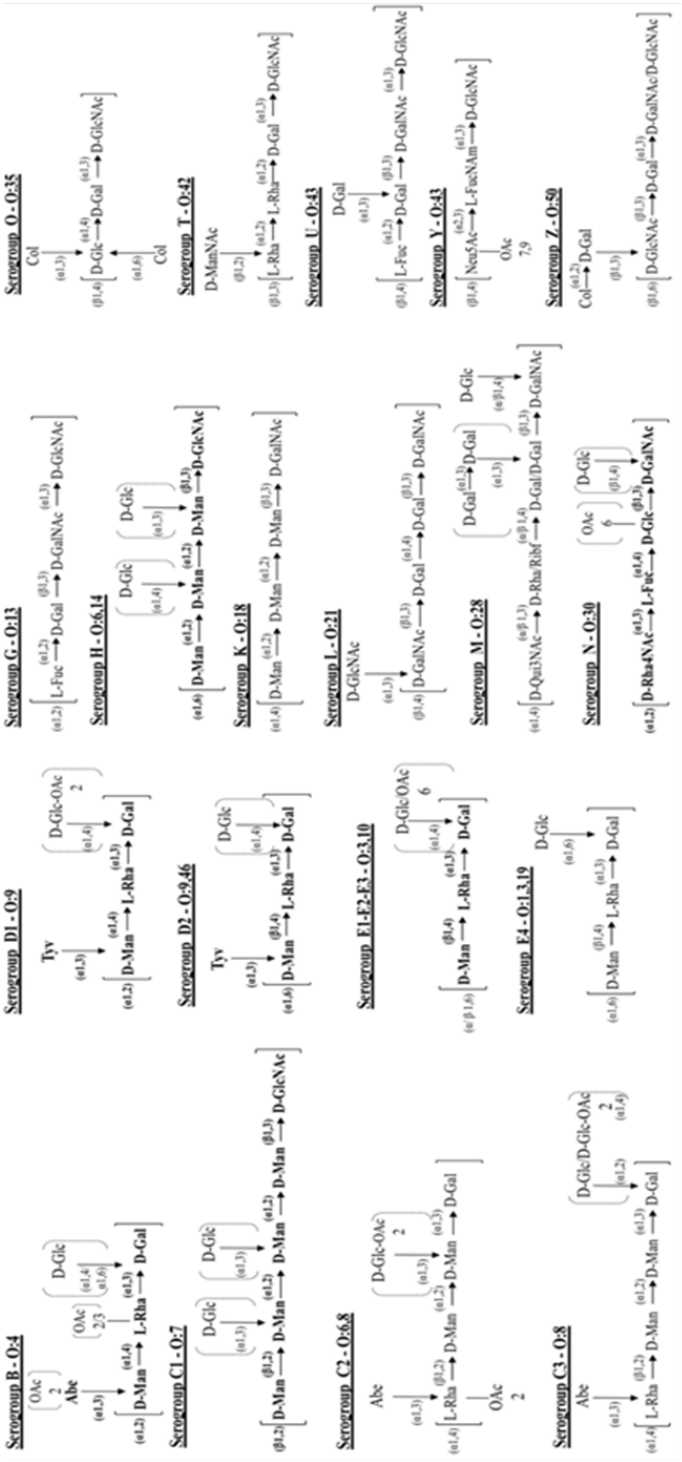


**Fig. S1**: *S. enterica* O-units structure of the modelled serogroups B, C (C1, C2, C3), D (D1, D2), E (E1, E4) and non-modelled serogroups G, H, K, L, M, N, O, T, U, Y and Z. Abbreviations: Abe, abequose; Col, colitose; D-Gal, D-galactose; D-GalNAc, 2-Acetamido-2-deoxy-D-glucose; D-Glc, D-glucose; D-GlcNAc, 2-Acetamido-2-deoxy-D-glucose; D-Man, D-mannose; D-ManNAc, 2-Acetamido-2-deoxy-D-mannose; D-Qui3NAc, 3-Acetamido-3-deoxy-D-quinovose; D-Rha4NAc, 4-Acetamido-4-deoxy-D-rhamnose; L-Fuc, L-Fucose; L-FucNAm2-Acetimidoylamino-2-deoxy-L-fucose; L-Rha, L-rhamnose; Neu5AC, N-acetyl neuraminic acid; OAc, O-acetyl group; Tyv, tyvelose. Square brackets represent a single O-unit structure. Arrows represent the type of linkages between the monosaccharides within the O-unit. O-units in bold are the same structurers found in different serotypes studied to represent a specific serogroup. O-unit structures not in bold are the structurer found when only one serotype was studied to represent a specific serogroup. Curved brackets represent additional monosaccharides and/or O-acetyl groups in the O-unit structures reported in specific serotypes: in serogroup B, several O-unit structures described in different *S.* Typhimuruium strains; in serogroup C1 several O-unit structures reported in different serotypes (*S.* Ohio, *S.* Thompson and *S.* Livingstone); in serogroup D1 different O-unit structures reported in *S.* Enteritidis strains [Figure adapted from Knirel, 2011; Liu et al., 2014; Micoli et al., 2014].

**Supplementary Table S1.** *Salmonella enterica* isolates used in this study, 2002-2015.

| Serogroups (no. isolates) | | Serotype (no. isolates)[a] | Source(s) (no. isolates)[c] | Country(ies) (no. isolates)[d] |
|---|---|---|---|---|
| B-O:4 (122) | | Typhimurium (35)[b] | A (1); E (1); Cte (1); H (24); Otf (2); P (2); Pie (2); Pt (2) | PT (22); AU (13) |
| | | 1,4,[5],12:i:- (38)[b] | A (3); E (1); Cte (1); H (22); Otf (2); P (3); Pie (3); Pt (1); Pte (1); W (1) | PT (20); AU (18) |
| | | Stanley (19) | H (11); Pt (7); Ww (1) | PT (3); AU (16) |
| | | Derby (7) | H (4); P (3) | PT (7) |
| | | Heidelberg (8) | H (2); Pt (6) | PT (3); IFP (5) |
| | | Saintpaul (4) | H (1); Pt (3) | PT (4) |
| | | Brandenburg (4) | H (2); P (2) | PT (4) |
| | | Chester (3) | A (1); Hv (2) | AG (3) |
| | | Agona (1) | Pt (1) | PT (1) |
| | | Budapest (1) | Aq (1) | PT (1) |
| | | Bredeney (1) | H (1) | PT (1) |
| | | II 4,12:l,w:z39 (1) | Wr (1) | AG (1) |
| C (108) | C1-O:7 | Infantis (20) | H (11), P (3); Pte (6) | PT (5); AU (16) |
| | | Rissen (20) | A (1); H (10), Otf (1); P (6); Pie (2) | PT (20) |
| | | Virchow (5) | H (2); Pie (2); Pte (1) | PT (5) |
| | | Mbandaka (20) | E (1); H (10); Otf (5); Pt (1); Pte (3) | PT (4); AU (16) |
| | | Braenderup (1) | Pte (1) | AG (1) |
| | | Thompson (1) | C (1) | PT (1) |
| | C2-O:6,8 | Newport (21) | A (1); Aq (1); H (12); Otf (1); Pt (6) | PT (5); AU (16) |
| | | Bovismorbificans (6) | H (6) | PT (6) |
| | | Hadar (5) | H (1); Pt (4) | PT (5) |
| | | Muenchen (3) | H (3) | PT (3) |
| | | Goldcoast (2) | H (1); P (1) | PT (2) |
| | C3-O:8 | Kentucky (1) | H (1) | PT (1) |
| | | Istanbul (1) | Pt (1) | PT (1) |
| | | Brikama (1) | H (1) | PT (1) |
| | | Sindelfingen (1) | Otf (1) | IFP (1) |
| D (43) | D1-O:9 | Enteritidis (34) | A (1); C (2); H (20); Otf (4); Pt (4); Pte (3) | PT (20); AU (14) |
| | | Ndolo (3) | H (3) | PT (3) |
| | | Houston (2) | H (1); Otf (1); | PT (2) |
| | | Dublin (1) | H (1) | PT (1) |
| | | II 9,46:z:- (1) | A (1) | AG (1) |
| | D2-O:9,46 | Linguere (1) | Aq (1) | PT (1) |
| | | Guerin (1) | Aq (1) | PT (1) |
| E (33) | E1-E2-E3-O:3,10 | Anatum (3) | C (1); H (1); P (1) | PT (3) |
| | | Give (3) | Otf (1); P (2) | PT (3) |
| | | London (3) | Otf (1); H (2) | PT (3) |
| | | Weltevreden (2) | Otf (2) | IFP (2) |
| | | Muenster (1) | A (1) | AG (1) |
| | | Uganda (1) | Pt (1) | PT (1) |
| | | Freiburg (1) | C (1) | PT (1) |
| | E4-O:1,3,19 | Senftenberg (19) | E (1); H (8); Otf (2); Pt (4); Pte (4) | PT (3); AU (16) |
| G-O:13 (3) | | Marshall (1) | H (1) | PT (1) |
| | | Poona (2) | A (1); H (1) | PT (1); AG (1) |
| H-O:6,14 (1) | | Sundsvall (1) | A (1) | AG (1) |
| K-O:18 (2) | | Cerro (2) | Ww (2) | PT (1); AG (1) |
| L-O:21 (1) | | Minnesota (1) | Pt (1) | IFP (1) |
| M-O:28 (3) | | Pomona (3) | H (3) | PT (3) |
| N-O:30 (2) | | Urbana (1) | H (1) | PT (1) |
| | | II 30:z29:z6 (1) | Wr (1) | AG (1) |
| O-O:35 (2) | | Adelaide (1) | H (1) | PT (1) |
| | | II 35:z39:1,7 (1) | A (1) | AG (1) |
| T-O:42 (1) | | II 42:b:enxz15 (1) | Wr (1) | PT (1) |
| U-O:43 (1) | | II 43:z29:e,n,x (1) | Pte (1) | AG (1) |
| Y-O:48 (1) | | IIIa 48:z4,z23:- (1) | W (1) | PT (1) |
| Z-O:50 (2) | | II 50:b:z39 (1) | A (1) | AG (1) |
| | | IIIb 50:i:z53 (1) | H (1) | PT (1) |

[a]*Salmonella* subspecies II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*.

