

FACTEURS D'ADAPTATION DE SALMONELLA AUX MÉTAUX LOURDS ET ANTIBIOTIQUES DANS LES ENVIRONNEMENTS DE LA FILIÈRE PORCINE EN FRANCE (SALMETAL-R).

HEAVY METALS AND ANTIBIOTIC RESISTANCE OF SALMONELLA IN THE PIG INDUSTRY IN FRANCE: FROM BREEDING TO HUMAN INFECTIONS (SALMETAL-R).

Etablissement **ENVA - Ecole Nationale Vétérinaire d'Alfort**

École doctorale **Agriculture, Alimentation, Biologie, Environnement et Santé**

Spécialité **Microbiologie**

Unité de recherche **LSA Laboratoire de Sécurité des Aliments - ANSES**

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Salmonella, résistance aux métaux lourds et aux antibiotiques, caractérisation génomique et phénotypique, co-sélection

Salmonella, resistance to heavy metals and antibiotics, genomic and phenotypic characterization, co-selection

Description de la problématique de recherche - Project description

Les métaux lourds et les antibiotiques sont utilisés depuis des décennies dans la filière porcine pour la santé des animaux et des aliments. Le cuivre, le zinc et l'argent sont, ou ont été, particulièrement utilisés comme compléments alimentaires en alternative aux antibiotiques et comme antimicrobiennes dans cette filière. L'utilisation d'antibiotiques est un sujet d'importance croissante en raison de l'émergence de résistances chez les bactéries présentes chez les animaux qui représente une menace pour la santé publique. Bien que la cooccurrence de gènes impliqués dans la résistance aux métaux lourds et aux antibiotiques a été décrite chez des nombreux bactéries Gram négatif, on connaît peu sur le sujet pour Salmonella, et plus particulièrement pour les sérovars Typhimurium et son variant monophasique qui représentent la moitié des souches isolées dans la filière porcine en France depuis une dizaine d'années. Des phénomènes de co- ou cross-résistance pourraient être à l'origine de la sélection et la persistance de ces sérovars le long de la filière. Le manque d'études sur la corrélation entre métaux lourds, antibiotiques et Salmonella est préoccupant, surtout en France où cette bactérie cause une grande partie des cas de maladies d'origine alimentaire principalement liés à la viande de porc.

Par le projet SaMETAL-R, nous voulons : i/ explorer le potentiel de dissémination des gènes de résistance aux cuivre, zinc et argent dans une vaste collection de salmonelles isolées tout au long de la chaîne de production de la filière porcine et confirmer phénotypiquement les résistances conférées susceptibles de participer à la sélection/persistance de ces souches ; ii/ identifier des possibles phénomènes de co-résistance ou cross-résistance entre les métaux lourds (cuivre, zinc et argent) et les antibiotiques le plus fréquemment utilisés dans la filière porcine et iii/ déterminer les niveaux d'expression de gènes impliqués dans ces résistances lors de la réponse à l'exposition aux antibiotiques et/ou métaux lourds d'intérêt aux concentrations utilisées sur le terrain.

Acquérir une meilleure compréhension de la résistance des salmonelles aux métaux lourds ainsi que des connexions entre cette résistance et leur sensibilité aux antibiotiques ouvrira la voie à des recommandations efficaces visant à entraver le développement de ces souches bactériennes.

Heavy metals and antibiotics have been used for decades in the pig industry to ensure animal health and food safety. Copper, zinc and silver are, or have been, particularly used as food supplements or even as alternatives to antibiotics because of their antimicrobial potential in this sector. The emergence of antibiotic resistance among zoonotic pathogenic bacteria in farmed animals constitutes a threat to public health and requires rational use of antibiotics in livestock. Although the co-occurrence of genes involved in resistance to heavy metals and antibiotics has been described in few Gram-negative bacteria, this phenomenon has been less studied for Salmonella, and particularly for serovars Typhimurium and its monophasic variant which represent half of the strains isolated in the pig industry in France over the past ten years. Co- or cross-resistance events could be at the origin of the selection and persistence of these serovars along the sector. The lack of studies on the correlation between resistance to heavy metals and antibiotics in Salmonella is worrying, especially in France where it is the main pathogenic bacteria responsible of food-borne illnesses linked to pork meat consumption.