[b]Clinically-relevant and emergent clones of *S.* 1,4,[5],12:i:- ("European clone" n=27, PFGE-types-A, B, C/ST34; "South European clone" n=6, PFGE-types-E, F, G, Q/ST19; "Spanish clone" n=6, PFGE-types-O, X/ST19) and *S.* Typhimurium (DT104 clone" n=9, PFGE-types-A, Q/ST19; "OXA-30-producing" n=6, PFGE-type-T/ST19; "European clone" n=16, PFGE-type-B, C, J; Others n=2, PFGE-types-AT, Z) serotypes were also included in FTIRS-ATR (Antunes et al., 2017; Mourão et al., 2014, 2016; unpublished data).

[c]A, animals; Aq, aquacultures; C, community laboratories; Cte, cattle and cattle environment; E, environment; H, hospital patients; Hv, Healthy volunteers; Otf, other type of food; P, pork; Pie, pigs and piggery environment; Pt, poultry; Pte, poultry and avian environment; W, bathing or drinking water; Wr, river water; Ww, wastewater.

[d]AG, Angola; AU, Austria; PT, Portugal; IFP, NTS of imported food products from Brazil or Vietnam.

**Supplementary Table S2.** Confusion matrix for the *S. enterica* serogroups PLSDA discrimination model (values are in %).

| FTIRS-ATR method | Classical method | | | | |
|---|---|---|---|---|---|
| | **B** | **C** | **D** | **E** | **SUM** |
| **B** | 39.78 | 0.00 | 0.00 | 0.00 | - |
| **C** | 0.00 | 35.48 | 0.00 | 0.00 | - |
| **D** | 0.00 | 0.00 | 13.59 | 0.00 | - |
| **E** | 0.00 | 0.00 | 0.39 | 10.75 | - |
| **SUM** | - | - | - | - | 99.60 |

**Supplementary Table S3.** Confusion matrix for the *S.* Typhimurium, *S.* 1,4,[5],12:i:-, *S.* Stanley and other B serotypes PLSDA discrimination model (values are in %).

| FTIRS-ATR method | Classical method | | |
|---|---|---|---|
| | ***S.* Typhimurium+ *S.* 1,4,[5],12:i- + *S.* Stanley** | **Other B serotypes** | **SUM** |
| ***S.* Typhimurium + *S.* 1,4,[5],12:i:- + *S.* Stanley** | 71.27 | 2.27 | - |
| **Other B serotypes** | 4.41 | 22.05 | - |
| **SUM** | - | - | 93.32 |

**Supplementary Table S4.** Confusion matrix for the *S.* Typhimurium, *S.* 1,4,[5],12:i:- and *S.* Stanley serotypes PLSDA discrimination model (values are in %).

| FTIRS-ATR method | Classical method | | |
|---|---|---|---|
| | ***S.* Typhimurium+ *S.* 1,4,[5],12:i-** | ***S.* Stanley** | **SUM** |
| ***S.* Typhimurium + *S.* 1,4,[5],12:i:-** | 68.66 | 14.17 | - |
| ***S.* Stanley** | 7.21 | 9.97 | - |
| **SUM** | - | - | 78.63 |

**Supplementary Table S5.** Confusion matrix for the *S.* Typhimurium and *S.* 1,4,[5],12:i:- serotypes PLSDA discrimination model (values are in %).

| FTIRS-ATR method | Classical method | | |
|---|---|---|---|
| | ***S.* Typhimurium** | ***S.* 1,4,[5],12:i:-** | **SUM** |
| ***S.* Typhimurium** | 44.52 | 14.91 | - |
| ***S.* 1,4,[5],12:i:-** | 7.65 | 32.91 | - |
| **SUM** | - | - | 77.43 |

**Supplementary Table S6.** Confusion matrix for the C1 and C2 groups PLSDA discrimination model (values are in %).

| FTIRS-ATR method | Classical method | | |
|---|---|---|---|
| | **C1** | **C2** | **SUM** |
| **C1** | 64.52 | 0.00 | - |
| **C2** | 0.00 | 35.48 | - |
| **SUM** | - | - | 100.00 |

**Supplementary Table S7.** Confusion matrix for the *S.* Rissen and other C1 serotypes PLSDA discrimination model (values are in %).

| FTIRS-ATR method | Classical method | | |
|---|---|---|---|
| | ***S.* Rissen** | **Other C1 serotypes** | **SUM** |
| ***S.* Rissen** | 27.00 | 5.55 | - |
| **Other C1 serotypes** | 14.03 | 64.45 | - |
| **SUM** | - | - | 91.45 |

**Supplementary Table S8.** Confusion matrix for the *S.* Enteritidis and other D1-D2 serotypes PLSDA discrimination model (values are in %).

| FTIRS-ATR method | Classical method | | |
|---|---|---|---|
| | ***S.* Enteritidis** | **Other D1-D2 serotypes** | **SUM** |
| ***S.* Enteritidis** | 76.92 | 6.62 | - |
| **Other D1-D2 serotypes** | 0.00 | 16.46 | - |
| **SUM** | - | - | 93.38 |

**Supplementary Table S9.** Confusion matrix for the E1-E2-E3 and E4 groups PLSDA discrimination model (values are in %).

| FTIRS-ATR method | Classical method | | |
|---|---|---|---|
| | **E1- E2-E3** | **E4** | **SUM** |
| **E1- E2-E3** | 45.45 | 0.00 | - |
| **E4** | 0.00 | 54.55 | - |
| **SUM** | - | - | 100.00 |

## 3.2.2. Assessing the potential of Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry for discrimination of clinically relevant *Salmonella* serogroups and serotypes

**Manuscript in final draft**

**Full-length Research Article**

# Assessing the potential of Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry for discrimination of clinically relevant *Salmonella* serogroups and serotypes

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**Abstract**

*Salmonella enterica* is one of the leading causes of foodborne disease worldwide, being strain subtyping required for epidemiological surveillance in the food chain and in public health. Thus, simpler, quick and low-cost alternative methods for *Salmonella* typing, as Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS), are required for routine use. In this study we assessed the potential of MALDI-TOF MS to discriminate the most frequent and clinically-relevant *S. enterica* serogroups and serotypes.

*S. enterica* isolates (n=183) of a well-characterized collection (2002-2015; different countries; origins), belonging to 42 serotypes and to most frequent serogroups [B-n=75/12 serotypes; C (C1, C2 and C3)-n=66/15 serotypes; D (D1 and D2)-n=25/7 serotypes and E (E1-E2-E3 and E4)-n=17/8 serotypes] were analysed by MALDI-TOF MS. Mass spectra were acquired from intact cells and analysed by Partial Least Square Discriminant Analysis (PLSDA) and/or Principal Component Analysis (PCA).

Under the experimental conditions tested in this work, MALDI-TOF MS did not provide B, C, D and E serogroup discrimination, neither among their sub-serogroups (C1/C2/C3 and D1/D2). However, some discrimination within E serogroup, E1-E2-E3 and E4 (E4-all *S.* Senftenberg), was observed (78.8% of correct assignments). Regarding serotype discrimination, this approach revealed some discriminations with distinct percentage of correct assignment, namely: *S.* 1,4,[5],12:i:- from remaining B serotypes (83.7%); *S.* Rissen (90.6%) and *S.* Virchow (76.9%) from remaining C1 serotypes; *S.* Newport (78.2%) and *S.* Bovismorbificans (90.0%) from remaining C2 serotypes and *S.* Enteritidis (D1) from the remaining D1/D2 serotypes (80.3%).

Globally, MALDI-TOF MS was not suitable for discrimination of the most clinically-relevant *Salmonella* serogroups (B, C, D and E) under the experimental conditions tested. Of remark, the good discrimination rates were achieved for relevant serotypes (e.g. *S.* Enteritidis, *S.* 1,4,[5],12:i:- and *S.* Rissen), opening the possibility for further methodological improvements.

**Keywords:** *Salmonella enterica*, MALDI-TOF MS, chemometrics, discrimination, typing.

## 1. Introduction

*Salmonella enterica* is one of the leading causes of foodborne diseases worldwide, representing a major public health burden, even for high-income countries (1,2). *S. enterica* is transmitted to humans by a wide range of foodstuffs, mainly of animal origin, and is frequently involved in endemic or epidemic scenarios worldwide (2,3). Therefore, the identification and discrimination of *S. enterica* strains at infra-species level is crucial for epidemiological surveillance and investigation in the food chain and in public health, (e.g. food-borne outbreaks) and for *Salmonella* control programmes in the food-chain (4,5,6). Several typing methods have been applied for *S. enterica* discrimination, namely serotyping, phage typing, Multilocus Sequence Typing (MLST), Pulsed-Field Gel Electrophoresis (PFGE), Multiple Locus Variable-number Tandem Repeat Analysis (MLVA), and more recently Whole-Genome Sequence (WGS). Those methods provide infra-species discrimination at different levels, but globally, they are time-consuming, expensive, laborious, and may require technical expertise (7,8). Of remark, conventional serotyping methodology, which is based on agglutination reactions with specific antisera and surface antigens, namely the somatic O-antigen and flagellar H-antigens (phases 1 and 2), is still the first approach and gold standard for *S. enterica* typing. (9). Additionally, the serotypes are determined by specific combinations of O-antigen and H-antigens, resulting in more than 2600 serotypes described (9,10). Still, the major clinically-relevant serotypes, frequently causing human infections worldwide, have been limited to *S.* Enteritidis (serogroup D), *S.* Typhimurium and its monophasic variant *S.* 1,4,[5],12:i:- (serogroup B), as well as some emerging serotypes [e.g. *S.* Virchow, *S.* Rissen (serogroup C1), *S.* Newport, *S.* Bovismorbificans (serogroup C2)] (1,2,9). Despite its relevance, identification at serotype level is usually lengthy to obtain, depend on expertise and/or expensive reagents, being restricted to few reference laboratories. In addition, incorrect results by classical serotyping as well as by WGS were already recognized (7,8,11).

Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) is based on the ionization and separation of ions according to mass-to-charge (m/z) ratio, given a characteristic fingerprint of a specific microbial cell and reflecting mostly peptides and small proteins content as the ribosomal proteins (12). Furthermore, this simpler, quick and low-cost alternative spectroscopic technique is currently used for identification in some routine bacteriology laboratories. In addition, MALDI-TOF MS was already successfully explored as an alternative to discriminate several pathogenic bacteria causing foodborne or nosocomial infections at different taxonomic levels (13-18). To the best of our knowledge, the potential of MALDI-TOF MS for *S. enterica* typing by whole-cell approach has been barely explored (19-21).

In this study, we investigated in a comprehensive *Salmonella* collection, the suitability of MALDI-TOF MS, combined with appropriate chemometric methods, to discriminate the most frequent and clinically-relevant *Salmonella* serogroups and serotypes.

## 2. Material and Methods

### 2.1. Bacterial collection.

A total of 183 *Salmonella enterica* isolates belonging to *S. enterica* subsp. *enterica* (subsp. I) were analysed by MALDI-TOF MS. This collection comprises isolates from the most frequent and clinically-relevant serogroups, comprising B (n=75; B-Group O:4), C (n=66; C1-Group O:7; C2-Group O:6,8; C3-Group O:8), D (n=25; D1-Group O:9; D2-Group O:9,46) and E (n=17; E1-E2-E3-Group O:3,10; E4-Group O:1,3,19). These serogroups encompass 42 serotypes, including the three most frequent worldwide (*S.* Enteritidis, *S.* Typhimurium and its monophasic variant, *S.* 1,4,[5],12:i:-) and others that are emergent and have clinical relevance (e.g. *S.* Heidelberg, *S.* Infantis, *S.* Newport, *S.* Rissen) (Supplementary Table S1). The isolates were collected between 2002 and 2015 in several countries (Portugal, Angola and Austria), being the serotypes determined by classical serotyping at the National Reference Centre (INSA, Lisbon, Portugal) or Austrian Agency for Health and Food Safety (AGES, Vienna, Austria). *S.* Typhimurium, *S.* 1,4,[5],12:i:- and *S.* Enteritidis were also confirmed by a PCR assay (22). The isolates were selected from a well-characterized collection, based on clonal lineages (MLST and *Xba*I-PFGE), phenotypic/genotypic antibiotic resistance profiles and sources (human clinical cases, food products, food-animal production settings and the environment).

### 2.2. Spectral acquisition.

The mass spectra of the studied isolates were acquired from an overnight subculture on Mueller-Hinton agar after 18 h of grow at 37 ºC. The MALDI-TOF MS experiments were performed with intact cells, which were directly spotted onto MALDI ground steel target (AnchorChip™) and after complete dry overlaid with 1 µL of chemical matrix solution (α-cyano-4-hydroxycinnamic acid in 50% of acetonitrile and 2.5% of trifluoroacetic acid). Mass spectra were acquired in the linear positive mode at a laser (nitrogen) frequency of 20 Hz in the range of 2,000 to 20,000 Da with a Microflex III instrument (Bruker Daltonic, Bremen, Germany). Each recorded spectrum is the result of six series of 40 single laser shots in different locations. The experiments were performed in quadruplicate using four distinct spots of the MALDI target (instrumental replicates) at least in two different days (biological replicates) with two different bacterial cultures. External calibration of the mass spectra was performed using *Escherichia coli* DH5α standard peaks (BTS). The mass spectra acquisition

of all *Salmonella* isolates studied was performed at the Servicio de Microbiología of the Hospital Universitario Ramón y Cajal (Madrid, Spain).

### 2.3. Spectra pre-processing and modeling.

The mass spectra were firstly preprocessed using the FlexAnalysis software (Bruker Daltonics, Bremen, Germany) applying the "smoothing" and "baseline subtraction" procedures. Spectra with zero-line and low signal-to-noise (S/N) ratio were rejected after visual examination. Further, the spectra were processed with mean-centring and then analysed with the chemometric methods, the non-supervised Principal Component Analysis (PCA) and the supervised Partial Least Square Discriminant Analysis (PLSDA) (23,24). The PLSDA models were preferred, being the PCA used whenever the number of isolates belonging to each class was insufficient (less than 5 isolates) to develop a robust supervised method. All chemometric data analysis were performed with Matlab software version R2016a (MathWorks, Natick, MA) and the PLS Toolbox version 8.2.1 for Matlab (Eigenvector Research, Manson, WA). Additionally, all spectra were visually inspected in order to identify particular discriminatory peaks, at a given m/z ratio or regions of the spectra for the different serogroups and serotypes tested.

### 2.4. Isolates discrimination work-flow.

The rational of this study consisted firstly in the development of a PLSDA model to discriminate *S. enterica* most frequent serogroups (B, C, D and E). Subsequently, within each serogroup, spectra were modeled to discriminate the isolates according their sub-serogroup (C1/C2/C3, D1/D2 and E1-E2-E3/E4, except for serogroup B) and their serotypes (for all serogroups), a PCA or a PLSDA chemometric models depending on the available number of isolates.

## 3. Results

### 3.1. Spectra overview.

The position of the peaks was achieved from the mean mass spectra of *S. enterica* isolates from each serogroup. No spectral variability was observed among the replicates of each isolate (data not shown). Globally, the analysis of the mass peaks, showed that the peaks were mainly concentrated into the range m/z 2,000 and 11,000 Da (Figure 1). The mean mass spectra of the four serogroups included in this study present a very similar pattern (Figure 1).

### 3.2. Discrimination between *S. enterica* serogroups isolates.

The mass spectra of the *Salmonella* isolates belonging to the serogroups B, C, D and E were modelled through a PLSDA model, which revealed the absence of defined clusters. Spectra of all serogroups were in fact widely dispersed across de scores map of the PLSDA model (Figure 2), which corroborates the already referred very similar peak pattern observed among the serogroups (Figure 1).

### 3.3. Discrimination within *S. enterica* serogroup B isolates.

Regarding to the mass spectra of serogroup B isolates, they were modelled for serotype discrimination through a PCA model. The isolates were quite disperse (data not shown) with two barely defined clusters, one containing *S.* 1,4,[5],12:i:- and another including the isolates belonging to other serotypes (data not shown). In this context, a PLSDA model was performed in order to access the potential for discrimination between *S.* 1,4,[5],12:i:- and the remaining B serotypes. The scores map of the PLSDA model (encompassing 58.9% of the spectral variability) revealed two defined clusters (Figure 3A) with 88.7% of correct serotypes assignments obtained from the confusion matrix of the model (Supplementary Table S2.) Subsequently, an additional PLSDA was attempted to discriminate *S.* Typhimurium and *S.* Stanley from the remaining B serotypes. The scores map (encompassing 77.3% of the spectral variability) revealed two distinct clusters (Figure 3B) and 94.9% of correct assignments were achieved from the corresponding confusion matrix (Supplementary Table S3). An additional PLSDA model was developed to discriminate *S.* Typhimurium and *S.* Stanley serotypes. The scores map (encompassing 86.6% of the spectral variability) has shown two quite defined clusters (Figure 3C) with a percentage of correct predictions obtained from the confusion matrix of 85.3% (Supplementary Table S4). Finally, we attempted to discriminate *S.* Derby, *S.* Heidelberg and the remaining B serotypes. The scores map (encompassing 77.6% of the spectral variability) presented three clusters (Figure 3D) with 83.7% of correct serotype assignments (Supplementary Table S5).

### 3.4. Discrimination within *S. enterica* serogroup C isolates.

Serogroup C isolates mass spectra were modeled through a PCA model aiming the discrimination between C1, C2 and C3 sub-serogroups but no defined clusters were observed (data not shown). Further PCA models were developed to attempt serotypes discrimination, either comprising all C serogroup isolates (C1, C2 and C3) and isolates of a single sub-serogroup for each C1 and C2 (not for C3 sub-serogroup due to the low number of isolates). No clearly defined clusters were observed (data not shown). Nevertheless, in the PCA of C1 sub-serogroup serotypes two barely defined clusters were observed, one

corresponding to *S.* Rissen serotype and other to the remaining C1 serotypes. In this context, a PLSDA model was developed aiming the discrimination of *S.* Rissen from the remaining C1 serotypes. The scores map (encompassing 84.7% of the spectral variability) has shown two clusters with some overlap (Figure 4A) with a percentage of correct predictions obtained from the confusion matrix of 90.6% (Supplementary Table S6). An additional PLSDA model was attempted to discriminate *S.* Virchow from the remaining C1 serotypes, showing two clusters (encompassing 77.9% of the spectral variability) with some overlap (Figure 4B) and percentage of correct predictions obtained from the confusion matrix of 76.9% (Supplementary Table S7). Other C1 serotypes discrimination was attempted with no success (data not shown).

Similar to C1 sub-serogroup, the C2 sub-serogroup serotypes PCA model revealed two barely defined clusters, one containing *S.* Newport and other the remaining C2 serotypes. With the same purpose, a PLSDA model was developed for their discrimination, presenting two clusters with some overlap (Figure 5A) (10 LVs encompassing 81.2% of the spectral variability) and a percentage of correct predictions of 78.2% (Supplementary Table S8). Then, the discrimination of *S.* Bovismorbificans form the remaining C2 serotypes was attempted through an additional PLSDA model, revealing two clusters with some overlap (Figure 5B) (encompassing 86.6% of the spectral variability) and 89.8% of correct predictions from the confusion matrix (Supplementary Table S9). No additional C2 sub-serogroup serotype discrimination was possible (data no shown).