In this context, the SaMETAL-R project aims to: i/ identify resistance genes to copper, zinc and silver and explore their genetic context and dissemination potential in large collection of Salmonella isolated along the pig production chain as well from human infections, and confirm

the resistance phenotypes likely implicated in the selection/persistence of these strains; ii/ identify possible phenomena of co-resistance or cross-resistance between heavy metals (copper, zinc and silver) and the most used antibiotics in the pig industry and iii/ study these resistances during exposure to heavy metals and/or antibiotics of interest at the concentrations used in the field.

Gaining a better understanding of heavy metal resistances in Salmonella as well as the interconnections with antibiotic resistances will pave the way for effective recommendations aimed at hindering the development of these resistant strains for the pig industry.

Thématique / Contexte

Attached to the two research entities, you will be welcomed for 18 months at Anses in the Food Safety Laboratory (Maisons-Alfort, 94700) and 18 months at INRAE Centre-Val-de-Loire (Nouzilly, 37380) in the research unit UMR1282 Infectiologie & Santé Publique.

In Anses, you will join the Salmonella and Listeria Unit (SEL)/Research Team. The SEL unit includes 30 people divided into three teams responsible for developing surveillance, reference and research activities on two bacterial pathogens involved in food-borne human infections: Salmonella and Listeria.

In INRAE, you will be part of the research group on Genomic Plasticity, Biodiversity and Antimicrobial Resistance (10 permanent staff) which carried out fundamental research on mobile genetic supports spreading antimicrobial resistance.

PhD information and candidature : Sabrina Cadel-Six (Tél 0149772719, sabrina.cadesix@anses.fr) Benoît Doublet (Tél. 0247427295, benoit.doublet@inrae.fr)

Heavy metals and antibiotics have been used for decades in the food industry to protect plants or animals from harmful pathogens and guarantee the production of healthy and safe food for the consumer.

Heavy metals are dense chemical elements naturally present in the environment, however some of them, such as copper, zinc and silver, have also been used in the agri-food sector, particularly in the pork industry, for decades. In breeding, zinc oxide has been widely used at relatively high concentrations for piglet weaning following the antibiotic use reduction, in order to prevent the development of digestive infections (diarrhea) before its ban by EU due to accumulation in soils (ecotoxicity). Copper is used as dietary supplements added to animal feed. This compound is necessary for certain biological functions and can be beneficial for animal health, however the concentrations at which it is administered, although regulated (Commission Implementing Regulation (EU) 2018/1039 of 23 July 2018), are underestimated in breeding because it is also present in the straw and seeds consumed by pigs, being used as a herbicide and fungicide in agriculture. In processing workshops, silver and zinc, due to their antimicrobial properties, are also used as active ingredients in biocides such as LIFE (<https://biocid-anses.fr/biocid#!>) or integrated into coatings to ensure hygienic surfaces (PolyVision). Copper, zinc and silver are therefore introduced by operators in the pig industry and accumulate throughout the production chain from livestock to food industry workshops as well as in finished products.

The use of antibiotics in the swine industry is a subject of increasing importance due to its implications for animal health, food safety and antibiotic resistance. Historically, antibiotics have been used in pig farming to prevent and treat bacterial infections, but their inappropriate use has led to the emergence of resistant bacteria present in animals. These resistant bacteria, through the food chain, can infect humans and represent a threat to public health. Since 2012, France has implemented measures to reduce the use of antibiotics in the pig industry, especially for medically-important antibiotics. Today, breeders must declare the quantities of antibiotics used in pig farming and microbiological criteria are monitored in slaughterhouses ((EC) No. 2073/2005). The data collected from these surveillance systems makes it possible to assess the evolution of antibiotic use and antibiotic resistance of harmful microorganisms in the pig sector. Through this surveillance system in 2021, an average Salmonella prevalence of 4.6% was recorded in slaughterhouses, ranging from 2.7% to 11.9% on cutting parts. A study carried out by the Pork Institute (Ifip) and ANSES showed that more than half of the Salmonella strains isolated in 2021 belong to the monophasic variant of Typhimurium (TVM) ST34 which carries a transposon providing resistances to tetracycline, broad-spectrum penicillins, aminoglycosides and sulfonamides (pHd by M. De Sousa Violante, 2022), antibiotics widely used in the pig sector in Europe in past decades.

The co-occurrence of genes involved in resistance or in the metabolism of heavy metals and antibiotics has been described in some Gram-negative bacteria (Vats et al., 2022, review). However, knowledge remains very limited for Salmonella regarding their levels of resistance to heavy metals, the implicated mechanisms/genes and also the co- or cross-resistance phenomena to other molecules such as antibiotics.