### 3.5. Discrimination within *S. enterica* serogroup D isolates.

PCA models were developed with D serogroup isolates mass spectra, first to discriminate D1 from D2 sub-serogroups and then to discriminate among D1 and D2 serotypes. No defined clusters were observed for D1 and D2 sub-serogroup discrimination (data not shown). The PCA model for serotypes discrimination revealed two well defined clusters, one corresponding to *S.* Houston isolates and other to the remaining D1 and D2 serotypes (Figure 6A). No PLSDA was tried due to the low number of *S.* Houston isolates. Furthermore, a possible discrimination of *S.* Enteritidis isolates (D1) from other serotypes belonging to D1 and D2 sub-serogroups was attempted by a PLSDA model due to the relevance of this serotype. The scores map of the PLSDA model (encompassing 91.6% of the spectral variability) revealed two clusters with some overlap (Figure 6B) and the corresponding confusion matrix revealed 80.3% of correct assignments (Supplementary Table S10).

### 3.6. Discrimination within *S. enterica* serogroup E isolates.

Mass spectra of serogroup E isolates were modelled through a PLSDA according to their sub-serogroups (E1-E2-E3 and E4). The PLSDA scores map (encompassing 86.6% of the spectral variability), evidenced two clusters with some overlap, and including some dispersion of E4 isolates (Figure 7). The corresponding confusion matrix leads to 78.8% of correct assignments (Supplementary Table S11).

## 4. Discussion

In this work the suitability of MALDI-TOF MS for *S. enterica* typing as an alternative to classical serotyping was explored in a wide and diverse (serogroups, serotypes, clones and origin) collection. MALDI-TOF MS potential for infra-species discrimination has already been explored in diverse bacterial species with different degrees of success (25-28) being their routine application for such controversial.

In this study it was possible to observed that the great majority of mass peaks (above 95%) were concentrated in 2,000 to 10,000 Da region, as observed in previous studies (15,17,18), including in *Salmonella* (20,29). Globally, we demonstrated that MALDI-TOF MS coupled with appropriated chemometric tools is an alternative and reliable method to accurately discriminate some clinically-relevant *Salmonella* serotypes (*S.* Enteritidis, *S.* 1,4,[5],12:i:-, *S.* Rissen). It was not possible to correctly predict the four *Salmonella* most frequent and relevant serogroups (B, C, D and E), including the closely related sub-serogroups (C1, C2 and C3, D1 and D2). In fact, the serogroups and sub-serogroups classification are associated with the O-antigen composition (9), which are of polysaccharide nature (not protein content), which justify the absence of discrimination using MALDI-TOF MS. Indeed, the lack of MALDI-TOF MS potential to discriminate isolates at different taxonomic levels was already reported in previous studies with other bacteria (25,26,28).

The few available studies demonstrating the potential of MALDI-TOF MS for *S. enterica* typing presented some limitations, such as bacterial collection (size, origin and representativeness), sample processing (media growth conditions, requirement of extraction methods, matrix, m/z range analysed), experimental workflows (19-21,30) or even poor data analysis. Most of these studies solely rely on direct spectral analysis without the use of appropriate multivariate data analysis. For instance, Dieckmann and Malorny (2011) (19) reported several workflows with specific biomarker combinations for the classification of different serotypes, however, using a mass range of 2,000 to 40,000 Da, which makes their achievements difficult to reproduce, since mass spectra above 20,000 Da are not currently available in most common MALDI-TOF MS equipment for routine in microbiological laboratories (31). Therefore, the absence of a standardized methodology for

a MALDI-TOF MS technique compromise the desired reproducibility of the methodology and correct classification of *Salmonella* serotypes. Globally, some incongruent results were observed between those studies due to the fact that the pointed biomarkers peaks among them differ for a particular serotype or even serogroup (19-21,30). Indeed, with a detailed analysis of those studies with our obtained results allowed to conclude that our data is also not able to totally corroborate their pointed biomarkers.

For instance, in serogroup B, comparing our obtained peaks and the previous reported biomarkers peaks for specific serotypes, some consensus peaks were found but incongruences were also denoted. Dieckmann and Malorny (2011) study (19) reported three combined biomarker peaks for *S.* Typhimurium/*S.* 1,4,[5],12:i:- discrimination, but only one (m/z 7,097) was found in our isolates, being also the only one discriminatory peak pointed in Ojima-Kato et al (2017) (30). Actually, analyzing the H1- and H2-antigens flagellins (UniProt), we verified that they present mass spectra above 51,000 Da and 45,000 Da, respectively, that is, outside the mass range used in this study, which possible justify the absence of particular discriminatory peaks in this particular serotype and its monophasic variant. We consider that the absence of differentiation at the clonal level for those serotypes should be further explored, including a more robust collection since other studies already reported some success at this taxonomic level in other bacteria (13,15). In addition, Kang et al (2017) (20) reported not only that the m/z 4,500 and 6,013 peaks were present in all their *S.* Typhimurium and *S.* Derby isolates, respectively, but also were not exclusive of each serotype, which was also observed in our analysis. For *S.* Stanley identification, only one (m/z 6,484) from the suggested biomarker peaks (19) was observed in our isolates. For *S.* Heidelberg identification all reported biomarker peaks (m/z 7,139 and m/z 10,988) (19) were found in our study. Also, in our study, it was possible to find some putative specific peaks [*S.* Stanley (m/z 3,574), *S.* Derby (m/z 6,286 and m/z 7,128) and *S.* Heidelberg (m/z 5,497 and m/z 10,995)] not reported in other studies which could eventually justify the achieved serotypes discrimination by the PLSDA models.

Concerning the serotypes of serogroup C, discrepant results were also observed. Particularly, in *S.* Rissen, the ribosomal protein S8 was proposed by Ojima-Kato et al (2017) (30), as a biomarker protein (m/z 14,008), nevertheless we could not confirm that result because of the absence of signal in our mass spectra above m/z 11,000. Additionally, we detected specific peaks (m/z 5,440 and m/z 10,880) in *S.* Rissen isolates, not previously reported, which eventually could explain its discrimination with our PLSDA model from other C1 serotypes. Furthermore, in our *S.* Virchow isolates, of the three proposed combined biomarker peaks by Dieckmann and Malorny (2011) (19), only two, m/z 6,008 and m/z 7,097 were observed, being the last peak exclusively found in this serotype, which may justify the discrimination from other C1 serotypes achieved here. Moreover, m/z 6,512 peak

suggested as specific biomarker for *S.* Infantis identification (19) was present in our *S.* Infantis isolates, yet was also detected in *S.* Mbandaka isolates, as previously reported (30) and in our *S.* Newport isolates. Regarding C2 sub-serogroup, from biomarker peaks proposed previously for *S.* Hadar identification (19), only one was found and exclusively in our *S.* Hadar isolates (m/z 10,927). Additionally, the m/z 5,966 was found in *S.* Newport as previously reported (19), yet other peak was also found exclusively in our isolates from this serotype (m/z 6,466). In addition, in our study, it was observed the absence of m/z 3,599 in *S.* Newport and *S.* Bovismorbificans, present in all other C serotypes. These findings could justify the discrimination of *S.* Newport and *S.* Bovismorbificans from the other C2 serotypes obtained in this study.

Regarding the serogroup D, the m/z 6,036 peak (putative uncharacterized protein), pointed out previously as a biomarker for *S.* Enteritidis (19), was also observed in our *S.* Enteritidis isolates. Nevertheless, this peak was also found in three isolates of *S.* Ndolo, other D1 serotype. Furthermore, Ojima-Kato et al. (2017) also found this peak but not in all *S.* Enteritidis isolates (30), contrasting with what was previously suggested. In fact, the m/z 6,036 peak is not a consensus and exclusive peak for *S.* Enteritidis identification, and therefore could not be considered a serotype specific biomarker.

Finally, in serogroup E, the E4 sub-serogroup was composed with isolates belonging only to *S.* Senftenberg serotype. Therefore, E1-E2-E3/E4 discrimination could benefit from the inclusion of additional isolates from other E4 serotypes to better clarify if this is a sub-serogroup or serotype discrimination. Despite, serotypes from E serogroup were previously less explored, *S.* Senftenberg serotype (E4 sub-serogroup) was studied by Ojima-Kato et al (2017), which described several consensus peaks for its classification. Nonetheless only two of them were detected in our isolates (m/z 6,511 and m/z 7,110) (30).

These data highlight the difficulty to correctly classify the *S. enterica* serotypes by MALDI-TOF MS, which is corroborated with the analysis of the 56 ribosomal proteins. Using *S.* Typhimurium strain LT2 as reference (GenBank accession number AE006468.2) and bioinformatic tools [PATRIC Command Line Interface (www.patricbrc.org), UniProt (www.uniprot.org), PUBMED Basic Local Alignment Search Tool-BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi)], a high degree of ribosomal proteins conservation (97% to 100%) between the different *S. enterica* serotypes was observed, being differences even detected between different strains of the same serotype (data not shown).

## 5. Conclusions

We demonstrated that MALDI-TOF MS coupled with multivariate analysis was unable to discriminate the four most frequent and clinically-relevant *S. enterica* serogroups (B, C, D and E) under the experimental conditions tested. Nevertheless, this methodology

revealed some potential for the classification of particular serotypes, including clinically-relevant ones (*S.* Enteritidis, *S.* 1,4,[5],12:i:-, *S.* Rissen). Still, using MALDI-TOF MS for *Salmonella* identification at serogroup, serotype and clonal level continues to be a challenge. In contrasting, other spectroscopic methodologies, as Fourier Transform-Infrared Spectroscopy (FTIR) already revealed to be a reliable and alternative technique to accurately discriminate clinically-relevant *Salmonella* serogroups, sub-serogroups and particular serotypes (*S.* Rissen, *S.* Enteritidis and *S.* Senftenberg) (32). Therefore, further studies on MALDI-TOF MS experimental conditions might be explored in order to obtain a standardized methodology with a clear reproducibility and robustness of the analysis. New insights on MALDI-TOF MS high-resolution typing are currently mandatory to be applied in clinical routine and food safety contexts (e.g. outbreak detection) to provide a quickly tracking and control of clinically-relevant *Salmonella* dissemination.

**Acknowledgements**

The authors would like to thank to Franz Allerberger and Christian Kornschober from Austrian Agency for Health and Food Safety (AGES) for providing the Salmonella isolates from Austria and to the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Food- and Water-borne Infections Study Group (EFWISG).

**Funding**

This work was supported by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Food- and Water-borne Infection Study Group (EFWISG) Study Group Research Grant - ESCMID Executive Committee [Proposal #8168 - "Discrimination of *Salmonella* non-typhoid high-risk clones by high-throughput spectroscopic techniques (MALDI-TOF and FTIR-ATR)"]; the European Union FEDER funds (POCI/01/0145/FEDER/007728) and National Funds (FCT/MEC, Fundação para a Ciência e Tecnologia and Ministério da Educação e Ciência) under the Partnership Agreement PT2020 [UID/MULTI/04378/2013]. Joana Campos is supported by a Ph.D. fellowship from FCT [grant number SFRH/BD/93091/2013)]. Clara Sousa was funded through the NORTE-01-0145-FEDER-000024 – "New Technologies for three Health Challenges of Modern Societies: Diabetes, Drug Abuse and Kidney Diseases". Joana Mourão is supported by a post-doc fellowship from project NORTE-01-0145-FEDER-000024.

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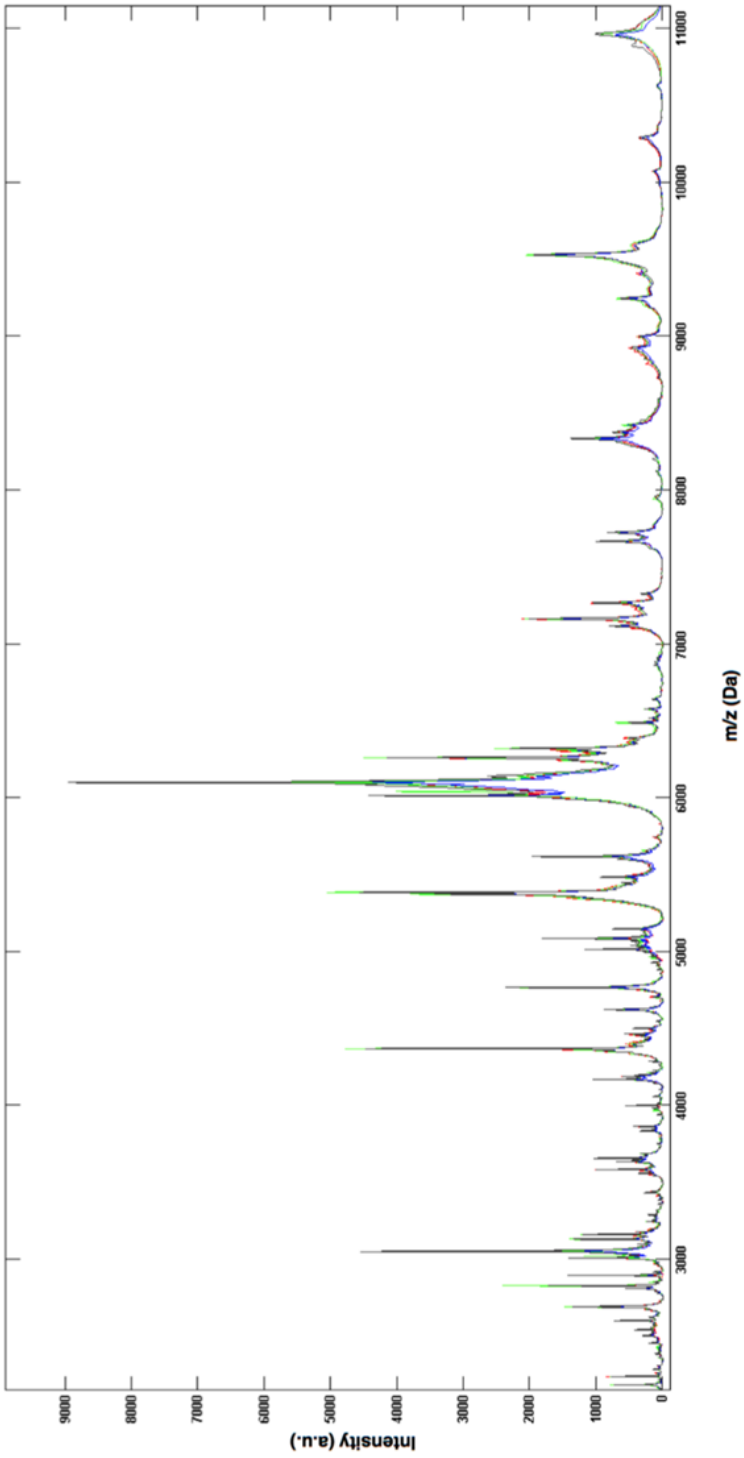


**Figure 1.** Mean mass spectra of the four most frequent *Salmonella enterica* serogroups, B, C, D and E in the region mass spectral m/z 2000 to 11000.

Legend: —— B, —— C ($C_1$-$C_2$-$C_3$), —— D ($D_1$-$D_2$) and —— E ($E_1$-$E_2$-$E_3$-$E_4$).

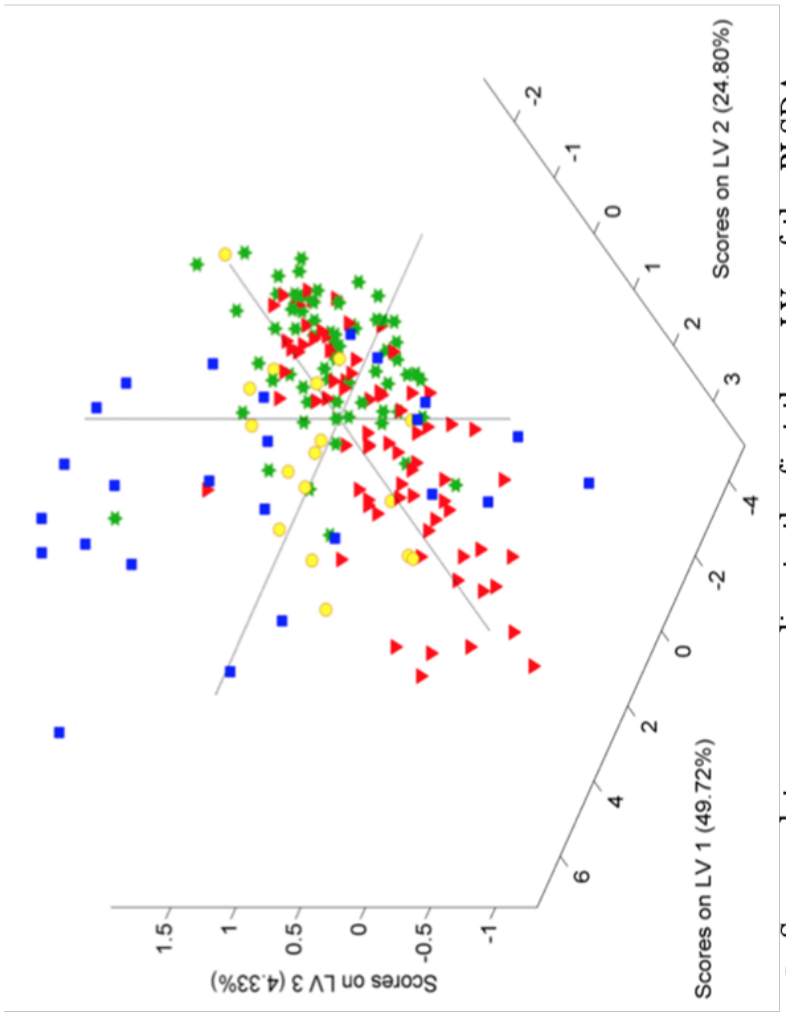


**Figure 2.** Scores plot corresponding to the first three LVs of the PLSDA regression model of the B, C, D and E serogroups isolates using the m/z 2000 to 11000 mass spectral region.

Legend: B, C ($C_1$-$C_2$-$C_3$), D ($D_1$-$D_2$) and E ($E_1$-$E_2$-$E_3$-$E_4$) serogroups.