Co-resistance: co-resistance corresponds to the accumulation of different resistance genes coding for different specific mechanisms that are clustered on the same genetic element, and thus can spread together in bacterial populations and benefit to bacteria under selection pressure (Vats et al., 2022, review). Long-term persistence of heavy metals in animals, livestock and industrial environments can exert selective pressure and thus results in co-selection of both resistances to heavy metals and antibiotics. Recent studies, conducted by ANSES as part of the ACTIA FASTYPERS Joint Technological Unit (2022/27) on a panel of 18 serovars, the most frequently isolated in France, including TVM, revealed the presence of resistance to heavy metals such as copper, silver, arsenic and mercury in the genomes of different serovars (Grandjean et al., 2023). Despite the evidence of a significant number of genes involved in metabolism and resistance to heavy metals, the chromosomal or mobile nature of these elements still remains to be elucidated and the genotype-phenotype correlation remains poorly characterized to date. Indeed, when the presence of resistance genes is essential to develop resistance to a particular compound, confirmation of its expression can only be obtained by the phenotypic determination of resistance levels using *in vitro* tests. As part of the H2020 COMPARE project, we demonstrated through Bayesian analysis on a panel of 218 Salmonella TVM ST 34 genomes that this clonal population first acquired the copper and silver resistance operons via the integration of SGI-4 before acquiring resistances to tetracyclines, broad-spectrum penicillins, aminoglycosides and sulfonamides (Cadel-Six et al. 2021). Despite these results on resistance to different heavy metals and antibiotics, no statistical association analysis has been carried out to date to compare all phenotypic resistance data generated with the content and diversity of resistance genes (heavy metals/antibiotics) identified in this panel.

Cross-resistance: Cross-resistance is defined as a single mechanism providing resistance to several molecules of different families (Vats et al., 2022, review). Cross-resistance mechanisms between metals and antibiotics could intervene such as the joint action of efflux pumps. As part of the UMT FASTYPERS, we were able to demonstrate, through transcriptomic studies, two different mechanisms of action of copper resistance in *Salmonella* Typhimurium and TVM strains involving the expression of genes coding for different copper efflux pumps and an increased capacity of TVM to survive in the presence of copper, especially in anaerobic conditions (Grandjean et al., 2023). Despite these results, we did not carry out phenotypic determination experiments, nor inactivation kinetics in the presence of different concentrations of antibiotics and/or heavy metals to determine the levels of expression of several candidate genes during the response to exposure to antibiotics and/or metals. These experiments are necessary to better understand the resistance mechanisms in the case of cross-resistance mechanisms.

There is a lack of descriptive and fundamental studies (genomic and phenotypic) on the possible correlation between resistance to heavy metals and antibiotics in *Salmonella*.

This lack of knowledge is even more problematic since, in France, *Salmonella* is responsible of 44% of collective food-borne illness in 2021. Moreover, pork meat is one of the main sources of contamination. France is the 3rd pork producing country in Europe and in 2021 pork remains the most consumed meat in France before poultry and beef (Ifip, 2022).

Objectifs

The SalMETAL-R v2.0 project aims to:

- (i) Phenotypically confirm heavy metals resistance that may participate in the selection/persistence of *Salmonella* strains along the pig industry and explore the potential for dissemination of copper, zinc and silver resistance genes. We will use a collection of 350 isolated strains (from breeding to meat samples) as well as isolates from patients to document the potential for genomic resistance to heavy metals and antibiotics in major *Salmonella* serovars isolated from the pig industry in France, to identify co-resistances and determine whether co-occurrence of resistance genes (heavy metals / antibiotics) are statistically significant. Genomic analysis will be conducted to predict the potential for dissemination (eg horizontal transfer) of genetic elements carrying resistance genes (plasmids, integrative elements). Long reads sequencing (Nanopore) will be performed on strains carrying plasmids/integrative elements conferring resistance to both heavy metals and antibiotics. Copper, zinc and silver resistance levels will be explored phenotypically for TVM strains isolated during food poisoning between 2018 and 2022 (pork meat) by minimum inhibitory concentrations (MIC) in liquid medium to confirm the predicted phenotype *in silico*.
- (ii) Examine potential interactions between different resistance mechanisms to heavy metals (copper, zinc and silver) and the most used antibiotics (only co-occurrence vs cross-resistance mechanisms). To explore these two phenomena, we will phenotypically study the levels of resistance to heavy metals of interest for a representative panel of epidemic strains isolated during foodborne illnesses that occurred in France between 2018 and 2022 resistant to tetracycline antibiotics, broad-spectrum penicillins, aminoglycosides and sulfonamides. We will perform phenotypic determination experiments (potential interaction between antibiotics and copper, silver and zinc) and perform inactivation kinetics in the presence of different concentrations of antibiotics and/or heavy metals.
- (iii) Determine the expression levels of several candidate genes during the response to exposure to antibiotics and/or heavy metals of interest. To better understand the mechanisms of resistance in the case(s) of cross-resistance mechanisms, we will perform real-time quantitative PCR (RT-qPCR) experiments targeting known genes to be involved in heavy metal metabolic pathways.