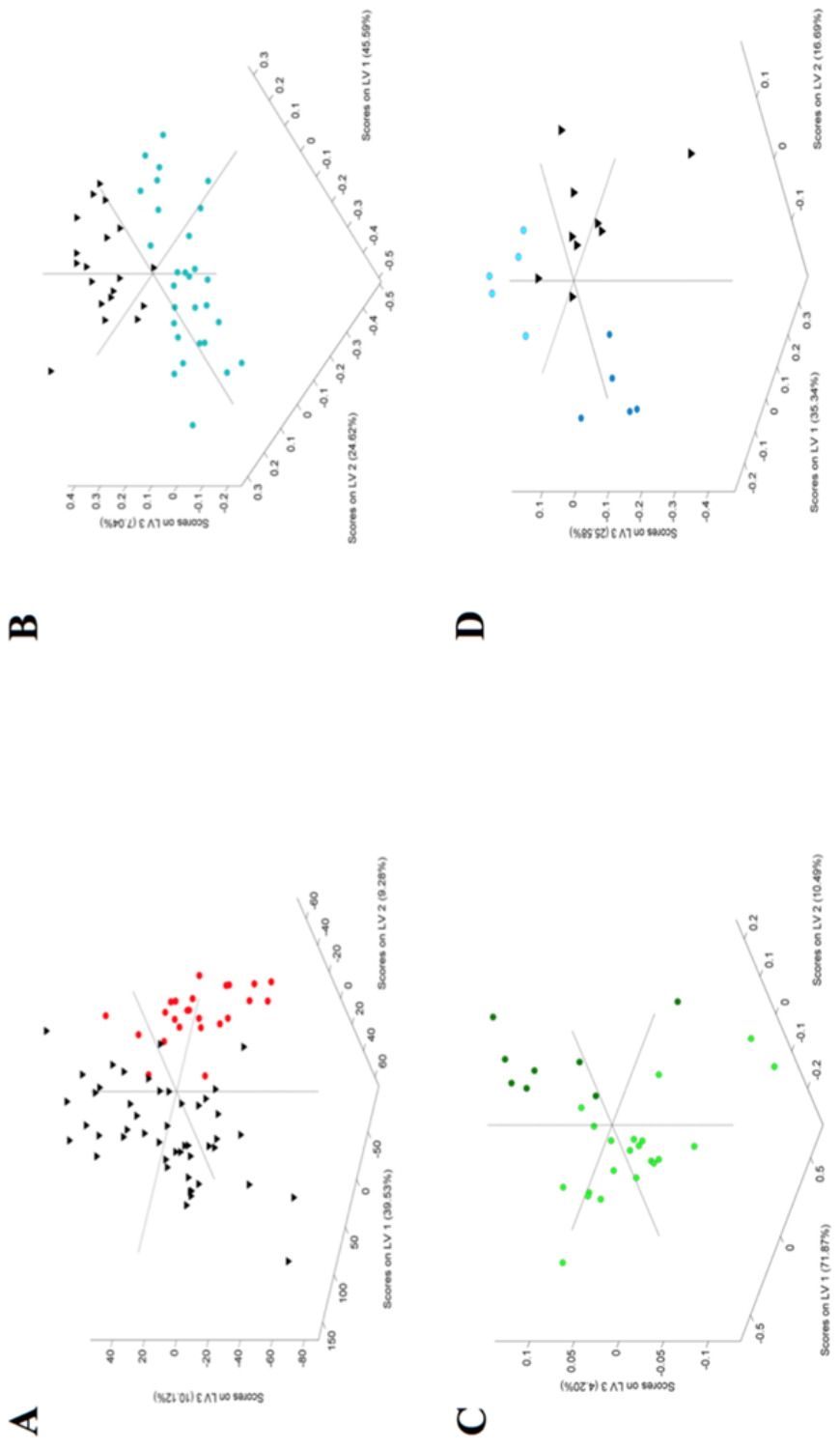


**Figure 3.** Scores plot corresponding to the first three LVs of the PLSDA regression model of B serogroup isolates using the m/z 2000 to 11000 mass spectral mass spectral region. **A)** Legend: ● *S.* 1,4,[5],12:i:- and ▼ other B serotypes. **B)** Legend: ● *S.* Typhimurium, *S.* Stanley and ▼ other B serotypes. **C)** Legend: ● *S.* Typhimurium and ● *S.* Stanley. **D)** Legend: ● *S.* Derby and ● *S.* Heidelberg ▼ other B serotypes

**Figure 4.** Scores plot corresponding to the first three LVs of the PLSDA regression model of C1 serogroup isolates using the m/z 2000 to 11000 mass spectral region. **A)** Legend: ■ *S.* Rissen and ▼ other C1 serotypes. **B)** Legend: ■ *S.* Virchow and ▼ other C1 serotypes.

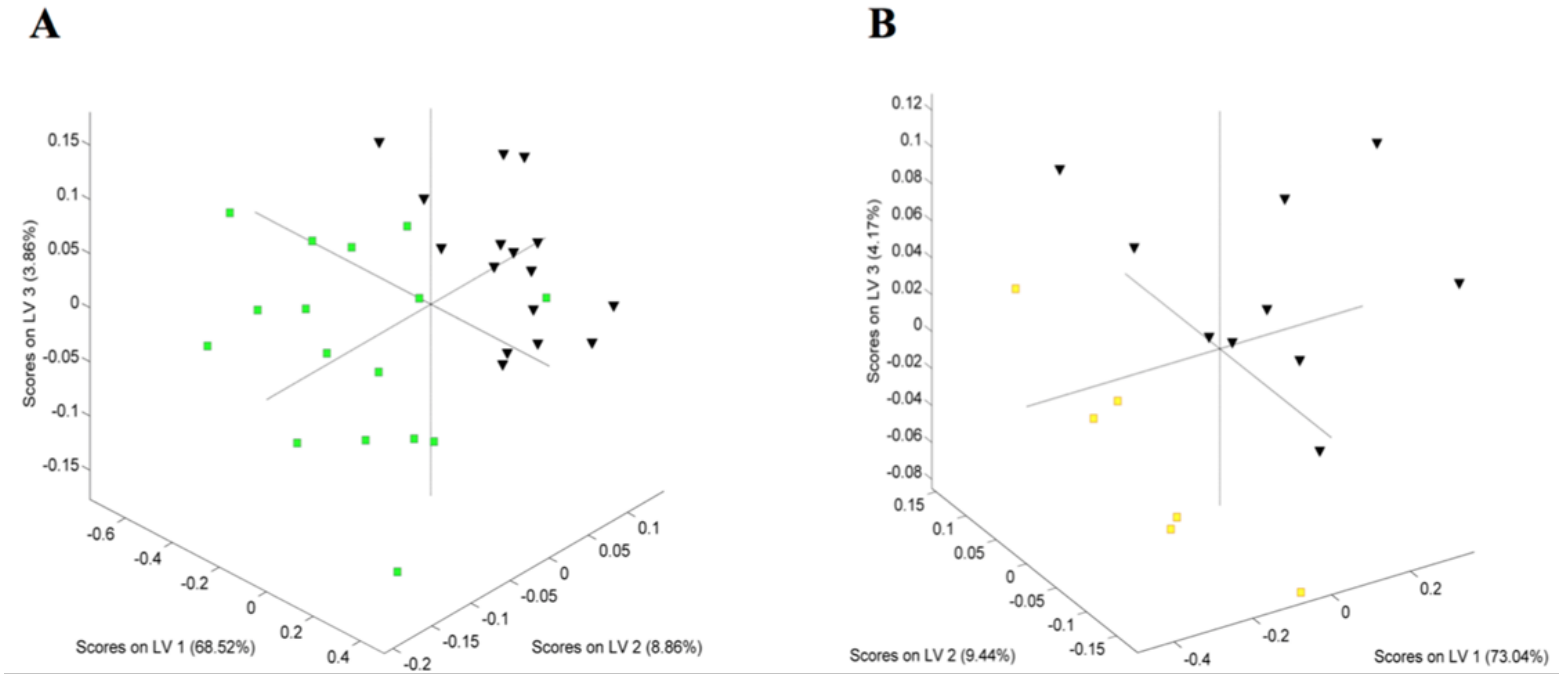


**Figure 5.** Scores plot corresponding to the first three LVs of the PLSDA regression model of C2 serogroup isolates using the m/z 2000 to 11000 mass spectral region. **A)** Legend: ■ *S.* Newport and ▼ other C2 serotypes. **B)** Legend: ■ *S.* Bovismorbificans and ▼ other C2 serotypes.

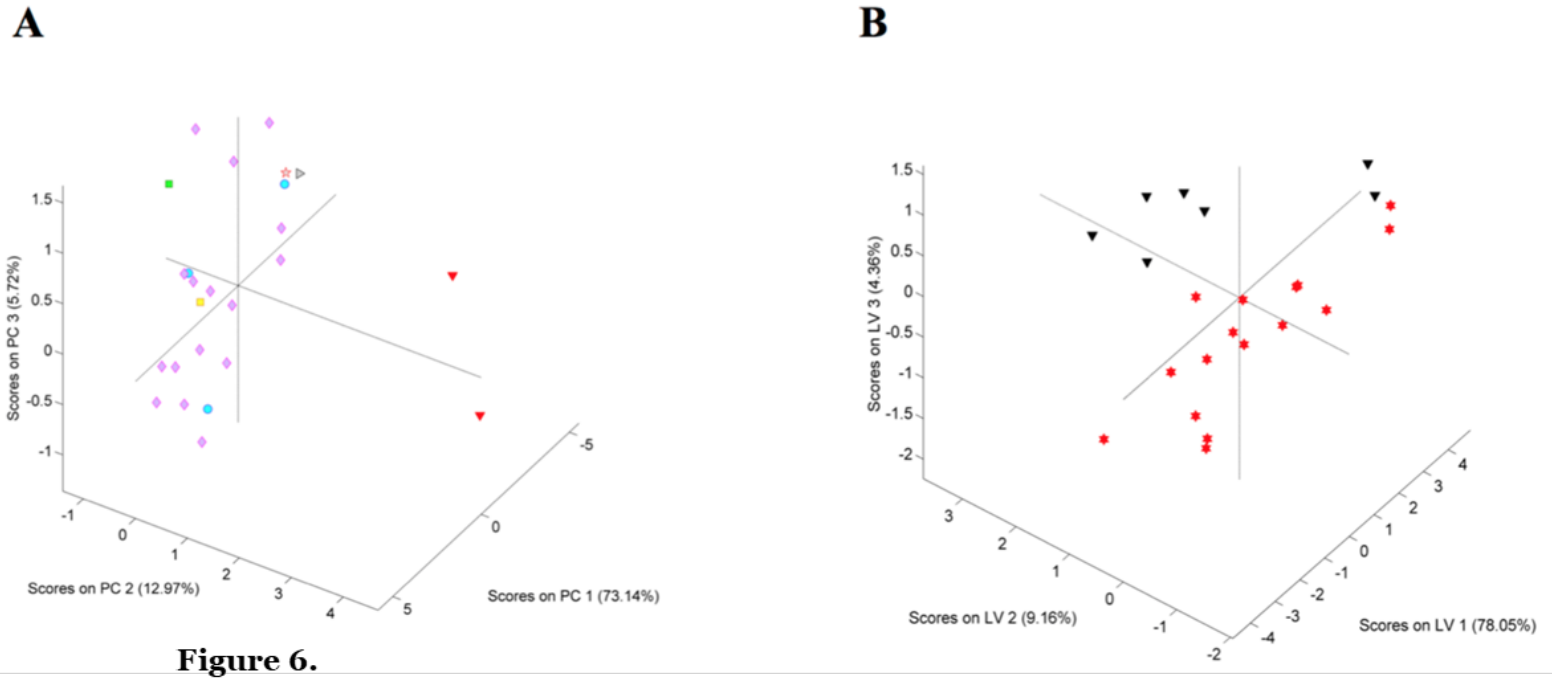


**Figure 6.**

**A)** Scores plot corresponding to the first three PCs of the PCA model of D serogroup isolates using the m/z 2000 to 11000 mass spectral region.

Legend: ▼ *S.* Houston serotype, ◇ *S.* Enteritidis, ● *S.* Ndolo, ☆ *S.* II 9,46:z:-, ▷ *S.* Dublin, ■ *S.* Linguere and ■ *S.* Guerin.

**B)** Scores plot corresponding to the first three LVs of the PLSDA regression model of D serogroup isolates using the m/z 2000 to 11000 mass spectral region.

Legend: ★ *S.* Enteritidis and ▼ other D non-*S.* Enteritidis serotypes.

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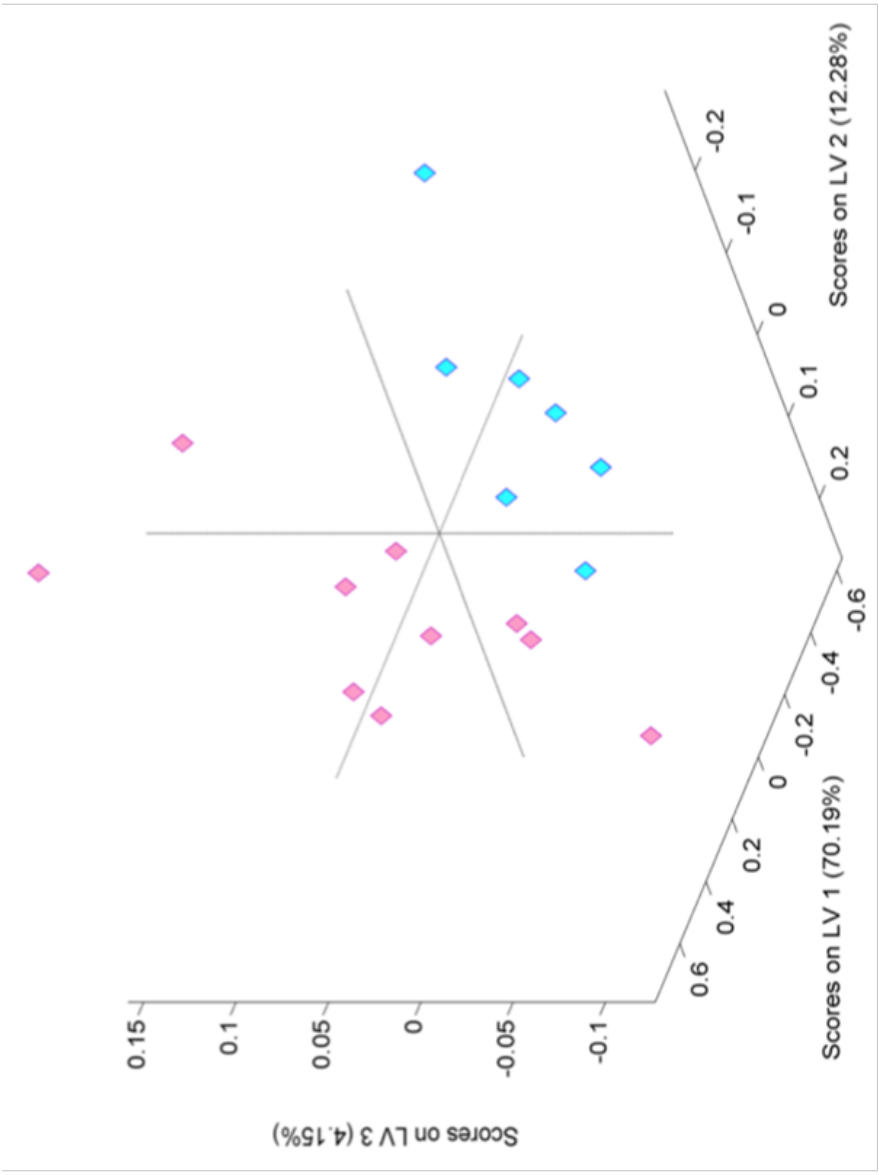


**Figure 7.** Scores plot corresponding to the first three LVs of the PLSDA regression model of E serogroup isolates using the m/z 2000 to 11000 mass spectral region. Legend: ◆ E1-E2-E3 and ◆ E4 serogroups.

**Supplementary Information**

**Supplementary Table S1.** *Salmonella enterica* isolates used in this study, 2002-2015.

| Serogroups (no. isolates) | | Serotypes[a] (no. isolates) | Source(s)[b] (no. isolates) | Country(ies) (no. isolates)[c] |
|---|---|---|---|---|
| B-O:4 (75) | | Typhimurium (21) | E (1); Cte (1); H (15); Otf (2); P (1); Pie (1) | PT (15); AU (6) |
| | | 4,[5],12:i:- (25) | A (2); E (1); Cte (1); H (14); P (3); Pie (2); Pt (1); W (1) | PT (15); AU (10) |
| | | Stanley (7) | H (5); Pt (2) | PT (3); AU (4) |
| | | Derby (5) | H (3); P (2) | PT (5) |
| | | Heidelberg (5) | H (2); Pt (3) | PT (2); IFP (3) |
| | | Saintpaul (2) | H (1); Pt (1) | PT (2) |
| | | Brandenburg (2) | H (1); P (1) | PT (2) |
| | | Chester (1) | Hv (1) | AG (1) |
| | | Agona (1) | Pt (1) | PT (1) |
| | | Budapest (1) | Aq (1) | PT (1) |
| | | Bredney (1) | H (1) | PT (1) |
| | | II 4,12:l,w:z39 (1) | Wr (1) | AG (1) |
| C (66) | C1-O:6,7 | Infantis (7) | H (4), P (2); Pte (1) | PT (3); AU (4) |
| | | Rissen (8) | H (3), P (3); Pie (2) | PT (8) |
| | | Virchow (5) | H (2); Pie (2); Pte (1) | PT (5) |
| | | Mbandaka (8) | H (5); Otf (2); Pt (1) | PT (4); AU (4) |
| | | Thompson (1) | C (1) | PT (1) |
| | | Braenderup (1) | Pte (1) | AG (1) |
| | C2-O:6,8 | Newport (16) | A (1); Aq (1); H (10); Otf (1); Pt (3) | PT (5); AU (11) |
| | | Bovismorbificans (6) | H (6) | PT (6) |
| | | Hadar (5) | H (1); Pt (4) | PT (5) |
| | | Muenchen (3) | H (3) | PT (3) |
| | | Goldcoast (2) | H (1); P (1) | PT (2) |
| | C3-O:8 | Kentucky (1) | H (1) | PT (1) |
| | | Istanbul (1) | Pt (1) | PT (1) |
| | | Brikama (1) | H (1) | PT (1) |
| | | Sindelfingen (1) | Otf (1) | IFP (1) |
| D (25) | D1-O:9 | Enteritidis (16) | A (1); H (9); Otf (2); Pt (3); Pte (1) | PT (8); AU (8) |
| | | Ndolo (3) | H (3) | PT (3) |
| | | Houston (2) | H (1); Otf (1); | PT (2) |
| | | Dublin (1) | H (1) | PT (1) |
| | | II 9,46:z:- (1) | A (1) | AG (1) |
| | D2-O:9,46 | Linguere (1) | Aq (1) | PT (1) |
| | | Guerin (1) | Aq (1) | PT (1) |
| E (17) | E1-E2-E3-O:3,10 | Anatum (1) | P (1) | PT (1) |
| | | Give (1) | P (1) | PT (1) |
| | | London (1) | H (1) | PT (1) |
| | | Weltevreden (1) | Otf (1) | IFP (1) |
| | | Muenster (1) | A (1) | AG (1) |
| | | Uganda (1) | Pt (1) | PT (1) |
| | | Freiburg (1) | C (1) | PT (1) |
| | E4-O:1,3,19 | Senftenberg (10) | H (5); Pt (3); Pte (2) | PT (3); AU (7) |

[a]*Salmonella* subspecies II, *S. enterica* subsp. *salamae*;

[b]A, animals; Aq, aquacultures; C, community laboratories; Cte, cattle and cattle environment; E, environment; H, hospital patients; Hv, Healthy volunteers; Otf, other type of food; P, pork; Pie, pigs and piggery environment; Pt, poultry; Pte, poultry and avian environment; W, bathing or drinking water; Wr, river water.

[c]AG, Angola; AU, Austria; PT, Portugal; IFP, NTS of imported food products.