Méthode

Fundamental microbiology, molecular biology, bacterial genomics and bioinformatics

Résultats attendus - Expected results

The expected results are:

- knowledge of the potential for dissemination of resistance genes to copper, zinc and silver and in conjunction with the antibiotics most frequently used in the pig industry;
- the deposit in open access databases of the complete genomes of candidate strains from the pork industry production chain;
- the phenotypic confirmation of resistance to copper, silver and zinc which participate in the selection and persistence of *Salmonella* strains isolated along the pig industry and the determination of the levels of resistance to these metals of interest for a panel representative of strains;
- the determination of co- or cross-resistance to copper, zinc and silver with regard to tetracycline antibiotics, broad-spectrum penicillins, aminoglycosides and sulfonamides (the most frequently used in the pig industry);
- knowledge of the expression levels of several candidate genes during the response to exposure to antibiotics and/or metals at the concentrations used in breeding and in processing workshops in order to identify possible common metabolic pathways.

Références bibliographiques

- J. Hawkey, S. Le Hello, B. Doublet, S. Granier, R. Hendriksen, F. Fricke, P.-J. Ceysens, C. Gomart, H. Billman-Jacobe, K. E. Holt, and F.-X. Weill. (2019) Global phylogenomics of multidrug resistant *Salmonella enterica* serotype Kentucky ST198. *Microbial Genomics*. 5: doi: 10.1099/mgen.0.000269.
- J. Kiss, M. Szabó, A. Hegyi, G. Douard, K. Praud, I. Nagy, F. Olasz, A. Cloeckert, and B. Doublet. (2019) Identification and characterization of oriT and two mobilization genes required for conjugal transfer of *Salmonella* Genomic Island 1. *Frontiers in Microbiology*. 10:457. doi: 10.3389/fmicb.2019.00457

- B. Doublet, K. Praud, T. Nguyen-Ho-Bao, M. Argudin Regueiro, S. Bertrand, P. Butaye, and A. Cloeckaert. (2014) Extended-spectrum- β -lactamase- and AmpC- β -lactamase-producing d-Tartrate-positive *Salmonella enterica* serovar Paratyphi B from broilers and human patients in Belgium, 2008-2010. *Journal of Antimicrobial Chemotherapy*. 69:1257-1264.
- Cadel-Six S, Cherchame E, Douarre P-E, Tang Y, Felten A, Barbet P, Litrup E, Banerji S, Simon S, Pasquali F, Gourmelon M, Mensah N, Borowiak M, Mistou MY and Petrovska L (2021) The Spatiotemporal Dynamics and Microevolution Events That Favored the Success of the Highly Clonal Multidrug-Resistant Monophasic *Salmonella* Typhimurium Circulating in Europe. *Front. Microbiol.* 12:651124. <https://doi.org/10.3389/fmicb.2021.651124>
- Grandjean Hugo, Douarre Pierre-Emmanuel, Romero Karol, Tran Mai-Lan, Mazurais Clément, Jitaru Petru, Cadel-Six Sabrina. Resistance of *Salmonella* to heavy metals, a key element for dominance in the agro-food industry in Europe?. ARAE - 9th Symposium on Antimicrobial Resistance in Animals and the Environment, Jul 2023, Tours, France. <https://hal.science/hal-04150303> and <https://arae2023.symposium.inrae.fr/abstract-book>
- Prakriti Vats, Ujjwal Jit Kaur, Praveen Rishi (2022) Heavy metal-induced selection and proliferation of antibiotic resistance: A review. *Journal of Applied Microbiology*, Volume 132, Issue 6, 1 June 2022, Pages 4058–4076. <https://doi.org/10.1111/jam.15492>
- A. Tamilselvi & Govindasamy Mugesh (2008) Zinc and antibiotic resistance: metallo- β -lactamases and their synthetic analogues. *JBIC Journal of Biological Inorganic Chemistry* volume 13, pages 1039–1053 <https://doi.org/10.1007/s00775-008-0407-2>
- Branchu P, Charity OJ, Bawn M, Thilliez G, Dallman TJ, Petrovska L and Kingsley RA (2019) SGI-1 in Monophasic *Salmonella* Typhimurium ST34 Is a Novel ICE That Enhances Resistance to Copper. *Front. Microbiol.* 10:1118. doi: 10.3389/fmicb.2019.01118
- C-S Holzel, C Muller, K Harms, S Mikolajewski, S Schafer, K Schwaiger, J Bauer Wan et al., 2012. Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environmental Research* 113, 21–27. doi:10.1016/j.envres.2012.01.002
- IFIP-Institut du porc, 2022, Porc par les chiffres, la filière porcine en France, dans l'UE et le monde, Ed 2022-2023.