**Supplementary Table S2.** Confusion matrix for the *S.* 4,[5],12:i:- and other B serotypes PLSDA discrimination model (values are in %).

| **MALDI-TOF MS method** | **Classical method** | | |
|---|---|---|---|
| | ***S.* 4,[5],12:i-** | **Other B serotypes** | **SUM** |
| ***S.* 4,[5],12:i:-** | 29.83 | 6.35 | - |
| **Other B serotypes** | 4.96 | 58.87 | - |
| **SUM** | - | - | 88.70 |

**Supplementary Table S3.** Confusion matrix for the *S.* Typhimurium, *S.* Stanley and other B serotypes PLSDA discrimination model (values are in %).

| **MALDI-TOF MS method** | **Classical method** | | |
|---|---|---|---|
| | ***S.* Typhimurium + *S.* Stanley** | **Other B serotypes** | **SUM** |
| ***S.* Typhimurium + *S.* Stanley** | 59.73 | 4.80 | - |
| **Other B serotypes** | 0.27 | 35.20 | - |
| **SUM** | - | - | 94.93 |

**Supplementary Table S4.** Confusion matrix for the *S.* Typhimurium and *S.* Stanley serotypes PLSDA discrimination model (values are in %).

| **MALDI-TOF MS method** | **Classical method** | | |
|---|---|---|---|
| | ***S.* Typhimurium** | ***S.* Stanley** | **SUM** |
| ***S.* Typhimurium** | 61.10 | 5.80 | - |
| ***S.* Stanley** | 9.90 | 24.20 | - |
| **SUM** | - | - | 85.30 |

**Supplementary Table S5.** Confusion matrix for the *S.* Derby, *S.* Heidelberg and other B serotypes PLSDA discrimination model (values are in %).

| **MALDI-TOF MS method** | **Classical method** | | | |
|---|---|---|---|---|
| | ***S.* Derby** | ***S.* Heidelberg** | **Other B serotypes** | **SUM** |
| ***S.* Derby** | 28.57 | 0.29 | 0.57 | - |
| ***S.* Heidelberg** | 0.00 | 21.29 | 8.43 | - |
| **Other B serotypes** | 0.00 | 7.00 | 33.86 | - |
| **SUM** | - | - | - | 83.72 |

**Supplementary Table S6.** Confusion matrix for the *S.* Rissen and other C1 serotypes PLSDA discrimination model (values are in %).

| **MALDI-TOF MS method** | **Classical method** | | |
|---|---|---|---|
| | ***S.* Rissen** | **Other C1 serotypes** | **SUM** |
| ***S.* Rissen** | 25.50 | 4.90 | - |
| **Other C1 serotypes** | 4.50 | 65.10 | - |
| **SUM** | - | - | 90.60 |

**Supplementary Table S7.** Confusion matrix for the *S.* Virchow and other C1 serotypes PLSDA discrimination model (values are in %).

| **MALDI-TOF MS method** | **Classical method** | | |
|---|---|---|---|
| | ***S.* Virchow** | **Other C1 serotypes** | **SUM** |
| ***S.* Virchow** | 59.25 | 7.36 | - |
| **Other C1 serotypes** | 15.75 | 17.63 | - |
| **SUM** | - | - | 76.88 |

**Supplementary Table S8.** Confusion matrix for the *S.* Newport and other C2 serotypes PLSDA discrimination model (values are in %).

| **MALDI-TOF MS method** | **Classical method** | | |
|---|---|---|---|
| | ***S.* Newport** | **Other C2 serotypes** | **SUM** |
| ***S.* Newport** | 39.50 | 11.30 | - |
| **Other C2 serotypes** | 10.50 | 38.70 | - |
| **SUM** | - | - | 78.20 |

**Supplementary Table S9.** Confusion matrix for the *S.* Bovismorbificans and other C2 serotypes PLSDA discrimination model (values are in %).

| **MALDI-TOF MS method** | **Classical method** | | |
|---|---|---|---|
| | ***S.* Bovismorbificans** | **Other C2 serotypes** | **SUM** |
| ***S.* Bovismorbificans** | 40.00 | 10.20 | - |
| **Other C2 serotypes** | 0.00 | 49.80 | - |
| **SUM** | - | - | 89.80 |

**Supplementary Table S10.** Confusion matrix for the *S.* Enteritidis and other D1-D2 serotypes PLSDA discrimination model (values are in %).

| **MALDI-TOF MS method** | **Classical method** | | |
|---|---|---|---|
| | ***S.* Enteritidis** | **Other D1-D2 serotypes** | **SUM** |
| ***S.* Enteritidis** | 59.21 | 16.50 | - |
| **Other D1-D2 serotypes** | 3.25 | 21.00 | - |
| **SUM** | - | - | 80.25 |

**Supplementary Table S11.** Confusion matrix for the E1-E2-E3 and E4 groups PLSDA discrimination model (values are in %).

| **MALDI-TOF MS method** | **Classical method** | | |
|---|---|---|---|
| | **E1- E2-E3** | **E4** | **SUM** |
| **E1- E2-E3** | 42.00 | 13.17 | - |
| **E4** | 8.00 | 36.83 | - |
| **SUM** | - | - | 78.83 |

Chapter 4

# CONCLUSIONS

The **Conclusions** that can be extracted from this Thesis achievements are summarized as follows:

**Use of human critical antibiotics in intensive food-animal production and global food trade are important food-chain drivers for the emergence and spread of antibiotic resistant non-typhoidal *Salmonella* (NTS)**

1) A recognized high use of polymyxins in Portuguese intensive animal food-production, primarily in pigs, was reflected by the detection of the *mcr*-1 gene, conferring colistin resistance, particularly in isolates from pork products and human infections. Our data also indicate a long-term dissemination of *mcr-1,* at least since 2011, with possibility of transmission to other bacteria sharing the same ecological niches. The association of *mcr-1* with transferable and broad host range plasmids (IncHI2 and IncX4) supports the relevance of horizontal transfer in the spread of colistin resistance. Moreover, *mcr-1* acquisition by two successful pig-associated multidrug-resistant clones of *S.* 1,4,[5],12:i:- and *S.* Rissen might further enhance their expansion in a context of high colistin use. Of note, co-tolerance to copper presented by those clones raises questions about the efficacy of recently suggested metal-based interventions (e.g. copper) to reduce the use of colistin and contain *mcr-1* dissemination.

2) The *oqxAB*, an unusual fluoroquinolone resistance gene in Europe, also conferring resistance to antimicrobials commonly used in animal production, was identified in two clinical ciprofloxacin-resistant *S.* Typhimurium/ST34 isolates. The finding of similar strains, IncHI2 plasmids and *oqxAB* genetic environment related to that found in human/animal settings in Asia, suggests a possible role of animal and food global trade for their transmission. Also, the concomitant presence of genes encoding resistance to antibiotics and biocides/metals widely used in farm animals suggests a potential role of different animal management practices, followed in Asia or even here, in the successful selection of *S.* Typhimurium/ST34. These data highlight the need of a continuous surveillance to contain further transmission of these PMQR genes into new hosts or different settings, facilitated by international human and animal/food travel.

3) This study evidenced the introduction into the EU, through imported poultry products, of uncommon extended-spectrum cephalosporins-resistant *Salmonella* serotypes (*S.* Minnesota/ST548 and *S.* Heidelberg/ST15), some associated with invasive human infections in which antibiotics are critical for their treatment,

4) carrying *bla*$_{CMY-2}$ on epidemic plasmids (IncA/C-ST2 or IncI1-ST12). Of note was the observation of one of the CMY-2-producing *S.* Heidelberg isolates belonging to an American epidemic clone. These data also raise questions about EU food regulations se setting a food safety microbiological criterion for fresh poultry meat covering only *S.* Enteritidis and *S.* Typhimurium (including the monophasic variant), not taking into account other emergent poultry related-serotypes uncommon in Europe.

**Understudied food-producing systems and geographical regions are potential reservoirs and transmission routes of NTS serotypes.**

5) A frequent occurrence of diverse *Salmonella* serotypes was observed during summer season in the two trout farms, the second major fish farmed species in EU. Identical strains upstream, within and downstream trout tanks pointed out the river water supplying the aquacultures as the source of NTS contamination. Transmission of NTS from aquaculture water to fish farmed was also evidenced. Despite the absence of *mcr* genes, colistin resistant *Salmonella* with new mutations in *pmrAB* genes was observed, possibly reflecting the high use of colistin in animal food-production in Portugal. Hence, here was demonstrated the risk of *Salmonella* transmission to humans associated with farmed trout consumption or contact with contaminated aquaculture fish farms, the fastest growing food-producing sector worldwide.

6) A high occurrence and diversity of NTS serotypes in different non-clinical sources (healthy humans, aquatic environments, farm and wild animals) of Benguela (Angola) were observed, extending the knowledge about the reservoirs and possible transmission routes in a geographical region with commercial or travel relationships with Europe. The detection of putative new *S. enterica* subsp. *salamae* serotypes from diverse potential sources (wild life and river water), also suggest that this region seems to be a hotspot of *S. enterica* diversity. Our data emphasizes the need to clarify the role of unusual or new serotypes as cause of human salmonellosis.

**Developments on *Salmonella enterica* discrimination at infra-species level by high-throughput typing approaches.**

7) FTIRS, coupled with multivariate chemometric analysis, was able to discriminate with high accuracy *Salmonella* belonging to clinically-relevant B, C, D and E serogroups. The sub-serogroups discrimination was also successfully achieved for, C1, C2 and C3, and for E1-E2-E3 and E4. Appropriate serotype discrimination was obtained for most *S.* Rissen from the remaining C1 serotypes and for *S.* Enteritidis from the remaining D1/D2 serotypes. Here was firstly demonstrated a correlation between the FTIRS serogroup discrimination and the O-antigen unit composition.

8) Conversely, MALDI-TOF MS, under the experimental conditions tested, demonstrated a limited potential for the discrimination of the most frequent and clinically-relevant *S. enterica* serogroups, which may be explained by the high conservation degree of *S. enterica* ribosomal proteins. Nevertheless, good discrimination rates were achieved for relevant serotypes (*S.* Enteritidis, *S.* 1,4,[5],12:i:-, *S.* Rissen), paving a way for further methodological improvements.

9) Overall, in this work, FTIRS demonstrated to be a more reliable and alternative high-throughput technique than MALDI-TOF MS to assist *Salmonella* typing at serogroup, sub-serogroup and serotype levels, with potential to be applied in routine microbiology laboratories. Still, a limited discriminative potential for *S.* Typhimurium and *S.* 1,4,[5],12:i:-, clones was verified by both techniques.

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