Précisions sur l'encadrement - Details on the thesis supervision

Co-Direction de thèse : Benoît Doublet (Tél. 0247427295, benoit.doublet@inrae.fr)

Conditions scientifiques matérielles et financières du projet de recherche

- 36-month doctoral contract: obtained by AAP Anses-INRAE 2023
- Isolation and sequencing of short and long reads strains + Phenotypic resistance tests and horizontal transfer tests + valorization and travel: SalmoBOND project (funding obtained CASDAR 2023); AMR surveillance project OO1-Action4 (funding obtained from Partnership Animal Health and Welfare).

Objectifs de valorisation des travaux de recherche du doctorant : diffusion, publication et confidentialité, droit à la propriété intellectuelle,...

The results of this thesis project will be promoted in the following ways:

- Co-signed articles:

In professional journals for stakeholders in the pig sector, veterinary laboratories, food industry manufacturers, and training centers (i.e. Notebook of IFIP).

In peer-reviewed scientific journals: EuroSurveillance, Applied and Environmental Microbiology, Frontiers in Microbiology, Food Microbiology, International Journal of food microbiology, Foodborne Pathogens and Diseases, etc.

- Oral and digital presentations:

The results will be presented at information days and international scientific conferences (JRP, IAFP, FoodMicro 2026, I3S 2026, ARAE 2027) or national (Société Française de Microbiologie). The website of the Anses *Salmonella* network (<https://reseau-salmonella.anses.fr/fr/minisite/salmonella/accueil>) will be used to present the project and its main results.

- Thesis manuscript and defence: The PhD student will write a thesis manuscript that presents the entire results of the research in a comprehensive and detailed way. The thesis will be published and made publicly available.

Collaborations envisagées

National network:

- Institut Pasteur, National Reference Center for *Salmonella*
- Ifip (Pig Institute)

In addition, several Anses laboratories are participating in the SaIMETAL-R project: Anses-Laboratory of Ploufragan-Plouzané-Niort, HQPAP Unit ; Anses-Laboratory of Fougères, LNR Antimicrobial Resistance and AB2R unit.

These collaborators represent direct partners in this project, having been involved upstream in the construction of the collection of strains which will be studied as part of the thesis project.

Profil et compétences recherchées - Profile and skills required

- Diplôme Master II en microbiologie et/ou bioinformatique
- Connaissances requises : Microbiologie fondamentale et génomique bactérienne
- Expérience appréciée : Bactériologie et biologie moléculaire (culture, extraction ADN/ARN, PCR...) ; gestion de grandes collections de microbes et de données.
- Compétences : Travail en équipe, autonomie, rigueur, esprit critique, réactivité, bon sens de l'organisation.

- Une expérience dans l'utilisation du système Linux (scripts bash, python et R)
- Des compétences en statistique seraient un plus.
- Solides compétences en communication écrite et orale

- Master II diploma in microbiology and/or bioinformatics
- Knowledge required: Fundamental microbiology and bacterial genomics
- Appreciated experience: Bacteriology and molecular biology (culture, DNA/RNA extraction, PCR...); management of large microbial and data collections.
- Soft skills: Teamwork, autonomy, rigor, critical thinking, responsiveness, good organizational skills.
- Experience in using Linux system (bash, python and R scripts) skills
- Experience in statistics would be a plus.
- Strong written and oral communication skills

Dernière mise à jour le 12 avril 2024